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Mixed fermentation of blueberry pomace with *L. rhamnosus GG* and *L. plantarum-1*: Enhance the active ingredient, antioxidant activity and health-promoting benefits

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ABSTRACT

Studies on the development of non-dairy probiotic foods and beverages are emerging. The optimal *Lactobacillus* and carbon resources were screened to improve the viability of probiotics in blueberry pomace. During fermentation, the total titratable acid and the viable counts were measured and peaked at 24 h, 15.75 mM and 11.59 Log CFU mL^{-1} respectively. Lactic acid content increased from 2.361 mg mL^{-1} to 6.334 mg mL^{-1}, while citric acid content was decreased significantly. Additionally, the antioxidant activity was improved, which may be attributed to the increase of total phenols and flavonoids up to 4629.21 μg GAE mL^{-1} and 404.99 μg RE mL^{-1}. Simulated gastrointestinal digestion *in vitro* showed that the total polyphenols were decreased slightly, while anthocyanins were increased. We also studied the cholesterol-lowing capability of fermented BPL and found that the cholesterol-clearance rate could reach 67.17%. Moreover, through mice weight-loaded swimming experiments, we observed that the physical strength of mice fed a fermented juice for one month was significantly better than that of the control group ($p < 0.05$). Therefore, this study provides a high value application of blueberry pomace and the fermented blueberry pomace with probiotics as a new type of probiotic food can furnish potentially value to human health.

1. Introduction

Nowadays, keeping a balanced and reasonable diet, especially those containing natural and healthy foods, has attracted more and more attention. Therefore, food industries are very aware of the importance of making available products with specific health benefits, most of them deriving from fruit, including blueberry (Martins et al., 2017).

Blueberries are widely known to be rich in phenols, including anthocyanins, flavonols, and proanthocyanidins (Wang et al., 2017). However, because blueberries are very vulnerable to mechanical damage, microbial decay and water loss, they are prone to deterioration, resulting in their short shelf life. (Paniagua et al., 2014). To solve these problems, researchers have used blueberries into a variety of processed products, including fruit juice and wine. Inevitably, a large amount of waste by-products, such as fruit residues, is yielded in the production process. For a long time, blueberry pomace has been underrated, although it is reported that it retains many anthocyanins and phenols. It has been demonstrated that blueberry pomace has a wide diversity of bio-activities such as antioxidant, antimicrobial, lipid-lowering, life-prolonging, anti-inflammatory, and cancer-preventive (De Souza et al., 2014; Folmer et al., 2014). So, it can be proved that blueberry pomace is a good ingredient for functional foods.

The biotransformation carried out by microorganisms, such as probiotics, is often used for the extraction and reuse of active ingredients. Probiotics, deemed to be live microorganisms, if administered in enough amount in the daily diet, may result beneficial to the host (Salmerón et al., 2015). Microorganisms must be alive and in large numbers, usually at doses of more than 10^9 cells per day (Prado et al., 2008). The great majority of probiotic strains belong to *Lactobacillus* and the *Bifidobacterium*, especially to the *Lactobacillus* genus (Stancu et al., 2014). The potential effect of probiotics has been extensively studied, and especially its cholesterol-lowering function has attracted wide attention (El-Gawad et al., 2005; Usman and Hosono, 2000). In addition to dairy products, more and more fermented beverage based

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on fruits and grains are marketed, which have solved two main weaknesses of lactose intolerance and cholesterol content with respect to fermented dairy products (Heenan et al., 2004). Those fruit-based fermented beverages not only play a part in nutrition, regulation of intestinal flora balance, but also have some other exceptional functions (He et al., 2016). For example, some studies showed that citrus fermented with *L. plantarum YIT 0132* could alleviate symptoms of perennial allergic rhinitis (Harima-Mizusawa et al., 2016). According to Zhu et al. (2016), the combination of blueberry and probiotics has a good curative effect on alcoholic fatty liver, while Johnson et al. (2013) found blueberry-blackberry fermented beverages exhibit excellent anti-inflammatory ability.

In view of the nutritional value of blueberry pomace and the pleasant taste and odor of some organic acids and aromatic substances produced during its fermentation, the aim of this study was to produce a fermented beverage with special nutritional value and antioxidant properties by optimizing strains, carbon sources and processing technology. The total phenols, flavonoids, anthocyanins and antioxidant capacity before and after fermentation have been compared. We also explored the cholesterol-lowering effect and the bio-availability of gastrointestinal digestion *in vitro*. In addition, the weight-loaded swimming experiment in mice was conducted to explore the anti-fatigue effect of the liquid fermented blueberry pomace.

2. Materials and methods

2.1. Sample

In this work, blueberry pomace from Wallin Blueberry Industry Co. Ltd, in Tsingdao City (China) was used. The pomace was squeezed out of blueberry wine, and the obtained blueberry pomace is a mixture, including sarcocarp, peel, seeds, and carpopodium. The dried blueberry pomace was ground by delete a small-scale vertical ultrafine pulverizer, filtered through a 100-mesh sieve and then stored at −40 °C for analysis.

2.2. Microbial cultures stock preparation

The following probiotics strains were graciously supplied by Laboratory of Functional Dairy Food and Probiotic Engineering (Ocean University of China): *Probiotic L. rhamnosus GG, L. plantarum-1, and L. plantarum-2*. All microbial cultures were stored frozen at −20 °C in MRS medium containing 20% glycerol in sterile screw cap tubes.

