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Bio-functional properties and anti-inflammatory activity on the RAW macrophage cell line of *Tungrymbai*, a traditional fermented soy food of Meghalaya

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Tungrymbai is an ethnic fermented soy food that is popular amongst the Meghalaya's Khasi and Garo tribes. In our study, we tried to standardise the traditional fermented soy product with well-characterised *Lactobacillus* cultures and studied its bio-functionalities. *Tungrymbai*, a fermented soy product, was prepared and studied for up to eight days for its biofunctional properties, total phenolic contents, isoflavone bioconversion, and anti-inflammatory activity on cell culture study during the storage study. Two lactic acid bacteria, namely K4E (*Lacticaseibacillus rhamnosus* KX950834.1) and K14 (*Lactobacillus helveticus* KU644578.1), were used for the preparation of the *Tungrymbai*. The storage study of *Tungrymbai* was carried out for eight days and was sampled every two days for further analysis. The sensory scores were greater than six up to day 6, whereas beyond that point, the scores began to decline. The pH of the *Tungrymbai* decreased to 5.34 with an increase in storage time, whereas the acidity increased to 0.054% lactic acid. The LAB counts were found to be highest during the initial time of the study (7.83±0.33 log CFU/mL). The maximum level of ACE inhibitory activity was noted during the initial day of the study to be 56.70±1.35%. Anti-diabetic activities such as α-glucosidase, α-amylase, and anti-lipase activities were performed for *Tungrymbai*. The greatest level of α-amylase inhibition was 34.07±2.31% observed during the initial time of the study, whereas α-glucosidase inhibitory activity was found highest on the eighth day of the study to be 37.67±1.24%, and lipase inhibitory activity increased with an increase in storage time up to the sixth day of the study (22.23±0.18%), which significantly decreased on the eighth day of the study. Antioxidative activity was found highest on the sixth day of the study, i.e., 73.65±0.54%. The total phenolic content of *Tungrymbai* decreased with an increase in storage time and was found to be highest on the initial day at 2.08±0.031mg/mL. Aglycone isoflavones, i.e., Daidzein and Genistein were estimated for *Tungrymbai*, where Diadzein content decreased with an increase in storage time, while Genistein content increased up to the fourth day of the study as 38.16±4.84%, which was significantly decreased on the sixth and eighth days of the study, respectively. MTT tests revealed that the lowered dosage of *Tungrymbai* exhibited no cytotoxicity in Raw 264.7 macrophages cell line.

Keywords: α-glucosidase, α-amylase, Anti-lipase, Isoflavone, Phenolic contents, Lactobacillus

The most prevalent, widely grown legumes in the world and generally regarded as a functional diet are soybeans (*Glycine max* L.)[1]. For several decades, the development of ethnic fermented foods has been eaten and linked with heritage and customs. This demonstrates the native inhabitants of Meghalaya depending on the traditional fermented foods rich with beneficial microorganisms other than processed drinks or foods[2]. Similar to Southeast Asians, Meghalaya's Khasi tribes consumed primarily fermented soybeans, bamboo and fish as part of their regular diet. There are many similarities between the northeastern part of India and the nations of Southeast Asia, not only with regard to the environment and the people's ancestry but also with regard to their eating behaviours. In the Khasi Hills of Meghalaya, a fermented plant protein known as *Tungrymbai* is made using soybeans. The production and consumption of soy based sticky food mostly represent the deeply ingrained eating customs associated with the ethnic groups living in Meghalaya and provide the local population with an affordable source of high protein food. Only the Khasi tribe

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prepares *Tungrymbai* using indigenous technology[3]. Soybean is high in lipids (20–30%), proteins (40–50%), and carbs (26–30%) compared to other grains or legumes (22%–33% protein)[4]. Green soybean seeds contain a sizable number of hydrophobic amino acids, which have reportedly been a source of ACE angiotensin-converting enzyme I peptides, which inhibit angiotensin-converting enzymes[1]. Inhibiting ACE results in a reduction in the concentration of angiotensin II, a peptide that causes vasoconstriction, and a simultaneous elevation in the levels of bradykinin, a peptide that induces vasodilation. Consequently, this leads to a general decrease in blood pressure especially important for people suffering from hypertension and cardiovascular diseases. The glycinin (11S globulin) and β-conglycinin (7S globulin) make about 65–85% of the total soybean proteins, whereas lipoxygenases, lectins, trypsin inhibitors, and amylases constitute additional minor proteins/glycoproteins[5]. An initial analysis revealed that the glycinin proteolysis produced four ACE I peptides[6]. In addition to proteins, soybeans are rich in isoflavones, including Daidzein and Genistein[7,8]. These substances contain anti-allergic and anti-inflammatory properties that reduce the probability of cancer and cardiovascular disease cell growth[8,9].

Understanding the microorganisms involved in the natural fermented soy-based food, *Tungrymbai* will help in providing the nutritional biological active compounds during fermentation. With the addition of Lactic Acid Bacteria (LAB), the fermentation process boosts a product's nutritional values by helping the number of vitamins and the biological values of hydrolysed proteins. Choosing starter cultures used in functional food production must take into consideration both the technological and functional qualities of isolated *Lactobacillus* species from fermented foods[10]. LAB has the ability to exhibit a variety of high-molecular-mass chemical substances that are antibacterial, like bacteriocins that efficiently inhibit food-borne pathogens and low molecular mass molecules like H_2O_2, CO_2, and diacetyl[11]. Studies have reportedly revealed that physiologically active peptides extracted from the hydrolysates of soy protein possess the capacity to decrease blood pressure *in vivo* and have ACE-inhibitory activity[12]. ACE, the key renin-angiotensin system enzyme, is inhibited by soy meals fermented with LAB, which has an anti-hypertensive impact[13]. *Lactobacillus* is widely recognised due to its potential probiotic effects on human health. They play a significant part in maintaining a healthy individual's microbiota and can reduce the risk of reactive oxygen species (ROS) production after meals[14]. The bioavailability of isoflavones is increased by *Lactobacillus* spp. fermentation in the majority of soybean based dietary items. Foods include isoflavones as glucosides, which are glucose-conjugated isoflavones that are physiologically inert and incapable of being taken in through the gut wall until they are processed. These glucosides help with protein digestion, supply more calcium-soluble nutrients, enhance digestive health, and strengthen the defence mechanism. For this study, *Tungrymbai* was prepared on a laboratory scale under sterile conditions with more hygienic conditions. In this study, we highlighted the importance of a storage study on the bio-functional characteristics, including ACE inhibition, antioxidant activity, anti-diabetic activities and biotransformation of *Tungrymbai* isoflavones, as well as the anti-inflammatory activity of fresh *Tungrymbai* in the RAW 264.7 macrophage cell line.

Materials and Methods

LAB used for preparation of *Tungrymbai*

Two LABs were used in the investigation, viz., (*Lacticaseibacillus rhamnosus* KX950834.1) designated as K4E and (*Lactobacillus helveticus* KU644578.1) designated as K14, which were isolated from the Garo tribes' traditional fermented food items in Meghalaya, India. In a de Man Rogosa Sharpe broth at 37°C, the *Lactobacillus* cultures were grown overnight and it is a selective media for Lactobacillus cultures. *Lactobacillus* biomass production was performed using a whey-based cheese medium, whereas active cultures of *Lactobacillus* were inoculated at a 5% rate in cheese whey supplemented with 1% each of yeast extract (Himedia, India) and proteose peptone (Himedia, India). Further incubation was performed until abundant development was attained. After centrifuging the culture cells for 20 min at 4500 rpm under 4°C, they were collected and washed twice with PBS (phosphate buffer saline). After that, cells were suspended in PBS with the cell counts adjusted to roughly 10^8 CFU/mL and stored below 4°C.

