Strategies for improvement of gamma-aminobutyric acid (GABA) biosynthesis via lactic acid bacteria (LAB) fermentation

Abstract

Gamma-aminobutyric acid (GABA) is a non-protein amino acid widely distributed in nature and extensively explored for its numerous physiological functions and effects on metabolic disorders. Lactic acid bacteria (LAB) are one of the most important GABA producers vigorously pursued due to their high GABA content and generally regarded as safe (GRAS) status that allows for direct formulation in various GABA-enriched food products. To meet the strict requirements of the food and nutraceutical industries, biosynthesis of GABA is typically preferred than the chemical synthesis route. The production of GABA is varying among various strains of LAB and affected by different fermentation conditions. Hence, optimizing the fermentation conditions to enhance the activity of the key enzyme, glutamic acid decarboxylase is essential to maximizing the GABA production. In this paper, the beneficial effects of GABA on human health and its applications in fermented food products are reviewed. A particular emphasis is given to the biosynthetic approach of GABA by various LAB species via microbial fermentation route. Efficient strategies for enhancement of GABA production through optimization of fermentation conditions, mode of fermentation, two steps fermentation, co-culturing approach, immobilization technique and genetic engineering are elaborately discussed.

Keywords: Gamma-aminobutyric acid, lactic acid bacteria, fermentation, biosynthesis, GABA-enriched food, non-protein amino acid

1.0 Introduction

Gamma-aminobutyric acid (GABA) is a four carbon non-protein amino acid where the amino group placed on γ-carbon with 103.1 g/mol relative molecular weight (Xu et al., 2017; Dhakal et al., 2012). GABA is synthesized by glutamic acid decarboxylase (GAD) in the presence of a co-enzyme, pyridoxal-5'-phosphate (PLP). GAD is the key enzyme in GABA synthesis that functions to catalyse the irreversible reaction of α-decarboxylation of L-glutamic acid to GABA (Yogeswara et al., 2020; Wu et al., 2018). In early 1880s, the first chemical synthesis of GABA was recorded and it was known as a natural metabolic product of plant and microbe while in 1950, GABA in a mammalian brain system that acts as a major inhibitory neurotransmitter was discovered (Lei et al., 2014; Wu et al., 2018).

Due to its widespread physiological and pharmacological effects in human, GABA related studies have been extensively conducted over the past several decades (Xu et al., 2017; Ochoa-de la Paz et al., 2021). This has opened new perspectives and breakthrough strategies in the development of more effective GABA-based therapeutic treatments for various health diseases such as brain, nervous system and cardiovascular disorders, diabetes, cancer and asthma (Diez-Gutiérrez et al., 2020; Grewal, 2020). Bacteria, yeasts, fungi, molds, animals, and plants (such as tea leaves, mulberry leaves and tomato) have been widely examined as natural GABA sources (Sahab et al., 2020). Nevertheless, GABA from microbes has also been extensively explored because the parameters affecting the GABA production are much easier to be manipulated than plants which have low concentrations of GABA and animal's GABA that could not breach the blood brain barrier which inhibit its functions towards human body (Diana et al., 2014b; Sahab et al., 2020).

Enzymatic or whole cell biocatalysis and microbial fermentation are the two major biosynthetic approaches to increase the GABA production (Xu et al., 2017). In an enzymatic or whole cell biocatalysis route, isolated GADs or recombinant GAD-expressing host strains are utilized as catalysts in the production of GABA (Cui et al., 2020). The successful GABA synthesis in these systems results from the enzymatic characteristics of the selected GADs (Xu et al., 2017). However, microbial fermentation is the most favored approach for GABA synthesis due to the simple procedure and low substrate cost with high transformation ratio (Gomaa, 2015). Furthermore, due to increasing demands for naturally-made nutrient sources, GABA produced through the fermentation of naturally-occurring microorganisms is highly preferred than the chemically-synthesized GABA (Shan et al., 2015). This is because GABA

produced by microorganisms are naturally presence in food production processes, low-cost, safe and can be commercialized as functional food products (Diez-Gutiérrez et al., 2020).

Lactic acid bacteria (LAB), *Escherichia coli*, and *Streptococcus salivarius* are among the most studied microorganisms for obtaining a naturally synthesized GABA (Xu et al., 2017). However, due to their wide spread availability, unique physiological properties and most importantly, their safe (GRAS) status, many researchers have shifted their attentions towards LAB for GABA production (Lei et al., 2014). Numerous LAB species isolated from various sources, especially fermented foods have been shown to have ability to biosynthesize GABA with production capacity depending upon species and strains (Cui et al., 2020).

The yield of GABA from LAB is mainly affected by several factors such as the activity of GAD and the fermentation conditions. These parameters include pH, temperature, incubation time, size of inoculum and composition of the media (Rayavarapu et al., 2021; Diez-Gutiérrez et al., 2020). Meanwhile, mode of fermentation (Kook & Cho, 2013), two steps fermentation (Peng et al., 2013), co-culturing approach (Wang et al., 2021), immobilization technique (Hsueh et al., 2021) and genetic engineering (Lyu et al., 2017) are some strategies established to improve the GABA production to meet the commercial demand.

2.0 GABA Health Benefits

The vast variety of health benefits resulting by GABA, urge researchers and industries to improve the productivity rate of GABA especially in food, nutraceutical (Diez-Gutiérrez et al., 2020) and pharmaceutical products (Ngo et al., 2019). The significant health benefits of GABA to human, include lowering anxiety, depression, and schizophrenia. symptoms (Flores-Ramos et al., 2017; Hinton et al., 2019; Sarawagi et al., 2021; Schür et al., 2016). GABA has certain physiological roles, such as neurotransmission, hypotensive activity, diuretic effects, tranquilizer effects, analgesic effects and anti-diabetic effects (Lei et al., 2014; Wu et al., 2018; Xu et al., 2017). GABA also helps to maintain a healthy cardiovascular system in human where it can control blood pressure and heart rate by expending the blood vessel (Gomaa, 2015). The metabolism of brain cells is activated by GABA by raising oxygen content and promoting cerebral blood flow and eventually inhibit the secretion of antidiuretic hormone, vasopressin (Kook & Cho, 2013). It also has an impact on asthma and respiratory regulation (Diana et al., 2014a). GABA helps to improve the secretion of growth hormones, plasma level, synthesis of proteins in the brain, prevention of chronic alcohol-related illnesses (Liao et al., 2013), improve

decision making and boost immunity (Moore et al., 2021). In women, there is a relation between progesterone, GABA, and mood behavior. Thyroid hormones and GABA systems are also regulated (Diana et al., 2014a).

GABA acts as an insulin secretagogue which prevents diabetic in human (Siragusa et al., 2007; Wan et al., 2015). GABA uptake and extracellular GABA levels are found to be lowered in diabetic and hyperglycemic patients, which exacerbates neuronal damage by lowering the GABA-mediated inhibitory function. Gabapentin is a GABA analogue used to treat diabetic neuropathy (Huang et al., 2014). In the meantime, GABA reduced blood glucose level in rats was studied by Ohmori et al., (2018). GABA tea was also reported to be able to reduce the diabetes-induced autophagy and diabetic-induced apoptosis in the cerebral cortex. This indicates that GABA tea treatment has the potential to prevent diabetic brain abnormalities.

GABA also inhibit cancer cell proliferation via apoptosis and could slow or prevent the invasion and spread of cancer cells, including mammary gland, colon, and hepatic cancer cells (Huang et al., 2014). GABA has also been shown to be able toinhibitleukemia cell proliferation (Hong et al., 2021). Gao et al. (2019) investigated on the effects of pinocembrin (natural flavonoid) on the growth of ovarian cancer cells and the expression of cadherin and GABA type B that was helpful in the treatment of epithelial ovarian cancer. Through GABA type B receptor, is has also been proven to be able to inhibit the growth of lung cancer (Schuller et al., 2008). Furthermore, a clinical study that was conducted on 89 patients with breast cancer stage 1 and 2, showed that GABA had a significant prognostic value in breast cancer (Brzozowska et al., 2017). The results revealed that cancer patients with a high level of GABA had a significantly longer survival period (131.2 months) compared to patients with low level of GABA and lack of e-cadherin immunoexpression (98.1 months).

GABA has the capability to regulate neurological disorders. Several studies have demonstrated that lack of GABA-mediated inhibition might set off a chain of events that results in neuronal damage and cell death (Antony et al., 2010; Ochoa-de la Paz, 2021). Numerous neurological disorders such as epilepsy, seizures, convulsions, Huntington's disease, and Parkinsonism would be the consequences of low GABA concentration below a certain level in the brain (Cho et al., 2007). Okada et al. (2000), found that treating these neurological diseases with a daily oral dosage of rice germ containing 26.4 mg GABA was successful. Undoubtedly, GABA levels in the brain increased as a result of yoga asana sessions and it is a possible

treatment for certain autonomic diseases (Streeter et al., 2010; van Aalst et al., 2020). Menopausal and premenopausal women are more likely to experience this (Diana et al., 2014a; Wang et al., 2019).

