

Research

Evaluation of antioxidant activity and fermentation properties of potential probiotic strain *Lactiplantibacillus plantarum* HY7720 in plant-based materials

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Abstract

People on vegan diets are at risk of being deficient in varied nutrients such as vitamin B₁₂ and certain amino acids. In this study, we investigated vitamin B₁₂-producing lactic acid bacteria (LAB) as well as the probiotic and antioxidant properties. *Lactiplantibacillus plantarum* HY7720 was screened from 22 strains of LAB that were isolated from different plant foods, and its growth ability and extracellular vitamin B₁₂-producing capacity in vitamin B₁₂-deficient medium were investigated. To determine whether HY7720 functions as a probiotic, survival rate in the simulated gastrointestinal tract and adhesion property to human intestinal epithelial cells of HY7720 were compared with positive control, *Lacticaseibacillus rhamnosus* GG (LGG). Moreover, the results showed that HY7720 recovered the gene expression levels of tight junction-associated proteins (TJPs), including TJP1, TJP2, occludin (OCLN), and claudin-1 (CLDN1) and inhibited the secretion levels of pro-inflammatory cytokines, including interleukin (IL)-6 and IL-8, in tumor necrosis factor (TNF)- α -stimulated Caco-2 cells. Furthermore, we verified that HY7720 exhibit the antioxidant potential, by showing its intracellular reactive oxygen species (ROS) scavenging ability in hydrogen peroxide (H₂O₂)-stimulated Caco-2 cells. The ability of HY7720 to ameliorate H₂O₂-induced cytotoxicity in Caco-2 cells was inhibited by mitogen-activated protein kinase (MAPK) inhibitors, indicating that its antioxidant responses are related to extracellular signal-regulated kinase (ERK) and c-JUN N-terminal kinase (JNK). This study also investigated the nutritional qualities of three plant-based materials (brown rice, white rice, and soy milk) fermented using HY7720. Collectively, HY7720 could be used as a promising probiotic strain for the prevention of nutritional deficiencies among people on vegetarian diets.

Article Highlights

- *Lactiplantibacillus plantarum* HY7720 is a promising probiotic candidate and fermentation starter for people on vegetarian as well as omnivorous diets.
- *L. plantarum* HY7720 has an extracellular vitamin B₁₂-producing capacity.
- *L. plantarum* HY7720 can help the body's probiotic and antioxidant function.

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1 Introduction

Vegetarian and vegan diets lead to a low intake of saturated fatty acids and animal protein and high intake of fiber and phytochemicals [1]. However, whether the health benefits of vegetarian diets exceed the risks of nutrients deficiencies has not been cleared. In addition, the numerous studies have emphasized that vegetarian diets are at risk of vitamin B₁₂ (cobalamin) deficiency in growing children and adolescents [2]. Moreover, half of the vegans and 7% of vegetarians were categorized as vitamin B₁₂ deficient in the cross-sectional analysis involving 689 men [3]. Vitamin B₁₂, generally supplied in animal-source foods, is synthesized by specific archaea or bacteria, not by plants [2]. Vitamin B₁₂ is necessary for synthesis of nucleic acids, erythrocytes, and nucleoprotein [4]. Recently, as increasing number of people who follow the vegetarian and vegan diets and improve their health through intaking balanced nutrition, the interests of vitamin B₁₂ have been growing steeply in the food industry [5].

A diversity of fermented foods and beverages, including kimchi, jangajji, and makgeolli, in major source of beneficial lactic acid bacteria (LAB) in different regions of Korea are produced. The fermented products are known for various pro-healthy benefits owing to the presence of LAB with probiotic, antioxidant, and antibacterial properties. These properties are primarily demonstrated to probiotics belonging to LAB, particularly *Lactobacillus* [6]. In addition, the fermented products have a high content of vitamins, minerals, and dietary fibers. Accordingly, a variety of researches have attempted to evaluate the health-promoting constituents and investigate metabolic processes of the probiotics for application in agriculture, nutrition, and pharmaceutical industries [7].

Probiotics is generally expected to prevent intestinal barrier dysfunction, providing maintenance of the host's health. Thus, it is essential to assess whether a novel LAB strain has protective effect on injury and disruption of intestinal barrier for validating its potential application as probiotic strain in food and pharmaceutical industries. Injury of intestinal epithelial cell leads to increase of intestinal permeability, which is closely related to the development of inflammatory diseases [8]. Tight junctions (TJs), intercellular adhesion protein complexes between epithelial cells, play crucial roles in regulation of epithelial barriers through paracellular pathway [9]. The various studies have reported that the dysfunction of intestinal barrier is associated with disturbance in the expression and translocation of TJ proteins (TJPs) [10]. A study by J.-D.Schulzke has reported that the number of TJ chains is reduced, accompanied by alternation of the expression levels of TJ proteins, in the intestinal tissues from patients with inflammatory bowel disease (IBD) [11]. Therefore, gene regulatory effect of a novel probiotic on intestinal TJ components, including TJP1, TJP2, occludin (OCLN), and claudin-1 (CLDN1), indicates that it plays potential role in maintenance of gut barrier. In addition, pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 are known as risk factors related to the pathogenesis of IBD and colorectal cancer. Hence, it is also important to evaluate the novel probiotic has the inhibitory effects on release of the pro-inflammatory cytokines in model of intestinal injury for using therapeutic implication or preservative solution for diverse intestinal disorders.

The certain probiotics play also antioxidant roles by alleviating oxidative stress. Oxidative stress is an imbalance condition between the generation and removal of reactive oxygen species (ROS) [12]. The long-term ROS exposure cause the development of various intestinal diseases, including IBD, ischemic intestinal injury, and colorectal cancers [13]. As antioxidants, the modes of action of probiotics are belong to chelating metal ion, producing antioxidant metabolites, up-regulating anti-oxidase activities, and regulating signaling pathways [14].

Lactiplantibacillus plantarum has a variety of beneficial properties for host, including improving immune system, balancing intestinal microecology, and increasing absorption of nutrients [15]. Owing to its functions, *L. plantarum* has been widely used in the dairy and food industry as the probiotic. Probiotics must survive in gastrointestinal environment if they colonize in small intestinal and regulate intestinal flora, thereby providing beneficial effects on human health [16]. *Lactobacillus* species are known to be resistant to acid condition intrinsically [17]. However, even strains belong to the same species exhibit a diversity of metabolic capacities and probiotic properties, showing strain-specific variability [18]. Therefore, it is important to precisely examine its certain probiotic potentials for application of industrial strain [19].

