FACULTEIT
BIO-INGENIEURSWETENSCHAPPEN

SCREENING OF GABA-PRODUCING LACTIC ACID BACTERIA AND INCREASING THE GABA CONTENT IN SOYMILK

Aantal woorden: 19775

Nills Parmentier
Studentennummer: 01401063

Promotor: Prof. dr. ir. Katleen Raes
Tutor: MSc. Phuong Hong Le

Masterproef voorgelegd voor het behalen van de graad Master in de industriële wetenschappen: Biochemie

Academiejaar: 2017 – 2018

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Kortrijk, juni 2018

The author,

Nills Parmentier

The promotor,

Prof. dr. ir. Katleen Raes

The tutor,

MSc. Phuong Hong Le

ACKNOWLEDGEMENTS

"Success consists of going from failure to failure without loss of enthusiasm" – Winston Churchill

This quote brings me back to the first month of my thesis, where everything seemed to fail, and my results were negative. If it was not for my enthusiasm and curiosity, I would not be writing the acknowledgements right now. So, I call this thesis, and with it the ending of four wonderful years, a success. This enthusiasm was fuelled by a lot of people, to whom I want to express my deepest and sincerest thanks.

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Juni, 2018 Nills Parmentier

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ABBREVIATIONS

2-ME	β-mercaptoethanol
BCG	Bromocresol green
CFU	Colony forming units
DM	Diabetes mellitus
DW	Dry weight
EDTA	Ethylene diamine tetraacetic acid
FW	Fresh weight
GABA	γ-aminobutyric acid
GABA-TA	γ-aminobutyric acid transaminase
GAD	Glutamic acid decarboxylase
Glu	L-glutamic acid
GRAS	Generally recognized as safe
HHP	High hydrostatic pressure
HPLC	High performance liquid chromatography
ifGH	Immuno functional growth hormone
irGH	Immuno reactive growth hormone
IS	Internal standard
LAB	Lactic acid bacteria
MRS	Man Rogosa Sharpe
MSG	Monosodium glutamate
NA	Nutrient agar
NB	Nutrient broth
OC	Overnight culture
OPA	o-phtaldialdehyde
PLP	Pyridoxal - 5′ – phosphate
SSADH	Semialdehyde dehydrogenase

ABSTRACT

In this study, a total of 17 LAB strains were screened for GABA producing ability, both qualitative and quantitative. These that produced the largest quantity of GABA were used to ferment soymilk.

Trial tests with *Lb. plantarum* and *Lb. brevis* in MRS with 200 mM MSG at 30°C resulted in 254.5 ± 3.7 mg/L and 48.9 ± 2.1 mg/L, respectively after 96 h. Moreover, addition of 0.25 mM PLP at the start of fermentation increased GABA production. After 72 h, 378.8 ± 0.6 mg/L and 58.4 ± 8.3 mg/L GABA were obtained with *Lb. plantarum* and *Lb. brevis*, respectively.

These fermentation parameters were then used for all 17 LAB strains, GABA was analysed after 96 h. Two strains produced the highest quantity of GABA: i.e., *Lc. lactis subsp. cremoris* produced 603.2 ± 12.9 mg/L with a conversion rate of 4.23% and *Lb. sakei*, produced 643.5 ± 42.7 mg/L with a conversion rate of 4.36%. Because these strains produced the most GABA, they were implemented to ferment soymilk.

Initially, GABA content was analysed in raw soymilk and soymilk with 2% glucose after 24 h of fermentation at 30°C. No significant difference in GABA producing ability was found between strains and media.

For both strains in soymilk, GABA content increased linear until 16 h of fermentation, prolonging the fermentation time did not increase the GABA content. Soymilk separation occurred around 4 h of fermentation, at this point only half of the amount of GABA obtained after 24 h was produced.

Finally, a significant effect of PLP was observed after 4 h of fermentation. Addition of 0.25 mM PLP increased GABA production by *Lb. sakei* with 341.5% and 1413% in soymilk without and with 2% glucose respectively. For *Lc. lactis subsp. cremoris*, this increase was 158.3% and 632.7%.

SAMENVATTING

In deze thesis werden 17 melkzuurbacteriën getest op hun mogelijkheid om GABA te produceren. Deze die de hoogste productie vertoonden, werden gebruikt om sojamelk te fermenteren.

Proeftesten met *Lb. plantarum* en *Lb. brevis* in MRS met 200 mM MSG bij 30°C zorgden voor een GABA-productie van 254.5 ± 3.7 mg/L en 48.9 ± 2.1 mg/L respectievelijk na 96 uur. Bovedien zorgde de additie van 0.25 mM PLP in het begin van de fermentatie voor een verhoogde GABA-productie. Na 72 uur werd 378.8 ± 0.6 mg/L en 58.4 ± 8.3 mg/L GABA bekomen na fermentatie met *Lb. plantarum* en *Lb. brevis* respectievelijk.

De fermentatieparameters werden gebruikt voor alle 17 stammen, GABA werd na 96 uur gemeten. Twee stammen zorgden voor de hoogste GABA-productie: *Lc. lactis subsp. cremoris* produceerde 603.2 ± 12.9 mg/L met een rendement van 4.23% en *Lb. sakei*, produceerde 643.5 ± 42.7 mg/L met een rendement van 4.36%. Daar deze stammen voor de hoogste productie zorgden, werden ze gebruikt om sojamelk te fermenteren.

Eerst werd GABA bepaald in sojamelk met en zonder 2% glucose na 24 uur fermentatie bij 30°C. Geen significant effect werd gevonden tussen de stammen en media.

Voor beide stammen steeg de GABA-concentratie lineair tot en met 16 uur, verlengen van de tijd verhoogde de GABA-concentratie niet. Het schiften van de sojamelk gebeurde na 4 uur fermentatie, hier was ongeveer de helft aan GABA geproduceerd als de concentratie na 24 uur.

Tenslotte werd een significant effect van PLP gevonden na 4 uur. Toevoegen van 0.25 mM PLP deed de GABA-productie door *Lb. sakei* stegen met 341.5% en 1413% in sojamelk zonder en met 2% glucose respectievelijk. Voor *Lc. lactis subsp. cremoris* was deze stijging 158.3% en 632.7% in sojamelk zonder en met 2% glucose respectievelijk.

INTRODUCTION

Nowadays the focus of food consumption is shifting from satisfaction to promoting a good and healthy life. People are becoming more aware of their health and the food industry goes along by producing so called functional foods. By doing so, they can differentiate themselves and increase profits. The global market for functional foods was worth € 108 billion in 2016 (Grand View Research, 2016). One category of the functional foods are the soy-based products.

Soybeans (*Glycine max*) are a good source of protein, dietary fibre, iron and vitamin B and are regarded as good meat-substitutes. They also contain bio-active compounds such as phytosterols and γ-aminobutyric acid (GABA). Several studies suggest that GABA may have beneficial effects on the human body, it could lower blood pressure, cholesterol levels, reduce psychological stress and has an antidiabetic effect (Inoue *et al*., 2003; Yamakoshi *et al*., 2007; Nakamura *et al*., 2009; Purwana *et al.*, 2014).

Consumption of products containing GABA could be implemented in a healthy diet, without burdening the consumer with medication.

However, concentrations are too low to exert beneficial effects. Therefore, increasing the concentration has gained interest lately. These methods focus on the enzyme that makes this conversion of glutamic acid (Glu) to GABA possible, glutamic acid decarboxylase (GAD). The main methods are germination and fermentation. Other methods include high-hydrostatic pressure (HHP) and ultrasound.

Germination is a natural process of plant growth where oxygen uptake is increased, and protein hydrolysis occurs. Because glutamic acid is the most abundant amino acid in soybeans, it can be used to produce GABA (Luo *et al.*, 2017). Several treatments to increase the GABA content during germination are hypoxia, cold stimulation, high-hydrostatic pressure (HHP) and ultrasound.

Fermentation by lactic acid bacteria (LAB) is an interesting route as well, it results in an acidic pH, thus preventing possible pathogens to grow. The low pH triggers a defense mechanism where glutamic acid is converted to GABA to raise intracellular pH. Additionally, LABs are generally recognized as safe (GRAS). With the right choice of LABs, they could arrive alive in the intestines and exert beneficial effects, the fermented product could then be regarded as probiotic (Woraharn *et al.*, 2014).

Fermentation of soybeans is already done to produce tempeh, soy sauce, natto and fermented soymilk. The fermentation process could be optimized to convert the glutamic acid in soybeans, therefore increasing GABA content. Fermentation parameters such as pH, temperature, strain, medium composition and fermentation time all contribute to the properties of the final product. Indeed, GABA content could be maximized, but the product also needs to be appealing and nutritional for the consumer.

The aims of this study are to:

a) Screen several LABs for their ability to convert Glu to GABA;
b) Use a selection of these LABs to enrich soymilk with GABA by fermentation;
c) Study the change of pH, titratable acid (TA), CFU/ml and GABA concentration as a function of fermentation time and the effect of additives in broth and soymilk.

LITERATURE

1 γ-aminobutyric acid

1.1 Glutamic acid decarboxylase

The key in GABA synthesis is the enzyme glutamic acid decarboxylase (GAD) (EC 4.1.1.15). It catalyzes the irreversible decarboxylation of L-glutamate to GABA and CO_2 and uses a proton in the process (Figure 1.1). The cofactor of this enzyme is the active form of vitamin B_6, pyridoxal - 5' - phosphate (PLP) (Feehily & Karatzas, 2013).

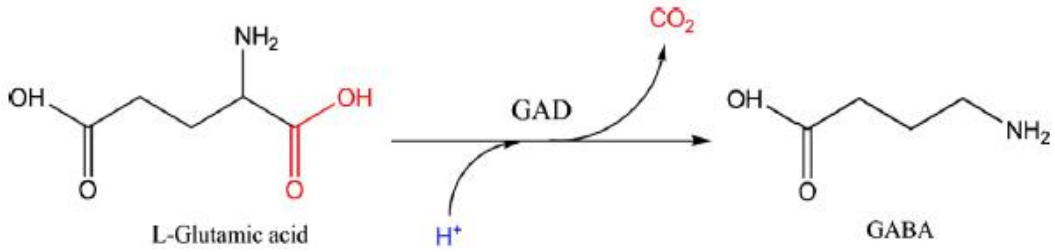


Figure 1.1: Synthesis of GABA through decarboxylation of L-glutamate (Yu et al., 2011)

In the GABA synthesis process, the first step is the interaction of PLP with the active center of GAD. The stabilization is achieved by binding at three specific nitrogen-residues of the enzyme. The phosphate-group interacts with a Gly-residue (1), the pyridinium-nitrogen interacts with an Asp-residue (2) and the carbon forms a double bond with the amine-nitrogen of a Lys-residue (3), forming an imine (Figure 1.2a) (John, 1995).

The next step is a so-called imine exchange, here the binding with Lys will be switched for Glu. This is stabilized by a hydrogen bond between the phenol-group and the imine-nitrogen (Figure 1.2b) (Seebeck & Hilvert, 2003).

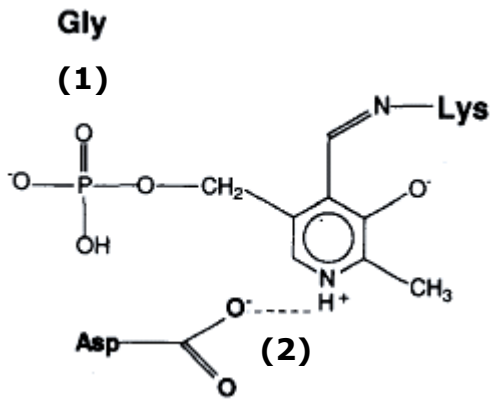


Figure 1.2a: Interaction of PLP with amino-acid residues of GAD in the active centre (John, 1995)

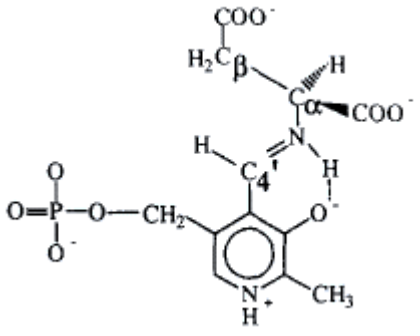


Figure 1.2b: Binding of PLP with Glu by an imine-bond (John, 1995)

Finally, the imine-nitrogen draws electrons from the carboxyl-group and breaks the bond (1), releasing CO_2. The carbanion intermediate is stabilized by the resonance of the phenol group (2) and the negative charge on the alpha-carbon is neutralized by a proton (3) (Figure 1.3). The formed GABA is switched with the Lys-residue and the enzyme is ready to catalyze the next reaction (Seebeck & Hilvert, 2003).

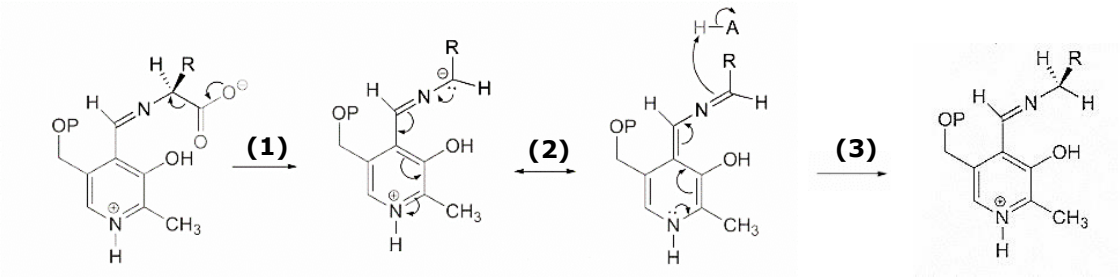


Figure 1.3: Decarboxylation of an amino-acid (top) by PLP (bottom) (Seebeck & Hilvert, 2003)

1.2 GABA in mammals

In mammals, the conversion of Glu to GABA plays a neurological role. GABA acts as an inhibitory neurotransmitter where it regulates mental health.

GAD in mammals is found in two isoforms, GAD65 and GAD67 with the number indicating their molecular weight in kDa. Being isoforms implies that the enzymes are structurally similar and perform the same biological role. In the case of GAD, the two isoforms are located in different parts of the cell. GAD65 is a membrane associated enzyme whereas GAD67 is found in the cytosol (Fenalti & Buckle, 2010). Also, GAD67 seems to be distributed equally throughout the cytoplasm (Lamigeon *et al.*, 2001), while GAD65 is mostly found in the nerve terminals (Martin & Rimvall, 1993). This statement relies on the fact that GAD65 focusses on GABA production for neurotransmission whereas GABA produced through GAD67 is only used in the GABA shunt (Pinal & Tobin, 1998).

Both enzymes form dimers with the monomeric unit consisting of a N-terminal, a C-terminal and a PLP-binding site. Two active sites are located in the heart of the PLP-binding domain (Fenalti *et al.*, 2007).

The cofactor PLP is constantly bound to the cytosolic GAD67, providing GABA continuously which is needed for the GABA shunt. GAD65 however is auto inactivated, meaning no PLP is bound. Only in response to stress GAD65 will be activated and provide additional GABA (Kash *et al.*, 1999; Fenalti & Buckle, 2010).

