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RESEARCH ARTICLE

Valorization of leftover green tea residues through conversion to bioactive peptides using probiotics-aided anaerobic digestion

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Abstract

Bioactive peptides (BPs) are protein fragments that benefit human health. To assess whether leftover green tea residues (GTRs) can serve as a resource for new BPs, we performed *in silico* proteolysis of GTRs using the BIOPEP database, revealing a wide range of BPs embedded in GTRs. Comparative genomics and the percentage of conserved protein analyses enabled us to select a few probiotic strains for GTR hydrolysis. The selected probiotics digested GTRs anaerobically to yield GTR-derived peptide fractions. To examine whether green tea (GT) peptide fractions could be potential mediators of host–microbe interactions, we comprehensively screened agonistic and antagonistic activities of 168 human G protein-coupled receptors (GPCRs). NanoLC-MS/MS analysis and thin-layer chromatography allowed the identification of peptide sequences and the composition of glycan moieties in the GTRs. Remarkably, GT peptide fractions produced by *Lactiplantibacillus plantarum* APsulloc 331261, a strain isolated from GT, showed a potent-binding activity for P2RY6, a GPCR involved in intestinal homeostasis. Therefore, this study suggests the potential use of probiotics-aided GTR hydrolysates as postbiotic BPs, providing a biological process for recycling GTRs from agro-waste into renewable resources as health-promoting BPs.

INTRODUCTION

Green tea (GT) extracts are a source of functional ingredients such as epigallocatechin-3-gallate (EGCG) in cosmetics and functional foods. In addition, polysaccharides extracted from GT as dietary fibre are used as nutritional supplements to boost immunity (Singh et al., 2011). However, a large amount of leftover green tea residues (GTRs) are agro-waste that still need to be valorized as value-added products (Nille et al., 2021). Therefore, beyond conventional functional ingredients such as polyphenols and polysaccharides, alternative

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options are needed for green and sustainable treatment of protein-rich GTRs to produce high value-added products and reduce environmental pollution caused by GTR waste (Wang et al., 2014).

Dietary proteins are a rich source of bioactive peptides (BPs) that play a crucial role in human physiology and metabolism (Kang et al., 2020; Shahidi & Zhong, 2019). BPs are specific protein fragments often released through hydrolysis by digestive enzymes and/or microbial fermentation in the gastrointestinal (GI) tract (Buchwald et al., 2014; Giordano et al., 2014; Padhi et al., 2014). Recent evidence supports the notion that BPs have various physiological functions, such as antihypertensive, antioxidant, antimicrobial, and anti-obesity effects (Fosgerau & Hoffmann, 2015; Jin et al., 2018; Kang et al., 2020). Some peptides ameliorate metabolic disorders by regulating extracellular signalling receptors, and attract attention in the global market as medicines (Muttenthaler et al., 2021). Key classes of such small peptide ligands include hormones, neurotransmitters, antigens, cytokines, and growth factors (Hruby, 2002), which mediate these biological functions through G protein-coupled receptors (GPCRs) (Giacometti & Buretić-Tomljanović, 2017). Recent studies have indicated that among the various metabolic compounds, BPs play important roles in mediating host cell signals related to human physiology and metabolism via various GPCRs (Levy et al., 2017; Muratspahic et al., 2019). In this regard, the functional role of BPs in dietary proteins has received significant attention in the nutraceutical industry because these BPs can provide nutritional benefits and health-promoting properties as natural active compounds.

Lactic acid bacteria (LAB) could be excellent biocatalysts for the use of GTRs as an untapped source of novel BPs. Owing to their proteolytic activity ascribed to their amino acid auxotrophy, LAB can release various BPs (Lee et al., 2022), biogenic amines (Raveschot et al., 2018), and free amino acids, which are required for bacteria in the human GI tract (Christiansen et al., 2008; Griffiths & Tellez, 2013; Hebert et al., 2008). Some LAB strains also have a different set of proteolytic systems, thereby producing a variety of antimicrobial peptides to prevent infectious pathogens and fermentation products that are beneficial for the host immunological response and intestinal gut microbiota (Ouwehand et al., 2002; Van Niel et al., 2002; Zocco et al., 2006). Furthermore, LAB, which colonize the end of the human small intestine (Sandine, 1979), are known to be safe and beneficial among microbes in fermented food and the GI tract (Gilliland, 1990; Tannock, 2004). Because LAB can provide various metabolite profiles depending on what food humans eat, their metabolic properties for modifying dietary components influence human health and disease (Levy et al., 2017; Vernocchi et al., 2020). Microbiota-specific metabolites, including short-chain fatty acids and BPs, play an important role in human health via host-gut microbiota crosstalk (Fan & Pedersen, 2021; Pasolli et al., 2020). These unique physiological and biochemical features make probiotics suitable biocatalysts for the production of BPs.

The present study attempted to use probiotics as biocatalysts to valorize leftover GTRs from agro-waste into useful BPs. Building a new biorefinery pathway using unused byproducts will facilitate waste management and resolve sustainable development challenges (Irla & Wendisch, 2022; Leong et al., 2021; Yeo et al., 2018). We further discuss the potential impact of GT peptides on health and diseases via host–microbe interactions.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions

Fourteen probiotic strains were obtained from the Korean Culture Center of Microorganisms (KCCM) in South Korea (Table S1). *Lactiplantibacillus plantarum* APsulloc 331261, isolated from tea leaves at Osulloc Dolsongi Tea Garden (33° N, 126° E), was obtained from Amore Pacific. Probiotic species were cultured as previously described (Lee et al., 2022). Briefly, the bacterial species were cultured in anaerobic serum bottles sealed with butyl-rubber stoppers at 37°C under a gas mixture of 80% N_2, 10% CO_2, and 10% H_2. Each strain was activated in Difco™ *Lactobacilli* Man, Rogosa & Sharpe (MRS) broth and transferred to the appropriate defined medium (DM) (Table S2) (Saguir & de Nadra, 2007). In the late exponential phase (2.0×10^7 cells/ml), bacterial cells were inoculated into freshly prepared DM and incubated for 48 h. Bacterial growth was monitored by measuring the turbidity at 600 nm using a spectrophotometer (Biochrom Libra S70; Biochrom). For direct cell counting, a Neubauer chamber (depth, 0.1 mm × area, 0.0025 mm^2; Paul Marienfeld GmbH & Co. KG) with a phase-contrast microscope (Olympus BX43; Olympus) was used.

For bacterial substrate utilization profiles and proteolytic pattern analysis, the appropriate DMs supplemented with 0.5% GTR was used. For bacterial growth in media supplemented with GTR, pH-driven protein solubilization and precipitation was performed (refer to section pH-driven GTR solubilization and precipitation).

