Probiotic Potential of Lactobacillus Strains on the Adipogenesis of 3T3-L1 Cells

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Abstract

Obesity is the most common health problem in developed countries and is considered a significant risk factor for many major human diseases. This study aimed to evaluate the inhibitory effect of lactic acid bacteria isolated from the human vagina on adipocyte differentiation in 3T3-L1 preadipocytes. We screened 26 strains of lactic acid bacteria to test their effects against obesity. Among the tested strains, Lactobacillus gasseri MG2855 exhibited a lipase inhibitory activity of 11.84% and inhibited relative lipid content of 3T3-L1 cells (96.21%) at a concentration of 1,000 μg/mL. The survival rate of L. gasseri MG2855 in 0.3% bile salt was 88.47%, and the survival rate after incubation for 3 h in pH 3.0 was 78.64%. L. gasseri MG2855 showed higher sensitivity to erythromycin, chloramphenicol, tetracyclin, and cephalothin in 16 antibiotic sensitivity tests. These results demonstrate that L. gasseri MG2855 can be employed as a potential probiotic with anti-obesity effects.

Keywords: Lactic acid bacteria; Antiobesity; Probiotic; Lactobacillus gasseri

Introduction

Lactic acid bacteria (LAB) possess special physiological activities and are generally regarded as safe bacteria (GRAS). LAB have been widely used in the production of a number of fermented foods, particularly in dairy and vegetable products with functional and probiotic properties [1]. Recently, as the consumer demand for natural and chemical preservatives has increased, a novel and alternative method for the preservation of human and animal foods is required. The term “probiotic” is described as a feed supplement with beneficial effects, which promotes a healthy lifestyle and improves the quality of human beings by maintaining and improving normal organ functions [2]. Furthermore, probiotics exhibit potent antagonist effect on pathogens in the gastrointestinal tract [3]. LAB produces essential antimicrobial metabolites that exterminate other pathogenic bacteria. There are numerous microorganisms that can classify as probiotics, including those that belong to the Lactobacillus and Bifidobacterium genera. Lactobacilli are considered enteric organisms and are used in the industrial processing of fermented dairy, meat, vegetable, and cereal products. According to the World Health Organization, for an organism to be classified as a probiotic, it has to comply with the principles related to their safety and biological properties. In terms of safety, the probiotic should not exhibit any pathogenicity, should not be able to transfer antibiotic-resistance genes, and should sustain genetic stability.

In spite of the current public awareness of obesity, the cases continue to increase. Obesity is the most common health problem in developed countries and is considered a significant risk factor for many major human diseases such as heart disease, cancer, arthritis, and diabetes [4]. Obesity develops because of an increase in the number of fat cells and their lipid content as a result of adipocyte differentiation (adipogenesis). The primary role of adipocytes involves the synthesis and storage of triglycerides during periods of calorific excess. As a result of adipogenesis, the size or number of the fat cells increases and lipid drops accumulate inside the cell [4]. Thus, preadipocyte cell lines are useful models for investigating the adipogenic process. An enhanced understanding of the process of adipogenesis would help in preventing the initiation and progression of adipogenesis, which leads to obesity and obesity-related diseases in humans. For a microorganism to be qualified for use as a probiotic, it should exhibit beneficial effects, including the modulation of immune responses [5] and anti-carcinogenic and anti-oxidative activities [6]. Moreover, certain LAB has been found to be effective in regulating the number of fat cells in adipose tissue in overweight adults [7] and in a diet-induced obese animal model [8].

The aim of this work was (a) to screen LAB strains with potentially significant anti-obesity activity and (b) to study the characteristics related to the probiotic potential of these microorganisms in vitro.

Materials and Methods

Cell culture

3T3-L1 preadipocytes were purchased from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) in an incubator at 37°C with 5% CO2. To induce adipocyte differentiation, two days post-confluence, 3T3-L1 preadipocytes (day 0) were stimulated for 48 h (day 2) with a standard induction cocktail (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/mL insulin; MDI) and herbal extracts (250 μg/mL) and maintained for 4 days (day 6) in DMEM supplemented with 10% FBS and 1 μg/mL insulin.