Fermentation using probiotics can be an efficient nutritional and preservative strategy to improve the quality of food products. *L. plantarum*, widely used as a starter culture of fermented food, has been known that it improves organoleptic characteristics of products [20]. Furthermore, it has abilities to synthesis a variety of bioactive compounds, including riboflavin, folic acid, and γ -aminobutyric acid, in the fermented foods using the species [21]. In addition, its enzymatic

hydrolysis can raise the protein bioavailability by increasing the content of free amino acids [22]. According to the findings, *L. plantarum* has a potential in development of nutritional and functional properties in fermented foods.

The aim to this study is to suggest a novel probiotic as a suitable alternative to vegetarian or vegan and present potential to its application in plant-based products. Herein, we selected the *L. plantarum* HY7720 that exerted high potential as vitamin B₁₂ producing bacteria and examined its probiotic and antioxidant properties. We also verified that its fermentative characteristics, including fortification of deficient nutrition in vegan diets and antioxidant activity, in plant-derived ingredients.

2 Materials and methods

2.1 Media and treatments

Modified eagle medium (MEM), antibiotic–antimycotic solution, fetal bovine serum (FBS), and trypsin–EDTA were purchased from Gibco (Grand Island, NY, USA). Recombinant human TNF- α protein was obtained from R&D Systems (Minneapolis, MN, USA). Hydrogen peroxide (H₂O₂), α -amylase from human saliva, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, bile extract porcine, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) diammonium salt, potassium persulfate, ERK inhibitor (PD98059), JNK (SP600125), and p38 inhibitor (SB203580) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) was purchased from Invitrogen (Waltham, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and IL-8 were purchased from BD Biosciences (San Diego, CA, USA).

2.2 Isolation and preparation of bacterial strains

Twenty-two LAB strains were isolated from fermented plant food collected from a variety of regions in Korea and stored in a microbial strain library at hy Co., Ltd. (Yongin, Republic of Korea) and used in the present study. Each sample was aseptically mixed with PBS with the ratio of 1:9 and homogenized using a stomacher. 1 mL of the samples was serially diluted and spread onto MRS agar. After incubation for 2 days at 37 °C, representative colonies were inoculated into MRS broth and each culture were stored with sterilized 10% skim milk at – 80 °C. *Lactocaseibacillus rhamnosus* GG (ATCC53103) (LGG) was obtained from the American Type Culture Collection (Manassas, VA, USA). LAB strains were obtained by streaking each freezer stock onto de Man, Rogosa and Sharpe (MRS) agar plates and incubating at 37 °C for 72 h. Single colonies were inoculated into MRS medium and incubated overnight at 37 °C. The cultures were centrifuged and the pellets were washed twice with sterile phosphate-buffered saline (PBS). Subsequently, the cell pellets were resuspended in PBS saline, or plant-based materials (brown rice, whited rice, and soy milk) for respective experiments.

2.3 Selection of vitamin B₁₂-producing lactic acid bacteria

The single-colony cultures were inoculated into 3 mL of vitamin B₁₂-free modified chemically defined media (CDM) based on a previous study with some modification [23]. After incubation for 3 days at 37 °C, *Lactiplantibacillus plantarum* HY7720, which was originally isolated from fermented vegetable foods, and showing exceptional absorbance at 600 nm, was preliminarily determined as a vitamin B₁₂ producer.

2.4 Quantitative analysis of vitamin B₁₂

To investigate extracellular vitamin B₁₂ derived from HY7720, the cells were incubated overnight at 37 °C in vitamin B₁₂-free modified CDM, and the supernatant was centrifuged at 13,000 $\times g$ for 20 min. The vitamin B₁₂ content of the supernatant was analyzed via high-performance liquid chromatography (HPLC) using a slightly modified method of a previous study [24]. The supernatant was filtered using a 0.22 μm membrane filter and stored in the dark. The operating conditions were as follows: column, Supelco Discovery C18 column (250 mm \times 4.6 mm I.D., 5 μm); flow rate, 0.5 mL/min; column temperature, 30 °C; injection volume, 10 μL ; wavelength of detector, UV 254 nm (Agilent 1260 G4212B); mobile phase, 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B); gradient, 0–2 min 20% A, 2–3 min 20–28% A, 3–11 min 28–35%, 11–19 min 35–43% A, 19–20 min 43–100% A, 20–22 min 100–100% A, 22–26 min 100–20% A, and 26–36 min 20% A.

2.5 Survivability in the gastrointestinal tract simulation model

The probiotic potential of HY7720 was determined by measuring survival rate in the simulated gastrointestinal tract (GIT) model system. The simulated digestion fluids, including simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), were prepared with electrolyte stock solutions, enzymes, bile extract, CaCl_2 , and water, as previously described [25]. The each simulated digestion fluids used as follows: SSF, 15.1 mmol/L KCl, 3.7 mmol/L KH_2PO_4 , 13.6 mmol/L NaHCO_3 , 0.15 mmol/L $\text{MgCl}_2(\text{H}_2\text{O})_6$, and 0.06 mmol/L $(\text{NH}_4)_2\text{CO}_3$; SGF, 6.9 mmol/L KCl, 0.9 mmol/L KH_2PO_4 , 25 mmol/L NaHCO_3 , 47.2 NaCl, 0.1 mmol/L $\text{MgCl}_2(\text{H}_2\text{O})_6$, and 0.5 mmol/L $(\text{NH}_4)_2\text{CO}_3$; and SIF, 6.8 mmol/L KCl, 0.8 mmol/L KH_2PO_4 , 85 mmol/L NaHCO_3 , 38.4 NaCl, and 0.33 mmol/L $\text{MgCl}_2(\text{H}_2\text{O})_6$. The cell culture suspensions of HY7720 and LGG, a well-known probiotic strain, were mixed with SSF (75 U/mL α -amylase, 0.75 mM CaCl_2) to adjust pH to 7.0 (50:50, v/v). After 2 min of incubation at 37 °C, the mixtures were mixed with SGF (1000 U/mL porcine pepsin, 0.075 mM CaCl_2) to adjust pH to 3.0 (50:50, v/v) and incubated for 2 h at 37 °C with continuous shaking. Subsequently, the mixtures were mixed with SIF (40 U/mL porcine pancreatin, 0.3 mM CaCl_2 , 0.24% bile extract) to adjust pH to 7.0 (50:50, v/v). Aliquots of these mixtures obtained at the end of each step were used to estimate the survival rates.