Fractionation of GT hydrolysates

After 48 h of incubation, the bacterial culture broths were centrifuged at 8000 *g* for 20 min at 4°C. The cell-free supernatant was 20-fold concentrated at 70°C for 24 h and centrifuged at 100,000 *g* for 1 h at 4°C to remove the precipitate. The resulting supernatant was then filtered through a 0.2 μm pore-size membrane (Millipore). The filtrate was fractionated through stepwise ultrafiltration

using 10 and 1 kDa cutoff discs (Millipore) to yield high molecular weight (HMW, >10 kDa), middle molecular weight (MMW, 1–10 kDa), and low molecular weight (LMW, <1 kDa) fractions. The fractions were lyophilized and stored at −70°C until use. Each fraction (100 mg) was dissolved in 20 mM Tris–HCl (pH 7.0) and loaded onto a Superdex 30 prep-grade (pg) column (200 ml) pre-equilibrated with the same buffer in a Biologic Duo-Flow FPLC system (Bio-Rad) at a rate of 1.5 ml/min. Eluents were monitored by measuring the absorbance at 210 nm, and each peak fraction was pooled, lyophilized, and stored at −20°C until use. Based on the standard curve obtained using proteins/peptides of known sizes (Figure S1), 1–10 kDa peptide fractions were pooled and used for further analysis.

pH-driven GTR protein solubilization and precipitation

For the analysis of bacterial growth and residual GT protein pellets, cells and proteins were separated by changing pH. The pH of the bacterial culture broth was adjusted by adding NaOH at a final concentration of 0.5 N. The culture broths were centrifuged at 13,000 *g*, and the cells were harvested. The cell pellets were suspended in 10 mM Tris–HCl (pH 8.0) to monitor bacterial growth. HCl was added to cell-free supernatants at a final concentration of 2 N to precipitate solubilized GT proteins. The supernatants were centrifuged at 13,000 *g* to obtain GT protein pellets. Pellets were dried overnight at 70°C, and their dry weights were measured.

Enzymatic deglycosylation

To analyse glycopeptide sequences from GTR hydrolysates, we separated them into *N*-glycans and peptides using PNGase F from *Elizabethkingia miricola* (Promega) (Zacharius et al., 1969) according to the manufacturer's instructions. *N*-glycan fractions were further analysed by thin-layer chromatography (TLC), and peptide sequences were identified using LC–MS/MS.

Peptide identification by LC–MS/MS

To identify peptides in GTR hydrolysates, an UltiMate 3000 RSLC nano system coupled to a Q-Exactive Orbitrap HF-X mass spectrometer (Thermo Fisher Scientific) was used in direct injection mode, as described previously (Lee et al., 2022). GTR hydrolysates containing peptides (3 μg) were loaded onto a trap column (internal diameter, I.D. 75 μm × 2 cm, packed with Acclaim PepMap 100 C18, 3 μm, 100 Å; Thermo Fisher Scientific) and eluted onto an analytical column (I.D. 75 μm × 50 cm, packed with PepMap RSLC C18, 2 μm; Thermo Fisher Scientific) at a flow rate of 0.27 μl/min. The mobile phase consisted of 0.1% (v/v) formic acid (FA) in water (solvent A) and 0.1% (v/v) FA in acetonitrile (ACN) (solvent B). MS analysis of peptide eluents was performed on a Q-Exactive Orbitrap HF-X mass spectrometer in the positive-ion mode. The normalized collision energy was 27% for the MS2 analysis.

Data analysis

For data analysis, the MS-GF+ search was used. First, the MS spectra were processed to convert the raw data into peak lists (.mzML format). Processed spectra were compared using the target decoy database search strategy to the GT sequences of *Camellia sinensis* (UniProt Proteome ID: UP000327468) and *Lacticaseibacillus paracasei* JCM 8130 (National Center for Biotechnology Information [NCBI] GenBank assembly accession: GCA_000829035.1), *L. plantarum* DSM 20174 (GCA_014131735.1), or an in-house protein sequence library of *L. plantarum* APsulloc 331261 (Arellano et al., 2020). For the MS-GF+ search, oxidation (+15.99) of methionine and acetylation (+42.01) of the peptide *N*-terminal were set as variable modifications. The precursor m/z tolerance was ±10.0 ppm, and the fragment m/z tolerance was ±20.0 ppm. A list of peptides was obtained, with a false discovery rate (FDR) of less than 1%. For the analysis of peptide bioactivities, previously characterized sequences and associated bioactivities were retrieved from the BIOPEP database (Minkiewicz et al., 2019).

Thin-layer chromatography

Thin-layer chromatography (TLC) was used to determine the composition of plant glycans. Samples (100 μg) were applied to a TLC plate (TLC aluminium sheets, 20 × 20 cm, Silica gel 60 F_{254}; Merck). The carbohydrates were separated in a solvent system consisting of 1-butanol, 2-propanol, ethanol, and water (45:45:5:5) in two consecutive ascents. The spots were visualized using potassium permanganate ($KMnO_4$; 1.5 g of $KMnO_4$ and 10 g of K_2CO_3 in 0.0625% NaOH). Carbohydrates were identified compared with the standards migration (mannose, *N*-acetyl glucosamine, galactose, and fucose; Sigma Aldrich).

GPCR β-arrestin assay

GPCR-binding activities were measured using the gpcrMax panel provided by Eurofins Profiling Services (Eurofins DiscoverX) (Olson & Eglen, 2007). Peptide fractions (1–10 kDa, 1 mg/ml) were tested for their agonistic and antagonistic activities in a cell-based assay of

168 GPCR targets. Briefly, cells were incubated with the samples or appropriate agonists to induce responses. Signals were generated by adding PathHunter detection reagent cocktail, followed by incubation for 1 h at 25°C in a dark room and detection of chemiluminescent signals. Agonistic activities were expressed as the % activity relative to the baseline (0%) and maximal control ligand response (100%) values. Antagonistic activities were expressed as the % activity relative to control agonist response (0%) and baseline (100%) values.

To analyse the dose-dependent agonistic activities toward P2RY6 or GPR35, PathHunter® eXpress β-arrestin GPCR assays (Eurofins DiscoverX) were performed according to the manufacturer's instructions. Briefly, Chinese hamster ovary (CHO)-K1 eXpress cells for P2RY6 or GPR35 were plated in 96-well plates at 6.6×10^3 cells/well and incubated at 37°C and 5% CO_2 for 48 h. Then, CHO-K1 P2RY6 or GPR35 eXpress cells were treated with 1 to 10 kDa GT peptide fractions from *L. plantarum* ATCC 14917, *L. plantarum* APsulloc 331261, and *L. paracasei* JCM 8130. UDP and zaprinast were used as the control ligands for P2RY6 and GPR35, respectively. Cells treated with test samples or control ligands were incubated at 37°C for 90 min, followed by 1 h of incubation with the detection reagent at room temperature. Agonistic activities were measured and calculated as above.

Bioinformatics tools

For *in silico* proteolysis of GT, the amino acid sequences of GT were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/) using the following keywords: *Camellia sinensis* [organism] and green tea. BIOPEP (https://biochemia.uwm.edu.pl/biopep-uwm/) (Minkiewicz et al., 2019) was used as the database for BP.

The conserved proteins between a pair of genomes were determined by aligning all the protein sequences of one genome with those of another using the percentage of conserved proteins (POCP) method and implemented in the R module (Qin et al., 2014). Default parameters were used for POCP analyses (*e*-value = 1 e^{-5}, sequence identity = 0.4, alignment length = 0.5). Hierarchical clusters were selected based on a 50% similarity cut-off. The proteolytic enzymes were obtained from the MEROPS database (Rawlings, 2010).

