Lactiplantibacillus plantarum LM1001 improves digestibility of branched-chain amino acids in whey proteins and promotes myogenesis in C2C12 myotubes

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Running Title: *L. plantarum* LM1001 promotes myogenesis in myotubes

Lactiplantibacillus plantarum LM1001 improves digestibility of branched-chain amino acids in whey proteins and promotes myogenesis in C2C12 myotubes

Abstract

Lactiplantibacillus plantarum is a valuable potential probiotic species with various proven health-beneficial effects. *L. plantarum* LM1001 strain was selected among ten strains of *L. plantarum* based on proteolytic activity on whey proteins. *L. plantarum* LM1001 produced higher concentrations of total free amino acids and branched-chain amino acids (BCAA: Ile, Leu, and Val) than other *L. plantarum* strains. Treatment of C2C12 myotubes with whey protein culture supernatant (1%, 2% and 3%, v/v) using *L. plantarum* LM1001 significantly increased the expression of myogenic regulatory factors, such as Myf-5, MyoD, and myogenin, reflecting the promotion of myotubes formation ($p<0.05$). *L. plantarum* LM1001 displayed β-galactosidase activity but did not produce harmful β-glucuronidase. Thus, the intake of whey protein together with *L. plantarum* LM1001 has the potential to aid protein digestion and utilization.

Keywords: *Lactiplantibacillus plantarum* LM1001, digestibility, branched-chain amino acids, myogenesis, C2C12 myotubes

Introduction

Lactiplantibacillus plantarum is a commensal microorganism in the human gastrointestinal (GI) tract and is also commonly found in many fermented foods (Kausik et al., 2009). Owing to its long history of safe use, *L. plantarum* has been acknowledged as "Generally Regarded as Safe (**GRAS**)" by the United States Food and Drug Administration (**FDA**) and as suitable for the "Qualified Presumption of Safety" by the European Food and Safety Authority (**EFSA**) (Liu et al., 2018).

A variety of health-beneficial effects of *L. plantarum* have been previously demonstrated. The administration of *L. plantarum* FBT215 significantly lowered the Firmicutes/Bacteroidetes ratio in healthy mice and effectively alleviated colonic inflammation (Chang et al., 2021; Lee et al., 2023). *L. plantarum* 200655 decreased oxidative stress in HT-29 cells and improved the texture attributes of yogurt such as water-holding capacity and viscosity (Kariyawasam et al., 2023). D-Galactose-mediated oxidative stress was suppressed while hepatic glutathione peroxidase activity was promoted in aged mice with the administration of *L. plantarum* C88 (Li et al., 2012). Some *L. plantarum* strains, such as *L. plantarum* TWK10 and *L. plantarum* HY7715, improved muscle mass and exercise performance in a rodent model, but details underlying the mechanism for attenuation of sarcopenia are still not certain (Chen et al., 2016; Lee et al. 2021). The authors postulated that *L. plantarum* HY7715 might contribute to improving the rate of protein digestion and absorption.

Whey proteins are side-stream products of cheese manufacture and are highly availability as a dietary protein source. Whey proteins contain a high concentration of branched-chain amino acids (**BCAA**: Leu, Ile, and Val, 22.3%) which play an important role in skeletal muscle synthesis compared to casein (20.3%), soy (17.5%), and wheat protein (14.1%) (Morifugi et al., 2009). According to Park et al. (2018), protein

74 supplementation improves muscle mass and physical performance in malnourished older
75 adults, but its efficacy depends on the level and type of protein. They reported that protein
76 supplementation at a level of 1.5 g/kg/day provided a beneficial effect on the prevention
77 of sarcopenia. The supplementation of hydrolyzed whey protein isolate showed greater
78 improvement in lean muscle mass and muscle strength during 10 wks of resistance
79 training in bodybuilders compared to caseins (Cribb et al., 2006). These results suggest
80 that hydrolyzed low-molecular-weight peptides containing Leu possibly accelerate the
81 utilization of amino acids for muscle synthesis compared to intact whey proteins.

82 The proteolytic activity of lactic cultures plays an important role in flavor and
83 texture development and the liberation of bioactive peptides in the production of
84 fermented foods (Satilmis et al., 2023). Kim et al. (2023) reported that the administration
85 of milk protein and a probiotic strain with high proteolytic activity significantly improved
86 the digestibility of proteins in mice. This suggests that a combination of protein and
87 probiotic culture might improve the transport of amino acids for muscle synthesis.

88 The objective of this study was to screen potential probiotic strains for their
89 ability to improve the bioavailability of BCAA in whey proteins for muscle synthesis. To
90 achieve this goal, a promising *L. plantarum* strain was selected based on digestibility and
91 BCAA production from whey proteins, and the effect of the selected probiotic strain (*L.*
92 *plantarum* LM1001) on myogenesis was evaluated using C2C12 myoblasts.

93

94 **Materials and Methods**

95 **Isolation and characterization of *L. plantarum* LM1001**

96 *L. plantarum* LM1001 was isolated from kimchi, Korean fermented vegetables,
97 as previously described (Bae et al., 2022). Briefly, homogenized kimchi was diluted in
98 phosphate-buffered saline (**PBS**) and plated on de Man-Rogosa-Sharpe agar (**MRS** agar;

BD Difco, Franklin Lakes, NJ, USA). To isolate *Lactobacillus* species, the colonies isolated from MRS agar were spread on bromocresol purple (BCP) containing MRS agar. Yellow colonies on BCP agar plate were cultured again in MRS agar. For the characterization of the isolated strain, a single purified colony was enriched in MRS broth and further analyzed by 16S rRNA sequencing. The identified Gram-positive and catalase-negative *L. plantarum* strain was named LM1001.

