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Health-Promoting Role of Fermented Pigeon Pea (*Cajanus cajan* L (Mill)) Milk Enriched with γ-aminobutyric Acid (GABA) Using Probiotic *Lactiplantibacillus plantarum* Dad-13

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Abstract: This study aimed to enhance γ-aminobutyric acid (GABA) in pigeon pea milk (CCM). The drink was prepared from germinated pigeon pea and fermented using the probiotic *Lactiplantibacillus plantarum* Dad-13. Various nutrients significantly increased the GABA content in pigeon pea milk, i.e., sucrose 3% (4409 mg/L), monosodium glutamate (MSG) 1% (59,562 mg/L), and whey 4% (5283 mg/L), respectively. Glutamate decarboxylase (GAD)-encoding genes were identified in the genome of the strain. The strain carried only one *gad*B gene, and no other *gad* genes were found in the genomes when compared with other strains. During fermentation, various metabolites, including organic acids, amino acid derivatives, and flavonoids, were detected. These metabolites may promote anti-inflammatory activity in cytokines such as TNF-α and IL6. In conclusion, the development of fermented pigeon pea enriched with GABA using probiotic *L. plantarum* Dad-13 shows promising potential as a functional food that can promote health benefits and help prevent diseases.

Keywords: pigeon pea; GABA; *L. plantarum* Dad-13; functional foods; anti-inflammatory

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1. Introduction

Fermented foods have been a part of the human diet for thousands of years and involve indigenous microbes to provide unique characteristics as well as nutritive values. In recent decades, the development of functional foods based on fermentation techniques has attracted several researchers. Lactic acid bacteria (LAB) play a significant role during food fermentation and their use represents a safe and natural method of food preservation [1]. LAB also provide several important metabolites such as vitamins, amino acids, fatty acids, biogenic peptides, and GABA [2,3]. GABA is a non-protein amino acid which plays an important role as an inhibitory neurotransmitter in the mammalian nervous center [4].

GABA is synthesized via a decarboxylation reaction of L-glutamic acid, and the reaction is catalyzed by glutamate decarboxylase (GAD, E.C.4.1.1.15) which requires pyridoxal 5′-phosphate as a cofactor [5]. GABA has been reported to modulate brain functions and reduce anxiety, promote sleep, and enhance mood [6]. The administration of 46.7 mg/mL of GABA significantly improved neurological disorders in mice [7]. These studies also suggest that high doses of GABA intake may be necessary for the efficacy of GABA. In

human trials, Okada et al. demonstrated that the daily consumption of rice germ, containing 26.4 mg of GABA, three times a day was significantly effective in depression and sleeplessness in menopausal women [8]. In addition, microbial GABA production by *L. buchneri* MS, isolated from kimchi, showed a complete protection of neuronal cells against neurotoxicant-induced cell death [9]. Several studies have reported that GABA exhibits anticancer, antiobesity, and antihypertensive activities [10–12].

The use of LAB as a starter culture is a safe and more natural approach for GABA production, primarily due to the GRAS status of LAB. GABA production in LAB is part of their natural defense under acidic conditions, facilitated by the glutamate-dependent-acid-resistant system (GDAR) [4,13]. During the formation of GABA, the system utilizes an intracellular proton along with glutamate and exchanges GABA for a different glutamate substrate, resulting in an increase in intracellular pH through proton consumption [14]. GABA is primarily secreted into the extracellular environment, with the yield varying among different strains and species [15–18]. Additionally, the yield is influenced by the organization and biochemical properties of GAD genes [19,20]. Several LAB and probiotic strains, including *L. pentosus*, *L. brevis*, *L. plantarum*, and *L. lactis*, are capable of producing GABA [13,21]. The strain *L. plantarum* Dad-13 is a probiotic strain isolated from *dadih*, a fermented buffalo milk from West Sumatra, Indonesia. The strain exhibits probiotic properties, including a resistance to gastrointestinal conditions and high concentrations of bile salts, antibacterial activity, the ability to survive and colonize the human gastrointestinal tract, and stimulate gastrointestinal function in healthy adults [22,23]. The GABA-producing ability of this strain has not been assessed. However, the use of this probiotic strain in the production of GABA-enriched fermented foods may offer advantageous effects.

The development of GABA-enriched foods using LAB, particularly from plant-based sources, has attracted the attention of food scientists. This interest stems from the insufficient GABA content in the human diet and the growing awareness of the importance of a healthy lifestyle. Additionally, LAB-produced bioactive compounds in foods have demonstrated various health-promoting effects, including anti-inflammatory, antihypertensive, antiobesity, and anticancer activities [24,25]. Pigeon pea (*Cajanus cajan*) is a legume that is often underutilized in the human diet due to its poor preference and long cooking times [26]. The use of LAB has been extensively applied in plant-based fermentation (grains and beans) mainly to improve nutritional content. Therefore, the development of pigeon pea milk enriched with GABA, by the probiotic *L. plantarum* Dad-13, is of great interest for the promotion of human health. This study aimed to develop pigeon pea milk containing GABA using the probiotic strain *L. plantarum* Dad-13. In addition, we also investigated the metabolite profile of fermented pigeon pea milk during fermentation, the anti-inflammatory activity of fermented pigeon pea milk on cytokines (IL6, IL10, TNF-α), as well as the organization of the glutamate decarboxylase gene (*gad*) in the genome of *L. plantarum* Dad-13.