1.3 GABA in micro-organisms

In micro-organisms, the GAD system protects the organism against acidity (Dhakal & Bajpai, 2012). This is accomplished by the cooperation between GAD and a Glu/GABA antiporter, although this is not limited by one enzyme or antiporter. For example, *E. coli* possesses two decarboxylases (GadA & GadB) and one antiporter (GadC) (Smith *et al.*, 1992) while *Lb. reuteri* has one decarboxylase (GadB) and two antiporters (GadC1 & GadC2) (Su *et al.*, 2011).

Firstly, extracellular Glu is exchanged for intracellular GABA through the antiporter. Then, GAD decarboxylates Glu and adds a proton to the α-carbon. The resulting molecule is GABA (Karatzas *et al.*, 2012)

GABA can then be exchanged for Glu to repeat the cycle, or it can be broken down by the GABA shunt. This shunt catabolizes GABA to succinate with the aid of two enzymes: GABA-transaminase (GABA-TA, EC 2.6.1.19) and succinate semialdehyde dehydrogenase (SSADH, EC 1.2.1.16). GABA-TA converts GABA to succinate semialdehyde (SSA) where the amino group is incorporated in α-ketoglutarate to form Glu. SSA is then oxidized by SSADH to succinate and is used in the Krebs cycle (Figure 1.4) (Feehily & Karatzas, 2013).

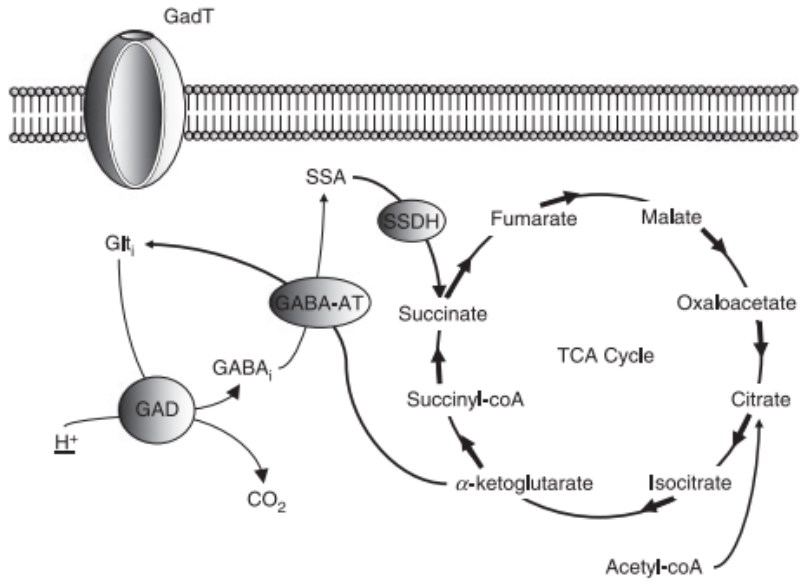


Figure 1.4: Catabolism of intracellular GABA to succinate through the GABA shunt (Feehily & Karatzas, 2013)

1.3.1 Acid resistance

How the above process contributes to acid resistance is not yet fully understood, although a model has been proposed (Figure 1.5). In this model, GAD is divided in GAD_i and GAD_e. The former converts intracellular Glu while the latter converts extracellular Glu. Under acidic conditions (pH < 4.5), the antiporter imports Glu which is converted to GABA by GAD_e. During this conversion, an intracellular proton (H^+) is incorporated in the Glu molecule to form GABA. This proton cannot be released again due to the highly stable bond. Therefore, it can be concluded that a proton has been removed from the cell resulting in an increase in intracellular pH. This GABA is then exchanged for extracellular Glu to close the cycle. Also intracellular Glu is converted to GABA by GAD_i and can be used in the GABA shunt pathway as mentioned above (Karatzas *et al.*, 2012).

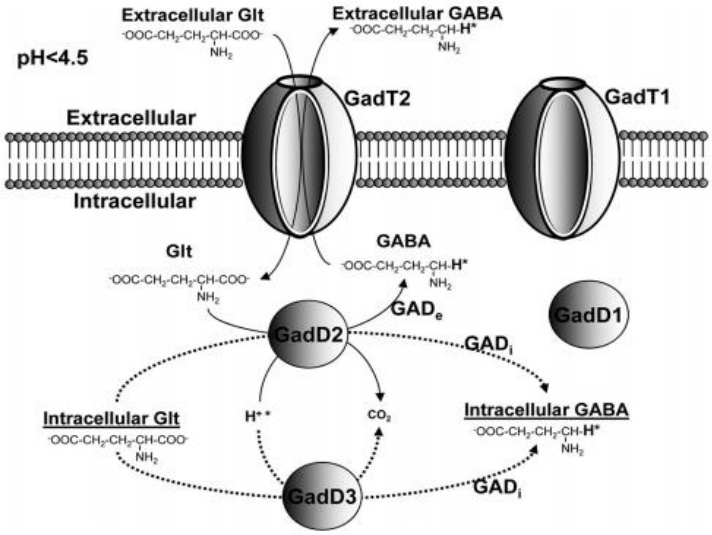


Figure 1.5: Suggested model of the GAD mechanism under acidic conditions (pH < 4.5) (Karatzas et al., 2012)

1.3.2 Futile proton hypothesis

However, this model fails to explain the acid resistance under low pH conditions (< pH 4.0). Because the carboxyl group of Glu and GABA has a pKa-value of 4.0, this chain will mainly be protonated when the pH drops below 4.0. The GAD system then imports a protonated Glu that, after conversion to GABA, releases a proton in the cell. This counteracts the release of a proton by the export of GABA and is known as the futile proton hypothesis (Feehily & Karatzas, 2013).

2 Soybeans

2.1 Nutritional value of soybeans

Soybeans consist primarily of carbohydrates and proteins. Besides, also a considerable amount of fat is found (Table 2.1). The amino acid composition reveals that Glu is the most abundant amino acid in soybeans (Table 2.2).

Table 2.1: Gross composition in soybeans (g/100 g FW) (adapted from Banaszkiewicz, 2011)

Component	***Content (g/100 g FW)***
Water	5.6 - 11.5
Crude protein	32 - 43.6
Fat	15.5 - 24.7
Carbohydrates	31.7 - 31.85
Crude ash	4.5 - 6.4

Table 2.2: Amino acid composition in soybeans (g/100 g DW) (Kovalenko et al., 2006)

Amino acid	***Content (g/100 g DW)***
Alanine	1.79
Arginine	3.17
Aspartic acid	4.79
Cysteine	0.70
Glutamic acid	7.66
Glycine	1.77
Histidine	1.15
Isoleucine	1.94
Leucine	3.26
Lysine	2.69
Methionine	0.61
Phenylalanine	2.16
Proline	2.04
Serine	1.92
Threonine	1.62
Tryptophan	0.50
Tyrosine	1.53
Valine	2.06

2.1.1 Carbohydrates

The main carbohydrates are the cell wall related sugars pectin, cellulose and hemicellulose. The oligosaccharides raffinose and stachyose are non-digestible for humans, but support the growth of probiotic LAB strains such as *Lb. acidophilus* and *Lb. delbruecki subsp. bulgaricus* (Banaszkiewicz, 2011). However, losses up to 50% of carbohydrates have been reported during soaking (Egounlety & Aworh, 2003).

2.1.2 Protein

The major protein in soybeans is the globular protein glycenin, contributing for 30% of the total protein content. Also, a 2*S* albumin (Gly m 2*S* albumin) was found which is used as a storage protein until germination. This family of storage proteins is known as food allergens because of their binding action on immunoglobulin E (Moreno & Clemente, 2008).

2.1.3 Fats

The fat fraction mainly exists as oil with the essential fatty acids linoleic acid (55%) and α-linolenic acid (8%) being the most common. Because of the unsaturated character of both, soybeans can become rancid by lipid oxidation. Other fats are phospholipids and fat soluble tocopherols (Liu, 1997).

2.1.4 Other components

Soybeans contain a reasonable amount of isoflavones (3 mg/g DW), which are structurally similar to estradiol (Zhang *et al.*, 2014). Because they can bind on mammalian estrogen receptors, they are often called phytoestrogens. Therefore, the intake of phytoestrogens could be linked to a healthy life (Kudou *et al.*, 1991) but simultaneously raises the question on how it acts on the human hormonal system (Fang *et al.*, 2001).

Also, up to 400 mg/100g DW of phytosterols are found, which have a cholesterol-lowering effect (Law, 2000). Saponins are also present and could exert anti-cancer effects (Yoshiki *et al.*, 1998). Finally, soybeans are a good source of iron. It has been shown that iron bound to ferritin is as much absorbed as iron from meat, and the presence of phytate has little effect on the absorption of ferritin-iron (Lönnerdal, 2009).

2.2 Use of soybeans

The goal of this study is to find a strain capable of producing high amounts of GABA and implement this to ferment Glu-rich foods, in this case soymilk. Soymilk is prepared by soaking dried soybeans in water and grinding them with water (ratio 1:10) afterwards. Soybeans can be used to prepare other foods as well, such as tofu, tempeh and soy sauce.

Tofu is the result of coagulating soymilk and pressing the curds together in special moulds. To initiate the coagulation, Mg- and/or Ca-salts, enzymes or acids are added. Coagulation can also be achieved by fermentation (Hou *et al.*, 1997).

Tempeh is made from whole soybeans that, after fermentation by fungi, form a cake. The production of tempeh is mainly done with fungi of the genus *Rhizopus* (Keuth & Bisping, 1994).

Soy sauce is a mixture of soaked soybeans and roasted grains. The fermentation is carried out by *Aspergillus* moulds who produce cellulases and proteases so, cellulose and proteins can be hydrolysed respectively. Also, bacteria are inoculated and will use the liberated sugars to produce organic acids. The dark colour is the result of Maillard reaction between the amino acids and reducing sugars. (Gao *et al.*, 2011).

2.3 Methods to increase GABA content

The production of GABA in soybeans, and in plants in general, is also carried out through GAD after an increase in cytosolic Ca^{2+}. This increase is a result of stressors like hypoxia, acidic pH, heat and cold or pressure. The produced GABA is then used for metabolic and physiological processes to shield the plant from the stress (Shelp *et al.*, 1999).

The initial GABA content in raw soybeans varies. Ko *et al.* (2013) reported a GABA concentration of 20 mg/100 g DW whereas Ueno *et al.* (2010) only found 9.27 mg/100 g DW soybeans. This difference among soybean varieties could be explained by the stresses the soybean had to endure from growth to analysis. Various mechanisms for the accumulation of GABA have been studied (Table 2.3). These are germination, fermentation and innovative processes such as HHP) and ultrasound (Poojary *et al.*, 2017). Germination and fermentation are the two most promising techniques.

Table 2.3: Methods to increase the GABA content in soybeans or soymilk

Product	Method	Initial GABA content [a]	GABA content after treatment [a]	X times increased	Source
Soybeans	Germination (6 h)	10.5 mg/100 g	62.8 mg/100 g	6.0	Matsuyama *et al.* (2009)
Soybeans	Germination (5 days)	8.9 mg/100 g	328.0 mg/100 g	42.7	Wang *et al.* (2015)
Soymilk	30 h fermentation at 42°C with 4 LAB strains: *Lb. casei, Lb. acidophilus, S. thermophilus, Lb. bulgaricus* and *B. longum*	9.4 mg/100 mL	36.2 mg/100 mL	3.9	Tsai *et al.* (2006)
Black soybean milk (+ 1% MSG, 1% brown sugar and 0.1% peptone)	48 h fermentation at 37°C by *Lb. brevis* FPA 3709	20.0 mg/100 mL	542.0 mg/100 mL	27.1	Ko *et al.* (2013)
Soybeans	HHP of 200 MPa for 10 min at 20°C	9.6 mg/100 g	43.3 mg/100 g	4.7	Ueno *et al.* (2010)
Soybeans soaked in 0.08 g/mL Glu for 2 days	HHP of 200 MPa for 10 min at 25°C	13.4 mg/100 g	70.0 mg/100 g	5.2	Ueno *et al.* (2013)
Soybeans	Ultrasound treatment of 300 W for 30 min at 25°C followed by 5 days germination	83.1 mg/100 g	119.3 mg/100 g	1.4	Yang *et al.* (2015)

[a] *GABA content was measured on DW*

2.3.1 Fermentation

As mentioned in section 2.2, fermentation of soybeans is mainly done to produce tempeh, soy sauce and tofu. The strain *R. microsporus* shows a good potential to increase GABA content while making tempeh, 370 mg/100 g DW was obtained by Aoki *et al.* (2003). However, when fermentation of soymilk with LABs is aimed, the solubility of the proteins will change depending on the ionic strength and pH as demonstrated by Lakemond *et al.* (2000) (Figure 2.1). Because of lactic acid production, the solubility of glycenin will change and could denaturate and/or precipitate resulting in a lack of structure and nutritional loss. Moreover, Glu would not be usable for the fermentation.

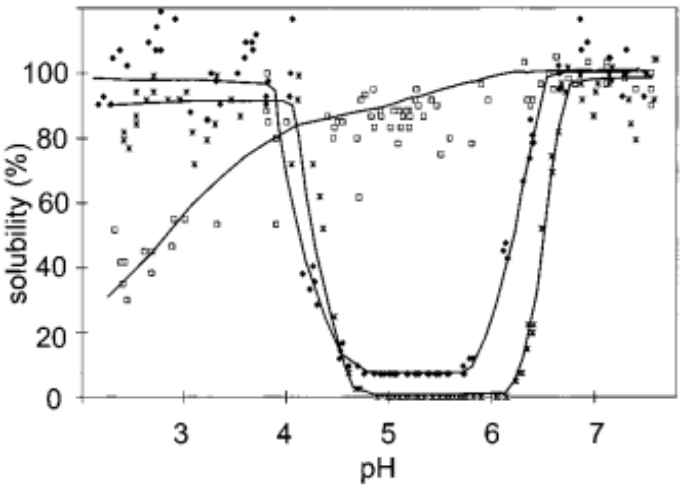


Figure 2.1: Solubility of soy glycenin as function of pH at different ionic strengths of 0.5 (□) 0.2 (◇) and 0.03 (*) (Lakemond et al., 2000)

The first requirement is the ability of the LAB strain(s) to grown on soymilk carbohydrates. Known strains capable of growing are *Lc. Lactis*, *Lb. casei*, *Lb. acidophilus* and *S. thermophilus* (Donkor *et al.*, 2007). Then, the strains need to convert Glu to GABA in the process. This system will only work in the low pH-range. The low pH would preferably be due to lactic acid to avoid the use of additives. Finally, proteolysis must be carried out before this low pH is reached to prevent aggregation and precipitation.

Tsai *et al*. (2006) fermented soymilk for 30 h at 42°C with five strains. The pH remained constant for the first 5 h, then the pH decreased rapidly to reach 4.3 after 10 h where it remained constant for another 20 h. To initiate protein breakdown, a protease known as Prozyme 6 was added after 5 h. The fermentation was also carried out without the addition of Prozyme 6 (Figure 2.2).

The addition of Prozyme 6 significantly increased the free amino acid concentration. After 30 h, GABA content reached 361.1 mg/100 g with Prozyme 6 and 198.4 mg/100 g without. This method could be used to ferment soymilk and enrich it with GABA. However, the authors did not give any information about the consistency and organoleptic properties during or after the fermentation.