Proteolytic activity of *L. plantarum* strains on whey proteins

The medium was prepared by dissolving 50 g of whey protein concentrate (**WPC**) (Marquez Brothers International, Inc., Hanford, CA, USA), 5 g of tryptone (Gibco, Paisley, UK), 2.5 g of yeast extract (BD Difco, Detroit, MI, USA), and 1 g of glucose (Daejung Chemicals, Siheung, Korea) in 1 L of distilled water. The medium was sterilized in an autoclave at 95℃ for 10 min. To assess proteolytic activity, *L. plantarum* strains were cultured in MRS and harvested by centrifugation (13,572×g, 4℃, 10 min) and washed twice with PBS. The washed bacteria were resuspended in PBS to approximately 10 Log CFU/mL and 1% (v/v) of the suspension was inoculated into WPC medium (the final concentration of *L. plantarum* strain was adjusted to 8 Log CFU/mL). The same volume of PBS was used instead of resuspended bacteria as a control. The mixture was incubated at 37℃ for 48 h under shaking at low speed. The protein concentration of initial WPC and cultured WPC was measured by the bicinchoninic acid (**BCA**) assay (Smith et al., 1985). The ratio of WPC degradation was calculated as follows:

$$\text{Proteolytic activity (\%)} = 100 - (A_{48h} / A_0) \times 100$$

where, A_0 is the initial protein concentration of WPC medium, and A_{48h} is the protein concentration of WPC medium after 48 h incubation. *L. plantarum* ATCC14917 (type strain) was used as a control.

Preparation of WPC culture supernatant using *L. plantarum* LM1001 (LP-WPC)

After incubation of WPC medium in the presence of *L. plantarum* LM1001, the culture supernatant was obtained by centrifugation (15,928×g, 4℃, 10 min). The supernatant was collected, filtered through a 0.22 μm syringe filter (Adventec, Tokyo, Japan) and named LP-WPC. LP-WPC was stored at -20℃ until use.

Identification of genes encoding the proteolytic system of *L. plantarum* LM1001

The genomic DNA of *L. plantarum* LM1001 was extracted using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit (Takara Bio, Kusatsu, Japan). The DNA sequencing library was constructed using single molecular real-time sequencing technology (Pacific Biosciences, Menlo Park, CA, USA). The hierarchical genome assembly process for *de novo* assembly was performed using the Celera Assembler (Macrogen, Seoul, Korea). In order to compare the proteolytic genes of *L. plantarum* LM1001 with those of *L. plantarum* ATCC 14917, as the reference strain, complete genome sequences of both strains were obtained from the NCBI microbial genome database (https://www.ncbi.nlm.nih.gov/ genome).

Free amino acid analysis

The free amino acid content of LP-WPC was determined by the method of Tang et al. (2023) with a slight modification. Briefly, an equal volume of TCA solution (10%, v/v) was added to LP-WPC. The mixture was placed at room temperature for 60 min and centrifuged at 18,472×g for 10 min. The supernatant was filtered through a 0.22 μm syringe filter (Adventec) prior to HPLC analysis. An Agilent 1260 Infinity HPLC system equipped with a diode array detector (Agilent, Santa Clara, CA, USA) was used for

149 analysis. The separation was carried out by means of an Agilent Zorbax Eclipse AAA
150 column (4.6 mm × 150 mm, 3.5 μm) using a mobile phase consisting of 0.1% formic acid
151 in water (v/v) as mobile phase A and 0.1% formic acid in acetonitrile (v/v) as mobile
152 phase B. Gradient elution was performed as follows: 0 min, 10% B; 1 min, 20% B; 10
153 min, 40% B; 40 min, 50% B; 42 min, 100% B; 57 min, 100% B; 60 min, 90% B; 70 min,
154 10% B. The flow rate was set to 0.4 mL/min, and the column oven temperature was
155 maintained at 40℃. The UV detection was performed at 338 nm. The quantification of
156 amino acid was performed using the external standard. Each sample was analyzed in
157 triplicate with three independent determinations.
158
159 **Probiotic properties**
160 The probiotic properties of *L. plantarum* LM1001 including its resistance to
161 gastric and bile salt conditions, adhesion to intestinal epithelial cells, and auto-
162 aggregation were measured as described previously (Bae et al., 2023). Briefly, cultured
163 *L. plantarum* LM1001 was incubated at 37°C either in MRS broth containing pepsin
164 (0.3%, pH 2.5, 2 h) or oxgall (0.3%, 24 h) and the viable cells were counted by spread
165 plate method on MRS agar. For adhesion test, HT-29 intestinal epithelial cells (ATCC,
166 Manassas, VA, USA) were harvested at 80% confluence, and the harvested cells were
167 seeded (1×10^5 cells/well) and incubated to form a monolayer. The HT-29 monolayer
168 was treated with *L. plantarum* LM1001 (8 Log CFU/mL) for 2 h without antibiotics. After
169 washing nonadherent bacteria with PBS, and viable cells were counted. In the auto-
170 aggregation test, cultured *L. plantarum* LM1001 was washed with PBS and adjusted to
171 have an A_{600} of 0.5. After incubated at 37℃ for 24 h, the absorbance of upper suspension
172 was measured at 600 nm. The aggregation (%) was calculated as follows:
173 $[(A_{0h} - A_{24h}) / A_{0h}] \times 100$

Where A_{0h} is the initial absorbance at 600 nm, and A_{24h} is the absorbance at 600 nm after 24 h.

Enzyme-producing activity

The intrinsic enzyme-producing activities of *L. plantarum* LM1001 were determined using the API ZYM kit (BioMérieux, Marcy-l'Etoile, France) according to the manufacturer's guidelines.

