A Novel Antidiabetic Food Produced via Solid-State Fermentation of Tartary Buckwheat using *L. plantarum* TK9 and *L. paracasei* TK1501

Running Title: Antidiabetic Food from Buckwheat Fermented by Probiotics

Lei Feng¹, Yufeng Xie¹, Chenmiao Peng, Yuxiaoxue Liu, Haikuan Wang*  
State Key Laboratory of Food Nutrition and Safety, College of Biotechnology, Tianjin University of Science and Technology, Tianjin, People’s Republic of China

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**SUMMARY**

Diabetes is a chronic metabolic disease characterized by hyperglycemia and a number of potential complications that significantly reduce the patient’s quality of life. In this study, an antidiabetic functional food was produced from Tartary buckwheat (TBW) fermented using *L. plantarum* TK9 and *L. paracasei* TK1501. The results of an orthogonal experimental design indicated the three factors with the largest effects on the growth of *L. plantarum* TK9 and *L. paracasei* TK1501 in solid-state fermentation (SSF) were in the order: water ratio > inoculum size > time of fermentation. Under the optimal fermentation conditions comprising a 1:1.5 water ratio, 24h of SSF and a

¹ These authors contributed equally to this work  
*Corresponding author: E-mail: hkwang@aliyun.com

ORCID IDs: 0000-0003-0746-3174 (Feng), 0000-0001-6373-3023 (Xie), 0000-0002-1815-8055 (Peng), 0000-0001-5432-8595 (Liu), 0000-0001-7548-6146 (Wang)
1·10⁷ CFU/g inoculum, the TBW fermented with *L. plantarum* TK9 and *L. paracasei* TK1501 yielded viable probiotic counts of (2.26±0.68)·10⁹ and (3.34±0.35)·10⁹ CFU/g, respectively. The nutritional potential, as well as antioxidant and antidiabetic properties of ethanolic extracts the from fermented TBW were investigated. The highest α-glucosidase inhibitory activity, with an IC₅₀ of 0.51 mg/mL, was observed in fermented TBW with *L. plantarum* TK9. However, fermented TBW with *L. paracasei* TK1501 was characterized by the highest DPP-IV inhibition, with an IC₅₀ of 2.47 mg/mL. Therefore, both fermentation with *L. plantarum* TK9 and *L. paracasei* TK1501 has the potential to yield a product that can help regulate the levels of blood glucose as part of a diabetic diet.

**Key words:** tartary buckwheat, fermentation, α-glucosidase inhibition, dipeptidyl peptidase IV inhibition, antidiabetic

**INTRODUCTION**

Diabetes is understood to constitute a metabolic disorder characterized by chronic hyperglycemia due to either insufficient insulin production (Type 1 diabetes) or insulin resistance (Type 2 diabetes mellitus; T2DM) (1). It is predicted that the global number of Type 2 diabetes (T2D) patients will exceed 300 million by 2030 (2). Dietotherapy is the most basic therapy to diabetes, which makes the production of food suitable for diabetics especially important.

In recent years, there is renewed interest in the utilization of TBW (*Fagopyrum tataricum* (L.) Gaertn.) due to its antihyperglycemic benefits (3). This antidiabetic effect of TBW is related to its unique chemical composition (4). It was discovered that
TBW possesses higher concentrations of certain bioactive phytochemicals than common buckwheat (5). Moreover, the total dietary fiber content of TBW seeds was found to be 26 %, with 0.54 % soluble and 24 % insoluble fibers (6). Furthermore, TBW is an important source of phenolic acids and flavonoids. The phenolic acids identified in TBW include p-hydroxybenzoic, protocatechuic, caffeic, chlorogenic, gallic, ferulic, p-coumaric, syringic, and vanillic acid (7). Flavonoids are the most crucial component of total buckwheat polyphenols as well as the most important health-promoting factors. In TBW sees, rutin is the major flavonoid, followed by quercetin (8). Flavonoid compounds have remarkable antioxidant effects, providing various health benefits such as anti-hypertension, anti-diabetic and anti-cancer effects (9). TBW is an excellent source of these phytochemicals, and it is in fact bitter due to its large flavonoid content.

