Oral Probiotic Activities and Biosafety of *Lactobacillus gasseri* HHuMIN D

Soyon Mann
Seoul National University
Myeong Soo Park
Bifido Co. Ltd.
Tony V. Johnston
Middle Tennessee State University
Geun Eog Ji
Seoul National University
Keum Taek Hwang
Seoul National University
Seockmo Ku (seockmo.ku@mtsu.edu)
Middle Tennessee State University https://orcid.org/0000-0003-2661-5073

Research

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Abstract

Background

Specific probiotic bacteria may have inhibitory effects against oral pathogens. *Lactobacillus* spp. have been researched worldwide and are used in probiotics, but due to difficulties with laboratory cultivation of and experimentation on oral microorganisms, there are few reports of *Lactobacillus* spp. being isolated from the oral cavity and tested against oral pathogens. This research aimed to determine the safety and inhibitory impacts of *L. gasseri* HHuMIN D as a potential oral probiotic with biotherapeutic capabilities against oral pathogens.

Results

The microbial and genetic characteristics of *L. gasseri* HHuMIN D were evaluated in this research. A 5% dilution of *L. gasseri* HHuMIN D culture supernatant exhibited 88.8% inhibition against halitosis-producing anaerobic microorganisms and *L. gasseri* HHuMIN D exhibited powerful inhibitory effects on the growth of every harmful oral bacterium tested. Unfortunately, other oral bacteria affected the growth of *L. gasseri* HHuMIN D, suggesting a poor proliferative response to most co-cultures. Hydrogen peroxide production by *L. gasseri* HHuMIN D reached 802 µmol/L after 12 hours and gradually diminished until 24 hours. It efficiently aggregated with *P. catoniae* and *S. sanguinis*, and completely supressed *S. mutans*-manufactured artificial dental plaque. *L. gasseri* HHuMIN D's KB cell adhesion capacity was 4.41 cells per cell, and the cell adhesion of *F. nucleatum* and *S. mutans* diminished strongly in protection and displacement assays. *L. gasseri* HHuMIN D was evaluated for safety using ammonia and biogenic amine development, hemolytic property and mucin degradation testing, antibiotic susceptibility, and whole genome sequencing (WGS).

Conclusion

Our results suggest that *L. gasseri* HHuMIN D may be a safe, bioactive, lactobacterial food ingredient, starter culture, and/or probiotic microorganism for human oral health.

Introduction

Oral diseases are acute and chronic, with a high prevalence globally, and oral health is a primary component of human health sustenance. Oral health is associated with a wide variety of illnesses and disorders including dental caries, periodontal disease, tooth loss, oral cancer, oral symptoms of HIV infection, oral-dental trauma, and birth defects such as noma and cleft lip. Oral diseases cause suffering and disability to millions of Americans, costing taxpayers billions of dollars annually, according to the Centers for Disease Control and Prevention (CDC). According to a survey published in 2019, more than 40 percent of US adults report having felt pain in their mouths, and more than 80 percent have had at least one cavity by age 34. The nation spends more than $124 billion annually on dental-care services. About 34 million hours of schooling and over $45 billion in revenue are lost every year due to dental emergencies that require unplanned treatment [1]. The 2015 Global Burden of Disease Survey estimated that about 3.5 billion people worldwide experience oral illnesses such as dental caries, periodontal disease, edentulism and extreme tooth loss [2]. The World Health Organization (WHO) reports that millions of individuals suffer immensely from unhealthy oral cavities and that, as a result, the financial burden of society is growing. Over the past 30 years, the global burden of tooth decay has remained largely unchanged and the total burden of oral disease on facilities will rise as a result of population growth and aging. The WHO also points out a correlation between socioeconomic status (income, occupation and education level) and the prevalence and severity of oral diseases. Since the Tokyo Declaration on Dental Care and Oral Health for Healthy Lifetime, numerous oral health enhancement practices, including discussion of the overall value of oral health, the improvement of

dental treatment systems, and the establishment of oral health programs, have been introduced by academia and industry [3]. In most high-income countries the lifetime prevalence of dental caries has decreased over the past 40 years, but the same prevalence has risen in low- and middle-income countries. As a consequence, rates of dental caries have increased worldwide [4]. Dental caries and oral disease in South Korea were ranked second and sixth, respectively, by out-patients in the 2017 National Health Insurance Service survey, were in the Top 10 ambulatory care frequency and expenditure ranking, and the number of patients is rising every year [5].

One oral disease, dental caries, is caused by acid, formed by bacteria attached to the tooth's surface, which corrodes the enamel and dentine of teeth. *Streptococcus mutans* are Gram-positive, facultative anaerobic bacteria with a clear association with early dental caries in humans and animals. Insoluble glucan, combined by glucosyltransferase (GTase) in foods with sucrose and produced by *S. mutans*, has a high rate of adhesion to tooth and firm oral surfaces, and plays an important role in the development of dental caries. Oral microorganisms attach glucans produced through biochemical processes to form dental plaques and produce lactic acid (and others) during carbohydrate metabolism [6, 7].

Halitosis is defined as “stink through the mouth or nasal cavity” or “unpleasant odor from the mouth.” This so-called “bad breath” is caused by volatile sulfur compounds (VSCs) such as hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) [8]. VSCs are produced by the decaying action of decomposing epithelial cells and white blood cells, which are eliminated by oral anaerobic bacteria. The production of volatile sulfur compounds is generated from amino acids such as cysteine and methionine, which contain sulfur. Bacterial genera that produce hydrogen sulfide from cysteine are *Peptostreptococcus*, *Eubacterium*, *Selenomonas*, *Centipeda*, *Bacteroides*, *Fusobacterium*, and *Prevotella*, and genera that make methyl mercaptan from methionine are known to be *Fusobacterium*, *Bacteroides*, *Porphyromonas*, and *Eubacterium* [8, 9]. In particular, *F. nucleatum*, a major bacterium for producing hydrogen sulfide or methyl mercaptan, is a gram-negative anaerobic bacterium that causes periodontitis and halitosis.

Many studies have been published on the inhibition of harmful oral bacteria for oral health [10, 11, 12, 13]. In spite of the fact that antibiotics, including penicillin, erythromycin, and tetracycline are effective in the prevention of oral disease, they have not been utilized in humans due to increased antibiotic resistance after long-term use [14]. Other studies have sought to inhibit oral periodontal bacteria using natural materials [13, 15, 16], inhibit GTase activity involving sucrose glucan formation [17], or use sucrose-alternative sweeteners that *S. mutans* are unable to metabolize [18]. Zinc ions, chlorhexidine (CHX) and cetylpyridinium chloride (CPC) and metal salts have been found to be effective in inhibiting the growth of halitosis-causing bacteria or in reducing the production of volatile sulfur compounds. Zinc chloride has a high affinity to sulfur, so thiol groups are oxidized which suppresses the precursors of volatile sulfur compounds. In this way, volatile sulfur compounds are rendered non-volatile, and halitosis is eliminated. Additionally, zinc chloride in the oral cavity serves an anti-microbial function as well as a halitosis mitigating capability by eliminating oral anaerobic bacteria [19]. Chlorhexidine, which is frequently used as a disinfectant in dental clinics, kills bacteria when applied to tooth enamel or the salivary pellicle and is widely used as a disinfectant in patients with periodontitis and to minimize halitosis [20]. Unfortunately, these strategies affect all oral microorganisms or have a short period of action, making lasting effects difficult to achieve [21].

Microorganisms usually present in the oral cavity are responsible for protective reactions to fungi such as *Candida*, pathogenic microorganisms such as purulent bacteria, and viruses [22]. A prevention strategy which suppresses the growth of all microorganisms present in the oral cavity is inappropriate for the prevention of oral disease. A healthy oral cavity can be maintained by promoting advantageous bacteria in the oral cavity and inhibiting destructive bacteria that cause oral disease [23]. Additional factors that reduce the effectivity of oral treatments include dilution and the lack of physical contact with all oral surfaces. It is not uncommon for liquid dental products to be quickly swallowed without making contact with all oral surfaces, reducing the effectiveness of the product. Saliva also dilutes these products,

rendering treatments effective for only around 2 hours, after which harmful oral bacteria rapidly grow again. Accordingly, the advancement of more effective, pragmatic and stable methods of prevention of dental caries are necessary [21].

Probiotic bacteria have been used for decades to support and preserve the quality of health. *Lactobacillus* species are the best-known probiotics and are a significant part of the natural human body flora, especially in the oral, digestive, and vaginal tracts. Host protection against pathogenic microorganisms is one of the major strengths of probiotics [24]. This research focuses on a lactic acid bacterium which may prevent dental caries and periodontal diseases by inhibiting oral harmful bacteria. The probiotics market was estimated to be USD 49.4 billion in 2018 and is projected to grow at a CAGR of 7.0%, reaching USD 69.3 billion by 2023. Consumption of probiotic food and beverages has increased, and consumer understanding of the potential benefits of probiotics has also increased [25]. Products containing prebiotics, probiotics or symbiotics continue to appear on the oral health market in addition to oral hygiene products containing fluorides and oral biofilm protection components [26]. Recently, interest by experts and consumers in pre-and probiotics has expanded, increasing the significance of lactic acid bacteria products' safety.

The Korean Ministry of Food and Drug Health requires manufacturers to submit information on the sources of microorganisms used in foods and drugs to allow for food product authorization and regulatory review for pharmaceutics. It has also developed a standard framework for quality assurance of these goods. The National Institute of Food and Drug Safety Evaluation has also laid out standard guidelines for the quality assurance of probiotics products [27]. An estimation of the safety level of isolated bacteria for novel clinical probiotic use is important for the identification and elimination of possible clinical pathogens.

In this study, *Lactobacillus* was evaluated as a means of controlling the oral microorganisms which cause oral disease. *Lactobacillus* candidates that inhibit the growth of harmful oral bacteria were collected from the saliva and feces of adults over 20 years of age. One competitor species was identified, and safety tests were carried out following Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO)- suggested studies and other published safety tests to verify its safety for academic and commercial applications [28].

Materials And Methods

Isolation of lactic acid bacteria inhibiting oral harmful bacteria

Samples of saliva were taken from 200 adults over 20 years of age with little or no supragingival plaque, enrolled in Seoul National University's Food Microbiological Research Laboratory (IRB No. 1907/003-018). Before brushing and before meals, samples were taken in the morning, combined with sterile 70 percent glycerol (1:1), and refrigerated before tested. Fecal samples were taken from 28 people over 20 years of age recruited from the Catholic University of Korea, Seoul St. Mary's Hospital (IRB KC17TNSI0570). Feces was collected in stool containers and stored frozen until analyzed. *Lactobacillus* sp. with excellent hydrogen peroxide production were selected using the process defined by Eschenbach et al [29] and John F. T. Spencer [30]. In keeping with their methods, two solutions, A and B, were prepared to add tetra methyl benzidine (3.3', 5.5'-tetra methyl benzidine or TMB) agar as a screening agent. Solution A consisted of 3 mL of DMSO (dimethyl sulfoxide, Sigma, St Louis, MO, USA) and 12.5 mg of TMB (Sigma, St Louis, MO). Solution B (0.5 mg/mL or 100 U/mL peroxidase) consisted of 1 mL of sterilized water and 0.5 mg of peroxidase (Sigma, St Louis, MO, USA). de Man-Rogosa-Sharpe (MRS, BD Difco™, Franklin Lakes, NJ, USA) agar was sterilized at 121 ℃ for 15 min and brought to 45 ℃. 0.6 mL of Solution A and 0.2 mL of Solution B per 10 mL of MRS agar was added to create the final selective agar medium. The mixture was poured and allowed to solidify on plates. Each selection plate, therefore, had a concentration of 1 mM TMB and 10 µg/mL (2 U/mL) of peroxidase.

To isolate lactic acid bacteria producing hydrogen peroxide, 100 µL or µg of each obtained sample was diluted serially with sterilized phosphate buffered saline (PBS, pH 7.4). The diluted solution was then distributed across the TMB

selective agar plates. After 48 hours of anaerobic incubation at 37 °C, the samples were exposed to 15–30 minutes of oxygen to allow for indicator response. Horseradish peroxidase induces oxidation of TMB by bacteria via the production of hydrogen peroxide, forming a blue colony. If the colony was blue, it was assessed as producing hydrogen peroxide (+). Navy blue was considered to be strongly positive (+ + +), blue to be positive (+ +), sky blue to be weakly positive (+), and if there was no change in color, negative (-). Ten single colonies of blue color formed on each medium were randomly chosen for multiple streaking onto new TMB agar plates to separate 500 single colonies.

Lactobacillus colonies were segregated utilizing *Lactobacillus* collection agar (LBS, BD DifcoTM, Franklin Lakes, NJ, USA) and a broth dilution test was performed to select bacteria that displayed the greatest antibacterial activity against oral microorganisms.

In total, 50 isolates were incubated at 37 °C in 5 ml of traditional MRS broth for 24 hours, and the supernatants were recovered by centrifugation at 4 °C for 5 minutes at 25,000×g. To remove the antimicrobial influence of organic acids, the supernatants were adjusted to pH 6.5 ~ 7 with 1M syringe sterilized (0.45 μm) NaOH and stored at -20 °C before analysis. Two hundred μL of MRS broth was added to 96-well plates (SPL Life Sciences Co., Ltd. Pocheon, Korea) and 11 types of oral microorganisms were inoculated into each well. Ten μL of each isolated strain cultivation supernatant was applied to individual wells, and the plates were anaerobically incubated at 37 °C. The wells were read at 600 nm for up to 24 hours using a microplate reader (Epoch2, BioTek, Winooski, VT, USA).

One isolated *Lactobacillus* was designated as "HHuMIN D" based on excellent antibacterial activity against oral microorganisms. 16S rRNA sequencing was conducted to genetically identify the isolated bacteria, followed by base-sequence molecular phylogenetic analysis. The organism was identified as *L. gasseri* and named " *L. gasseri* HHuMIN D ".

Bacterial strains and growth conditions

In this study, four oral anaerobic bacteria (*Fusobacterium nucleatum* KCOM 1001, *Porphyromonas gingivalis* KCOM 2796, *Prevotellaintermedia* KCOM 2889, and *Porphyromonas catoniae* KCOM3169) and seven oral facultative anaerobic bacteria (*Streptococcussobrinus* KCOM1157, *Streptococcus mitis* KCOM 1356, *Streptococcus oralis* KCOM 1493, *Streptococcus gordonii* KCOM 1788, *Streptococcus sanguinis* KCOM 2167, *Streptococcus parasanguinis* KCOM 2522, and *Streptococcus mutans* KCTC3065) from the Korean Collections of Oral Microbiology were utilized.

F. nucleatum, P. gingivalis, *P. intermedia*, and *P. catoniae* KCOM3169 were individually incubated in KCOM broth (Brain Heart Infusion (BHI, BD Difco™, Franklin Lakes, NJ, USA) with 0.5% yeast extract (BD Difco™, Franklin Lakes, NJ, USA), 0.05% Cysteine HCl-H2O (Sigma, St Louis, MO, USA), 0.025% Resazurin (Sigma, St Louis, MO, USA), 5 mg/mL Hemin solution (Sigma, St Louis, MO, USA), and 10 mg/mL vitamin K1 solution (Sigma, St Louis, MO, USA) for 24 hours at 37 °C under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). *S. sobrinus* KCOM1157, *S. mitis* KCOM 1356, *S. oralis* KCOM 1493, *S. gordonii* KCOM 1788, *S. sanguinis* KCOM 2167, *S. parasanguinis* KCOM 2522, and *S. mutans* KCTC3065 were individually grown in BHI broth under aerobic conditions for 24 hours at 37 ℃ before use. *L. gasseri* HHuMIN D was inoculated into MRS broth and incubated at 37 ° C for 24 hours. The culture broth of *L. gasseri* HHuMIN D was placed in 50% (v/v) glycerol solution and stored at -80 °C. Prior to use in each experiment, strains were subcultured twice in the appropriate medium. *Weissella* cibaria was used as a control probiotic *lactobacillus* in this study, and the culture conditions were the same as *L. gasseri* HHuMIN D.