Effect of LP-WPC on myogenesis in C2C12 myoblasts

C2C12 mouse myoblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in complete Dulbecco's modified Eagle medium (DMEM; Welgene, Daegu, Republic of Korea), which contains 10% fetal bovine serum (**FBS**, Welgene) and 1% penicillin-streptomycin (Welgene), at 37℃ in a 5% CO_2-humidified incubator. To induce myogenic differentiation, C2C12 cells (5×10^4 cells/well) were plated in a 6-well plate and cultured for 3 days in complete medium. When the cells reached 80% to 90% confluency, the medium was switched to differentiation medium containing 2% horse serum (MB Cell, Republic of Korea) and 1% antibiotics to induce myotube differentiation. LP-WPC (1, 2, and 3%) was treated to C2C12 cells, and the effect on the expression of myogenic regulatory factors, such as myogenic factor 5 protein (**Myf-5**) (Abcam, Cambridge, UK), myoblast determination factor 1 (**MyoD**) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), myogenin (Abcam), was analyzed using western blotting. Cytotoxicity of LP-WPC on C2C12 cells was monitored, and western blotting was conducted, as previously described (Bae et al., 2022).

Statistical analysis

Statistical analyses were performed using SPSS Statistics version 21 software (IBM, Armonk, NY, USA). When a significant difference ($p<0.05$) was found in the analysis of variance (ANOVA), Duncan's multiple comparison and Student`s *t*-test were conducted to determine the significant difference between treatment means.

Results and Discussion

Proteolytic activity of *L. plantarum* strains

To analyze the proteolytic activity, various *L. plantarum* strains were cultured in WPC medium, and changes in protein concentration were compared. As shown in **Fig. 1**, five strains resulted in a significant decrease in protein content after incubation ($p<0.05$, **Fig. 1A**). *L. plantarum* LM1001 strain displayed the greatest proteolytic activity among the tested strains (**Fig. 1B**).

There is no standard method for the evaluation of proteolytic activity of lactic acid bacteria strains, although the agar-well diffusion test has often been used as an index of proteolytic activity (Beganovic et al., 2013). However, the measurement of a clear zone diameter is not accurate enough as a quantitative assay for the determination of proteolytic activity. The BCA assay is a total protein assay. The principle of the assay is based on the reductive properties of the peptide bonds to reduce Cu^{2+} to Cu^{+} under alkaline conditions. When the Cu^{+} reacts with the organic dye, BCA, a purple complex is formed that can be measured spectrophotometrically at 562 nm. The extent of reduction of Cu^{2+} is also dependent on the protein composition, such as Trp, Tyr, Cys, and cystine (Smith et al., 1985). Wiechelman et al. (1988) reported that at least a tripeptide is required for complex formation between Cu^{+} and BCA. Thus, the decreased color intensity in the BCA assay was probably due to proteolysis-mediated peptide bond reduction, as the total moles of

oxidizable amino acids in the substrate (WPC) are the same. The protein content was consistently decreased by the addition of bromelain, a positive protease control (**Fig. 1A**).

Whey protein is an excellent dietary protein source for muscle synthesis and the prevention of sarcopenia (Devries and Phillips, 2015). The interplay between the host and gut microbiota influences protein metabolism by modifying metabolite production and amino acid homeostasis (Lin et al., 2017). Probiotics indirectly affect protein metabolism by altering the gut microbiota composition or directly improve protein digestion by promoting digestive enzyme activity in the gut (Wang and Ji, 2019). It has been demonstrated that the intake of protein together with probiotics with high proteolytic activity promoted digestibility and bioavailability of proteins (Jeon et al., 2023).

The proteolytic and peptidolytic activity varies depending on the bacterial species and strains. Table 1 shows the results of the whole genome sequencing (WGS) analysis of L. plantaraum LM1001. The number of the protease- and peptidase-related genes of L. plantarum LM1001 was 81, which was about twice as many as that of *L. plantaraum* ATCC 14917, the type strain of *L. plantarum* species. The high proteolytic genetic potential of L. plantarum LM1001 might be related to its superior proteolytic activity to other L. plantarum strains. In particular, L. plantarum LM1001 has 19 metalloprotease or metallopeptidase (MMP)-related genes containing one or two divalent metal ions, such as Zn2+, Ca2+, Mg2+, and Cu2+ in active sites (Hasan et al., 2021). Based on these results, L. plantarum LM1001 was selected as a strong WPC hydrolytic strain and used for further study. In addition, proteolytic activity of lactic acid bacteria varied depending on the type of protein such as soy protein, casein, and whey protein. L. plantarum LM1001 showed strong proteolytic activity especially on WPC (data not shown).

Changes in free amino acids and BCAA contents by fermentation of WPC with *L.*

***plantarum* strains**

Changes in total free amino acids and BCAA content in the WPC culture supernatant were determined after TCA (10%, v/v) precipitation of LP-WPC. As shown in **Table 2**, the total free amino acids and BCAA content were significantly increased by fermentation with various *L. plantarum* strains. This implies that proteolysis of protein molecules occurred by proteases/peptidases released from *L. plantarum* strains. LMW peptides and free amino acids generated by the metabolism and transformation of intact dietary protein in the gut are readily absorbed by the gut endothelial cells and improve the bioavailability of WPC.

BCAA plays an important role in muscle biosynthesis. In particular, Leu promotes protein synthesis through the activation of mechanistic target of rapamycin complex 1 (**mTORC1)** (De Bandt, 2016). Leu supplementation improved the regeneration of skeletal muscles, decreased the inflammation area, and increased the number of proliferating satellite cells in muscles from old rats (Pereira et al., 2015). The concentration of BCAA (including Leu) in the culture supernatant was significantly increased by fermentation, and it varied among the *L. plantarum* strains. The cultures fermented with *L. plantarum* LM1001 showed the highest total free amino acids (4,261 μg/mL) and BCAA (2,208 μg/mL) content. *L. plantarum* LM1206 and *L. plantarum* LM1205 strains also produced high levels of free amino acids but they were similar to those of *L. plantarum* ATCC 14917, the type strain.

