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# Development of Nutritionally enriched Soymilk using a Mixed Probiotic Fermentation by *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b

***Minnu Sasi*[1,2*], *Sandeep Kumar*[1,3] and *Anil Dahuja*[1]**
[1]*Division of Biochemistry, ICAR—Indian Agricultural Research Institute (New Delhi), India.*
[2]*Dev Bhoomi Uttarakhand University, Naugaon (Uttarakhand), India.*
[3]*Automation and Plant Engineering Division,*
*ICAR- National Institute of Secondary Agriculture, Ranchi (Jharkhand), India.*

*(Corresponding author: Minnu Sasi*)*
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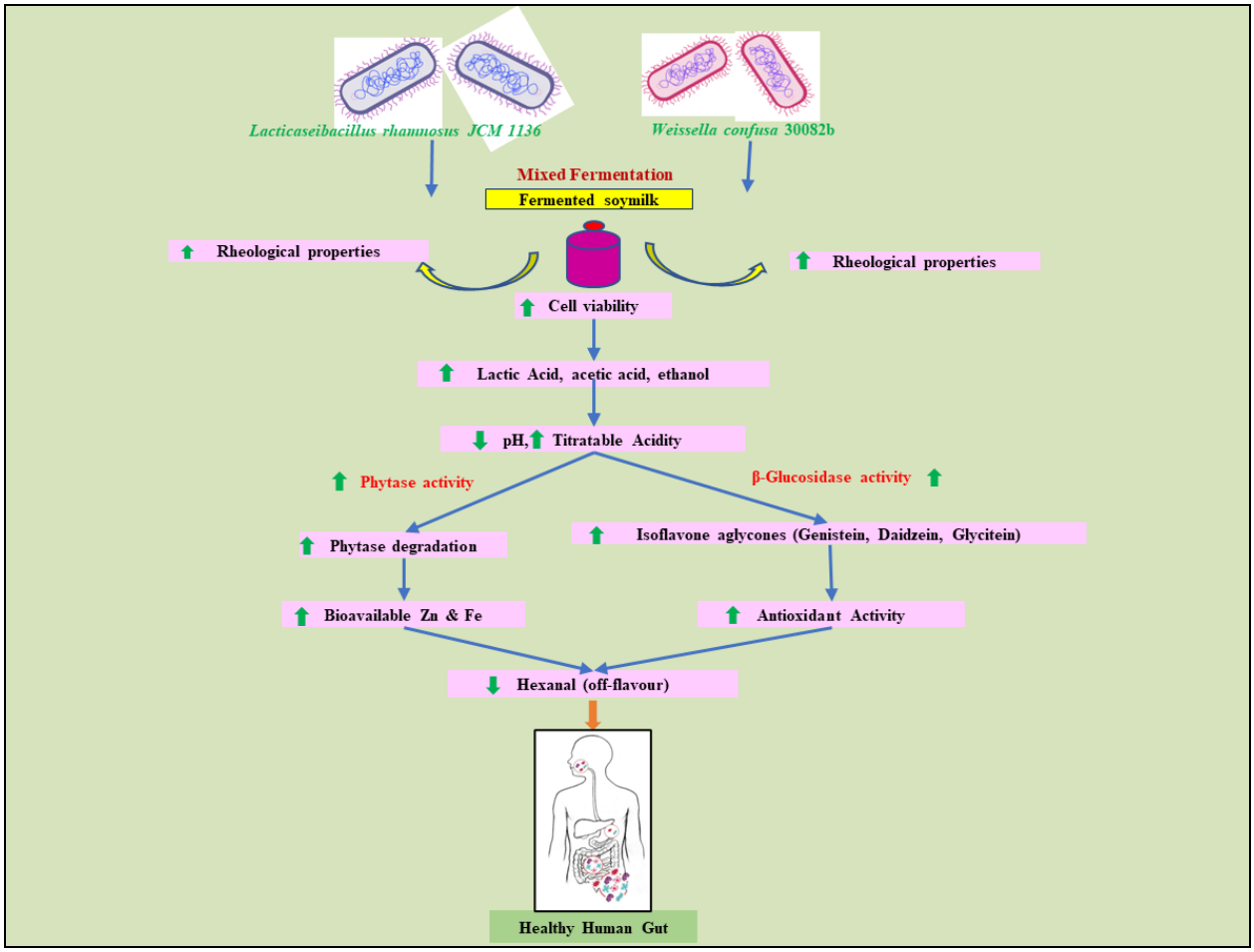
**ABSTRACT: The soybean products, on account of the presence of health-giving compounds like isoflavones, phospholipids, and polyunsaturated fatty acids, have immense potential to enhance human health. Single strain probiotic soy foods may exhibit many challenges associated to their growth, survival, viability, stability and functionality in food processing, storage and consumption as well as changes of sensory characteristics of probiotic foods. Mixed culture fermentations are generally implemented to stimulate the production of nutritional compounds and enzymes resulting from positive mutual interactions between the bacteria, called protocooperation. With this aim, potential techno-functional properties of soymilk fermented with mixed cultures of *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b have been studied. The investigations on the effect of probiotic fermentation after 6, 24, 30, 48 h and 7$^{th}$ day of storage indicate a decrease in pH (5.76 to 4.76), an increase in titratable acidity (0.19% to 0.47%), an increase in TAO (33.13-67.86%), reduction in phytic acid (1.185-0.339g per 100g), increase in free mineral content- Fe (0.31-2.08µg/ml) & Zn (0.26-1.15µg/ml), bioconversion of isoflavone glycosides into more bioavailable aglycones (daidzein-18.708-99.24; glycitein- 18.586- 43.41; genistein-20.778-67.64µg/10ml), detected for unfermented control and 1%(v/v) mixed culture fermented soymilk, respectively. The viable count of bacteria was maintained up to 3.32 × 10$^8$ CFU/ml for mixed cultures after 7 days of storage at 4ºC. However, the values of nutritive factors were observed to be augmented and anti-nutritional factors seemed to be reduced after mixed culture fermentation. Hence, mixed culture fermentation with *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082 bis an ideal strategy to guide consumers towards appropriate prophylactic and therapeutic uses of probiotics that deliver the desired beneficial health effects.**

**Keywords:** Lactic acid bacteria; mixed fermentation; nutraceutical potential; mineral bioavailability; phytic-acid; isoflavones.

