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Supplementation of *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* in Soymilk to Develop Non-Dairy Functional Food

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A B S T R A C T

Soymilk is one of the popular plant-based beverages among the consumers due to its high nutritive value and multifarious health benefits. However, its beany flavor limits it consumption to only health-conscious population. Fermentation by probiotic microorganisms may increase its nutritive value and provide a pleasant taste profile leading to increased consumer acceptability worldwide. With reference to this, in current study, we have supplemented probiotic organisms (*Lactiplantibacillus plantarum* JSD25 and *Pediococcus pentosaceus* JSB11) into soymilk. The inoculum size (8%) was optimized and both the probiotic isolates have shown more than 7 $\log_{10}$ CFU/mL of viability even after 28th day of refrigerated storage. Moreover, they have also shown more than 6 $\log_{10}$ CFU/mL of viability when exposed to gastro-intestinal simulation. Then physico-chemical characterization of probiotic supplemented soymilk samples was done and minimum alterations were observed in pH, titratable acidity, sugar, protein, fat content and viscosity in soymilk sample supplemented with *P. pentosaceus*. Further, in case of sensory evaluation, higher consumer acceptance was observed in soymilk supplemented with *P. pentosaceus* followed by soymilk supplemented with L. plantarum with reference to control soymilk. Therefore, the present study results indicate that both the organisms are suitable for the development of probiotic supplemented soymilk.

Introduction

Over the past few decades, researchers have emphasized in all sections of the food product development to address the changing needs and to meet the present demands of consumers all over the world. Increasing urbanization has accelerated the demand of new alternatives of healthy foods and hence, the development of innovative functional foods or beverages is the recent trend in food research (Sethi *et al.,* 2016). Among the regularly consumed beverages; milk is the most common example that is relished by human population since ages due to its high nutritive value and flexibility in satiating appetite. However, clinical reports have revealed that some constituents of milk are associated with deleterious health effects such as milk protein allergies, lactose intolerance and coronary heart diseases because of its high cholesterol. As a result, plant-based milk and milk products may be a better choice for consumers looking for dairy-free alternatives. These products are a good source of minerals, non-allergic proteins, essential fatty acids etc. and unlike regular milk products, they lack cholesterol, saturated fatty acids, and lactose (Kundu *et al.,* 2018). Plant-based beverages are generally water-soluble and resemble bovine milk in appearance (Silva *et al.*, 2020). They are generally classified into five different categories such as Cereal based (oat milk, rice milk, corn milk, spelt milk), Legume based (soy milk, peanut milk, lupin milk, cowpea milk), Nut based (almond milk, coconut milk, hazelnut milk, pistachio milk, walnut milk), Seed based (sesame milk, flax milk, hemp milk, sunflower milk) and Pseudo-cereal based (quinoa milk, teff milk, amaranth milk) (sethi *et al.*, 2016). Among these categories from the past few years, a great attention was given to soybean based products and soymilk is one of the most important and accepted product worldwide. Soymilk is prepared by the traditional method in which the whole soybeans are soaked in water and then extracted with water. This extract is further boiled and filtered through cheese cloth and the final filtered product obtained can be taken directly or can be flavored with syrup to be used as a drink (Ang *et al.*, 1985). Popularity of soymilk is attributed to its high nutritive value as soybeans are one of the best sources of plant protein among all other cereals and legumes. Soybean contains protein (38-40%), carbohydrates (28-30%) and fat (18%) wherein 70% of total soy proteins are glycinin and β-conglycinin. Other components present in soybeans include varying concentration of isoflavone, high levels of minerals (iron, calcium, zinc etc.) and many vitamins (vitamin E, B3, B6, B9 etc.) (Mazumder and Hongsprabhas, 2016).

Recently, soy proteins and isoflavone have gained lots of attention by researchers because of their multifarious health benefits. Soy proteins are believed to reduce the blood cholesterol through up-regulating LDL receptors and increasing bile acid excretion in feces. Two major isoflavones present in soybean "genistein" and "daidzein" are responsible for increasing the activity of enzymes involved in fatty acid β-oxidation (Eslami and Shidfar, 2019). Soybeans are also reported to improve bone health and possess anti-cancer and anti-diabetic activity (Cao *et al.*, 2019).

In Eastern countries, soymilk is always been consumed as a pleasant and nutritious drink without association with cow's milk. However, despite of its high nutritive value and health benefits, it has not gained much popularity in the western countries mainly because of its beany flavor and higher cost than cow milk. It is used only as a milk substitute by a limited group of people having lactose intolerance (Ang *et al.*, 1985). Many efforts were taken in this direction to eliminate this beany flavor of soymilk and make it more acceptable. One of such approaches is to heat the soybeans either before or during the initial processing to inactivate lipoxygenase enzyme responsible for producing off flavors. A second approach is extraction of lipids to remove the substrate that leads to development of undesirable flavors, and a possible third approach is the use of fermentation process to improve flavors. Among them, the third approach has been quite successful with fungi such as *Rhizopus oligosponts*, *Neurospora sitophila*, and *Aspergillus oryzae* to produce fermented soybean foods. Moreover, *Bacillus subtilis* natto is also a successfully used bacterial strain in the preparation of soy products (Mital & Steinkraus, 1979) and strains of *Lactobacillus*, *Leuconostoc*, *Pediococcus* etc. can also be used for soymilk fermentation (Rasika *et al.*, 2021).

With reference to this, the present study is focused on the supplementation of probiotic microorganisms into commercially available soymilk to investigate their survival for one month at refrigerated storage period. Further, these samples were also analyzed for their physico-chemical characteristics to observe possible changes in their properties due to probiotic supplementation.

Materials and Methods

Chemicals and Reagents

Sofit soymilk (procured from local supermarket, Anand, Gujarat), MRS broth, agar powder, 3,5-dinitrosalicylic acid (DNSA), copper sulfate ($CuSO_4$), sodium potassium tartrate, folin ciocalteu reagent, sodium hydroxide (NaOH), phenolphthalein indicator, chloroform, methanol. All chemicals were of analytical grade and purchased from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

Microbial Analysis of Probiotic Supplemented Soymilk

Optimization of inoculum size of probiotics

Both the identified probiotic cultures (*Lactiplantibacillus plantarum* and *Pediococcus pentosaceus*) were activated in 10 mL MRS broth and supplemented in 50 mL of soymilk in different concentrations such as 2, 4, 6, 8 and 10 % (% *v/v* of 10^8 CFU/mL culture). These probiotic supplemented soymilks were stored at refrigerated temperature and at every 24 h time interval, the viability of cultures was determined using spread plate method till 72 h of storage. The plates were incubated at 37°C for 24 h; after incubation, the plates were observed for bacterial growth and the viability was determined in terms of $\log_{10}$ CFU/mL. Probiotic inoculum size showing significant viability after 72 h of refrigerated storage was selected for further study.

Determination of viability of probiotics in soymilk

Two soymilk systems were prepared by inoculating *Lactiplantibacillus plantarum* and *Pediococcus pentosaceus* culture, respectively. Both the systems were kept at refrigerated temperature for one month of storage period. After 24 h of storage the viability was determined for both the soymilk systems. The probiotic soymilk samples were withdrawn from the systems and proceeded for spread plate method. The plates were incubated at 37°C for 24 h and viability was determined in terms of $\log_{10}$ CFU/mL. This process was repeated at weekly interval till one month of refrigerated storage period and the viability was determined in $\log_{10}$ CFU/mL.

Determination of viability of probiotics in soymilk after simulation treatment

Probiotic supplemented soymilk samples were treated with simulated gastric and intestinal juice to determine the probiotic viability in gastro-intestinal tract. After 24 h of storage at refrigerated temperature, 1 mL of probiotic supplemented soymilk samples were treated with 5 mL of simulated gastric juice (for 2 h), intestinal juice (for 4 h) and complete 6 h gastro-intestinal simulation was given. For the same, after 2 h of gastric simulation, the gastric juice was replaced with intestinal juice and incubated further for 4 h at 37°C under shaking condition at 100 rpm. After the incubation, the viability of the probiotic cells was determined in terms of $\log_{10}$ CFU/mL. This process was repeated at weekly time interval till one month of refrigerated storage period.