Oral intake of natural or biosynthetic GABA has been shown to exert beneficial effetcs on sleep disturbances such as insomnia (Hepsomali et al., 2020). For an example, the amounts of GABA in a variety of teas have been proven to provide a sleep-inducing effect (Diana et al, 2014a). In a double-blind, randomized trial, a fermented rice germ extract containing GABA helped to improve sleep efficiency and reduce sleep latency in insomnia patients (Yu et al., 2020). After taking 100 mg GABA for 60 minutes, volunteers' brains had a large increase in alpha wave activity and a substantial decrease in beta wave activity, indicating that this neurotransmitter could promote relaxation and reduce anxiety. GABA was also demonstrated to have a sleep-inducing effect through animal studies where serotonin and melatonin act as sleep inducers (Kook & Cho, 2013). A recent study also revealed that GABA extracted from the fermented rice germ can normalize caffeine-induced sleep disorders in mice (Yu et al., 2020).

GABA has the ability to protect against chronic kidney disease and improved nephrectomy-induced oxidative stress as well as enhancing liver and renal function (Chen et al., 2016; Ngo and Vo, 2019; Sasaki et al., 2006). GABA has also been shown to diminish rheumatoid arthritis inflammation and to lessen the metabolic response to ischemia events (Diana et al., 2014a). GABA increased collagen and hyaluronic acid synthesis in human dermal fibroblasts (Saraphanchotiwitthaya & Sripalakit, 2018). Furthermore, due to its water-holding capacity and hygroscopic qualities, GABA exhibited a moisturizing impact which can be used in the cosmetics industry. GABA is also being looked into as an anti-aging supplement (Cuypers et al., 2018; Ma et al., 2020). GABA was also shown to improve wound healing in rats by lowering inflammation and stimulating the proliferation of fibroblasts (Saraphanchotiwitthaya & Sripalakit, 2018). To date, even though GABA has vast promising health benefits to human, the full functions and effects of GABA within human are still unknown and hence attracting continuous research and discovery throughout the world.

3.0 GABA from Microorganisms

GABA is widely distributed in nature that could be found in plants (Li et al., 2021), animals (Tillakaratne et al., 1995), and microorganisms (Luo et al., 2021). GABA produced

within the mammalian brain system could not breach the blood brain barrier or only a trace amount could do that, which prevents the flow of GABA throughout the body to exert the health benefits within human (Moore et al., 2021). GABA contents in fruits and vegetables are naturally very low (the used wet weights ranging from 0.03 to 2.00 mol/g) that is not enough to have a biological activity (Kook & Cho, 2013). Meanwhile, GABA produced via chemical synthesis (Liu et al., 2017) is less preferred due to its negative impact on environmental pollution and it involves complex purification methods which are cost ineffective (Sarasa et al., 2020; Ke et al., 2016). Therefore, researchers and industries are nowadays focusing on utilizing a more promising microbial method to produce GABA (Grewal, 2020; Dhakal et al., 2012). GABA production using microbe system can be easily controlled (Cui et al., 2020). Furthermore, another added value of utilizing microorganisms for GABA production are their faster growth rate and less cultivation space required in comparison to plants.

GABA can be synthesized through an irreversible decarboxylation reaction within various microorganisms such as bacteria, yeast and fungi due to the presence of GAD enzyme and pyridoxal 5-phosphate (PLP) co-factor (Lei et al., 2014). GADs have diverse structural and functional characteristics in different microorganisms. GAD can be found from a wide range of microbes, including *E. coli*, lactic acid bacteria (LAB), *S. salivarius*, and *Bacillus megaterium*, *Aspergillus oryzae*, *Pyrococcus horikoshii*, *Neurospora crass Monascus purpureus* and *Rhizopus microspores* (Xu et al., 2017). GABA in bacteria is produced by two ways which are direct intake of L-glutamate as a substrate that will be catalyzed by GAD enzyme to produce GABA (Lyu et al., 2018; Yuan et al., 2019), and another way is GABA production that involves bypassing certain key steps of triacarboxylic acid (TCA) cycle (Xu et al., 2021; Toro and Pinto, 2015). The GABA production mechanism in bacteria is illustrated in Figure 1. Briefly, the existing GABA in the mitochondrial matrix is converted into succinate semialdehyde (SSA) which is irreversibly oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH) that entered the TCA cycle, and eventually α-ketoglutamic acid is produced (Kumar & Punekar, 1997). Glucose as a substrate through the glycolysis pathway istransformed into pyruvate before it is converted into Acetyl-CoA which then through the TCA cycle produces α-ketoglutamic acid. L-glutamate is formed when α-ketoglutamic acid is catalyzed by the glutamate dehydrogenase (GDH). The glutamate is then decarboxylated to produce GABA using the GDH enzyme (Figure 1).

The GABA production in yeast and fungi is similar as in plant where the mechanism involves cytosolic glutamate decarboxylase, polyamine degradation, or a non-enzymatic

mechanism that involve proline or L-lysine (Ramos-Ruiz et al., 2019). During the investigation of amino acid composition in *Rhodotorula glutinis* (red yeast), GABA was detected to be present as a non-protein amino acid fraction (Krishnaswamy and Giri, 1953). In wild-type *Neurospora crassa*, GABA was found to be functionally involved in the early phase of spore germination before diminishing (Schmit and Brody, 1975). GABA was observed in filamentous fungi such as *Aspergillus nidulans* (Biratsi et al., 2021) and *Aspergillus niger* (black aspergillus) during acidogenesis (Kubicek et al., 1979; Tong et al., 2019). *Aspergillus oryzae*, isolated from soy sauce koji was also capable of producing GABA as the strain possess a gene for glutamate decarboxylase (Ab Kadir et al., 2016).

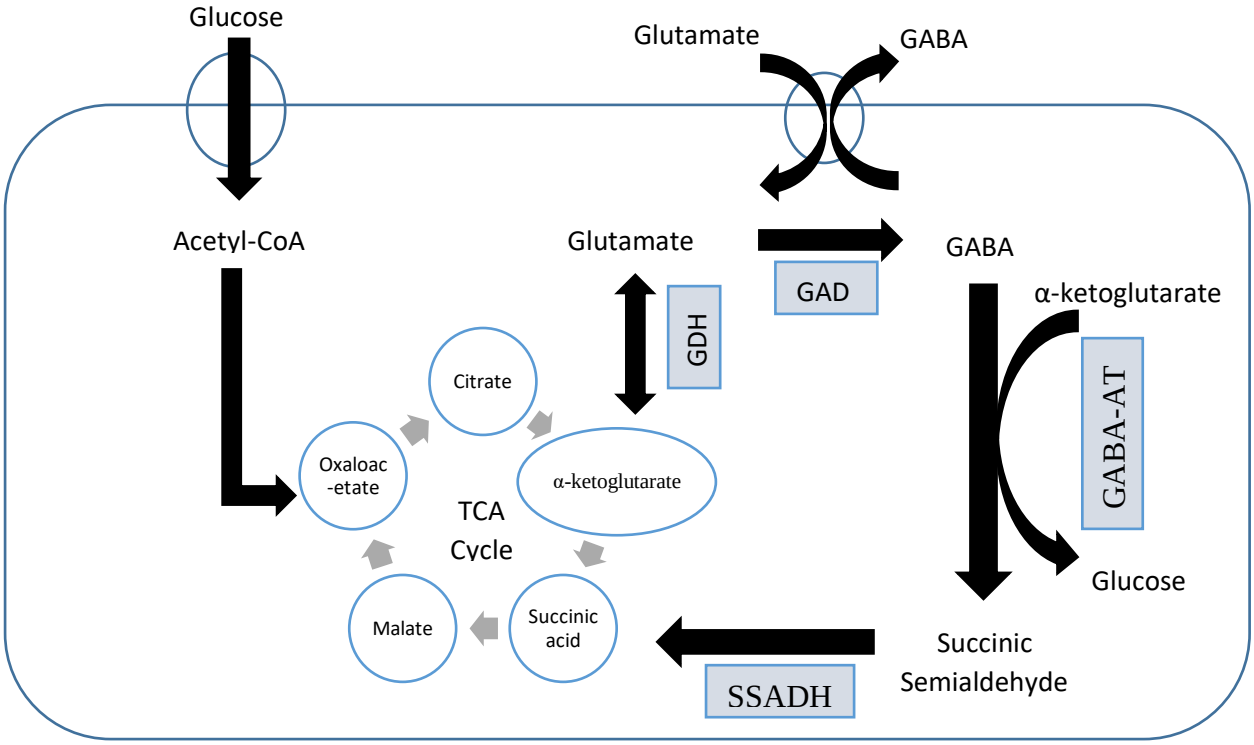

Figure 1. GABA production mechanism in bacteria. GDH, L-glutamate dehydrogenase; GAD, glutamate decarboxylase; GABA-AT, GABA aminotransferase; SSADH, succinate semialdehyde dehydrogenase.

4.0 GABA-producing LAB

Lactic acid bacteria (LAB) are gram-positive bacteria mainly producing lactic acid that are commonly found in fermented foods, vegetables, and in both human and animal intestines (Mora-Villalobos et al., 2020; Fazilah et al., 2019; Othman et al., 2018). They create a wide range of antibacterial compounds, including organic acids, hydrogen peroxide, and bacteriocin, in addition to flavor development and food preservation (Hajar & Hamid, 2013; Md Sidek et al., 2018; Jawan et al., 2020; Othman et al., 2017).