**Abbreviations:** Lactic acid bacteria (LAB); anti-nutritional factor (ANF); total antioxidant activity (TAO); colony-forming units (CFU); 1,1′-diphenyl-2-picrylhydrazyl (DPPH); De Man, Rogosa and Sharpe medium (MRS); Ultra-high Performance Liquid Chromatography (UPLC); titratable acidity (TA).

**Summary of components discussed:** As a result of mixed fermentation by *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b strains, there was an enhanced synthesis of lactic acid, acetic acid, ethanol, and $CO_2$ which lowered the pH of fermented soymilk and augmented the titratable acidity. As a result of enhanced viability, the hydrolytic enzyme activity- β-glucosidase was increased, which resulted in the bioconversion of isoflavone glycosides to aglycones such as genistein and daidzein aglycones which are strong antioxidants were capable of enhancing the antioxidant activity of soymilk. The increased activity of phytase enzyme resulted in the conversion of phytic acid to free phosphate. The breakdown of phytate aided in enhancing the concentration of bioavailable Fe and Zn in the fermented soymilk. The amount of hexanal, a volatile compound causing off-flavour was exceptionally reduced. The co-inoculation of *Weissella confuse* 30082b and *L. rhamnosus* JCM 1136 could reproduce the metabolic and microbial processes of fermentation, enhancing the nutritional and sensory quality of the fermented soymilk, which its the intake can improve our gut health.




## INTRODUCTION

Plant-based or non-dairy milk alternatives are a quick-growing segment in innovative and functional food product development across the globe because of the high demand for more nutritious and sustainable foods. Concerted research efforts are required in the coming years to generate better-quality food products with upstanding consumer acceptability and superior nutritional, sensory, and functional properties. Soymilk is a native source of oligosaccharides with lower degrees of polymerization, such as Raffinose Oligosaccharides (RFOs) - raffinose and stachyose (Sasi *et al.*, 2022). Soy protein has the highest quality among vegetable proteins with a lower cost than animal proteins establishing it as a prime constituent that can be conditioned into many flavorsome protein foods (Qin *et al.*, 2022). Also, soy products are identified as a virtuous source of phytochemicals comprising isoflavones, tocopherols, and anthocyanins, imparting various health-promoting effects (Serna- Saldivar, 2022). Furthermore, soy foods can be a magnificent substitute for milk products, particularly for Lacto-vegan and lactose-intolerant communities as soy- foods lack disaccharide-lactose. Also, soybean contains a high concentration of anti-nutritional components like phytate, saponins, and trypsin inhibitors which impede the bio accessibility and bioavailability of nutritional factors in soy products (Suprayogi *et al.*, 2022). Enzymatic oxidation of polyunsaturated fatty acids like linoleic acid and linolenic acid by lipoxygenase genes (Lox) is identified as a major source of the be any flavor (Nedle *et al.*, 2021).

More creative valorization methods target to recuperate beneficial bioactive components from plant-based substitutes for nutraceuticals. The mixed-culture fermentation media always encompasses more than one type of known or unknown microorganisms. It is a persuadable and ingenious process for the bioconversion of multiple substrates into better quality products in varying concentrations, depending on the microbe species, composition, and type of substrate and operational conditions of fermentation (Bevilacqua *et al.*, 2020). Mixed cultures are the rule in nature. Mixed fermentations also enhance the bacterial growth rate, propound better protection against pathogens, and dispense essential nutrients for optimal performance through cooperative interactions. Also, single fermentations cannot provide a comprehensive view of the actual biochemical attributes of fermentation (Hesseltine, 1992).

Strains of the *Lactobacillaceae* family are gram-positive, often homofermentative, facultatively anaerobic friendly bacteria, inhabit our digestive, urinary, and genital systems, and do not cause any disease. At the same time, *Weissella* sp. is a gram-

positive facultatively anaerobic bacteria, often heterofermentative, placed within the family *Lactobacillaceae* with a morphology that ranges from spherical or lenticular cells to irregular rods. Monoinoculation attains a stable quality of the product but reduced flavor. During vegetable fermentation, dominant species are switched from less acid-tolerant heterofermentative to more acid-tolerant homo-lactic species within a week. This phenomenon affects the carbon metabolic pathway and the quality of fermented products. *Lacticaseibacillus rhamnosus* JCM 1136 strain belonging to the *Lactobacillaceae* family can activate homo-lactic fermentation, found in various fermented dairy products, and generate a harsh sour flavor (Bharath Kumar *et al.,* 2023; Lee *et al.,* 2021). *W. confusa* are opportunistic bacteria, and several studies have been done on the safety of this bacterial species, indicating their probiotic potential. The Senate Commission on Food Safety has validated the use of *W. confusa* in food (Vogel *et al.,* 2011). *Weissella* sp. is one of the predominant species during Kimchi fermentation and can commence hetero-lactic fermentation generating a pleasant aromatic flavor (Mun *et al.,* 2020).

During fermentation, short-chain fatty acids such as acetate, propionate, butyrate, and lactic acids help in the concomitant reduction of the pH, which results in the augmentation of total titratable acidity (TA) and which activates the hydrolytic enzymes like β-glucosidases and phytases (Markowiak and Slizewska 2017). Soymilk is an abundant source of hydrophilic antioxidants like isoflavonoids especially genistein & daidzein, water-soluble vitamins (B – complex), and lipophilic antioxidants like fatty acids and lipid-soluble vitamins (A & E) (Ma and Huang 2014). The bioavailability of isoflavones is influenced by their chemical form in foods. Their biological activity of isoflavones is partly ascribed to the structural similarities with the primary physiologically relevant estrogen –17β-estradiol. Isoflavones bind to and activate intracellular estrogen receptors: ERα and ERβ and, mimicking the effects of estrogen, are commonly referred to as phytoestrogens (Miadokova, 2009). Isoflavone glucosides like daidzin, genistin, glycitin, and their respective acetyl and malonyl glucosides are considered to possess reduced bioactivity and therefore less absorbable and bioavailable. Meanwhile, isoflavone aglycone metabolites, especially genistein, glycitein, and daidzein have higher bioactivity due to their structure and are more bioavailable than glycosides. The anti-nutritional properties of phytate area scribed to its powerful chelating activity due to its six reactive P-esters (Rahate *et al.,* 2021). A high amount of phytic acid decreases the absorption of multivalent cations, such as magnesium (Mg), calcium (Ca), zinc (Zn), and iron (Fe) as they are particularly susceptible to forming insoluble and indigestible complexes with phytate (Samtiya *et al*., 2021).