Starter cake preparation for *Tungrymbai*

The formulation and development of defined starter cultures were carried out by following Dung[15] and Mishra *et al.*[16] with requisite modifications. The batch culture technique used for fermenting the starter cake

for *Tungrymbai* involved the inoculation of 100g rice powder with cell suspensions of K4E and K14 (previously adjusted to 10^8 CFU/mL), followed by the addition of *Scoparia dulcis* extract. *Scoparia dulcis* extract was added due to its valued medicinal properties, which can enhance ACE-inhibition, antioxidant capacity, and antimicrobial potential. These attributes are significant in traditional medicine and fermentation processes among tribal communities. Its inclusion in *Tungrymbai* is based on its anti-inflammatory, antioxidant, antimicrobial, and hepatoprotective effects, making it both a flavour enhancer and a contributor to health benefits in fermented product[16]. Dough was prepared using sterile distilled water under aseptic conditions in the Laminar air flow chamber. Kneaded dough was rolled flat forming small starter cakes and was transferred into sterile petri plates. The fermentation and drying of the starter cakes were carried out at an ambient temperature of 40°C. The fermentation then proceeded without further agitation or intervention, maintaining a static environment. This batch culture method allowed for controlled fermentation and development of the starter culture within the rice powder substrate (Fig. 1; Fig. 2). This ready to use starter cake was then stored under refrigerated conditions (7±2°C) till further use.

Composition and preparation method of *Tungrymbai*

A local variety of soybean seeds obtained from the *Iewduh* local market in Khasi Hills, Meghalaya, was used. Batch culture fermentation was employed in the preparation of *Tungrymbai*. This method involves fermenting a fixed amount of cooked soybeans with a starter cake without adding or removing any material

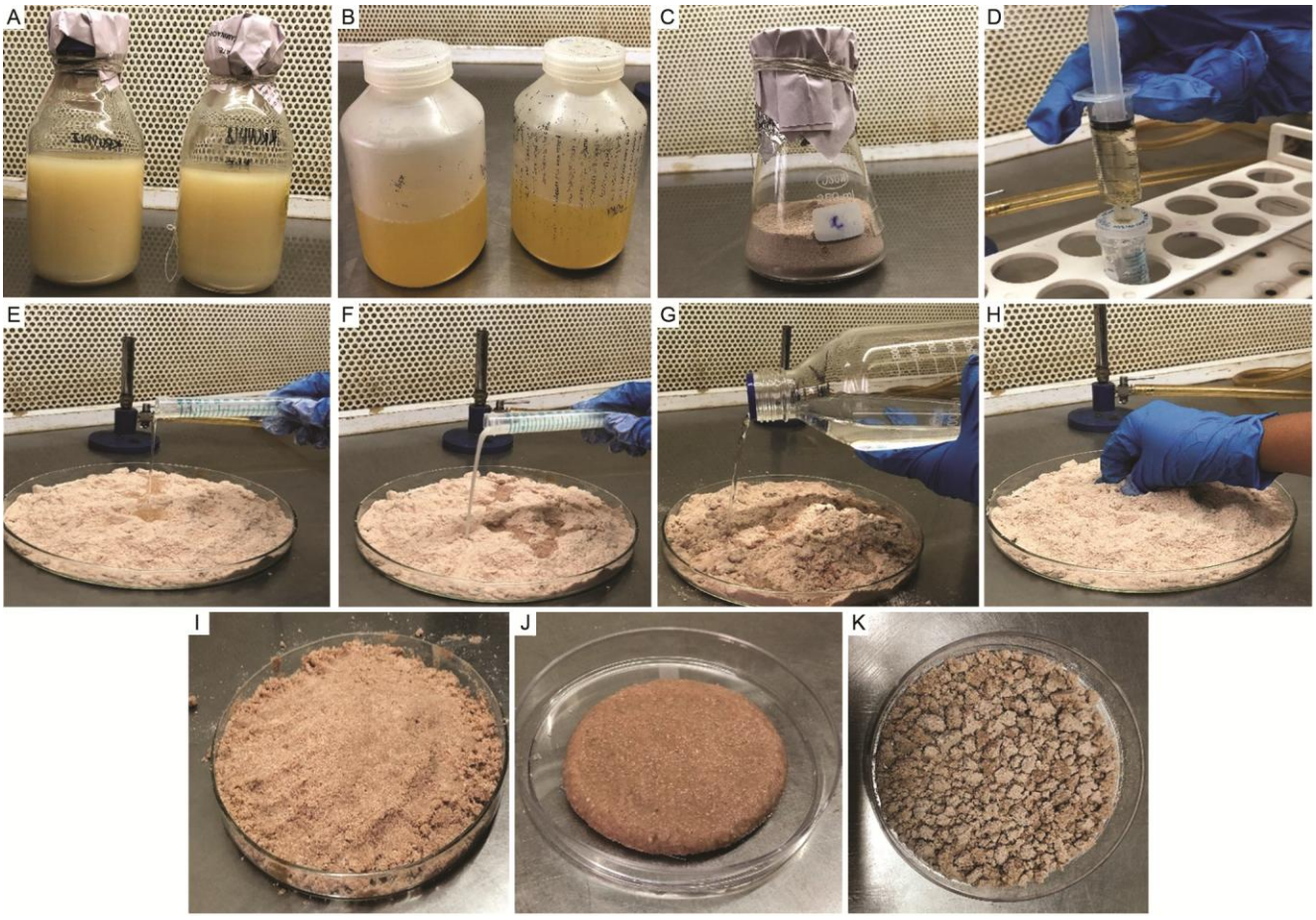

Fig. 1 — Preparation of starter cake for *Tungrymbai*. (A) Growth of culture in cheese whey, (B) Centrifuged the over grown cultures at 5000 rpm for 15 min and washed the pellet twice with saline solution, (C) Autoclaved rice powder, (D) preparation of filter sterilised S. dulcis extract using 0.45µm syringe filter, (E) Transferred the plant extract to rice flour, (F) Culture added to the rice flour, (G) Required amount of sterile water is added, (H) Preparation of starter cake by kneading a dough, (I) Kneaded dough rolled flat forming small starter cakes-starter culture, (J) Transferred small cakes in sterile Petri plates for drying and (K) Oven dried starter cake at 40°C for 4 days.

throughout the fermentation process. The fermentation, conducted for three to four days at 37°C without agitation, allows for precise control over factors like temperature and time, leading to the development of desired flavours, textures, and acidity characteristic of *Tungrymbai*. The fermentation was conducted at an ambient temperature of 37°C. During the fermentation period of three to four days, intermittent shaking was not applied. The fermentation process was allowed to occur without agitation. The entire fermentation process, including the addition of starter cake and mixing with soybeans, was carried out under sterile conditions to ensure the purity and quality of the fermentation[15] (Fig. 3; Fig. 4).

Storage study of *Tungrymbai*

Physicochemical properties

pH and titratable acidity of the *Tungrymbai* were determined as per the methods followed by Mishra *et al*.[16]. Sample was prepared by homogenising 10g of soybean sample with 90mL of sterile distilled water. 5mL of this sample was taken and mixed with 10mL of distilled water and two drops of phenolphthalein indicator was added. Each sample was titrated with 0.1N NaOH solution until the sample turned light pink in colour. The reading was taken accordingly and recorded for each sample. The percent acidity was expressed in terms of % lactic acid. The titratable acidity was calculated as percent lactic acid as follows:

% of acidity = [mL of NaOH used] x [0.1N NaO] x [0.09g (milliequivalent factor)] x [100] g of sample used.

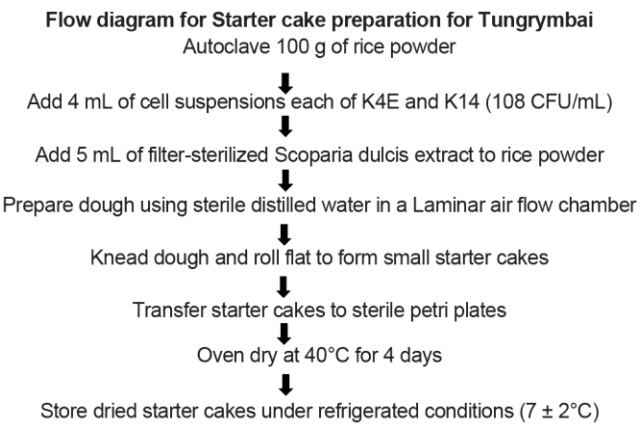

Fig. 2 — Flow diagram for starter cake preparation

Microbial analysis

Total *Lactobacillus*, yeast and mould, and coliform counts of *Tungrymbai* were being carried out for 8 days under refrigerated conditions (7±2°C) on MRS (de Man Rogosa Sharpe) agar, PDA (Potato dextrose agar), and EMB (Eosin methylene blue) agar respectively. Log CFU/mL was used to denote total viable counts.