Physico-chemical Analysis of Probiotic Supplemented Soymilk

Three different systems were prepared, Control soymilk (C), soymilk with *L. plantarum* (DS) and soymilk with *P. pentosaceus* (BS). Different physico-chemical parameters such as pH, titratable acidity (TA), sugar content, protein content, fat content, and viscosity were analyzed using standard methods. The sugar content was estimated by DNSA (3, 5-dinitrosalicylic acid) method (Sumner & Sisler, 1944) using glucose as a standard. For the same, the samples were acid hydrolyzed prior to analysis. The total protein content was determined by Folin-Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as a standard. The total fat content was determined in terms of gm % using Bligh and Dyer method (Bligh & Dyer, 1959). The pH was determined using digital pH meter ("*Systronics"* digital pH meter 335) and the acidity was determined by titrimetric method with 0.02 N

NaOH solution using phenolphthalein as an indicator (AOAC, 2019). Moreover, viscosity of the soymilk samples was measured using a Brookfield DV-III ultra-viscometer (AMETEK Brookfield, USA) at 25°C with spindle S61 at 150 rpm and 10-15% torque for 30 s of cycle, the shear stress was 4-5 D/cm2 and the shear rate was 190-200 per min. Viscosity of soymilk samples were measured with reference to distilled water. All the samples were kept at 25°C prior to the analysis and all the analysis were done at weekly time interval till one month of refrigerated storage of soymilk samples.

Sensory Evaluation of probiotic soymilk

Twenty-one untrained panelists, consisting of 6 males and 15 females between the ages of 20–45 years have participated in a sensory evaluation study. All the participants were instructed to fill the questionnaire and provide an informed consent form. Those participants having allergy or intolerance to soy or any medical illness were excluded from the study.

All the panelists were provided three cups, labelled A (control soymilk), B (soymilk with *L. plantarum*) and C (soymilk with *P. pentosaceus)*. Participants have indicated their ratings for colour, flavor, taste, texture, aroma and overall acceptability for each sample using a five-point hedonic scale ranging from 1 (extremely dislike) to 5 (extremely like).

This sensory evaluation was done at weekly time interval till one month of storage period and from the overall results the consumer acceptance was determined.

Statistical Analysis

All experimental measurements were repeated independently in triplicate, and the results were expressed as the mean ± standard deviation and evaluated by performing the analysis of variance (ANOVA) and Tukey's test, both considering the significance level of $P < 0.05$. The analysis was performed using MS Excel.

Results and Discussion

Microbial Analysis of Probiotic Supplemented Soymilk

Optimization of inoculum size of probiotics

To optimize inoculum size of probiotic microorganisms in soymilk sample, variable inoculum size (2-8%) was taken. Maximum growth was observed with 6% inoculum size for both the probiotic organisms (Fig. 1a & 2a). When inoculum size was lower (2-4%), low viability ($<10^7$ CFU/mL) was observed whereas, in higher inoculum size (8-10%), curdling of soymilk sample was observed. So, 6% inoculum was selected for further study for both the probiotic isolates (Fig. 1b & 2b). Similar kind of results were observed by Sharma *et al.,* (2021) where they have observed very significant viability (10^6 CFU/mL) of *P. acidilactici* BD16 in soymilk only with 2% *v/v* inoculum size. They observed that there was a drastic enhance in growth from 10^4 to 10^8 CFU/mL during the initial time but after 10 h of soymilk fermentation, the cell count started declining subsequently may be due to scarcity of the nutrients. Likewise, Ranadheera *et al.,* (2012) have optimized the inoculum size of *P. jensenii* 702 for yogurt preparation and observed that higher inoculum (10^8 CFU/g) leads to higher viability (~10^8 CFU/g) and lower level of inoculum (10^6 CFU/g) resulted in $<10^6$ CFU/g of cell viability.

Determination of viability of probiotics in soymilk

Functional food development not only requires high viable cell count in initial phase but that high viability should also be maintained during the whole storage period. Therefore, viability of both the cultures i.e. *L. plantarum* (D25) and *P. pentosaceus* (B11) was determined at weekly interval for one month of refrigerated storage period (Fig. 3a). In case of *L. plantarum*, the viability was increased from 7.2 ± 0.003 to 8.7 ± 0.003 $\log_{10}$ CFU/mL while for isolate *P. pentosaceus* the viability was maintained from 7.03 ± 0.010 to 7.42 ± 0.002 $\log_{10}$

CFU/mL during one month of storage at 4°C as shown in Fig. 3b. So, the results indicate that both the isolates have maintained their viability throughout the storage time and as per the reports, to provide health benefits, it is mandatory to maintain at least 6 log_{10} CFU/mL probiotic microbial count at the time of consumption which was observed in this study (Li *et al.,* 2014).

Similarly, Wang *et al.,* (2004) have supplemented two different probiotic microorganisms (*S. thermophilus* and *B. longum*) into soymilk in two different ways i.e. freeze dried cells with initial cell count of 8.08 and 7.36 log CFU/g and spray dried cells with initial cell count 7.51 and 7.07 log CFU/g for *S. thermophilus* and *B. longum*, respectively. They observed the viability of freeze-dried *S. thermophilus* was retained up to 7.76±0.22, 7.71±0.15 and 7.59±0.07 while the viability of spray dried cells was declined to 6.97±0.13, 6.78±0.06 and 6.70±0.11 in laminated pouch, glass bottle and pet bottle type of storage container, respectively. Similar observation was reported for *B. longum* where 7.18±0.16, 7.13±0.12 and 7.05±0.12 log CFU/g of viability was observed in case of freeze-dried cells and 6.83±0.13, 6.82±0.16 and 6.60±0.03 log CFU/g of viability was observed for spray dried cells in laminated pouch, glass bottle and pet bottle type of storage container, respectively. Further, Ebhodaghe *et al.,* (2012) have stored the *B. longum* supplemented soymilk at refrigerated as well as room temperature and observed a similar kind of results in both the cases. Initially, an increased was observed in the viability, which declined from 21st day onward. In addition, Battistini *et al.,* (2018) observed the viability of *B. animalis* Bb-12, *S. thermophilus* and *L. acidophilus* La-5 supplemented in soymilk after 28 days of storage. They observed the viable cell count of both *B. animalis* Bb-12 and *S. thermophilus* was higher than 8 log CFU/mL while the cell count of *L. acidophilus* La-5 was decreased to 6.4 log CFU/mL after 28 days of storage. This decrease in viability supports the fact that low temperature of refrigeration inhibits the growth of lactic acid bacteria which grow well at temperature between 20-40°C with an optimum temperature between 30-32°C (Falade *et al.,* 2015). Besides this, other factors are also responsible for declining growth of bacteria such as depletion of nutrition, lowering of pH due to fermentation, etc. (Taghizadeh *et al.,* 2018).

Determination of viability of probiotics in soymilk after simulation treatment

Food is the most common delivery system for any probiotic bacteria and it can protect them from harsh acidic conditions and enhance the gastro-intestinal survival as addition of food will balance the pH of the gastric contents (Wang *et al.,* 2009). After observing a significant viability till the one month of storage, we have also studied the viability after simulation treatment. We have treated the probiotic-supplemented soymilk with simulated gastric juice (for 2 h), simulated intestinal juice (for 4 h) and gastro-intestinal simulation (for 6 h) and the viability was determined at weekly interval for one month of refrigerated storage (Fig. 4a & 5a). Both the probiotic microorganisms incorporated in soymilk have shown more than 6 log CFU/mL as shown in Fig 4b and 5b even after the 4th week of storage. In a similar study, Bao *et al.,* (2012) have supplemented 12 different strains of *L. plantarum* in soymilk and observed their viability after treating it with simulated gastric and intestinal juice. They observed five strains **(**IMAU10120, IMAU20029, IMAU70004, IMAU60171, IMAU60042) exhibited fairly good acid tolerance and they have shown survival ranges from 7.3-3.5 log CFU/mL even after 24 h of treatment. They have also studied intestinal simulation for 4 h with different bile salt concentrations (range 0.3-1.8%) and observed that total eight strains (IMAU70089, IMAU60171, IMAU70004, IMAU70042, IMAU10156, IMAU40126, IMAU10120, and IMAU60042) have shown significant survival (>3 log CFU/mL) at 1.8% bile salt concentration. Moreover, Ribeiro *et al.,* (2014) have added free and microencapsulated *L. acidophilus* LA-5 cells in soy yoghurt which were simulated by gastric juice (pH 3.0) and intestinal juice (pH 7.0; 1% bile). They have observed reduction of only 0.38 log cycle after gastric simulation and no reduction after intestinal simulation depicting high resistance of *L. acidophilus* LA-5 towards gastro-intestinal simulation.

