This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Anti-obesity activity of the water extract of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 fermented soy milk products

Running Title: Anti-obesity of soy milk fermented with lactic acid bacteria

Meng-Chun Cheng\textsuperscript{a, b}, Tsung-Yu Tsai\textsuperscript{b*}, Tzu-Ming Pan\textsuperscript{a*}

\textsuperscript{a} Department of Biochemical Science & Technology, National Taiwan University, Taipei, Taiwan.

\textsuperscript{b} Department of Food Science, Fu Jen Catholic University, New Taipei City, Taiwan.

*Corresponding Author:

Dr. Tzu-Ming Pan, Professor; Tel: +886-2-3366-4519 ext 10; Fax: +886-2-3366-3838; E-mail: tmpan@ntu.edu.tw

Dr. Tsung-Yu Tsai, Associate Professor; Tel: +886-2-2905-2539; Fax: +886-2-2905-2540; E-mail: tytsai@mail.fju.edu.tw
Abstract

The anti-obesity activity of water extract of soy milk fermented with *Lactobacillus paracasei* subsp. *paracasei* NTU 101 (W101) was investigated. A high-fat diet (HFD) was used to induce obesity in rats, and the effects of daily W101 feeding (8 weeks) were observed. Rats fed the HFD and supplemented with low-dose W101 (LW101, 15 mg kg\(^{-1}\) body weight day\(^{-1}\)) or high-dose W101 (HW101, 150 mg kg\(^{-1}\) body weight day\(^{-1}\)) had significantly reduced final body weight in comparison with that of the HFD group. W101 decreased the formation of lipid plaques in the aorta, reduced adipocyte cross-sectional area and diameter, and reduced levels of CCAAT/enhancer-binding protein β (C/EBPβ), peroxisome proliferator associated receptor γ (PPARγ), and C/EBPα. Regarding lipogenesis regulation in adipocytes, W101 suppressed heparin-releasable lipoprotein lipase (HR-LPL) in adipose tissues and inhibited lipid absorption, thereby reducing lipogenesis. *Lactobacillus paracasei* subsp. *paracasei* NTU 101-fermented soy milk may be used to develop health foods that prevent obesity.

Keywords: obesity, *Lactobacillus paracasei* subsp. *paracasei* NTU 101 (NTU 101), fermented soy milk, CCAAT/enhancer-binding protein (C/EBP), peroxisome proliferator associated receptor γ (PPARγ)
Introduction

Obesity, defined as abnormal lipid accumulation, is a health concern in developing and developed countries.\(^1\) At the cellular level, adipocyte number is increased by proliferation of preadipocytes in obese individuals, which is followed by differentiation of the cells into mature adipocytes, after which the cells accumulate triacylglycerol and become enlarged.\(^2\) During the differentiation process, increased CCAAT/enhancer-binding protein β and δ (C/EBPβ and C/EBPδ) activity induces transcription of C/EBPα and peroxisome proliferator activated receptor γ (PPARγ).\(^3\)

Therefore, inhibition of adipocyte differentiation and accumulation are important targets for preventing obesity and its associated conditions.

Probiotics are live microbial additions to the diet.\(^4\) During the past 3 decades, \textit{Lactobacillus} and \textit{Bifidobacterium} have received attention as probiotic organisms and have been associated with health-promoting effects, and have therefore been incorporated into a great range of dairy food products.\(^5\) We isolated a lactic acid bacterium from native newborn infant feces in Taiwan that was identified as \textit{Lactobacillus paracasei} subsp. \textit{paracasei} NTU 101 (NTU 101). The NTU 101 strain has probiotic characteristics, survives well at low pH, and tolerates high bile concentrations.\(^6\) NTU 101-fermented milk decreased serum and liver total cholesterol levels by 23.5-30.1% in Syrian hamsters with hypercholesterolemia.\(^7\) In addition,
mixtures of milk and soy milk fermented with NTU 101 prevented or slowed hyperlipidemia-induced oxidative stress and atherosclerosis. Studies show that the anti-obesity activity of milk/soy milk mixtures fermented with NTU 101 may result from increased levels of daidzein and genistein. Dietary supplement with NTU 101-fermented soy skim milk can attenuate bone loss in OVX mice and aging-induced BALB/c mice and possibly lower the risk of osteopenia or osteoporosis in aging. Moreover, NTU 101-fermented milk-soy milk prevents acute gastric ulcers by enhancing superoxide dismutase activity and prostaglandin E2 synthesis. We have also demonstrated that NTU 101-fermented milk has antihypertensive activity in spontaneously hypertensive rats. NTU 101 and their fermented products are protective factors against dental caries development. Taken together, the studies described above suggest that NTU 101 could be utilized in the development of functional fermented foods.

