Bioscience of Microbiota, Food and Health Vol. 43 (2), 120–127, 2024

Full Paper

# Transcriptional profiling of geniposide bioconversion into genipin during gardenia fructus extract fermentation by *Lactobacillus (Lactiplantibacillus) plantarum* SN13T

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Received August 30, 2023; Accepted November 26, 2023; Published online in J-STAGE December 18, 2023

***Lactiplantibacillus plantarum* SN13T is a probiotic plant-derived lactic acid bacterium that can grow in various medicinal plant extracts. In this study, we fermented an aqueous extract of gardenia fructus, the fruit of a medicinal plant, with SN13T, such that the bioactivity of the extract was potentiated after fermentation to suppress the release of inflammatory mediators, such as nitric oxide (NO), reactive oxygen species (ROS), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), as well as downregulate inflammatory genes in lipopolysaccharides (LPS)-stimulated RAW 264.7 cells. This increased antioxidant and anti-inflammatory activity was mediated through bioconversion of the iridoid glycoside geniposide to its aglycone genipin via the supposed hydrolytic action of β-glucosidases harbored by SN13T. In the complete genome of SN13T, ten putative genes encoding β-glucosidases of glycosyl hydrolase (GH) family 1 organized among eight gene operons were identified. Transcriptional profiling revealed that two 6-phospho-β-glucosidase genes, *pbg9* and *SN13T_1925*, located adjacently in the gene operon *SN13T_1923*, were transcribed significantly more than the remaining genes during fermentation of the gardenia extract. This suggests the role of these β-glucosidases in bioconversion of geniposide to genipin and the subsequent enhanced bioactivity of the gardenia fructus extract after fermentation with SN13T.**

**Key words: *Lactiplantibacillus plantarum* SN13T, gardenia fructus, geniposide, genipin, 6-phospho-β-glucosidase**

## INTRODUCTION

Fermentation is a valuable biotechnology that can be exploited to develop novel plant-based foods with improved health-promoting properties [1]. Lactic acid bacteria (LAB) species such as *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, and *Pediococcus* spp. derived from different plant sources mainly dominate spontaneous plant fermentation, as they inherit many ecological niche-specific metabolic enzymes. Bioactive compounds such as glycosides, antioxidants, phenolic compounds, and dietary fibers are abundantly present in medicinal plants and allow species and strain-specific LAB to follow various metabolic routes [2]. The plant fermentation process induced by LAB involves the decomposition and/or bioconversion of complex phytochemicals into bioavailable and bioactive compounds via the action of microbial enzymes, such as glycosyl hydrolase, phenolic acid decarboxylase, reductase, and esterase, that concentrates functional microbial metabolites with beneficial consequences for human health [3].

The Food and Agriculture Organization of the United Nations (FAO) has adopted "live microorganisms which when administered in adequate amounts confer a health benefit on the host" as the definition for probiotics and provided guidelines for the selection of probiotics, like strain identification by phenotypic and genotypic methods, *in vitro* tests, and animal studies for functional characterization, safety assessment, and double-blind, randomized, placebo-controlled human trials or other appropriate designs with sample sizes and primary outcomes appropriate to determine if a strain/product is efficacious [4]. In our library of over 1,200 LAB strains isolated from many plant sources, such as fruits, vegetables, flowers, and medicinal herbs, *L. plantarum* SN13T, which was isolated from banana leaves, is a probiotic strain fulfilling such criteria. The strain SN13T (GenBank accession no. AP019815.1) was observed to be much more resistant to artificial gastric juices and bile than animal-derived LAB strains [5]. Previous double-blind, randomized trials have demonstrated that this strain improves constipation and liver function by significantly reducing the serum γ-glutamyl

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(Supplementary materials: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2480/)



doi: 10.12938/bmfh.2023-066

transpeptidase (γ-GTP) value and altering the composition of the gut microbiota [5, 6]. Similarly, the oral administration of live SN13T cells improved alcohol poisoning symptoms by restoring intestinal microbiota and reducing nonvolatile putrefactive amines [7]. Furthermore, SN13T can grow vigorously in different herbal extracts, as it harbors β-glucosidase enzymes [8]. When it was grown in aqueous extracts of the medicinal herbs *Artemisia princeps* Pampanini and *Mentha arvensis*, different bioactive compounds were generated that increased their therapeutic potentials [8–10].

In this study, we speculated that SN13T also produces a bioactive compound when grown in an aqueous extract of gardenia fructus, i.e., the fruit of *Gardenia jasminoides* J. Ellis (Rubiaceae). For centuries, it has been used as a folk medicine in Asian countries for the treatment of inflammation, jaundice, fever, hepatic disorders, etc. [11]. Geniposide, the major iridoid glycoside in gardenia fructus, is known to have anti-inflammatory effects [12]. Genipin, the aglycone of geniposide, is metabolically produced from the geniposide by bacterial enzymes and absorbed in the intestine, as shown in Fig. 1 [13]. Genipin has been demonstrated to have anti-inflammatory activity stronger than that of geniposide, suggesting that genipin, rather than geniposide, is the major anti-inflammatory component of the gardenia fruit [11]. In fact, the anti-inflammatory function of gardenia fructus extract was reported to be improved through treatment with β-glucosidase [14]. Hence, the aim of this study was to demonstrate that *L. plantarum* SN13T fermentation of gardenia fructus extract increases its bioactivity by bioconversion of geniposide to genipin, followed by transcriptional profiling of the associated β-glucosidase genes.

