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ORIGINAL ARTICLE

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Isolation, characterization, and utilization of γ-aminobutyric acid (GABA)-producing lactic acid bacteria from Myanmar fishery products fermented with boiled rice

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Abstract γ-Aminobutyric acid (GABA)-producing lactic acid bacteria (LAB) were isolated from four types of Myanmar traditional fermented fishery products with boiled rice. All of them belonged to the genus *Lactobacillus*, and comparison of the effects of these representatives on GABA accumulation in fermented fishery products with boiled rice revealed that *Lactobacillus farciminis* D323 is the most effective strain as a starter culture. These results may contribute to the development of traditional fermented fishery products with functional properties. In addition, this study is the first to show in detail the distribution of GABA-producing LAB in Southeast Asian fermented fishery products.

Keywords γ-Aminobutyric acid · Fermentation · *Lactobacillus* · Fermented fishery products · Myanmar

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Introduction

γ-Aminobutyric acid (GABA) is a four-carbon, nonprotein amino acid that is widely distributed in nature [1]. GABA has been proved to be an inhibitory neurotransmitter in mammalian brains [2]. In addition, GABA has multiple physiological functions such as tranquilizing, diuretic, and hypotensive activities [3, 4]. Furthermore, it has been reported that administration of GABA-enriched food is effective for regulation of depression, sleeplessness, and autonomic disorders [5]. It also has a hypotensive effect [6, 7], and can be used for improvement of chronic alcohol-related symptoms [8], stimulation of immune system cells [9], and prevention of diabetic conditions [10]. Therefore, the benefits of GABA on human health have recently attracted increased attention in the food industry, and several GABA-enriched functional foods are currently manufactured, for instance, tea leaves treated unaerobically [11], rice germs soaked in water [12], red mold rice [13], tempeh-like fermented soybeans [14], and dairy products such as yogurt [15], fermented milk products [7], and cheese [16, 17].

Various microorganisms, including bacteria such as lactic acid bacteria (LAB), fungi, and yeast, that produce GABA have been reported. There have been numerous investigations into GABA-producing LAB from various viewpoints, such as microbiological taxonomy, GABA production from nutrient medium or various fermentation ingredients, and the biochemical and genetic characterization of glutamate decarboxylase (GAD) (EC 4.1.1.15), which catalyzes the primary biosynthesis of GABA, namely α-decarboxylation of L-glutamic acid. Ueno et al. [18] purified and characterized GAD of *Lactobacillus brevis* IFO 12005, and a more detailed genetic study on this GAD has recently been performed [19]. In addition, this

strain was also demonstrated as a GABA producer from alcohol distilled lees [20]. An earlier report described the GABA production and enzymatic properties of GAD of the *Lb. paracasei* isolated from a Japanese fermented crucian carp, *funa-zushi* [21, 22]. Moreover, some researchers reported GABA-producing *Lb. buchneri* [23], *Lb. brevis* [24, 25], and *Lb. plantarum* [26] in *kimchi*, a Korean fermented vegetable product. *Lb. brevis* was also isolated from fresh milk and *plaa-som*, a Thai fermented fish product [27, 28]. As mentioned earlier, *Lactobacillus* species are the main GABA-producing LAB; however, other LAB species have also been described as GABA producers. *Lactococcus lactis* has been reported as a producer of carbon dioxide and GABA during cheese ripening [16, 29]. The detailed characteristics of GAD of *Lc. lactis* have been elucidated [30].

Lactic acid bacteria are some of the most commonly used microorganisms in food fermentation, and have been used not only in natural fermentation, but also are often used as a starter culture to accelerate and direct the fermentation of various types of food fermentations [31]. Previously, various studies on the development of fermented food containing high levels of GABA using a starter culture of GABA-producing LAB were reported as follows: *Lactobacillus* sp. L13 was demonstrated as a preferred starter culture for the accumulation of GABA in the Japanese pickled turnip [32]. *Lb. brevis* was also used for the fermentation of GABA-enriched blackberry juice [33]. In addition, a selection of GABA-producing LAB to be used as a starter culture for cheese making [17], and milk fermentation and cheese manufacturing using *Lc. lactis* and *Streptococcus salivarius* subsp. *thermophilus* Y2 [15, 16, 34] have been reported. These investigations focused on pickled vegetables, fruit products, and dairy products, and we are currently unaware of any reports on the application of GABA-producing LAB in fermented fishery products.