RESULTS AND DISCUSSION

Design of bio-based recycling technology to valorize GTR into value-added products

Green tea contains not only carbohydrate polymers but also proteins as primary constituents (Horanni & Engelhardt, 2013) (Figure 1A), thereby releasing high levels of free amino acids, including theanine, and other functional ingredients such as EGCG and EGC, during extraction and infusion procedures (Juneja et al., 1999). After harvesting GT leaves, primary catechin and caffeine were initially extracted using previously optimized extraction and infusion procedures (Kim et al., 2009) (Step 1 in Figure 1B). Subsequently, the first leftover GTRs underwent thermal extraction to extract water-soluble heterogeneous GT polysaccharides as bioactive compounds (Chung et al., 2019) (Step 2 in Figure 1B). Next, insoluble GT dietary fibres were further extracted from the second leftover GTRs using alkaline extraction to yield the third leftover GTRs rich in proteins (Step 3 in Figure 1B) (Zhang et al., 2014, 2016). The total amino acid contents in the protein hydrolysate of 3rd GTR extracts varied from 6.8 to 33.79 mg/g, with the amino acid composition determined by the amino acid analyser (Table S3). Remarkably, the third GTR extracts contained high levels of essential amino acids (e.g., phenylalanine and methionine), together with polar and non-polar amino acids (Table S3). This result indicates that these GTRs are useful for further utilization as valuable protein resources for high-value-added products.

Toward this end, beyond conventional GT uses, we attempted to valorize protein-rich GTRs as renewable resources to produce BPs exerting diverse physiological functions as cellular signalling molecules in human physiology and metabolism. The GTRs used in this study contained approximately 60% crude protein and the remaining 40% consisted of dietary fibre, fat, and other compounds (Figure 1C). Harvesting of beneficial BPs from GTRs requires an integrated approach to predict the potential health benefits of dietary sources and screen appropriate proteolytic systems (Kang et al., 2020). Accordingly, we designed a biological process using a bottom-up approach to produce repertoires of health-promoting BPs from leftover GTR rich in proteins using anaerobic fermentation with LAB (Step 4 in Figure 1B).

In silico analysis revealed potential BP embedded in leftover green tea residues

To assess whether leftover GTRs can serve as resources to produce high-value-added products, *in silico* digestion of GTRs was performed with various proteases, and they were analysed for potential BPs (Figure 2A). Protein sequences of *Camellia sinensis* (UniProt ID: UP000327468) were subjected to *in silico* proteolysis using the Expasy Peptide Cutter (https://web.expasy.org/peptidecutter/) with 28 proteases well-characterized for their P1 sites. Remarkably, *in silico* proteolysis revealed 770 putative BPs embedded in GTRs belonging to 26 different BP categories, ranging from angiotensin-converting enzyme (ACE) inhibitors to neuropeptides (Figure 2A). In addition, differential BP spectra were obtained using

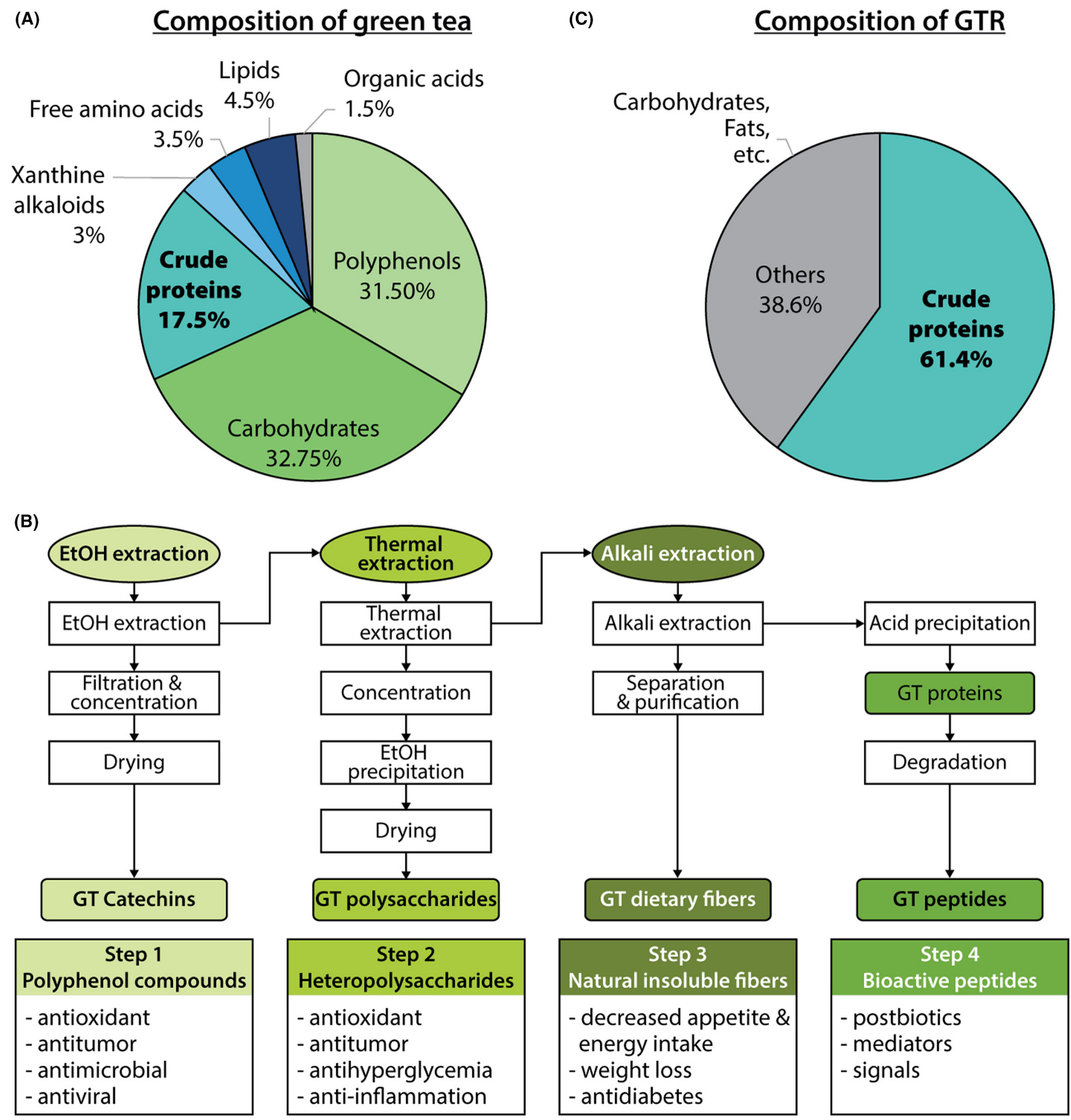

FIGURE 1 Valorization strategy for increasing the value chain of green tea (GT) residues through microbial routes to value-added products. (A) GT has the highest carbohydrate content, followed by polyphenols and crude proteins (Chacko et al., 2010). (B) Major ingredients in GT such as polyphenols, polysaccharides, vitamins, and minerals are extracted through a series of downstream processes, resulting in the formation of leftover GT residues. Such GT residue wastes rich in proteins can be further processed through probiotics-aided anaerobic digestion, converting them into postbiotic BPs. (C) GTR used in this study were composed of 61.4% crude proteins.

mammalian and bacterial protease activities (Figure 2B). While human gastrointestinal (GI) enzymes (e.g., trypsin, chymotrypsin, and pepsin) exclusively produce hypolipidemic, antiviral, opioid inhibitor, and anticancer peptides, bacterial proteases exclusively release anti-inflammatory peptides. This result indicates that bacterial proteases can serve as biocatalysts to produce novel BPs derived from GTRs.

