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Development of γ-aminobutyric acid (GABA)-rich yoghurt using *Tetragenococcus halophilus* strain KBC isolated from a commercial soy sauce *moromi*

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Abstract

γ-aminobutyric acid (GABA) has been increasingly incorporated in fermented foods to generate "Superfood". Recently, soy sauce *moromi* has been proven to contain a microbial strain that can produce an immunostimulatory compound, GABA. In this research, *Tetragenococcus halophilus* strain KBC (THSKBC) was utilised to ferment milk and enhance GABA production in simplified yoghurt fermentation. Yoghurt fermentation was performed on milk inoculated with THSKBC (F1), yoghurt starter (F2), and THSKBC and yoghurt starter (F3), respectively. The fermentation period of F1 was averaged at 21 hrs compared to both F2 and F3, which was complete after 8 hrs. A spectral HPLC analysis verified that the GABA content of three formulations and yoghurt produced from F3 had the highest GABA concentration of 205.17 mg/L (p <0.05) compared to F1 and F2, which were 78.95 mg/L and 70.31 mg/L, respectively. Thirty panellists were selected for sensory analysis to assess the appearance, colour, odour, taste, texture, and overall acceptability of GABA-yoghurts. The general acceptance of GABA-yoghurts shows F1 and F2 are insignificant (p > 0.05), but F3 was significantly different from commercial yoghurt (p < 0.05). Therefore, this study proved the potential of THSKBC isolated from soy sauce *moromi* in fermenting milk for GABA-rich yoghurt as future food.

1. Introduction

Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that exists naturally in animals, plants, and microorganisms (Hepsomali *et al*., 2020). Generally, biosynthesis of GABA comprises of three main reactions catalysed by cytosolic enzyme glutamate decarboxylase (GAD, E.C.4.1.1.15), metabolised in the mitochondria and succinate by mitochondrial enzymes GABA-T (E.C.2.6.1.19) and SSADH (succinic semi-aldehyde dehydrogenase) (E.C.1.2.2.16) *in vivo* by a metabolic pathway known as GABA shunt pathway (Sita and Kumar, 2020). Besides that, glutamate decarboxylase (GAD, EC 4.1.1.15) and diamine oxidase (DAO, EC 1.4.3.6) activity can be enhanced under stressful conditions; hypoxia, salt stress, heat or cold shock, drought, and mechanical injury to promote GABA formation (Yang, Guo and Gu, 2013). Microorganisms are a significant GABA source and are able to produce GABA from L-glutamic acid by decarboxylation catalysed by glutamic acid decarboxylase (GAD), with pyridoxal 5′ - phosphate (PLP) as a cofactor (Park *et al.*, 2021). In addition, GABA is also a functional amino acid that plays a significant role in many biological activities such as a major inhibitory neurotransmitter in the mammalian central nervous system, and endurance of microorganisms in an acidic environment and involves as a substrate in tricarboxylic acid (TCA) cycle in plants (Rashmi *et al.*, 2018).

Currently, GABA is well known to exist in various foods such as green tea, soybean, germinated brown rice, kimchi, cabbage pickles, yoghurt, and other fermented food (Ngo and Vo, 2019). In recent years, GABA-enriched foods have been actively promoted due to their positive effects on health, blood pressure regulation, and pain alleviation (Liao *et al.*, 2017). However, due to low GABA content in natural animal- and plant-based food

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products (Chang *et al*., 2016), many foods containing GABA cannot meet people's requirements (Cui *et al.*, 2020). Based on the potential of the trio bacteria, *Aspergillus oryzae NSK* (from *koji*), *Bacillus* sp. (from *moromi*), and stringent anaerobic *Tetragenococcus* sp. (from *moromi*), various local Malaysian soy sauce manufacturers were reported to be capable of manufacturing GABA (Wan-Mohtar *et al.*, 2020). The compositions and capacities of the microorganisms in soy sauce are regulated by synthetic biology to produce novel and future food products (Lv *et al.*, 2021).

