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RESEARCH ARTICLE

Lactobacillus fermentation improved the nutrient value and hypoglycemic activity of chickpea milk

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ABSTRACT

In this work, the effects of *Lactobacillus* fermentation on the nutrient content and hypoglycemic ability of chickpea milk were studied. Specifically, the fermentation of *Lactobacillus acidophilus* ATCC 4356 (LA) and *Lactobacillus plantarum subsp. plantarum* CICC 20279 (LP) reduced the contents of soluble sugar and protein in chickpea milk. But both single bacteria fermentation (SBF, LA or LP) and mixed bacteria fermentation (MBF, LA and LP) significantly increased the contents of some bioactive substances, such as dietary fiber, free amino acids, total polyphenols and total flavonoids. Moreover, LA fermented chickpea milk showed the best inhibition of α-glucosidase (highest inhibition rate up to 92.91%) and the MBF samples showed the best inhibition of α-amylase (highest inhibition rate up to 33.33%). In addition, free phenolics, conjugated phenolics, and total phenolics were all remarkably increased in chickpea milk after LA fermentation. The three types of phenolic compounds (free, conjugated, and bound) showed greater inhibition of α-glucosidase than α-amylase, and the conjugated phenolics exhibited the best inhibitory effects on both enzymes. From the results, *Lactobacillus* fermentation process may effectively improve the nutritional values and potential hypoglycemic activity of chickpea milk.

Keywords: *Lactobacillus*; Chickpea milk; Nutrient composition; Hypoglycemic activity

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease caused by genetic factors, environmental factors and living habits, which is mainly characterized by hyperglycemia. Currently, pharmacotherapy is still the mainstay of treatment. The therapeutic strategies can be roughly divided into two categories: promoting insulin secretion to improve glucose metabolism or delaying carbohydrate degradation by inhibiting the activity of metabolic enzymes. (Abirami et al., 2014). The majority of therapeutic medications have significant or modest side effects, such as adverse reactions to hypoglycemia, and liver damage caused by long-term use (Liday, 2011). Consequently, researchers are committed to finding non-pharmacological methods that can reduce blood sugar levels, and dietary interventions such as fermented foods have been widely studied as a safer way (Baruah et al., 2022).

Chickpea (*Cicer arietinum*) is a kind of bean with a long edible and medicinal history, accounting for 17% of the total legumes in the world. Chickpeas have high nutritional value because they are rich in a variety of plant proteins, vitamins minerals and essential amino acids (Paredes-López et al., 1991). Chickpeas and their isolated ingredients can assist in the treatment of bronchitis, mucositis and dyspepsia, while having antioxidant, hypoglycemic, hypolipidemic and other probiotic effects (Arcan and Yemenicioğlu, 2007; Pittaway et al., 2007).

Naturally occurring phenolic substances confer chickpeas some hypoglycemic properties. It has been reported that polyphenols in plants may have a synergistic effect in inhibiting starch hydrolase, which could achieve a certain hypoglycemic function by inhibiting the activity of amylase (Apostolidis et al., 2007; Kim et al., 2005). Isoflavones such as chickpea biochanin A and formononetin are important parts of phenolic components in chickpeas (Zhao et al., 2009). A considerable number of phenolics are present in the cell wall matrix and combine with macromolecular substances such as pectin, cellulose and structural proteins, which are more difficult to be used by the body than free phenolics (Yeo and Shahidi, 2017). To increase the nutritional value of chickpeas, it is speculated that

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microbial fermentation may be an effective way to improve the bioavailability of these substances. Some researchers pointed out that probiotic bacteria like *Lactobacillus* and *Bifidobacterium* could biotransform plant substrates, boosting the content of phenolic components and their bioactivity while also degrading the proteins into free amino acids (Wang et al., 2003; Wei et al., 2007).

Lactic acid bacteria (LAB) are a kind of bacteria that can use fermentable carbohydrates to produce a large amount of lactic acid, which are widely distributed in nature and has important physiological functions. Fermentation is a common method for legume treatment, resulting in an effective increase in the content of some active substances such as free amino acids and phenolics through biotransformation (Sanjukta and Rai, 2016). Study has shown that solid-state fermentation could effectively release phenolic compounds in sorghum and improve the bioavailability of sorghum nutrients (Espitia-Hernández et al., 2022). The nutritional characteristics and antioxidant capacity of soybean milk were greatly improved by mixed fermentation of LAB and kombucha bacteria. (Peng et al., 2022). The application of LAB to ferment beans is a new trend in bean food processing. In the process of fermentation, some bioactive substances produced by microorganisms provide fermentation products with high nutritional value.