Physico-chemical Analysis of Probiotic Supplemented Soymilk

Determination of sugar content of soymilk

The choice of probiotic microorganism for soymilk supplementation is very crucial because there are very limited microorganisms that can ferment the typical sugars of soymilk i.e. stachyose, raffinose and sucrose. On one side utilization of these sugars by probiotics have tendency to reduce flatulence in intestinal tract and therefore improve the digestibility while on the other side this can lead to increase in acidity due to lowering of pH (Mital & Steinkraus, 1979). In this study, we have analyzed the sugar content of probiotic supplemented soymilk at weekly intervals till one month of storage. The initial sugar concentration was 0.99±0.1% which was reduced to 0.87±0.14% and 0.88±0.15% in soymilk supplemented with *P. pentosaceus* and *L. plantarum*, respectively (Fig 6). This low reduction in sugar concentration suggests lower fermentation of soymilk by probiotics, which lead to the least alteration and high product stability. Likewise, Wang *et al.,* (2013) have also reported reduction in glucose concentration from 83.47 ± 1.06 mM to 56.65 ± 0.59 mM and fructose concentration from 0.59 ± 0.04 mM to 0.29 ± 0.01 mM upon 18 h of soymilk fermentation by *L. casei*. Similar kind of reduction was also reported by Giri *et al.,* (2018) who compared dairy cream cheese spread (DCCS) with probiotic soy cheese spread (PSCS) where sugar concentration was observed 27.6 ± 1.21 and 19.8 ± 0.64 mg/g in DCCS and PSCS, respectively.

Determination of protein content of soymilk

Plant-based protein beverages have attracted the interest of consumers and researchers as a health-promoting functional food (Qamar *et al.,* 2020). Soy protein either as intact or more commonly as functional proteins contributes many potential health benefits such as reduction in chronic diseases like obesity, cardiovascular disease and immune disorders (Xiao & Shah, 2021). We have also determined the protein content of probiotic supplemented soymilk at weekly time intervals for one month of storage period. After 4 weeks of refrigerated storage, no change was observed in protein concentration of probiotic-supplemented soymilk with reference to control soymilk as shown in Fig 7. Likewise, Singh and Vij (2017) have supplemented *L. plantarum* C2 in soymilk, they observed protein hydrolysis along with incubation time and the highest proteolysis (477.9±29.24 µg serine/ml) was observed after 24 h. In contrast, Ebhodaghe *et al.,* (2012) have observed a gradual increase in protein content from 1.76 to 1.96 % in soymilk supplemented with *B. longum* and this increase may be because of the increase in the production of proteinaceous biomaterial by probiotics.

Determination of fat content of soymilk

Fat content of probiotic as well as control soymilk was determined regularly at weekly time intervals till one month of storage period as shown in Fig. 8. Until 3rd week of storage, no significant change was observed in control as well as in probiotic soymilk, but after 4th week of storage, fat content of soymilk with *P. pentosaceus* was declined to 5.0±0.27% as compared to control (5.8±0.28%) and *L. plantarum* (6.0±0.11%) supplemented soymilk. However, no major decline was observed so we can conclude that there was no significant alteration in fat content of probiotic soymilk with reference to control. Similarly, Ahsan *et al.,* (2020) have compared the fat content of fermented (*L. acidophilus*) and non-fermented soymilk where they observed that fermentation lead to decrease in fat content from 1.94±0.046 % in non-fermented to 1.53±0.037 % in fermented soymilk. They have suggested that this decrease might be due to breakdown of fat molecules into fatty acids and glycerol as well as utilization of these fatty acids as an energy source by probiotic microorganism. In contrast, Gamba *et al.,*

(2020) have observed an increase in fat content of fermented (3.87 ± 0.12%) soymilk kefir in comparison to non-fermented (3.51 ± 0.18%). Likewise, Ahanian *et al.,* (2014) have reported that use of fermented soymilk has increased the fat content from 6.896±0.152 to 7.596±0.020% in sesame ice-cream. Similar observation was also reported by Rinaldoni *et al.,* (2012) in case of soy yogurt preparation wherein the fat content was observed to increase from 4.5 ± 0.6 g/L to 15.5 ± 0.5 g/L.

Determination of pH of soymilk

Alteration in pH of any functional food is directly related to microbial activity. Higher the microbial activity or fermentation, lower the pH due to increase in the production of organic acids (Manus *et al.,* 2021). The pH of probiotic soymilk samples was determined using pH meter at weekly interval with reference to control soymilk. The initial pH of all the three soymilk samples remained almost similar but after 4^{th} week of storage a significant decrease was observed in case of soymilk supplemented with *L. plantarum* (Fig. 9). On the 28^{th} day, the pH of control soymilk was 7.48±0.013 whereas the pH of soymilk with *L. plantarum* was decreased up to 7.33±0.009. However, the pH of soymilk having *P. pentosaceus* (7.47±0.006) was observed almost similar to the control soymilk. This suggest higher activity of *L. plantarum* in soymilk in comparison to *P. pentosaceus* which indicates that different probiotic species have different capability of fermenting soymilk (Sharma *et al.,* 2021). This observation was supported by Li *et al.,* (2012) where they fermented soymilk with six different probiotic strains and they reported that all the six probiotic strains were able to ferment soymilk but their activity potentials were different. *L. rhamnosus* GG took the shortest time of 9.5 h to reach final pH 4.5 while *L. acidophilus* NCFM took the longest time of 21 h. Other four strains (*L. casei* Zhang, *B. animalis* V9, *B. animalis* BB12 and *L. casei* Shirota) took 12-16 h of time to ferment soymilk. This indicates that each and every probiotic has different fermentation characteristics. Additionally, Battistini *et al.,* (2018) have fermented soymilk with three probiotic strains (*S. thermophilus*, *B. animalis* Bb-12 and *L. acidophilus* La-5). These fermented soymilk samples were supplemented with prebiotics and no alterations was observed in pH after 28 days of fermentation. They observed that the pH of control soymilk (4.48 ± 0.05), probiotic soymilk with fructo-oligosaccharide (4.46 ± 0.09), probiotic soymilk with inulin (4.40 ± 0.06) were almost similar.

Moreover, Taghizadeh *et al.,* (2018) have developed a probiotic soymilk chocolate mousse and observed a significant decrease in pH of mousse with *L. paracasei* (from 6.04±0.04 to 5.05±0.01) after 21 days of storage. However, the pH drop-rate in mousse with *B. lactis* (6.92±0.03 to 5.74±0.01) and *L. acidophilus* (6.90±0.02 to 5.35±0.02) were lower than *L. paracasei*. Further, Falade *et al.,* (2015) have prepared yoghurt using soymilk and Bambara groundnut milk and stored it for 9 days at 7°C. They observed a significant decrease in pH from 4.97 to 4.20 in soy yoghurt and a decrease from 5.21 to 4.11 in Bambara yoghurt after 9 days of storage and suggested that soymilk is an appropriate carrier for probiotic product preparation.

Determination of titratable acidity (TA) of soymilk

The hydrolysis and fermentation reactions by probiotic bacteria contribute to acidification of the food, which can be observed by determining the titratable acidity (Montanari *et al.,* 2020; Salmerón *et al.,* 2015). In the current study, we have determined titratable acidity of probiotic soymilk along with the soymilk control sample to confirm whether the fermentation process has occurred during the storage or not (Fig 10). After 4^{th} week of storage at refrigerated temperature, the acidity of control soymilk (0.06±0.03 %) and *P. pentosaceus* supplemented soymilk (0.06±0.01%) was observed similar while the soymilk with *L. plantarum* has showed 0.08±0.04% acidity. This minor rise in acidity is may be due to utilization of soymilk sugars by *L. plantarum*. On a similar line, Mridula &