2. Materials and Methods

2.1. Materials

Pigeon pea was obtained from a local market and the beans were vacuum packed prior to use. The strain *L. plantarum* Dad-13 was obtained from FNCC (Food and Nutrition Culture Collection), Center of Food and Nutrition Studies Universitas Gadjah Mada, Yogyakarta, Indonesia. The strain *L. plantarum* Dad-13 was inoculated in de Man, Rogosa, and Sharpe (MRS) broth and incubated at 37 °C for 24–48 h. GABA standard was obtained from Sigma Aldrich (St. Louis, MO, USA). All chemicals were of the highest grade.

2.2. Production of Fermented GABA Pigeon Pea Milk (CCM)

Pigeon peas were cleaned and washed using tap water to remove impurities. Pigeon peas (250 g) were allowed to soak for 6 h in 1 L of distilled water at room temperature. After soaking, the water was drained and the beans were dried using paper towels. The beans were allowed to germinate for 36 h at room temperature in dark conditions. After

germination, the beans were ground using a blender (Phillips, Jakarta, Indonesia) with a ratio of 1:7 of tap water. The resulting mixture was filtered through a cheesecloth to obtain raw CCM. The raw CCM was pasteurized at 90 °C for 20 min to eliminate the endogenous microorganism. In total, 200 mL of CCM was poured into a 250 mL conical flask. Subsequently, additional nutrients such as MSG (0–1%), whey isolate (0–4%), and sucrose (0–3%) were added into the CCM drink. The mixtures were then sterilized at 121 °C for 5 min prior to fermentation to eliminate spoilage microorganisms. Freshly prepared CCM milk was inoculated with *L. plantarum* Dad-13 (5% *v*/*v*) with an approximate final cell density of 9 log CFU/mL. Fermentation was carried out in a closed container at 30 °C for 48 h without shaking. The GABA production in fermented CCM was further determined. The total LAB in CCM was determined using a spread plate technique on MRS agar (Oxoid, MA, USA). The pH and acidity of CCM were determined using a pH meter (Ohaus, NJ, USA) and titration according to AOAC (1995).

2.3. Determination of GABA

The concentration of GABA in the CCM drink was determined using ultra performance liquid chromatography (UPLC Acquity H-Class, Waters Corporation, Milford, MA, USA) with a PDA detector. The samples were hydrolyzed using 6 N HCL and followed by a derivatization of the samples and the GABA standard for UPLC analysis [27]. The samples were derivatized using the Tag ultra-derivatization kit (Waters, Milford, MA, USA) following the manufacturer's instructions. The derivatized samples were then injected into the Acquity UPLC H class. The mobile phase of UPLC consists of AccQ. Tag Ultra Eluent A 100%; Accq. Tag Ultra Eluent B (Aquabides 90:10); Aquabides Eluent C; AccQ. Tag Ultra Eluent B 100%. The operating system was used at a flow rate of 0.5 mL/min and at a temperature of 49 °C with a wavelength of 260 nm.

2.4. Determination of Metabolite Profile

The metabolite profile in CCM was determined using UPLC-QTOF-MS/MS analysis. The analysis was performed using ACQUITY UPLC with the BEH column (1.8 µm, 2.1 × 50 mm; Waters, Wayland, MA, USA). The eluent system used a binary gradient with mixture A consisting of water and formic acid (99.9:0.1) and mixture B consisting of acetonitrile and formic acid (99.9:0.1). The linear gradient was set from 2 to 95% B (0–15 min), and the flow rate was 0.2 mL/min. Metabolite profiling was conducted by coupling the ACQUITY UPLC system to the Quadropole time-of-flight mass spectrometer Xevo G2-S QToF (Waters, Wayland, MA, USA) with an electrospray ionization interface (ESI) in the positive ionization mode. Nitrogen was used as the desolvation gas and set to 350 °C with a flow rate of 793 L/h. The collision energy was adjusted to a low voltage (4 V), and the ramp collision energy voltages were 25–60 V, respectively. The mass analysis range was 50–1200 *m*/*z*. The precise molecular mass and molecular formula were analyzed using MassLynx 4.1 (Waters, Wayland, MA, USA).

2.5. Bacterial Genomic Extraction and Bioinformatic Analysis

Genomic DNA was extracted using the SDS method [28]. The harvested DNA was analyzed via agarose gel electrophoresis using a QubitR 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA). Genome annotation was performed using the online program Rapid annotation using Subsystem Technologies (RAST) SEED. Additionally, the program was used to determine the orientation of the *gad* gene https://rast.nmpdr.org/ (accessed on 27 March 2023). Genome assembly was conducted using SOAP denovo software to contig with SPAdes-3.12.0 (Linux, Belton, MO, USA) in a Linux environment [29]; two different K-mers (99 and 127) were selected for assembly.

