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A comprehensive investigation into the production of gamma-aminobutyric acid by *Limosilactobacillus fermentum* NG16, a tuna gut isolate

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ABSTRACT

Gamma-aminobutyric acid (GABA), a four-carbon non-protein amino acid, is widely known to have multiple physiological functions. The present study aimed to investigate the cultivation parameters for GABA production by a lactic acid bacteria (LAB) strain isolated from a tuna gut sample. Among 60 tuna gut LAB, only 7 *Limosilactobacillus fermentum* isolates, i.e. NG01, NG12, NG13, NG14, NG16, NG23, and NG27, were capable of GABA fermentation, with NG16 being the most potent GABA producer. The GABA production by isolate NG16 was therefore thoroughly characterised. The optimal batch culture conditions for GABA production were an initial cell density of 5×10^6 CFU mL^{-1}, a monosodium glutamate concentration of 2%, an initial pH of 7, a fermentation temperature of 35 °C, and an incubation time of 96 h. Under this cultivation conditions, NG16 produced a maximum GABA yield of 25.52 ± 0.41 mM.

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KEYWORDS

tuna gut, GABA, *Limosilactobacillus fermentum*, lactic acid bacteria, optimisation

1. INTRODUCTION

Gamma-aminobutyric acid (GABA) is a non-protein amino acid, which is widely present in nature including animals, plants, and microorganisms. It plays an important role in the central nervous system as an inhibitory neurotransmitter that may regulate blood pressure and diuretic and tranquiliser effects (Somkuti et al., 2012). In addition, it possesses antioxidant, hypolipidemic, and anti-inflammatory properties (Diana et al., 2014). Fermentation processes can increase the GABA content in food, and recent studies have focused on the development of GABA containing functional foods (Ratanaburee et al., 2013). A variety of microorganisms can be used as starter cultures for GABA fermentation. Among these, lactic acid bacteria (LAB) are the most popular because of their GRAS (generally recognized as safe) status. Many LAB, such as *Lactococcus lactis* (Laroute et al., 2016), *Companilactobacillus futsaii* (Sanchart et al., 2017), *Corynebacterium glutamicum*, *Lactiplantibacillus plantarum* (Yang et al., 2015), and *Streptococcus thermophilus* (Han et al., 2020), have the capacity to produce high amounts of GABA.

The GABA production capacity of LAB depends on the specificity and activity of various intracellular enzymes. Glutamic acid decarboxylase (GAD, EC 4.1.1.15) consists of the GAD enzyme (encoded by *gadA* or *gadB*) and the glutamate/GABA antiporter *GadC* (Yunes et al., 2016). GABA is formed by the decarboxylation of L-glutamate transported into a cell through *GadC* by GAD with pyridoxal-50-phosphate (PLP) as a cofactor. GABA is then released into the culture medium by *GadC*. In addition, alpha-ketoglutaric acid generated in the TCA cycle is converted into L-glutamate by L-glutamate dehydrogenase (GDH, EC 1.4.1.4). Conversely, GABA can be degraded to succinic semialdehyde through the activity of GABA aminotransferase (GABA-AT, EC 2.6.1.19). This complex intracellular enzyme system results in a strain-specific GABA production activity (Cui et al., 2020), which is influenced by cultivation conditions (Sanchart et al., 2017). Therefore, scrutinous strain selection and optimisation of fermentation conditions are of paramount importance to obtain high GABA yields.

Fish sauce is a popular fermented product in Vietnam and many other Asian countries. Its production involves metabolic activities of fish gut microbiota, among which LAB play a vital role as they enhance flavour and quality of the fermented product. In the present study, we identify and optimise the cultivation conditions for GABA production by a LAB strain derived from tuna gut.

