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Probiotic characterization of Curd Developed from Soy Milk

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ABSTRACT: Soy milk is one of the best alternatives for non-dairy milk. It is free of cholesterol and lactose and might be used as a beverage for vegans, lactose-intolerant and milk-allergic patients. But consumption of soy milk is limited, because of its beany flavor and presence of oligosaccharides. To overcome these limitations, lactic acid fermentation is the best way to improves digestibility of milk constituents. In the present study, probiotic soy curd was developed by using *Lactobacillus acidophilus* as starter culture. The prepared soy curd was evaluated for their probiotic characterization along with antimicrobial activity and antibiotic resistance. The prepared soy curd possesses all tested probiotic characteristics. The soy curd was also found antagonistic activity against selected human enteric pathogens and resistance for selected antibiotics.

Keywords: Antimicrobial, Antibiotic resistance, *Lactobacillus acidophilus,* Probiotic, Soymilk, Soy curd.

INTRODUCTION

Economically soybean (*Glycine max*) is the most important bean in the world as it provides vegetable protein for millions of people; used as an ingredients for various chemical products and also a potential source of bioactive peptides. It is a very good source of low-cost protein. It contains about 40% protein, which is notably valuable as it contains sufficient lysine and limiting in the Sulphur containing amino acids. Presence of lysine helps in overcomes the lysine deficiency of cereals. Besides proteins, soybean also contains 35% carbohydrates in form of little starch (less than 1%) and hexose. (Karr-Lilienthal *et al.*, 2005). Soybean is also a good source of dietary fibers. Dietary fiber increases the water-holding capacity of stools in humans and it also increases stool bulk, stool softness and transit time that helps in reduction of hemorrhoids, diverticulosis and other lower GI tract diseases. Previous study confirmed the association of soybean and its constituent with reduction of cardiovascular disease (Rimbach *et al.*, 2008), prostate cancer and breast cancer reduction (Steiner *et al.*, 2008) and bone health improvement (Zhang *et al.*, 2007). This health claims supports to place soy foods in functional food category as it showed medicinal as well as nutritional importance. By soaking and grinding of soybeans, boiling the mixture and filtering out the remaining particulates, soy milk is prepared. It is a plant-based drink which may be used as an alternative to the vegan and lactose intolerance people (Lin *et al.*, 2004). In nutritional aspects, soymilk provides decent amount of all the nutrients, which one can found in raw soybean. In comparative study, both soy milk and cow milk shows presence of proteins, carbohydrate, fat and water in approx same amount, but the primary difference in both milk is type of fat present. Soy milk contains high amount of mono-unsaturated and poly-unsaturated fatty acids whereas cow milk contains high amount of saturated fatty acids (Icier *et al.*, 2015). Studies showed that consuming products which are high in saturated fatty acids may increase the risk of cardiovascular diseases whereas mono-unsaturated and poly-unsaturated fatty acids regulates cholesterol level and alleviating cardiovascular inflammations. Based on free of cholesterol and lactose, soy milk might be used as a beverage for vegans, lactose-intolerant and milk-allergic patients. But because of beany flavor and the presence of oligosaccharides namely stachyose and raffinose, the consumption of soy milk is limited (Li *et al.*, 2014). These oligosaccharides are non-digestible to the human and may cause flatulence. Another disappointment with soy milk or soybean is presence of phytic acids. This acid is negatively charged and forms a complex with positively charged proteins and make it unavailable for other reactions. To overcome these limitations, lactic acid fermentation is the best way (Espinoza and Navarro 2010). Lactic acid fermentation improves digestibility of milk constituents (Chou and Hou 2000). Ayurveda, the Indian medicine system also recommends using curd, a fermented milk product, in varieties of illness. In the last few decades, the attention towards lactic acid bacteria for food manufacturing is increased due to the association of these bacteria with health- promoting effects (Narayan *et al.,* 2021). Lactic acid bacteria are classified as probiotics designated as “GRAS" organisms (Generally Recognized as Safe). Probiotics are defined as the group of microorganisms which possess health benefits to the host when consumed in an appropriate amount (Joint FAO/WHO/ OIE, 2003). In Indian Subcontinent, curd is the traditional fermented milk product and consumed in different forms such as shrikhand, lassi, misti doi or kadhi. Basically, curd is produced by addition of lactic starters in milk. A good quality curd is obtained after incubation of nine hours at 32ºC when mixed starter culture is used and in case of pure starter culture, curd is obtained after incubation of 12 hours at 30ºC. During the fermentation, milk proteins hydrolyzed, pH drops and the viscosity increases. In addition, production of bacterial metabolites contributes to the taste as well as the health promoting properties. Curd is the oldest health beneficial fermented product having well documented health effects for GI conditions, such as diarrheal diseases, constipation, colon cancer, *Helicobacter pylori* infection, IBD, lactose intolerance and allergies (Yadav *et al.*, 2007). It is reported that curd prepared from mixed probiotic starter culture may helps in activation of non-specific immune system and also protects from *Shigella dysenteriae* infections (Singh and Kansal 2003). Another study also suggested delay in the progression of chemical and dietary induced diabetes after consumption of probiotic curd (Yadav *et al.,* 2006). Taking into consideration of the above points, the present study focused on the development of curd from soy milk by using *Lactobacillus acidophilus* as starter culture and probiotic characterization of the developed soy product.