Evaluation of the effect of *L. gasseri* HHuMIN Don oral microorganisms

To test the impact of *L. gasseri* HHuMIN D on oral microorganisms, viable cell counts were carried out after co-culture using selective agar plates. Oral anaerobic and facultative anaerobic bacteria were inoculated at 5% into individual 5ml conical tubes containing 2 mL of KCOM broth and BHI broth. Then, *L. gasseri* HHuMIN D was inoculated at 5% into the same tubes. The tubes were incubated at 37 °C for 24 hours, followed by viable cell counts for each bacterium to validate

the effect of *L. gasseri* HHuMIN D on their growth by serial dilution and plating on KCOM agar for oral anaerobes, MSB (Mitis Salivarius Sucrose Bacitracin, Sigma-Aldrich, St. Louis, MO, USA) agar for oral facultative anaerobic bacteria and LBS agar (Sigma-Aldrich, St. Louis, MO, USA) for *L. gasseri* HHuMIN D.

Hydrogen peroxide production test

L. gasseri HHuMIN D was subcultured twice in MRS broth at 37 °C without agitation and incubated at 37 °C on a shaker table at 50 rpm for up to 24 hours. The culture medium was centrifuged for 10 minutes at 4 ° C at 2000×g, and the supernatant extracted. Supernatants were colorimetrically analyzed using Pierce Quantitative Peroxide Assay Kits (aqueous-compatible formulation, Thermo Fisher Scientific, Waltham, MA, USA). Two hundred µL of working reagent and 20 µL of supernatant were placed into individual cells of a 96-well microplate and allowed to develop for fifteen minutes. The optical density of each well was then determined at 595 nm with a microplate reader (Epoch2, BioTek, Winooski, VT, USA). A 1mM (1000µM) solution of hydrogen peroxide was produced to standardize hydrogen peroxide production by diluting a 30% H_2O_2 supply 1:9000 (11µL of 30% H_2O_2 into 100mL of ultrapure water). The 1 mM hydrogen peroxide solution was then serially diluted 1:2 with sterile water (100 µL of sterile water + 100 µL of 1 mM hydrogen peroxide). Two hundred µL of the working reagent was then added to 20 µL of the diluted H_2O_2 standard in microplate wells and the absorbance was assessed via plate reader at 595 nm.

Susceptibility assay of the bacteriocin produced by *L. gasserie* HHuMIN D to hydrolytic enzymes

Proteins, carbohydrates and lipolytic enzymes were purchased from Sigma Aldrich (St. Louis, MO, USA) and used according to the manufacturer's instructions. Lipase (4307 U/mg) and trypsin (13,50 U/mg) in 50 mM Tris-HCl buffer (pH 7.5), α-chymotrypsin (83.9 U/mg) and carboxypeptidase A (73 U/mg) in 50 mM Tris-HCl (pH 8.0), pepsin (3.280 U/mg) in 10 mM citrate (pH 6.0), α-amylase (519 U/mg) in 0.1 M sodium phosphate (pH 7.0) and proteinase K (30 U) /mg) in 0.01 M Tris-HCl (pH 7.9), 0.05 M EDTA and 0.5% SDS buffer were prepared at a concentration of 20 mg/mL. The enzymes were added to the culture supernatant at 10% (2 mg/mL) and reacted at 37 ℃ for 150 minutes except for proteinase K, which was reacted at 45 ℃ for 12 hours. The remaining bacteriocin activity was measured using a microtiter plate for oral harmful bacteria. The group not treated with an enzyme solution under the same conditions was used as a negative control [31].

Coaggregation assays

L. gasseri HHuMIN D coaggregation was tested using oral microorganisms according to a modified Handley et al [32] spectrophotometric assay. *L. gasseri* HHuMIN D was harvested by centrifuging the culture medium at 4000×g for 15 minutes. Pellets were washed with Cisar's buffer (1 mM Tris (hydroxymethyl) aminomethane (pH 8.0), 100 µM $CaCl_2$, 100 µM $MgCl_2$, and 0.15 M NaCl) three times, and resuspended to approximately 10^9 cells/mL. One ml of the coaggregation pairs were vortexed for 10 seconds and incubated with soft stirring at 110 rpm at 37 °C for 30 min. The coaggregations were then allowed to remain at room temperature for 3 minutes after incubation. One half ml of supernatant was removed from the culture and the optical density at 660 nm was determined using a microplate reader (Epoch2, BioTek, Winooski, VT, USA).

Coaggregation was determined using the decrease in absorbance vs. the control. The proportion of coaggregation was determined using the equation by Handley et al (1987):

$$\text{Coaggregation (\%)} = \frac{OD_{660}(\text{X control} + \text{Y control})/2 - OD_{660}(\text{X} + \text{Y})}{OD_{660}\ (\text{X control} + \text{Y control})/2} \times 100$$

where X control and Y control represent OD_{660} of the two microorganisms in the control tubes and (X + Y) is the mixture of organisms.

Effect of *L. gasseri* HHuMIN D on the formation of artificial dental plaque by *S. mutans*

To validate the degree to which *L. gasseri* HHuMIN D prevents the development of artificial dental plaque by *S. mutans*, beaker wire tests following the protocol of Yu, et al [33] were conducted. Five percent sucrose was added to 40 ml of broth consisting of equivalent quantities of BHI and MRS broth and the pH was adjusted to 6.9 to 7.0 by the addition of 0.1 M MOPS buffer. 1×10^8 *S. mutans* and 1×10^8 *L. gasseri* HHuMIN D cells were inoculated alone or in combination in the medium. Stainless steel orthodontic wires (4 cm length, 0.016-inch, 1 mm dia, Oramco, Glendora, CA, USA) were suspended on conical tubing and immersed in the broth. The weights of the artificial dental plaques produced on the wires were calculated after 12 hours of incubation at 37 ° C while shaking at 30 rpm.

Adhesion test

Adhesion assay of *L. gasseri* HHuMIN D

Preparation of adhesion assay

KB cell line oral epithelial cells (KCLB 10017) were acquired from the Cell Line Bank of Korea (Seoul, Korea). KB cells were cultured in RPMI-1640 Media (Sigma-Aldrich, St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and 1% (v/v) antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO_2 at 37 ℃. For the adhesion efficiency assay, KB cells were inoculated into a minimum critical medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) containing 10 percent (v/v) FBS at $1Í10^5$ cells/well. Upon transfer to a 24-well multi-culture dish (Thermo Fisher Scientific, St Peters, MO, USA), the cells were grown for 18 hours at 5% CO_2 at 37 °C.

L. gasseri HHuMIN D was grown for 18 hours in MRS broth at 37 °C and the bacterial concentration was adjusted to 10^8 CFU/mL (measured at 600 nm). Isolated bacteria were centrifuged at 8000×g for 3 minutes and washed with PBS three times. The washed bacteria were resuspended in MEM media without antibiotics. The number of cells measured by hemocytometer (Paul Marienfeld, Lauda-Königshofen, Germany) was about 6 log cells per well.

Inoculation of isolated bacteria into cells

L. gasseri HHuMIN D cells were added to the 24-well culture dish cells containing KB cells at about 6 log cells per well and incubated for 1 hour at 37 °C and 5% CO_2. To extract unattached cells, the suspension was eliminated after incubation and the 24-well plates were washed three times with PBS (pH 7.4). The cell monolayer was washed twice with PBS to further eliminate unadhered bacteria. For 10 minutes, 200 µL trypsin/EDTA (Welgene, Daegu, Korea) was applied to the cell monolayer, and the cells and attached bacteria were collected. MG cell Genomic DNA Extraction SV kits (Doctor Protein, Seoul, Korea) were used to remove the genomic DNA (gDNA) of the attached bacteria in the extracted pellets. Pending analysis, the final volume of all DNA samples (200 µL) was stored at -20 °C. The number of bacteria adhered to the KB cells was determined using qRT-PCR.

Competition between *L. gasseri* HHuMIN Dand pathogens for cell adhesion

Protection and displacement assays were conducted to assess the competition between *L. gasseri* HHuMIN D and harmful oral bacteria (*F. nucleatum* and *S. mutans*) using modified versions of a previously reported process [34]. In protection assays, 1 mL ($1Í10^8$ CFU/mL) of *L. gasseri* HHuMIN D was applied to 24-well culture dishes loaded with KB

cells and incubated for 1 hour at 37 °C and 5% CO_2 for 1 hour. The suspension was removed after incubation, and the cell monolayer was washed twice with PBS. Next, 1 mL (1Í10^8 CFU/mL) of a harmful oral bacteria suspension (*F. nucleatum* and *S. mutans*) was applied to the wells and incubated for 1 hour at 37 °C and 5% CO_2. The suspension was removed, and the cell monolayer was washed twice with PBS to extract bacteria that had not been adhered. Two hundred µL of trypsin/EDTA (Welgene, Daegu, Korea) was added to the cell monolayer and allowed to stand for 10 minutes, after which the cells and attached bacteria were gathered.

In displacement assays, 1 mL (1Í10^8 CFU/mL) of a harmful oral bacteria suspension (*F. nucleatum* and *S. mutans*) was applied to 24-well KB cells and incubated for 1 hour at 37 °C and 5% CO_2 for 1 hour. The suspension was removed after incubation, and the monolayer cell was washed twice with PBS. Next, 1 mL (1Í10^8 CFU/mL) of *L. gasseri* HHuMIN D was applied to the wells and incubated for 1 hour at 37 °C and 5% CO_2. The suspension was removed after the second inoculation, and the cell monolayer was washed twice with PBS to extract bacteria that had not been adhered. Two hundred µL of trypsin/EDTA (Welgene, Daegu, Korea) was added to the cell monolayer and allowed to stand for 10 minutes, after which the cells and attached bacteria were gathered.

Wells containing only *F. nucleatum* and *S. mutans* were used as controls, and three replications of both test types were performed. The number of bacteria appended to each KB cell was determined using real-time PCR. An MG cell Genomic DNA Extraction SV kit (Doctor Protein, Seoul, Korea) was used to extract the genomic DNA (gDNA) of the attached bacteria in the harvested pellets. The final volume of 200 µL of all DNA samples was held at -20 °C before analysis.

Quantitative detection of bacteria using real-time PCR

The collected gDNA was used to quantitatively analyze each bacterium that was attached to the KB cells using real-time PCR. The isolated bacteria (*L. gasseri* HHuMIN D) and harmful bacteria (*F. nucleatum* and *S. mutans*) were cultured for 18 hours in each medium at 37 °C for standardization of PCR conditions, and the number of bacteria in the standard strain was determined. Next, gDNA was isolated from 1 ml of the same culture solution using MG cell Genomic DNA Extraction SV kits (Doctor Protein, Seoul, Korea), and serially diluted from 10^1 to 10^8 to build a standard curve for real-time PCR quantification. Standard curves were drawn using bacterium-specific primers, and the bacteria were quantified to test cell adhesion from the extracted DNA. A standard curve was drawn using primers specific to each bacterium, and bacteria were quantified from the extracted DNA to measure cell adhesion. The primers used to amplify the bacteria are shown in Table 1. Real-time PCR was conducted on 96-well plates plates (SPL Life Sciences Co., Ltd. Pocheon, Korea) using tepOnePlus™ Real Time PCR systems (Thermo Fisher Science, St Peters, MO, USA). The PCR reaction mixture contained 1×TB Green™ Premix Ex Taq™ (Takara, Japan), 10 µM of each primer, 2 µL of DNA and distilled water to a final volume of 20 µL. Real-time PCR conditions for the first denaturation were 10 minutes at 95 °C, and then 40 reaction cycles in the polymerase chain were conducted at 95 °C for 15 seconds and 60 °C for 1 minute. Analysis of the results was carried out using StepOne ™ and StepOnePlus ™ Software v2.3 (Thermo Fisher Scientific, St Peters, MO, USA). The number of bacteria was calculated by converting the value of the cycle threshold (CT) into the standard curve.

Safety assessment of *L. gasseri* HHuMIN D

Ammonia production test

L. gasseri HHuMIN D was cultured anaerobically in BHI media at 37 °C for 5 days. The production test for ammonia by catalyzed indophenol reaction was conducted [35]. To assess the production of ammonia, the culture medium was centrifuged (2236R centrifuge; Labogene Aps, Lillerød, Denmark) at 10,000×g for 30 minutes to isolate the supernatant, which was adjusted after extraction to pH 7.0 with 1 N NaOH.

Two solutions were prepared: Solution A consisted of 0.01 g of sodium nitroferricyanide dihydrate (Sigma, St. Louis, MO, USA) and 2 g of phenol (Sigma, St. Louis, MO, USA) in 200 mL LC-MS grade water, and Solution B consisted of 1 g of sodium hydroxide and 0.08 g of sodium hypochlorite in 200 mL LC-MS grade water. One hundred µL of *L. gasseri* HHuMIN D supernatant was dispensed into 96-well plates, followed by 10 µL each of solutions A and B, and incubated for 1 hour at 20°C. Using a microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA) the optical diameter was measured at 625 nm. Uninoculated BHI medium was used as a control and a standard curve was used to measure ammonia concentration. *Enterococcus faecium* ATCC19433 supernatant was employed as a positive control.

Biogenic amine production test

L. gasseri HHuMIN D biogenic amine analysis was conducted using the method of Kim et al [36]. Four biogenic amines, cadaverine (≧ 97.0%), histamine (≧ 97.0%), putrescine (≧ 98.5%), and tyramine (≧ 99.0%), were purchased from Sigma-Aldrich (St. Louis, MO, USA), as were 1.7-diaminoheptane (internal standard; ISTD, 98%, Cat. # D174708), dansyl chloride (≥99.0%, Cat. # 39220), and L-proline (≥99.0%, Cat. # P0380). Whatman No. 4 filter paper was purchased from Whatman Intl., Ltd (Maidstone, UK). Sodium carbonate (99.0%, Cat. # 433401201), ether (99.0%, Cat. # 33475S1280), and acetone (99.7%, Cat. # A0108) were purchased from Samchun Pure Chemical Co., Ltd. (Pyeongtaek, Korea).