The objective of this study was to investigate the effects of fermentation of *Lactobacillus acidophilus* ATCC 4356 (LA) and *Lactobacillus plantarum subsp. plantarum* CICC 20279 (LP) on the nutrient content and hypoglycemic activity of chickpea milk. Moreover, the conjugated phenolics, bound phenolics and free phenolics in unfermented/fermented chickpea milk were isolated. The changes of fermentation on the content and hypoglycemic activity of the three types of phenolics were further analyzed.

MATERIALS AND METHODS

Materials

Chickpeas obtained from supermarkets (Changji, Xinjiang, China). Gallic acid and Rutin were purchased from Tanmo Quality Control Technology Co. (Changzhou, Jiangsu, China). Total Dietary Fiber Kit (TDS) (Dublin, Ireland). α-Glucosidase and α-amylase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glucose, sodium carbonate, ninhydrin, phenol, folin-phenol were procured from Kermel (Tianjin, China). Coomassie Brilliant Blue G-250, ethanol, 4-Nitrophenyl β-D-glucopyranoside (PNPG), soluble starch (MACKLIN, Shanghai, China). 3,5-dinitrosalicylic acid solution (DNS), bovine serum albumin (Solarbio, Beijing, China).

Strains and cultural conditions

L. acidophilus ATCC 4356 was preserved by Beijing Chuanglian Biotechnology Co. (Beijing, China). *L. plantarum subsp. plantarum* CICC 20279 was obtained from the China Center of Industrial Culture Collection (Beijing, China). The strains were passaged three times every 18 h in De Man-Rogosa-Sharpe broth (Oxoid) incubated at 37 °C for activation. Lactobacilli cells were collected by centrifugation (5000 ×g, 10 min, 4℃), washed thrice, and suspended in sterilized phosphate buffer saline (PBS) before use.

Preparation and fermentation of chickpea milk

Chickpeas were soaked overnight at room temperature before being ground into chickpea milk. The mass to volume ratio of chickpeas to water is 1:10 (w/v). The chickpea milk was sterilized with autoclave (Shanghai bosun Industrial Co., Ltd., Shanghai, China) at 121°C for 20 min. The inoculum amount was 1% for each of the single bacterial fermented (SBF) groups, and the mixed bacterial fermented (MBF) group of LA and LP 1:1(v/v) were inoculated with 1% dose (The viable counts of lactobacilli cells were about 1.0×10^{8} CFU/mL). All samples were incubated at 37℃ (Fig. 1).

Determination of viable counts and pH value

Viable bacteria were counted by the method of plate counting (Hyronimus et al., 2000). The pH values of all samples were measured using the PHS-3C digital acidity meter (INESA Science Instrument Co., Ltd., Shanghai, China) (Ombaka et al., 2021).

Determination of soluble sugar

Fermented chickpea milk was collected every 4 h and the centrifugal supernatant was lyophilized (8000 × g, 15 minutes, 4 °C; FD-1A-50 freeze drier, Beijing, China). The content of soluble sugar in freeze-dried powder was determined by sulfuric acid-phenol method described by Xiao et al. (2019). The standard curve was prepared using glucose.

Determination of dietary fiber

The dietary fiber content of unfermented/fermented chickpea milk lyophilized powder was determined based on the standard procedure of TDS.

Determination of soluble protein

The soluble protein content of sample solution (2.5 g/L) was determined using the Bradford assay according to Li and Wang (2021). The standard curve was drawn with bovine serum albumin as the standard.

Determination of free amino acids

The centrifugal supernatant (8000 ×g, 15 min, 4°C) of unfermented/fermented chickpea milk was used as

