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ORIGINAL ARTICLE

Heat-treated *Limosilactobacillus fermentum* LM1020 with menthol, salicylic acid, and panthenol promotes hair growth and regulates hair scalp microbiome balance in androgenetic alopecia: A double-blind, randomized and placebo-controlled clinical trial

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Abstract

Background: Androgenetic alopecia (AGA) is a common and chronic problem characterized by hair follicle miniaturization.

Aims: In this study, heat-treated *Limosilactobacillus fermentum* LM1020 (HT-LM1020) was investigated in human follicle dermal papilla cell (HFDPC), scalp tissue, and clinical trials for patients with AGA.

Patients/Methods: Cell proliferation and the expression of cyclins and cyclin-dependent kinases (CDKs) were measured in HFDPC. The relative gene expression of 5α-reductase and growth factors were investigated in hair scalp. This double-blind, randomized, placebo-controlled clinical trial was conducted over 24 weeks. Primary efficacy was evaluated by measuring hair density, and secondary efficacy was assessed by experts and self-assessment. Changes in the microbiota of the hair scalps were analyzed using 16S metagenome amplicon sequencing.

Results: HT-LM1020 promoted cell growth ($p < 0.001$) and cyclin B1 expression, and it reduced 5α-reductase and induced fibroblast growth factor 7 (FGF7), FGF10, and epithelial growth factor7 (EGF7) ($p < 0.001$). In the clinical trial, the experimental group demonstrated an increase in hair density from 133.70 to 148.87 n/cm^2 at Week 24 ($p < 0.001$), while also expressing satisfaction with their hair density, reduced hair loss, and hairline. At Week 24, the total ratio of lactic acid bacteria operational taxonomic unit (OTU) in the scalp increased from 6.65% to 26.19%. At the same period, placebo-controlled group decreased *Staphylococcus caprae* OTU from 77.95% to 14.57% while experimental group decreased from 65.80% to 41.02%.

Conclusions: These present results showed that HT-LM1020 was a co-effector of ingredients for anti-hair loss contributing to cell proliferation and the expression of CDKs.

KEYWORDS
16S metagenome amplicon sequencing, androgenetic alopecia, clinical trial, *Limosilactobacillus fermentum*, postbiotics

1 | INTRODUCTION

Androgenetic alopecia (AGA) occurs in both men and women with aging.[1,2] AGA can lead to miniaturization of hair follicles[3] and hair loss at the top and front of the scalp,[1] and is typically classified into male-pattern hair loss (MPHL) and female-pattern hair loss (FPHL).[1–3] MPHL recedes from the bitemporal front hairline to the vertex, whereas FPHL diffuses the thinning of hair across the entire scalp.[1] The main mechanisms of AGA include genetic predisposition, androgens, inflammation, and environments.[2]

The U.S. Food and Drug Administration (FDA) has approved two pharmacological agents, minoxidil and finasteride.[1–3] Minoxidil prolongs the anagen phase of hair, which promotes hair regrowth[1,3] and improves blood supply to hair follicles.[3] Oral administration of finasteride inhibits the enzyme 5α-reductase, which is responsible for converting testosterone to dihydrotestosterone (DHT). However, minoxidil and finasteride are associated with adverse reactions, such as scalp dryness, skin irritation, and dizziness, in some patients.[1,2] Other non-approved treatments for spironolactone, low-level laser therapy, light-emitting diode devices, platelet-rich plasma, and exomes were also reported adverse reactions.[1] For these reasons, recent studies focused on the role of scalp microbiome and regulation for treatment of alopecia.[4]

Menthol, salicylic acid, and panthenol are commonly used in hair products. Menthol soothes skin discomfort[5] and provides relief through a cooling effect on the scalp.[6] Salicylic acid is a keratolytic agent that reduces flakes on the hair on the scalp,[7] and panthenol acts as a skin-regenerating and hydrating agent.[8] However, menthol, salicylic acid, and panthenol are defined as hair loss conditioners, not growth-promoting agents such as minoxidil and finasteride.[9]

This study aimed to isolate novel lactic acid bacteria (LAB), *Limosilactobacillus fermentum* LM1020, and heat-treated *L. fermentum* LM1020 (HT-LM1020), to investigate their hair growth-promoting effects in human follicle dermal papilla cells (HFDPC) and human scalp tissue. Moreover, a double-blind, randomized, placebo-controlled clinical trial was performed to evaluate the application of HT-LM1020 as a cosmetic ingredient for anti-hair loss with menthol, salicylic acid, and panthenol. In addition, changes in the microbiome diversity and balance in hair scalps were observed using 16S rRNA gene metagenomic sequencing.

2 | MATERIALS AND METHODS

2.1 | Isolation of *L. fermentum* LM1020

The sourdough was obtained from a local bakery in Jinju, South Korea. Sourdough was cut into 10 g pieces and homogenized in 90 mL of phosphate-buffered saline (PBS) using a stomacher. Homogenized samples were collected in sterilized tubes and spread onto de Man-Rogosa-Sharpe (MRS) agar (BD, Franklin Lakes, NJ, USA). The plates were then incubated at 37°C for 48 h. After incubation, opaque white colonies were isolated and further incubated to measure acid production, catalase activity, and Gram stain.[10] The catalase-negative, Gram-positive, acid-producing rod-type strain was named LM1020 and was further identified by 16S rRNA gene sequencing as *L. fermentum*. *L. fermentum* LM1020 was stored at −80°C in an MRS medium containing 20% glycerol until the experiments. The 16S rRNA sequence information is shown in Table S1.

2.2 | Preparation of HT-LM1020

HT-LM1020 was prepared by the Department of Production of Lactomason (Jinju, Korea) according to previously described methods.[10] Lyophilized HT-LM1020 (product number S-210826-1) was stored at −80°C until further investigation.

2.3 | Cell culture

The HFDPC was obtained from PromoCell (Heidelberg, Germany). The cells were maintained in follicular dermal papilla cell growth medium (PromoCell) supplemented with growth factors (0.04 mL/mL fetal calf serum, 0.004 mL/mL bovine pituitary extract, 1 ng/mL human recombinant fibroblast growth factor (FGF), and 5 μg/mL human recombinant insulin) (PromoCell) at 37°C in a humidified atmosphere containing 5% CO_2. HFDPCs were seeded in cell culture flasks at the recommended density (5000–10000 cells/cm^2) and did not exceed passage 8.

2.4 | Measurement of relative cell proliferation

The HFDPCs were seeded in 96-well cell culture plates at a density of 1×10^4 cells/well. After 24 h of incubation, the growth medium was replaced with a fresh medium without growth factors and synchronized overnight. Prior to treatment with HT-LM1020, the culture medium was replaced with fresh growth medium containing growth factors. The HT-LM1020-treated cells were incubated for 24 h, and cell proliferation was measured.[11]

Relative cell proliferation was measured using thiazolyl blue tetrazolium bromide (MTT) (Alfar aesar, Haverhill, MA, USA) solution.[10] Briefly, HT-LM1020-treated cells were washed twice with PBS, and cell debris was removed. Washed HFDPC were treated with 0.5 mg/mL MTT solution dissolved in the growth medium and incubated for 4 h. After 4 h, MTT formazan was dissolved in dimethyl sulfoxide, and absorbance was measured at 570 nm using a

microplate reader (SpectraMax iD3, Molecular Devices, San Jose, CA, USA). Relative cell proliferation was measured by comparing the absorbance of the control (non-treated HFDPC). The effects of 0.003% of menthol (Sigma-Aldrich, Burlington, MA, USA), 0.0025% of salicylic acid (Sigma-Aldrich), and 0.002% of panthenol (Sigma-Aldrich) on cell proliferation were compared.