Diabetes has become a major disease worldwide, and it can lead to serious complications, causing enormous damage to quality of life and health. The main target in the treatment of diabetes is the control of blood glucose levels (10). Several contemporary classes of hypoglycemic agents seem to provide an effective treatment for diabetes (11). Among them, inhibitors of dipeptidyl peptidase IV (DPP-IV) are becoming a hot topic in the development of new hypoglycemic drugs (12). The glucagon-like peptide-1 (GLP-1) can have a longer half-life due to DPP-IV inhibition, and reach the threshold of glucagon homeostasis (1). A number of synthetic DPP-IV inhibitors have shown promising results in the treatment of T2D (13). The α-glucosidase inhibitors, being the best-tolerated antidiabetics, have a main effect of reducing α-glucosidase in the microvilli in order to delay the absorption of glucose and fructose, lower the postprandial blood glucose spikes and reduce the
requirement for insulin injections (12, 14). However, such drugs have side effects, such as flatulence and diarrhea, and there is a demand for fermented food with DPP-IV inhibitory and α-glucosidase inhibitory properties for the oral treatment of T2D patients (15).

SSF has received much interest recently as an alternative to the more costly submerged fermentation (SmF) because of its potential to convert inexpensive agro-industrial solid residues and plant matter into a variety of valuable products (16). This research uses TBW as substrate for SSF of probiotics, with the aim to increase the contents of functional components as well as the nutritional and health-promoting value of the final product by complex metabolic reactions of microorganisms. Therefore, the objective of this study was to evaluate the effect of SSF on the chemical composition of TBW, as well as on its antioxidant and antihyperglycemic activities.

MATERIALS AND METHODS

Microorganisms and materials

The microorganisms were deposit in the China General Microbiological Culture Collection Center in Beijing. The CGMCC accession number of L. plantarum TK9 is 11891, and that of L. paracasei TK1501 is 13130.

The TBW samples used in this study were manufactured by Chinese Yunnan Province. TBW grains were cleaned and stored in darkness in polyethylene containers at room temperature.

Preparation of microbiological cultures
L. plantarum TK9 and L. paracasei TK1501 were activated in 10 mL of MRS broth (Oxoid, Basingstoke, UK) at 37 °C for 18 h, using 1 % inocula. The A\textsubscript{600} of resulting culture of L. plantarum TK9 was between 1.0-1.2, with viable counts of (3.50±0.35)×10\textsuperscript{8} CFU/mL; the A\textsubscript{600} of L. paracasei TK1501 was between 1.4-1.6, with viable counts of (5.50±0.40)×10\textsuperscript{9} CFU/mL. The cultures were centrifuged (GL20A, Xiangyi, Hunan, China) at 5000×g for 10 min, the supernatants were discarded, and the bacterial cells resuspended in sterile saline solution and adjusted to 1×10\textsuperscript{9} CFU/mL. The thus obtained suspensions were applied as inocula for SSF.

Optimization of fermentation conditions using orthogonal experimental design

Table 1 shows the influence factors and level values selected in this study. The Orthogonal method was used to help analyze the performance of the fermented TBW and determine the level (water ratio, inoculum size, time) of influence of factors affecting the total viable counts of the probiotic bacteria.

TBW grain substrate (40g) and sterile distilled water (by mass per volume) were mixed in conical flasks (250 mL, Deschem, Changshu, China) as shown in Tables 2 and 3, autoclaved at 121 °C for 20 min, and cooled to 37 °C before the addition of the required amounts of L. plantarum TK9 or L. paracasei TK1501 starter cultures. The inoculated substrates were fermented in a 37 °C incubator (SHKE6000-1CE, Thermo Fisher Scientific, Waltham, USA). Afterwards, samples from the the optimal combinations were freeze-dried using a DW3 freeze dryer (Heto-Holten A/S, Denmark) and stored at -20°C for further analysis. The native unfermented samples (inoculated with the same volume of sterile saline) collected at 0 h were used as the
negative control. SSF was performed in triplicate. SPSS software version 22.0 was used to perform the statistical analysis (17).

Table 1

Table 2

Table 3

Determination of the total viable counts

Total viable counts of bacterial cells were assessed according to the method described by Zhang et al. (18). The fermented TBW (10 g) was homogenized for 15 s in 90 mL of sterilized physiological saline (0.85 %) in a blender (JT-C, Jintian, Luohe, China). Total viable counts of *L. plantarum* TK9 and *L. paracasei* TK1501 were made using a pour plate method and MRS agar (Oxoid, Basingstoke, UK) after serial dilution in maximum recovery diluents. Serial dilutions were prepared in sterilized physiological saline and 1 mL of the appropriate dilution was poured on plates in triplicate. The poured plates of *L. plantarum* TK9 were incubated at 37 °C for (48±2) h. The cultures of *L. paracasei* TK1501 were incubated at 37 °C for (60±2) h. The colonies were then counted, and the viable counts were expressed as colony forming units per gram (CFU/g) of the sample.

