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Effect of Lactic Acid Bacteria Fermentation on Antioxidation and Bioactivity of Hawthorn Pulp

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Abstract. Mixed fermentation was carried out for 12 h at 37 °C using *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Lactobacillus casei*. The effects of lactic acid bacteria (LAB) fermentation on the antioxidant activity and phytochemical concentration of hawthorn pulp (HP) were investigated by comparing the IC₅₀ value (μL/mL) of HP before and after lactic acid bacteria fermentation. Fermented hawthorn pulp (FHP) showed higher DPPH free radical scavenging ability, ABTS free radical scavenging ability and hydroxyl free radicals. Fermented hawthorn pulp (FHP) has higher Fe³⁺ reducing ability using the absorbance values; LAB fermentation significantly increased the content of free phenol and flavonoids in hawthorn pulp, and the content increased by 34.14% and 53.15 % respectively.

1. Introduction

Hawthorn (*Crataegus pinnatifida*) is also called "mountain red", which belongs to the *Rosaceae* plant. It can promote the secretion of digestive juice, increases appetite, and has many medicinal values such as stomach and elimination. It has been reported that phenolic compounds such as proanthocyanidins, hyperoside, vitexin, chlorogenic acid, erythroic acid, isoquercetin, epicatechin and isoquercitrin are highly contained in hawthorn [1]. Phenolic compounds in foods have neuroprotective, cardioprotective, anti-inflammatory, anti-oxidant, cancer chemopreventive, immunomodulatory and antipyretic properties that are equally beneficial to human health [2]. The antioxidant properties of Hawthorn and its accompanying health benefits have also been widely reported [3]. However, these products are characterized by high sugar, which severely limits the consumption of consumers. Therefore, in recent years, probiotic bacteria such as LAB have been added to juice to increase the probiotic effect of the juice and enrich the taste of the beverage [4]. For this reason, it is indeed necessary to apply technology to improve the shelf life, nutrition, sensory quality and health benefits of hawthorn to promote commercial production for economic and health benefits.

Fermentation of juice by probiotics can increase the viability of bacterial cells and improve the functional aspects of the food matrix [5]. In addition to processing fruits and vegetables into fermented beverages to increase probiotic effect and sensory, it has been reported that fermentation can increase the concentration and bioavailability of polyphenolic compounds in fermented beverages [6]. There are few studies on the effects of LAB fermentation on the antioxidant and bioactivity of hawthorn pulp.



Therefore, in order to improve the utilization of hawthorn and product diversity, this paper aims to study the effects of LAB fermentation on the antioxidant activity and bioactivity of HP.

2. Materials and methods

2.1. Chemicals and plant material

Folin-Ciocalteu phenol and rutin reagents were obtained from Shanghai Yuanye Biological Technology Co., Ltd (Shanghai, China). Hawthorn dry was purchased from Laiwu Wanbang Food Co, Ltd. (Laiwu, China).

2.2. Activation of starter culture

Three strains of *Lactobacillus* (CICC 20265, CICC 20248 and CICC 20241) were purchased from CICC (Beijing, China). The strain was activated according to the method described by Kwaw et al [6] with minor modifications. Briefly, the two-loop culture was inoculated into 100 mL of MRS liquid medium and activated at 37 °C for 24 h. The activated strain was then transferred to 100 mL of MRS liquid medium at a 2 % inoculation amount, cultured to mid-log phase, and the culture was centrifuged at $4390 \times g$ at 4 °C. (TDZ5-WS, Xiangyi Centrifuge Instrument Co., Ltd., Changsha, Hunan, China) 10 min. Microbial cells were collected and washed with 0.1 % sterile NaCl.

2.3. Preparation and fermentation of hawthorn pulp

The insect-free hawthorn was selected and washed with water. The cleaned hawthorn was beaten in a food grade juicer for 10 min (material to water ratio 1: 15). 100 mL of hawthorn pulp was placed in a 250 mL Erlenmeyer flask and autoclaved at 121 °C for 15 min. The HP was prepared. Activated lactic acid bacteria (1: 1: 1 ratio, 10 % inoculum) were inoculated into sterile haw pulp and incubated for 12 h in the 37 °C incubator (log phase late). Obtained fermented hawthorn pulp (FHP).