A recent publication showed greater beneficial effects from soy milk which containing complex combinations of nutrients and bioactive compounds. Soypro, a new soy milk fermented with lactic acid bacteria, decreased levels of low density lipoprotein cholesterol in rats with high-fat diet (HFD)-induced obesity and might partially inhibit adipocyte differentiation. In the present study, Sprague-Dawley (SD) rats with HFD-induced obesity were used to investigate the anti-obesity and
hypolipidemic effects of the water extract of \textit{Lactobacillus paracasei} subsp. \textit{paracasei} NTU 101-fermented soy milk (W101). To further elucidate the mechanism underlying the anti-obesity and hypolipidemic effects of W101, we measured obesity factors, including weight gain, feed efficiency (weight gain divided by food intake), fat pad weight, crude body fat, adipocyte number, lipolysis activity, heparin-releasable lipoprotein lipase (HR-LPL) activity, and expression of transcription factors related to adipose tissue.
Materials and methods

Chemicals

Acrylamide, aprotinin, ammonium persulfate, and β-nitrophenyl butyrate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Lactobacilli MRS broth and Bacto-agar were purchased from Difco Co. (Detroit, MI, USA). Non-genetically modified soybean (Glycine max (L.) Merrill BB50) was obtained from Chuan Gui Bio-Organic Co. (Taoyuan, Taiwan). Monoclonal mouse anti-actin monoclonal antibody was purchased from Chemicon International Inc. (Temecula, CA, USA). Monoclonal C/EBPα and C/EBPβ antibody were purchased from Cell Signaling Technology Inc. (Boston, MA, USA). Polyclonal PPARγ antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA).

Preparation soy milk and LAB-fermented soy milk

The soybeans were cleaned and soaked in the deionized water for 8 h at 25 °C. Grinding the swollen soybeans with water (1 : 8 w/w, dry soybean basis) in a food blender. The mixture was filtered through a defatted cotton sheet and recovered the soy milk. The soy milk was heated in a water bath at 90 °C for 1 h. L. paracasei subsp. paracasei NTU 101 was used in this study. The strain was cultured on MRS medium. The statement reinoculation of MRS broth with 1% LAB (9.06 ± 0.03 log10
CFU mL\(^{-1}\)) were inoculated into 1 L soy milk and cultured at 37 °C for 2 days. After fermentation, the product was freeze-dried (SDF-25 freeze-dryer, Chang Jung Business Co., Feng-Jen, Taiwan) in order to obtain the dry powders. The dry soy milk powder was extracted with water in a rotary shaker at 150 rpm for 1 h at 37 °C and then filtered through Whatman No. 42 filter paper.