All LAB strains with GAD genes have the ability to synthesize GABA (Yogeswara et al., 2020). To date, GAD systems with a variety of genetic organization are extensively reported in various LAB species such as *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Streptococcus* (Cui et al., 2020). These GAD systems are in the chromosome, but some are found located in their plasmid. pH is one of most important parameters in the production of GABA because the biosynthesis mechanism of GABA is closely related to the pH (Kook and Cho, 2013). GAD is involved in maintaining the cellular pH under acidic environments (Park et al., 2014). Hence, acidizing the cellular pH of the cultivation media is a way to enhance the GABA production since GAD needs H^+ ions to synthesize GABA. The existence of the *gdh* gene in LAB, is responsible for the safe and environmental friendly production of biologically active glutamic acid (L-glutamic acid) which is the precursor for GABA production (Zareian et al., 2012). However, most LAB are not capable of synthesizing enough L-glutamate for the biosynthesis of GABA and hence, the supplementation of MSG that will be hydrolyzed to L-glutamate is essential (Cui et al., 2020).

Most GABA-producing LAB have been isolated from food sources since LAB are known to have the ability to utilize glutamate which is present in food ingredients to counteract the excess acidity that arises from the conversion of sugars to lactic acid (Laroute et al., 2016). GABA which is formed as a by-product of the fermentations is the main interest for the development of a wide range of commercial products and GABA-enriched food products are of the particular importance (Sahab et al., 2020). Among various LAB species, *Lactobacillus* spp. such *L. brevis*, *L. buchneri*, *L. bulgaricus*, *L. futsaii*, *L. helveticus*, *L. plantarum*, *L. paracasei*, or *L. paraplantarum* are the most predominant species that have been studied for the production of GABA (Yogeswara et al., 2020, Lim et al., 2018). Nowadays, research on GABA production by LAB strains at commercial production scale is also progressing. For an example, 660 mM of GABA (100% conversion yield) was successfully produced by *L. sakei* B2-16 (isolated from *Kimchi*) using rice brand extracts medium in scale-

up production from 5 L to 300 L and finally a 5000 L fermenter (Kook et al., 2010b). Table 1 further summarizes several LAB strains that were reported to be good GABA producers.

Table 1. LAB producers for GABA production.

LAB strain	Source of isolation	GABA content	Reference
Lactobacillus brevis	Kimchi	18.76 mM	Lim et al., 2017
	Traditional mountain Malga cheese	84 ± 37 mg/kg	Carafa et al., 2019
	Horreh fermented food	553.5 ppm	Behbahani et al., 2020
	Aji-narezushi (Japan traditional fermented food)	721 ± 38 mg/100 mL	Barla et al., 2016
	Quinoa sourdough	270 mM	Villegas et al., 2016
Lactobacillus buchneri	Japan traditional fermented food aji-narezushi	665 ± 27 mg/100 mL	Barla et al., 2016
Lactobacillus delbrueckii subsp. *bulgaricus*	Italian cheese	63 mg/kg	Siragusa et al., 2007
Lactobacillus fermentum	Sourdough	5.494 g/L	Rayavarapu et al., 2021
Lactobacillus futsaii	Thai fermented shrimp (Kung-Som)	135 mg/L/h	Sanchart et al., 2018
Lactobacillus otakiensis	Traditional Pico cheese	659 mg/L	Ribeiro et al., 2018
Lactobacillus paracasei	Yogurt	1070.09 mg/kg	Zarei et al., 2018
	Traditional Pico cheese	584 mg/L	Ribeiro et al., 2018
Lactobacillus paraplantarum	Traditional Pico cheese	48 mg/L	Ribeiro et al., 2018
Lactobacillus plantarum	Doogh	170.492 mg/kg	Zarei et al., 2018
	Stomach of honeybee Apis dorsate	83.65 mg/100g yoghurt	Hussin et al., 2021
	Fermented fish products	15.74 g/L	Tanamool et al., 2020
	Traditional Pico cheese	937 mg/L	Ribeiro et al., 2018
Lactococcus garvieae	Traditional Pico cheese	39 mg/L	Ribeiro et al., 2018
Lactococcus lactis	Camel's milk	457 mg/kg	Redruello et al., 2021
	Traditional Pico cheese	62 mg/L	Ribeiro et al., 2018
Leuconostoc citreum	Traditional Pico cheese	29 mg/L	Ribeiro et al., 2018

Leuconostoc mesenteroides	Traditional Pico cheese	49 mg/L	Ribeiro et al., 2018
Pediococcus acidilactici	Cheese	91.09 mg/kg	Zarei et al., 2018
Pediococcus pentosaceus	Fermented fish sauce (mam nem)	27.9 ± 0.42 mM	Thuy et al., 2021
Streptococcus thermophilus	Yogurt-sake	1096 µM	Ohmori et al., 2018
	Nostrano cheese	91 ± 28 mg/kg	Carafa et al., 2019
Weissella hellenica	Ika-koujizuke and ika-kurozukuri (Japan traditional fermented food)	718 ± 33 mg/100mL, 769 ± 21 mg/100mL	Barla et al., 2016

5.0 GABA Enriched Fermented Foods

Fermented foods represent an excellent source of dietary GABA (Sahab et al., 2020; Diana et al., 2014a). GABA-enriched foods are essential in human diet because although the human body can naturally produce its own GABA, the production is often hindered either by lack of vitamins, zinc or estrogen or excess of salicylic acid and food additives (Aoshima and Tenpaku, 1997). In comparison, higher amounts of GABA are present in fermented foods than naturally found in plant-derived foods (Oh et al., 2005). Hence, GABA-enriched products such as beverages, snacks, and supplements are nowadays widely sold on the market. GABA-enriched food products are manufactured by either blending the GABA compound directly into the food or by employing microorganisms to biosynthesize GABA during the fermentation process (Ab Kadir et al., 2016).

Many research studies are directing towards utilizing GABA from LAB since they have unique physiological properties and are generally regard as safe (GRAS) (Lei et al., 2014) . In addition, GADs from LAB have attained a lot of interest mainly because most LABs are typically thought to be harmless with high GAD activity unlike *E. coli* that has a bacterial endotoxin which is not safe to consume despite also having a relatively high GAD activity (Zhao et al., 2016). Thus, to be used as food additives, GABA from *E. coli* must be first separated from the cells by downstream processing while GABA from LAB can be used without separation (Lopes et al., 2010). To develop fermented foods with high amounts of GABA, beside employing LAB with high GAD activity, the concentration of glutamic acid in the food matric should also be high enough (Quílez and Diana, 2017).

GABA-enriched cheeses are reported to provide multiple health benefits. For an example, a daily intake of cheddar cheese containing between 10 to 19 mg of GABA for 12

weeks helped to reduce blood pressure in human subjects (Redruello et al., 2021, Pouliot-Mathieu et al., 2013, Inoue et al., 2003). In the preparation of cheese by a combination of two LAB strains, *S. thermophilus* 84C *and L. brevis* DSM 32386, milk that initially contained 1.9 mg/kg GABA was increased to 91 mg/kg GABA at the end of ripening (20 days) (Carafa et al., 2019). The pH was swiftly reduced from ~6.5 to ~5.2 within 2 days of ripening and the value was retained until the end of ripening period. At the end of the ripening process, a change of microbiota was noted in the cheese samples with a significant decrease in *Streptococcaceae* (from 56 to 30%), *Enterobacteriaceae* (from 25% to 11 %) and an increase of *Enterococcaceae* (from 27% to 47%). Recently, Redruello et al., (2021) isolated six GABA-producing *L. lactis* subsp. *lactis* strains from raw camel's milk that have a good potential to be individually used as starter cultures for GABA-enriched cheeses. Glutamate, salt to moisture ratio and pH were among the critical parameters that need to be optimized for high GABA production by *L. lactis* subsp. *lactis* in cheeses (Gardner-Fortier et al., 2013).

Yoghurt rich in GABA is another value added functional dairy product that provides various health benefits such as cardiovascular health (Abd El-Fattah et al., 2018). Among the major obstacles for high GABA production in food matrix such as yogurt includes the need for high concentrations of glutamate (between 30 to 500 mM) that produce unfavorable salty or savory taste and the presence of PLP cofactor (between 20 to 200 µM) that is costly (Zhang et al., 2014, Ohmori et al., 2018). A significant increase in GABA content (from 59 mg/100g to 112.95 mg/100 g) was observed over 21 days of cold storage of yogurt preparation by two starter cultures, *L. plantarum* Taj-Apis362 strains (Hussin et al., 2021). Likewise, yogurt cultured with *L. cremoris* and *L. lactis* O-114, *L. helveticus* Lh-B 02 and *L. rhamnosus* B-1445 showed an increase in GABA content from 5.71 mg/100g to 10.33 mg/100g after 14 days of cold storage (Abd El-Fattah et al., 2018). The presence of glutamic acid that is released from microbial proteolysis helps to enhance the GABA content in yogurt during the cold storage. Beside the activity of GAD and the concentration of glutamate, the viability of LAB also positively influenced the GABA production (Shan et al., 2015). However, prolonging the refrigerated storage up to 28 days, may reduce the GABA content (Hussin et al., 2021). This observation may be attributed to the presence of *Saccharomyces cerevisiae* (a spoilage microbe) that utilized or degraded GABA by GABA-permease enzymes (Ando et al., 2016, Tofalo et al., 2020, Hernández et al., 2018).