L. plantarum strains displayed different proteolytic characteristics and provided different bioactivities and sensory impacts depending on the amino acid profile produced (Satilmis et al., 2023). Chu et al. (2023) demonstrated that the administration of *L. plantarum* CCFM405 alleviated Rotenone-induced Parkinson's disease by modulating gut microbiota and BCAA biosynthesis. This suggests that administration of appropriate

274 probiotics plays an important role in the generation of desirable metabolites such as
275 BCAA.
276
277 **Probiotic properties of *L. plantarum* LM1001**
278 The probiotic properties of *L. plantarum* LM1001, including its resistance to
279 pepsin and bile salt, adhesion to HT-29 cells, and auto-aggregation, were evaluated. As
280 shown in **Table 3**, *L. plantarum* LM1001 showed more than 99% acid tolerance and 73%
281 bile salt tolerance, respectively. The tolerance of *L. plantarum* LM1001 to bile salt was
282 significantly higher than that of *L. plantarum* ATCC 14917 ($p < 0.05$), whereas the
283 adherence ability to HT-29 cells and auto-aggregation (%) of *L. plantarum* were
284 comparable to those of the *L. plantarum* type strain.
285 Probiotics are live microorganisms that contribute to host health, and the abilities
286 to survive and colonize in the GI tract environment are some of the major criteria for the
287 selection of probiotics (Pramanik et al., 2023). The attachment of *Lactobacillus* spp. to
288 human enterocytes occurs through the cell surface components of microorganisms such
289 as cell-surface collagen-binding proteins and cell wall-anchored proteins (**CWAP**) (Bae
290 et al., 2023). CWAP contains an amino acid motif consisting Leu-Pro-X-Thr-Gly
291 (LPXTG, X: any amino acid), and cleavage of the Thr residue by sortase (**Srt**) enzymes
292 mediates cell wall attachment to human epithelial cells (Zhang et al., 2015). LPXTG and
293 Srt-related genes were identified in the WGS analysis of *L. plantarum* LM1001, and these
294 genes could contribute to the adhesion of probiotics.
295
296 **Enzyme-producing activity**
297 Probiotic strains produce enzymes, which influence the utilization of nutrients.
298 **Table 4** indicates the intrinsic enzyme-producing activities of *L. plantarum* LM1001. *L.*

plantarum LM1001 showed 13 enzyme activities, such as alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, and β-galactosidase. Among these enzyme activities, β-galactosidase activity can alleviate lactose intolerance by catalyzing the hydrolysis of lactose into glucose and galactose. Whey protein powder for muscle growth is typically produced from WPC and contains 15% lactose (dry weight basis). The combined ingestion of whey protein powder and *L. plantarum* LM1001 might relieve discomfort derived from lactose maldigestion. In addition, *L. plantarum* LM1001 did not display β-glucuronidase activity which alters xenobiotic availability in the gut and is associated with an increase in the risk of colon cancer (Sears and Garrett, 2014).

Consistent with the results of the present study, *L. plantarum* Ln4 and *L. plantarum* G72 strains displayed β-galactosidase activity and did not produce β-glucuronidase, whose activity is harmful to human health (Son et al., 2017). The genes encoding β-galactosidase and glycoside hydrolase were also identified in the WGS analysis (β-galactosidase, β-galactosidase small subunit, glycosidase hydrolase family 25, family 78 glycoside hydrolase catalytic domain, and glycoside hydrolase family 1, 13, 65, and 73 proteins).

Effect of LP-WPC on myogenic differentiation in C2C12 cells

The effect of LP-WPC on the differentiation of skeletal muscle cells was determined using C2C12 myoblasts. As shown in **Fig. 2**, the treatment with more than 5% sample (WPC vs. LP-WPC culture broth) showed less than 80% cell viability in the MTT assay. Thus, the non-cytotoxic concentrations of the sample were selected as 1, 2, and 3%. LP-WPC treatment at the level of 2% and 3% of medium significantly upregulated the protein expression of all myogenic regulator factors, such as Myf-5, MyoD, and myogenin in C2C12 cells ($p < 0.05$, **Fig. 3**). Conversely, there were no significant changes

in the expression of myogenic factors by treatment with WPC broth (**Supplement 1**). These results suggest that LP-WPC can enhance myogenic activity in myoblasts.

Myogenesis is a process that regulates the complex growth and maturation of muscle tissue, including the proliferation, differentiation and maturation of myoblasts (Allen et al., 1979). Myoblasts with a single nucleus are fused to form multinucleated structures, and MyoD and Myf-5 play pivotal roles in myoblast commitment and myogenic differentiation (Sabourn and Rudnicki, 2000). Myogenin contributes to muscle homeostasis as a secondary myogenic regulator. Mutation of *myog* in zebrafish caused a decrease in muscle mass and muscle fiber size (Ganassi et al., 2018).

The increased expression of myogenic regulators in response to LP-WPC is probably due to the generation of BCAA in the culture supernatant. The supplementation of BCAA promoted mTOR1 signaling and simultaneously activated the autophagy function of muscle cells in patients with liver disease (Tsien et al., 2015). The activation of mTORC1 signaling is regulated by various factors such as mechanical and endocrine stimuli, intercellular energy level, and the availability of amino acids (Bar-Peled and Sabatini, 2014). Atherton et al. (2009) examined the effect of essential amino acid (**EAA**) on protein synthesis in C2C12 skeletal muscle cells and found that Leu significantly promoted mTOR and 4E-binding protein 1(4EBP1) signaling, but other EAA had no effect on anabolic signaling.