**Animals treatment**

Thirty-five male Sprague Dawley rats (6-week old) were obtained from BioLasco Co. (Taipei, Taiwan), individually housed in stainless steel screen-bottomed cages, and allowed free access to standard laboratory chow (Ralston Purina, St. Louis, MO, USA) and water. Animals were subjected to 12 h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C (Protocol complied with guidelines described in the “Animal Protection Law”, amended on Jan. 17, 2001 Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, ROC). The rats were administered samples (1 mL) and randomly assigned to one of the following diets for 8 weeks: (a) normal chow diet (ND; 4.5% fat, 3.34 kcal g\(^{-1}\)), (b) high-fat diet was prepared in pellet form consisting of 26.7% butter powder (Gene Asia Biotech Co., Ltd., Nang-Tou, Taiwan) in normal chow (HFD group; 30% fat, 4.85 kcal g\(^{-1}\)), (c) positive control (PC group; 290 mg kg\(^{-1}\) body weight day\(^{-1}\)) + HFD,
(d) low-dose W101 (LW101 group; 15 mg kg\(^{-1}\) body weight day\(^{-1}\)) + HFD, and (e) high-dose W101 (HW101 group; 150 mg kg\(^{-1}\) body weight day\(^{-1}\)) + HFD. The Chai Li Won AliShan Oolong tea (Uni-President Vietnam Co., Ltd., Tainan, Taiwan) was a health food in Taiwan which has been shown to have anti-obesity effects (Health Food No. A00159). The positive control treatment was Chai Li Won AliShan Oolong tea (290 mg kg\(^{-1}\) body weight day\(^{-1}\)) which contained catechins (39.68 mg) and tea polyphenols (99.20 mg). Supplementation with catechins has been shown to suppress HFD-induced body fat accumulation by modulating lipid metabolism and reduce the risk of coronary disease.\(^{18-21}\)

During the experimental period, animals were allowed free access to food and water. At the end of the experimental period, the animals were sacrificed by carbon dioxide asphyxiation after fasting for 12 h. The visceral fat and subcutaneous fat pad were removed and weighed. Portions of the epididymal fat pad were immersed in 10% formaldehyde for histological inspection, and the other portions were frozen immediately in liquid nitrogen and stored at -80 °C for analysis of lipolysis and heparin-releasable lipoprotein lipase (HR-LPL) activity. The liver tissue was rinsed with sterile phosphate buffered saline (PBS) to remove blood, frozen on dry ice, and stored at -80 °C. The experiment was reviewed and approved by the Animal Care and
Research Ethics Committee of Fu Jen Catholic University (IACUC approved No: A10121).

Feed efficiency

The body weight and food consumption of each animal were recorded weekly. The feed efficiency was calculated for each rat using the following formula: grams of body weight gain per grams of total food intake × 100%.

Soudan IV stain of aortic plaque in artery

The aorta was dissected out, opened longitudinally from heart to the iliac arteries. The 2% Sudan IV was used to stain the lipid-rich lesions on the surface of aorta, and then washed with gradient methanol (100%, 90%, 80%, 70%, 60%) and PBS. Images were captured by a digital camera. The aortic surface area and its stained plaque area (red) were selected and analysed by the Posterize program of Photoshop 7.0 software (Adobe Systems Incorporated, San Jose, CA, USA). The aortic plaque percent (%) was calculated as the following formula: pixel of stained plaque area per pixel of whole aorta × 100%.

Determination of body fat
The fat pads were divided into three parts: visceral fat pad, subcutaneous fat, and crude carcass fat. And for the present study, the total visceral fat mass represents the sum of mesenteric, epididymal, and perinephric fat as Barzilai et al. (1997) showed; the subcutaneous fat were the sum of fat around the groin and lumbar; the carcass was defined as the entire shaved rat torso minus the visceral fat and subcutaneous fat. Following the method of Lima-Leopoldo et al. with slight modifications, carcasses were individually wrapped and frozen at -20 °C. At a later date, each carcass were dried at 80 °C for 4 h, followed by drying at 105 °C until constant weight (5-6 d, typically). The dried carcass were chopped into small pieces and grinded, and the crude carcass fats were quantified following the AOAC method. The percentage of body fat in each carcass was calculated by the following formula: grams of the crude carcass per fat grams of final body weight × 100%.