2.6 Adhesion assay to intestinal epithelial cells

The adhesion ability to human intestinal epithelial cells was assessed as previously reported [26]. Briefly, human colonic carcinoma Caco-2 cells were obtained from the ATCC and maintained in MEM supplemented with 10% FBS and 1% antibiotic–antimycotic at 37 °C under 5% CO_2 . Caco-2 cells were plated onto 24-well plates (1.0×10^5 cells/well). When the cells reached 100% confluency, the medium was replaced with MEM without FBS. HY7720 and the reference strain LGG were diluted in PBS and inoculated into Caco-2 cells at a density of 1.0×10^8 cells/mL/well. After 2 h of incubation at 37 °C, the cells were washed four times with PBS and treated with 0.05% trypsin–EDTA for 5 min to separate them from the plates. The cell viability of HY7720 and the reference strain was determined using MRS agar plates.

2.7 Reverse transcription-PCR (RT-qPCR) analysis

Caco-2 cells were plated at 1.0×10^5 /well in 12-well plates and maintained in a cell culture medium (MEM supplement with 10% FBS and 1% antibiotic–antimycotic). To obtain mature differentiated monolayer, the culture medium was changed with fresh medium every 2–3 days for a total of 21 days. The differentiated cells were pretreated with or without 1.0×10^7 CFU/mL of HY7720 or LGG in a serum-free medium for 1 h, followed by TNF- α (100 ng/mL) treatment for 72 h. Phosphate-buffered saline (PBS) was used vesicle control. Total RNA was extracted using an Easy-BLUE™ Total Extraction kit (iNtRON Biotechnology Inc., Seongnam, Republic of Korea). cDNA was synthesized from 1 μg of total RNA using an Omniscript® Reverse Transcription kit (Qiagen, Hilden, Germany). The mRNA levels were quantified using QuantStudio 6 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and a TaqMan Universal PCR Master Mix (Applied Biosystems). The levels of TJP1 (Hs01551861_m1), TJP2 (Hs00910543_m1), OCLN (Hs00170162_m1), CLDN1 (Hs00221623_m1), and GAPDH (Hs99999905_m1) transcripts were quantified using gene-specific primers.

2.8 Cytokine measurement

The culture media were collected under the conditions described above and used to measure the levels of IL-6 and IL-8 using ELISA kits according to the manufacturer's instructions.

2.9 ABTS⁺ radical scavenging assay

ABTS⁺ radical scavenging activities of HY7720, LGG, and the fermented samples were determined using the modified method of a previous study [27]. Briefly, to prepare ABTS radical cation stock solution, 7 mM of ABTS was mixed with 2.4 mM of potassium persulfate in equal quantities and allowed to react in the dark at room temperature for 16 h. The stock solution was diluted with distilled water appropriately to show absorbance of 1.0 ± 0.02 at 734 nm. All samples and the positive control (L-ascorbic acid) were reacted with the working solution in the dark at room temperature for 15 min, and absorbance was measured at 734 nm. Antioxidant activity was calculated as follows: antioxidant activity (%) = $[1 - (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{vehicle sample}} - A_{\text{vehicle blank}})] \times 100$.

2.10 Intracellular ROS scavenging assay

Caco-2 cells (2.0×10^4 /well; 96-well plate) were pretreated with HY7720 or LGG (2.0×10^5 CFU/mL) for 1 h, followed by H_2O_2 treatment (500 μ M) for 1 h. The cells were incubated with 10 μ M of H_2 DCFDA solution in the dark at 37 °C for 30 min and washed with PBS. DCF fluorescence was measured (excitation 485 nm, emission 528 nm) using a Synergy HTX multi-mode reader (BioTek Instruments, Inc., Winnoski, VT, USA).

2.11 Lactate dehydrogenase (LDH) leakage assay

Caco-2 cells (2.0×10^4 /well; 96-well plate) were pretreated with specific MAPK inhibitors (20 μ M of PD98059, 20 μ M of SP600125, or 10 μ M of SB203580) for 30 min, prior to HY7720 or LGG (2.0×10^5 CFU/mL) treatment. After 1 h incubation, the cells were treated with 1 mM of H_2O_2 for 2 h, and the culture media were collected and subjected to LDH measurement. A CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI, USA) was used following the manufacturer's instructions.

2.12 Fermentation of plant-based materials

To investigate the effects of HY7720 on the fermentation quality of plant-based materials, brown rice (BR) and white rice (WR) extracts were obtained from Eco trading co., Ltd. (Seongnam, Republic of Korea), and soy milk (SM) was obtained from hy Co., Ltd. All fermentation solutions of these planted-based materials were diluted with distilled water in a 1:10 (*w/w*) ratio, and then centrifuged. The fermentation solutions were inoculated with a pure culture of HY7720 at a final concentration of 1.0×10^9 CFU/mL and cultured at 37 °C for 24 h. After centrifugation of the samples (3 plant-based materials before fermentation and 3 plant-based materials after fermentation), the supernatants were filtered using 0.22 μ m pore-sized membrane filter to examine different parameters.

2.13 Analytical determination of lactate and amino acids

The level of lactate in the samples was quantified via HPLC using the method of a previous study [28]. The operating conditions were as follows: column, Aminex HPX-87H (Bio-Rad, 300 mm \times 7.8 mm); flow rate, 0.6 mL/min; column temperature, 50 °C; injection volume, 20 μ L; wavelength of detector, UV 210 nm; and mobile phase, 4 mM of sulfuric acid. The level of amino acids was analyzed using an L-8500 high-speed amino acid analyzer (Hitachi Ltd., Tokyo, Japan) with #2622SC (Hitachi, 4.6 mm I.D. \times 60 mm), as described previously [29]. For the pretreatment, the samples and 5% trichloroacetic acid (TCA) were mixed in equal quantities and centrifuged at 10,000 rpm for 15 min, followed by acid hydrolysis with 0.02 N of hydrochloric acid (HCl). The operating conditions were according to the manufacturer's instructions.