The strains were reactivated by means of double passages on MRS in an incubator at 37 °C when needed. All strains were obtained by centrifugation (4000 rpm, 5 min, 4 °C), washed twice with sterile 0.85% NaCl, and re-suspended to reach the final cell density of 0.60 that correspond to 9.00 Log CFU mL^{-1}. The cell density was measured at 600 nm. These cultures were inoculated for fermentation.

2.3. Screening of LAB

Single *Lactobacillus* strains, and mixed strains (every two of three *Lactobacillus* strains mentioned above) with the ratio of 1:1 (v:v) were added to blueberry pomace liquid (pomace and distilled water with the ratio of 1:20 (v:v)) respectively with a concentration of 7.5 Log CFU mL^{-1} and incubated at 37 °C for 24 h.

2.4. Optimization of carbon sources

Glucose, fructose, maltose and sucrose were added to blueberry pomace liquid (hereinafter referred to as BPL), which was inoculated with 7.5 Log CFU mL^{-1} of mixed LAB and incubated at 37 °C for 24 h.

2.5. Fermentation of blueberry pomace liquid

The BPL consisted of the blueberry pomace and distilled water in a ratio of 1:20 (v:v), involving carbon source. The initial pH of the BPL was adjusted to 6.2 with Na_2CO_3. After sterilization at 90 °C for 10 min, BPL was inoculated with 7.5 Log CFU mL^{-1} of LAB and incubated at 37 °C for 28 h. Viable cell counts, pH, the total titratable acid and other chemical composition of samples were determined every 4 h. Viable cell counts were obtained by the plate count method through diluting samples serially with sterile water. The pH of fermented BPL was measured by a digital pH meter (Mettler Toledo DELTA 320, Swiss).

2.6. Determination of active ingredient

2.6.1. Total titratable acidity and organic acids measurements

Total titratable acidity (TTA) was measured by potentiometric titration method, whose pH was adjusted to 6.2 with 0.1 M NaOH.

Quantitative analysis of organic acids (lactic acid, citric acid, acetic acid, oxalic acid, succinic acid) were carried out by HPLC (Agilent Technology, USA) equipped with a K–2600UV–visible detector at 210 nm and separated in Agilent ZORBAX-SB C18 column (4.6 mm × 250 mm, 5 μm) at 35 °C. The isocratic elution was performed with 97% 0.02 M KH_2PO_4 and 3% methanol as a mobile phase for 20 min at 0.5 mL min^{-1}. The samples were filtered and diluted by filtration using a cellulose ester acetate membrane.

2.6.2. Determination of total flavonols (TF)

Total flavonoid contents of the samples were determined according to the method of Xie et al. (2015). Rutin standard solution and samples were diluted to 5 ml with 30% ethanol solution in 10 mL plugged test tubes. 0.3 mL of 5% NaNO2 solution was added in turn and shaken for 5 min, and after that, 0.3 mL of 10% AI (NO3)3 solution was mixed and shaken for 6 min. Finally, 4 mL of 1.0 M NaOH solution was added, and the volume was fixed with 30% ethanol solution to 10 mL. The solution was shaken well and let rest for 10 min. Absorbance was measured at 510 nm. The TF content was calculated using the calibration curve of rutin solution and expressed as milligrams of rutin equivalent (RE) per milliliter of the sample.

2.6.3. Determination of total phenolic acids (TP)

TP of the samples were estimated using the method described by Porto and Natolino (2018). According to this method, 1 mL Folin-Ciocalteu reagent, 4 mL Na_2CO_3 (7.5%) and 4 mL water were added into the fermented beverage. After a 10-min incubation at 75 °C, absorbance was read at 765 nm. Gallic acid was used as standard, and the concentration of TP was expressed as weight of gallic acid equivalent (GAE) per milliliter of sample.

2.6.4. Determination of total anthocyanin (TA)

Based on the research of Porto and Natolino (2018), TA was determined by the differential pH method. A buffer solution with pH 1.0 was prepared with KCl and hydrochloric acid. Another buffer solution with pH 4.5 was prepared with NaAc and hydrochloric acid. The absorbance of the diluted sample was determined at 520 nm and 700 nm after adding 9 mL buffer solution with pH 1.0 and 4.5 respectively and heated in a water-bath at 40 °C for 40 min. The content of anthocyanin was calculated by the following formula:

$$\textit{the content of anthocyanin}\ (mg/L) = (A \times Mr)/(\varepsilon \times 1) \times Df \times 1000$$

$$A = (A520\ nm\ pH\ 1.0 - A700\ nm\ pH\ 1.0) - (A520\ nm\ pH\ 4.5 - A700\ nm\ pH\ 4.5)$$

Mr is the relative molecular mass of cypermethrin-3-glucoside (449.2); ε is the molar extinction coefficient of cypermethrin-3-glucoside (26900 mol^{-1}); Df is the dilution factor (total dilution multiple of

the sample).

2.7. Determination of the antioxidant activity

2.7.1. Total reducing power assay (TRP assay)

The reducing power was determined on the basis of the method of Ferreira et al. (2007). Different concentrations (500 μL) of the fermentation fluid, 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide were mixtured carefully and incubated at 50 °C for 30 min. After adding 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged at 650 rpm for 10 min. The upper layer (2.5 mL) was blended with 2.5 mL deionized water and 0.5 mL of 0.1% ferric chloride. The absorbance was determined at 700 nm.