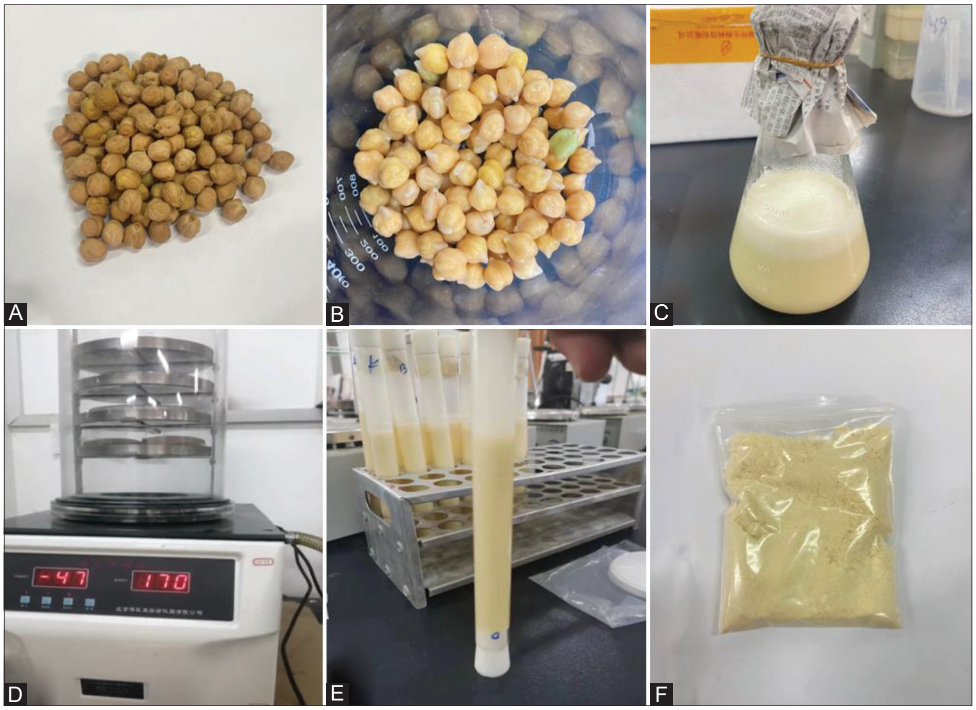

Fig 1. Images of the preparation and fermentation process of chickpea milk. (A) Chickpeas. (B) Chickpeas soaked overnight. (C) Chickpea milk. (D) Freeze-drying process. (E) Fermentation of chickpea milk. (F) Freeze-dried powder.

sample solution. The method of ninhydrin colorimetry was operated to determine the free amino acids content of samples (Chen et al., 2007). The isoleucine solution was used as a standard.

Determination of total polyphenol

The Folin-Ciocalteu method described by Zhang et al (2016) was taken to implement the extraction and determination of total polyphenol. The standard curve was plotted with the gallic acid solution as the standard.

Determination of total flavonoids

The colorimetric method was adopted to quantify the content of total flavonoids. (Granato et al., 2016) Additionally, the extraction treatment of flavonoids in unfermented/fermented chickpea milk was appropriately modified. Briefly, 0.5 g lyophilized sample solution (dissolved in 60 mL of 60% ethanol) was evaporated at 70°C. After being filtrated, the sample solution of flavonoid was obtained by fixing the volume of concentrated solution to 100 mL. The standard curve was constructed using rutin.

Determination of α-glucosidase inhibition rate

The α-glucosidase decomposed 4-Nitrophenyl β-D-glucopyranoside (PNPG) to produce 4-nitrophenol, which had the maximum absorption peak at 405 nm. The α-glucosidase inhibition of samples were characterized by measuring the change of absorbance value (Zhang et al., 2007). The following equation was exploited to calculate the inhibition rate:

$$Inhibition\% = \left\{1 - \frac{A_{405\ sample} - A_{405\ sample\ blank}}{A_{405\ control} - A_{405\ blank}}\right\} \times 100 \qquad (1)$$

Where $A_{sample\ blank}$ is the absorbance without α-glucosidase; $A_{control}$ is the absorbance without sample; A_{blank} is the absorbance without sample and α-glucosidase.

Determination of α-amylase inhibition rate

α-Amylase catalyzes the hydrolysis of starch to reduce sugar, which reacts with DNS under alkaline conditions to form brownish-red amino compounds, the products of which have characteristic absorption peaks at 540 nm (Cardullo et al., 2020), and the enzyme inhibition is quantified by the following equation:

$$Inhibition\% = \left\{1 - \frac{A_{540\ sample} - A_{540\ sample\ blank}}{A_{540\ control} - A_{540\ blank}}\right\} \times 100 \qquad (2)$$

Where $A_{sample\ blank}$ is the absorbance without α-amylase; $A_{control}$ is the absorbance without sample; A_{blank} is the absorbance without sample and α-glucosidase.

Extraction of different types of phenolics

Three different types of phenolics (free, conjugated, and bound) were extracted from 2 g of unfermented/fermented chickpea milk lyophilized powder, following the previously reported procedure (Bei et al., 2017), with the slight difference that the final sample solutions were all fixed to 10 mL with 50% methanol.