## MATERIALS AND METHODS

### *Bacteria culture and fermentation conditions*

Lactic acid bacterial strain *L. plantarum* SN13T, isolated previously from a plant source—banana leaves—was grown at 37°C overnight in MRS broth (Merck, Darmstadt, Germany). After cultivation, the bacterial cells were collected by centrifugation at 8,000 × g for 10 min.

The fruit of *G. jasminoides* J. Ellis (Rubiaceae), i.e., gardenia fructus (5 g), was purchased from Kojima Kampo Co., Ltd. (Osaka, Japan) and extracted by suspending and boiling it in 100 mL of distilled water for 30 min. After cooling to room temperature, it was centrifuged at 5,000 × g for 10 min and filtered with a 0.22 μm membrane filter (Advantec Toyo Kaisha Ltd., Tokyo, Japan) to obtain an aqueous extract of gardenia fructus (GF). Then, overnight *L. plantarum* SN13T cells (approximately $3 \times 10^9$ colony-forming units/mL) obtained by centrifugation were inoculated into the GF extract and incubated at 30°C with shaking at 120 rpm. After 24 hr, the fermented extract (fGF-SN13T) was finally collected by centrifugation at 5,000 × g for 10 min and subsequent filtration.

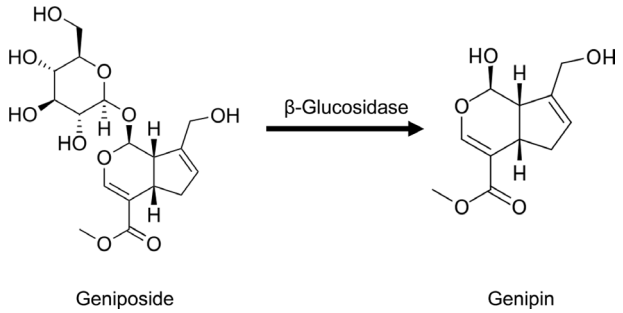


**Fig. 1.** Chemical structures and enzymatic conversion of geniposide into genipin.

For RT-qPCR experiments of bacterial β-glucosidase genes, overnight *L. plantarum* SN13T cells were incubated with GF extract for 0, 2, 8, or 24 hr at 30°C with shaking at 120 rpm.

### *Measurement of total phenolic content*

The Folin–Ciocalteu colorimetric method was used to determine the total phenolic content (TPC) in the fermented and unfermented extract. Briefly, 100 μL of 10% Folin–Ciocalteu reagent was added to 10 μL of each extract. Then, 90 μL of sodium carbonate (1N) solution was added. After incubation for 1 hr, the absorbance was measured at 765 nm. The TPC was expressed in μg/mL of the gallic acid equivalent (GAE) [15].

### *Cell culture and treatment*

Murine macrophage-like cell line RAW 264.7 cells (RRID: CVCL_0493) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL of penicillin/streptomycin by incubation in a humidified 5% $CO_2$ atmosphere at 37°C. After the medium was exchanged with fresh DMEM containing 0.5% FBS, 1 μg/mL LPS (Lipopolysaccharides from *Escherichia coli* O55:B5, Sigma-Aldrich, St. Louis, MO, USA) was added to stimulate the cells in the presence or absence of the extracts at a 1% (*v/v*) final concentration, and the cells were then incubated for 5 hr for quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) experiments or 24 hr to determine the concentrations of nitric oxide (NO), reactive oxygen species (ROS), and the cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α).

### *Measurement of cell viability, intracellular ROS, NO, and inflammatory cytokines*

RAW 264.7 cells at a density of $1.8 \times 10^5$ cells per well were incubated with LPS (1 μg/mL) in the presence or absence of the extracts for 24 hr. Cell viability was determined using a CCK-8 kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions, with CCK-8 solution (10 μL) added to each well. The cells were incubated for another 2 hr, and the absorbance at 450 nm was measured. The percentage of viable cells was determined as a value relative to untreated cells. Similarly, for the measurement of intracellular ROS, after incubation for 24 hr, the cells were treated with 10 mM dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min at 37°C and washed twice with phosphate-buffered saline. DCF fluorescence was measured at excitation and emission wavelengths of 485 and 530 nm, respectively [16]. Thus, the relative fluorescence unit (RFU) value obtained represented the intracellular ROS level and was expressed as the percent of LPS-only treated cells. The production of NO was determined by mixing equal volumes of the 24 hr cell culture supernatants and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.2% naphthyl ethylenediamine dihydrochloride). After 5 min at room temperature, the absorbance at 550 nm was measured. The NO concentration was calculated from the standard curve of sodium nitrite and finally

doi: 10.12938/bmfh.2023-066 

expressed as a percent of LPS-only treated cells [17]. Finally, the concentrations of the inflammatory cytokines IL-6 and TNF-α in the 24 hr cell supernatants were determined with their respective enzyme linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA, USA) and were expressed as the percent of LPS-only treated cells.

### *RNA extraction and qRT-PCR analysis*

RNA extraction and qRT-PCR were performed separately for RAW 264.7 cells treated with LPS and/or extracts and bacterial cells incubated in GF, in order to study the gene expression of LPS-stimulated inflammatory mediators and the gene expression of β-glucosidase genes, respectively.