Bio-functional properties of *Tungrymbai* under refrigerated storage conditions

Determination of ACE inhibitory activity

The percentage measurement of *Tungrymbai*'s ACE inhibitory activity was done using the substrate Hippuryl-L-histidyl-L-leucine (HHL), applying Dineshbhai *et al.*[17] methodology with a few modifications. 50µL of a 5mM HHL solution were combined with 200µL of the supernatant sample. To this mixture, 500µL of deionised water was added, and ACE enzyme was added to the reaction in a volume of 20µL (4mU in 250µL), and the reaction

Fig. 3 — Preparation of *Tungrymbai*. (A) Overnight soaked Soybeans, (B) Washed and strained Soybeans, (C) Autoclaved soybeans added with defined starter cake, (D) Product after fermentation: *Tungrymbai*

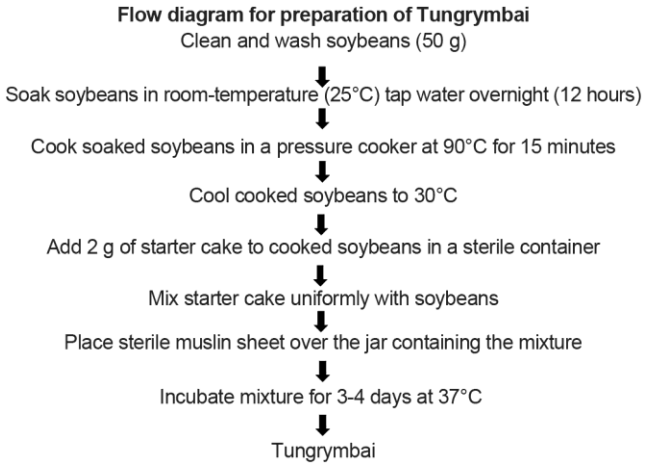

Fig. 4 — Flow diagram for preparation of *Tungrymbai*

was then incubated for 30 min at 37°C. The reaction was stopped after incubation with the addition of 1mL of chilled hydrochloric acid (1N), and the mixture was then re-incubated for 30 min at 37°C. The mixture was centrifuged for 10 min at 5000 rpm after adding 1.7mL of ethyl acetate. Using a micropipette, the upper layer of the biphasic system was collected at 100°C and evaporated for 10 min. Hippuric acid remnants were diluted in 2mL of deionised water, and the mixture's absorbance was measured using a spectrophotometer with a blank at 228nm. As a control, a sample of unfermented soy was retained.

Assessment of anti-diabetic activities of *Tungrymbai*

α-Amylase inhibition activity

The inhibition of α-amylase approach described by Kinariwala *et al.*[18] was applied with a few minor modifications. A reaction mixture was made up of 200µL of *Tungrymbai* supernatant centrifuged at 5000 rpm for 10 min, 400µL of phosphate buffer (pH 6.8), 20µL of α-amylase (2U/mL), and 15 min of pre-incubation at 37°C. Then, 200µL of 1% soluble starch was added, and the mixture was incubated at 37°C for an additional 20 min. 2mL of DNSA colour reagent was prepared freshly and added to the reaction mixture, which was then heated at 100°C for 10 min. A spectrophotometer (Systronics, India) was used to measure the absorbance at 540nm. The results were shown as a percentage of total inhibition as follows:

$$\alpha\text{-Amylase inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

where, A_c is the absorbance of control and A_s is the absorbance of test sample.

α-Glucosidase inhibition activity

Inhibition of α-glucosidase activity was carried out after Shukla *et al.*[19]. 100µL of *Tungrymbai* supernatant (pre-incubated for 10 min at 37°C) was included in a reaction mixture containing 2.5µL of α-glucosidase enzyme (1U/mL), 1.5mL of phosphate buffer (100mM, pH 6.8), and 500µL of 4-Nitrophenyl-β-D-glucopyranoside (PNPG, 5mM). It was incubated at 37°C for 20 min. 100µL of sodium carbonate (Na_2CO_3, 0.1M) was added to stop the reaction process. At 405nm, p-nitrophenol absorbance was determined spectrophotometrically. The inhibitory activity was determined by applying the formula:

$$\alpha\text{-Glucosidase inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

where, A_c is the absorbance of control and A_s is the absorbance of test sample.

Lipase inhibition activity

Lipase inhibition activity was performed following Khakhariya *et al.*[20]. A reaction mixture was prepared by adding 100µL of *Tungrymbai* supernant, 1.7mL of phosphate buffer (100mM, pH 6.8), 2µL of pancreatic lipase (0.5mg/mL), and 100µL of 4-methylumbelliferyl oleate (4MUO, 0.25mM) and incubated at 37°C for 30 min. To stop the process, 100µL of sodium citrate solution (0.1M) was added. Spectrophotometrically, the 4MU-induced fluorescence was detected at 260nm.

$$\text{Lipase inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

where, A_c is the absorbance of control and A_s is the absorbance of test sample.

Total Antioxidative capacity

The ability of a chemical to neutralise the stable ABTS (2, 2-Azino-bis, (3-ethylbenzothaizoline-6-sulfonic acid), Sigma-Aldrich, USA) radical was used to evaluate the radical-scavenging potential of different cultures. Using the Das *et al.*[21] method, the antioxidative activity was measured. The ability of the samples to scavenge free radicals was calculated using the equation shown below:

$$\text{ABTS radical scavenging activity (\%)} = [(A_{\text{control}} - A_{\text{sample}})/ (A_{\text{control}})] \times 100$$

Where, A_{Sample} is the absorbance of sample and $A_{Control}$ is the absorbance of control sample.

Total Phenol content

Total phenolic content was measured following the protocol given by Mishra *et al.*[22], with minor modifications. The sample was produced by

collecting the supernatant and centrifuging it for 10 min at 10,000 rpm. The sample was filtered through a 0.45µm syringe filter after being 50 times diluted. A 1:1 diluted amount of Folin-Ciocalteau reagent was applied to 1mL of filtrate. After adding 10mL of 7.5% sodium carbonate, the liquid was thoroughly shaken. It was then held at ambient temperature in the dark for a further 60 min, and OD at 750nm was measured using a spectrophotometer. Gallic acid was used as the benchmark. Milligrams of gallic acid equivalents (GAE) per 100 millilitres (mg/100mL) are a unit used to express the results.

Extraction of isoflavones

According to the instructions provided by Lodha *et al*.[23], isoflavones from *Tungrymbai* were extracted. Sampling of the *Tungrymbai* product was carried out for eight days of the study, wherein samples were collected at an interval of two days. Using a freeze dryer, the samples were further freeze-dried (HyperCOOL HC3055, India). In order to fill a 15mL falcon tube, 500mg of the freeze-dried material were diluted in 10mL of acetonitrile. The samples were extracted for two hours at room temperature on a rotary shaker. The tube was then filled with 3.5mL of HPLC grade water, transferred to a 15mL centrifuge tube, and centrifuged for 20 min at 5000 rpm. A 0.45µm syringe filter was used to filter the clear supernatant after it had been collected in a fresh tube. The sample was then manually injected into the RP-HPLC, and chromatograms with a wavelength of 260nm were produced.

Separation and quantification of isoflavones by RP-HPLC

Shimadzu HPLC is outfitted with a 7725i, an SPD-20A wavelength detector, and an LC20 HPLC pump. The analysis of isoflavones was performed using a Shimadzu LC-20 manual injector with a 20µL loop, a diode array ultraviolet (UV-260nm) detector, and a SeQuant ZIC-cHILIC (Mark, Germany) column (PEEK 250×4.6mm, 3mm, 100 Å pore size). Using an isocratic run with mobile phase (0.05% TFA in 50% of 100mM ammonium acetate and 50% methanol) with a flow rate of 0.5mL/min, isoflavones were eluted over a period of 35 min. A 0.45µm Millipore (Bangalore) membrane filter was used to vacuum filter and degas all solvents.