2.7.2. Measurement of ferric reducing antioxidant power (FRAP assay)

The ferric reducing antioxidant power (FRAP) assay was modified slightly in accordance with the method described by Chang et al. (2018). FRAP working fluid was made up of 0.3 M acetate buffer solution (pH 3.6), 0.01 M 2,4, 6-tripyridine triazine and 0.02 M $FeCl_3.6H_2O$ in a ratio of 10:1:1 (v:v:v), and preheated by a water bath at 37 °C before use. After mixing 0.1 mL diluted fermentation liquid with 3 mL FRAP working fluid for 5 min, the absorbance value was detected at 595 nm against a blank containing all the reagents but the samples.

2.8. Simulated in vitro gastrointestinal (GI) digestion

The simulated gastric fluid (SGF): the pH of 0.2% NaCl solution was adjusted to 3.5, 2.5 and 1.5 with 0.1 M HCl, and then pepsin was added to the solution to ensure the final concentration was 3.0 g L^{-1}. The solution was filtered and sterilized with a 0.22 μm microfiltration membrane. The fermented BPL was mixed with SGJ at a ratio of 1:10 (v:v) and shaken at 37 °C for 2 h at 100 rpm min^{-1}. The number of viable bacteria, active ingredients, and antioxidant activity were measured later.

The simulated intestinal fluid (SIF): the pH value of 6.8% KH_2PO_4 solution was adjusted to 7.0 ± 0.1 with 0.1 M NaOH. By adding a certain amount of trypsin, the concentration of trypsin was 10 g L^{-1}, and a 0.22 μm membrane was used for filtration and sterilization. The fermented BPL was added into the SIF in a ratio of 1:10 (v:v) and shaken at 37 °C for 4 h at 100 rpm min^{-1}. The number of viable bacteria, active ingredients, and antioxidant activity were measured later.

The simulated bile salt (SBS): The concentration of MRS medium was 0.05%, 0.1%, 0.2%, 0.3% and 0.4% through adding porcine bile salt. Sterilization of SBS was obtained at 121 °C for 15 min for standby use. The fermented BPL in the proportion of 10% was added to the solution of various concentrations and incubated at 37 °C for 4 h. The viable cell counts were detected by the plate count method in MRS agar and expressed as Log CFU mL^{-1} (Valero-Cases and Frutos, 2015; Roberts et al., 2018).

2.9. Determination of cholesterol decrease in vitro

Cholesterol decrease *in vitro* was carried out with OPA, which was quoted from Liong and Shah (2005) with slight modification. Fermented BPL was inoculated into the MRS-THIO-OX-CHOL medium at 10% inoculation volume, and then cultured at 37 °C for 24 h. During the incubation, samples were collected at 0 h, 6 h, 12 h, or 24 h, centrifuged at 3500 rpm min^{-1} for 10 min. 0.25 mL of supernatant was added and fully shaked with 0.1 mL of 1 mg mL^{-1} phthalaldehyde solution. After 10 min of static reaction, 2 mL of mixed acid solution (H_2SO_4 mixed with glacial acetic acid in a ratio of 1:1 (v:v)) was added, and then the reaction solution was placed in 96-well plate for 10 min. OD value was measured at 550 nm by enzyme labeling instrument. The cholesterol content in fermentation juice was determined according to the fitting equation of cholesterol standard curve, and the final cholesterol removal rate (%) was determined according to the following formula.

$$V(\%) = (B - A)/B \times 100\%$$

A is the cholesterol content in the supernatant of each strain after fermentation (μg);
B is the cholesterol content in the supernatant of each strain before fermentation (μg).

2.10. Measurement of the anti-fatigue activity

2.10.1. Animals and treatment

Male C57BL/6J mice 12-week-old were purchased from Pengyue Experimental Animal Breeding co. LTD, in Jinan City (China). The mice (27 ± 1 g of the weight) were adapted to the animal laboratory for 1 week with a standardized environment of 22–24 °C, 45–65% humidity and a 12/12-h light-dark cycle. Experiment was conducted in accordance with the guidelines of the Animal Ethics Committee of Ocean University of China. All experiments met the requirements of the National Laboratory Animal Act (PR China).

2.10.2. Weight-loaded swimming test

After 1-week adaptation, the mice were randomly separated into three groups (6 mice each): blank control group (sterile water); positive control group (Red Bull, a famous anti-fatigue drink in China) and experimental group (fermented BPL). According to the design, the dosage of gavage was 10% of the body weight of each mouse per day for 4 weeks. The weight and food intake of mice were recorded every 3 days.

30 min after the last gavage, mice were placed in a plastic cask with a height of 30 cm and a diameter of 30 cm, containing 20 cm deep water (about 23 °C). A piece of play-dough, about 10% of the body weight, was loaded on the tail roots of mice. As soon as they entered the water, their limbs would struggle violently. After a period of time, the mice would give up and float with their heads over the water for breathing, accompanied by slight limb movements. An exhaustive swimming time was recorded as the time when rats failed to rise to the surface to breathe after 5 s (Lin et al., 2014).

2.11. Statistical analysis

All experiments and analysis were made in triplicate. The results were expressed as mean ± standard deviation. ANOVA and Duncan's multiple range test using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) were utilized for the determination of significant differences at $P < 0.05$.

