



# Selection and Characterization of Probiotic Bacteria Exhibiting Antiadipogenic Potential in 3T3-L1 Preadipocytes

Chul Sang Lee<sup>1,2</sup> · Mi Hyun Park<sup>3</sup> · Sae Hun Kim<sup>1</sup>

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## Abstract

Abnormal adipocyte growth, distinguished by an increase in cell numbers and cellular differentiation, is regarded as a major pathological characteristic of obesity. Thus, inhibition of adipogenic differentiation in adipocytes could prevent obesity. Recently, certain probiotic strains have been reported to regulate lipid metabolism *in vitro* and/or *in vivo*. In this backdrop, this study aimed to investigate basic probiotic properties and potential antiobesity characteristics of mouse 3T3-L1 preadipocytes. Six lactic acid bacteria (LAB) strains were prescreened for their cholesterol-lowering activity, antioxidant activity, and survival at low pH and in a solution containing bile salts. These six strains were investigated for antiadipogenic activity by employing 3T3-L1 mouse preadipocytes. 3T3-L1 cells were treated with selected strains during the differentiation process. *Lactobacillus johnsonii* 3121 and *Lactobacillus rhamnosus* 86 were found to be more capable of reducing triglyceride and lipid accumulation, as compared to control group, which are fully differentiated 3T3-L1 adipocytes. These strains also inhibited adipocyte differentiation by downregulating the adipogenic transcription factor in 3T3-L1 adipocytes. Taken together, these results indicate that *L. johnsonii* 3121 and *L. rhamnosus* 86 could potentially act as probiotic bacteria and prevent fat accumulation by regulating adipogenesis-related markers.

**Keywords** Obesity · Adipogenesis · 3T3-L1 preadipocytes · Probiotics · Lactic acid bacteria

## Introduction

Obesity, characterized by excessive fat storage in tissues, presents excessive risks to an individual's health, and is, therefore, considered a major risk factor for morbidity and mortality worldwide. Moreover, obesity is associated with diseases such as type 2 diabetes, hypercholesterolemia, hypertension, and hepatic steatosis, termed as the metabolic syndrome (MS), which is a crucial problem that needs to be resolved for improved quality of life [1]. Obesity occurs from a combination of less active lifestyle and a failure to reduce dietary energy intake in line with reduced total energy expenditure arising from reduced physical activity

[2]. Recently, lockdown due to the coronavirus disease 2019 (COVID-19) pandemic have caused disruptions in daily lifestyles, thereby increasing the global prevalence of obesity [3].

Lactic acid bacteria (LAB) are lactic acid-producing gram-positive bacteria that have been exploited in association with various health benefits and are widespread in nature. The most common health-promoting LABs are classified as probiotics. Probiotics are live microorganisms that provide beneficial health effects to the host when administered in adequate amounts [4]. Numerous beneficial effects of probiotics, such as anticonstipation, antiosteoporotic, and antiobesity effects, are frequently suggested by many research studies [5–7]. The health benefits of probiotic bacteria are usually explained by the protective effects of the interaction of consumed probiotics with commensal gut microbiota in the host [8]. It is, therefore, essential for LABs to withstand low pH conditions in the stomach and the presence of secreted bile salts in the small intestine [9]. Moreover, all probiotics meant for consumption must undergo thorough safety checks, including the absence of harmful metabolite (such as urease and gelatinase) secretion [10].

✉ Sae Hun Kim  
saehkim@korea.ac.kr

<sup>1</sup> College of Life Sciences and Biotechnology, Korea University, Seoul 02841, Republic of Korea

<sup>2</sup> Institute of Life Science and Natural Resources, Korea University, Seoul 02841, Republic of Korea

<sup>3</sup> Maeil Central Research Laboratory, Pyeongtaek, Gyeonggi-do 17714, Republic of Korea

Gut microbiota (GM) is defined as the community of bacteria inhabiting the gastrointestinal (GI) tract that has a complicated and mutually beneficial relationship with the host. Recently, the control on host gut microbiota exerted by probiotics has emerged as an important factor affecting health in the host, including obesity [11]. Dysbiosis, a form of microbial imbalance induced by a high-fat diet, is one of the most important factors in the development of obesity [12]. Colonization of intestinal microbes from obese mice to germ-free mice resulted in increased body weight gain and fat accumulation in the latter, thereby providing robust evidence of the association between obesity and GM [13]. Taken together, employing probiotic bacteria to target and alter high-fat diet-induced disturbance of gut microbiota might be an effective approach to treat obesity.

The present work was conducted in order to screen novel potential probiotics by testing basic probiotic properties such as resistance to low pH and bile salts. We have previously prescreened 61 strains out of 151 probiotic candidates based on their survival rate in a low pH environment. Therefore, the objective of this study was to confirm the survivability of these prescreened strains and explore their adaptation to the bile salt environment. Moreover, their cholesterol-lowering and antioxidant activities were probed to identify strains capable of exerting antiadipogenic effects in 3T3-L1 adipocyte cells *in vitro*.

## Materials and Methods

### Preparation of Bacterial Strains

The bacterial strains used in this study were obtained from the Probiotics Research Laboratory, CKDBio Research Institute (Ansan, South Korea) and the Department of Food Bioscience and Technology, Korea University (Seoul, Korea). Strains were individually grown in Man, Rogosa and Sharpe (MRS) broth (BD Co., Franklin Lakes, NJ, USA) for 18 h at 37 °C. The strains were sub-cultured thrice prior to each experiment.

### Estimation of Acid and Bile Tolerance

Tolerance to acid and bile conditions was assessed as reported previously [14], with minor modifications. Activated bacterial strains were harvested by centrifugation (10,000×g, 5 min, 4 °C) and washed twice in phosphate-buffered saline (PBS) prior to inoculation in acidified and bile-supplemented MRS broth. To evaluate the ability of the strains to grow under acidic conditions, MRS broth was acidified to pH 2.5 and supplemented with 1000 U mL<sup>-1</sup> of pepsin (Sigma-Aldrich, St. Louis, MO, USA). The washed bacterial cells were inoculated into the acidified broth and

incubated for 3 h at 37 °C. Acid tolerance was evaluated by comparing the number of colonies at 0 h with that at 3 h after incubation. The ability of strains to grow in the presence of bile was evaluated using MRS broth containing 0.3% oxgall (Sigma-Aldrich, St. Louis, MO, USA). The broth was inoculated with the washed bacterial cells and incubated for 24 h at 37 °C. The viability of strains in the bile condition was assessed by comparing the number of colonies at 0 h with that at 24 h after incubation. A 100-μL aliquot of each sample was spread in triplicate onto MRS agar plates in order to count the number of viable bacterial colonies on the surface. Acid and bile tolerance rates were evaluated by calculating the survival rate after 3 and 24 h of incubation at 37 °C, respectively.

### Safety Evaluation

To evaluate bacterial safety, urease and gelatin liquefaction activities of the strains were assessed. A gelatin medium containing 12% gelatin (Sigma-Aldrich, St. Louis, MO, USA) was inoculated with activated strains at a concentration of 1% and incubated for 48 h at 37 °C. Gelatin liquefaction of strains was assessed by storing medium in a refrigerator for 24 h and checking whether gelatin was hydrolyzed or not. Urease activity of the strains was assessed in urea agar media containing 2% urea (Sigma-Aldrich, St. Louis, MO, USA) and 0.0012% phenol red (Sigma-Aldrich, St. Louis, MO, USA). Each strain was streaked in urea agar media and incubated for 48 h at 37 °C. Media was kept under observation to check whether the color changed from yellow to pink. Change in media color to pink indicates that the strain has the ability to hydrolyze urea.