Genome-wide analysis of probiotics as biocatalysts for the production of BPs from GTRs

Acid-tolerant LABs were selected as workhorses for the digestion of GTRs. To choose potential LAB strains capable of producing a wide range of BPs, the genome sequences of 14 representative probiotics approved for

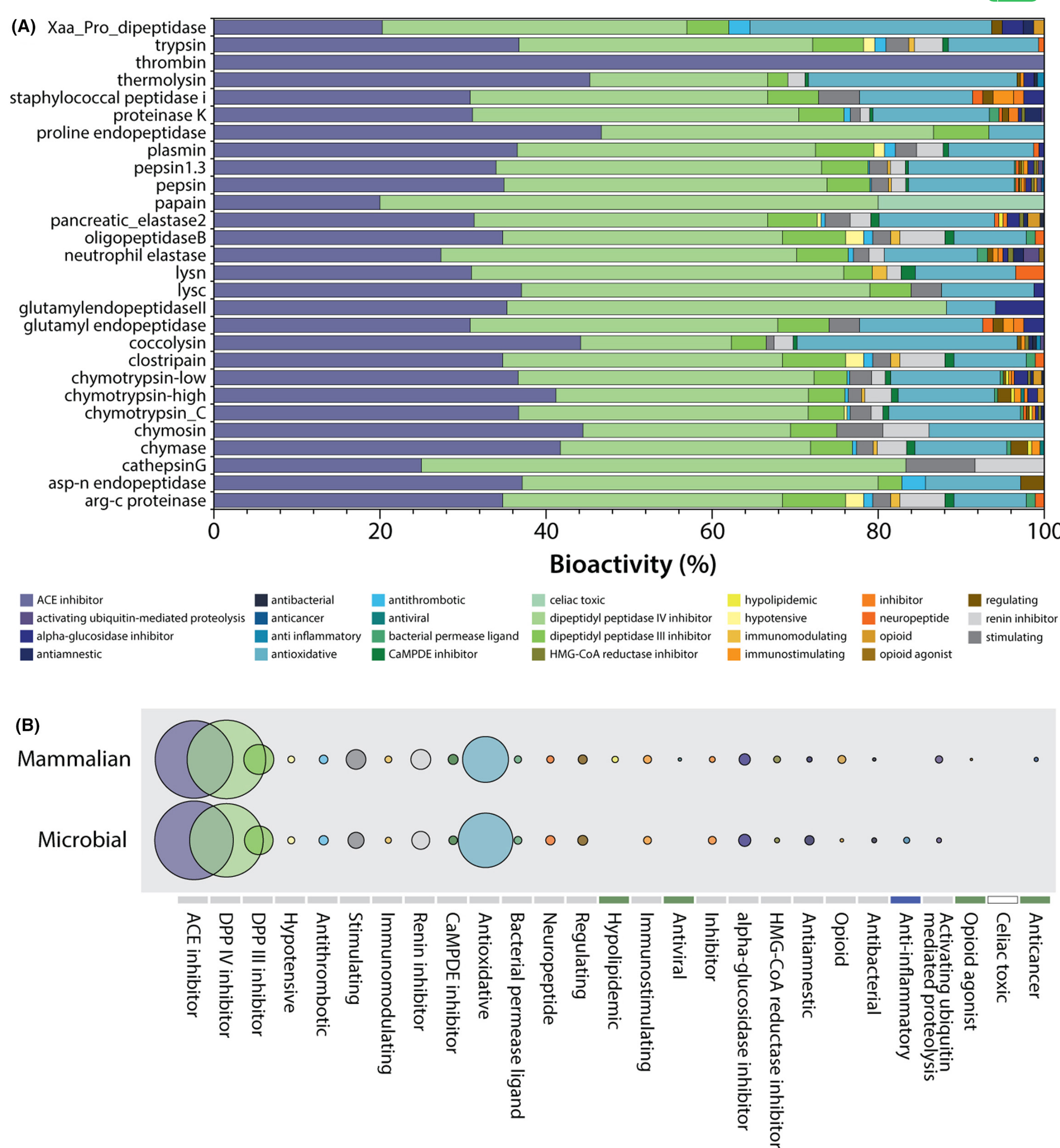

FIGURE 2 *In silico* analysis of bioactivities of peptides from green tea (GT) residues. (A) Differential bioactivity profiles of potential bioactive peptides (BPs) released from *in silico* GT protein digestion by proteases. Proteins of *Camellia sinensis* (UniProt proteome ID: UP000327468) were digested using *in silico* enzymatic digestion with 27 proteases with characterized cleavage sites. Of the digested peptides, 770 putative bioactive peptides were matched to the BIOPEP database. Peptides with an angiotensin-converting enzyme (ACE) and dipeptidyl peptidase IV (DPP IV) inhibiting activities were most widely distributed, followed by those with anti-oxidative activities. (B) The distribution of BPs was distinguished between mammalian and bacterial proteases. Hypolipidemic, antiviral, opioid inhibitor, and anti-cancer activities were exclusive to peptides produced from mammalian enzymes (green), whereas anti-inflammatory activities were exclusive to those produced from microbial enzymes (blue).

uses in functional foods by the Ministry of Food and Drug Safety, Korea were compared to analyse their genome contexts (Table S1). Genomic sequences were investigated based on their amino acid sequence similarity using pairwise POCP analysis. The 14 probiotics were then classified into seven distinct clusters (Figure SA2), consistent with previous comparative genomics (Sun et al., 2015).

We further selected all functional genes encoding proteases and/or peptidases in these LAB strains

to characterize their proteolytic systems using the MEROPS database. Accordingly, we compared the protease profiles of the probiotic strains based on the presence or absence of proteases and peptidases. Remarkably, clusters of proteolytic enzymes in the probiotics were identical to the clusters identified by POCP analysis (Figure S2B). These results imply that LAB strains belonging to the same cluster are likely to exert similar proteolytic activities. In addition, the catalytic types of proteolytic enzymes showed cluster-specific patterns (Figure S2C). For instance, the species in cluster 2 (C2; *E. faecalis*) had the highest number of proteases and peptidases of the metallo (M), serine (S), and cysteine (C) catalytic types. Species belonging to C6 (*L. casei*, *L. rhamnosus*, and *L. paracasei*) had a higher number of M proteases than other types, whereas species belonging to C5 had a similar numbers of proteases corresponding to M, S, and C catalytic types. The specific protease and peptidase profiles of the probiotics examined in this study indicate that strains belonging to each cluster have different proteolytic activities for GTRs, implying that their substrate utilization by GTRs and their product profiles may differ from one another. Thus, these results indicate that the metabolic enzymes of LAB species from separate clusters could have different GTR-degrading capacities.

L. plantarum is a potential candidate for the production of low molecular weight GT peptides

To cross-validate the genome-based clustering of LABs, the effects of GTRs on bacterial growth and GTR hydrolysis patterns were investigated (Figure 3). Fourteen LAB strains were grown in appropriate DM supplemented with 0.5% (w/v) GTRs as substrates under anaerobic conditions for 48h to investigate changes in bacterial cell yields (Figure 3A). Species belonging to C2 (*E. faecalis*), C3 (*L. plantarum*), and C4 (*L. fermentum*, *L. reuteri*, and *L. salivarius*) commonly showed significant increases in cell yields upon supplementation with GTRs, whereas other clusters showed differential patterns within the same cluster. For instance, *L. acidophilus* in C5 and *L. rhamnosus* in C6 showed non-significant changes in cell yields, whereas other species in the same clusters showed significant increases in cell yields. Such differential effects of GTRs on bacterial growth suggest that LABs produce distinct GTR hydrolysates.