Overall, in this study, mixed culture fermentations with probiotic bacteria were manifested to enhance the microbial stability and fitness, shelf life, and safety of the fermented food products. Also, the potential ability of mixed culture fermentation to improve the sensory properties, bioavailability of nutrients, and yield of targeted bioactive compounds either through microbial synthesis or by augmenting the digestibility of the fermented product was studied.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *L. rhamnosus* JCM 1136 strain from *Lactobacillaceae* Family with probiotic properties was procured from the Japan Collection of Microorganisms RIKEN Bio Resource Research Center, Kyoto Japan. Despite reports of *Weissella*-related infections, the evolving mechanistic findings suggest that *Weissella* are clinically treatable bacteria with emerging antimicrobial and probiotic benefits ranging from oral health, skin care, obesity, and inflammatory diseases to cancer (Ahmed *et al.,* 2022). A novel strain of *W. confusa* 30082b isolated from healthy human fecal samples (age group 21-40) at National Centre for Cell Science (NCCS) Pune, India and 16S rRNA gene-based identification and probiotic characterization for the *Weissella* group was carried out (Joglekar *et al.,* 2022). The strains were stored at −80°C in the presence of glycerol (649 g/l) and activated in MRS (De Man, Rogosa, and Sharpe) broth (procured from Sigma Aldrich Chemicals Pvt Ltd, USA) twice before use. MRS broth containing the strain *L. rhamnosus* was incubated at 37°C, and the one inoculated with *W. confusa* 30082b at 30°C for 48 h. The viable count of bacteria in activated MRS broth was determined to be $10^7$ CFU/ml using the plate count method before their inoculation in soymilk. Washing of the bacterial cells with 5ml of DMEMgenta solution was done before inoculating the soymilk to avoid interference with the culture medium residues. Soybean seeds of variety-Pusa 1213 were collected from the Division of Genetics and Plant Breeding (Pulse Section), ICAR- IARI, harvested in December 2019.

Isoflavone standards- daidzin, genistin, glycitin, daidzein, genistein, and glycitein were procured from Sigma-Aldrich (USA), while the phytic acid assay kit was purchased from Megazyme (Bray, Ireland). DPPH (1,1′-diphenyl-2-picrylhydrazyl), tetrahydrofuran, and the rest of the analytical grade reagents were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd, New Delhi, India.

***Preparation of Fermented Soymilk.*** To prepare fermented soymilk, soybean seeds (400 g) were soaked for 15 h in double-distilled water. The dehulled soybean seeds were crushed using warm distilled water with a seed-water ratio of 1: 10. The mixture was strained using a clean muslin cloth, and soymilk was collected. Further, it was sterilized by autoclaving at 121ºC for 15min and cooled to room temperature. Sterilized soymilk was inoculated with (1%v/v) cultures of *L. rhamnosus* and *W. confusa* 30082b mixed in a ratio of 1:1 with an average concentration of $10^7$ CFU/ml. Soymilk-containing strains with *L. rhamnosus* and *Weissella confusa* 30082b (1%v/v) at 30°C for 48 h were stirred at 250rpm to obtain fermented soymilk. The microaerophilic environment was maintained by

sparging the soymilk with 0.05v/v/min filter-sterilized (0.22$\mu$m) nitrogen. Periodic sample collection (0, 12, 24, 30, and 48 h after fermentation and on the 7$^{th}$ day of refrigeration) was carried out to detect the growth of the strains and to estimate the biochemical parameters. The fermentation was done in a bioreactor (Applikon-Model: Bio Console ADI 1025, India) in the Microbiology Division, ICAR-IARI where the ambient temperature, pH, redox potentials, and oxygen levels were managed.

***Measurement of pH and Titratable Acidity***. pH was constantly displayed by the 405-DPAS probe in the fermenter. The TA was measured by the AOAC (Association of Official Analytical Chemists, 1984) method and expressed in percentage lactic acid. To 10ml of fermented soymilk, 10ml of distilled water was added. To the mixture, 4-5 drops of phenolphthalein indicator were added and then the solution was titrated against 0.1 N NaOH. Once the solution reached its endpoint, the color changed to pink due to the presence of a phenolphthalein indicator. The percentage of lactic acid equivalent was calculated by

$$\% \text{ Lactic acid} = \frac{\text{ml of } 0.1\,\text{N NaoH required for neutralization} \times 0.009 \times 100}{\text{Volume of soymilk} \times \text{Specific gravity}}$$

Volume of soymilk × Specific gravity
Where The volume of soymilk = 10ml
Specific gravity = ~1.018 g/ml

***Determination of Total Antioxidant Activity (TAO).*** The antioxidant activity of fermented soymilk, both hydrophilic and lipophilic fractions was detected using DPPH (2, 2-diphenyl-1-picrylhydrazyl) method (Rodriguez- Roque *et al.,* 2013). To 5ml fermented/ unfermented soymilk (control) 10ml of methanol was added. The mixture was centrifuged at 6,000rpm for 20min at 4$^{o}$C. The supernatant was identified as the hydrophilic fraction with water-soluble compounds. To the residue obtained after centrifugation, 10 ml of tetrahydrofuran was added. The mixture was again centrifuged at 6,000rpm for 20min at 4$^{o}$C. The second supernatant was identified as the lipophilic fraction with lipid-soluble compounds. For further analysis, aliquots of 200µl of hydrophilic or lipophilic fractions were added to 3.8ml of methanolic solution of DPPH (0.025 g/l). The homogenous mixture was shaken vigorously for a few seconds and withheld in dark for half an hour. The absorbance was measured at 515nm against a methanol blank. Results were expressed in the percentage of inhibition of DPPH radical and calculated using the following equation:

$$\% \text{ DPPH inhibition} = (\text{Control abs} - \text{Soymilk sample abs}) / \text{Control abs} \times 100$$

Where,
Control abs = The absorption by hydrophilic or lipophilic extracts of fermented soymilk
Soymilk sample abs= The absorption by hydrophilic or lipophilic extracts of fermented soymilk

