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Original Article

Bio-nutritional aspects of *Tungrymbai*, an ethnic functional fermented soy food of Khasi Hills, Meghalaya, India

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SUMMARY

Background & aims: Tungrymbai is a fermented soy product which is a sticky food that exhibits unique flavour and texture which is exclusively made by the local Khasi tribes of Meghalaya (India). A similar kind of product was prepared under laboratory conditions followed by shelf-life study and analysis of its bio-functional attributes with comparison to the traditionally made product.
Methods: The traditional practice for the preparation of *Tungrymbai* was closely monitored and a similar product preparation was made in laboratory conditions using indigenous *Lactobacillus* cultures. The shelf life study (physicochemical, microbial and organoleptic attributes) was conducted for 4 days for the *Tungrymbai* products. Further, the bio-functional properties such as ACE-inhibitory, antioxidative activity and biotransformation of Isoflavones were analysed for laboratory made *Tungrymbai* products with inoculation of *Lactobacillus* cultures as well as without inoculation (control).
Results: Out of all the quality attributes of the various *Tungrymbai* samples, *Tungrymbai* with 1% cell biomass of *Lactobacillus* culture in combinations was mostly preferred by the sensory panellists than other samples with a sensory score >6. ACE inhibitory activity

Abbreviations: ABTS, 2,2′ - azino-bis (3-ethlybenzothiazoline-6-sulphonic acid); ACE, Angiotensin converting enzyme; CO_2, Carbon di-oxide, DPPH; 2, 2-diphenyl-1-picrylhydrazyl, FRAP; Fluorescence recovery after photobleaching, H_2O_2; Hydrogen peroxide, IC50; Half maximal inhibitory concentration, LAB; Lactic acid bacteria, MRS; De Man, Rogosa and sharpe agar; NCBI, National centre for biotechnology information; ROS, Reactive oxygen species; RP-HPLC, Reverse phase high performance liquid chromatography.

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of Tungrymbai prepared under laboratory condition increased from 30.75% at day 0–83.55% at day 5, followed by a reduction after day 10 (36.98%) and the antioxidative activity was maximum at day 0 (99.14%) followed by a decrease at day 2 (81.01%). After fermentation of 4 days, production of soy isoflavones in agyclones were 34.70% genistein and 29.37% daidzein for traditionally made *Tungrymbai* and 55.44% genistein and 86.58% daidzein for laboratory made *Tungrymbai* infused with *Lactobacillus* cultures.

Conclusion: Hence, the *Lactobacillus* culture combinations of *Lactobacillus rhamnosus* K4E and *L. helveticus* K14 could serve as potential candidates for the preparation of novel ethnic fermented foods in Meghalaya with more improved bio-functionality.

1. Introduction

For thousands of years, ethnic fermented foods have been prepared, consumed and correlated to culture, traditions thereby revealing the intellectual richness associated with the indigenous people of Meghalaya with respect to their ability to prepare microbial products for varied purposes in addition to food and beverages [1]. For people of South-East Asia, preparation and consumption of sticky, non-salty, flavoursome fermented soybean foods are of traditional heritage and culture and they remain a distinct food culture of the people [2]. It is not only in terms of the climate and the ancestry of people, but also in their food habits there is a strong similarity between north-eastern region of India and south-eastern Asian countries. The regular diet of the Khasi tribes of Meghalaya constitutes of mainly fermented soybean, fish and bamboo just like the Southeast Asians.

A fermented vegetable protein prepared from soybean (*Glycine max* L. Meri) is termed as *Tungrymbai* in Khasi Hills of Meghalaya. Soybean is fermented at the household and village level and as though the tribal art related to their heritage is gradually dying out and since the process is tedious and because of that, the fermented product is easily available at the market. Soybean based sticky food serves as a cheap source of high protein food in local diet and preparation and consumption of this food mainly reflects the deep rooted food culture linked with the various ethnic communities residing in Meghalaya. Preparation of *Tungrymbai* is exclusively practiced by the Khasi tribal people using indigenous technology [2]. It is presumed that the process of fermentation boosts up the nutritional quality of any product by enhancing the amount of vitamins and protein solubility if supplemented with Lactic acid bacteria (LAB) and brief idea of the microorganisms associated during natural fermentation of *Tungrymbai* would help to establish genetic as well as bio-functional resources engaged with the fermentation process. Functional as well as technological attributes of *Lactobacillus* spp. isolated from fermented foods are important criteria for selection of starter cultures to be used in the manufacture of functional foods [3].

LAB bears the potential of exhibiting various antibacterial elements such as low molecular mass compounds such as H_2O_2, CO_2, diacetyl and high molecular mass compounds like bacteriocins [4,5] which effectively causes the inhibition food borne pathogens. Soy foods when fermented with LAB shows antihypertensive effect by inhibition of angiotensin converting enzyme (ACE), the primary enzyme in the renin-angiotensin system [6]. As per reports, studies indicated that biologically active peptides extracted from the hydrolysates of the soybean protein had the ACE-inhibitory activity and the potential to improve hypertension in vivo [7]. Among lactic acid bacteria, *Lactobacillus* have gained popularity for their potential probiotic effects in human health and they are important members of the healthy human microbiota with the efficacy of possessing antioxidative activity, and are able to decrease the risk of accumulation of ROS during the ingestion of food [8]. Fermentation by *L.* spp. improves the bioavailability of Isoflavones in most soy based food products isoflavones are

in the form of glucosides, which are conjugated with glucose, biologically inactive, and not absorbed through the intestinal wall until or unless fermented thereby assisting in digestion of protein, providing of more soluble calcium, enhances intestinal health, and supports immune system. *Tungrymbai* is a fermented soybean product popularly consumed in Meghalaya, particularly by the ethnic *Khasi* tribes. *Tungrymbai* has typical flavour and taste. In the study, traditional method for preparing *Tungrymbai* has been depicted and tried to improve the method under laboratory scale maintaining hygienic conditions. Pre-cooked and post cooked methods were adopted for the preparation of *Tungrymbai* as per their traditional practices under laboratory conditions. Pre-cooked *Tungrymbai* is generally prepared in a house hold level and sold in local market and post cooked *Tungrymbai* is a ready to eat product and preferred to consume with rice in ethnic tribes of Meghalaya. In this study, we report the comparative study of traditional and laboratory made *Tungrymbai* on the basis of shelf life analysis and biofunctional properties such as ACE-inhibitory, antioxidative activity and biotransformation of isoflavones.