### Estimation of Cholesterol-Lowering Activity

A colorimetric assay to assess the cholesterol-lowering activity of strains was conducted [15], with minor modifications. MRS-THIO broth was prepared by adding 0.2% sodium thioglycolate (Sigma Aldrich, St. Louis, MO, USA) and 0.3% oxgall (Sigma-Aldrich, St. Louis, MO, USA) to MRS broth. The broth was further supplemented with filter-sterilized cholesterol micelles (1 mg mL<sup>-1</sup> in 0.4 M sucrose solution) at a final concentration of 100 μg mL<sup>-1</sup>. The broth was inoculated with 1% of the selected strains and incubated for 20 h at 37 °C. Uninoculated broth was used as a negative control. After incubation, cells were removed by centrifugation (10,000×g, 10 min, 4 °C) and total cholesterol content of the supernatant was measured using a colorimetric method [16], with minor modifications. The supernatant (2 mL) was mixed with 3 mL of 97% ethanol (Sigma-Aldrich, St. Louis, MO, USA) and 2 mL of 50% potassium hydroxide (w/v), and then heated at 60 °C for 10 min. After cooling to room temperature, 5 mL of hexane was added and

the resultant mixture was vortexed. Next, 4 mL of the hexane layer was transferred into a tube and dried using nitrogen gas. *o*-Phthalaldehyde (Sigma-Aldrich, St. Louis, MO, USA) (0.55 mg dissolved in 1 mL of acetic acid) was added to the tube and incubated for 10 min. Further, 2 mL of sulfuric acid was added and mixed for 1 min. Absorbance was measured spectrophotometrically at 550 nm using a VersaMax™ Tunable Microplate Reader (Molecular Devices, San Jose, CA, USA). To determine the concentration of cholesterol, the recorded  $A_{550}$  values were compared with a standard curve. Results were expressed as micrograms of cholesterol per milliliter. The cholesterol-lowering activity was calculated using the following equation:

$$\text{Cholesterol-lowering activity (\%)} = \frac{\text{Control cholesterol}(\mu\text{g}) - \text{Sample cholesterol}(\mu\text{g})}{\text{Control cholesterol}(\mu\text{g})} \times 100$$

### Estimation of Antioxidant Activity

To investigate the antioxidant activity of selected strains, live cells of activated strains were assessed by the DPPH method [17], with minor modifications. An aliquot of bacterial live cell (800  $\mu\text{L}$ ) was added to 1 mL of a 0.2 mM methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA) reagent. The suspension was mixed thoroughly and allowed to stand for 30 min at 37 °C in dark. After incubation, the supernatant from each sample was collected by centrifugation (10,000 $\times$ g, 1 min, 4 °C). The absorbance of the supernatant was measured spectrophotometrically at 517 nm using a VersaMax™ tunable microplate reader (Molecular Device, San Jose, CA, USA). For comparison, PBS and 0.2 mM ascorbic acid (Sigma-Aldrich, USA) were used as negative and positive controls, respectively. The antioxidant activity was expressed as the percentage of DPPH radical scavenging activity using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{OD}_{517(\text{control})} - \text{OD}_{517(\text{sample})}}{\text{OD}_{517(\text{control})}} \times 100$$

### Cell Culture

For the cell experiments, *Leuconoc mesenteroides* 7 (99% identity: accession no. CP003101), *Lactobacillus rhamnosus* 86 (99% identity: accession no. NR\_113332.1), *Lactobacillus sakei* 105 (99% identity: accession no. CR936503), *Lactobacillus casei* 911 (99% identity: accession no. KP326371.1), *Lactobacillus johnsonii* 3121 (99% identity: accession no. NR\_117574.1), and *Lactobacillus plantarum* MA2 (99% identity: accession no. NR\_113338.1) were selected based on their probiotic potentials. The mouse pre-adipocyte cell line 3T3-L1 was obtained from the American

Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GE Healthcare, Chicago, IL, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Dublin, Ireland) and 1% (v/v) antibiotic solution (penicillin and streptomycin solution, 100 U mL<sup>-1</sup>) (GE Healthcare, Chicago, IL, USA). Cells were incubated at 37 °C and 5% CO<sub>2</sub> in a humidified atmosphere, and the culture medium was replaced with fresh DMEM every 2 days. Cells were transferred at 60% confluence using 1 mL of trypsin–EDTA (GE Healthcare, Chicago, IL, USA). Cell differentiation was performed in 6-well cell culture plates (Sigma-Aldrich, St. Louis, MO, USA). Cells were seeded at a density of 8  $\times$  10<sup>4</sup> cells/well in maintenance

medium (DMEM containing 10% FBS and 1% antibiotic solution) and grown to 100% confluence. At 100% confluence, the culture medium was replaced with fresh maintenance medium and incubated for 48 h to induce cell arrest. After 48 h, the maintenance medium was replaced with induction medium (DMEM + 10% (v/v) FBS + 1% antibiotic solution + 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO, USA) + 1  $\mu\text{M}$  dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) + 10  $\mu\text{g mL}^{-1}$  of insulin). After 2 days, induction medium was replaced with growth medium (DMEM + 10% (v/v) FBS + 1% antibiotic solution + 10  $\mu\text{g mL}^{-1}$  of insulin). After an additional 2 days of incubation, the growth medium was changed every 2 days. At 8 days post-induction of differentiation, cells were harvested and analyzed. For the in vitro experiments, bacterial cells were heat-killed by heating each grown strain in PBS for 10 min at 95 °C. Each of the heat-killed strains in PBS was added to the growth medium on days 0–8 at a concentration of 10<sup>8</sup> colony-forming unit (CFU) mL<sup>-1</sup>.

### Cell Viability (MTT Assay)

The cytotoxicity of the selected strains was assessed by MTT assay. The mouse pre-adipocyte 3T3-L1 cells were seeded into 96-well cell culture plates (Sigma-Aldrich, St. Louis, MO, USA) at a density of 1  $\times$  10<sup>4</sup> cells/well and incubated at 37 °C and 5% CO<sub>2</sub> condition. After 24 h, the medium was replaced with maintenance medium containing each of the heat-killed strains at a concentration of 10<sup>8</sup> CFU mL<sup>-1</sup> and incubated for 24 or 48 h. The medium was subsequently removed, and the remaining cells were used for the MTT assay. A 10- $\mu\text{L}$  aliquot of a 12 mM stock solution of

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) and phenol-red free DMEM (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubation for 3 h at 37 °C and 5% CO<sub>2</sub>, the supernatant was discarded, followed by the addition of 50 µL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to each well for dissolution of the formed formazan crystals in viable cells. The culture plate was then incubated for 30 min in dark. The absorbance of each well was measured at 575 nm using a VersaMax™ tunable microplate reader (Molecular Device, San Jose, CA, USA).

### Oil Red O Staining

After induction of differentiation, cells were washed twice with PBS, followed by the addition of 2 mL of paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO, USA) to each well. After incubation for 5 min at room temperature, PFA was replaced with fresh PFA to fix the cells and incubated for 1 h. Next, the cells were washed thrice with PBS. Finally, cells were stained with 0.3% oil red O (Sigma-Aldrich, St. Louis, MO, USA) in isopropanol (Sigma-Aldrich, USA) for 1 h, and then washed twice in PBS. Stained cells were examined under a light microscope, and images were recorded. To quantify lipids in cells, oil red O stained lipid droplets were dissolved with 100% isopropanol for 10 min with gentle shaking. The absorbance of the eluted solution was measured at 500 nm using 100% isopropanol as a blank with a VersaMax™ tunable microplate reader (Molecular Device, San Jose, CA, USA).

### Triglyceride Assay in 3T3-L1 Cells

Triglyceride (TG) accumulation in 3T3-L1 cells was measured using a Triglyceride Quantification Colorimetric/Fluorometric Kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions.

### RNA Isolation and qRT-PCR of mouse pre-adipocyte 3T3-L1 cells

Total RNA from the 3T3-L1 cells was isolated using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Final RNA concentration and quality were determined by ultraviolet absorbance using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Gene expression levels were quantified by quantitative real-time PCR (qRT-PCR) using SYBR® Green (Sigma-Aldrich, St. Louis, MO, USA) and a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The GAPDH gene was analyzed as a housekeeping gene, and each qPCR reaction was run in triplicate in a single 96-well reaction plate. Relative gene expression was determined from the mean value of the three replicates and calculated using the inbuilt Bio-Rad CFX Manager software (Bio-Rad, Hercules, CA, USA). Primer sequences used in the study are shown in Table 1 [18–21].