In Myanmar, there are various types of traditional fermented fishery products, such as fermented fish or shrimp with boiled rice, fish sauce, and fish paste, which are known to be a necessary part of the dietary habits of the Myanmar people as seasonings or side dishes. Among them, fermented fishery products with boiled rice are mainly prepared using four types of fishery products, with different names depending on the type of fishery product used. They are called *ngachin*, *pazun-chin*, *ngagyin-chin*, and *ngaphae-chin* when tinfoil barb *Puntius schwanenfeldi*, speckled prawn *Metapenaeus monoceros*, rohu *Labeo rohita*, and featherback fish *Notopterus notopterus* are used as ingredients for these fermented products, respectively. Although Myanmar's traditional fermented fishery products are very popular and consumed widely in the country, there are only a few early studies, including microbiological studies, on fish paste and fish sauce [35, 36]. On the other hand, there are no detailed reports on LAB or GABA-producing LAB in these fermented fishery products.

To contribute to the development of traditional fermented fishery products with functional properties, we focused on screening GABA-producing LAB that are suitable for the fermentation of traditionally fermented fishery products. For this purpose, we used four types of Myanmar fermented fishery products with boiled rice with less focus on the microbiological viewpoint. We also describe the distribution of GABA-producing LAB in these products and the productivity of each isolate. Moreover, GABA-producing LAB were used as starter cultures in the manufacturing of their original fermented fishery products to confirm the accumulation of GABA.

Materials and methods

Fermented fishery products for isolation of GABA-producing LAB

A total of 11 samples containing four types of fermented fishery products with boiled rice were purchased from a market in Yangon City, Myanmar in 2006 and 2008, as shown in Table 1. Tinfoil barb and speckled prawn were used for fermentation, and the products were called *ngachin* (N) and *pazun-chin* (PC-1 and PC-2), respectively. In addition, rohu and featherback fish were also used for fermentation of *ngagyin-chin* (NGC-1 to 4) and *ngaphae-chin* (NPC-1 to 4), respectively. These purchased samples were transported to our laboratory by air and immediately stored at 4°C.

Chemical properties of fermented fishery products

The pH values were measured using a pH meter (M-11; Horiba, Kyoto, Japan), and the salt concentrations were measured using a NaCl meter (C-121; Horiba, Kyoto, Japan). Before these measurements, one gram of each fermented fishery product was mixed well with 9 ml distilled water and homogenized. For the amino acid analysis, 5 g of a fermented product was homogenized with 4 volumes of 6% perchloric acid (PCA) and centrifuged at 18,800×*g* for 10 min at 4°C. The obtained supernatant was neutralized by adding 5 N KOH to adjust the pH to 7.0 and filtered through 0.22-μm cellulose acetate membranes. The neutralized supernatants were analyzed for GABA content using a JLC-500 automatic amino acid analyzer (JEOL, Tokyo, Japan). Unless otherwise stated, all experiments were performed in triplicate.

Table 1 Chemical properties and distribution of isolated GABA-producing LAB in four types of fermented fishery products

Fermented products	pH	NaCl	GABA amount of fermented products (mg/100 g)	No. of isolates	No. of GABA producers	Isolation medium
Tinfoil barb (*Ngachin*, N)	4.8 ± 0.1	3.8 ± 1.1	193.4 ± 6.5	9	1	NaCl 5%-GYP
Speckled prawn (*Pazun-chin*, PC-1)	4.3 ± 0.1	4.1 ± 0.8	26.2[a]	13	4	NaCl 5%-GYP
Speckled prawn (*Pazun-chin*, PC-2)	3.9 ± 0.1	3.9 ± 0.3	0.5 ± 0.1	12	0	NaCl 10%-GYP
Rohu (*Ngagyin-chin*, NGC-1)	4.2 ± 0.2	6.6 ± 0.4	4.0 ± 6.5	12	0	NaCl 7%-MRS
Rohu (*Ngagyin-chin*, NGC-2)	4.5 ± 0.1	8.6 ± 1.7	1.5 ± 2.1	12	0	NaCl 7%-MRS
Rohu (*Ngagyin-chin*, NGC-3)	4.2 ± 0.3	7.2 ± 0.4	10.0 ± 13.6	12	2	NaCl 7%-MRS
Rohu (*Ngagyin-chin*, NGC-4)	4.3 ± 0.2	7.1 ± 0.2	1.9 ± 1.9	12	2	NaCl 7%-MRS
Featherback fish (*Ngaphae-chin*, NPC-1)	3.8 ± 0.1	6.9 ± 0.5	0.3 ± 0.5	12	0	NaCl 3%-MRS
Featherback fish (*Ngaphae-chin*, NPC-2)	4.1 ± 0.1	4.1 ± 1.4	0.2 ± 0.4	12	0	NaCl 3%-MRS
Featherback fish (*Ngaphae-chin*, NPC-3)	3.9 ± 0.2	5.2 ± 0.6	ND	12	0	NaCl 3%-MRS
Featherback fish (*Ngaphae-chin*, NPC-4)	3.9 ± 0.1	5.1 ± 0.6	ND	12	3	NaCl 3%-MRS

Data expressed as mean ± standard deviation (SD) from three independent experiments