***In-vitro* cell culture study**

The RAW 264.7 murine macrophage cells were obtained from the National Cell Science Centre (NCSC), Pune (Maharashtra, India). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10% penicillin-streptomycin, and 10% l-glutamine. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. *Escherichia coli* lipopolysaccharide (LPS) was procured from Cusabio Biotech (China) for use as a stimulus. Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and IL-1beta were also acquired from Elabscience (USA). HiMedia Laboratories (India) supplied MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) for cytotoxicity assessment.

Cytotoxicity of fermented soybean food *Tungrymbai* (FSFT) in RAW 264.7 macrophages

According to Khare *et al*.[24], RAW 264.7 cells were used for an MTT cell viability experiment. For this, 1×10^4 RAW 264.7 cells/well were planted on a 96 well plate and maintained in a humidified 5% CO_2 environment. The cells became confluent after being cultured for 24h, and then different concentrations of FSFT (8, 4, 2, 1, 0.5, and 0.25mg/mL) were added for another 24h. Each well was then filled with 100µL of MTT (5mg/ml in phosphate-buffered saline, pH 7.4), and each well was incubated for an additional 4h until a purple colour appeared. After removing the supernatant and allowing each well to receive 100µL of DMSO to dissolve the formazan salt, the optical density was measured at 570nm using a 96 well microtitre plate reader (M200 PRO, Tecan Life Science). The results were presented as the percentage of viable cells compared to the control group.

Nitric oxide assay

RAW 264.7 cells were seeded in a 48 well plate at a density of 1×10^5 cells/well and stimulated with FSFT at 2, 1, 0.5 and 0.25mg/mL, as well as 1µg/mL LPS, for 18h. After a 16h incubation period, 150µL of the culture supernatant was mixed with 100µL of Modified Griess reagent, which contained 1% sulfanilamide (0.1% sulfuric acid) and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid. The solution's optical density (OD) was measured at 540nm in a microplate reader after 30 min of incubation. The percentage of NO inhibition compared to control was estimated based on the amount of nitrite found in culture supernatants. The formula (OD of test/OD of positive control)×100 was used to determine the percent NO generated.

***In-vitro* pro-inflammatory cytokine analysis**

The supernatants obtained from the aforementioned interventions were kept at -80°C for further analysis and used to measure the concentrations of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 using commercial ELISA kits in accordance with the manufacturer's institution (Elabscience, USA). At 450nm, the absorbance/optical density (OD) generated was measured. A four-parameter logistic curve with standard concentration and OD values was plotted to determine the results.

Organoleptic evaluation

Ten Sensory Panelists from Northeastern Hill University (NEHU), Department of Rural Development and Agricultural Production at the Tura Campus in Meghalaya, who are predominantly from the Khasi Hills and were accustomed to the consumption of *Tungrymbai*, were requested to rate the *Tungrymbai* sample on a 9 point hedonic scale for taste, aroma, colour, texture, mouth feel, and overall acceptability. Sensory evaluations of *Tungrymbai* samples were stored at temperatures between 6-8°C for eight days.

Statistical analysis

The findings of three investigations have been expressed using SEM (mean standard error). Testing was done in triplicate. A one-way ANOVA was used for the analysis of variance, and Duncan's test with a 95% confidence interval was used to look for any changes between sample means that were statistically significant. One-way ANOVA and Tukey's *post-hoc* test were both utilised in the cell culture study. To examine the data, Graph Pad Prism 8.0 Software Inc. (La Jolla, CA, USA) was used.

Results and Discussion

Physicochemical properties (pH and acidity)

Among the biologically beneficial elements present in soybeans are isoflavones, soyasaponins, lignans, derivatives of cinnamic acid, terpenes, and sterols. During fermentation, soy's chemical constituents are altered and reduced. During fermentation, LAB utilises the soy complex oligosaccharides present in soybean products and converts them into simpler compounds, which further leads to the synthesis of lactic acid. The pH of the product as a whole is lowered by lactic acid. pH and acidity for *Tungrymbai* were measured for eight days, and the results were taken at every two day interval. The pH of *Tungrymbai* significantly decreased from 6.94 to 5.34 with an increase in storage time, whereas the acidity significantly increased from 0.018% to 0.054% lactic acid at eight days of study (Fig. 5). Soybean and quinoa were used to develop a plant-based yoghurt, and it's physicochemical, rheological, sensory, and functional qualities were assessed by Huang *et al.*[25]. The yoghurt prepared from soyabean showed a pH range of 4.09±0.01 to 4.18±0.01 during the first 21 days of the storage trial carried out under refrigeration (4°C). Chun *et al.*[26] examined how salt concentrations affected the fermentation process of *Doenjang*, a customary fermented soybean paste from Korea. The pH during the beginning was found to be 6.1 to 6.3, which gradually decreased with an increase in fermentation time. The pH of the 9% salt *Doenjang* samples decreased to 4.8 at 130 days. *Shuidouchi*, a traditional Chinese fermented soybean, was examined

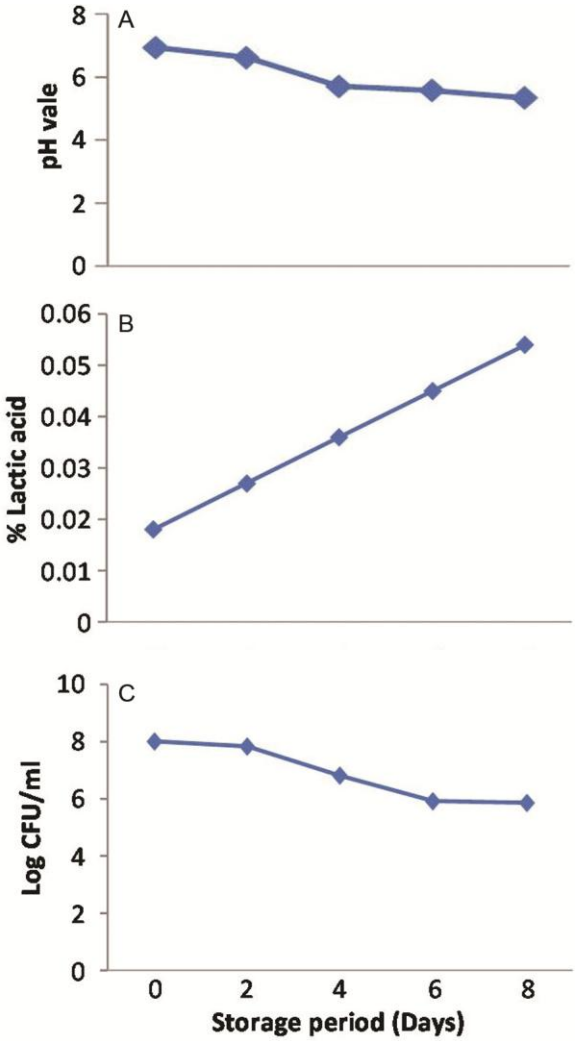

Fig. 5 — pH, acidity and LAB counts of *Tungrymbai*

for its microbial diversity and chemical composition by Chen *et al.*[27]. From southwest China, 11 *Shuidouchi* samples were obtained, whose pH ranged from 5.23±0.04 to 7.27±0.04. According to Lee *et al.*[28], a starter culture of *Lactobacillus plantarum* reduced biogenic amines during the fermentation of miso. The pH of the miso decreased from 6.2 at the initial time to 4.9 after forty days of study. Chun *et al.*[26] and Lee *et al.*[28] also found that pH decreased as storage time increased, which may be related to the lactic acid production by the LAB employed in the study's lactic acid production. According to a study by Falade and Akinrind[29], the titratable acidity of fermented soybean samples gradually increased over the course of the fermentation process. Additionally, on the fifth day of fermentation, titratable acidity dramatically decreased from 0.019% observed on the fourth day to 0.015%, perhaps as a result of the raised pH. Kwon *et al.*[30] observed that the fermented soybean product *Cheonggukjang* showed a titratable acidity of 4.49±1.73% after 2 days of fermentation. Ng'ong'ola-Manani[31] found the initial titratable acidity of lactic acid bacteria-fermented soyabean paste in the range of 0.16% to 0.20%, which significantly increased threefold after 24h. In our investigation, an increase in acidity was found as storage duration increased, which was similarly shown by Falade and Akinrind[29] and Ng'ong'ola-Manani[31].