2. MATERIALS AND METHODS

2.1. Isolation of lactic acid bacteria

Entire gut fractions of 40 freshly caught tuna were used to isolate LAB. Samples were homogenised in Ringer's solution (Sigma-Aldrich, Milan, Italy), serially diluted, plated onto MRS agar

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(Oxoid, Milan, Italy), and anaerobically incubated at 37 °C for 48 h. Colonies were randomly selected, Gram stained, and catalase tested. Gram-stain-positive and catalase-negative isolates were considered to be presumptive LAB and were stored at –80 °C for further analysis.

2.2. Preparation of inoculants

LAB isolates were grown at 37 °C for 24 h in MRS broth in screw cap tubes. Cell pellets obtained after centrifuging (Universal 320R, Hettich GmbH & Co. KG, Tuttlingen, Germany) 24 h old cultures at 12,000 *g* for 5 min at 4 °C were resuspended in Ringer's solution. OD_{600} values were used to standardise cell suspensions as inoculants.

2.3. Screening of GABA-producing LAB

LAB isolates were grown for 24 h in MRS broth in screw cap tubes containing 1% (*w/v*) monosodium glutamate (MSG) with an initial pH of 6.2 and at 37 °C. The GABA content in the supernatant obtained after centrifuging (Universal 320R, Hettich GmbH & Co. KG, Tuttlingen, Germany) at 12,000 *g* for 5 min at 4 °C was analysed by HPLC as described below.

2.4. Identification of LAB isolates

Isolates were grown on MRS agar under anaerobic conditions at 37 °C for 48 h. Third-generation bacterial cells were harvested and identified following a MALDI-TOF MS method described previously (Thuy et al., 2021).

2.5. Optimisation of culture conditions for GABA production

The factors studied included MSG concentration (0, 0.5, 1, 1.5, and 2%), initial cell density (5×10^5, 10^6, 5×10^6, 10^7, and 5×10^7 CFU $mL^{-1)}$, initial pH (4, 5, 6, 7, 8, and 9), culture temperature (30, 35, 40, 45, and 50 °C), and incubation time (24, 48, 72, 96, and 120 h). When assessing the effect of one culture condition on GABA production the other factors were kept constant.

2.6. Quantification of GABA content

The supernatants obtained after centrifugation (Universal 320R, Hettich GmbH & Co. KG, Tuttlingen, Germany) of cultures at 12,000 *g* for 5 min at 4 °C was 10-fold diluted with deionised water. The dissolved proteins in the supernatants were removed by adding 1 mL of 3% sulfosalicylic acid and centrifuging at 6,000 r.p.m. for 5 min. GABA from the resulting supernatants was then derivatised by dabsylation with 4 mM 4-dimethylaminoazobenzen-4-sulfonyl chloride at 70 °C for 20 min and quantified by using an HPLC method as described previously (Thuy et al., 2021).

2.7. Statistical data analysis

All data are reported as mean ± SD from triplicate experiments. One-way ANOVA, Tukey's HSD and Duncan's multiple range tests were used to compare the means. A difference was considered statistically significant if $P \leq 0.05$. All analyses were conducted using SPSS v. 16.0 (SPSS Inc, Chicago, IL USA).

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3. RESULTS AND DISCUSSION

3.1. Screening of GABA-producing LAB

The screening of multiple LAB isolates is essential to identify GABA-producing strains. Among 60 tuna gut isolates tested, only seven, i.e. NG01, NG12, NG13, NG14, NG16, NG23, and NG27, were capable of GABA production, with NG16 as the most potent GABA producer (Fig. 1). Upon initial screening in MRS broth (comprising an initial cell density of 10^7 CFU mL^{-1}, 1% monosodium glutamate, pH 6.2, 37 °C) for 24 h, the GABA content in spent culture medium produced by this NG16 isolate was 13.21 ± 0.31 mM.

