Anti-adipogenic effect of *Lactobacillus fermentum* MG4231 and MG4244 through AMPK pathway in 3T3-L1 preadipocytes

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Abstract This study evaluated the anti-adipogenic effects and mechanisms underlying the action of *Lactobacillus fermentum* MG4231 and MG4244 strains on adipogenesis and lipid accumulation in 3T3-L1 preadipocytes. Treatment with cell-free extracts (CFEs) from the two strains reduced lipid accumulation and intracellular triglyceride production in 3T3-L1 adipocytes by more than 50%. The inhibitory effects of *L. fermentum* on lipid accumulation were mediated by the downregulation of FAS and aP2 resulting from the inhibition of PPAR γ and C/EBP α gene expression. Moreover, AMPK and HSL phosphorylation was upregulated by CFE treatment. These results indicated that the anti-adipogenic and lipolysis activities of *L. fermentum* strains were caused by increased AMPK and HSL

phosphorylation. Both strains displayed high leucine arylamidase and β -galactosidase enzymatic activity, with excellent adhesion to epithelial cells. Therefore, we identified *L. fermentum* as potential new probiotics for the prevention of obesity.

Keywords *L. fermentum* · MG4231 · MG4244 · Adipogenesis · Probiotics

Abbreviations

CFE	Cell-free extract
DEX	Dexamethasone
IBMX	Isobutylmethylxanthine
DMEM	Dulbecco modified Eagle's medium
FBS	Fetal bovine serum
TG	Triglyceride
MTT	Tetrazolium bromide salt
PPAR γ	Peroxisome proliferator-activated receptor γ
C/EBP α	CCAAT/enhancer-binding protein α
FAS	Fatty acid synthase
aP2	Adipose-specific fatty acid-binding protein
SREBP1	Sterol regulatory element-binding protein 1
LPL	Lipoprotein lipase
ACC	Acetyl CoA carboxylase
AMPK	AMP-activated protein kinase
p-AMPK	Phospho-AMP-activated protein kinase
HSL	Hormone-sensitive lipase
p-HSL	Phospho-hormone-sensitive lipase

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Introduction

Obesity is caused by excessive energy accumulation and metabolic imbalance. Public awareness regarding the problem of obesity is rising, but the number of overweight

or obese patients continues to increase worldwide (Blüher, 2019). Obesity is associated with several factors, including eating habits, lack of activity, stress, and endocrine system changes. Obesity is considered a key risk factor for serious metabolic diseases, such as type 2 diabetes, dyslipidemia, cardiovascular disease, and cancer (Schwartz et al., 2017). Therefore, regulating lipid metabolism and preventing obesity can help lower the risk of metabolic diseases. Body fat associated with obesity can be regulated by adipogenesis, lipolysis, lipogenesis, and fatty acid oxidation (Włodarczyk and Nowicka, 2019).

Adipocyte differentiation (adipogenesis) is a complex process that produces mature adipocytes involving various signaling molecules, transcription factors and specific genes (Gregoire et al., 1998). During adipogenesis, adipocytes undergo complex changes in cell morphology, gene expression, and triglyceride (TG) accumulation. Differentiated cells continue to multiply, accumulate lipid droplets, and grow into mature adipocytes (Ghaben and Scherer, 2019). The CCAAT/enhancer-binding protein α (C/EBP α) and peroxisome proliferator-activated receptor γ (PPAR γ) genes are activated in the early stages of differentiation, by specific adipocyte-transcription factors (Mandard et al., 2004; Rosen et al., 2009). PPAR γ and C/EBP α synergistically activate adipocyte-specific genes such as adipose-specific fatty acid-binding protein (aP2), fatty acid synthase (FAS), lipoprotein lipase (LPL), and leptin (Brun et al., 1996). Therefore, an understanding of the mechanisms that regulate adipocyte differentiation may be useful in the treatment of obesity and various complications associated with obesity.