**HR-LPL activity assay**

The sample of epididymal fat pads weighting 0.1 g were placed in 1 mL of Krebs Ringer bicarbonate buffer (20 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgSO₄ • 7H₂O, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 2% BSA; pH 7.4) supplemented with 10 U/mL heparin at 37 °C for 1 h. LPL activity was measured according to the previous study on the basis of its esterase property using
p-nitrophenyl butyrate as a substrate.\textsuperscript{29} The TG hydrolase activity with synthetic TG substrates is inhibited by molar sodium chloride, and these properties have been used to distinguish LPL activity from the activities of other lipases in plasma.\textsuperscript{30,31} Therefore, HR-LPL activity was calculated from the productivity of p-nitrophenol using the following formula: 
\[ C (\mu \text{M}) = \frac{[A_{400}(0.15 \text{ M NaCl})-A_{400}(1 \text{ M NaCl})]}{0.012}, \]
where \(A_{400}(0.15 \text{ M NaCl})\) and \(A_{400}(1 \text{ M NaCl})\) are the absorbances of released p-nitrophenol at 400 nm in 0.15 M and 1 M NaCl assay buffer, respectively, and 0.012 is the micromolar extinction coefficient of p-nitrophenol.

**Adipose tissue histology**

The adipose tissue samples were fixed in buffered 10% formaldehyde solution and embedded in paraffin. The tissues in paraffin wax were cut into 5 µm sections, and then stained with haematoxylin and eosin for routine observations.\textsuperscript{32} The cross-sectional areas and diameter were examined via light microscopy (Opticphot-2; Nikon, Tokyo, Japan) and used a computerized image analyser (Leica Q500MC; Leica, Nussloch, Germany) for image analysis.

**Cecum lipid analyses**
The cecum were collected after sacrificing, the cecum content were extracted with methanol : chloroform (2 : 1, v : v) using a previously described method. The lipophilic layer from the extraction was collected and dried under vacuum. The cecum triacylglycerol (TG) was measured by commercial kits (TR-210 for TG, Randox Laboratories Ltd., Antrim, U.K.).

**Serum insulin, total cholesterol, and triacylglycerol measurements**

Serum insulin level was assayed by ELISA kit (Mercodia, Winston Salem, NC, USA). Serum TC and TG levels were determined by commercial kits (CH-201 for TC and TR-210 for TG, Randox Laboratories Ltd.).

**Statistical Analysis**

All experiments were performed on groups of seven animals. Data are presented as means ± standard deviation. The statistical significance in the biochemical effects was determined by one-way analysis of variance (ANOVA) using the general linear model procedure of SPSS 19.0 statistics software (Chicago, IL, USA), followed by ANOVA with the Duncan’s tests. All comparisons are made relative to HFD group, and the significant differences are indicated as *p < 0.05, **p < 0.01, ***p < 0.001, respectively.
**Results**

**Effect of W101 on body weight changes and feed efficiency**

The initial average body weight of the groups did not differ significantly ($p > 0.05$); however, the weight of the animals fed with HFD increased significantly throughout the experimental period (Fig. 1). The groups that received high-dose and low-dose W101 showed significantly suppressed weight gain after 2 and 8 weeks in comparison with the HFD group ($p < 0.05$). Furthermore, dietary supplementation of the HFD with Chai Li Won AliShan Oolong Tea, low-dose W101, and high-dose W101 significantly reduced final body weight after 8 weeks of feeding in comparison with that of the HFD group (by about 25%, 8%, and 13%, respectively; $p < 0.05$). The total food intake was significantly increased by the high-fat diet ($p < 0.001$) (Table 1). The HFD-induced gain in body weight was much more significant than that induced by a normal diet ($p < 0.001$), potentially because of the higher unit calorie level of the HFD (normal diet, 3.34 kcal g$^{-1}$; high-fat diet, 4.85 kcal g$^{-1}$). In order to normalize the change in weight to food intake, we calculated feed efficiency. The feed efficiency of the HFD group was twice that of the ND group, while the feed efficiency of the PC, LW101, and HW101 groups was significantly decreased in comparison with that of the HFD group ($p < 0.05$) (Table 1).
Effect of W101 on fat weight and fat cell size

As shown in Fig. 2, the visceral fat, subcutaneous fat, total fat weight, and crude carcass fat percentage in the HFD group were significantly elevated in comparison with those of the ND group (\(p < 0.001\)). Treatment with HW101 significantly decreased visceral fat, total fat, and crude carcass fat percentage in comparison with those of the HFD group (by about 28%, 26%, and 16%, respectively; \(p < 0.05\)).