2.6. Anti-Inflammatory Activity

The anti-inflammatory activity of fermented CCM was assessed using an ELISA kit (GAMA Biotech, Malang City, Indonesia) following the manufacturer's instructions. After

centrifugation at 2000 RPM for 20 min, the samples were adjusted to pH 7.2–7.4 using PBS buffer. Next, 40 µL of the samples was added to the wells, followed by the addition of 10 µL of IL6, IL10, and TNF-α. The mixture was then combined with 50 µL of streptavidin-HRP and incubated at 37 °C for 60 min. Subsequently, the plates were washed with buffer, and 50 µL of substrate solution A and solution B were added to each well. After incubating for 10 min at 37 °C in the dark, 50 µL of stop solution was added, and the optical density (OD) at 450 nm was measured within 10 min of adding the stop solution.

2.7. Statistical Analysis

Statistical analysis was performed using IBM SPSS (Ver. 25, Inc., Chicago, IL, USA). A one-way ANOVA and the Multiple Duncan Range Test were used to determine the significant differences ($p < 0.05$) among treatments. The results are expressed as mean ± standard deviation (SD). All experiments were performed at least in duplicate.

3. Results and Discussion

3.1. Effects of Nutrients on GABA Production

Due to the beneficial effects of GABA on human health, numerous attempts have been made to enhance GABA production during food fermentation. Figure 1 illustrates the critical role of nutrient fortification, including glutamate, carbon source, and nitrogen source, prior to fermentation in GABA production.

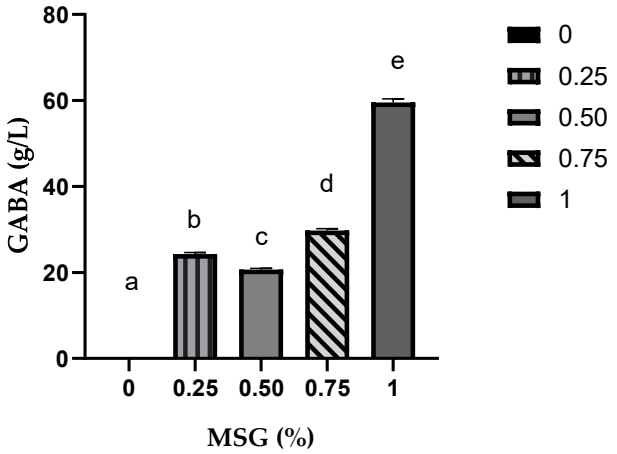

Figure 1. Effect of MSG on GABA production in fermented CCM. The results are expressed as mean ± SD. Different letters above the bars indicate significant differences at $p < 0.05$.

The addition of L-glutamate in the form of MSG is a crucial factor in increasing GABA production during fermentation. Currently, MSG is the most important additive for significantly enhancing GABA production due to its affordability and widespread availability. Different concentrations of MSG (ranging from 0% to 1%) were added to the CCM, and it was found that the addition of 1% MSG significantly improved GABA production during a 48 h fermentation period ($p < 0.05$). Among the different concentrations tested, 1% MSG resulted in the highest GABA content in the CCM, reaching 60 g/L after 48 h of fermentation. This finding highlights the notable increase in GABA content compared to other concentrations and suggests that 1% MSG should be selected for further experimentation. Previous studies have reported that a higher concentration of MSG up to 100 mM could improve GABA production in *L. plantarum* FNCC 260 during fermentation, with a GABA concentration of 1226 mg/L at 96 h of fermentation [27]. In contrast, high concentrations of MSG have been found to have adverse effects on several species and strains of LAB, including *S. thermophillus* Y2, *L. paracasei* NFRI 7415, and *L. brevis* CRL 1942 [30–32]. Higher levels of glutamate can be toxic to bacterial cells and suppress the expression of the gadB gene [18].

In this study, different concentrations of whey powder were added to CCM (Figure 2). The addition of 4% whey significantly ($p < 0.05$) improved GABA yields in CCM, resulting in 5.2 g/L of GABA, which is 2.5 times higher than that obtained with 1% whey. A similar study by Kittibunchakul et al. also demonstrated that the addition of 4% isolated soy protein increased GABA yield (3.8 mg/100 mL) in fermented brown rice milk [21]. The interaction between whey and GABA production is primarily attributed to the cross-linking of β-lactoglobulin through amidation and acetylation reactions, which may enhance the solubility of the protein [33].