**Determination of Phytic Acid.** Phytic acid extraction was carried out from freeze-dried fermented soymilk according to the simple, quantitative method described in Megazyme (Bray, Ireland). Initially, fermented soymilk was stirred vigorously for 4 h at room temperature with hydrochloric acid (0.66 M). The aliquot of the supernatant obtained was neutralized by mixing 0.5ml of sodium hydroxide solution (0.75 M). 1 ml of the mixture is centrifuged at 13,000rpm for 10min. To 100 µl the supernatant obtained, 600µl of distilled water and 200µl of solution 1 (buffer) were added. Then. incubation at 40$^{o}$C for 15 mins was carried out after 40µl of phytase enzyme suspension was added to the tube. Once the first incubation is over, solution-3 buffer and alkaline phosphatase suspension were added to the same reaction mixture and kept for incubation again at the same conditions mentioned above. The reactions were terminated by mixing 300µl Trichloroacetic acid (50%w/v). After stopping the reaction, the mixture was subjected to centrifugation at 13000rpm for 10min. 1ml of the supernatant obtained was transferred to another tube and 500µl of color reagent solution containing Ascorbic Acid (10%w/v) and Ammonium molybdate (5%w/v) was carried out. Again, the solution was thoroughly mixed by vortexing, and incubation was done at 40$^{o}$C for 1 hour. The reactions were transferred to the cuvettes and spectrophotometric reading was observed at 655nm. The total phosphate released is given as phytic acid per 100g of freeze-dried sample. The phosphorus calibration curve was prepared according to the Megazyme protocol.

$$\text{The concentration of phosphorus} = \frac{\text{mean M} \times 20 \times \text{F} \times \Delta\text{A phosphorus [g/100g]}}{10\,000 \times 1.0 \times \text{v}}$$

Where:
M' = mean value of phosphorus standards [µg/ΔA phosphorus]
20 = original sample extract volume [ml]
F = dilution factor= 28.6
ΔA = absorbance change of sample between total phosphorus and free phosphorus
10 000 = conversions from µg/g to g/100 g
1.0 = weight of original sample material [g]
v = sample volume (used in the colorimetric determination)

***Estimation of free Fe and Zn.*** The free Fe and Zn contents in fermented soymilk were detected with the help of a flame atomic absorption spectrometer (Model-AA7000, Shimadzu Corporation, Japan) at the Phipps laboratory, Division of Soil science and Agricultural Chemistry, ICAR-IARI. Filtration of fermented soymilk was done using a double layer of Whatman Filter paper 42. The standard solutions of Fe used were 0, 0.2, 0.5, 1.0, 1.5, and 2.0 ppm and for Zn were 0, 0.2, 0.4, 0.6, 0.8, and 1 ppm. The concentrations of minerals recorded were converted to milligrams (mg) of the

minerals by multiplying with the dilution factor and dividing by 1000, as follows:

Absorbency (ppm) × dry weight × D

$$MW = \frac{\text{Absorbency (ppm)} \times \text{dry weight} \times D}{\text{Weight of sample} \times 1000}$$

Weight of sample × 1000

The dilution factor for Zn and Fe was 100.

***Estimation of soy-isoflavones using the UPLC method.*** Isoflavone profiling of fermented soymilk was detected by Ultra-high Performance Liquid Chromatography - DAD (Shimadzu Nexera X2 series analytical system (Shimadzu, Japan). Six target isoflavones analytes- daidzin, genistin, glycitin, daidzein, genistein, and glycitein were differentiated on a UPLC system Shim-Pack G1ST $C_{18}$ column (2µ; 2.1 × 150mm) by PDA (Photo Diode Array) which was operated between 200 and 800nm. The mobile phase was solvent B (38% acetonitrile and 0.1% formic acid), and solvent A (10% acetonitrile and 0.1% formic acid). The flow rate of solvents was 0.58ml/min in an isocratic mode (injection volume 4µl, column temperature- 40°C). The program was continued for 10min. Soy- isoflavones extraction from fermented soymilk was carried out by adding4ml of absolute methanol (100%) to 1mlof sample and kept for incubation at 70°C for 30min. The tubes were subjected to centrifugation at 20°C at 13000rpm for 30min. Then, the supernatant obtained was filtered on a membrane filter (0.45µm) and collected in UPLC vials. The elution gradient followed: 0% B was continued for 10 sec, later augmented to 90% B from 10sec to 1min, then 90% B for 5min, and then 0% B for the next 5min. Finally, the column was re-equilibrated with 15% B for 2min. The chromatograms were detected at 254nm and quantitative analysis was based on their retention time and peak areas. Calibration curves for genistin, glycitin, daidzin, genistein, glycitein, and daidzein were obtained using concentrations of 0.2–20.00µg/ml. Data were compared with calibration curves of each isoflavone, and results were expressed as sµg of isoflavone/10ml of soymilk.

***Viable cells count.*** The viable cell count of*L. rhamnosus* and *W. confusa* 30082 bin fermented soymilk was determined using the spread plate method. 0.85% m/v NaCl solution was used to dilute the sample. Serial dilution of the samples was carried out and the diluted samples were spread on MRS agar plates with a sterile glass spreader. The plates inoculated in triplicates with the bacterial cultures were incubated overnight at 37°C/30°C. The colony counts were enumerated and calculated using the formula:

CFU/ml = (Number of colonies)/(volume added to culture plate × dilution factor)

Where,

Number of colonies = Number of countable colonies

Volume added to culture plate = 50µl

Dilution factor = $10^{-n}$

***Determination of volatile compound- Hexanal.*** To estimate the amount of hexanal in fermented soymilk, gaseous samples were collected from headspace and injected into the gas chromatograph (Thermo-scientific Tri-plus500, India). The separation of volatiles was performed on a ZB-WAX capillary column (30 m×0.25 mm×0.25µm). The column temperature was 40° C for 5min in the beginning, then inflated to 130° C at a rate of 10°C/min for 1min, and then up to 210° C at a rate of 5° C /min for 3min and inflated to 240°C at a rate of 8° C /min and was maintained at 240° C for 3min. The carrier gas used was Helium at a flow rate of 1ml/min.

**Statistical Analysis.** The results were obtained for each treatment in triplicates and were represented as means ± standard deviations. The correlations (r) between all biochemical parameters including pH, TA, TAO, phytic acid, free Fe and Zn contents, viable count of bacteria, hexanal of unfermented control, and fermented soymilk with each bacterial strain were measured.