3. Results and discussion

3.1. Screening of LAB

Development of new functional food demands several important factors to be considered; the most important of which is the bioactive component (Gupta et al., 2010). The three species of lactobacilli cultured in BPL added exogenously with 1% glucose showed an excellent growth with an inoculation amount of 2% (v/v) at 37 °C (Fig. 1). Obviously, BPL is an appropriate medium for the reproduction of these three probiotics. In all cases, the number of cells fermented for 24 h was higher than 10^{10} CFU mL^{-1}. Many other researchers found that the fermentation with mixed strains was better than that with a single strain. But in our study, the number of live bacteria in mixed fermentation group was slightly less than mono-strain fermentation, except for the combination of *L. rhamnosus GG* and *L. plantarum-1*. The compound culture technology of probiotics can not only promote the transformation of chemical composition, but also improve the sensory quality of the fermented juice. In addition, the symbiosis and/or synergism of different cultures are also available in some cases (Bujna et al., 2018).

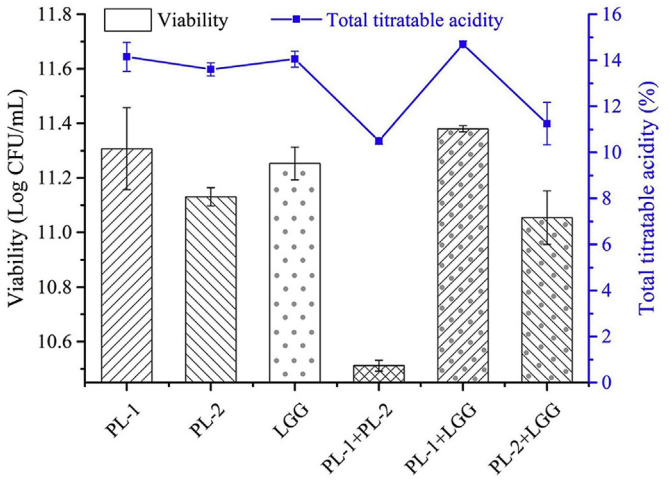

Fig. 1. Effect of *Lactobacillus* stains on fermentation.

Therefore, in this research, we selected the mixed fermentation by 1:1 (v:v) of *L. rhamnosus GG* and *L. plantarum-1*, although there was no significant difference of viability and acidity in L. plantarum-1 and the mixture ($P > 0.05$).

3.2. Optimization of carbon sources

For better growth of probiotics in BPL, carbon and nitrogen sources are usually added exogenously. We chose glucose and yeast extract as carbon and nitrogen sources to observe the growth of probiotics in these two environments. According to the pH and the titratable acid showed in Supplementary Table 1, the reproductive capacity of probiotics in the carbon source additive group was notably much stronger than that in the original solution and nitrogen source addition group. The previous results indicated that BPL supplemented with nitrogen source was unable to sustain the growth of probiotics because of insufficient nutrients. Therefore, we focused on studying the effect of the type and the dosage of carbon sources on the fermentation.

The results showed that viability and total titratable acidity (TTA) were markedly influenced by the type of carbon source (Fig. 2). It is widely known that carbon source is one of the six major nutrient elements. Lactic acid bacteria can produce acid by utilizing carbon sources, but their abilities to use carbohydrates are different. Sugar was used in the order of glucose, fructose, sucrose and maltose. It may be that sucrose and maltose need to be hydrolyzed into monosaccharides before use by bacteria, which leads to a low utilization rate of sucrose and maltose. As shown in Fig. 2, different contents of glucose in BPL and fermented at 37 °C for 24 h were checked: we found that the

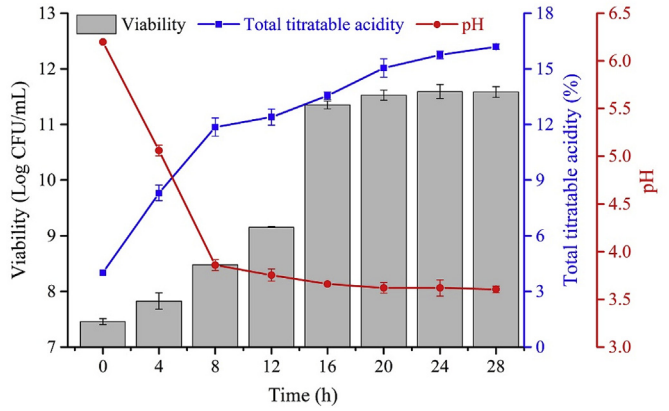

Fig. 3. Viability (A) of *L. rhamnosus* GG and *L. plantarum*-1, pH (B) and total titratable acidity (C) in fermented BPL during 28 h of fermentation.

number of viable bacteria grew up first and then decreased with the increase of glucose content. Especially when the amount of glucose reached 1.5%, the number of viable bacteria reached a maximum of 11.19 Log CFU mL^{-1}. At the same time, their pH and TTA showed the lowest and highest values respectively. As a consequence, 1.5% glucose was preferred as an excellent food additive for BPL fermentation.