2. Materials and methods

2.1. Bacterial strains, growth conditions and sample preparation

The cultures used in the study, namely *Lactobacillus rhamnosus* K4E and *Lactobacillus helveticus* K14 (NCBI GenBank Accession No. KX950834 and KU644578) were isolated from the various ethnic fermented foods (fermented fish, fermented bamboo shoots and fermented soybeans) of Meghalaya, India which were deposited in Animal Science Laboratory, Department of Rural Development and Agricultural Production, North-Eastern Hill University, Tura Campus, Meghalaya, India [9]. These isolates were propagated in De Man, Rogosa and Sharpe agar (MRS) medium for 24 h at 37 °C respectively. These isolates were studied for probiotic potentials earlier and selected due to their maximum probiotic characteristics for this study [10].

2.2. Preparation of Tungrymbai (traditional method in Khasi tribes of Meghalaya, India)

The traditional practice for the preparation of *Tungrymbai* was closely monitored and observed in a stepwise manner by visiting a village (Pahamsyiem) in Nongpoh, Ri-Bhoi district of Khasi Hills, Meghalaya, India as depicted in Fig. 1. Around 500 g of soybean seeds were soaked in a pan submerged with water and kept for around 16–18 h. The water was discarded and the soybeans were transferred into a cleaned utensil and then boiled water was added into it. After that, soybeans were boiled for 2–3 h until it softens at 95 °C by changing water twice. The excess water was drained off and cooled to temperature at 26 °C. Then, boiled soybeans were transferred to a bamboo basket aligned with the fresh leaves of "*slamet*" (local name in Khasi) (*Pyrnium* sp.). 3–4 pieces of hot charcoal blocks were placed on the boiled soybeans and was fully wrapped with "*slamet*" *(Pyrinium* spp.*)* leaves for providing the smoky flavor. Bamboo leaves were used to cover the fully wrapped soybeans with *slamet* leaves. The basket was placed inside a jute bag and was tied firmly. Then the basket was hung over on the fire place (oven) and fermented at 30°–40 °C for a period of 3–4 days. After fermentation, the pre-cooked product was taken out by gently opening the bamboo basket and removing the *slamet* leaves. The fermented soybeans (*Tungrymbai)* were crushed/grinded uniformly in a mortar-pestle that serves as a pre-cooked *Tungrymbai.*

2.3. Preparation of Tungrymbai like product under laboratory conditions

As shown in Fig. 2, about 300 g soybean was cleaned, washed and soaked in tap water overnight at room temperature and the soybeans were not dehulled. Soybeans were boiled at 90 °C for 30 min and distributed into three parts containing 100 g each and then cooled to 25 °C.

1st part (Control): Cooked soybeans were placed inside the sterile bamboo basket packed with *slamet (P.* spp.*)* leaves and was wrapped with bamboo leaves and then the basket was covered by

sterile muslin cloth and incubated for 3–4 days at 37 °C in an incubator. This served as a control without any inoculation (*Lactobacillus* cultures); 2nd part (*Tungrymbai* with 1% cell biomass): Cooked soybeans were inoculated with 1% cell biomass of *Lactobacillus* cultures in combination, uniformly mixed with the soybeans and placed inside the sterile bamboo basket added with *slamet (P.* spp.*)* leaves and wrapped with bamboo leaves and then the basket was covered by sterile muslin cloth and incubated for 3–4 days at 37 °C; 3rd part (*Tungrymbai* with 2% cell biomass): Cooked soybeans were inoculated with 2% cell biomass of *Lactobacillus* cultures in combination, uniformly mixed with the soybeans and placed inside sterile bamboo basket added with *slamet (P.* spp.*)* leaves and wrapped with bamboo leaves and then the basket was covered by sterile muslin cloth and incubated for 3–4 days at 37 °C.

2.4. Shelf-life study of laboratory made Tungrymbai *like product*

2.4.1. Organoleptic evaluation

Staff and students of Department of Rural Development and Agricultural Production of North-Eastern Hill University, Tura Campus, Meghalaya who are basically residents of Khasi Hills and are used to consume *Tungrymbai* (fermented soybeans) as part of their diet, were requested to evaluate for assessing the three *Tungrymbai* samples on the basis of aroma, taste, colour, mouth feel, texture and overall acceptability using a 9-point hedonic scale. The sensory evaluation of the three *Tungrymbai* samples were stored at refrigerated conditions (6°C–8 °C) up to 4 days.

2.4.2. Physicochemical properties

For pH determination, pH of the *Tungrymbai* samples was determined and the titratable acidity was evaluated as per the methods adapted from Thokchom & Joshi [11].

2.4.3. Microbial analysis

Total *Lactobacillus* counts (in MRS agar), coliform counts (in EMB agar), yeast and mould (in SCA agar) counts of pre-cooked *Tungrymbai* samples were analysed up to 4 days under refrigerated conditions (6°C–8 °C) and the viable cell counts were expressed as log CFU/ml [11].

2.5. Bio-functional properties of the functional fermented Tungrymbai *under different storage conditions*

2.5.1. Determination of ACE inhibitory activity (in vitro)

The ACE inhibitory activity of the laboratory made *Tungrymbai* (with or without addition of cultures) was estimated spectrophotometrically. The extent of inhibition is calculated as follows: $(A_{control}\text{-}A_{sample})/A_{control} \times 100$.

Where, $A_{control}$ = the absorbance without ACE inhibitory component, A_{sample} = the absorbance in the presence of ACE and ACE inhibitory component.

Inhibition is expressed as the concentration of component that inhibits 50% of ACE activity (IC50) and 1 Unit of ACE inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions [12].

2.5.2. Total antioxidative capacity

Total radical scavenging capacity was based on the ability of a compound to scavenge the stable ABTS radical in 5 min by adapting the method of Hati et al. [13] with few modifications. The antioxidative activity of the tested samples was calculated by determining the decrease in the absorbance at different time intervals using the following equation: $E = (A_C\text{-}A_T)/A_C \times 100$.Where, A_C and A_T are respective absorbance of $ABTS^+$ and tested samples, was expressed as inhibition percentages.