2.5 | Western blot analysis

HFDPC were seeded in 6-well cell culture plates at a density of 4×10^5 cells/well and treated with HT-LM1020, as described in Section 2.4. The HT-LM1020-treated cells were lysed by PRO-PREP™ lysis buffer (iNtRON Biotechnology, Seongnam, Korea) with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The lysed cells were centrifuged at 13000*g* for 30 min at 4°C. After centrifugation, supernatants were collected in a clear tube and protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

Capillary western blotting was performed using JESS, an automated western blotting system (ProteinSimple, San Jose, CA, USA).[10] Briefly, each protein sample was diluted to 0.8 mg/mL and separated in a 12–230 kDa capillary cartridge (ProteinSimple) according to the manufacturer's instructions. The capillaries were blocked using blocking solution accompanied by a capillary cartridge and incubated with primary and secondary antibodies sequentially. Protein expression was visualized using enhanced chemiluminescence, and the intensity of the bands was analyzed using ImageJ software.

Antibodies against cyclin B1, D1, E1, and cyclin-dependent kinase (CDK) 2 were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies against CDK4 and CDK6 were purchased from GeneTex (Irvine, CA, USA). The anti-rabbit secondary horseradish peroxidase (HRP) antibody was obtained from ProteinSimple.

2.6 | Preparation of human scalp tissue sample and treatment

The specimen was anonymized, discarded tissue sample obtained from surgery. For tissue culture, subcutaneous fat was removed from the human scalp tissue using wash solution (0.1 M PBS containing 1000 U/mL penicillin (Gibco, Waltham, MA, USA), 1 mg/mL streptomycin (Gibco), and 25 μg/mL fungizone (Gibco, pH 7.4), and the tissue was cut into 1 cm × 1 cm piece. The tissue specimens were cultured in semi-solid agarose medium containing William's E medium (Invitrogen, Waltham, MA, USA) supplemented with 2 mM glutamine (Sigma-Aldrich), 10 ng/mL hydrocortisone (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), and 0.4% agarose (Sigma-Aldrich).[12] The surfaces of the tissue specimens were treated with 20 μL of HT-LM1020 (1×10^8 and 1×10^9 cells/mL) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Medium replacement and HT-LM1020 treatments were performed every 24 h, and tissue specimens were harvested at 72 h.

2.7 | Preparation of human scalp tissue sample and treatment

The relative gene expressions of 5α-reductase, FGF7, FGF10, and epithelial growth factor (EGF) in human scalp tissue were evaluated using quantitative Reverse Transcription PCR (qRT-PCR). Briefly, human scalp tissue specimens were homogenized using a TissueLyser II (Qiagen, Hilden, Germany). Total RNA was extracted using RNAiso Plus (Invitrogen), according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific) and reverse transcribed using an RNA to cDNA EcoDry Premix Kit (Takara Bio, Kusatsu, Japan). The synthesized cDNA, Taqman Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA), and Taqman primer of target (5α-reductase 1; Hs00971645_g1, FGF7; Hs00940253_m1, FGF10: Hs00610298_m1, EGF: Hs01100002_m1, Applied Biosystems) were used for the qRT-PCR experiments. The expression level of each gene was normalized to that of the housekeeping gene GAPDH (Hs02786624_g1, Applied Biosystems) and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.8 | Clinical trial

2.8.1 | Study design

This was a double-blind, randomized, placebo-controlled trial. Observations were performed at 0, 8, 16, and 24 weeks. The clinical trial was conducted from November 4, 2021, through June 10, 2022. All participants were adequately informed about the clinical research and voluntarily consented to participate in the study.

2.8.2 | Subjects

The criteria for the subject recruitment and outline of the clinical trials were based on "Guidelines for Human Clinical Trials of Cosmetics Aimed at Alleviating Hair Loss Symptoms" from the Ministry of Food and Drug Safety of the Republic of Korea.

In accordance with these guidelines, this study was conducted in men and women between the age of 18–54 years with AGA. Participants were evaluated using the Basic and Specific (BASP) classification, with males based on the Norwood classification and females based on the Ludwig classification. Participants of the basic type included individuals with M1, C1, or U1. Participants of a specific type included individuals with at least V1 or F1, Norwood classification of at least II, and Ludwig classification of at least Grade I.

Participants who used topical hair restorers, applied scalp and hair thickening agents, and underwent scalp reduction and hair transplant procedures during the past month were excluded, as were participants who had undergone hair loss treatment for the past 6 months.

A total of 27 males and 23 females were enrolled in the clinical trial (mean aged: 39.84). Fifty participants were randomly allocated to either the placebo-controlled group or the experimental group. Of the 50 participants, 6 were excluded based on the exclusion criteria.

2.8.3 | Preparation of hair tonic product

The products for the clinical trial were prepared by HBNine (Cheongju, Korea). The experimental group was administered hair tonics containing 5% HT-LM1020, 0.3% menthol, 0.25% salicylic acid, and 0.2% panthenol. The placebo-controlled group used hair tonics that excluded menthol, salicylic acid, and panthenol. During the clinical trial, all participants applied an appropriate amount of the product on their scalp before bedtime. The participants used same shampoo once a day before product application.

2.8.4 | Irritation test of HT-LM1020

Before the clinical trial, an irritation test was performed on volunteers. Briefly, 20 μL of hair tonic was added to the IQ Ultimate chamber (Chemotechnique MB Diagnotics AB) and applied to the skin using 3 M microspore tape. After 24 h, the skin irritation level was assessed by experts.

2.8.5 | Efficacy assessment

The primary efficacy assessment was based on hair density measurement, and the secondary efficacy assessments were based on visual assessment by the investigator and subject questionnaires. The hair density was measured using a phototrichogram. A circular area with a diameter of 1 cm^2 was shaved on the balding area, and a tattoo with a diameter of 1 mm was placed in the center to mark the testing area. Folliscope 5.0 (LeadM, Seoul, Korea) was used at every visit to measure the number of hairs (total hair count; number/cm^2) within a 1 cm diameter, with the tattoo used as a reference point. The investigators (professor, Severance Hospital, Yonsei University) conducted a visual assessment by filming the crown area and front hairline of the participants at 8, 16, and 24 weeks after using the test product. The evaluation was conducted on a 7-point scale (+3, significantly improved; +2, improved; +1, slightly improved; 0, no change; −1, slightly worse; −2, worse; and −3, significantly worse). High-resolution photographs of the crown area and frontal hairline were obtained using a digital camera (Canon EOS 650D; Canon, Tokyo, Japan). Reliability is measured using the Intraclass Correlation Coefficient (ICC) between the two investors.[13] The subjects completed a subjective questionnaire at Weeks 8, 16, and 24 after using the test product. The evaluation was conducted on a 7-point scale (+3, significantly improved; +2, improved; +1, slightly improved; 0, no change; −1, slightly worse; −2, worse; and −3, significantly worse).

2.8.6 | Safety evaluation

Adverse reactions were assessed at every visit, and all adverse reactions were recorded in the case report.