LAB sample and treatment

After culturing, the isolated strains were harvested in a refrigerated centrifuge (1,100 × g for 3 min at 4°C) and washed three times with 0.1× PBS.

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distilled water to remove the MRS broth. The washed isolated strains were freeze-dried and resuspended in phosphate buffered saline (PBS; pH 7.2) at a concentration of 10 mg/mL and homogenized for 50 sec followed by a 1 min rest period (repeated 3 times) using a sonicator (VCX 400, Sonic and Materials Inc., CT, USA). The suspension was centrifuged at 1,100 × g for 15 min at 4°C. The 3T3-L1 cells were treated with 1,000 μg/mL concentrations of the supernatant.

**Probiotic activity**

The method of Lee et al. [9] for lipase activity determination was modified in this study. Pancreatic lipase activity was measured using porcine pancreatic lipase (Sigma, USA). For the enzymatic reaction, 0.1 mg/mL of the sample solution dissolved in water, 0.167 mM p-nitrophenylpalmitate (PNP; Sigma, USA) solution, and 0.061 M (pH 8.5) Tris-HCl buffer were mixed in the well of a plate, and then 0.3 mg/mL of the lipase solution was added to initiate the reaction. After incubation at 25°C for 10 min, its absorbance was measured at 400 nm.

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**Cell differentiation and sample treatment**

The cells were cultured as described in the “Cell Culture” section. To examine the effects of the isolated strains on adipocyte differentiation, the medium was treated every alternate day until the end of the experiment on day 8. For the positive control, cells were cultured in the same media containing a drug-free vehicle and baicalin (100 μM), reported to be an adipogenesis inhibitor [10].

**Cell viability and oil red O staining**

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. 3T3-L1 preadipocytes were treated with selected strains at concentrations of 0, 100, and 1,000 μg/mL. After 24 h, 20 μL of MTT solution was added, and cells were incubated at 37°C for 4 h. The supernatant was then discarded, and 200 μL of dimethyl sulfoxide was added. Absorbance was measured on a microplate reader (Bio-Rad Model 550; Hercules, CA, USA) at 546 nm to obtain the percentage of viable cells.

Eight days after inducing differentiation, the media was discarded, and the cells were washed twice with PBS and fixed with 3.7% formalin for 15 min. To visualize the lipid droplets, the fixed cells were washed twice with 60% isopropanol (in PBS) and then stained with a 0.5% Oil Red O solution for 40 min at room temperature. The Oil Red O solution was then removed, and the cells were washed twice with deionized water before being photographed. For quantification of Oil Red O uptake, cells were incubated with isopropanol, and the optical density of the solution was measured at 540 nm. The percentage of the cells stained with Oil Red O relative to the control wells containing the cell culture medium without compounds was calculated as sample OD/Control OD (with baicalin) × 100.

**Biochemical tests and identification**

Selected isolates were identified by Gram staining, conventional biochemical tests [11], and sequencing of the 16S ribosomal RNA gene using universal primers (518F and 800R). PCR and 16s rRNA sequencing were outsourced to Macrogen Co. (Daejeon, Korea). Biochemical tests and identification based on 16S rRNA gene sequences [12]. Basic Local Alignment Search Tool (BLAST) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to compare the sequences obtained with available DNA sequences registered in the database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Sequences were aligned using the PHYDIT program (http://plaza.snu.ac.kr/~jchun/phydit/), and alignments were manually corrected. A phylogenetic tree was constructed with the neighbor-joining method using the MEGA 5.0 software [13].

**Results and Discussion**

**Inhibitory effect on lipase activity**

Several approaches have been reported for the prevention and treatment of obesity [18]. Among these, both natural and synthetic
pancreatic lipase inhibitors are effective in preventing obesity, which is likely due to the inhibition of intestinal lipid absorption [19]. Therefore, lipase inhibitors are used for designing drugs for the treatment of obesity and acne [20]. After incubating in MRS broth at 37°C for 18 h, 26 strains exhibited lipase inhibitory activity from the 221 strains tested. Anti-lipase activities of the selected strains were repeatedly measured in duplicates. Table 1 show that the MG2211 and MG2333 strains exhibited highest lipase inhibitory activity among the 26 selected strains.