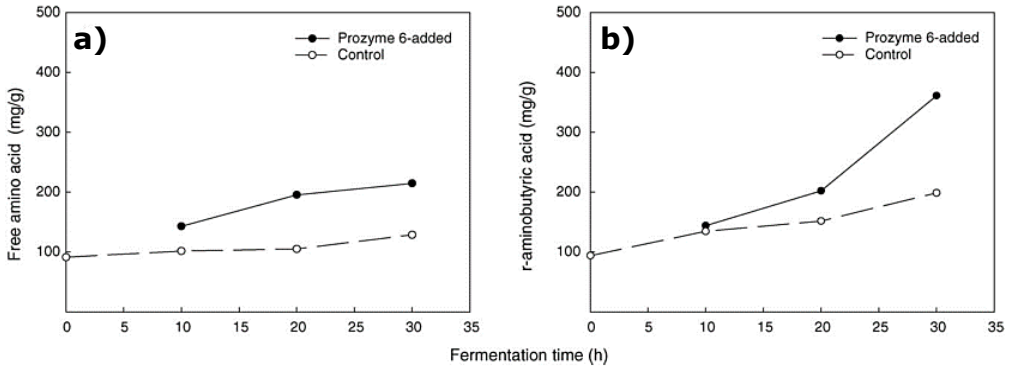


Figure 2.2: Free amino acids (a) and GABA content (b) in soymilk as a function of fermentation time with and without the addition of Prozyme 6 (Tsai et al., 2006)

Another experiment, performed by Ko *et al*. (2013), used *Lb. brevis* FPA 3709 to ferment black soybean milk. After optimisation, the highest GABA content was obtained when 1% monosodium glutamate (MSG), 1% brown sugar and 0.1% peptone was added to the milk. The fermentation was carried out for 48h at 30°C and resulted in 542 mg/100 mL GABA, effectively increasing the GABA content with 96.3%.

2.3.2 Germination

Germination is a process in which the plant grows from a seed. During this process, water is absorbed and the aerobic respiration is increased to provide energy. Germination is best carried out at an optimum temperature, depending on the type of plant (Raven *et al.*, 1999). The increased respiration during the germination process results in protein hydrolysis that leads to the liberation of amino acids. Because Glu is the most abundant amino acid in soybeans, it can be used to produce GABA and thus increase the content during germination (Luo *et al.*, 2017).

Matsuyama *et al.* (2009) analyzed the effects of soybean germination and soaking on the GABA content. First, all seeds were soaked in water at 40°C for 2 h. Then, seeds were germinated or soaked for 6 h and 24 h in the dark at 25°C. They found that germination for 6 h increased the GABA content six-fold. Soaking the seeds for 24 h resulted in the highest increase, 8 times higher than the GABA content of the control seed (Figure 2.3).

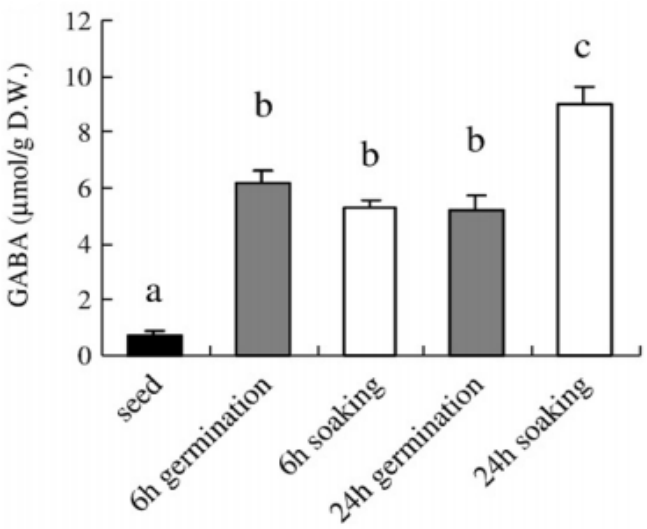


Figure 2.3: Effect of 6 h and 24 h germination and soaking on the GABA content in soybeans (Matsuyama et al., 2009)

2.3.3 Innovative processing: HHP

HHP is a technique in which a fluid is compressed with pressures ranging from 100 to 1200 MPa. This technique is already used for the inactivation of micro-organisms. As a result, HHP enhances food preservation while, unlike thermal processing, maintains taste and nutritional value of the product to some extend (Sanchez-Moreno *et al.*, 2009). As for enzymes, applying pressure results in reversible, irreversible or partial unfolding of the enzyme. Depending on the structure of the active site, this leads to an increase or decrease in activity (Chakraborty *et al.*, 2014).

For GAD, it appears that high pressure increases its activity. Ueno *et al.* (2010) soaked soybeans in water and applied a pressure of 200 MPa, effectively increasing GABA content from 9.23 mg/100 g DW to 43.3 mg/100 g DW. The mechanism behind the increase is not yet understood. However, it could either be linked with the structural change of GAD or a change in the cell environment. As mentioned earlier, GAD activity increases with Ca^{2+}-uptake and high pressure could induce structural changes in Ca^{2+}-channels. Glu and GABA transport could be affected as well, which in turn increases Glu uptake. Another explanation is an increase in cytosolic H^+ due to physical damage to organelles. This activates the GAD system and increases GABA content (Poojary *et al.*, 2017).

2.3.4 Innovative processing: ultrasound

Ultrasonication is the process in which soundwaves with a frequency higher than 20 kHz are used. Soundwaves are longitudinal waves, constantly compressing and decompressing the medium therefore resulting in an alternating high- and low-pressure field (Rokhina *et al.*, 2009). Applying these soundwaves affects homogenization, enzyme activity and extractability in an indirect way (Poojary *et al*., 2017).

The pressure waves can affect cells and their enzymes, in the same way HHP does. The increase or decrease in enzyme activity will depend on parameters such as duration and applied energy, which are typically in the range of 25-100 W and no longer than 60 minutes (Basto *et al.*, 2007). Additionally, altered membrane permeability can increase mass transport (Rokhina *et al.*, 2009).

Yang *et al.* (2015) used ultrasonication to increase GABA content in soybeans. Therefore, 100 seeds were dispersed in 160 mL water and put in an ultrasonic bath. They applied ultrasonic soundwaves of 40 kHz for 30 minutes at 25°C. The energy of the waves ranged from 0 to 300 W. Afterwards, the soybeans were germinated for 5 days. The soybeans not treated with ultrasonic waves had a GABA content of 83.1 mg/100 g DW, whereas those treated with 300 W showed 119.3 mg GABA /100 g DW. However, they did not investigate the cause of this increase but suggested that the increase in GAD activity was the cause. Also, the higher germination rate could increase GABA synthesis.

3 Fermentation parameters affecting GABA synthesis

Fermentation has a great potential to increase the GABA content. Fermenting soybeans and soybean byproducts is already done worldwide, and GABA is a mere byproduct of this process. So large-scale production, after optimization, of GABA through the GAD system could be used by the food industry. However, the highest GABA yield depends on the used LAB strain, as well as the fermentation conditions (Table 3.1). These conditions include pH, temperature, fermentation time, initial Glu concentration, PLP concentration and the use of additives (Li *et al.*, 2010). An optimum between these parameters could potentially result in an applicable and cost-effective fermentation for the food industry.

Table 3.1: GABA content after different fermentation parameters with various LABs

Strain	Medium	Additives	T (°C)	pH	Time (h)	GABA	Source
Lb. brevis NCL912	MRS broth + 3% MSG	none	30	5.0	48	15.4 g/L	Li *et al.* (2008)
Lb. buchneri	MRS broth + 5% MSG	1% NaCl 1 % glucose	30	5.0	36	25.9 g/L	Cho *et al.* (2007)
Lb. plantarum	Buckwheat, amaranth, chickpea & quinoa flours (1:1:5.3:1)	0.1 mM PLP	30	5.0	24	0.5 g/kg	Coda *et al.* (2010)

3.1 pH

The pH of the environment is crucial for GABA synthesis, it acts as a stressor for the micro-organisms to increase alkalinity. However, it appears that this is strain-related. Barla *et al.* (2016) reported a GABA production by *Lb. brevis* AN1-5 around 700 mg/100 mL at a pH level of 3.0, whereas *Lb. brevis* AN2-2 only produced 100 mg/100 mL at the same pH. The optimum pH for GAD activity is not the same as for the growth (Komatsuzaki *et al.*, 2005), so the pH should be regulated in between. Table 3.2 shows the difference in pH for an optimum GAD activity and an optimum growth. It should be noted that the pH will decrease during fermentation due to the production of lactic acid. Therefore, the pH must be adjusted regularly to maintain the desired pH.

Table 3.2: pH ranges for optimum GAD activity and optimum growth for different LABs

Strain	Optimum pH of GAD	Optimum pH of growth	Source
Lb. brevis Lb 85	4.0 – 5.0	4.0 - 6.0	Shi *et al.* (2014)
Lb. plantarum ATCC 14917	4.0 - 5.0	3.2 – 4.0	Shin *et al.* (2014)
Lc. lactis subsp. lactis	4.7	5.0 – 6.0	Nomura *et al.* (1999)

An optimum pH value of 5.0 for growth was found by Li *et al.* (2010) using *Lb. brevis* NCL912. After 36 h of fermentation, the biomass at pH 6.0 was higher than the biomass at pH 5.0, but GABA production was higher at pH 5.0 (Figure 3.1). The decrease in biomass around 24 h at pH 5.0 and the simultaneous increase in GABA production shows that the intracellular enzyme remains active, even when the equilibrium between dying and growing is shifted to dying. The GABA produced at a certain pH level will depend on the GAD activity at that given pH as well as the amount of micro-organisms.

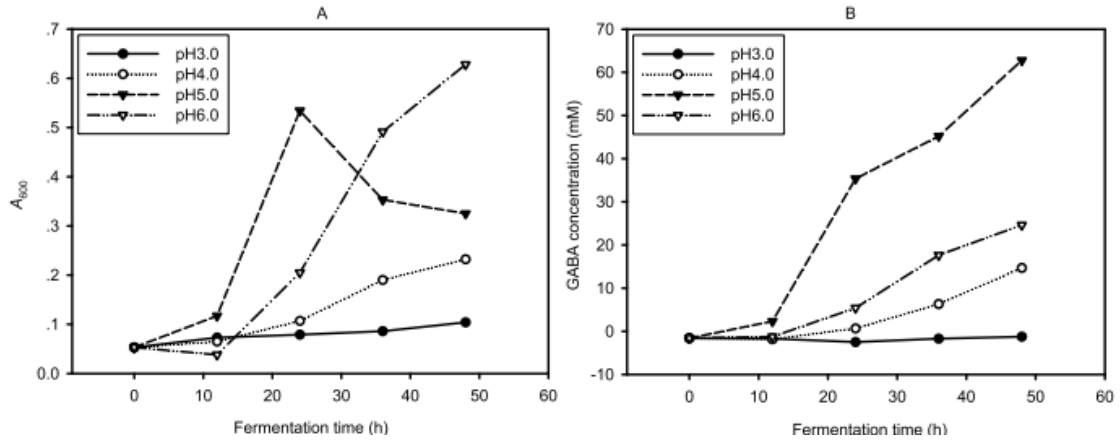


Figure 3.1: Effect of pH on biomass (A) and GABA yield (B) by Lb. brevis NCL912 (Li *et al.*, 2010)

3.2 Temperature

The biocatalytic activity of enzymes increases with increasing temperature and decreases rapidly after their optimum. Because of protein denaturation, the optimum temperature is usually not higher than 40°C (Berg & Tymoczko, 2002). Besides an optimum temperature for GABA activity, the micro-organisms have an optimum growth temperature. The strain *Lb. brevis* NCL912 showed an equal GABA production at 30°C and 35°C whereas the cell density was less at 30°C. Also growth of the strain was possible at 45°C although GABA production was not (Li *et al.*, 2010). In the optimization of the fermentation parameters by Xiaoxue *et al.* (2009), they found that *Lc. lactis subsp. lactis* produced 7.2 g/L GABA at a temperature of 31.8°C even when the optimum growth temperature is between 37°C to 40°C (Ahmed *et al.*, 2006).

3.3 Fermentation time

It takes time for the micro-organisms to get used to their new environment and start the fermentation. GABA production generally increases with the fermentation time, but a higher fermentation time leads to a loss of biomass. This is generally caused by the depletion of the necessary components of the medium or due to the inhibitory effect of a high GABA concentration or other metabolites (Madigan *et al.*, 2015).

As shown in Figure 3.2a, an optimum of 1.76 mM (181.5 mg/L) of GABA was produced after 60 h of fermentation by *Lb. plantarum* Taj-Apis362. The fermentation parameters were fixed: 50 mM Glu, pH 5.0 and incubation at 30°C (Tajabadi *et al.*, 2015).

It appears that besides the fermentation time itself, the time of substrate addition is important as well (Figure 3.2b). Xiaoxue *et al.* (2009) found that GABA yield was highest when MSG was added at the start of the fermentation by *Lc. lactis subsp. lactis*. The yield was lowest when MSG was added after 24 h and 96 h.

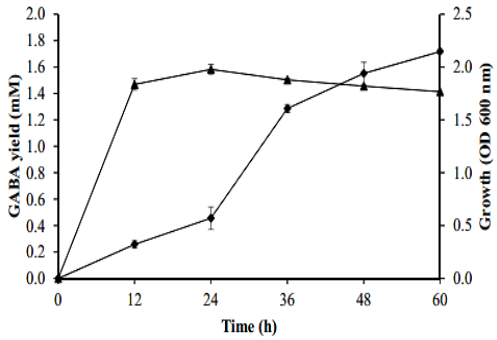


Figure 3.2a: Effect of fermentation time on growth (▲) and GABA production (◆) by Lb. plantarum Taj-Apis362 (Tajabadi et al., 2015)

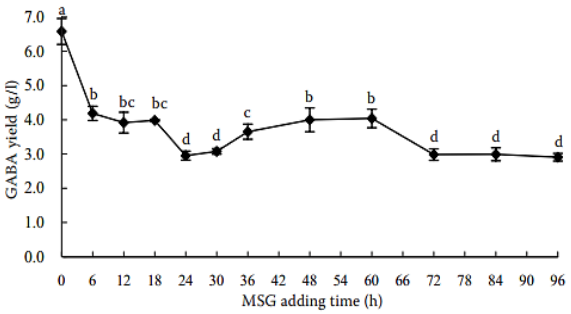


Figure 3.2b: Effect of MSG adding time on GABA yield by Lc. lactis subsp. lactis (Xiaoxue et al., 2009)

3.4 Glu concentration

The presence of Glu, or its sodium salt monosodium glutamate (MSG), is necessary to produce GABA. It also increases biomass through maintaining cytoplasmic pH and thus helping the cells to survive. The indirect effect of Glu on the pH is shown in Figure 3.3c, the addition of 0.75 M Glu caused the pH to increase or at least be constant. When no Glu was added, the pH decreased to 3.5. The addition of 1.0 M Glu kept the pH around 5.0 but resulted in a low cell density and GABA yield as seen in Figure 3.3a and Figure 3.3b, respectively. Addition of 0.75 M of Glu resulted in the highest GABA yield and pH (Li *et al.*, 2010).