Yoghurt is a semi-solid fermented milk product produced by thermophilic lactic acid bacteria starting cultures, *Streptococcus* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Sarkar, 2019). It is also a high-nutrient probiotic food composed of abundant nutrients such as protein, vitamin B2, vitamin B12, calcium, magnesium, potassium, and zinc to improve health (Cifelli *et al*., 2020). Besides that, yoghurt is one of the probiotic-based food in demand in the growing market globally and is regarded as a food product with various health benefits (Koirala and Anal, 2021). The processes involved in major industrial types of yoghurt manufacturing are as follows; blending necessary ingredients, pasteurisation and heat treatment of milk to kill unwanted microorganisms, homogenisation, preparation of bulk starter, the addition of starter and fermentation, cooling of yoghurt, stirring and pumping to obtain the final desired product (Chandan, 2017). The fermentation involved is called lactic acid bacteria (LAB) fermentation. During the fermentation process, lactic acid is produced by the bacterial cultures from lactose in milk, which lowers the pH and causes the coagulation of milk protein, giving it a viscous gel-like shape (Nagaoka, 2019).

Lactic acid bacteria (LAB) fermentation has been applied in the dairy industry for decades. The application cooperates with species belonging to gram-positive bacteria; *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Gemechu, 2015). The bacteria are the principal metabolic end-product of LAB fermentation via carbohydrate metabolisation (Othman *et al.*, 2017). Besides that, LAB strains are regarded as generally-regarded-as-safe (GRAS) microorganisms and have been utilised as highly efficient and environmentally friendly alternatives to produce GABA-enriched fermented foods (Cataldo *et al.*, 2020).

Tetragenococcus halophilus is a lactic acid bacterium with a coccus shape with a low guanine and cytosine content that is gram-positive, non-motile, non-spore-forming, facultatively anaerobic, and mildly halophilic (Chun *et al.*, 2019). This strain is vital in producing acetic acid, formic acid, benzaldehyde, methyl acetate, ethyl 2-hydroxypropanoate, 2-hydroxy-3-methyl -2-cyclopenten-1-one, and 4-hydroxy-3-methoxybenzaldehyde, which are all critical aroma compounds in soy sauce (Devanthi *et al.*, 2018).

In the present study, *T. halophilus* strain KBC (THSKBC) isolated from a commercial soy sauce manufacturer in Perak, Malaysia (Yee *et al.*, 2021) was used to ferment milk into yoghurt. This approach allowed further understanding of THSKBC and its capability of fermenting milk as well as its potential contributions to boost GABA production and improve sensory properties of yoghurt.

2. Materials and methods

2.1 Microorganism isolation and bacteria culture

2.1.1 Isolation of Tetragenococcus halophilus strain KBC

Tetragenococcus halophilus strain KBC (THSKBC) was obtained from the bacteria isolation in the Functional Omics and Bioprocess Development Laboratory, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Kuala Lumpur, Malaysia (Yee *et al.*, 2021). The strain was previously isolated from the *moromi* sample from Kwong Bee Chun Soy Sauce Sdn. Bhd., Kamunting, Perak, Malaysia.

2.1.2 Cultivation of Tetragenococcus halophilus strain KBC

From the stock cultures, a loopful of THSKBC culture was streaked on the MRS agar plate containing 0.5% (w/v) $CaCO_3$ (Bendosen Laboratory, Bendosen, Norway), 5% (w/v) NaCl (food grade) under sterile conditions, and incubated for two days at 25°C. This method was performed in triplicate to obtain a pure isolated culture on the MRS agar plate. The developed THSKBC colonies were further used for bacteria inoculum.

2.1.3 Inoculation of Tetragenococcus halophilus strain KBC (THSKBC) for yoghurt making

To prepare THSKBC inoculum suspension, three loopful of THSKBC colonies were transferred into a 50-mL centrifuge tube containing 25 mL of distilled water. The THSKBC colonies and distilled water were mixed using a vortex device.

2.1.4 Bacteria cell count

Once the bacteria inoculum suspension was prepared, the approximate number of bacteria was measured by comparing the turbidity of the bacteria suspension with 0.5 McFarland standard. The absorbance of bacteria suspension was standardised to 0.5 McFarland standard. The absorbance should be in a range of 0.08 to 0.1 to obtain an approximate cell density of 1.5×10^8 CFU/mL following the method by Dalynn Biologicals (2014). Then, 2 mL of bacteria suspension was micro-pipetted into a cuvette after being resuspended using a micropipette. The bacteria cell concentration was determined and adjusted to an acceptable absorbance reading at 0.08 to 0.1 by using a spectrophotometer (Jenway 7305, United Kingdom) at 600 nm.