2.4 Determination of antioxidant activity in vitro

2.4.1 DPPH radical scavenging activity. The 2, 2-diphenyl-1-picrylhydryl scavenging activity (DPPH%-SA) of the hawthorn pulp before and after fermentation was evaluated using the method described by Kwaw et al [6] with some modifications. A 0.2 mmol/L DPPH solution was prepared with absolute ethanol and stored in a brown bottle at low temperature. 2 mL of different concentrations of the sample were added to 2 mL of DPPH free radical solution, the chamber was incubated at 37 °C for 30 min in the dark, and the absorbance was measured at 517 nm (UV-755B, Shanghai Youke Instrument Co., Ltd., Shanghai, China). Calculate the DPPH clearance of the sample using the formula below:

$$\text{DPPH clearance rate (\%)} = \left(\frac{A_0 - A_1 + A_2}{A_0} \right) \times 100 \%$$

Where A_0 is the absorbance of 2 mL absolute ethanol and 2 mL DPPH, A_1 is the absorbance of the sample group, A_2 is the absorbance of 2 mL of the sample and 2 mL of absolute ethanol.

2.4.2 ABTS radical scavenging activity. The samples were tested for ABTS radical scavenging ability according to the method of Wang et al [7] and slightly modified. Briefly, the ABTS stock solution consisted of potassium persulfate (2.45 mmol/L) and ABTS solution (7 mmol/L) and was kept at 4 °C for 12 h. The absorbance of the ABTS stock solution was diluted to 0.70 ± 0.02 prior to measurement. Then, 0.03 mL of the sample was added to 3.0 mL of the ABTS reaction solution and incubated for 10 min in the dark. After the incubation, the absorbance of the mixture was measured at 734 nm. The clearance rate of the sample was calculated according to the following formula:

$$\text{ABTS clearance rate (\%)} = \left(\frac{A_0 - A_s}{A_0} \right) \times 100 \%$$

Where A_0 is the absorbance of the control group (deionized water instead of the sample), A_s is the absorbance of the sample group.

2.4.3 Hydroxyl radical scavenging activity. The ability of the sample to scavenge hydroxyl radicals was determined by the method of Chen et al [8]. 1 mL of phenanthroline (0.75 mmol/L) and 2 mL of PBS buffer (0.2 mmol/L, pH = 7.4) were aspirated and thoroughly mixed, and 1 mL of FeSO_4 (0.75 mmol/L) was added and mixed. Add 1 mL of H_2O_2 (0.01 %), 1 mL of different concentrations of the sample solution, mix thoroughly, after 60 min at 37 °C in a water bath, and then measure at 536 nm with a spectrophotometer. Calculate the scavenging rate of hydroxyl radicals according to the following formula:

$$\text{Hydroxyl radical scavenging rate (\%)} = \left(\frac{A_s - A_p}{A_b - A_p} \right) \times 100 \%$$

Where A_s is the absorbance of the sample set, A_p is the absorbance of the blank of the sample, and A_b is the absorbance of the blank of H_2O_2 .

2.4.4 Fe^{3+} reducing power. The reducing ability of the hawthorn pulp was determined by the method of Wu et al [9], and slightly modified. 1 mL of the sample solution, 0.2 mL of PBS solution (0.2 mol/L, pH = 6.6) and 2.5 mL of potassium ferricyanide solution (1 %) were added to the test tube, mixed uniformly, and then bathed at 50 °C for 20 min, after cooling. Add 2.5 mL of trichloroacetic acid solution (10 %), and mix and let stand for 10 min. 2.5 mL of the above mixed solution was added to a stoppered tube, and then 2.5 mL of distilled water and 1 mL of a ferric chloride solution (0.1 %) were added, mixed uniformly, and allowed to stand for 10 min, and the absorbance was measured at 700 nm. The higher the absorbance value indicates the stronger the reducing ability of the sample.

2.5 Phytochemical concentration assay

2.5.1 Extraction of free phenolic substances. Refer to the method of Paiva et al [10] with slight modifications. The 10 g sample was placed in a 100 mL centrifuge tube, 10 mL of 80 % frozen acetone solution was added and sonicated for 60 min in a 500 W ultrasonic cold water bath (Beijing Hongxianglong Bio-Technology Co., Ltd.). Then, it was centrifuged at $4390 \times g$ for 10 min (TDZ5-WS, Xiangyi Centrifuge Instrument Co., Ltd., Changsha, Hunan, China), and the supernatant was taken out. The residue was re-extracted once, the supernatant was combined, filtered, and steamed to a dry state at 45 °C and made up to 50 mL with ultrapure water. The extracted samples were stored at -20 °C for storage.

2.5.2 Extraction of bound phenolic substances. According to the description of Finchiario et al [11] and slightly modified. The remaining precipitate in 2.5.1 was added to 40 mL of 4 mol / L NaOH solution, and filled with an appropriate amount of nitrogen, sealed and shaken for 3 h. Add 6 mol/L HCl solution to adjust the pH to 1, and remove the residue by suction filtration. The mixture was extracted 5 times with 2 volumes of ethyl acetate, and steamed to a dry state at 45 °C. The residue was dissolved in water to a volume of 10 mL, and the extract was placed in a refrigerator at -20 °C for free storage.

