Antioxidative, ACE inhibitory and antibacterial activities of soy milk fermented by indigenous strains of lactobacilli

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Abstract
Soy milk, a derivative of soybean, is an alternative to dairy beverage, but its acceptability is limited worldwide due to unpleasant beany flavour. Fermentation may, however, improve the nutritional and sensory values of soy milk. Thus, post-fermentation improvement in functional attributes of soy milk were investigated via antioxidant, ACE inhibitory, and antimicrobial activities in this study using five test and one control strains of lactobacilli. Results indicated that soy milk fermented by Lactobacillus rhamnosus strain C25 (LR C25) effectively scavenged more than 60% of the ABTS, DPPH and Hydroxyl radicals generated in vitro models. Moreover, all the strains showed significantly higher (p < 0.05) antioxidant activity as compared to unfermented soy milk in all three assays performed. Further, Lactobacillus plantarum strain C6 (LP C6) fermented soy milk displayed significantly higher (p < 0.05) percent ACE inhibitory activity (68.40 ± 0.93%) as compared to other tested Lactobacillus isolates, reference strain and unfermented soy milk. It was also observed that LP C6 strongly inhibited the growth of indicator strain of E. coli in the agar well diffusion assay. These strains can therefore be further explored in the preparation of beneficial soy foods and bioactive food supplements for wellbeing.

KEYWORDS
ACE-inhibitory, Antioxidant, Bioactive peptides, Isoflavones, Soybean

1 | INTRODUCTION

Economically, soybean (Glycine max L. Merrill) is the world’s most important leguminous crop, supplying millions of people with vegetable protein, and hundreds of chemical ingredients. Traditionally, soybean is used to make various fermented and unfermented products such as miso, soy cheese, soy sauce, soy yogurt, tamari, tempeh, and soymilk, especially in the Asian subcontinent (Jayachandran & Xu, 2019; Tamang, 2015). Among them, soy milk is an aqueous extract of soybeans produced by soaking, grinding, and filtering. Soy milk is a perfect emulsion of oil, water, and protein. It includes sufficient proteins, iron, unsaturated fatty acids and other nutrients and low fat and carbohydrates (Singh, Vij, & Hati, 2014). Soy milk is also one of the richest sources of isoflavones, generally referred to as phytoestrogens because of their resemblance to estrogens (Cao, Green-Johnson, Buckley, & Lin, 2019).

Although, soy milk is a perfect nutrient supplement, but its acceptability limited worldwide due to beany flavor and flatulence. Fermentation is now recognized as the best way to reduce soy milk’s beany taste and flatulence along with enhancing soy milk’s functional properties by increasing bioactive components and decreasing antinutritional components. In addition, fermentation is used to improve the bioavailability of soy vitamins, minerals, isoflavones and proteins (Cao et al., 2019; Singh & Vij, 2018). Refer to their reported beneficial
effects on human nutrition and health, fermented soybean products are getting more attention now days.

Last few decades witnessed the use of lactic acid bacteria as a probiotic and a starter organism for the development of health beneficial foods. Although, *Bacillus subtilis* and *Aspergillus* are the microorganisms that are widely used to ferment various soy products (Jayachandran & Xu, 2019), now specific strains of lactic acid bacteria have been recognized as a starter for the fermentation of soy milk (Dobreva, Dragnev, Mladenova, & Danova, 2019). Lactic acid bacteria can degrade soy proteins into simpler forms like oligopeptides, di-peptides, and tri-peptides during fermentation resulting in improving the functionality of the protein. Enzymatic machinery of lactic acid bacteria can hydrolyze soy complex oligosaccharides which are principally responsible for its beany flavor (Hati et al., 2013). Fermentation also enriches soy milk with functional attributes. It has reported that fermented soybean possess potent antioxidant (Singh & Vij, 2018) and angiotensin-converting enzyme (ACE) inhibitory activities (Wu & Ding, 2002), which are the major cause of most of the new generation disorders such cancer, cardiovascular and other related diseases. In this way, soy fermented milk and their bioactive components can be a possible alternative for the prevention of several cardiovascular and life-style diseases. This study was therefore designed to determine the functional attributes of soy milks fermented by selected strains of Lactobacillus. Three important activities viz. antioxidant, antihypertensive and antimicrobial were selected to appraise the possibility to develop a soy based functional fermented product.