Conclusion

Adequate protein consumption and physical exercise are major strategies to improve muscle mass. WPC is widely used in protein powder formulations. *L. plantarum* LM1001 was selected based on the digestibility and potential to generate BCAA from WPC. The addition of LP-WPC to C2C12 myoblasts significantly increased the expression of Myf-

5, MyoD, and myogenin reflecting a promotion in the formation of myotubes. *L. plantarum* LM1001 displayed β-galactosidase activity but did not produce β-glucuronidase. Thus, the intake of whey protein together with *L. plantarum* LM1001 has the potential to aid protein digestion and utilization. The effect of this combination on muscle mass in an animal model is currently underway.

Conflicts of Interest

Youngjin Lee, Yoon Ju So, Woo-Hyun Jung, Tae-Rahk Kim, and Minn Sohn are employees of Lactomason. Industry employees are involved in the study of probiotic characterization, However, they had no role in the interpretation of data or publication processes.

Author contribution

Conceptualization: Imm J-Y, Kim T-R, and Shon M, Data curation: Jeong Y-J, Investigation: Lee Y, So Y-J, Jung W-H, and Jeong Y-J, Writing - original draft: Lee Y, So Y-J, Jung W-H, and Jeong Y-J, Writing - review & editing: Imm J-Y.

IRB/IACUC approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

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472 **Table 1. The proteolytic genes in *L. plantarum* LM1001 and ATCC 14917**

Description	Gene Name	Strain	
		L.plantarum LM1001	*L. plantarum* ATCC 14917
Aminopeptidase	*pepC/pepN/pepN*	0	3
Aminopeptidase p family protein	*ypdF*	2	1
ATP-dependent clp endopeptidase proteolytic subunit		1	0
ATP-dependent clp protease ATP-binding subunit	*clpC/clpE/clpX*	4	3
ATP-dependent protease clp protease protease proteolytic subunit	*clpP*	0	2
ATP-dependent protease ATPase subunit	*clpY*	1	2
ATP-dependent protease subnit HslV	*hslV*	0	1
ATP-dependent zinc metalloprotease	*ftsH*	0	1
Beta-barrel assembly-enhancing protease	*bepA*	0	1
C1 / C40 / C69 family peptidase	*pepE/pepD*	7	0
Carboxy-terminal processing protease CtpA	*ctpA*	0	1
Clp protease		2	0
Cpbp family intramembrane metalloprotease		13	0
D-alanyl-d-alanine carboxypeptidase/D-alanyl-D-alanine dipeptidase	*dacA/ddpX*	2	2
dipeptidase	*pepD/pepDA*	2	4
Extracellular zinc metalloproteinase		1	0
Family deacylase		2	0
Family metallo-endopeptidase		2	0
Gamma-D-glutamyl-L-lysine dipeptidyl-peptidase	*ykfC*	0	1
gamma-d-glutamyl-meso-diaminopimelate peptidase		1	0
gnat family n-acetyltransferase		1	0
guanosine monophosphate reductase		1	0
ld-carboxypeptidase	*mccF*	2	0
Lipoprotein signal peptidase	*IspA*	0	1
m1 / m15 family metallopeptidase	*pepN/ddpX*	3	0
m3 family oligoendopeptidase		1	0
matrixin family metalloprotease		3	0
Methionine aminopeptidase	*Map*	0	1
Neutral endopeptidase	*pepO*	0	1
Oligoendopeptidase F_ plasmid	*pepF1*	1	1
Peptidase E / m13 / m23	*pepE/pepO*	2	1
Peptidase propeptide and ypeb domain protein		1	0
Peptidase proteolytic subunit		1	0
Peptidase s41		1	0
Peptidase t	*prpT*	1	1
Peptide cleavage export abc transporter		1	0
Peptidoglycan endopeptidase		1	0
Phage endopeptidase		1	0
Prepilin peptidase	*comC*	1	0
Proline iminopeptidase	*fpaP/peplP*	1	1
Proline-specific peptidase family protein		1	0
Protease HtpX	*htpX*	0	1
Putative dipeptidase		0	1
Putative L_D-transpeptidase YciB	*yciB*	0	3
putative protease YdeA	*ydeA*	0	1
Regulator of sigma-W protease RasP	*rasP*	0	1
Rhomboid family intramembrane serine protease	*gluP*	1	1
Ribosomal-processing cysteine protease Prp		1	0
Rip metalloprotease	*rasP*	1	0
Serine hydrolase		2	0
Serine protease Do-like HtrA	*htrA*	0	1
Signal peptidase I / Signal peptidase II	*spsB / ispA*	4	2
Transpeptidase/transpeptidase family protein		5	0
Xaa-pro dipeptidyl-peptidase	*pepX*	1	1
Zinc metallopeptidase		2	0
Number of proteolytic genes		81	41

473

474 **Table 2. Changes in total free amino acids and BCAA content in WPC medium after**
475 **fermentation of various *L. plantarum* strains.**

476

Strain	Amino acids content (μg/mL)				
	Free amino acids	BCAA			
		Val	Ile	Leu	Total
Control	2,019±39^{a}	165±4^{a}	111±2^{a}	331±8^{a}	608±13^{a}
L. plantarum ATCC 14917	3,639±135^{ef}	681±15de	494±16^{ef}	648±24^{e}	1,823±49^{c}
L. plantarum LM1001	4,261±195^{g}	752±26^{g}	574±27^{g}	882±10^{g}	2,208±62^{e}
L. plantarum LM1202	3,420±96de	692±23^{ef}	479±10^{e}	633±4^{e}	1,804±32^{c}
L. plantarum LM1203	3,047±120^{b}	630±34^{cd}	424±10^{bcd}	551±7^{b}	1,605±50^{b}
L. plantarum LM1204	3,417±104de	694±31^{ef}	447±13^{d}	641±12^{e}	1,781±53^{c}
L. plantarum LM1205	3,634±162^{ef}	743±39^{fg}	479±17^{e}	705±14^{f}	1,927±67^{d}
L. plantarum LM1206	3,734±127^{f}	743±32^{fg}	513±12^{f}	716±3^{f}	1,972±44^{d}
L. plantarum LM1209	3,039±177^{b}	632±39^{cd}	435±21^{cd}	540±17^{b}	1,607±74^{b}
L. plantarum LM1210	3,121±98^{bc}	632±26^{cd}	403±6^{b}	580±5^{c}	1,614±27^{b}
L. plantarum LM1211	3,323±156^{cd}	624±4^{c}	508±24^{f}	606±19^{d}	1,739±73^{c}

477

478 Data are shown as means ± SDs of thee independent experiments. BCAA: Branched-chain
479 amino acids; Free amino acids: Ser+Asp+Glu+Thr+Gly+Tyr+Ala+Met+Phe+Lys. Different
480 superscript indicate statistical significance in the same columns ($p<0.05$).