2.2 Preparation of GABA-yoghurt

Dutch Lady pasteurised fresh milk, and Dutch Lady low-fat yoghurt was acquired in the local market and used to prepare GABA-yoghurt. Yoghurt starter culture containing *L. delbrueckii* ssp. *Bulgaricus* and *S. thermophilus* was obtained from the Dutch Lady low-fat yoghurt with natural flavour. The milk was pasteurised at 82°C for 20 mins and cooled to 43°C (Ibrahem and El Zubeir, 2016). Then, 200 mL of the pasteurised milk was poured into three different jars coded as follows: milk inoculated with 10 mL THSKBC; formulation 1 (F1), milk inoculated with 5 mL yoghurt starter culture; formulation 2 (F2), and milk inoculated with 10 mL THSKBC and 5 mL yoghurt starter culture; formulation 3 (F3). All three formulations were incubated at 43°C until coagulation occurred. When coagulation was observed, the yoghurts were immediately stored in a refrigerator at 4°C. The experiment was repeated in independent triplicate for each formulation.

2.3 Determination of GABA content in yoghurt by High-Performance Liquid Chromatography

The yoghurt samples' GABA content was determined according to the method by Hussin *et al.* (2020) with a slight modification. Yoghurt samples were centrifuged at 9025.9 *g* for 15 mins, and the collected supernatants were filtered through a 0.22-m pore-size nylon filter (Fisher Scientific, Brecon, UK). Next, the supernatant was injected into the HPLC machine and a Hypersil Gold C-18 column supported the process (250×4.6 mm I.D., particle size 5 µm; Thermo Scientific, Meadow, UK). The mobile phase used was the mixture of 60% solution A (aqueous solution of 8.205 g sodium acetate, 0.5 mL triethylamine and 0.7 mL acetic acid in 1000 mL) adjusted to pH 5.8, 28% solution B (deionised water), and 12% solution C (acetonitrile). The mobile phase was adjusted at 0.6 mL/min, and the HPLC separation was detected at 254 nm at room temperature. The GABA content of the sample was determined by comparing the graph peak with the standard curve of GABA. The standard curve was obtained by running 4 different concentrations of pure GABA (0.125 mg/L, 0.5 mg/L, 0.75 mg/L, and 1.0 mg/L) through HPLC machine.

2.4 Sensory evaluation of GABA-yoghurt

Sensory analysis was conducted using 30 panellists consisting of students and staff from the Faculty of Science, Universiti of Malaya. Panellists were asked about health issues such as lactose intolerance and/or allergies to apicultural products as a criterion for research participation (Santos *et al.*, 2020). Before the examination, all participants were required to sign an informed consent form and an informed consent with allergies (Chen *et al.*, 2018). F1, F2, F3, and commercial yoghurt (C1) were served to the panellists randomly and labelled with a 3-digit code. The sensory attributes; appearance, colour, odour, taste, texture and overall acceptability were evaluated using a 9-point hedonic scale (1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like or dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, 9 = like extremely). The degree of yoghurt colour ranges from green or black spots to shiny milky white, the rank of taste and odour were covering from metallic taste and abnormal odour to sour and sweet flavour and odour, and the level of appearance ranges from rough, lumpy and bubbly to smooth texture and no bubbles (Zhi *et al.*, 2018).

2.5 Statistical analysis

Statistical analysis was conducted using IBM SPSS version 26 statistics software. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons was performed. The values were expressed as a mean ± standard deviation to determine the significant differences among means for all testing at $p < 0.05$.

3. Results

3.1 Isolation and cultivation of Tetragenococcus halophilus strain KBC

Based on Figure 1, THSKBC was isolated from soy sauce *moromi*, which was obtained from Kwong Bee Chun (KBC Sdn. Bhd.) Soy Sauce in Kamunting, Perak, Malaysia. THSKBC was identified as cocci shape gram-positive bacteria by performing a gram stain procedure, and isolated colonies were obtained on MRS agar (Figure 2). Figure 2 also shows the absorbance obtained from the THSKBC strain is 0.109, which is in the range between 0.08 to 0.1 at 600 nm.

3.2 GABA yoghurt production

According to the result presented in Figure 3(A), F1 had the longest time spent for milk fermentation (21 hrs) at 43°C among the formulations. In addition, both F2 and