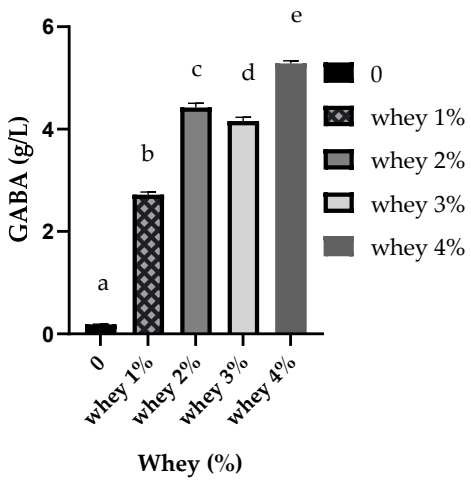

Figure 2. Effect of whey on GABA production in fermented CCM. The results are expressed as mean ± SD. Different letters above the bars indicate significant differences at $p < 0.05$.

The addition of carbon sources, such as sucrose, played a crucial role in promoting cell growth and GABA yields during fermentation. Specifically, the addition of 3% sucrose significantly ($p < 0.05$) improved GABA yields, resulting in 4.4 g/L of GABA during fermentation (Figure 3). In contrast, the addition of 1% and 2% sucrose only slightly increased GABA production, and to a lesser extent, suggesting that a higher concentration of sucrose was required to enhance the yield. It is important to note that the optimal carbon source for GABA production may vary depending on the strain. For example, maltose was found to be the best carbon source for GABA production in *L. brevis* K203 [34], glucose for *L. plantarum* HU-C2W [35], and 4% sucrose for *L. sakei* B2–16 [36]. Considering these findings, the nutrients with the highest GABA production (MSG 1%, whey 4%, sucrose 3%) were selected for further studies to produce the fermented CCM drink.

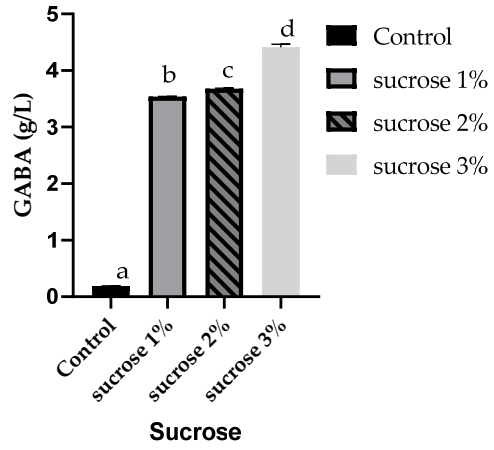

Figure 3. Effect of sucrose on GABA production in fermented CCM. The results are expressed as mean ± SD. Different letters above the bars indicate significant differences at $p < 0.05$.

3.2. GABA Production in Fermented CCM and Growth Profile of L. Plantarum Dad-13

The three nutrients that gave optimum GABA production were mixed to produce fermented CCM. GABA production and the growth profile of *L. plantarum* Dad-13 during CCM fermentation are presented in Figure 4. The probiotic *L. plantarum* Dad-13 was able to grow in CCM during fermentation and led to pH reduction due to lactic acid formation (Figure 5). GABA production was greatly enhanced at an acidic condition (pH 3.8) and affected by the cell growth. This result is in line with a previous study by Yogeswara et al. where maximum GABA production was observed at pH 4.0 [27]. Similarly, *L. buchnerii* optimally produced GABA at pH 5.0 [9]. The results suggest that GAD activity is stimulated under acidic conditions and under the stress of the cells [4,15]. Maximum GABA production was obtained at 12 h of fermentation with 5.6 g/L of GABA followed by the growth of *L. plantarum* Dad-13 (8. 27 log cfu/mL). Afterwards, we observed that GABA production was decreasing at 24 h of fermentation and steadily decreased at the end of fermentation (48 h). However, we observed that *L. plantarum* Dad-13 showed optimum growth at 24 h of fermentation and slightly decreased by one log cycle at 48 h of fermentation. GABA production cannot be maintained during fermentation due to GABA transaminase (GABA-T) activity, which degrades GABA. GABA-T facilitates GABA degradation using either pyruvate or α-ketoglutarate as the amino acceptor. The conversion of succinic semialdehyde to succinate is irreversible, and is catalyzed by succinic semialdehyde dehydrogenase [37]. In the present study, GABA production was positively correlated with the growth of *L. plantarum* Dad-13.

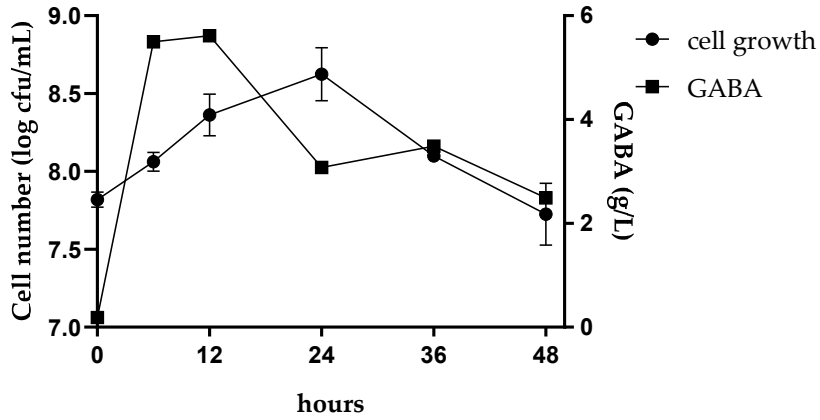

Figure 4. Growth profile and GABA production of *L. plantarum* Dad-13 in CCM during fermentation. The results are expressed as mean ± SD.