The total RNA from RAW 264.7 cells treated for 5 hr with LPS and the unfermented or fermented extract and *L. plantarum* SN13T cells grown in GF extract for 0, 2, 8 or 24 hr were isolated using a NucleoSpin RNA plus kit (Macherey–Nagel GmbH and Co. KG, Dueren, Germany). ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) was used for gDNA removal and reverse transcription, according to the manufacturer's instructions. qRT-PCR was conducted with a CFX Maestro 2.3 real-time PCR system (Bio-Rad, Hercules, CA, USA) using a KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). All qPCR reactions were conducted under the following conditions: an initial 2 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 10 sec at 60°C. Relative mRNA expressions were normalized to the respective housekeeping genes, i.e., *gapdh* for RAW 264.7 cells and *ldh* for SN13T cells. Gene expression was analyzed using the ΔΔCT method. All qPCR assays amplified a single product, as determined by melting curve analysis. The primers used for RAW 264.7 cells and SN13T cells are shown in Tables 1 and 2, respectively.

### *HPLC analysis of GF and fGF-SN13T*

HPLC analyses were performed according to a previous study, with slight modifications [9, 10]. Briefly, aliquots (2.5 μL) of the GF and fGF-SN13T extracts were applied to high-performance liquid chromatography (HPLC) with a JASCO system (JASCO Corporation, Tokyo, Japan) and a YMC-Pack ODS-AQ (150 × 4.6 mm, 5 μm, 12 nm) column (YMC, Kyoto, Japan). After the column was equilibrated with water containing 0.1% trifluoracetic acid, gradient elution was performed with 0 to 10%, 10 to 40%, and finally 40 to 60% acetonitrile over 20 min, 30 min, and 10 min sequentially at a flow rate of 1 mL/min. The elution profiles were monitored at an absorbance of 238 nm. The chromatogram of the GF extract was compared with that of the fermented extract, fGF-SN13T. Geniposide and genipin in the extracts were identified using the respective analytical standards, and their concentrations were determined by standard curves at an absorbance of 238 nm.

### *Sequence data analyses*

A search of putative gene sequences annotated with β-glucosidase was performed for the whole genome sequences of *L. plantarum* SN13T using the in silico MolecularCloning (R) Genomics Edition version 6.0.30D software. Homology searches and primer designs were performed with the BLAST algorithm on the National Center for Biotechnology Information server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Predicted gene operons were obtained from the Prokaryotic Operon DataBase [18]. Multiple alignments were made using the Clustal Omega program (https://ebi.ac.uk/Tools/msa/clustalo/) on the EBI site after the retrieval of sequences from the GenBank database.

### *Statistical analysis*

Data were analyzed with GraphPad Prism 8.0.1. Data for all assays and the qRT-PCR analysis of the RAW 264.7 cells are

**Table 1.** Primers used for RAW 264.7 cell qRT-PCR in this study

| Primer | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| ***gapdh*** | GACATCATACTTGGCAG | CTCGTGGAGTCTACTGGT |
| ***inos*** | GGTGTTGAAGGGGTAGCTGA | ATCATGGACCACCACACAGC |
| ***sod 2*** | GTGACTTTGGGTCTTTTGA | GCTAACATTCTCCCAGTT |
| ***il-1β*** | ATGGCAACTGTTCCTGAACTCAACT | CAGGACAGGTATAGATTCTTTCCTTT |
| ***il-6*** | ACAGGTCTGTTGGGAGTGGTATC | CTCTCTGCAAGAGACTTCCATCC |
| ***tnf-α*** | AGCCCCCAGTCTGTATCCTT | CTCCCTTTGCAGAACTCAGG |

qRT-PCR: quantitative real-time reverse-transcription polymerase chain reaction.

**Table 2.** Primers used for β-glucosidase gene expression of SN13T in this study

| Primer | Forward (5′–3′) | Reverse (5′–3′) |
|---|---|---|
| ***ldh*** | GCCGACGAAGGGGTTAAGAA | GTAGGTATCGAGGGCAGCAC |
| ***SN13T_2594*** | TCGAAGCCGATGGCAGTATC | TTCAAGTCTGCGCCGTTAGT |
| ***SN13T_2593*** | TCATGACTGCTGGTCGCAAT | AGTCGTAGTCCGAGTCACCA |
| ***SN13T_2384*** | CTTCAGATTGGCACGATGCG | TTCTCACCGAAGCGAACGAA |
| ***SN13T_2270*** | GGTAAGGGCCTGAGTATCGC | GTTTCGGCATCTTCATCCGC |
| ***SN13T_2112*** | GCAAAAGCTGTTGGTCGGTT | TTGAACAACCCCGTCCGAAT |
| ***SN13T_1926*** | GGACGATCTTGACGCGGTAT | CGTATCCGTCACACTACCCG |
| ***SN13T_1925*** | GGCTGCCAACCAAGTTGAAG | TAGCGTGTGAAGTCCGCAAT |
| ***SN13T_1841*** | ATTGATGGTCCCAAAGCGGT | AATTCCCTTCGCTAACGCCA |
| ***SN13T_1353*** | CCACGGCGTATACCCAATCA | CATCAGCCAGGCGAAAATCG |
| ***SN13T_0875*** | TACGCAACCACTTTTGCTGC | AGCAGCAACGGGTATCAACA |

 

presented as the mean values from three independent assays in triplicate, and error bars indicate the standard deviation. Data for the qRT-PCR analysis of β-glucosidase genes of SN13T are presented as the mean values from two independent assays in triplicate, and error bars indicate the standard deviation. The significance of differences was determined via ANOVA followed by a *post hoc* Tukey test, and differences with $p<0.05$ were considered statistically significant.