ND not detected

[a] $n = 2$

Isolation of GABA-producing bacteria

An enrichment culture was carried out to isolate GABA-producing LAB. Five grams of each of the fermented products was inoculated into 35 ml de Man, Rogosa, and Sharpe (MRS) broth [37] or glucose yeast extract polypepton (GYP) broth [18] containing 5% monosodium glutamate (MSG) adjusted to initial pH of 7.0. An appropriate amount of NaCl corresponding to each of the fermented products was added to each enrichment culture medium; the detailed conditions are shown in Table 1. After 3 days of incubation at 27°C, an aliquot of the culture broth was transferred to a new liquid medium for further cultivation. This procedure was repeated three times in total, and the final culture broth was diluted serially and then spread on 1.2% agar medium corresponding to each enrichment culture broth. After incubation at 27°C for 1 week under anaerobic condition using an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan), individual colonies were isolated and purified. These isolates were stored at −80°C in MRS broth containing 20% glycerol (w/v).

Confirmation of GABA production of isolates

All the isolates were cultured in 3 ml 5% MSG-MRS broth (pH 7.0) at 27°C for 2 days. The GABA-producing abilities of the isolates were then confirmed using thin-layer chromatography (TLC). One microliter of supernatant from each culture broth was spotted onto a silica plate (Silicagel 60 F_{254}; Merck, Darmstadt, Germany). TLC was conducted using a solvent mixture [1-butanol:acetic acid:distilled water (3:2:1)], and a spot of GABA was treated on a hot plate for a few minutes after being detected by ninhydrin spray (Wako, Tokyo, Japan). In addition, the GABA-producing abilities determined from the TLC results were reconfirmed using an automatic amino acid analyzer as follows: GABA-producing candidates were cultured once again in 3 ml 5% MSG-MRS broth supplemented with 0.1 mM pyridoxal-5′-phosphate (PLP) or without PLP at 27°C for 6 days. Culture supernatants were mixed with 4 volumes of 6% PCA and centrifuged at 18,800 × *g* for 10 min at 4°C. The supernatant was neutralized by adding 5 N KOH and filtered through 0.22-μm cellulose acetate membranes. The neutralized supernatants were analyzed for GABA content using the automatic amino acid analyzer described above. All experiments were performed in triplicate.

Phenotypical identification of GABA-producing LAB

Gram stain, cell form, and cell motility were observed by light microscopy (BX 51; Olympus, Tokyo, Japan). Catalase activity was tested with 3% H_2O_2. The acid production from D-glucose was determined using MRS agar medium (pH 7.0) on the basis of a clear zone indicating $CaCO_3$ dissolution, and MRS broth with a Durham's tube was used to determine gas production from D-glucose. To determine the oxygen requirement for growth, the isolates were incubated anaerobically on MRS agar medium in a GasPack system. The growth behavior under different NaCl and temperature conditions was also determined using MRS broth. The fermentation of various carbohydrates was determined using API CH strips with API CHL medium (Bio Merieux, Lyon, France). The LAB strains were grown in the MRS broth at 27°C for 24 h. The MRS

broth was centrifuged at 5,000×*g* for 10 min at 4°C, and the collected cells were washed twice with distilled water. The washed cells were suspended in 2 ml distilled water and inoculated into the API CH strips. The strips were incubated at 27°C for 48 h, and then the isolates were identified using apiweb version 1.2.1 (Bio Merieux, Lyon, France) after the acid formation was determined visually.

Genotypical identification of GABA-producing LAB

Deoxyribonucleic acid (DNA) was extracted by the method of Marmur [38]. Oligonucleotides 5′-AGT TTG ATC CTG GCT AG-3′ (primer 27F) and 5′-GTT ACC TTG TTA CGA CTT C-3′ (primer 1492R) were used as primers for amplification of the 16S rRNA gene via polymerase chain reaction (PCR) using a Takara r*Taq* gene amplification PCR kit (Takara Bio, Shiga, Japan) [39]. The PCR products were purified by polyethylene glycol (PEG) precipitation. Then, the PCR products for sequencing were prepared using BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA, USA), and DNA sequencing was carried out using an Applied Biosystems 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequence results were compiled from overlapping sequence data using the GENETYX computer program (GENETYX, Tokyo, Japan). The resulting sequences were compared with known sequences using the basic local alignment search tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/).