L. gasseri HHuMIN D was grown anaerobically in MRS broth with 0.05 percent (w/w) L-cysteine-HCl (Sigma, St. Louis, MO, USA) at 37 ℃ for 18 hours. Next, 25 mL 0.1 N HCl was added to 5 mL of *L. gasseri* HHuMIN D supernatant and blended for 5 minutes. The supernatant was separated by centrifugation (2236 R centrifuge; Labogene Aps, Lillerød, Denmark) at 10,000×g for 15 minutes. The gathered supernatant was filtered through a 20–25 µm membrane filter (Whatman Int'l., Ltd., Maidstone, UK) and 1 mL of filtrate was combined with 0.1 mL of 0.01% (w/v) 1.7-diaminoheptane, 0.5 mL of saturated sodium carbonate solution, and 1 mL of 1% dansyl chloride. The mixture was incubated in a water bath (WBC 1510A; Jeio Tech. Co., Ltd., Seoul, Korea) after thorough mixing, with light blocked at 45 ℃ for 1 hour. By adding 0.5 mL of 10 percent proline and 5 mL diethyl ether at room temperature and allowing it to stand for 5 minutes, the remaining dansyl chloride was removed. The supernatant was concentrated using a Scanvac Speed Vacuum Concentrator (Labogene Aps, Lillerød, Denmark) at 1500 rpm and 20 ° C until dry for high performance liquid chromatography (HPLC) analysis. Next, the dry pellets were resuspended in 1 mL of acetonitrile (Sigma-Aldrich, St. Louis, MO, US). After filtering through a 0.22-µm filter membrane, the resuspended sample and standard were analyzed. A Thermo Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, St Peters, MO, USA) with a VDSpher C-18 column (4.6 Í 250 mm, 5 µm) (VDS Optilab Chromatographie Technik GmbH, Berlin, Germany) was used to analyze the samples. The mobile phase was (A) acetonitrile, (B) distilled water (0-1 min, A: B = 40: 60; 1 - 25 minutes, A: B = 100: 0; 25 - 35 minutes, A: B = 60: 40). The injection volume was 20 µL, the flow rate was 0.8 mL/min, the column temperature was 30 ℃ (constant), and the UV detection wavelength was 250 nm.

Hemolytic test

Hemolysis testing of *L. gasseri* HHuMIN D was conducted according to the method of Kim et al [36]. Hemolysis was observed by anaerobic incubation of *L. gasseri* HHuMIN D in BL agar (BD Difco™, Franklin Lakes, NJ, USA) for 2 days at 37 °C with 5 percent sheep blood. As a positive control, *Listeria ivanovii* subsp. ivanovii ATCC 19119 was incubated on the same agar for 2 days under aerobic conditions at 37 °C. The presence or absence of light visible through both sides of the plates indicated hemolysis. Strains that did not produce green areas around the colonies (α-hemolysis) or did not cause hemolysis in the blood plate (γ-hemolysis) were considered nonhemolytic. Strains having a clear zone around the colonies were classified as β-hemolytic bacteria.

Mucin degradation test

L. gasseri HHuMIN D degradation of mucins was estimated using the method of Kim et al [36]. Type III porcine stomach mucin was purchased from Sigma-Aldrich (St. Louis, MO, USA). As a negative control, MRS medium was used without a carbon source. Four MRS mediums with different carbon sources were prepared for analysis: 0.5% (w/v) mucin, 1.0% (w/v) mucin, 0.5% (w/v) glucose, and 1% (w/v) glucose. Each broth was inoculated with *L. gasseri* HHuMIN D and anaerobically incubated 2 days at 37 °C. Strain growth was estimated using a microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA) at 0, 12, 24, 36, and 48 hours by measuring the optical density at 550 nm. By subtracting the final optical density value of each sample from the original optical density value, the growth in each broth was determined.

Antimicrobial susceptibility test

Research has shown that antibiotic resistance may be present in *Lactobacillus* spp. [37]. Since antibiotic resistance can be transmitted via plasmids, the evaluation of antibiotic resistance is an important consideration for the safety assessment of *Lactobacillus* spp. [38]. *L. gasseri* HHuMIN D antimicrobial susceptibility was estimated using the method of Kim et al [36]. Twenty-three antimicrobial compounds were tested in this experiment: ampicillin sodium salt, bacitracin, carbenicillin disodium salt, cephalothin sodium salt, chloramphenicol, clindamycin hydrochloride, dicloxacillin sodium salt hydrate, erythromycin, gentamicin sulfate, kanamycin sulfate, lincomycin, methicillin, metronidazole, mupirocin, neomycin sulfate, penicillin G, phosphomycin disodium salt, polymyxin B sulfate salt, rifampicin, streptomycin sulfate salt, tetracycline, trimethoprim–sulfamethoxazole (trimethoprim), and vancomycin hydrochloride.

All antimicrobials were purchased from Sigma (St. Louis, MO, USA) except vancomycin hydrochloride, which was purchased from USP, Bethesda, Maryland, USA. Before being added to LSM-Cys broth (LAB susceptibility test medium with L-cysteine, consisting of 90 percent IST and 10 percent MRS broth medium), each antibiotic powder was dissolved in a suitable diluent, sterilized and filtered. IST was purchased from Kisan Bio Co., Ltd. (Mb cell I so-Sensitest Broth, Seoul, Korea), and MRS was purchased from Becton, Dickinson and Company (BD Difco™ MRS *Lactobacilli* broth, Franklin Lakes, NJ, USA). The ISO10932: 2010 minimal inhibitory concentration (MIC) values for the 20 antibiotics tested on *L. gasseri* HHuMIN D were used.

For this experiment, LSM-Cys (LAB susceptibility test) medium containing 0.03% (w/v) L-cysteine HCl was used with 96-well plates. Upon diluting serially twice, the antibiotic broth used ranged from 0.0032 - 1024 g/mL. When tested with a microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA), the inoculum was adjusted to a turbidity of 0.16 - 0.2 at 625 nm. The final concentration was approximately 3Í10^8 cfu/mL. Finally, the microorganisms were inoculated at a rate of 0.2% in the LSM-Cys media and 50 µL of each diluted medium was inoculated into each well. There was no negative control. The dilute medium was anaerobically incubated at 37 °C for 48 hours. The MIC was visually determined by comparing the test samples to the control; antibiotic susceptibility was considered to be the prevention of bacterial growth by 80% or more.

Complete genome sequencing, assembly, and annotation of *L. gasseri* HHuMIN D

Chunlab, Inc. (Seoul, Korea), which used PacBio Sequel Systems (Pacific Biosciences, Menlo Park, CA, USA) and analyzed using CLgenomicsTM and EZBioCloud Apps services (Chunlab, Seoul, Korea), conducted whole genome sequencing of *L. gasseri* HHuMIN D. General gene information was investigated by EZBiodCloud Apps and functional annotations for the expected genes were analyzed using RefSeq protein (NR; NCBI), Clusters of Orthologous Groups of proteins (COG), EggNOG, SEED, Swiss-Prot, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases [39-42].

Safety evaluation with complete genome sequence

Analysis of virulence factors was carried out through the VirulenceFinder 2.0 Website, which is a part of the openly accessible web-based tool for whole genome sequencing (WGS) analysis hosted by the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/, 2014). The predicted *L. gasseri* HHuMIN D genes were compared with the

comprehensive antibiotic resistance database (CARD, http://arpcard.mcmaster.ca) for identifying antibiotic resistance (https://card.mcmaster.ca). The ability to synthesize biogenic amines and aggregate platelets was searched using full genome sequencing of *L. gasseri* HHuMIN D via CLGenomics (ChunLab, Seoul, South Korea, 2004) through matching with the KEGG pathway.

Statistical analysis

Data are expressed as mean ± SEM by SPSS software (IBM SPSS Statistics, Version 25, SPSS Inc., Chicago, IL, USA). Differences between the means were measured by one-way ANOVA using Duncan's multiple range test. Statistical significance was assumed at $p < 0.05$.

Results And Discussion

Identification of hydrogen peroxide-generating *lactobacilli*

The screening of *Lactobacillus* progressed in a three-step process. First, 500 strains with excellent development of hydrogen peroxide were isolated from saliva and feces. Second, *Lactobacillus* sp. were collected using LBS agar plates to sort through the 500 isolated strains. A total of 50 isolates were identified. Finally, the 50 isolates were tested for their strength against oral microorganisms, and *L. gasseri* HHuMIN D was the most effective. In fact, *L. gasseri* HHuMIN D severely inhibited the growth of oral anaerobia, which are known to cause halitosis (data not shown).

L. gasseri HHuMIN D was shown to produce hydrogen peroxide and suppress oral microorganisms. 16S rRNA gene sequencing of *L. gasseri* HHuMIN D showed that it was a *Lactobacillus* sp. Multiple alignment of 16S rRNA base sequences of isolated bacteria with the *Lactobacillus* sp. 16S rRNA sequence was obtained through the similarity matrix listed on GenBank. This genealogy research showed a 99% 16S rRNA homology; *L. gasseri* HHuMIN D shares 99% homology with *L. gasseri*.

Lactobacillus species isolated from the oral cavity vary widely and representative oral lactic acid bacteria include *L. acidophilus, L. casei, L. fermentum, L. gasseri, L. johnsonii, L. rhamnosus, L. reuteri, L. salivarius* and *L. vaginalis* [43-46]. These probiotic strains are normally used in dairy and health/functional food products. These results indicate that candidate probiotic *Lactobacillus* strains are present in the oral cavity. The current research confirms the probiotic capabilities of *L. gasseri* HHuMIN D. The antimicrobial effect of 5% of HHuMIN D supernatant was clearly demonstrated in the control of anaerobic oral bacteria causing halitosis. *P. catoniae, P. intermedia, F. nucleatum,* and *P. gingivalis were* inhibited by 90%, 89 ± 1%, 88 ± 1%, and 88 ± 1%, respectively. *S. mutans, a* dental caries-inducing bacterium, was inhibited by 60 ± 2%. *S. mitis* and *S. gordonii,* which cause periodontitis, were inhibited by 22 ± 2% and 19 ± 5%, respectively. Antimicrobial activity was lower against oral facultative anaerobic bacteria (*S. sobrinus, S. sanguinis, S. parasanguinis*, and *S. oralis*). On the other hand, the antimicrobial effect of 5% of *W. cibaria* culture supernatant showed relatively weaker antimicrobial activity than *L. gasseri* HHuMIN D (Table 2).

Lactobacillus culture medium neutralization and disinfection is an experimental process used to monitor the antibacterial activity of bacteria metabolites, such as bacteriocin. In another study, sterile neutral *S. mutans* ATCC 25175 supernatant showed >80% inhibition of *L. paracasei* strains [47]. Another study confirmed that *Lactobacillusgasseri*, which produces gassericin A, has inhibitory properties against a wide range of oral pathogens, including carcinogenic and periodontal pathogens [48]. In this study, the strong inhibitory effect of the culture supernatant of *L. gasseri* HHuMIN D against oral anaerobic pathogens was confirmed, and *L. gasseri* HHuMIN D's significant antibacterial activity was confirmed.

Inhibition effect of *L. gasseri* HHuMIN D on oral microorganisms

Oral lactic acid bacteria are known to inhibit harmful oral bacteria by the production of various antibiotics [49]. *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Streptomyces* spp. are the majority of the oral probiotics studied to date*. L. gasseri* has also been studied as an oral probiotic and there are results similar to the findings of this research that *L. gasseri* is effective against harmful oral bacteria [50,51].

The interactions between *L. gasseri* HHuMIN D and oral anaerobic (*F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *P. catoniae*) and facultative anaerobic bacteria (*S. sobrinus* KCOM1157, *S. mitis* KCOM 1356, *S. oralis* KCOM 1493, *S. gordonii* KCOM 1788, *S. sanguinis* KCOM 2167, *S. parasanguinis* KCOM 2522, and *S. mutans* KCTC3065) are shown in Fig. 1. All harmful oral bacteria were strongly inhibited by *L. gasseri* HHuMIN D, but interaction with oral microorganisms also impaired the proliferation of *L. gasseri* HHuMIN D. Compared to the control, *L. gasseri* HHuMIN D showed poor growth in most co-cultures. Among them, the lowest viable cell counts occurred when *L. gasseri* HHuMIN D was co-cultured with *P. intermedia*.

A previous study on the co-culture of oral anaerobia (*F. nucleatum, P. gingivalis*) and beneficial oral bacteria *Weissella cibaria* showed that *F. nucleatum* reduced the number of viable cells up to 1.5 Í 10^8 CFU/mL, and *P. gingivalis* reduced the number of viable cells to <10^5 CFU/mL, compared to controls [21]. In this experiment *L. gasseri* HHuMIN D inhibited *F. nucleatum* more effectively. Additionally, *L. gasseri* HHuMIN D inhibited *P. intermedia*, *P. catoniae* and seven facultative anaerobic bacteria (*S. sobrinus* KCOM1157, *S. mitis* KCOM 1356, *S. oralis* KCOM 1493, *S. gordonii* KCOM 1788, *S. sanguinis* KCOM 2167, *S. parasanguinis* KCOM 2522, and *S. mutans* KCTC3065) quite effectively.

In another study, *Lactobacillus salivarius*, *Lactobacillus fermentum,* and the fermentation broths of these bacteria showed a definite inhibitory effect against harmful periodontal bacteria. *Lactobacillus* spp. showed greater direct antibacterial effects against harmful oral bacteria than their microbial supernatants. As the number of lactic acid bacteria and the concentration of the fermentation broth increased, the antibacterial effect also increased [52]. The current research results are similar to those of Chen et al., [52] : *L. gasseri* HHuMIN D's antibacterial effect was greater than *L. gasseri* HHuMIN D's supernatant antibacterial effect. This indicates the direct inhibition of harmful bacteria by *L. gasseri* HHuMIN D and indirect inhibition by the metabolites it produces [45, 53]. *L. gasseri* HHuMIN D strongly inhibited all harmful oral bacteria, but the supernatant of this bacteria inhibited only anaerobic oral bacteria and had different effects. The combination of these two actions efficiently kills pathogenic microorganisms in the oral environment. The results of this study indicate that *L. gasseri* HHuMIN D can affect various oral diseases caused by periodontal bacteria. Consuming probiotic or lactic acid bacteria-containing health/functional foods can prevent or treat periodontal disease [45].

Accumulation of hydrogen peroxide by *L. gasseri* HHuMIN D

Oral *Lactobacillus* spp. produces bacteriocins, lactic acid and hydrogen peroxide, which act as a defense against pathogens [54,55]. The generation of hydrogen peroxide is a typical function of *Lactobacillus* spp. (*L. bulgaricus, L. lactis*, and *L. plantarum*) isolated from the oral cavity. Several studies have shown that these beneficial bacteria inhibit the growth of pathogenic micro-organisms such as *Staphylococcus aureus* [56]*, Pseudomonas* spp*.* [57], and psychrotrophic bacteria [58,59]. In the process of separating *Lactobacilli* from saliva and feces, colony-colored strains were visually chosen using selective agar plates for *Lactobacillus* with outstanding production of hydrogen peroxide. A Pierce Quantitative Peroxide Assay Kit (aqueous-compatible formulation, Thermo Fisher Scientific, Waltham, MA, USA) was used to test quantitatively using the colorimetric process (Fig. 2). The hydrogen peroxide produced by *L. gasseri* HHuMIN D increased continuously from 0 µmol/L to 802 µmol/L after 3 hours. After 12 hours, the concentration of hydrogen peroxide decreased steadily to 24 hours. *L. johnsonii* and *L. gasseri* have been shown to produce hydrogen peroxide at rates between 400 and 1400 µmol/L [60]. Other research found that *S. sanguis* released hydrogen peroxide up to 30 µmol/L, *S. oralis* released hydrogen peroxide up to 640 µmol/L and both inhibited plaque formation by inhibiting *S. mutans* proliferation. The application of lactoperoxidase and thiocyanate to oxygen-supplied cultures revealed that *S. mutans* was inhibited, but did not affect the growth of, *S. sanguis* and *S. oralis* [61]. Additional research contends that

H_2O_2 produced by beneficial bacteria can decrease halitosis by inhibiting the growth of *F. nucleatum* and reducing the VSC produced by oral anaerobic bacteria [62].