2.8.7 | Compliance evaluation

All participants used the test product once a day for 24 weeks and recorded their use on a compliance log to evaluate compliance using the following equation:

$$\text{Compliance}\,(\%) = \frac{\text{Actual amount of usage}}{\text{Required amount of usage}} \times 100$$

2.9 | 16S metagenome amplicon sequencing

The scalp hair microbiomes of 10 clinical trial participants in this clinical trial were analyzed. The specimens were harvested using a sterile cotton swab (NBgene-SKIN, Noble Bio, Hwaseong, Korea) enclosed in nucleic acid transport medium. Specimens were transported under 4°C and extracted DNA within 24 h.[14]

DNA extraction and library construction were performed by Macrogen (Seoul, South Korea). Briefly, genomic DNA was extracted from each sample and amplified according to the 16S Metagenomic Sequencing Library Preparation (part # 15044223 Rev. B, Illumina, CA, USA). The V3–V4 region of the 16S rRNA gene amplicons were used for library construction using the Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit (Illumina). Paired-end sequencing (2 × 300 bp) was performed on a MiSeq platform (Illumina).[15]

Library trimming and taxonomic classification were performed by InsilicoGen (Yongin, Korea). Taxonomic classification was performed using the SILVA ribosomal RNA and Greengenes-formatted databases. Operational taxonomic unit (OTU) was detected at a 0.03 genetic distance (with 97% sequence similarity).[16] The sequence data were deposited in the Sequence Read Archive under the accession number PRJNA1012497.

2.10 | Statistical analysis

Statistical analyses were performed using the SPSS Statistics version 25 software (IBM, Armonk, NY, USA).

Mean values were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple-range test, Tukey's range test, and Games–Howell post hoc test at $p < 0.05$, including cell proliferation, western blot analysis, and gene expression.

In the clinical trial, the hypothesized mean difference for intergroup significance was between Weeks 0 and 24 or between the placebo-controlled and experimental groups. The homogeneity test of hair density measurements at Week 0 between the placebo-controlled and experimental groups was conducted using a parametric method, the independent samples *t*-test, because the data satisfied the normality assumption of the normality test. Intra-group comparisons of hair density were performed using repeated-measures ANOVA followed by post hoc testing with Bonferroni correction (parametric method) or the Mann–Whitney *U* test (non-parametric method). Intergroup comparisons of categorical variables, such as visual assessments and questionnaire evaluations, were analyzed using the Mann–Whitney *U* test for the ordinal scale.

3 | RESULTS

3.1 | Cell proliferation and cell cycle-related protein expression in HT-LM1020-treated HFDPC

Figure 1 shows the relative cell proliferation and cell cycle-related protein expression in HT-LM1020-treated HFDPC. The relative cell proliferation increased to 136.94%, 146.06%, 154.71%, 153.32%, 155.49%, and 153.01% at 1×10^3 to 1×10^8 cells/mL of HT-LM1020 ($p<0.001$). In addition, above 1×10^5 cell/mL HT-LM1020-treated groups were distinguished from the control, 1×10^3 cells/mL, and 1×10^4 cells/mL treated groups (Figure 1A). Compared with the hair conditioner ingredient, menthol decreased the cell viability of HFDPC to 91.27% ($p<0.01$) and salicylic acid and panthenol did not

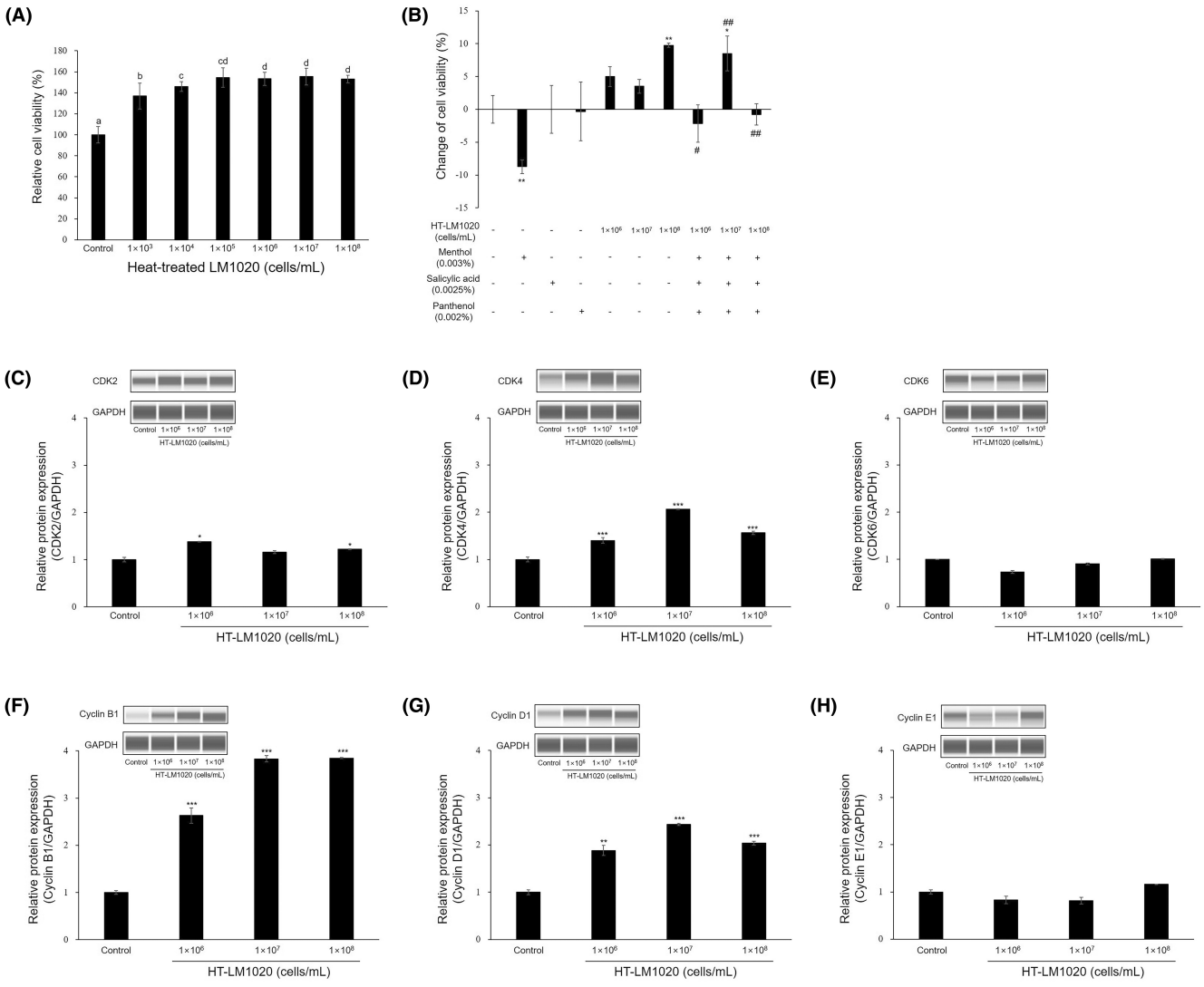

FIGURE 1 Cell proliferation effect and cell cycle-related proteins regulation of heat-treated *Limosilactobacillus ferementum* LM1020. Data are shown as mean ± standard deviation of three independent experiments. (A) Relative cell proliferation of human follicle dermal papilla cell (HFDPC). The different letters indicated significant difference as assessed by Duncan's multiple-range test. (B) Change of relative cell viability of HFDPC. Significant differences compared to control are indicated by asterisks (*$p<0.05$; **$p<0.01$) and significant differences compared to menthol are indicated by sharp ($^{\#}p<0.05$; $^{\#\#}p<0.01$). (C–H), protein expression of cyclins and cyclin-dependent kinases. Significant difference compared to control are indicated by asterisks (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