Preparation of trial fermented products using three representative GABA-producing LAB

All experiments were performed in quadruplicate. Before preparations of the trial fermented products, all cookware was sterilized. Three types of fermented fishery products with boiled rice were prepared experimentally at approximately 300 g, laboratory scale using the method traditionally used in Myanmar. Trial fermentation experiments were performed twice. In the first trial, tinfoil barb (*ngachin*, N-T1), speckle prawn (*pazun-chin*, PC-T1), and rohu (*ngagyin-chin*, NGC-T1) were fermented with boiled rice using GABA-producing isolates, namely strains D323, F311, and K35 as starter cultures, respectively. In the second trial, tinfoil barb (*ngachin*, N-T2), speckle prawn (*pazun-chin*, PC-T2), and rohu (*ngagyin-chin*, NGC-T2) were also fermented with boiled rice using strain D323. The process is described as follows: Fresh fish or shrimp were purchased from the fish market in Yangon City. They were washed, eviscerated, cut into small pieces, and then salted at 10:1 weight ratio of fishery products to salt. For the preparation of boiled rice, long-grain rice was cooked with a rice cooker. After salting for 3 h, boiled rice was added at the ratio of salted fishery products to boiled rice of 2:1 by weight. Lyophilized cells (0.3 g) of each starter culture (approximately 10^{10} cfu/g) were inoculated, and then the mixture was fermented in a plastic jar for 6 days at room temperature. During this fermentation process, degassing was not performed. The GABA content of trial fermented products was measured using the automatic amino acid analyzer described above.

Statistics

The statistical significance of GABA contents of trial fermented fishery products was evaluated using Student's *t* test, and differences between trial products were compared by one-way analysis of variance (ANOVA) using Statistic version 6.0 (Statsoft, Tulsa, OK, USA) and Tukey's multiple-range test at $P < 0.05$.

Results

Chemical properties of the fermented fishery products

Table 1 presents the basic chemical properties of the four types of fermented fishery products used for the screening of GABA-producing LAB. The pH values of these fermented fishery products were kept under acidic conditions of 3.8–4.8, and the NaCl concentrations were between 3.8% and 8.6%. The lowest pH was found in fermented featherback fish (NPC-1) at 3.8, and the highest pH was 4.8 in fermented tinfoil barb (N). On the other hand, the highest NaCl concentration was 8.6% for fermented rohu (NGC-2), whereas the lowest was 3.8% for fermented tinfoil barb (N). The amount of GABA in fermented tinfoil barb was 193.4 mM, whereas in fermented speckled prawn, fermented rohu, and fermented featherback fish, it ranged from 0.5 to 26.2 mM, 1.5 to 10.0 mM, and ND to 0.3 mM, respectively.

Screening of GABA-producing LAB

The number of GABA-producing LAB isolated from the four types of fermented fishery products with boiled rice are indicated in Table 1. A total of 130 bacteria were obtained from the enrichment culture broth, and among them, 12 isolates showed GABA production from the TLC results (Fig. 1); all of them were confirmed as GABA producers using the automatic amino acid analyzer. A GABA producer (strain D323) was isolated from the fermented tinfoil barb sample (N), and four producers (F311, F332, F341, and F342) were also isolated from a sample of fermented speckled prawn (PC-1). Moreover, four strains (K35, K39, K42 and K47) and three strains (P41, P43 and P44) were also isolated from the two

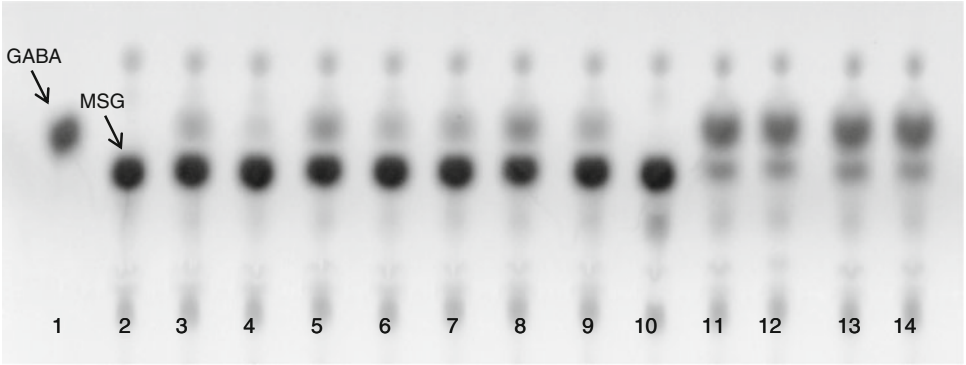

Fig. 1 GABA production of isolated LAB determined by TLC. Analyses were performed after incubation for 2 days in MRS medium. *Lane 1* 1% GABA solution, *lane 2* 5% MSG in MRS broth, *lane 3* strain K35, *lane 4* strain K39, *lane 5* strain K42, *lane 6* strain K47, *lane 7* strain P41, *lane 8* strain P43, *lane 9* strain P44, *lane 10* strain D323, *lane 11* strain F311, *lane 12* strain F332, *lane 13* strain F341, *lane 14* strain F342

samples of fermented rohu (NGC-3 and NGC-4) and a sample of fermented featherback fish (NPC-4), respectively. However, we could not isolate any GABA producers from the remaining fermented products.