As one of the major sources of food all over the world, cereal-based products enriched with GABA have attracted much attention. Daily intake of 30 g of breakfast cereal flasks with

the inclusion of quinoa, brand or malt flour that enriched with GABA (66, 90 and 258 ppm, respectively) was evidenced to reduce high blood pressure (Joye et al., 2011). Kim et al., (2019) investigated on the effect of rice brand and raisin liquid for the preparation of GABA-rich sourdough via a three-stage fermentation process. In 42 hours of fermentation, they observed the number of naturally occurring LAB and yeast cells were gradually increased. The repeated fermentation significantly helped to increase the GABA content (from ~275 mg/100 g to 575 mg/100g) in the sourdough. Among the eighteen lactobacilli strains used in the preparation of breads in amaranth and wheat flour (incubated at 30 °C for 6 hours), two of the strains (*L. brevis* A7 and *L. farciminis* A11) demonstrated high GABA producing capability that was up to 39 mg/kg (Venturi et al., 2019). In comparison, GABA concentrations in sourdoughs prepared from wheat flour were higher than the amaranth flour. This could be due to the higher glutamic acid content in the wheat flour. It was also observed that all the tested LAB strains were able to acidify the doughs with the final pH values recorded for wheat sourdoughs (pH ranged from 3.5 to 4.1) were lower than the amaranth flour (pH ranged from 4.1 to 5.1). *L. brevis* A7, *L. farciminis* A11, *L. farciminis* B7 and *L. rossiae* Ga12 were among the most acidifying strains in both flours. Meanwhile, Diana et al. (2014b) showed the GABA production in sourdough prepared using *L. brevis* CECT 8183 (isolated from artisan Spanish cheese) attained 98.2 mg/100 mL after 48 hours of fermentation in optimal conditions.

Nowadays, beverages fortifying with GABA are also being broadly explored due to its potential health benefits (Quílez and Diana, 2017). The production of GABA through the fermentation of strawberry juice by *Levilactobacillus brevis* CRL 2013 yielded high GABA production (262 mM) (Cataldo et al., 2020a). From the *in-vivo* studies in mice, this GABA enriched strawberry juice was shown to have an immunomodulatory property that was capable of differentially modulating the inflammatory response triggered by TLR4 activation. Fermentation of mulberry juice with the addition of 2% (w/v) MSG by *L. brevis* F064A resulted in a maximum GABA content of 3.31 ± 0.06 mg/mL (Kanklai et al., 2021). In the meantime, mulberry juice fermented with a co-culture starter, *L. plantarum* BC114 and *S. cerevisiae* SC125 produced higher GABA content (2.42 g/L) compared to single cultures using *S. cerevisiae* SC125 (1.45 g/L) (Zhang et al., 2020). Several other GABA-enriched fermented foods, such as dairy products, beverages, cereal-based products, seafood, meat, vegetables, legumes and plant leave have been made by employing GABA-producing LAB as starters are further summarized in Table 2.

Table 2: GABA-enriched foods.

Food product	LAB starter strain	GABA content	References
Dairy products			
Cheese	Mixed cultures of *S. thermophilus* 84C and *L. brevis* DSM 32386	91 ± 22 mg/Kg	Carafa et al., 2019
Cheese	*L. lactis* strains (as starter) and *L. plantarum* TAUL1588 (as adjunct)	296.75 mg/kg cheese	Renes et al., 2019
Yogurt	*L. plantarum* Taj-Apis362	83.65 mg/100 g	Hussin et al., 2021
Fermented milk	Mixed cultures of *L. lactis* DIBCA2 and *L. plantarum* PU11	144.5 mg/kg	Nejati et al., 2013
Fermented milk	*L. lactis* L-571 or *L. lactis* L-572	~86 mg/L	Santos-Espinosa et al., 2020
Fermented skim milk	*L. lactis* FGL0007	431.42 µg/mL	Cha et al., 2018
Fermented chickpea milk	*L. plantarum* M-6	537.23 mg/L	Li et al., 2016
Fermented buffalo milk (Dadih)	*L. plantarum* N5	500 mM	Harnentis et al., 2019
Cereal-based products			
Fried sourdough bread (bhatura)	*L. lactis* subsp. *lactis*	226.22 mg/100 g	Bhanwar et al., 2013
Sourdough bread	*L. brevis* CECT 8183	98.2 mg/100 mL	Diana et al., 2014b
Monascus-fermented rice	*L. plantarum 8014*	29.9 mg/g	Li et al., 2020
Beverages			
Cherry-kefir drink	*L.* sp. *Makhdzir Naser-1*	3.818 mg/mL	Gharehyakheh, 2021
Fermented litchi juice	*L. plantarum* HU-C2W	134 mg/100 mL	Wang et al., 2021
Fermented mature coconut water	Mixed cultures of *L. acidophilus* B0258, *L. brevis* VM1, *L. casei* B0189, and *L. plantarum* B0103	~600ppm	Abdul Rahman et al., 2018
Fermented mushroom beverage (*Hericium erinaceus)*	*L. fermentum* HP3	2.44 mg/mL	Woraharn et al., 2016
Red seaweed beverage	*L. plantarum* DW12	4000 mg/L	Ratanaburee et al., 2011

Black raspberry juice	*L. brevis* GABA100	22.6 ± 2.4 mg/mL	Kim et al., 2009
Meat & seafood-based products			
Fermented sausage (beef and pork)	*L. brevis* Y8	61.47 mg/kg	Yu et al., 2017
Thai fermented pork sausage (Nham)	Mixed cultures of *L. namurensis* NH2 and *P. pentosaceus* HN8	4051 mg/kg	Ratanaburee et al., 2013
Fish sauce from giant masu salmon	*L. plantarum* N10	4.5 mg/100 mL	Taoka et al., 2019
Vegetable, legume, leaf			
Thai fermented Vegetables (Som-pak)	*L. plantarum L10-11*	15.74 g/L	Tanamool et al., 2020
Soymilk	*L. fermentum*	5.54 g/L	Rayavarapu et al., 2021
Soymilk	*L. brevis* FPA 3709	2.45 ± 0.30 mg/mL	Ko et al., 2013
Adzuki beans	Mixed cultures of *L. lactis* and *L. rhamnosus*	68.2 mg/100 m L	Liao et al., 2013
Groundnut brittle	*L. lactis* subsp. *lactis*	816 mg/g	Batra et al., 2018
Fermented mulberry leaf powder	*L. pentosus* SS6	54.96 mg/g	Zhong et al., 2019

6.0 Strategies to enhance GABA production from LAB

GABA is known to be synthesized chemically or biologically (Shan et al., 2015). In comparison, biosynthetic approaches that involve enzymatic or whole-cell biocatalysis and microbial fermentation may be more promising than the chemical methods since the reaction procedure is simpler involving mild reaction conditions and high catalytic efficiencies as well as being sustainable and environmental friendly (Shan et al., 2015; Xu et al., 2017). The involvement of hazardous or corrosive chemical substances, low selectivity, undesirable by-products and environmental pollution problems are among the reasons that chemical synthesis methods are less suited for GABA production, especially in food industries (Li et al., 2010a). Besides, the separation of GABA from by-products and organic solvents in the chemical approach is very challenging since both product and by-products has similar molecular weight

and the product solubility is similar to most organic solvents (Wang et al., 2016). In the meantime, the complexity and high purification costs are the main factors for microbial fermentation to be favored over the enzymatic biocatalysis route for GABA production (Grewal, 2020). Microbial fermentation is considered the most successful biological approach for GABA production due to its convenience, inexpensive substrate usage and a high transformation ratio (Gomaa, 2015). In general, the rate of GABA production by LAB can be enhanced by employing suitable strategies such as mode of fermentation, optimization of fermentation parameters, improvement of the fermentation technique as well as the genetic engineering approach.

6.1 Fermentation modes

The primary goals of fermentation are cost effective and simple production of biomass that allows the highest yield of the targeted product. As the simplest lab operation system, the production of GABA is widely studied in batch mode rather than fed batch and continuous modes (Cui et al., 2020). Nevertheless, fed batch and continuous fermentation modes are often the preferred mode of operation at industrial scale since it allows for higher yield and productivity (EL Moslamy, 2019; Ming et al., 2016). The lower yield observed in the batch fermentation is mainly due to the usage of substrates such as MSG that at certain concentrations could inhibit the cell growth and yield of GABA (Li et al., 2010b). In addition, MSG may also result in a rapid increase of pH due to decarboxylation that may not be an ideal condition for LAB (Wang et al., 2018). Meanwhile, in a fed batch fermentation mode, a proper initial substrate concentration could be added and subsequently more substrate is added with a suitable feeding strategy during the fermentation course, resulting no inhibition in the cell growth and allowing for higher GABA production.

GABA biotransformation by MSG induced resting cells of *L. brevis* SIIA11021 (isolated from Chinese traditional pickled vegetables) was previously reported in batch and fed-batch fermentations (Lei et al., 2014). A yield of 134 mM was attained in the batch mode operated under optimal conditions (pH 4.7, 30°C) while a significant enhancement with a final yield of 440 mM was achieved in the fed-batch mode under similar conditions. To further increase the batch yield of GABA by *L. brevis* NCL912 (maximum yield of 345.83 mM) (Li et al., 2010b), a fed batch mode was developed under optimized conditions (32ºC, pH 5.0, 100 rpm) with glutamate feeding at 12 hours (280.70 g) and 24 hours (224.56g) (Li et al., 2010c).