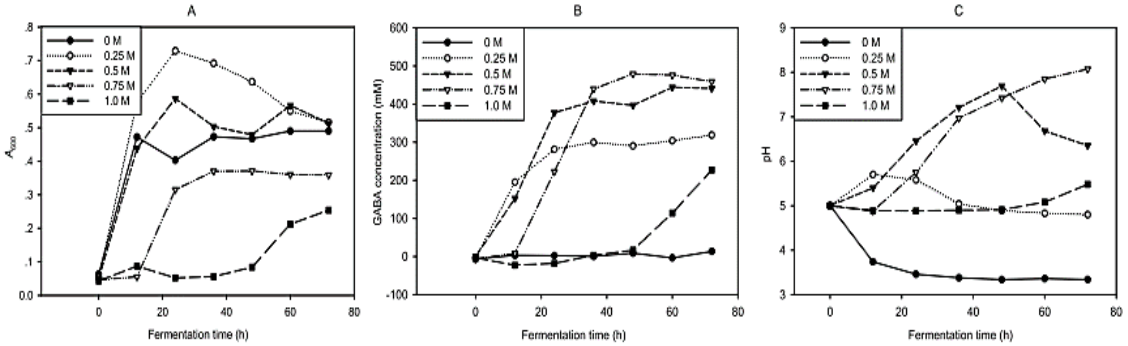


Figure 3.3: Influence of MSG on (a) cell density, (b) GABA yield and (c) pH of fermentation in MRS by Lb. brevis NCL912 (Li *et al.*, 2010)

Glu conversion varies between strains and is correlated with other parameters. The highest GABA yield (7.2 g/L) by *Lc. lactis subsp. lactis* was achieved by adding 15.0 g/L MSG and incubating at 31.8°C with an initial pH of 7.1 (Xiaoxue *et al.*, 2009). *Lb. plantarum* Taj-Apis362 produced 737.3 mg/L of GABA when 73.2 g/L of MSG was added in MRS broth of pH 5.31 and incubated at 36°C (Tajabadi *et al.*, 2015).

3.5 PLP concentration

Another factor affecting GABA synthesis is the addition of the cofactor PLP. However, this depends on the ability of the strain to synthesize the cofactor for themselves and the form of the enzyme (apo or holo). This was seen in the study of Li *et al*. (2010) with the strain *Lb. brevis* NCL912. Addition of 10 µM of PLP to MRS broth did not increase GABA production or affect cell growth as seen in Figure 3.4.

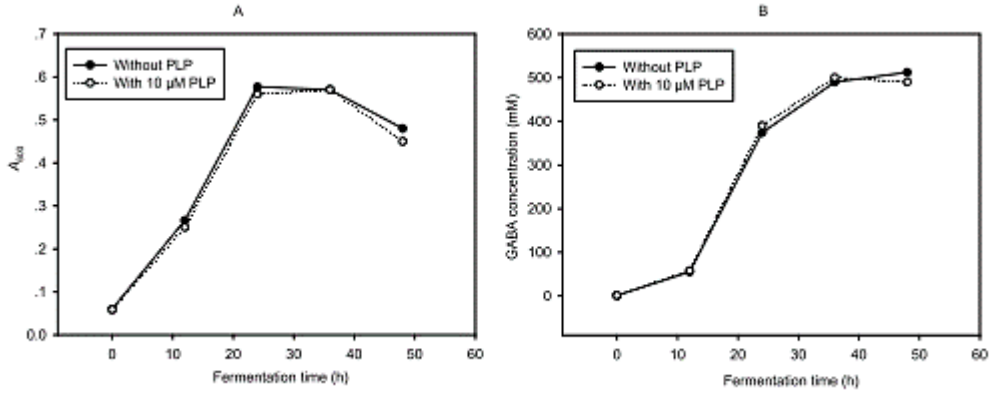


Figure 3.4: Effect of PLP on cell growth (A) and GABA yield (B) of Lb. brevis NCL912 (Li et al., 2010)

The strain *Lb. paracasei* NFRI 7415 however, benefits from the addition of 10 µM PLP whereas 100 µM PLP results in a lower GABA yield (Figure 3.5). The cell growth was in both cases slightly lower (Komatsuzaki *et al.*, 2005).

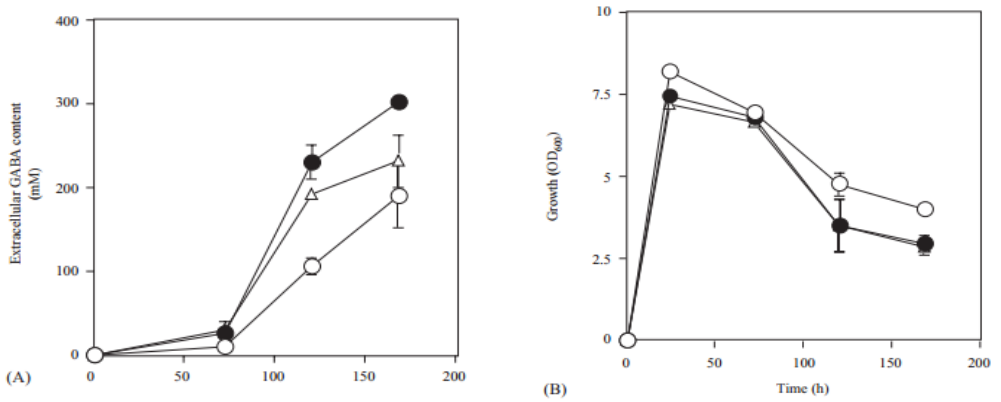


Figure 3.5: Effect of PLP on GABA yield (A) and cell growth (B) by Lb. paracasei NFRI 7415 (o No PLP, ● 10 µM PLP, Δ 100 µM PLP) (Komatsuzaki et al., 2005)

So, GAD in *Lb. brevis* NCL912 most likely occurs as a holoenzyme, were PLP is constantly bound and additional PLP has little to no effect. When the enzyme occurs as apoenzyme in some strains, additional PLP may have a positive effect (Fan *et al.*, 2012).

3.6 Additives

The way micro-organisms grow will mostly depend on the nutrient composition of the culture, so the composition of the medium can influence GABA yield. The main additives are nitrogen and carbon sources, especially the addition of 1.25% glucose seems to improve GABA production (Li & Cao, 2010). The main nitrogen sources are yeast extract, soya peptone and beef extract. Addition of 2.5% of each of these compounds resulted in 20.6 g/L GABA production by *Lb. brevis* (Li *et al.*, 2008).

Another way to improve GAD activity is by adding sulfate ions. These ions increase the hydrophobic interactions between the subunits of the GAD enzyme. Ammonium sulfate appears to increase the activity more than sodium sulfate or magnesium sulfate (Li & Cao, 2010).

The effect of activators was tested by Sa *et al.* (2015) on the isolated GAD enzyme of *Lb. sakei* A156. GAD activity was increased with $MnCl_2$, $CaCl_2$, and $MgCl_2$ whereas $CuSO_4$ caused a decrease of GAD activity. Similar reports were found by Lee *et al.* (2017) with a recombinant GAD from *Enterococcus avium* M5 (Table 3.3).

Table 3.3: Effect of additives recombinant GAD activity of Enterococcus avium M5 (adapted from Lee et al. (2017))

Activator (2 mM)	**Remaining activity (%)**
Control	100
$CaCl_2$	124.3 ± 2.1
$CoCl_2$	98.4 ± 3.4
$CuSO_4$	56.8 ± 2.4
$FeCl_3$	99.2 ± 1.6
KCl	97.8 ±2.1
$MgCl_2$	102.3 ± 1.2
$MnCl_2$	116.4 ± 1.4

4 Positive effects of GABA in mammals

GABA is not able to fully cross the blood-brain barrier (Kuriyama & Sze, 1971), so ingested GABA will not exhibit neuronal effects. However, several studies reported the positive effects of GABA-enriched food (Table 4.1) indicating that GABA can enter the bloodstream via the intestines.

Table 4.1: Functions of GABA-enriched food in mammalian subjects with GABA content, adapted from Wu & Shah (2017)

Subject	Type of food	GABA content	Function	Source
Mildly hypertensive patients	GABA-rich fermented milk	10 ~ 12 mg/100 mL	Blood-pressure-lowering effect	Inoue *et al.* (2003)
Spontaneously hypertensive rats	GABA-rich soy sauce	1.0 g/100 mL	Reduced overall cardiovascular risk	Yamakoshi *et al.* (2007)
Healthy male	GABA-rich chocolate	0.28 g/100 g	Psychological stress-reducing effect	Nakamura *et al.* (2009)
Forced swimming rat	GABA-rich fermented black soybean milk	542 mg/100 mL	Anti-depressant effect	Ko *et al.* (2013)
Male	Pure GABA	N.A.	Increased human growth hormone	Powers *et al.* (2008)

(N.A.: not applicable)

4.1 GABA uptake in humans

The uptake of amino acids happens in the small intestines through protein carriers. However, no specific carriers are found for GABA. Thwaites *et al.* (2000) concluded that GABA shares a Na-dependent transporter, more specific the β-alanine carrier. This symport requires energy thus it is coupled with an ATPase. This symport could potentially carry GABA across the epithelial cell wall into the blood stream where it can reach the organs and exert its beneficial effect(s).

4.2 Antidiabetic effect of GABA

Diabetes mellitus (DM) is a disorder caused by high blood sugar for an extended amount of time. This is caused by either a too low insulin production by the pancreas (Type 1 DM), the inability of cells to respond to the insulin (Type 2 DM) or when women develop high blood sugar during pregnancy (gestational diabetes). Type 1 DM is mainly caused by genetics whereas type 2 is caused by lifestyle factors such as poor diet and a lack of physical activity (Gardner *et al.*, 2011). In 2014, approximately 422 million people worldwide had some form of diabetes and it was the cause for 1.6 million deaths worldwide in 2015 (WHO, 2016).

The pancreas is an organ that has two distinctive functions: the processing of food to make them available for absorption (exocrine functions) and other tasks regarding endocrinal metabolism. This endocrine pancreas is composed of glands called the islets of Langerhans which are divided in five cell types. Two of them are the most common, α-cells (20-25%) which produce glucagon and β-cell (65-70%) which produce insulin and GABA.

The main function of the hormone insulin is to capture and store carbohydrates, protein and fat throughout the body. Its counterpart glucagon however, releases the stored energy in the form of glucose and the ketone bodies acetoacetic acid and β-hydroxybutyric acid (Goodman, 2003). Glucagon inhibits insulin release and vice versa. It appears that GABA also has the ability to inhibit glucagon secretion, even when insulin secretion is limited (Figure 4.1) (Gardner *et al.*, 2011, Crandall & Shamoon, 2016).

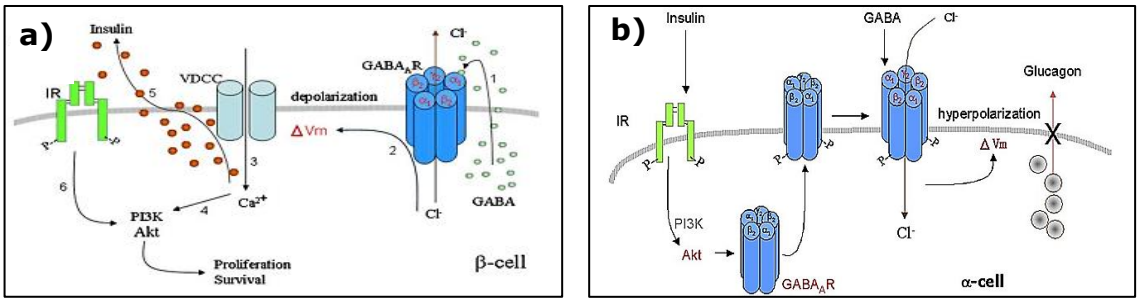


Figure 4.1: a) Effect of GABA on β-cells, depolarization of the cell membrane and influx of Ca^{2+} b) Effect of insulin and GABA on α-cells, inhibition of the glucagon secretion (adapted from Xu et al. (2006) & Soltani et al. (2011))

Now in the case of type I DM, more than 70% of the β-cells are destroyed and little to no insulin is present. GABA-release from the β-cells is also limited. This destruction is caused by insulitis, an auto-immune mechanism in which the β-cells are removed by B- and T-lymphocytes (Campbell-Thompson *et al.*, 2016). When β-cells are limited, so is insulin resulting in a rise in glucagon and an elevated blood sugar level (hyperglycemia). More so, the body is unable to store energy and continues to release glucose, fatty acids and amino acids from the corresponding tissues.

This can be reversed by applying insulin (Soltani *et al.*, 2011) but an alternative and promising treatment is the use of GABA through the mechanism mentioned above.

Purwana *et al.* (2014) transplanted human α- and β-cells in diabetic mice. The mice were treated with or without 6 mg/mL GABA in drinking water. Mice treated with water showed a β-cell count of approximately 45% whereas those treated with GABA had around 60% β-cells (Figure 4.2A). At the same time, insulin levels were twice as high in mice treated with GABA (Figure 4.2B) and glucagon levels were five times lower (Figure 4.2C). Similar results were obtained by Tian *et al.* (2013).

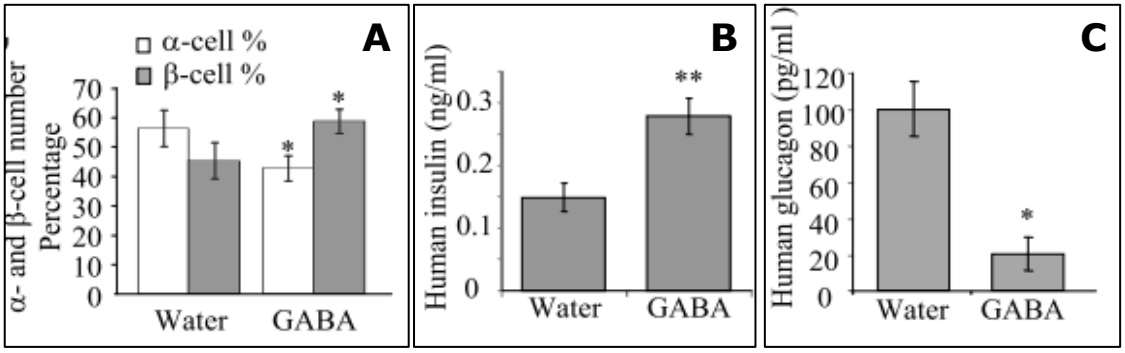


Figure 4.2: Effect of GABA on A) α- and β-cell number, B) insulin and C) glucagon in diabetic mice adapted from (Purwana et al., 2014)

4.3 Blood-pressure lowering effect

The pressure that the circulating blood exerts on the blood vessels is called blood-pressure. It has a maximum during the heart beat (systole) and a minimum between two heart beats (diastole). The unit is millimeters mercury (mmHg) and is written as: systolic blood-pressure/diastolic blood-pressure. A normal blood-pressure is around 120/80 mmHg (Appel *et al.*, 2006).

Blood-pressure is mainly affected by three factors that influence each other. First, the so called cardiac output. This is the amount of blood pumped in the aorta in a given time. Secondly, blood volume which can be affected by a high sodium intake. Finally, the resistance in the blood-vessels which is mainly affected by the radius of the vessel and blood viscosity, which increases resistance (Guyton, 1981). These effects are controlled by physiological and biochemical processes, one if which is the release of noradrenaline. Here, GABA can play a role.

Noradrenaline is released as a neurotransmitter in the brain, or as hormone by the adrenal glands to control the fight-or-flight response. This is done by increasing blood flow and cardiac output which results in a higher blood pressure. Simultaneously, the feeling of stress is increased. The plasma concentration of noradrenaline in healthy individuals ranges from 0.9 - 2.4 nmol/L whereas in hypertensive patients it occurs in a range of 1.2 - 3 nmol/L (Gardner *et al.*, 2011). Several studies show that GABA can inhibit the release of noradrenaline and lower blood-pressure.