Microbial analysis

The LAB counts were taken every 2 days until the eighth day of storage study of *Tungrymbai* stored at refrigerated conditions (7±2°C). There was a finding that LAB counts decreased considerably ($P \leq 0.05$) with an increase in time. The highest count was observed during the beginning of the study (8.01 ± 0.36 log CFU/mL), which was found to be on par with the second-day counts (7.83±0.33 log CFU/mL) (Fig. 5). The samples examined during the shelf life research of laboratory produced *Tungrymbai* were devoid of coliforms. This might be due to the production of lactic acid during fermentation, which lowers the pH and increases the acidity, ultimately inhibiting the Gram-negative coliforms. Kim *et al.*[32] studied traditional Korean fermented soybean paste (*Doenjang*) and found a correlation between physiochemical quality traits and consumer hedonic perception. The *Doenjang* samples prepared by the traditional method showed 6.6–7.0 log CFU/g for the range of total plate counts, whereas the one prepared using the Japanese method showed a significantly lower bacterial count of 2.6 log CFU/g. Nguyen *et al.*[33] observed that the *L. brevis* counts during the time of encapsulation using different carriers and drying methods remained 6.1 to 6.4 log CFU/mL which gradually decreased to 4.4 to 5.1 log CFU/mL upon storage at 4°C on 21st day. Li *et al.*[34] observed that during the preparation of an okara derived beverage using different starter cultures, the *Lacticaseibacillus paracasei* 6244 culture group exhibited the most rapid growth post-fermentation, with a viable bacteria count of 3.53×10^8 CFU/mL. Subsequently, the cultures containing *Lacticaseibacillus rhamnosus* S24, *Lacticaseibacillus paracasei* 6244, and *Lactobacillus acidophilus* 11,073 exhibited the viable counts of 2.77×10^8 CFU/mL, while the *Lacticaseibacillus rhamnosus* S24 alone showed the viable count of 1.77×10^8 CFU/mL. Over time, there was a gradual decrease in the total number of viable probiotic microorganisms across all samples. Particularly, the *Lacticaseibacillus rhamnosus* S24 culture group exhibited the most significant decline, dropping from 3.47×10^7 CFU/mL to 1.77×10^8 CFU/mL. A decrease in lab counts was noticed by Nguyen *et al.*[33] and Li *et al.*[34], which were in line with our results.

Bio-functional properties of the *Tungrymbai* under refrigerated storage conditions

Determination of ACE inhibitory activity

Peptidyl dipeptide hydrolase ACE (EC 3.4.15.1) is crucial for the physiological control of blood pressure. To produce the strong vasoconstrictor angiotensin II that is a component of the renin-angiotensin system, ACE cleaves the C-terminal dipeptide of angiotensin I. This causes an increase in blood pressure. Furthermore, ACE deactivates bradykinin, an important vasodilator, raising blood pressure. As a result, ACE activity should be decreased in order to reduce angiotensin II production and boost bradykinin levels, which will regulate blood pressure[35,36]. Food-derived ACE-inhibitory peptides have increasingly gained popularity as kinder and safer alternatives for blood pressure regulation. The ACE-inhibitory activity of *Tungrymbai* ranged from 27.80% to 56.70%. With longer storage times, ACE inhibitory activity significantly decreased. The highest activity was observed during the initial day (56.70±1.35%) of fermentation and the lowest on the eighth day (27.80±0.50%) of study (Fig. 6). Chourasia *et al.*[37] demonstrated that soy chhurpi fermented with

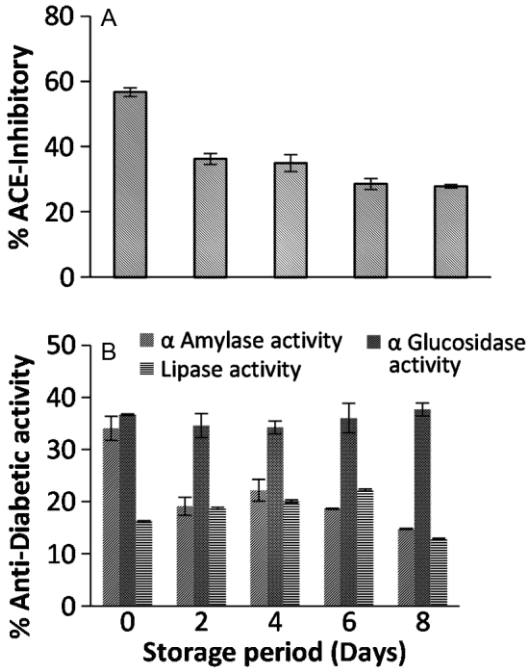

Fig. 6 — Bio-functional properties of *Tungrymbai* during storage under refrigeration condition (7±2^{0}C)

L. delbrueckii WS4 gave a maximum level of ACE inhibition of 10.5±0.3% when compared to soy chhurpi made from soymilk without any fermentation. Novel angiotensin I converting enzyme inhibitory peptides were discovered by Daliri *et al.*[38] in isolates of soy protein that have undergone *Pediococcus pentosaceus* SDL1409 fermentation. Low molecular weight peptides from fermentate showed 65.1±0.78% ACE inhibitory activity. ACE-inhibitory peptide was investigated as a potential functional food by Wang *et al.*[39]. The sample's ACE-inhibitory activity was discovered to be 34.1±1.3%, whereas individual fraction 1's ACE-inhibitory activity (molecular weight <6000Da) and fraction 2's (molecular weight>6000Da) were found to be 49.6±1.5% and 5.8±1.1%, respectively. ACE-inhibitory activity was higher in our study compared to that of Chourasia *et al.*[37] and Wang *et al.*[39].

Assessment of anti-diabetic activities of *Tungrymbai*

α-Amylase inhibition activity

Amylase inhibitors can function as carbohydrate blockers, decreasing carbohydrate digestion and absorption in the gastrointestinal tract. Additionally, in most instances, the inhibitory mechanism of protein to amylase occurs by directly inhibiting the active centers of numerous subsites of the enzyme[40]. The α-amylase inhibition activity for *Tungrymbai* ranged from 14.80% to 34.07%. The highest activity was seen at the initial time (34.07±2.31%) of *Tungrymbai* fermentation. The α-amylase inhibition activity on the second day (19.13±1.72%), the fourth day (22.17±2.09%), and the sixth day (18.63±0.09%) were significant and found to be at par with each other (Fig. 6). According to Khakhariya *et al.*[20], fermented buffalo milk containing *Lacticaseibacillus paracasei* (M11) showed α-amylase inhibition activity in the range of 57.09% to 79.14%, with the maximum activity seen at 48h. Inhibitory, anti-inflammatory, and antidiabetic effects of fermented camel milk were all examined by Shukla *et al.*[41]. The *L. plantarum* KGL3A fermented camel milk had α-amylase inhibition activity that ranged from 55.64% to 80.94%, with the peak activity being shown at 48h. According to Kinariwala *et al.*[18], ten cultures were tested for their potential to suppress growth, with *Lacticaseibacillus rhamnosus* M9 showing the most activity (71.05%) and *Limosilactobacillus fermentum* M10 shows the lowest activity (1.45%).