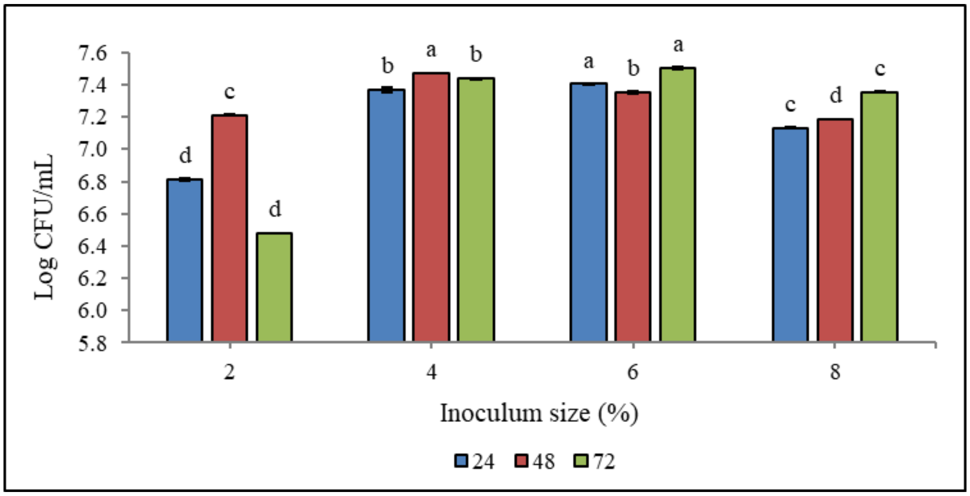
Sharma, (2015) have developed a probiotic soymilk with supplementation of *L. acidophilus*- NCDC14 and this was further enriched with the addition of sprouted cereal flour of wheat, barley, pearl millet and green gram along with an oat meal as a stabilizer. They observed that acidity of these probiotic beverages was dependent on the level of sprouted cereal flour and specially soymilk. As the level of soymilk increases the acidity level decreases. Besides this, the acidity values for different plant based beverages also depend on the probiotic strain used for fermentation. To confirm this Salmerón *et al.,* (2015) have studied three different cereal beverages with three different probiotic organisms and determined their titratable acidity as 0.12 ± 0.01% (*L. acidophilus*), 0.08 ± 0.02 (*L. plantarum*), 0.11 ± 0.01% (*L. reuteri*) for barley beverage, 0.13 ± 0.02% (*L. acidophilus*), 0.12 ± 0.02% (*L. plantarum*), 0.14 ± 0.01% (*L. reuteri*) for malt beverage and 0.07 ± 0.02% (*L. acidophilus*), 0.05 ± 0.01% (*L. plantarum*), 0.04 ± 0.01% (*L. reuteri*) for oat beverages. These results were also supported by Le *et al.,* (2020), who have observed different titratable acidity of soymilk fermented with different probiotic culture such as 12.6 ±0.2% with *W. cibaria* FB069 and 14.8±0.4% with *L. rhamnosus* GG.

Moreover, Montanari *et al.,* (2020) have prepared probiotic peanut milk and soymilk where they observed that acidity was increased from 0.34±0.01 to 1.1±0.4% in peanut milk and an from 0.53±0.03 to 1.5±0.1 in soymilk after 42 days of storage. Additionally, Hauly *et al.,* (2005) have developed probiotic yoghurt with *L. bulgaricus* and *S. thermophilus* where the acidity of soymilk yoghurt increased from 0.38 to 0.67% after 7 days of storage

Determination of viscosity of soymilk

The utilization of soymilk protein and carbohydrate by probiotic bacteria increases the acidity of soymilk, which sometimes results in protein agglutination. This might be responsible for change in the apparent viscosity and other rheological characteristics of soymilk (Kumari *et al.,* 2021). To confirm any protein agglutination in our probiotic soymilk samples, we have determined the viscosity of probiotic supplemented samples as well as control soymilk. The initial viscosity of soymilk samples was observed as 2.2±0.007, 2.2±0.007 and 2.3±0.07 for control soymilk, *P. pentosaceus* supplemented soymilk and *L. plantarum* supplemented soymilk, respectively. No significant change was observed in any of the soymilk samples till 2nd week of storage but after 3rd week of storage, minor rise in viscosity was observed in case of soymilk supplemented with *L. plantarum*. Similar observations were also notified after 4th week of storage where the viscosity of control soymilk, *P. pentosaceus* supplemented soymilk and *L. plantarum* supplemented soymilk were observed as 2.6±0.013, 2.7±0.007 and 3.1±0.00 cP, respectively (Fig. 11). These results depict more protein agglutination in the soymilk sample supplemented with *L. plantarum* in comparison to *P. pentosaceus* supplemented soymilk sample. In reference to this, Montanari *et al.,* (2020) have explained that the soy-based beverage would show higher viscosity because of its higher protein and fiber content. However, this is not the case for all times probably due to the instability of soymilk constituents. The viscosity of soy extracts can be affected by the number of crosslinking between partially hydrolyzed polysaccharides and partially denatured proteins both soluble and insoluble. Rossi *et al.,* (1999) have fermented soymilk with four different combinations of probiotic strains and determined its viscosity as 210, 189, 302 and 155 cP for *E. faecium* & *L. jugurti, E. faecium* & *L. acidophilus, S. thermophilus* & *L. delbrueckii* sp. *bulgaricus* and *E. faecium* supplemented soymilk samples, respectively. Similarly, Bao *et al.,* (2012) have also reported that viscosity of soymilk markedly increased with increase in fermentation time and reach 1,000– 1,400 cP at the end of the fermentation period. With this high viscosity, curdling of soymilk sample was observed and that was also confirmed by determining the viscosity of soymilk curd supplemented with *Lactobacillus acidophilus* and *Bifidobacterium lactis* which was ranged between 1467-1640 cP (Joel *et al.,* 2019).

Fig.1a Optimization of inoculum size of *L. plantarum* for soymilk



Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for variable inoculum size at different time intervals.

Fig.1b Viability of *L. plantarum* in soymilk with 6% inoculum size with variable time interval a) 24 h b) 48 h c) 72 h

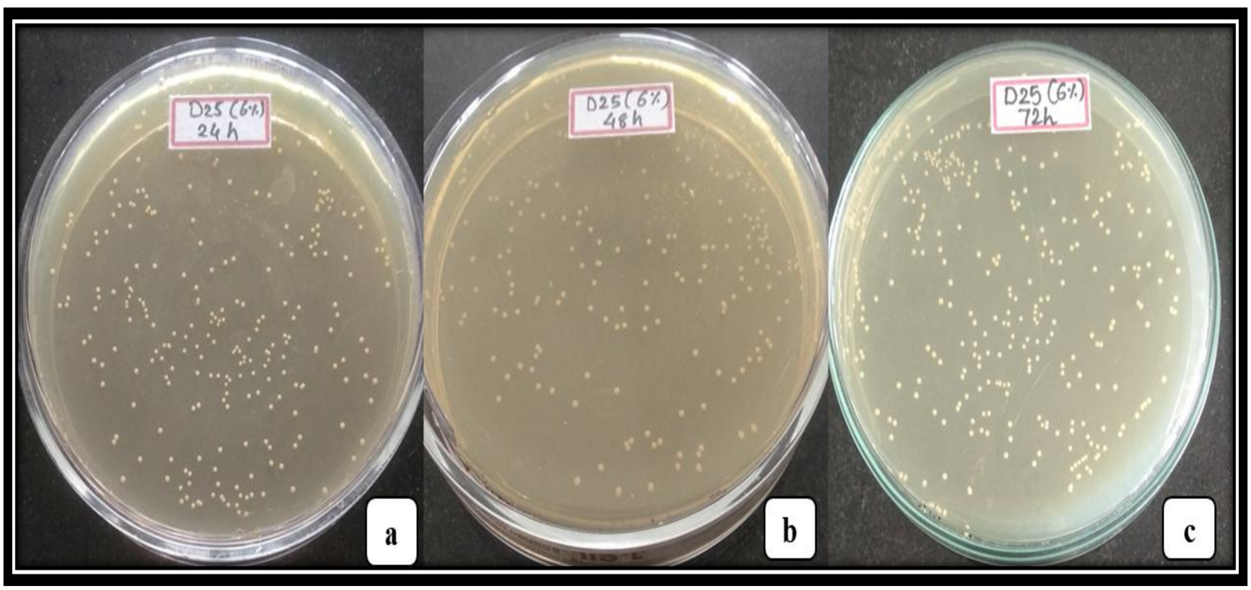
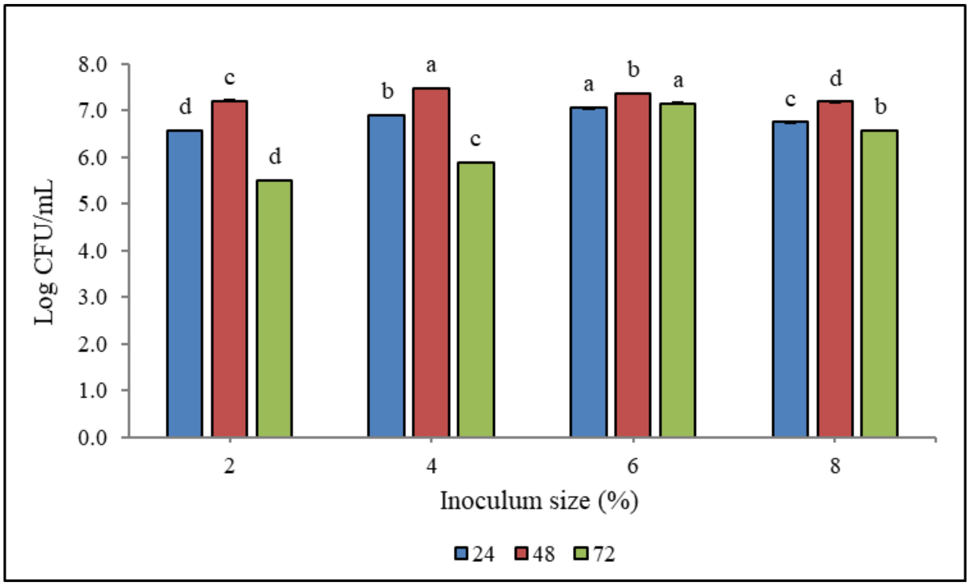


