

PROBIOTIC COMPOSITIONS FOR TREATMENT OF HAIR LOSS

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 63/281, 887, filed November 22, 2021, the contents of which are hereby incorporated by reference in
5 the entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Hair loss is a particularly prevalent condition among the general population. In the US alone, more than 40 million men and 25 million women suffer from hair loss due to a variety of reasons such as illness, medical treatment, and simply aging. Beginning at age 35
10 with a 40% hair loss rate for men and growing to a 70% loss rate for men age 80, the majority of the world-wide population will need professional services to help them restore their hair. Although less susceptible to hair loss due to aging, by the time they reach age 60, greater than 80% of all women will suffer from notable signs of hair loss manifested in thinning hair and/or loss of hair volume.

15 [0003] Medical conditions and medical treatments also account for a significant portion of the causes for hair loss. For example, chemotherapeutic agents administered to cancer patients often lead to significant or even completely loss of hair on the patients' head and face. While hair loss due to chemotherapy is mostly temporary in nature, in small number of cases, however, regrowth of lost hair may not be achieved to the full extent. Another example of
20 medical condition-induced hair loss has been observed in the ongoing pandemic of COVID-19 caused by SARS-CoV2. Among patients who have recovered from COVID-19, some will develop a profound hair loss. This is particularly notable among patients suffering from the so-called long COVID. These patients persistently exhibit one or more clinical symptoms of the disease even after they have cleared the virus and have been tested negative (*e.g.*, by
25 polymerase chain reaction or PCR for SARS-CoV2) at least 4 weeks ago.

[0004] Due to the cultural significance assigned to the head hair, a strong need has always existed for hair restoration and hair loss prevention. A variety of techniques have been in regular use, including the use of medications, scalp treatments, laser hair therapy, as well as other surgical or non-surgical procedures. These available techniques often involve high cost,
30 low efficacy, and, in some cases, a substantive level of technical complexity. Thus, there is

an urgent need for new, effective, and easy-to-use methods for treating or preventing hair loss. The present invention fulfills this and other related needs by identifying beneficial gut microorganisms in an effort to formulate new compositions and devise new methods that are effective for preventing or treating hair loss as well as enhancing hair restoration among
5 individuals, who may be adults or children and suffering or at risk of hair loss, due to varying causes including infectious diseases such as COVID-19.

BRIEF SUMMARY OF THE INVENTION

[0005] The present inventors discovered in their studies the certain gut microbial species can promote and enhance hair growth among individuals who suffer from reduced or thinning
10 hair due to various conditions such as infectious illness (*e.g.*, viral or bacterial infection such as SARS-CoV-2), thus provide an important utility in the treatment of hair loss, a condition of high prevalence in the general population. The microorganisms so identified now serve as the basis of new methods and compositions for prophylactic and therapeutic applications.

[0006] In a first aspect, the present invention provides a composition that is useful for
15 treating hair loss in a human subject, an adult or child. The composition comprises an effective amount of (1) *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof; and (2) at least one physiologically acceptable excipient. In some embodiments, the composition consists essentially of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof plus one or more physiologically
20 acceptable excipients, optionally with one or more prebiotics. In some embodiments, the composition contains no detectable quantity of other bacteria of the *Bifidobacterium* species. In some embodiments, the composition consists essentially of an effective amount of (1) a combination of *Bifidobacterium longum* and *Faecalibacterium prausnitzii*; and (2) one or more physiologically acceptable excipients; and optionally (3) one or more prebiotics. In
25 some embodiments, the *Bifidobacterium longum* is a strain *Bifidobacterium longum* HK003 deposited with the Guangdong Microbial Culture Collection Center (GDMCC) under Accession Number GDMCC 61784. In some embodiments, the *Bifidobacterium longum* or *Faecalibacterium prausnitzii* is in the form of viable cells. In some embodiments, the

bacteria are in the form of or non-viable cells, such as heat-inactivated or chemically inactivated cells. In some embodiments, the amount of each of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* is about 1.7×10^7 - 1×10^{10} CFU for adults and about 8.5×10^6 - 5×10^9 CFU for children in daily intake. In some embodiments, the composition is formulated for oral ingestion, for example, in the form of a food or beverage item.

[0007] In the second aspect, the present invention provides a method for treating hair loss in a subject, who may be an adult or a child. The method includes the step of administering to the subject an effective amount of the composition described above and herein: in other words, comprising an effective amount of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof along with at least one physiologically acceptable excipient. In some embodiments, the administering step comprises administering to the subject one single composition comprising both *Bifidobacterium longum* and *Faecalibacterium prausnitzii* in an effective amount. In some embodiments, the administering step comprises administering to the subject a first composition comprising *Bifidobacterium longum* in an effective amount, and administering to the subject a second composition comprising *Faecalibacterium prausnitzii* in an effective amount. In some embodiments, the administering step comprises oral ingestion of the composition described above and herein comprising an effective amount of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof plus at least one physiologically acceptable excipient. In some embodiments, the administering step comprises oral ingestion of the composition prior to food intake, or shortly after food intake, or at the same time of food intake (e.g., with a meal). In some embodiments, the subject has recovered from COVID-19, for instance, the subject is a long-COVID patient.

[0008] In a related aspect, the present invention provides a novel use of a composition for treating hair loss in a subject. The composition comprises an effective amount of (1) *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof; and (2) at least one physiologically acceptable excipient. In some embodiments, the composition consists essentially of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof plus one or more physiologically acceptable excipients, optionally with

- one or more prebiotics. In some embodiments, the composition contains no detectable quantity of other bacteria of the *Bifidobacterium* species. In some embodiments, the composition consists essentially of an effective amount of (1) a combination of *Bifidobacterium longum* and *Faecalibacterium prausnitzii*; and (2) one or more
- 5 physiologically acceptable excipients; and optionally (3) one or more prebiotics. In some embodiments, the *Bifidobacterium longum* is a strain deposited with the Guangdong Microbial Culture Collection Center (GDMCC) under Accession Number GDMCC 61784. In some embodiments, the *Bifidobacterium longum* or *Faecalibacterium prausnitzii* is in the form of viable cells. In some embodiments, the bacteria are in the form of or non-viable cells,
- 10 such as heat-inactivated or chemically inactivated cells. In some embodiments, the amount of each of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* is about 1.7×10^7 - 1×10^{10} CFU for adults and about 8.5×10^6 - 5×10^9 CFU for children in daily intake. In some embodiments, the composition is formulated for oral ingestion, for example, in the form of a food or beverage item.
- 15 **[0009]** In a third aspect, the present invention provides a kit for treating hair loss in a subject comprising a plurality of containers, each containing a composition comprising an effective amount of *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof. In some embodiments, the kit includes two compositions, each comprising an effective amount of *Bifidobacterium longum* or *Faecalibacterium prausnitzii*
- 20 along with one or more physiologically acceptable excipients. In some embodiments, the compositions are in the form of a powder, liquid, paste, cream, tablet, or capsule. In some embodiments, the compositions are formulated for oral administration or for local deposit as means for delivery.
- [0010]** In a fourth aspect, the present invention provides a newly isolated strain of
- 25 *Bifidobacterium*, *Bifidobacterium longum* HK003, deposited with the Guangdong Microbial Culture Collection Center (GDMCC) under Accession Number GDMCC 61784, as well as compositions comprising *B. longum* HK003, and methods of their use. In one embodiment, a composition is provided, which comprises the *Bifidobacterium longum* of this invention. For example, such a composition is in the form of a freeze-dried powder comprising the *B.*

longum. In some embodiments, the freeze-dried powder is produced by freeze-drying the *B. longum* preserved in a lyoprotectant comprising gelatin, skimmed milk powder, trehalose, and sucrose. In one embodiment, the lyoprotectant comprises about 1.5% (weight/volume or w/v, expressed in 1.5 grams of solid dissolved in 100 ml of solvent, *e.g.*, water) of gelatin, 5 about 15% (w/v) of skimmed milk powder, about 15% (w/v) of trehalose, and about 5% (w/v) sucrose. In some embodiments, such a composition is in the form of a capsule or microcapsule in which the *B. longum* is encapsulated with a carrier material comprising soybean protein isolate and konjac gum.

[0011] In one embodiment, a method of reducing inflammation is provided by way of 10 administration to a subject in need thereof an effective amount of (1) the *B. longum* of this invention or (2) the composition comprising the *B. longum* of this invention. In some embodiments, the effective amount of the *B. longum* of this invention or (2) the composition comprising the *B. longum* of this invention reduces inflammatory cytokine levels in the subject. In some embodiments, the effective amount of the *B. longum* of this invention or (2) 15 the composition comprising the *B. longum* of this invention reduces pathogenic bacteria levels in the gastrointestinal tract of the subject. Such pathogenic bacteria may include *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *enterococcus faecalis*, or any combination thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

20 **[0012] Figure 1. Alopecic condition in mice after each treatment. Figure 1A,** Schematic diagram of treatment interventions in mice. In Group 1 - Water, mice were treated with normal drinking water throughout the experiment. In Group 2 - P80, mice were treated with 1% P80 throughout the experiment. In Group 3 - P80+Abx, mice were treated with 1% P80 throughout the experiment, and additionally treated with Abx at week 13 and left for 2 25 weeks without treatment for observation. In Group 4 - P80+Abx+FMT, mice were treated with 1% P80 throughout the experiment, and additionally treated with Abx+FMT at week 13 (n = 10; except n = 8 for Group 1). **Figure 1B,** Mice after P80 administration before treatment challenges. In water group, no mouse showed sign of hair thinning or development

of alopecia. In each of P80, Abx and FMT group, 40% of mice (4 out of 10) showed sign of alopecia after 12 weeks of P80 administration. **Figure 1C**, Pre- and Post-Abx treatment. Alopecic mice increased from 40% to 70% (7 out of 10) with increased alopecic severity after Abx treatment. **Figure 1D**, Pre- and Post-FMT treatment. Before treatment
 5 interventions, 4 of 10 mice (prevalence 40%) showed sign of alopecia in FMT group. After Abx-FMT, 4 mice showed sign of hair-regrowth as indicated by green arrows. **Figure 1E**, The statistical analysis of the alopecia score in water, P80, post-Abx and post-FMT groups. FMT significantly recovered the severity of alopecia after 2-weeks treatment, while the alopecia in Abx group is even worse when compared to P80 group. The alopecia score was
 10 determined by area of alopecia affected on the dorsal body. The alopecia scoring scale can be found in **Figure 10D**.

[0013] Figure 2. Alopecia scores and microbial compositions in mice after each treatment intervention. Figure 2A, Four treatment inventions altered microbial compositions in each group of mice (n = 4). **Figure 2B**, Principal Coordinates Analysis
 15 (PCoA) of faecal microbiome in mice from each treatment group. **Figure 2C**, Discriminative analysis in the microbiome of alopecic and non-alopetic mice. Microbial compositions were differentiated with a cut-off of significant level (LDA score ≥ 2). **Figure 2D**, Relative abundance of *Akkermansia muciniphila* under the *Verrucomicrobia* phylum in each treatment group. Boxplots of relative abundance of *Akkermansia muciniphila* in 4 treatment groups
 20 were shown as mean $\pm$ s.e.m. (n = 4). In box plots, middle lines in the boxes represent the median, where lower and upper limits correspond to the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. * $P < 0.05$, Kruskal-Wallis test corrected for multiple comparisons followed by a Dunn's test.

**[0014] Figure 3. Condition of dorsal fur in Group 3 - P80-treated mice before and
 25 after FMT (extended experiment). Figure 3A**, Images of alopecic condition was captured before and after FMT. **Figure 3B**, Result of alopecia score pre- and post-FMT expressed as mean $\pm$ s.e.m. Each dot represented one mouse (n=10). Alopecia score was scored visually according to a 0–6 scoring system (**Figure 10D**) * $P < 0.05$, Mann-Whitney test. **Figure 3C**. PCoA analysis of selected mice from water, pre- and post-FMT treated group (n = 4). **Figure**

3D, Microbial compositions in P80-treated mice pre- and post-FMT. FMT altered the microbial compositions in 4 alopecic mice which has subsequently demonstrated hair-regrowth after the treatment. Each bar represented the microbial composition of one mouse (n = 4). **Figure 3E**, Discriminative analysis (LDA score ≥ 2) in the microbiome of P80-treated alopecic mice pre- and post FMT (n = 4).

[0015] Figure 4. Significantly differentiated metabolic pathways between alopecic and non-alopetic mice. **Figure 4A**, Discriminative analysis separated metabolic pathways between alopecic and non-alopetic mice, threshold criteria was set as LDA score ≥ 3 (n = 20). **Figure 4B**, Examples of the most significant metabolic pathways were visulised with species stratification between alopecic and non-alopetic mice (FDR $q = 0.007879$) (n = 20). **Figure 4C**, Discriminative analysis separated GO terms between alopecic and non-alopetic mice, threshold criteria was set as LDA score ≥ 2.5 (n = 20). **Figure 4D**, Heatmap visualization of full GO terms in all treatment groups. Cell colors were differentially visulised by Log₂RPK (n = 20).

[0016] Figure 5. Metabolic parameters in mice from each treatment group. **Figures 5A and 5C**, GTT and ITT were performed in mice after each treatment. Blood glucose levels were measured at 15, 30, 60, 90 and 120 min. Compared with mice who had P80 group, breakdown of blood glucose was significantly faster in mice who had antibiotics cocktail (**** $P < 0.0001$) and FMT groups (**** $P < 0.0001$). **Figures 5B and 5D**, Area under the curve (AUC) was calculated from GTT and ITT tests. Compared to P80 group, resulting AUC of blood glucose concentration was smaller in antibiotics (P80+Abx, **** $P < 0.0001$) and FMT groups (P80+FMT, **** $P < 0.0001$) respectively. (A and B) Results of GTT and GTT AUC in each treatment group mean $\pm$ s.e.m. (n = 9, except n = 8 for water-treated group). Blood glucose levels were measured at 15, 30, 60, 90 and 120 min. Significance level in GTT was determined by * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$, two-way ANOVA corrected for multiple comparisons followed by a Tukey test. Significance level in GTT AUC was determined by * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$, one-way ANOVA corrected for multiple comparisons followed by a Tukey test. (C and D) Results of ITT and ITT AUC in each treatment group mean $\pm$ s.e.m. (n = 9, except n = 8 for water-treated group). Blood

glucose levels were measured at 15, 30, 60, 90 and 120 min. Significance level in ITT was determined by $*P<0.05$, two-way ANOVA corrected for multiple comparisons followed by a Tukey test. Significance level in ITT AUC was determined by $*P<0.05$; $**P<0.01$, one-way ANOVA corrected for multiple comparisons followed by a Tukey test.

- 5 [0017] **Figure 6. Relative abundance of SCFA- and BCAA- producing bacteria in each treatment group.** **Figures 6A-H**, Relative abundance of each SCFA- and BCAA- producing bacterium represents mean $\pm$ s.e.m. ($n = 4$). In box plots, middle lines in the box represent the median, where lower and upper limits correspond to the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. $*P<0.05$, Kruskal-Wallis test
- 10 corrected for multiple comparisons followed by a Dunn's test. For relative abundance of pre- and post-FMT, each plot represents mean $\pm$ s.e.m. ($n = 4$). $*P<0.05$, Mann-Whitney test.

- [0018] **Figure 7. Skin histology in mice.** **Figure 7A**, Images of mice after receiving P80 + PBS or P80 + *B. longum* treatment. **Figure 7B**, HE analysis in the skin tissues of water, P80 + PBS and P80 + *B. longum* treated mice. Scale bar, 100 μ m. **Figures 7C and D**, alopecia score and statistical analysis in HE images. Alopecia scores were expressed as mean $\pm$ s.e.m. Each dot represented one mouse ($n=6$). Alopecia score was scored visually according to a 0–6 scoring system (**Figure 10D**). $*P<0.05$, Mann-Whitney test. Statistical analysis in HE images were determined by mean $\pm$ s.e.m. ($n = 6$). $*P<0.05$; $**P<0.01$; $***P<0.001$, one-way ANOVA corrected for multiple comparisons followed by a Sidak test.

- 20 [0019] **Figure 8. LEfSE analysis comparing the relative abundance of bacteria in hair loss group and controls.** Eight species are depleted and 38 species are enriched in patients recovered from COVID-19 with hair loss symptom (hair loss group, $n=13$) compared to non-COVID-19 controls (control, $n=24$) at 6 months after illness onset.

- [0020] **Figure 9. Relative abundance of *Faecalibacterium prausnitzii* was significantly lower in hair loss group.** Relative abundance was estimated from shotgun metagenomic sequencing. Relative abundance of *Faecalibacterium prausnitzii* at 6 months after illness onset was compared between patients recovered from COVID-19 with hair loss symptom
- 25

(hair loss group, n=13) and non-COVID-19 healthy controls (control, n=24) (Mean 1.84% vs 4.78%, respectively, $P < 0.01$, Wilcoxon test).