2 | MATERIALS AND METHODS

2.1 | Materials and microorganisms

All chemicals and reagents were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). Nutrient agar (NA), de-Man Rogosa and Sharpe (MRS) and agar base were procured from Hi-Media, Mumbai, India. Soybean, variety JS-335, was purchased from local market of Karnal (India). Five *Lactobacillus* strain viz. *L. plantarum* C6 (LP C6), *L. rhamnosus* C8 (LR C8), *L. rhamnosus* C25 (LR C25), *L. rhamnosus* C28 (LR C28), and *L. rhamnosus* C34 (LR C34) used in this study were isolated from cheese (Singh & Vij, 2018). One reference lactic strain *Lactobacillus helveticus* NCDC 288 was procured from the National Collection of Dairy Cultures (NCDC), Karnal, India. Pathogenic indicator bacterial strains were procured from the American Type Culture Collection (ATCC), USA, National Collection of Type Cultures (NCTC), UK, Microbial Type Culture Collection (MTCC), India, and National Collection of Dairy Cultures (NCDC), India (Table 1).

2.2 | Fermentation of soy milk

Soy milk was prepared according to the method mentioned by Singh and Vij (2017). Fermentation was carried out using 1% inoculum of the strains, *Lactobacillus plantarum* C6 (LP C6), *Lactobacillus rhamnosus* C8 (LR C8), *Lactobacillus rhamnosus* C25 (LR C25), *Lactobacillus rhamnosus* C28 (LR C28), *Lactobacillus rhamnosus* C34 (LR C34) and *Lactobacillus helveticus* (NCDC 288), separately at 37°C for 24 h. Fermented hydrolysates were prepared by centrifuge (12,000 ×g/10 min/4°C) soy milk after 24 h of fermentation. The obtained hydrolysates were filter-sterilized by passing through 0.22 μm sterile Millex syringe filter.

2.3 | Antioxidative activity

ABTS radical scavenging capacity (ABTS RSC) was assessed by method of Re et al. (1999) with slight modifications. A 10 μl aliquot of fermented hydrolysate was added to 990 μl ABTS solution, prepared by mixing ABTS with potassium persulphate. The decrease in the absorbance of solution was recorded for 10 min at 734 nm using UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The results were expressed as; ABTS RSC (%) = (A734 nm blank - A734 nm sample/A734 nm blank) × 100.

DPPH radical scavenging activity (DPPH RSC) was analyzed by the method given by Brand-Williams, Cuvelier, and Berset (1995) with some modifications. A 250 μl aliquot of hydrolyzate was added to an equal amount of DPPH working solution in an amber color eppendorf and incubated at 37°C for 1 h. The absorbance of the solution was measured at 515 nm (UV-1800 spectrophotometer) against methanol by taking distilled water as a blank. The results were expressed as; DPPH RSC (%) = (A515 nm blank - A515 nm sample/A515 nm blank) × 100.

Hydroxyl radical scavenging assay was carried out by Fenton reaction method (He, Luo, Cao, & Cui, 2004) with some modifications. Reaction mixture was prepared by mixing 1 ml brilliant green (0.435 mm) with 2 ml FeSO₄ (0.5 mm) and 1.5 ml of 3% H₂O₂. A 1 ml aliquot of hydrolyzate was added to reaction mixture and incubated at room temperature for 20 min. The change in absorbance was measured at 624 nm by UV-1800 spectrophotometer. The results were expressed as; Hydroxyl radical scavenging activity (Hydroxyl RSC) (%) = (A624 nm blank - A624 nm sample/A624 nm blank) × 100.