Probiotics are living microorganisms that improve the microbial environment in the human gastrointestinal tract and have beneficial effects on the health of the host. Numerous microorganisms are classified as probiotics, most of which belong to the *Lactobacillus* and *Bifidobacterium* genera (Nagpal et al., 2012). Probiotics are mainly used to treat gastrointestinal disorders such as diarrhea, irritable bowel syndrome, constipation, and lactose intolerance. Recent studies have focused on identifying the beneficial functions of probiotics, such as their anti-diabetic, immune enhancing, and anti-cancer effects (Kerry et al., 2018). Some probiotics have potential anti-obesity effects related to the regulation of lipid and glucose metabolism (Castro-Gonzalez et al., 2019; Miyoshi et al., 2014; Wu et al., 2015). The anti-obesity effects of probiotics have been attributed to changes in gut bacteria, as well as increased absorption of intestinal energy, permeability, and energy metabolism (Kobyliak et al., 2016). Therefore, the intestinal microbiota plays an important role in host metabolism, and its composition and regulation may be promising targets for preventing obesity.

In our previous studies (Kang et al., 2018), the MG4231 and MG4244 strains were isolated by evaluating lipase activity and adipogenesis in 3T3-L1 cells (a total of 221 strains were isolated from human origin). These two strains corresponded to gram-positive and rod-type homo bacteria, and the results of a sugar fermentation test and 16S rRNA analysis identified them as strains of *L. fermentum*. These strains were highly resistant to artificial gastric juice and bile salts, and their auto-aggregation rates were higher than 60%. All of these strains have been proven to be safe as raw materials for use in food, and they have been confirmed as probiotics. In a preliminary study, we identified the beneficial effects of these strains in mice fed high-fat diets. Based on these findings, the present study investigated the mechanism responsible for the anti-adipogenic activities of cell-free extract (CFE) from the *L. fermentum* MG4231 or MG4244 strains using 3T3-L1 preadipocytes. In addition, by evaluating their intracellular adhesion ability and enzyme activity, we identified these strains as probiotics with potential anti-obesity effects.

Materials and methods

Materials

3T3-L1 cells (KCLB 10092.1) and HT-29 cells (KCLB 21739) were obtained from the Korea Cell Line Bank (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640, fetal bovine serum (FBS), and penicillin–streptomycin mixture were purchased from Gibco Inc. (Grand Island, NY, USA). The reverse transcription kit and SYBR green master mix were obtained from Applied Biosystems (Foster City, CA, USA). Other reagents such as phosphate buffered saline (PBS), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), tetrazolium bromide salt (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers used for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) were purchased from Bioneer (Seoul, Korea). Antibodies, against PPAR γ , C/EBP α , FAS, aP2, AMP-activated protein kinase (AMPK), phospho-AMP-activated protein kinase (p-AMPK), hormone-sensitive lipase (HSL), phospho-hormone-sensitive lipase (p-HSL), and β -actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Preparation of CFE

Lactobacillus fermentum strains (MG4231 and MG4244) were obtained from MEDIOTEN Co., Ltd. (Jecheon, Korea). Each strain was activated by culturing in MRS

broth for 18 h at 37 °C. The cultures were harvested in a refrigerated centrifuge (15,000× *g* for 15 min at 4 °C) and washed three times with distilled water to completely remove the remaining broth. To prepare CFE, each strain was lyophilized and resuspended in PBS at a concentration of 3 mg/mL. The strains were then grinded three times for 60 s using an ultrasonic grinder. The pulverized strains were filtered through a 0.2 µm syringe filter, and the filtrates were maintained at − 80 °C until use (Saadatzaheh et al., 2013).

Cell cytotoxicity assay

The cell cytotoxicity of CFEs from each strain in 3T3-L1 preadipocytes was determined by measuring cell viability using an MTT assay. Briefly, 3T3-L1 preadipocytes were seeded in a 96-well plate at 1×10^5 cells/mL and incubated for 24 h. Each well was then treated with 10, 25, 50, 75 or 100 µg/mL CFE for 24 h. Cells were then treated with 20 µL of MTT solution (5 mg/mL) and then incubated at 37 °C in a 5% CO₂ atmosphere for 4 h. The supernatant was then removed and 100 µL of DMSO was added to each well to dissolve the formazan crystals. Absorbance at 540 nm was measured using a microplate reader.