3.2. Identification of LAB isolates

Sixty tuna gut isolates were characterised using MALDI-TOF MS. The obtained mass spectra were compared with those of taxonomically well characterised reference strains in an in-house built reference database (Wieme et al., 2014; Lappa et al., 2021). Isolates were considered identified when their spectra clustered among taxonomically well characterised reference strains of a single LAB species, as described by Wieme et al. (2014). The mass spectra of the isolate NG16 coded as R-66963 belonged to a cluster that was assigned to *Limosilactobacillus fermentum*.

3.3. Effect of culture conditions on GABA production by *L. fermentum* NG16

3.3.1. Initial cell density. Previous studies showed that the dynamics of GABA fermentation by LAB species is greatly affected by initial cell density (Ratanaburee et al., 2013; Thuy et al., 2021). We, therefore, investigated the impact of using different initial cell densities of *L. fermentum* NG16 on its extracellular GABA production. Initial cell densities in MRS broth ranged from

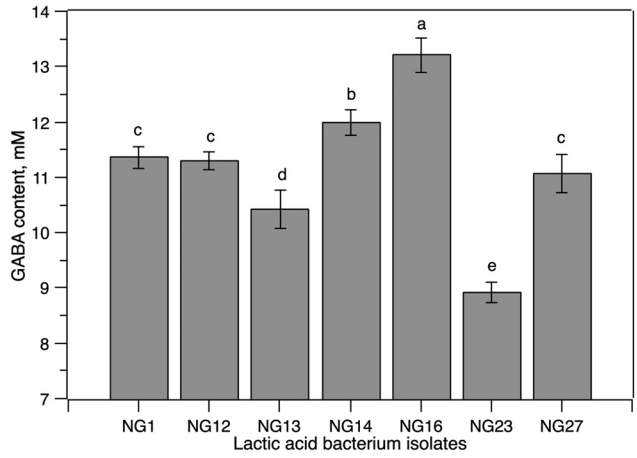

Fig. 1. GABA production by 7 lactic acid bacterium isolates. Bars with different letters differ significantly at $P < 0.05$

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5×10^5 to 5×10^7 CFU mL^{-1}, while other conditions were kept constant, including MSG of 1% (w/v), temperature of 37 °C, initial pH of 6.2 and fermentation time of 24 h. An initial cell density of 5×10^6 CFU mL^{-1} yielded the highest GABA content in the spent culture medium, i.e. 16.30 ± 0.11 mM (Fig. 2). The lower GABA yields obtained at cell density of 5×10^5 CFU mL^{-1} and 10^6 CFU mL^{-1} likely resulted from the lower number of cells present, which might even had to adapt to a nutrient-rich environment, resulting in a delayed GABA peak yield (Thuy et al., 2021). Higher cell densities, in contrast, may provoke stress (Bunch, 1994), which leads to a decrease in GABA production. In comparison, *Levilactobacillus namurensis* NH2 and *Pediococcus pentosaceus* HN8 at a cell density of 10^6 CFU g^{-1} in a Thai fermented pork sausage produced a maximum GABA amount of 4.501 mg kg^{-1}, which was higher than those at cell densities of 10^7 and 10^8 CFU g^{-1} (Ratanaburee et al., 2013).

3.3.2. Monosodium glutamate concentration. In many microorganisms including LAB, L-glutamate is converted to GABA via a decarboxylation reaction catalysed by GAD. As most LAB are unable to synthesise enough L-glutamic acid for GABA production (Cui et al., 2020), we supplemented various concentrations of monosodium glutamate (MSG) to the culture medium of *L. fermentum* NG16. The results showed that with an increase in MSG concentration from 0 to 2%, the yield of GABA proportionally increased as well (Fig. 3). This suggested that the increase in MSG concentration stimulated the production of GABA via the GABA shunt pathway (Feehily et al., 2013). The GABA content reached a maximum (18.13 ± 0.12 mM) at an MSG concentration of 2% (*w/v*). Various microorganisms have different optimal concentrations of MSG. For example, *L. brevis* CRL 1942 produces the highest amount of GABA at the optimal MSG content of ~4.6% (*w/v*) (Villegas et al., 2016), while the optimal glutamate concentration for GABA production by *Lacticaseibacillus paracasei* NFRI 7415 is 8.46% (*w/v*) (Komatsuzaki et al., 2005).