2.6. Estimation of bioconversion of isoflavones during fermentation in Tungrymbai

2.6.1. Sample preparation and extraction

Sample was prepared by following the methodology of Hati et al. [14]. High Performance Liquid Chromatography (HPLC) was performed to determine the amount of glucosides in the form of daidzin

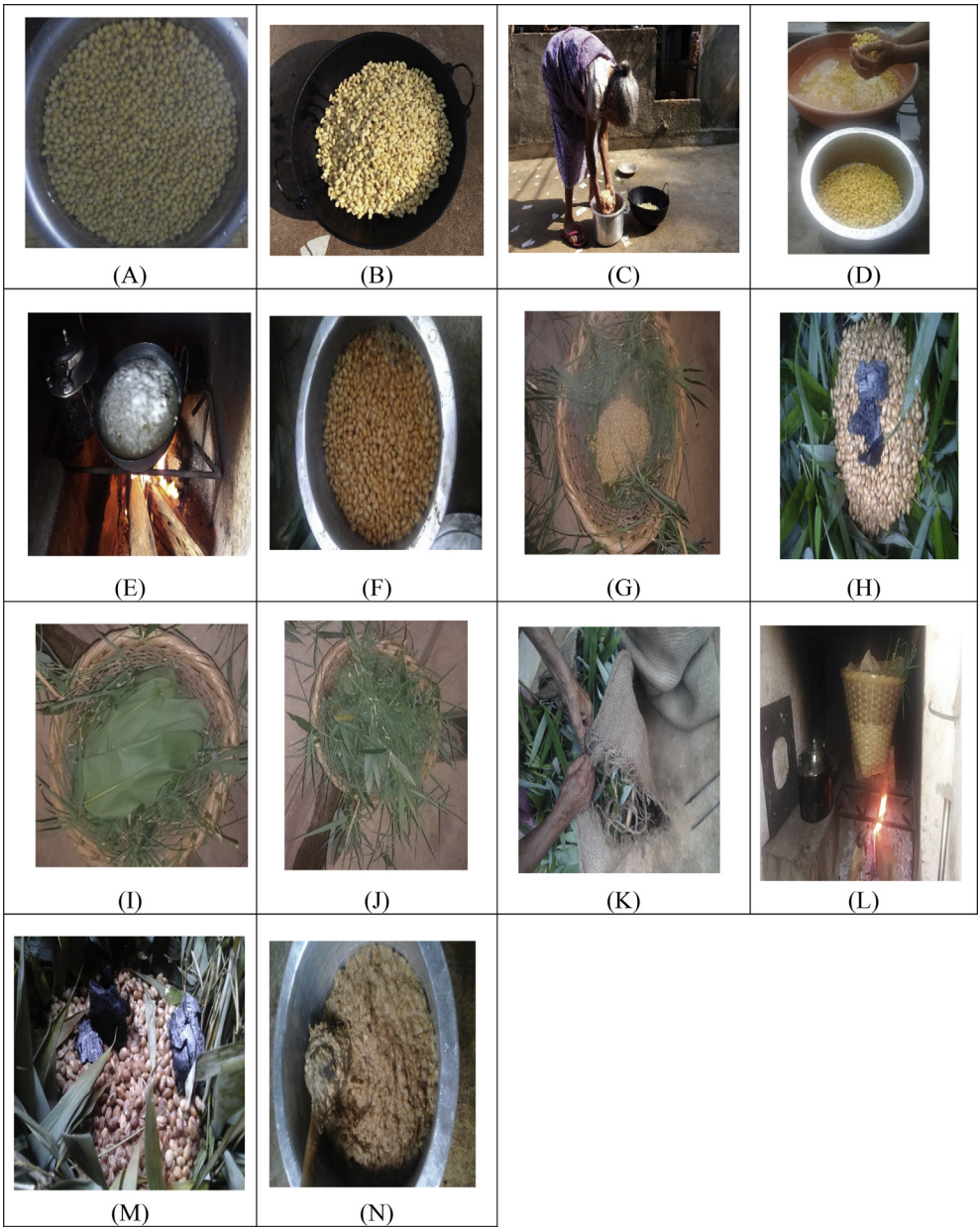

Fig. 1. *Tungrymbai* (fermented soybeans) prepared traditionally by the Khasi tribes. (A)- Around 500 g of soybean seeds were soaked in a pan submerged with water and was kept for around 16–18 h. (B) & (C) - The water was discarded and the soybeans were transferred into a cleaned utensil and boiled water was added into it. (D) & (E) - Soybeans were boiled for 2–3 h until it softens at 95 °C by changing water twice. (F)- The excess water was drained off and was lowered to temperature at 26 °C. (G)- Boiled soybeans were transferred to a bamboo basket aligned with the fresh leaves of "*slamet*" (local name in Khasi) (*Pyrnium sp.*). (H)- 3–4 pieces of hot charcoal blocks were placed on the boiled soybeans and was fully wrapped with "*slamet*" *(Pyrinium spp.)* leaves for providing the smoky flavour. (I) & (J) - Bamboo leaves were spreaded uniformly over the fully wrapped soybeans with *slamet* leaves. (K) - The basket was placed inside a jute bag and was tied firmly. (L) - Then the basket was hung over on the fire place (oven) and fermented at 30°–40 °C for a period of 3–4 days. (M) - After fermentation, the pre-cooked product was taken out by

and genistin whereas aglycones in the form of daidzein and genistein in both *Tungrymbai* samples (with or without addition of cultures). The extraction of isoflavones from *Tungrymbai* samples was carried out in triplicates by adapting the method from Otieno et al. [15] with a few alterations.

2.6.2. Separation and quantification

Isoflavones analysis was carried out in Shimadzu HPLC equipped with LC20 at HPLC Pump, with SPD-20A Wavelength Detector, 7725i Shimadzu LC-20 manual injector with 20 ml loop, diode array ultraviolet (UV-260 nm) detector, SeQuant ZIC-cHILIC (Mark, Germany) column (PEEK 250 3 4.6 mm, 3 mm, 100 Å pore size) were used as per the method of Hati et al. [14]. *Tungrymbai* supernatant as obtained as per the above method was then loaded in a 20 μl volume into the manual injector and analysed.

2.7. Statistical analysis

All the data presented here are the average of three independent assays and the results obtained were expressed as mean ± standard deviation (M±SD). One way analysis of variance (ANOVA) was applied and comparison was made through Bonferroni's test with least significant difference of $P \leq 0.05$ using IBM SPSS Statistical program (Ver. 20).

3. Results

The Khasi tribes of Meghalaya, India have been using microbes unknowingly for various food preparations including the preparation of *Tungrymbai* traditionally [2]. *Tungrymbai* is traditionally prepared at house hold level under natural conditions with undefined micro-flora and hence, quality and safety of products are in concern. Hence, almost similar *Tungrymbai* like product was developed under laboratory conditions using *Lactobacillus* culture isolated from ethnic fermented foods of Meghalaya, India in combinations under aseptic conditions.