2.14 Microbial growth analyses

To compare the growth ability of HY7720 in media containing nitrogen source derived from a plant or animal source, all tested media were prepared based on MRS medium with modifications. The specific recipe was as follows: glucose, 20 g/L; yeast extract, 15 g/L; complex nitrogen source, 10 g/L; dipotassium hydrogen phosphate, 2 g/L; sodium acetate, 5 g/L; diammonium hydrogen citrate, 2 g/L; magnesium sulfate, 0.01 g/L; manganese sulfate, 0.04 g/L; and polysorbate 80, 1 g/L. The following nitrogen sources were tested: soy peptone such as Hy-Soy™ Kosher IPS (Kerry Bio-Science, Norwich, NY, USA) and HSP-349 (The Tatua Co-Operative Dairy Co., Ltd., Morrinsville, New Zealand), Broadbean peptone (Solabia Group, Pantin, France), casein peptone such as Bacto™ Tryptone from casein (Beckton Dickinson, CA, USA) and HCP-321 (The Tatua Co-Operative Dairy Co., Ltd.), and lactalbumin peptone (Tatua-2016, The Tatua Co-Operative Dairy Co., Ltd.). The growth curves were recorded at 37 °C by measuring absorbance at 600 nm (OD_{600}), and the maximum specific growth rate and the slope of the exponential growth phase ($\mu_{max} = \Delta \ln(OD_{600})/\Delta t$) were determined.

2.15 Molecular characterization of bacterial isolates

Whole genome sequencing (WSG) were conducted at Sanigen Inc. (Seoul, Republic of Korea). The genomic DNA of *L. plantarum* HY7720 was sequenced with Illumina MiSeq and Oxford Nanopore MinION platforms. For Illumina sequencing,

the extracted genomic DNA was fragmented using Covaris M220 (Covaris, MA, USA). The sheared DNA were used to prepare a WGS library with an average insert size of 550 bp using a TruSeq Nano DNA Sample Prep kit (Illumina, CA, USA). The library was sequenced on an Illumina MiSeq platform using 300 bp paired-end sequencing mode. For nanopore sequencing, a MinION sequencing library was prepared using the Nanopore Ligation Sequencing Kit (SQK-LSK110; Oxford Nanopore Technologies, Oxford, UK). The library was sequenced with an R9.4.1 MinION flow cell (Flongle) for a 24 h run using MinKNOW with the default settings (MinKNOW core 5.0.0, Guppy 6.0.6). The qualified sequencing data were de novo assembled with Unicycler v0.4.8. The genome was annotated using Prokka v 1.14.6 and their coding sequence were identified. Based on the virulence factor database (VFDB), we surveyed the HY7720 isolates for the presence of four virulence factors such as hemolysin/cytolysin (*cylA*), aggregation substance (*asa1*), hyaluronidase (*hyl*), and gelatinase (*gelE*).

2.16 Antibiotic susceptibility of bacterial isolates

HY7720 was evaluated for its susceptibilities to seven antibiotics according to the guidelines followed by the European Food Safety Authority (EFSA). The minimal inhibitory concentrations (MICs) of ampicillin, gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, and chloramphenicol were then determined using MIC Test Strip (Liofilchem, Teramo, Italy) according to the manufacturer's instructions. The MIC value was the point where the zone of inhibition that intersects each strip.

2.17 Statistical analysis

All data are expressed as mean \pm standard deviation (SD). Significant differences were identified using one-way analysis of variance (ANOVA), followed by Newman–Keuls test (multiple groups) or Student's t-test using GraphPad Prism v5 (San Diego, CA, USA).

3 Results and discussion

3.1 HY7720 is a novel extracellular vitamin B₁₂-producing *Lactiplantibacillus* strain

Vegetarian or vegan diets may be selected for ethical, health, or religious reasons. In recent years, the demands for vegetarian or vegan diets have risen sharply owing to their health benefits in terms of prevention of several diseases, such as cardiovascular disease, type 2 diabetes, and cancer [30]. However, since vegetarians are more likely to have a higher socio-economic status and to follow a health-conscious lifestyle with regular exercise and no smoking, it is not clear whether the benefits of vegetarian diets overcome the risks of nutritional deficiencies, including deficiencies in vitamin B₁₂, certain amino acids, iron, and omega-3 [2]. In particular, several studies have highlighted the health risks of vitamin B₁₂ deficiency due to veganism and vegetarianism [2, 3, 31]. As previously stated, major strains that have high natural production level of vitamin B₁₂ are *Pseudomonas freudenreichii* and *P. denitrificans* [32]. Additionally, several studies reported that certain *Lactobacillus* species show ability to produce vitamin B₁₂, including *Limosilactobacillus reuteri*, *Lactobacillus coryniformis*, *Lactiplantibacillus plantarum*, and *Lactobacillus rossiae* [33]. Since intracellular environment is a main barrier to in situ fortification of these strains in foods, it is important to verify their ability for extracellular production [24]. In this study, we tested the growth of 22 LAB strains from plant-based sources in CDM medium without vitamin B₁₂, but all strains did not grow. After that, the growth of these strains was evaluated using the vitamin B₁₂-free modified CDM medium (contained cobalt, an essential component of vitamin B₁₂) (Supplementary Table 1). After discovering HY7720 had the highest growth in that medium, we verified that HY7720 produced 38 μ g/L of vitamin B₁₂ (CN-Cbl) in the vitamin B₁₂-free modified CDM broth extracellularly (Supplementary Fig. 1). Although CN-Cbl is a physiologically inactive form of cobalamin in contrary to adenosylcobalamin (Ado-Cbl) and methylcobalamin (Met-Cbl), CN-Cbl has higher stability toward heat and light than the active forms; therefore, it is considered most utilized commercial form of vitamin B₁₂ [34]. However, since vitamin B₁₂ production of HY7720 is less than industrial vitamin B₁₂-producing strains, further examination is necessary to enhance its production of extracellular vitamin B₁₂. The genome of HY7720 consists of 6 contigs with total size of 3,508,619 bp and a GC% of 44.15%. The average nucleotide identity (ANI) gave a value of 98.95% between HY7720 and *L. plantarum* CIP 103151 (NR_104573.1), identifying the strain as *L. plantarum*.