MATERIAL AND METHODS

Procurement and processing of soybean seeds. High quality wholesome and mature soybean seeds were procured from reliable source near SHUATS, Prayagraj. After procurement, soybean seeds were sundried and kept in air tight plastic container for future work.

Preparation of soy milk. To prepare soy milk, blending method (Afroz *et al.*, 2016) was followed. 100g of soybean seeds was soaked in 1.0L of distilled water at room temperature (28°C). After 14-16h, the soak water was drained off and the seeds were blanched at 98°C for 30min. 0.3% sodium bi-carbonate was added in water to remove the bitterness and anti-nutrition factors from seeds. The drained seeds were hand washed thoroughly to remove their husk and then placed in a warring blender. After that 600ml of boiled distilled water at 87-90°C was added in seeds and then blended for 10-15 min at low speed. The boiled water inactivated the enzyme, lipoxygenase during blending. The resulting slurry was filtered through two layers of muslin cloth and boiled at 100°C for 10-15 min with constant stirring (Fig. 1).

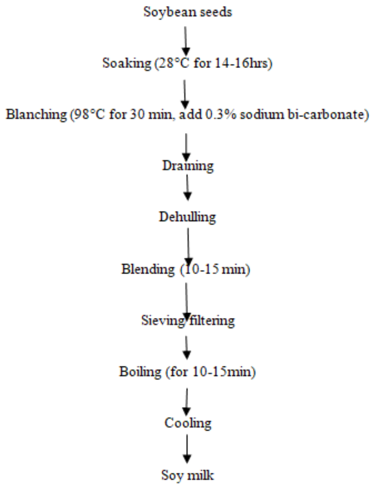

Fig. 1. Flow chart of soy milk preparation.

Selection and storage of Starter culture. For preparation of soy curd, *Lactobacillus acidophilus* was selected as starter culture. The selected lactic acid bacterial culture was isolated and identified in previous study.

Preparation of probiotic soy curd. 100ml of sterile soy milk was boiled at 90°C for 15 minutes and poured into sterile glass jars. The probiotic soy curd was prepared by inoculating with 1% starter culture *i.e.*, *Lactobacillus acidophilus*. The preparation of probiotic soy curd was kept for incubation at 45°C for 24-48 hours (Fig. 2).

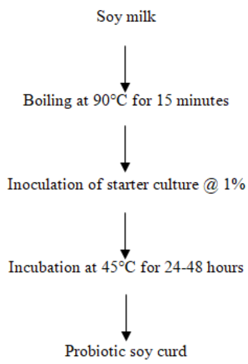

Fig. 2. Flow chart for preparation probiotic soy curd.