3.1. Shelf-life study of laboratory made Tungrymbai *like product*

The *Tungrymbai* samples were evaluated for aroma, taste, colour, mouth feel, texture and overall acceptability during storage periods (1, 2, 3 and 4 days) at refrigerated conditions (6°–8 °C) by a panel of eight experienced judges. Out of all the quality attributes of the *Tungrymbai* samples as shown in Table 1, *Tungrymbai* sample with 1% cell biomass was mostly preferred by the sensory panellists than other samples (control and *Tungrymbai* with 2% cell biomass). Table 1 shows the organoleptic evaluation of three *Tungrymbai* like products (samples infused with 1% cell biomass, 2% cell biomass of *Lactobacillus* culture combinations and a control where no bacterial inoculation was done). As presented in Table 1, the sensory scores were >6 for all the three samples till day 2 but the scores started to deteriorate after day 3 for *Tungrymbai* with 2% cell biomass and the control sample with a sensory score of <6 which cannot be considered for consumption further. For *Tungrymbai* with 1% cell biomass, the sensory scores were >6 till day 3 but the product started deteriorating from day 4 and hence from the study, *Tungrymbai* with 1% cell biomass was preferred over other 2 samples with storage life till day 3. One way ANOVA followed by Bonferroni's post-Hoc test showed significant difference ($P < 0.05$) between the three *Tungrymbai* samples in terms of the sensory parameters (aroma, taste, colour, mouth feel, texture and overall acceptability) as mentioned in Table 1.

The decrease in pH and the increase in titratable acidity were non-significant ($P > 0.05$) during the storage periods (1, 2, 3 and 4 days) of different *Tungrymbai* samples observed at refrigerated conditions (6°–8 °C) as conducted by one way ANOVA followed by Bonferroni's post-Hoc test, presented in Table 2. In order to analyse the counts of spoilage organisms (if present), microbial analysis was included in

gently opening the bamboo basket and removing the *slamet* leaves. (N) - The fermented soybeans (*Tungrymbai)* were crushed/grinded uniformly in a mortar-pestle. This serves as a pre-cooked *Tungrymbai* consumed with rice as a part of their regular meals (especially in winter season).

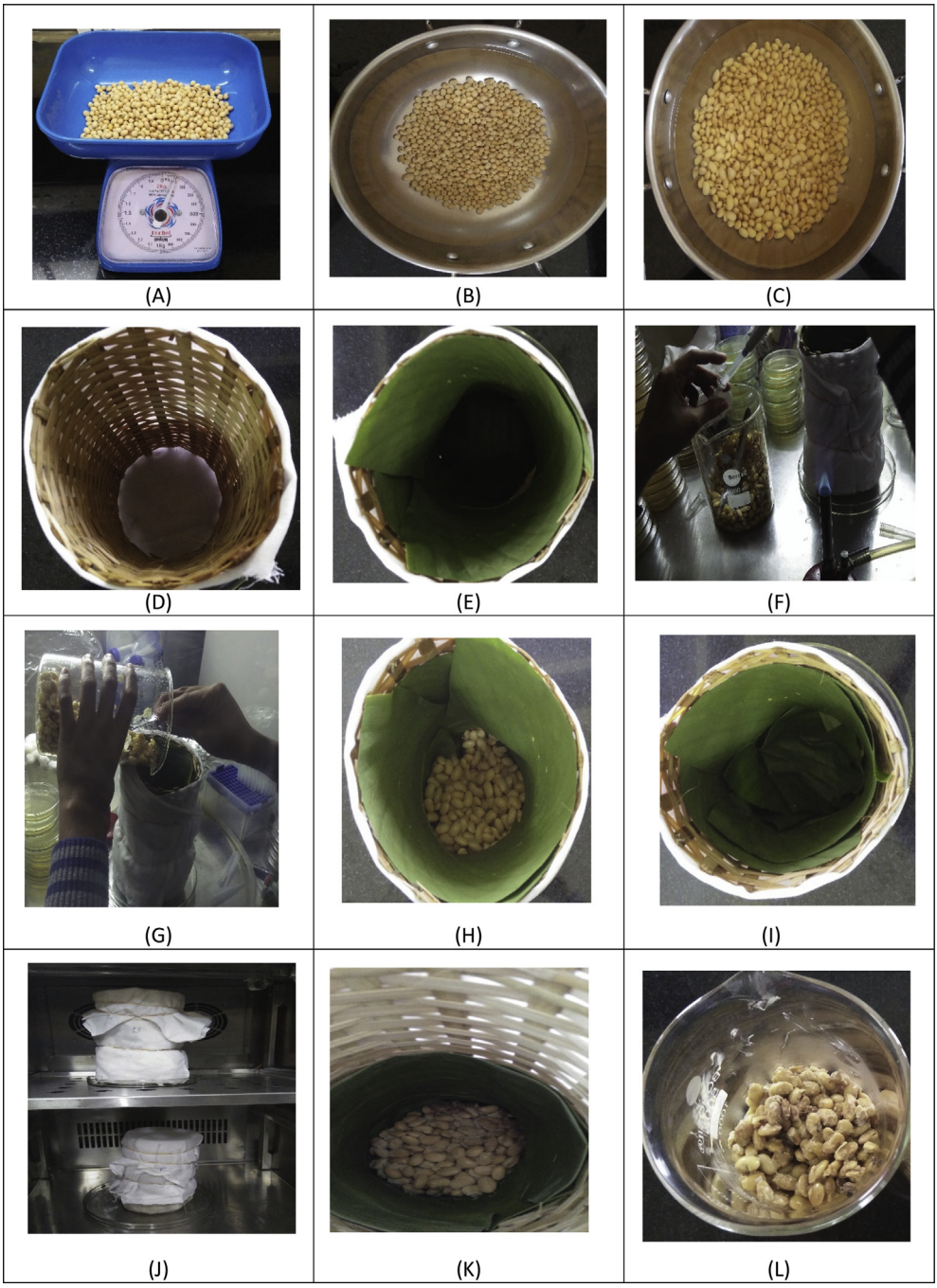

Fig. 2. Pre-cooked *Tungrymbai* prepared under laboratory conditions. (A) - 300 g soybeans weighed in balance; (B) - soybeans soaked for 16–18 h in 1 L of distilled water at room temperature (28 °C) in a beaker; (C) – soaking and germination of soy beans; (D) and (E)- Leaves of *Pyrinium spp.* (*Slamet*) was used to aligned the bamboo basket; (F) and (G) & (H)-Addition of 1% and 2% cell biomass respectively into the sterile bamboo basket after mixing; (I)- The soybeans with biomass was covered with slamet leaves (*Pyrinium* sp.); (J)- *Tungrymbai* samples covered with muslin clothes kept for fermentation for 3–4 days at 37 °C in an incubator; (K) and (L)- Pre-cooked fermented *Tungrymbai* samples after fermentation.