Inoue *et al.* (2003) used fermented milk containing 10–12 mg GABA/100 mL. Test subjects were divided in two groups, the first group took 100 mL of milk each day for 12 consecutive weeks. The second group was given a placebo. After 12 weeks, the systolic blood pressure decreased with 17.4 mmHg in subjects who took the fermented milk. Diastolic blood-pressure decreased with 7.2 mmHg. After 12 weeks the subjects no longer took the fermented milk and the blood-pressure increased again (Figure 4.3).

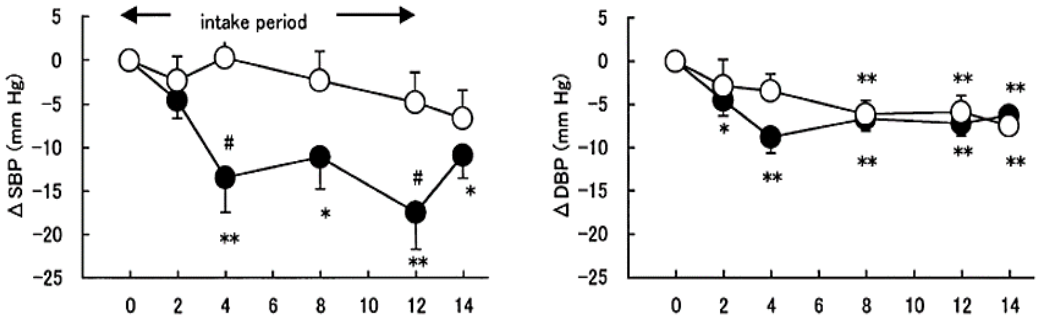


Figure 4.3: Change in blood-pressure (systolic = ΔSBP, diastolic = ΔDPB) in subjects who took fermented milk enriched with 10 – 12 mg GABA/100 mL (●) and who took a placebo (o) each day for 12 weeks (Inoue et al., 2003)

4.4 Other beneficial effects

Although more studies need to be performed, GABA has shown to inhibit the growth of cancer cells. More specific cholangiocarcinoma, a form of cancer in epithelial cells of the bile duct (Huang *et al.*, 2013). They injected human cholangiocarcinoma cells in mice. Every day, the mice were treated with 1000 mg/kg GABA dissolved in 0.9% NaCl for 5 weeks. After 35 days, the volume of the tumor in the control group grew to 0.62 ± 0.03 cm^3 whereas the volume in the treated group was only 0.34 ± 0.03 cm^3. The mechanism is still being studied but GABA appears to activate an immune defense pathway, which slows the cancer progression.

Lowering cholesterol levels is also associated with GABA-rich food, brown rice for example contains approximately 0.12 mg GABA/g. A 6-week diet with 24 h pre-germinated brown rice lowered low density lipoprotein-cholesterol (LDL-c) with 47% in hypercholesterolemic rats. However, the effects could also be due to other functional compounds such as γ-oryzanol and dietary fibre (Roohinejad *et al.*, 2010).

GABA has shown to increase the concentration of two isoforms of human growth hormone in men, immunoreactive growth hormone (irGH) and immunofunctional growth hormone (ifGH). This hormone is a key in the growth, reproduction and regeneration of cells. Powers *et al*. (2008) conducted an experiment in which men between 18 and 30 years old were given 3 g of GABA, blood samples were tested for irGH and ifGH. After 30 minutes of ingestion, irGH and ifGH had risen 200% and 175% respectively.

MATERIALS AND METHODS

5 Materials

5.1 Chemicals and reagents

Qualitative GAD screening	***Quantitative GAD screening***
LAB strain(s)	LAB strain(s)
L-glutamic acid (Glu)	Na_2HPO_4
γ-aminobutyric acid (GABA)	KH_2PO_4
Triton X-100	β-mercaptoethanol (2-ME)
NaCl	Ethylene diamine tetraacetic acid (EDTA)
Bromocresol green (BCG)	L-glutamic acid (Glu)
	Pyridoxal-5'-phosphate (PLP)

Fermentation and screening	***HPLC analysis***
LAB strain(s)	Ortho-phtaldialdehyde (OPA)
Man Rogosa Sharpe (MRS) agar	β-mercaptoethanol (2-ME)
Man Rogosa Sharpe (MRS) broth	Norvaline (internal standard)
Nutrient broth (NB)	Sodium acetate
Nutrient agar (NA)	Acetonitrile
Monosodium glutamate (MSG)	MeOH
Pyridoxal-5'-phosphate (PLP)	L-Glutamic acid
Soymilk (soybean:water; 1:10 (v/v))	γ-aminobutyric acid (GABA)

Plate count	***Titratable acid***
LAB strain(s)	Oxalic acid
Man Rogosa Sharpe (MRS) agar	NaOH
Man Rogosa Sharpe (MRS) broth NaCl (0.90%)	

5.2 Lactic acid bacteria

All LABs used are form the order Lactobacillales except for one strain which belongs to genus of *Propionibacterium* (*P*) in the order of Actinomycetales. The genera *Lactobacillus* (*Lb*) and *Pediococcus* (*Pc*) belong to the family of Lactobacillaceae, *Leuconostoc* (*L*) belongs to the family of Leuconostocaceae and *Lactococcus* (*Lc*) belongs to the family of Streptococcaceae.

Species	***LMG number***	***T_{opt} (°C)***
Lb. acidophilus	9433	37
Lb. brevis	11437	30
Lb. casei	6904	30
Lb. curvatus	9198	30
Lb. farciminis	9200	30
Lb. mali	6899	30
Lb. plantarum subsp. plantarum	6907	30
Lb. reuteri	9213	37
Lb. rhamnosus	25859	37
Lb. sakei	18175	30
Lb. viridescencs	3507	30
Lc. lactis subsp. cremoris	6897	30
L. mesenteroides	6908	30
Pc. damnosus	11484	30
Pc. pentosaceus2	10740	30
Pc. pentosaceus1	11488	30
P. freudenreichii	16410	37

5.3 Fermented food samples

Fermented food samples were bought on the Vietnamese market, so the fermentation process was not known. However, these products are usually obtained after natural fermentation. Plant-based food samples were: fermented tofu, germinated soybean and fermented eggplant. Animal-based food samples were: fish sauce, fermented anchovy fish and fermented shrimp.

6 Methods

6.1 Growth of LABs

Seventeen different species of LABs were provided in a glycerol stock, preparation of the inoculum was done in MRS broth. The medium was prepared by dissolving 52 g of MRS in 1 L of distilled water at 60°C. The broth was divided over test tubes (9 mL per tube). The tubes were autoclaved for 15 minutes at 121°C and stored at 4°C for further use. The strains were grown at their optimum temperature, initially for 24 h.

Then, the test tubes were visually checked for turbidity. Some strains showed growth after 24 h while others grew well only after 48 h. Then, two mother cultures of each strain were made by taking 100 µL of the 1st generation and adding it to 9 ml of MRS broth. The mother cultures were incubated for 24 h and were stored at 4°C to use when necessary.

6.2 Fermented products

Six fermented food samples were obtained in a glycerol stock, preparation of the inoculum was done in MRS broth. The same procedure was done as for the growth of LABs.

6.3 Screening of GAD activity in LABs

6.3.1 Qualitative method: rapid colorimetric activity assay

Firstly, GABA-producing strains were screened by a qualitative colorimetric method for their GAD activity. The qualitative determination of GAD activity was performed according to the protocols by Woraharn *et al*. (2014), Cotter & Gahan (2001) and Jilly & Schreckenberger (1984). The test was performed on 17 LAB strains and 6 fermented food samples.

Principle: The qualitative determination of GAD activity is based on the colour change of a pH indicator, bromocresol green (BCG). The pH indicator is protonated below pH values of 3.8 and has a yellow colour. It deprotonates above pH 3.8 and has a blue-green colour at pH values of 5.4 and higher, where it is stabilized by resonance (Figure 6.1) (Sabadini & Carvalho, 2013).

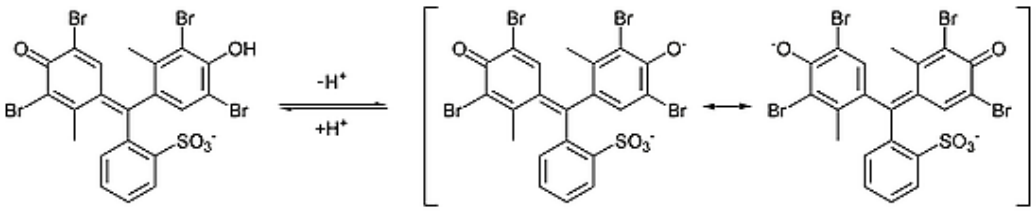


Figure 6.1: Schematic representation of the action of the pH indicator BCG (Sabadini & Carvalho, 2013)

The increase in pH is due to GAD, which incorporates one proton in the substrate L-glutamic acid to produce GABA and CO_2 (Yu *et al.*, 2011). A green or blue color is considered a positive result for GAD activity whereas a yellow color is a negative result.

For this test, three overnight cultures (OC) of each strain were used: two served as sample and one as control. Firstly, 5 mL of OC was transferred in a 15 mL conical screw cap test tube. The tubes were centrifuged at 22°C for 10 min at 4000 rpm. Then, the supernatant was discarded and the bacterial pellets were washed with 5 mL of 0.90% NaCl solution. The tubes were centrifuged again under the same conditions. The supernatant was discarded and 500 µl of test solution was added to all tubes, except the control tubes. To the control tubes, 50 µL of 100 mg/L GABA solution was added. To have an equal volume, only 450 µL test solution was used. The pellets were suspended by vortexing.

The test solution for the assay consisted of 0.1 g MSG, 30 µL of Triton X-100, 9 g of NaCl and 5 mg of BCG dissolved in 100 mL of distilled water. After adding the test-solution, the tubes were incubated in a warm water bath for 4 hours at 37 °C. The colour development was checked hourly.

A similar test was performed with 1 mL of OC to see whether the colour could be seen better, these were washed with 1 mL of 0.90 % NaCl solution.

6.3.2 Quantitative method: enzyme extraction

The quantitative determination of GAD activity was performed according to the protocols used by: Guo & Yang (2012), Bai *et al.* (2009) and Wang *et al.* (2010) to extract the enzyme. The test was performed on 17 LAB strains and 6 fermented samples.

Principle: The goal of this experiment was to extract the GAD enzyme and to use it to produce GABA from Glu. Therefore, bacterial cells were disrupted and homogenised. During the procedure, the enzyme was exposed to the environment, so the experiment must be performed in a buffer suitable for the chosen enzyme. In this case a 70 mM potassium phosphate buffer pH 5.8 was used. To inhibit the oxidation of thiol-groups present on the enzyme, 2-ME was added to the buffer. EDTA acts as a chelating agent for Ca^{2+}- and Mg^{2+}-ions that could inhibit or activate the enzyme, leading to false positive or false negative results.

Extraction step: The first test was performed with an OC of the strain *Lb. brevis*. Therefore, 5 mL of OC was centrifuged at 4000 rpm for 10 minutes at 22°C. The supernatant was discarded and the pellets were washed with 10 mL of 0.90 % NaCl-solution. The tube was centrifuged again under the same conditions, and the supernatant was discarded. Then, the bacterial pellets were suspended in 15 mL of extraction buffer. This was a potassium phosphate buffer (70 mM, pH 5.8) containing 2 mM 2-ME, 2 mM EDTA and 0.25 mM PLP. The mixture was homogenized in an ice bath for 5 minutes using the Ultra Turrax.

The tube was centrifuged for 20 minutes at 4000 rpm and 4°C. The supernatant containing the enzyme was collected for further analysis and was stored at -18°C.

Reaction step: To test the GABA producing capacity, an amount of substrate was added to the enzyme extraction. Since this test was performed for the first time, the ratio of enzyme and substrate (E/S) giving the highest yield of GABA, was not yet known. The substrate consisted of potassium phosphate buffer (50 mm, pH 5.6) containing 100 mM L-glutamic acid and 0.25 mM PLP. Next, this ratio was fixed and the homogenisation time was also checked (Table 6.1).

Table 6.1: Setup to determine GABA production through GAD of Lb. brevis

Strain	V_{sample} (mL)	V_{buffer} (mL)	Time homogenized (min)	$V_{extract}$ (µL)	$V_{substrate}$ (µL)
Lb. brevis	5	15	5	450	450
Lb. brevis	5	15	5	600	300
Lb. brevis	5	15	5	300	600
Lb. brevis	10	10	2	1000	500
Lb. brevis	10	10	5	1000	500

For the initial test, the first tube contained 450 µL enzyme solution and 450 µL substrate (E/S = 1/1), the second 600 µL enzyme and 300 µL substrate (E/S = 2/1) and the last one 300 µL enzyme and 600 µL substrate (E/S = 1/2). The tubes were incubated for 2 h at 40°C and the reaction was terminated at 90°C for 5 minutes. The suspension was then centrifuged at 4000 x *g* for 15 minutes at 22°C.

The test was performed again, but now the homogenisation time was tested. Two tubes were used, one was homogenized for 2 minutes and the other one for 5 minutes.

6.4 Screening of GABA production: analysis in broth

This experiment focused on the GABA production by two strains: *Lb. plantarum* and *Lb. brevis*, both chosen for their high GABA production according to Dhakal & Bajpai (2012). The GABA concentration was analysed in the broth. The influence of MSG, PLP and growth medium on GABA production was investigated.

The initial setup consisted of 12 Erlenmeyer flasks, each containing 80 ml of medium. Six contained MRS broth, the other six nutrient broth (NB). Three Erlenmeyer flasks of each medium were 1% inoculated with *Lb. plantarum* or *Lb. brevis*. Then, these three flasks were used to evaluate the effect of additives. The first one contained MSG (200 mM) and PLP (0.25 mM), only 200 mM MSG was added to the second one. The third one contained no additives.

The initial pH of the media was not adjusted, this was pH 7.0 for NB and pH 6.45 for MRS broth. Table 6.2 shows an overview of the setup.

Table 6.2: Setup to test the effects of additives on GABA production, pH and growth

Setup	Code	Strain	Medium	pH	MSG (mM)	PLP (mM)
1	PMGP	*Lb. plantarum*	MRS	6.45	200	0.25
2	PMG	*Lb. plantarum*	MRS	6.45	200	/
3	PM	*Lb. plantarum*	MRS	6.45	/	/
4	PNGP	*Lb. plantarum*	NB	7.00	200	0.25
5	PNG	*Lb. plantarum*	NB	7.00	200	/
6	PN	*Lb. plantarum*	NB	7.00	/	/
7	BMGP	*Lb. brevis*	MRS	6.45	200	0.25
8	BMG	*Lb. brevis*	MRS	6.45	200	/
9	BM	*Lb. brevis*	MRS	6.45	/	/
10	BNGP	*Lb. brevis*	NB	7.00	200	0.25
11	BNG	*Lb. brevis*	NB	7.00	200	/
12	BN	*Lb. brevis*	NB	7.00	/	/

All flasks were autoclaved and additives were added afterwards. The flasks were inoculated (1 %) with the OC. They were incubated for 96 h at 30°C, while shaking. Every 24 h a sample volume was taken and used to measure pH, growth and GABA. The test was performed in duplicate.