Probiotic characterization of prepared soy curd. Enumeration of viable cells in soy curd. 1 ml of prepared soy curd was transferred aseptically into 9 ml sterile peptone water, mixed thoroughly and serially diluted up to 10^6 using 9 ml peptone water blanks. 0.1 ml of diluted sample was inoculated on MRS agar plate. After incubation, the total viable count was calculated as CFU/ml using following formula

$$\text{CFU/ml} = \frac{\text{Average no.of colonies} \times \text{Dilution Factor}}{\text{Volume of culture plated}}$$

Resistance to low pH: MRS broth tubes of varying pH, *i.e.*, pH 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 was prepared and incubated with 1ml soy curd sample and incubated at 37°C for 24 hrs. After incubation, 0.1ml inoculums of each tube was poured to MRS agar medium by pour plate method and incubated at 37°C for 48hrs. The pH resistance of probiotic soy curd was estimated by calculating and comparing bacterial colony counts (CFU/ml) in MRS agar plate with varying pH (Tambekar and Bhutada 2010).

Resistance to bile salt. For bile tolerance assay, soy curd was inoculated into 10mL of fresh MRS broth with varying concentration of bile oxgall (0.5, 1.0, 1.5 and 2%) and without (control) bile oxgall and was incubated anaerobically at 37°C for 48h, after which 0.1ml sample from each tube was spread-plated on MRS agar and incubated anaerobically at 37°C for 24 h. After incubation, resistance to bile salt of soy curd was assessed by colony counts (CFU/mL) on the plates. Bile tolerance was estimated by comparing viable cell counts in MRS with and without bile oxgall.

Phenol tolerance. For the determination of phenol tolerance, test tubes containing MRS broth was adjusted with different concentration (0.1 to 0.4%) of phenol. After sterilization, each test tube was inoculated with probiotic curd and incubated at 37°C for 24h. After 24hrs of incubation, 0.1ml sample from each tube was poured to MRS agar medium by pour plate method and incubated at 37°C for 48 hrs. After incubation, phenol tolerance of soy curd was assessed by colony counts (CFU/mL) on the plates (Hoque *et al.*, 2010).

Antimicrobial activity. The prepared soy curd was screened for antimicrobial activity against pathogens namely *Bacillus cereus*, *Shigella flexnerii*, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas* sp. by agar well diffusion method. Suspension of soy curd and test pathogen was prepared separately by dissolving them in sterile distilled water. Suspension of each test pathogens was spread onto the nutrient agar, into which 6mm deep wells was dug. About 100µl of soy curd suspension was poured into each well, and nutrient agar plates were incubated for 37°C at 24hrs. Inhibition zone diameter was measured.

Determination of antibiotic resistance. 5 antibiotic discs (Vancomycin, Gentamycin, Chloramphenicol, Streptomycin and Neomycin) were used to determine the antibiotic susceptibility of soy curd using discs diffusion method on MRS agar plates. A 100µl suspension of soy curd suspension was spread on MRS agar plates. The antibiotic discs were placed on the surface of agar plate with help of a sterile forceps. For each antibiotic disc separate plate were used. The plate was incubated at 37°C for 24 hours. After that incubation inhibition zone was recorded.

RESULTS AND DISCUSSION

Enumeration of viable cells in the prepared soy curd. It is very important to make sure the survival of selected lactic acid bacteria during production and storage of soy curd. There are various intrinsic (such as water activity, pH, titrable acidity, oxygen), processing (such as incubation temperature and time, storage conditions) and microbiological parameters (such as probiotic strain selected and employed, proportion of inoculation) which effects the viability of selected lactic acid bacteria into prepared soy curd. Therefore, in the present study, the viable cell count of prepared soy curd was calculated in different day intervals *i.e.*, 0 days, 7 days, 14 days, 21 days and 30 days. The viable cell count was presented as CFU/ml × 10^6. At 0 day the count was 2.2 which were increased after 7 days and at 7 day the viable cell count was 3.1. The VCC was decreased after 7 days of preparation. The recorded viable cell count was 1.9, 1.0 and 0.8 on 14th, 21th, and 30th day after preparation (Fig. 3). The results are in agreement with research conducted by D'Alessandro *et al.* (2023) in which soy beverages fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* showed a significantly decrement in cell load viability after 14 days of storage.