Preparation of extracts

The freeze-dried samples from the optimal combination in the orthogonal experiment and the negative control were ground to a powder using an M20 Universal Mill (IKA, Staufen, Germany). Then, 10 g of the freeze-dried SSF-powder was
extracted with 200 mL of 70 % (v/v) ethanol for 2 h in an ultrasonic extractor (KH-600TDV, Hechuang, Kunshan, China). Afterwards, the samples were centrifuged (Multifuge X1R, Thermo Fisher Scientific, Waltham, USA) at 25,155×g and 4 °C for 10 min, and the supernatants collected. The residue was then suspended in 100 mL of 70 % (by volume) ethanol, ultrasonicated and centrifuged under the same conditions. The supernatants were combined, filtered through Whatman No. 1 paper (GE Healthcare, USA) and freeze-dried. An aliquot comprising 5 mg of the freeze-dried sample was stored at -20 °C and dissolved in 1 mL of phosphate buffer (0.1 M, pH=6.8) immediately before analysis.

Chemical analysis

Determination of the total phenolics content

The content of total phenolics in the extracts was determined by a modified Folin-Ciocalteu method (19). Briefly, 100 μL of each diluted extract was mixed with 500 μL of Folin-Ciocalteu reagent (Sangon Biotech, Shanghai, China) and 6 mL of distilled water, and shaken for 1 min. Afterwards, 2 mL of a 15 % (by mass per volume) Na₂CO₃ (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) solution was added to the mixture, shaken once again for 2 min, and the volume of the solution adjusted to 10 mL with distilled water. Finally, the mixture was incubated in the dark for 2 h at room temperature. The absorption at 750 nm was recorded on an InfiniteM200 PRO multifunctional microplate reader (Tecan, Männedorf, Switzerland) against a solution without sample as blank (100 μL of 70 % ethanol instead of the test samples). A standard curve /1/ was prepared using gallic acid (Sigma-Aldrich, St. Louis, USA). Samples were independently analyzed in triplicate and the TPC was
expressed as milligrams of gallic acid equivalents (GAE) per gram of extract (mg GAE/g).

\[ A = 0.4789c \pm 0.0303 \quad \text{/1/} \]

where \( A \) is the absorbance at 750 nm and \( c \) is the concentration (\( c = 0.2 - 1 \) mg/mL, \( R^2 = 0.9978 \)).

**Determination of the total flavonoid content**

Determination of the total flavonoid content (TFC) followed a published colorimetric method (20) with slight modifications as follows: The mixture included 0.3 mL NaNO\(_2\) (Damao chemical reagent factory, Tianjin, China) solution (5\% by mass per volume), 0.6 mL AlCl\(_3\) (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) solution (10\%, by mass per volume), 2 mL NaOH (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) solution (1 M) and 1 mL diluted sample solution. The volume of the mixture was adjusted to 10.0 mL with distilled water. After 5 min of incubation at room temperature, the absorption at 507 nm was recorded against the mixture without sample solution as a blank. The TFC was expressed as rutin (Yuanye Biological Technology, Shanghai, China) equivalents (mg rutin/g) using the calibration curve /2/ of rutin.

\[ A = 0.636c \pm 0.0388 \quad \text{/2/} \]

where \( A \) is the absorbance at 507 nm and \( c \) is the concentration (\( R^2 = 0.9974 \)).

**Determination of \( \alpha \)-glucosidase inhibition**

The \( \alpha \)-glucosidase inhibition of the samples was assessed according to the method described by Zhu et al. (12) with slight modifications. Briefly, the reaction
mixture contained 25 μL of 10 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) (Sigma-Aldrich, St. Louis, USA) and 25 μL of the sample pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of 50 μL α-glucosidase solution (0.16 U/mL, Sigma-Aldrich, St. Louis, USA) diluted with 0.1 M phosphate buffer, and incubated at 37 °C for 30 min. The reaction was terminated by adding 100 μL of 0.1 M Na₂CO₃. The enzymatic activity was quantified based on measurements of the absorbance of the samples at 405 nm on a Multiskan MK3 plate reader (Thermo Fisher Scientific, Waltham, USA). Each test sample was analyzed in technical triplicate, and the absorbance values were corrected against sample blanks in which α-glucosidase was replaced with phosphate buffer. The positive control (α-glucosidase activity with no inhibitor) and negative control (no α-glucosidase activity) were prepared by substituting phosphate buffer instead of the sample or instead of the sample and the α-glucosidase solution, respectively. The α-glucosidase inhibition rate (α-GIR) was calculated as:

\[
α - \text{GIR} (\%) = \left( 1 - \frac{A(\text{sample}) - A(\text{blank})}{A(\text{positive control}) - A(\text{negative control})} \right) \times 100
\]

**Determination of DPP-IV inhibition**

DPP-IV inhibitory activity was assessed according to the method described by Zeng et al. (12) with some modifications. Briefly, 25 μL Gly-Pro-p-nitroanilide (6 mM, Sigma-Aldrich, St. Louis, USA) and 25 μL TBW sample (or 25 μL PBS as a control) were mixed and pre-incubated at 37 °C for 10 min. The reaction was initiated by adding 50 μL DPP-IV from porcine kidney (3·10⁻⁴ U/L, ≥10 U/mg protein) (Sigma-
Aldrich, St. Louis, USA) and the mixture was incubated at 37 °C for 60 min. The reaction was terminated by adding 100 μL 1 M sodium acetate buffer (pH=4.0), and the absorbance of the samples at 405 nm was measured on a plate reader. Each sample was analyzed in technical triplicate, and the absorbance values were normalized to sample blanks in which DPP-IV was replaced with Tris–HCl buffer (0.1 M, pH=8.0). The negative control (no DPP-IV activity) and positive control (DPPIV activity with no inhibitor) were prepared by substituting Tris–HCl buffer (100 mM, pH=8.0) instead of the sample or instead of the DPP-IV solution and the sample, respectively. Diprotin A (Sigma-Aldrich, St. Louis, USA) was used as a standard inhibitor. The DPP-IV inhibition rate (DIR) was calculated as follows:

\[
DIR(\%) = \left(1 - \frac{A(\text{sample}) - A(\text{sampleblank})}{A(\text{positivecontrol}) - A(\text{negativecontrol})}\right) \times 100
\]

RESULTS AND DISCUSSION

Statistical analysis of the results obtained using orthogonal experimental design

The most important factors to be considered in the development of a functional food are the bioactive components. In this study, a solid-state-fermented TBW-based probiotic product was developed, and hence the final viable probiotic cell count should be considered. Thus, the fermentation parameters were optimized in order to attain a high growth rate of the lactic acid bacteria (LAB) in TBW. We designed an orthogonal experiment with three factors and four levels (Table 1). The results were utilized to find the optimal fermenting conditions and analyze the relationship between the factors and systemic performance of the fermentation (Tables 2 and 3).
Ki represents the sum of the corresponding test results when the level number is i for any factor, and ki= K_i/4 (4 is the number of levels). The larger the R value (R= K_{max}- K_{min}), the greater the influence of this factor on the viable count. We found that the R value of the water ratio (A), inoculum size (B), time of fermentation (C) for TK9 and TK1501 was R=0.345, 0.305, 0.330 and R=0.6575, 0.4375, 0.5025, respectively. The effect of the factors on the number of viable bacteria was in the order A>C>B. Thus, the optimal fermentation conditions were a 1:1.5 water ratio, a 24 h fermentation period and 1·10^7 CFU/g inoculum size (A1C2B2). Subsequently, analysis of variance (ANOVA) was used to determine the significance of the model (Tables 4 and 5). The p-values were used as a tool to check the significance of each coefficient; the smaller the p-value, the bigger the significance of the corresponding coefficient. The corresponding p-values of <0.05 suggested that the water ratio, inoculum size, and time of fermentation are significant terms. Therefore, a small variation of their values will alter the total viable count comparatively much. Under the optimal conditions, the highest viable counts of TBW fermented with *L. plantarum* TK9 and *L. paracasei* TK1501 was (2.26±0.68)·10^9 CFU/g and (3.34±0.35)·10^9 CFU/g, respectively.