[0021] Figure 10. Footage of alopecic mice and alpha diversities in all treatment

groups. Figure 10A, Close footage of a P80-induced alopecia mouse. To confirm reproducibility of the result, new breed of 3 weeks old female mice (born from a different mother) were fed with 1% P80 for 13 weeks. The representative mouse was anesthetized by isoflurane before skin collection. **Figures 10 B and C**, Alpha-diversities in the murine faecal microbiome. Boxplots of biological diversities in 4 treatment groups were shown as mean $\pm$ s.e.m. ($n = 4$). In box plots, middle lines in the boxes represent the median, where lower and upper limits correspond to the 25th and 75th percentiles, and whiskers represent the minimum and maximum values. $*P < 0.05$, Kruskal-Wallis test corrected for multiple comparisons followed by a Dunn's test. **Figure 10D**, Criteria for scoring alopecia score to each mouse²².

[0022] Figure 11A, Images of P80-administered mice fed with standard chow or soya-

deprived diet and their produced stools. **Figure 11B**, Alopecia score, body weight gain and food intake. Statistical analysis in alopecia scores, body weight gain and HE images.

Alopecia scores were determined by mean $\pm$ s.e.m. ($n = 16$, except $n = 10$ for soya-deprived diet group). $***P < 0.001$, Mann-Whitney test. Body weights were recorded weekly in both diets fed mice for 13 weeks mean $\pm$ s.e.m. ($n = 16$, except $n = 10$ for soya-deprived diet group). $****P < 0.0001$, two-way ANOVA corrected for multiple comparisons followed by a

Sidak test. Food intakes were determined by mean $\pm$ s.e.m. ($n = 16$, except $n = 10$ for soya-deprived diet group). $*P < 0.05$, Mann-Whitney test. **Figure 11C**, Representative HE images in mice fed with two different diets. **Figure 11D**, Statistical analysis in HE images.

Statistical analysis in HE images was determined by mean $\pm$ s.e.m. ($n = 16$, except $n = 10$ for soya-deprived diet group). $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$, Mann-Whitney test.

[0023] Figure 12. Pre-inoculation of *Akkermansia muciniphila* exacerbated P80-

induced alopecia. Figure 12A, Schematic diagram of P80 administration and *A.*

muciniphila inoculation. **Figure 12B**, Images of mice after receiving P80 or Abx + Akk +

P80. **Figure 12C**, Alopecia scores were determined by mean $\pm$ s.e.m. (n = 6 for P80 group, n = 8 for Akk group). *** $P < 0.001$, Mann-Whitney test.

[0024] **Figure 13. Significantly differentiated molecular functions and biochemical reactions in between alopecic and non-alopecic mice.** **Figure 13A**, Discriminative analysis separated KO terms in between alopecic and non-alopecic mice, threshold criteria was set as (LDA score ≥ 2.5) (n = 20). **Figure 13B**, Heatmap visualization of full KO terms in all treatment groups (n = 20). **Figure 13C**, Discriminative analysis separated MetaCyc reaction terms in between alopecic and non-alopecic mice, threshold criteria was set as (LDA score ≥ 3) (n = 20). **Figure 13D**, Heatmap visualization of full KO terms in all treatment groups. Cell colors were differentially visulised by Log2RPK (n = 20).

[0025] **Figure 14. Schematic diagram of proof-of-concept experiment using BI as probiotic treatment.**

[0026] **Figure 15. Body weights and skin histology in CYP-treated mice.** **Figure 15A**, An image of mice after receiving CYP treatment. **Figure 15B**, Appearances of CYP + PBS treated and CYP + *B. longum* treated mice after depilation for 14 days. Mice fed with water were used as the control group. Scale bar, 100 μ m. **Figure 15C**, HE results for mice treated with CYP + PBS and CYP + *B. longum*. **Figure 15D**, Statistical analysis of body weight gain, alopecia scores and hair follicles (HF)-associated parameters for measurement of alopecia severity. **Figure 15E**, presentative HE images for skin and statistical analysis of skin thickness measured from HE images. Body weights were recorded daily in CYP + PBS and CYP + *B. longum* treated mice for 14 days mean $\pm$ s.e.m. (n = 6). Two-way ANOVA corrected for multiple comparisons followed by a Sidak test. Alopecia scores were determined by mean $\pm$ s.e.m. (n = 6). ** $P < 0.01$, Mann-Whitney test. Statistical analysis in HE images was determined by mean $\pm$ s.e.m. (n = 6). * $P < 0.05$; **** $P < 0.0001$, Mann-Whitney test.

[0027] **Figure 16. Animal study design.** Female C57 mice (7 weeks, telogen phase) were depilated by wax strips (Veet, USA) to induce the anagen phase of the hair cycle. At day 9

after depilation, the CIA model were induced by a single intraperitoneal injection of Cyclophosphamide.

[0028] Figure 17. CYP-induced alopecia in C57BL/6 mice. Fig. 17A The normal black hair started to re-appear in the blank control mice without CYP at day 10 after depilation.

5 **Fig. 17B** Representative images of mice at 23 days post-depilation. **Fig. 17C** Quantification results of pigment intensity analysis of mice dorsal skin. The grayscale images of the mice depilated area was quantified using intensity analysis in ImageJ software (NIH, USA) and expressed as a fold change of pigmented dorsal skin in relative to control group. (n=5,5,9,10,10/group, respectively). The statistical significance of the differences between
10 compared groups was tested by Wilcox-test and designed by "****"=0.001, "***"=0.01, "**"=0.05, "NS"=2.

[0029] Figure 18A Representative HE of skin sections from CIA mice at day 25 post-depilation. **Figure 18B** Quantification results of HE staining from mice dorsal skin. Original magnification. Scale bar, 200 μ m. Approximately 30 hair follicles per mouse were analyzed,
15 and representative H&E images were selected from each group. The statistical significance of the differences between compared groups was tested by Wilcox-test and designed by "****"=0.001, "***"=0.01, "**"=0.05, "NS"=2.

**[0030] Figure 19 Pan-genome was constructed using Roary based on the core and accessory genes showing phylogenetic relatedness of the isolates by blue (present) and
20 white (absent) fragments. *indicates target genome.**

[0031] Figure 20 Growth curve of *B. longum* HK003 in MRS medium without polysorbate-80.

**[0032] Figure 21 Growth curve of *B. longum* HK003 in RCM broth supplemented with galactooligosaccharide. (A) Growth phase at 6-24 hours. (B) Exponential equation
25 was determined.**

[0033] Figure 22 Colony morphology of *B. longum* HK003. (A) *B. longum* HK003 colony on RCM agar. (B) Appearance of *B. longum* HK003. (C) *B. longum* HK003 in RCM liquid media.

- [0034] Figure 23 Cell morphology of *B. longum* HK003 observed by Scanning Electron Microscopy (SEM). (A) rod-shaped. (B) irregular Y-shaped.
- [0035] Figure 24 OD 600nm measurement of *B. longum* HK003 grown under anaerobic and aerobic conditions.
- 5 [0036] Figure 25 Growth curve of *B. longum* HK003 using various carbon source.
- [0037] Figure 26 Freeze-dried powder of *B. longum* HK003.
- [0038] Figure 27 Microencapsulation of *B. longum* HK003 with whey protein isolate and gum Arabic.
- [0039] Figure 28 Microencapsulation of *B. longum* HK003 with soybean protein isolate and gum Arabic.
- 10 [0040] Figure 29 Bile resistance of *B. longum* HK003 microencapsulated with soybean protein and gum Arabic.
- [0041] Figure 30 Antibiotic resistance of *B. longum* HK003 evaluated by disc diffusion method.
- 15 [0042] Figure 31 Antibiotic resistance of other *Bifidobacteria* strains evaluated by disc diffusion method.
- [0043] Figure 32 Plasmid extraction followed by gel electrophoresis confirmed absence of plasmid in *B. longum* HK003.
- [0044] Figure 33 *B. longum* HK003 reduced gene expression level of LPS-induced IL-6 in NCM460 cells. NCM460 cells were exposed to LPS with or without addition of live *B. longum* HK003 (BL HK003) and a commercial *B. longum* DSM 16603 (BL Commercial) for 24 hours. NCM460 without exposure to LPS or *B. longum* was used as blank control (Blank). Expression level of IL-6 was determined by quantitative real-time PCR. The columns represent average expression level of IL-6 relative to that of actin from triplicate
- 20 measurements. Results are expressed as mean $\pm$ SD. *P<0.05; **P<0.01; ***p<0.001. Blank
- 25

– no treatment; LPS – LPS alone; BL HK003 – LPS with *B. longum* HK003; BL Commercial – LPS with *B. longum* DSM 16603.

[0045] **Figure 34 *B. longum* HK003 reduced gene expression level of LPS-induced IL-1beta in NCM460 cells.** NCM460 cells were exposed to LPS with or without addition of live *B. longum* HK003 (BL HK003) and a commercial *B. longum* (DSM 16603, BL Commercial) for 24 hours. NCM460 without exposure to LPS or *B. longum* was used as blank control (Blank). Expression level of IL-1beta was determined by quantitative real-time PCR. The columns represent the average ratio of cytokines and actin from triplicate measurements. Results are expressed as mean $\pm$ SEM. *P<0.05; **P<0.01; ***p<0.001.

10 Blank – no treatment; LPS – LPS alone; BL HK003 – LPS with *B. longum* HK003; BL Commercial – LPS with *B. longum* DSM 16603.

[0046] **Figure 35 *B. longum* HK003 reduced gene expression level of LPS-induced IL-6 in NCM460 cells through its metabolites.** NCM460 cells were exposed to LPS with or without addition of live *B. longum* HK003 (BL HK003), heat-killed *B. longum* HK003 (Heat-kill BL HK003) and the conditioned medium of *B. longum* HK003 (BL HK003 Supernatant) for 24 hours. NCM460 without exposure to LPS, live or heat-killed *B. longum*, or its conditioned medium was used as blank control (Blank). Expression level of IL-6 was determined by quantitative real-time PCR. The columns represent the average ratio of cytokines and actin from triplicate measurements. Results are expressed as mean $\pm$ SEM.

20 *P<0.05; **P<0.01; ***p<0.001. Blank – no treatment; LPS – LPS alone; BL HK003 – LPS with live *B. longum* HK003; Heat-kill BL HK003 – LPS with heat-killed *B. longum* HK003; BL HK003 Supernatant – LPS with *B. longum* conditioned medium.

[0047] **Figure 36 *B. longum* HK003 reduced gene expression level of LPS-induced IL-1beta in NCM460 cells through its metabolites.** NCM460 cells were exposed to LPS with or without addition of live *B. longum* HK003 (BL HK003), heat-killed *B. longum* HK003 (Heat-kill BL HK003) and the conditioned medium of *B. longum* HK003 (BL HK003 Supernatant) for 24 hours. NCM460 without exposure to LPS, live or heat-killed *B. longum*, or its conditioned medium was used as blank control (Blank). Expression level of IL-1beta

was determined by quantitative real-time PCR. The columns represent the average ratio of cytokines and actin from triplicate measurements. Results are expressed as mean $\pm$ SEM.

*P<0.05; **P<0.01; ***p<0.001. Blank – no treatment; LPS – LPS alone; BL HK003 – LPS with live *B. longum* HK003; Heat-kill BL HK003 – LPS with heat-killed *B. longum* HK003;
 5 BL HK003 Supernatant – LPS with *B. longum* conditioned medium.

[0048] Figure 37 Antimicrobial effect of *B. longum* HK003 against ATCC pathogenic

bacteria. Bacterial spot 1: *B. adolescentis* (strain from internal collection); Bacterial spot 2: *B. bifidum* (strain from internal collection); Bacterial spot 3: *B. longum* HK003; Bacterial spot 4: *B. pseudocatemulatum* (strain from internal collection); Bacterial spot 5: *B.*

10 *adolescentis* BA02 (DSM 18351); Bacterial spot 6: *B. bifidum* BB01 (DSM 22892); Bacterial spot 7: *B. longum* (DSM 16603); Bacterial spot 8: *B. pseudocatemulatum* (DSM 20438).

DEFINITIONS

[0049] As used herein, the term “**SARS-CoV-2** or severe acute respiratory syndrome coronavirus 2,” refers to the virus that causes Coronavirus Disease 2019 (COVID-19). It is
 15 also referred to as “COVID-19 virus.”

[0050] The term “**post-acute COVID-19 syndrome (PACS)**” or “**long COVID**” is used to describe a medical condition in which a patient who has recovered from COVID, as indicated by a negative PCR report at least 2 weeks prior (*e.g.*, from at least 3 or 4 weeks earlier), yet continuously and stably exhibits one or more symptoms of the disease without any notable
 20 progression. The symptoms may include respiratory (cough, sputum, nasal congestion/ runny nose, shortness of breath), neuropsychiatric (headache, dizziness, loss of taste, loss of smell, anxiety, difficulty in concentration, difficulty in sleeping, sadness, poor memory, blurred vision), gastrointestinal (nausea, diarrhea, abdominal pain, epigastric pain), dermatological (hair loss), or musculoskeletal (joint pain, muscle pain) symptoms, as well as fatigue.

25 **[0051]** As used herein, the term “**hair loss**” describes a condition in which a patient’s hair follicles on his or her head suffer from diminished ability or have lost entirely their ability to regenerate hair or sustain hair growth, as evidenced by reduced number or strand of hair, reduced volume of hair, and/or reduced rate of hair growth or regrowth/replacement.

[0052] The term "**inhibiting**" or "**inhibition**," as used herein, refers to any detectable negative effect on a target biological process, such as RNA/protein expression of a target gene, the biological activity of a target protein, cellular signal transduction, cell proliferation, presence/level of an organism especially a micro-organism, any measurable biomarker, bio-parameter, or symptom in a subject, and the like. Typically, an inhibition is reflected in a decrease of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater in the target process (*e.g.*, a subject's bodyweight, or the blood glucose/ cholesterol level, or any measurable symptom or biomarker in a subject, such as an infection rate among subjects by a pathogenic infectious agent), or any one of the downstream parameters mentioned above, when compared to a control. "Inhibition" further includes a 100% reduction, *i.e.*, a complete elimination, prevention, or abolition of a target biological process or signal. The other relative terms such as "suppressing," "suppression," "reducing," and "reduction" are used in a similar fashion in this disclosure to refer to decreases to different levels (*e.g.*, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater decrease compared to a control level) up to complete elimination of a target biological process or signal. On the other hand, terms such as "activate," "activating," "activation," "increase," "increasing," "promote," "promoting," "enhance," "enhancing," or "enhancement" are used in this disclosure to encompass positive changes at different levels (*e.g.*, at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, or greater such as 3, 5, 8, 10, 20-fold increase compared to a control level in a target process, signal, or parameter).

[0053] As used herein, the term "**treatment**" or "**treating**" includes both therapeutic and preventative measures taken to address the presence of a disease or condition or the risk of developing such disease or condition at a later time. It encompasses therapeutic or preventive measures for alleviating ongoing symptoms, inhibiting or slowing disease progression, delaying of onset of symptoms, or eliminating or reducing side-effects caused by such disease or condition. A preventive measure in this context and its variations do not require 100% elimination of the occurrence of an event; rather, they refer to a suppression or reduction in the likelihood or severity of such occurrence or a delay in such occurrence.

[0054] The term “**severity**” of a disease refers to the level and extent to which a disease progresses to cause detrimental effects on the well-being and health of a patient suffering from the disease, such as short-term and long-term physical, mental, and psychological disability, up to and including death of the patient. Severity of a disease can be reflected in the nature and quantity of the necessary therapeutic and maintenance measures, the time duration required for patient recovery, the extent of possible recovery, the percentage of patient full recovery, the percentage of patients in need of long-term care, and mortality rate.

[0055] A “**patient**” or “**subject**” receiving the composition or treatment method of this invention is a human, including both adult and juvenile human, of any age, gender, and ethnic background, who may not have been diagnosed with any particular disease or disorder (*e.g.*, have not had a positive nucleic acid and/or antibody test result for COVID-19) but is in need of preventing or treating hair loss. Typically, the patient or subject receiving treatment according to the method of this invention to treat hair loss is not otherwise in need of treatment by the same therapeutic agents. For example, if a subject is receiving the composition according to the claimed method, the subject is not suffering from any disease that is known to be treated by the same therapeutic agents. Although a patient may be of any age, in some cases the patient is at least 40, 45, 50, 55, 60, 65, 70, 75, 80, or 85 years of age; in some cases, a patient may be between 40 and 45 years old, or between 50 and 65 years of age, or between 65 and 85 years of age. In contrast to an “adult,” who is at least 18 years of age and often older, a “child” subject is one under the age of 18 years, *e.g.*, about 5-17, 9 or 10-17, or 12-17 years old, including an “infant,” who is younger than about 12 months old, *e.g.*, younger than about 10, 8, 6, 4, or 2 months old.