Fig.2a Optimization of inoculum size of *P. pentosaceus* for soymilk



Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for variable inoculum size at different time intervals.

Fig.2b Viability of *P. pentosaceus* in soymilk with 6% inoculum size with variable time interval a) 24 h b) 48 h c) 72 h

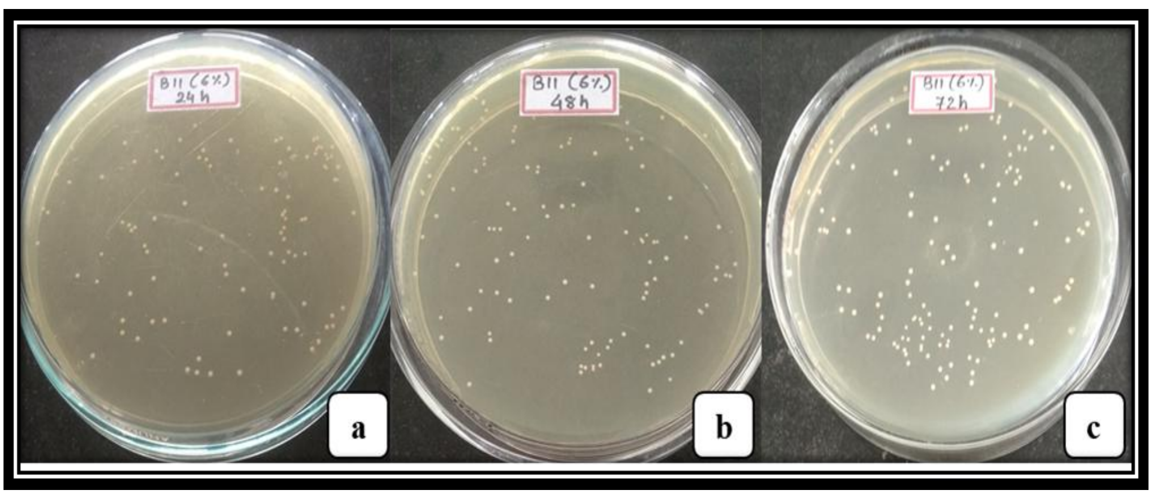


Fig.3a Viability of probiotic organisms in soymilk

a) *L. plantarum* (D25) b) *P. pentosaceus* (B11)

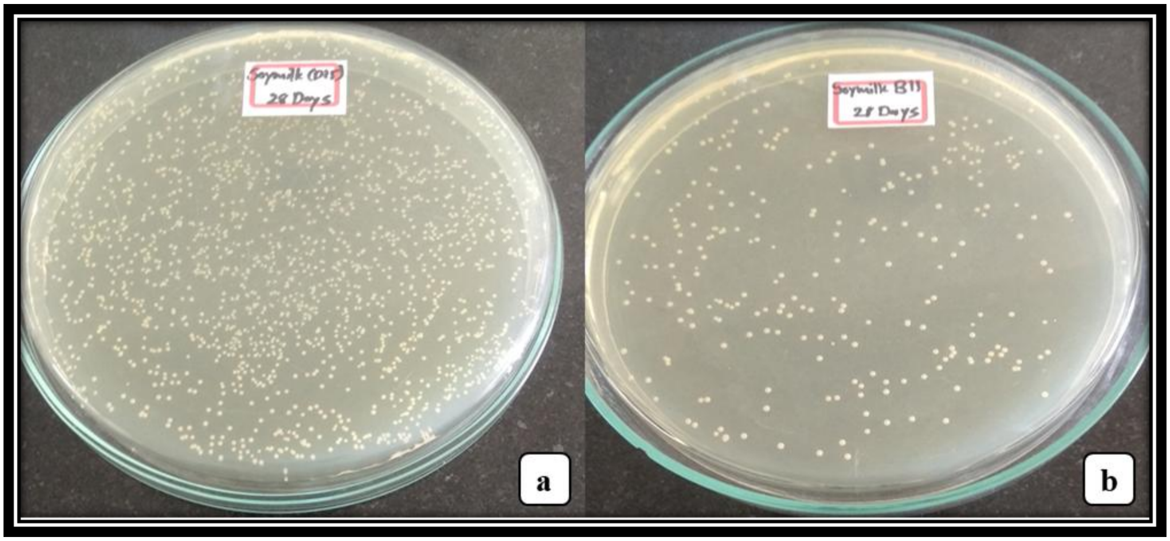
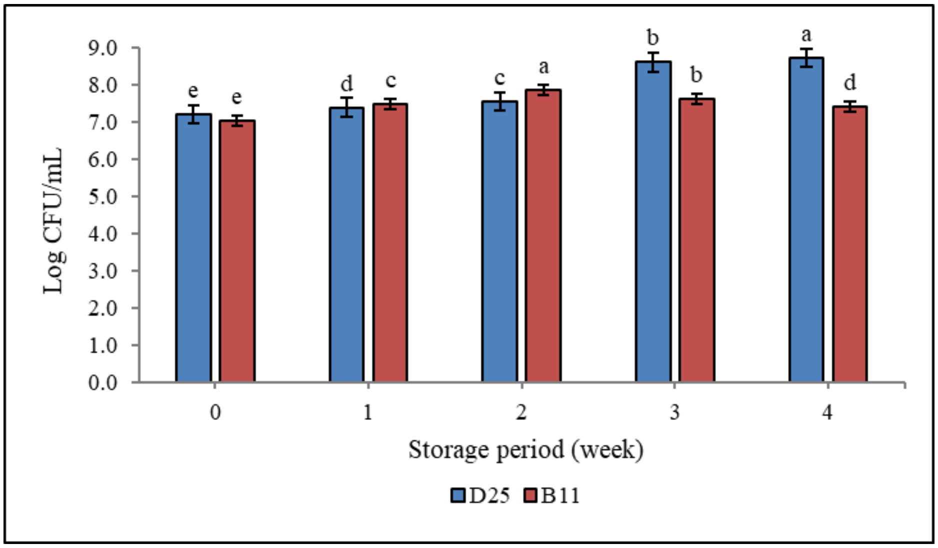


Fig.3b Viability of *L. plantarum* (D25) and *P. pentosaceus* (B11) in soymilk



Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for viability of different probiotic with different storage period.