L. gasseri HHuMIN D exhibits fairly good hydrogen peroxide production ability and is predicted to suppress harmful anaerobic bacteria that are vulnerable to hydrogen peroxide by continuous production. Further experimentation will be necessary to specifically investigate the effect of *L. gasseri* HHuMIN D's H_2O_2 on oral anaerobic bacteria.

Susceptibility of the bacteriocin produced by *L. gasserie* HHuMIN D to hydrolytic enzymes

Several types of hydrolytic enzymes were added to the partially purified bacteriocin solution at a concentration of 10%, reacted, and the residual activity of the antibacterial substance was measured. Antibacterial activity was completely lost by proteinase K, trypsin, and α-chymotrypsin treatment, indicating that the antibacterial active substance is proteinaceous, and possibly bacteriocin (Table 3). The fact that the activity was not lost by pepsin is possibly an enzyme concentration issue or indicative of the lack of a specific cleavage site for pepsin to react at. Since α-amylase did not affect the antibacterial activity, the carbohydrate moiety was either not present in the antibacterial molecule or not related to the antibacterial activity. Bacteriocins in which antibacterial activity is lost only with proteolytic enzyme treatment have previously been reported. For example, the same results were found in the case of *Bacillus licheniformis* [63].

The greatest advantage of bacteriocin is that it is composed of proteins or peptides. Bacteriocin is decomposed by proteolytic enzymes in the digestive tract of the human body, so it is considered to be non-toxic and non-persistent [64-67].

Coaggregation of *L. gasserie* HHuMIN D and oral microorganisms

In this research we evaluated the coaggregation of *L. gasseri* HHuMIN D with various oral microorganisms and found that *L. gasseri* HHuMIN D showed the highest cohesion with *P. catoniae* (70%) and *P. intermedia* (28 ± 5%). There was no cohesion with *P. gingivalis* or *F. nucleatum.* Amongst aerobic oral bacteria, *L. gasseri* HHuMIN D coaggregated with *S. sanguinis* best (74 ± 2%), followed by *S. gordonii* (62 ± 1%), *S. mutans* (49 ± 4%), *S. mitis* (46 ± 3%), *S.sobrinus* (37 ± 2%), *S. oralis* (30 ± 2%), and *S. parasanguinis* (6 ± 3%) However, the coaggregation of *W. cibaria* was relatively weaker than that of *L. gasseri* HHuMIN D (Table 4). *L. gasseri* HHuMIN D generally has higher coaggregation with oral facultative anaerobic bacteria than oral anaerobic bacteria with the exception of *P. catoniae*, which indicates that *L. gasseri* HHuMIN D possesses effective inhibitory capacity against oral facultative anaerobic bacteria. In another study evaluating the accumulation of 4 strains of human streptococci and 6 strains of lactobacillus used in consumer products, all probiotic strains showed the ability to aggregate with oral pathogens, but the degree of aggregation was different for each strain and dependent on time [68]. Therefore, it is safe to state that only some strains of *Lactobacillus* can co-aggregate and not all *Lactobacillus* spp. co-aggregate with harmful bacteria.

There are more than 500 bacterial species in the oral cavity, of which 15-20 bacterial species are known to produce toxic materials and are directly implicated in the evolution of various forms of periodontal diseases [69]. Many of these harmful bacteria are also highly capable of coagulating with other microorganisms, live in the oral cavity and create toxic substances constantly, thus harming oral health [70]. Although the mechanism of aggregation has not been identified, the ability of probiotics to coagulate with harmful bacteria is important as a secondary function to support the main function of probiotics. Oral probiotics, which adhere well to oral mucosa and dental tissues, effectively inhibit oral disease and the growth of harmful oral bacteria [71,72].

L. gasseri HHuMIN D's ability to coagulate with harmful bacteria is essential for creating and sustaining a stable oral cavity. Beneficial bacteria with high coaggregation are not readily eliminated by saliva and physical removal from the oral cavity, since they can be rapidly attached to oral periodontal bacteria in the oral cavity. Additionally, harmful bacteria are exposed directly to antimicrobials such as bacteriocins, lactic acid, and hydrogen peroxide through direct coaggregation with beneficial bacteria [73].

Effect of *L. gasseri* HHuMIN Don the formation of artificial dental plaque by *S. mutans*

S. mutans, a facultative anaerobic coccus in the oral cavity, produces lactic acid as an end-product of glycolysis using sucrose as a substrate, and secretes glucosyl transferase (GTF; EC 2.4.1.5) to make insoluble glucan, a glucose polymer [74]. These glucans are insoluble mucous substances which cling to the tooth surface to help bind bacteria to the tooth surface and cause enamel and dentin degradation through retention of the organic acid produced. *S. mutans* binds to the tooth surface, creates high amounts of acid by digestion of carbohydrates and erodes the enamel of the tooth, inducing dental caries [75].

To determine the inhibition by *L. gasseri* HHuMIN D of the formation of dental plaque by *S. mutans*, a beaker wire test was performed (Fig. 3). Artificial dental plaques formed on calibration wire suspended in *S. mutans* inoculated broths, but such plaques were not developed in *L. gasseri* HHuMIN D and *W. cibaria* inoculated broths. No artificial dental plaque was formed on calibration wire in broth co-cultured with *S. mutans* and *L. gasseri* HHuMIN D, indicating 100% inhibition (Table 5).

In one study, *S. mutans* created artificial dental plaque was suppressed by 53% by *E. faecium* T7 co-culture [33]. In another experiment, *Lactobacillus* lactis 1370 suppressed plaque formation by 95% [76]. Compared to other organisms tested, *L. gasseri* HHuMIN D strongly inhibits dental plaque formation by *S. mutans*. *L. gasseri* HHuMIN D whole genome sequencing revealed the presence of the glucosyltransferase gene (*gtfD*) that generates GTF-S (P49331), a strongly branched water-soluble beta-glucan synthetic enzyme (alpha 1,6-glucose). *L. gasseri* HHuMIN D only produces water-soluble glucan and prevents plaque production by inhibiting the growth of harmful bacteria in the oral cavity at the same time. *S. mutans* plaque formation in the oral cavity is affected by many factors, one of which is the glucan binding domain (GBD) in the *S. mutans* GTF enzymes (GTF-I, GTF-S, GTF-SI) that generate glucan, a major plaque element. Based on the type, the glucan produced has various effects on the GBD, such that the form and volume of glucan eventually released are different [77]. The water-soluble glucan produced by *L. gasseri* HHuMIN D mediated the GBD of water-insoluble glucan produced by *S. mutans* in the co-culture of *L. gasseri* HHuMIN D and *S. mutans* while inhibiting the overall development of water-insoluble glucan. Several researchers have isolated and analyzed other species of *Lactobacillus* that produce water-soluble glucans such as those produced by *L. gasseri* HHuMIN D [78,79].

Adhesion test

Adhesion assay of *L. gasseri* HHuMIN D

Harmful oral bacteria may bind to epithelial cells in the oral cavity, which can endanger oral health if the number of bacteria is high. We assessed *L. gasseri* HHuMIN D's ability to prevent the adhesion of harmful oral bacteria to KB cells, a typical oral epithelial cell, using inhibition, competition, and displacement assays. Oral epithelial cell monolayer testing is one of the methods used to identify beneficial bacteria and has also been used as a guide to test the attachment of beneficial bacteria to the oral epithelium. According to an earlier study, if the number of attached bacteria per KB cell is 1.5 or more, the attachment capacity is considered to be very strong (+ + + +) and if the number of attached bacteria is 1.5 to 1, the adhesion capacity is considered to be strong (+ + +). If the number of bacteria attached is 1 to 0.5, the ability to adhere is moderate (+ +) and if the number of the bacteria attached is less than 0.5, the ability to adhere is considered weak (+) [80]. The KB cell adhesion ability of *L. gasseri* HHuMIN D was determined to be 4.41 ± 1.4 cells per cell– very strong. *F. nucleatum* showed a very high adhesion capacity of 18.35 ± 4.0, and *S. mutans* showed a strong adhesion capacity of 2.57 ± 0.1 (Table 6). Strains with strong adhesion are likely to adhere to oral epithelial cells, form colonies, and inhibit the attachment of pathogenic bacteria to epithelial cells. In a previous analysis, the adhesion abilities of harmful bacteria were measured for *P. intermedia* from $4.1Í10^4$ ± $2.7Í10^4$ to 152 ± 57 per 10^5 KB cells, for *P. gingivalis* at $9.6Í10^5$ ± $1.0Í10^5$, and for *E. coli* at 278 ± 133 [80]. In another study, *F. nucleatum*'s KB cell adhesion at the initial dose was from 19.2 ± 0.3% to 1.5 ± 0.4, suggesting that *F. nucleatum* has very strong adhesion capabilities [81].

Competition between *L. gasseri* HHuMIN Dand oral microorganisms for cell adhesion

The ability of *L. gasseri* HHuMIN D to inhibit cell adhesion by harmful bacteria was assessed by conducting protection and displacement assays. In the protection assays, *L. gasseri* HHuMIN D was allowed to bind to KB cells before introducing harmful oral bacteria. *L. gasseri* HHuMIN D decreased cell adhesion by *F. nucleatum* and *S. mutans* by 63% and 71%, respectively, and that of *F. nucleatum* and *S. mutans* by 100% and 90%, respectively. *L. gasseri* HHuMIN D strongly inhibited *F. nucleatum* adhesion and it seems that the cell adhesion of *L. gasseri* HHuMIN D might also have been lowered by competition with the harmful bacteria (Table 7).

The attachment of *L. gasseri* HHuMIN D after the attachment of harmful bacteria was measured in the displacement assays. With *F. nucleatum* and *S. mutans*, the cell adhesion of *L. gasseri* HHuMIN D decreased to 70% and 73%, respectively. However, the attachment of harmful bacteria decreased by 89% and 90%, respectively. Taken together, these two results showed a stronger ability to inhibit the binding of harmful bacteria when first bound to *L. gasseri* HHuMIN D. This suggests that *L. gasseri* HHuMIN D is more effective in preventing than inhibiting the attachment of oral harmful bacteria. Consequently, continuous intake is likely necessary to enable adhesion of *L. gasseri* HHuMIN D to highly concentrated oral cavity cells. Other researchers have theorized that there is competition for common adhesion receptors between harmful bacteria and beneficial oral bacteria [82] and that antibacterial or antiadhesive factors produced by beneficial bacteria inhibit oral harmful bacteria from adhering after the beneficial bacteria aggregate [83].

Safety evaluations of *L. gasseri* HHuMIN D

As a potential novel probiotic bacterium, *L. gasseri* HHuMIN D was evaluated for its potential as a clinical pathogen on the basis of its phenotypic properties and genomics.

Ammonia production

The evaluation of ammonia production confirmed the safety of *L. gasseri* HHuMIN D; no ammonia was produced. On the other hand, *Enterococcus faecium* ATCC19433, a positive control, produced 109 ± 7 μg/mL of ammonia. *L. gasseri* HHuMIN D's ammonia production is below the level of concern in South Korea's Ministry of Food and Drug Safety's milk product quality [27]. Microorganisms may create various poisonous substances by nitrogen derivatives through decomposing the proteins, peptides and amino acids in saliva or food [84]. When a microorganism enters the large intestine, it is able to generate poisonous substances such as phenol, ammonia and indole by decomposition of proteins [85]. Ammonia formed by microorganisms is known to migrate to the liver and cause cell damage cofactors and chronic hepatic damage. The production of ammonia from microorganisms is closely related to human health and must be assessed to demonstrate the safety of commercial probiotics. According to Vince and Burridge [86], *Clostridia, Enterobacter, Bacillus* spp., and Gram-negative anaerobes create large amounts of ammonia. Furthermore, certain *Streptococci, Micrococci*, and Gram-positive non-spore forming anaerobes release small quantities of ammonia, and Gram-positive aerobic rods generate trace amounts of ammonia. Certain strains of *Lactobacillus* can produce small amounts of ammonia during growth.

Evaluation of biogenic amine production

L. gasseri HHuMIN D did not produce cadaverine, histamine, putrescine, or tyramine. Since ammonia and/or BAs are used as a quality indicator for fermented foods, *L. gasseri* HHuMIN D's absence of ammonia and BA activity suggests that *L. gasseri* HHuMIN D is suitable for use in the manufacture of fermented and non-fermented foods. Biogenic amines (BAs) derived from amino acids are common anti-nutritional compounds in animals and humans. Fresh meat, potatoes, and cheese commonly contain these compounds. Ingestion of massive amounts of BAs may cause symptoms similar to significant allergic reactions. BAs have been identified as causative agents in many cases of food poisoning and are critical from a hygienic point of view because they can induce a variety of pharmacological reactions [87]. BAs are

involved in numerous mammalian metabolic and intracellular processes, such as synaptic transmission, modulation of blood pressure, allergic reactions, and management of cellular growth. Probiotic bacteria, commonly used in the food industry, produce BAs through microbial metabolic activities such as decarboxylation and protein molecule transamination [88].

Hemolytic property test

L. ivanovii developed β-hemolysis colorless zones around colonies in BL agar added 5% sheep blood but *L. gasseri* HHuMIN D cultivated in the same medium did not reveal colorless zones around the colonies (Fig. 4). Therefore, *L. gasseri* HHuMIN D does not cause hemolysis. *The Guidelines for the Evaluation of Probiotics in Food*, produced by FAO and WHO joint research, states, “If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required” [28, 89]. The hemolytic characteristics of microorganisms are an important measurement criterion for the safety of bacteria since they may liquefy/degrade red blood cells and ultimately cause anemia and edema. Among probiotics, *Lactobacillus* spp. are graded as α-hemolytic microorganisms [90]. According to research [91], several *Lactobacillus* spp. (*L. sakei* MBSa1 bac+, *L. curvatus* MBSa3 bac+ and *L. lactis* 368 bac–) demonstrate strong β-hemolysis.

Mucin degradation

Several research teams have studied the mucous dissolving capacities of human pathogenic bacteria since 1980, and mucus dissolving capacity is now considered a measure of microbial virulence and microbial toxicity [92-95]. The intestinal mucus gel coating is a membrane made of glycoproteins and is an essential part of the intestine. Even as it acts as a biological shield from microorganisms, the intestinal mucus of infants and immunocompromised hosts are capable of bacterial translocation. This bacterial translocation is regarded one of the most critical probiotic safety tests due to the risk for septicemia and bacteremia endocarditis [96]. Although most microorganisms do not exhibit mucolytic activity, several studies have reported that certain microorganisms do and the genes that induce mucin degradation enzymes have been identified [97]. Various intestinal pathogens are known to hydrolyze glycoprotein-based mucus gel layers and possess the ability to metabolize mucus-derived monosaccharides [98]. The 'Guidelines for the Evaluation of Probiotics' of the FAO/WHO does not offer guidelines on the assessment of the mucin-decomposing function of probiotics [28], but the Steering Committee of the Norwegian Scientific Committee for Food Safety declared that decreased bowel mucin development or increased mucin degradation should be subject to examination because it may have harmful effects for patients [99]. While there is no need for a safety assessment for mucin deterioration in the oral cavity, this study was performed to take into consideration the potential for ingestion and entry to the intestine by *L. gasseri* HHuMIN D. *L. gasseri* HHuMIN D was also assessed for the potential of translocation by in vitro mucolytic assays.