481

482 **Table 3. Probiotic properties of *L. plantarum* LM1001**

483

Probiotic properties (%)		Strain	
		L. plantarum LM1001	*L. plantarum* ATCC 14917
Resistance (%)	pH 2.5 pepsin (0.3%)	100±0[a]	98±1[b]
	Bile salt (0.3%)	74±1[a]	65±0[b]
Auto-aggregation (%)		54±3[a]	57±2[a]
Adhesion rate to HT-29 cell (%)		83±1[a]	85±1[a]

484 Data are shown as means ± SDs of thee independent experiments. Different superscripts
485 indicate significant difference in the same row ($p<0.05$).

486

487 **Table 4. Enzyme-producing activity of *L. plantarum* LM1001**

488

Enzyme	Enzyme activity
Alkaline phosphatase	Positive
Esterase (C4)	positive
Esterase Lipase (C8)	positive
Lipase (C14)	negative
Leucine arylamidase	positive
Valine arylamidase	positive
Crystine arylamidase	positive
Trypsin	negative
α-chymotrypsin	negative
Acid phosphatase	positive
Naphtol-AS-BI-phosphohydrolase	positive
α-galactosidase	positive
β-galactosidase	positive
β-glucuronidase	negative
α-glucosidase	positive
β-glucosidase	positive
N-acetyl-β-glucosaminidase	positive
α-mannosidase	negative
α-fucosidase	negative

489

490

491 **Figure captions**

492

493 **Fig. 1. Changes in protein concentration after incubation of WPC with various *L.***
494 ***plantarum* strains (A) and their protein digestibility percentage (B).** Reconstituted WPC
495 (5%, w/v) was incubated for 48 h in the presence of individual *L. plantarum* strains**.** Bromelain
496 (5 mg/mL) was used as the positive control. Data are shown as the means ± SD of three
497 independent experiments. *p<0.05, ** p<0.01, and *** p<0.001 compared to 0 h protein
498 contents. Different letters indicate significant difference between groups (p<0.05).

499

500 **Fig. 2. Effect of WPC (A) and LP-WPC (B) culture supernatant on cell viability of C2C12**
501 **myotubes.** Data are shown as the means ± SD of three independent experiments. *p<0.05, **
502 p<0.01, and *** p<0.001 compared to control (open bar).

503

504 **Fig. 3. Effect of LP-WPC medium on myogenic differentiation in C2C12 cells.** (A)
505 Representative images of western blotting. Relative protein expression of (B) Myf-5, (C)
506 MyoD, and (D) myogenin in LP-WPC treated C2C12 cells. Data are shown as the means ± SDs
507 of thee independent experiments. *p<0.05, and ** p<0.01, compared to control group.

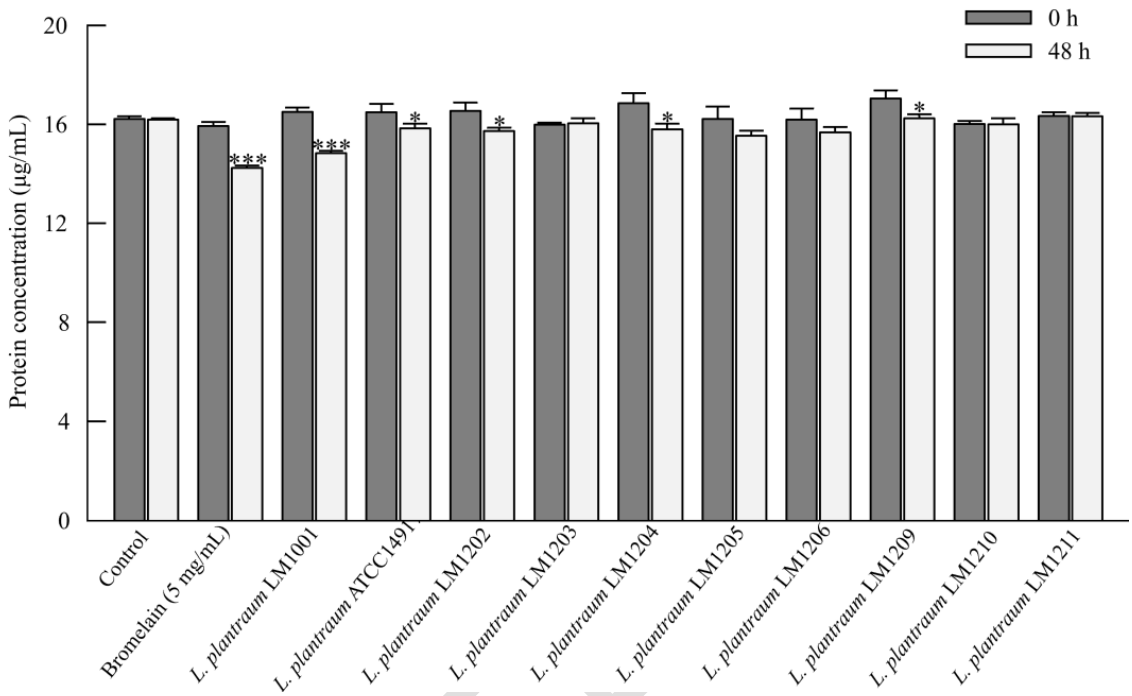
508

509

510

Fig. 1

(A)