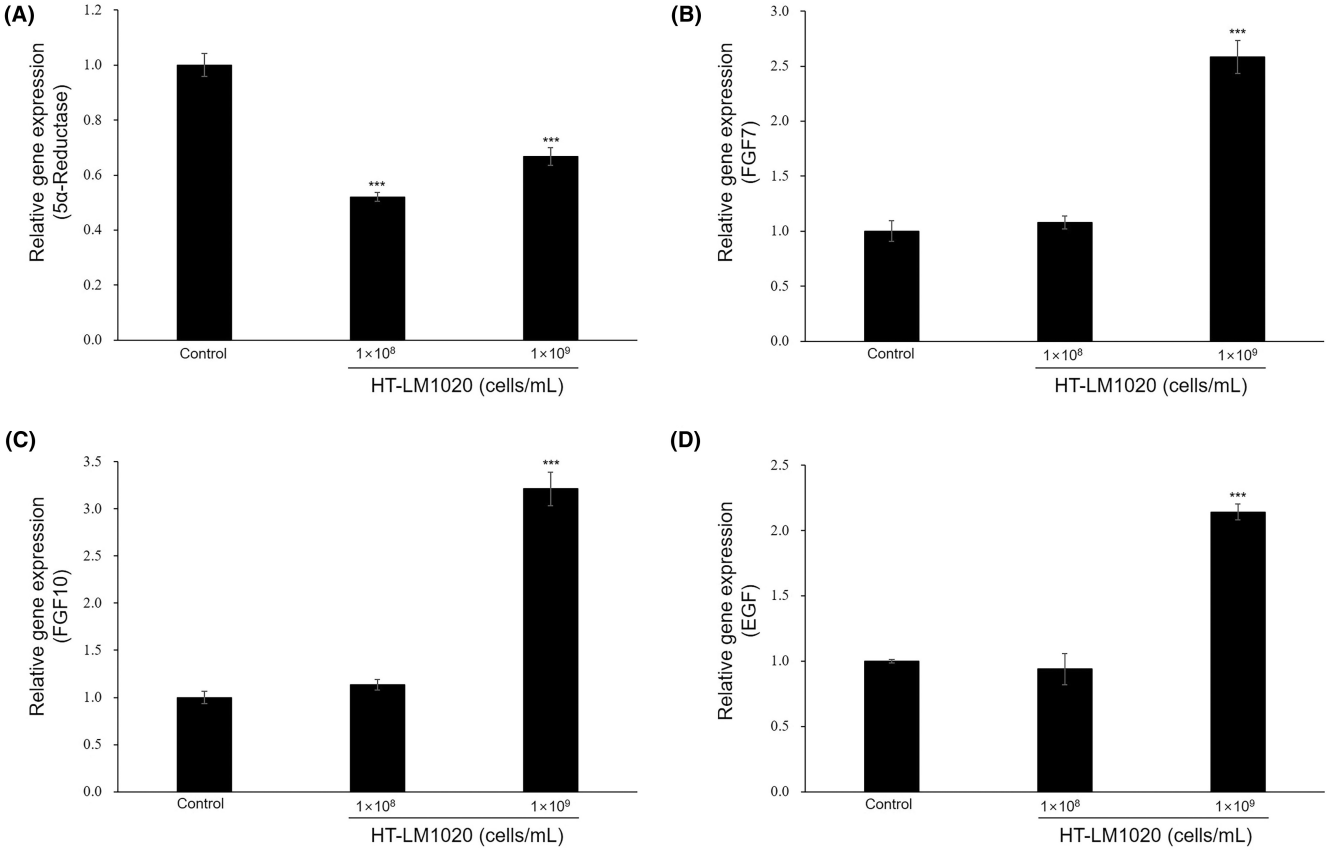

FIGURE 2 Effect of heat-treated *Limosilactobacillus fermentum* LM1020 in human scalp tissue. (A) Relative gene expression of 5α-reductase. (B) Relative gene expression of fibroblast growth factor 7. (C) Relative gene expression of fibroblast growth factor 10. (D) Relative gene expression of epidermal growth factor. Significant difference compared to control are indicated by asterisks (***$p < 0.001$).

showed significant changes. As shown in Figure 1B, HT-LM1020 attenuates menthol toxicity. The 1×10^6, 1×10^7, and 1×10^8 cells/mL HT-LM1020 co-treated with menthol, salicylic acid, and panthenol showed 97.85%, 108.50%, and 99.21% cell viability, respectively. These changes were indicated that HT-LM1020 mitigated cytotoxicity of menthol ($p < 0.05$) (Figure 1B).

The protein expression of CDK4 increased to 1.40-, 2.07-, and 1.56-fold at cell concentrations of 1×10^6, 1×10^7, and 1×10^8 cells/mL in the HT-LM1020 HFDPC treated groups, respectively ($p < 0.001$) (Figure 1D). CDK2 was significantly increased at 1×10^6 and 1×10^8 cells/mL HT-LM1020 ($p < 0.05$), whereas CDK6 did not show a significant increase compared with the control. Cyclin B1 and cyclin D1 increased to 3.85- and 2.04-fold at the 1×10^8 cells/mL treatment level ($p < 0.001$), respectively, whereas cyclin E expression did not show a significant increase at the 1×10^8 cells/mL treatment level (1.16-fold).

3.2 | Change of 5α-reductase and growth factors in HT-LM1020-treated human scalp tissue

The relative gene expression of 5α-reductase, FGF7, FGF10, and EGF in human scalp tissue was shown in Figure 2. HT-LM1020 reduced 5α-reductase gene expression to 0.52- and 0.67-fold at cell concentrations of 1×10^8 and 1×10^9 cell/mL, respectively ($p < 0.001$). None of the growth factors significantly increased in the 1×10^8 cell/mL HT-LM1020-treated tissue sample; however, 1×10^9 cells/mL HT-LM1020 induced gene expression of FGF7 (2.58-fold), FGF10 (3.21-fold), and EGF (2.14-fold) ($p < 0.001$).

3.3 | Clinical trial

3.3.1 | Participants' summary

The placebo-controlled group ($n = 21$) and experimental group ($n = 23$) were subjected to a clinical trial, as described in Figure 3. The mean age of patients in the clinical group ($n = 44$) was 39.21. The average compliance rate for the test products was 95.37%.

3.3.2 | Safety assessment

Before clinical trial, skin irritation was not observed by HT-LM1020. Until Week 24, no adverse effects were detected in either the placebo-controlled or experimental groups.