### Statistical Analysis

IBM SPSS Statistics software version 25.0 (IBM Corp, Armonk, NY, USA) was used to analyze the data. One-way analysis of variance was used to compare sample means. Multiple comparisons of means were performed using Tukey's post hoc test.  $P < 0.05$  was considered statistically significant.

## Results

### Evaluation of Basic Probiotic Properties in LABs

Probiotic functionality depends on the ability of the strain to confer health advantages on a host upon oral consumption of viable cells. Therefore, we studied the tolerance of bacterial strains to acidic conditions (artificial gastric juice) and the

**Table 1** Primer sequences used in qRT-PCR analysis

Gene	Sequence	Ta (°C)	Reference
PPAR $\gamma$	F: 5'-GGA AGA CCA CTC GCA TTC CTT-3'	61.0	[19]
	R: 5'-GTA ATC AGC AAC CAT TGG GTC A-3'		
C/EBP $\alpha$	F: 5'-GCG GGA ACG CAA CAA CAT C-3'	61.0	[18]
	R: 5'-GTC ACT GGT CAA CTC CAG CAC-3'		
aP-2	F: 5'-AAG GTG AAG AGC ATC ATA ACC CT-3'	58.5	[20]
	R: 5'-TCA CGC CTT TCA TAA CAC ATT CC-3'		
GAPDH	F: 5'-GTA TGA CTC CAC TCA CGG CAA A-3'	58.5	[21]
	R: 5'-GGT CTC GCT CCT GGA AGA TG-3'		



presence of bile salt (artificial intestinal stress). We set a cutoff value of 0.5 for the log CFU value at 3 h subtracted from that at 0 h, to indicate intolerance to low pH conditions. The same cutoff value was also considered for the bile salt condition. Previously, we have prescreened 61 strains out of 151 candidates that qualified for further investigation based on this cutoff value for resistance to acid and bile salts (data not shown). In order to increase the accuracy of identifying potential probiotics from the prescreened strains, we double-checked the acid and bile salt tolerance of the 61 prescreened strains (Figs. 1a–c and 2a–c). In the main screening process, the tolerance rates to acid and bile conditions were evaluated by calculating the survival rate after 3 h in acidic broth and 24 h in bile-supplemented broth. Twenty out of the 61 strains exhibited a high survival rate (more than 95%) after incubation in an acidic environment for 3 h (Fig. 1a). Moreover, these 20 strains also showed adaptability to high bile conditions, as evident from the more than 120% survival rate (Fig. 2a). Thereafter, the safety of the prescreened strains was verified using urease and gelatinase agar, and all 61 strains were evaluated to be safe (Table 2).

### Evaluation of Cholesterol-Lowering Activity in LABs

In order to confirm the functionality of the 61 strains, we assessed their cholesterol lowering activity in the presence of 0.3% oxgall (Fig. 3a–c). All strains were capable of removing cholesterol from the MRS-THIO broth after 18 h of incubation. However, the extent of cholesterol reduction was strain-specific. The top 20 of the 61 strains exhibited over 27% reduction in cholesterol concentration (Fig. 3a). Moreover, *Lactobacillus plantarum* MA2 and *L. johnsonii* 3121 were identified to exhibit the highest cholesterol-lowering activities (over 50%) in MRS-THIO broth.

### Evaluation of Antioxidant Activity in LABs

We further examined the functional properties of the 61 prescreened strains by measuring the radical scavenging activity of DPPH (Fig. 4a–c). DPPH is a widely used chemical compound for evaluating the antioxidant activity of cell-free supernatant of bacteria. *Leuconostoc mesenteroides* 7, *Lactobacillus casei* 911, and *Lactobacillus sakei* 105 exhibited over 55% DPPH scavenging activities. Moreover, the top 20 strains were also characterized by over 30% DPPH scavenging capacity, which was taken as the minimum cutoff value for in vitro experiments (Fig. 4a).

### Evaluation of Cytotoxicity of LABs in 3T3-L1 Cells

Further investigations were continued by narrowing down the 61 strains to 6 probiotic candidates that qualified in the top 20 with respect to the parameters of functionality

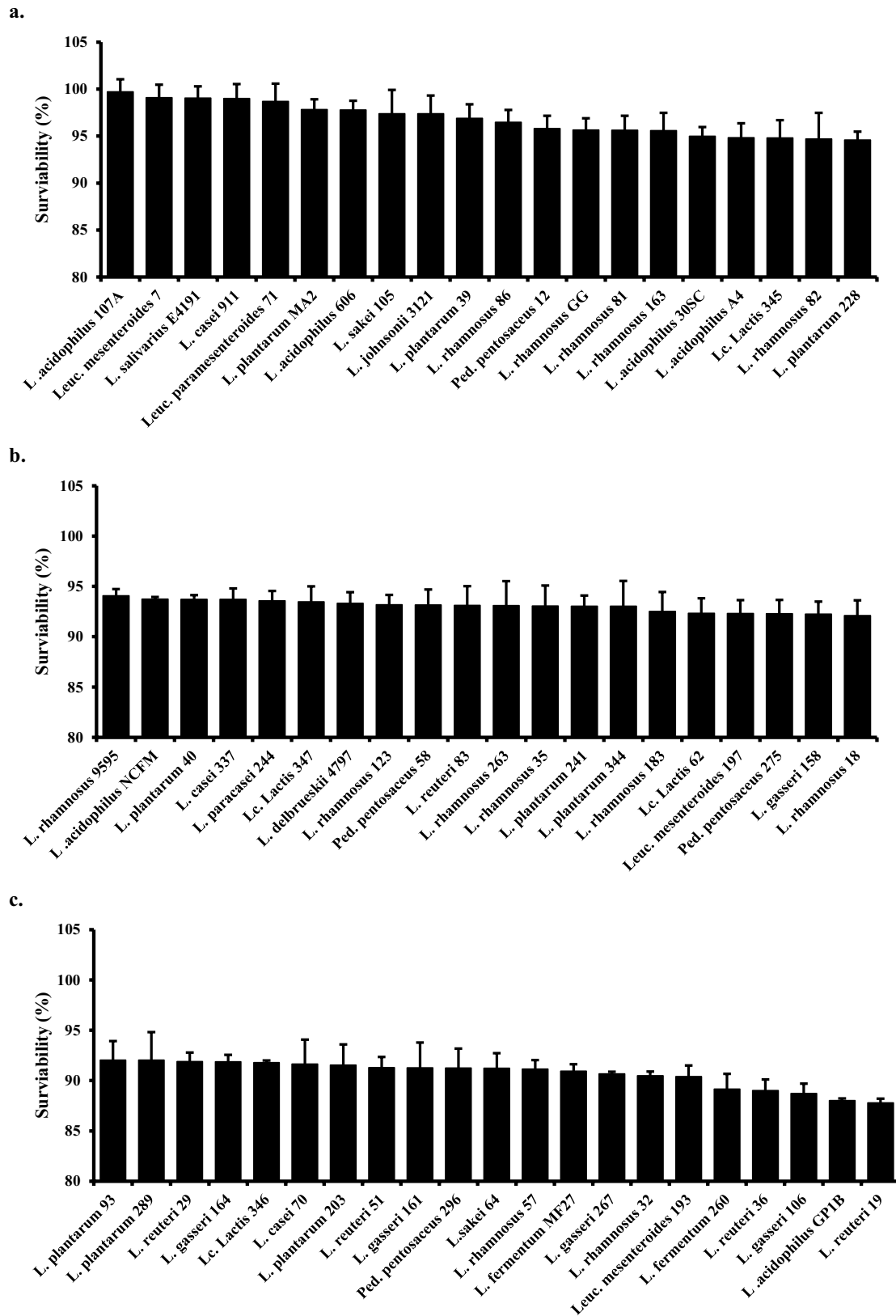
(acid tolerance, bile tolerance, cholesterol-lowering activity, and DPPH scavenging activity) explored in this study. Therefore, *L. johnsonii* 3121, *L. rhamnosus* 86, *L. plantarum* MA2, *L. sakei* 105, *L. casei* 911, and *Leuc. mesenteroides* 7 were selected for in vitro experiments using 3T3-L1 cells. The MTT assay was used to determine cytotoxic effect of the six heat-killed probiotic candidates on 3T3-L1 cell proliferation (Fig. 5). Our results showed that none of the six heat-killed strains affected the viability of 3T3-L1 cells. Culturing cells with these six heat-killed strains did not result in significant differences, compared to the control group, at both 24 and 48 h of incubation ( $P > 0.05$ ). These data indicate that the six probiotic candidates displayed negligible cytotoxicity and caused no damage to the cells.