## RESULTS AND DISCUSSION

***pH and Titratable acidity during fermentation and storage.*** During fermentation of soymilk with 1%(v/v) mixed cultures of *L. rhamnosus* and *W. confusa* 30082b, a gentle decrease in pH was observed from 6h (5.76) to 48h (4.76) of fermentation. There is a slight increase from 4.76 to 5.03 till 7 days of refrigeration (Fig. 1). Meanwhile, the percentage of titratable acidity increased from 6 hours (0.19%) to 7 days (0.47%) of fermentation (Fig. 2). In a similar co-culture fermentation, the pH value reached less than 4.50 in all cultures done with different combinations of *L. acidophilus*, *Pediococcus acidilactici*, and *Saccharomyces cerevisiae* with an initial concentration of 7 log CFU/ml of inoculum was enough to reduce the pH in peanut- soymilk. Also, the titratable acidity of peanut soymilk augmented from 0.07% to 0.49% after 24 h of fermentation with the mixed culture fermentation with all the aforementioned microorganisms (Santos et al., 2014). There is an inverse correlation between TA and pH ($P$<0.01; r= -0.97) and this trend of decrease in pH while an increase in TA has been reported earlier (Obadina *et al*., 2013).

***Hydrophilic and lipophilic antioxidant activity.*** In the soymilk fermented with 1%(v/v) mixed cultures of *L. rhamnosus* and *W. confusa* 30082b, the percentage of total antioxidant activity was observed to increase, which ranges from 63.66% (with 33.60% for hydrophilic and 30.06% for lipophilic fractions) from 6h to 67.86% (37.70% for hydrophilic and 30.15% for lipophilic fractions) till 30h of fermentation. There was a slight decrease of % DPPH inhibition from 58.75% (30.11% for hydrophilic and 28.64% for lipophilic fractions) to 54.32% (29.16% for hydrophilic and 25.16% for lipophilic fractions) in 48h and 7 days of fermentation, respectively. Compared to the unfermented control, the TAO of mixed culture fermented soymilk was persistently high at all time intervals (Fig. 3). In similar research conducted on fermented red bayberry pomace, there is enhanced TAO (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$)15.04 mM and %DPPH inhibition-94.37) for co-culture fermentations with *Lactobacillus* and acetic acid bacteria combined with yeast strains, compared with individual inoculations (ABTS- 9.61-13.06 mM and % DPPH inhibition- 91.34- 94.06) (Zhu *et al.*, 2020). The augmented TAO can be because the

fermentation of mixed probiotics can coordinate with each other and aids in producing anabolic and catabolic new phenolic metabolites, organic acids, and polysaccharides (Zhu *et al.*, 2020).

***Total Phytic Acid Content in fermented soymilk.*** In the soymilk fermented with 1%(v/v) mixed cultures of *L. rhamnosus* and *W. confusa* 30082b, the amount of phytic acid was maintained at reduced levels from 0.374 – 0.329g/100g (~3.16- 3.60-fold decrease) from 6 hours to 7 days of fermentation as compared to unfermented control (Fig. 4). In a study conducted by Obeah *et al.*, 2017, in cassava peels fermented with a mixture of *S. cerevisiae*, L. *delbruckii*, and *L. coryneformis*, there was a significant reduction in the phytic acid content from 1044 mg/100g (unfermented) - 788 mg/100g (mixed fermentation). However, it is evident from our results that phytic acid was lower in mixed culture compared to the unfermented control. Mixed culture fermentation showed the most pronounced phytate-lowering effect because of the higher cell biomass and combined action of additive bacterial phytases and other enzymes, making the multistep transformations like phytate to phosphate a more straightforward process (Bevilacqua *et al.*, 2020). There was a negative correlation between Fe content and phytic acid (r= -0.96** $P<0.01$) and AO with phytic acid (r= -0.96** $P<0.01$).

***Mineral Content in Fermented Soymilk.*** In the soymilk fermented with 1%(v/v) mixed cultures of *L. rhamnosus* and *W. confusa* 30082b, both Fe and Zn content was observed to be higher than the unfermented control. The Fe content varies from 0.75 to 2.08µg/ml (upto 6.70-fold increase) from 6h to 48h and then decreases to 1.86µg/ml (6.01-fold increase) until 7 days of storage (Fig. 5). Meanwhile, the Zn content varies from 0.62 to 1.15µg/ml (upto 4.42-fold increase) from 6h to 48h and then decreases to 0.93µg/ml (3.57-fold increase) until 7 days of storage (Fig. 6). The maximum Fe and Zn content was 2.08 and 1.15µg/ml, respectively, at 48 h of fermentation. The phytate/mineral molar ratios are used to predict the inhibitory effect of phytate on the bioavailability of minerals. If the phytate/Fe molar ratio is higher than 1, it could impair Fe bioavailability in humans, whereas for Zn, if the phytate/Zn molar ratio is higher than 5, the bioavailability of Zn could be less than 50% (Garcia-Mantrana *et al.*, 2015).

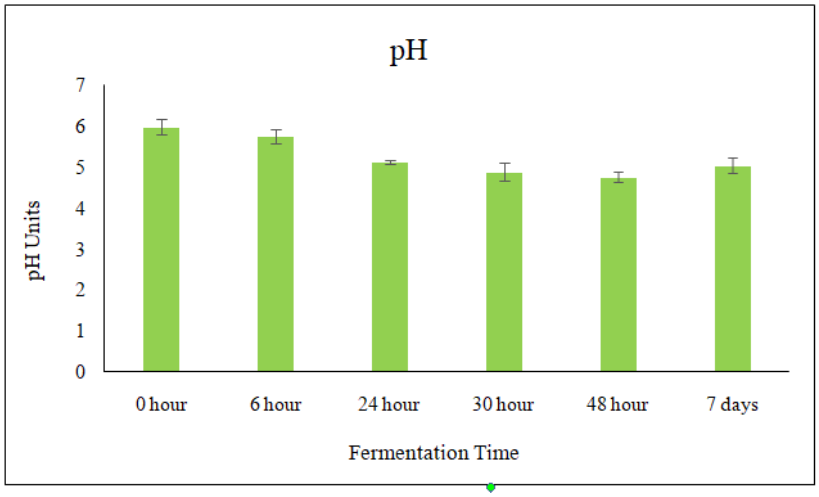



**Fig. 1.** Change in pH of soymilk after fermentation with mixed culture. pH change (in pH units) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.