The effects of the test substances on fat cell cross-sectional area and diameter are shown in Fig. 3. The cross-sectional area and diameter of fat cells in the epididymal fat pads were significantly increased in the HFD group (\(p < 0.001\) vs. the ND group). The HW101 group showed significantly reduced adipocyte cross-sectional area and diameter in comparison with the HFD group (\(p < 0.001\)) (Fig. 3). These results show that W101 inhibited adipocyte cell volume expansion in rats fed an HFD diet.

Effect of W101 on atherosclerotic plaques

Atherosclerotic plaques in the aorta cause the formation of atheromatous lesions in atherosclerosis. As illustrated in Fig. 4, aortic lipid plaque area was increased in the HFD-induced rats (about 7-fold that of the ND group). The formation of lipid plaques in the aorta was significantly decreased by LW101 and HW101 in comparison with
the rate observed in the HFD group (p < 0.05) (Fig. 4). And the standard deviation of LW101 was resulting in individual differences.

Effect of W101 on serum biochemical values

The serum total cholesterol (TC) and triacylglycerol (TG) contents of the HFD group were significantly greater than those of the ND group (p < 0.01) (Fig. 5A). In comparison with those of the HFD group, the TC and TG contents of the LW101 group were decreased by about 12% and 14%, respectively, while the TC and TG contents of the HW101 group were significantly decreased by about 23% and 21%, respectively (p < 0.01). As shown in Fig. 5B, supplementation with LW101 and HW101 significantly lowered TC and TG concentrations in the liver in comparison with those of the HFD group (p < 0.01). Although 8 weeks of HFD feeding did not result in fasting hyperglycemia in any of the experimental groups (data not shown), offspring receiving the HFD had fasted serum insulin concentrations significantly higher than those of rats fed a normal diet (p < 0.001) (Fig. 5C); however, dietary supplementation of the HFD with LW101 and HW101 significantly decreased serum insulin concentrations (p < 0.05). These data indicate that W101 might suppress the development of hyperlipidemia and hyperinsulinemia by regulating serum levels of lipids and insulin.
Effect of W101 on lipolysis activity, heparin-releasable LPL activity, and lipid content in cecum

Lipases hydrolyze TG in adipose tissue and impede lipogenesis. As shown in Fig. 6A, the HFD group had significantly increased glycerol release in comparison with that of the ND group (p < 0.05). The glycerol release of the LW101 and HW101 groups was decreased, as indicated by reduced the total fat weight (Fig. 2A) in HFD-induced rats.

Lipoprotein lipase is involved in import of TG-derived fatty acids by adipose tissue for storage. The LW101 and HW101 treatment groups showed significantly decreased HR-LPL activity (by about 25% and 23%, respectively), in comparison with that of the HFD group (p < 0.01) (Fig. 6B). Lipid content in the cecum is illustrated in Figs. 6C and 6D. Treatment with LW101 and HW101 significantly increased TC and TG contents in the cecum (p < 0.01 vs. the HFD group). These results show that W101 inhibited lipogenesis by reducing lipid content in cecum, in addition to suppressing HR-LPL activity in adipose tissue.

Effect of W101 on C/EBPβ, PPARγ, and C/EBPα protein levels

We used Western blots to test whether the reduction of epididymal fat pads in the rats treated with W101 was accompanied by changes in the expression of transcription
factors C/EBPβ, PPARγ, and C/EBPα. As shown in Fig. 7, W101 significantly and dose-dependently reduced C/EBPβ expression in comparison with that of the HFD group (p < 0.05), but no change was observed in the protein expression of C/EBPα and PPARγ. Studies investigating the importance of C/EBPβ and C/EBPδ have demonstrated that loss of one or both of these factors can lead to decreased adipose mass. Tanaka et al. (1997) demonstrated that the induction of C/EBPα and PPARγ does not always require C/EBPβ, but co-expression of C/EBPα and PPARγ is not sufficient for complete adipocyte differentiation in their absence. These data collectively suggest that supplementation with W101 attenuates HFD-induced body weight gain, which is attributable to fat mass reduction, possibly by reducing adipogenesis.