3.2 HY7720 shows resistance in simulated gastrointestinal tract and adherence ability to human intestinal cells

Probiotics provide health benefits for the host when they are taken in adequate amounts; as living microorganisms, they help maintain the balances of intestinal microflora. Although various *Lactobacillus* species are known to have probiotic properties, only certain strains with ability to survive in the gastrointestinal tract can be considered probiotic strains [35]. Indeed, survivability and ability to adhere to the intestinal epithelium are the main criteria used to select probiotic strains [36]. The survival rates of HY7720 and LGG were not affected under the conditions of the oral phase. HY7720 had a similar gastric survival rate to LGG. Moreover, HY7720 showed non-significantly higher resistance to intestinal conditions than LGG (Fig. 1A). We also estimated the ability of the two strains to adhere to human intestinal epithelial Caco-2 cells. As shown in Fig. 1B, HY7720 exhibited a significantly higher ability to adhere to the Caco-2 monolayer than the reference strain LGG. In addition, seven antibiotics were tested according to the EFSA guidance, and HY7720 was found to be susceptible to all antibiotics (Supplementary Table 2). The WGS analysis also showed that the virulence genes, including *cylA*, *asa1*, *hyl*, and *gelE* were not detected in HY7720 isolates.

3.3 HY7720 inhibits TNF- α -induced down-regulation of tight junction-associated genes and pro-inflammatory cytokines production in Caco-2 cells

The pro-inflammatory cytokine TNF- α triggers an increase in epithelial barrier permeability by interrupting TJs [37]. Additionally, previous studies have reported that TNF- α leads to the activation of the nuclear transcription factor kappa B (NF- κ B), followed by the expression of diverse pro-inflammatory genes [38]. Thus, TNF- α is widely used to induce an increase in intestinal epithelial TJ permeability and intestinal inflammation. In this study, we observed that HY7720 significantly increased the mRNA expression levels of TJ components, including scaffolding molecules, zonula occludens (ZO) (also known as *TJPs*), and transmembrane proteins (*OCN* and *CLDN1*), were reduced by TNF- α in Caco-2 cells (Fig. 2A–D). LGG treatment also tended to attenuate TNF- α -induced down-regulation of tight junction-associated genes. However, there was no statistical significance in *TJP1* and *TJP2* expression levels. The recovery effects of ZO, *OCN*, and *CLDN1* expression levels by HY7720 were in line with a previous research showing the maintenance effects of their protein expression in a murine colitis model by probiotic mixtures [39]. HY7720 and LGG also significantly reduced the secretion levels of the pro-inflammatory cytokines, IL-6 and IL-8, which were increased by TNF- α in Caco-2 cells (Fig. 2E, F). Our data suggest that HY7720 can be used to reinstate the intestinal epithelial barrier, providing a novel treatment option for gut barrier dysfunction.

3.4 HY7720 is protective against oxidative stress-induced cytotoxicity through regulation of ERK and JNK

The roles of probiotic formulations in promoting antioxidation have also been highlighted in a number of studies [14, 40]. LGG has been proven in previous studies to have good antioxidant capacity [40]. In our work, to unveil if HY7720

Fig. 1 Survivability of HY7720 and LGG in the gastrointestinal tract simulation model and adherence ability to Caco-2 cells. **A** The survival rates of each step were estimated compared to the initial number of each strain. **B** Adhesion ability was determined as the percentage of adhesion to Caco-2 cells. Data are represented as mean \pm SD of three independent experiments. ** $p < 0.01$ and *** $p < 0.001$ when compared to the LGG group

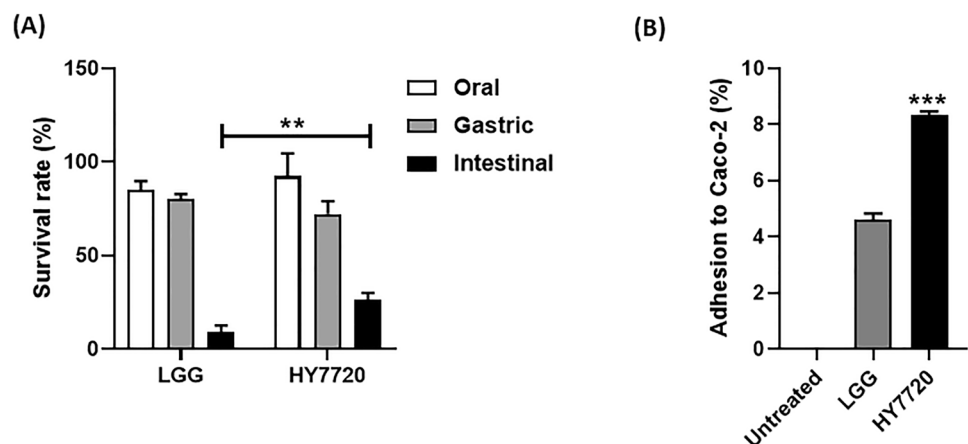
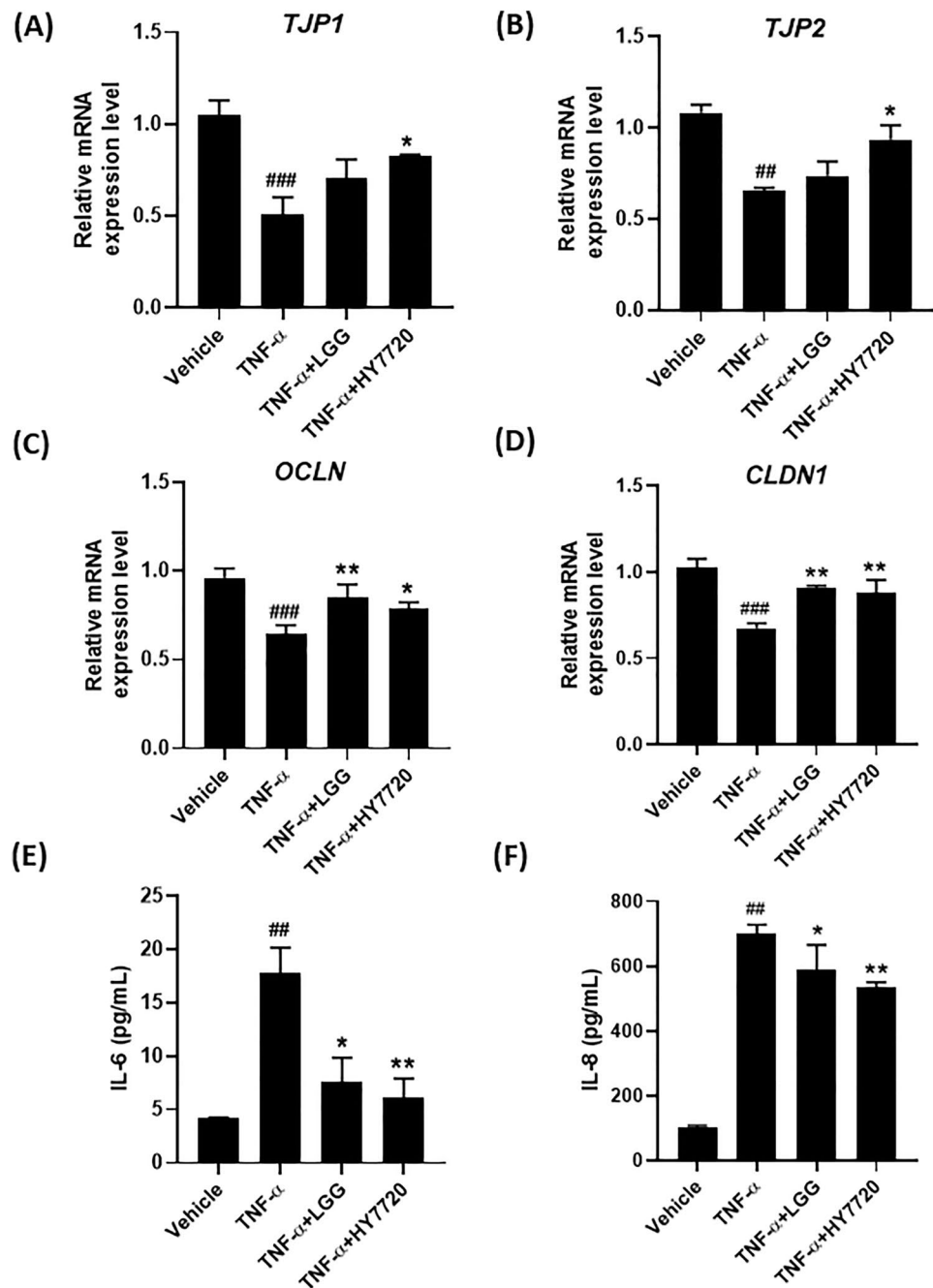