The highest GABA concentration (1006 mM) was accomplished after 48 hours of fermentation. Similarly, Kook & Cho (2013) established a fed batch fermentation to enhance the GABA production by *L. brevis* B3-18 (isolated from kimchi) that managed to produce significantly higher GABA concentration (1000 mM) as compared to the batch fermentation (660 mM).

A semi continuous fermentation with the implementation of cell recycling, offers another interesting approach for an industrial scale of GABA production. Recently, Hsueh et al., (2021) established a semi continuous fermentation by immobilization of *L. brevis* RK03 on a ceramic porous "Power Material" (PM) for GABA production. The highest GABA production of 55 010 mg/L was attained in a medium that was adjusted to pH 5.0 after three cycles. They also found that the MSG conversion rate was near completion (97%) after 25 consecutive fermentations that lasted for 1200 hours with the optimum GABA production of 55 g/L. Earlier, an invention on a semi continuous fermentation for GABA production by LAB was patented by China Agricultural University (2015). The LAB was fermented in a mode of segmental substrate addition (Man, Rogosa and Sharpe (MRS) gelatin culture medium), segmental of temperature control and segmental of oxygen control to obtain the highest GABA of 38.7 g/L in a total fermentation time of 106 hours.

6.2 Optimization of Fermentation parameters

There are several important parameters needed to be optimized for high yield GABA production in fermentation such, pH, temperature, incubation period, initial substrate concentration and type of medium (Li et al., 2010a). Table 3 summarizes several examples of optimized fermentation conditions for GABA production by various LAB.

Table 3. Optimized conditions for fermentation of GABA production by various strains isolated from different sources.

Strain	Source of isolation	Optimized condition	GABA yield	Reference
***L. plantarum* EJ2014**	Rice bran	pH: 6.5, 30 ˚C, 22.5 g/L MSG, 100 g/L yeast extract, 10 g/L dextrose, 72 h	19.8 g/L	Park et al., 2021
***L. plantarum Taj*-Apis362**	Honey stomach (*A. dorsata*) in Malaysia	pH: 5.3, 36 ˚C, 497.97mM (initial) MSG, 60 h	0.73 g/L	Tajabadi et al., 2015
***L. futsaii* CS3**	*Kung-Som*	pH: 4.5, 37 ˚C, 25 mg/mL, 48 h	6.3 g/L	Sanchart et al., 2017
***L. brevis* SIIA11021**	Chinese traditional pickled vegetables	pH: 4.7, 30 ˚C, 100 g/L MSG, 1.3 % (v/v) ethanol, 24 h	45.4 g/L	Lei et al., 2014
***L. brevis* RK03**	Red bigeye fish (*Priacanthus macracanthus*)	pH: 4.5, 30 ˚C, 1% (w/v) glucose, 2.5% (w/v) yeast extract; 2 ppm each of CaCO3, MnSO4, and Tween 80, 10 µM PLP, 650 mM MSG, 88 h	62.523 mg/L	Wu et al., 2018
***L. brevis* NM101-1**	Egyptian dairy products	pH: 5, 35 ˚C, 750 mM MSG, 200 µM PLP, 72 h	17.4 g/L	Gomaa, 2015
***L. plantarum* DSM749**			14.5 g/L	

***L. plantarum* NDC75017**	China traditional fermented dairy products	pH: 4.5, 36 ˚C, 80 mM MSG, 18 µM, 48 h	3.15 g/L	Shan et al., 2015
***L. lactis lactis* NCDO2118**	Vegetables origin (France)	pH: 6.5, 30 ˚C, 5 g/L MSG, 20 g/L glucose, 5 g/L arginine, 20 g/L malate, 14 h	0.9 g/L	Laroute et al., 2016
***L. brevis* CRL 2013**	Andean Amaranth and Real Hornillos quinoa sourdoughs	pH: 6.5, 30 ˚C, 267 mM MSG, de Man, Rogosa & Sharpe (MRS)-20 g/L each glucose and fructose, 72 h	27.3 g/L	Cataldo et al., 2020b
***L. brevis* TISTR 860**	Fermented spider weed	pH: 5, 30 ˚C, MRS, 2% (w/v) MSG, 10% (w/v) non-germinated red kidney bean milk, 2% (w/v) glucose, 1% (w/v) peptone, 12 days	4.6 g/L	Saraphanchotiwitthaya, & Sripalakit, 2018
***L. brevis* CRL 1942**	Andean Amaranth and Real Hornillos quinoa sourdoughs	pH:6.5, 30 ˚C, 270 mM MSG, 144 h	26.3 g/L	Villegas et al., 2016

MRS: de Man Rogosa & Sharpe, MSG: Monosodium glutamate, PLP: Pyridoxal 5 Phosphate, h: Hours

6.2.1 Effect of pH on LAB

pH is the most important parameter in the production of GABA in comparison to the other fermentation conditions (Kook and Cho, 2013). pH plays an essential role in the GABA production by regulating the activity of GAD during the fermentation process (Lin, 2013, Yang et al., 2008). In general, different strains have different optimal pH to reach the maximum yield of GABA (Dhakal et al., 2012). However, most of the LAB strains isolated from the fermented foods such as yogurt, kimchi, paocai, cheese and tea preferred acidic pH (Sharafi and Nateghi, 2020). GAD in LAB is only active under acidic conditions with the maximum yield of GABA obtained at the pH ranging from 4.0 to 6.0 and could lose its activity or denature at higher pH (Rashmi et al., 2018; Li et al., 2010a). In the beginning of the LAB fermentation, the pH will acidify due to the substrate uptake and organic acid production (Behbahani et al., 2020). In addition, without or with a very low concentration of initial glutamate, the pH of LAB culture dropped to below 3.5 due to the production and accumulation of acidic substances such lactic acid, acetic acid and other organic acids (Li et al., 2010a). Nonetheless, in the log phase and close to the stationary growth phase, the pH increased logarithmically due to the H^+ consumption resulting from the GABA production (Behbahani et al., 2020). The role of GADs is activated when the cytosolic condition is acidic, in which the presence of glutamate facilitates the counter reaction that raise back the pH to protect the cells from the acidic condition whilst GABA is being produced. This process is known as a coupling reaction (Li et al., 2010a).

The GAD activity in *L. brevis* was found to be generally high over a wide range of acidic pH values, with optimum values of pH between 4.2 - 4.6 (Xu et al., 2017). In an optimization study using Box-Behnken's Response Surface Methodology (RSM), *L. brevis* TD10 grown in MRS media was found to reach the highest amount of GABA (19.9 g/L) at pH 4.65 (Sharafi and Nateghi, 2020). In another report, RSM model indicated pH (initial pH of 4.74) was the most significant factor for GABA production by *L. brevis* HYE1 that resulted in an experimental value of 18.76 mM which was in an agreement with the predicted value of 21.44 mM (Lim et al., 2017). The highest GABA production by *L. plantarum* L10-11 was recorded in an initial pH range of MRS medium from 5.0 to 6.0 but was dramatically decreased at pH below 4.0 or above 8.0 (Tanamool et al., 2020). Meanwhile, *L. brevis* RK03 that was grown in two different media, MRS and MRS mixed with glutamic acid at initial pH values ranging from 2.5 to 6.5 reached the highest GABA production at pH 4.5 yielding, 983 mg/L and 25 g/L, respectively (Wu et al., 2018). It was also observed that the pH of the culture medium changed with the incubation time and beside GABA, the initial pH was found to

influence the amount of the final cell biomass. While investigating the GABA productivity by three strains, *L. brevis*, *L. buchneri* and *W. hellenica* strains, the initial pH of the medium was adjusted to 3, 4, 5 and 6 and this pH was kept adjusted at every 12 hours for 50 hours fermentation period (Barla et al., 2016). It was found that the GABA productivity reached to 200-800 mg/100 mL at neutral pH while the lowest productivity was at pH 3.

Despite most LAB strains being active at acidic pH however, there are some LABs reported to have an optimum GABA production at an initial pH of above 6.0. For example, *L. lactis* strain B (isolated from Chinese traditional cabbage kimchi) produced the most GABA (reached maximum value of 7.2 g/L) when the pH was between 7.0 to 8.0, which is a weak alkaline pH range (Lu et al., 2009). Recently, Cataldo et al. (2020b) conducted a study to evaluate the effect of an early acidification of media (initial pH of MRS media was adjusted to pH 4.0, 5.0, 5.8, 6.4, 6.7 and 7.25) on GABA production by *L. brevis* CRL 2013. They found that the GABA production was not affected by pH values between 5.8 to 7.25 but decreased when the initial pH was set at below 5.8.