Determination of the different types of phenolic content

The procedure was the same as for determining total polyphenol. The samples were replaced with different types of phenolics that were extracted above.

Determination of the hypoglycemic activity of different types of phenolics

The method was the same as that for the determination of α-glucosidase and α-amylase inhibition. The samples were replaced with different types of phenolics extracted above.

Statistical analysis

The mean ± standard deviation (SD) of more than twice determinations was utilized to express the results of the recorded experiments. Data were analyzed by one-way analysis of variance (ANOVA). Significance of differences tests was performed using Duncan's multi-interval test ($p = 0.05$) or independent samples T-test ($p = 0.05$).

RESULTS AND DISCUSSION

Viable counts and pH changes in fermented chickpea milk

The primary metabolites of the glucose metabolism process of LAB are organic acids. The pH of chickpea milk fermented by different strains decreased during the fermentation process until it reached a stable period after 20 h as reflected in Fig. 2. The lower intracellular pH and the lower ΔpH at the cell membrane confer a good environment for fermentative bacteria like LAB to grow and reproduce. The high ΔpH promotes a large toxic accumulation of fermentative acid anions, while the persistent decrease in intracellular pH may pose a potential limitation to various aspects of bacterial proliferation. A dramatic decrease in pH may result in the eventual collapse of the cells (Cook and Russell, 1994; McDonald et al., 1990). Therefore, when the pH value decreased to a certain extent, the acid production of the cell almost stopped, and the pH value no longer changed. In this assay, it was found that the pH values of two SBF groups were lower than that of MBF group after 20 h.

The viable counts in the samples fluctuated during fermentation. The decline might be due to the fact that the growth of *Lactobacillus* was inhibited by organic acids produced during fermentation. The increase in viable counts might be for the reason that the microorganisms had developed a certain adaptation to acid stress and formed some complex mechanisms for survival (Jiang et al., 2015; Nezhad et al., 2015). The changes in viable counts of the MBF samples lagged behind the SBF samples, and the viable counts in SBF samples were higher than that in the MBF samples in the first 20 h. A recent study found that protein induction and repression may have a correlation with the fact that yeast and LAB developed a proteomic response to acid stress, which might be the internal mechanism of bacterial growth fluctuations (Adewara and Ogunbanwo, 2022). The viable counts in the three fermentation samples were all higher than 1.0×10^9 CFU/mL at 24 h, indicating that the two strains of this experiment could effectively proliferate in chickpea milk.

Effects of fermentation on the soluble sugar and dietary fiber contents of chickpea milk

Products such as lactic acid, ATP, acetic acid, and CO_2 are produced by LAB through carbon sources during the process of bacterial proliferation (Kandler, 1983), which may account for the continuous decrease of soluble sugar content in the early stage of the fermentation (0-20 h). But the soluble sugar content increased (Fig. 3) at the end of the fermentation (24 h), probably due to the production of extracellular polysaccharides by the bacteria during

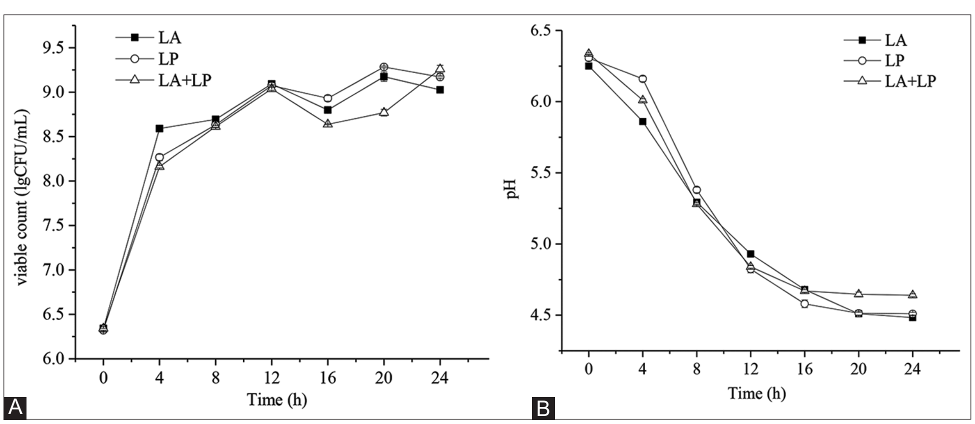

Fig 2. Viable counts (A) and pH value (B) of chickpea milk fermented by different *Lactobacillus* strains.