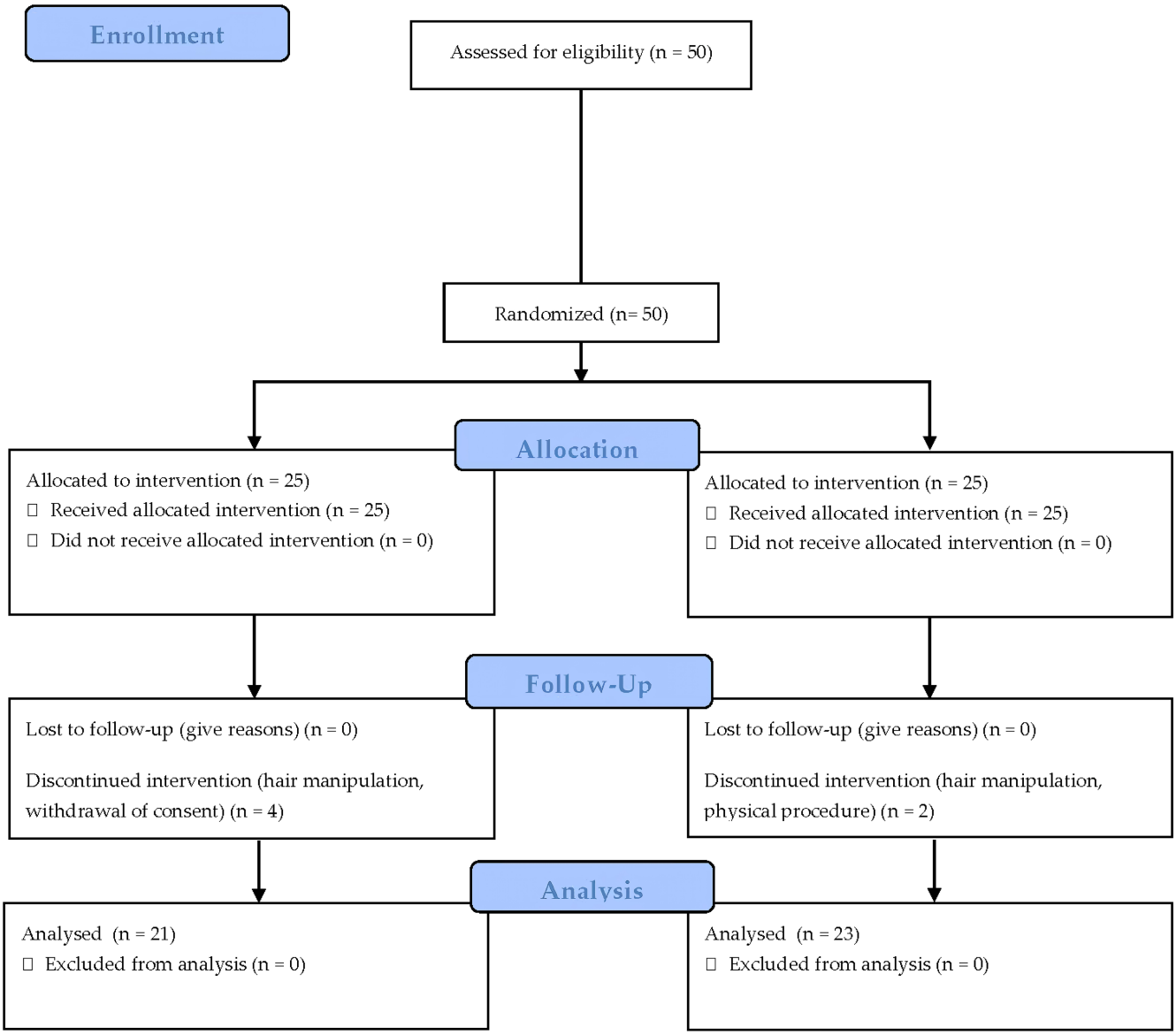

FIGURE 3 Consolidated Standards of Reporting Trials flow diagram of the study.

3.3.3 | Hair density (primary efficacy outcome)

Table 1 shows changes in hair density during the clinical trial. At Week 0, placebo-controlled group showed 137.3 n/cm^2 of hair density. During the clinical trial, the hair density of placebo-controlled group decreased to 137.0, 135.0, and 133.6 n/cm^2 at Week 8, Week 16, and Week 24, respectively. In contrast, the experimental group showed an increase in hair density from 133.7 to 137.7, 139.6, and 140.9 n/cm^2 at 8, 16, and 24 weeks, respectively ($p < 0.001$). The phototrichogram results are illustrated in Figure 4, which shows a representative image of the participants.

3.3.4 | Investigator visual assessment (secondary efficacy outcome)

Table 2 shows investigator visual assessment of crown area and front hairline through high-resolution photograph. At Week 8, significant difference was not detected between placebo-controlled group and experimental group ($p = 0.591$). However, proceeding from clinical trial, significant difference was evaluated between the placebo-controlled group and experimental group at Week 16 ($p = 0.027$) and Week 24 ($p = 0.023$). The ICC for reliability was measured as 0.916 ($p < 0.001$). The presentative photograph of crown area and front hairline is shown in Figure 5.

TABLE 1 Change of hair density in participants.

Variables	Placebo-controlled group	*p* value[a]	Experimental group	*p*-value[a]
Hair density (n/cm^2)				
Week 0	137.3 ± 21.9	-	133.7 ± 24.7	-
Week 8	137.0 ± 21.2	1.000	137.7 ± 24.6	<0.001
Week 16	135.0 ± 20.9	0.019	139.6 ± 25.0	<0.001
Week 24	133.6 ± 21.0	0.002	140.9 ± 24.8	<0.001
Variables	**Placebo-controlled group**		**Experimental group**	***p* value[b]**
Change of hair density (n/cm^2)				
Week 8	−0.3		4.0	<0.001
Week 16	−2.3		5.9	<0.001
Week 24	−3.7		7.2	<0.001

Note: Data are shown as mean ± standard deviation of each group.

[a]Significant differences were indicated comparing to Week 0.

[b]Significant differences were indicated comparing to placebo-controlled group.