Fig.4a Viability of *L. plantarum* in soymilk after simulation treatment

a) Gastric simulation b) Intestinal simulation c) Gastro-intestinal simulation

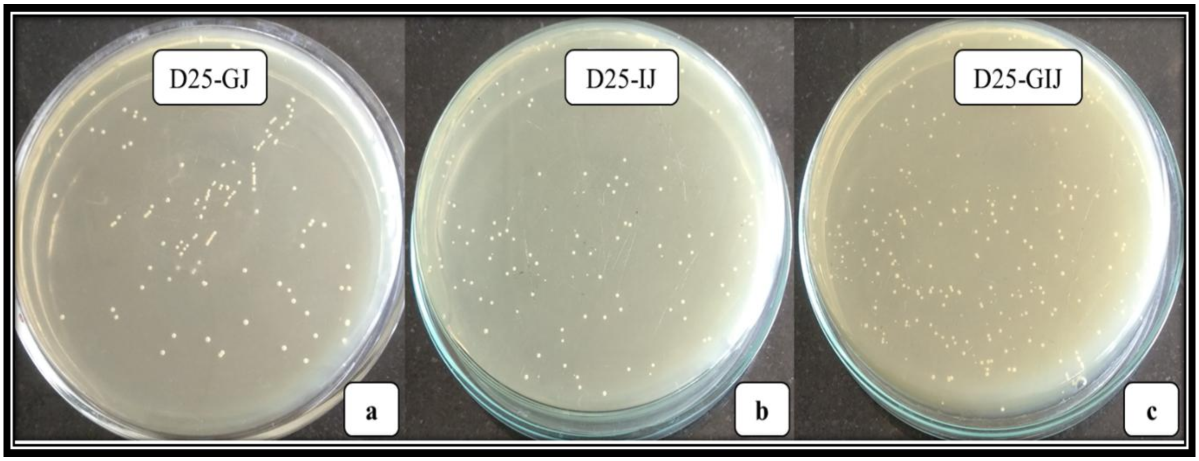
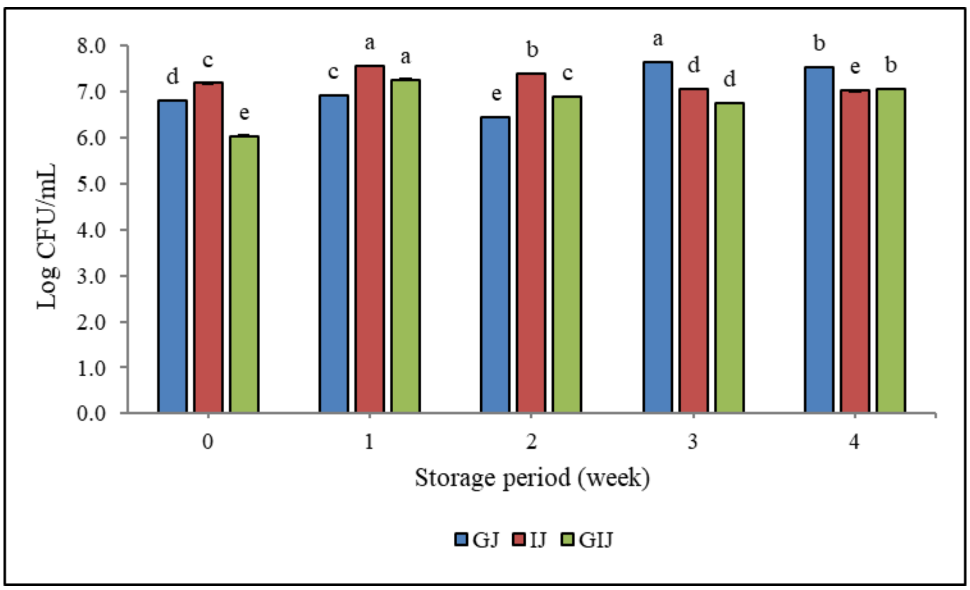


Fig.4b Viability of *L. plantarum* (D25) incorporated in soymilk after simulation



Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for different simulation treatment.

Fig.5a Viability of *P. pentosaceus* in soymilk after simulation treatment

a) Gastric simulation b) Intestinal simulation c) Gastro-intestinal simulation

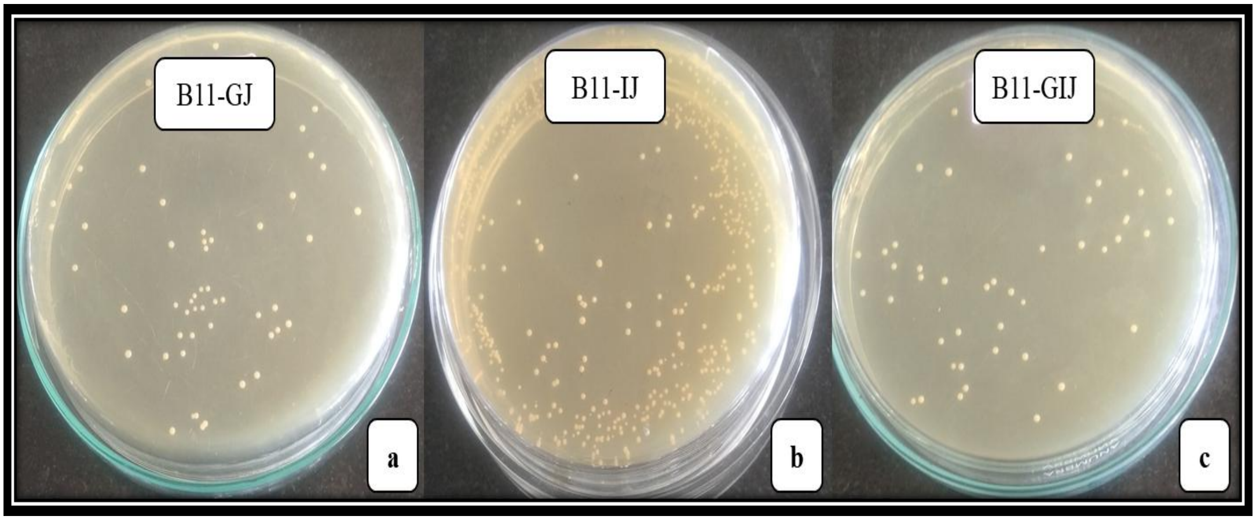
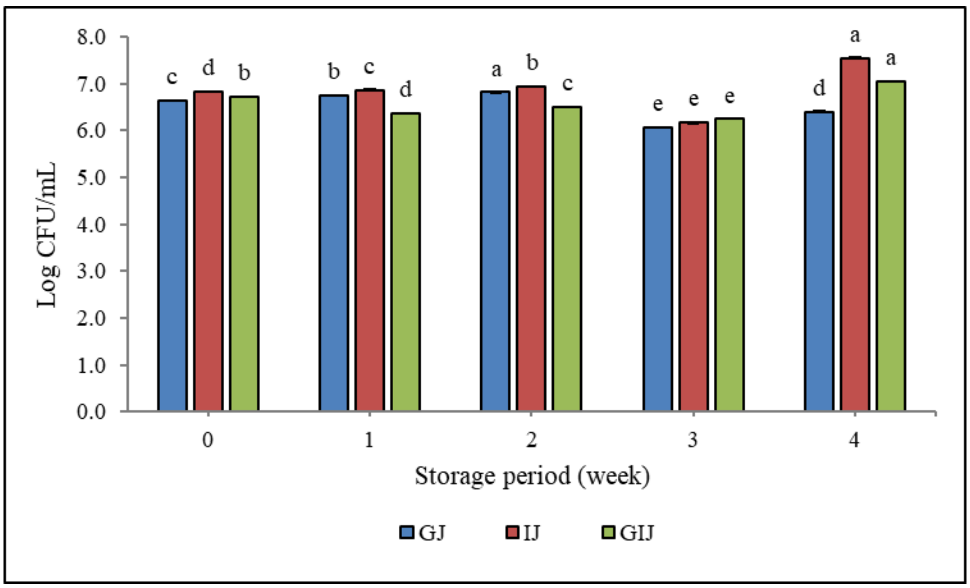
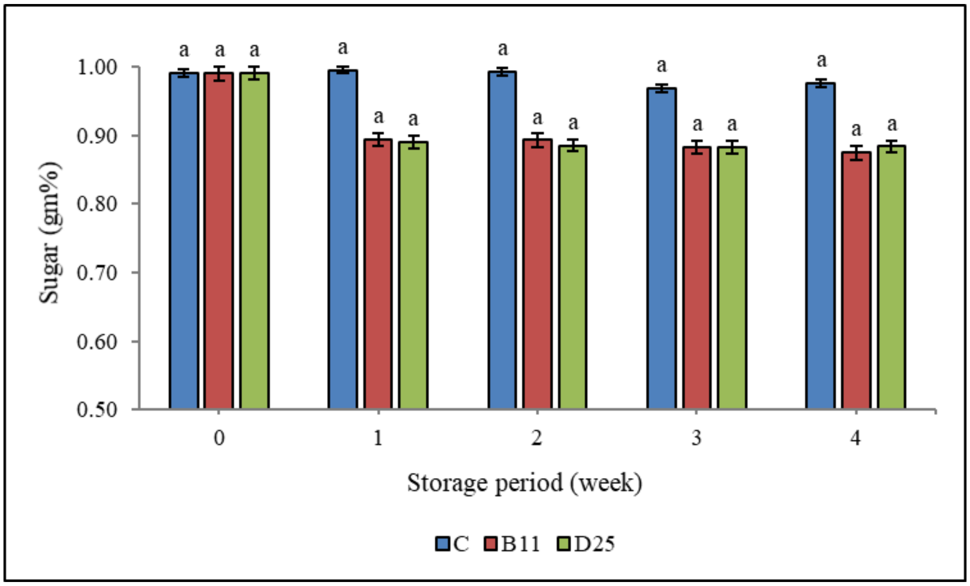