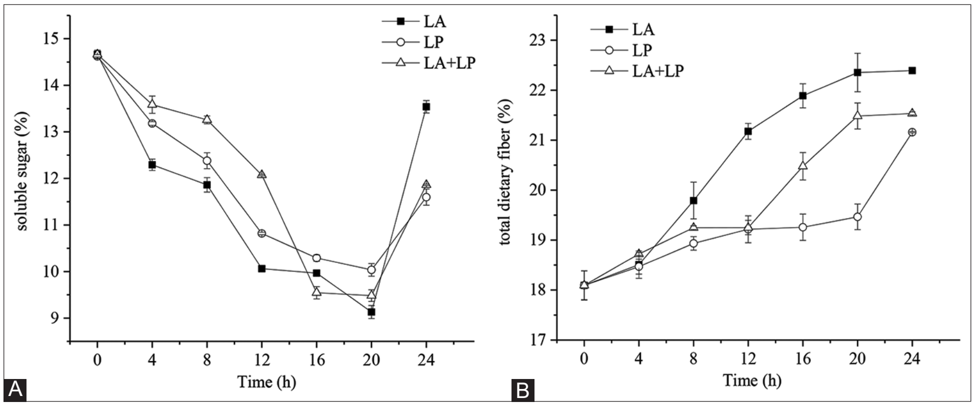

Fig 3. Soluble sugars (A) and total dietary fiber content (B) in chickpea milk fermented by different strains.

this period. It had been reported that both *Lactobacillus acidophilus* and *Lactobacillus plantarum* could produce extracellular polysaccharides (Deepak et al., 2016; Dilna et al., 2015).

The maintenance of human health is closely related to the indispensable role played by dietary fiber, which is of great significance in the treatment of some chronic cases such as hyperlipidemia and hypertension. In our study, both SBF and MBF increased the dietary fiber content in chickpea milk. The samples fermented by LA for 24 h had the highest dietary fiber content of 22.39%. Zhang et al. (2022) reported that the proportion of soluble dietary fiber and insoluble dietary fiber in hulless barley increased by solid-state fermentation of beer yeast and *Lactobacillus plantarum*. In obese rodent models, as well as in kids and adults, diets with high dietary fiber had shown the ability to enrich beneficial intestinal bacteria and offered hope for reducing the negative microbial alterations caused by high-sugar and high-fat diets (Tuplin et al., 2022). Therefore, increasing the content of dietary fiber by fermentation in this experiment would be conducive to improving various functionalities of chickpea milk.

Effects of fermentation on the soluble protein and free amino acids contents of chickpea milk

Fig. 4 presented the changes of soluble protein and amino acid content in chickpea milk during the whole fermentation process. The content of soluble protein in different samples decreased gradually, and the MBF group had the lowest soluble protein content (0.74 ±0.02%) at the end of fermentation. Meanwhile, both SBF and MBF increased the content of free amino acids in chickpea milk, and the content was positively correlated with fermentation time.

The opposite change trend of protein content and free amino acids content could be explained by the following reasons. Firstly, proteins can be decomposed and utilized as a nitrogen source required for microbial growth and reproduction. Secondly, small molecular acids such as lactic acid and acetic acid produced by *Lactobacillus* during fermentation would change the pH of the system, resulting in the flocculation and precipitation of the protein due to reaching the isoelectric point. Additionally, active substances produced by microbial fermentation such as protein hydrolases degrade protein into small molecule peptides or free amino acid (Garavand et al., 2023; Sanjukta and Rai, 2016; Shi et al., 2014). In addition, transaminases produced during *Lactobacillus* fermentation could synthesize amino acids. Compared with macromolecular protein, free amino acids are more easily absorbed and utilized by the human body.

Effects of fermentation on the total polyphenol and flavonoids content of chickpea milk

Polyphenols are a kind of plant secondary metabolites which exist widely in plants. In addition to being a natural antioxidant, it also possesses multiple substantial pharmacological functions, such as clear human free radicals, anti-cancer, anti-aging, hypoglycemic and hypolipidemic. The hypoglycemic effect can be achieved by inhibiting carbohydrate hydrolases (McCue et al., 2005). The Fig. 5 showed that the total polyphenol content of all three experimental groups reached the maximum after 20 h of fermentation, with the highest polyphenol content of 1.78 μg/mg in the MBF group. The total flavonoids content of all three experimental groups also reached the maximum after 20 h of fermentation, with the highest flavonoids content of 0.871 μg/mg in the LP fermentation group. The metabolic activity of microorganisms is strongly linked to the increase or release of mostly natural phenolic substances bound in the matrix of plant cell walls.