[0056] The term “**effective amount**,” as used herein, refers to an amount that produces intended (*e.g.*, therapeutic or prophylactic) effects for which a substance is administered. The effects include the prevention, correction, or inhibition of progression of the symptoms of a particular disease/condition and related complications to any detectable extent, *e.g.*, incidence of disease, infection rate, one or more of the symptoms of a viral or bacterial infection and related disorder (*e.g.*, hair loss resulted from COVID-19). The exact amount will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known

techniques (see, e.g., Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); and Pickar, *Dosage Calculations* (1999)).

5 [0057] The term “**about**” when used in reference to a given value denotes a range encompassing $\pm 10\%$ of the value.

[0058] A “**pharmaceutically acceptable**” or “**pharmacologically acceptable**” excipient is a substance that is not biologically harmful or otherwise undesirable, *i.e.*, the excipient may be administered to an individual along with a bioactive agent without causing any undesirable biological effects. Neither would the excipient interact in a deleterious manner with any of
10 the components of the composition in which it is contained.

[0059] The term “**excipient**” refers to any essentially accessory substance that may be present in the finished dosage form of the composition of this invention. For example, the term “excipient” includes vehicles, binders, disintegrants, fillers (diluents), lubricants, glidants (flow enhancers), compression aids, colors, sweeteners, preservatives,
15 suspending/dispersing agents, film formers/coatings, flavors and printing inks.

[0060] “**Prebiotics**” are nutrients that can be degraded by microorganisms in a person’s gastrointestinal tract to generate short-chain fatty acids. Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and trans-galacto-oligosaccharides (TOS) are the most common prebiotics. Fermentation of prebiotics by gut microbiota produces short-chain fatty
20 acids (SCFAs), including lactic acid, butyric acid, and propionic acid.

[0061] The term “**consisting essentially of**,” when used in the context of describing a composition containing an active ingredient or multiple active ingredients, refers to the fact that the composition does not contain other ingredients possessing any similar or relevant biological activity of the active ingredient(s) or capable of enhancing or suppressing the
25 activity, whereas one or more inactive ingredients such as physiological or pharmaceutically acceptable excipients may be present in the composition. For example, a composition consisting essentially of active agents (for instance, *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a combination thereof) effective for treating or preventing

hair loss in a subject is a composition that does not contain any other agents that may have any detectable positive or negative effect on the same target process (*e.g.*, hair follicles' ability to sustain hair growth or regrowth) or that may increase or decrease to any measurable extent of the severity of the target condition among the receiving subjects.

5 DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0062] This invention describes the use of specific bacterial species (*B. longum* or *F. prausnitzii* or a combination thereof), optionally in further combination with one or more prebiotics, for improving or restoring hair growth in a subject, who may be an adult or a child, suffering from hair loss. The practical application of the invention includes development and manufacturing of commercial food products or health supplements containing the pertinent bacterial species, for example in the form of a powder, tablet, capsule, or liquid, which can be taken alone or added to food or beverages, especially in connection with other active agents or efforts promoting hair growth at or around the same time.

15 II. Pharmaceutical Compositions and Administration

[0063] The present invention provides pharmaceutical compositions comprising an effective amount of *B. longum* or *F. prausnitzii* or a combination thereof, optionally in further combination with one or more prebiotics for promoting or enhancing hair regrowth or restoration in a person to reduce hair loss, *e.g.*, as a result from a viral or bacterial infection such as COVID-19. Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* **249**: 1527-1533 (1990).

25 **[0064]** The pharmaceutical compositions of the present invention can be administered by various routes, *e.g.*, systemic administration via oral ingestion or local delivery using a rectal suppository. The preferred route of administering the pharmaceutical compositions is oral administration at daily doses of about 10^6 to about 10^{12} CFU for each of *B. longum* and *F.*

prausnitzii or a combination thereof at a weight ratio of about 0.1 to about 1. Optionally, one or more prebiotics may be further administered to the subject, either in one single composition or in multiple compositions. Additionally, the composition may be formulated in a daily dosage format comprising *B. longum* or *F. prausnitzii* or a combination thereof in the total amount of about 0.1 to about 20 grams, about 0.2 to about 15 grams, about 0.5 to about 12 grams, or about 1 to about 5-10 grams. The appropriate dose may be administered in a single daily dose or as divided doses presented at appropriate intervals, for example as two, three, four, or more subdoses per day. The duration of administration may range from about 2 weeks to about 12 months, *e.g.*, about 4 week to about 6 months, or about 8 weeks to about 12 weeks, or for any length of time in order to achieve adequate hair regrowth or restoration.

[0065] For preparing pharmaceutical compositions containing *B. longum* or *F. prausnitzii* or a combination thereof, optionally with a prebiotic compound, one or more inert and pharmaceutically acceptable carriers are used. The pharmaceutical carrier can be either solid or liquid. Solid form preparations include, for example, powders, tablets, dispersible granules, capsules, cachets, and suppositories. A solid carrier can be one or more substances that can also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, or tablet disintegrating agents; it can also be an encapsulating material.

[0066] In powders, the carrier is generally a finely divided solid that is in a mixture with the finely divided active component, *e.g.*, *B. longum* or *F. prausnitzii* or a combination thereof, optionally further in combination of one or more prebiotics. In tablets, the active ingredient is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired.

[0067] For preparing pharmaceutical compositions in the form of suppositories, a low-melting wax such as a mixture of fatty acid glycerides and cocoa butter is first melted and the active ingredient is dispersed therein by, for example, stirring. The molten homogeneous mixture is then poured into convenient-sized molds and allowed to cool and solidify.

[0068] Powders and tablets preferably contain between about 5% to about 100% by weight of the active ingredient(s) (namely *B. longum* or *F. prausnitzii* or a combination thereof, optionally further in combination with one or more prebiotics). Suitable carriers include, for example, magnesium carbonate, magnesium stearate, talc, lactose, sugar, pectin, dextrin, starch, tragacanth, methyl cellulose, sodium carboxymethyl cellulose, a low-melting wax, cocoa butter, and the like.

[0069] The pharmaceutical compositions can include the formulation of the active ingredient(s), *B. longum* or *F. prausnitzii* or a combination thereof, optionally further in combination with one or more prebiotics, with encapsulating material as a carrier providing a capsule in which the active ingredient(s) (with or without other carriers) is surrounded by the carrier, such that the carrier is thus in association with the active ingredient(s). In a similar manner, sachets can also be included. Tablets, powders, sachets, and capsules can be used as solid dosage forms suitable for oral administration.

[0070] Liquid pharmaceutical compositions include, for example, solutions suitable for oral administration or local delivery, suspensions, and emulsions suitable for oral administration. Sterile water solutions of the active component (*e.g.*, *B. longum* or *F. prausnitzii* or a combination thereof, optionally further in combination with one or more prebiotics) or sterile solutions of the active component in solvents comprising water, buffered water, saline, PBS, ethanol, or propylene glycol are examples of liquid or semi-liquid compositions suitable for oral administration or local delivery such as by rectal suppository. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents, and the like.

[0071] Sterile solutions can be prepared by dissolving the active component (*e.g.*, *B. longum* or *F. prausnitzii* or a combination thereof, optionally further in combination with one or more prebiotics) in the desired solvent system, and then passing the resulting solution through a membrane filter to sterilize it or, alternatively, by dissolving the sterile active component in a previously sterilized solvent under sterile conditions. The resulting aqueous

solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9, and most preferably from 7 to 8.

- 5 [0072] Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of an active agent sufficient to effectively promote or enhance hair growth in the recipient.

III. Additional Therapeutic Agents

- 10 [0073] Additional known therapeutic agent or agents may be used in combination with an active agent such as *B. longum* or *F. prausnitzii* or a combination thereof, optionally further in combination with one or more prebiotics such as FOS, GOS, or TOS, in the practice of the present invention for the purpose of promoting hair growth or restoration for the purpose of preventing or treating hair loss resulted from another medical condition such as an infectious
- 15 disease or associated disorder caused by a viral or bacterial infection, for example, COVID-19. In such applications, one or more of these previously known effective therapeutic agents for the underlying condition (e.g., COVID-19) can be administered to patients concurrently with an effective amount of the active agent(s) either together in a single composition or separately in two or more different compositions.
- 20 [0074] For example, drugs and supplements that are known to be effective for use to prevent or treat COVID-19 include ivermectin, vitamin C, vitamin D, melatonin, quercetin, Zinc, hydroxychloroquine, fluvoxamine/fluoxetine, proxalutamide, doxycycline, and azithromycin. Any one or more of these medications or supplements may be used in combination with the active agents (such as *B. longum* or *F. prausnitzii* or a combination
- 25 thereof) of the present invention to facilitate effective treatment of COVID-19, especially its potential long-term symptoms typically recognized as long-COVID symptoms and therefore enhance the hair restoration results in the patient as a part of full recovery from the disease. In particular, the combination of Zinc, hydroxychloroquine, and azithromycin and the

combination of ivermectin, fluvoxamine or fluoxetine, proxalutamide, doxycycline, vitamin C, vitamin D, melatonin, quercetin, and Zinc have demonstrated high efficacy in both COVID prophylaxis and therapy. Thus, these known medication/supplement or nutraceutical combinations can be used in the method of this invention along with the active components of *B. longum* or *F. prausnitzii* or a combination thereof, optionally further with one or more prebiotics such as fructooligosaccharides and the like.

IV. Kits

[0075] The invention also provides kits for preventing and treating hair loss in individuals, including those who may have suffered from an infectious disease such as COVID-19, according to the method disclosed herein. The kits typically include a plurality of containers, each containing a composition comprising one or more of *B. longum* or *F. prausnitzii* or a combination thereof. Optionally, additional container(s) may be included in the kit providing composition(s) comprising one or more of ingredients selected from one or more prebiotics such as FOS, GOS, and TOS. Further, additional agents or drugs that are known to be therapeutically effective for prevention and/or treatment of the underlying disease (*e.g.*, COVID-19), including for ameliorating the symptoms and reducing the severity of the disease, as well as for facilitating recovery from the disease (such as those described in the last section or otherwise known in the pertinent technical field) may be included in the kit. The plurality of containers of the kit each may contain a different active agent/drug or a distinct combination of two or more of the active agents or drugs. The kit may further include informational material providing instructions on how to dispense the pharmaceutical composition(s), including description of the type of patients who may be treated (*e.g.*, human patients who suffer from hair loss, for example, as a result of a previously diagnosed disease such as COVID-19), the dosage, frequency, and manner of administration, and the like.

25

EXAMPLES

[0076] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially the same or similar results.

Example I. Modulation of gut microbiota improves alopecia in mice**BACKGROUND**

[0077] Hair loss affects millions of people worldwide and can have devastating effects on an individual's psychoemotional well-being. There are many causes of hair loss including genetics, inflammation, psychological stress, or use of drugs such as chemotherapy. The research group led by the present inventors discovered that consumption of polysorbate-80 (P80, a commonly used dietary emulsifier) promotes hair loss in mice when coupled with soybean-deprived diet. Dietary emulsifier, even at a low concentration, has been shown to cause low-grade inflammation in the gut by impacting the gut microbiota¹. Chemotherapy is another known cause of hair loss. Chemotherapy-induced alopecia (CIA) is one of the most distressing treatment-related adverse effects. An estimated 65% of patients undergoing classic chemotherapy will experience hair loss². It has been shown that chemotherapy could adversely affect the gut microbiota, inducing acute dysbiosis³. It is thus hypothesized that restoration of the gut microbiome homeostasis could potentially treat or prevent hair loss or promote hair growth. Recently, fecal microbiota transplant (FMT), the delivery of complete microbiome communities to restore gut microbiome homeostasis, has been reported to have positive result in alopecia areata (AA)^{4, 5}, demonstrating the beneficial effect of gut modulation therapy to treating hair loss. Results of FMT are often dependent on the quality of donor stool, which consist of a mixture of undefined microbiota from different kingdoms, metabolites and digested materials from the donor⁶. The results are therefore variable and unpredictable. Selected and defined consortium of microbes might produce more controlled and predictable outcome^{6, 7}. The present invention addresses the need for a hair loss treatment that is based on a defined composition of bacteria.

METHODS**25 Mice**

[0078] All the animal handling procedures in this work were approved by Animal Experimentation Ethics Committee (AEEC) at the Chinese University of Hong Kong (Ref No. 18-154-MIS and 20-151-MIS). For P80 treatment, female C57BL/6J mice were weaned at 3

weeks old and housed under specific pathogen-free (SPF) conditions. For chemotherapy experiment, 8 weeks old female C57BL/6J mice were used instead.

P80-induced alopecia mouse model

[0079] Chronic P80 consumption coupled with soybean-deprived diet promotes alopecia in mice. The soya-deprived diet (Advanced protocol[®] Select Rodent 50 IF/6F Auto 5V0F, LabDiet, St. Louis, MO, USA) used managed formulation of nutrient contents, which contained no soybean meal and alfalfa. The phytochemicals that managed in this diet was targeted to be 50 ppm total. The crude fiber was 2-fold lower than the common laboratory rodent diet 5001^{1, 8, 9}. Emulsifying agent P80 was purchased from Sigma (Sigma, St. Louis, Missouri)¹. At week 0, mice were co-housed for 1 week (not shown in figure) before being assigned to different treatment groups with *ad libitum* access to food at week 1. All experimental groups of mice (Groups 2-4, **Figure 1A**) were given P80 (1%) in the drinking water with *ad libitum* access for a duration of 12 weeks, whereas the control group mice (Group 1, **Figure 1A**) were given *ad libitum* access to normal water.

Treatment of antibiotics and FMT in P80-administered mice

[0080] To explore the effect of gut microbiota in alopecia, mice in Groups 3 and 4 (**Figure 1A**) were treated with antibiotics and FMT respectively after 12 weeks of P80 administration. Group 3 mice, parallel to P80 administration, were additionally administered 3-day antibiotics treatment and left for 2 weeks of observation without any treatment (Group 3, **Figure 1A**). The antibiotics cocktail comprised ampicillin (0.5 mg/mL, Sigma), gentamicin (0.5 mg/mL, Sigma), metronidazole (0.5 mg/mL Sigma), neomycin (0.5 mg/mL, Sigma), vancomycin (0.25 mg/mL, MP Biomedicals) and sucralose (4 mg/mL, Sigma). Group 4 mice, parallel to P80 administration, were additionally administered 3-day antibiotics treatment followed by 1 infusion of FMT (Group 4, **Figure 1A**), and left for 2 weeks of observation without any treatment.

Faecal microbiota transplantation

[0081] On the day of performing FMT, all faecal samples from the water group (Group 1, **Figure 1A**) of the same time point (*i.e.*, week 13) were thawed, mixed and vortexed for 3 min

by sterile PBS¹⁰. The mixed fecal solution was settled by gravity for 2 min. The supernatant was collected, which was subsequently administered to mice by oral gavage at the dose of 100ul for each mouse.

DNA extraction in faecal samples

- 5 [0082] Approximately 100 mg stool sample was vortexed with 1 ml ddH₂O and then centrifuged at 13,000×g for 1 min. Supernatant was discarded and pellet remaining was resuspended in 800 µL TE buffer (pH 7.5) supplemented with 1.6 µl 2-mercaptoethanol and 500 U lyticase (Sigma), and incubated at 37 °C for 90 min. After incubation, the sample was centrifuged at 13,000×g for 2 min and supernatant was discarded. After this pre-processing,
- 10 purified DNA was extracted from the pellet using Maxwell® RSC PureFood GMO and Authentication Kit (Promega), following manufacturer's instructions. In brief, 1 ml of CTAB buffer was added to the pellet and vortexed for 30 s, then the sample was heated at 95°C for 5 min. After heating, the sample was vortexed with bead beading (Biospec, 0.5mm for bacteria and 0.1mm for fungi, 1:1) at maximum speed for 15 min. Following this, 40 µl proteinase K
- 15 and 20 µl RNase A were added and incubated at 70°C for 10 min. Supernatant was obtained by centrifuging at 13,000×g for 5 min and subsequently pipetted to the Maxwell® RSC cassette for DNA extraction.

Metagenomic sequencing

- [0083] Extracted DNA were prepared for library construction and shot-gun metagenomic
- 20 sequencing (150 bp paired-end) on the Illumina NovaSeq 6000 platform at Novogene, Beijing, China. An average of 59,700,498 (± 7,015,926) raw reads (6G data) per sample were obtained.