For a detailed analysis of the extent of GTR hydrolysis, strains representing each cluster were selected. Cell-free supernatants of *S. thermophilus* (C1), *E. faecalis* (C2), *L. plantarum* (C3), *L. salivarius* (C4), *L. delbrueckii* (C5), *L. paracasei* (C6), and *B. longum* (C7) were analysed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3B). SDS-PAGE patterns show that the proteolytic patterns varied depending on the seven LAB strains examined. Interestingly, the cell-free supernatants of *L. plantarum* (C3), *L. salivarius* (C4), and *L. paracasei* (C6) displayed pronounced amounts of GTR hydrolysates compared with those of other strains, such as *E. faecalis* (C2), *S. thermophilus* (C1), *L. gasseri* (C5), and *B. longum* (C7), which showed marginal hydrolyzing effects.

To further characterize the GTR hydrolysates produced by LABs, we concentrated and fractionated the culture supernatants of the three GTR-hydrolyzing strains (i.e., *L. paracasei*, *L. plantarum*, and *L. salivarius* representing C6, C3, and C4, respectively) by ultrafiltration to yield HMW (>10 kDa), MMW (1–10 kDa), and LMW (<1 kDa) GTR hydrolysates. Each fraction was subjected to calibrated size-exclusion chromatography (Figure 3C). The gel filtration profiles indicated that the GTR hydrolysates contained a wide range of lower molecular weight peptide fractions. Of the three, *L. plantarum* and *L. salivarius* showed a decrease in HMW GTR proteins, whereas *L. paracasei* showed relatively large amounts of HMW GTR proteins after bacterial culture. Consequently, the culture supernatant of *L. plantarum* showed an increase in LMW GTR proteins, and that of *L. salivarius* showed an increase in MMW GTR proteins. These results indicate that *L. plantarum* and *L. salivarius* produced <1 and 1–10 kDa GT peptide fractions under anaerobic conditions. Moreover, *L. plantarum* appeared to be the most prominent producer of GTR hydrolysates with peptides as primary products.

As the three strains exhibited differential protein degradation patterns, all three strains had differential proteolytic systems (Figure S3) and substrate utilization patterns (Figure S4). According to the data obtained from the MEROPS database, the three species commonly possess 55 proteases and peptidases and 5 to 31 unique proteases and peptidases. Moreover, analysis of substrate utilization patterns indicated that carbohydrates serve as primary nutrients for all three species, regardless of the presence or absence of GTRs. However, unlike *L. paracasei*, *L. plantarum* and *L. salivarius* exhibited a relatively high extent of protein utilization. The residual amounts of proteins in *L. plantarum* and *L. salivarius* culture broths corresponded to less than 50% of the initial amounts (Figure S4). Taken together, size-exclusion chromatographic and substrate utilization profiles indicate that these LAB species have different GTR hydrolytic capabilities, presumably owing to different repertoires of proteases, which coincides with the POCP analyses.

Differential utilization of GTRs by *L. plantarum* strains

Based on our findings, *L. plantarum* was chosen as the workhorse to degrade GTR and produce GT peptides.

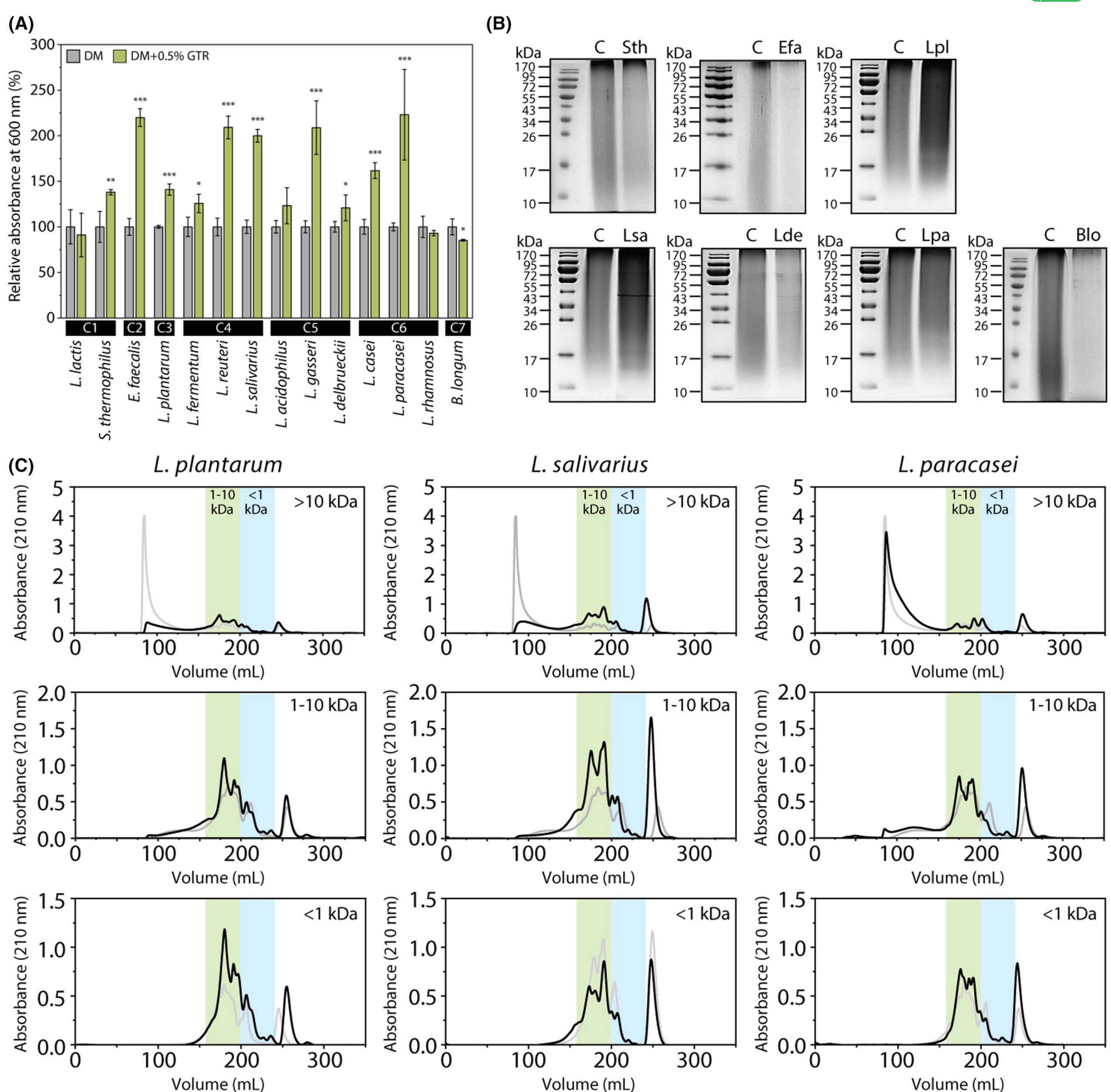

FIGURE 3 Utilization and degradation of green tea (GT) residue proteins by probiotics. (A) Probiotics cultured in media supplemented with 0.5% GT residues showed differential changes in cell yield. Statistical differences were examined using an unpaired student's *t*-test. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$, compared with the untreated control. Values are represented as mean ± SD ($n = 3$). (B) Representative probiotic species from each cluster show differential degradation of GT residues, as analysed through SDS-PAGE. GT residues hydrolyzed by probiotics are on the right lanes and those not hydrolyzed are provided as negative controls on the left lanes. *E. faecalis*, *S. thermophilus*, *L. plantarum*, *L. salivarius*, *L. paracasei*, *L. gasseri*, and *B. longum* represent cluster 1 to 7 in sequential order. (C) Size-exclusion chromatograms of high molecular weight (>10 kDa), middle molecular weight (1–10 kDa), and low molecular weight (<1 kDa) fractions of GT residue protein hydrolysates produced by *L. plantarum*, *L. paracasei*, and *L. salivarius*. Green and blue regions represent the 1 to 10 kDa and <1 kDa fractions, respectively, calculated based on the standard curves in Figure S1.