The fermentation of *Lactobacillus* caused the disruption of the chickpea cell wall structure, thus releasing a certain amount of phenolic compounds, or inducing the synthesis of phenolic bioactive compounds, which may explain the increase in total polyphenol content (Hur et al., 2014). In

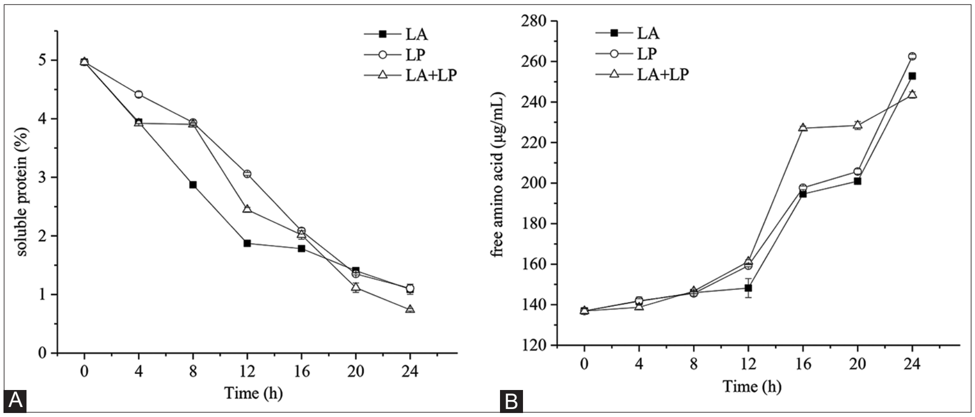

Fig 4. Effects of fermentation with different strains on the soluble protein (A) and free amino acids content (B) of chickpea milk.

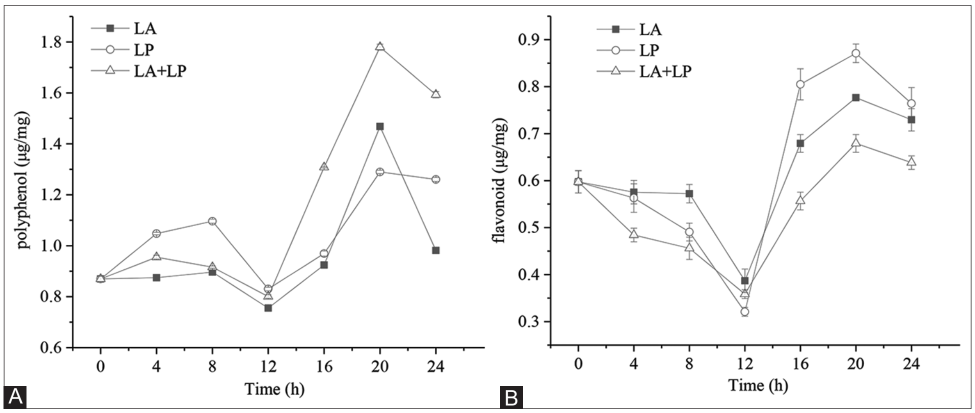

Fig 5. Effects of fermentation with different strains on the polyphenol (A) and flavonoid content (B) of chickpea milk.

addition, the microorganisms themselves could synthesize some phenolic compounds in the process of growth and reproduction (Georgetti et al., 2010). The decline of total polyphenol might be due to the condensation of phenolics and proteins to form precipitation, or some substances produced by *Lactobacillus* reduced the phenolic content to ensure their growth (Rodríguez et al., 2009).

Effects of fermentation on the hypoglycemic activity of chickpea milk

It was a proven way to achieve the hypoglycemic effect by inhibiting carbohydrate hydrolases, such as α-glucosidase and α-amylase (Cardullo et al., 2020). The previous experimental results (Fig. 2-Fig. 5) showed that chickpea milk fermented by *Lactobacillus* at 20 h and 24 h had higher nutritional value and more phenolic substances. On this basis, to further investigate a more efficient fermentation method that could improve the hypoglycemic ability of chickpea milk, the inhibition rates of α-glucosidase and α-amylase of fermented chickpea milk at 20 h and 24 h were investigated.

As shown in Fig. 6(a), the α-glucosidase inhibition rates of fermented chickpea milk were all significantly higher than that of unfermented chickpea milk, consistent with the findings of other studies that the hypoglycemic activity increased with fermentation (Wan et al., 2019; Zhang et al., 2018). The best inhibition effect was observed in chickpea milk fermented by LA for 24 h, with the inhibition rate of 92.91%. From Fig. 6(b), it can be seen that chickpea milk of MBF had the best inhibition effect on α-amylase. It could be found that both unfermented and fermented chickpea milk showed a better inhibitory effect on α-glucosidase than on α-amylase. These results demonstrated that *Lactobacillus* fermentation could enhance the hypoglycemic ability of chickpea milk.