Discussion

Obesity disorders involved lipid accumulation in adipocytes following excessive caloric intake that exceeds the body's metabolic requirements, and such disorders increase mortality and morbidity due to numerous related diseases. White adipose tissue (WAT) and brown adipose tissue are the two morphologically and functionally distinct types of adipose tissue. WAT is able to store excess calories in the form of TG. When cells require energy, such as during periods of fasting, these needs are
largely met by fatty acids and glycerol formed from lipolysis of stored TG.\textsuperscript{40}

Therefore, the TG content within cells reflects the balance between lipogenesis and lipolysis, which also determines cell size.\textsuperscript{9}

The rodent HFD model has been used commonly to study visceral obesity because the pathogenesis of obesity in the model is similar to that in humans.\textsuperscript{38,41} The beneficial effect of lactic acid bacteria on metabolic syndrome in obese rodents has been reported recently. Administration of \textit{Lactobacillus gasseri} NT can reduce fat synthesis in HFD-induced mice,\textsuperscript{42} and the ability of \textit{L. paracasei} ST11 to reduce body weight and abdominal fat weight in rats has been shown.\textsuperscript{43} In addition, SM 101 causes decreases in feed efficiency, body weight, and body fat pad weight of 53.2\%, 49.7\% and 55.9\%, respectively.\textsuperscript{9} Moreover, NTU 101 and SM 101 also reduce the average radius of adipocytes and increase the number of small adipocytes.\textsuperscript{9,44}

We observed an increase in body weight in HFD rats (Fig. 1), which is a hallmark of obesity. We found that W101 improved HFD-induced body weight gain, feed efficiency, fat weight, body crude fat percentage, adipocyte diameter, and adipocyte cross-sectional area. Visceral fat accumulation is a major risk factor for several diseases, including diabetes, hyperlipidemia, hypertension, and arteriosclerosis.\textsuperscript{42} In the present study, we demonstrated that W101 decreased visceral fat weight in HFD-induced rats (Fig. 2). Lactic acid bacteria as novel probiotics alter
body adiposity,\textsuperscript{9,45} suggesting that the strain NTU 101, a probiotic, when it grown in soy milk may generate metabolites responsible for the effect observed in the rats.

The most important event in the onset of atherosclerosis-associated cardiovascular diseases is rupturing of atherosclerotic plaques.\textsuperscript{46} HFD feeding induces pronounced plaque formation throughout the aorta.\textsuperscript{47} Administration of W101 and PC inhibited aortic lipid accumulation in comparison with that of the HFD group (Fig. 4). We also showed that serum and liver lipids were decreased by W101 and PC treatment (Figs. 5A and 5B). It might be regulated by reducing in activities of enzymes related to hepatic fatty acid synthesis.\textsuperscript{48} Inhibition of HR-LDL activity in the fat pads and lipid content in the cecum of HFD-induced rats by the W101 treatment contributed to these decreases in lipid accumulation and absorption, and thus inhibited fat pad accumulation and reduced fat pad size (Figs. 6A, 6C and 6D).

Adipogenesis is highly regulated by two primary adipogenic transcription factors, PPARγ and C/EBPs.\textsuperscript{49} C/EBPβ is induced early to transactivate expression of PPARγ and C/EBPα, which are master transcription factors for terminal adipocyte differentiation.\textsuperscript{50} W101 inhibited C/EBPβ protein expression in the early stages of adipocyte differentiation and further protein expression of PPARγ and C/EBPα, thus reducing adipocyte differentiation. However, the PC group began inhibiting differentiation only at the PPARγ expression differentiation stage. Regarding
lipogenesis regulation in mature adipocytes, the PC group had increased glycerol release in comparison with the ND group and suppressed the HR-LPL activity, whereas W101 only decreased HR-LPL activity by reducing lipogenesis.