Fig.5b Viability of *P. pentosaceus* (B11) incorporated in soymilk after simulation



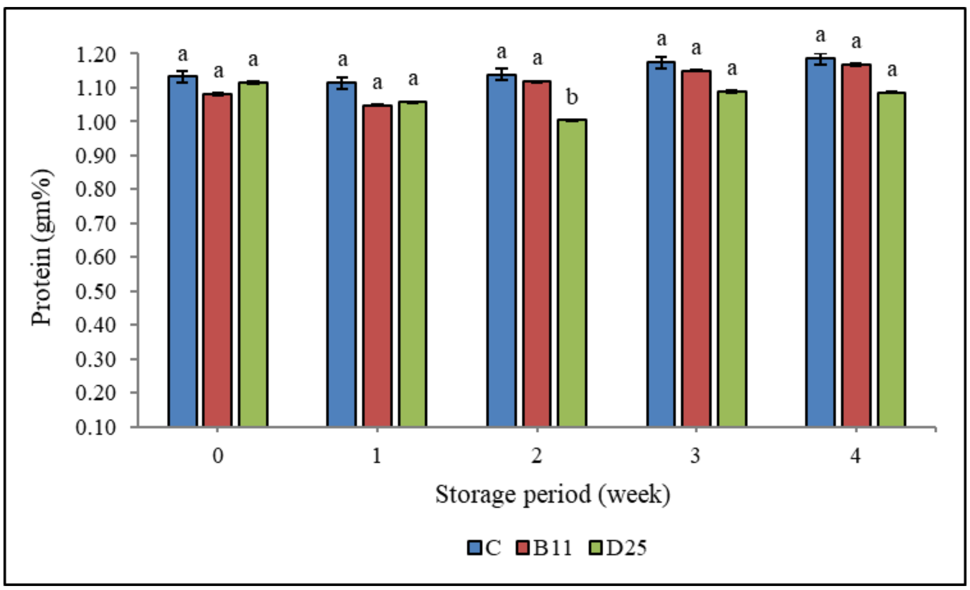
Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for different simulation treatment

Fig.6 Sugar content in probiotic soymilk with storage



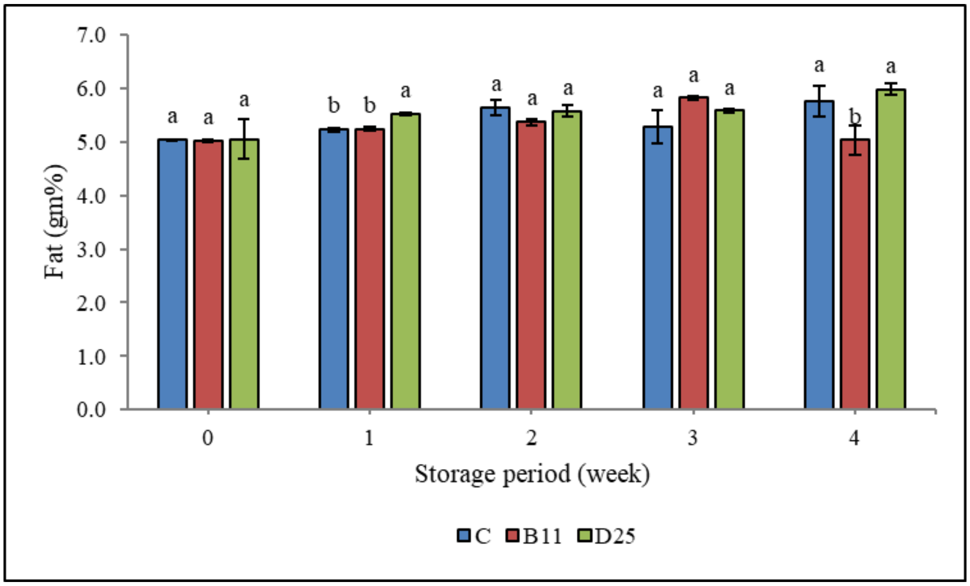
Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for sugar content in soymilk samples at variable storage period.

Fig.7 Protein content in probiotic soymilk with storage



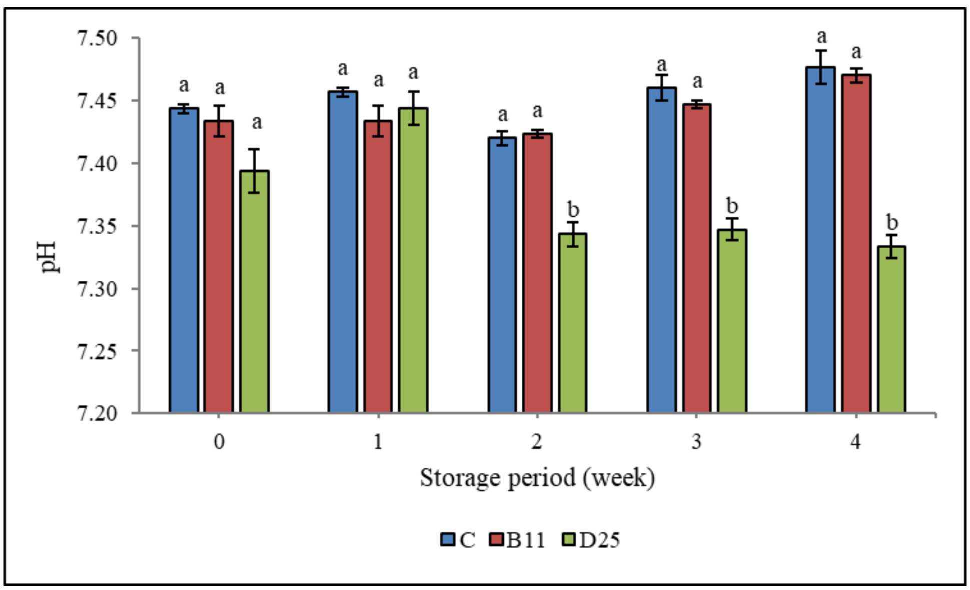
Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for protein content in soymilk samples at variable storage period.

Fig.8 Fat content in probiotic soymilk with storage



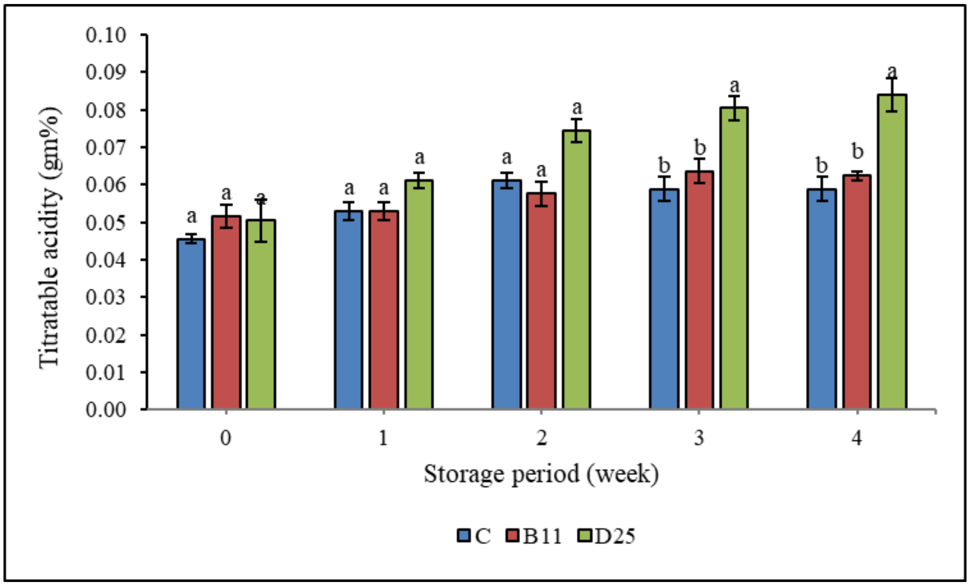
Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for fat content in soymilk samples at variable storage period.