3.3. Fermentation of blueberry pomace liquid

The above-mentioned optimum conditions were selected to evaluate the quality during the fermentation of BPL. As it is shown from the microbial reproduction in Fig. 3, from 0 h to 16 h, the number of live bacteria was in a state of crazy growth, increasing by 4 Log CFU mL^{-1}. After 16 h, microorganisms grew slowly; and the overall trends became stable after 24 h with the viable count approaching 11.5 Log CFU mL^{-1}. Kun et al. (2008) chose carrot juice as a raw material to produce probiotic food with *Bifidobacterium* strains (*B. lactis Bb-12, B. bifidum B7.1, and B3.2*), and 10^7 CFU mL^{-1} initial cell concentrations resulted in 10^8 CFU mL^{-1} after 6 h of incubation. Salmerón et al. (2015) reported that cell viability of cereal-based beverages fermented with three different lactobacilli strains (*L. acidophilus, L. plantarum,* and *L. reuteri*) at 37 °C for 10 h were all around 8.0 Log CFU mL^{-1}, which was markedly lower than that in our study. This may be due to the different utilization of fermentation substrates by different strains, resulting in different growth conditions. In a few words, BPL is an excellent medium for *Lactobacillus*.

In virtue of the metabolic activity of probiotics, short-chain fatty acids (SCFA) were produced, which increased the acidity of the medium, accordingly. After 12 h of fermentation, the pH of fermented

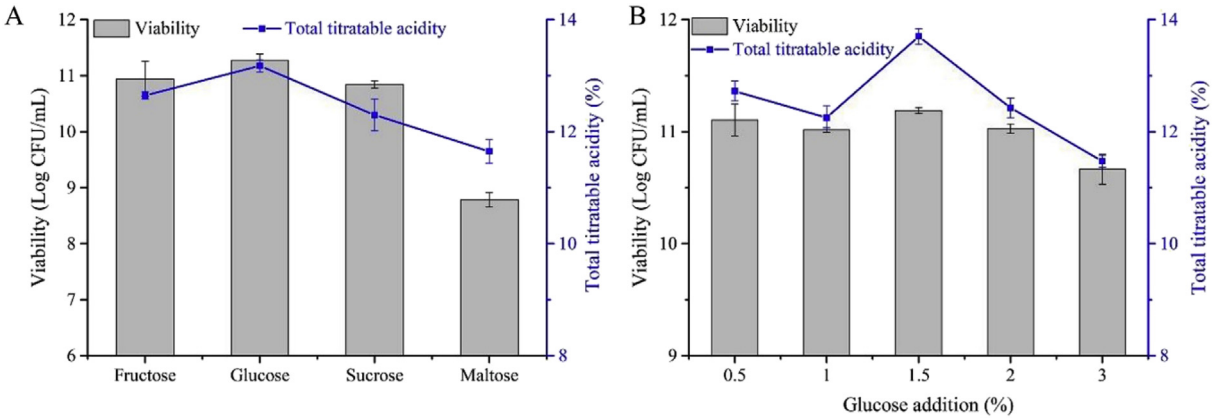

Fig. 2. Effect of supplementing with different carbon sources (A) and different concentration of glucose (B) on viability and total titratable acidity.

Table 1
Changes in the content of organic acid during fermentation.

Fermentation time (hours)	Organic acid (mg mL^{-1})				
	Lactic acid	Acetic acid	Tartaric acid	Oxalic acid	Citric acid
0	2.361 ± 0.062^{g}	0.809 ± 0.000^{g}	1.698 ± 0.008^{a}	0.660 ± 0.005^{a}	0.480 ± 0.039^{bc}
4	2.469 ± 0.001^{g}	0.809 ± 0.010^{g}	1.702 ± 0.006^{a}	0.751 ± 0.004^{a}	0.488 ± 0.002^{a}
8	3.508 ± 0.066^{f}	4.893 ± 0.206^{f}	1.664 ± 0.009^{b}	0.582 ± 0.024^{c}	0.393 ± 0.038^{d}
12	4.653 ± 0.058^{e}	8.451 ± 0.181^{e}	1.702 ± 0.007^{c}	0.696 ± 0.023^{a}	0.322 ± 0.002^{b}
16	4.965 ± 0.125^{d}	9.420 ± 0.387^{d}	1.678 ± 0.004^{d}	0.683 ± 0.010^{b}	0.243 ± 0.005^{b}
20	5.235 ± 0.306^{c}	10.261 ± 0.952^{c}	1.675 ± 0.005^{d}	0.640 ± 0.007^{b}	0.217 ± 0.045^{c}
24	5.861 ± 0.008^{b}	12.204 ± 0.026^{b}	1.679 ± 0.005^{e}	0.685 ± 0.053^{b}	0.027 ± 0.000^{b}
28	6.334 ± 0.010^{a}	13.378 ± 0.298^{a}	1.682 ± 0.007^{e}	0.660 ± 0.002^{b}	0.027 ± 0.000^{bc}

Data expressed as mean ± Standard deviation.
Means that do not share the same letter are significantly different at $p < 0.05$.

juice was dropped from 6.2 to 3.8 (Fig. 3), below which the growth of most food spoilage bacteria can be inhibited (Costa et al., 2013). On the contrary, TTA increased gradually along with fermentation and stabilized after 24 h (Fig. 3), which was mainly ascribed to the transformation of sugars into organic acids by lactic acid bacteria to reduce pH and increase acidity (Salmerón et al., 2015).