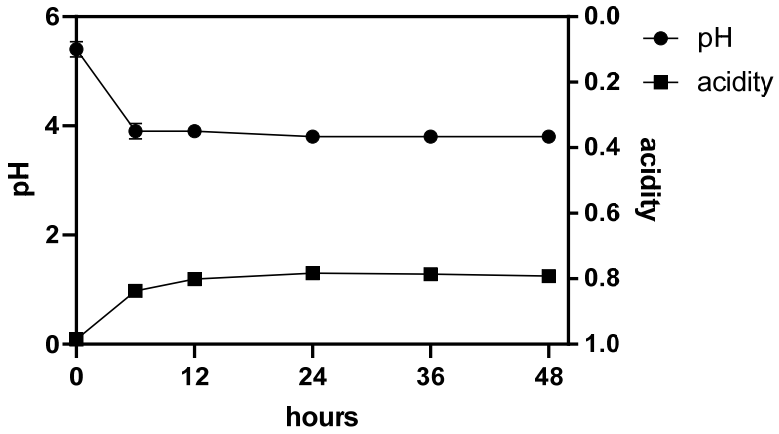

Figure 5. pH and acidity of fermented CCM during fermentation. The results are expressed as mean ± SD.

In this study, we observed that the fortification of single nutrients in a fermented CCM drink yielded greater GABA concentrations compared to a mixture of nutrients.

This may be due to an antagonist interaction among nutrients, suggesting that proper formulation is needed to optimize GABA production in fermented CCM. Furthermore, the fortification of whey protein may contribute to a reduction in GABA, since GABA tends to form linear or ring structure oligomers in the presence of whey protein, which may contribute to GABA losses [33]. Generally, GABA production in fermented CCM was higher than that in other plant-based drinks such as grape must fermented by *L. plantarum* DSM 19463 (930 mg/L) [15], and mature coconut water fermented by *L. plantarum* DW 12 (1280 mg/L) [38]. This indicates that GABA production is affected by species/strains as well as the medium's composition. Furthermore, in a study involving diet-induced obese mice, treatment with 12 mg/mL of GABA demonstrated the ability to ameliorate oxidative stress and dysfunction in thyroid hormones. This suggests that GABA administration can potentially act as a preventive measure against obesity [39]. In addition, Okada et al. revealed that a daily consumption of 26.4 mg of GABA led to the recovery of sleeplessness and depression symptoms in 65% of patients [8]. Furthermore, the presence of a high number of viable cells, exceeding 7 log cfu/mL, in fermented CCM can have beneficial effects on the gastrointestinal tract (GIT) of humans. This number of cells meets the requirement for probiotic consumption as stated by the World Health Organization (WHO).

3.3. Organization of GAD Genes of L. plantarum Dad-13

GAD plays a crucial role in the synthesis of GABA and is predominantly located in the cytoplasm. LAB-possessing *gad* genes have the ability to produce GABA. However, it should be noted that the *gad* system and the organization of the *gad* operons can vary among different species of LAB [40]. The organization of GAD-encoding genes was compared among three strains: *L. plantarum* Dad-13, *L. plantarum* WCFS1, and *L. brevis* ATCC 367 (Figure 6). In the case of *L. plantarum* Dad-13 and *L. plantarum* WCFS1, a transcriptional regulator gene called *gad*R, which has the potential to activate *gad*B genes responsible for glutamate decarboxylase production [41], is located between phosphoenolpyruvate kinase (ATP) and a hypothetical protein. On the other hand, the *gad*B genes are situated between phosphoenolpyruvate kinase and gamma-D-glutamate-meso-diaminopimelate muropeptidase gene. In addition, the genome of *L. plantarum* Dad-13 did not contain the antiporter gene (*gad*C) next to the *gad*B gene, as similarly reported for another *L. plantarum* strain [42].

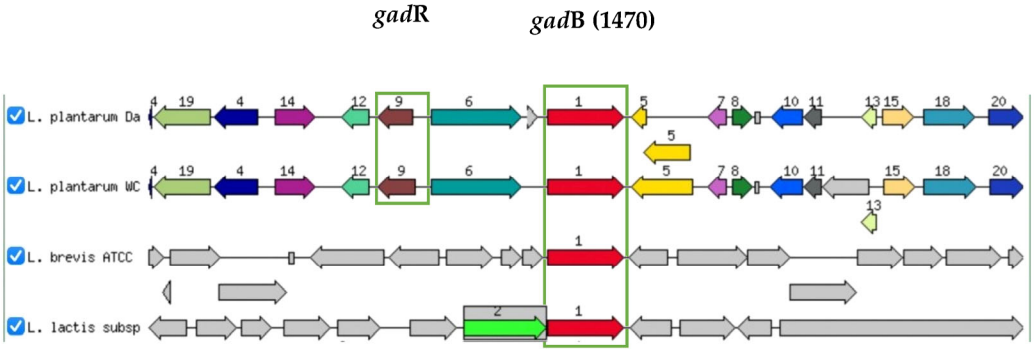

Figure 6. *gad* genes organization in *L. plantarum* Dad-13. 1; glutamate decarboxylase; transcriptional regulator (*gad*R; gene number 9); phosphoenolpyruvate kinase (gene number 6); gamma-D-glutamate-meso-diaminopimelate muropeptidase (gene number 5).