6.5 Screening of GABA production by LABs

The GABA production of 17 different LAB strains after 96 h incubation was tested. This was done in MRS broth containing MSG (200 mM) and PLP (0.25 mM) as the optimum result from section 6.3. All flasks were autoclaved and additives were added afterwards. The flasks were inoculated (1 %) with the corresponding OC. They were incubated at their optimum growth temperature, while shaking. After 96 h the flask was analysed for GABA content and residual Glu concentration to determine the conversion rate. Therefore, the samples were diluted 100 times with 75% EtOH. The test was performed in duplicate.

6.6 GABA production in soymilk by LABs

6.6.1 Soymilk preparation

Soymilk was prepared by soaking dried soybeans in water and grinding them afterwards. Soaking the beans results in water absorption and an increase in softening and cooking quality (Sanches de Lima *et al.*, 2014).

To prepare a soymilk medium, soybeans were soaked in distilled water with a ratio 1:10 (w/v) for 12 - 15 h at 22°C. Then, the beans were rinsed with distilled water and grinded for 2 minutes with a ratio of raw bean to water (60°C) 1:10 (w/v). The soymilk was then filtered through a two-layer cheese cloth and pasteurised for 10 minutes at 90°C.

6.6.2 GABA production in soymilk – 24 h fermentation

The initial test was performed with two strains of LAB showing a high GABA production in MRS (from section 6.5), i.e. *Lb. sakei* and *Lb. lactis subsp. cremoris*. Three flasks were prepared for each strain: one with soymilk and PLP (0.25 mM), one with soymilk containing 2% glucose and PLP (0.25 mM) and one with MRS containing MSG (200 mM) and PLP (0.25 mM) to observe differences (Table 6.3).

Table 6.3: Setup for GABA production in soymilk & MRS after 24 h incubation at 30 °C

Strain	Medium	Additives	PLP
Lb. sakei	Soymilk	/	0.25 mM
Lb. sakei	Soymilk	2 % glucose	0.25 mM
Lb. sakei	MRS	200 mM MSG	0.25 mM
Lb. lactis subsp. cremoris	Soymilk	/	0.25 mM
Lb. lactis subsp. cremoris	Soymilk	2 % glucose	0.25 mM
Lb. lactis subsp. cremoris	MRS	200 mM MSG	0.25 mM

Firstly, soymilk was prepared according to section 6.6.1. After cooling to 30°C, PLP and glucose were added to the corresponding flasks. Then, the flasks were inoculated (1%) with OC. They were incubated at 30 °C for 24 h, while shaking.

The flasks were analysed for GABA content, growth, pH and titratable acidity (TA). The test was performed in duplicate.

6.6.3 Soymilk – function of fermentation time

The same setup and analysis was performed as described in section 6.6.2. However, samples were taken after 0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, 16 h and 24 h to follow the changes in properties of fermented soymilk as function of fermentation time.

6.6.4 GABA production in soymilk – effect of PLP

To test the effect of PLP on growth, GABA production, TA and pH, a fermentation was done with and without the addition of 0.25 mM PLP (Table 6.4). The flasks were inoculated (1%) and incubated at 30°C for 4 h (the optimum fermentation time as the result from section 6.5.3) while shaking.

Table 6.4: Setup for the analysis of the effect of PLP on pH, titrate, growth and GABA production

Strain	**Medium**	**Additives**	**PLP**
Lb. sakei	Soymilk	/	0.25 mM
Lb. sakei	Soymilk	/	/
Lb. sakei	Soymilk	2% glucose	0.25 mM
Lb. sakei	Soymilk	2% glucose	/
Lb. sakei	MRS	200 mM MSG	0.25 mM
Lb. sakei	MRS	200 mM MSG	/
Lb. lactis subsp. cremoris	Soymilk	/	0.25 mM
Lb. lactis subsp. cremoris	Soymilk	/	/
Lb. lactis subsp. cremoris	Soymilk	2% glucose	0.25 mM
Lb. lactis subsp. cremoris	Soymilk	2% glucose	/
Lb. lactis subsp. cremoris	MRS	200 mM MSG	0.25 mM
Lb. lactis subsp. cremoris	MRS	200 mM MSG	/

7 Analysis methods

7.1 Colony count

Colony count was carried out by serial dilution and streak plate method, all under sterile conditions.

A serial dilution was prepared in sterile 0.90 % NaCl tubes each containing 9 mL. Adding 1 mL of sample to a tube diluted the sample 10 times (dilution 10^1). Adding 1 mL of this tube to the next one dilutesd the sample 10 times more (dilution 10^2) and so on. This was done until the desired dilution was achieved. For each sample, a total of three consecutive dilutions were plated.

For the streak plate method, MRS agar was made by adding 1.5% bacteriological agar to MRS broth and boiling it. After autoclaving, the agar was divided over petri dishes so each one contained approximately 15 mL. The plates were dried in a laminar flow until the agar was solidified. Then, 100 µL of the needed dilution was streaked over the plate using a cell spreader.

The plates were incubated upside down for 48 h at the desired temperature, afterwards the number of colonies were counted.

7.2 pH measurement

The pH was measured by using a calibrated pH meter.

7.3 Titration

The amount of titratable acid was determined by titration with NaOH 0.1 M with phenolphthalein as indicator. Therefore, the exact concentration of the titrant needed to be checked with oxalic acid.

$$H_2C_2O_4 + 2\,NaOH \rightarrow Na_2C_2O_4 + 2\,H_2O \qquad \text{(r 7.1)}$$

To use a volume of 25 ml of NaOH 0.1 M, approximately 0.16 g of oxalic acid was needed. The moles of oxalic acid need to be divided by a factor two because one mole of oxalic acid reacts with two moles of NaOH.

$$m_{ox.acid} = \frac{C_{NaOH} * V_{NaOH} * MM_{ox.acid}}{2} = \mathbf{0.1576\ g} \qquad \text{(eq 7.1)}$$

With the known concentration and used volume of the titrant to reach the endpoint, the TA in the samples could be calculated with the following formula.

$$C_{TA} = \frac{C_{NaOH} * V_{NaOH}}{V_{sample}} \qquad \text{(eq 7.2)}$$

The mixture consisted of 5 mL sample, 5 drops of phenolphthalein and 50 mL of distilled water to increase the volume. TA was expressed as % lactic acid using following formula.

$$\%\ lactic\ acid = \frac{C_{lactic\ acid} * MM_{lactic\ acid}}{\rho_{lactic\ acid} * 10} \quad \text{(eq 7.3)}$$

7.4 GABA analysis by HPLC

Sample preparation and GABA analysis were performed by the method implemented by Verscheure (2017).

7.4.1 Sample preparation

The media used for the fermentations (MRS, NB and soymilk) contain amino acids, peptides and protein that can interfere with the analysis. Therefore, Glu and GABA were extracted from the samples using 75% EtOH.

Firstly, 5 mL of EtOH 75 % was added to 0.5 mL of sample in a conical screw cap test tube. The tube was shaken for 15 min and was centrifuged for 10 min at 4000 rpm at 22°C. Then, the supernatant was filtered through a 0.45 µm membrane filter to avoid clogging the injection port or column. The filtered sample was stored in the freezer at -18°C until analysis.

7.4.2 Analysis

To quantitatively analyse GABA, a reaction of the GABA with o-phthaldialdehyde (OPA) was done, which results in a fluorescent product. This reaction was catalyzed by 2-ME at pH 9. The isoindole product was measured at an excitation wavelength (λ_{ex}) of 340 nm and an emission wavelength (λ_{em}) of 455 nm using a fluorescence detector (Figure 7.1).

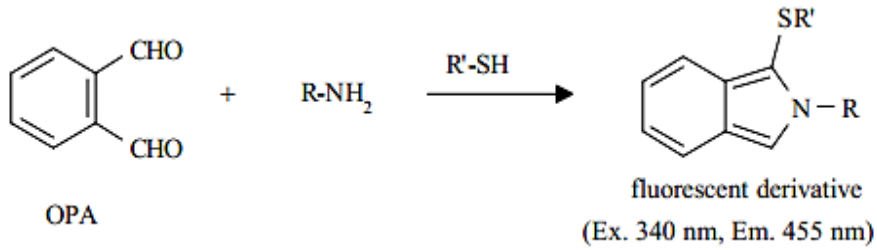


Figure 7.1: Reaction of o-phtaldialdehyde with an amino acid (Coppex, 2000)

The derivatization mixture consisted of 114 µL derivatization agent (5 mL of OPA, 1.25 mL of 0.5 M borate buffer, pH 9.9 and 125 µL of 2-ME), 36 µL of sample and 10 µL of 25 mg/L norvaline. The mixture reacted for 2 minutes at 22°C in the dark. Norvaline served as internal standard (IS). The derivatization solution was stable for one week when stored at 4°C in the dark.

The analysis was performed through a gradient elution with a flow of 1 mL/min (Table 7.1). The mobile phase consisted of two solvents, solvent A being 0.05 M sodium acetate; pH 7.2 and solvent B being 0.1 M sodium acetate, acetonitrile and MeOH (46:44:10, v/v/v; pH 7.2). Before the analysis, the column (Nucleodur 100-5 C_{18}ec column, 5µm; 150mm x 4,6mm) was prewashed with the solvents.

Table 7.1: Gradient of the mobile phase for GABA analysis with HPLC

Time (min)	Solvent A (%)	Solvent B (%)
0	80	20
24	0	100
34	0	100
39	100	0
54	100	0

The retention time of Glu was estimated at 4.5 minutes, GABA around 12 minutes and the IS norvaline at 15.5 minutes. The limit of detection for Glu was 0.36 mg/L and for GABA was 0.51 mg/L.

7.4.3 Calculations

The concentration of Glu and GABA in the samples were calculated through a standard curve. The curve was made by analysing known concentrations of Glu and GABA in 75% EtOH, being 1, 5, 10, 25, 40, 50, 60, 75 and 100 mg/L. To account for losses during the sample preparation and/or injection, the peak area of the amino acid was divided by the peak area of the internal standard norvaline. The curve was obtained by plotting these ratios as a function of the concentration.

Because the derivatization agent was only stable for one week, the standard curve had to be measured again every week.

7.4.4 Conversion rate

For the experiment described in section 6.5, glutamic acid concentration in the broth was analysed to calculate the conversion rate. Knowing that the initial concentration was 200 mM, the conversion rate could be calculated using following formula.

$$Conversion\ rate\ (\%) = \frac{Initial\ c_{Glu}\ (mM) - c_{Glu}\ after\ 96\ h\ (mM)}{c_{GABA}\ (mM)}\ x\ 100\ \%$$

7.5 Data analysis

The experiments were performed in duplicate, unless stated otherwise, of which mean and standard deviation were calculated.

RESULTS AND DISCUSSION

8 Results and discussion

8.1 Screening of GAD activity in LABs

8.1.1 Qualitative method: rapid colorimetric activity assay

To test the presence of GAD in the LAB strains, a colorimetric activity assay was performed. The development of a blue colour indicated a high activity whereas a yellow colour indicated a low activity. Colour development was checked after 4 h incubation at 37°C and linked to an activity according to Figure 8.1. Table 8.1 summarizes the results obtained from 17 LAB strains and 6 fermented samples

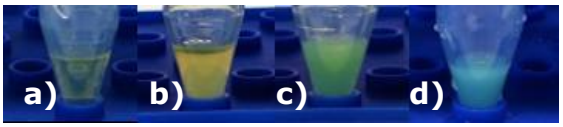


Figure 8.1: Colour development after 4 h incubation at 37°C, a) no activity, b) low activity, c) medium activity and d) high activity

Table 8.1: Visual interpretation of the activity of 17 LAB strains and 6 fermented samples through the rapid colorimetric activity assay

Strain/product	**Colour**	**Activity after 4 h at 37°C**
Lb. acidophilus	Clear - Yellow	None - Low
Lb. reuteri	Yellow	Low
Lc. lactis subsp. cremoris	Yellow	Low
Lb. brevis	Green	Medium
Lb. plantarum	Green	Medium
Lb. rhamnosus	Green	Medium
Lb. sakei	Green	Medium
L. mesenteroides subsp.	Green	Medium
P. freudenreichii subsp.	Green	Medium
Pc. pentosaceus1	Green - Blue	Medium - High
Pc. pentosaceus2	Green - Blue	Medium - High
Lb. casei	Blue	High
Lb. curvatus	Blue	High
Lb. farciminis	Blue	High
Lb. mali	Blue	High
Lb. viridescencs	Blue	High
Pc. damnosus	Blue	High
Fermented eggplant	Clear	None
Fermented tofu	Yellow - Green	Low - Medium
Germinated soybean	Green	Medium
Fermented shrimp	Green - Blue	Medium - High
Fermented anchovy fish	Blue	High
Fish sauce	Blue	High

Of the 17 strains tested, all of them showed at least a low activity with *Lb. acidophilus* being the one with the lowest colour development. A total of 8 strains developed a blue or greenish-blue colour indicating a high activity. For the fermented samples, only fermented eggplant did not develop a colour. Low activities could be due to the micro-organisms used for the fermentation of the different samples were not able to produce the enzyme or produced the enzyme under different circumstances. Another explanation is that the LABs can produce GAD, but the strains and/or GAD were not fully active during the test. However, the strain(s) used in the fermentation of food products are not known so further research is needed. The strains from the fermented products could be isolated and their fermentation parameters could be tested.

It should be noted that all tested strains were grown at their optimum temperature, but the enzyme assay was performed at 37°C according to the protocol. The enzyme of some strains could show a different activity when incubated at lower (or higher) temperatures (Li *et al.*, 2010).

This qualitative method gives a first indication of the activity of GAD, especially if one is not sure if the strain produces the enzyme. However, this method lacks power to distinguish the colours from each other.

For example, the enzyme of *Lb. curvatus* showed a blue colour and thus a high activity. The pH of the reaction solution was 4.17, but according to Sabadini & Carvalho (2013), a blue colour develops above pH 5.40. So, the colour of *Lb. curvatus* was in fact a colour in between green and blue but it was perceived as blue. However, this could also be due to interfering compounds.

Because this method was not accurate enough for the determination in a fermentation broth, it was decided to measure the activity quantitively. However, further developments of the method could increase the accuracy of the method and could be a powerful tool for screening GABA producing strains.

8.1.2 Quantitative method: enzyme extraction

To measure GABA quantitively, an extraction of GAD from LABs was performed. A reaction mixture was obtained after adding Glu as a substrate and PLP as cofactor of GAD. The mixture was incubated for 2 h at 30°C and GABA was analysed by HPLC. First, an optimisation for the volume of extraction buffer and the homogenisation time was performed.

- Optimisation of extraction buffer volume

The first extraction was performed with broth fermented by *Lb. brevis*. The sample volume was 5 mL and the buffer volume was 15 mL. The mixture was homogenized for 2 minutes and 450 µL was added to 450 µL substrate. Although Glu (t_R = 4.882 min) and IS (t_R = 16.246 min) were observed, no GABA peak (t_R = ± 12 min) was found (Figure 8.2). Similarly, negative results were obtained with the other two enzyme/substrate (E/S)-ratios, E/S = 1/2 and E/S = 2/1. Because no optimal ratio was known, ratio E/S of 1/2 was chosen for the other extractions so the enzyme had enough substrate.