0 h
48 h
Protein concentration (μg/mL)
20
16
12
8
4
0
***
***
*
*
*
*
Control
Bromelain (5 mg/mL)
L. plantraum LM1001
L. plantraum ATCC1491
L. plantraum LM1202
L. plantraum LM1203
L. plantraum LM1204
L. plantraum LM1205
L. plantraum LM1206
L. plantraum LM1209
L. plantraum LM1210
L. plantraum LM1211

(B)

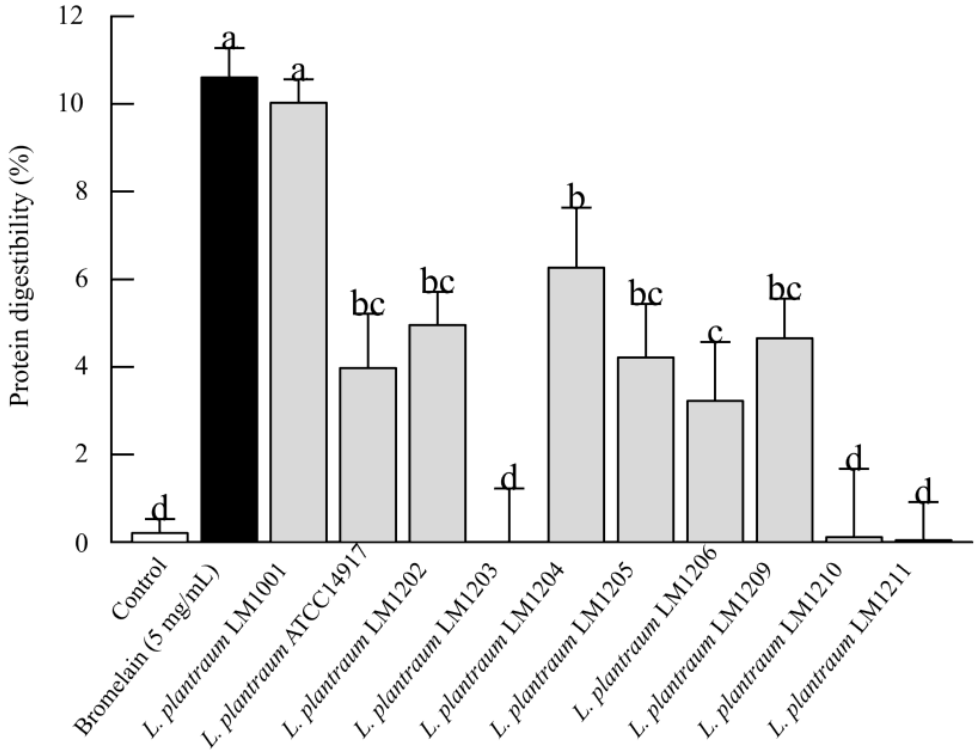
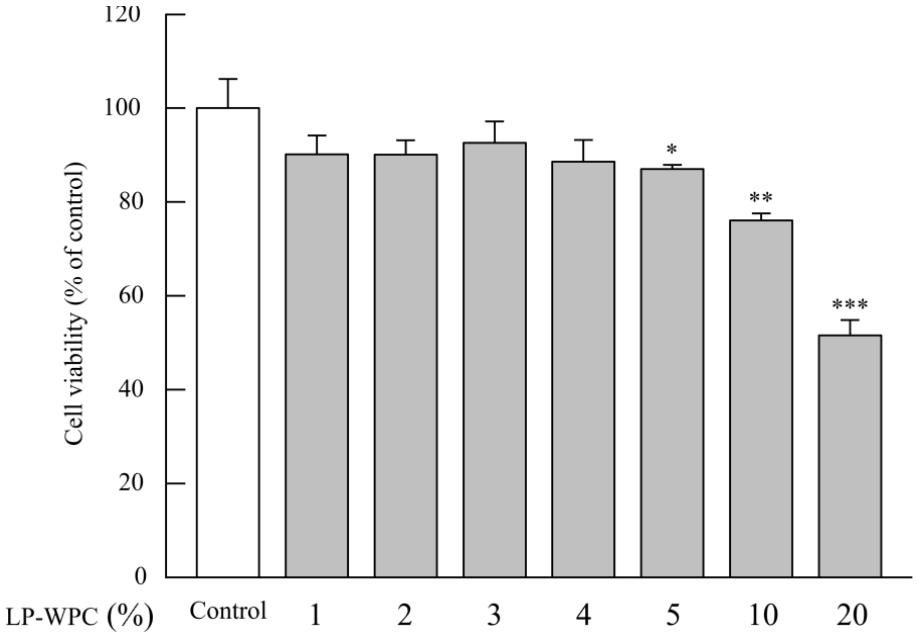

Protein digestibility (%)
12
10
8
6
4
2
0
a
a
bc
bc
d
b
bc
c
bc
d
d
Control
Bromelain (5 mg/mL)
L. plantraum LM1001
L. plantraum ATCC14917
L. plantraum LM1202
L. plantraum LM1203
L. plantraum LM1204
L. plantraum LM1205
L. plantraum LM1206
L. plantraum LM1209
L. plantraum LM1210
L. plantraum LM1211

Fig. 2

(A)


120
100
80
60
40
20
0
Cell viability (% of control)
*
**
***
LP-WPC (%)
Control
1
2
3
4
5
10
20

(B)