Table 1
Organoleptic evaluation of pre-cooked *Tungrymbai* samples.

Parameters	Storage periods (days)	*Tungrymbai* (Control)	*Tungrymbai* with 1% cell biomass	*Tungrymbai* with 2% cell biomass
Aroma	1	7.11 ± 0.40^{a}	7.33 ± 0.52^{a}	6.70 ± 0.81^{b}
	2	6.66 ± 0.54^{b}	7.15 ± 0.50^{a}	6.15 ± 0.75^{b}
	3	5.33 ± 0.40^{c}	6.33 ± 1.11^{b}	5.83 ± 0.40^{c}
	4	4.50 ± 0.44^{d}	5.50 ± 0.54^{c}	4.33 ± 0.41^{d}
Taste	1	7.15 ± 0.45^{a}	7.54 ± 0.22^{a}	6.66 ± 0.07^{b}
	2	6.91 ± 0.16^{b}	7.22 ± 0.08^{a}	6.22 ± 0.66^{b}
	3	5.16 ± 0.05^{c}	6.83 ± 1.05^{b}	5.35 ± 0.40^{c}
	4	4.50 ± 0.33^{d}	5.22 ± 0.55^{c}	4.83 ± 0.44^{d}
Color	1	7.11 ± 0.40^{a}	7.66 ± 0.25^{a}	6.33 ± 0.45^{b}
	2	6.66 ± 0.11^{b}	7.45 ± 0.07^{a}	5.83 ± 0.08^{c}
	3	5.22 ± 0.50^{c}	6.66 ± 1.09^{b}	5.33 ± 0.25^{c}
	4	4.50 ± 0.22^{d}	5.89 ± 0.35^{c}	4.44 ± 0.31^{d}
Mouth feel	1	7.10 ± 0.81^{a}	7.46 ± 0.28^{a}	6.45 ± 0.47^{b}
	2	6.83 ± 0.88^{b}	7.22 ± 0.33^{a}	6.23 ± 1.05^{b}
	3	5.82 ± 0.56^{c}	6.76 ± 0.06^{b}	5.66 ± 0.26^{c}
	4	4.66 ± 0.28^{d}	5.88 ± 0.43^{c}	4.36 ± 0.64^{d}
Texture	1	7.15 ± 0.44^{a}	7.64 ± 0.25^{a}	6.45 ± 0.07^{b}
	2	6.55 ± 0.77^{b}	7.12 ± 1.01^{a}	6.18 ± 0.66^{b}
	3	5.26 ± 0.06^{c}	6.55 ± 1.07^{b}	5.50 ± 0.40^{c}
	4	4.55 ± 0.36^{d}	5.53 ± 0.33^{c}	4.61 ± 0.44^{d}
Overall acceptability	1	7.41 ± 0.64^{a}	7.88 ± 0.21^{a}	6.60 ± 0.81^{b}
	2	7.11 ± 0.57^{a}	7.45 ± 0.47^{a}	6.22 ± 0.75^{b}
	3	5.33 ± 0.32^{c}	7.15 ± 1.10^{a}	5.24 ± 0.40^{c}
	4	4.84 ± 0.40^{d}	5.40 ± 0.77^{c}	4.38 ± 0.41^{d}

Values are mean ± SD of eight independent determinations ($n = 8$) of each sample.
Values bearing different superscripts in each column differ significantly ($P < 0.05$).

Table 2
Physicochemical analysis of pre-cooked *Tungrymbai* samples.

Parameters	Storage periods (days)	*Tungrymbai* (Control)	*Tungrymbai* with 1% cell biomass	*Tungrymbai* with 2% cell biomass
pH	1	6.10 ± 0.015^{a}	6.05 ± 0.015^{a}	6.00 ± 0.025^{a}
	2	5.98 ± 0.055^{a}	5.91 ± 0.022^{a}	5.88 ± 0.030^{a}
	3	5.89 ± 0.051^{a}	5.84 ± 0.031^{a}	5.75 ± 0.015^{a}
	4	5.78 ± 0.011^{a}	5.74 ± 0.025^{a}	5.63 ± 0.035^{a}
TA (%lactic acid)	1	0.061 ± 0.010	0.095 ± 0.050	0.095 ± 0.015
	2	0.076 ± 0.050	0.102 ± 0.061	0.111 ± 0.012
	3	0.087 ± 0.011	0.116 ± 0.015	0.120 ± 0.030
	4	0.103 ± 0.035	0.127 ± 0.033	0.133 ± 0.021

Values are mean ± SD of three independent determinations ($n = 3$) of each sample.
Values bearing different superscripts in each column differ significantly ($P < 0.05$).

the 4 day storage study. There was a significant ($P < 0.05$) increase in the *Lactobacillus* counts over the storage study (1, 2, 3 and 4 days) for the three different *Tungrymbai* samples at refrigerated conditions (6°–8 °C) with a stable growth on the 4th day of the shelf life study due to exhaustion of nutrients correlating with storage time as depicted in Table 3. There was complete absence of coliforms observed during the storage periods that signifies that the *Tungrymbai* products were safe from faecal contamination. Yeast and Mould growth was observed on the last day (4th) of storage may be due to result of contamination or inefficient sterilization claiming that these fermented soy product can be stored up to 3 days under refrigeration conditions. Moreover, it was also observed that the *Lactobacillus* counts were highest in post-cooked *Tungrymbai* sample (I) with cell count of 5.639 ± 0.200 log CFU/ml and viable *Lactobacillus* counts was absent in the post-cooked *Tungrymbai* sample (II) except only few

Table 3
Microbial analysis of pre-cooked *Tungrymbai* samples.