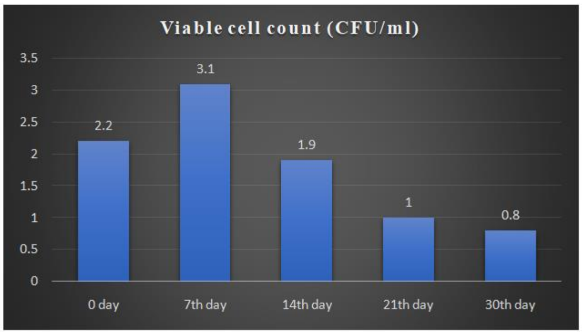

Fig. 3. Enumeration of viable cells in the prepared soy curd.

Resistance to pH. In the present study, soy curd was examined for growth in different pH range from pH 1 to pH 10, in which growth was observed in the range of pH 3 to pH 8 whereas no growth was observed in pH 1, pH 2, pH 9 and pH 10. In pH 7 the recorded CFU/ml was the same as CFU recorded with control whereas when pH values decrease, the CFU/ml also decreased. The counted CFU/ml for soy curd was 0.7 × 10^6, 1.3 × 10^6, 1.9 × 10^6, 2.9 ×10^6, 3.5 ×10^6 and 2.6 ×10^6 with pH 3, pH 4, pH 5, pH 6, pH 7 and pH 8 respectively whereas control showed 3.5 ×10^6 CFU/ml (Fig. 4). Previously Kumari *et al.* (2022) also screened acid tolerance activity in lactic acid bacteria namely *L. rhamnosus* NCDC953 and *L. reuteri* NCDC958 for development of fermented soy milk product. The current findings were also supported by Gunyakti and Asan-Ozusaglam (2019); Bhushan *et al.* (2021). In the present study, when pH increases or decreases from optimum pH *i.e.*, pH 7, the CFU/ml was also decreased. These findings are in agreement with the results found by Dunne and Mahony (2001), where *Lactobacillus* strains showed viability when exposed to pH values of 2.5–4.0 but displayed loss of viability when pH values lowers.

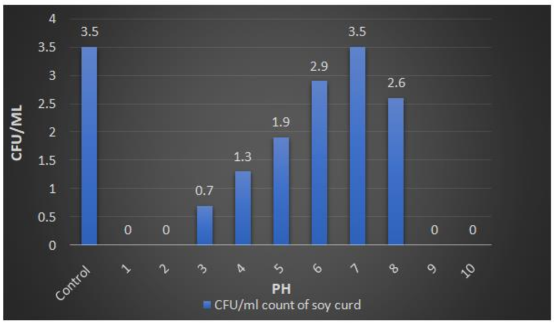

Fig. 4. Resistance to pH.

Resistance to bile salt. In the present study, soy curd showed growth in different tested bile concentrations *i.e.*, 0.5%, 1.0%, 1.5% and 2.0% but the counts were decreased when bile salt concentration increases. The counted CFU/ml for soy curd were 2.7×10^6, 1.8×10^6, 1.4×10^6 and 1.9×10^6 with 0.5%, 1.0%, 1.5% and 2.0% bile salt concentration respectively whereas control (0% bile concentration) showed 3.5×10^6 CFU/ml (Fig. 5). The results are supported by the study done Kumari *et al.* (2022) in which bile salt tolerance in lactic acid bacteria namely *L. rhamnosus* NCDC953 and *L. reuteri* NCDC958 for development of fermented soy milk product was showed for 0.3% to 1% concentration of bile salt. Oh *et al.* (2018) also supports the present findings.