## RESULTS

### *Increased bioactivity of GF extract induced by fermentation with* L. plantarum *SN13T*

As shown in Fig. 2a, TPC, which was measured in terms of the gallic acid equivalent (μg/mL), increased time dependently when the GF extract was fermented with *L. plantarum* SN13T. There was no change in TPC after only 2 hr of fermentation, but it significantly increased from 359.3 μg/mL to 403.5 μg/mL and then 496.3 μg/mL after 8 hr and 24 hr, respectively. It was observed that as TPC increased, the bioactivity of the GF extract in reducing LPS-induced NO production in RAW 264.7 cells also increased time dependently with SN13T fermentation, with no significant changes in the cell viability of the LPS-treated cells in the presence of the unfermented or fermented GF extract. As shown in Fig. 2b, the unfermented GF extract and 2-hr fermented GF extract could reduce NO production to 78% and 73%, respectively. After 8 hr or 24 hr fermentation, the extract could significantly reduce the NO level to 8% of that of the LPS-treated cells. Similarly, production of other LPS-induced pro-inflammatory mediators, such as intracellular

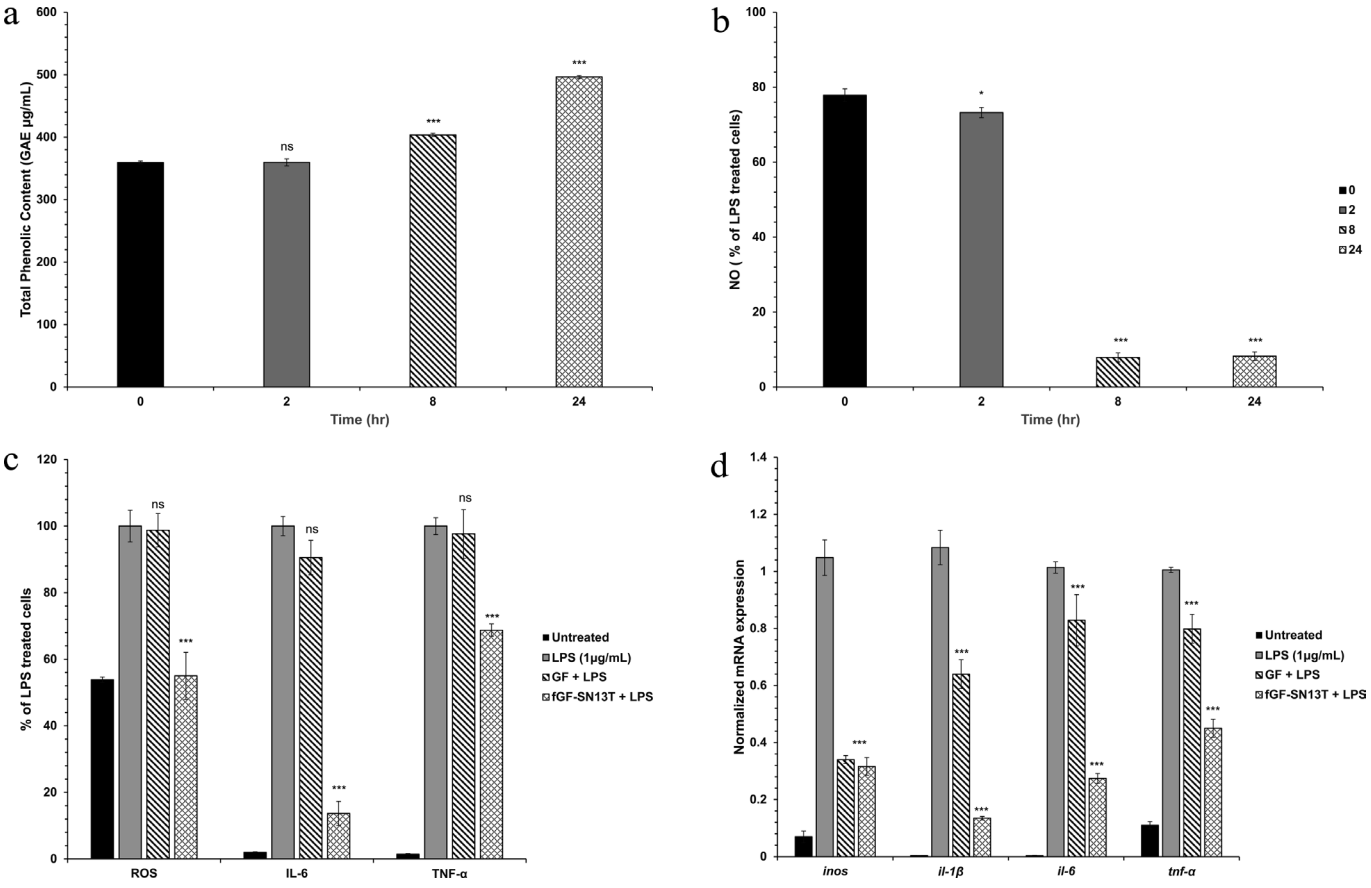


**Fig. 2.** (a) Total phenolic content (TPC) measured in the gallic acid equivalent (GAE-μg/mL) of GF extract after 0, 2, 8, or 24 hr of fermentation. Data represent the mean value from three independent assays in triplicate, and error bars represent +/− standard deviation. ***$p<0.001$ vs. 0 hr and ns: nonsignificant. (b) Nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells in the presence of GF extract after 0, 2, 8, or 24 hr of fermentation, expressed as the percent of LPS-treated cells. Data represent the mean value from three independent assays in triplicate, and error bars represent +/− standard deviation. $p<0.05$ and ***$p<0.001$ vs. 0 hr. (c) Intracellular ROS, IL-6, and TNF-α production in LPS-stimulated RAW 264.7 cells in the presence of GF or fGF-SN13T, expressed as a percent of LPS-treated cells. Untreated: RAW 264.7 cells without LPS or sample extracts, LPS (1 μg/mL): RAW 264.7 cells treated with 1 μg/mL LPS without sample extracts, GF + LPS: RAW 264.7 cells treated with 1 μg/mL LPS in presence of unfermented Gardenia extract and fGF-SN13T + LPS: RAW 264.7 cells treated with 1 μg/mL LPS in presence of 24-hr fermented Gardenia extract with SN13T. Data represent the mean value from three independent assays in triplicate, and error bars represent +/− standard deviation. ***$p<0.001$ vs. 0 hr or LPS (1μg/mL), ns: nonsignificant. (d) Normalized mRNA expressions of the inflammatory genes *inos*, *il-1β*, *il-6*, and *tnf-α* in LPS-stimulated RAW 264.7 cells in the presence of GF or fGF-SN13T. Untreated: RAW 264.7 cells without LPS or sample extracts, LPS (1 μg/mL): RAW 264.7 cells treated with 1 μg/mL LPS without sample extracts, GF + LPS: RAW 264.7 cells treated with 1 μg/mL LPS in presence of unfermented Gardenia extract and fGF-SN13T + LPS: RAW 264.7 cells treated with 1 μg/mL LPS in presence of 24-hr fermented Gardenia extract with SN13T. Data represent the mean value from three independent assays in triplicate, and error bars represent +/− standard deviation. ***$p<0.001$ vs. LPS (1 μg/mL).

doi: 10.12938/bmfh.2023-066 