Cell growth levels were evaluated after incubation by measuring the absorption of quinary-modified MRS media (basal medium (glucose-free MRS basal medium with 0.5% mucin, 1.0% mucin, 0.5% glucose, and 1.0% glucose) at a wavelength of 550 nm. Basic sugars (glucose, fructose, maltose, and sucrose) were added to inhibit the development of mucinase through catabolic repression. However, this can lead to negative effects since mucinolytic enzymes can still be produced. Glucose, which is widely used as a source of carbon in MRS media, was therefore omitted from all broths to prevent this flaw.

If *L. gasseri* HHuMIN D had the ability to produce mucinases, through mucin degradation it would be able to survive and grow aggressively in the presence of mucin and no other carbohydrate sources. As illustrated in Fig. 5, *L. gasseri* HHuMIN D growth was actively induced by the addition of glucose as the carbon source. But growth was not observed when mucin was added instead of glucose. These results show that *L. gasseri* HHuMIN D does not use mucin as a source of carbon for growth.

Antibiotic susceptibility

As shown in Table 8, *L. gasseri* HHuMIN D was sensitive to ampicillin, carbenicllin, cephalothin, chloramphenicol, clindamycin, dicloxacillin sodium salt hydrate, erythromycin, lincomycin, methicillin, penicillin G, tetracycline, and vancomycin (MICs ranged from 0.01 to 4 μg/mL). There was general resistance to bacitracin, gentamicin, katamycin, metronidazole, neomycin, polymyxin B, phosphomycin, streptomycin, and trimethoprim-Sulfamethoxazole (all MICs were greater than 32 μg/mL).

To distinguish antibiotic tolerance from antibiotic-sensitivity, microbiological cut-off values for the antibiotic tolerance of microorganisms used as food were defined by the European Food Safety Authority [100]. Except for gentamicin, streptomycin, and kanamycin, the MIC values of *L. gasseri* HHuMIN D were less than or equal to the cut-off values proposed by the EFSA. In a study of *Lactobacillus* spp.'s susceptibility to 23 antibiotics, some lactobacilli showed resistance to kanamycin, vancomycin, and chloramphenicol. The authors hypothesized that strains with these genes did not necessarily indicate cause for concern about the transition of antibiotic resistance and could be used in food and medicinal formulations as natural biopreservatives [101]. Ampicillin, erythromycin, vancomycin, chloramphenicol, and clindamycin were low compared to the cut-off values proposed by EFSA. Antibiotic resistance can be transmitted via plasmids, the assessment of antibiotic resistance is a significant criterion for assessing *Lactobacillus* sp. safety [102]. Therefore, we used WGS to genetically identify the antibiotic resistance gene of *L. gasseri* HHuMIN D and ascertain the possibility of transmission to other bacteria through the presence or absence of a plasmid.

Whole genome sequencing (WGS) of *L. gasseri* HHuMIN D

The size of the entire gene sequence of *L. gasseri* HHuMIN D was 2,066,663 bp and the GC composition ratio was 34.9%. The average GC content of *Lactobacillus* spp. is 46.61%, *L. gasseri* HHuMIN D is lower than the average GC content of *Lactobacillus* spp. The number of rRNA genes and tRNA genes were 7 and 63, respectively. The number of coding sequences (CDSs) was 2,015, and the average of the coding sequence length was 923.9 bp. Figs. 6 and 7 show a genetic map of *L. gasseri* HHuMIN D and a functional classification based on COG.16S rRNA analysis can be used to analyze the microbial population composition by analyzing short nucleotide sequences but cannot be used to explain the microbial genome's functional and physiological details [103]. Whole genome sequencing (WGS) is a technique which studies the functional aspects of a microorganism by sequencing a microorganism's entire genome and comparing it to a gene previously identified [104].

Bacteriocin gene analysis through the WGS

Bacteriocin is a proteinaceous bacterial product with bactericidal activity [105]. Any Gram-positive bacteria that produce bacteriocin are considered to be active against Gram-negative bacteria [106]. This broad spectrum of compounds is important in preventing the growth of harmful bacteria and alleviating disease. They are produced by specific bacteria of the lactic acid bacteria, including lactococci, lactobacilli, and pediococci [107].

The whole genome sequence of *L. gasseri* HHuMIN D was verified through CLgenomicsTM and EZBioCloud applications to see if this strain qualifies for GRAS status. All results are summarized in Table 9. We found that the antimicrobial activity against oral harmful bacteria may be the result of one or more of three bacteriocin genes in which the antimicrobial activity of *L. gasseri* HHuMIN D was found: hlv, lafA, **lafX.** *The***WGS cannot determine which of these genes are responsible for inhibitory activity against the pathogens tested and there is no data for reference because there are insufficient screening studies on bacteriocins produced by *L. gasseri* HHuMIN D against harmful oral bacteria. Further studies on bacteriocins produced by *L. gasseri* HHuMIN D and their effects on harmful oral bacteria are needed.**

Since 1980, the *L. acidophilus* group has been classified by DNA-DNA homology into six homologous species: *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* and *L. johnsonii* [108,109]. Out of these six species *L. gasseri* is thought to be the primary member inhabiting the human intestine, and studies on the bacteriocins produced by this bacterium have been conducted [110-112]. Lactacin F is the best-studied bacterocin in the LAB class Ⅱb. Gassericin T

is a 2-component bacteriocin consisting of GatA and GatX and of the lacticin-F family; it is known as the primary bacteriocin developed by *L. gasseri* strains [113]. Gassericin T is heat-stable (121°C, 10 min), pH-tolerant (pH 2–11) and bactericidal against several food poisoning gram-positive bacteria such as *Bacillus cereus, Listeria monocytogenes, and Staphylococcus aureus* [110]. Helveticin J was first studied in *L. helveticus*. Helveticin J can be used as an indicator of closely related species, is active at neutral pH under aerobic or anaerobic conditions and is sensitive to proteases and heat (30 min at 100 °C) [114]. These 2 bacteroicins are good candidate biopreservatives.

Biogenic amines production gene analysis through the WGS

Biogenic amines are substances that have one or more amine groups that are used in eukaryotic cells as precursors to hormones, alkaloids, nucleic acids, and proteins. BAs are naturally produced by animals, plants, and microorganisms, but foods containing high amounts of BAs induce excessive gastric acid production in the body, improve cardiac efficiency, and can contribute to migraines and hypertension. BAs are an essential component in both qualitative and quantitative aspects of foods and beverages. Typical BAs include histamine, tyramine, putrescine, cadaverine, and β-phenyl ethylamine. These compounds are formed by histidine, tyrosine, ornithine, lysine, and β-phenylalanine decarboxylase and decarbonation reactions, so they can exist in acidic conditions. Traditionally, BAs have been used as indicators of microbial activity in food, and high levels of BAs in food implies deterioration in the quality of food.

The whole genome sequence of *L. gasseri* HHuMIN D was scanned via the CLgenomicsTM and EZBioCloud applications for genes that generate enzymes engaged in the metabolic pathways that create biogenic amines. Fig. 8 demonstrates the biosynthetic mechanism of different biogenic amines.

No genes were identified that produce tyrosine decarboxylase (which generates tyramine from tyrosine), L-tryptophan decarboxylase (which generates tyramine from tyrosine, histamine from histidine, tryptamine from tryptophan, and phenylethylamine from phenylalanine), lysine decarboxylase (which generates cadaverine from lysine), histidine decarboxylase (which generates histamine from histidine), carbamoyl-phosphate synthase (which generates ornithine from NH3), ornithine carbamoyl transferase (which generates citruline from ornithine), argininosuccinate synthase (which generates L-arginosuccinate from citruline), argininosuccinate lyase (which generates arginine from L-arginosuccinate), arginase (which generates ornithine from arginine), polyamine oxidase (which generates putrescine from spermidine), spermine synthase (which generates spermine from spermidine), spermidine synthase (which generates methylthioadenosine from S-adenosylmethionine) (Figs. 8), and genes were identified that produce glutaminase (which generates NH3 from glutamine), ornithine decarboxylase (which generates putrescine from ornithine), S-adenosylmethionine synthetase (which generates S-adenosylmethionine from methionine) (Figs. 8).

Glutamine can be converted to NH3 through glsA or GLS and converted NH3 can be converted to ornithine through CPS1. As a result of confirming the mechanism, L. gasseri HHuMIN D can be converted from glutamine to NH3 via glsA or GLS but cannot be converted to ornithine since there is no CPS1 thereafter. If ornithine exists, it can be converted via the urea cycle into citriuline, L-arginosuccinate, and arginine; the associated genes have not been found in the *L. gasseri* HHuMIN D genome. Polyamines such as putrescine, spermidine and spermine are produced using ornithine as the starting substrates through different pathways (Fig. 8). Except for methionine adenosyltransferase (MAT) and putrescine biosynthetic genes (ODC1, speC, speF), no genes encoding polyamine biosynthetic enzymes were found in the *L. gasseri* HHuMIN D genome. Contrary to the genetic results, the production amount seems to be very small, as bioamines are not measured in BA production capacity evaluation.

Platelet aggregation

It has been reported that platelet aggregation induced by some lactic acid bacteria occurs in endocarditis, so it is a significant part of microbiological safety assessment. Phosphatidylserine plays a crucial role in the development of blood

clotting and thrombus, so the genes involved in the metabolic pathways that create this compound were evaluated. *L. gasseri* HHuMIN D does not have any genes that produce phosphatidylserine in the metabolic pathway (Fig. 9)

Virulence factor

Virulence genes lend pathogenicity to microorganisms. *L. gasseri* HHuMIN D was assumed to be non-pathogenic using VirulenceFinder 2.0, a program that enables pathogenic and non-pathogenic bacteria to be differentiated using data from the whole genome sequence in order to classify potential virulence genes within the genome. The *L. gasseri* HHuMIN D genome sequence was compared with the genomic sequences of four noted pathogens (*Escherichia coli, Enterococcus, Listeria*, and *Staphylococcus aureus*). Virulence factors evaluated included *Escherichia coli* shiga toxin gene and *Staphylococcus aureus* exoenzyme genes, host immune alteration or evasion genes and toxin genes. The *L. gasseri* HHuMIN D genomic sequencing revealed no virulence factors or toxic or pathogenic genes.

Antibiotic resistance and associated genes

L. gasseri HHuMIN D genes related to antibiotic resistance were discovered using the ideal and strict algorithms of CARD (Table 10). The genome of *L. gasseri* HHuMIN D was found to contain 16 putative genes associated with resistance to beta-lactams (7), bacitracin (1), aminoglycoside (1), aminocoumarin (1), lincomycin (1), polymyxin (1), macrolide (2) and multi antibiotic (2). Antibiotic resistance assays showed that *L. gasseri* HHuMIN D was resistant to gentamicin, streptomycin, and kanamycin, but genes associated with antibiotic resistance were not detected. Although penicillin, bacitracin, and lincomycin genes linked to resistance have been identified, the organism itself has demonstrated low resistance in antibiotic resistance assays. It must be noted that the *L. gasseri* HHuMIN D genome and the phenotype for antibiotic resistance do not match completely. It should be considered that the *L. fermentum* OK genome and the phenotypes of antibiotic resistance may not exactly fit. For DNA replication, GyrA is necessary. Multidrug efflux transporters are involved in several detoxifying activities in cells and are widely distributed across many forms of *Lactobacillus* spp. [115]. There are also safety factors regarding the use of antibiotic-resistant strains, due to the possibility of transferring antibiotic-resistance genes to intestinal pathogens [116]. Consequently, whole genome sequencing was used to determine whether the antibiotic-resistant genes of *L. gasseri* HHuMIN D could be transmitted through plasmids to other bacteria. No gene capable of delivering antibiotic resistance in the whole genome of *L. gasseri* HHuMIN D was found (data not shown). Since genes cannot be spread, antibiotic resistance to *L. gasseri* HHuMIN D is known to be inherent or normal. Several studies have documented that tolerance to aminoglycoside groups such as gentamicin, streptomycin, kanamycin and neomycin is suspected to be intrinsic to *Lactobacillus* spp. and is due to the absence of cytochrome-mediated electron transport that mediates drug absorption [117,118].

Conclusion

L. gasseri HHuMIN D culture supernatant inhibited halitosis producing anaerobic microorganisms 88.8% and antimicrobial activity by *L. gasseri* HHuMIN D against deleterious oral bacteria was strong. Multiplication of *L. gasseri* HHuMIN D was impaired when co-cultured with other oral bacteria. *L. gasseri* HHuMIN D continuously released hydrogen peroxide after 12 hours to 802 μmol/L and gradually decreased until 24 hours. *P. catoniae*, and *S. sanguinis* were aggregated with *L. gasseri* HHuMIN D, and *L. gasseri* HHuMIN D decreased the manufacture of artificial dental plaque from *S. mutans* by 100 %. The KB cell adhesion ability of *L. gasseri* HHuMIN D was 4.41 cells per cell and the cell adhesion of *F. nucleatum* and *S. mutans* decreased dramatically in protection and displacement assays. These characteristics of *L. gasseri* HHuMIN D are thought to indirectly help inhibit harmful oral microorganisms. *L. gasseri* HHuMIN D showed direct inhibitory effects on the growth of oral pathogens. *L. gasseri* HHuMIN D does not produce biogenic amines and ammonia. Hemolysis and mucin decomposition activity were not observed and antibiotic resistance testing and WGS analysis confirmed that there was no antibiotic resistance or genes associated with biogenic amines, platelet aggregation, virulence or antibiotic resistance. Three genes that express bacteriocin were identified. These

assessments demonstrated compliance of the *L. gasseri* HHuMIN D with EFSA and FDA requirements. This study suggests that *L. gasseri* HHuMIN D may be effective in inhibiting harmful oral bacteria and be used as a natural probiotic, a functional food, or a food for wellbeing.

Abbreviations

L. gasseri: *Lactobacillus gasseri*; *F. nucleatum*: *Fusobacterium nucleatum*; *P. gingivalis*: *Porphyromonas gingivalis*; *P. intermedia*: *Prevotella intermedia*; *P. catoniae*: *Porphyromonas catoniae*; *S. sobrinus*: *Streptococcus sobrinus*; *S. mitis*: *Streptococcus mitis*; *S. oralis*: *Streptococcus oralis*; *S. gordonii*: *Streptococcus gordonii*; *S. sanguinis*: *Streptococcus sanguinis*; *S. parasanguinis*: *Streptococcus parasanguinis*; *S. mutans*: *Streptococcus mutans*; WGS: Whole genome sequencing; WHO: World Health Organization; GTase: Glucosyltransferase; VSCs: Volatile sulfur compounds; H_2S: Hydrogen sulfide; CH_3SH: Methyl mercaptan; CHX: Chlorhexidine; CPC: Cetylpyridinium chloride; FAO: Food and Agriculture Organization of the United Nations; TMB: Tetra methyl benzidine; DMSO: Dimethyl sulfoxide; MRS: de Man-Rogosa-Sharpe; PBS: Phosphate buffered saline; LBS: *Lactobacillus* selection; BHI: Brain Heart Infusion; MSB: Mitis Salivarius Sucrose Bacitracin; FBS: Fetal bovine serum; MEM: minimum critical medium; CFU: Colony-forming unit; CT: Cycle threshold; Trimethoprim : Trimethoprim–sulfamethoxazole; LSM-Cys: LAB susceptibility test medium with L-cysteine; MIC: Minimal inhibitory concentration; COG: Clusters of Orthologous Groups of proteins; KEGG: Kyoto Encyclopedia of Genes and Genomes; CGE: Center for Genomic Epidemiology; CARD: Comprehensive antibiotic resistance database; GTF: Glucosyl transferase; gtf: Glucosyltransferase; GBD: Glucan binding domain; BAs: Biogenic amines; CDSs: Coding sequences; MAT: Methionine adenosyltransferase

Declarations

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Not applicable.