2.5.3 Determination of total phenolic content. The total phenolic content (TPC) was determined using the Folin-Ciocalteu method, with reference to the method of Berker et al [12] and slightly modified. Briefly, 1 mL of the extract and 5 mL of Folin-Ciocalteu reagent were mixed uniformly and allowed to stand for 1 min; then 4 mL of 7.5 % sodium carbonate solution was added; after thorough mixing, the volume was adjusted to 10 mL mark with distilled water. The mixture was allowed to stand at 25 °C for 1 h, and its absorbance was read at 760 nm using a UV spectrophotometer (UV-755B, Shanghai Youke Instrument Co., Ltd, Shanghai, China). Based on a calibration curve using gallic acid, TPC is

expressed in mg GAE/100 g FW. The total phenolic content (TPC) of the sample was calculated from the standard curve: $Y = 8.8631x - 0.0018$, $R^2 = 0.9997$.

2.5.4 Determination of total flavonoids content. The total flavonoid content (TFC) was determined using the method described by Hani et al [13] with minor modifications. 1 mL of the extract and 5 % sodium nitrite 0.7 mL were added to a 25 ml stoppered test tube, shaken and allowed to stand for 6 min; then 10 % aluminum nitrate solution 0.7 mL was added, shaken and allowed to stand for 6 min; Add 10 mL of 1 mol/L sodium hydroxide, and make up to volume with pure water. After mixing, let stand for 15 min. The absorbance A was measured at 510 nm, based on a calibration curve using rutin, expressed as mg RE/100 g FW. The total flavonoid content (TFC) of the sample was calculated from the standard curve: $Y = 12.741x - 0.0059$, $R^2 = 0.999$.

3. Results and discussion

3.1. Effect of Lactic Acid Bacteria Fermentation on DPPH radical scavenging rate

As shown in Figure 1, DPPH radical scavenging rate of fermented hawthorn pulp increased significantly, and showed a dose-dependent relationship with the increase of sample concentration. In the concentration range of the test, the DPPH free radical scavenging rate of the fermented hawthorn pulp was about 1.1 to 1.3 times that of the hawthorn pulp. By calculation, the IC_{50} of DPPH free radical scavenging rate of fermented hawthorn pulp was 4.401 $\mu\text{L/mL}$, and the IC_{50} of hawthorn pulp was 6.049 $\mu\text{L/mL}$. The difference between the two was significant ($p < 0.05$).

3.2. Effect of Lactic Acid Bacteria Fermentation on ABTS radical scavenging rate

As shown in Figure 2, ABTS radical scavenging rate of fermented hawthorn pulp increased significantly, and showed a dose-dependent relationship with the increase of sample concentration. The ABTS free radical scavenging rate of the fermented hawthorn pulp was about 1.1 to 1.4 times that of the hawthorn pulp. By calculation, the IC_{50} of ABTS free radical scavenging rate of fermented hawthorn pulp was 70.042 $\mu\text{L/mL}$, and the IC_{50} of hawthorn pulp was 83.125 $\mu\text{L/mL}$. The difference between the two was significant ($p < 0.05$).

3.3. Effect of Lactic Acid Bacteria Fermentation on hydroxyl radical scavenging rate

It can be seen from Figure 3 that the hydroxyl radical scavenging rate of the hawthorn pulp after fermentation increased significantly and was dose-dependent with the increase of the sample concentration. The hydroxyl radical scavenging rate of the fermented hawthorn pulp was about 1.2 to 1.4 times that of the hawthorn pulp. By calculation, the IC_{50} of hydroxyl radical scavenging rate of fermented hawthorn pulp was 1.220 $\mu\text{L/mL}$, and the IC_{50} of hawthorn pulp was 3.266 $\mu\text{L/mL}$. The difference between the two was significant ($p < 0.05$).

3.4. Effect of Lactic Acid Bacteria Fermentation on Fe^{3+} reducing power

It can be seen from the Figure 4, Fe^{3+} reducing power of fermented hawthorn pulp increased significantly, and showed a dose-dependent relationship with the increase of sample concentration. The Fe^{3+} reducing power of the fermented hawthorn pulp was about 1.2 to 1.3 times that of the hawthorn pulp. There was a significant difference in Fe^{3+} reducing power compared to the hawthorn pulp before and after fermentation ($p < 0.05$).