### Effect of LABs on Antiadipogenic Capacities of 3T3-L1 Cells

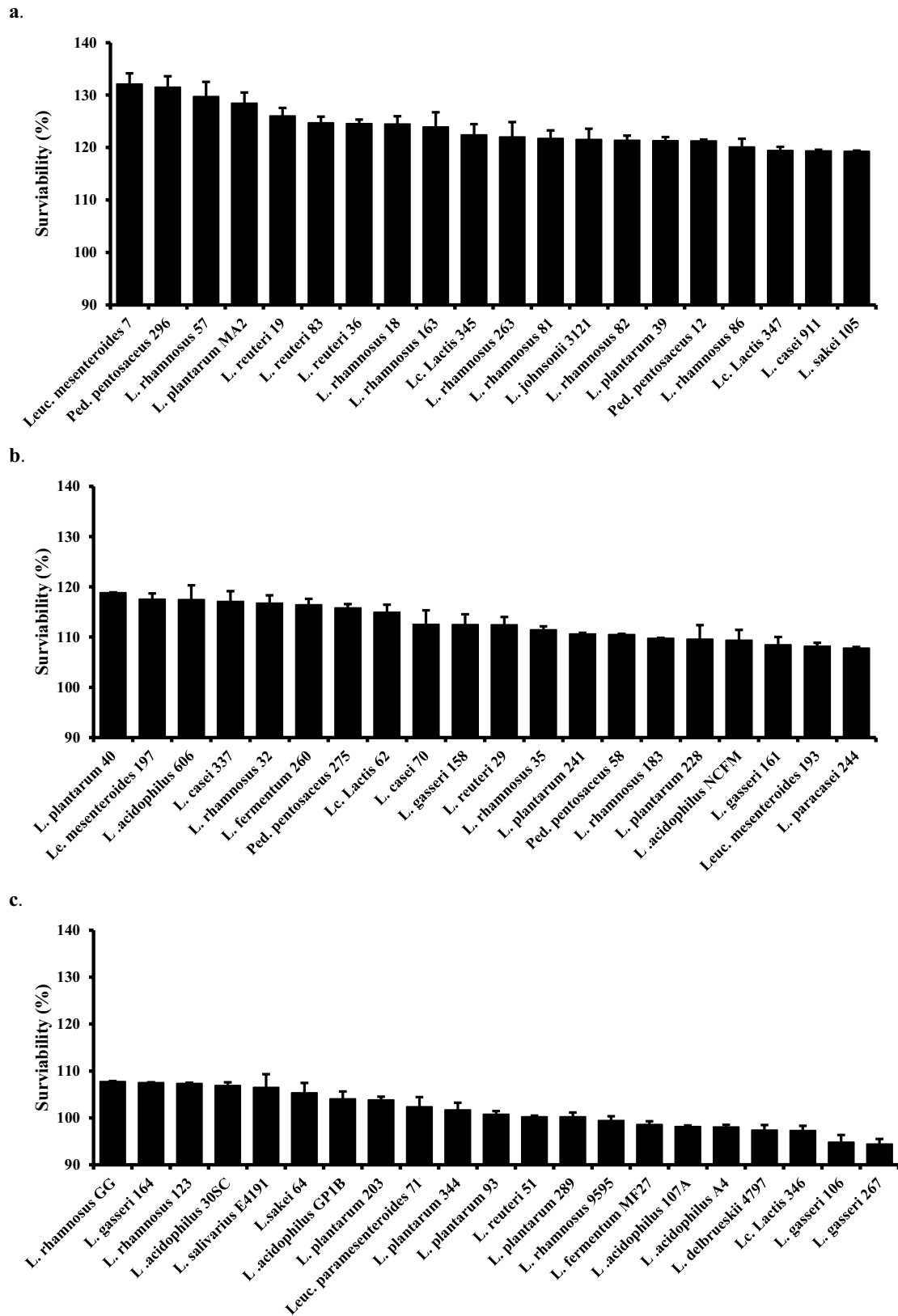
In order to evaluate the antiadipogenic effects of the heat-killed bacterial strains, the 3T3-L1 pre-adipocytes were treated with or without the heat-killed probiotic candidates for 8 days (Fig. 6a–c). The differentiated 3T3-L1 cells were subsequently stained with oil red O for visualization of lipid droplets and treatment with *L. rhamnosus* 86 and *L. johnsonii* 3121 were found to significantly inhibit lipid accumulation (Fig. 6c). The microscopy-based imaging and examination of oil red O-stained lipid droplets were performed by eluting in isopropanol. These data showed that *L. rhamnosus* 86 and *L. johnsonii* 3121 significantly downregulated the intracellular lipid content compared to control 3T3-L1 cells ( $P < 0.05$ ) (Fig. 6b). Moreover, the decreased lipid accumulation activity was also supported by trends in the TG content in 3T3-L1 cells (Fig. 6a). Both *L. rhamnosus* 86 and *L. johnsonii* 3121 were capable of significantly reducing the TG content compared to the control (Fig. 6a). Taken together, these results suggest that treating 3T3-L1 cells with heat-killed *L. rhamnosus* 86 and *L. johnsonii* 3121 during differentiation could lead to inhibition of adipogenesis by suppressing lipid accumulation.

### Effect of LABs on mRNA Expression Levels of Adipogenesis-Related Markers in 3T3-L1 Cells

Based on the downregulation of lipid and TG accumulation tested on the six probiotic candidates, *L. rhamnosus* 86 and *L. johnsonii* 3121 were selected for further qRT-PCR analysis. The mRNA expression levels of adipocyte differentiation markers, including peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), CCAAT/



**Fig. 1** Acid tolerance of the 61 LAB strains sorted according to decreasing overall survivability. **a** Top 1 to 20. **b** Top 21 to 40. **c** Remaining 41 to 61. Results are expressed as mean ± SE ( $n=3$ )



**Fig. 2** Bile tolerance of the LAB strains sorted by decreasing overall survivability. **a** Top 1 to 20. **b** top 21 to 40. **c** Remaining 41 to 61. Results are expressed as mean  $\pm$  SE ( $n=3$ )