Adipocyte differentiation and treatment with CFE

3T3-L1 cells were seeded in 6-well plates at a density of 1×10^5 cells/mL. Post-confluent 2 day (D0), the medium was replaced with differentiation cocktail medium (MDI; 0.5 mM IBMX, 1 µM DEX, and 10 µg/mL insulin) in DMEM supplemented with 10% FBS and the cells were incubated for 2 days. On days two (D2) and four (D4), the medium was replaced with DMEM containing 10 µg/mL insulin and the cells were incubated for 2 days. On day six (D6), the medium was replaced with DMEM and cells were incubated for two days. Differentiation was completed on day eight (D8). The CFE from each strain was treated with 10, 25, and 50 µg/mL along with the medium.

Oil Red O staining

Intracellular lipid accumulation in differentiated 3T3-L1 cells was measured by Oil Red O staining. Briefly, differentiated 3T3-L1 cells were washed twice and fixed with a 3.7% formaldehyde solution for 15 min. Subsequently, the formaldehyde was removed, the cells were washed three times with PBS, and then stained with filtered Oil Red O solution for 30 min at room temperature. Oil Red O solution was added to each well, and each well was then washed three times with PBS. Cells were observed under an optical microscope. Oil Red O quantification was

performed by extracting the dye with 100% isopropanol and measuring the absorbance at 540 nm using a microplate reader.

TG contents

The TG content of differentiated 3T3-L1 cells was measured using a commercial TG assay kit (Asan-Pharmaceutical, Seoul, Korea). Protein concentrations were measured using the BCA method.

qRT-PCR analysis

The mRNA expression level of transcription factors and target genes related to adipogenesis in 3T3-L1 cells was quantified by qRT-PCR. Total RNA from differentiated 3T3-L1 cells was extracted using Trizol reagent. The extracted RNA was reverse-transcribed into cDNA using a reverse-transcription kit. qRT-PCR analysis was performed using the SYBR Green PCR master mix. The sequences of the qRT-PCR primers used in this study are provided in Table 1. The qRT-PCR amplification conditions were as follows: 40 cycles of 30 s at 95 °C, 5 s at 95 °C, and 30 s at 60 °C. Relative quantification of gene expression was calculated relative to β-actin.

Western blot analysis

The expression of obesity-related proteins in 3T3-L1 cells was determined by western blotting. Briefly, differentiated 3T3-L1 cells were scraped with lysis buffer containing 25 mM Tris-HCl buffer (pH 7.6). Proteins in the lysates were quantified using the BCA method. The protein (20 µg) was mixed with 10% SDS loading buffer, separated by electrophoresis on 10% SDS polyacrylamide gel and transferred to a PVDF membrane, which was blocked for 90 min at room temperature with 5% skim milk in TBST buffer. After incubating the membrane for 20 h at 4 °C, the membrane was incubated with primary antibodies against PPARγ, C/EBPα, FAS, aP2, p-AMPK, AMPK, p-HSL, HSL, or β-actin. After washing three times with TBST buffer, the membrane was further incubated with goat anti-rabbit IgG-HRP-conjugated secondary antibody for 90 min at room temperature and washed three times with TBST buffer. Proteins were visualized using an ECL detection kit. The expression level of target proteins was determined by comparison to β-actin protein levels.

Enzyme activity

Enzyme activity of the *L. fermentum* strains was measured using the API ZYM kit. According to the manufacturer's instructions, the strains were cultured in MRS broth for

Table 1 Sequences of oligonucleotides used in qRT-PCR

Gene	Accession ID	Primer	Sequence (5' to 3')	Annealing temperature (°C)
PPAR γ	NM_01127330.2	F	CAGGTCAGAGTCGCCCCG	60
		R	TCAGCAACCATTGGGTCAGC	
C/EBP α	NM_001287514.1	F	CCCTTGCTTTTGCACCTCC	59
		R	TGCCCCATTCTCCATGAAC	
FAS	NM_007988.3	F	CAAGTGTCCACCAACAAGCG	59
		R	GGAGCGCAGGATAGACTCAC	
aP2	NM_024406.3	F	GGATTTGGTCACCATCCGGT	59
		R	CCAGCTTGTACCATCTCGT	
β -actin	NM_007393.5	F	AGAGGATGTGGATGGAGGCT	59
		R	TCCACTATGATCTCCAGCGA	