ROS and the cytokines IL-6 and TNF-α, was also significantly decreased by treatment with the 24-hr fermented extract (fGF-SN13T) but not by treatment with the unfermented GF extract, as shown in Fig. 2c. Moreover, Fig. 2d shows that LPS-induced inflammatory genes, such as *inos*, *il-1β*, *il-6*, and *tnf-α*, could be significantly downregulated in the presence of the unfermented or 24-hr fermented GF extracts. The normalized expression of the inflammatory cytokine genes was reduced by more than twofold by the fGF-SN13T extract, suggesting that its bioactivity was clearly higher than that of the unfermented GF extract.

### *HPLC analysis of GF and fGF-SN13T*

We performed an HPLC analysis of the unfermented GF extract and 24 hr-fermented fGF-SN13T extract and identified their major constituents as geniposide and genipin, respectively, by comparing them with their analytical standards at the absorbance of 238 nm, as shown in Fig. 3a–3c. Geniposide and genipin were observed to be eluted at 27.5 min and 30.3 min, respectively. In the GF extract, geniposide was found to be the major peak, with no detection of genipin. Genipin was the major peak in fGF-SN13T, while a minor peak of geniposide was also detected. Hence, we measured the concentrations of geniposide and genipin in the GF extract before fermentation and after 2, 8, and 24 hr of fermentation, as shown in Fig. 3d. The initial concentration of geniposide in the unfermented GF extract was determined to be 2,295 µg/mL, and the concentration decreased significantly to 1,450 µg/mL and then 304 µg/mL after fermentation for 8 hr and 24 hr, respectively. Furthermore, genipin was only detected after 8 hr of fermentation, and its concentration increased from 204 µg/mL at 8 hr to 515 µg/mL at 24 hr of fermentation.

### *Identification of β-glucosidase genes in* L. plantarum *SN13T*

On searching the complete genome of *L. plantarum* SN13T (accession number: AP019815.1) for the annotated β-glucosidase genes, fourteen putative genes were found. Among them, the *nagZ* gene of locus *SN13T_1949*, two genes of loci *SN13T_1846* and *SN13T_1842*, and the *gcnA* gene of locus *SN13T_1824* were identified as glycosyl hydrolase (GH) family 3, family 4, and family 20 glucosidases, respectively, while the remaining were identified as belonging to GH family 1 (GH1). Using the Prokaryotic Operon DataBase, the ten putative GH1 β-glucosidase genes were predicted to be organized among eight operons, as shown in Table 3. The β-glucosidase genes were usually accompanied by phosphotransferase system (PTS) and transcription regulators in the operon.

### *Transcriptional profiling of GH1 β-glucosidase genes of* L. plantarum *SN13T during gardenia fructus fermentation*

To identify the genes associated with the bioconversion of geniposide to genipin, we performed a time-dependent transcriptional profiling of the ten putative GH1 β-glucosidases of *L. plantarum* SN13T during GF extract fermentation. The heatmap in Fig. 4a shows that two β-glucosidases present adjacently in loci *SN13T_1926* (*pbg9*) and *SN13T_1925* were