Effects of fermentation on the content of different types of phenolics and their hypoglycemic capacity

Chickpea contains a wide range of phenolic compounds. Inhibition of carbohydrate hydrolase activity by phenolics is a potential way to control carbohydrate digestion and regulate postprandial blood glucose (Kim et al., 2005). From above results, it was found that, in the LA fermented chickpea milk (20 h), the content of dietary fiber, free amino acids, total polyphenol and flavonoids increased. And the LA fermented chickpea milk (20 h) also showed good α-glucosidase inhibitory effect. Thus, the three types of phenolics in the LA fermented chickpea milk (20 h) were isolated for further investigation.

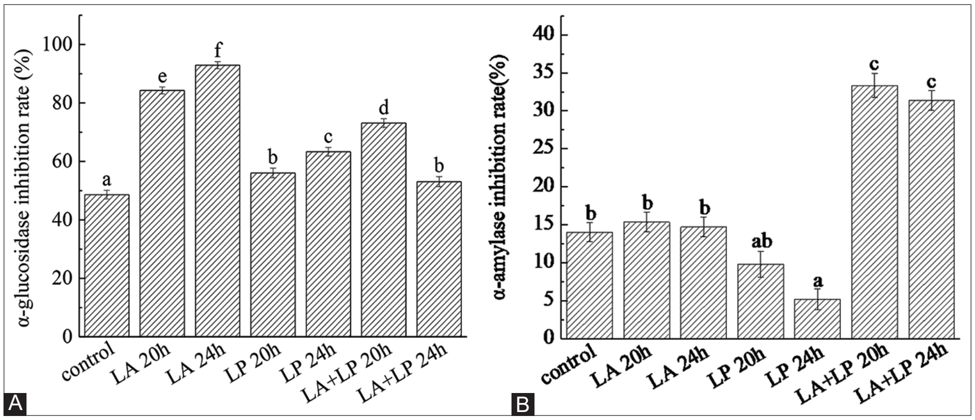

Fig 6. α-glucosidase (A) and α-amylase (B) inhibition of chickpea milk fermented by different strains. a, b, c represents significant differences between data (p< 0.05).

Phenolics in legumes can usually be divided into three types: conjugated, bound, and free. Conjugated phenolics refers to phenolic esterified sugar and other low molecular weight compounds. Bound phenolics are bound to the cytoderm in the form of covalent bonds (ester bonds or ether bonds) (Acosta-Estrada et al., 2014; Bhanja et al., 2009; Liu, 2007). To further study the changes in the content of different types of phenolics in chickpea milk by fermentation, the conjugated phenolics, bound phenolics, and free phenolics were extracted separately and their hypoglycemic abilities were also compared.

Content of different types of phenolics

The content of conjugated phenolics and free phenolics increased dramatically after fermentation as shown in Fig. 7. In unfermented chickpea milk, the bound phenolics had highest content of 7.76±0.65 μg/mL. After fermentation, the content of free phenolics was significantly higher than the other two types (16.07±0.17 μg/mL). Sánchez-Magaña et al. (2014) applied *Rhizopus oligosporus* to chickpea. It was confirmed that the solid-state biotransformation enhanced the free and total phenolic content. McCue and Shetty (2004) reported that solid-state fermented soybeans containing oligosporangium or shiitake could release a large number of free phenolic compounds. Cheng et al. (2013) indicated that β-glucosidase produced by fungal fermentation catalyzed the release of soybean substrates in isoflavone aglycones, thus altering the phenolic composition. Our results confirmed that *Lactobacillus* fermentation improved the phenolic composition of chickpea milk, resulting in an increase in the content of free phenolics, conjugated phenolics and total phenolics in chickpea milk.