Total phenolics content during TBW fermentation

During the fermentation of TBW with LAB, the composition of the TBW changes under the influence of bacterial metabolism. Hence, fermentation affected the bioactive constituents (Table 6). In TBW extracts TPC increased from (243.05±13.96)
mg GAE/g dry extract in the native unfermented sample to (251.8±10.37) mg GAE/g in the extract from *L. plantarum* TK9-fermented TBW. The TPC of *L. paracasei* TK1501-fermented TBW was (241.5±5.42) mg GAE/g dry extract, without significant changes compared to the native unfermented material. A similar TPC in TBW had been reported by Ran et al. (21) and Zhu et al. (5). Those results might be explained by the fact that the levels of bioactive compounds can be modified during fermentation by the metabolic activity of microbes (22). Some reports stated that fermentation with *L. plantarum* is an efficient process for increasing the concentration of phenolic compounds in fermented cowpea flour (23, 24). The β-glucosidase enzyme produced during fermentation is thought to catalyze the hydrolysis of complex polyphenols to yield simpler and biologically more active compounds, leading to an increase of the total phenolic content (25). In addition, some reports suggested that the higher antioxidant activity might be due to the presence of phenolic components (26).

~Table 6~

**Total flavonoids content during TBW fermentation**

The TFC of the different samples is shown in Table 1. The TFC of the native unfermented TBW sample, as well as the samples fermented with *L. plantarum* TK9 and with *L. paracasei* TK1501 was (25.54±1.141), (25.644±1.428) and (25.67±0.406) mg/g, respectively. No significant differences in the TFC were found between the native unfermented TBW and the preparations fermented with *L. plantarum* TK9 and *L. paracasei* TK1501. Importantly, this also means that there was no loss of TFC
during fermentation. Moreover, recent reports stated that the TFC of TBW is much higher than that of common buckwheat (12, 27).

**Inhibition of α-glucosidase activity**

Diabetes is a chronic metabolic disorder mainly characterized by high blood glucose levels. Therefore, treatment of diabetes mainly focuses on reducing the fluctuations of blood glucose in order to reduce the subsequent complications. This has led to the development of α-glucosidase inhibitors as oral anti-diabetic agents that can postpone the digestion and absorption of carbohydrates, and thus reduce postprandial hyperglycemia, which are are widely used in treatment of diabetes (28).

In this study, the extracts of TBW fermented using the two different strains were able to inhibit α-glucosidase in a concentration-dependent manner (Fig. 1). All tested TBW extracts at a concentration of 1 mg/mL showed α-glucosidase inhibitory activities ranging from 29.25 to 75.82 %, with the higher value obtained using TBW fermented with *L. plantarum* TK9. This extract at 0.008, 0.04, 0.1, 0.2, 0.4, 0.8, and 1.0 mg/mL displayed α-glucosidase inhibitory activities of (0.03±0.02), (0.32±0.15), (2.28±0.35), (20.23±1.62), (42.75±0.34), (68.91±0.05) and (75.82±0.15) %, respectively, with an IC$_{50}$ value of 0.51 mg/mL. By contrast the extract of native TBW at the same concentrations had inhibitory activities of (1.54±7.82), (4.91±1.59), (5.73±4.01), (17.85±2.19), (22.63±1.47), (44.12±1.33) and (60.54±0.64) %, respectively, with an IC$_{50}$ value 0.87 mg/mL. The extract fermented with *L. paracasei* TK1501 was even less effective, with respective inhibitory activities of (2.73±5.89), (5.69±3.67), (7.17±1.58), (7.11±1.58), (10.41±5.73), (17.11±6.95) and (29.25±0.48) %, with an IC$_{50}$ value >1 mg/mL. The IC$_{50}$ value of the positive control (acarbose) for α-
glucosidase inhibition was 0.85 mg/mL (26). Earlier studies have reported that TBW inhibits α-glucosidase (29). Based on the relationship between bioactive components and α-glucosidase inhibition, TFC and TPC are considered to play an important role in the inhibitory activity of TBW (21). Some reports have suggested that TFC is related to α-glucosidase activity inhibition (30). Moreover, quercetin possesses a higher inhibitory activity on α-glucosidase than rutin (4). Therefore, we suspected that the L. plantarum TK9 fermentation process enhanced the quercetin content. It has already been confirmed that certain strains of Lactobacillus have inhibitory potential against a range of α-glucosidases and at least one β-glucosidase (31). It was found that compared to L. paracasei TK1501, L. plantarum TK9 fermented product has a significantly higher inhibitory effect on α-glucosidase. The inhibition of intestinal α-glucosidase is a known strategy to regulate blood glucose (32). Although synthetic inhibitors of intestinal α-glucosidase have been widely used in the clinic, there are considerations concerning cost and side effects. Consequently, there is demand for alternatives from natural plant, animal, and probiotic sources (33-35), and many bacteria have been shown to have inhibitory activity. Cell-free supernatants of six strains of L. plantarum showed inhibitory activity ranging from 24.96 to 41.81 % (36). Notably, the strain TK9 investigated in this study was even more active than these six strains. Its comparatively high inhibitory activity suggested that TBW fermented with L. plantarum TK9 may have dual beneficial effects on glycaemia regulation by reducing the intestinal absorption of carbohydrates. Similarly, it has been reported that L. plantarum NCU116 and carrot juice fermented with NCU116 had the potential to regulate blood glucose levels (37). The TBW fermented with L. plantarum TK9 had very satisfactory α-glucosidase inhibitory activity, and it merits
commercial exploration as an efficient agent for the management of glucose metabolism.