α-Glucosidase inhibition activity

When bound to the active site of α-glucosidase, α-glucosidase inhibitors can form complexes with greater binding affinity than carbohydrate α-glucosidase complexes. Their structures are comparable to those of disaccharides or oligosaccharides. As a result, the small intestine's brush border site's carbohydrate hydrolysis and glucose absorption are inhibited[42]. The ability of *Tungrymbai* to inhibit α-glucosidase ranged from 34.23% to 37.67%. Maximum inhibitory activity was seen on the eighth day (37.67±1.24%) of the study, whereas the fourth day observed 34.23±1.22% activity, which was low compared to other days (Fig. 6). Gao *et al.*[43] used the *Bacillus amyloliquefaciens* SY07 strain to research the glucosidase inhibitory action of fermented Okara broth. The α-glucosidase inhibitory activities ranged from 24.72 to 47.07%, with the highest inhibition found in fermenting Okara and the lowest in fermented Okara+Luria-Bertani broth. Aqueous extracts made from the ethanol of *Kochujang* samples have an average 29.6% inhibitory efficacy against α-glucosidase, according to Yang *et al.*[44]. When fermenting soymilk with *Lactobacillus plantarum* P1201 and testing for antioxidant and digestive enzyme inhibitory characteristics, Lee *et al.*[45] noticed changes in the concentrations of conjugated linoleic acid and isoflavones. In comparison to SPHM (soybean

powder hydrolysate milk) extracts, all fermented soybean powder hydrolysate milk (FSPHMs) had higher α-glucosidase inhibitory activity. Between SPHM and FSPHM (11.3 → 55.8%), the Shinhwa cultivar demonstrated the largest increase of 45%.

Lipase inhibition activity

A vital digestive enzyme called pancreatic lipase transforms triglycerides into monoglycerides and free fatty acids. Total cholesterol levels in the body are lowered when the lipase enzyme is inhibited, which plays a role in treating obesity[46]. The lipase inhibition activity of *Tungrymbai* ranged from 12.93% to 22.23%. The sixth day of study showed significantly the highest activity (22.23±0.18%), followed by the fourth day (20.07±0.28%), and the second day (18.73±0.18%) of activity (Fig. 6). Khakhariya *et al.*[47] found that the *Limosilactobacillus fermentum* KGL4 strain and *Saccharomyces cerevisiae* WBS2A fermented buffalo milk had the highest lipase inhibitory activity at 48h, at 61.79±2.14% and 73.85±1.19%, respectively. Shukla *et al.*[19] explored the anti-diabetic effects of *Lacticaseibacillus paracasei* M11-fermented camel milk. The lipase inhibitory activity increased with an increase in incubation time, from 54.26% observed at 12h to 65.46% detected at 48h. A study by Choi *et al*.[48] focused on the anti-obesity properties of soygerm isoflavones fermented by *Bifidobacterium breve.* It was observed that lipase inhibitory activity increased with an increase in fermentation time. 100ppm of fermented soygerm isoflavone glycosides inhibited pancreatic lipase activities up to 30% in the enzyme reaction mixture.

Total Antioxidative capacity

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2), and hydroxyl radical (OH), are produced during cellular metabolism. They were involved in cell signalling, apoptosis, gene expression, and ion transport. However, when these ROS molecules are produced in excess or cellular defences are insufficient, biomolecules such as proteins, lipids, and nucleic acids can be destroyed by the oxidative stress process. Such damage may result in a range of age-related degenerative disorders, including cancer, Parkinson's disease, and Alzheimer's disease. A few years ago, researchers examined the availability of probiotic microorganisms as natural antioxidants. Lactobacilli make up a substantial component of probiotics, and their antioxidant potential has been demonstrated in various research studies[49,50]. Antioxidative action for *Tungrymbai* ranged from 70.29% to 73.65%. The highest activity was detected on the sixth day (73.65±0.54%), which was found on par with the fourth day (72.85±0.44%) and the second day (72.16±0.07%) of the study (Fig. 7). According to Lee *et al*.[51], the antioxidant capacity of fermented soybeans increased as the fermentation period increased. Overall, the antioxidant activity increased from 33.1% (initial day) to 78.1% (ninth day). Santos *et al*.[52] observed that the antioxidant activity increased in fermented Okara by 20.65 mmol Trolox equivalent/g after 72 h of fermentation, up from 9.74mmol Trolox equivalent/g. Dai *et al*.[53] investigated how solid-state fermentation could increase the nutrient content and biological activity of soybean meal. The antioxidative activity increased from 7.62% to 81.08% with an increase in the concentration of fermented soybean meal from 0.5 to 8mg/mL.

Total Phenol content

Phenolic chemicals, which are found in all plants, are a crucial component of human dietary habits and

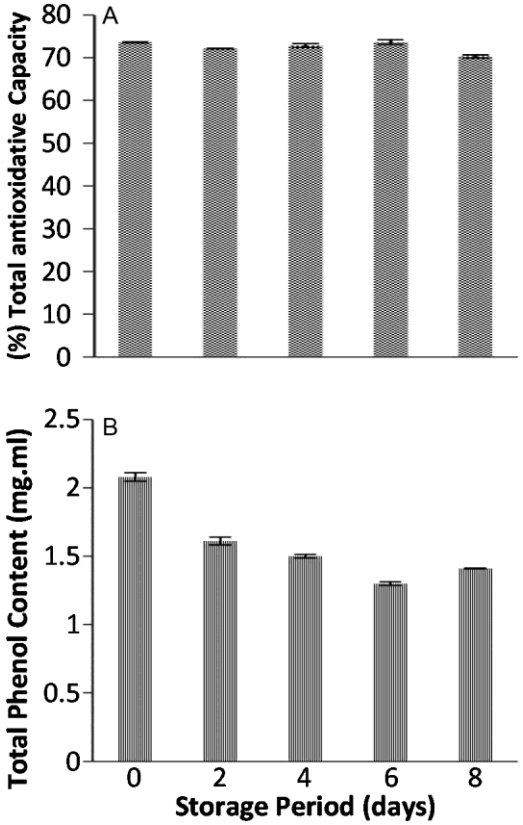

Fig. 7 — Total antioxidant activity and Total phenol content in *Tungrymbai*

are of great interest due to their antioxidant characteristics and potential health benefits. The phenol content of *Tungrymbai* ranged from 1.30mg/mL to 2.08mg/mL. The phenol content of *Tungrymbai* was found to be highest at the initial time (2.08±0.031mg/mL) of product preparation. There was a significant decrease in phenol content after eight days of study (Fig. 7). Katuwal *et al*.[54] observed a decreasing pattern of phenolic content in fermented soybean food Kinema with an increase in incubation time. The overall amount of phenolic was discovered to be 66.32±3.54mg GAEs/g at 24h of time, which was then reduced to 30.08±4.17mg GAEs/g after 72h. Sanjukta *et al*.[55] detected total phenolic contents in fermented soybeans in the range of 7.9–8.4mg GAE/g for yellow soybeans and 6.9–7.5mg GAE/g for black soybeans. Natural soybean cuisines from northeast India called *Tungrymbai* and *Bekang* were examined for their functional qualities by Chettri and Tamang[56]. The highest total phenolic content was found in the *Tungrymbai* sample (3.7±0.1mg GAE/g) and in the *Bekang* sample (4.2±0.3mg GAE/g). By increasing the overall nutritional qualities of the fermented product, the fall in polyphenol concentration during fermentation may enhance digestibility and also raise the bioavailability of minerals. The lowering of polyphenols is a result of fermentation with various microorganisms[22].