Next, the GTR-degrading capabilities of the type strain and *L. plantarum* APsulloc 331261, isolated from GT, were compared. Their genomic contexts were similar, with an orthoANI value of 99.06% (Table S4). The major differences in their genes were related to carbohydrate metabolism and cell membrane transport (Figure S5). Remarkably, their growth physiology under different oxygen concentrations and media compositions revealed noticeable differences under anaerobic conditions in protein-rich media (i.e., MRS) (Figure S6).

To compare their growth physiology and GTR-utilizing capabilities, bacterial culture samples from GTR-supplemented media were treated with NaOH, followed by HCl, to conduct pH-driven protein solubilization and precipitation (Figure 4A). Both strains showed a significant increase in cell yield when GTR

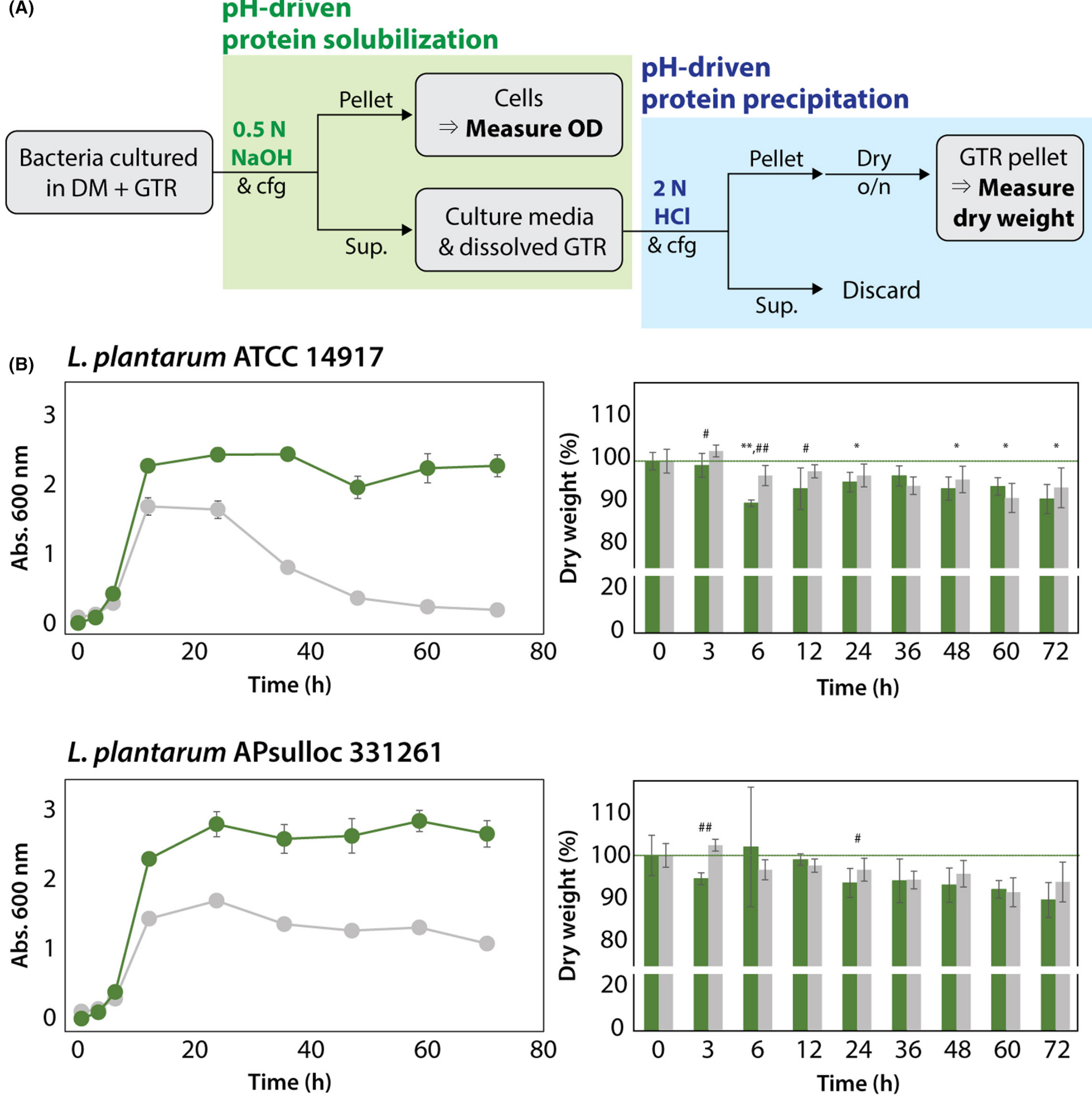

FIGURE 4 Growth characteristics of *L. plantarum* ATCC 14917 (type strain) and *L. plantarum* APsulloc 331261 in media with green tea (GT) residues. (A) pH-driven protein solubilization and precipitation to analyse bacterial cell yields and residual GT residue pellets. Culture broths were first treated with NaOH to solubilize GT proteins. Cells in pellets were used to analyse cell numbers. Cell-free supernatants were treated with HCl to precipitate residual GT residue proteins. Pellets were dried, and their dry weights were measured. (B) Growth profiles (left) of *L. plantarum* ATCC 14917 and *L. plantarum* APsulloc 331261 and residual GT residue pellets over time (right) are shown.

was supplemented in the media, but the extent was greater in *L. plantarum* APsulloc 331261 (Figure 4B, left panel). Moreover, both strains showed a decrease in leftover GT proteins in the culture supernatants over time (Figure 4B, right panel). After 24 h of culture, GT proteins, as measured by dry weight, gradually decreased until 70 h. These results imply that *L. plantarum* APsulloc 331261 is more likely to utilize GTR as a nutrient for growth and produce GT peptides.

Biochemical and biophysical analyses revealed *N*-glycosylated GT peptides from anaerobic fermentation of GTRs by *L. plantarum* strains

The GT peptide fractions of *L. plantarum* and *L. paracasei* showed a marked decrease in the levels of HMW peptides and an increase in MMW and LMW peptide levels compared with those in the control (DM), as determined by SDS-PAGE (Figure 5A). The hydrolytic