Authors' contributions

Soyon Mann designed the experiment under the supervision of Geun Eog Ji, Myeong Soo Park and Seockmo Ku. Soyon Mann performed microbial experiments. Soyon Mann, Geun Eog Ji, Myeong Soo Park, Keum Taek Hwang and Seockmo Ku performed the literature research and analyzed data. Tony V. Johnston, Keum Taek Hwang and Seockmo Ku edited and revised the manuscript. Seockmo Ku and Tony V. Johnston participated this work based on a non-disclosure research agreement between Middle Tennessee State University and BIFIDO Co., Ltd. All authors discussed drafts and approved the final manuscript for publication.

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Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Soyon Mann, Tony V. Johnston, Keum Taek Hwang and Seockmo Ku declare no conflicts of interest. Myeong Soo Park and Geun Eog Ji are directly employed by BIFIDO Co., Ltd., and they hold BIFIDO Co., Ltd. stocks as a CTO and CEO respectively.

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Tables

Table 1 Oligonucleotides used as primers for real-time PCR

Bacteria name	Oligonucleotide sequence		Length	Reference
Lactobacillus	Forward	TGG AAA CAG RTG CTA ATA CCG	21	[119]
	Reverse	GTC CAT TGT GGA AGA TTC CC	20	
Fusobacterium nucleatum	Forward	AGA GTT TGA TCC TGG CTC AG	20	[120]
	Reverse	GTC ATC GTG CAC ACA GAA TTG CTG	24	
Streptococcus mutans	Forward	CTA CAC TTT CGG GTG GCT TG	20	[121]
	Reverse	GAA GCT TTT CAC CAT TAG AAG CTG	24	

Table 2 Inhibition of periodontal bacteria by *L. gasseri* HHuMIN D supernatant

Strains	Inhibition (%)	
	W. cibaria	*L. gasseri* HHuMIN D
Fusobacterium nucleatum KCOM 1001	5±1[a]	**88±1**[a]
Porphyromonas gingivalis KCOM 2796	11±3[a]	**88±1**[a]
Prevotella intermedia KCOM 2889	13±1[a]	**89±1**[a]
Porphyromonas catoniae KCOM3169	14±2[a]	**90±0**[a]
Streptococcus sobrinus KCOM1157	8±1[a]	**60±2**[b]
Streptococcus mitis KCOM 1356	17±2[b]	4±4[c]
Streptococcus oralis KCOM 1493	22±5[b]	4±6[c]
Streptococcus gordonii KCOM 1788	0±0[c]	19±5[d]
Streptococcus sanguinis KCOM 2167	0±0[c]	2±3[c]
Streptococcus parasanguinis KCOM 2522	12±3[a]	2±2[c]
Streptococcus mutans KCTC3065	0±0[c]	22±2[d]

Values are expressed as the mean ± standard deviation. Means not sharing a common letter are significantly different groups at $p<0.001$. (n=3)

Table 3 Susceptibility of *L. gasserie* HHuMIN D culture supernatant antimicrobial activity to various hydrolytic enzymes

Enzyme	Activity
Control (no-enzyme)	+++
Proteinase K	-
Trypsin	-
α-Chymotrypsin	-
Pepsin	+++
α-Amylase	+++

Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, and *Porphyromonas catoniae* were used as indicators. Degree of growth inhibition by OD (600nm): +++, value similar to control; ++, value weaker than control; -, no growth. Culture supernatant was mixed with enzyme solutions at a final concentration of 1 mg/ml.

Table 4 Coaggregation reactions between *W.* cibaria or L*. gasseri* HHuMIN D and various periodontal bacteria

Strains	Coaggregation (%)	
	W. cibaria	*L. gasseri* HHuMIN D
Fusobacterium nucleatum KCOM 1001	4±2[a]	2±2[a]
Porphyromonas gingivalis KCOM 2796	0±1[a]	0[a]
Prevotella intermedia KCOM 2889	0±2[a]	28±5[b]
Porphyromonas catoniae KCOM3169	0±1[a]	70±0[c]
Streptococcus sobrinus KCOM1157	20±1[b]	37±2[b]
Streptococcus mitis KCOM 1356	2±0[a]	46±3[d]
Streptococcus oralis KCOM 1493	0±1[a]	30±2[b]
Streptococcus gordonii KCOM 1788	11±6[b]	62±1[c]
Streptococcus sanguinis KCOM 2167	50±3[c]	74±2[e]
Streptococcus parasanguinis KCOM 2522	0±3[a]	6±3[a]
Streptococcus mutans KCTC3065	29±4[d]	49±4[d]

Values are expressed as the mean ± standard deviation. Means not sharing a common letter are significantly different groups at p<0.001. (n=3)

Table 5 Effect of *L. gasseri* HHuMIN D on the formation of artificial plaque by *Streptococcus mutans* on wires using BHI media containing 5% sucrose

Tested bacterial strain	Plaque weight (mg)
S. mutans	105±5[a]
W. cibaria	0±0[b]
L. gasseri HHuMIN D	0±0[b]
W. cibaria + *S. mutans*	53.4±12[c]
L. gasseri HHuMIN D + *S. mutans*	0±0[b]

The values are expressed as the mean ± standard deviation. Means not sharing a common letter are significantly different groups at p<0.001. (n=3)

Table 6 Adhesion ability to KB cells of tested strains

Strains	No. of CFU recovered
L. gasseri HHuMIN D	4.41 ± 1.4[a]
F. nucleatum KCOM1001	18.35 ± 4.0[b]
S. mutans KCTC3065	2.57 ± 0.1[a]

The values are expressed as the mean ± standard deviation for triplicate samples of lysates from the infection of 10^5 KB cells by 10^8 bacteria. Means not sharing a common letter are significantly different groups at p<0.01. (n=3)

Table 7 The number of experimental bacteria and pathogens bound to KB cells in adhesion ability, protection assays and displacement assays

	Strains	No. of CFU recovered [a] ($\times 10^5$)	Adhesion inhibitory rate (%)
Positive Control	*L. gasseri* HHuMIN D	4.41 ± 1.4	
	F. nucleatum KCOM1001	18.35 ± 4.0	
	S. mutans KCTC3065	2.57 ± 0.1	
Protection assays[b]	(HHuMIN D→KCOM1001) HHuMIN D	1.64 ± 0.2**	63
	(HHuMIN D→KCOM1001) KCOM1001	0.05 ± 0.0***	100
	(HHuMIN D→KCTC3065) HHuMIN D	1.27 ± 0.6**	71
	(HHuMIN D→KCTC3065) KCTC3065	0.25 ± 0.2****	90
Displacement assays[c]	(KCOM1001→HHuMIN D) HHuMIN D	1.32 ± 0.4**	70
	(KCOM1001→HHuMIN D) KCOM1001	2.10 ± 0.8***	89
	(KCTC3065→HHuMIN D) HHuMIN D	1.18 ± 0.3**	73
	(KCTC3065→HHuMIN D) KCTC3065	0.27 ± 0.1****	90

[a] Values represent the means ± the standard deviations for triplicate samples of lysates from the infection of 10^5 KB cells by 10^8 bacteria. Compared with the positive control group: * = p<0.05, ** p<0.01, *** = p<0.001, **** = p<0.0001. (n=3).

[b] Protection assays are a method of investigating how harmful bacteria are inhibited from attaching by selected bacteria which are already attached.

[c] Displacement assays are a method of investigating the degree of attachment by selected bacteria after harmful bacteria are already attached.

Table 8 Antimicrobial susceptibility (MIC values) of *L. gasseri* HHuMIN D

(MIC values, μg/mL)

Classification	Antimicrobial agent	Cut-off of *Lactobacillus* (EFSA[a])		Strain	
		Lactobacillus obligate homofermentative[b]	*Lactobacillus* obligate heterofermentative[c]	*L. gasseri* HHuMIN D	*E. faecium* ATCC 29212
β-Lactam group	Penicillin G			0.063	0.5
	Carbenicllin (disodium salt)			0.5	8
	Methicillin			2	16
	Ampicillin (sodium salt)	1	2	0.25	0.25
	Dicloxacillin sodium salt hydrate			0.5	4
Aminoglycoside group	Gentamicin (sulfate)	16	16	32	256
	Streptomycin (sulfate salt)	16	64	64	> 256
	Kanamycin (sulfate)	16	32	512	256
	Neomycin (sulfate)			256	1024
Cephem group	Cephalothin (sodium salt)			1	16
Tetracycline group	Tetracycline	4	4	4	32
Peptide group	Polymyxin B (sulfate salt)			>1024	>1024
	Bacitracin			64	
Macrolide group	Erythromycin	1	1	0.5	8
Synthetic antimicrobial group	Metronidazole			>256	>256
The other group	Vancomycin (HCl)	2[d]	n.r.	0.5	2
	Chloramphenicol	4	4	2	8
	Lincomycin (hydrochloride)			4	
	Rifampicin			<0.125	0.5
	Clindamycin (hydrochloride)	1	1	0.5	>16
	Phosphomycin (disodium salt)			>1024	32
	Mupirocin			16	64

	Trimethoprim-Sulfamethoxazole (Co-trimoxazole)	>256	32

n.r. = not required.

[a] Data from EFSA (2012)

[b] including *L. delbrueckii, L. helveticus, L. gasseri*

[c] including *L. fermentum*

[d] not required for *L. salivarius*

Table 9 Genes and genetic characteristics of *L. gasseri* HHuMIN D bacteriocin on the basis of whole genome sequencing results

Gene	Length	Product/Function	Database hit
hlv	996	Bacteriocin helveticin-J	UniProtKB: P22294
lafA	228	Bacteriocin lactacin-F subunit LafA	UniProtKB: P24022 NCBI: LJ_RS03150
lafX	198	Bacteriocin lactacin-F subunit LafX	UniProtKB: Q48509

Table 10 Putative antibiotic resistance genes in the *L. gasseri* HHuMIN D genome

Gene ID	Name	Length	COG_name	KEGG_name	Product	Category
HHuMIND_00032	GLIA	1452	2814	22134	multidrug resistance protein	multi antibiotic resistance gene
HHuMIND_00185	cusR, copR, silR	666	0745	07665	transcriptional regulatory protein	beta-lactam antibiotic resistance gene
HHuMIND_00391	mdlB, smdB	1794	1132	18890	probable multidrug resistance ABC transporter ATP-binding/permease protein YheH	multi antibiotic resistance gene
HHuMIND_00573	aacC	810	2746	00662	aminoglycoside N(3)-acetyltransferase	aminoglycoside antibiotic resistance gene
HHuMIND_00588	mrcA	2379	0744	05366	peptidoglycan glycosyltransferase	beta-lactam antibiotic resistance gene
HHuMIND_00697	pbpB	2151	0768	08724	penicillin-binding protein 2B	beta-lactam antibiotic resistance gene
HHuMIND_00746	sotB	1383	0477	08159	lincomycin resistance protein LmrB	lincomycin antibiotic resistance gene
HHuMIND_00878	mrdA	2100	0768	05515	serine-type D-Ala-D-Ala carboxypeptidase	beta-lactam antibiotic resistance gene
HHuMIND_01245	gtrB, csbB	933	0463	20534	undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase	polymyxin antibiotic resistance gene
HHuMIND_01314	parE	1959	0187	02622	DNA gyrase subunit	aminocoumarin antibiotic resistance gene
HHuMIND_01342	abcA, bmrA	996	1132	18104	xenobiotic-transporting ATPase	beta-lactam antibiotic resistance gene
HHuMIND_01640	ddpF	678	1136	02032	macrolide export ATP-binding/permease protein MacB	macrolide antibiotic resistance gene
HHuMIND_01864	pbp1b	2829	0744	03693	penicillin-binding protein 1A	beta-lactam antibiotic resistance gene
HHuMIND_01995	ABC.CD.P	1869	0577	02004	bacitracin export permease protein BceB	bacitracin antibiotic resistance gene
HHuMIND_02034	cpoA	1044	0438	13678	alpha-galactosylglucosyldiacylglycerol synthase	beta-lactam antibiotic resistance gene
HHuMIND_02075	metN	690	1135	02071	macrolide export ATP-binding/permease protein MacB	macrolide antibiotic resistance gene

Figures

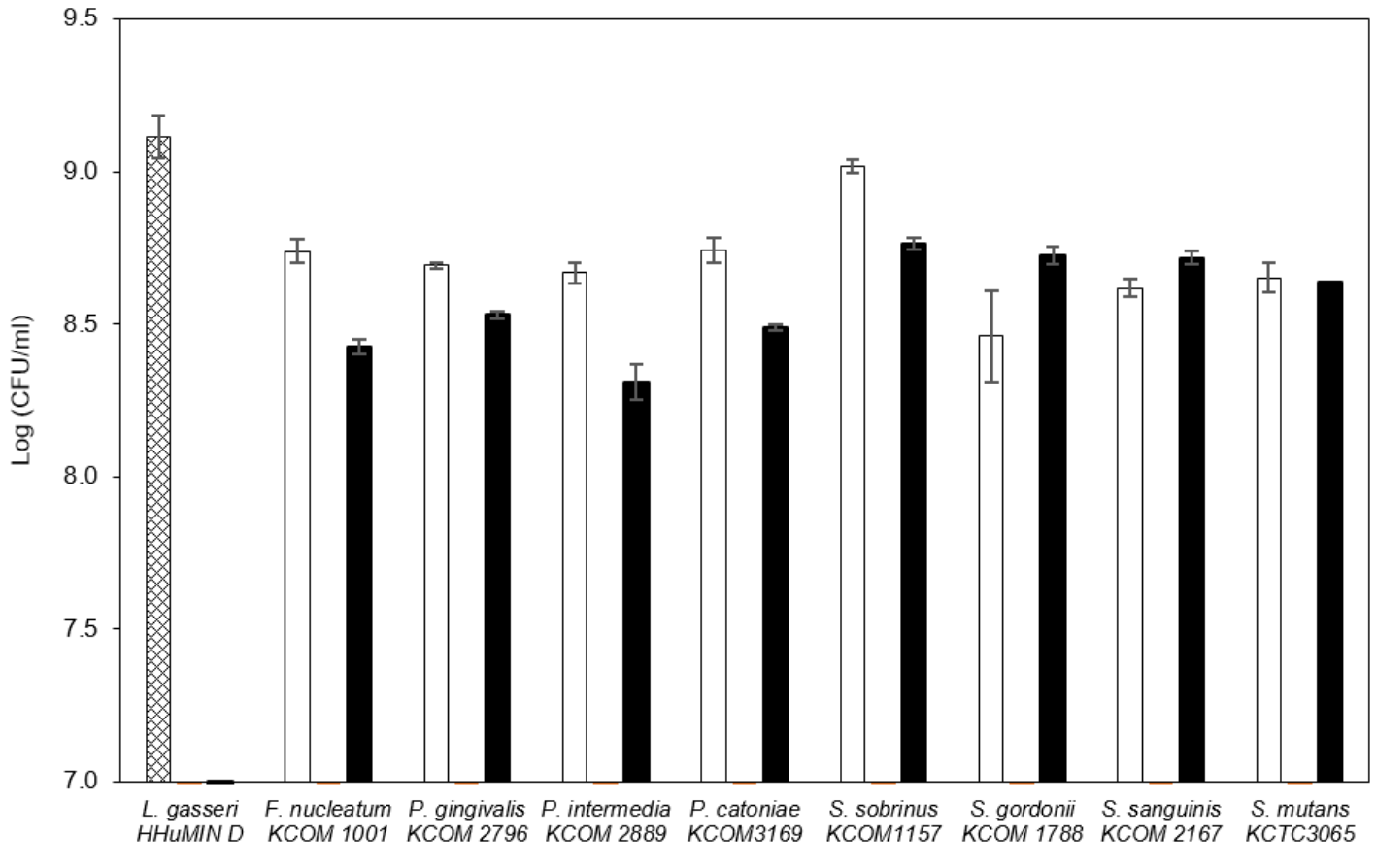

Figure 1

Inhibitory effect of L. gasseri HHuMIN D on the proliferation of various periodontal bacteria. ▨, L. gasseri HHuMIN D; □, Periodontal bacteria group; ▨, Periodontal bacteria in mixed culture; ■, L. gasseri HHuMIN D in mixed culture. Log 7 and below means <105.