Inhibitory effect of isolated strains on adipocyte differentiation and cell viability

Adipogenesis is a highly regulated, complex differentiation process, in which preadipocytes are transformed into differentiated adipocytes, and is triggered by the coordinated signaling of growth factors, cytokines, and hormones [21]. In this study, we screened the 26 selected strains to test their potential anti-adipogenic activity. 3T3-L1 adipocytes were cultured and differentiated in DMEM containing 10% FBS for 6-8 days in the absence or presence of the samples (at a final concentration of 1,000 μg/mL), according to the differentiation protocols. As shown in Table 1, MG2444 demonstrated the highest relative lipid content (80.72%) on adipogenesis of 3T3-L1 preadipocytes, as assessed by intracellular triglyceride droplet staining with Oil Red O. In addition, MG2700 (82.11%), MG5003 14.47 90.61 86.11 88.07, MG2988 17.35 105.57 154.43 67.15, MG2388 17.35 111.69 145.38 87.93, MG3066 17.11 171.43 114.34 91.17, MG2155 17.11 189.9 94.27 93.32, MG5001 15.79 96.46 83.3 77.54, MG5011 15.79 97.59 107.26 72.45, MG2344 15.79 138.66 112.04 110.61, MG5009 11.84 95.97 69.63 62.79, MG4001 11.84 88.4 88.07 86.11, MG5018 10.53 86.69 81.15 74.85, MG2899 11.84 97.57 80.55 76.85, MG5006 11.84 99.6 66.83 64.30, MG2311 12.24 97.57 80.55 76.85, MG2199 11.22 80.72 107.84 82.79, MG5003 10.53 92.26 81.15 74.85, MG4003 10.53 92.08 101.7 100.4, MG4203 10.53 96.45 116.6 102.2.

Table 1: Anti-lipase activity of isolated lactic acid bacteria.
Tolerance to low pH and bile salts

Stomach and execute their health effects as viable potent cells on reaching the colon [25]. The effects of highly acidic pH (3.0) on the survival of the nine selected strains over different incubation periods are shown in Figure 1. The population densities of eight selected strains were found to be <6 log CFU/mL after exposure to a pH of 3.0 for 3 h, except for MG4002. In particular, MG4001, MG2211, MG2311, MG2444, and MG4203 showed very high resistance to a pH of 3.0 with final viable populations exceeding 7 log CFU/mL. In fact, research has shown that Lactobacillus strains are resistant to pH varying between 2.5 and 4.0. The work of Maragkoudakis et al. [26] showed that the viability of the Lactobacillus strains of dairy origin was not affected by a pH of 3.0. However, in the study by Prasad et al. [27], only four LAB from the 200 strains isolated from dairy origin were resistant to a pH of 3.0 (≥ 80%) (Figure 1).

Resistance of LAB to bile salts is also an important factor for their colonization [28]. As shown in Figure 2, the viable counts of all selected strains were found to be >8 log CFU/mL after exposure to 0.3% (w/v) bile salts for 24 h, with low reduction in viable counts (<1 log CFU/mL). However, MG4003, MG2211, and MG2700 exhibited a lower tolerance to bile salts, and their population densities reduced by more than 2 log CFU/mL after 24 h of exposure to bile salts (Figure 2).

Auto aggregation

In general, the probiotic strains showed higher auto aggregation abilities. All nine selected strains tested showed higher percentages of aggregation after 5 h of incubation Figure 3. MG4002 and MG2855 showed the highest auto aggregation percentages at 37°C. The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains that are used as probiotics. Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces. In most cases, aggregation ability is related to cell adherence properties [29] (Figure 3).