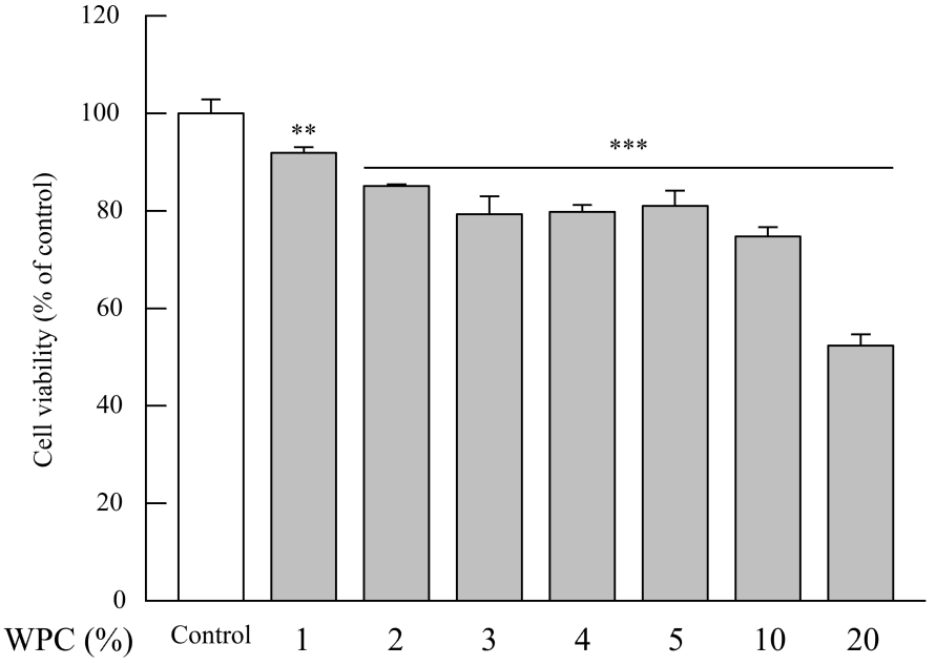
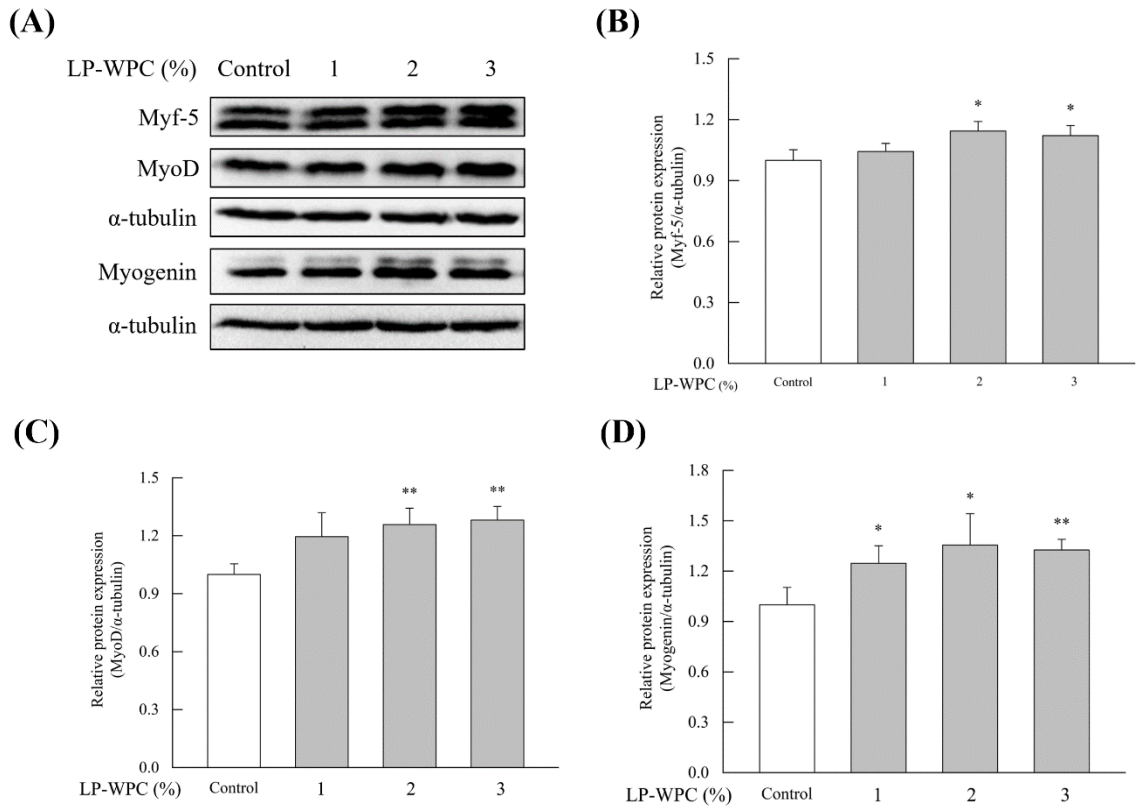

120
100
80
60
40
20
0
Cell viability (% of control)
**
***
WPC (%)
Control
1
2
3
4
5
10
20

Fig. 3


(A)
LP-WPC (%) Control 1 2 3
Myf-5
MyoD
α-tubulin
Myogenin
α-tubulin
(B)
Relative protein expression (Myf-5/α-tubulin)
LP-WPC (%) Control 1 2 3
(C)
Relative protein expression (MyoD/α-tubulin)
LP-WPC (%) Control 1 2 3
(D)
Relative protein expression (Myogenin/α-tubulin)
LP-WPC (%) Control 1 2 3

Supplement 1

Effect of WPC medium on myogenic differentiation in C2C12 cells. (A) Representative images of Western blotting. Relative protein expression of (B) Myf-5, (C) MyoD, and (D) myogenin in WPC treated C2C12 cells. Data are shown as the means ± SDs of thee independent experiments.