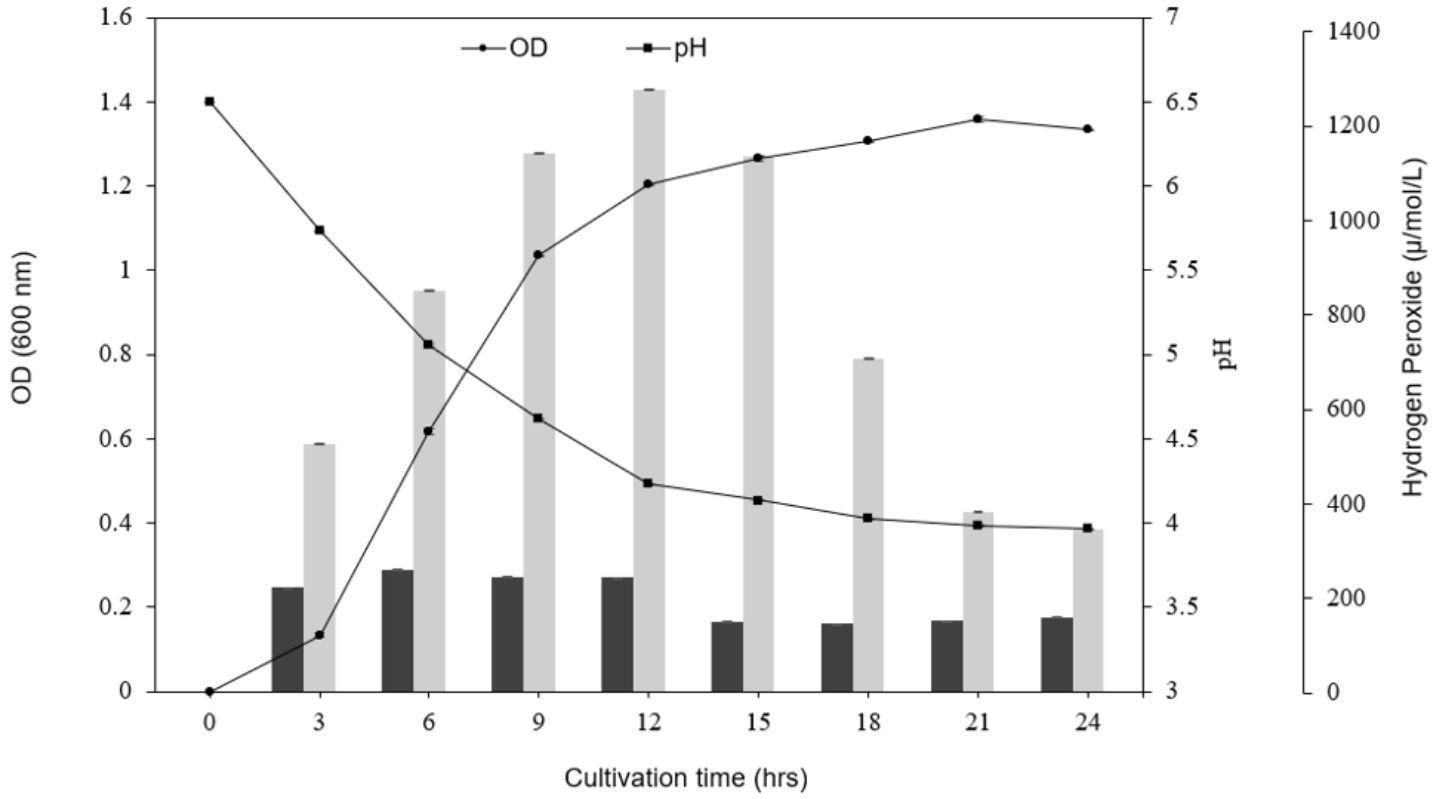

Figure 2

Optical density (●), pH (■) and accumulation of hydrogen peroxide of L. gasseri HHuMIN D (light gray) and W. cibaria (dark gray) cultivated in MRS medium (pH6.5) at 37 °C under anaerobic conditions.

Figure 3

Effect of L. gasseri HHuMIN D on the formation of artificial plaque by S. mutans on wires using BHI media containing 5% sucrose. Artificial plaque formed on wires in S. mutans single culture (A), L. gasseri HHuMIN D single culture (B) and L. gasseri HHuMIN D / S. mutans co-culture culture (C) are shown.

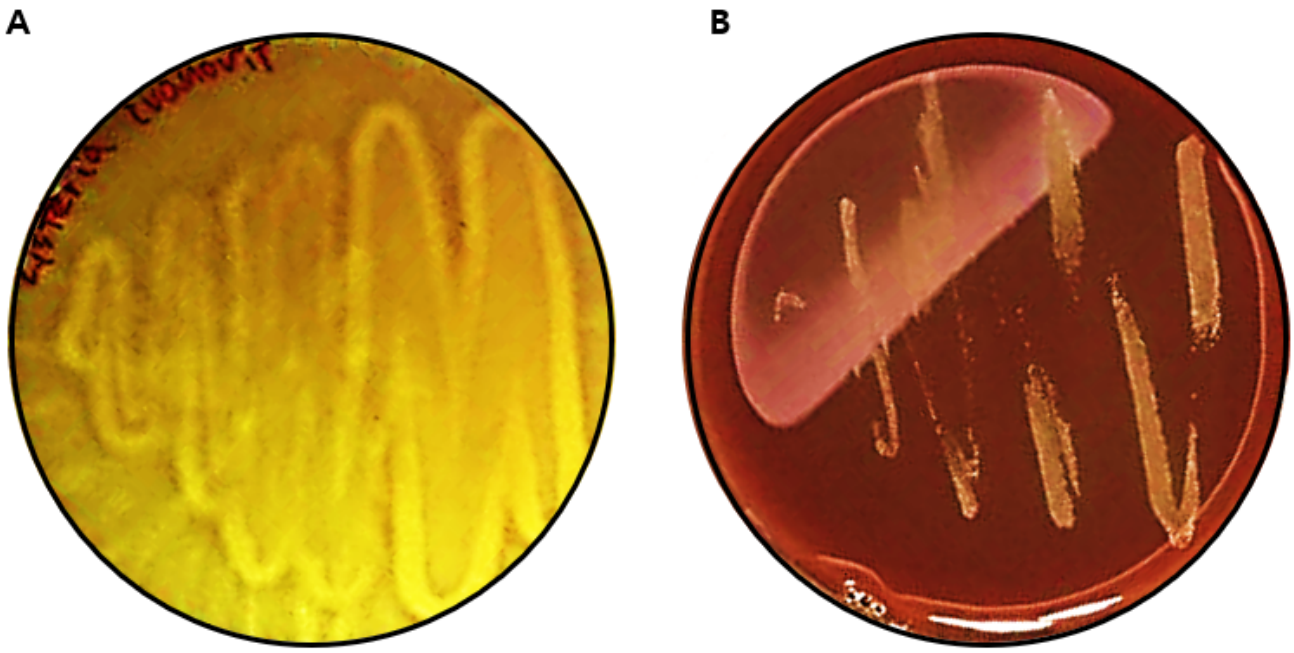

Figure 4

Hemolysis activity of L. ivanovii subsp. ivanovii ATCC 19119 ((A); positive control, back light) (beta hemolytic) and L. gasseri HHuMIN D ((B); back light). Purified culture strains were streaked on 5% blood agar plate. After 24 hours, the changes of plate color were observed. L. gasseri HHuMIN D growth with no blood cell lysis.

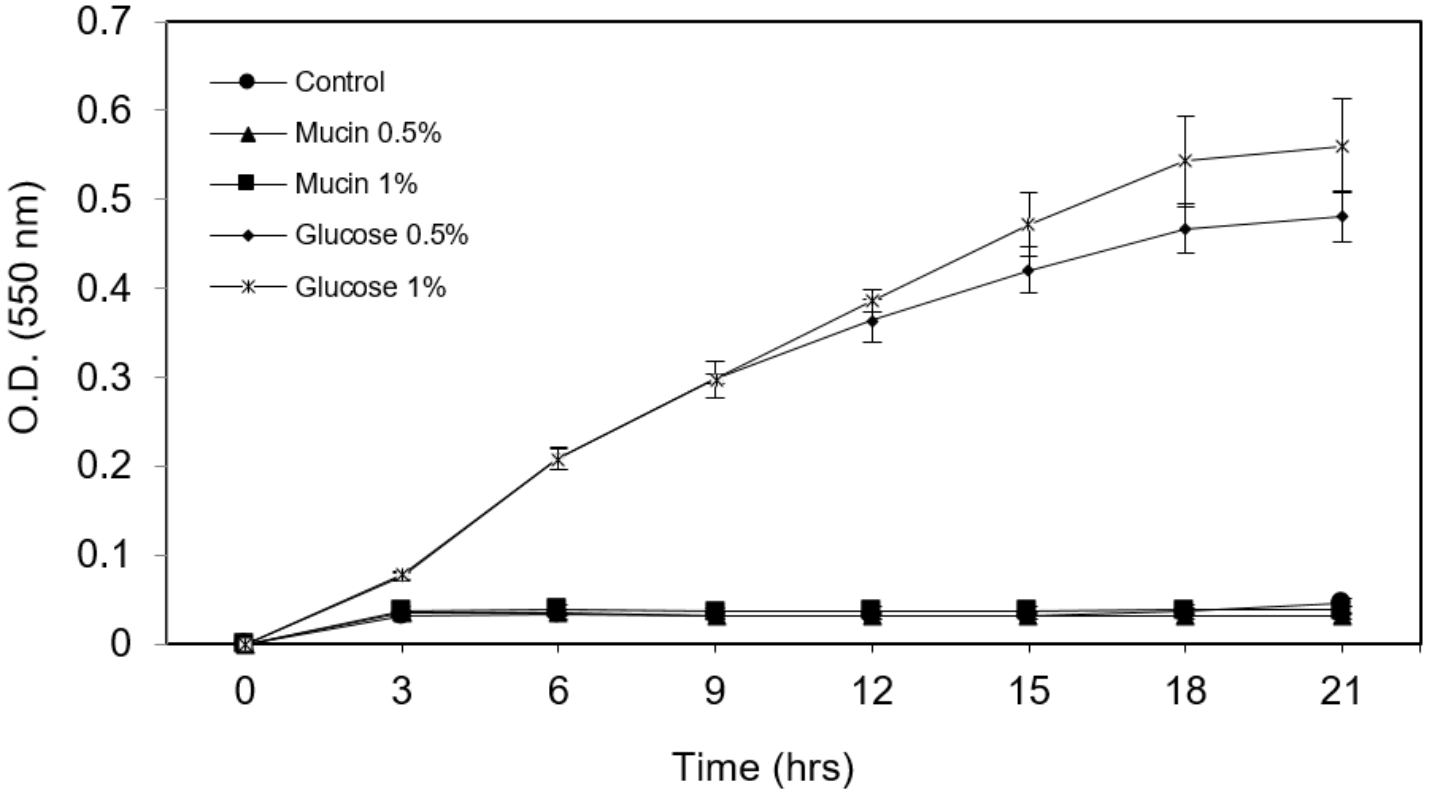

Figure 5

The effect of different concentrations (0, 0.5 and 1% [w/v]) of mucin and dextrose on L. gasseri HHuMIN D in modified MRS, evaluated by optical density (550 nm) and recorded after 3 to 21 hours.

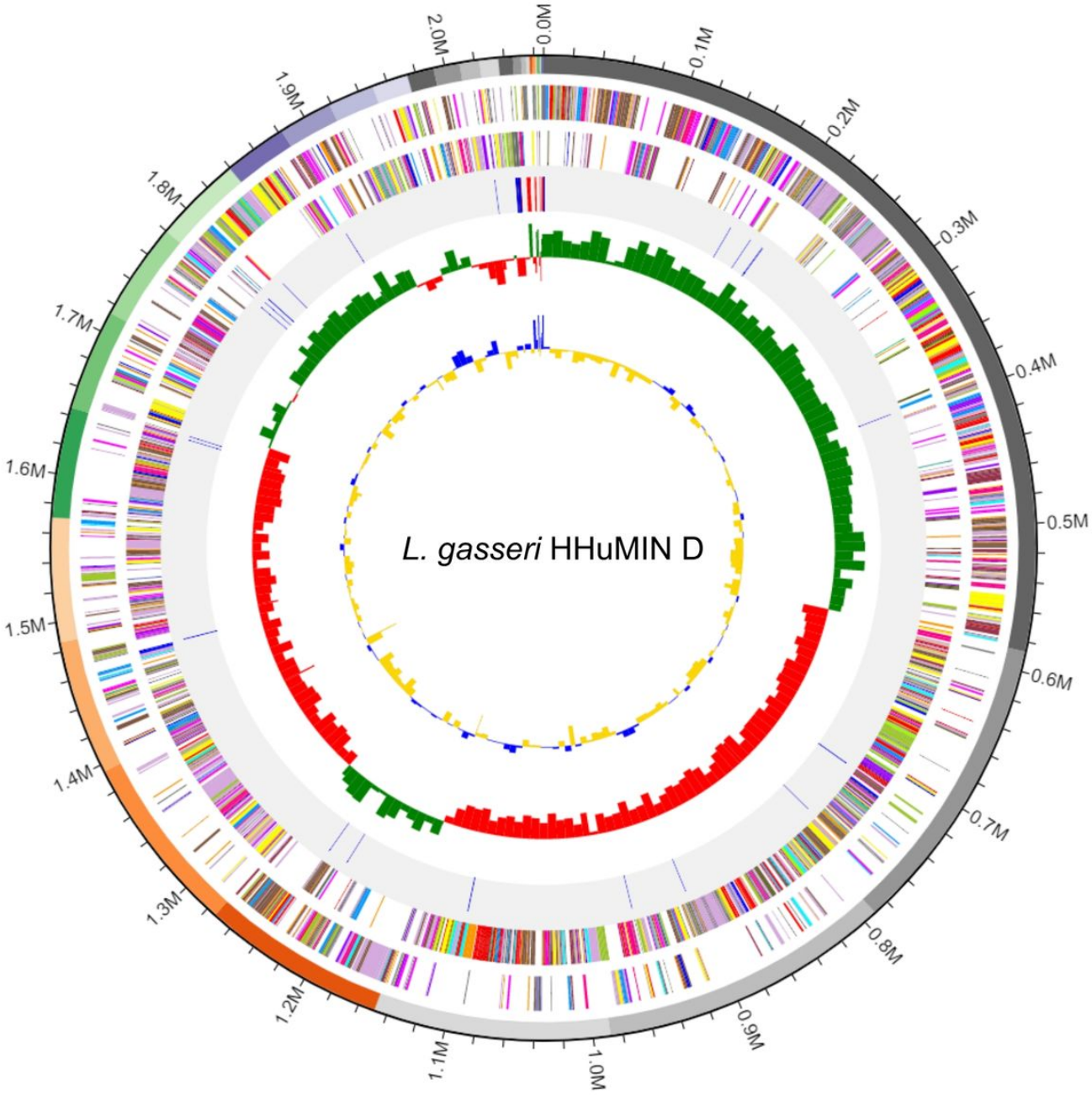

Figure 6

Genome map of L. gasseri HHuMIN D. Whole genome sequencing of L. gasseri HHuMIN D was performed by Chunlab, Inc. (Seoul, Korea), which used PacBio Sequel Systems (Pacific Biosciences, Menlo Park, CA, USA) and analyzed using CLgenomicsTM and EZBioCloud Apps programs (Chunlab, Seoul, Korea). The gene content circular image consists of five circles, and each circle shows the information of rRNA/tRNA, Reverse CDS, Forward CDS, GC Ratio and GC skew from the outside to the inside.