6.2.2 Incubation Temperature

Incubation temperature is another important factor influencing the production of GABA by LAB (Behbahani et al., 2020). Besides biocatalytic activity, temperature also affects the thermodynamic equilibrium of reactions. Temperature ranging from 30 to 37 ˚C is the ideal temperature for GABA production while most LAB could not withstand the temperature above 45˚C that consequently influenced their ability to produce GABA (Li et al., 2010a). However, the GADs from some LAB strains such as *L. brevis* possess higher catalytic rate at temperatures between 30 to 50 ˚C (Yu et al., 2012). It was reported at temperatures between 30°C and 35°C, *L. plantarum* DSM19463 generated the most GABA (59 M/h) (Di Cagno et al., 2010). It was also reported that increasing the temperature from 30°C to 37°C did not affect the accumulation of GABA in the fermentation broth that was observed within 72 hours of incubation period of *L. plantarum* L10-11 (Tanamool et al., 2020). From the optimization study using RSM, the highest GABA of 170 mg/kg was achieved from the cultivation of *L. plantarum* at 37°C in MRS broth (Zarei et al., 2018). *L. paracasei* NFRI 7415 also produced the maximum GABA of 302 mM at 37°C, but at 43°C, the GABA production and cell growth were substantially reduced (Komatsuzaki et al., 2005). Likewise, the maximum GABA yield of 4.2 g/L was achieved at 37 ºC from the fermentation of *L. fermentum* in a soymilk medium (Rayavarapu et

al., 2021). However, increasing the temperature to 40 ºC decreased the GABA production as well as the cell count of *L. fermentum*. In contrast, Thuy et al., (2021) reported an increase of GABA production by *P. pentosaceus* MN12 from 13 mM to 25 mM with the increase of temperatures from 30ºC to 45ºC, respectively. However, further increasing the temperature to 50ºC significantly reduced the GABA content to 15 mM.

6.2.3 Incubation Time

The incubation time has significant effect on the amount of GABA production. For LAB strains, it was shown that GAD started converting to GABA during the late logarithmic growth phase and at its best in the stationary phase (Hayakawa et al., 2004, Kook & Cho, 2013). During the 72 hours incubation time course of GABA production by *L. fementum*, GABA was slowly produced at 24 hours and reached the maximum concentration of 4.6 g/L at 48 hours (during its stationary growth phase) (Rayavarapu et al., 2021). Assessment throughout the fermentation time course revealed that *L. brevis* RK03 (grown in G broths containing 1% glucose; 2.5% yeast extract; 2 ppm each of $CaCO_3$, $MnSO_4$, and Tween 80; 10 μM PLP and 650 mM MSG) produced the highest GABA yield (62 g/L) at 88 hours and obtained slightly lower yields (approximately 57 to 60 g/L) in prolong incubation periods of 96, 104 and 112 hours (Wu et al., 2018). In the meantime, the maximum generation of GABA was achieved by *L. plantarum* DSM19463 (Di Cagno et al., 2010) and *L. paracasei* NFRI 7415 (Komatsuzaki et al., 2005) after 72 and 144 hours of fermentation, respectively.

6.2.4 Size of Inoculum

Effect of inoculum sizes from 1 to 4 % was evaluated for GABA production by *L. fermentum* in a soymilk medium with supplementation of glucose (Rayavarapu et al., 2021). The highest yield of GABA of 3.8 ± 0.01 g/L was obtained at inoculum size of 1 % and viable cell count of 5.8 ± 0.3 log cfu/mL. *L. pentosus* SS6 fermentation in mulberry leaf powder produced the greatest amount of GABA at inoculum size of 7% (Zhong et al. (2019). Meanwhile, Wu et al., (2018) studied the effect of *L. brevis* RK03 cell inoculum from 1 $x10^7$ to $1x10^9$ CFU/mL on the production of GABA in two different media, MRS and MRS with the addition of 550 mM glutamic acid. The highest yield of GABA was obtained in the

fermentation with 1x10^9 CFU/mL inoculum resulted in 773 mg/L (in MRS) and 14443 mg/L GABA (in MRS with glutamic acid).

6.2.5 Carbon and Nitrogen Sources

LAB production of GABA by fermentation is affected by the composition of media particularly carbon and nitrogen sources. Recently, Rayavarapu et al. (2021) investigated on the effect of carbon (glucose, sucrose, lactose, and maltose) and nitrogen (MSG, peptone, yeast extract, beef extract) sources supplementation to the soymilk media GABA production by *L. fermentum*. They found that among other carbon and nitrogen sources tested, glucose and MSG strongly affected the GABA production. Increasing the concentration of glucose from 0.5 to 1.0 % significantly increased the yield of GABA from 4 to 4.7 g/L which was in correlation with the cell count that was also increased from 1.2 x 10^9 to 6.26 x 10^9 CFU/mL. However, increasing glucose to 2%, drastically decreased the yield of GABA to 0.8 g/L suggesting there was a strong substrate inhibitory effect. High concentration of glucose leads to high osmotic pressure shrinking the cells causing inhibition of cell growth as well as GABA production (Nguyen et al., 2008).

Zhong et al. (2019) studied the effect of carbon (glucose, saccharose, xylose) and nitrogen (peptone, K_2HPO_4 and L-sodium glutamate) sources at different concentrations to the production of GABA by *L. pentosus* SS6 in mulberry leaf powder (5%). The highest yields of GABA were attained using saccharose at 5 and 10% (~ 35 mg/g GABA) and K_2HPO_4 at 1.6% (~30 mg/g GABA). Among the carbon source (glucose, lactose, sucrose, maltose, galactose, glycerol, molasses, starch, dextrin1 (DE 8-10), dextrin2 (DE10-12), brown sugar, golden sugar) and nitrogen sources (yeast extract, tryptone, soya peptone, beef extract, peptone, ammonium thiocyanate, alumiuium nitrate, ammonium nitrate, ammonium molybodate, ammonium persulfate, urea, diammonium hydrogen phosphate) screened in the single parameter optimization study, 1% of glucose and 1% of peptone yielded the maximum GABA production by *L. brevis* RK03 (Wu et al., 2018). Meanwhile, using an optimized synthetic medium consisting of 10 g/L glucose (as carbon source) and 100 g/L yeast extract (as nitrogen source) and 2.25% MSG, an enhanced production of GABA reaching 20 g/L was successfully attained by *L. plantarum* EJ2014 (Park et al., 2021).

From the screening of 36 nitrogen sources (13 types of yeast extract, bovine heart extract, beef extract, bovine liver extract, tryptone, 2 types of soy peptone, yeast peptone, egg

albumin extract, gelatin peptone, bacterial peptone, 2 types of tryptones, 3 types of casein peptones, protease peptone, fish peptone, beef peptone, 2 types of soy peptones, polypeptone, and lactoalbumin hydrolysate) on GABA production by *L. brevis* NCL912, Wang et al. (2018) discovered only five nitrogen sources (yeast extract 02-12C, 02-12A, FM405, FM408 and soy peptone Fp410) showed relatively high GABA production. Further optimizing these five nitrogen sources in a range of 10 to 75 g/L, the highest GABA formation (~170 g/L) was attained in the medium containing 25 g/L yeast extract FM408. In the meantime, the other reports on *L. brevis* production of GABA demonstrated the presence of 2% yeast extract or casein peptone (Binh et al., 2014) and 1 % peptone (Saraphanchotiwitthaya and Sripalakit, 2018) had facilitated the increase of the GABA synthesis.

6.2.6 Initial Concentration of MSG

Theoretical production yield of GABA can be represented by how much glutamic acid which is supplemented as MSG has been converted into GABA by decarboxylation reaction (Behbahani et al., 2020). GABA is started to be synthesized once the MSG that is added in the fermentation medium directly enters the cells by their antiporter system in the cell membrane (Kook and Cho, 2013). Nevertheless, adding a suitable initial MSG concentration is important because at high concentration of glutamate could inhibit the growth of LAB and consequently limit the yield of GABA (Li et al., 2010b).

The ideal initial MSG concentration varies among different LAB strains (Li et al., 2010a). *L. paracasei* NFRI 7415 obtained a GABA concentration of 161 mM in the presence of 500 mM glutamate (Komatsuzaki et al., 2005). Shi et al. (2017), developed an efficient bioconversion of a mixed substrate consisting of L-glutamic acid (80 g/L) and MSG (240 g/L) into GABA by *L. brevis* TCCC13007 in form of resting cells generating 201 g/L GABA. Behbahani et al. (2020) showed the production of GABA by *L. brevis* A3 linearly increased with the increase in initial MSG concentration. As expected, the GABA production was significantly low in the low concentrations of whey and MSG that were supplemented as the carbon and nitrogen sources, respectively. Under optimized culture conditions (4.95 % MSG, 14.95 % whey, 37ºC), the maximum GABA production of 53.5 ppm was obtained after 48 hours cultivation of *L. brevis* A3. Effect of MSG concentrations between 0.5 to 5.0 % (w/v) on GABA production by *L. plantarum* L10-11 in MRS broth was previously studied by Tanamool et al. (2020). They observed an increase in GABA accumulation (15.74 g/L) with the increase

of MSG concentrations up to 4% (w/v). In another study, the yield of GABA and GAD activity of *L. plantarum* NDC75017 was enhanced by the supplementation of MSG up to 75 mM but declined at above this concentration (Shan et al., 2015). High concentration of MSG may inhibit the cell growth of LAB and subsequently reduce the GABA production. Likewise, the contents of GABA by *P. pentosaceus* MN12 were proportionally increased from 0 to 60 mM of MSG with an optimum GABA value of 17.6 ± 0.24 mM but decreased with 90 and 120 mM of MSG addition (Thuy et al., 2021). The reduction of GABA at high concentration of MSG may be due to the elevation in osmotic pressure of the medium that affected the metabolism of cells and the production of GABA (Villegas et al., 2016). In the biosynthesis of GABA by *L. lactis* strain B, the highest yield was obtained when MSG was added at the start of fermentation, but the GABA yield decreased when MSG was added at 6 hour intervals of fermentation period (Lu at el., 2009). This observation shows that beside initial concentration, the time of MSG addition also has an important effect on the GABA productivity.