GABA productivity of isolates

The GABA production of the twelve isolates after incubation for 6 days is shown in Fig. 2. The amounts of GABA produced in the MRS broth reached 97.5–798.0 mM, and the highest amount was produced by strain F311. All the isolates were divided into two groups on the basis of their GABA productivity. Eleven strains showed high GABA productivity, which ranged from 472.7 to 798.0 mM, whereas strain D323 produced a small amount of GABA (97.5 mM). The effect of addition of PLP to the culture medium on GABA productivity was also examined, because GAD has been described as a PLP-dependent enzyme [40]. The GABA productivity of all the isolates seemed to be enhanced by the addition of PLP, and the level of increase was different depending on the type of isolate. In particular, the GABA production of strain D323 increased 5.1-fold and reached 493.6 mM in the growth medium containing PLP.

General phenotypic characterization of GABA-producing LAB

The twelve GABA-producing bacteria were Gram-positive, catalase-negative, and homofermentative rod-shaped bacteria. They can grow in 0%, 3%, 5%, 7%, and 10% NaCl media. In addition, eleven isolates could grow at 15°C and 45°C, whereas only one isolate, strain D323, could not grow above 40°C. These results indicate that all the GABA producers should be classified into one genus, *Lactobacillus*, on the basis of the identification protocols used for lactic acid bacteria [41].

16S rRNA gene sequencing analysis of GABA-producing LAB

Among the 12 isolated GABA producers, three strains were used for processing trial fermentation products on the laboratory scale. Therefore, an additional taxonomical study of these representatives was performed with detailed fermentation potential tests using 16S rRNA gene sequencing analysis and API rapid CH fermentation strips. First, we performed 16S rRNA gene sequencing analysis to clarify the taxonomic positions of strains F311, K35, and D323 from the phylogenetic viewpoint. The 16S rRNA gene of strain F311 showed 99.9% sequence similarity to that of strain K35 with a single base difference in 1,488 positions. From comparison with the sequences of the type strains of *Lactobacillus* species in the public DDBJ database, strains F311 and K35 were shown to be closely related to both *Lb. plantarum* JCM 1149^T (D79210) and *Lb. pentosus* JCM 1558^T (D79211). The comparison of the 16S rRNA gene sequence revealed no difference between strain F311 and *Lb. plantarum* JCM 1149^T (D79210), and only a single difference (99.9% similarity) between strain F311 and *Lb. pentosus* JCM 1558^T (D79211) in 1,488 positions. In addition, no nucleotide difference was observed between strain K35 and *Lb. pentosus* JCM 1558^T (D79211), and only a single nucleotide difference (99.9% similarity) was found between strain K35 and *Lb. plantarum* JCM 1149^T (D79210). On the other hand, the 16S rRNA gene sequence of strain D323 showed 100% similarity to *Lb. farciminis* DSM 21850 (AJ417499) in 1,505 positions. There are twenty unknown nucleotides in the sequence of *Lb. farciminis* ATCC 29644^T (M58817). Therefore, realignments were carried out using the revised sequence excluding these ambiguous nucleotides, and a significantly high similarity between strain D323 and *Lb. farciminis* ATCC 29644^T (M58817) was observed with only one nucleotide difference in 1,495 positions.

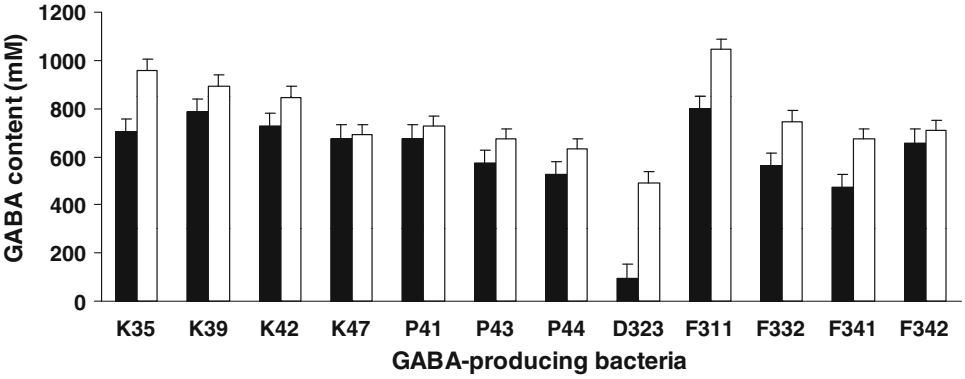

Fig. 2 GABA productivity of LAB isolated from Myanmar fermented fishery products; GABA amounts were determined using an amino acid analyzer after incubation for 6 days in MRS medium with 0.1 mM PLP (*white*) or without PLP (*black*). Data expressed as mean ± SD from three independent experiments