Organic acids are natural compounds in fruits, which have an important influence on the organoleptic properties and stability of fruit juice. In our research, the fermentation juice mainly contained acetic acid, lactic acid, tartaric acid, oxalic acid, and citric acid. As fermentation proceeded, production of acetic acid was more intensive than that of lactic acid; about 6.3 mg mL^{-1} lactic acid and 13.6 mg mL^{-1} acetic acid was detected (Table 1). Although it was said that acetic acid had an unpleasant flavour attribute, there was no pungent flavor in our production. Fugelsang (2007) described that lactic acid bacteria metabolize citric acid to produce lactic acid, diacetyl, acetoin, and acetic acid, which was onfirmed by the citric acid content decrease from 0.480 mg mL^{-1} to 0.027 mg mL^{-1} in our study.

Phenolic metabolites are known to have many potential benefits for human health, especially as strong antioxidants (Suntornsuk et al., 2002). According to Table 2, the total phenol content of fermented BPL increased from 1066.89 μg AGE mL^{-1} to 4269.21 μg AGE mL^{-1} and the total flavonoids from 81.71 μg RE mL^{-1} to 404.99 μg RE mL^{-1}. Kaprasob et al. (2017) fermented apple juice with *L. plantarum, L. casei* and *L. acidophilus*, and found there was only a slight increase in the total phenol content, from 118.43 mg GAE L^{-1} to 128.20 mg GAE L^{-1}. According to previous reports, the contents of many compounds, such as beta-carotene, polyphenols, and flavonoids, increased during the fermentation mechanism of lactic acid bacteria and yeast. In addition, there was an obvious decrease in the content of anthocyanin, from 15.41 μg mL^{-1} to 5.44 μg mL^{-1}. The same was found in Ubeda et al. (2013) studies, in which total anthocyanins decreased after acetic acid fermentation of strawberries. It was reported that anthocyanins were unstable compounds, and affected by many factors such as time, temperature, pH, etc (Clifford, 2000; Cavalcanti et al., 2011), resulting in changes of their chemical structures.

Table 2 indicated the change in antioxidant activity of BPL before and after fermentation. It showed that FRAP and TRP had a very slight increase, but with no obvious differences ($p > 0.05$). Ng et al. (2011) also found an increase in antioxidant activity in plant parts after fermentation, higher than that in our study, and they considered it depended on an increase in total phenolic compounds. Some studies suggested that fermentation can induce the structural decomposition of plant cell walls (Katina et al., 2007), which on its turn may release and/or induce the synthesis of various bioactive compounds. Polyphenols have been proven to be excellent antioxidants on account of 3′-4′-dihydroxy in their B ring; gallate in flavonoid C ring is also an important structure in metal ion chelation (Chu and Chen, 2006; Khokhar and Apenten, 2003). The increase of TPC above mentioned in our study may also be the main reason for the enhancement of antioxidant capacity. Besides, *Lactobacillus* also has antioxidant activity, and many lactic acid bacteria have enzymatic and non-enzymatic antioxidant mechanisms, which can minimize the production of reactive oxygen species (Lee et al., 2006). All these results certified that fermented BPL can be used as a functional beverage with good antioxidant activity.

3.4. Simulated in vitro gastrointestinal (GI) digestion

To estimate active ingredients, antioxidant activity and cell tolerance in the gastrointestinal environment, fermented BPL was exposed to SGF and SIF; the results were summarized in Table 2. The total phenol content of fermented BPL under different SGF conditions (pH 1.5, pH 2.5 and pH 3.5) decreased slightly from 4269.21 μg AGE mL^{-1} to 4209.99 μg AGE mL^{-1}, 4142.94 μg AGE mL^{-1} and 3838.62 μg AGE mL^{-1} respectively. These results were in accordance with data previously reported by Pérez-Vicente et al. (2002), showing a decrease in

Table 2
Comparison of the content of active ingredient, antioxidant activity and cell tolerance of BPL before and after fermentation, and in the process of simulating gastrointestinal digestion.

		Active ingredient			Antioxidant activity		Cell tolerance	
		Total phenolic (μg GAE mL^{-1})	Total flavonoid (μg RE mL^{-1})	Total anthocyanin (μg mL^{-1})	FRAP (mmol L^{-1} Trolox)	TRP (mmol L^{-1} Trolox)	Viable count (Log CFU mL^{-1})	Survival rate (%)
Before fermentation		1066.89 ± 47.21	81.71 ± 7.99	15.41 ± 0.17	205.15 ± 26.21	489.00 ± 9.62	/	/
After fermentation		4269.21 ± 103.30	404.99 ± 5.39	5.44 ± 0.09	269.82 ± 51.13	662.86 ± 18.01	11.59 ± 0.124	/
SGF	1.5	4209.99 ± 48.75^{a}	206.24 ± 6.59^{a}	19.20 ± 0.17^{a}	317.15 ± 31.61^{a}	680.60 ± 65.80^{a}	1.60 ± 0.00^{d}	0.00 ± 0.00^{c}
	2.5	4142.94 ± 289.10^{a}	202.36 ± 8.44^{a}	18.70 ± 0.33^{a}	321.08 ± 33.44^{a}	686.61 ± 66.24^{a}	9.46 ± 0.00^{c}	1.80 ± 0.00^{c}
	3.5	3838.62 ± 97.32^{b}	205.85 ± 7.47^{a}	18.87 ± 0.50^{a}	329.91 ± 22.54^{a}	723.91 ± 37.14^{a}	10.15 ± 0.00^{b}	8.86 ± 0.00^{b}
SIF	7.0	3190.77 ± 135.56^{c}	196.77 ± 3.31^{a}	9.27 ± 0.25^{b}	106.89 ± 36.81^{b}	651.13 ± 102.23^{a}	10.45 ± 0.01^{a}	18.99 ± 0.32^{a}

SGF, simulated gastric fluid; SIF, simulated intestinal fluid.
Data expressed as mean ± Standard deviation.
Means that do not share the same letter are significantly different at $p < 0.05$.