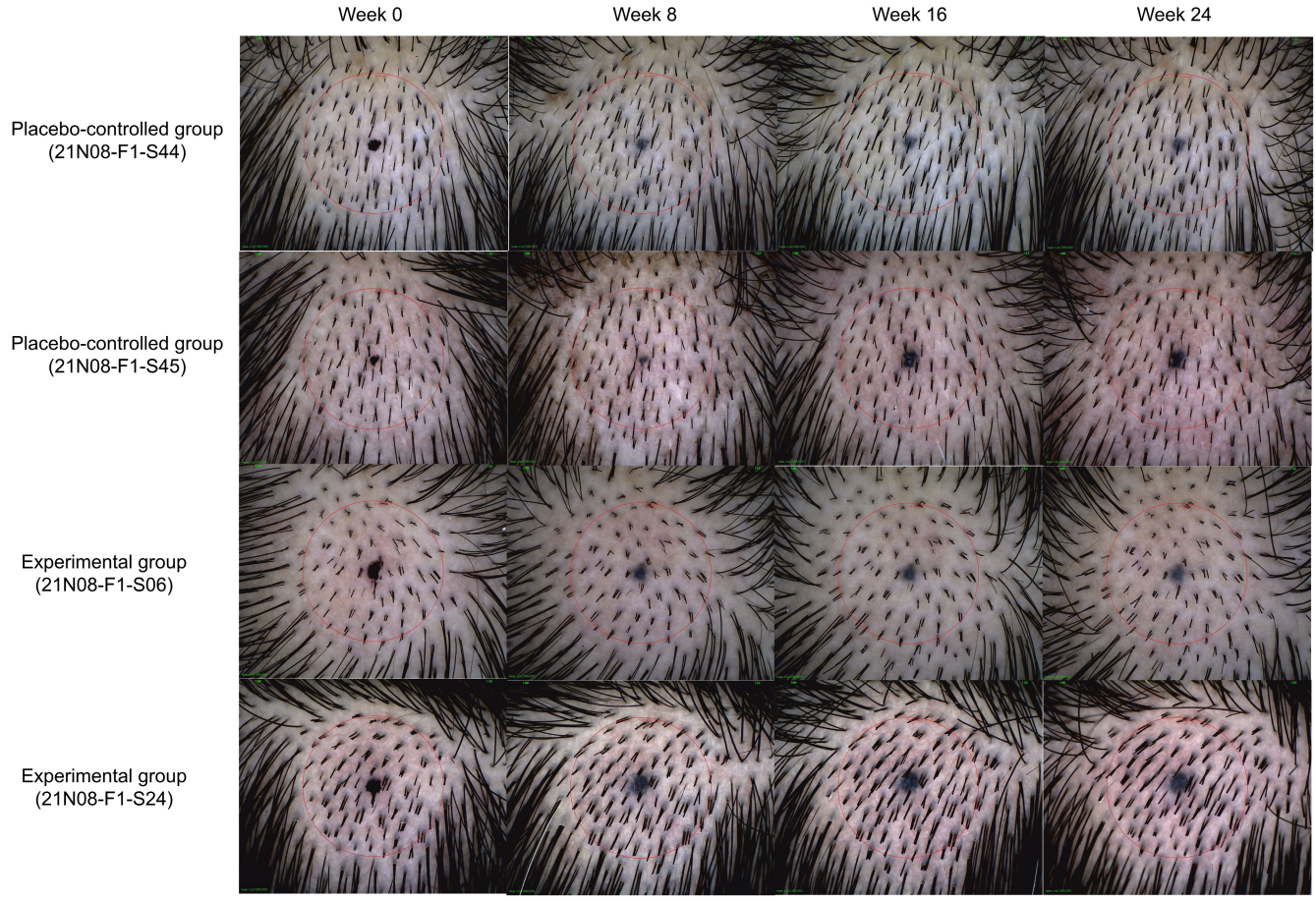

FIGURE 4 Phototrichogram of placebo-controlled group and experimental group. The phototrichogram images were obtained using folliscope.

3.3.5 | Satisfaction score (secondary efficacy outcome)

The participants' satisfaction scores are listed in Table 3. The experimental group reported hair density satisfaction scores of 0.913, 1.435, and 1.217 at Weeks 8, 16, and 24, respectively. These values were significantly different from those of the placebo-controlled group at Weeks 8 ($p = 0.007$) and 16 ($p = 0.001$). Satisfaction scores for hair loss steadily increased to 1.174 (at Week 8), 1.304 (at Week 16), and 1.522 (at Week 24). During the same period, the placebo-controlled group

TABLE 2 Investigator visual assessment by experts.

	Placebo-controlled group		Experimental group	
Variables	**Investigator 1**	**Investigator 2**	**Investigator 1**	**Investigator 2**
Average score				
Week 0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 8	0.048 ± 0.384	0.048 ± 0.218	0.043 ± 0.209	0.087 ± 0.288
Week 16	0.048 ± 0.218	0.000 ± 0.000	0.261 ± 0.541	0.261 ± 0.541
Week 24	−0.143 ± 0.478	−0.048 ± 0.384	0.261 ± 0.541	0.217 ± 0.518
Intraclass correlation coefficient[a]			0.916 ($p < 0.001$)	

Variables	**Placebo-controlled group**	**Experimental group**	***p*-value[c]**
Average score[b]			
Week 0	0.000 ± 0.000	0.000 ± 0.000	-
Week 8	0.000 ± 0.316	0.043 ± 0.209	0.591
Week 16	0.000 ± 0.000	0.261 ± 0.541	0.027
Week 24	−0.143 ± 0.478	0.217 ± 0.518	0.023

Note: Data are shown as mean ± standards deviation of each group.

[a]The reliability analysis was performed using intraclass correlation coefficients.

[b]Values were evaluated by experts through high-resolution photograph.

[c]Significant differences were indicated comparing to placebo-controlled group.

showed 0.476 ($p = 0.004$), 0.619 ($p = 0.008$), and 0.905 ($p = 0.012$) of satisfaction score, respectively. Regarding the satisfaction score of the front hairline, the experimental group showed significantly more satisfactory results at Week 16 ($p = 0.009$).

3.4 | Microbiota profiling

As shown in Figure 6, the microbiome niche differed between the placebo-controlled and experimental groups. In the placebo-controlled group, the OTU ratio of LAB increased from 9.48% to 57.48% and *Staphylococcus* ratio decreased from 90.52% to 42.16%. The experimental group showed an increase in the LAB OTU ratio (from 6.65% to 26.19%) and a decrease in the *Staphylococcus* OTU ratio (from 93.35% to 73.81%) (Figure 6A). However, the placebo-controlled group showed an overpopulation of *L. fermentum* CIP 102980 (8801 OTU) in one individual (21N08-F1-S04) at Week 24. This affected the microbiome diversity of the participants. As shown in Figure 6B, the Shannon index of the placebo-controlled group increased from 0.95 to 1.28, whereas that of the experimental group increased from 1.22 to 1.961 in the same period. The diversity of LAB in the placebo-control group decreased from 1.41 to 0.29, whereas that in the experimental group increased from 0.61 to 1.68 (Figure 6C). Additionally, the OTU of *Staphylococcus caprae* decreased from 77.95 to 14.57 in the placebo-controlled group. At the same period, experimental group decreased from 65.80 to 41.02 (Figure 6D).

4 | DISCUSSION

Recently, many researchers focused on LAB for regulating AGA or hair growth. Nam et al.[17] reported *Lacticaseibacillus paracasei* HY7015 promoted the growth of HFDPC and hair in a telogenic mouse model. The *L. paracasei* HY7015 stimulated IGF-1 in telogenic mouse while HT-LM1020 promoted FGF and EGF in human scalp tissue. Another study investigated metabolites from LAB for regulating AGA.[11] Yoon et al.[11] studied extracellular vesicles produced by *Leuconostoc holzapfelii* to heal wound areas and promote cell growth of HFDPC. Similar to present study, Tasi et al.[18] researched the effect of heat-treated *L. parasei* GMNL-653 for regulating scalp microbiome. Compared to previous studies, this study was the first trial of heat-treated LAB for regulating AGA including HFDPC and human scalp tissue study, clinical trial, and next-generation sequencing analysis.

Androgenetic hair originates from hair follicles that are located in the dermis. Hair follicles produce keratin to stimulate growth factors that play a critical role in hair growth regulation.[19] Hair follicles repeatedly undergo hair growth cycles consisting of anagen, catagen, and telogen phase.[5,8] The transition of the growth cycle is regulated by the dermal papilla, which is the signaling center for hair follicles.[8] Dermal papillae also supply nutrients and growth factors to hair follicles by penetrating blood vessels.[19] For example, insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and keratinocyte growth factor (KGF) are supplied to matrix keratinocytes to stimulate hair shafts during the anagen phase.[19] Thus, HFDPC proliferation plays an important role in the mitigation of AGA.