Carbohydrate fermentation profiles of GABA-producing LAB

The carbohydrate fermentation profiles of three representative strains are shown in Table 2. The results of the computer-aided identification based on the fermentation profiles using apiweb version 1.2.1 indicate that strains F311 and K35 have 99.7% and 98.8% identities to *Lb. plantarum* and *Lb. pentosus*, respectively. The profiles of strains F311 and K35 appeared to be similar, but the profile of strain D323 was different from those of strains F311 and K35. All three strains fermented D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, esculin, salicin, D-maltose, and D-trehalose. In addition to these carbohydrates, both strains F311 and K35 fermented L-arabinose, D-ribose, D-galactose, D-mannitol, arbutin, D-cellobiose, D-lactose, D-melibiose, and gentiobiose. Methyl-α-D-mannopyranoside, D-raffinose, D-turanose, and D-arabitol were fermented only by strain F311, and glycerol, sorbitol, methyl-α-D-glucopyranoside, and gluconate were fermented only by strain K35. On the other hand, strain D323 could not be identified as any specific *Lactobacillus* species owing to its ambiguous identification result, because *Lb. farciminis* was not found on the identification list in the instruction manual of API CH.

GABA accumulation in trial fermented products inoculated with GABA-producing LAB

Three isolates (strains D323, F311, and K35) were used as starter cultures to elucidate the accumulation of GABA while processing trial fermentation products. These individuals were selected from each of three fermented products containing higher amounts of GABA (N, PC-1, and NGC-3). In the first experiment, as indicated in Fig. 3, three representative strains were inoculated into trial fermented products corresponding to the isolation source of each strain. When strain D323 was inoculated into tinfoil barb (N-T1) as a starter culture, the highest GABA amount

Table 2 Carbohydrate fermentation profiles of three representative GABA producers isolated from Myanmar fermented fishery products

Substrate	Isolates		
	D323	F311	K35
Glycerol	−	−	+
L(+)-Arabinose	−	+	+
D-Ribose	−	+	+
D-Galactose	−	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Rhamnose	−	−	ND
D-Mannitol	−	+	+
D-Sorbitol	−	−	+
Methyl-α-D-mannopyranoside	−	+	−
Methyl-α-D-glucopyranoside	−	−	+
N-Acetylglucosamine	+	+	+
Amygdalin	−	+	ND
Arbutin	−	+	+
Esculin	+	+	+
Salicin	+	+	+
D-Cellobiose	−	+	+
D-Maltose	+	+	+
D-Lactose	−	+	+
D-Melibiose	−	+	+
D-Sucrose	−	−	ND
D-Trehalose	+	+	+
D-Raffinose	−	+	−
Gentiobiose	−	+	+
D-Turanose	−	+	−
D-Arabitol	−	+	−
Gluconate	−	−	+

Erythritol, D-arabinose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, dulcitol, inositol, inulin, D-melezitose, starch, glycogen, xylitol, D-tagatose, D-fucose, L-fucose, L-arabitol, 2-keto-gluconate, and 5-keto-gluconate were not fermented by three isolates

+, positive; −, negative; *ND* not determined

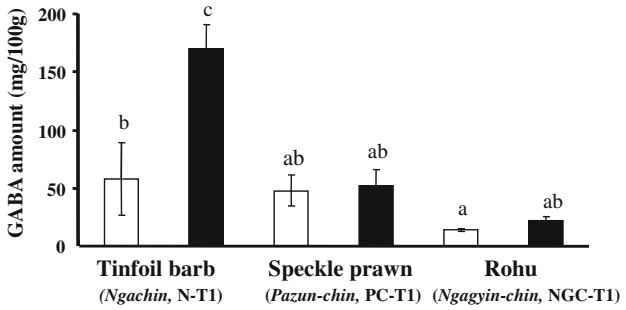

Fig. 3 Accumulation of GABA in three types of trial fishery products fermented with different LAB as starter cultures. N-T1, PC-T1, and NC-T1 were inoculated with strains D323, F331, and K32, respectively. Means with different letters within the same column are significantly different ($P < 0.05$). GABA amounts in samples inoculated with each strain are indicated by *black bars*, and GABA amounts in uninoculated samples are indicated by *white bars*. Data expressed as mean ± SD from four independent experiments