The genome of *L. brevis* ATCC 367 has two coding genes for GAD, one of which is located next to *gad*C and most likely acts as a operon including the transcriptional regulator (*gad*R) and glutamyl-tRNA synthetase, as previously reported for strain *L. brevis* NCL912 [43]. The genetic organization of the GAD system can also vary within different strains of the same species. For instance, in *S. thermophilus* strains ACA-DC 2, B59671, and TH1435, the *gad*B-*gad*C genes are found to be flanked by transposon elements. Additionally, S-ribosylhomocysteine lyase and ribonuclease Y are located upstream and downstream

of the gadB-gadC operon, respectively [44]. In addition, Yunes et al. reported that among 30 strains of *L. plantarum*, only one *gad*B gene was identified, and it showed a high expression in the early stationary phase at low pH [42].

3.4. Metabolite profile of Fermented CCM

Metabolite profiling during CCM fermentation was performed using UPLC-QTOF-MS/MS. In total, 47 metabolites were identified during fermentation. Among the identified metabolites, organic acids, peptides, amino acids, and their derivatives made up the largest proportion of 37.5% of the total number of identified metabolites, followed by flavonoids, fatty acid derivatives, and miscellaneous compounds accounting for 12.5%, 2.08%, and 16.7%, respectively.

A heatmap was generated to observe the changes in metabolite levels during fermentation. The different shades in the figure represent varying levels of metabolites, with the highly abundant ones depicted in red. It was observed that during the fermentation period at 6 and 12 h, only 16.7% of metabolites were detected. This suggests that the strain might have consumed sucrose and adapted to the fermentation environment during this period. The strain exhibited the ability to synthesize various amino acid derivatives and peptides, including leucine, aspartate, isoleucine, aspartame, and glutamate, which are crucial for cell growth. Additionally, organic acids such as lactate, N-(1-deoxy-1-fructosyl), and leucine, 2-(dimethylamino) ethyl methacrylate were produced during fermentation. Moreover, glutamate and phenylalanine serve as a substrate for GABA synthesis and contribute to umami taste and bitterness [45]. During the fermentation process, there is a noticeable shift in metabolite levels. At 24 h of fermentation, a significant abundance of organic acids, amino acids, and GABA was observed, constituting 52% of the compounds (Figure 7). This increase in amino acids, their derivatives, and peptides can be attributed to the extracellular proteolytic activity of the LAB. A number of studies have shown that GABA exhibits physiological effects to regulate blood pressure, relieve anxiety, and regulate blood sugar [10,46,47]. However, the level of GABA decreased over time due to the catabolic activity of the LAB [4]. During the fermentation process, it was observed that certain amino acids and organic acids decreased over time. However, there was an increase in other metabolites such as 4-hydroxyquinoline, indoleacrylic acid, and methylpirrolidine. These metabolites may play a significant role in suppressing inflammation, and also exhibit effectiveness against Mycobacterium tuberculosis. Additionally, they showed a strong cytotoxicity effect on non-tumorigenic HEK cells [48].

A number of flavonoids were found in abundance and increased at 36 h and 48 h of fermentation such as cyanidin, galangin, 6-beta-D-glucopyranosyl-8-beta-D-ribopyranosylapigenin, kaempferol, and 3′,4′, 7-trihydroxy-flavone. Common phenolic compounds found in beans include flavonoids, anthocyanins, flavonols, flavanols, isoflavones, flavanones, proanthocyanidins, and tannins. These compounds are present in various forms, including free forms, insoluble conjugate forms, and bound forms with oligosaccharides and peptides [49]. Flavonoids are significant metabolites that are frequently associated with antioxidant activity, anti-inflammatory activity, and anticancer properties [50,51]. During fermentation, LAB can secrete hydrolytic enzymes that break down macromolecules, leading to an improved release of soluble and insoluble phenolic compounds from the plant cell wall [52]. Fermentation with *L. plantarum* 21802 has been shown to improve the flavonoid profile in grape juice, with an increase of 26.83% observed in flavonoids such as rutin, quercetin, and isoquercetin [52]. However, it is worth noting that James et al., (2020) reported a decrease in flavonoid content during the fermentation of pigeon pea, possibly due to the activity of β-glucosidase from *L. plantarum* [53]. These findings suggest that the impact of fermentation on flavonoid content can vary depending on the substrate and microbial species involved. Similar variations in flavonoid profiles have been observed in other fermented foods, such as mung beans [54], fermented *Moringa olifiera* [55], Koji [56], and coffee beans [57].