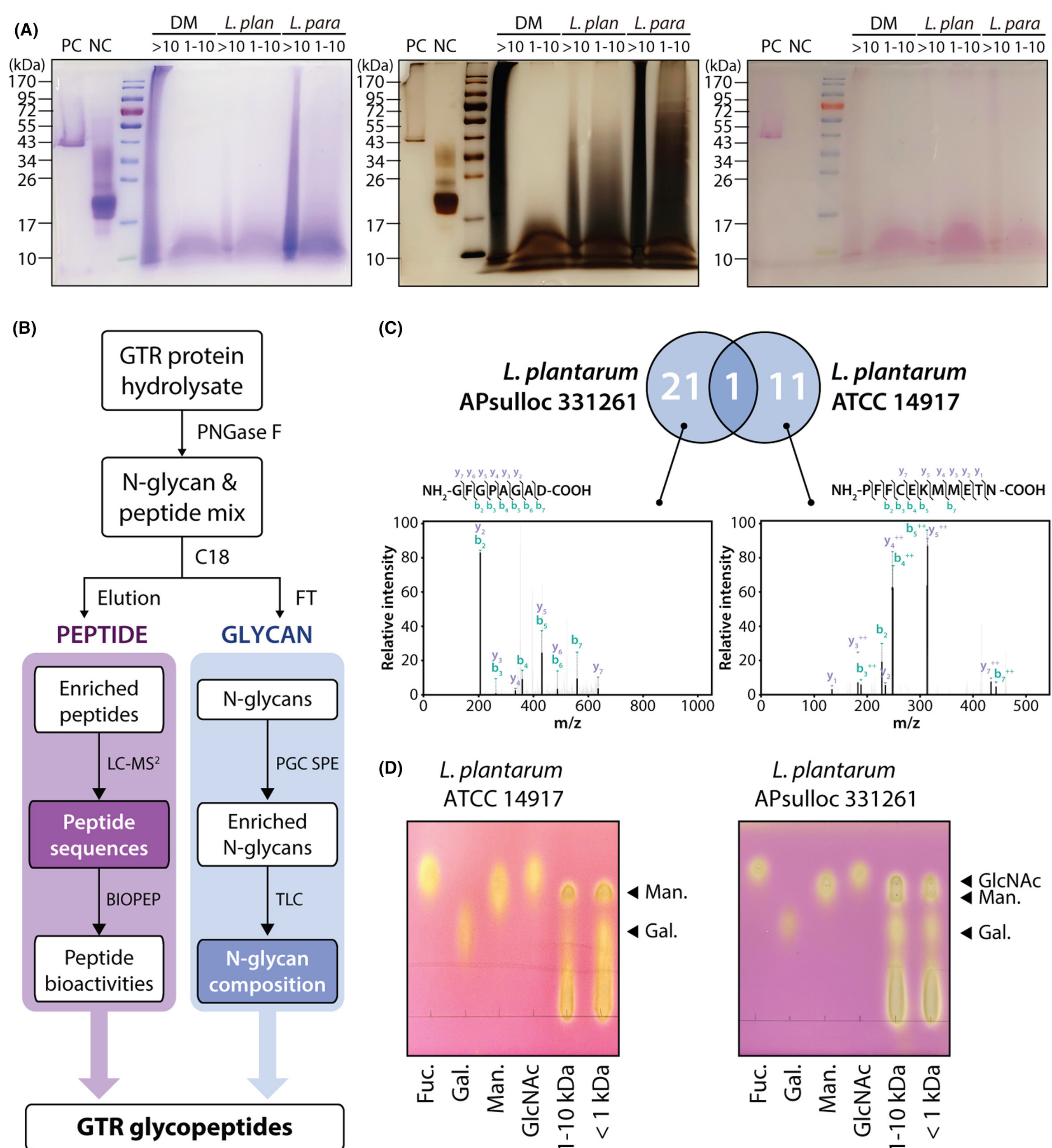

FIGURE 5 Analysis of green tea (GT) glycopeptide profiles produced by *L. plantarum*. (A) Protein (Coomassie blue R-250 and silver nitrate) and glycoprotein (periodic acid-Schiff) staining of GT glycopeptide fractions on SDS-PAGE gels. Glycopeptide fractions in control media and those produced by *L. paracasei* JCM 8130 and *L. plantarum* ATCC 14917 were loaded on each lane (50 μg of >10 kDa and 500 μg of 1–10 kDa). (B) Analysis workflow of GT glycopeptides. Glycopeptide fractions were deglycosylated using PNGase F, and glycans and peptides were analysed using TLC and LC–MS/MS analysis, respectively. (C) Differential peptide profiles produced by *L. plantarum* ATCC 14917 and *L. plantarum* APsulloc, as analysed by LC–MS/MS. representative MS spectra for GT peptides and fragment ions of detected peptides produced by *L. plantarum* strains (D) TLC to identify glycan composition in GT glycopeptides. Standard compounds (fucose, galactose, mannose, and *N*-acetyl glucosamine) and hydrolyzed GT glycopeptide samples were run on silica gel in butan-1-ol/propan-2-ol/ethanol/water (45:45:5:5) solvent system. Potassium permanganate ($KMnO_4$) was used as a detection reagent.

activity of *L. paracasei* was somewhat lower than that of *L. plantarum*, as seen by the distribution of protein bands. Notably, all peptide bands of GTR hydrolysates were severely smeared on SDS-PAGE gels, presumably because plant-derived proteins are *N*-glycosylated (Kornfeld & Kornfeld, 1976; Strasser, 2016). The GT peptide fractions used in this study were indeed heavily glycosylated, as examined using different staining