Isoflavone estimation

Daidzein, genistein, and glycitin are three free aglycone isoflavones, as well as their malonyl glucosidic, glucosidic, and acetyl glucosidic conjugates. There are twelve main isoflavone components altogether. Unless they have been fermented, isoflavones in soybeans commonly take the form of glucosides and occasionally take the form of aglycones. Aglycone forms, namely diadzein and genistein, were estimated during the storage study of *Tungrymbai* for 8 days. The daidzein content was found in a range of 64.55 to 75.63%. Diadzein content decreased non-significantly when the storage period increased from zero to eight days. Genistein content was found in the range of 19.83 to 48.10%. The amount of genistein increased significantly up to the fourth day during the storage study, from 38.16±4.84% observed on the zeroth day to 48.10±7.25% observed on the fourth day, which were found to be on par with each other. Then, a decrease in genistein content was observed on the sixth day (27.35±3.66%) and the eighth day (19.83±0.67%), which were found to be on par with each other (Fig. 8). The impact of various fermentation conditions on the isoflavone concentration and antioxidant capacity of soy tempeh was examined by Lo *et al*.[57]. The results showed a significant increase in diazein and genistein contents as fermentation time increased. There were increases in diadzein (553.34±3.39mg/kg) and genistein (306.46±4.13mg/kg) contents during the fourth day of fermentation when compared to the initial day (diadzein: 49.53±0.91mg/kg; genistein: 65.05±1.73mg/kg). Out of four selected *Lactobacillus* strains, Lodha *et al*.[23] observed that the *Limosilactobacillus plantarum* (KGL3A) strain had the highest levels of isoflavone biotransformation. After 24h of fermentation, the amount of daidzein and genistein in soymilk (AAU, NRC37) increased to 92.74% and 93.31%, respectively. According to Hati *et al*.[58], lactic cultures demonstrated isoflavone bioconversion at a rate of 2% while fermenting soymilk that had been treated with a concentrate of whey protein (WPC70). *Lactobacillus bulgaricus* NCDC (09) produced 6.9 (49.42%) and 9.7 (23.49%) fold higher amounts of aglycones (genistein and diadzein), whereas *L. helveticus* MTCC 5463 (V3) produced 7 (47.47%) and 9 (22.83%) fold higher amounts of aglycones over the control sample (unfermented soymilk supplemented with WPC70). Isoflavones have been linked to several health benefits. Isoflavones present in soy food can help protect blood vessel health, relieve menopausal symptoms, reduce breast cancer chances, and lower cholesterol and glucose levels. Foods containing isoflavones may help to preserve cellular health by raising the body's antioxidant levels[59].

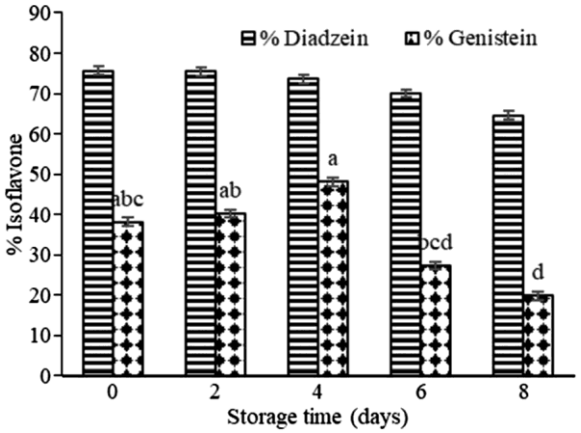

Fig. 8 — Diadzein and genistein content in *Tungrymbai* during storage study

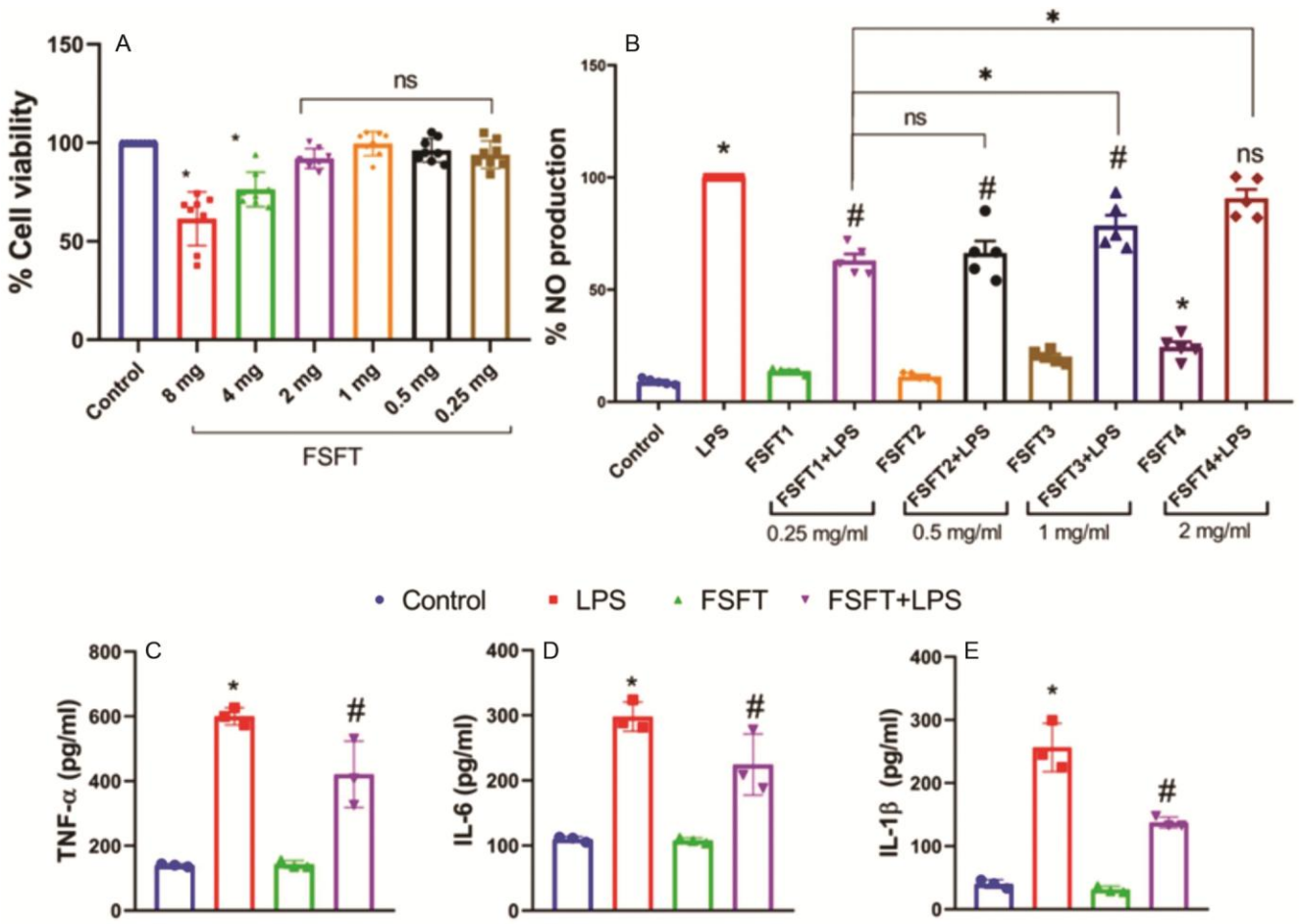

Fig. 9 — Effect of the fermented soybean food *Tungrymbai* (FSFT) on (A) cell viability, (B) nitric oxide productions, (C) TNF-α, (D) IL-6, and (E) IL-1β measured in the supernatants of LPS-stimulated RAW 264.7 macrophages

Effect of FSFT on RAW 264.7 cells viability

FSFT 100% viability was applied to RAW 264.7 cells at doses of 2, 1, 0.5 and 0.25mg/mL (Fig. 9A). The effect of FSFT on cell viability was cytotoxic at 8 and 4mg/mL. Therefore, we conclude that a concentration between 2 and 0.25mg/mL would be suitable for nitric oxide estimation.

FSFT attenuate LPS-induced TNF-α, IL-6 and IL-1β cytokine levels in RAW 264.7 macrophages

According to the study, LPS-stimulated macrophages had significantly higher levels of nitric oxide than macrophages in the control group. FSFT at dosages of 0.25, 0.5, 1 and 2mg/mL was used to avoid this incidence, as depicted in Fig. 9B. Nevertheless, the dose of 0.25mg/mL exhibited the most favourable suitability for subsequent investigation due to its comparable reduction in nitric oxide relative to the higher doses of FSFT.

Low dose of FSFT prevented LPS-induced NO and Pro-inflammatory cytokine production by the RAW 264.7

In vitro analysis of FSFT revealed that LPS-stimulated RAW 264.7 macrophages had much lower amounts of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (Fig. 9C-E). The macrophages produced more TNF-α, IL-1β, and IL-6 after being stimulated with LPS. Administration of FSFT at a concentration of 0.25mg/mL decreased the synthesis of pro-inflammatory markers. Hence, the findings indicate that FSFT demonstrated considerable anti-inflammatory efficacy, as demonstrated by a notable reduction in pro-inflammatory cytokine levels caused by LPS, without inducing any cytotoxic effects.