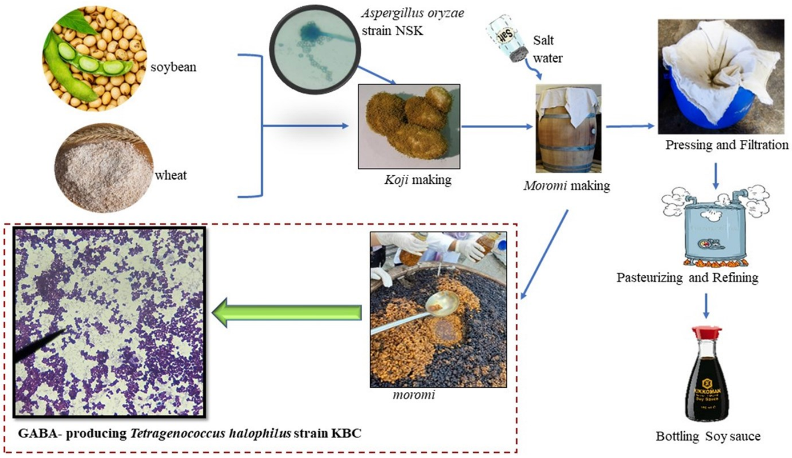

Figure 1. Graphical abstract of soy sauce production

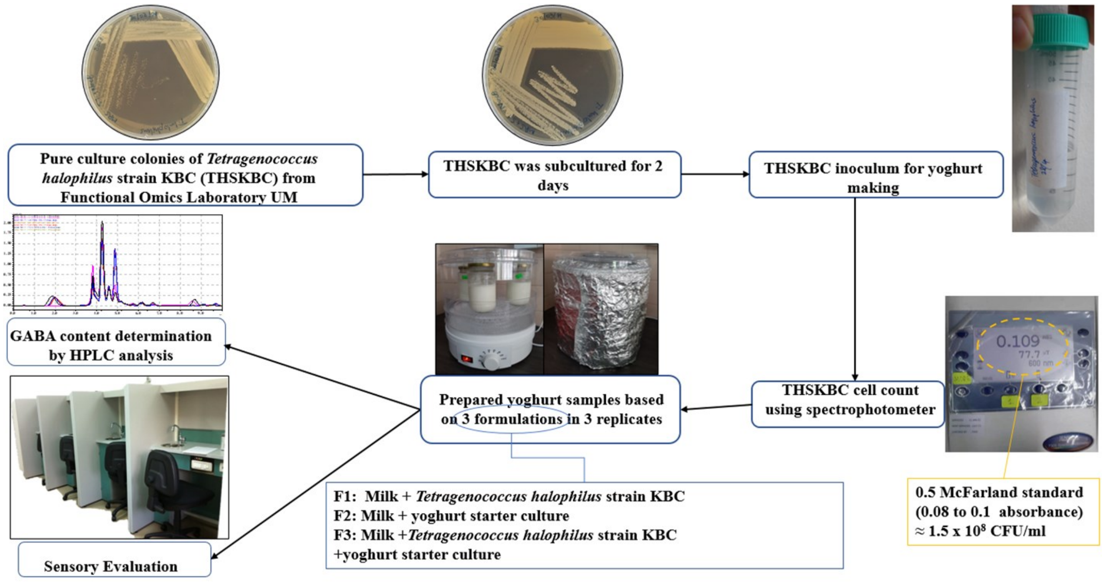

Figure 2. Graphical abstract of isolation and cultivation of *Tetragenococcus halophilus* strain KBC (THSKBC), GABA yoghurts production and analysis of HPLC and sensory evaluation.

F3 took 8 hrs for milk fermentation at 43°C. Moreover, both F2 and F3 showed no difference in fermentation time without the addition and addition of THSKBC. Even though F1 had the longest fermentation time, THSKBC was able to ferment milk and produce yoghurt.

3.3 GABA production by different formulations of GABA yoghurts

The GABA content in all three formulations was analysed by using High-Performance Liquid Chromatography (HPLC). The GABA content (mg/ L) of yoghurt samples produced from each formulation was shown in Figure 3(B). The mean GABA concentration in yoghurt samples made from F1 was 78.95 mg/L, which was slightly higher than the 70.31 mg/L found in yoghurt samples made from F2. The maximum or higher GABA concentration (205.17 mg/L) was found in yoghurt samples generated from F3, which was significantly higher than F1 and F2. Although both F2 and F3 showed the same fermentation time with no addition and addition of THSKBC, the GABA concentration of F3 depicted a significant level (205.17 mg/L) compared to F2 (70.31 mg/L). This analysis showed that THSKBC could produce GABA. In addition, the strain also can enrich the GABA content in yoghurt with the addition of a