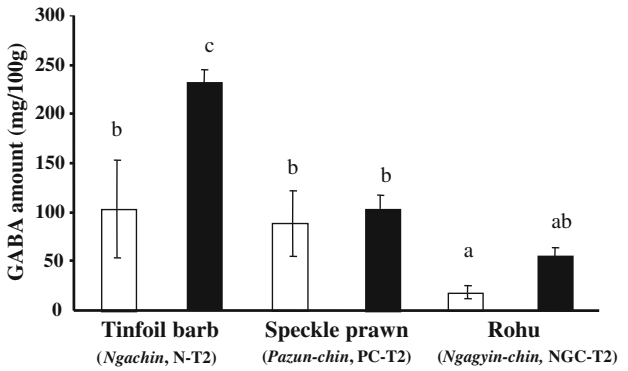

Fig. 4 Accumulation of GABA in three types of trial fishery products fermented with strain D323 as a starter culture. Means with different letters within the same column are significantly different ($P < 0.05$). GABA amounts in samples inoculated with strain D323 are indicated by *black bars*, and GABA amounts in uninoculated samples are indicated by *white bars*. Data expressed as mean ± SD from four independent experiments

was detected in the final fermentation product, reaching 169.7 mg/100 g. On the other hand, a smaller amount of GABA accumulation was observed in fermented speckled prawn (PC-T1) inoculated with strain F311 and in fermented rohu (NGC-T1) inoculated with strain K35. Comparison of the GABA amounts in each of the reference products that were not inoculated with starter cultures revealed that there were markedly significant differences in the GABA amounts between tinfoil barb (N-T1) fermented with and without strain D323. These results suggested that strain D323 is the most effective as a starter culture in fermented fishery products with boiled rice. Therefore, a second trial processing the fermented products was performed to confirm the GABA accumulation effect of strain D323 (Fig. 4). When three types of fermented products inoculated with strain D323 [tinfoil barb (N-T2), speckled prawn (PC-T2), and rohu (NGC-T2)] were compared, the highest GABA amount was obtained in fermented tinfoil barb (N-T2), reaching 231.9 mg/100 g, where significant increases were also found in the inoculated and noninoculated products. In the case of the other two fermentations inoculated with strain D323, a higher GABA amount was found in fermented speckled prawn (PC-T2), whereas no effect on GABA accumulation was observed in comparison with the reference product. Although the GABA amount was low, there was a significant difference between the inoculated and noninoculated products of fermented rohu (NGC-T2).

Discussion

Traditional fermented fish products are classified into two groups, namely the more widely known fish/salt formulations, such as fish paste and sauce, and fish/salt/carbohydrate blends [42]. Among them, fermented fishery products with boiled rice, which are called by different names locally, are manufactured and consumed widely in East and Southeast Asia. Except for the Myanmar fermented products used in this study, they are known to include, for example, *pla-ra*, *plaa-som*, *pla-chom*, and *kung-chom* in Thailand, *burong-isda* in the Philippines, *phaak* in Cambodia, and *funa-zushi* in Japan. All the Myanmar fermented fishery products used in this study had acidic pH ranging from 3.8 to 4.8 owing to the LAB. On the other hand, the highest NaCl concentration was 8.6%, whereas the lowest was 3.8%. The pH and NaCl concentration of other fishery products fermented with boiled rice in neighboring countries have previously been shown. The fermented fish product *pla-ra* was found to have high NaCl concentration around 11.5–23.7%. In contrast, the fermented small fish product *pla-chom* and the small shrimp product *kung-chom* had NaCl concentrations similar to those of our products, ranging from 3.2% to 9.4% (w/w) [43]. *Pla-ra*, *pla-chom*, and *kung-chom* were also found to be acidic, with pH ranging from 4.3 to 5.6, 4.2 to 6.2, and 3.9 to 4.7, respectively. Despite the geographic distance, the properties of these products seemed to be similar to those of the Japanese *funa-zushi*. Kubo et al. [44] analyzed four types of Japanese *funa-zushi* and reported that these products contain 2.1–5.8% (w/w) NaCl with pH from 3.7 to 4.0.

In this study, 12 GABA-producing LAB were isolated from four types of Myanmar fermented fishery products with boiled rice in accordance with the results of both TLC and amino acid analysis. These producers accounted for approximately 10% of the total number of isolates, and they were isolated from five fermented samples. These results indicate that GABA-producing LAB are widely distributed in Myanmar fermented fishery products with

boiled rice. In a previous study, GABA-producing *Lb. paracasei* was isolated from Japanese crucian carp fermented with boiled rice [21]. Moreover, *Lb. brevis* was also isolated as a GABA producer from *plaa-som*, a Thai fermented fish product with boiled rice [28]. Although this finding in *plaa-som* encouraged us to undertake our investigation, their detailed results concerning *Lb. brevis* have not been clarified at present. Therefore, our report is not only the first to show that GABA-producing LAB are distributed in Myanmar fermented fishery products, but also the first detailed one concerning GABA-producing LAB in Southeast Asia fermented fishery products with boiled rice.