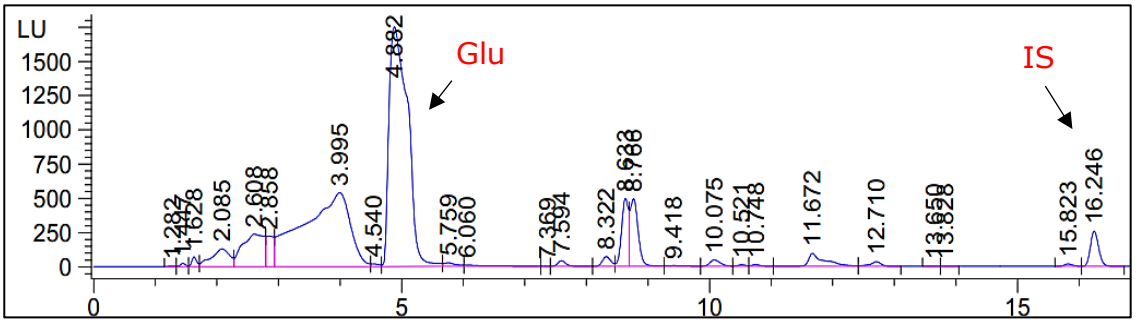


Figure 8.2: Chromatogram obtained after extraction of GAD from Lb. brevis, 5 mL sample and 15 mL buffer homogenized for 2 minutes with E/S-ratio 1/2

Next, the experiment was performed again with 10 mL of broth culture and 10 mL of buffer, making the extract more concentrated. A peak of Glu (t_R = 4.831 min) and the IS (t_R = 16.225 min) was observed but GABA was still absent (Figure 8.3). Therefore, homogenization time was increased to 5 minutes.

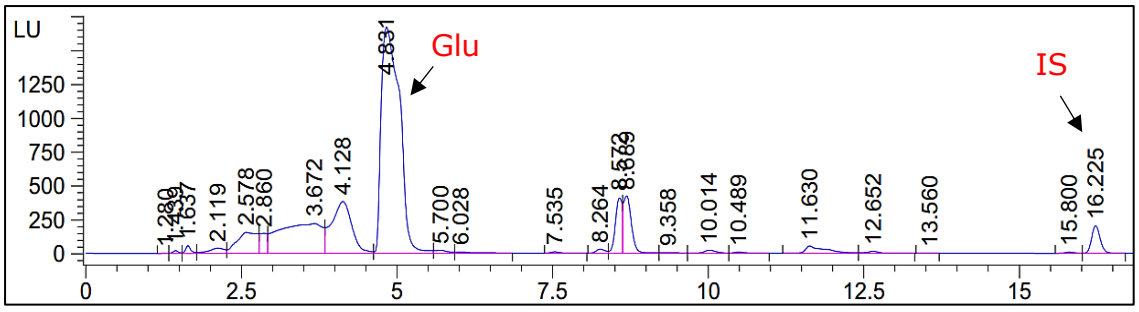


Figure 8.3: Chromatogram obtained after extraction of GAD from Lb. brevis, 10 mL sample and 10 mL buffer homogenized for 2 minutes with E/S ratio 1/2

- Optimisation of homogenisation time

Now, the homogenisation time was increased to 5 minutes. Again, no GABA production was observed (Figure 8.4).

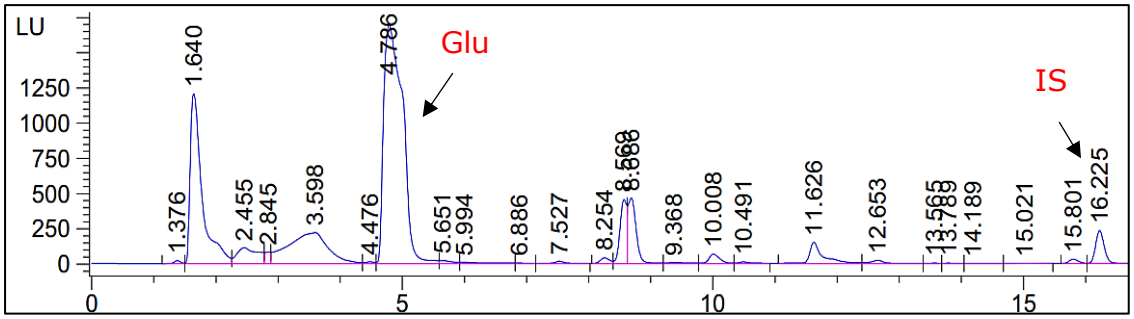


Figure 8.4: Chromatogram obtained after extraction of GAD from Lb. brevis, 10 mL sample and 10 mL buffer homogenized for 5 minutes with E/S-ratio 1/2

Apparently, the enzyme did not produce GABA under the used circumstances or no enzyme was present at all. The protocol was followed strictly; all temperatures and incubation times were respected. However, the protocol used was for the extraction of GAD from soybeans (Guo & Yang (2012); Bai *et al.* (2009); Wang *et al.* (2010)). So, it is possible that the enzyme was not extracted at all or was inactivated during the process.

The most likely reason is the fact that the MRS medium used for growth of the strains did not contain free MSG. Thus, bacteria did not produce GAD to convert MSG to GABA. Moreover, there was no stress condition (heat shock, low pH) which is one of the main factors to stimulate the production of GAD during culturing LABs. Also, an incubation time of 2 h could be too short to produce the enzyme. Therefore, a trial test was performed with 24 h incubation of the extracted sample and substrate. However, also no GABA peak was found in the chromatogram.

Instead of extracting the enzyme, it was decided to perform a fermentation and to analyse the GABA content in broth.

8.2 Screening of GABA production: analysis in broth

Because the extraction of GAD from bacterial cells was unsuccessful, a fermentation was performed in broth. The produced GABA was then analysed using the HPLC. The initial tests consisted of fermentations of *Lb. plantarum* and *Lb. brevis* in MRS and NB. In both media, 200 mM MSG and 0.25 mM PLP were added.

Samples were taken every 24 h and were tested for pH, CFU and GABA content. First, samples from fermentation in NB were analysed. No GABA was found, even after 96 h fermentation by both LAB strains (Figure 8.5). Therefore, these samples were only analysed once because they were of no further importance.

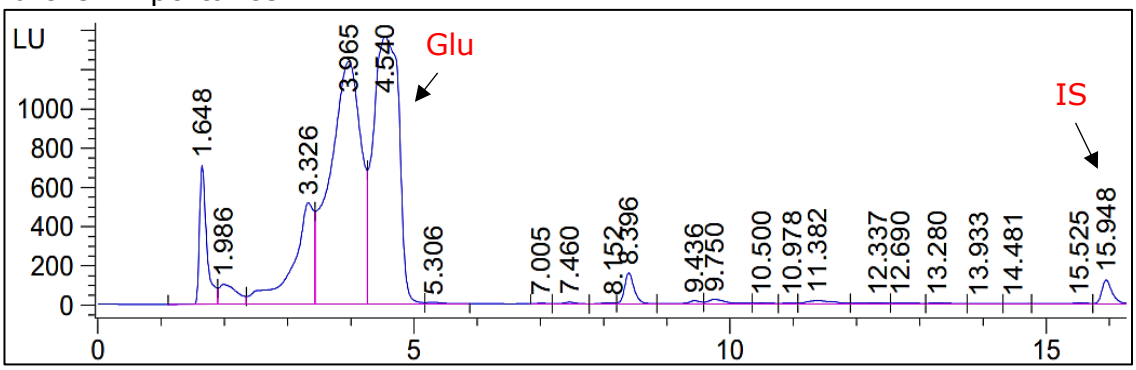
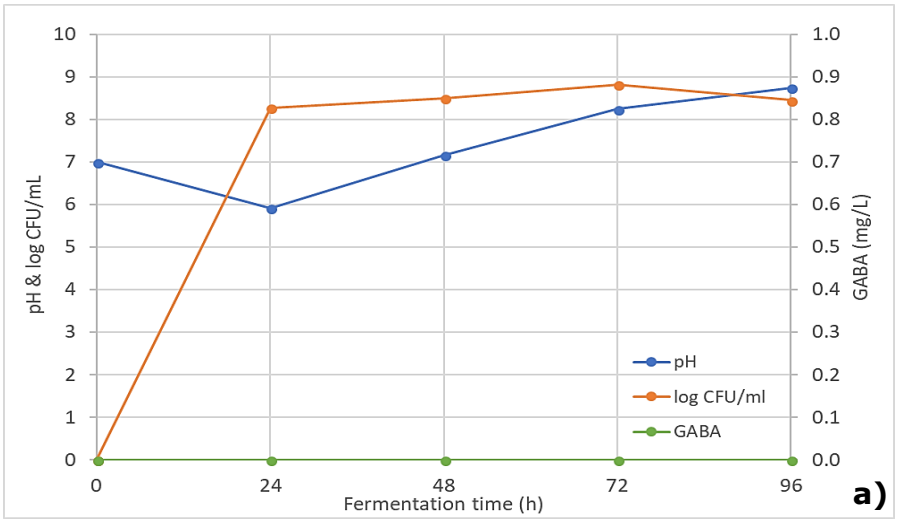


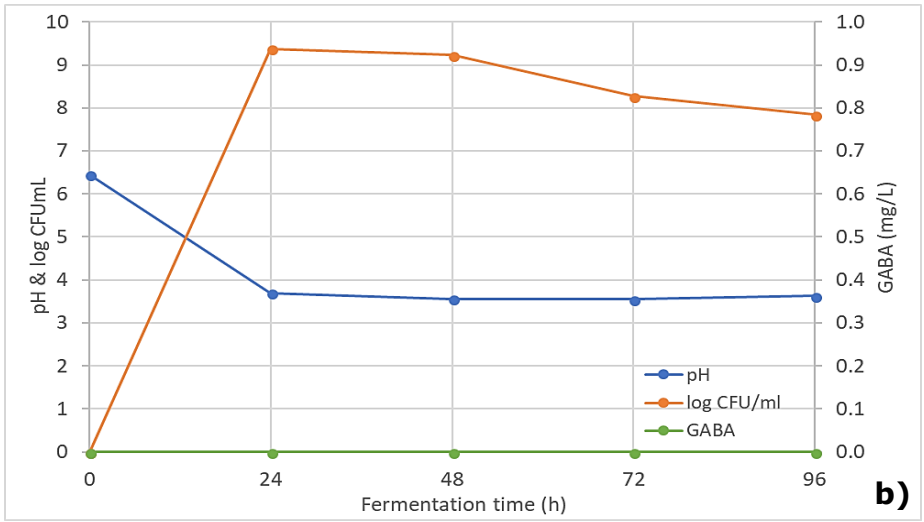
Figure 8.5: Chromatogram obtained after 96 h fermentation of Lb. plantarum in NB with 200 mM MSG and 0.25 mM PLP

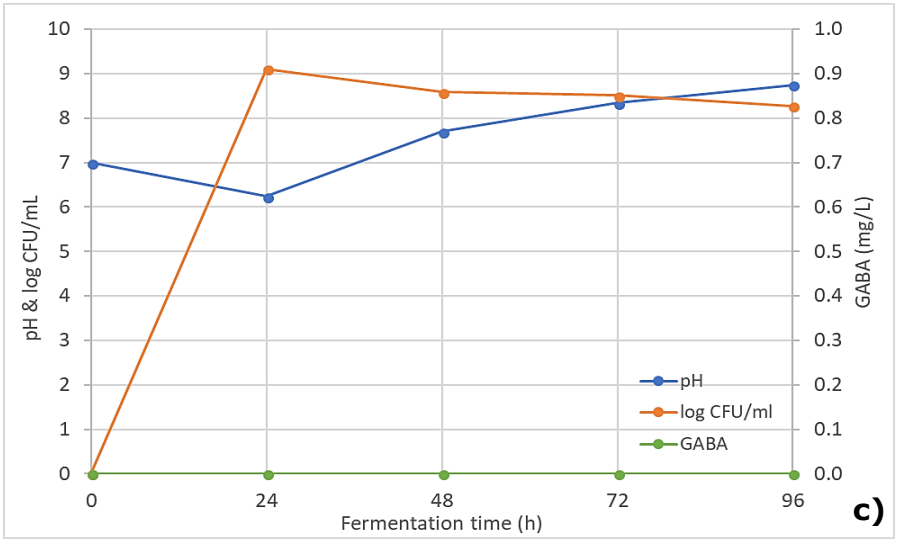
Then, samples in MRS were analysed where no MSG was added. Because of this, no GABA could be formed which corresponds with the observations (Figure 8.6). These samples were also analysed once. Thus, it can be concluded that *Lb. plantarum* and *Lb. brevis* did not produce GABA in NB with and without MSG and in MRS without MSG.

In NB flasks fermented with *Lb. plantarum*, the pH drops from pH 7.0 to 5.92 after 24 h. It then increased to 8.75 after 96 h fermentation. The pH in NB fermented with *Lb. brevis* followed a similar pattern. NB did not contain glucose, so no lactic acid could be produced. As a result, the pH did not drop to pH 4.5 which otherwise would have activated the GAD system.

In MRS without MSG, the pH dropped drastically after 24 h of fermentation. It reached a final pH of 3.63 with *Lb. plantarum* and 4.03 with *Lb. brevis*. Because of the low pH, the strains would normally activate the GAD, as the mechanism is activated around pH 4.5 (Karatzas *et al.*, 2012), but MSG was absent. The growth curve of *Lb. plantarum* hinted that the GAD-system was not activated. The curve started to decline after 48 h fermentation. *Lb. brevis* however, did not seem to be influenced by the low pH. This corresponds with observations by Barla *et al.* (2016) where *Lb. brevis* grew well at pH values around pH 3.0.







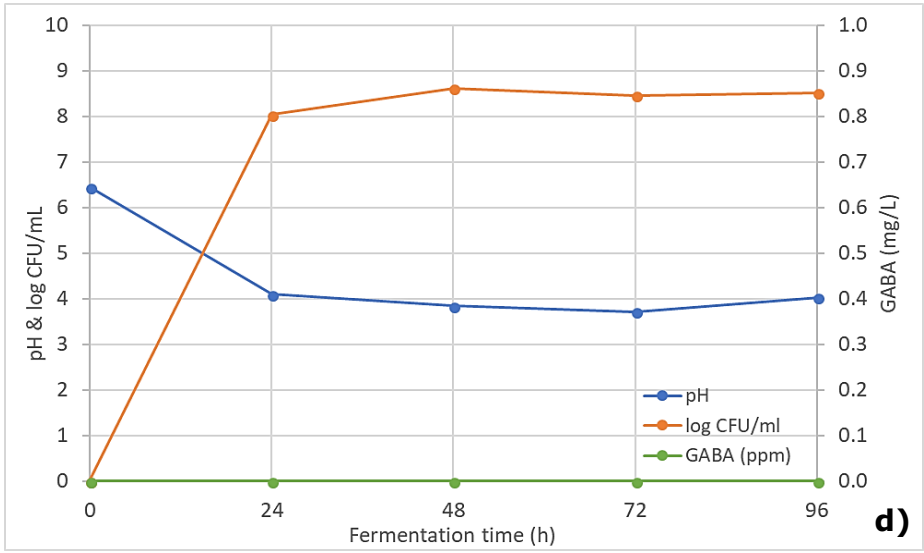


Figure 8.6: Course of pH, growth and GABA production as a function of time for Lb. plantarum (a) in NB with 200 mM MSG and (b) in MRS; for Lb. brevis (c) in NB with 200 mM MSG and (d) in MRS (N=1)

Finally, the samples fermented in MRS with MSG added were analysed and all showed GABA production (Table 8.2).