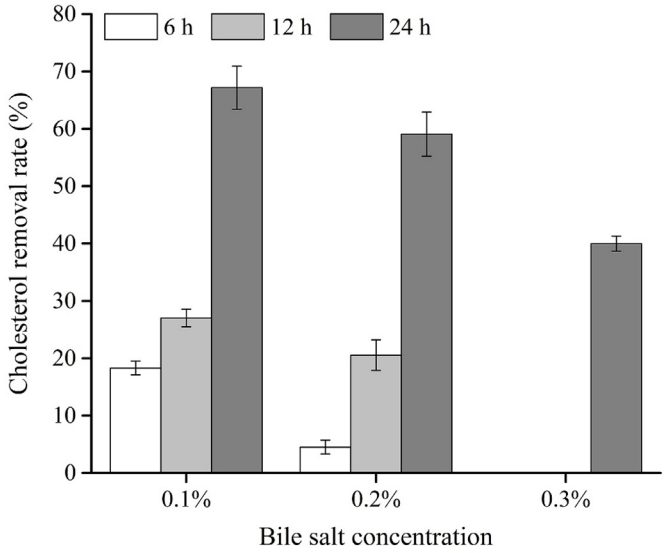

Fig. 4. Cholesterol clearance rate of fermented BPL beverage at different bile salt concentration.

TPC in pomegranate juice. Compared with SGF, the amount of TPC in SIF was marginally lower. The bioavailability of phenolic compounds could be influenced by many factors, such as interactions with proteins, carbohydrates, fibers and fats (D'Archivio et al., 2010). Similarly, Hou et al. (2018) deemed that the decrease of TPC content during digestion may be owing to the binding reaction between protease and phenolic substances. However, Correa et al. (2014) thought the pancreatin in the digestive samples simulating intestinal digestion did not affect the degradation of polyphenols. These standpoints just revealed the variations of the amount of total phenolics influenced by digestion in our study. Likewise, the total flavonoids content showed a significant decline during incubation under intestinal conditions. And there was no significant difference in TFC at three different pH of SGF. It could be attributed to ring-fission of flavonoids during digestion, where the C-ring was degraded resulting in the formation of several phenolic acids (Manach et al., 2004).

Contrary to the TFC and TPC, the content of total anthocyanins went up sharply after SGF and SIF treatments, even coming up to 19.20 mg L^{-1} at pH 1.5, four times as much as that of fermented BPL. This was in line with the report of Stanisavljević et al. (2015) And Spencer et al. (2000) attributed it to the partial degradation of proanthocyanidin oligomers to anthocyanins after gastric and pancreatic digestion. In relation to this, anthocyanins are able to survive under acidic gastric conditions (McDougall et al., 2005), because the low pH in the stomach are conducive to the conversion of anthocyanins into its most stable flavylium cation form (Celep et al., 2015).

The acidity resistance of probiotics is not only essential to resistance to gastric stress, but also a prerequisite for the production of acidic probiotics food. So the pH of simulated gastric fluid is an important factor affecting the survival rate of probiotics entering intestine through the stomach. The initial number of bacterial colonies of fermented juice was estimated at 11.59 Log CFU mL^{-1}. The viable cell count was observed a decrease after incubation for 2 h in SGF in pH 3.5 and pH 2.5, respectively by 1.44 Log CFU mL^{-1} and 2.13 Log CFU mL^{-1}. Like many other studies, the survival rate of probiotics at pH 1.5 was small enough to be negligible. The above results showed that pH was the main factor affecting the survival of the probiotic *L. rhamnosus GG* and *L. plantarum-1* in stomach, and along with the decrease of pH, the survival rate of probiotics was also reduced.

Since it has been widely recognized that material, possessing high reducing power, could be regarded as antioxidants, at least *in vitro*, the measurement of total reducing power and ferric reducing antioxidant power were detected to investigate the antioxidant capacity of fermented BPL after gastrointestinal digestion. The results of the present study demonstrated that fermented BPL exhibited a slight increase antioxidant activity with the increase of pH in SGF condition, while there was a sharp decline in FRAP by 60.38% in SIF. pH modifications may lead to alterations in structure and conformation of phenolic compounds, consequently affecting antioxidant activity. Thus, pH variations in the different compartments employed in the *in vitro* digestion procedure and different buffer systems used in the test solution will affect the evaluation of antioxidant activity. A number of documents have demonstrated the strong correlation between phenolic content and antioxidant activity. However, in this study, the TPC did not change significantly after gastric digestion, while the antioxidant activity showed a slight increase. It could be attributed to the fact that the anabolism and catabolism of new phenolic compounds or metabolites are difficult to detect by current techniques of analysist. So it remains to be explored what substances play a leading role in the oxidation resistance of fermented BPL.