~Fig. 1~

**Inhibition of DPP-IV activity**

Dipeptidyl-peptidase IV (DPP-IV) is a highly specialized aminopeptidase that appears to be a major physiological modulator of a number of regulatory peptides, neuropeptides and chemokines (38, 39). GLP-1 is a gastrointestinal hormone that can reduce the appetite, suppresses glucagon secretion, stimulate insulin secretion and reduce gastric emptying (13). It has been reported that GLP-1 levels were reduced after a mixed meal and an oral glucose load in patients with T2D (40). GLP-1 is rapidly metabolized by the enzyme dipeptidyl peptidase IV (DPP-IV) (41). Preliminary clinical data have shown the potential of DPP-IV inhibitors in treating T2D (42). The need to develop safe DPP-IV inhibitors has led to increased attention to natural sources. Recently, natural sources as diverse as dietary proteins, medicinal plants, and marine life have been confirmed to have DPP-IV inhibitory effects (43). All extracts used in our research showed DPP-IV inhibitory activity in a concentration-dependent manner. The TBW extracts (4 mg/mL) showed DPP-IV inhibitory potential, among which the extract of TBW fermented with *L. paracasei* TK1501 displayed the greatest inhibition (77.21±1.87) %, followed by *L. plantarum* TK9 (57.34±2.13) % and native non-fermented TBW extract (39.88±1.41) %. Fig. 2 shows the inhibitory effects of the three different TBW samples (native, *L. plantarum* TK9 and *L. paracasei* TK1501) at different concentrations (0.2-5 mg/mL), with IC₅₀ values >5, 3.45 and 2.47 mg/mL, respectively. Unlike the results of α-glucosidase inhibition, the extract of TBW
fermented with *L. paracasei* TK1501 showed the best inhibition of DPP-IV activity. The positive control diprotin A had an IC$_{50}$ of 6.36 μmol/L, and behaved as a competitive inhibitor, which was in agreement with the literature (12, 21). Our work represents the first report on the inhibition of DPP-IV activity by TBW. We speculated that effective inhibitory components might not be related to TPC or TFC in view of our previous results. Similarly, some reports suggested that the strains themselves were the most likely producer of the inhibitory compounds (1, 12, 21, 44). The results of this study suggest that fermented TBW has antihyperglycemic properties, and hence might provide a new dietotherapy food for the control of diabetes.

~Fig. 2~

**CONCLUSIONS**

In this study, two TBW samples fermented using pure cultures of *L. plantarum* TK9 and *L. paracasei* TK1501, respectively, were evaluated for their chemical composition as well as their antioxidant and antihyperglycemic activities, compared to native unfermented TBW.

There was little difference between fermented TBW and the native unfermented material regarding TPC and TFC. Moreover, the extracts of TBW fermented with *L. plantarum* TK9 and *L. paracasei* TK1501 showed remarkable α-glucosidase and DPP-IV inhibitory effects, respectively. Taken together, the data indicate good potential of fermented TBW for application in the production of antidiabetic functional foods.

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Table 1. Levels and factors affecting the SSF of TBW.

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<tr>
<td>Water ratio (by mass per volume)</td>
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<tr>
<td>Inoculum size/(CFU/g)</td>
<td>B</td>
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<td>Time/h</td>
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Table 2. Orthogonal in terms of different factor levels (values 1-4): the experimental results for factors A, B, C with the measured TBW + *L. plantarum* TK9.