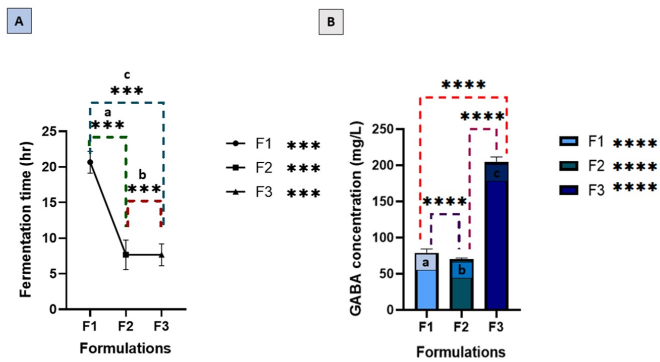

Figure 3. (A) Fermentation time (hr) of GABA yoghurts from different formulations (F1, F2 and F3). Symbols: ***$p<0.05$, indicates significant difference between formulations. The different letter indicates formulations are significant to each other. The dotted line shows the formulations are significant to each other. (B) GABA concentration (mg/L) of GABA yoghurts from different formulations (F1, F2 and F3). Symbols: ****$p<0.05$, indicates significant difference between formulations. The different letter indicates formulations are significant to each other. The dotted line shows the formulations are significant to each other.

yoghurt starter and THSKBC producing a high concentration of GABA.

3.4 Sensory evaluation

Figure 4 shows the sensory attributes, colour, odour, taste, texture, and overall acceptability. F1 had the highest taste score (6.20) compared to other formulations. In addition, F3 scored the lowest in appearance, colour, odour and texture (4.77, 6.17, 6.03, and 5.37, respectively), whereas F2 ranked the weakest in taste (5.47). As can be seen, F3 appeared lumpy, less shiny form, and less appetising to the panellists, contributing to the lowest score in overall acceptability (5.30). In general, commercial yoghurt; C1 obtained the highest appearance, colour, odour and overall acceptability scores (7.70, 7.63, 7.00, and 6.60, respectively). C1 received the highest overall acceptability score (6.60), followed by F1 and F2 (5.87). All in all, among the GABA yoghurts, F1 achieved the highest scores for appearance, colour, odour, taste and texture (5.60, 6.53, 6.30, 6.20, and 5.73, respectively) compared to F2 and F3.

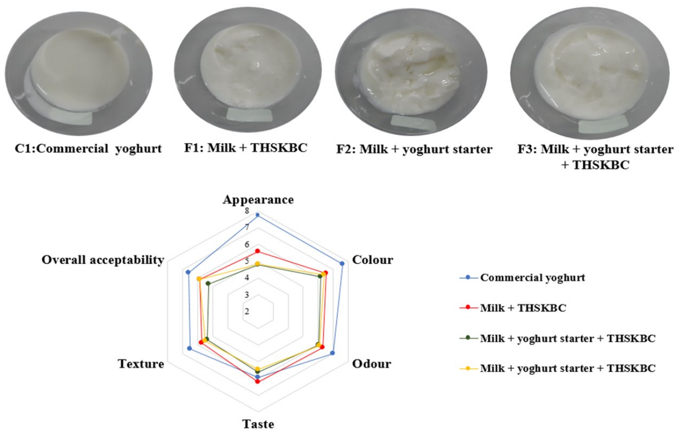

Figure 4. Radar chart of sensory evaluation of commercial yoghurt and different formulations of GABA yoghurts. 30 panellists examined the sensory test to evaluate colour, odour, taste, texture, and overall acceptability using the 9-point hedonic scale.

4. Discussion

4.1 Milk fermentation between THSKBC and other starter cultures

Fermentation time is determined by the strains utilised, the physical state of the microbial combination (liquid or lyophilised culture), and the desired acidity level in the finished yoghurt (Baglio, 2014). The comparison of temperature and time spent for milk fermentation between THSKBC and other starter cultures was shown in Table 1. *Tetragenococcus halophilus* strain KBC indicated the most prolonged time taken for milk fermentation (21 hrs) at 43°C compared to all previous studies. A study by Yilmaz-Ersan and Kurdal (2014) reported *Streptococcus thermophilus and Lactobacillus bulgaricus* took the shortest incubation time (3 hrs) at 42°C followed by *Streptococcus thermophilus strains* and *Lactobacillus delbrueckii* ssp. *bulgaricus* strains consumed 3.5-4.5 hrs at 42°C (Han *et al.*, 2014). Among all studies, the incubation time for the combination of *L. delbrueckii* ssp. *bulgaricus, Streptococcus. thermophilus, and L. rhamnosus GG* consumed the most extended time around 16 hrs at 42°C (Jia *et al.*, 2016).