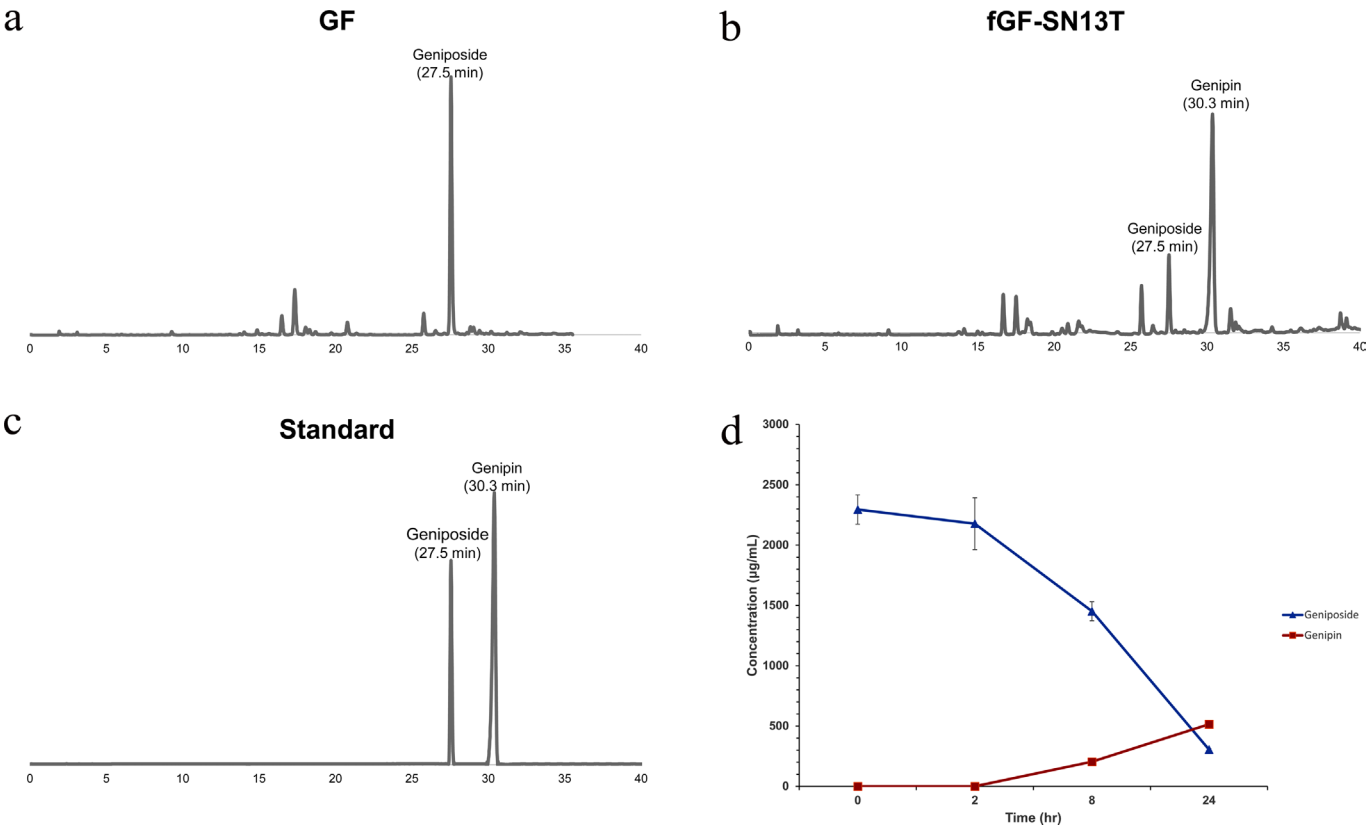


**Fig. 3.** (a) High-performance liquid chromatography (HPLC) chromatograms of GF; detected at 238 nm. (b) HPLC chromatograms of fGF-SN13T; detected at 238 nm. (c) HPLC chromatograms of analytical standards of geniposide and genipin; detected at 238 nm. (d) Concentrations (µg/mL) of geniposide and genipin measured in GF after 0, 2, 8, and 24 hr of fermentation, as determined from HPLC standard curves. Data represent the mean value from three independent assays in triplicate, and error bars represent +/− standard deviation.

doi: 10.12938/bmfh.2023-066 

more time-dependently upregulated than the other genes, with pbg9 being the most expressed gene at 24 hr during GF fermentation. Furthermore, these two genes, which are located in the same operon, i.e., *SN13T*_1923, were significantly overexpressed compared with genes in other operons at 24 hr of fermentation, as shown in Fig. 4b. The glyco-hydro superfamily β-glucosidase gene of *SN13T_1925* is located downstream of the BglB superfamily β-glucosidase *pbg9* gene and upstream

**Table 3.** Putative GH1 β-glucosidase genes of *L. plantarum* SN13T (GenBank accession no. AP019815.1) and their predicted organization in operons

| Operon name | Gene | Locus | COG gene | Putative function |
|---|---|---|---|---|
| *SN13T_2591* | - | *SN13T_2591* | COG1440 | PTS cellobiose-specific component IIB |
| | *celA* | *SN13T_2592* | COG1447 | PTS cellobiose-specific component IIA |
| | ***pg5*** | ***SN13T_2593*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| | **-** | ***SN13T_2594*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| *SN13T_2384* | **-** | ***SN13T_2384*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| | - | *SN13T_2385* | COG1455 | PTS cellobiose-specific component IIC |
| | - | *SN13T_2386* | COG1440 | PTS cellobiose-specific component IIB |
| *SN13T_2269* | *celD* | *SN13T_2269* | COG1455 | PTS cellobiose-specific component IIC |
| | ***pbg1*** | ***SN13T_2270*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| | - | *SN13T_2271* | COG2188 | Transcriptional regulators |
| *SN13T_2111* | - | *SN13T_2111* | COG1040 | NA |
| | - | *SN13T_2112* | COG1940 | Transcriptional regulator/sugar kinase |
| | ***bg1*** | ***SN13T_2113*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| *SN13T_1923* | *bglG5* | *SN13T_1923* | COG3711 | Transcriptional anti-terminator |
| | - | *SN13T_1924* | COG2190 | PTS IIA components |
| | **-** | ***SN13T_1925*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| | ***pbg9*** | ***SN13T_1926*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| *SN13T_1838* | - | *SN13T_1838* | COG3538 | Uncharacterized conserved protein |
| | - | *SN13T_1839* | COG0383 | Alpha-mannosidase |
| | - | *SN13T_1840* | COG1940 | Transcriptional regulator/sugar kinase |
| | ***bg1*** | ***SN13T_1841*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| *SN13T_1352* | - | *SN13T_1352* | COG1940 | Transcriptional regulator/sugar kinase |
| | **-** | ***SN13T_1353*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| | *pts8C* | *SN13T_1354* | COG1455 | PTS cellobiose-specific component IIC |
| *SN13T_0875* | **-** | ***SN13T_0875*** | **COG2723** | **β-glucosidase/6-phospho- β-glucosidase/ β-galactosidase** |
| | - | *SN13T_0876* | COG2190 | PTS IIA components |
| | *bglG2* | *SN13T_0877* | COG3711 | Transcriptional anti-terminator |

Information was obtained from the Prokaryotic Operon DataBase.
Bold print highlights β-glucosidase genes.