PPAR γ peroxisome proliferator-activated receptor- γ , C/EBP α CCAAT/enhancer binding protein α , FAS fatty acid synthase, aP2 adipocyte protein 2

24 h, inoculated into API strips, and then incubated at 30 °C for 4 h to confirm the degree of staining. The activity of 19 enzymes was evaluated on a 5-level scale according to the color intensity.

Adherence activity

The intestinal adhesion ability of the two *L. fermentum* strains were evaluated using HT-29 cells (a human colorectal adenocarcinoma cell line). Briefly, HT-29 cells (1×10^5 cells/mL) were aliquoted into 6-well plates and incubated in RPMI medium without FBS and antibiotics at 37 °C in a 5% CO₂ atmosphere for 24 h. Each strain (1×10^8 CFU/mL) was inoculated into HT-29 cells and incubated at 37 °C. Non-adherent cells were removed, and the adherent cells were detached with trypsin–EDTA solution, washed with PBS (pH 7.0), and then cultured on MRS agar plates. The following equation calculated the rates of adhesion of *L. fermentum* strains: adhesion rate (%) = (number of viable cells/initial number of cells) $\times$ 100.

Statistical analysis

Results are presented as mean $\pm$ standard deviation for experiments performed in triplicate. Statistical analysis was performed using SPSS version 22 (IBM Corp., Endicott, NY, USA). Statistical significance was assessed using one-way analysis of variance (ANOVA) with Duncan's multiple range tests. *p* values < 0.05 were considered statistically significant.

Results and discussion

Cytotoxicity of *L. fermentum* MG4231 and MG4244 strains

Probiotic bacteria secrete few substances into the culture supernatant and express others on the cell surface (Ricci et al., 2018; Smid and Kleerebezem, 2014). CEF is suitable for investigating the function of living cells using metabolites, enzymes and cell surface extracts owing to its stability in some biological reactions, such as the action of antibiotics and inflammation (Zhang et al., 2005). Therefore, CFE was used in this experiment as an alternative to direct administration of probiotics. The fibroblast cell line of 3T3-L1 was used as an in vitro model for evaluating the feasibility of a probiotics having an inhibitory effect on adipocyte differentiation. Intracellular CFEs from the MG4231 or MG4244 strain of *L. fermentum* (10–100 μ g/mL) were used to treat 3T3-L1 preadipocytes, and significant cytotoxicity was observed when treating with concentrations > 75 μ g/mL (Fig. 1A). Thus, in subsequent experiments, CFE treatment were performed at 50 μ g/mL or less.

Effects of *L. fermentum* MG4231 and MG4244 strains on adipocyte differentiation

Adipogenesis is a highly regulated and complex differentiation process induced by growth factor, cytokine, and hormones signaling pathways (Mota et al., 2017). *Lactobacillus* is known to inhibit adipocyte differentiation in in vivo and in vitro models (Jang et al., 2019; Zhang et al., 2016). In this study, the effects of *L. fermentum* MG4231 and MG4244 strains on lipid accumulation in differentiated 3T3-L1 cells was assessed by Oil Red O staining on differentiation D8. 3T3-L1 adipocytes stained with Oil Red O solution were observed under an optical

Fig. 1 Effects of CFEs from *L. fermentum* MG4231 or MG4244 strains on lipid accumulation in 3T3-L1 adipocytes. **A** Cell viability, **B** Oil Red O staining, **C** Lipid accumulation and **D** TG content. Lipid accumulation and TG content in 3T3-L1 adipocytes was evaluated by Oil Red O staining and a TG assay kit. Results are presented as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences between means at $p < 0.05$ as assessed by Duncan's multiple range test

microscope (Fig. 1B). As a result, more lipid droplets were induced in the cells treated with MDI only, and lipid accumulation was also significantly increased. However, each CFE treatment with MG4231 or MG4244 inhibited lipid accumulation in a dose-dependent manner (20.2 or 46.6% at 25 μ g/mL and 60.0 or 52.3% at 50 μ g/mL, respectively; Fig. 1C).