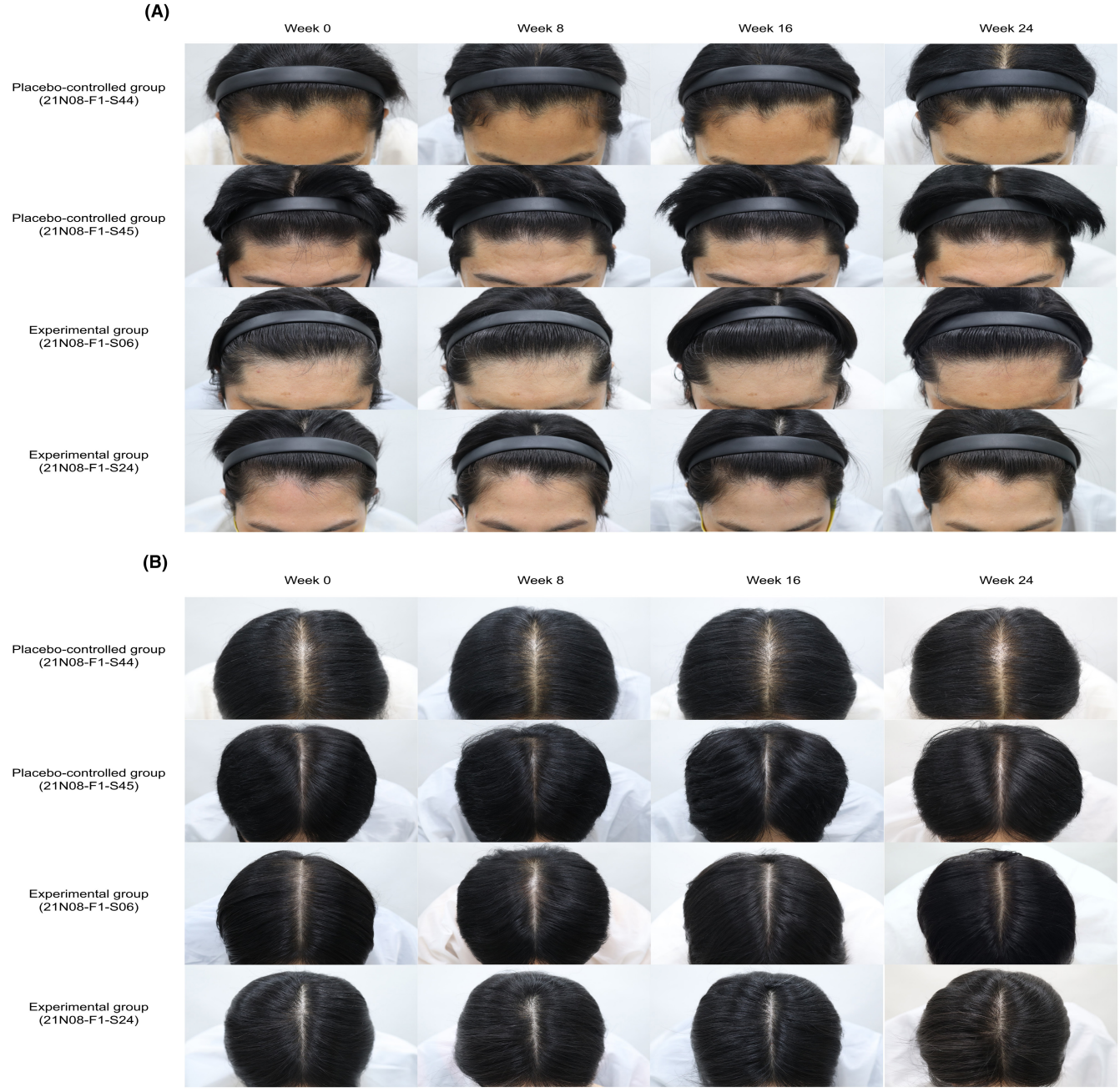

FIGURE 5 High-resolution photograph of crown area (A) and front hairline (B). Crown area and front hairline were observed using digital camera.

Mammalian cell division and death are controlled by the cell cycle, which is essential for maintaining tissue homeostasis. Cyclins and CDKs are major families of proteins that control cell cycle. The mammalian cyclin family is not fully understood, but a few types of cyclins (A-, B-, C-, D-, and E-type) have defined roles in cell cycle progression. Each cyclin binds to a catalytic partner to form cyclin-CDK complexes. Distinct cyclin-CDK complexes activate different stages of the cell cycle, including DNA replication, chromosomal segregation, and mitotic exit. Cyclin D-CDK4/6 enters the cell cycle from quiescence (G0 phase), the G1 to S phase transition, and initiates DNA replication.[20] Cyclin D-CDK4/6 induces phosphorylation of retinoblastoma protein (Rb) and disrupts the Rb/E2F interaction to activate cyclin E.[21] Cyclin E activates CDK2, and the cyclin E-CDK2 complex initiates DNA replication.[20,21] The cyclin B-CDK1 complex plays an important role in mitotic exit.[22] The cyclin B-CDK1 complex is activated during the late G2 and early M phases and phosphorylates essential proteins for organelle segregation, mitochondrial activity and fission, nuclear envelope breakdown, and chromosomal segregation.[20] In late metaphase, cyclin B degradation is initiated by an anaphase-promoting complex for mitosis and cytokinesis.[20,22] Thus, cyclin B1 accumulation results in efficient mitotic events during cell division. In this study, the relative protein expression

TABLE 3 Comparison of satisfaction score between placebo-controlled group and experimental group.

Satisfaction index	Week	Placebo-controlled group	Experimental group	*p*-value[a]
Satisfaction of hair density on the crown area	Week 8	0.238 ± 0.625	0.913 ± 0.848	0.007
	Week 16	0.571 ± 0.811	1.435 ± 0.788	0.001
	Week 24	0.952 ± 1.071	1.217 ± 0.600	0.396
Satisfaction of hair loss	Week 8	0.476 ± 0.981	1.174 ± 0.778	0.004
	Week 16	0.619 ± 0.973	1.304 ± 0.703	0.008
	Week 24	0.905 ± 0.889	1.522 ± 0.730	0.012
Satisfaction of front hairline	Week 8	0.333 ± 0.796	0.609 ± 0.783	0.198
	Week 16	0.476 ± 1.078	1.217 ± 0.795	0.009
	Week 24	0.810 ± 1.078	1.217 ± 0.902	0.169

Note: Data are shown as mean ± standard deviation of each group.

[a]Significant differences were indicated comparing to placebo-controlled group.