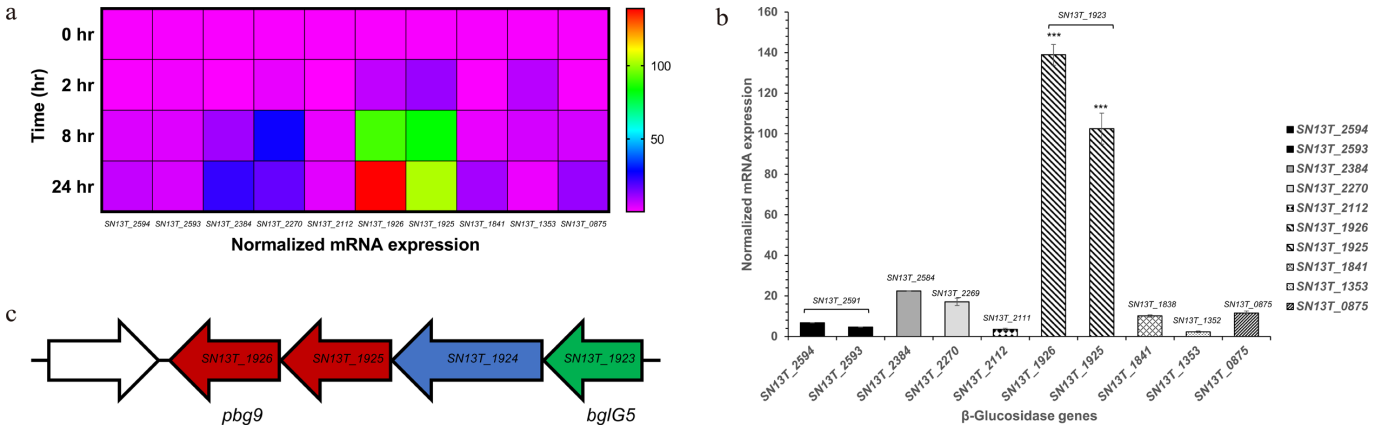


**Fig. 4.** (a) Heatmap showing normalized mRNA expressions of ten putative GH1 family β-glucosidase genes expressed in *L. plantarum* SN13T during the fermentation of *Gardenia fructus* extract at 0, 2, 8, and 24 hr. (b) Normalized mRNA expressions of β-glucosidase genes expressed in *L. plantarum* SN13T during the fermentation of *G. fructus* extract at 24 hr. The gene operons of corresponding genes are given above each bar. ***p<0.001 vs. all other genes; Data represent the mean value from two independent assays in triplicate, and error bars represent +/− standard deviation for qRT-PCR analysis. (c) Cartoon representing the synteny of candidate β-glucosidase genes functional during *G. fructus* fermentation in the SN13T genome. Genes are represented as arrows and are not to scale. Red arrows indicate candidate β-glucosidase genes, the blue arrow represents a PTS component–encoding gene, the green arrow represents the gene encoding the anti-termination protein bglG5, and the white arrow represents flanking genes not involved in glucoside hydrolysis.

doi: 10.12938/bmfh.2023-066 

of two genes encoding PTS IIA components and transcriptional anti-terminator *bgl5G* genes, as depicted in Fig. 4c. These β-glucosidase genes, i.e., *pbg9* and *SN13T_1925*, demonstrated about 96% homology to the *bglH2* and *bglH3* genes of *L. plantarum* ATCC 8014, respectively, which harbored a similar polycistronic *bgl* gene operon (see alignments in Supplementary Figs. 1 and 2, respectively).

## DISCUSSION

In the present study, we demonstrated that when *L. plantarum* SN13T is grown in GF extract, it raised the TPC and potentiated the bioactivity, suppressing LPS-induced inflammatory mediators such as NO, ROS, IL-6, and TNF-α along with their gene expressions in RAW 264.7 cells. The strain SN13T has also been previously reported to produce IL-8-inhibiting molecules, such as catechol and seco-tanapartholide C, when grown in another medicinal herb extract, *A. princeps* Pampanini [8]. It also produced the phenolic acid metabolite dihydrocaffeic acid by upregulating hydroxycinnamate reductase genes when grown in a *M. arvensis* extract and, thus, potentiated its bioactivity [9, 10]. Similarly, in our previous study, fermentation of the medicinal herb extract *Paeonia radix* Alba with *Lactobacillus brevis* 174A was found to enhance its antioxidant and anti-inflammatory bioactivity by producing the gallic acid metabolite pyrogallol and increasing the TPC [15]. Some plant-derived LAB strains could also produce the anti-bacterial and anti-biofilm metabolite 3-phenyllactic acid when fermented in medicinal plant extracts [19]. Collectively, these studies emphasize that medicinal plant extracts can be conveniently exploited as fermentation media for plant-derived LAB to develop functional foods with enhanced bioactive properties.