The severity of obesity is related to the differentiation, expansion, and TG accumulation of adipocytes in adipose tissue (Rizzatti et al., 2013). Adipocytes evolved to accumulate TG for intracellular energy storage, and fully mature adipose tissue mainly consists of mature adipocytes filled with TG droplets (Iwamoto et al., 2018). In this study, the effects of CFE from the MG4231 or MG4244 strains on intracellular TG production in 3T3-L1 adipocytes were evaluated using the TG assay kit on differentiation D8 (Fig. 1D). The TG content in untreated or MDI-treated cells was 53.5 and 134.8 mg/dL, respectively. However, treatment with each CFE (50 μ g/mL) of MG4231 or MG4244 caused a significant reduction in TG accumulation to 85.2 or 65.5 mg/dL, corresponding to a reduction of 36% and 51.4% compared to the MDI-treated cells, respectively. The degree of adipocyte differentiation inhibition was dependent on the concentration of CFE treatment and was enhanced by MG4244 compared to MG4231. Kim et al. (2018) reported that *L. plantarum* K10 treatment (100 μ g/mL) inhibited adipocyte differentiation of 3T3-L1 cells by 32%. Exopolysaccharide (EPS) is a component of the lactic acid bacteria (LAB) cell envelope or is a primary or secondary metabolite that accumulates in a viscous form outside the cell wall during fermentation. Recently, EPS has attracted attention as a physiologically functional substance involved in immune regulation, hypocholesterolemia, antioxidant activity, anti-tumor activity and anti-atherosclerotic effect (Kleerebezem et al., 2010; Patten and Laws, 2015). Park et al. (2011) have reported the anti-adipogenesis effect of *L. plantarum* KY1032 cell extract in 3T3-L1 cells. Zhang et al. (2016) reported that an intraperitoneal injection of EPS extracted from *L. rhamnosus* GG reduced lipid metabolism imbalance. Although further studies are needed to determine the active components of cell extracts of these strains, these results suggest that the *L. fermentum* MG4231 and

MG4244 strains effectively reduced lipid and intracellular TG accumulation in differentiated 3T3-L1 cells.

Effects of *L. fermentum* MG4231 and MG4244 strains on mRNA expression of adipogenic genes

Adipose tissue mass can be reduced by inhibiting the generation of lipids, which are regulated by various adipogenic transcription factors (Prusty et al., 2002). Therefore, these adipogenic factors are recognized as essential factors for reducing obesity (Lazar et al., 2018). In this study, the expression of specific adipogenic genes in 3T3-L1 cells was measured by qRT-PCR. In MDI-treated cells, the levels of PPAR γ and C/EBP α were increased by 2.5- and 1.8-fold, respectively, compared to untreated cells (Fig. 2A, B). However, treatment with each CFE of MG4231 or MG4244 reduced the expression of these genes in a dose-dependent manner. Upregulation of these genes increases lipid droplets in the adipocyte cytoplasm and results in complete differentiation (Rapold et al., 2013). As shown in Fig. 2C, D, FAS levels were also significantly increased in MDI-treated cells compared to untreated cells. However, in cells treated with CFE > 25 μ g/mL, FAS expression was significantly reduced. In addition, aP2 expression was reduced by treatment with CFE > 25 μ g/mL for MG4231 or 50 μ g/mL for MG4244. A previous study has shown that *L. gasseri* SBT2055 reduces the expression of fat-producing genes such as FAS, ACC1, and SREBP1 (Ogawa et al., 2015). Therefore, these results confirmed that the inhibitory effect of *L. fermentum* MG4231 and MG4244 strains on lipid accumulation was caused by downregulation of FAS and aP2 as a result of PPAR γ and C/EBP α inhibition.