Quality control and decontamination

- [0084] Obtained raw reads were quality-filtered and decontaminated by KneadData
- 25 (v0.7.2). Java8 (v1.8.0_152-release), Bowtie2 (v2.3.4.3) and Trimmomatic (v0.39.1) were built as prerequisites to support KneadData running. Any leading or trailing N-bases and other bases that had Phred quality scores of 3 or below, cut each sequence read with a 4-base sliding window trimmer that required minimum average quality scores of 15 were trimmed,

and any sequence reads that had 50 bases or fewer were removed. Checking adapter sequences in paired-end reads for maximum mismatch count, simple and palindromic matches of 2, 10 and 30 bases, respectively, with a library of universal Illumina TruSeq3-PE-2.fa adapter sequences were cut. Surviving reads were then delivered to Bowtie2 for
5 decontaminating host DNA that were from the C57BL/6J mouse genome.

Taxonomic classification

[0085] Clean reads were classified using Kraken2 (v2.0.7-beta) against the archaea, bacteria, plasmid, viral, fungi and protozoa custom database from NCBI RefSeq. Clean reads were mapped to the lowest common ancestor of all reference genomes with exact k-mer
10 match. Abundance table at species level were imported into R (v3.5.0), analyzed and visualised with phyloseq package (v1.24.2). Principle coordinate analysis (PCoA) was performed using the Bray-Curtis Dissimilarity metric.

Chemotherapy-induced alopecia

[0086] At week 0, mice were co-housed for 1 week (not shown in figure) before being
15 assigned to different treatment groups with *ad libitum* access to food at week 1. Mice were intraperitoneally injected with 100 mg/kg cyclophosphamide (CYP) (Sigma, St. Louis, Missouri) at week 1, 5, 9, 13^{11, 12}. On the day of the fourth CYP injection, hairs on the mice dorsal skin were depilated to enter the anagen phases consistently. The CYP-treated mice were then subjected to PBS or BI treatment for 14 consecutive days following the fourth CYP
20 administration (Group 4 and 5, **Figure 14**). Mice fed with water during the whole experiment were regarded as the control group (Group 1, **Figure 14**). Skin conditions were monitored and body weights were measured daily.

Skin harvest and histology

[0087] For harvesting dorsal skin, mice were initially anaesthetized with 1% inhalatory
25 isoflurane. Skin tissues were surgically removed and fixed in 10% formalin solution for 24 h. Ethanol at 70% concentration was used to preserve skin tissues before embedding. Skin tissues were embedded in paraffin block and stored at 4°C before sectioning. The paraffin blocks were sectioned at 5 µm thickness and stained with haematoxylin and eosin (H&E)¹.

Histomorphometry was performed by Image J¹³. Counting of subcutaneous follicles, measurement of skin thickness and assessment of growth phases of each hair follicle were performed in (x4) magnification images.

Isolation, culture and oral gavage of Bifidobacterium longum

- 5 [0088] BI was isolated from a fresh fecal sample donated by a healthy individual at the Department of Microbiology, Prince of Wales Hospital, Hong Kong. Approximately 500 mg faecal sample was diluted in 15 ml sterile PBS and vortexed thoroughly. Approximately 100 ul of the vortexed solution was spread on M2GSC agar plates. Freshly grown colonies were picked by sterile needles and prepared for matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis. Ethanol/formic acid extraction procedure was carried out, followed by charging of MALDI target plate with the extract and overlaying with α -cyano-4 hydroxy-cinnamic acid matrix solution. BI was identified by matching the MALDI-TOF MS mass spectra to the reference of MALDI BioTyper 1.1, software (Bruker Daltonik GmbH, Bremen, Germany). For culture and oral gavage, single colony was picked and cultured aseptically in brain heart infusion (BHI) broth and spread on BHI agars in an anaerobic chamber (85% N₂, 10% H₂, and 5% CO₂). Before oral gavage, the liquid culture was prepared in the anaerobic chamber at 37°C to obtain a concentration at OD = 0.7¹⁴. Then, the culture media was centrifuged at 5000 g, 4 °C for 15 min and resuspended with fresh PBS. On the day of oral gavage, each mice was gavaged with 200 ul of the suspended liquid culture¹⁵.

Skin harvest and histology

- 25 [0089] For harvesting dorsal skin, mice were initially anaesthetized with 1% inhalatory isoflurane. Skin tissues were surgically removed and fixed in 10% formalin solution for 24 h. Ethanol at 70% concentration was used to preserve skin tissues before embedding. Skin tissues were embedded in paraffin block and stored at 4°C before sectioning. The paraffin blocks were sectioned at 5 μ m thickness and stained with haematoxylin and eosin (H&E)¹. Histomorphometry was performed by Image J¹³. Counting of subcutaneous follicles,

measurement of skin thickness and assessment of growth phases of each hair follicle were performed in (x4) magnification images.

Statistical Analysis

- [0090] The D'Agostino–Pearson omnibus test was used to verify normality of all data.
- 5 Significance was determined using t-tests, one-way ANOVA corrected for multiple comparisons followed by a Tukey test or Sidak test, two-way ANOVA corrected for multiple comparisons followed by a Tukey test or Sidak test (GraphPad Prism software, version 6.01). For data that are not verified by D'Agostino–Pearson omnibus test or in non-parametric distributions, Mann-Whitney test and Kruskal-Wallis test followed by the Dunnett post hoc
- 10 test were used for comparisons of two groups or multiple groups. For data in functional analysis, *Q-value* was determined by Kruskal-Wallis test followed by false discovery rate (FDR) correction.

RESULTS

Embodiment 1: Antibiotics exacerbated while FMT reversed P80-induced alopecia

- 15 [0091] After 12 weeks of P80 treatment, 40% of mice (4 out of 10) in each P80-administered group (Groups 2-4, **Figure 1A**) developed hair thinning and alopecia (**Figure 1B and Figure 10A**). In contrast, no sign of alopecia was observed in control mice receiving water instead of P80 (Group 1, **Figure 1A and 1B**). These data demonstrate that P80 could induce alopecia in mice.
- 20 [0092] At week 13, parallel to P80 administration, mice in Group 3 were additionally administered 3 days of antibiotic cocktail. Two weeks after antibiotics administration, alopecic mice increased from 40% to 70% (7 out of 10). Their alopecia score was also higher than that of mice that received P80 only without antibiotics treatment (Group 3 vs Group 2, mean $\pm$ s.e.m 2.7 ± 0.4726 vs 1.3 ± 0.3667 , $P < 0.05$) (**Figure 1C and E**). This indicates
- 25 antibiotics exacerbate P80-incuded alopecia.
- [0093] At week 13, mice in Group 4 received a single infusion of FMT using stool microbiome from Group 1 preceded by a 3-day antibiotics treatment (**Figure 1A**). Two weeks after FMT, all 4 alopecic mice demonstrated hair-regrowth (**shown in green arrows**,

Figure 1D). Compared to Group 1 (water), alopecia score of mice in Group 2 (P80, $P<0.05$) and Group 3 (P80+Abx, $P<0.0001$) but not Group 4 (P80+Abx+FMT) is significantly higher than that of Group 1 (water) (**Figure 1E**), indicating FMT reversed P80-induced alopecia.

[0094] To identify bacteria species associated with alopecia, stool samples at week 15 (post Abx or Abx+FMT treatment, **Figure 1A**) were collected from alopecic mice selected from Group 2 and 3 ($n=8$), and non-alopeic mice selected from Group 1 and 4 ($n=8$) for metagenomics sequencing. LEfSE (LDA score ≥ 2) analysis showed that *Bifidobacterium longum* (Bl) was significantly enriched in non-alopeic mice compared to alopecic mice (**Figure 2C**).

10 Embodiment 1 (Extended experiment): *Bifidobacterium longum* was engrafted after FMT

[0095] It is hypothesized that bacterial species that are engrafted after FMT may contribute to hair regrowth. To identify specific bacterial species, an additional FMT were performed to mice in Group 3 (**Extended Experiment, Figure 1A**) as an extended experiment. Mice showed hair regrowth and lowered alopecia score after FMT (**Figure 3A and 3B**). Stool samples were collected pre- and post-FMT for metagenomics sequencing. We found that bacterial species including Bl and *Faecalibacterium prausnitzii* (Fp) were engrafted after FMT (**Figure 3E and Figure 6**). These bacteria engrafted after FMT treatment were reported to have SCFA-producing properties.

20 Embodiment 2 (Part 1): Oral gavage of *Bifidobacterium longum* reversed P80-induced alopecia

[0096] It is inferred that Bl might play a role in reversing alopecia in P80-administered mice. To address this hypothesis, a proof-of-concept model was conducted by supplementing Bl for 2 weeks in P80-induced alopecic mice (Group 1-3, **Figure 14**). The Bl used was a novel strain, *Bifidobacterium longum*_HK003 that was isolated from human feces (Accession No. GDMCC 61784). In this experiment, mice were treated with water (Group 1) or P80 (Group 2 and 3) for 12 weeks, then further received water (Group 1), PBS (Group 2) or Bl (Group 3) for 2 weeks. At week 15, alopecia score was scored visually according to a 0–6 scoring system (**Figure 10D**), and skin tissues were collected for H&E analysis (**Figure 14**).

The emergence of atrophic follicles referring to the atrophic diseases in animals is described as “Skin, Follicle - Atrophy”¹⁶. This pathological condition is characterized by reduced sizes and numbers of pilosebaceous units in the dermis and occasionally accompanied by malformation of follicular units and chronic inflammation¹⁶. Other chemical agents, for instance chemotherapeutics were also reported to induce follicular dystrophy and miniaturization in mice, but no inflammation was involved¹¹.

[0097] At week 15, mice treated with P80+BI obtained a significantly lower alopecia score than mice treated with P80+PBS (Group 3 vs Group 2, mean $\pm$ s.e.m 0.5 ± 0.2236 vs 2 ± 0.4472 ; $P < 0.05$, **Figure 7C**), indicating that BI could rescue alopecia induced by P80.

Looking at skin histology, the inventors found that number of hair follicles (HF) in anagen (rapid growth) phase ($p < 0.01$, **Figure 7C**), size of HF ($p < 0.05$, **Figure 7C**) and skin thickness ($p < 0.05$, **Figure 7D**) were significantly reduced, while number of atrophic HF ($p < 0.001$, **Figure 7C**) were significantly increased in P80+PBS treated mice but not in P80+BI treated mice when compared to water treated mice. Treatment with BI significantly reduced number of atrophic HF (Group 3 vs Group 2, $p < 0.05$, **Figure 7C**) and increased skin thickness (Group 3 vs Group 2, $p < 0.01$, **Figure 7D**).

Embodiment 2 (Part 2): Oral gavage of *Bifidobacterium longum* reverses chemotherapy-induced hair depigmentation and alopecia

[0098] To explore whether the therapeutic effect of BI in hair loss could be expanded to other existing alopecia models, chemotherapy-induced alopecia (CIA) experiment was conducted in mice as a relevant biological model of drug induced alopecia^{12, 17} (Group 4 and 5, **Figure 14**). Following three separate intraperitoneal injections of cyclophosphamide (CYP), mice showed modest weight loss, fur depigmentation and alopecia before depilation at week 13 (**Figure 15A**). Before the fourth injection of CYP, all the mice were depilated to enter anagen phases consistently. The mice were then subjected to the last injection of CYP, followed by a 14-consecutive-day treatment intervention of either PBS (placebo) or BI through oral route respectively. After 14 days of interventions, mice that received PBS (control) appeared thin, weak and scarce of hair on the dorsal body, whereas mice that

- received BI treatment appeared relatively more energetic and displayed modest hair regrowth (**Figures 15B and 15C**). After treatments, mice that received BI had mildly reduced weight loss and obtained a lower alopecia score compared to mice receiving PBS only (mean $\pm$ s.e.m 0.5 ± 0.2236 vs 2.333 ± 0.3333 , $P < 0.01$) (**Figure 15D**). Histologically, HFs were lost or
- 5 miniaturized in the CYP + PBS group, along with reduced skin thickness (**Figures 15E and 15F**). In contrast, mice that received BI treatment showed an increase of elongated anagen follicles with growing hair shafts ($P < 0.0001$), sizes of HFs ($P < 0.05$), total number of HFs in subcutis ($P < 0.05$), skin thickness ($P < 0.05$) but reduced number of atrophic HFs ($P < 0.05$) (**Figures 15E and 15F**).
- 10 **[0099]** In summary, this study shows that BI promoted hair-regrowth in two alopecic models (P80- and CYP-induced alopecia). The therapeutic effect of BI is replicable in both models, as demonstrated by its hair re-growth effect in alleviating P80-induced hair loss, as well as its protecting effect in defending CYP-induced follicular damage in the skin.

Example II. Depletion of bacterial species in people with hair loss

15 **METHODS**

Study Cohort

- [0100]** Medical records and stool samples were collected from a total of 106 COVID-19 patients and followed until six months after discharge. Patients could be discharged if they fit either one of the below criteria: two clinical specimens of the same type (*i.e.*, respiratory or
- 20 stool) tested negative for nucleic acid of SARS-CoV-2 by RT-PCR taken at least 24 hours apart or tested positive for SARS-CoV-2 antibody. Serial stool samples were obtained from 68 of 106 patients who had 6 months follow-up after clearance of SARS-CoV-2. The presence of 30 most commonly reported post-COVID symptoms including hair loss^{18, 19} was assessed at 6 months after illness onset. Post-acute COVID-19 syndrome (PACS) was
- 25 defined as at least one persistent symptom which cannot be explained by alternative diagnosis four weeks after clearance of SARS-CoV-2. Gut microbiome compositions were characterized using shotgun metagenomics.

Stool DNA extraction and sequencing

[0101] Detailed methods are described in Zuo *et al*²⁰. Briefly, DNA was extracted from 0.1 g of homogenised faecal samples using the Maxwell RSC PureFood GMO and Authentication Kit and a Maxwell RSC Instrument nucleic acid extraction platform (Promega, Wisconsin, USA) according to manufacturer's instructions. Sequencing libraries were prepared from extracted DNA using the Nextera DNA Flex Library Prep Kit (Illumina, California, USA) and sequenced on an Illumina NovaSeq 6000 System (2×150 bp) at the Centre for Gut Microbiota Research, Chinese University of Hong Kong. Raw sequence data generated for this study are available in the Sequence Read Archive under BioProject accession: PRJNA714459.

Bioinformatics

[0102] Raw sequence data were quality filtered using Trimmomatic V.39 to remove adaptor and low-quality sequences and decontaminated against human genome (Reference: hg38) by Kneaddata (V.0.7.2, web site: bitbucket.org/biobakery/kneaddata/wiki/Home). Following this, microbiota composition profiles were inferred from quality-filtered forward reads using MetaPhlAn3 version 3.0.5. GNU parallel²¹ was used for parallel analysis jobs to accelerate data processing.

Statistical analysis and inferring gut microbiota composition

[0103] Associations of specific microbial species with patient parameters were identified using the linear discriminant analysis effect size (LEfSe) and the multivariate analysis by linear models (MaAsLin) statistical frameworks implemented in the Huttenhower Lab Galaxy instance (web site: huttenhower.sph.harvard.edu/galaxy/).

RESULTS

Faecalibacterium prausnitzii was associated with hair loss identified by linear model

[0104] From 1 February to 31 August 2020, a total of 106 recovered COVID-19 patients were recruited and followed up for six months from three regional hospitals in Hong Kong. Median age was 61 years (Interquartile range (IQR): 33 – 62 years) and most were females (n=56; 52.9%). Amongst the patients, type 2 diabetes mellitus (10.4%) was the most

common co-morbidity followed by hypertension (7.5%) (**Table 1**). Majority of cases had mild to moderate COVID-19 (81.9%) during hospitalisation and 25 patients (23.6%) received antibiotic during hospitalisation. Overall, post-acute COVID-19 syndrome (PACS) was reported in 81.1% (n=86) and 76.4%(n=81) of patients with COVID-19 at 3 months and 6 months, respectively. The most commonly reported symptoms at 6 months after illness onset were fatigue (31.3%), followed by poor memory (28.3%), hair loss (21.7%), anxiety (20.8%) and difficulty in sleeping (20.8%).

Table 1. Clinical characteristics of the 106 recovered COVID-19 patients

Male, n (%)	50 (47.1)
Age, years (IQR)	61 (33 – 62)
Non-smokers, n (%)	62 (75.6)
Presence of any co-morbidities, n (%)	45 (42.5)
Types of co-morbidities	
Diabetes Mellitus	16 (15.7)
Hypertension	18 (17.0)
Hyperlipidaemia	12 (11.3)

10

[0105] Associations of single bacteria taxa with different categories of symptoms were tested using multivariate association with linear model (MaAsLin). Different symptomatology was associated with different gut microbiome patterns. Butyrate-producing species *Faecalibacterium prausnitzii* (Fp) was significantly depleted in patients who had hair loss at 6 months.