3.3.3. Initial pH. The pH of the culture medium is a key parameter affecting GABA production by LAB as it affects not only bacterial growth but also the activity of the GAD system (Cui et al.,

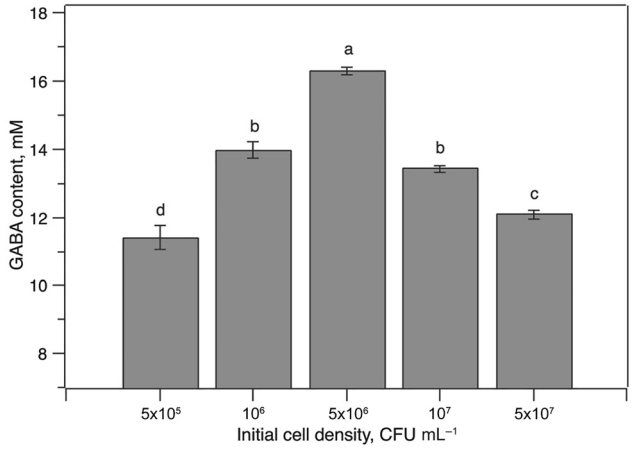

Fig. 2. GABA production by *L. fermentum* NG16 with different initial cell densities. Bars with different letters differ significantly at $P < 0.05$

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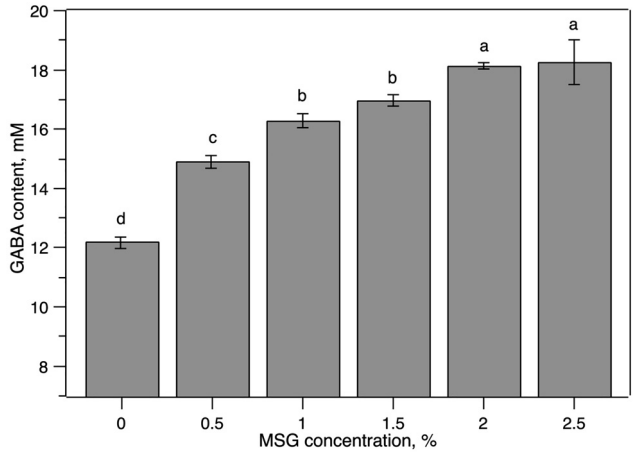

Fig. 3. GABA production by *L. fermentum* NG16 with different monosodium glutamate concentrations. Bars with different letters differ significantly at $P < 0.05$

2020). The C-terminal region of GAD appeared associated with the enzyme's pH dependency (Yu et al., 2012). We, therefore, investigated the effect of initial pH on GABA synthesis by *L. fermentum* NG16. The results demonstrated that the GABA content in spent culture medium increased significantly with an increase in initial pH from 5 to 7 (Fig. 4). The highest GABA yield of 19.88 ± 0.33 mM was obtained at initial pH 7. At initial pH 8, GABA production decreased to 16.79 ± 0.18 mM. This reduction may be due to the inhibition of microbiological growth in the alkaline medium. It should be noted that the production of lactic acid during the fermentation process will cause a decrease in pH of the culture medium, which will affect both