Microbial load (log CFU/ml)	Storage periods (days)	*Tungrymbai* (Control)	*Tungrymbai* with 1% cell biomass	*Tungrymbai* with 2% cell biomass
Total *Lactobacillus* count	1	7.67 ± 0.017^{a}	7.88 ± 0.034^{a}	7.93 ± 0.061^{a}
	2	7.82 ± 0.078^{a}	8.18 ± 0.043^{b}	8.20 ± 0.069^{b}
	3	7.88 ± 0.003^{a}	8.22 ± 0.008^{b}	8.24 ± 0.019^{b}
	4	7.92 ± 0.015^{a}	8.25 ± 0.029^{b}	8.28 ± 0.022^{b}
Yeast and Mold count	1	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
	2	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
	3	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
	4	1.33 ± 0.010^{a}	1.57 ± 0.020^{a}	2.13 ± 0.016^{a}
Coliform count	1	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
	2	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
	3	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml
	4	Absent in 1 ml	Absent in 1 ml	Absent in 1 ml

Values are mean ± SD of three independent determinations ($n = 3$) of each sample.
Values bearing different superscripts in each column differ significantly ($P < 0.05$).

spore forming bacteria. This could be due to the presence of endospore that survived the heat generated from cooking temperature. *Lactobacillus* spp. was unable to survive in post-cooked *Tungrymbai* sample (II) because of anti-bacterial activities of natural spices of ginger and garlic and that could inhibit the growth of *Lactobacillus*. Moreover, the cooking practices adopted for consumption of fermented product also may be responsible for the elimination of *Lactobacillus* in post-cooked *Tungrymbai* sample (II). Comparatively, *Lactobacillus* sp. survived in post-cooked *Tungrymbai* sample (I) as the fermented soybeans were not fried or cooked along with the spices but the microbial load was less as compared to the pre-cooked fermented soybean samples due to the addition of spices that are likely to possess effective anti-bacterial activities.

3.2. *Bio-functional properties of laboratory made* Tungrymbai *under different storage conditions*

As compared to the *Tungrymbai* prepared without addition of culture, ACE inhibitory activity of *Tungrymbai* prepared under laboratory condition with *Lactobacillus* cultures increased from 30.75% at day 0–83.55% at day 5 followed by a reduction from day 8 (44.04%) to day 10 (36.98%) claiming to possess the highest ACE inhibitory activity. Hence, from the above study it was found that potent ACE inhibitory peptides were produced at day 5 during storage at 4 °C, which were highly significant ($P < 0.05$). However, the values decreased sharply between day 8 and 10 indicating further proteolytic degradation of peptides (Fig. 3). It was found that *Tungrymbai* prepared under laboratory condition (with *Lactobacillus* cultures) showed higher inhibition compared to the *Tungrymbai* product made without *Lactobacillus* cultures and hence, it can be presumed that extremely potent ACE inhibitory peptides were produced at day 5 during storage at 4 °C. However, the values decreased sharply between day 8 and 10 indicating further proteolytic degradation of peptides. Since, soy protein is good quality protein and produce peptides upon hydrolysis by proteolytic enzymes. ACE inhibitor of *Tungrymbai* is because of production of peptides that are released during hydrolysis of soy proteins by proteases produced by *Lactobacillus* culture in combinations used in the study. Significantly, laboratory made *Tungrymbai* (incorporated with *Lactobacillus* cultures) showed higher ACE inhibitory activity ($P < 0.05$) than *Tungrymbai* made without *Lactobacillus* cultures.

Using ABTS radical scavenging method [13] the antioxidant activities of the laboratory made *Tungrymbai* (with and without *Lactobacillus* cultures) were analysed during different storage periods (0, 2, 5, 8 and 10 days) as depicted in Fig. 4a and Fig. 4b. For laboratory made *Tungrymbai* (with *Lactobacillus* cultures), at day 0, the antioxidative radical scavenging activity was 99.14% followed by 81.01% at day 2; at day 5, the inhibition percentage was 73.13% followed by 71.23% at day 8 and 55.60% at day 10 with temperature of 6 °C and the results were statistically significant ($P < 0.05$) over the period of 10 days. Comparatively, laboratory made *Tungrymbai* with *Lactobacillus* cultures showed highest antioxidative capacity proving to be a potential antioxidant, suppressing production of the

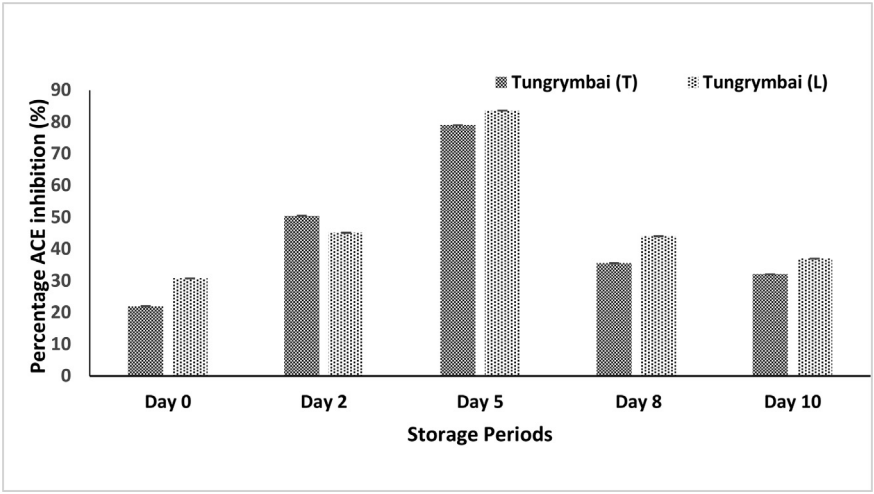

Fig. 3. ACE inhibitory activity of laboratory made *Tungrymbai* (T- control; L- *Lactobacillus* cultures infused) samples on various storage periods. *Values are mean ± standard deviation of triplicate determinations (n = 3). The differences between the traditional and lab made *Tungrymbai* were statistically significant ($P < 0.05$).

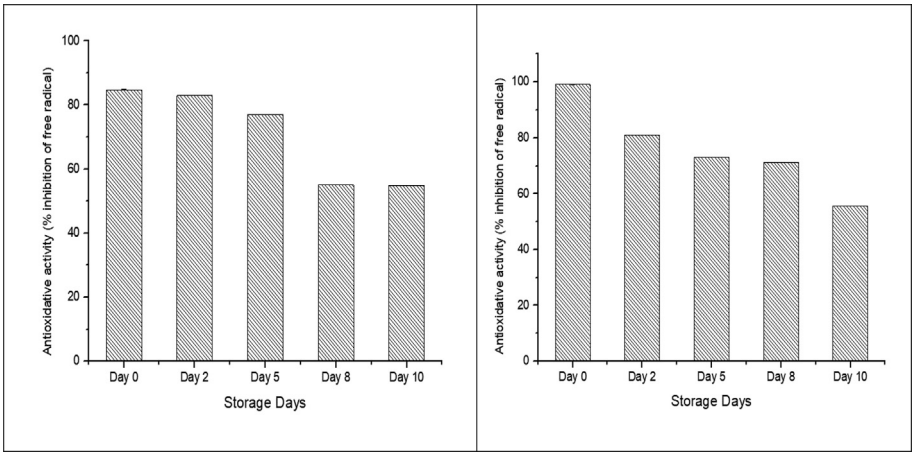

Fig. 4. a Antioxidative activity (% inhibition of free radical) of *Tungrymbai* (without *Lactobacillus* cultures) [Traditional based] **b**. Antioxidative activity (% inhibition of free radical) of fermented *Tungrymbai* like product under Laboratory condition (with *Lactobacillus* cultures).