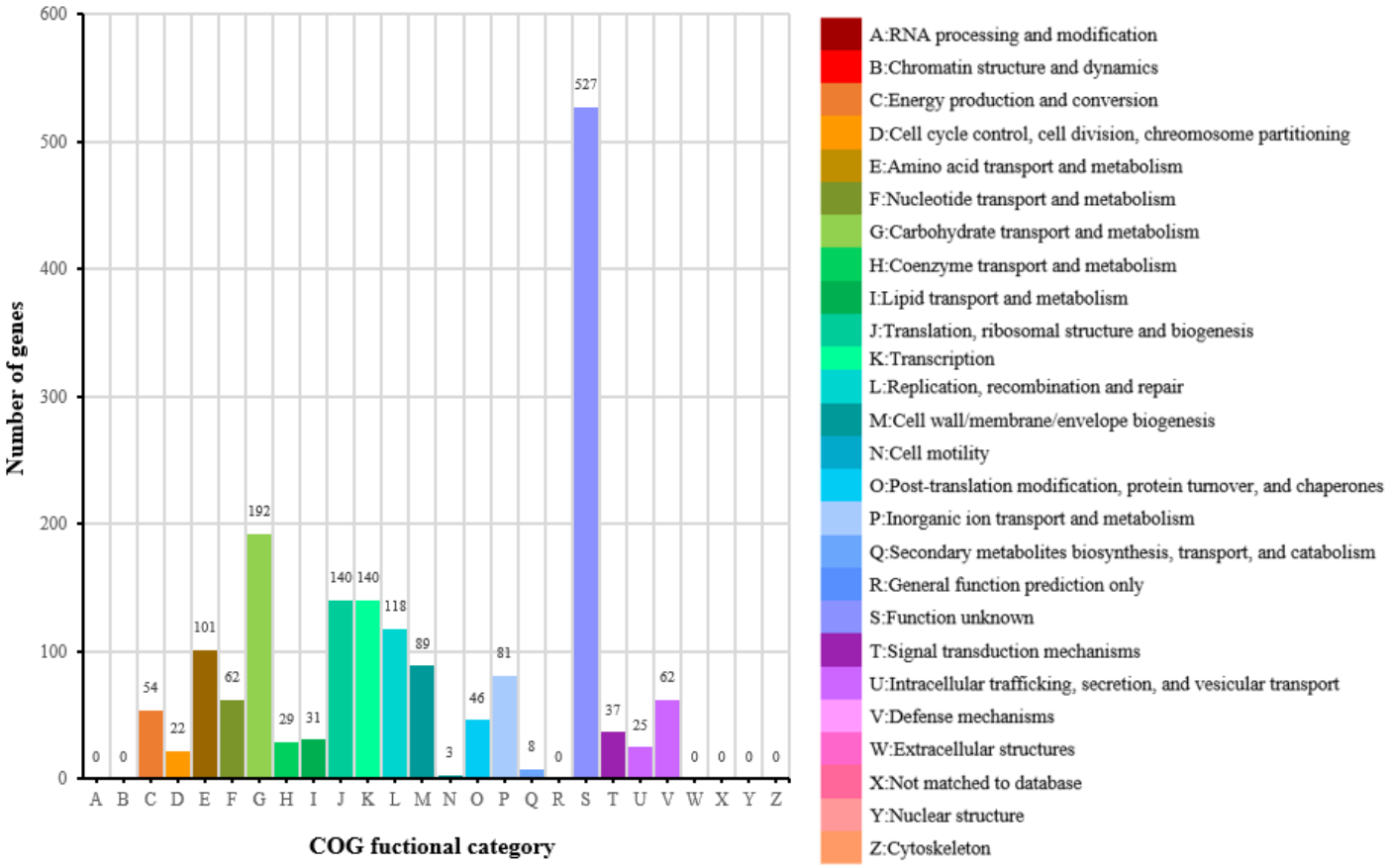

Figure 7

Functional categories based on EggNog/COG of genome of L. gasseri HHuMIN D. Whole genome sequencing of L. gasseri HHuMIN D was performed by Chunlab, Inc. (Seoul, Korea), which used PacBio Sequel Systems (Pacific Biosciences, Menlo Park, CA, USA) and analyzed using CLgenomicsTM and EZBioCloud Apps programs (Chunlab, Seoul, Korea). The function of each protein was found through homology comparison with NCBI's RefSeq Database, and functional categories were completed by synthesizing information such as COG classification by NCBI COG, SEED classification by FIGfam, and EC classification etc. The figure is a combination of the assignments of each COG and the functional category (25 categories, which are often designated from A to Z) using EggNog.

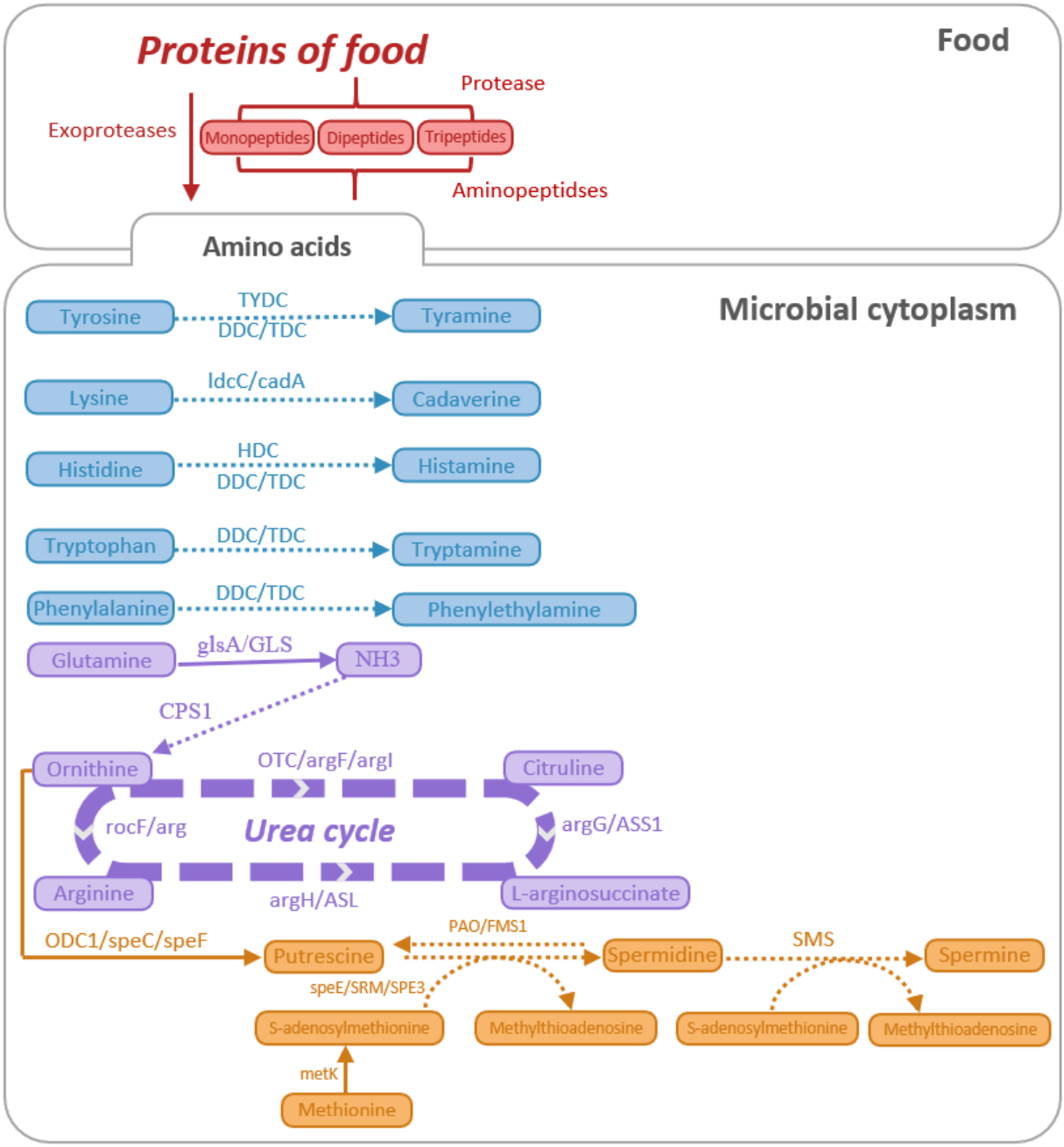

Figure 8

Biosynthetic pathways of different biogenic amines by L. gasseri HHuMIN D. Solid arrows indicate that the biosynthesis pathways of biogenic amine is effective and dashed arrows indicate invalid: TYDC, tyrosine decarboxylase; DDC/TDC, L-tryptophan decarboxylase; ldcC/cadA, lysine decarboxylase; hdc, HDC, histidine decarboxylase; glsA/GLS, glutaminase; CPS1, carbamoyl-phosphate synthase; OTC/argF/argI, ornithine carbamoyl transferase; argG/ASS1, argininosuccinate synthase; argH/ASL, argininosuccinate lyase; rocF/arg, arginase; ODC1/speC/speF, ornithine decarboxylase; PAO/FMS1, polyamine oxidase; SMS, spermine synthase; speE/SRM/SPE3, spermidine synthase; and metK, S-adenosylmethionine synthetase. Whole genome sequencing of L. gasseri HHuMIN D was performed by Chunlab, Inc. (Seoul, Korea), which used PacBio Sequel Systems (Pacific Biosciences, Menlo Park, CA, USA) and analyzed using CLgenomicsTM and EZBioCloud Apps programs (Chunlab, Seoul, Korea). Biosynthetic pathways of different biogenic amines were analyzed by matching with the KEGG pathway using complete genome sequencing of L. gasseri HHuMIN D via CL GenomicsTM

(ChunLab, Seoul, South Korea, 2004). The activities of related genes and substances produced can be inferred through the biosynthetic pathways provided by KEGG.

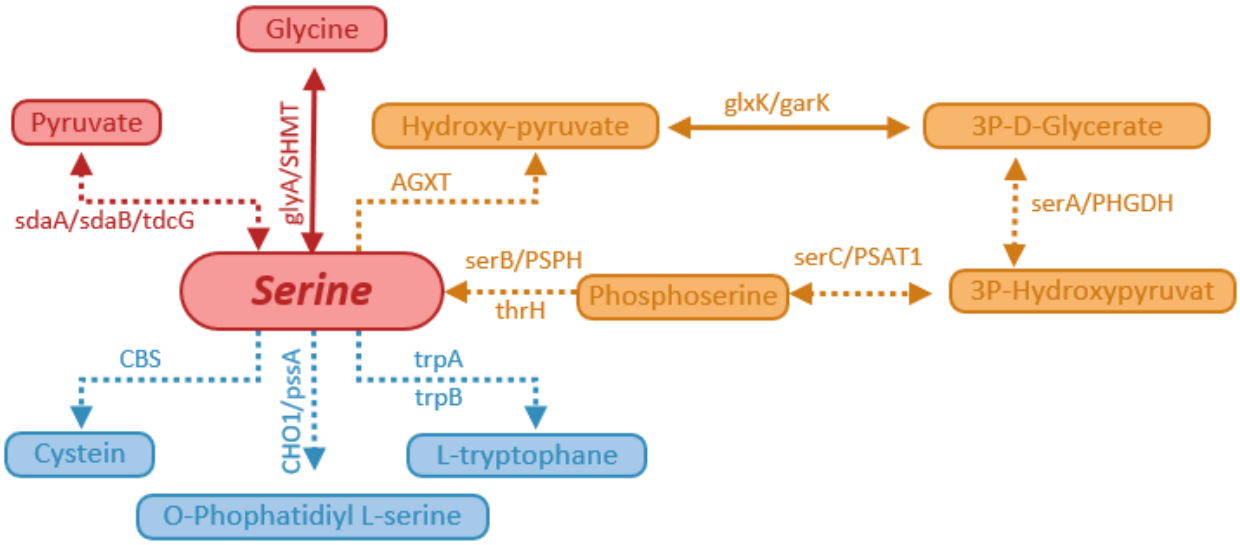

Figure 9

Glycine, serine, and threonine metabolism pathways of L. gasseri HHuMIN D. Solid arrows indicate that the metabolism pathways of glycine, serine, and threonine are effective and dashed arrows indicate invalid: sdaA/sdaB/tdcG, L-serine dehydratase; glyA/SHMT, glycine hydroxymethyltransferase; AGXT, alanine-glyoxylate transaminase; glxK/garK, glycerate 2-kinase; serA/PHGDH, D-3-phosphoglycerate dehydrogenase; serC/PSAT1, phosphoserine aminotransferase; serB/PSPH, phosphoserine phosphatase; thrH/phosphoserine, homoserine phosphotransferase; CBS, cystathionine beta-synthase; CHO1/pssA, CDP-diacylglycerol—serine O-phosphatidyltransferase; trpA, tryptophan synthase alpha chain; and trpB, tryptophan synthase beta chain. Whole genome sequencing of L. gasseri HHuMIN D was performed by Chunlab, Inc. (Seoul, Korea), which used PacBio Sequel Systems (Pacific Biosciences, Menlo Park, CA, USA) and analyzed using CLgenomicsTM and EZBioCloud Apps programs (Chunlab, Seoul, Korea). Biosynthetic pathways of glycine, serine, and threonine were analyzed by matching with the KEGG pathway using complete genome sequencing of L. gasseri HHuMIN D via CL GenomicsTM (ChunLab, Seoul, South Korea, 2004). The activities of related genes and substances produced can be inferred through the biosynthetic pathways provided by KEGG.