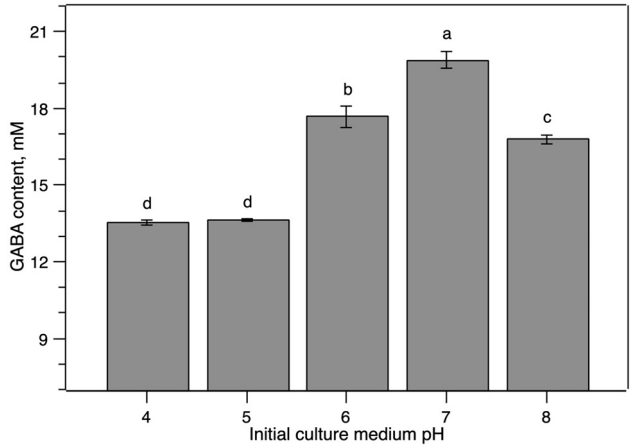

Fig. 4. GABA production by *L. fermentum* NG16 at different initial culture medium pH values. Bars with different letters differ significantly at $P < 0.05$

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growth and activity of the GAD system. Thus, it was possible that strain *L. fermentum* NG16 had optimal growth or optimal GAD activity at initial pH 7 given that highest GABA yield was obtained at this initial pH value. In comparison, Komatsuzaki et al. (2005) reported that an initial pH of 5 was optimal for GABA production by *L. paracasei* NFRI 7415, while *L. brevis* GABA100 produced a maximum amount of GABA at initial pH 3.5 in the fermentation of black raspberry juice (Kim et al., 2009).

3.3.4. Culture temperature. Together with culture pH, fermentation temperature is also a key factor affecting GABA production by LAB (Cui et al., 2020). In the present study, *L. fermentum* NG16 was inoculated into MSR broth at various culture temperatures, while initial cell density, MSG concentration, and initial pH were kept at 5×10^6 CFU mL^{-1}, 2% (w/v), and 7, respectively. The GABA content in spent culture medium was quantified after 24 h of fermentation. The concentration of GABA increased markedly from 30 to 35 °C, reaching a peak of 21.29 ± 0.59 mM, but decreased gradually with further increase in incubation temperature (Fig. 5). The temperature may affect GABA production by acting on cell physiology or GAD activity. In this study, the temperature above 35 °C may have negative effect on cell growth, resulting in a reduction of GABA accumulation in the spent culture medium. Besides, incubation temperature may also affect GAD system in *L. fermentum* NG16, which needs to be further evaluated as the effect of temperature on GAD activity varies with LAB strains. For example, Shin et al. (2014) demonstrated that optimal temperature for the activity of GAD in *L. plantarum* ATCC 14917 was 40 °C, while Liu et al. (2021) documented that GAD purified from *L. brevis* F109-MD3 had an optimal temperature for its activity at 65 °C. In fact, different strains even of the same species produce maximum amount of GABA at different temperatures. For instance, the *L. brevis* strain GABA 100 produced a higher amount of GABA at a fermentation temperature of 30 °C compared to 37 or 25 °C (Kim et al., 2009), while the optimal temperature for GABA synthesis by *L. brevis* CRL 1942 was 37 °C (Villegas et al., 2016). In the present study, the highest GABA

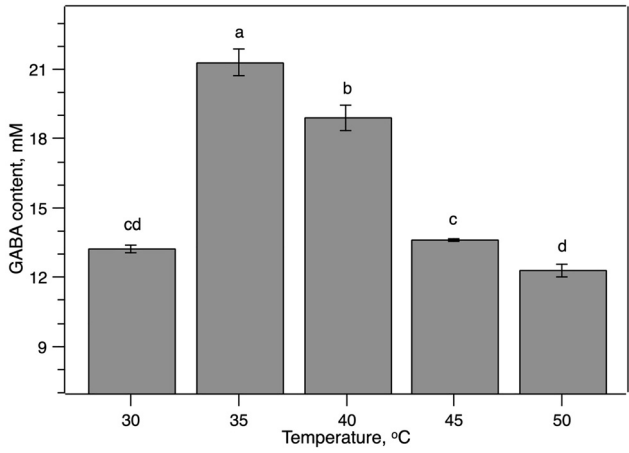

Fig. 5. GABA production by *L. fermentum* NG16 at different culture temperatures. Bars with different letters differ significantly at $P < 0.05$