**Table 2** Strain numbers and safety evaluations of the probiotic candidates using urease and gelatinase agar

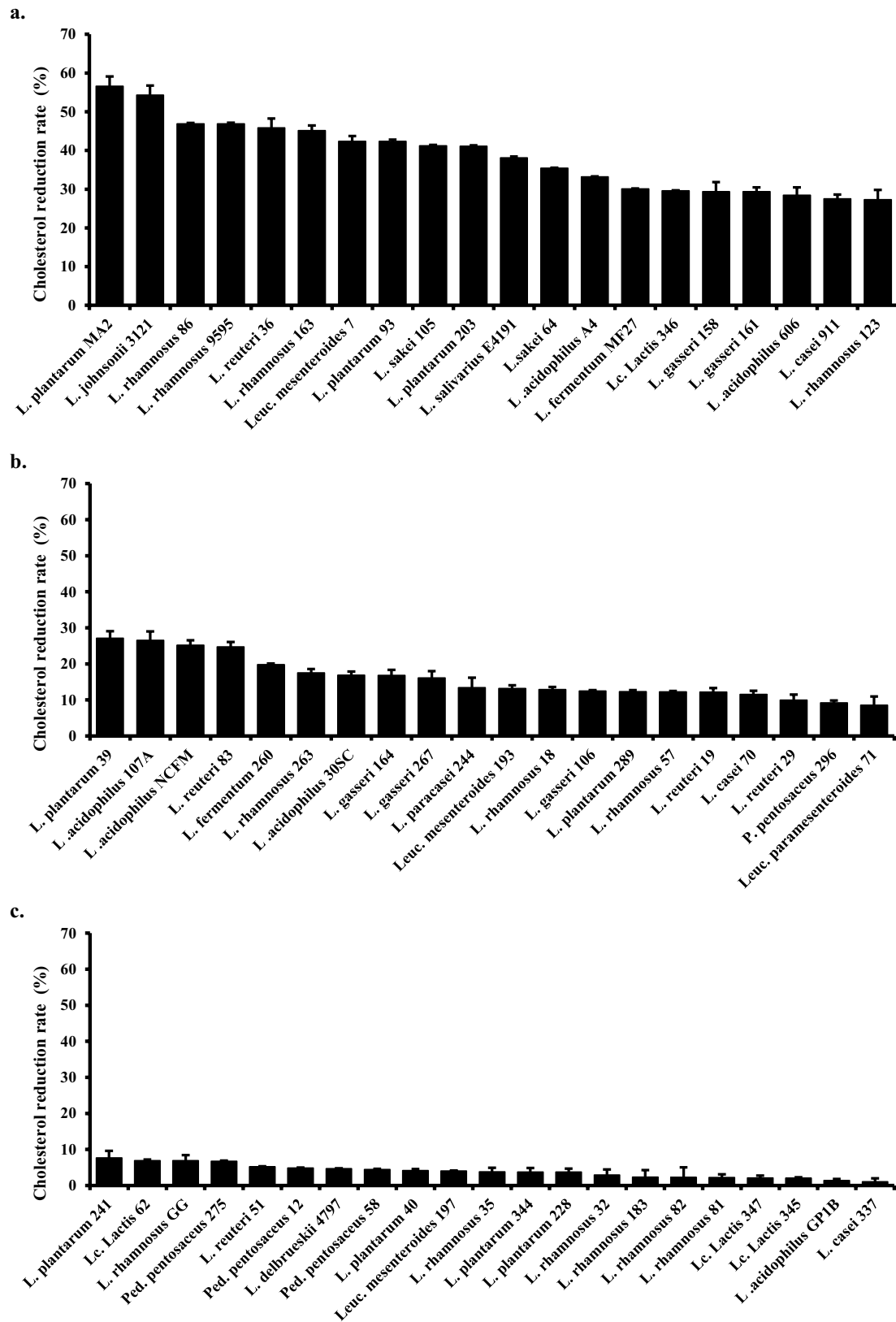
Strain	Urease test	Gelatinase test	Strain	Urease test	Gelatinase test
<i>Lactobacillus acidophilus</i> 107A	-	-	<i>Lactobacillus reuteri</i> 51	-	-
<i>Lactobacillus acidophilus</i> 30SC	-	-	<i>Lactobacillus reuteri</i> 83	-	-
<i>Lactobacillus acidophilus</i> 606	-	-	<i>Lactobacillus rhamnosus</i> 123	-	-
<i>Lactobacillus acidophilus</i> A4	-	-	<i>Lactobacillus rhamnosus</i> 163	-	-
<i>Lactobacillus acidophilus</i> GP1B	-	-	<i>Lactobacillus rhamnosus</i> 18	-	-
<i>Lactobacillus acidophilus</i> NCFM	-	-	<i>Lactobacillus rhamnosus</i> 183	-	-
<i>Lactobacillus casei</i> 337	-	-	<i>Lactobacillus rhamnosus</i> 263	-	-
<i>Lactobacillus casei</i> 70	-	-	<i>Lactobacillus rhamnosus</i> 32	-	-
<i>Lactobacillus casei</i> 911	-	-	<i>Lactobacillus rhamnosus</i> 35	-	-
<i>Lactobacillus delbrueskii</i> 4797	-	-	<i>Lactobacillus rhamnosus</i> 57	-	-
<i>Lactobacillus fermentum</i> 260	-	-	<i>Lactobacillus rhamnosus</i> 81	-	-
<i>Lactobacillus fermentum</i> MF27	-	-	<i>Lactobacillus rhamnosus</i> 82	-	-
<i>Lactobacillus gasseri</i> 106	-	-	<i>Lactobacillus rhamnosus</i> 86	-	-
<i>Lactobacillus gasseri</i> 158	-	-	<i>Lactobacillus rhamnosus</i> 9595	-	-
<i>Lactobacillus gasseri</i> 161	-	-	<i>Lactobacillus rhamnosus</i> GG	-	-
<i>Lactobacillus gasseri</i> 164	-	-	<i>Lactobacillus sakei</i> 64	-	-
<i>Lactobacillus gasseri</i> 267	-	-	<i>Lactobacillus sakei</i> 105	-	-
<i>Lactobacillus johnsonii</i> 3121	-	-	<i>Lactobacillus salivarius</i> E4191	-	-
<i>Lactobacillus paracasei</i> 244	-	-	<i>Lactococcus lactis</i> 345	-	-
<i>Lactobacillus plantarum</i> 203	-	-	<i>Lactococcus lactis</i> 346	-	-
<i>Lactobacillus plantarum</i> 228	-	-	<i>Lactococcus lactis</i> 347	-	-
<i>Lactobacillus plantarum</i> 241	-	-	<i>Lactococcus lactis</i> 62	-	-
<i>Lactobacillus plantarum</i> 289	-	-	<i>Leuconostoc mesenteroides</i> 193	-	-
<i>Lactobacillus plantarum</i> 334	-	-	<i>Leuconostoc mesenteroides</i> 197	-	-
<i>Lactobacillus plantarum</i> 39	-	-	<i>Leuconostoc mesenteroides</i> 7	-	-
<i>Lactobacillus plantarum</i> 40	-	-	<i>Leuconostoc paramesenteroides</i> 71	-	-
<i>Lactobacillus plantarum</i> 93	-	-	<i>Pediococcus pentosaceus</i> 12	-	-
<i>Lactobacillus plantarum</i> MA2	-	-	<i>Pediococcus pentosaceus</i> 58	-	-
<i>Lactobacillus reuteri</i> 19	-	-	<i>Pediococcus pentosaceus</i> 275	-	-
<i>Lactobacillus reuteri</i> 29	-	-	<i>Pediococcus pentosaceus</i> 296	-	-
<i>Lactobacillus reuteri</i> 36	-	-			

enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ), and adipocyte protein 2 (aP2), were evaluated in 3T3-L1 preadipocytes treated with *L. rhamnosus* 86 and *L. johnsonii* 3121 (Fig. 7). All three genes were significantly down-regulated in pre-adipocytes, which confirms that the differentiation process was successfully induced in mature adipocytes. Interestingly, 3T3-L1 cells treated with *L. rhamnosus* 86 and *L. johnsonii* 3121 showed significantly downregulated gene expression levels of PPAR $\gamma$ , C/EBP $\alpha$ , and aP2 ( $P < 0.05$ ), which represent the key adipogenic transcription factors related to adipocyte differentiation. These results indicate that *L. rhamnosus* 86 and *L. johnsonii* 3121 decrease the amount of intracellular lipid accumulation in 3T3-L1 adipocytes by downregulating the expression levels of genes related to adipogenesis.