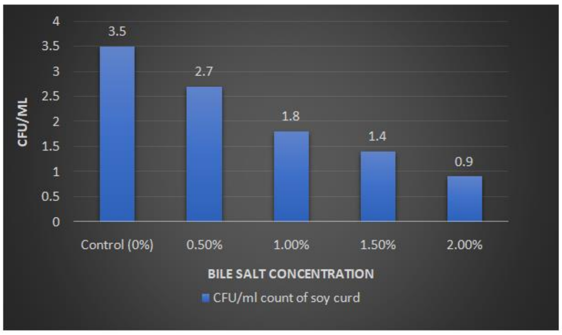

Fig. 5. Resistance to bile salt.

Phenol Tolerance. In the present study, soy curd was examined for phenol tolerance with different phenol concentrations *i.e.* 0.1%, 0.2%, 0.3% and 0.4%. Prepared soy curd showed growth in all tested concentrations of phenol. The CFU/ml was recorded 3.2×10^6, 2.9×10^6, 2.5×10^6 and 2.0×10^6 with 0.1%, 0.2%, 0.3% and 0.4% phenol concentration. The control (0% phenol) showed 3.5×10^6 CFU/ml. The CFU/ml was decreased when the concentration of phenol increased (Fig. 6). The present findings are in agreement with study done by Rahman *et al.* (2016) in which phenol tolerance in LAB with 0.1% to 0.2% concentration was recorded but the growth of LAB was reduced as concentration increased from 0.3% to 0.4%.

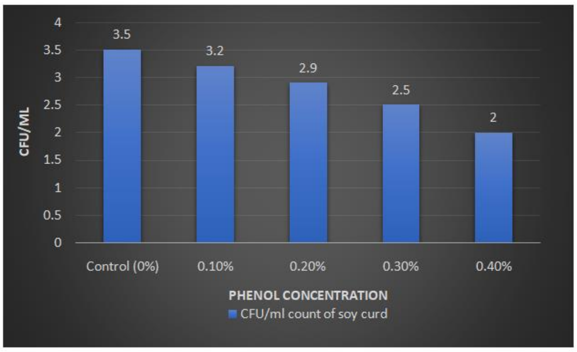

Fig. 6. Phenol Tolerance.

Hemolytic activity. Hemolytic activities of soy curd were evaluated on blood agar plates. Soy curd did not showed α-hemolytic and ß-hemolytic activity when grown on Columbia blood agar plates. The prepared soy curd showed γ hemolytic, *i.e.*, negative, or no hemolytic activity. The present findings are in agreement with study done by Kumari *et al.* (2022); Bhushan *et al.* (2017); Peres *et al.* (2014).

Antimicrobial activity of prepared probiotic soy curd. Antibacterial activity of prepared soy curd against test pathogens (*Bacillus cereus*, *Shigella flexnerii*, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas* sp.) were studied by following the agar well diffusion method. Soy curd showed inhibition of the growth of all tested pathogens. The highest zone of inhibition was recorded against *Bacillus cereus* while the lowest zone of inhibition was recorded against *Listeria monocytogenes*. The zone of inhibition recorded for prepared soy curd was 30 mm against *Bacillus cereus*, followed by *Escherichia coli* (ZOI=29mm), *Shigella flexnerii* (ZOI=27mm), *Pseudomonas sp.* (ZOI=21mm) and *Listeria monocytogenes* (ZOI=19mm) (Fig. 7). The results are in agreement with research conducted by D'Alessandro *et al.* (2023) in which soy beverages fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* showed antagonistic activity against intestinal pathogens namely *Escherichia coli* H10407, *S. choleraesuis* and *Y. enterocolitica*. Another study done by Narayan *et al.* (2021) also reported antibacterial activity of soymilk fermented with *L. rhamnosus* GG, *L. plantarum* MTCC 25432 and 25433 against pathogens namely *Staphylococcus aureus*, *E. faecalis*, *E. coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Salmonella enterica*. The present study outcomes was also supported by Hati *et al.* (2018). Antibacterial activity of fermented soy milk was also reported by Mishra *et al.* (2019) against *Escherichia coli, bacillus subtilis, Listeria monocytogenes, Salmonella typhi and Staphylococcus aureus.*