RAW 264.7 macrophages were employed in investigating anti-inflammatory properties, given their status as immune effector cells that have significant importance in the host's immune system[60]. Lipopolysaccharide (LPS) was used to activate macrophages and encourage the production of nitric

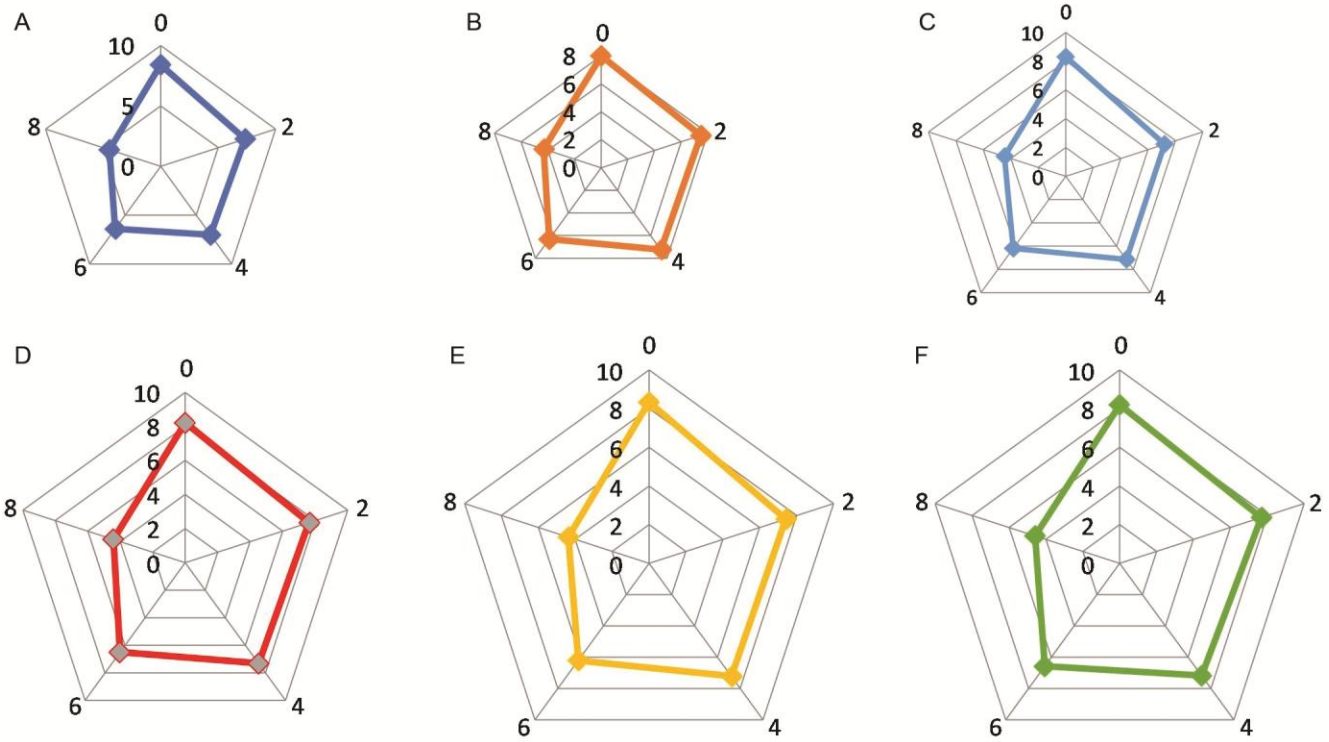

Fig. 10 — Organoleptic evaluation of *Tungrymbai* (A) Aroma, (B) Taste, (C) Colour, (D) Mouth feel, (E) Texture & (F) Overall acceptability

oxide and other pro-inflammatory cytokines and mediators[61]. Lipopolysaccharide (LPS) is associated with gram-negative bacteria, leading to the induction of immune cell activation and subsequent systemic inflammation[62]. Furthermore, it has been documented that the macrophage inflammatory model caused by lipopolysaccharide (LPS) is often regarded as the most exemplary classic inflammatory model[63,64]. Upon stimulation with lipopolysaccharide (LPS), a series of incidents is initiated, causing the release of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and other similar molecules[65]. The release of nitric oxide is also observed as a mediator in this inflammatory response. Nitric oxide (NO) stimulates the inflammatory response and interacts with Toll-like receptor 4 (TLR-4), which is found on the surface of macrophages, to directly activate NF-κB[66]. Within this investigation, FSFT was found to significantly reduce the generation of inflammatory cytokines and mediators in RAW 264 when administered. LPS was used to excite 7 macrophages. The results collected from the study indicate that FSFT plays a part in mediating the reduction of inflammation. Our findings are consistent with those of Lyu *et al*.[67], Kawahara *et al*.[68] and Prado *et al*.[69], who investigated the anti-inflammatory properties of fermenting soybean products in RAW 264.7 cells. By reducing the production of pro-inflammatory cytokines, we conclude that FSFT may function as effective anti-inflammatory peptides.

Organoleptic evaluation

The *Tungrymbai* samples were evaluated by a group of nine sensory panelists for their overall acceptability across storage periods and for their aroma, taste, colour, mouth feel, texture, and other attributes (0, 2, 4, 6 and 8 days) at refrigerated conditions (7±2°C). The organoleptic assessment of the *Tungrymbai* is displayed in Fig. 10. As seen in Fig. 10, the sensory scores were greater than six up to day 6, whereas beyond that point, the scores began to decline. The current study findings are in line with Chettri & Tamang[70] sensory evaluation of *Tungrymbai* that had been developed in a laboratory. They employed different strains and mixes of *Bacillus* spp., such as *B. pumilus* and *B. licheniformis*. *Tungrymbai* made using a combination of starters was discovered to have better organoleptic qualities in terms of flavour, aroma, texture, and overall acceptance. Tamang *et al*.[71] also provided a description of the native skills possessed by the Khasi

tribes in the northeastern region in relation to the cooking of traditional fermented soybean foods. Soybeans have been used by many Asian cultures as seasonings and side dishes, both individually and in addition to fermented foods[72].

Conclusion

We have developed *Tungrymbai*-a fermented soy-based product with well characterised *Lactobacillus* cultures having probiotic potential. This product will help the society for betterment of the lifestyle. In the storage analysis of *Tungrymbai*, it had been noted that ACE inhibitory activity, total phenolic contents, α-amylase inhibitory activity, and daidzein content were found to be highest during the initial time of the study. Lipase inhibitory activity and genistein content were also found to be highest during the fourth day of the storage study. The *Lactobacillus* cultures i.e., K4E and K14, along with the *Scoparia dulcis* herbal extract, could increase the activity of α-glucosidase inhibition, lipase inhibitory activity, and antioxidant capacity in *Tungrymbai*. These *Lactobacillus* cultures has the potential to develop functional fermented Traditional soy bean food with health benefits. We have studied the biofunctional properties (anti-hypertensive and anti-diabetic) of the developed product in *in vitro* conditions. Further, clinical studies are required to validate the health claims for this traditional fermented soy food.

Ethics approval and consent to participation

The study underwent scrutiny and received approval from the Ethical Committee of Northeastern Hill University, identified by Reference No. TC/RDAP/DBT Twinning/Sens./2023-01, dated: 10.05.2023, focusing on Investigations Involving Human Subjects. Each panelist provided documented informed consent to engage in the study, affirming that no harm is linked to the consumption of the product within the parameters of this research.

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Conflict of interest

Authors declare no conflict of interest

Authors' contributions

SH, BKM, KKK, KR, BB: conceptualised the study, designed experiments, supervised data collection and the study; RM, BB, KR: analysed data, prepared the draft manuscript; SH, SV, KKK, MB: read and edited the manuscript. All authors read and approved the manuscript.

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