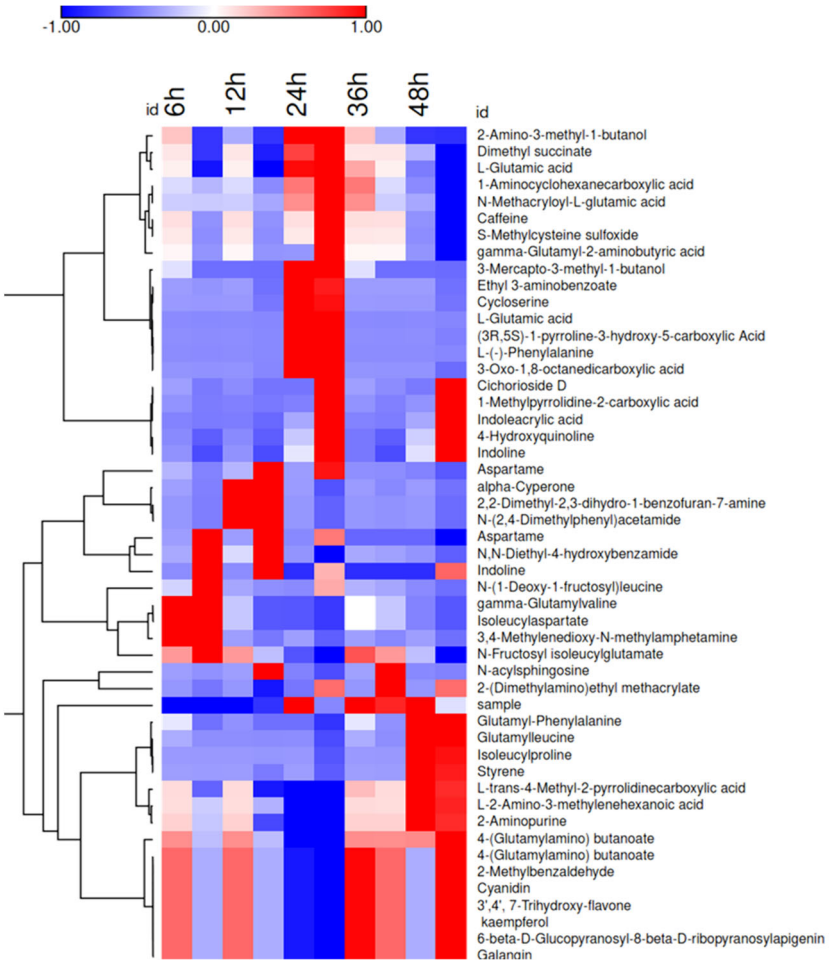

Figure 7. Metabolite profile of fermented CCM during fermentation.

3.5. Anti-Inflammatory Activities

To determine the possible health-promoting effects of fermented CCM, its anti-inflammatory activity was determined (Figure 8). Fermentation time has been found to significantly affect the anti-inflammatory activity ($p < 0.05$) of fermented CCM by suppressing the proinflammatory cytokines TNF-α and IL6. Fermented CCM at a fermentation period of 48 h showed a significant decrease in TNF-α and IL6 levels. Additionally, fermented CCM improved the levels of anti-inflammatory cytokine IL10 during fermentation. This suggests that fermentation time can impact the anti-inflammatory activity of fermented CCM. The increase in IL10 levels can help to reduce the levels of proinflammatory cytokines. The expression of IL10 has been observed in several probiotic strains, including *L. plantarum* and *L. rhamnosus* [58].

The anti-inflammatory activity of fermented foods has been shown in several studies. As demonstrated by Kim et al. (2010), Korean soy sauce decreased proinflammatory cytokines TNF-α and nitric oxide (NO) in a dose-dependent manner [59]. TNF-α and IL6 are major proinflammatory cytokines which contribute to the development of type 2 diabetes and are also associated with a higher risk of metabolic syndromes, such as hypertension and atherosclerosis [60]. During fermentation, the production of health-modulating metabolites, such as organic acids, can have various beneficial effects. Organic acids have been shown to reduce the secretion of proinflammatory cytokines and suppress the accumulation of reactive oxygen species (ROS) in intestinal enterocytes [51]. Amino acid derivatives, such as GABA or alanine, and dimethyl succinate also contribute to the immunomodulatory and anti-tumor activity found in fermented CCM [45]. In addition, several flavonoid constituents found in fermented CCM, such as kaempferol (flavonol) and

cyanidin (anthocyanidin), showed anti-inflammatory activities via different mechanisms such as the inhibition of transcription factor NF-kB, cytokines GATA-3, and the activator of transcription 6 (STAT-6) [50]. Moreover, flavonoids also suppress cAMP phosphodiesterase, which is crucial for regulating different cell functions during inflammation and promoting inflammation at a higher level [50].