Conclusion

In conclusion, W101 improved HFD-induced obesity, hyperlipidemia and hyperinsulinemia. The results of this study show that W101 was able to significantly decrease body weight gain, feed efficiency, fat weight, body crude fat percentage, adipocyte diameter, and adipocyte cross-sectional area. This inhibitory effect was dependent on the actions of W101 regulation of differentiation, in which W101 inhibited C/EBPβ protein expression in the early stages and further decreased C/EBPα expression. Regarding lipogenesis regulation in mature adipocytes, W101 increased lipase activity and decreased HR-LPL activity, thereby reducing lipogenesis (Fig. 8).

Furthermore, W101 had a significant effect on suppressing lipid content in cecum. W101 improved hyperlipidemia and hyperinsulinemia by attenuating the insulin levels and atherosclerotic plaques in the aorta in HFD-induced rats. Our results suggest that W101 may be used to develop health foods to prevent obesity.
We would like to express our gratitude to Mr. James Chang, Chuan Gui Bio-Organic Co., who kindly provided soybean (Glycine max (L.) Merrill BB50).
Conflict of Interest

The authors declare no competing financial interest.
References


**Figure legends**

**Fig. 1.** Effects of LW101 and HW101 on body weight in male SD rats fed a high-fat diet. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei NTU 101;* HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.

**Fig. 2.** Effects of LW101 and HW101 on the visceral fat, subcutaneous fat, total fat pad (A), and crude carcass fat percentage (B) in male SD rats fed a high-fat diet. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei NTU 101;* HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.

**Fig. 3.** Effects of LW101 and HW101 on fat cell size by histopathologic (A), diameter (B), and cross-sectional area (C) of male SD rats fed a high-fat diet. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea);
LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.

**Fig. 4.** Effects of LW101 and HW101 on atherosclerotic plaques are indicated by the red dye in the graph (A), and the proportion of the area taken up by the atherosclerotic plaques in the aorta of male SD rats fed a high-fat diet (B). ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.

**Fig. 5.** Effects of LW101 and HW101 on serum and liver lipid parameters (A, B) and serum insulin (C) of male SD rats fed a high-fat diet. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; HW101: high-dose of water extract of soy milk fermented by *L. paracasei*
subsp. *paracasei* NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.

**Fig. 6.** Effects of LW101 and HW101 on lipolysis activity (A) and heparin-releasable LPL activity in fat pads (B); the cecum TC (C) and TG (D) in male SD rats fed with HFD. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.

**Fig. 7.** Effects of LW101 and HW101 on C/EBPβ, PPARγ, and C/EBPα protein expressions in adipose of male SD rats fed a high-fat diet. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.
Fig. 8. Proposed mechanism of W101 of lipogenesis in male SD rats fed a high-fat diet.
Table 1. The effects of LW101 and HW101 on total food intake, body weight gain, and feed efficiency in male SD rats fed a high-fat diet

<table>
<thead>
<tr>
<th></th>
<th>Total food intake (kilocalories)</th>
<th>Weight gain (g)</th>
<th>Feed efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>5634.7 ± 306.0***</td>
<td>179.7 ± 21.4***</td>
<td>10.7 ± 1.2***</td>
</tr>
<tr>
<td>HFD</td>
<td>8101.2 ± 357.2</td>
<td>350.0 ± 52.8</td>
<td>21.0 ± 3.1</td>
</tr>
<tr>
<td>PC</td>
<td>8014.8 ± 337.9</td>
<td>184.1 ± 27.4***</td>
<td>11.1 ± 1.6***</td>
</tr>
<tr>
<td>LW101</td>
<td>8097.3 ± 834.9</td>
<td>290.2 ± 55.4*</td>
<td>17.5 ± 3.6*</td>
</tr>
<tr>
<td>HW101</td>
<td>8061.2 ± 136.3</td>
<td>267.4 ± 41.3**</td>
<td>16.1 ± 2.3**</td>
</tr>
</tbody>
</table>

The rats were administered samples (1 mL) for 8 weeks. ND: normal chow diet; HFD: high-fat diet; PC: positive control (Chai Li Won AliShan Oolong tea); LW101: low-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101; HW101: high-dose of water extract of soy milk fermented by *L. paracasei* subsp. *paracasei* NTU 101. *p < 0.05, **p < 0.01, ***p < 0.001, significantly different from HFD group.
Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8.