Prior to the application of GABA-producing LAB for GABA accumulation in trial fermented fishery products, we carried out several experiments to clarify GABA productivity and the taxonomical characteristics of our isolates. Addition of PLP to the culture media was also examined in relation to GABA productivity, because PLP has been described as a necessary coenzyme of GAD [40]. Therefore, we expected that addition of PLP to the culture medium would affect the GABA production of our isolates. In previous study, Komatsuzaki et al. [21] described that even 0.01 mM PLP effectively enhanced the GABA production of *Lb. paracasei* isolated from *funa-zushi*. Therefore, although our isolates were identified as a different species, in our experiment for the enhancement of GABA production, 0.1 mM PLP was supplemented to the culture media, which was expected to be an adequate amount. GABA producers in this study were divided into two groups on the basis of their GABA production. In the case of strain D323, addition of PLP contributed markedly to the enhancement of GABA productivity. In contrast, the effects of PLP addition on GABA production in the other eleven isolates seemed to be smaller than that in the case of strain D323. PLP addition was found to effectively promote GABA production in the culture medium during incubation with *Lb. paracasei* [21]. However, PLP has not always been shown to effectively increase GABA production in all LAB described previously as GABA producers. Jun et al. [27] reported that the activity of the purified GAD of *Lb. brevis* is slightly increased by addition of PLP, and they also mentioned a possible reason, namely that the strong integration of PLP with GAD of this strain results in a holoenzyme, causing minimal enhancement of enzymatic activity. Denaturation of PLP in the culture medium during cultivation was also considered as a reason for the minimal enhancement of GABA production of *St. salivarius* subsp. *thermophilus* Y2 [34].

From the results of the phenotypic characterization, all the GABA producers isolated in this study belonged to the genus *Lactobacillus*. In addition, 16S rRNA gene sequencing results and carbohydrate fermentation ability determined using API CH indicate that three representative strains (F311, K35, and D323) having different GABA productivities can be identified as *Lb. plantarum*, *Lb. pentosus*, and *Lb. farciminis*, respectively. These *Lactobacillus* species, including *Lb. plantarum*, *Lb. pentosus*, and *Lb. farciminis*, were previously isolated from various types of similar fermented products in Thailand [43].

In this study, trial fermented fishery products were processed using three representative strains (F311, K35, and D323), which were isolated from different types of fermented fishery products. Therefore, in the first experiment, we used them as starter cultures for the trial fermentation corresponding to the origin of each isolate, and the highest GABA accumulation was observed in the fermented tinfoil barb product (N-T1) inoculated with strain D323. In addition, in the second experiment, in which strain D323 was used as the starter culture, the highest amount of GABA accumulated in the fermented tinfoil barb product (N-T2) as well as in the former product (N-T1). These results indicate that strain D323 is a useful starter culture in terms of GABA accumulation, and can be used as a starter culture for manufacturing of GABA-enriched tinfoil barb fermented in boiled rice. At present, the reason why strain D323 is so useful for GABA accumulation in tinfoil barb fermentation is unknown, because suitable and optimal culture conditions for GABA production in vitro are not currently understood, although the behaviors of starter cultures are known to be affected by various complex factors in the fermentation environment. Therefore, detailed investigation of strain D323, in terms of the effect of culture conditions on GABA productivity under various broth culture conditions, and the biochemical and enzymatic characterization of GAD, as well as gene cloning, are underway. Moreover, detailed study on other GABA producers, such as strains F311 and K35 in comparison with strain D323, should be performed.

We are currently unaware of any reports on GABA amount in Myanmar fermented fishery products. Therefore, we also investigated GABA amount in several samples of four types of fermented fishery products with boiled rice purchased in Myanmar. As shown in Table 1, among them, the highest GABA amount was detected in fermented tinfoil barb, compared with those in fermented speckled prawn, fermented rohu, and fermented featherback fish. These results indicate that the isolation source of strain D323, which was shown to be a useful starter culture in this study, corresponds to the fermented fishery product containing the highest GABA amount. Although tinfoil barb fermentation itself might provide a suitable environment for GABA accumulation because of some factors, these results suggested that GABA producers belonging to *Lb. farciminis*, such as strain D323, contribute to GABA accumulation during tinfoil barb fermentation owing to the

wide distribution of *Lb. farciminis*, just like in Thai fermented fishery products described previously [43]. In addition to that, GABA producers were isolated in the fermented product containing no GABA (NPC-4). However, in most cases GABA producers were not isolated in the fermented products containing small amounts or no GABA.

In conclusion, this study is the first to show in detail the distribution of GABA-producing LAB in Southeast Asian fermented fishery products. In addition, to our knowledge, this is the first time that GABA-producing LAB has been applied as a starter culture for the fermentation of fishery products. The accumulation of GABA in the Myanmar fermented tinfoil barb product in boiled rice (*ngachin*) using strain D323 as a starter culture appears to be of considerable interest for the manufacturing of traditional fishery products. These findings may contribute to enhancing the health benefits and increasing the commercial value of traditional fermented fishery products.

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