Fig. 2 Effects of HY7720 on mRNA expression of tight junction-associated proteins and production of pro-inflammatory cytokines in TNF-treated Caco-2 cells. Relative mRNA levels of **A** TJP1, **B** TJP2, **C** OCLN, and **D** CLDN1 were normalized against that of GAPDH. **E** IL-6 and **F** IL-8 secretion in the culture media was determined using ELISA kits. Data are represented as mean \pm SD of three independent experiments. ## p < 0.01 and ### p < 0.001 when compared with the vehicle group. * p < 0.05 and ** p < 0.01 when compared to the TNF- α group



had the potential to be an antioxidant, the antioxidant activities of HY7720 and LGG were evaluated by measuring their ABTS⁺ radical scavenging activity. At low and high concentrations of HY7720, ABTS⁺ radical scavenging capacity was $68.79 \pm 0.11\%$ and $93.71 \pm 8.66\%$, respectively, showing that HY7720 expressed higher antioxidant activity than LGG (Fig. 3A). In addition, we examined its intracellular ROS scavenging activity and anti-cytotoxicity in H₂O₂-treated Caco-2 cells. HY7720 and LGG significantly restored the intracellular ROS level and LDH leakages, which are markers of apoptosis, increased by H₂O₂ in Caco-2 cells (Fig. 3C). Moreover, we hypothesized that HY7720 regulated MAPKs among the antioxidant signaling pathways known to be regulated by probiotics. Interestingly, we found that inhibiting ERK and JNK activation blocked the cytoprotective effect of HY7720 against apoptotic cell death induced by H₂O₂, but inhibiting the p38 inhibitor did not reveal this effect (Fig. 3D). A previous study reported that surface layer proteins from *Lactobacillus* strains can elevate ERK phosphorylation, thereby decreasing apoptotic cell death [41]. Also, in line with our findings, a previous study reported that a certain *Lactiplantibacillus plantarum* strain activates only two MAPKs (ERK and JNK) in porcine intestinal epithelial cells, promoting the expression of human defense