Fig.9 pH of probiotic soymilk with storage



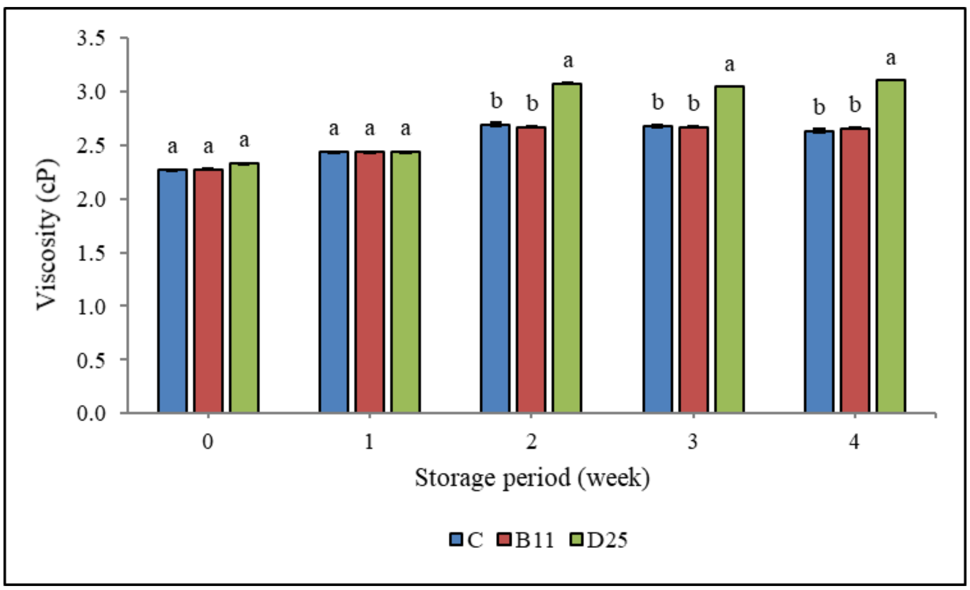
Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for pH of soymilk samples at variable storage period.

Fig.10 Titratable acidity of probiotic soymilk with storage



Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for acidity of soymilk samples at variable storage period.

Fig.11 Viscosity of probiotic soymilk with storage



Different letters above the bars denote statistically significant differences at $p<0.05$ by tukey's test for viscosity of soymilk samples at variable storage period.

Fig.12 Sensory evaluation of probiotic soymilk supplemented with *L. plantarum* and *P. pentosaceus*

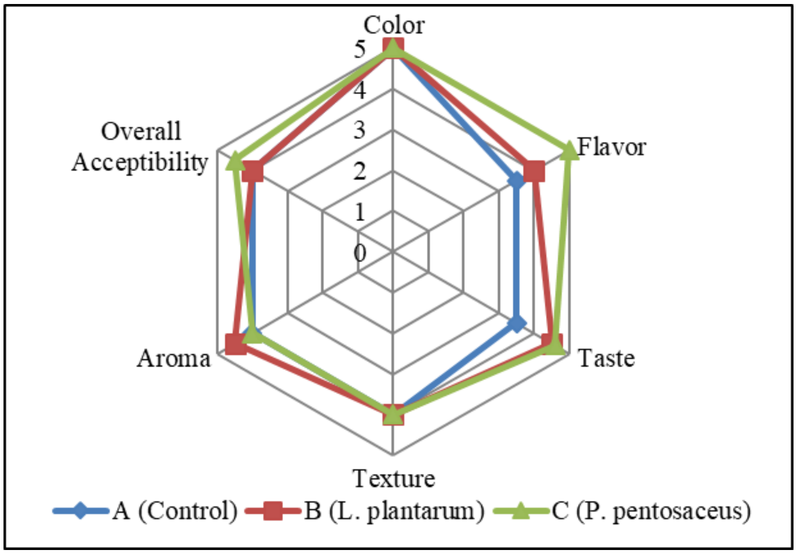
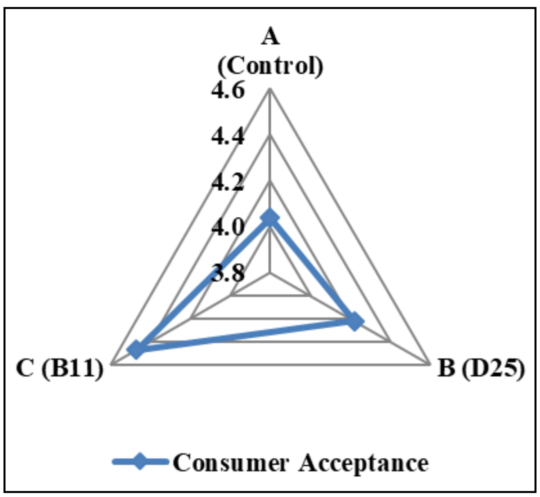


Fig.13 Consumer acceptance of probiotic soymilk supplemented with *L. plantarum* (D25) and *P. pentosaceus* (B11)



Sensory Evaluation of Probiotic Soymilk

Sensory evaluation is an important tool to determine the human responses towards food product with minimum biasness and influences to consumers' perceptions (Lascano *et al.,* 2020). Therefore, to determine consumer acceptance of probiotic supplemented soymilk samples, we have done sensory evaluation of these samples at weekly time interval till one month of storage. The results of sensory evaluation indicated that the colour and texture of soymilk did not change after one month of storage period; however, in flavor, taste and aroma, minor change was observed due to fermentation by probiotics (Fig. 12). From the data obtained after 4th week of storage, we have observed that, soymilk with supplementation of *P. pentosaceus* has shown more consumer acceptance than the control soymilk and *L. plantarum* supplemented soymilk sample as shown in Fig. 13. This overall consumer acceptance indicates more stability of *P. pentosaceus* supplemented soymilk than soymilk with supplementation of *L. plantarum*. Likewise, Fernanda *et al.,* (2018) have shown that the soymilk supplemented with *L. bulgaricus* obtained 94.5% of positive acceptance (score of 6, 7, 8 and 9 points), 4% of rejection (score of 3 and 4), and only 1.5% of indifference zone (score of 5 points) on 1-9 hedonic scale for overall impression attribute. So, they concluded that the product presents an adequate sensory characteristics and potential to be marketed as probiotic soymilk. Further, Bao *et al.,* (2012) have also determined the sensory properties of soymilk supplemented with six different *L. plantarum* strains (IMAU10156, IMAU40126, IMAU70004, IMAU60042, IMAU60171 and IMAU10120) and observed that strain IMAU10156 and IMAU10120 were better than those of the other four strains. Even after 28 days of storage, both the strains have shown stable taste and flavor of soymilk. Although, they have observed that the fermented characteristics of soymilk by *L. plantarum* IMAU10120 were not remarkable but the taste and flavor scores were favorable and as suggested by Hauly *et al.,* (2005), taste is the key factor for acceptance of any fermented beverage by consumers. Moreover, Joyce *et al.,* (2021) have also analyzed the fermented soymilk with sensory property on a 9-point Hedonic scale and they observed high acceptance by consumers even though the soymilk fermentation was poor and they suggested that this may be due to the absence of beany flavor in fermented soymilk. In this study, we have also observed a better flavor, taste and aroma in the soymilk sample supplemented with probiotics and more acceptance of that by consumers in comparison to control soymilk.

Soymilk is a very nutritive alternative of regular cow milk and it is also a suitable food matrix for the growth of probiotic bacteria. Both the probiotic organisms *Lactiplantibacillus plantarum* and *Pediococcus pentosaceous* have shown significant viability in soymilk for one month of refrigerated storage period. Fermentation by these organisms have not shown any significant changes in physico-chemical property of soymilk. This indicates that these probiotic organisms can be explore further for the development of non-dairy functional food. Moreover, after sensory evaluation, both the probiotic supplemented soymilk samples have shown more consumer acceptance than control soymilk sample and the soymilk supplemented with *Pediococcus pentosaceous* was the most preferred soymilk by the consumers. From the results of this study, we can conclude that probiotic supplemented soymilk has the potential to be marketed as a novel functional food beverage.

Conflicts of Interest

The authors declare that they have no known conflicts of interest in the publication.

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