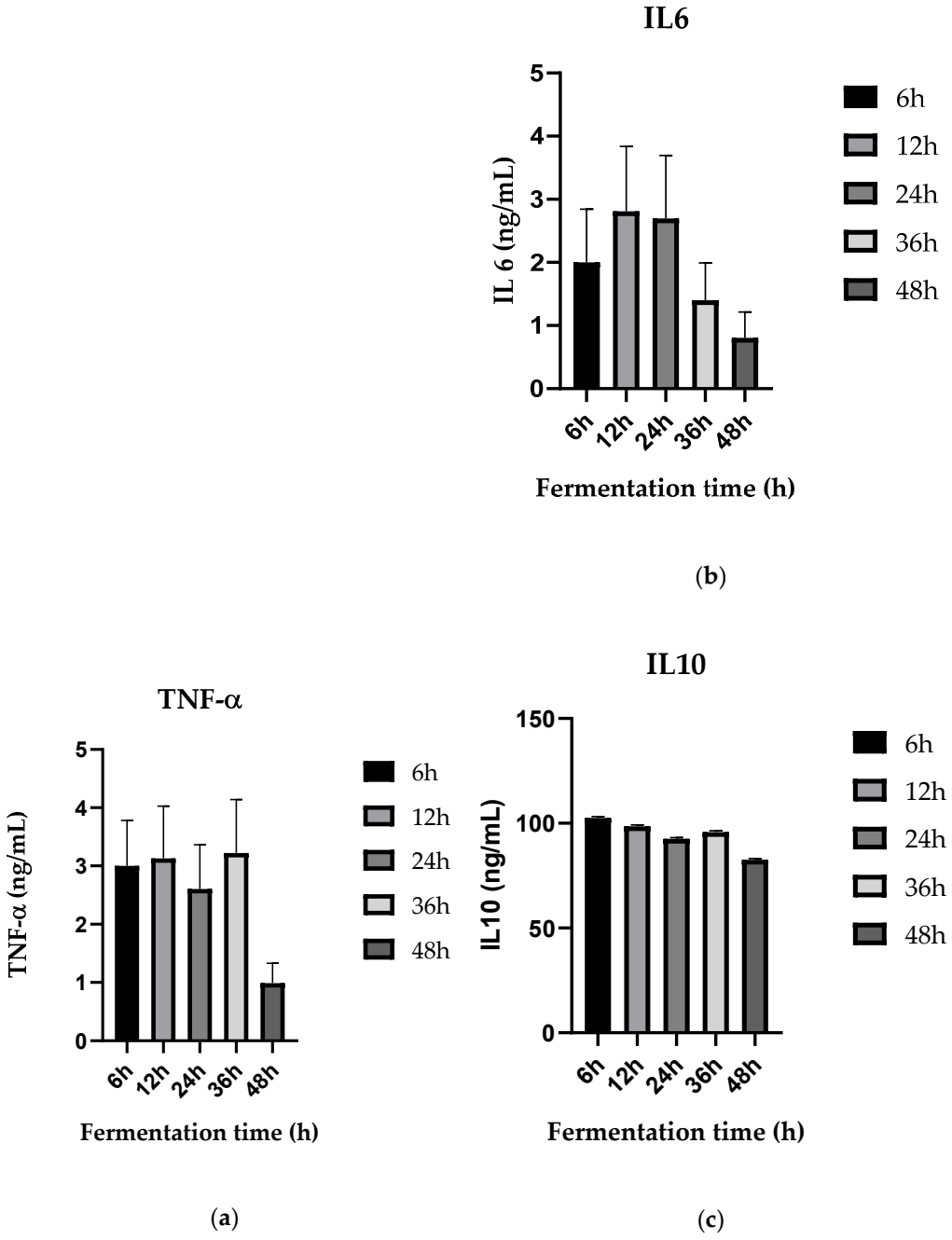

Figure 8. Anti-inflammatory effect of fermented CCM with different fermentation time. The effect of fermented CCM on cytokines TNF-α (**a**), IL6 (**b**), and IL10 (**c**), respectively.

4. Conclusions

This study focused on the enhancement of GABA levels and the proliferation of probiotic cells in fermented pigeon pea milk (CCM) using the probiotic strain *L. plantarum* Dad-13. The supplementation of sucrose, MSG, and whey isolate significantly increased GABA levels in fermented CCM. The presence of *gad*B genes in the strain played a crucial role in GABA synthesis. The fermentation process also resulted in the generation of

various beneficial metabolites, including organic acids, amino acids, and flavonoids, which contribute to the anti-inflammatory activities of fermented CCM. This study discovered that utilizing underutilized food materials, such as pigeon pea, adds value in terms of health and economic aspects. The development of fermented plant-based milk products, like CCM, provides a nutritional alternative for individuals with lactose intolerance and malnutrition. However, further research using in vivo methods and human trials is needed to investigate the health benefits associated with consuming fermented CCM.

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