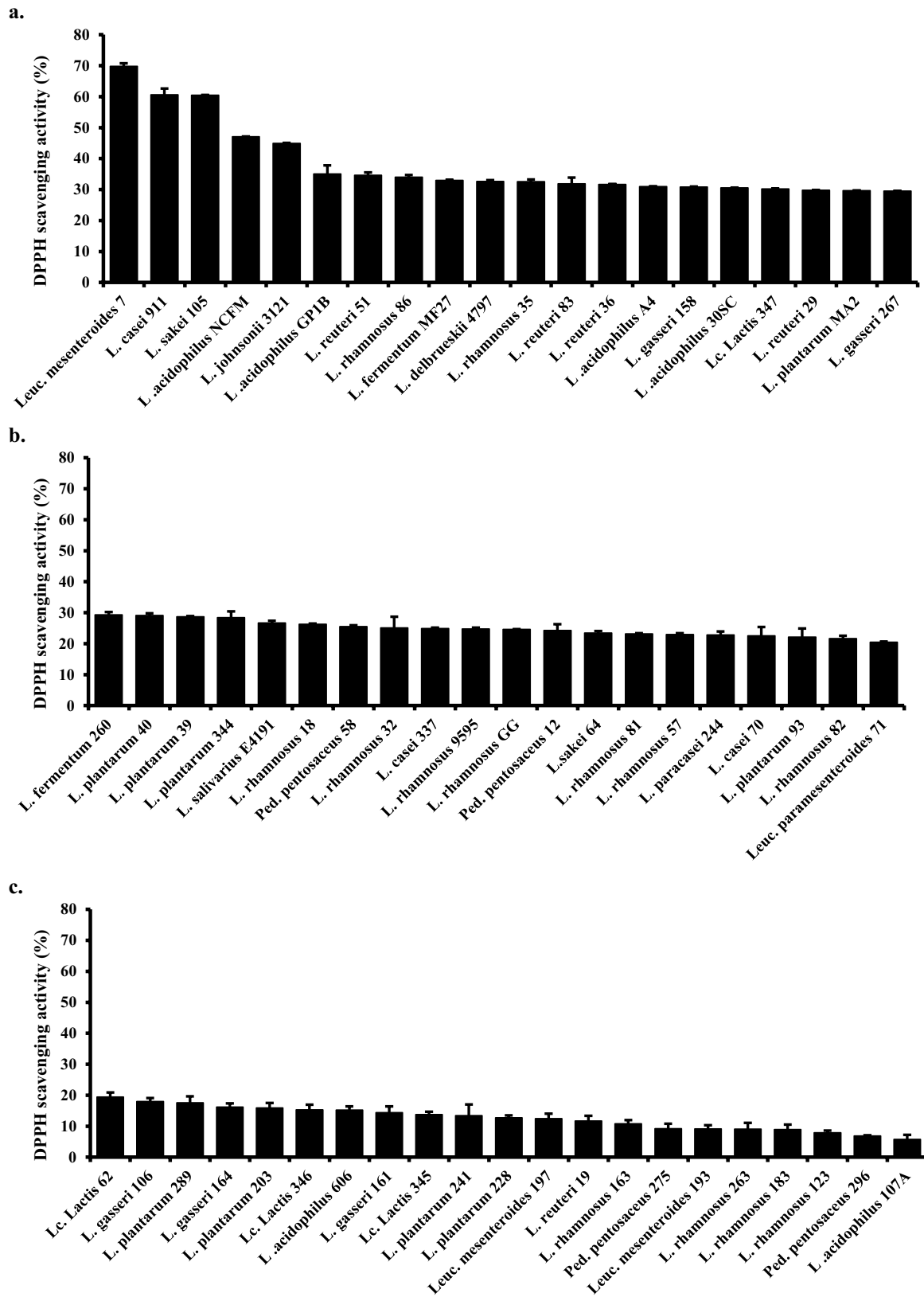
## Discussion

The present study was designed to identify novel probiotics with antiadipogenic capacity. Bacteria must be able to survive acidic conditions in the stomach and resist bile acids in order to exert their beneficial effect on the host and function as probiotics [9]. Therefore, in this study, artificial stress conditions of the human GI tract were mimicked. The first stress condition that probiotic bacteria face is the low pH environment in the stomach where gastric acid is secreted [22]. The average pH of secreted gastric acid is 2.5. This can damage the cell membrane of the bacteria and kill it before it reaches the gut and exerts its beneficial effects. Next, bacteria must resist the acidic bile conditions prevalent at the start of the small intestine; this is another crucial requirement for bacteria to act as probiotics. Bile is known to play

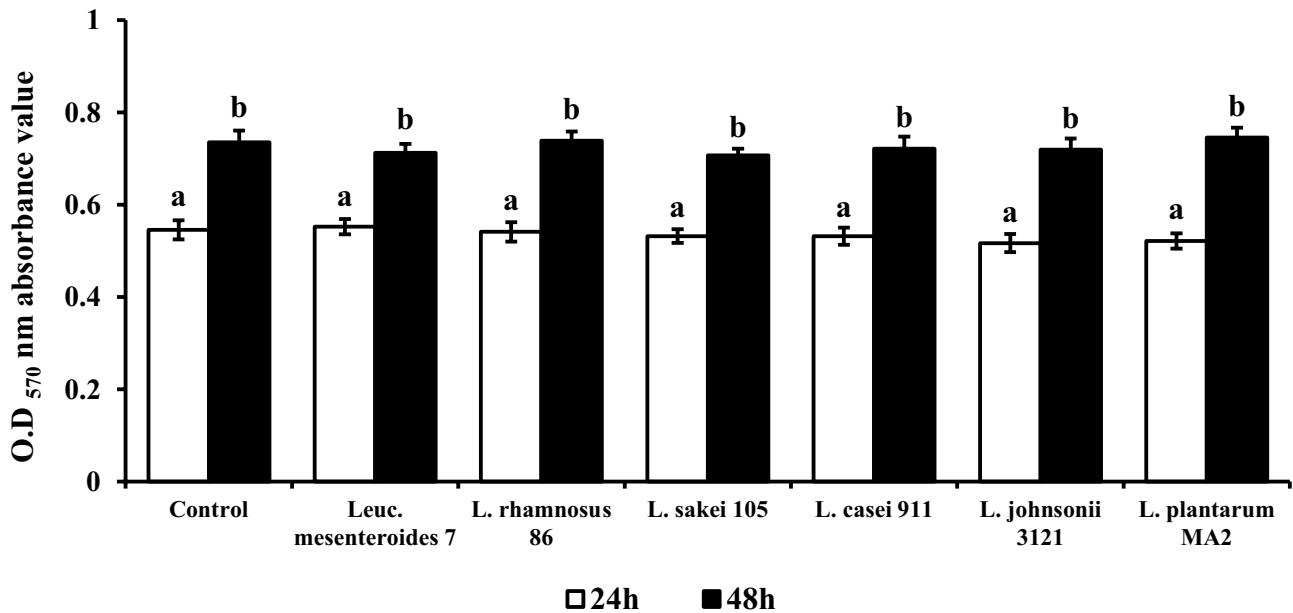




**Fig. 3** Cholesterol-lowering activities of the 61 LAB strains sorted by decreasing reduction rate. **a** Top 1 to 20. **b** Top 21 to 40. **c** Remaining 41 to 61. Results are expressed as mean  $\pm$  SE ( $n=3$ )