radical cation in a concentration dependant manner to scavenge the $ABTS^{+}$. The total antioxidative capacity showed that $ABTS^{+}$ assay basically measured the relative activity of the *Lactobacillus* culture combinations incorporated in the laboratory made *Tungrymbai* to scavenge the $ABTS^{+}$ generated in aqueous phase presuming to contribute to the reduction of the ferryl myoglobin radical over the period of 0, 2, 5, 8 and 10 days successfully, as compared to the *Tungrymbai* made without *Lactobacillus* cultures. The variation amongst the different storage days were found to be highly significant ($P < 0.05$) for antioxidative efficacy of the laboratory made *Tungrymbai* (with *Lactobacillus* cultures) as compared to the *Tungrymbai* without *Lactobacillus* cultures based. The results demonstrate the time-dependency of the reaction and the influence of the selected time-point of measurement on the reported antioxidant

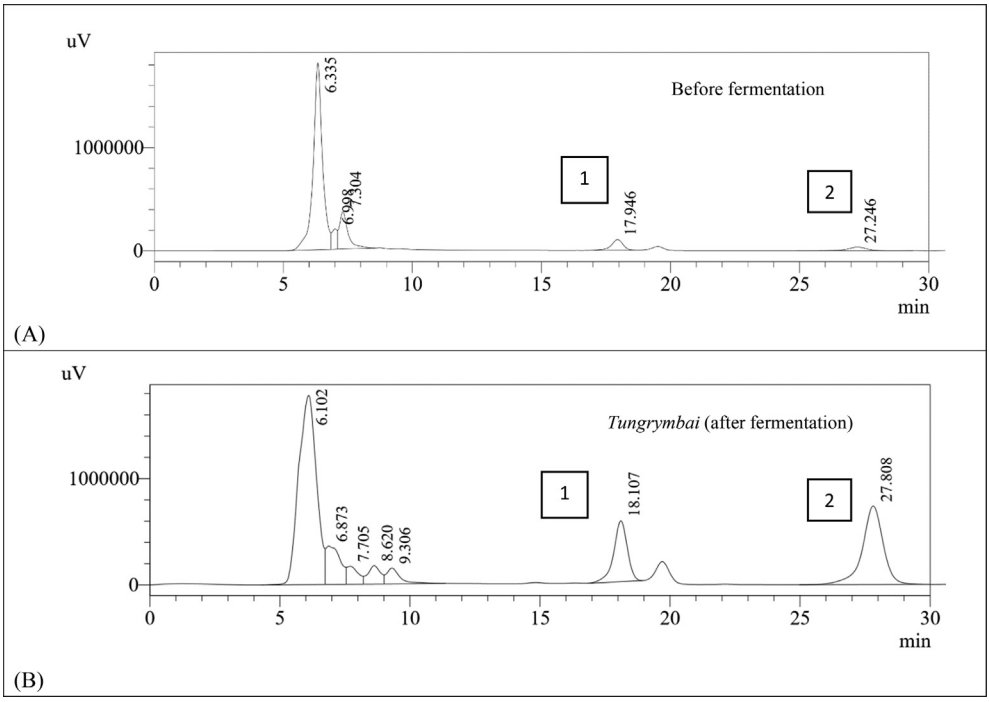

Fig. 5. (A)- Biotransformation of isoflavones in *Tungrymbai* (un-inoculated) (1: Daidzein and 2: Genistein); (B)- Biotransformation of isoflavones in laboratory made *Tungrymbai* with *Lactobacillus* cultures (1: Daidzein and 2: Genistein).

activity; thus the determinants of the antioxidant activity are the extent of reduction and rate of reduction of the radical.

The isoflavones genistin, daidzin, genistein, and daidzein were separated and analysed using RP-HPLC analysis. The most abundant form of isoflavones are glycosides (genistin and daidzin) which are converted to the corresponding aglycones (genistein and daidzein) during the fermentation of *Tungrymbai*. After fermentation for 4 days, in case of *Tungrymbai* (without *Lactobacillus* cultures) agyclones constituted of 34.70% genistein and 29.37% daidzein (Fig. 5a) and there was a maximum bioconversion from glucosides to aglycones for laboratory made *Tungrymbai* (with *Lactobacillus* cultures) with 55.44% genistein and 86.58% daidzein (Fig. 5b) after 24 h of fermentation removing of sugar moiety to produce their respective aglyconic forms. The glycosides (genistin and daidzin) were constituted of 79.37% and 34.70% before fermentation and after fermentation the converted to the corresponding aglycones with 86.58% genistein and 55.44% daidzein. Comparatively, Genistin contributed to the greatest concentration of glycoside isoflavones in laboratory made *Tungrymbai* (with *Lactobacillus* cultures).

4. Discussion

In the present study, the findings are in agreement with Chettri &Tamang [16] who reported the sensory evaluation of laboratory prepared *Tungrymbai* and *Bekang* which were familiar with these traditional fermented soy products of North-eastern regions of Meghalaya, India. They employed the strains of *Bacillus* spp. and their combinations as such as *B. licheniformis, B. pumilus*. Organoleptically, *Tungrymbai* prepared by using starter D (mixture of *Bacillus subtilis* TS2:B24, *B. licheniformis* TSB:B13 and *B. pumilus* TSA:B15) scored highest in taste, aroma, texture and general acceptability. Tamang et al. [17] also reported the indigenous knowledge of north-eastern Khasi tribes associated with the

production of ethnic fermented soybean foods. In Asia, soybeans have been employed by various tribes into different food patterns on their own and also uniformed with fermented products for being used as seasonings or side dishes [18].