Our results also showed that during the fermentation of GF with *L. plantarum* SN13T, the iridoid glycoside geniposide, which was the major bioactive principle, was time-dependently hydrolyzed into its aglycone genipin. The detection of genipin and the increased bioactivity of the fermented extract can be clearly correlated. The anti-inflammatory effects of both geniposide and genipin have been reported to act via inhibition of the 5-lipoxygenase and NF-κB pathways, respectively [12, 13]. However, one study pinpointed stronger activity of genipin and regarded genipin, rather than geniposide, as the major anti-inflammatory component of GF [11]. In fact, geniposide is hydrolyzed to genipin via the action of β-glucosidase produced by intestinal or fecal microbiota, and the *in vitro* enzymatic treatment of GF extract also improves its bioactivity [14, 20]. Thus, some studies have reported that a combination of geniposide or GF extract with *Lactobacillus* strains enhanced their functional properties, such as anti-oxidative stress and anti-inflammation in septic mice, an antioxidant effect in mouse liver injury, and an anti-proliferative effect in oral cancer cells [21–25]. Meanwhile, other studies have succeeded in enhancing the concentration of genipin using the immobilized bacterial enzyme glycosyl hydrolase family 3 β-glucosidase from *L. antri* or using immobilized fungal cells of *Trichoderma reesei* [25, 26]. In this regard, the fermentation of GF with β-glucosidase harboring *Lactobacillus* strains can be conveniently exploited for the effective bioconversion of geniposide to genipin.

β-glucosidase enzymes are GHs that mediate the release of glycosyl monomers such as β-D-glucose from various disaccharides, oligosaccharides, and alkyl- and aryl-β-D-glucosides [27]. In LAB, β-glucosidase activity is widespread, as it plays a substantial role in carbohydrate metabolism and releases a wide range of secondary plant metabolites from their β-D-glucosylated precursors, thus improving the flavor of fermented foods and the bioavailability of health-promoting, antioxidative metabolites. Hydrolysis of the phenolic glucoside oleuropein in olives, the isoflavones genistin and daidzin in soybeans, the anthocyanins malvidin and delphinidin, and the cyanogenic glucoside linamarin in cassava are notable examples of β-glucosidase activities that are beneficial in food fermentation [28]. Thus, some *L. plantarum* strains have been investigated for β-glucosidase activities as well as identification and isolation of β-glucosidase-encoding genes [29, 30]. In the complete genome of *L. plantarum* SN13T, we identified 10 putative genes encoding GH1 β-glucosidases among a total of 14 genes annotated as β-glucosidases. Most of these genes were accompanied by phosphotransferase system (PTS) EII components and transcriptional regulators in their gene operons. GH1 comprises enzymes with a number of known activities, including both β-glucosidase (EC 3.2.1.21) and 6-phospho-β-glucosidase (EC 3.2.1.86) [31]. In the published genome of *L. plantarum* WCFS1, 11 genes putatively encoding GH1 enzymes with phospho-β-glucosidase functionality were identified, nine of which were located adjacent to genes encoding β-glucoside/cellobiose-specific EII components. This confirms that PTS-related glycosidase genes are frequently organized in the same operon as genes encoding EII (ABC) components specific to the corresponding substrate. The transcriptional anti-terminator (BglG) is encoded by *bglG*, which is inactive in its phosphorylated state. BglG is activated through dephosphorylation to the substrate during the uptake of β-glucosides and thus prevents transcription termination [28].

Among the ten putative genes encoding GH1 β-glucosidases, the time-dependent transcription profile revealed that two genes of locus *SN13T_1925* and *SN13T_1926* (*pbg9*), located adjacently in operon *SN13T_1923*, were significantly upregulated as compared with the remaining genes when *L. plantarum* SN13T was grown in GF extract, suggesting that this operon might be associated with the bioconversion of geniposide to genipin. These genes showed close resemblances to *bglH2* and *bglH3* genes encoding 6-phospho-β-glucosidases of *L. plantarum* ATCC 8014 arranged in a similar polycistronic *bgl* gene operon. Among them, *bglH3* was reported to be a new candidate gene, and both genes were transcribed by *L. plantarum* C11C8, specifically during table olive fermentation, implying that phospho-β-glucosidase activity could be a metabolic strategy undertaken by *L. plantarum* to adapt in an environment poor in free sugars, as in the case of table olives [31].

In conclusion, plant-derived *L. plantarum* SN13T mediates the bioconversion of geniposide to genipin by the transcription of two 6-phospho-β-glucosidase-encoding genes located in a specific gene operon, which enhances the antioxidant and anti-inflammatory activity of GF extract during fermentation. Hence, it is concluded that the exploitation of medicinal herb extracts as fermentation broths for plant-derived LAB is a significant technique for enhancing their functional features.

doi: 10.12938/bmfh.2023-066 

## AUTHOR CONTRIBUTIONS

Conceptualization—Shrijana Shakya and Narandalai Danshiitsoodol; methodology—Shrijana Shakya and Narandalai Danshiitsoodol; formal analysis—Shrijana Shakya; writing, original draft preparation—Shrijana Shakya; writing, review and editing—Shrijana Shakya, Narandalai Danshiitsoodol, Masafumi Noda, and Masanori Sugiyama; supervision—Narandalai Danshiitsoodol. All authors have read and agreed to the published version of the manuscript.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ Supplementary material.

## CONFLICT OF INTEREST

There are no conflicts of interest.

## ACKNOWLEDGEMENT

We thank the Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University, for the permission to use their equipment.

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