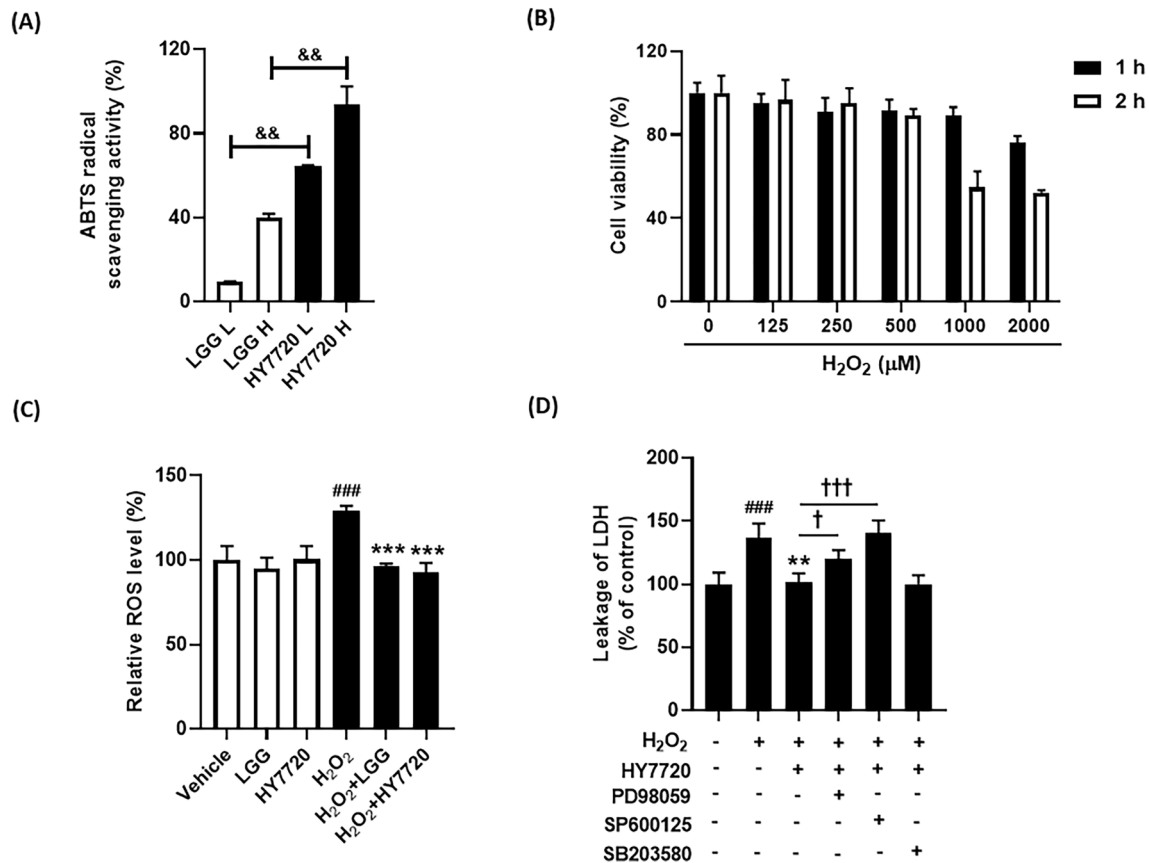


Fig. 3 Antioxidant effects of HY7720 on H₂O₂-induced oxidative stress in Caco-2 cells. **A** Radical scavenging activity of HY7720 and LGG as determined by ABTS assay **B** Cell viability was evaluated via MTT assay. **C** The ROS levels are expressed as percentages of DCF fluorescence intensity to that of control cells with vehicle. **D** Cytotoxicity was determined by measuring the activity of LDH released from damaged cells. Data are represented as mean \pm SD of three independent experiments. ### p < 0.001 when compared to the vehicle group, ** p < 0.01 and *** p < 0.001 when compared to the H₂O₂ group, † p < 0.05 and ††† p < 0.001 when compared to the HY7720 + H₂O₂ group. PD98059: ERK inhibitor; SP600125: JNK inhibitor; SB203580: p38 inhibitor

protein (HDP) [42]. Therefore, our data suggest that the protective effects of HY7720 against oxidative injury are regulated by different signaling pathways.

3.5 Fermentation with HY7720 improves the nutritional qualities and antioxidant activities of plant-based materials

We also examined whether fermentation with HY7720 enhances the nutritional status of plant-based foods as a compensatory strategy for vegetarianism or veganism (Table 1). Here, production of lactate, as a main organic acid, increased during the fermentation of plant-based materials with HY7720, as expected. After the fermentation of each solution of brown rice extract, white rice extract, and soy milk (BR, WR and SM, respectively), the levels of lactate increased markedly. Furthermore, lysine and methionine are considered to be the major limiting essential amino acids among the nutrients known as being deficient in plant-based diets in general. In addition, it is reported that lysine and methionine intakes account for the largest differences between vegetarians and omnivores, which are about 48% and 43% less in vegetarians, respectively [43]. In this study, the free-lysine contents were increased in the plant-based samples (BR, WR, and SM) after fermentation, unlike methionine. The levels of free methionine were not detected either before or after fermentation. The elevations of free lysine could be explained as being a consequence of bacterial enzymatic hydrolysis. In addition, it could not be completely excluded that the results were obtained due to the lysine biosynthetic capability of HY7720. However, it is necessary to perform further investigation to determine lysine biosynthesis by HY7720. Previous study has demonstrated that *L. plantarum* JSA 22, isolated fermented soybean foods, has ability to synthesize lysine using aspartate as a precursor in rice germ milk [44]. Accordingly, to elucidate that HY7720 is a suitable starter for

Table 1 Changes in chemical composition and antioxidant activity of the plant-based materials during fermentation

Measurement	BR		WR		SM	
	0 h	24 h	0 h	24 h	0 h	24 h
Lactate (g/L)	0.01 ± 0.00	14.89 ± 4.51***	0.01 ± 0.00	9.51 ± 4.21***	0.01 ± 0.07	11.21 ± 0.83***
Lysine (µg/mL)	ND	8.59 ± 0.02***	ND	8.76 ± 0.03***	ND	9.93 ± 0.05***
Vitamin B ₁₂ (µg/L)	1.703 ± 0.01	ND	ND	ND	ND	10.45 ± 0.05***
VCEAC (µg/mL)	9.77 ± 0.00	14.05 ± 0.01**	4.55 ± 0.09	7.82 ± 0.10***	9.48 ± 0.76	10.26 ± 0.04

Data are represented as mean ± SD of three independent experiments. The vitamin C equivalent antioxidant capacity (VCEAC) values were determined using vitamin C standard curves. ** $p < 0.01$ and *** $p < 0.001$ when compared to 0 h group. ND, not detected; BR, brown rice; WR, white rice; SM, soy milk

fortified-lysine product, further studies are needed to identify its lysine biosynthetic pathway. Our data, at least, demonstrate that fermentation using HY7720 enhances the level of lysine, which, as a highly bioavailable form, can benefit the nutritional status of vegetarians or vegans.

Moreover, the levels of vitamin B₁₂ were compared before and after fermentation using HY7720 in all plant-based materials. Interestingly, among the three solutions, only SM was the solution with increased vitamin B₁₂ content after fermentation. The level of vitamin B₁₂ reduced in fermented BR than in uncultured BR. In the case of WR, the level of vitamin B₁₂ was not detected either before or after fermentation. Furthermore, we hypothesized that the antioxidant activities would increase in the plant-based materials after fermentation by HY7720. According to previous studies, antioxidant activities could be enhanced in foods fermented by *Lactiplantibacillus plantarum* strain, highlighting its functional effects [45]. We verified that fermentation using HY7720 markedly increased antioxidant activities in BR and WR, but its effect seemed less important in SM. Collectively, these results indicate that *L. plantarum* HY7720 is not only a probiotic formulation but also has potential in the development of fermented foods and beverages with health benefits. More specifically, our findings support the proposition that *L. plantarum* HY7720 would be used as a proper probiotic starter into plant-based juice for prevention of nutritional deficiencies that may occur in vegan or vegetarian. Further study is needed to evaluate the optimal fermentation conditions for each plant-based sample or combination and its sensory characteristics and shelf life to validate acceptable quality.