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<th>B</th>
<th>C</th>
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<td>1.60·10⁹</td>
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<tr>
<td>11</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1.72·10⁹</td>
</tr>
</tbody>
</table>
Table 3. Orthogonal in terms of different factor levels (values 1-4): the experimental results for factors A, B, C with the measured TBW + L. paracasei TK1501.

| Experiment No. | A | B | C | Viable count*/
|---------------|---|---|---| (CFU/g) |
| 1             | 1 | 1 | 1 | $3.20 \cdot 10^9$ |
| 2             | 1 | 2 | 2 | $3.34 \cdot 10^9$ |
| 3             | 1 | 3 | 3 | $2.98 \cdot 10^9$ |
| 4             | 1 | 4 | 4 | $2.68 \cdot 10^9$ |

* Each value in the table represents the mean (n=3)
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>$2.70 \times 10^9$</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>$2.95 \times 10^9$</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>$2.47 \times 10^9$</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>$2.57 \times 10^9$</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>$2.44 \times 10^9$</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>$2.77 \times 10^9$</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>$2.85 \times 10^9$</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>$2.86 \times 10^9$</td>
</tr>
<tr>
<td>13</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>$1.72 \times 10^9$</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>$2.75 \times 10^9$</td>
</tr>
<tr>
<td>15</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>$2.75 \times 10^9$</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>$2.35 \times 10^9$</td>
</tr>
</tbody>
</table>

| K_1 | 12.2 | 10.06 | 11.35 |
| K_2 | 10.69 | 11.81 | 11.65 |
| K_3 | 10.92 | 11.05 | 10.74 |
| K_4 | 9.57 | 10.46 | 9.64 |

| k_1 | 3.05 | 2.515 | 2.8375 |
| k_2 | 2.6725 | 2.9525 | 2.9125 |
| k_3 | 2.73 | 2.7625 | 2.685 |
| k_4 | 2.3925 | 2.615 | 2.41 |

* Each value in the table represents the mean (n=3)
Table 4. Analysis of variance and regression analysis for *L. plantarum* TK9

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water ratio (A)</td>
<td>0.293</td>
<td>3</td>
<td>0.098</td>
<td>7.005</td>
<td>0.02</td>
</tr>
<tr>
<td>Inoculum size</td>
<td>0.199</td>
<td>3</td>
<td>0.066</td>
<td>4.763</td>
<td>0.05</td>
</tr>
<tr>
<td>Time (C)</td>
<td>0.220</td>
<td>3</td>
<td>0.073</td>
<td>5.266</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**SS**: Sum of square; **F**: variance ratio; **P**: probability

Table 5. Analysis of variance and regression analysis for *L. paracasei* TK1501

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water ratio (A)</td>
<td>0.873</td>
<td>3</td>
<td>0.291</td>
<td>9.785</td>
<td>0.01</td>
</tr>
<tr>
<td>Inoculum size</td>
<td>0.434</td>
<td>3</td>
<td>0.145</td>
<td>4.870</td>
<td>0.48</td>
</tr>
<tr>
<td>Time (C)</td>
<td>0.492</td>
<td>3</td>
<td>0.197</td>
<td>6.631</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**SS**: Sum of square; **F**: variance ratio; **P**: probability
Table 6. The chemical composition of native unfermented and fermented TBW.

<table>
<thead>
<tr>
<th></th>
<th>TPC/(mg GAE/g) *</th>
<th>TFC/(mg/g) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native TBW</td>
<td>243.05±13.96</td>
<td>25.54±1.141</td>
</tr>
<tr>
<td>TBW + <em>L. plantarum</em> TK9</td>
<td>251.8±10.37</td>
<td>25.64±1.428</td>
</tr>
<tr>
<td>TBW + <em>L. paracasei</em> TK1501</td>
<td>241.5±5.42</td>
<td>25.67±0.406</td>
</tr>
</tbody>
</table>

*TPC: Total phenolics content; TFC: Total flavonoids content. Each value in the table represents the mean ±SD (n=3), (P<0.05).

Fig. 1 α-Glucosidase inhibition activity of TBW ethanol extracts obtained from SSF. Each value corresponds to the mean of three independent replicates with error bars indicating the standard deviations.
Fig. 2 DPP-IV inhibition activity of TBW ethanol extracts obtained from SSF. Each value corresponds to the mean of three independent replicates with error bars indicating the standard deviations.