15

Bacterial species associated with hair loss identified by LEfSE

[0106] To confirm these findings, another method was applied to identify bacterial species associated with hair loss in patients recovered from COVID-19. Using LEfSE analysis, the bacteria composition in patients recovered from COVID-19 with hair loss symptom (n=13) was compared against non-COVID-19 controls (n=24) at 6 months after illness onset. Eight species including Fp were depleted in recovered patients with hair loss.

20

Fp as beneficial bacteria for treating or preventing hair loss or promoting hair growth

[0107] In animal model [Embodiment 1 (Extended experiment)], it has been shown that Fp was engrafted after FMT in P80-treated alopecic mice, in which hair regrowth was observed. In line with this, it was discovered that Fp was depleted in patients recovered from COVID-19 with hair loss symptom. Therefore, Fp can be used as an active agent to treat or prevent hair loss or to promote hair growth.

Example III. Oral gavage of *Bifidobacterium longum*, *Faecalibacterium prausnitzii* and FMT promotes hair cycle progression in chemotherapy-induced alopecia

Animals

[0108] Healthy 6-week-old female C57BL/6 mice were obtained from the Laboratory animals services center, The Chinese University of Hong Kong. The mice were acclimatized for one week before starting the experiment. This study was approved by the Animal Experimentation Ethics Committee (AEEC) from The Chinese University of Hong Kong.

CYP-induced alopecia (CIA) mice model and tissue collection

[0109] Seven-week-old Female C57BL/6 mice were depilated by wax strips (Veet, USA) to induce anagen development of unparalleled homogeneity and synchrony over the entire depilated back of the mouse. The back skin of the telogen mice was pink and would be darkened with anagen initiation, as the melanogenic activity of hair follicles was closely related to the hair cycle. At day 9 after depilation, alopecia in this mouse model was induced by a single intraperitoneal injection of Cyclophosphamide (CYP, 130mg/kg body weight) (Sigma-Aldrich, C0768)²³. Before the injection of CYP, the mice were orally gavaged with PBS, *Bifidobacterium longum* HK003 (Accession No. GDMCC 61784), *Faecalibacterium prausnitzii* (ATCC, 27768) and stool samples from three health human donors (FMT group), respectively (**Figure 16**). For FMT group, to eradicate commensal bacteria, drinking water was supplemented with neomycin (0.5g/L, Sigma), metronidazole (0.5g/L, Sigma), ampicillin (0.5g/L, Sigma), and vancomycin (0.25g/L, Sigma) for 7 days prior to the stool gavage of the FMT group. Mice were observed daily for the presence of alopecia. Photographs were taken twice a week. Grayscale images were subjected to intensity analysis where the pigment

intensity of depilated area in each group was quantified using the ImageJ software and expressed as the fold change of the pigment intensity relative to that of the blank control group on day 23 post-depilation. A higher pigment intensity indicates an earlier transition to anagen phase and thus a faster progression of hair cycle. Skin sections were harvested at day 5 25 post-depilation and stools were collected twice a week.

Bacteria and FMT treatment

[0110] Both strains were cultured in Yeast Casitone Fatty Acids Broth, making up to 2×10^8 CFUs/ml and 2ml bacteria solution was centrifuged and resuspended in 100 μ l PBS, which was orally gavaged to mice daily. For FMT group, 1 g of the stool from health human donor 10 was suspended in 5 ml PBS and 100 μ l suspension was orally gavaged to mice every other day for 5 times.

Histology

[0111] The skin specimens were fixed in 4% Paraformaldehyde (PFA) solution for at least 24 hours at 4 C° before dehydration and paraffin embedding, then processed for routine 15 histology using Hematoxylin and Eosin staining. The skin samples were sectioned which sectioned at 5 μ m depth along the longitudinal and cross-sectional axes of the hair follicles. The number and percentage of telogen hair follicles in each skin layer were analyzed.

Stool specimen analysis

[0112] Fecal bacterial DNA was extracted by Maxwell® RSC PureFood GMO and 20 Authentication Kit (Promega) with modifications to increase the yield of DNA from the mice stool. The extracted fecal DNA was used for ultra-deep metagenomics sequencing via Illumina Novoseq 6000 (Novogen, Beijing, China). Profiling of the composition of bacterial communities was performed on metagenomic trimmed reads via MetaPhlAn3.

RESULTS

CYP-induced alopecia in C57BL/6 mice

[0113] The hair growth cycle consists of three phases: a resting telogen phase, where C57BL/6 skin is a pale pink color, an active hair growth anagen phase, where the skin

becomes dark gray or black, and finally, a catagen phase where hair growth stops, and the skin transitions back to the telogen phase, returning to a pale pink color. The dark pigmentation during the transition from telogen phase to anagen phase may result from the collection of melanin in the hair follicles, in preparation for new hair growth during the anagen phase.

[0114] In this experiment, wax strips were applied on the dorsal skin of 7 week old mice to remove the hair, of which all dorsal skin hair follicles were in telogen, as evidenced by the homogenous pink skin color at the depilated area. This immediately induced synchronous anagen development over the depilated back of the mice from all groups at day 0. The CYP-induced alopecia (CIA) model was established by a single intraperitoneal injection of CYP (130mg/kg body weight) at day 9 post-depilation. As demonstrated by the blank control group, hair was fully regrown by day 15 after depilation. In CIA model, no hair regrowth was observed until day 15 after depilation and the skin of depilated area appear gray (**Figure 17A**).

15 ***Supplementation of *B. longum*, *F. prausnitzii* and FMT promotes hair cycle progression as demonstrated by increased pigmentation in depilated area***

[0115] Depilated area of mice was observed and photographed for 2 weeks after CYP treatment. As illustrated in **Figure 17B**, on day 23 post-depilation, full hair growth maintained in the blank control group (without CYP), whereas only regrowth of a few abnormal gray hairs was observed in the CYP control mice without any other treatment. As shown in **Figure 17B**, mice treated with Bl, Fp and FMT prior to CYP showed darker skin color at the depilation area compared to that of the CYP control mice without any other treatment. This indicated that supplementation with Bl, Fp and FMT prior to CYP treatment accelerated transition to anagen phase of hair cycle.

25 [0116] This visual observation was verified by analyzing the pigment intensity of grayscale images of the depilated area using the ImageJ software (NIH, USA). The pigment intensity of the depilated area in each group was quantified and expressed as fold change using that of blank control mice as reference. At day 23 post-depilation, the blank control group (without

CYP) showed the highest intensity while the CYP control group showed the lowest. All 3 treatment groups (Bl, Fp and FMT) showed significantly higher intensity compared to CYP control ($p < 0.05$ respectively; Wilcoxon test; **Figure 17C**). Such increased intensity indicates enhanced hair growth as skin pigmentation in mice is coupled with the hair cycle progression.

5 Only during the growing phase do hair follicles actively generate pigment, causing the skin to appear black. These results indicate that Bl, Fp and FMT promote telogen-to-anagen phase conversion of hair follicles in the CIA model.

Supplementation of *B. longum*, *F. prausnitzii* and FMT promotes hair cycle progression as demonstrated by histology

10 **[0117]** It is known that around day 17 after depilation the follicles spontaneously start to undergo regression (catagen) to enter the resting phase (telogen) around day 20 after depilation²³. At day 23 post-depilation, we harvested the skin specimen from mice and analyzed the histology of hair follicles to quantify the percentage of hair follicles in telogen phase. As expected, almost all hair follicles reached telogen phase in blank control group

15 (without CYP treatment) at day 23 post-depilation. In contrast, a higher anagen to telogen hair follicle ratio was observed in CYP control, indicating the delay of the hair cycle progression in CYP treated mice (**Figure 18A**). Compared to CYP control group, a higher percentage of telogen hair follicles was observed in Bl, Fp and FMT treated group (**Figure 18A**). Moreover, the quantification results of HE staining from mice dorsal skin showed

20 significantly higher percentage of telogen hair follicles in Bl treated group than that of CYP control group (**Figure 18B**, $p < 0.05$, Wilcoxon test). This indicates Bl, Fp and FMT promotes hair growth in CIA mice model.

METHODS AND COMPOSITIONS FOR TREATING HAIR LOSS

[0118] In view of the above findings, a method and composition are provided for treating

25 or preventing hair loss or promoting hair growth, which relates to the use of a probiotic bacteria, *Bifidobacterium longum* (Bl) or *Faecalibacterium prausnitzii* (Fp) or their combination as the active agent(s). The probiotic bacteria, Bl, maybe commercially available Bl but is preferably a specific strain *Bifidobacterium longum* HK003 as deposited with the

Guangdong Microbial Culture Collection Center (GDMCC) under Accession No. GDMCC 61784. The composition may comprise an effective amount of B1 only, Fp only, combination of B1 and Fp, or with the addition of prebiotics that could boost the viability of B1 or Fp through the digestive tract. The probiotic bacteria B1 and/or Fp may be in the form of viable cells or non-viable cells such as killed cultures, mixtures of viable and non-viable cultures.

[0119] This method and composition may be used to enhance the hair growing effect of other agent. In this case, the method or composition may further includes administering to the subject one or more agents known to effectively induce hair growth.

Dosage

[0120] It is preferable that the strain or composition is administered at least one per week over a period of at least 2 weeks. The strain or composition can be administered several times or once daily. The composition generally comprise of the strain in an amount between about 1.67×10^7 - 1×10^{10} CFU for adults and about 8.5×10^6 - 5×10^9 CFU for children (Table 2). Dosage of about 1.67×10^7 CFU at least once daily is preferred for both adults and children.

Table 2 Suggested dosage of the strain or composition

Probiotic	Adult	Children
<i>Bifidobacterium longum</i>	$1.67 \times 10^7 \sim 1 \times 10^{10}$ CFU	$8.5 \times 10^6 \sim 5 \times 10^9$ CFU
<i>Faecalibacterium prausnitzii</i>	$3.5 \times 10^8 \sim 1 \times 10^{10}$ CFU	$1.75 \times 10^7 \sim 5 \times 10^9$ CFU

CFU: colony forming unit

Form of the Composition

[0121] The composition can be incorporated into a food composition, for instance by dry mixing the components of the synbiotic composition successively, together or as a premix, into a food composition, following regular processing techniques. For example, the food product can be powdered beverage, milk-based product, yoghurt, yoghurt melts, ice-cream, dry cereal mix and porridge.

Usage

[0122] The composition, and the resulting food composition comprising such composition, may be for use in the prevention and/or treatment of hair loss, including the promotion of the hair regrowth and the inhibition of the hair loss. For example, in a subject planning to receive chemotherapy, the composition can be administered at least 7 days before the initiation of chemotherapy and continue throughout the chemotherapy, to prevent or reduce hair loss caused by the chemotherapy. As another example, in a subject experiencing hair loss regardless of cause, the composition can be administered to induce hair regrowth. This subject may further receive a test to determine the level of BI or Fp in the gut prior to receiving the composition to guide the dosage, and to determine whether the subject is a suitable candidate to receive this composition for treating hair loss. If the level of BI is high, the subject may not be a suitable candidate for a BI based composition. If the level is of Fp is high, the subject may not be a suitable candidate for a Fp based composition.

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Example IV. New Strain of *Bifidobacterium longum*

[0123] This invention provides a newly isolated strain of *Bifidobacterium longum* and the method to culture this strain. This strain exhibited good acid tolerance, which is an important property of probiotic. This strain has shown health-promoting activities including the promotion of hair growth.

BACKGROUND

[0124] The beneficial effects of consuming microorganisms such as probiotics on human health has been well recognized. Probiotics is commonly defined as live microorganisms which, when administered in adequate amounts, confer a health benefit to the host ¹. Recent developments have provided growing evidence regarding the therapeutic potential of selected microorganisms to prevent or treat diseases, in addition to confer a general health benefit. The term “live biotherapeutic products” (LBP) has been used to describe these microorganisms with pharmaceutical expectations. *Bifidobacterium longum* has a long history of safe use in human food and confer various health benefit to the host. However, it should be recognized that each strain of the same species has unique and different properties and that their beneficial effects cannot be extrapolated to other strain ². Here, we isolated a new strain of *B. longum*, *B. longum* HK003, from a healthy individual and provided methods for culturing it. This strain has been deposited with the Guangdong Microbial Culture Collection Center (GDMCC) under the Accession Number GDMCC 61784.

15 Genetic Characteristics of *Bifidobacterium longum* HK003

[0125] The invention relates to a *Bifidobacterium longum* strain, *B. longum* HK003 (GDMCC 61784), isolated from a female healthy individual and is not previously described. It can be identified by genomic and phenotypic characteristics. This taxonomic unit is differentiated from other *B. longum* strains by possessing specific genetic features in the genome. These genes that are specific to *B. longum* HK003 are listed in **Table 3**. One way to genetically characterize *B. longum* HK003 is outlined in Experiment 1.

Table 3 Genes Specific to *B. longum* HK003

Gene	Annotation
araQ_8	L-arabinose transport system permease protein AraQ
aruH_2	Arginine--pyruvate transaminase AruH
cytR_2	HTH-type transcriptional repressor CytR
dasC_1	Diacetylchitobiose uptake system permease protein DasC
dnaA_2	Chromosomal replication initiator protein DnaA
dnaN_2	Beta sliding clamp
gdh_2	NADP-specific glutamate dehydrogenase

group_2057	IS256 family transposase ISBlo5
group_393	IS256 family transposase ISBlo8
group_7658	Iron-sulfur cluster carrier protein
gyrA_3	DNA gyrase subunit A
gyrB_3	DNA gyrase subunit B
hypBA1_1	Non-reducing end beta-L-arabinofuranosidase
ilvC_2	Ketol-acid reductoisomerase (NADP(+))
lacF_5	Lactose transport system permease protein LacF
lrpC_2	HTH-type transcriptional regulator LrpC
noc_2	Nucleoid occlusion protein
oatA_3	O-acetyltransferase OatA
recF_2	DNA replication and repair protein RecF
rnpA_2	Ribonuclease P protein component
rpmH_2	50S ribosomal protein L34
rsmG_2	Ribosomal RNA small subunit methyltransferase G
ugpA	sn-glycerol-3-phosphate transport system permease protein UgpA
yhjE	Inner membrane metabolite transport protein YhjE
yidC_2	Membrane protein insertase YidC

Phenotypic Characteristics, Safety Profile and Health Benefits of *B. longum* HK003

- [0126] Phenotypic characteristics of *B. longum* HK003 is outlined in Experiments 2-8. *B. longum* HK003 can be grown in both Reinforced Clostridial Medium (RCM) and De Man Rogosa Sharpe agar medium (MRS). It is generally considered that adequate amount of live bacteria should reach the human intestine for them to exert beneficial effects. Therefore, high viable counts and survival rates during stomach passage are necessary. *B. longum* HK003 is characterized by its good acid tolerance, with the ability to withstand acidic pH (pH=3) for up to 2 hours. The method of fermentation and enhancement of *B. longum* HK003 viability through freeze-drying and microencapsulation are illustrated in Example 9-11. We demonstrated that *B. longum* HK003 is safe for consumption, as outlined in Experiments 12-15. Antimicrobial resistance (AMR) gene analysis showed that *B. longum* HK003 only have AMR genes for 2 antibiotics, which are not clinically relevant antibiotics according to EFSA guidance³. Acute oral toxicity test showed that *B. longum* HK003 is non-toxic according to China National Food Safety Standard GB 15193.3-2014. *B. longum* HK003 exhibited

antibacterial effect against *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis*. *B. longum* HK003 also elicited anti-inflammatory effect through reduction of pro-inflammatory cytokines, IL-6 and IL-1beta, as illustrated in Experiments 16-17.

EXPERIMENTS

5 **Experiment 1 - Genomic analysis**

- [0127] The genomic DNA from isolated strain, *B. longum* HK003, was extracted using the QIAamp DNA Mini Kit according to the manufacturer's instructions, and subject to genome sequencing with the Illumina NextSeq 4000 platform (2x150 bp) at Beijing Genomics Institute (BGI, Shenzhen, China). The sequencing data were quality-checked and filtered.
- 10 The forward and reverse reads were then assembled de novo using SPAdes, which resulted in 45 contigs of 2,228,649 bp with a GC content of 59.99%. The quality of the genome assemblies and contamination score were assessed using CheckM. In order to compare the genomes between strain HK003 and other *B. longum* strains, the whole genome sequence of 49 publicly available *B. longum* strains were obtained from NCBI database (**Table 4**). The
- 15 genome sequence of *B. longum* HK003 and the other 49 strains were subjected to annotation using Prokka. Prokka is an annotation tool to achieve a rich and reliable annotation of genomic bacterial sequences. The Prokka annotation of the genome sequence for the HK003 strain contained 1824 coding sequences (CDS), 61 RNAs and 1 repeat. The genome size and number of CDSs in the HK003 strain was comparable to other *B. longum* strains in the NCBI
- 20 genome database. These studies support the identity of *B. longum* HK003 as a novel strain belonging to the *B. longum* species.