2.4 | ACE inhibitory activity

ACE inhibitory activity was analyzed according to the method given by Cushman and Cheung (1971) with minor modifications. A 50 μl hydrolyzate was mixed with 50 μl of ACE (50 mU/ml) and pre-incubated for 10 min at 37°C. Then 150 μl of 4.15 mm HHL (Hippuryl-L-Histidyl-L-Leucine) solution was mixed with above mixture and incubated for 30 min at 37°C. The reaction was terminated by the addition of 500 μl of 1 M HCl. The hippuric acid liberated by the ACE was then extracted with 1.5 ml ethyl acetate by centrifugation at 3000 x g for 10 min followed by heat evaporation at 95°C for 10 min. The residue containing hippuric acid was dissolved in 1 ml of deionized water and the absorbance of the solution was measured at 228 nm (UV-1800 spectrophotometer). For blank all components were prepared according to the same method but without ACE.
Antioxidative activity of fermented soy milks was evaluated by three different radical scavenging assays, ABTS, DPPH and hydroxyl, considering the absence of a universal method to evaluate the total antioxidant activity of one food product. In case of ABTS RSC, all the Lactobacillus isolates along with a reference culture displayed remarkable ability, between 63 to 70% (Figure 1a). Notably, LP C6 showed highest, 70.48 ± 1.78%, ABTS RSC among all the bacterial strains studied. Likewise, ABTS RSC of LP C25, LR C28, LR C34 and LR C8 were 68.64 ± 1.08, 67.92 ± 1.67, 67.34 ± 1.23 and 63.09 ± 1.88%, respectively. Essentially, the ABTS RSC is based on the reduction of pre-formed radical monocation of ABTS generated by oxidation with potassium persulfate (Re et al., 1999). Addition of our fermented hydrolyzate of soy milk to this pre-formed radical cation reduces it color to an extent in a 10 min period depending on the antioxidant activity. All the Lactobacillus isolates showed significantly higher (p < 0.05) ABTS RSC from unfermented soy milk (Figure 1a); indicated some compounds may produced during fermentation having radical scavenging potential.

Likewise, the percent DPPH RSC of LR C25 (64.0544 ± 1.10) and LR C28 (63.88 ± 0.71) was almost similar, both showed significantly higher (p < 0.05) activity among the tested bacterial strains and unfermented control (Figure 1b). Conversely, LP C6 and LR C34 were observed with lower DPPH RSC in comparison to reference bacterial strain NCDC 288. Moreover, no significant difference in percent DPPH RSC was found between LR C8 and NCDC 288. In DPPH RSC assay fermented hydrolyzates were allowed to react with a stable DPPH radical in a methanol solution. The reduction of DPPH radical was followed by monitored in decrease of absorbance at 550 nm (Brand-Williams et al., 1995). Thus, two of our strains LR C25 and

### TABLE 1  Antibacterial activity of soy milks fermented by lactobacilli

| Strains | Salmonella enterica (NCTC 6017) | Sigella dysenteriae (NCDC 107) | Listeria monocytogenes (ATCC 15313) | Staphylococcus aureus (MTCC 1144) | Escherichia coli (ATCC 25922) | Bacillus cereus (ATCC 14579) |
|---------|---------------------------------|-------------------------------|-----------------------------------|-------------------------------|-----------------------------|-----------------------------|
| LP C6   | +                               | +                             | ++                                | ++                            | +++                         | +                           |
| LR C8   | +                               | ++                            | +                                 | +                             | -                           | ++                          |
| LR C25  | +                               | +                             | +                                 | +                             | +                           | +                           |
| LR C28  | ++                              | +                             | +                                 | ++                            | ++                          | +                           |
| LR C34  | +                               | +                             | +                                 | +                             | +                           | +                           |
| NCDC288 | +                               | –                             | +                                 | ++                            | +                           | –                           |
| Soy Milk (UF) | –                       | –                             | –                                 | –                             | –                           | –                           |

<7 mm, no activity (–); 8–10 mm, weak inhibition (+); 11–14 mm, moderate inhibition (++); 15–20, strong inhibition (+++).
control (Figure 1c). Overall, all the Lactobacillus strains in our study were efficiently able to inhibit more than 50% of hydroxyl radicals. Differences in radical scavenging activity of soy milks may be due to generation of diverse free radicals in all the three assays performed.