3.6 Nitrogen sources derived from plant ingredients support the growth of HY7720 better than that animal ingredients

Food products that are suitable for vegans do not contain animal-derived sources. According to the vegan definition agreed upon by the consumerism ministers of the German federal states, vegan foods also do not have ingredients and processing aids from animal origin added at all steps of their production [46]. Thus, to investigate the possibility of industrialization for vegan probiotic products, we compared the growth parameters (μ_{\max} and OD_{\max}) during culture of HY7720 in plant-derived and animal-derived nitrogen sources media. As shown in Fig. 4, the nitrogen-based media derived from the hydrolysis of soy or broadbean performed better than the media based on animal-derived nitrogen sources (casein or lactalbumin peptone) in terms of growth yield. Growth on the plant-derived nitrogen sources, HSP-349, Hy-Soy™ Kosher IPS, and Broadbean peptone, yielded a maximum population density of 0.871, 0.825, and 0.879, respectively, and a maximum specific growth rate of 0.780, 0.814, and 0.771, respectively (Table 2). Although the media based on Hy-Soy™ Kosher IPS showed a higher tendency for maximum specific growth and maximum population density than the media based on casein or lactalbumin peptone, there was no statistically significant relation. A variety of types of LAB have adapted to live and grow on plant microbiomes even though plants are generally thought to be poor settings for numerous microbes. In this study, it has not been determined the relationship between certain factors, such as nutrients and metabolites, affecting the growth parameters of HY7720 and its origin. However, our findings imply that the HY7720 is adapted for growth on specific plants, especially legumes. Further development of media for growing HY7720 on the basis of the above data may lead to its utilization in probiotic manufacturing process that follows the vegan ethos.

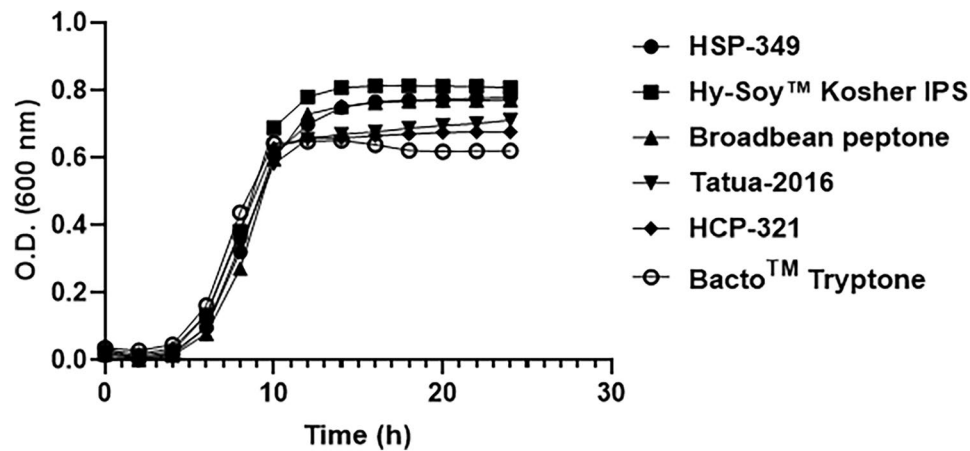


Fig. 4 Growth curves of HY7720 in plant-derived, or animal-derived, nitrogen-based media at 37 °C, as recorded with an optical density (OD) at 600 nm. The growth media are named as each complex nitrogen source. The plant-derived, nitrogen-based media include soy peptone (HSP-349 and Hy-Soy™ Kosher IPS) and broad bean peptone. The animal-derived, nitrogen-based media include casein peptone (Bacto™ Tryptone and HCP-321) and lactoalbumin peptone (Tatua-2016)

Table 2 Parameters for growth of HY7720 in plant-derived or animal-derived nitrogen source media

Medium	μ_{\max}	OD _{max}
HSP-349	0.871 ± 0.008 ^{#/†}	0.780 ± 0.016 ^{*/###/†††}
Hy-Soy™ Kosher IPS	0.825 ± 0.008	0.814 ± 0.021 ^{***/###/†††}
Broadbean peptone	0.879 ± 0.110 ^{*/###/††}	0.772 ± 0.011 ^{***/###/†††}
Tatua-2016	0.721 ± 0.045	0.712 ± 0.021
HCP-321	0.669 ± 0.007	0.677 ± 0.022
Bacto™ Tryptone	0.634 ± 0.088	0.621 ± 0.017

The growth media are named as each complex nitrogen source. The plant-derived, nitrogen-based media include soy peptone (HSP-349 and Hy-Soy™ Kosher IPS) and broad bean peptone. The animal-derived, nitrogen-based media include casein peptone (Bacto™ Tryptone and HCP-321) and lactoalbumin peptone (Tatua-2016). Data are represented as mean ± SD of three independent experiments. * $p < 0.05$ and *** $p < 0.001$ when compared to the Tatua-2016, # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ when compared to the HCP-321, † $p < 0.05$, †† $p < 0.01$, and ††† $p < 0.001$ when compared to the Bacto™ Tryptone. μ_{\max} : maximum specific growth rate; OD_{max}: maximum population density

4 Conclusion

This study showed that *Lactiplantibacillus plantarum* HY7720 has the ability to produce extracellular vitamin B₁₂ and shows potential probiotic and antioxidant properties. Fermentation with HY7720 also enhances the nutritional qualities, such as lysine and vitamin B₁₂, and antioxidant properties of plant-based materials. In addition, we verified that plant-based media provide a good alternative to animal-based media for growing HY7720. Therefore, HY7720 could be suggested as a potential probiotic supplement, as well as a starter culture candidate, in applications of plant-based products for vegetarian or vegan diets.

Author contributions Ju-Yeon Kim: conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, visualization. Eun Jung Choi: conceptualization, investigation. Woo Young Jang: conceptualization, investigation. Soo A Kim: investigation. Kyeong Heo: investigation. Heerim Kang: investigation. Jeanne Kang: investigation. Yong-Tae Kim: conceptualization, formal analysis, resources, writing—review and editing, supervision. Jae-Jung Shim: conceptualization, writing—review and editing, supervision. Jung-Lyool Lee: conceptualization, writing—review and editing, supervision. Jae-Hwan Lee: conceptualization, writing—review and editing, supervision.

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Data availability All data generated or analyzed in the current study are available on request to the corresponding author on reasonable request.

Declarations

Consent for publication The authors agree to publish this article.

Competing interests The authors declare no competing interests.

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