- [0128] The pan and unique genomes of strain HK003 and the other 49 strains were identified by Roary⁴. Whole genome sequencing of *B. longum* HK003 was compared to 49 publicly available *B. longum* strains in NCBI database. Maximum likelihood phylogenetic
- 25 tree based on the alignment of accessory genomes was generated using FastTree 2⁵. Pan-genome was constructed using Roary based on the core and accessory genes showing phylogenetic relatedness of the isolates by blue (present) and white (absent) fragments.

*indicates target genome. *B. longum* HK003 is most closely related to the strain *B. longum* subsp. *longum* strain BORI (Figure 19) 25 genes specific to HK003 was identified (Table 3).

Table 4 Publicly available *B. longum* strains download from NCBI

Strain name	GenBank
<i>Bifidobacterium longum</i> strain 51A	CP026999.1
<i>Bifidobacterium longum</i> strain 105-A	AP014658.1
<i>Bifidobacterium longum</i> strain 35624	CP013673.1
<i>Bifidobacterium longum</i> strain BAMA-B05	CP043002.1
<i>Bifidobacterium longum</i> strain BG7	CP010453.1
<i>Bifidobacterium longum</i> strain BIM B-813D	CP060493.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain BORI	CP031133.1
<i>Bifidobacterium longum</i> strain CACC 517	CP048001.1
<i>Bifidobacterium longum</i> DJO10A	CP000605.1
<i>Bifidobacterium longum</i> strain HN001	CP069278.1
<i>Bifidobacterium longum</i> strain I2-2-3	CP065397.1
<i>Bifidobacterium longum</i> strain JSRL02	CP046514.1
<i>Bifidobacterium longum</i> strain K2-21-4	CP065395.1
<i>Bifidobacterium longum</i> strain K5	CP072500.1
<i>Bifidobacterium longum</i> strain K15	CP072501.1
<i>Bifidobacterium longum</i> strain LC67	CP060588.1
<i>Bifidobacterium longum</i> strain LTBL16	CP034089.1
<i>Bifidobacterium longum</i> strain NBRC 114370	CP084012.1
<i>Bifidobacterium longum</i> NCC2705	AE014295.3
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> 157F	AP010890.1
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> CECT7210	LN824140.1
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> strain JCM 11347	CP062951.1
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> strain JCM 11660	CP062952.1
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> strain KCTC 5934	CP062937.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain AH1206	CP016019.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain B3	CP049770.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain B4	CP049769.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> BBMN68	CP002286.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain BCBL-583	CP083257.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> F8	FP929034.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> GT15	CP006741.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 7050	CP062949.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> JCM 7052	AP022379.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 7053	CP071683.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 7055	CP062964.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 11340	CP062945.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 11341	CP062950.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 11342	CP071684.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain JCM 11343	CP062940.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> KACC 91563	CP002794.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain KCTC 3420	CP062944.1

<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain KCTC 3421	CP071590.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain KCTC 5914	CP062947.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain NBRC 114494	CP084020.1
<i>Bifidobacterium longum</i> subsp. <i>longum</i> strain YS108R	CP029796.1
<i>Bifidobacterium longum</i> subsp. <i>suillum</i> strain JCM 19995	CP070996.1
<i>Bifidobacterium longum</i> subsp. <i>suillum</i> strain KCTC 15605	CP079202.1
<i>Bifidobacterium longum</i> strain VHProbi Y08	CP089302.1
<i>Bifidobacterium longum</i> strain W13	CP096771.1
<i>Bifidobacterium longum</i> strain ZJ1	CP040235.1

Experiment 2 - Growth of *B. longum* HK003

[0129] Reinforced Clostridial Medium (RCM) is a medium for the cultivation of anaerobes.

It can be used for the isolation and cultivation of anaerobic organisms such as *Bifidobacteria*.

5 deMan Rogosa Sharpe (MRS) medium is another medium commonly used for cultivation of *Bifidobacteria*⁶. Mupirocin and L-cysteine hydrochloride can be supplemented to MRS medium, which become inhibitory to a wide range of non-bifidobacteria such as *Bacilli*, *Lactococci*, and *Streptococci*. This selective culture media is suitable for enumeration of *bifidobacteria* in products or composition, especially when the *bifidobacteria* is not a
10 dominant component.

[0130] The original MRS medium (without mupirocin and L-cysteine hydrochloride) contain polysorbate-80 (p80), which has been shown to induce gut inflammation through perturbation of the gut microbiome⁷. We prepared a modified MRS medium by omitting p80 from the original MRS ingredients. The growth of *B. longum* HK003 in this MRS medium
15 without p80 at 37°C under anaerobic condition (Coy Anaerobic chamber, mix gas 90% N₂, 5% CO₂, 5% H₂) was evaluated. The growth curve of *B. longum* HK003 was determined by measuring the optical density (OD) of the *B. longum* HK003 culture every 6 hours for 48 hours with the length of wave $\lambda = 600$ nm. **Figure 20** shows that *B. longum* HK003 can be grown in MRS medium without p80, with the log phase at 6-24 hours, and reaching the
20 stationary phase at around 24 hours.

[0131] In addition, the original MRS medium also contain magnesium sulfate and manganese sulfate which are not suitable for human consumption⁸. We found that *B. longum*

HK003 can be grown in MRS medium when p80, magnesium sulfate and manganese sulfate are all eliminated (**Table 5**).

Table 5 Grown of *B. longum* HK003 (measured by OD 600nm) in different modified MRS medium

Medium	OD 600nm level of <i>B. longum</i> HK003			
	0 h	12 h	24 h	48 h
MRS medium without p80	0	0.43	1.19	1.38
MRS medium without p80, magnesium sulphate and manganese sulfate	0	0.40	1.10	1.32

5

[0132] In another experiment, the growth phase of *B. longum* HK003 that was never exposed to any freeze-drying was determined by measuring the optical density (OD) at the wavelength of 600nm. On a ratio of 1:9, the fresh culture was inoculated into RCM broth supplemented with galactooligosaccharide (GOS) purged with CO₂ in 250mL Hungate bottle under 37°C incubation shaker at 220 rpm for 48 hours. The OD reading was measured for every 6 hours. The growth curve was plotted and doubling time ($T_d = \ln(2)/B$) was calculated from the exponential equation $Y = Ae^{Bx}$. **Figure 21A** showed that *B. longum* HK003 grow well in RCM medium, with the log phase at 6 to 24 hours and the culture reaches the maximum growth OD 600nm level of 3.0. Exponential equation was determined to be $y = 0.2493e^{0.1003x}$, and the estimated doubling time, *i.e.*, the time per generation was estimated to be 414 min (6.9hr) (**Figure 21B**).

15

[0133] In the following experiments, these medium in the form of agar or broth were used.

20

25

- RCM: standard Reinforced Clostridial Medium
- MRS: standard deMan Rogosa Sharpe medium
- mMRS: MRS medium (MRS) modified by removing P80, magnesium sulfate and manganese sulfate
- mMRS-Cys: MRS medium modified by removing P80, magnesium sulfate and manganese sulfate, while adding 0.05% L-cysteine hydrochloride
- mMRS-MuCys: MRS medium modified by removing P80, magnesium sulfate and manganese sulfate, while adding 50mg/l mupirocin and 0.05% L-cysteine hydrochloride

Experiment 3 - Morphology of *B. longum* HK003

[0134] *B. longum* HK003 is anaerobic, Gram-positive, rod-shaped bacteria. Colonies on Reinforced Clostridial Media (RCM) agar plates supplemented with galactooligosaccharide (GOS) after 2-3 days anaerobic incubation (Coy Anaerobic chamber, mix gas 90% N₂, 5% CO₂, 5% H₂) exhibited the following characteristics: Size in diameter: 1.5-2.5 mm (medium); Color: white or light yellow; Opacity: opaque; Form: circular; Elevation: convex; Margin (edges): entire; Surface appearance: smooth and shiny (**Figure 22A and B**). Culture grown in liquid RCM medium after 2-3 days of anaerobic incubation demonstrated moderate to heavy turbidity with bacterial pellet formed at the bottom of tube (**Figure 22C**). Cell morphology of *B. longum* HK003 observed by scanning electron microscopy (SEM) is shown in **Figure 23**.

Experiment 4 - Acid tolerance test

[0135] To study acid tolerance, cultures of *B. longum* HK003 were grown in mMRS-MuCys broth at 37 °C for 24 hours. They were then sub-cultured into 10 ml of fresh mMRS-MuCys broth and incubated for another 24 hours. The cultures were centrifuged at 2000 g for 10 min at 4 °C, the pellets washed twice in sterile phosphate buffered saline (PBS, pH 7.2; Sigma) and resuspended in 1 ml of PBS. Subsequently, 0.1 ml of culture suspension was added separately into a series of tubes containing 2 ml of sterile PBS at pH 3. Hydrochloric acid (2 M) was used to adjust the pH of the PBS to pH 3. The tubes were incubated for 0, 0.5, 1, and 2 hours. After incubation, 0.1 ml from each tube was cultured on mMRS-MuCys agar plates and viable bacterial colonies were counted. All tests were done in duplicates (**Table 6**).

Table 6 Acid (pH=3) tolerance of *B. longum* HK003

Strain	0 h	0.5 h	1 h	2 h
<i>B. longum</i> HK003	2.0×10 ⁸ CFU/ml	2.3×10 ⁸ CFU/ml	2.5×10 ⁸ CFU/ml	1.8×10 ⁸ CFU/ml

Experiment 5 - Bile tolerance test

[0136] *B. longum* HK003 were grown for 24 hours in mMRS-MuCys broth, and 0.1 ml of the culture suspension was inoculated into tubes containing 10 ml of mMRS-MuCys broth and 0.3% chicken bile (Sigma). mMRS-MuCys broth without bile was used as controls. The

inoculated tubes were incubated at 37 °C for 0, 1, 2, 3, and 4 hours. After incubation, 0.1 ml from each tube was cultured on mMRS-MuCys agar plates and viable bacterial colonies were counted. All tests were done in duplicates (**Table 7**).

Table 7 Bile tolerance of *B. longum* HK003

Strain	0 h	1 h	2 h	3 h	4 h
<i>B. longum</i> HK003 (with bile)	2.0×10 ⁸ CFU/ml	1.5×10 ⁶ CFU/ml	3.0×10 ⁵ CFU/ml	2.5×10 ⁵ CFU/ml	2.0×10 ⁵ CFU/ml
<i>B. longum</i> HK003 (control)	2.0×10 ⁸ CFU/ml	2.2×10 ⁸ CFU/ml	2.4×10 ⁸ CFU/ml	2.4×10 ⁸ CFU/ml	2.5×10 ⁸ CFU/ml

5

Experiment 6 - Oxygen tolerance for *B. longum* HK003

[0137] The Relative Bacterial Growth Ratio (RBGR) was performed to investigate the effects of oxidative stress for *B. longum* HK003 and other isolated *Bifidobacterium* strains. On a 1:9 ratio, the fresh culture was inoculated aseptically to two separate 250mL Hungate
 10 bottles containing RCM broth supplemented with galactooligosaccharide (GOS) for aerobic and anaerobic growth. For aerobic growth, the bottle was plugged with sterile cotton wool whereas for anaerobic growth, the medium was deoxygenated, and the bottle sealed with butyl rubber. Inoculated bottles were incubated on a shaker at 220 rpm at 37°C for 48 hours. The OD 600nm reading was measured every 6 hours (**Figure 24**). The RBGR of the culture
 15 was determined by dividing the absorbency of aerobic growth by the absorbency of the anaerobic growth. *B. longum* HK003 grew poorly under aerobic condition with RBGR values closer to 0 (**Table 8**), highlighting the extreme sensitivity of *Bifidobacterium* to oxygen.

Table 8 The Relative Bacterial Growth Ratio (RBGR) of *B. longum* HK003

Organism type and strain	Growth (OD 600nm) 37°C, 24hr, 200rpm		RBGR
	Aerobically shaken	Anaerobically shaken	
<i>B. longum</i> HK003	0.57	2.94	0.19

20 RBGR values form a scale ranging from ∞ with obligate aerobes to 0 with obligate anaerobes

Experiment 7 - Enzymatic activity of *B. longum* HK003

[0138] Enzymatic reaction of *B. longum* HK003 was determined by RapID ANA II system containing different substrates included in the 10 wells of the test panel. Some of these wells are bifunction, totally providing 18 tests. The substrates included are 1) urea, 2) p-nitrophenyl- β ,D-disaccharide, 3) p-nitrophenyl- α ,L-arabinoside, 4) o-nitrophenyl- β ,D-galactoside, 5) p-nitrophenyl- α ,D-glucoside, 6) p-nitrophenyl- β ,D-glucoside, 7) p-nitrophenyl- α ,D-galactoside, 8) p-nitrophenyl- α ,L-fucoside, 9) p-nitrophenyl-N-acetyl- β ,D-glucosaminide, 10) p-nitrophenylphosphate, 11) leucyl-glycine- β -naphthylamide, 12) glycine- β -naphthylamide, 13) proline- β -naphthylamide, 14) phenylalanine- β -naphthylamide, 15) arginine- β -naphthylamide, 16) serine- β -naphthylamide, 17) pyrrolidonyl- β -naphthylamide, and 18) tryptophane. The test procedure and reactions interpretation are as described in the manufacturer's test protocol and interpretation guide for anaerobic cultures. Results are shown in **Table 9**.

Table 9 Enzyme activity of *B. longum* HK003

No	Test	Reactive ingredient	Enzymatic activity of <i>B. longum</i> HK003
1	URE	Urea	-
2	BLTS	p-Nitrophenyl- β ,D-disaccharide	-
3	α ARA	p-Nitrophenyl- α ,L-arabinoside	+
4	ONPG	σ -Nitrophenyl- β ,D-galactoside	+
5	α GLU	p-Nitrophenyl- α ,D-glucoside	+
6	β GLU	p-Nitrophenyl- β ,D-glucoside	-
7	α GAL	p-Nitrophenyl- α ,D-galactoside	+
8	α FUC	p-Nitrophenyl- α ,L-fucoside	-
9	NAG	p-Nitrophenyl-n-acetyl- β ,D glucosaminide	-
10	PO4	p-Nitrophenylphosphate	-
11	LGY	Leucyl-glycine- β -naphthylamide	-
12	GLY	Glycine- β -naphthylamide	-
13	PRO	Proline- β -naphthylamide	+
14	PAL	Phenylalanine- β -naphthylamide	+
15	ARG	Arginine- β -naphthylamide	+
16	SER	Serine- β -naphthylamide	+
17	PYR	Pyrrolidonyl- β -naphthylamide	-
18	IND	Tryptophane	-

15 "+" represents enzymatic activity being detected; "-" represents enzymatic activity not detected

Experiment 8 - Fermentation of carbohydrates by *B. longum* HK003

- [0139] The carbohydrate fermentation pattern of *B. longum* HK003 was determined. *B. longum* HK003 was grown for 24 hours in mMRS. 0.1 ml of the culture suspension was inoculated into tubes containing 10 ml of mMRS broth, in which the ingredient glucose was being replaced by 2.0 g/L of different carbohydrates. Cultures were allowed to grow under anaerobic condition incubation (Coy Anaerobic chamber, mix gas 90% N₂, 5% CO₂, 5% H₂) at 37°C. The growth curve was determined by measuring the optical density (OD) of these *B. longum* HK003 culture every 6 hours for 48 hours with the length of wave $\lambda = 600$ nm. It was shown that *B. longum* HK003 can grow in medium using xylan, arabinoxylan, β -glucan, agarose, carrageenan and arabinogalactan as sole carbon source. Growth curves *B. longum* HK003 in these media are shown in **Figure 25**. In contrast, *B. longum* HK003 cannot be grown when cellulose, lignin and galacturonic acid is the sole carbon source.

Experiment 9 - Scale-up and optimization of *B. longum* HK003 fermentation

Seed stock

- [0140] Seed stock (consisting of a single pure *B. longum* HK003 strain) in glycerol tube or powdered seed stock were inoculated into liquid tubes, and incubated at 37 °C for 24 hours under strict anaerobic condition (Coy Anaerobic chamber, mix gas 90% N₂, 5% CO₂, 5% H₂). Modified MRS medium supplemented with L-cysteine hydrochloride (mMRS-Cys) in the form of broth was used.

5L tank fermentation

- [0141] The liquid tube containing the seed culture was inoculated into a triangular flask of 250 ml medium and incubated for 12-18 hours at 37 °C under strict anaerobic condition. Prior to inoculation to 5L fermentation tank containing 2500ml medium, the pH of medium was adjusted to 6.8 and allowed to stabilize at 37°C for 12-18 h. After that, 120 ml of seed culture from the triangular flask was inoculated to the 5L fermentation tank to obtain a 4.5% (V/V) inoculum and cultured for 12-18 hours. Under these conditions, a final concentration of 6.25×10^9 CFU/ml was achieved. The resulting culture was then transferred to the 15L fermentation tank.