6.2.7 Addition of Pyridoxal 5-Phosphate (PLP)

The addition of pyridoxal 5-phosphate (PLP) (as GAD coenzyme) could affect the rate of GABA production in LAB (Sarasa et al., 2020). The effect of PLP at the concentration of 0 to 100 µM was previously investigated on the GABA production by *L. brevis* RK03 (Wu et al., 2018). The maximum yield of GABA (> 25 g/L) was obtained at 10 or 20 µM of PLP. When PLP was introduced at different time intervals of 0, 24, and 48-hours incubation of *S. salivarius* subsp. *thermophilus* Y2, 63, 66, and 73 g/L GABA were produced, respectively (Yang et al., 2008). This result shows that the addition time of PLP influenced the production of GABA suggesting that later addition of PLP (i.e, at 48 hours) might avoid the denaturation of PLP in the culture medium. However, Li et al. (2010a) reported that the addition of PLP in the study involving *L. brevis* NCL912 did not affect the yield of GABA due to the presence of enough PLP production within *L. brevis* NCL912.

6.2.8 Addition of Trace Elements

The addition of trace elements may have significant effect on the GABA production by LAB due to GADs activation capability. For an example, the addition of NaCl in MRS medium at concentrations up to 3% seemed to be able to enhance the GABA production by *L. plantarum*

L10-11 (Tanamool et al., 2020). This could be due to the activation of GAD in the osmotic stress induced by NaCl (Kanwal et al., 2014). Seo et al. (2013) showed the presence of $CaCl_2$ aid in the activation of GAD activity in *L. brevis* 877G. In contrast, the GAD activity was deactivated in the presence of KI, $AgNO_3$, $ZnCl_2$, $CuCl_2$, or Na_2SO_4 while NaCl exhibited no significant effect. Meanwhile, the supplementation of arginine and malate along with glutamate led to the highest GABA production (8.6 mM) and enabled an earlier production at pH 6.5 by *L. lactis* NCDO 2118 (Laroute et al., 2016).

In overcoming the inhibition effect on GABA formation that is often observed due to excessive MSG, Wang et al. (2018) examined the usage of L-glutamic as the substitute in the biosynthesis by *L. brevis* NCL912. They concluded L-glutamic acid is a promising alternative to MSG due to the high concentration of GABA (205 g/L) obtained. L-Glutamic acid may offer advantages over MSG since it has lower solubility and could be added one time (at the beginning of the cultivation) due to its sustained release and pH buffering capacity. They also investigated the effect of 20 potential trace elements (mannitol, sodium citrate, linoleic acid, $MnSO_4 \cdot H_2O$, KNO_3, KH_2PO_4, NaCl, $CaCl_2$, $FeCl_3 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, NH_4NO_3, Al $(NO_3)_3 \cdot 9H_2O$, VB_1, VB_6, VB_7, VB_{12}, NAD, $NADPNa_2$, Zn $(CH_3COO)_2 \cdot 2H_2O$) on the GABA production by *L. brevis* NCL912 and found only $MnSO_4 \cdot H_2O$ significantly improved the GABA production. Further optimizing the concentration of $MnSO_4 \cdot H_2O$ resulted in the maximum GABA attained in 25 mg/L $MnSO_4 \cdot H_2O$ while increasing the concentrations did not facilitate the GABA production.

The evaluation of Tween-80 as the growth stimulator of *L. brevis* RK03 showed the addition of 2 ppm Tween-80 was sufficient to achieve the maximum GABA production (Wu et al. 2018). Li et al. (2010) and Wang et al. (2018) also showed that Tween 80 (2 g/L) helped to trigger the production of GABA by *L. brevis* NCL912. Tween-80 may improve the cell membrane permeability and thus help to increase the efficiency of the glutamate-GABA antiporter to promote the GABA formation (Wang et al., 2018).

6.3 Fermentation Technique

6.3.1 Two-stages Fermentation

In certain fermentations, the optimum growth rate and production rate will be reached at different optimum parameters as encountered by Peng et al., (2013) during the GABA production by *L. brevis* CGMCC 1306. They found that the optimum growth rate was observed at pH 5.0, 35 ˚C while the optimum GABA production was at pH 4.5, 40 ˚C. This problem was sorted with a two-stage pH and temperature control technique, in which for the first 32 hours, the fermentation was controlled at pH 5.0, 35 ˚C to promote the growth of cell and then these parameters were changed to pH 4.5, 40 ˚C to increase the production of GABA. An amount of 475 mmol/L GABA was achieved at 72 hours using this two-stage pH and temperature control technique compared to one-stage technique that only produced 399 mmol/L of GABA. This strategy also prevented the cell growth inhibition by high initial substrate concentration because an appropriate initial concentration of substrate was introduced for first 32 hours during cell growth period whereas the post-inoculation of additional substrate after 56 hours increased the GABA production to 526 mmol/L.

The GABA biosynthesis under submerged fermentation is relatively related to the biochemical characteristics of the GAD in which its activity is retained even though the cells are no longer viable (Yang et al., 2006). This suggests that the GABA production can be increased by adjusting the cultivation parameters to suit the biotransformation of the LAB cells once the highest GAD production is reached. This hypothesis was proven by Yang et al. (2008) while investigating the production of GABA by *S. salivarius* subsp. *thermophilus* Y2. Initially the fermentation temperature was controlled at 37ºC which was the suitable condition for optimal GAD production and at 24 hours, the culture conditions were adjusted to 40ºC and pH 4.5 to allow for the optimal GAD reaction activity. At 48 hours, 0.02 mmmol/L PLP was added to the fermentation broth. The results showed this strategy greatly helped to enhance the GABA production yielding 80 g/L that was equivalent to 1.76-fold increment from the conventional one-stage fermentation technique.

6.3.2 Co-culturing Strategy

Co-culturing is a common technique in fermentation used to improve cell growth and the productivity of desired products (Wang et al., 2021). Usually, a group microbe or more

than one microbe that share similar characteristics or categorized under the same family will be used in this co-culturing process (Cui et al., 2020). The effects of one species towards another is usually studied in this technique because some species only act as a precursor producer for another to utilize it and produce the desire product. However, in some cases, there will be no correlation at all between the strains, only manipulating the co-culturing strategy to increase the productivity.

L. brevis reportedly yielded a high amount of GABA however it had low proteolytic activity that prevented its efficient application in milk fermentation (Wu et al., 2015). Hence, this problem was sorted by co-culturing *L. brevis* with convectional dairy starter cultures. These starter cultures help to breakdown the milk protein into peptides which will act as nutrition for the cell growth of *L. brevis* (Cui et al., 2020). To improve the GABA productivity of *L. plantarum, a* co-culturing approach with *L. lactis* subsp. *lactis* was implemented in the fermentation using cheese whey that resulted the maximum 366 mg/100 mL of GABA (Karimian et al., 2020). Previously, the protease producer, *L. sakei* 795 was fermented with a GABA-producing *L. brevis* 877G strain, successfully increasing the GABA production achieving 22.5 mM (Seo et al., 2013). Co-culture of activated high viscosity yogurt starter YC-X11 with highly proteolytic cultures of O-114, *L. rhamnosus B-1445*, and *L. helveticus* Lh-B 02 displayed a considerable role for increasing the GABA production resulting the highest GABA content of 10.3 mg/100mL in yogurt (Abd El-Fattah et al., 2018). *L. plantarum* K154 (isolated from kimchi) and *Leu. mesenteroides* SM (isolated from carrot juice) were employed to co-ferment a water dropwort homogenate enriched with sucrose and yeast, significantly increasing the GABA production (100 mM) compared to a fermentation utilizing only *L. plantarum* K154 (1.5 mg/mL) (Kwon et al., 2016). This result shows that both strains collaborated synergistically to produce GABA in which *Leu. mesenteroides* SM produced an acidic thick broth at the beginning of the fermentation providing a suitable condition for *L. plantarum* K154 to produce GABA.

Co-culturing *L. futsaii* CS3 and *Candida rugosa* 8YB in a two-step fermentation technique utilizing tuna condensate to provide a natural glutamic acid for the formation of GABA resulted in the highest GABA productivity of 135 mg/L/h (Sanchart et al., 2018). The first step of fermentation was responsible for L-glutamic acid generation while the latter part utilized the generated L-glutamic acid to produce the end-product, GABA. Other examples of GABA production using co-culture approach such as the production of 15 mM of GABA from co-culturing of *L. bulgaricus* IAM1120 and *S. thermophilus* IFO 13,957 (Watanabe et al.,

2011) and co-culture of *Bacillus subtillis* HA with *L. plantarum* K154 that produced 83 mM of GABA (Kim et al., 2014). Additionally, co-culturing of *L. plantarum* K154 and fungus *Ceriporia lacerate* produced 15.53 mg/mL GABA and other active compounds such peptides and polysaccharides in a cost-effective manner. Exopolysaccharides, protease, cellulose, and α-amylase that were abundant in *C. lacerate,* provided enough resources for the optimal growth of *L. plantarum* (Lee & Lee, 2014).