Effects of *L. fermentum* MG4231 and MG4244 strains on adipogenic protein expression

The effect of the MG4231 or MG4244 strains on the expression of adipogenic target proteins was investigated by western blot analyses. PPAR γ , C/EBP α , FAS, and aP2 levels were significantly increased in MDI-treated cells (Fig. 3A–D), which mirrored the expression pattern of their mRNAs. However, in cells treated with CFE, the expression of these proteins was significantly suppressed in a dose-dependent manner. Overall, the effects of the two strains on the expression of adipogenic proteins were almost identical, except for in relation to FAS.

AMPK functions in energy recognition and homeostasis and plays an important role in glucose and lipid metabolism. Activation of AMPK may inhibit adipogenesis and can be activated by leptin and adiponectin (Lee et al., 2019). Thus, mechanisms modulating AMPKs may be promising targets for treating obesity. HSL is considered a

Fig. 2 Effects of CFEs from *L. fermentum* MG4231 or MG4244 strains on mRNA expression in 3T3-L1 adipocytes. Relative expression levels of **A** PPAR γ , **B** C/EBP α , **C** FAS and **D** aP2 gene. The mRNA as assessed by qRT-PCR was quantified relative to the

expression of β -actin as an internal control. Results are presented as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences between means at $p < 0.05$ as assessed by Duncan's multiple range test

key enzyme that catalyzes lipolysis in white adipose tissue. Lipid droplets in mature adipocytes are regulated by TG influx through the function of LPL and TG outflow, which is regulated by adipose triglyceride lipase and HSL (Carmen and Víctor, 2006). HSL phosphorylation induces lipolysis in adipocytes (Djouder et al., 2010). In addition, in this study, the expression of these signaling proteins involved in fat production was assessed (Fig. 3F, G). In MDI-treated cells, p-AMPK and p-HSL protein expression was significantly reduced compared to their levels in untreated cells. On the other hand, in cells treated with CFE of MG4231 $> 25 \mu\text{g/mL}$ or MG4244 $> 10 \mu\text{g/mL}$, the expression of p-AMPK protein was restored and was significantly increased above the expression level in untreated

cell. In addition, treatment with CFE ($50 \mu\text{g/mL}$) from each strain restored p-HSL protein expression in MDI-treated cells. In a previous study, *L. acidophilus* NS1 improved lipid metabolism and insulin sensitivity through the AMPK signaling pathway (Park et al., 2018). The *Clostridium butyricum* MIYAIRI 588 has been reported to increase AMPK activity and relieve non-alcoholic fatty liver disease (Endo et al., 2013). Therefore, our results show that the *L. fermentum* MG4231 and MG4244 strains are involved in lipolysis by increasing HSL phosphorylation and exhibit anti-obesity effects mediated by the AMPK pathway in adipocyte differentiation and fat accumulation.

Fig. 3 Effects of CFEs from *L. fermentum* MG4231 or MG4244 strains on protein expression in 3T3-L1 adipocytes. **A** Representative western blots and quantification of expression levels of **B** PPAR γ , **C** C/EBP α , **D** FAS, **E** aP2, **F** p-AMPK/AMPK and **G** p-HSL/HSL

proteins. Protein expression was quantified relative to the expression of β -actin as an internal control. Results are presented as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences between means at $p < 0.05$ as assessed by Duncan's multiple range test

Enzyme activities of *L. fermentum* MG4231 and MG4244 strains

Generally, microorganisms produce enzymes and inhibit enzyme activity in various unique ways (Seo et al., 2018). Evaluating the activity of enzymes produced by LAB is essential to reveal their potential use as probiotics (Colombo et al., 2018; Plaza-Diaz et al., 2019). In this study, the enzymatic activity of the MG4231 or MG4244 strains was measured using the API ZYM kit. As shown in Table 2, the two strains showed that leucine arylamidase and β -galactosidase had the highest enzymatic activity (> 40 nM), followed by α -galactosidase (> 30 nM). β -Galactosidase (lactase) activity is essential for improving lactose intolerance in the intestine by hydrolyzing lactose into monosaccharides, glucose, and galactose. In contrast,

probiotics should not produce β -glucuronidase, which is recognized as a toxic enzyme involved in the development of colorectal cancer (Husain, 2010). β -glucuronidase hydrolyzes glucuronide into carcinogens such as glucuronic acid and aglycone (Bäumler and Sperandio, 2016). Glucuronide is removed from the liver as bile, but β -glucuronidase can release these toxic substances. Therefore, it is suggested that these strains are relatively safe *Lactobacilli*.