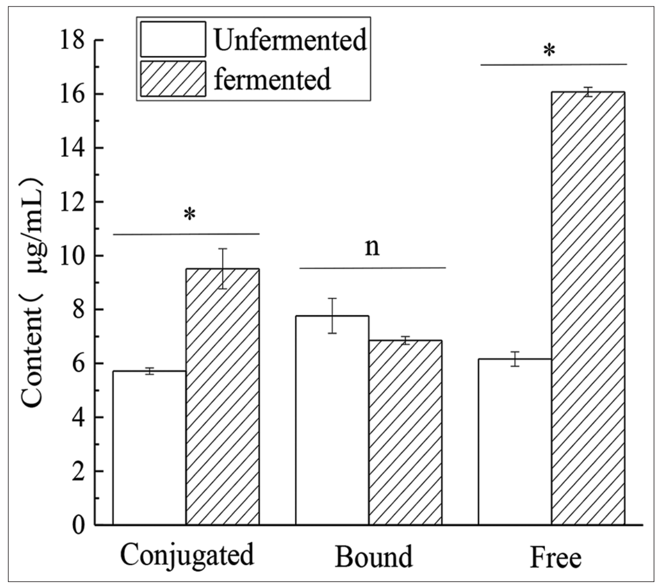

Fig 7. Conjugated, bound and free phenolic content in fermented (*L. acidophilus*) and unfermented chickpea milk. "n" means no significant difference between the same group of data, "*" means a significant difference (p < 0.05) between the same group of data.

Inhibitory activity of different types of phenolics on α-glucosidase and α-amylase

As shown in Fig. 8, the conjugated phenolics showed better inhibitory activity on α-glucosidase and α-amylase. Moreover, *Lactobacillus* fermentation increased the inhibitory activity of bound phenolics and free phenolics on α-glucosidase as well as the inhibitory rates of conjugated phenolics and bound phenolics on α-amylase. The content of bound phenolics did not increase significantly (Fig. 7), but their enzyme inhibition effect was enhanced, which was presumably related to the change in specific categories of bound phenolics. Additionally, the inhibition rate of phenolics extracts on α-glucosidase was higher than on α-amylase. Apostolidis and Lee (2010) had reported that the water extracts of *Ascophyllum nodosum* with high phenolic content had a better inhibitory effect on α-glucosidase than on α-amylase, which might provide an effective and less side-effective complementary treatment for postprandial hyperglycemia.

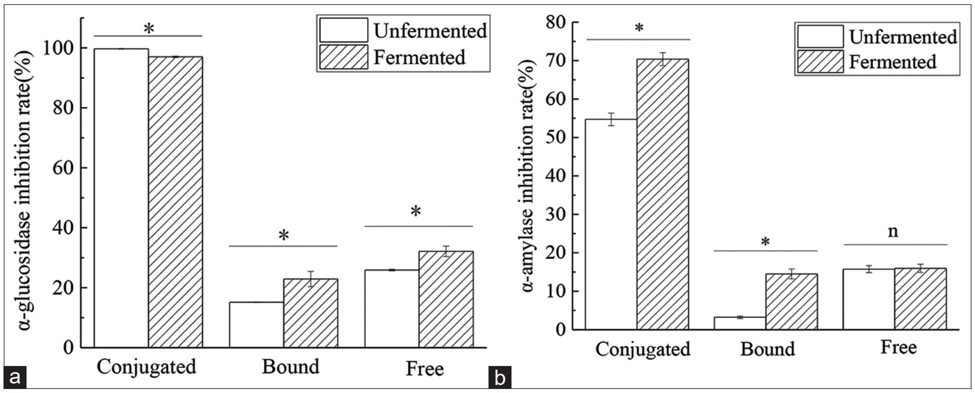

Fig 8. Inhibition rates of different forms of phenolics on α-glucosidase (a) and α- amylase (b). "n" means no significant difference between the same group of data, "*" means a significant difference (p< 0.05) between the same group of data.

CONCLUSION

All the findings demonstrated that both SBF and MBF in this work could improve the nutritional values and hypoglycemic effects of chickpea milk. The fermented chickpea milk contained more dietary fiber, free amino acids, total polyphenol and total flavonoids than the unfermented one. In addition, the fermentation of *L. acidophilus* ATCC 4356 increased the content of conjugated phenolics and free phenolics in chickpea milk. And the fermentation also enhanced the α-glucosidase inhibition rate of bound phenolics and free phenolics, as well as the α-amylase inhibition rate of conjugated phenolics and bound phenolics. Therefore, lactic acid bacteria fermentation would be a potential way to develop chickpea milk into functional food.

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Author Contributions

Meng-Xi Zhu: experiments conduction, data processing and writing. Chao Xu: experiments conduction. Yun-Peng Hao: resources and software processing. Jun Meng: project administration, funding acquisition, writing and editing. Jing-Li Wang: data processing. Shao-Bing Zhang: supervision. All authors read and approved the manuscript.

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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