15L tank fermentation

[0142] Prior to inoculation, the pH of medium in the 15L fermentation tank was adjusted to 6.9. The resulting culture from 5L fermentation tank was inoculated to 15L tank to obtain a 4-5% (V/V) inoculum. The inoculum was fermented under constant temperature and anaerobic condition. During fermentation, alkali (sterile NaOH solution) was added when the pH dropped to 6.0 to avoid further drop in pH. To control the fermentation process, samples were taken every 1-2 hours to determine OD, and were centrifuge to determine total sugar and total nitrogen in the supernatant. At 6 hours after inoculation, the number of viable bacteria in the fermentation broth was measured by standard plate count. At 12 hours after inoculation, the fermentation broth was centrifuged at 4 °C to obtain the end product of bacteria pellet.

Optimization of fermentation medium

[0143] To enhance the yield of *B. longum* HK003, various 1) saccharides as additional carbon source and 2) peptone as additional nitrogen source) were added to the base medium (mMRS-Cys) to investigate their effect in boosting the growth of *B. longum* HK003. All the tests were done in 15 L fermentation tank and viable bacteria count was measured by standard plate count as described above. In the base medium, glucose was the sole carbon source, while gelatin peptone, beef and yeast extract were the nitrogen source.

1) Additional carbon source

[0144] 2.5g/L of saccharides including Lactose (LAC), raffinose (RAF), stachyose (STA), galactooligosaccharide (GOS), fructooligosaccharide (FOS), isomaltooligosaccharide (IMO), xylooligosaccharide (XOS), were added to the base medium (mMRS-Cys), which contain 10g/L glucose as sole carbon source. We found that addition of LAC, GOS and FOS to mMRS-Cys could promote the growth of *B. longum* HK003 (**Table 10**).

Table 10 The viable concentration of *B. Longum* HK003 in fermentation medium supplemented with various saccharides

Carbon source	Viable concentration (CFU/ml)
Glucose (original 10g/L)	5.0×10^9
Lactose (2.5 g/L) + Glucose (original 10 g/L)	9.0×10^9
Raffinose (2.5 g/L) + Glucose (original 10 g/L)	5.5×10^9
Stachyose(2.5 g/L) + Glucose (original 10 g/L)	4.2×10^9
Galactooligosaccharide (2.5 g/L) + Glucose (original 10 g/L)	1.3×10^{10}
Fructooligosaccharide (2.5 g/L) + Glucose (original 10 g/L)	1.0×10^{10}
Isomaltooligosaccharide (2.5 g/L) + Glucose (original 10 g/L)	5.8×10^9
Xylooligosaccharide (2.5 g/L) + Glucose (original 10 g/L)	6.1×10^9

2) Additional nitrogen source

- 5 [0145] 5g/L of peptone including beef peptone, soybean peptone, and tryptone were added to the base medium which contain 10g/L of gelatin peptone as one of the nitrogen sources. We found that addition of beef peptone and tryptone could promote the growth of *B. longum* HK003 (Table 11).

10 **Table 11 The viable concentration of *B. Longum* HK003 in fermentation medium supplemented with various peptone**

Nitrogen source	Viable concentration (CFU/ml)
Gelatin peptone (original 10 g/L)	6.0×10^9
Beef peptone (5 g/L) + Gelatin peptone (original 10 g/L)	1.1×10^{10}
Soybean peptone (5 g/L) + Gelatin peptone (original 10 g/L)	6.9×10^9
Tryptone (5 g/L) + Gelatin peptone (original 10 g/L)	9.5×10^9

Experiment 10 - Optimal lyoprotectants for lyophilization of *B. longum* HK003

- [0146] Three different formulas of lyoprotectants were tested and compared (Table 12). In all experiments, lyoprotectant in the form of powder was added to bacteria suspension in 1:2 ratio (W/V). Bacterial cells were centrifuged (4,000 g, 10 min) and pellets were resuspended in 1/10 of the supernatant to obtain a 10 times concentrated solution. One milliliter of the solution was poured into plates and frozen at -80°C (at a rate of $-2^\circ\text{C}/\text{min}$) before being freeze-dried for 36-48 hours (Labconco, USA). Freeze-dried powder of *B. longum* HK003 using formula 3 as lyoprotectant is shown in Figure 26. After freeze-drying, viable bacteria

were measured in triplicate by standard plate count. The percentage of viable bacteria was 55-60%, 30-35% and 15-20% for formula 3, 2, and 1 respectively.

Table 12 Percentage of viable *B. longum* HK003 after freeze-drying in different formulas of lyoprotectants

Lyoprotectant	Composition	Percentage of viable <i>B. longum</i> HK003
Formula 1	1.5% (m/v) gelatin, 15% (m/v) skimmed milk powder, 20% (m/v) sucrose	15-20%
Formula 2	1% (m/v) gelatin, 10% (m/v) skimmed milk powder, 15% (m/v) trehalose, and 5% (m/v) sucrose	30-35%
Formula 3	1.5% (m/v) gelatin, 15% (m/v) skimmed milk powder, 15% (m/v) trehalose, and 5% (m/v) sucrose	55-60%

5 **Experiment 11 – Microencapsulation of *B. longum* HK003**

[0147] Two materials for microencapsulation were evaluated -

Material 1: 100 g whey protein isolate (WPI) and 50 g gum arabic (GA)

Material 2: 50 g soybean protein isolate (SPI) and 10 g konjac gum (KGM)

[0148] For each material tested, the said amount was added into a 1000 ml beaker,

10 followed by 1000 ml distilled water. The mixture was stirred at 1000 r /min for 4 hours to obtain the WPI-GA or SPI-KGM solution. In 480 ml of WPI-GA or SPI-KGM solution, 20 ml of bacterial suspension (12 log CFU / ml) was added, followed by 25 g of trehalose and 25 g of skimmed milk powder which act as cryoprotectants. The mixture was stirred evenly to obtain a homogenous solution. This embedded microcapsule solution was pre-frozen in a refrigerator at - 80 °C for 12 hours, which was then lyophilized in a freeze-dryer to obtain the microencapsulated powder of *B. longum* HK003 (**Figures 27 and 28**).

[0149] Bile resistance of the microencapsulated *B. longum* HK003 powder were evaluated as described in Experiment 5. We found that microencapsulation using material 2 but not material 1 protected *B. longum* HK003 against bile. Viability loss was only 1-1.5 log when Material 2 was used (**Figure 29**), while the viability loss was 3-4 log when Material 1 was used, or when no microencapsulation was applied (refer to Experiment 5, **Table 5**).

Experiment 12 - Antimicrobial resistance analysis

Antimicrobial resistance genes

[0150] Raw sequence reads acquired by whole genome sequencing were filtered and quality trimmed using Trimmomatic (v0.36). Short reads were assembled to contigs using
5 MEGAHIT (v1.2.9). The assembled contigs were screened for antimicrobial resistance genes (ARG) against CARD databases (v1.05) using ABRicate (v1.0.0) with default parameters.

[0151] Results from the evaluation indicate that out of 1600 antimicrobial resistance genes screened, only 2 putative genes *ileS* and *EF_Tu* related to resistance of mupirocin and elfamycin respectively were found (**Table 13**).

10 **Table 13 Antimicrobial resistance genes of *B. longum* HK003**

Gene_name	Resistance	START	END	COVERAGE	%COVERAGE	%IDENTITY
<i>ileS</i>	Mupirocin	60763	64061	23-3324/3324	99.04	82.83
<i>EF_Tu</i>	Elfamycin	971624	972821	2-1193/1194	98.66	75

[0152] Bifidobacteria are intrinsically resistant to mupirocin, an antibiotic that is being used in selective media for this genus. In one study, it has been shown that all 40 bifidobacterial strains tested exhibit a phenotype of generally high resistance to mupirocin⁹.
15 Homologs of the *ileS* genes were observed in many *B. longum* strains. All were dismissed as safety concerns. For elfamycin, the percentage identify of *EF_Tu* gene present in *B. longum* HK003 to the reference antibiotic resistance gene is only 75%. The risk of *B. longum* HK003 carrying transmissible antibiotic genes that actually confer antibiotic resistance against elfamycin is low. In addition, analysis of the genetic context by comparing the genome of *B.*
20 *longum* HK003 with that of other *B. longum* species from the NCBI database (**Table 2**) indicated that regions flanking the *ileS* and *EF_Tu* genes are highly conserved. Therefore, a potential transfer of these gene is considered to be unlikely. These findings indicated that *B. longum* HK003 does not present concerns for antibiotic resistance.

Antimicrobial susceptibility testing determined by disc diffusion method

25 [0153] Antibiotic susceptibility of *B. longum* HK003 was determined by the disc diffusion method. *B. longum* HK003 and other *Bifidobacterium* strains were suspended in Mueller

Hinton broth (Sensititre) with turbidity adjusted to 0.5 McFarland standard. The bacteria suspensions were streaked onto the 4mm thick RCM agar plates supplemented with galactooligosaccharide (GOS). Commercial discs (Oxoid) containing antibiotics agent relevant for the treatment of anaerobic bacteria was selected based on The European

5 Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendation. These include penicillin G (1 IU), piperacillin-tazobactam (30-6 µg), meropenem (10 µg), vancomycin (5 µg), clindamycin (2 µg), metronidazole (5 µg). Mupirocin (200 µg) was included as antimicrobial resistance gene for mupirocin was found in *B. longum* HK003. These antibiotics discs with a diameter of 6 mm were placed on the inoculated RCM agar

10 plates. The plates were then incubated anaerobically at 37°C for 24 hours. Antibiotic susceptibility was evaluated based on the diameter (in millimeters) of the growth inhibition zone around the disc (**Figure 30, Figure 31**), and classified as resistant (R), moderately susceptible (MS) or susceptible (S) according to the interpretative standard described by Charteris et al.¹⁰ (**Table 12**). It should be noted that there is no established EUCAST/

15 Clinical and Laboratory Standards Institute (CLSI) breakpoints for *Bifidobacterium* at present.

[0154] As with other bifidobacterial strains, *B. longum* HK003 was not resistant to penicillin G, piperacillin-tazobactam, vancomycin, but resistant to metronidazole according to the interpretive standard of Charteris *et al.*¹⁰. Interpretative charts were not available for meropenem and mupirocin, thus only the zone sizes were reported in **Table 14**. As there was

20 growth around the antibiotic disc of mupirocin, we regard *B. longum* HK003 as resistant to mupirocin. As for meropenem, since no growth of *B. longum* HK003 was observed around the disc, we regard *B. longum* HK003 as not resistant to meropenem. Overall, the antibiotic susceptibility profile of *B. longum* HK003 is comparable to other *Bifidobacterium* strains tested.

25 **Table 14 Zone of inhibition (mm) of *B. longum* HK003 and probiotics/type strains**

Strains	Zone of inhibition in mm (degree of susceptibility)						
	P1	TZP36	MEM10	VA5	DA2	MTZ5	MUP200
Isolated strain							
<i>B. longum</i> HK003	30 (S)	41 (S)	41*	26 (S)	44 (S)	9 (R)	6*

Probiotic/type strain							
<i>B. adolescentis</i> DSM18351	27 (MS)	45 (S)	31*	24 (S)	39 (S)	6 (R)	6*
<i>B. bifidum</i> DSM22892	23 (MS)	29 (S)	28*	21 (S)	39 (S)	6 (R)	6*
<i>B. longum</i> DSM16603	28 (S)	40 (S)	43*	25 (S)	41 (S)	6 (R)	32
<i>B. pseudocatenulatum</i> DSM20438	29 (S)	38 (S)	40*	24 (S)	35 (S)	6 (R)	6*

*Interpretative chart not available

P1 - Penicillin; TZP36 - Piperacillin-tazobactam; MEM10 - Meropenem; VA5 - Vancomycin; DA2 - Clindamycin; MTZ5 - Metronidazole; MUP200 - Mupirocin

5 Degree of susceptibility expressed as R (resistant), MS (moderately susceptible), or S (susceptible) ¹⁰

Minimum inhibitory concentration (MIC) of antibiotics

[0155] Minimal inhibitory concentration of *B. longum* HK003 was determined using Sensititre anaerobe plates. *B. longum* HK003 was susceptible to ampicillin, chloramphenicol, clindamycin, tetracycline, erythromycin, and vancomycin as evaluated based on the EFSA cut-off value (Table 15). The antibiotic susceptibilities of BL HK003 are overall similar to patterns of other bifidobacterial species.

Table 15 Minimum inhibitory values for *B. longum* HK003

Antibiotic		MIC in µg/mL	ESFA cut off values in µg/mL
AMP	Ampicillin	≤0.5	2
CHL	Chloramphenicol	≤2	4
CLI	Clindamycin	≤0.25	1
TET	Tetracycline	2	8
ERY	Erythromycin	≤ 1	1
VAN	Vancomycin	≤2	2
MERO	Meropenem	≤ 0.5	n.r.
MRD	Metronidazole	16	n.r.
MUPIRO	Mupirocin	>8	n.r.
MUP256	Mupirocin 256ug/mL	256	n.r.
AMOX	Amoxicillin	0.5	n.r.
AUG	Amoxicillin/Clavulanic Acid	≤0.5	n.r.
A/S	Ampicillin/Sulbactam	≤0.5	n.r.

TANS	Cefotetan	16	n.r.
FOX	Cefoxitin	8	n.r.
IMI	Imipenem	≤0.06	n.r.
MEZ	Mezlocillin	≤4	n.r.
MXF	Moxifloxacin	1	n.r.
PEN	Penicillin	0.5	n.r.
PIP	Piperacillin	≤4	n.r.
P/T	Piperacillin/Tazobactam	1	n.r.
RIF	Rifampin	>1	n.r.

* EFSA cut-off values for *Bifidobacterium* as listed in ‘Guidance on microorganisms used as feed additives or as production organisms’ (EFSA Journal 2018, 16(3):5206); n.r. = not required.

Experiment 13 - Absence of Virulence Gene

- 5 [0156] Whole genome sequence (WGS) of *B. longum* HK003 was analyzed for virulence genes. Whole genome sequence (WGS) of *B. longum* HK003 was analyzed for virulence genes. Raw sequence reads acquired by whole genome sequencing were filtered and quality trimmed using Trimmomatic (v0.36). Short reads were assembled to contigs using MEGAHIT (v1.2.9). The assembled contigs were screened for Virulence Gene against
- 10 VFDB 2022 (website: mgc.ac.cn/VFs/) using ABRicate (v1.0.0) with default parameters.
- [0157] In addition, raw sequencing reads of *B. longum* HK003 was also analyzed using VirulenceFinder,¹¹⁻¹³ which is a component of the publicly available web-based tool for whole-genome sequencing (WGS) analysis hosted by the Center for Genomic Epidemiology (CGE) (website: genomicepidemiology.org). The database detects homologous sequences
- 15 for the virulence genes related to *Escherichia coli*, *Enterococcus*, *Listeria*, and *Staphylococcus aureus* in WGS data. All 4 databases of *E. coli*, *Enterococcus*, *Listeria* and *S. aureus* was searched using the threshold for %ID and minimum length of 90% and 60% respectively. No virulence gene was found.

Experiment 14 – Absence of Plasmid

- 20 [0158] Presence of plasmid is associated with increased risk of horizontal transfer of virulence or AMR genes. Raw sequencing reads of *B. longum* HK003 was analyzed by

PlasmidFinder 2.1, a web-based tools for in silico detection of known plasmid^{13,14}. No plasmid was found by searching the “Gram Positive” database with the threshold for minimum identity and coverage set as 95% and 60% respectively.

[0159] To confirm the absence of plasmid in *B. longum* HK003, *B. longum* HK003 culture was subjected to plasmid DNA extraction using the typical alkaline lysis method with solutions 1, 2, 3¹⁵. TOP10 competent *E. coli* transformed with control plasmid was used as positive control. Briefly, 5mL of *B. longum* HK003 culture was centrifuged, and the cells were resuspended in 200 μ L of solution 1. The cell suspension was added with 10 μ L lysozyme (200 mg/mL) and incubated at 37 °C for up to 1 hour. Then, 200 μ L of solution 2 was added and the mixture was incubated on ice for 5 min. Next, 200 μ L of solution 3 was added, and the mixture was incubated on ice for 10 min. The cell debris was removed by centrifugation and the clarified cell lysate was recovered and extracted with 400 μ L of phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase was transferred to a fresh tube and plasmid DNA was precipitated using 1.2 volumes of isopropanol, washed with 100 μ L of 70% (v/v) ethanol and then resuspended in 1 μ L of RNase and 10 μ L of TE buffer with incubation at 37 °C for 5 min. The extraction was performed twice. Purified plasmid DNA was visualized after running with agarose gel electrophoresis. No band was observed for *B. longum* HK003 (**Figure 32**), confirming the absence of plasmid.