3.5. Determination of cholesterol decrease in vitro

Inhibition of cholesterol absorption in diet is an effective way to reduce cholesterol in blood (Ros, 2000). In recent years, many reports have confirmed that probiotics have the effect of reducing blood lipids. Besides, anthocyanins could reduce cholesterol by hydrophobically binding, increasing cholesterol excretion. (Yao et al., 2013). Therefore, fermented BPL, as a probiotic beverage rich in anthocyanins, should also have lipid-lowering effect. We measured the change of cholesterol clearance at different concentrations of bile salt by OPA over time. The results in Fig. 4 revealed that with the increase of bile salt concentration, the removal rate of cholesterol of fermented BPL decreased, but it was positively correlated with time and showed a significant difference in time ($p < 0.05$). After 24 h, the removal rates of cholesterol were 67.17%, 59.06% and 39.97%, respectively with 0.1%, 0.2% and 0.3% bile salts. However, with 0.3% bile salts, fermented BPL had almost no cholesterol scavenging capacity at 6 h and 12 h. In conclusion, fermented BPL had excellent cholesterol-lowering potential, but whether probiotics or other active substances played a major role remained to be discussed.

3.6. Measurement of the anti-fatigue activity

Fatigue involves many physiological and biochemical factors. One important factor causing exercise fatigue is the increase of free radicals, furthermore, exogenous dietary antioxidants could reduce exercise-induced oxidative stress and improve physiological status. Exercise endurance is an intuitive index for evaluating physical fatigue, while weight-loaded swimming is a common experimental exercise model for evaluating fatigue. As shown in Fig. 5, it was seen that the swimming time of mice in fermented BPL group was 31.27 ± 13.72 min, 4 more times longer than that of the blank control group. Moreover, the swimming time of fermented BPL was like the positive control, which indicated that the anti-fatigue capability of fermented BPL was equivalent to the positive control group. Although the anti-fatigue function of Red Bull differed from the fermented BPL, which relied mainly on the high concentration of caffeine. In previous study, it has been found that *L. plantarum* can improve swimming endurance, and endurance swimming time dose-dependently increased with increasing *L. plantarum* dose (Chen et al., 2016). Moreover, Xie et al. (2015) had proved that polyphenols and flavonoids are anti-fatigue components of okra pods. High levels of antioxidants in fermented products can limit excessive reactive free radicals *in vivo*, which can help to prevent and control diseases related to oxidative stress (Bujna et al., 2018). In addition, Fig. 5 also revealed the weight gain of mice. The weight of mice in fermented BPL and positive control groups decreased by an average of 2.29 g and 0.83 g, while there was a slight increase in control group

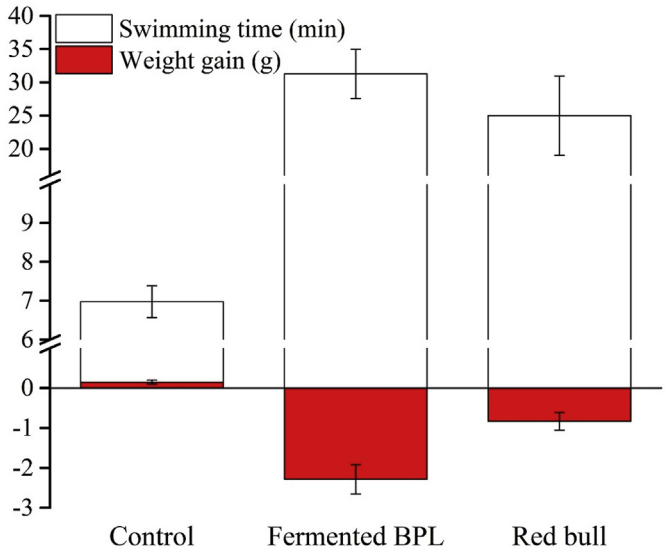

Fig. 5. The anti-fatigue effect of fermented BPL.

by 0.15 g. This may be attributed to the above-mentioned good cholesterol-lowering ability of the fermented BPL. We found the trend in weight gain was the opposite of swimming time, which reflecting the cholesterol-lowering properties of fermented BPL may have a correlation with anti-fatigue activity. Therefore, it provided an important basis for the development of fermented BPL as a new antioxidant and anti-fatigue compound, although the underlying specific mechanisms remained to be explored.

4. Conclusion

At present, probiotic fermentation products are mostly limited to dairy products, while the fermentation of vegetables and fruits is still less, letting alone fruit residue. This study showed that when PL-1 and LGG were selected as fermentation strains, and 1.5% glucose was added as a carbon source, the number of viable bacteria in fermented BPL reached 11.59 Log CFU mL^{-1}, and a certain amount of organic acids such as lactic acid and acetic acid were produced. It has been proved that the active ingredients and antioxidant capacity of blueberry pomace have been improved after fermentation. Meanwhile, it had excellent cholesterol clearance at 24 h under three concentrations of cholate. In addition, the fermented BPL also showed an outstanding performance on anti-fatigue. In the weight-bearing swimming experiment of mice, the swimming time of mice was 4 more times that of the blank control group. In short, probiotic fermentation of BPL is a kind of highly viable beverage with potential nutritional and special effects, and is of great help to the reutilization of blueberry pomace.

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Declaration of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.049.

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