Free radical scavenging capacities of soy milk may be principally due to bioactive components such as bioactive peptides, isoflavones, saponines and phenolic compounds, generated during fermentation by specific strains of lactobacilli. In this context, L. plantarum and L. rhamnosus strains were reported to actively grow in soy milk and enhances antioxidant activity by increasing phenolics and isoflavone aglycones (Subrota, Shilpa, Brij, Vandna, & Surajit, 2013; Xiao et al., 2015). Similar to our work, Xu and co-workers also studied antioxidant capacities of the twenty-seven fermented soybean products, who found highest activity in black bean product “Douchi”. In addition, eleven L. plantarum strains isolated from traditional Chinese fermented products were analyzed and found that L. plantarum C88 showed the highest hydroxyl radical and DPPH scavenging activities, with inhibition rates of 44.31% and 53.05%, respectively. Further, when L. plantarum C88 was administered to mice suffering from oxidative stress, the serum superoxide dismutase activity, glutathione peroxidase activity and the total antioxidant capacity in liver increased significantly (Xu, Du, & Xu, 2015). Similarly, Lactococcus acidophilus fermented soymilk also reported to improve antioxidant capacities in hyperlipidemia rats (Chen, Wu, Yang, Xu, & Meng, 2017). A cocktail of probiotic (Bifidobacterium bifidum, Lactobacillus casei, and Lactobacillus plantarum) fermented soymilk also reduced the production of reactive oxygen species in high-fat diet mice (Zhang, Wang, et al., 2017).

3.2 | ACE inhibitory activities of fermented soy milks

ACE inhibitory activity of fermented soy milk was investigated using HHL as a substrate and shown in Figure 2. The activity of LP C6, 68.40 ± 0.93%, was found significant higher (p < 0.05) from other tested Lactobacillus isolates, reference strain and unfermented soy milk. Additionally, the percent ACE inhibitory activity of LR C8, LR C25 and LR 28 was 56.52 ± 1.22, 65.52 ± 1.40 and 62.92 ± 0.52, respectively. The inhibition recorded by LR C8, LR C25 and LR C28 was significantly higher (p < 0.05) from reference strain NCDC 288. The ACE inhibitory activity of all the fermented soy milks was significantly higher (p < 0.05) in comparison to unfermented soy milk. It has been known that; several ACE inhibitory peptides are released from

FIGURE 1 | Antioxidative activity of fermented soy milks. Panel “a” ABTS radical scavenging capacity (ABTS RSC); panel “b” DPPH radical scavenging capacity (DPPH RSC); panel “c” Hydroxyl radical scavenging activity (Hydroxyl RSC). Graphs represents the mean ± SEM of each experiment performed in triplicate, values with different letters differ significantly (p < 0.05) in all panels.
S. aureus and milk against E. coli; highest antibacterial activity was displayed by LP C6 fermented soy in inhibition against tested pathogenic microorganisms (Table 1). The antibacterial activity of fermented soy milks showed weak to strong.

...found ACE inhibitory activity (41.66%) in fermented soy milk hydrolyzed (Mishra, Hati, Das, & Prajapati, 2019). Bhatnagar et al. (2018) also reported around 80% ACE inhibitory activity of lactobacilli fermented soy milk after 5 days of storage under refrigeration conditions. Moreover, unfermented soy milk used as control in our study did not show inhibition against any of the pathogenic microorganism (Table 1). Similar to our results Zhao and Shah (2014) also documented antibacterial effect of soy product against Gram-positive pathogenic bacteria. Likewise, in an interested study Abd El-Gawad, El-Sayed, El-Zeini, Hafez, and Saleh (2014) reported that E. coli cells were disappeared after three days of storage when co-inoculated with starter bacteria at the time of soy yoghurt preparation. Mishra and co-workers (2019) recently found antibacterial activity of flavored fermented soy milk against L. monocytogenes, B. subtilis, S. aureus, S. typhi and E. coli strains. Antimicrobial activities of the cell-free supernatant of soy milk fermented by L. helveticus has also found against B. subtilis and E. coli strains (Hati, Patel, & Mandal, 2018).