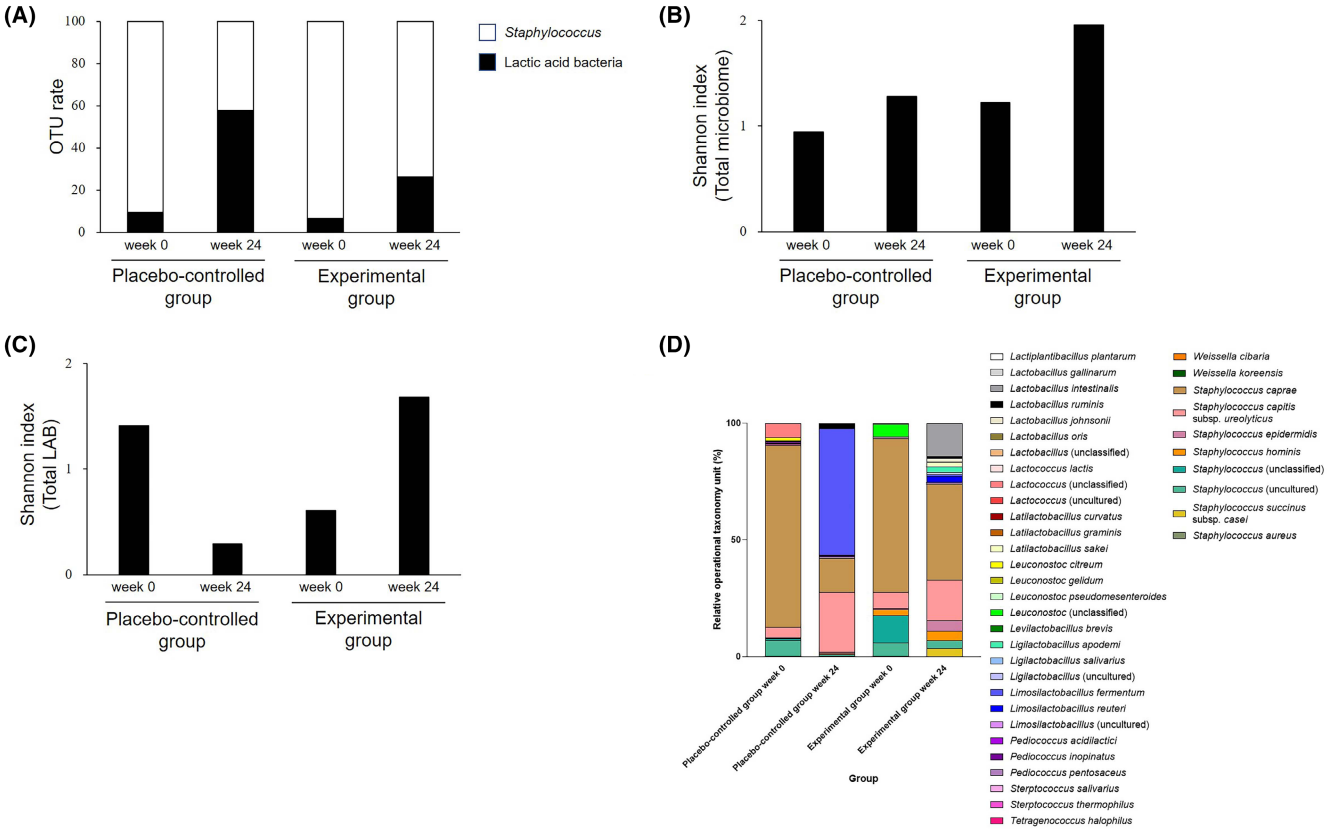

FIGURE 6 Change of microbiome niche in hair scalp after clinical trials. (A) Operational taxonomic unit (OTU) ratio of lactic acid bacteria (LAB) in hair scalp. (B) Shannon index value of hair scalp (in total microbiome). (C) Shannon index value of hair scalp (in LAB). (D) Comparison of OTU diversity in hair scalp.

of cyclin B1 significantly increased in HT-LM1020-treated groups ($p < 0.001$), whereas cyclin E1 expression levels did not significantly increase (Figure 1). These changes were presumed to have affected HT-LM1020-treated HFDPCs located at the G2/M checkpoint through the S phase.

AGA is generally triggered by DHT converted from testosterone by 5α-reductase.[1,2] DHT has a higher affinity for androgen receptors than testosterone[2] and results in follicle miniaturization.[3] Androgen also induced secretion of cytokines including transforming growth factor β1, interleukin-1α, and tumor necrosis factor-α. These cytokines shorten the anagen phase of hair growth, induce apoptosis in hair follicles, and eventually lead to hair loss.[2] In addition, androgen leads to the early appearance of telogen, resulting from the reduction of growth factors such as FGF, IGF-1, and

VEGF.[23] HT-LM1020 inhibited gene expression of 5α-reductase and upregulated growth factors ($p < 0.001$) in human hair scalp (Figure 2). These results indicate that HT-LM1020 promotes hair growth and affected to placebo-controlled group in clinical trial. While decreasing hair density in placebo-controlled croup, the satisfaction scores slightly increased. These symptoms were considered as placebo effect by HT-LM1020. However, the excessive concentration (1×10^8 cells/mL at HFDPC and 1×10^9 cells/mL at scalp tissue) of HT-LM1020 can show a decrease of effectiveness. This tendency was also observed in extracellular vesicles from *L. holzapfelii*.

The hair follicle is a territory for microbiomes on the hair scalp.[24] The microbial population on the scalp comprises *Cutibacterium*, *Staphylococcus*, *Malassezia*, and other microorganisms.[4,13] Recent studies have focused on dysbiosis of the scalp, resulting in hair and skin disorders, including infectious diseases.[24,25] Ho et al.[26] reported that miniaturized hair follicles induced by AGA were predominantly dominated by *Cutibacterium acnes* and upregulated immune response genes such as toll-like receptors and cluster of differentiation. According to Huang et al.,[27] the fungal genus composition changes in patients with AGA. For example, *Malassezia* and *Trichosporon* increased in AGA hair scalps, whereas *Fusarium* and *Epicoccum* showed diametric changes. Suzuki et al.[4] studied relation of scalp microbiome and sebum production in patients with AGA. The AGA groups higher *Corynebacterium* with free fatty acids while non-AGA groups had abundant *Malassezia globosa* with diglyceride. The microbiome changes have also been observed in not only AGA also alopecia areata (AA).[25,28] During AA, a decline in *S. caprae* ratio occurs in patients, whereas the number of *Cutibacterium* species increases.[28] This study focused potency of microbiome treatment for AGA using HT-LM1020. However, this study limited the relationship of LAB and *Staphylococcus*. For clear relationship of LAB and scalp microbiome in AGA, further investigations such as interaction between LAB and *Malassezia* are more needed. Although these limitations, HT-LM1020 containing product improved microbiome diversity in hair scalp and less decrease of *S. caprae* ratio than the placebo-controlled group (Figure 6). These results will be expected to improve hair condition in AGA scalp.

5 | CONCLUSION

In conclusion, our study demonstrated the potential of a novel cosmetic ingredient as a postbiotic. HT-LM1020 induced the proliferation of HFDPC cells by regulating the cyclin-CDK complex and growth factors, such as FGF and EGF. In addition, HT-LM1020 with menthol, salicylic acid, and panthenol showed significant improvement in patients with AGA, accompanied by microbiota regulation of the scalp. Menthol, salicylic acid, and panthenol do not induce hair growth; however, HT-LM1020 showed synergistic effects with these ingredients in attenuating AGA. These present results showed that HT-LM1020 was a co-effector of ingredients for anti-hair loss contributing cell proliferation and the expression of CDKs. In addition, the experimental had been approved by Korean Food and Drug Administration as a functional cosmetic for anti-hair loss.

AUTHOR CONTRIBUTIONS

Won-Young Bae and Tae-Rahk Kim contributed conceptualization of investigation. Won-Young Bae, Inhee Jung, and Young In Lee analyzed and performed the experiments. So Lim Shin, Jangmi Suk, and Ju Hee Lee contributed conceptualization of clinical trials, and all researchers contributed data validation and curation. Tae-Rahk Kim and Minn Sohn supervised the study. All researchers wrote and reviewed manuscript and revised before submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The 16S rRNA amplicon has been deposited at Sequence Read Archive under accession PRJNA1012497 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1012497). The authors confirm that the data of this study are available within the article.

ETHICS STATEMENT

A Specimen collection for human scalp tissue was approved by the Institutional Review Board of Severance Hospital, Yonsei University (approval number: 4-2021-1524). The clinical trial was approved by the Institutional Review Board of the Global Medical Research Center (GMRC), Seoul, Korea (approval number: GIRB-21O29-ET). The clinical trial was conducted at the Safety & Hair Research Center of GMRC (Seoul, Korea). Informed consent was obtained from all participants involved in the study. Before the experiment, the researchers explained the experimental process in detail and the experiment began after the participants signed a consent form.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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