Antibiotic susceptibility assay and hemolysis

Antibiotic overuse results in the development and dispersal of antibiotic resistance genes in a species, and such resistance genes are then transferred to other microorganisms. Thus, the sensitivity of probiotics to conventional antibiotics is a fundamental health-promoting characteristic [30]. Table 3 shows the antibiotic susceptibility results of selected strains, which were resistant to CTT, GM, K, S, CIP, NA, and VA. However, the selected strains were interpreted to be susceptible to AM, CF, SXT, C, and E. Several authors have found that the strains of L. plantarum are resistant to various antibiotics [31]. Wang et al. [32] reported that different isolated LAB strains were resistant to different classes of antibiotics. According to Tulumoglu et al. [33], all isolated L. fermentum strains were resistant to VA and some strains were susceptible to clindamycin. Angmo et al. [34] indicated that most Lactobacillus strains isolates were almost susceptible to all investigated antibiotics, with VA being an exception. L. fermentum isolate was also resistant to norfloxacin, S, K, NA, and VA. In the present study,
Figure 2: Survival of lactic acid bacteria with probiotic activities in the presence of 0.3% bile salt.

Figure 3: Comparison of the auto aggregation ability of selected strains.
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Table 3: Antimicrobial resistance of selected strains.

| Antimicrobials | Selected strains |
|----------------|------------------|
|                | MG4001 | MG4003 | MG4002 | MG2211 | MG2311 | MG2444 | MG4203 | MG2700 | MG2855 |
| β-Lactams      |        |        |        |        |        |        |        |        |        |
| Ampicillin (AM)| S      | S      | S      | S      | S      | S      | S      | S      | S      |
| Cefotaxime (CTX)| R      | R      | I      | R      | S      | S      | R      | R      | S      |
| Cefepime (CEP) | R      | R      | R      | S      | S      | S      | S      | R      | I      |
| Cefotetan (CTT)| R      | R      | R      | R      | R      | R      | R      | R      | I      |
| Cephalothin (CF)| S    | I      | S      | S      | S      | S      | S      | S      | I      |
| Aminoglycosides|        |        |        |        |        |        |        |        |        |
| Gentamicin (GM)| R      | R      | R      | R      | R      | R      | R      | R      | R      |
| Kanamycin (K)  | R      | R      | R      | R      | R      | R      | R      | R      | R      |
| Streptomycin (S)| R    | R      | R      | R      | R      | R      | R      | R      | R      |
| Quinolones and fluoroquinolones| | | | | | | | | |
| Ciprofloxacin (CIP)| R  | R      | R      | R      | R      | R      | R      | R      | R      |
| Nalidixic acid (NA)| R    | R      | R      | R      | R      | R      | R      | R      | R      |
| Sulphonamides   |        |        |        |        |        |        |        |        |        |
| Sulphamethoxazole/trimethoprim (SXT)| S  | S      | R      | S      | S      | S      | S      | S      | S      |
| Tetracyclines   | I      | I      | S      | I      | S      | S      | S      | S      | I      |
| Phenicols       |        |        |        |        |        |        |        |        |        |
| Chloramphenicol (C)| S    | S      | S      | S      | S      | S      | S      | S      | S      |
| Transpeptidation/translocation| | | | | | | | | |
| Erythromycin (E)| I      | S      | S      | I      | S      | S      | S      | S      | S      |
| Glycopeptide    |        |        |        |        |        |        |        |        |        |
| Vancomycin (VA) | R      | R      | R      | R      | R      | R      | R      | R      | S      |
| Nucleic acid inhibition| | | | | | | | | |
| Rifampin (RA)   | R      | R      | S      | I      | S      | I      | R      | S      | S      |

none of the selected strains exhibited hemolytic activity against human blood. Therefore, all strains were considered as safe and could be used as potential probiotic strains. This result was concurrent with previous reports of Gao et al. [35].

Conclusion

In conclusion, we investigated whether LAB isolated from the human vagina exhibited any anti-obesity activity such as inhibition of adipocyte differentiation of the 3T3-L1 cells. In the present study, we isolated potential probiotic strains having functional properties of food. L. gasseri MG2855 showed essential probiotic properties, including complete tolerance to acid and bile salts and high auto-aggregation activity. Furthermore, it was antibiotic resistant and did not cause human blood hemolysis. These results demonstrate that L. gasseri MG2855 could be an excellent anti-obesity probiotic.

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