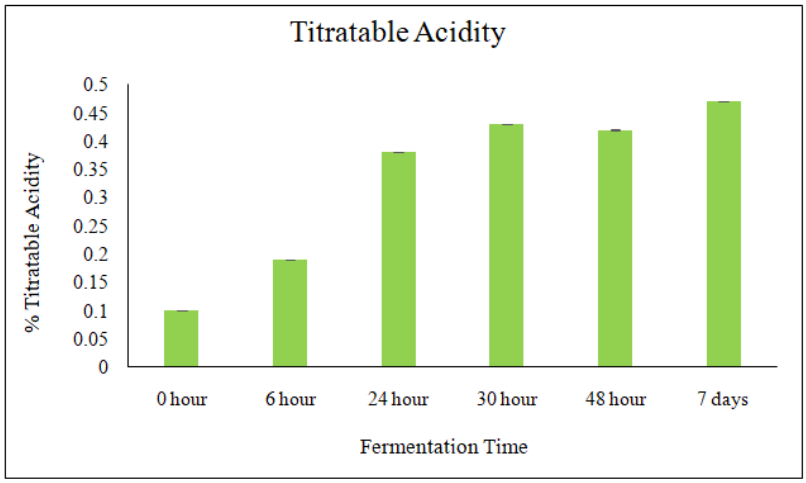



**Fig. 2.** Change in Titratable Acidity of soymilk after fermentation with mixed culture. Change in Titratable acidity (%) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.

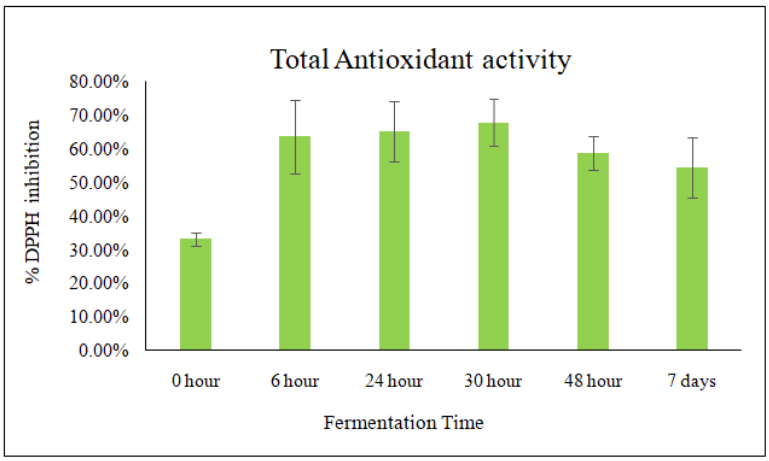



**Fig. 3.** Change in Total Antioxidant Activity of soymilk after fermentation with mixed culture. Change in total antioxidant activity (% DPPH inhibition) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.

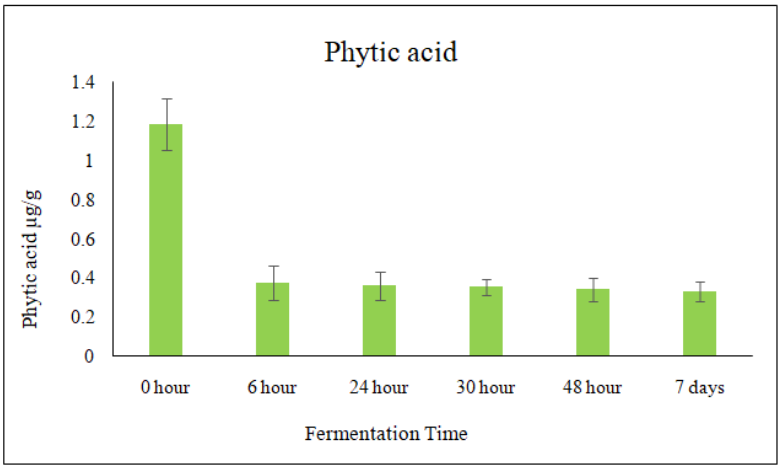



**Fig. 4.** Change in Phytic acid of soymilk after fermentation with mixed culture. Change in Phytic acid content (µg/g) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.

The values of the phytate-Fe ratio ranged from 0.4980 (6h) to 0.1630 (48h) for mixed culture, which is lesser than 1 and shows higher bioavailability of Fe. While the phytate- Zn ratio varied between 0.6032 (6h) to 0.2948 (48h), which is less than 5, it indicates that mixed culture facilitates better bioavailability of Zn. The higher content of Fe and Zn can be directly correlated to the reduction in phytic acid in the mixed culture fermentation, which is understood to have resulted from the combined action of phytases from both *Lactobacillus* and *Weissella* sp. The presence of a positive correlation was observed between Fe and Zn content with AO (r=0.92** *P*<0.01).

***Effects of probiotic fermentation on isoflavone bioconversion.*** The fermentation processes using mixed cultures of 1%(v/v) of *L. rhamnosus* and *W. confusa* 30082b showed a significant reduction in isoflavone glycosides content with consequent increment aglycones, compared to the soymilk control (Fig. 7 A, B, C). In the mixed fermentation, the number of aglycones formed was higher. The concentration of daidzein ranges from 44.458 (6 h) to 99.235µg/10ml(2.376- 5.304-fold) (24 h), then gradually decreases up to 45.093µg/10ml (2.410-fold) till 7 days of refrigeration. While the amount of genistein varied between 67.636 (6h) to 43.981µg/10ml (3.250- 2.11-fold) (7 days), showing a decreasing trend. The amount of aglycone glycitein progressively decreases up to 30.321 at 30h from 43.410µg/10ml at 6h (1.631- 2.335-fold) and then increases to 37.050µg/10ml (1.993-fold increase) when measured at 48$^{th}$ h of fermentation (Fig. 8). The total daidzein, genistein, and glycitein concentrations in mixed culture are 363.422, 297.090, and 203.900µg/10ml, respectively, which is highest in the case of daidzein compared to the unfermented control and second-largest for genistein after *L. plantarum* fermented soymilk. Our results prove that *L. rhamnosus* and *W. confusa* 30082b are excellent isoflavone bio transformers with higher efficiency when inoculated together. In a similar study, mixed culture fermentations with 9 *Lactobacillus* sp. strains showed the highest glucoside bioconversion due to the higher enzyme activities of cell-bound β-glucosidases (El-Shazly *et al.*, 2021).

***Viable Cell Count.*** In the soymilk fermented with 1%(v/v) mixed cultures of *L. rhamnosus* and *W. confusa* 30082b, the viable count of bacteria remained higher throughout the fermentation up to 7 days of storage.