Their antioxidant capacity was enhanced, this might be due to the glucoside glucosides of some glycosylated phenols produced by lactic acid bacteria after lactic acid bacteria fermentation [14], after that, the bound polyphenols were hydrolyzed by the glycosidase or esterase produced by the lactic acid bacteria to increase the free phenol content in the fermentation broth, thereby improving the antioxidant capacity of the sample after fermentation, thereby improving the antioxidant capacity of the sample after fermentation. In addition, during the fermentation process, some structural changes in

antioxidant components might occur, and some components with stronger electron donating ability might be formed to enhance their antioxidant capacity.

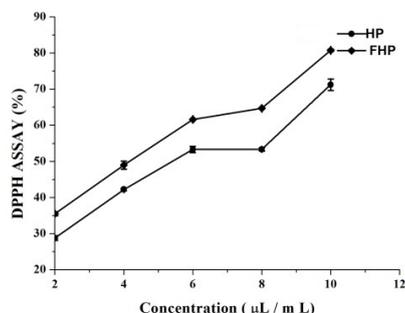


Figure 1. Changes of in DPPH radical scavenging rate of hawthorn pulp before and after lactic acid bacteria fermentation.

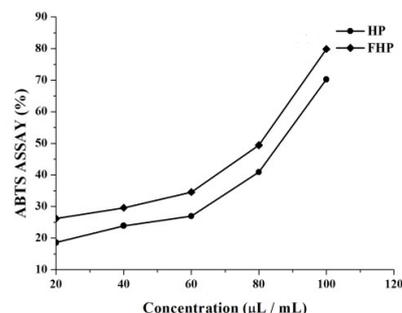


Figure 2. Changes of in ABTS radical scavenging rate of hawthorn pulp before and after lactic acid bacteria fermentation.

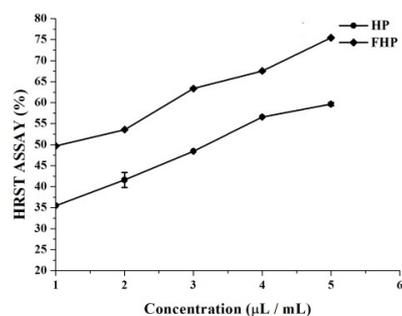


Figure 3. Changes of in hydroxyl radical scavenging rate of hawthorn pulp before and after lactic acid bacteria fermentation.

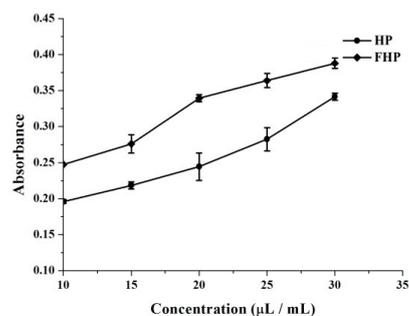


Figure 4. Changes of in Fe³⁺ reducing power of hawthorn pulp before and after lactic acid bacteria fermentation.

3.5. Effect of lactic acid fermentation on phytochemical concentration

Table 1 showed the changes of free phenol and bound phenol content, free flavonoids and bound flavonoids after the fermentation of hawthorn pulp. It could be seen from Table 1 that the content of free phenol and free flavonoids in the fermented hawthorn pulp increased significantly compared with the hawthorn pulp which was not fermented by lactic acid bacteria, which increased by 34.14 % and 53.15 %; while the content of bound phenol and bound flavonoids decreased significantly, reduced by 36.38 % and 61.29 % respectively. However, after fermentation by lactic acid bacteria, the total phenolic content and total flavonoid content of the hawthorn pulp was significantly increased. Lactic acid bacteria produced a variety of enzymes during the fermentation process [15], thereby causing the cleavage and acidification of glycosides and esters combined with phenolic acids, thereby releasing the bound phenols into free phenols.

Table 1. Changes of free phenols and flavonoids, bound phenols and flavonoids in hawthorn pulp before and after lactic acid bacteria fermentation.

	Polyphenol(mg GAE/100 g FW)		Flavonoid(mg RE/100 g FW)	
	Free state	Combined state	Free state	Combined state
HP	105.735±0.078a	15.95±0.028a	1.43±0.014a	0.5±0.014a
FHP	141.835±0.078b	11.695±0.021b	2.19±0.014b	0.31±0.014b

4. Conclusions

Lactic acid bacteria fermentation has a significant effect on the antioxidant activity and phytochemical concentration of hawthorn pulp. After the fermentation of lactic acid bacteria, the *in vitro* antioxidant activity of the hawthorn pulp was greatly improved. In addition, the content of free phenol and flavonoids in the hawthorn pulp was also significantly increased.

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