Experiment 15 - Acute Toxicity

[0160] Acute oral toxicity test was performed by Guangdong Detection Center of Microbiology according to China National Food Safety Standard GB 15193.3-2014. In brief, *B. longum* HK003 was inoculated into MRS medium and incubated at 36°C for 3 days under anaerobic condition. Cell concentration of the suspension was then adjusted to 2×10^8 CFU/ml – 2×10^9 CFU/ml with sterile 0.86% (w/v) saline. 5g of the test suspension was supplemented to 20ml with sterile water to obtain the final test solution. 10 male and 10 female specific pathogen free (SPF) Kunming mice were used in the study. Before the test, mice were fed with nothing except water for 6 hours. During the test, mice were randomly grouped, given 0.4ml/20g body weight of the test solution as a single dose, and fasted for 2

hours. All the mice were then fed with normal diet. Growth condition, signs of intoxication and time of mortality of the mice was individually recorded. All the mice were weighted on day 0, day 7 and day 14. During the 14 days observation, no abnormality including obvious intoxication symptoms or death was observed in any of the mice. The 50% Lethal Dose (LD₅₀) value of *B. longum* HK003 to SPF Kunming mice was greater than 500mg/kg body weight. Therefore, it is considered as actual non-toxic and meets the requirement of National Food Safety Standard.

Experiment 16 – in vitro study of anti-inflammatory effects of *B. longum* HK003

METHODS

10 *Cell culture*

[0161] Normal human epithelial cells NCM460 and INT were bought from ATCC (American Type Culture Collection, Manassas, VA, USA) and preserved in our lab. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, USA) supplied with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA) in cell culture incubator at 37°C and 5% CO₂.

Bacteria culture

[0162] *B. longum* HK003 was first isolated from a female with modified cooked meat medium (Oxoid, Thermo Fisher Scientific, USA). It was identified with Bruker biotyper (Billerica, Massachusetts, United States) and whole genome sequencing was performed to confirm the identity of the strain. Another *B. longum* strain (DSM 16603), obtained from commercial probiotic manufacturer, was included in the study for comparison. This strain is commonly used in food products or dietary supplements.

[0163] All *B. longum* was cultivated in reinforced Clostridial medium (RCM, Oxoid, Thermo Fisher Scientific, USA) overnight at 37 °C in hungate tubes filling with 100% CO₂. After overnight culture, bacteria were aliquoted into 50mL centrifuge tube in anaerobic chamber (Coy, USA) and centrifuged at 4000g for 15minutes. Bacteria cells were resuspended with supernatant at the concentration of 10⁷ CFU per 100 μL. The conditioned medium was obtained by filtering the remaining supernatant with 0.22μm membrane

(Milipore, Burlington, Massachusetts, United States) to remove all bacterial cells. Bacterial cells and conditioned medium were further used to co-culture with NCM460 cells.

Lipopolysaccharide (LPS) induced inflammation assay

[0164] Lipopolysaccharides from *E. coli* (LPS) was bought from Thermo Fisher Scientific to establish an endotoxin induced inflammation model. Normal human epithelial cells NCM460 were stimulated by the addition of 50 ng/mL LPS for 24 hours. For co-culture study, live bacteria culture at 10 multiplicity of infection (MOI), heat-killed bacteria at 10 MOI, or 10% (v/v) of supernatant (conditioned medium) was added immediately after addition of LPS. NCM460 cells without exposure to LPS was used as blank control. After the 24-hour incubation, NCM460 cells were collected for total RNA isolation and pro-inflammatory gene expression analysis by the real-time PCR method. All other solutions and equipment that were in contact with the cells had been autoclaved before use. Cells were handled in a biosafety cabinet.

Quantification of proinflammatory gene expression using real-time PCR

[0165] Total RNA was isolated from the treated cells with TRIzol reagent (TAKARA, Japan) following the manufacturer's instructions. RNA was quantified and qualified with the NanoDrop One spectrophotometer (Thermo Fisher Scientific, USA). Equal amounts of total RNA (0.5 µg) from each sample were reverse transcribed to cDNA with PrimeScript™RT Master Mix (TAKARA, Japan), following the manufacturer's protocol.

[0166] Real-time PCR was performed in a final volume of 10 µL including 2 µL sample cDNA with TB Green® Premix Ex Taq (TAKARA, Japan). The sequences of the used primers are listed in **Table 16**. The reaction mixtures were incubated for an initial denaturation at 94 °C for 5 min, followed by 40 PCR cycles: 40 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C with QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific, USA).

Table 16 Primers used in real-time PCR analysis

Primer	Primer sequences (5' → 3')	GC%
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Human IL-6 F	ACTCACCTCTTCAGAACGAATTG	43.48
Human IL-6 R	CCATCTTTGGAAGGTTGAGGTTG	47.83
Human IL-1beta F	AGCTACGAATCTCCGACCAC	55
Human IL-1beta R	CGTTATCCCATGTGTGCAAGAA	45.45
Human Actin F	CATGTACGTTGCTATCCAGGC	52.38
Human Actin R	CTCCTTAATGTCACGCACGAT	47.62

RESULTS

B. longum HK003 reduced LPS-induced IL-6 and IL-1beta gene expression

[0167] Gene expression levels of pro-inflammatory cytokines IL-6 and IL-1beta were detected with real-time PCR following the manual's instructions. As shown in **Figures 33** and **34**, gene expression levels of IL6 and IL-1 β were significantly increased in LPS-treated NCM460 cells (LPS) compared with the level in untreated controls (blank). Co-culture with *B. longum* HK003 (BL HK003) significantly reduced IL-6 and IL-1 β levels compared to LPS treated group (LPS). Meanwhile, no significant difference was observed between *B. longum* HK003 and the commercial *B. longum* strain DSM 16603 (BL Commercial, **Table 17**).

B. longum HK003 alleviated LPS-induced inflammation through its metabolites

[0168] To evaluate whether the anti-inflammatory was attributed to *B. longum* HK003's metabolites, LPS treated NCM460 cells were exposed to heat-killed *B. longum* HK003 and its conditioned medium. Heat-killed *B. longum* HK003 was prepared by heating live *B. longum* HK003 for 30 minutes then resuspended in PBS to remove its metabolites. Conditioned medium was obtained by collecting and filtering the supernatant of live *B. longum* HK003 culture with 0.22um membrane to remove all bacterial cells.

[0169] RNA extraction and real-time PCR were the same as in the experiment with live *B. longum* HK003. As shown in **Figures 35** and **36**, live *B. longum* HK003 (BL HK003) and its conditioned medium (BL HK003 Supernatant) significantly reduced gene expression level of IL-6 and IL-1 β , with no significant differences being observed between these 2 groups. On the other hand, reduction of IL-1 β but not IL-6 was observed upon exposure to heat-killed *B. longum* HK003. Effect of live *B. longum* HK003 and its conditioned medium for reduction

of IL-1 β is significantly stronger than that of heat-killed *B. longum* HK003 (**Figure 36**). These findings indicated that the anti-inflammatory effect of *B. longum* HK003 was mainly exerted through its metabolites.

Experiment 17 - Antibacterial effect of *Bifidobacterium* against pathogenic bacteria

(antimicrobial activity assay)

[0170] The inhibitory effect of *B. longum* HK003 against Gram-positive and Gram-negative pathogenic bacteria was investigated and compared to other *Bifidobacterium* obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) or from commercial probiotic manufacturer. Strains from commercial probiotic manufacturer are commonly used in food products or dietary supplements.

[0171] The bacterial strains used in the study are listed in **Table 17**. Pathogenic bacteria were obtained from the American Type Culture Collection (ATCC). Agar plates of Reinforced Clostridial Media (RCM) (Oxoid Ltd, UK) for *Bifidobacterium* sp. and Tryptic Soy (TS) (BD, Difco) for pathogenic bacteria, were inoculated. All *Bifidobacterium* strains and pathogenic bacteria were incubated at 37°C for 24-48 hours under anaerobic and aerobic conditions, respectively prior to the antimicrobial activity test.

Table 17 Information of bacterial strains used in this study

Bacterial species	Strain or Collection Number	Source	Gram-stain	Culture media
Isolated strains				
<i>Bifidobacterium longum</i> HK003	HK003	Human	Positive	RCM
Probiotic strains				
<i>Bifidobacterium adolescentis</i>	BA02 (DSM 18351)	Commercial	Positive	RCM
<i>Bifidobacterium bifidum</i>	BB01 (DSM 22892)	Commercial	Positive	RCM
<i>Bifidobacterium longum</i>	BL03 (DSM 16603)	Commercial	Positive	RCM
<i>Bifidobacterium pseudocatenulatum</i> (type strain)	DSM 20438	DSMZ	Positive	RCM
Pathogenic strains				
<i>Escherichia coli</i>	ATCC 25922	ATCC	Negative	TS
<i>Pseudomonas aeruginosa</i>	ATCC 27853	ATCC	Negative	TS
<i>Staphylococcus aureus</i>	ATCC29213	ATCC	Positive	TS

<i>Enterococcus faecalis</i>	ATCC 29212	ATCC	Positive	TS
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RCM- Reinforced Clostridial Media; TS- Tryptic Soy

- [0172] The antimicrobial activity test was performed using agar spot procedures with modifications. In a Petri dish containing 30 mL of RCM agar, 10 μ L of each *Bifidobacterium* culture (the equivalent of MarFarland 0.5) was spotted on one quadrant of the RCM agar plate, followed by incubation at 37°C for 24 h under anaerobic conditions (Coy Anaerobic chamber, mix gas 90% N₂, 5% CO₂, 5% H₂). After incubation, 10 mL of 0.75% TS soft agar containing pathogenic strains (the equivalent of MarFarland 0.5) was overlaid onto the RCM agar containing the spot growth of *Bifidobacterium* sp. The solidified plates were incubated at 37°C for 24 h under aerobic conditions. The formation of a clear/translucent halo zone around the growth of the *Bifidobacterium* was indicative of antimicrobial activity. The diameter of the growth inhibition zone was measured and expressed in millimetres. The entire experiment was done in duplicate.

RESULTS

- [0173] All of the *Bifidobacterium* tested showed a zone of inhibition against all pathogens except *B. bifidum* demonstrated no effect on *S. aureus* and *E. faecalis*. *B. longum* HK003 showed an overall better inhibition against pathogens compared to other *Bifidobacteria* strains (Table 18, Figure 37).

Table 18 Growth inhibition of pathogens by *Bifidobacterium* sp.

	Pathogenic strains			
	<i>Escherichia coli</i> ATCC 25922	<i>Pseudomonas aeruginosa</i> ATCC 27853	<i>Staphylococcus aureus</i> ATCC 29213	<i>Enterococcus faecalis</i> ATCC 29212
Isolated strains				
<i>Bifidobacterium longum</i> (BL HK003)	+++	+++	++	++
Commercial probiotic/ Type strains				
<i>Bifidobacterium adolescentis</i> (DSM 18351)	++	++	+	+
<i>Bifidobacterium bifidum</i> (DSM 22892)	+	+	-	-
<i>Bifidobacterium longum</i> (DSM 16603)	++	++	++	+

<i>Bifidobacterium pseudocatenulatum</i> (DSM 20438)	++	++	+	+
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(-) no inhibition

(+) zone of inhibition between 8 and 13mm

(++) zone of inhibition between 13 and 18mm

(+++ zone of inhibition >18 mm

- 5 [0174] All patents, patent applications, and other publications, including GenBank Accession Numbers and equivalents, cited in this application are incorporated by reference in the entirety for all purposes.

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WHAT IS CLAIMED IS:

1

1 **1.** A composition for use in treating hair loss in a subject comprising an
2 effective amount of (1) *Bifidobacterium longum* or *Faecalibacterium prausnitzii* or a
3 combination thereof; and (2) a physiologically acceptable excipient.

4 **2.** The composition of claim 1, which comprises no detectable amount of
5 other *Bifidobacterium* bacteria.

6 **3.** The composition of claim 1, consisting essentially of an effective
7 amount of (1) *Bifidobacterium longum*; (2) one or more physiologically acceptable excipients;
8 and optionally (3) a prebiotic.

1 **4.** The composition of claim 1, consisting essentially of an effective
2 amount of (1) *Faecalibacterium prausnitzii*; and (2) one or more physiologically acceptable
3 excipients; and optionally (3) a prebiotic material.

1 **5.** The composition of claim 1, consisting essentially of an effective
2 amount of (1) a combination of *Bifidobacterium longum* and *Faecalibacterium prausnitzii*;
3 and (2) one or more physiologically acceptable excipients; and optionally (3) a prebiotic
4 material.

1 **6.** The composition of claim 1, wherein the *Bifidobacterium longum* is a
2 strain deposited with Guangdong Microbial Culture Collection Center (GDMCC) under
3 Accession Number GDMCC 61784.

1 **7.** The composition of any one of claims 1-6, wherein the
2 *Bifidobacterium longum* or *Faecalibacterium prausnitzii* is in the form of viable cells or non-
3 viable cells.

1 **8.** The composition of any one of claims 1-6, wherein the amount of
2 *Bifidobacterium longum* or *Faecalibacterium prausnitzii* is about 1.7×10^7 - 1×10^{10} CFU for
3 adults and about 8.5×10^6 - 5×10^9 CFU for children in daily intake.

- 1 **9.** The composition of claim 8, which is formulated for oral ingestion.
- 1 **10.** The composition of claim 9, which is in the form of a food or beverage
2 item.
- 1 **11.** A method for treating hair loss in a subject, comprising administering
2 to the subject an effective amount of the composition of any one of claims 1-10.
- 1 **12.** The method of claim 11, wherein the subject is an adult.
- 1 **13.** The method of claim 11, wherein the subject is a child.
- 1 **14.** The method of claims 11-13, wherein the administering step comprises
2 administering to the subject one composition comprising *Bifidobacterium longum* and
3 *Faecalibacterium prausnitzii*.
- 1 **15.** The method of claim 12, wherein the administering step comprises
2 administering to the subject a first composition comprising *Bifidobacterium longum*, and
3 administering to the subject a second composition comprising *Faecalibacterium prausnitzii*.
- 1 **16.** The method of any one of claims 11-15, wherein the administering step
2 comprises oral ingestion of the composition.
- 1 **17.** The method of claim 16, wherein the administering step comprises oral
2 ingestion prior to or with food intake.
- 1 **18.** The method of any one of claims 11-17, wherein the subject has
2 recovered from COVID-19.
- 1 **19.** A kit for treating hair loss in a subject comprising a plurality of
2 compositions each comprising an effective amount of *Bifidobacterium longum* or
3 *Faecalibacterium prausnitzii* or a combination thereof.
- 1 **20.** The kit of claim 19, comprising two compositions each comprising an
2 effective amount of *Bifidobacterium longum* or *Faecalibacterium prausnitzii*.

1 **21.** The kit of claim 19 or 20, wherein the compositions are in the form of
2 a powder, liquid, paste, cream, tablet, or capsule.

1 **22.** An isolated strain of *Bifidobacterium longum* deposited with the
2 Guangdong Microbial Culture Collection Center (GDMCC) under the Accession Number
3 GBMCC 61784.

1 **23.** A composition comprising the *Bifidobacterium longum* of claim **22**.

1 **24.** The composition of claim **23**, which is a freeze-dried powder.

1 **25.** The composition of claim **24**, wherein the freeze-dried powder is
2 freeze-dried in a lyoprotectant comprising gelatin, skimmed milk powder, trehalose, and
3 sucrose.

1 **26.** The composition of claim **25**, wherein the lyoprotectant comprises
2 about 1.5% (w/v) of gelatin, about 15% (w/v) of skimmed milk powder, about 15% (w/v) of
3 trehalose, and about 5% (w/v) sucrose.

4 **27.** The composition of any one of claims **23-26**, which is
5 microencapsulated with a carrier material comprising soybean protein isolate and konjac gum.

1 **28.** A method of reducing inflammation in a subject comprising
2 administering to the subject an effective amount of (1) the *Bifidobacterium longum* of claim
3 **22** or (2) the composition of any one of claims **23-27**.

1 **29.** The method of claim **28**, wherein the effective amount of (1) the
2 *Bifidobacterium longum* of claim **22** or (2) the composition of any one of claims **23-27**
3 reduces inflammatory cytokine levels.

1 **30.** The method of claim **28**, wherein the effective amount of (1) the
2 *Bifidobacterium longum* of claim **22** or (2) the composition of any one of claims **23-27**
3 reduces pathogenic bacteria levels in the subject's gastrointestinal tract.

1 **31.** The method of claim **30**, wherein the pathogenic bacteria comprise
2 *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *enterococcus faecalis*.