6.3.3 Immobilization Technology

The immobilization concept and techniques for efficient biocatalyst (viable cells (microbial, plant, or animal) and enzymes) have introduced a new dimension to the constantly expanding the area of biotechnology (Lou et al., 2021). In particular, immobilized whole-cell systems have received a great attention because of the multiple advantages that they can offer compared to the free-living bacteria. The advantages of using immobilized whole-cell systems include higher productivity, higher cell survival than the free suspension, improved bacterial balance, enhanced plasmid stability, prevention of cell lysis, protection against bacteriophages and stressful conditions such as shear damage, as well as better generation and secretion of by-products (Aeron & Morya, 2017). Natural polymeric gels (i.e., carrageenan, calcium alginate, agar) (Halim et al., 2017) and synthetic polymers (i.e., polyacrylamide, polyvinyl, polyurethane) (Hsueh et al., 2017) have both been widely employed for whole-cell immobilization.

Different immobilisation methods have been developed to immobilise bacteria on carriers, and the immobilisation substance may alter bacterial growth and metabolite production (Hsueh et al., 2021). Kook & Cho, (2013) reported, a fed-batch mode involving immobilized *L. brevis* GABA 057 strains using isomalto-oligosaccharides added alginate bead that managed to transform 534 mM of MSG into 223 mM GABA within 48 hours. The addition of isomalto-oligosaccharides with alginate beads helped to increase the stability of bacterial cells and the GABA productivity. Lyu et al. (2019) established an immobilization system for an engineered GABA high-producing strain *L. brevis* Gad_{ADC14} mutant within gellan gum gel beads producing the highest GABA of 88 g/L after 10 successive fermentation cycles at pH 4.4 at 40 ˚C. The usage of an engineered bacteria immobilized in these natural support matrices effectively improved the thermo stability of the LAB cells as well as its productivity

Recently, the efficiency of immobilized *L. brevis* RK03 using three types of porous ceramic materials (namely All Clean (AL), Ocean Free (OF), and Power Material (PM)) was evaluated for the production of GABA (Hsueh et al., 2021). Among these, the fermentations of *L. brevis* RK03 immobilized in PM in a batch mode resulted in 96.5% conversation rate of MSG to GABA in 96 hours; while in a semi-continuous mode (operated with the similar optimal conditions) produced a higher GABA productivity with 86% conversation rate recorded after 288 hours within 5 cycles. Further scaling up of the optimized conditions of semi-continuous fermentation into 10 L fermenter demonstrated 97% conversation rate that was observed for 25 cycles (1200 hours) exhibiting 55 g/L GABA. From an earlier report, *L. brevis* RK03 was immobilized in hydrogel films (made of 93% 2-hydroxyethyl methacrylate (HEMA) / 3% polyethylene glycol diaceylate (PEGDA)) showing a maximum value of 98% conversion rate of MSG to GABA after 240 hours of fermentation (Hsueh et a., 2017). Nevertheless, this conversion rate was gradually declined to 84% after five cycles of semi-continuous mode within 420 hours of fermentation.

6.4 Genetic Engineering

Genetic engineering is another approach commonly used to improve GABA production via changes in the metabolic pathways of bacteria. Generally, there are two different genetic engineering approaches which are direct modulation approach and indirect modulation approach (physiology-oriented engineering). For direct modulation approach, alteration is directly focus on the key enzyme, GAD-encoding gene within the cells to enhance the decarboxylation activity of L-glutamic acid into GABA (Cui et al., 2020). In the meantime, indirect modulation approach focuses on the alteration of cell metabolism (excluding the GAD-encoding gene) that indirectly affect the cell growth and eventually improve the GABA production.

An engineered high GABA producing strain, *L. brevis* $GadA_{\Delta C14}$ was reported to produce a high yield of GABA (88 g/L) via whole cell immobilization method in gellan gum gel beads (Lyu et al., 2019). The strain was developed though overexpressing a C-terminally truncated GadA mutant, which allowed the enzyme to work in a near-neutral pH compared to the wild type. Extending the active pH range of intracellular GAD via engineering approach might be a viable option for reducing the harmful effects of slightly acidic and neutral environments on *L. brevis* $GadA_{\Delta C14}$ during GABA production. Co-expressing both *gad*B1 and

*gad*B2 from *L. brevis* into *C. glutamicum* managed to double up the GABA production (19 g/L) accomplished in the single-expressing strain (*gad*B1 or *gad*B2) (Shi et al., 2013). Meanwhile, the *gad*B2, *gad*C, and *gad*R complex from *L. brevis* Lb85 was genetically modified into *C. glutamicum* strain ATCC 13032 to produce GABA concentration up to 2.15 g/L after 72 hours of fermentation (Shi & Li, 2011).

Indirect modulation approach or also known as physiological-oriented engineering has significant effects on the metabolic pathway and end-product of the microbe even though the modulation was not directly made on the specific gene that control the metabolic pathway of the microbe (Zhang & Li, 2013). This approach increased the survival rate of microbe and retain the productivity at an optimum level under certain stressful conditions during fermentation or industrial applications such as shear stress, oxidative stress, extreme pH, and extreme temperature. As a result, increasing the physiological performances of industrially related strains through physiology-oriented engineering has become a significant technique widely applied to boost work efficiency (Lyu et al., 2017). LAB paradoxically produced reactive oxidative species (ROS) such as H_2O_2 which caused harmful effects towards the cell growth of LAB itself and eventually led to death (Lyu et al., 2016). To prevent the potentially harmful effects of H_2O_2 on cells, the gene *katE*, which encodes a heme-dependent catalase (CAT), was cloned from *L. brevis* CGMCC1306 and overexpressed on another wild type *L. brevis*. As the result, the wild type *L. brevis* with CAT expressed had higher survival rate (823-fold) and eventually produced 66.4 g/L of GABA.

By expressing *L. plantarum* GAD in *L. sakei* host cells, Kook et al. (2010a) managed to achieve a maximal GABA output of 27.3 g/L. Meanwhile, the genetically engineered GADs from *E.coli* that had E89A/H465A double mutant was shown to retain a substantially high catalytic activity at neutral pH however the triple mutant Q5D/V6I/T7E allowed the GADs to function at higher temperatures beside increasing the melting temperature (Ho et al., 2013). It was reported that the mutation on C-terminal of GADs in *L. brevis* CGMCC 1306 achieved a greater activity at near-neutral pH, while T17I/D294G/ E312S/Q346H mutant from *L. brevis* Lb85 attained 43.2- fold of wild type activity at pH 6.0 (Yu et al., 2012).

A phase in which LAB fermentation experiences a decreasing pH is the appropriate period for LAB to synthesize GABA however, the acidic condition may inhibit the cell growth (Cui et al., 2020). LAB counteract this situation with a number of acid resistance pathways; and the most common one is F_oF_1-ATPase pathway which control the intracellular pH by

developing the proton motive force and releasing intracellular protons (Wang et al., 2018). During GABA production, the generated protons will be utilised back by the GAD system, thus F_oF_1-ATPase pathway will become inefficient in regulating the intracellular pH (Cui et al., 2020). Therefore *L. brevis* NRA6 overexpressed with F_oF_1-ATPase-deficient and GAD was developed to redirect the protons influx towards GAD system and subsequently increased the GABA production (Lyu et al., 2017). It was reported that *L. brevis* NRA6 overexpressed with F_oF_1-ATPase-deficient and GAD obtained higher GABA yield (43.65 g/L) compared to the wild type strain.

7.0 Conclusions

Nowadays, GABA has emerged as a versatile bioactive compound universally used to develop numerous health-oriented products. Nonetheless, GABA intended for use in food and nutraceutical products must fulfil strict safety requirements and hence cannot be chemically synthesized. Therefore, a biotechnological approach for GABA production appears to be promising due to simple procedure, high catalytic efficiency, and environmental compatibility. In particular, microbial fermentation by LAB is one of the most favorable approaches to biosynthesize natural GABA mainly because the procedure is convenient relying on cheaper substrate consumption with high biotransformation rate. GABA production by LAB fermentation could be improved by selecting the best mode of fermentation (i.e., batch, fed batch and continuous), optimization of the fermentation conditions (i.e., pH, temperature, incubation time, size of inoculum, medium composition and initial substrate concentration, addition of PLP cofactor and other trace elements) and employing fermentation techniques (i.e., two-steps fermentation, co-culturing approach, immobilization technology and genetic engineering approachs) that are best suited to the selected GABA producer LAB strain. Cost effective, easy to scale-up and high-performance production as well as optimization through emerging biotechnological tools and techniques, will remain the focus of interests whilst exploring the potential of GABA as a health-related bioactive compound. While LABs are currently the most attractive group of microorganisms capable of producing GABA at high yields, there is always an opportunity for the discovery of novel GABA producing LAB strains with new and improved therapeutic efficacy. Furthermore, considering the growing interest for novel health-promoting and functional food products, continuous research and development should also be converging towards providing the accurate measure of efficacy and safety to

meet consumers expectations on quality and claimable health benefits of the GABA-enriched products. To achieve these goals requires confirmation from *in-vitro* and *in-vivo* experiments as well as clinical trials.

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