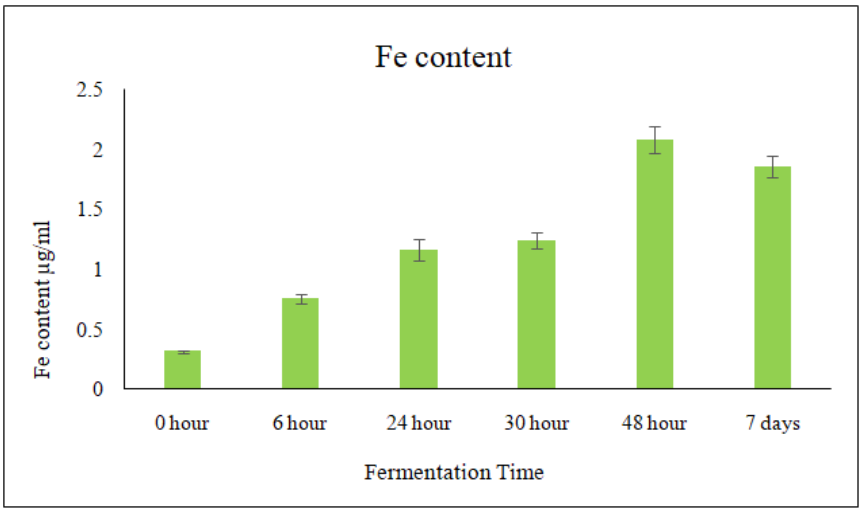



**Fig. 5.** Change in Fe content of soymilk after fermentation with mixed culture. Change in Fe content (µg/ml) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.

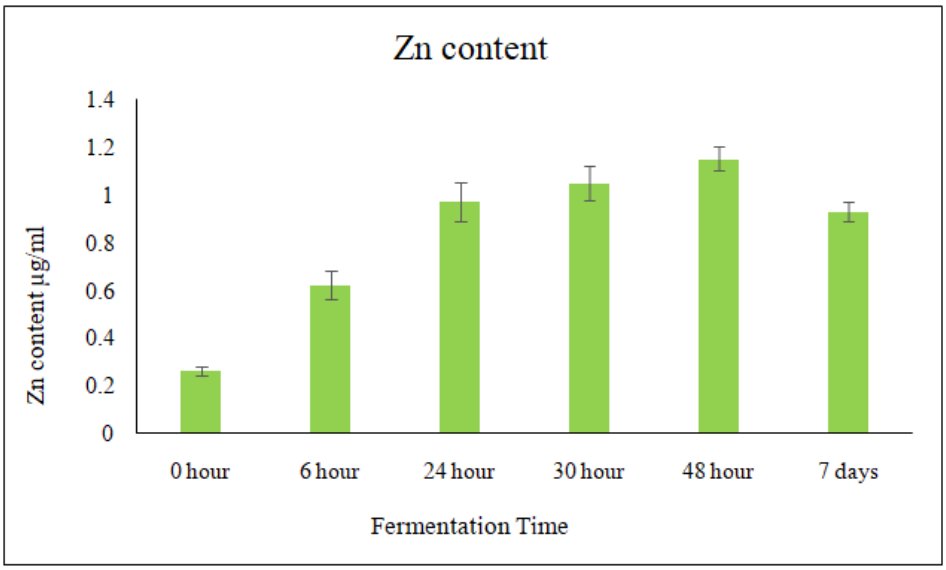
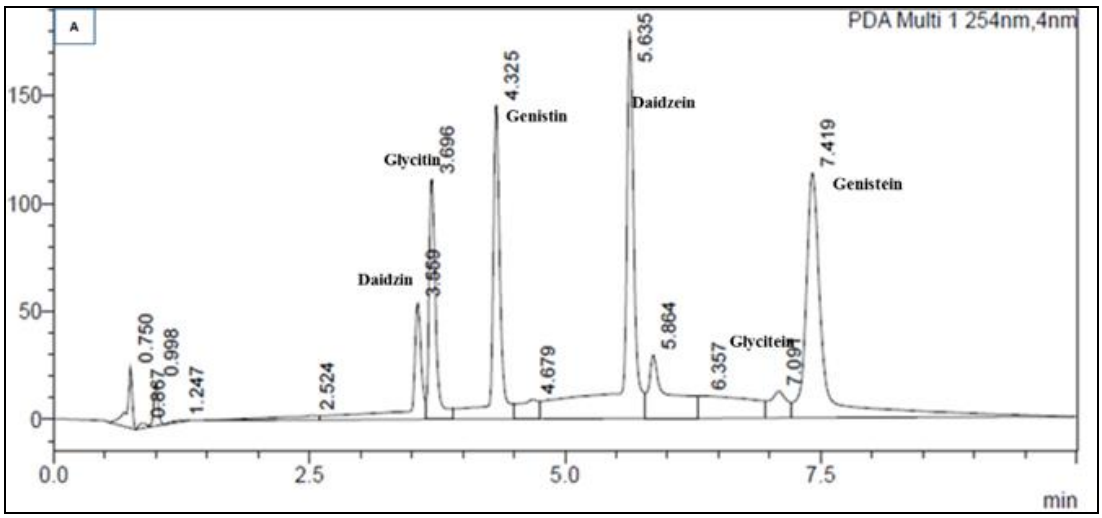



**Fig. 6.** Change in Zn content of soymilk after fermentation with mixed culture. Change in Zn content (µg/ml) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.