4.2 GABA production by different formulations of GABA -yoghurts

Different LAB species produce GABA in different ways and have diverse fermentation requirements (Kook and Cho, 2013). Table 2 compares GABA production from THSKBC isolated from soy sauce *moromi* and other isolated strains. THSKBC with yoghurt starter depicts higher GABA content (205 mg/L) compared to the study by Yee *et al.* (2021), which indicates THSKBC with 20% molasses produces lower GABA level (159.00 mg/L). Besides that, *Aspergillus oryzae* NSK showed the lowest GABA concentration (73.13 mg/L) (Ab Kadir *et al.*, 2016). However, in another study by Hajar-Azhari *et al.* (2018), and Wan *et al.* (2019), *A. oryzae* NSK gave higher GABA concentration (354.08 mg/L) and (3278.31 mg/L), respectively. Wan-Mohtar *et al.* (2020) also reported significant GABA concentrations for unoptimised and optimised *Bacillus cereus* strain KBC; 532.74 mg/L and 3393.02 mg/L.

4.3 Sensory evaluation

The primary fermentation process of milk into yoghurt resulted in various products with different sensory, organoleptic, and rheological features, including acid taste, smooth texture and viscous gel (Peng *et al.*, 2020). The lactic acid produced by the starter and aroma compounds naturally present in milk, and those produced by the fermentation process give yoghurt its well-defined sour flavour (Das *et al.*, 2019). Acetaldehyde is a metabolic product of carbohydrate metabolism and acts as a significant component in yoghurt's distinctive aroma and flavour (Ameen and Caruso, 2017). Besides that, consumers' preference for commercial yoghurt was predicted, given that the product's formulation included other substances such as corn syrup and flavourings that contributed to flavour and aroma (Santos *et al.*, 2020). On top of that, Pinto *et al.* (2021) also stated several consumers perceived ultra-processed food such as commercial low-fat yoghurt as 'healthier' than traditional processed food due to its weight loss benefit. Additionally, no sensory evaluation has been performed on THSKBC to date. Hence, the varied results between formulations showed different attributes value and the

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Table 1. Temperature and time spent for milk fermentation by various starter cultures.

Starter cultures	Temperature (°C)	Milk fermentation Time (hr)	References
Tetragenococcus halophilus strain KBC (THSKBC)	43	21	This study
S. thermophilus and *L. delbrueckii subsp. bulgaricus*	37-43	6-8	Corrieu and Béal (2016)
L. delbrueckii ssp. bulgaricus, Streptococcus. thermophilus, and L. rhamnosus GG	42	16	Jia *et al*. (2016)
Streptococcus thermophilus strains and *Lactobacillus delbrueckii ssp. bulgaricus*	42	3.5-4.5	Han *et al*. (2014)
Commercial starter YF-L812	42	5.5	Han *et al*. (2014)
Streptococcus thermophilus and *Lactobacillus bulgaricus*	42	3	Yilmaz-Ersan and Kurdal (2014)
Lactobacillus acidophilus and *Bifidobacterium lactis*	42	8	Yilmaz-Ersan and Kurdal (2014)
Lactobacillus acidophilus, Bifidobacterium lactis and *Lactobacillus casei*	40	10	Yilmaz-Ersan and Kurdal (2014)

Table 2. GABA concentration by isolated strains from various soy sauce sources.

Isolated strain	Soy sauce	GABA concentration (mg/L)	References
Tetragenococcus halophilus strain KBC (THSKBC)	*moromi*	205.17	*This study*
Tetragenococcus halophilus strain KBC (THSKBC)	*moromi*	159.00	Yee *et al*. (2021)
Bacillus cereus strain KBC (unoptimised)	*moromi*	532.74	Wan-Mohtar *et al*. (2020)
Bacillus cereus strain KBC (optimised)	*moromi*	3393.02	Wan-Mohtar *et al*. (2020)
Aspergillus oryzae NSK	*koji*	3278.31	Wan *et al*. (2019)
Aspergillus oryzae NSK	*koji*	354.08	Hajar-Azhari *et al*. (2018)
Aspergillus oryzae NSK	*koji*	73.13	Ab Kadir *et al*. (2016)

addition of the strain is acceptable to produce GABA yoghurt.

5. Conclusion

Based on the results, the THSKBC strain proved its potential to ferment milk and yield significant GABA concentration. Besides that, the sensory evaluation showed the highest scores of all attributes and overall acceptability for THSKBC. This indicated THSKBC isolated from soy sauce *moromi* is capable of developing fermenting milk for GABA-rich yoghurt.

Conflict of interest

The authors declare no conflict of interest.

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