Thokchom and Joshi [11] reported that pH, titratable acidity and lactic acid bacterial counts were maximum in pre-cooked *Tungrymbai* samples than the post-cooked which support the study. Almost similar kind of study has been reported by Sohliya et al. [1] where the population of lactic acid bacteria increased remarkably in *Tungrymbai* that was fermented for 3 days stored at room temperature. A similar work was reported by Thokchom & Joshi [11], where the microbial abundance dropped remarkably in the post-cooked *Tungrymbai* sample after exposure to cooking practices for all the counts other than the spore formers which was experienced in our study too. Moreover the important probiotic lactic acid bacteria such as *Lactobacillus* sp. were reduced after cooking which is indicative of the reduction of beneficial microbe from the traditional food. Detailed knowledge of the diverse microbial population including lactic acid bacteria and their respective isolation sources of the traditional indigenous fermented food as *Tungrymbai* (Khasi Hills, Meghalaya, India) will assist in achieving their genetic resources and developing their specific database thereby revealing their priority as probiotics with association to the ethnic traditional fermented foods by the indigenous tribes [1].

For treatment of hypertension, ACEs inhibitory peptides are considered as an ideal solution that can be obtained from the food proteins associated with fermented soy foods [19] since fermentation by lactic acid bacteria generates these functional peptides that exerts with antihypertensive impact if they surpass the gastrointestinal digestive system followed by reaching cardiovascular system in their active mode [20]. A similar finding was reported by Korhonen & Pihlanto [21] on increased concentration of an ACE-inhibitory peptide from a yoghurt-like product fermented with a *L. helveticus* strain. Jiang et al. [22], Kuba et al. [23] and Okamoto et al. [24,25] reported the ACE inhibitory activity expressed as IC50 (half maximal inhibitory concentration) values of 0.51 mg/ml for tempeh, 0.66 and 1.77 mg/ml for *tofuyo*, 3.44 and 0.17–17.80 mg/ml for soy sauce, 2.38, 5.35, and 1.27 mg/ml for *miso* paste, and 0.16, 0.19, 0.40, and 0.27–0.44 mg/ml for *natto*, 0.38 mg/ml for yak milk casein, respectively. ACE inhibitory activity of *natto* is about 1.3 times higher than that of steamed soybean [26]. Vermeirssen et al. [27] reported high ACE inhibitory activity after digestion (in vitro) of pea protein when fermented with *L. helveticus* which is at par with our study since soy and pea are both legumes.

Various ethnic fermented soybean foods of Asia has been reported with maximum antioxidative features; say, *natto* of Japan (soy food fermented by *Bacillus*) [28]; *chungkokjang, jang and kimchi* of Korea [18,29], *douchi* of China [30], *kinema* of India and Nepal [31], *bekang* and *tungrymbai* of India [32], *thuanao* of Thailand [33] and *tempe* mold of Indonesia [34]. Our study is in agreement with Hati et al. [12] who reported that *L. rhamnosus* C6 strain provided highest antioxidant activity in ABTS method, DPPH method and FRAP method (89.09, 50.09 and 801.25% inhibition. However, the strains *Lactobacillus* NCDC19 and NCDC17 showed good amount of inhibition in ABTS method (91.97, 90.16% inhibition) via ABTS radical scavenging method. In a study, it was reported that fermentation of soybean meal by *Bacillus subtlis* TK8 that was isolated from *Tua-nao* (a traditional fermented soybean of Thailand) succeeded in improving the ability for removing of $ABTS^{+}$ that might be due to generation of active elements from soybean acted upon the microorganisms leading to better scavenging impact [35]. The study reported by Amadao et al. [36] highlights the high $ABTS^{+}$ scavenging activities of few traditional soy foods (*Chungkukjang, Natto*) of Japan fermented by *Bacillus subtlis* with 14.9–26.6% inhibition. In a similar study, it was stated that soybean fermented by lactic acid bacteria and *Bacillus* sp. showed a high ABTS + radical-scavenging potential as compared to soybean crude extract on the basis of IC_{50} value with trolox as a reference [37].

The most active agylconic forms of soy isoflavones are daidzein and genistein that gets highly absorbed in the gastrointestinal tract with maximum quantities when compared to the inactive glucoside forms of soy isoflavones (daidzin and genistin) [38]. Our study was in agreement with Chen et al. [39] who reported isoflavone bioconversion from soymilk by incorporating *Lactobacillus paracasei* and *Bifidobacterium longum* as they increased from 52% to 60% aglycones over unfermented soymilk respectively after 48 h of incubation. Bacterial-induced hydrolytic deconjugation triggers transformation of isoflavone glucosides derived from soymilk into aglycone form. This process increases their retention in the gastrointestinal tract [40]. During fermentation, isoflavone glycosides are transformed to aglycones with a higher phytoestrogen activity [41] and in correlation to our study, by

employment of *Lactobacillus plantarum* and *Lactobacillus acidophilus*, production of active glycones from glucosides was interpreted from various traditional fermented foods [42]. In a study by Gardner et al. [43], it was stated that the content of genistein was higher in amount in the plasma of adults (healthy) who consumed traditional fermented soy foods (dosage concentration: 144 mg thrice a day) than that of individuals administered with soy isoflavone tablets (dosage concentration: 96 mg thrice a day). Similarly a study with a Korean traditional soybean paste (*Cheonggukjang*) generated the findings that prior to fermentation by *L. acidophilus* KCTC 3925, contents of daizein, glycitein, and genistein (aglyconic isoflavones) were approximately 152.7, 90.9, and 98.7 mg/kg, which were altered to 767.7, 156.3, and 289.3 mg/kg after a period of 60 h fermentation followed by an rise to 3,017.7, 576.7 and 663.7 mg/kg at 120 h fermentation [44], respectively and this very result is in concordance to our results mentioned above with a similar study with the report of Coward et al. [45] and Wang and Murphy [46] that genistein content was maximum in the traditional based fermented soybean-based foods as compared to the unfermented food.

In summary, the laboratory made fermented soy product (*Tungrymbai*) can be considered at par with the traditionally prepared *Tungrymbai* with similar kind of flavour and taste suggested by the panellists and the entire method was followed under hygienic conditions. *Tungrymbai* prepared under laboratory conditions showed different bio-functional properties such as Antioxidative and ACE inhibitory activities and biotransformation of soy isoflavones produced by the potent *Lactobacillus* cultures. Further, *Lactobacillus* cultures employed in the study could be considered as promising cultures for the preparation of various ethnic functional fermented soy foods enriched with soy aglycones produced by defined *Lactobacillus* cultures followed by validation of health claims by clinical trials in animal models.

Authorship

Sujit Das formulated the research questions, designed the study and carried it out. Birendra Kumar Mishra and Subrota Hati contributed as supervisors, formulated the research questions, and designed the study. BIF center, North-Eastern Hill University, Tura Campus, Meghalaya contributed in statistical consultant. All authors contributed in writing the article and approved it before submission.

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Conflicts of interest

None of the authors has any conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yclnex.2019.05.004.

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