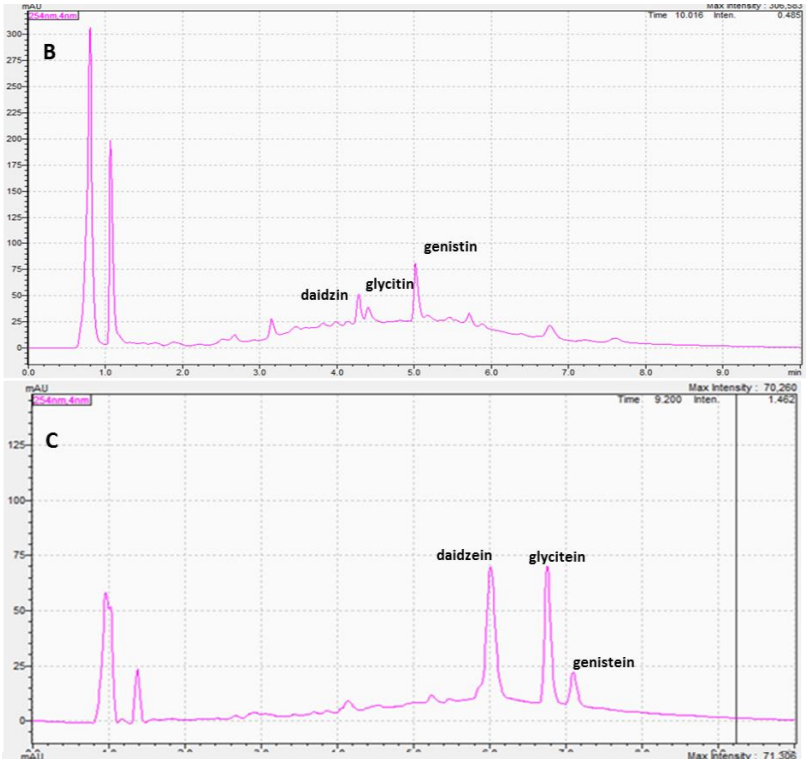



**Fig. 7.** Change in Isoflavone Aglycones of soymilk before and after fermentation with mixed culture (A) Isoflavone-standards peaks (B) Chromatograms of isoflavone aglycones (daidzein, genistein and glycitein) content (µg/10 ml) of unfermented control soymilk (C) Chromatograms of isoflavone aglycones (daidzein, genistein and glycitein) content (µg/10 ml) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation.

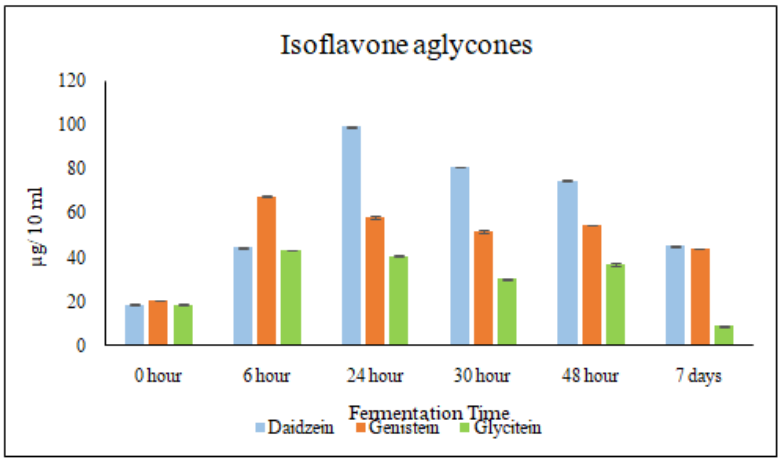



**Fig. 8.** Change in Isoflavone Aglycones of soymilk after fermentation with mixed culture. Change in isoflavone aglycones (daidzein, genistein and glycitein) content (µg/10 ml) of soymilk fermented with mixed cultures of 1% (v/v) *Lacticaseibacillus rhamnosus* JCM 1136 and *Weissella confusa* 30082b after 0, 6, 24, 30, 48 h of fermentation and 7 days of storage at 4°C.

At 6h of fermentation, 1.21 × $10^8$ CFU/ml was observed. It increased to 4.87 × $10^8$ CFU/ml till 30 h of fermentation. There was a slight decrease in the viable count from 4.67 × $10^8$ to 3.32 × $10^8$ from 48h to 7 days. The glucooligosaccharides and galactan exopolysaccharides produced by *Weissella* sp. can be a source of prebiotics and vitamins in the growth medium and can stimulate the growth of other strains like *Lactobacillus* sp. in a mixed culture fermentation through their synergistic effects (Cai *et al.*, 2021). There was a negative correlation between AO and phytic acid (r= -0.98*** $P$<0.001), CFU and phytic acid (r= -0.97** $P$<0.01).

**Hexanal content.** Depletion of green odorants like long-chain straight aldehydes, including hexanal, heptanal, and nonanal enhanced the mouthfeel, aroma, and consumer acceptance of the fermented soy drink while maintaining the nutritional profile of soy drink (Nedele *et al.*, 2021). In our studies, in the unfermented soymilk (control), the hexanal concentration was

observed to be 102.489±0.91ppm and was reduced up to 2.03 ± 0.05ppm. Similar research showing the reduction in the hexanal via mixed fermentation of lactic acid bacteria (LAB) and kombucha bacteria in fermented soymilk indicates that a mixed fermentation strategy can reduce the beany flavor and produce new flavours (Peng *et al.,* 2022).

## CONCLUSIONS

Mixed culture fermentations are generally implemented to stimulate the production of nutritional compounds and enzymes resulting from positive mutual interactions between the bacteria, called protocooperation. Our studies indicate that the interaction between these strains with homofermentative (*L. rhamnosus*) and heterofermentative (*W. confusa* 30082b) metabolisms increased the yield of metabolites which could stimulate the growth of these microorganisms, thus increasing the cell viability. As a result of fermentation, there was the production of lactic acid, acetic acid, ethanol, and $CO_2$, which combinedly reduced the pH of the soymilk from 6.0 to 4.5 while enhancing the titratable acidity from 0.10 to 0.33%. As a consequence, the action of β- glucosidases and phytases were found to increase, leading to the enhanced bioconversion of isoflavone glucosides to aglycones as well as phytic acid to free phosphate. The generation of genistein and daidzein aglycones which are potent antioxidants boosted the antioxidant activity of soymilk. The degradation of phytate, a metal chelating ANF, enhanced the bioavailable content of Fe and Zn in the fermented soymilk.

Overall, mixed culture fermentations with probiotic bacteria were manifested to enhance the microbial stability and fitness, shelf life, and safety of the fermented food products. The co-inoculation of *Weissella confusa* and *L. rhamnosus* could reproduce the metabolic and microbial processes of fermentation, enhancing the nutritional and sensory quality of the fermented products. Also, for the more efficacious utilization of soybean, which harbours a plethora of ANFs, it is desirable to carry out mixed culture fermentation to lower their concentrations. Also, it could improve the sensory properties, and microstructure and increase the bioavailability of nutrients and yield of targeted bioactive compounds either through microbial synthesis or by augmenting the digestibility of the fermented product. The drawback of commercial soy- probiotics is the inability to deliver sufficient probiotics to the acidic lower gastrointestinal tract, which entails developing a more peculiar target delivery system and proper formulations. The industrial production of probiotics is still challenging because of their prerequisite for a rich culture media anaerobic environment, maintaining an optimal fermentation process and assembling the final product while perpetuating its quality. In the future, to cope with the pitfalls of probiotic soy foods and enhance their functionality, a better understanding of the probiosis mechanisms and more advanced cultivation technologies need to be formulated.

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**Conflict of Interest.** None.

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