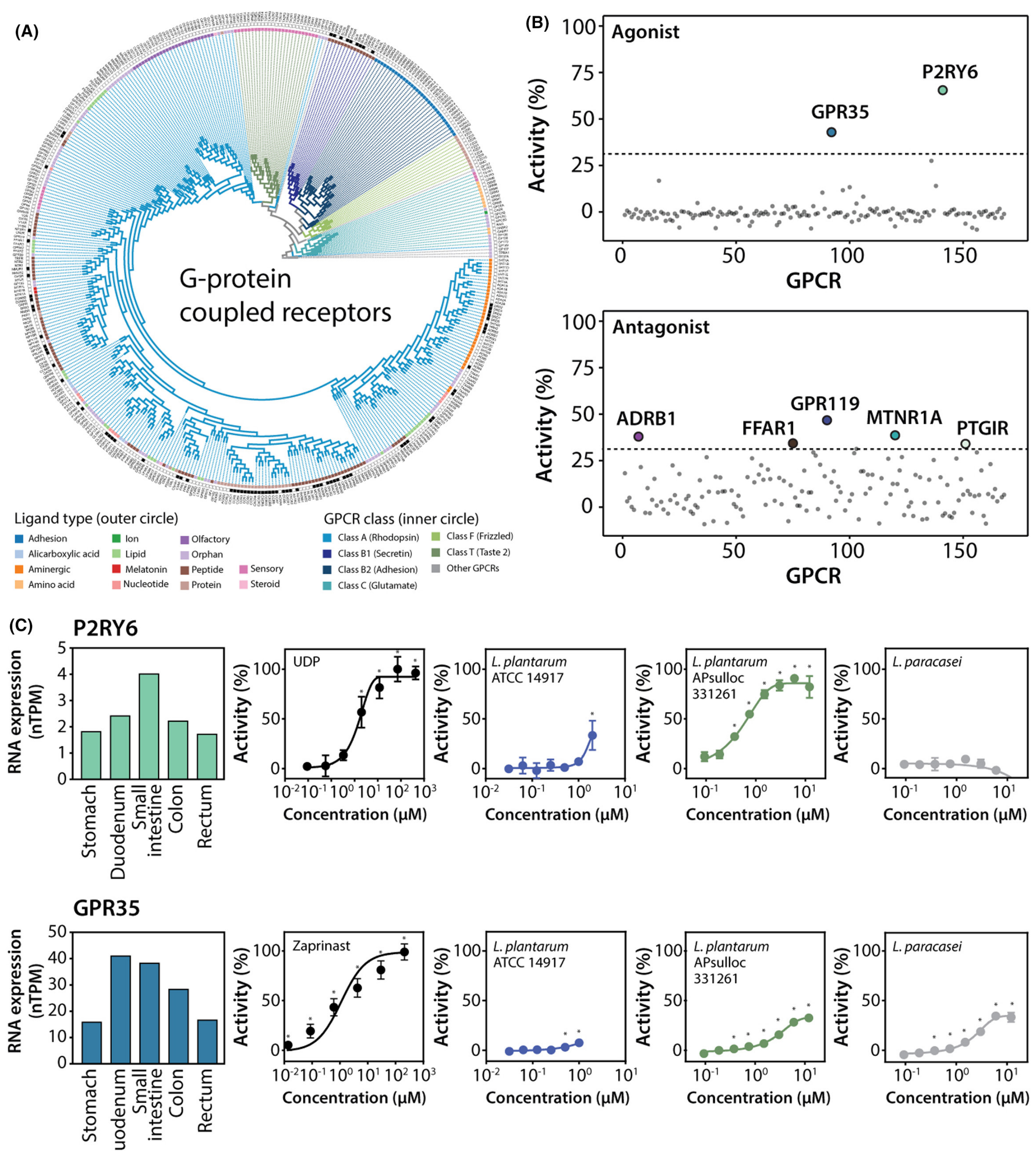

FIGURE 6 Differential agonistic activities of GT glycopeptides produced by three probiotic species. (A) A phylogenetic tree of 423 human GPCRs with their corresponding GPCR class and ligand types. The distribution of 168 GPCRs screened in this study are indicated by black squares. (B) GT glycopeptide fractions produced by *L. plantarum* ATCC 14917 were screened for their agonistic and antagonistic activities against 168 GPCRs. Pyrimidinergic receptor P2Y6 (P2RY6) and G protein-coupled receptor 35 (GPR35) showed the highest agonistic activities. (C) GT glycopeptides produced from three probiotic species were screened for their agonistic activities against P2RY6 and GPR35 (right panel), and these GPCRs were highly expressed in the duodenum and small intestine (left panel). *L. Plantarum* APsulloc 331261 showed clear agonistic activity toward P2RY6 in a concentration-dependent manner. Statistical differences were examined using an unpaired student's *t*-test. *$p < 0.05$, compared with the untreated control. Values represent mean ± SD (n = 3).

methods in SDS-PAGE analysis. The periodic acid-Schiff (PAS) staining showed that the GT peptides, especially those with low to middle molecular weights, were glycosylated (Figure 5A, right panel).

For convenience in the analysis of heavily *N*-glycosylated GT peptides, oligosaccharides were removed from peptides via deglycosylation using PNGase F, and the compositions and sequences of

deglycosylated peptides were analysed (Figure 5B). According to mass spectrometric analysis of deglycosylated GT peptides, the two *L. plantarum* strains (*L. plantarum* ATCC 14917 and *L. plantarum* APsulloc 331261) hydrolyzed GTR proteins to produce GT peptides. However, their peptide profiles varied and were mostly strain specific (Figure 5C and Table S5). Of the 33 GT-derived peptides identified, >97% were exclusive to a single strain. Such exclusive peptide profiles imply that the *L. plantarum* strains have differential GTR-degrading capabilities. Moreover, more than half of the deglycosylated peptides contained asparagine in their sequence, indicating a high probability of heavy *N*-glycosylation.

Various sugar moieties derived from deglycosylated *N*-glycans were detected on TLC plates. The presence of mannose, galactose, *N*-acetyl glucosamine, and fucose as major constituents of plant *N*-glycans was monitored to analyse the chemical composition of GTR *N*-glycans (Strasser, 2016). The GT peptides appeared to be glycosylated primarily by mannose, *N*-acetyl glucosamine, and galactose (Figure 5D). Taken together, we conclude that *L. plantarum* strains can produce *N*-glycosylated GT peptides from GTRs through anaerobic digestion, but their profiles vary considerably.

GTR hydrolysates revealed agonistic and antagonistic activities toward several GPCRs in human physiology and metabolism

To investigate the potential bioactivity of GT peptides, we screened GT peptides produced by *L. plantarum* ATCC 14917 against a panel of 168 GPCRs for their agonistic and antagonistic activities (Figure 6A,B). The 1–10 kDa GT peptide fractions of *L. plantarum* exhibited higher agonistic activities for two GPCRs (P2RY6 and GPR35), which corresponded to >30% of activation by control agonists, and higher antagonistic activities for five GPCRs (i.e., GPR119, MTNR1A, ADRB1, PTGIR, and FFAR1) (Figure 6B). The two GPCRs, pyrimidinergic P2RY6 (Salem et al., 2019) and rhodopsin-like GPR35 (Kaya et al., 2021; Quon et al., 2020), are highly associated with intestinal inflammation, suggesting that GT peptides from *L. plantarum* ATCC 14917, which are agonists of both GPCRs, might be involved in regulating intestinal homeostasis against colitis and colorectal cancers. Moreover, GTR hydrolysates also exhibited antagonistic activities against GPR119, which is a therapeutic target for type 2 diabetes mellitus (Overton et al., 2008; Shah, 2009), and for MTNR1A, which is approved for the treatment of sleep and mood disorders (Shiu et al., 2010).

We selectively chose P2RY6 and GPR35 to examine the effects of GT peptide fractions produced by other strains. Interestingly, the GT peptide fractions produced by LABs showed different binding activities toward GPCRs in a strain-dependent manner (Figure 6C). *L. plantarum* APsulloc 331261 showed distinct agonistic activity toward P2RY6 in a dose-dependent manner. Despite the low purity of the sample, this result highlights the potential of GT peptides to function as binding ligands for GPCRs, thereby communicating with host cells.

Taken together, the comprehensive screening of GTR hydrolysates for targeting GPCRs indicates that *N*-glycosylated GT peptides derived from LAB-aided GTR hydrolysates exhibit anti-inflammatory activity through GPCR binding activity. Furthermore, these data suggest that GT peptides could serve as BPs not only for inhibiting inflammation in the human gut but also for modulating sleep and mood disorders, potentiating the use of therapeutic agents for the treatment of immune and hormonal disorders.

CONCLUSIONS

The anaerobic digestion-aided generation of *N*-glycosylated GT peptides enabled us to potentiate the sustainable and green treatment of GTR agro-waste. Such a recycling strategy enables us to open new value chains of GTRs, which can be extended to other plant residues. The use of proteolytic LABs as biocatalysts allowed us to utilize GTRs as a resource for producing peptides with anti-inflammatory activities through GPCR binding activities. Therefore, anaerobic digestion valorizes GTR hydrolysates into health-promoting agents, potentiating the usability of GT peptides as nutraceuticals and cosmeceuticals.

AUTHOR CONTRIBUTIONS

J. Y. Lee, H. I. Hong, J. E. Lee, H. S. Jin, J. O. Chung, and D. W. Lee formulated the research plan. J. Y. Lee, H. I. Hong, J. E. Lee, Y. J. Hong, H. S. Jin, and J. O. Chung performed the experiments. J. Y. Lee, H. I. Hong, J. E. Lee, Y. J. Hong, H. S. Jin, H. W. Hwang, H. S. Jin, J. O. Chung, and D. W. Lee analysed the data. J. Y. Lee, H. I. Hong, J. E. Lee, and D. W. Lee wrote the manuscript. Y. D. Hong, W. S. Park, J. O. Chung, and D. W. Lee conceived, planned, supervised, and managed this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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