Adhesion abilities of *L. fermentum* MG4231 and MG4244 strains to HT-29 cells

Sufficient host interactions are required for probiotics to provide health benefits to the host gut (Gioacchini et al., 2017). The adhesion of probiotics to mucosal and epithelial

Table 2 Enzymatic activity of *L. fermentum* MG4231 and MG4244 strains

No.	Enzymes	<i>L. fermentum</i>	
		MG4231	MG4244
1	Control (blank) ¹⁾	0 ²⁾	0
2	Alkaline phosphatase	0	0
3	Esterase (C4)	3	3 ⁵⁾
4	Esterase lipase (C8)	3	3
5	Lipase (C14)	1 ³⁾	0
6	Leucine arylamidase	5	5 ⁷⁾
7	Valine arylamidase	3	3
8	Crystine arylamidase	1	0
9	Trypsin	0	0
10	α -chymotrypsin	0	0
11	Acid phosphatase	2 ⁴⁾	2
12	Naphtol-AS-BI-phosphohydrolase	4 ⁶⁾	3
13	α -galactosidase	4	4
14	β -galactosidase	5	5
15	β -glucuronidase	0	0
16	α -glucosidase	0	3
17	β -glucosidase	0	0
18	N-acetyl- β -glucosaminidase	0	0
19	α -mannosidase	0	0
20	α -fucosidase	0	0

Enzyme activities were recorded and assigned values from 0 (no activity) to 5 (maximum activity) at 10 nM intervals in the API-ZYM color reaction chart. ¹⁾Control: no substrate (blank), ²⁾0: no activity, ³⁾1: 5 nM, ⁴⁾2: 10 nM, ⁵⁾3: 20 nM, ⁶⁾4: 30 nM, ⁷⁾5: $\geq$ 40 nM

cells is essential for the production of enzymes, lactic acid, vitamins, and antimicrobial compounds (Zommiti et al., 2017). Therefore, the candidate probiotics must have the ability to adhere to intestinal epithelial cells. In this study, the in vitro adhesion of *L. fermentum* strains to HT-29 cells was investigated. *L. rhamnosus* GG, a well-established strain with high adhesion in vivo, was used as a positive control strain (Polak-Berecka et al., 2014). As shown in Table 3, the MG4231 and MG4244 strains exhibited sufficient binding to HT-29 cells at rates of $3.35 \pm 0.33\%$ and $4.00 \pm 1.40\%$, respectively, rates that are similar to the *L. rhamnosus* GG ($4.10 \pm 0.98\%$). However, the mean between the experimental groups was not statistically different. Therefore, the level of adhesion of the *L. fermentum* MG4231 and MG4244 strains to epithelial cells demonstrate their potential to be used as probiotics.

In conclusion, this study showed that the *L. fermentum* MG4231 and MG4244 strains significantly inhibited lipid accumulation and TG production in differentiated 3T3-L1 cells. The anti-obesity activity of these strains due to the repression of adipogenic-specific genes (PPAR γ , C/EBP α , FAS, and aP2) by upregulating of AMPK phosphorylation. In addition, these strains were found to strongly adhere to intestinal epithelial cells. Therefore, our study suggests that the *L. fermentum* MG4231 and MG4244 strains may be valuable probiotics candidates for the prevention of obesity.

Table 3 Adhesion ability of *L. fermentum* MG4231 and MG4244 strains

Subjects	<i>Lactobacillus</i> strains		
	<i>L. rhamnosus</i> GG	<i>L. fermentum</i>	
		MG4231	MG4244
Adhesion ability (%)	4.10 ± 0.98	3.35 ± 0.33	4.00 ± 1.40

Results are presented as mean $\pm$ SD for three independent experiments. Statistical significance between means was assessed using ANOVA with Duncan's multiple range test ($p < 0.05$)

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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