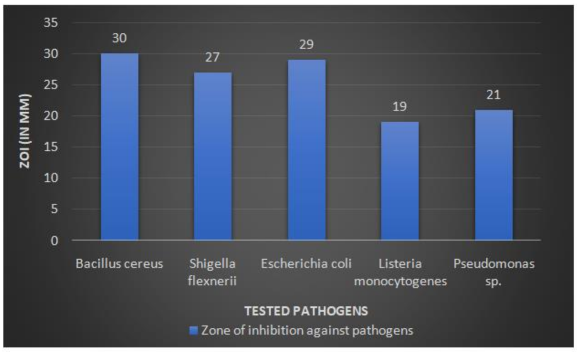

Fig. 7. Antimicrobial activity of prepared soy curd.

Determination of antibiotic resistance. Antibiotic resistance of prepared soy curd was studied by using the disc diffusion method. Recorded ZOI was used to determine the resistance or sensitivity of isolates for selected antibiotics viz., vancomycin, gentamycin, Chloramphenicol, Streptomycin and Neomycin. The recorded zone of inhibition was considered as sensitive if zone of inhibition was greater than 21mm and if zone of inhibition was lower than 15mm, this was considered as resistant. The prepared soy curd showed resistant towards vancomycin, chloramphenicol and streptomycin whereas towards gentamycin and neomycin it showed sensitivity (Fig. 8). Previously, Kumari *et al.* (2022) also studied the antibiotic susceptibility test for in lactic acid bacteria namely *L. rhamnosus* NCDC953 and *L. reuteri* NCDC958 for development of fermented soy milk product and recorded resistance against ciprofloxacin, nalidixix acid and co-trimaxazole by *L. rhamnosus* NCDC953 while *L. reuteri* NCDC958 showed resistant only against nalidixix acid and co-trimaxazole. Resistance in lactic acid bacteria toward ciprofloxacin, nalidixix acid and co-trimaxazole was also reported by Gunyakti and Asan-Ozusaglam (2019); Kaktcham *et al.* (2012); Sharma *et al.* (2017), respectively.

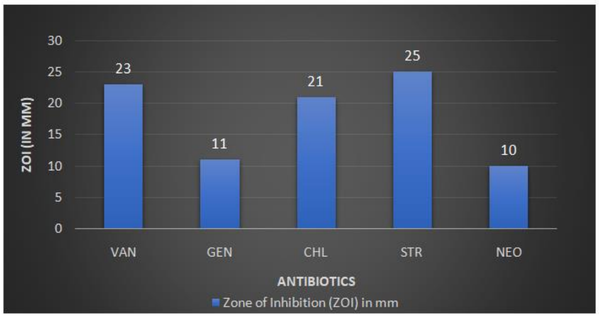

Fig. 8. Determination of antibiotic resistance (VAN = vancomycin, GEN=gentamycin, CHL=chloramphenicol, STR=streptomycin and NEO=Neomycin).

CONCLUSIONS

In the present study, soy curd was prepared by using *Lactobacillus acidophilus* as starter culture. After preparation of probiotic soy curd, probiotic potential of prepared soy curd was studied. The lactic acid bacteria *i.e., Lactobacillus acidophilus* showed all tested probiotic potentials in soy curd. It showed pH tolerance from pH 3 to pH 8, bile salt and phenol tolerance up to all tested concentration *i.e.*, 0.5% to 2% and 0.1% to 0.4% respectively. Viable cell count of prepared soy curd was found maximum for 7 days after preparation. The prepared soy curd showed antimicrobial activity against all tested pathogens. In antibiotic sensitivity profiling, the prepared soy curd showed antibiotic sensitivity towards vancomycin, chloramphenicol and streptomycin whereas towards gentamycin and neomycin, it showed resistant. On the basis of this finding, the study concluded that the prepared soy curd had probiotic potential.

FUTURE SCOPE

The results of this pilot study suggested that consumption of soy curd could be helpful and effective as it showed both nutritional and antimicrobial aspects. To clarify the safety and ambiguous aspects of probiotic soy curd administration in a human host, clinical trials are needed.

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Conflict of Interest. None.

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