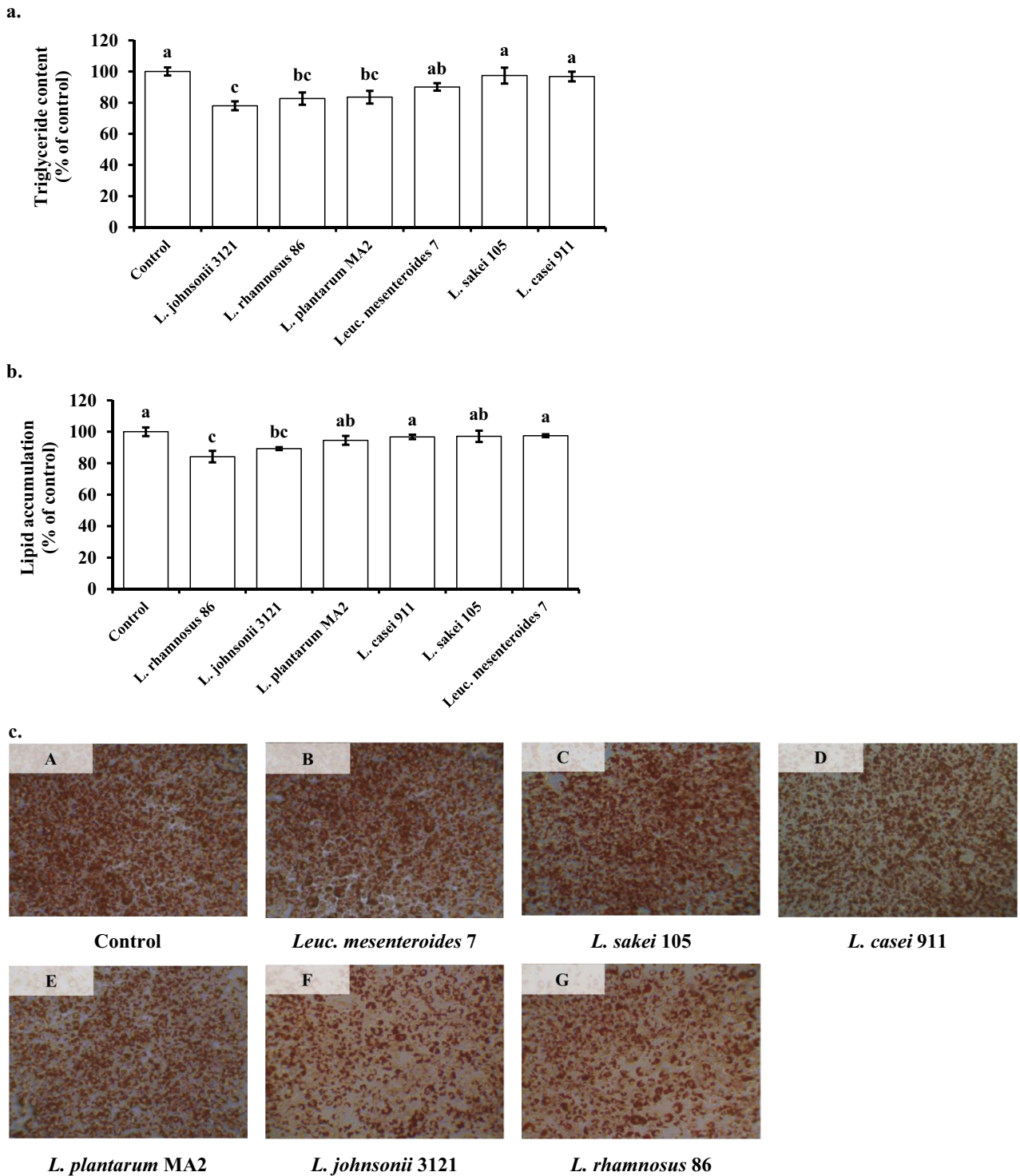
**Fig. 4** Antioxidant activities of the 61 LAB strains sorted according to decreasing DPPH scavenging activity. **a** Top 1 to 20. **b** Top 21 to 40. **c** Remaining 41 to 61. Results are expressed as mean  $\pm$  SE ( $n = 3$ )



**Fig. 5** Cytotoxicity of the six probiotic candidates on 3T3-L1 cells. Results are expressed as mean  $\pm$  SE ( $n=3$ ). Lowercase superscript letters (a, b) denote significantly different mean values in the same series ( $P<0.05$ )

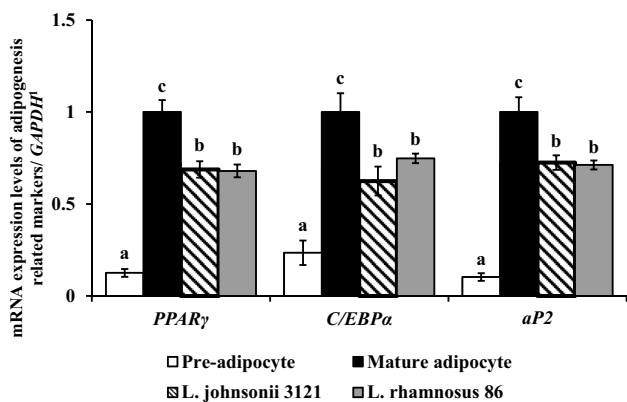
an essential role in specific and nonspecific gut defense systems, and the intensity of its inhibitory effect is determined primarily by the bile salt concentration [23]. On the other hand, bile salts can disrupt bacterial cell membranes, which is toxic to living bacterial cells; therefore, bile tolerance is considered an important characteristic of probiotics, which enables bacterial survival, growth, and subsequent positive action in the GI tract [24]. We have previously identified 61 strains, out of 151 candidates, that exhibit high resistance to bile and low pH conditions. The present work was carried out with the objective of selecting the top 20 strains out of the 61 prescreened ones, which show the highest tolerance to both low pH and bile salt environments. Several studies have demonstrated that some LABs have high survivability in bile salt containing medium due to the presence of genes coding for bile salt hydrolases [25]. Moreover, safety evaluation is another important characteristic needed to be considered while screening for potential probiotics. The absence of urease and gelatinase activities represents one of the crucial factors that underscore the safety of probiotic consumption. Urease is an enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide, thereby playing a key role in urea-related diseases such as urolithiasis [26]. Gelatinase is a protease that is capable of hydrolyzing gelatin, which may lead to tissue necrosis and damage to the body [27]. In our study, all 61 prescreened strains showed negligible action with respect to both of these factors. In order to confirm the functional characteristics of these 61 strains, their antioxidant and cholesterol-lowering activities were evaluated. We anticipated that the most important functional

ability needed for LABs to exert antiobesity effects could be cholesterol reducing activity. Kumar and colleagues have earlier suggested that cholesterol-lowering probiotics could be potential biotherapeutics for treating metabolic diseases [28]. Several LAB strains have been reported to possess the ability to assimilate and uptake cholesterol by adsorbing cholesterol into the cell wall, thereby assimilating it into the cell membrane [29–31]. Our study demonstrated that the top 20 strains exhibited over 27% reduction in cholesterol. Moreover, *L. plantarum* MA2 and *L. johnsonii* 3121 were capable of lowering cholesterol levels by more than 50%. For the second functionality, we evaluated the radical scavenging activity of the 61 strains. Since chronic oxidative stress is a major cause of metabolic diseases such as obesity, the antioxidant activity of LAB strains could be an ideal therapeutic method for treating obesity. Previous studies have shown that oxidative stress in accumulated fat mediates the development of obesity-associated MS via the dysregulation of adipocytokine production and increased ROS production [32–34]. The present observations have demonstrated that *Leuc. mesenteroides* 7, *L. casei* 911, and *L. sakei* 105 exhibited the highest antioxidant potential among the 61 strains. Based on these results and the cut-off values discussed above, we selected six strains, namely *L. johnsonii* 3121, *L. rhamnosus* 86, *L. plantarum* MA2, *L. sakei* 105, *L. casei* 911, and *Leuc. mesenteroides* 7, which were included in the top 20 strains that exhibited strong resistance to low pH and bile salt. Moreover, these six strains possessed greater cholesterol-lowering ability and higher efficiency in scavenging free radicals compared to the 41 strains that ranked below



**Fig. 6** Effects of the six probiotic candidates on triglyceride (TG) and lipid accumulation in 3T3-L1 cells. **a** TG accumulation in differentiated mature 3T3-L1 adipocytes. **b** Quantification of stained lipids with oil red O in differentiated mature 3T3-L1 adipocytes. **c** Lipid accumulation in differentiated mature 3T3-L1 adipocytes stained by oil red O. The red dot indicates the accumulated lipid. It can be seen