Figure 2: ACE-inhibitory activity of fermented soy milks. Graphs represents the mean ± SEM of each experiment performed in triplicate, values with different letters differ significantly (p < 0.05).

the inactive soybean precursor proteins by the action of microbial proteases during the fermentation (Singh & Vij, 2017). In this context, the higher ACE inhibitory activity of most of the strains as compared to unfermented soy milk indicated that some ACE inhibitory peptides were released during fermentation. Several previous studies support this statement that soy milk fermented by specific strains of lactic acid bacteria is a good source of ACE inhibitory peptides (Singh & Vij, 2017; Tsai, Lin, Pan, & Chen, 2006). Likewise, Tsai and co-workers (2006), documented ACE-inhibitory effect of tri-peptide (Val-Pro-Pro and Ile-Pro-Pro) in animal model obtained from fermented soy products. Similar to our study, more than 55% ACE inhibition was reported in fermented soybean extracts and soy whey medium (Lye, Kuan, Ewe, Fung, & Liong, 2009; Pyo & Lee, 2007). Moreover, Ma, Cheng, Yin, Wang, and Li (2013) reported more than 60% increment in ACE inhibitory activity followed by fermentation. A recent study also reported around 80% ACE inhibitory activity of lactobacilli fermented soy milk after 5 days of storage under refrigeration conditions (Mishra, Hati, Das, & Prajapati, 2019). Bhatnagar et al. (2018) also found ACE inhibitory activity (41.66%) in fermented soy milk hydrolysate of Lactobacillus paracasei CD4 strain. In another study, the ACE inhibitory activity of L. fermentum fermented soy milk was observed to reached 60% after 20 h of fermentation (Myagmardorj, Purev, & Batdorj, 2018).

3.3 | Antibacterial activities of fermented soy milks

Antibacterial activity of fermented soy milks showed weak to strong inhibition against tested pathogenic microorganisms (Table 1). The highest antibacterial activity was displayed by LP C6 fermented soy milk against E. coli. LP C6 showed moderate inhibition against L. monocytogenes and S. aureus. Moreover, LP C8 and LR C28 displayed moderate activity against S. dysenteriae, B. cereus, S. enterica, S. aureus and E. coli. LR C34 and NCDC 288 also showed moderate inhibition against S. aureus. Conversely, reference Lactobacillus strain NCDC 288 did not show activity against S. dysenteriae and B. cereus strains. The inhibition of the growth of pathogens may be through the generation of antimicrobial compounds, antimicrobial peptides, and organic acids during fermentation (Singh et al., 2015). Some previous reports support this statement that specific strain of Lactobacillus species such as L. plantarum and L. rhamnosus are able to produces some antimicrobial compounds (Lin & Pan, 2017; Zhang, Wu, et al., 2017). Also, unfermented soy milk used as control in our study did not show inhibition against any of the pathogenic microorganism (Table 1). Similar to our results Zhao and Shah (2014) also documented antibacterial effect of soy product against Gram-positive pathogenic bacteria. Likewise, in an interested study Abd El-Gawad, El-Sayed, El-Zeini, Hafez, and Saleh (2014) reported that E. coli cells were disappeared after three days of storage when co-inoculated with starter bacteria at the time of soy yoghurt preparation. Mishra and co-workers (2019) recently found antibacterial activity of flavored fermented soy milk against L. monocytogenes, B. subtilis, S. aureus, S. typhi and E. coli strains. Antimicrobial activities of the cell-free supernatant of soy milk fermented by L. helveticus has also found against B. subtilis and E. coli strains (Hati, Patel, & Mandal, 2018).

4 | CONCLUSION

Based on the results from this study we may infer that selected strains of Lactobacillus generated some biofunctional components during soy milk fermentation. The functional activities (antioxidative, ACE inhibitory and antibacterial) of fermented soy milk may be due to a specific bioactive component or cumulative effect of these bioactive compounds. The enhanced bioactivities of fermented soy milk in our study as compared to unfermented soy milk possibly support this statement. Therefore, these Lactobacillus isolates may be used for the development of health beneficial soy foods or bio-therapeutics. The present study did not, however, describe the bioactive components and specific mechanisms that regulate these effects. Further research may therefore be conducted to classify the biofunctional compounds in fermented soy milk and their impact in the in vivo system.

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CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTIONS

BPS design the study, performed the experiments, analyzed the data, and wrote the manuscript. BB reviewed and edited the manuscript. SV supervised the concept and reviewed manuscript.
ETHICS STATEMENT
This article does not contain any human and animal subjects for experiment.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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