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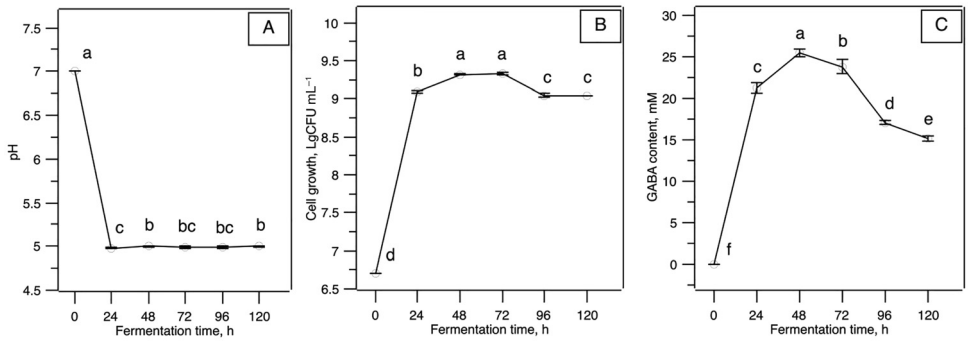

Fig. 6. Change of pH value (A), cell growth (B), and GABA concentration (C) during fermentation with *L. fermentum* NG16. Means with different letters differ significantly at $P < 0.05$

yield was obtained at a culture temperature of 35 °C, thus, this temperature was used for further experiments.

3.3.5. Time course study of pH, cell growth, and GABA production by* L. fermentum *NG16. Previously reported data showed that GABA accumulation in culture media is a function of fermentation time (Villegas et al., 2016; Thuy et al., 2021). Therefore, the effect of time on GABA yield as well as medium pH and cell growth of *L. fermentum* NG16 was investigated under optimal conditions of initial cell density, MSG concentration, initial pH, and fermentation temperature as determined above (Fig. 6). The results showed a concurrent increase in cell density and GABA content, which corresponded with a decrease in pH during the first 24 h. The pH decrease likely reflected lactic acid production by *L. fermentum* NG16. During the first 24 h of fermentation the pH value dropped sharply from 7 to around 5 and then remained stable until the end of the experiment at day 5 (Fig. 6A). The cell density increased sharply from around 7 to over 9 log CFU mL^{-1} during the first 24 h of fermentation. It then slowly increased further to reach a maximum of 9.334 ± 0.013 log CFU mL^{-1} after 72 h of fermentation after which it started to decline. The reduction in cell density after 72 h of fermentation (Fig. 6B) might be caused by nutrient depletion as well as autolysis of dead cells. Finally, during the first 24 h of fermentation, the GABA content increased proportionally with the increase in cell density and a decrease in pH, suggesting that GABA production occurred concurrently with lactic acid fermentation. In addition, there was a correspondence between GABA yield and cell density from 0 to 48 h of fermentation and a maximum level GABA concentration of 25.52 ± 0.41 mM was reached at 48 h of fermentation. After this 48 h period, GABA in the spent culture medium might be absorbed into the cells and degraded by GABA aminotransferase (Feehily et al., 2013) leading to a decrease in GABA yield (Fig. 6C).

4. CONCLUSIONS

Among 60 LAB isolates obtained from tuna gut samples, 7 were capable of GABA production. *L. fermentum* strain NG16 yielded the highest concentration of GABA in MRS broth

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supplemented with 1% (*w/v*) MSG, and its production of GABA was subsequently thoroughly characterised. The optimal batch culture conditions for GABA production by this strain were an initial cell density of 5×10^6 CFU mL^{-1}, an MSG concentration of 2% (*w/v*), an initial pH of 7, a fermentation temperature of 35 °C, and an incubation time of 48 h. Under these optimal conditions, a maximum GABA level of 25.52 ± 0.41 mM in spent culture medium was obtained. The results of the present study provided a basis for the development and production of functional foods containing a high level of GABA.

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