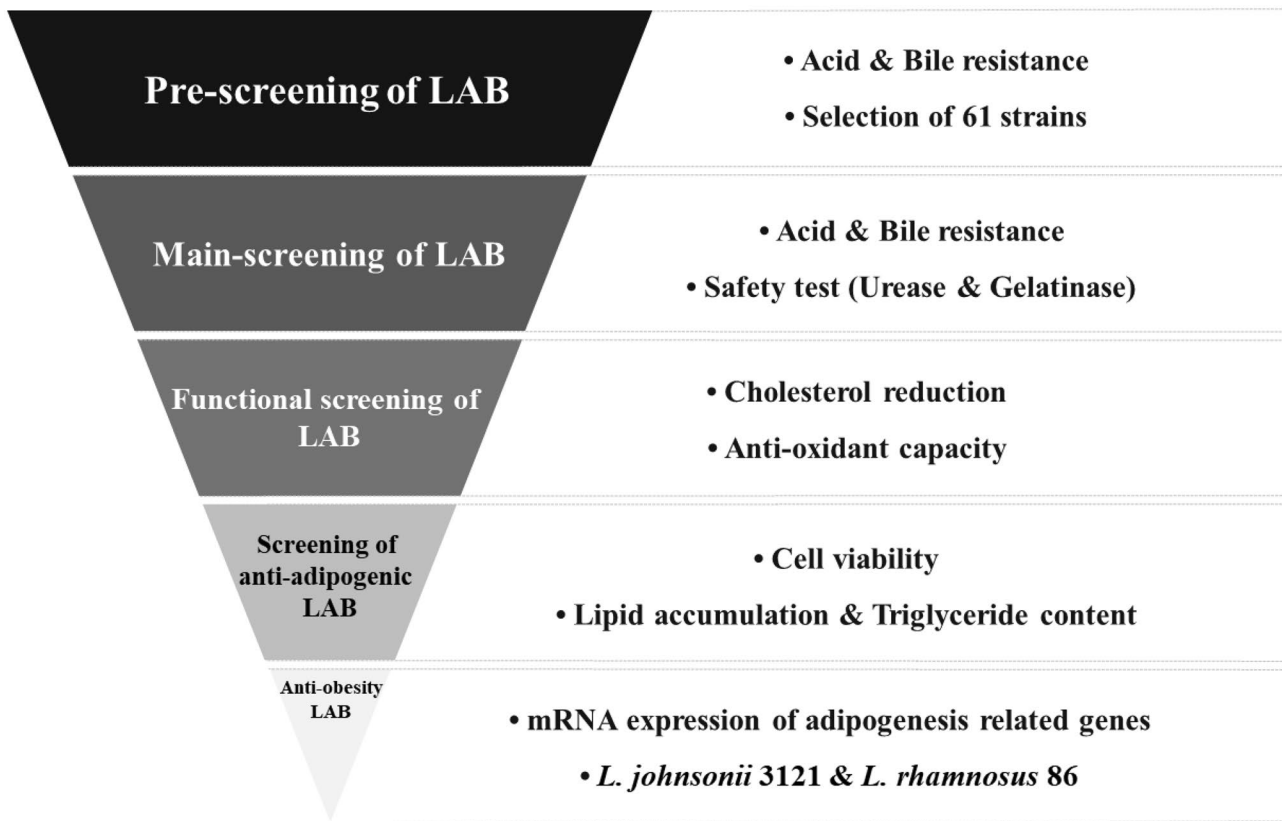
that heat-killed *L. johnsonii* 3121 and *L. rhamnosus* 86 administrated cells have significantly less red areas overall. (×100 magnification). Results are expressed as mean ± SE (n=6). Lowercase superscript letters (a, b, c) denotes significantly different mean values in the same series (P<0.05)



**Fig. 7** Effect of *L. rhamnosus* 86 and *L. johnsonii* 3121 on mRNA expression levels of adipogenesis-related markers (peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ), and adipocyte protein 2 (aP2)) in 3T3-L1 cells. Results are expressed as mean  $\pm$  SE ( $n=6$ ). Lowercase superscript letters (a, b, c) denote significantly different mean values in the same series ( $P < 0.05$ )

20. Since the objective of this study was to screen for probiotics with antiobesity effects, the cytotoxicity and lipid accumulation levels were examined using 3T3-L1 mouse pre-adipocyte cells. The proliferation of 3T3-L1 cells was

not affected by the six strains, indicating that none of these strains were cytotoxic to adipocytes. Further examination of the six strains was performed by confirming the decreased TG content and downregulated lipid accumulation in 3T3-L1 cells. Among the six strains, only *L. johnsonii* 3121 and *L. rhamnosus* 86 were capable of significantly decreasing the TG content and lipid accumulation in fully differentiated adipocytes. To confirm this antiadipogenic effect, the expression of adipogenesis-related genes was examined by qRT-PCR. Several studies have demonstrated the inhibition of adipogenesis in adipocytes on treatment with functional bacterial strains, which regulate adipogenic transcription factors [35, 36]. Similar findings were observed in our study. The qRT-PCR analysis revealed that both *L. johnsonii* 3121 and *L. rhamnosus* 86 significantly decreased the gene expression levels of PPAR $\gamma$ , C/EBP $\alpha$ , and aP2, which are known to control adipocyte differentiation [37]. The early stage of adipocyte differentiation is known to be regulated by PPAR $\gamma$  and C/EBP $\alpha$ , which are critical transcription factors in adipogenesis [38]. Moreover, PPAR $\gamma$  regulates expression of genes involved in lipid metabolism (such as aP2), thereby triggering intracellular fat accumulation [39]. Further, aP2 is a carrier protein of fatty acids which is expressed in mature adipocytes. Interestingly, mRNA expression and protein levels of aP2 are known to markedly increased on increased



**Fig. 8** Experimental design of the selection strategy employed for identifying probiotics with antiobesity properties

PPAR $\gamma$  gene expression, which highlights the role of PPAR $\gamma$  as an aP2 regulator [40]. Based on these results, our study clearly indicates that the inhibited adipogenesis and lipid accumulation effects exerted by heat-killed *L. johnsonii* 3121 and *L. rhamnosus* 86 in mouse preadipocyte cells are mediated by the suppression of key adipogenic transcription factors related to adipocyte differentiation.

## Conclusion

This study aimed to screen functional probiotic strains with antiobesity effects and identified *L. johnsonii* 3121 and *L. rhamnosus* 86 as potentially effective antiobesity probiotic strains. We established five steps to screen potential probiotic strains with antiobesity functions (Fig. 8). Sixty-one novel strains were prescreened from 151 candidates for their ability to show resistance to acidic and bile-containing environment. The functional aspects of these 61 strains were assessed in terms of their cholesterol-lowering and antioxidant activities. Based on the data regarding acid tolerance, bile tolerance, cholesterol reduction, and antioxidant activity, *L. johnsonii* 3121, *L. rhamnosus* 86, *L. plantarum* MA2, *L. sakei* 105, *L. casei* 911, and *Leuc. mesenteroides* 7 were selected for further studies, since these strains exhibited their probiotic potential by qualifying among the top 20 strains in all four parameters measured. Of the six strains, only *L. johnsonii* 3121 and *L. rhamnosus* 86 were found to be capable of lowering TG content and lipid accumulation in 3T3-L1 cells. Further, *L. johnsonii* 3121 and *L. rhamnosus* 86 could inhibit adipogenic differentiation in 3T3-L1 cells by downregulating the expression levels of adipogenic transcription factors PPAR $\gamma$ , C/EBP $\alpha$ , and aP2. Taken together, these results identify *L. rhamnosus* 86 and *L. johnsonii* 3121 as potential therapeutic agents possessing antiobesity effects. Moreover, in vivo trials are under progress in animal models of high-fat diet-induced obesity in order to further confirm their functionality as antiobesity probiotics.

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**Data Availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethics Approval** This article does not contain any studies with human or animal subjects.

**Conflict of Interest** The authors have no conflicts of interest to declare that are relevant to the content of this article.

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