Table 8.2: Course of pH, growth and GABA production as a function of time for Lb. plantarum (P) and Lb. brevis (B) in MRS (M) with or without the addition of MSG (G) and PLP (P) (N=2)

Code	***Unit***	***24 h***	***48 h***	***72 h***	***96 h***
PMGP	pH	4.22 ± 0.08	4.29 ± 0.11	4.36 ± 0.11	4.46 ± 0.09
	log CFU/mL	9.6 ± 0.4	9.4 ± 0.5	9.2 ± 0.4	9.0 ± 0.9
	GABA (mg/L)	20.9 ± 3.2	118.1 ± 5.5	378.8 ± 0.6	371.4 ± 1.5
PMG	pH	4.22 ± 0.08	4.29 ± 0.09	4.35 ± 0.11	4.45 ± 0.08
	log CFU/mL	9.6 ± 0.2	9.5 ± 0.2	9.4 ± 0.3	9.3 ± 0.3
	GABA (mg/L)	21.3 ± 2.8	68.0 ± 0.00	157.1 ± 4.9	254.5 ± 3.7
BMPG	pH	4.63 ± 0.04	4.39 ± 0.06	4.50 ± 0.03	4.58 ± 0.00
	log CFU/mL	9.5 ± 0.1	9.5 ± 0.1	9.5 ± 0.1	9.5 ± 0.3
	GABA (mg/L)	13.3 ± 0.8	19.0 ± 1.5	58.4 ± 8.3	50.1 ± 3.4
BMG	pH	4.65 ± 0.01	4.35 ± 0.08	4.47 ± 0.10	4.61 ± 0.06
	log CFU/mL	9.4 ± 0.3	9.1 ± 0.9	8.9 ± 0.9	8.9 ± 1.0
	GABA (mg/L)	9.4 ± 0.4	13.8 ± 2.7	16.5 ± 9.9	48.9 ± 2.1

Figure 8.7 shows how the GAD system shields *Lb. plantarum* and *Lb. brevis* from the low pH, which drops to 4.0 after 24 h fermentation. The pH remained constant until 96 h in all cases. Growth remained constant over the course of fermentation time, the slight decrease could be due to measurement errors. The strains remained in the stationary phase. This corresponds with the experiment by Tajabadi *et al.* (2015).

Regarding the GABA content, when MSG and PLP were added, it can be seen that the GABA content increased for the first 48 h of fermentation. Then it raised steadily to reach the maximum content at 72 h of fermentation, with 378.8 ± 0.6 mg/L and 58.4 ± 8.3 mg/L for *Lb. plantarum* and *Lb. brevis* respectively.

When prolonging the fermentation time to 96 h, GABA content produced by *Lb. plantarum* decreased slightly (371.4 ± 1.5 mg/L) as well as for L*b. brevis* (50.1 ± 3.4 mg/L). By contrast, in the fermentation with MSG added but without PLP, the GABA content produced by these strains increased regularly for the first 72 h. Then it jumped up to reach to the highest content at 96 h of fermentation. However, the GABA production when PLP was absent was lower than when PLP was present for *Lb. plantarum*, this was 254.4 ± 3.7 mg/L. *Lb. brevis* produced 48.9 ± 2.1 mg/L without PLP, so no effect of PLP was observed.

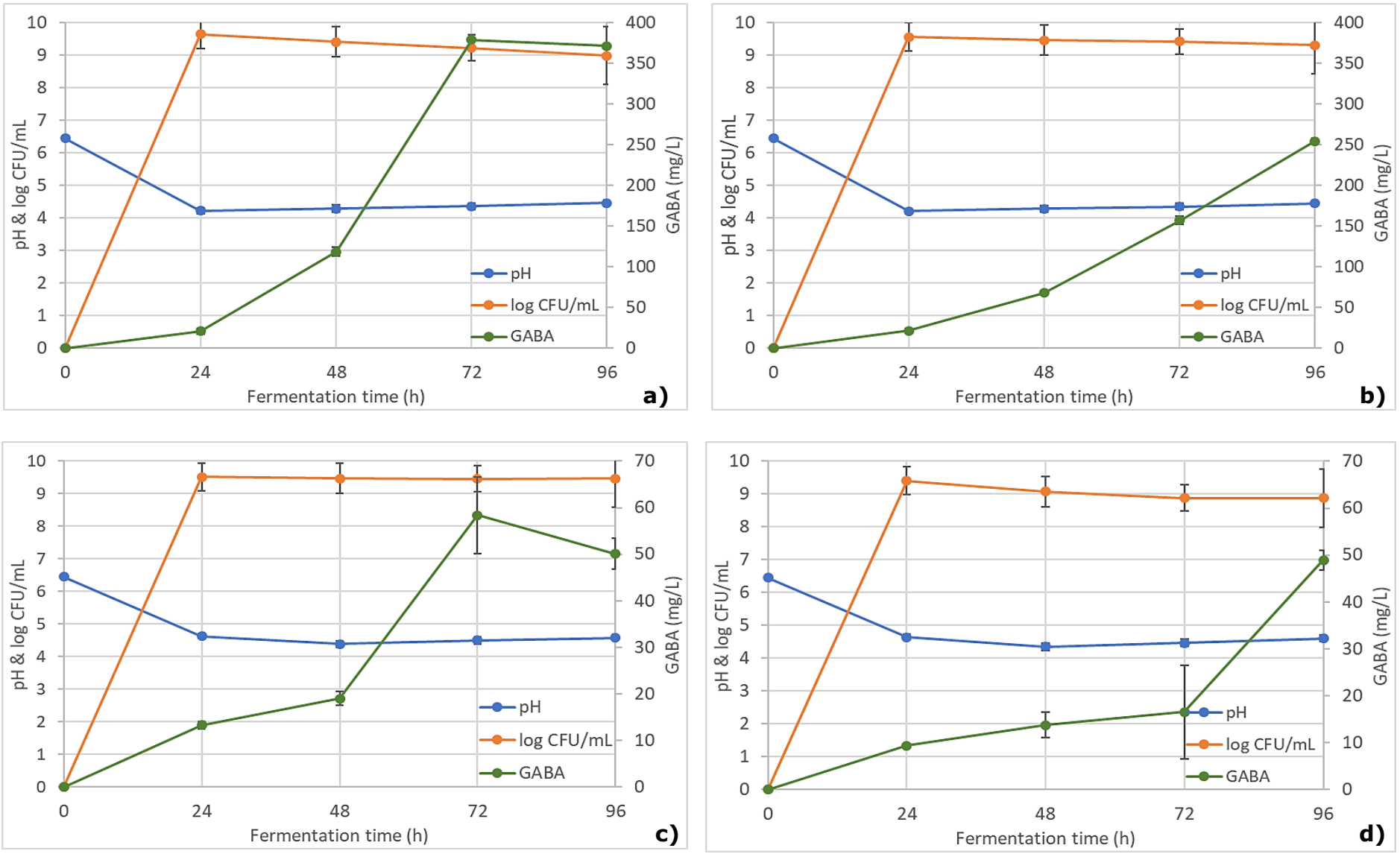


Figure 8.7: Course of pH, growth and GABA production as a function of time for Lb. plantarum in MRS with 200 mM MSG (a) with 0.25 mM PLP, (b) without 0.25 mM PLP and Lb. brevis in MRS with 200 mM MSG (c) with 0.25 mM PLP and (d) without 0.25 mM PLP (N=2)

There was a significant difference in GABA producing capacity between both strains. When the same additives were added, and the same fermentation time was used, the amount of GABA produced by *Lb. brevis* were much lower than by *Lb. plantarum.* Interestingly, the difference in GABA production was much higher when prolonging fermentation time. To compare between strains, *Lb. plantarum* produced 1.6 times more GABA with PLP and 2.3 times more without PLP after 24 h than *Lb. brevis*. This difference increased to 7.4 and 5.2 times with and without PLP respectively after 96 h of fermentation. So again, *Lb. plantarum* produced more GABA under these circumstances than *Lb. brevis*, regardless of the addition of 0.25 mM PLP.

As for the effect of PLP, a significant increase in GABA production was observed with the addition of 0.25 mM PLP. This difference was minor after 24 h of fermentation but started to increase with fermentation time. For *Lb. plantarum*, an increase of 74% and 141% of GABA were obtained after 48 h and 72 h fermentation, respectively, compared to incubations without PLP added. For *Lb. brevis* however, this effect was only observed after 72 h of fermentation where 253% more GABA was produced with 0.25 mM PLP compared to no PLP addition. The GABA content was stable after 96 h of fermentation. For these fermentation parameters, *Lb. plantarum* benefits more from the addition of 0.25 mM PLP than *Lb. brevis*.

Indeed, addition of 0.1 µM of PLP did not affect GABA production of *Lb. brevis* NCL912 (Li *et al*., 2010) but this concentration is 2500 times lower than the one used in this experiment. Fan *et al.* (2012) also found that 0.1 mM of PLP had no effect of GABA production on recombinant GAD from *Lb. brevis*, so it can be said that addition of 0.25 mM PLP had no effect on GABA production by this strain. This corresponds with the observations in this experiment. As for *Lb. plantarum* little is known about the effect of PLP on GABA production. One study found that addition of 0.1 mM PLP increased GABA production in sourdough fermentation by *Lb. plantarum* (Coda *et al.*, 2010b). The observations in this experiment also showed a clear increase in GABA production by *Lb. plantarum* with the addition of PLP.

The difference between the strains regarding PLP could be explained by the appearance of GAD as described in the literature study (Fan *et al.*, 2012). Huang *et al.* (2007) found that GAD from *Lb. brevis* was mostly present as apo-enzyme where after binding to PLP, remained a holo-enzyme. Therefore, addition of PLP had little to no effect. As for *Lb. plantarum*, it could be that the interaction between apo-GAD and PLP is more easily broken and addition of PLP increases the GABA production.

GABA production by both strains was much lower than most experiments found in literature. However, because the parameters were fixed and not optimized, this could be expected.

Li *et al*. (2010) were able to produce 35.7 g/L with *Lb. brevis* NCL912 under optimized conditions whereas Park & Oh (2005) only produced 825 mg/L with *Lb. brevis* OPY-1 with a non-optimized process. The latter is still 14 times more than the concentration obtained in this experiment.

For *Lb. plantarum* however, more GABA was obtained than the experiment performed by Tajabadi *et al.* (2015). They produced 181.5 mg/L of GABA after 60 h with *Lb. plantarum* Taj-Apis362 in optimized conditions, in this experiment 378.8 mg/L was obtained after 72 h in non-optimized conditions.

It should also be noted that the used strains in literature are usually isolated from Glu-rich fermented foods. Therefore, these strains are already used to convert Glu to GABA whereas the strains used in this experiment are not.

So, to increase GABA production with the strains used in this experiment, it would be better to grow them in MRS with MSG before the fermentation. Also, the fermentation conditions need to be optimised for each strain to maximize GABA production.

8.3 Screening of GABA production by LABs

To maintain the same fermentation conditions for all strains, 200 mM MSG and 0.25 mM PLP were chosen to add into MRS broth. Seventeen LAB strains were incubated for 96 h at their optimum temperature to investigate the GABA producing capacity. Their GABA production is given in Table 8.3. However, because the concentration of Glu was too high, it could not be measured with the standard curve. Therefore, the samples were diluted 100 times. This was only performed for the second replicate, hence no standard deviation for Glu_{96h} is given.

Of the 17 LAB strains tested, three produced the highest amount of GABA after 96 h incubation. These were *Lb. plantarum, Lc. lactis subsps. cremoris* and *Lb. sakei* with 391.2 ± 26.5 mg/L, 603.2 ± 12.9 mg/L and 643.5 ± 42.7 mg/L, respectively. However, only a conversion rate of Glu to GABA of 2.48%, 4.23% and 4.36%, respectively was observed. *Lb. sakei* for example used 71.63% of the Glu in the medium, but only 4.36% of this was used to produce GABA. This indicates that a lot of optimization needs to be done to increase the conversion yields. The effect of initial MSG and PLP concentration, temperature and pH should be tested for each strain so more of the Glu will be used for GABA production instead of using it for other metabolic processes.

Table 8.3: GABA production in MRS with MSG (200 mM) and PLP (0.25 mM) by 17 LAB strains after 96 h fermentation at their optimum temperature (N=2)

Strain	$GABA_{96h}$ (mg/L)			Glu_{96h} (g/L)	Glu_{96h} used (%)	Glu_{96h} left (%)	Conversion rate (%)
Pc. damnosus	22.8	±	4.6	14.1	52.14	47.86	0.21
Lb. farciminis	30.9	±	12.3	12.6	57.20	42.80	0.26
Lb. curvatus	37.7	±	16.6	15.2	48.94	51.06	0.37
Lb. brevis	43.5	±	16.8	13.7	53.31	46.69	0.40
Lb. acidophilus	44.3	±	12.8	14.9	49.48	50.52	0.43
Lb. reuteri	47.5	±	28.4	16.3	44.62	55.38	0.52
Lb. rhamnosus	49.7	±	16.0	14.3	51.33	48.67	0.47
L. mesenteroides	51.9	±	20.1	7.7	73.66	26.34	0.34
Lb. viridescencs	52.1	±	13.4	12.7	56.81	43.19	0.44
Pc. pentosaceus2	56.2	±	13.8	14.8	49.72	50.28	0.55
Lb. mali	57.6	±	10.0	14.2	51.70	48.30	0.54
Pc. pentosaceus1	63.0	±	9.5	12.5	57.51	42.49	0.53
P. freudenreichii	69.1	±	37.6	18.0	38.85	61.15	0.86
Lb. casei	125.7	±	12.5	15.1	48.69	51.31	1.25
Lb. plantarum	391.2	±	26.5	6.4	76.45	23.55	2.48
Lc. lactis subsp. cremoris	603.2	±	12.9	9.1	69.18	30.82	4.23
Lb. sakei	643.5	±	42.7	8.4	71.63	28.37	4.36

The low conversion rates could be explained by the chosen parameters. Firstly, the parameters were the same for all strains but each of the strains has an optimum pH, temperature and initial MSG concentration. Subsequently, each GAD has optimum conditions as well.

For *Lb. brevis* BH2 isolated from kimchi, optimum parameters of 5% MSG, 30°C and 48 h of fermentation in MRS resulted in 20.1 g/L GABA with 73% conversion rate (Kim *et al.*, 2007). Compared to this experiment, the only difference was the MSG concentration (3%) and the strain. *Lb. brevis* BH2 was isolated from kimchi, were Glu is abundantly present. The strain used in this experiment was isolated from silage. Without the presence of MSG, the strains were not triggered to produce GABA and did not use GAD in such a way to shield themselves from low pH. Only when they were inoculated with the presence of MSG, GAD production was initiated and thus could have delayed GABA production. In another experiment, performed by Moo-Chang *et al.* (2010), GABA production with recombinant GAD from *Lb. sakei* B2-16 was optimised. This resulted in 27.6 g/L GABA with 100% conversion. Here, 12% MSG was added in an optimised MRS medium. So, the low conversion rates are most probably due to the unoptimized fermentation parameters.

In this experiment, the final pH was not measured, so no information was available about this. Most likely, the pH continued to drop as the fermentation progressed. No significant effects of this were observed on *Lb. brevis* and *Lb. plantarum* in section 6.4, but could have played a role on other strains. Also, the initial MSG concentration could have been too high for some strains, only half was used by the LABs after 96 h. Therefore, this concentration could have inhibited cell growth and subsequently GABA production in some strains, however this is not certain.

Because of the high GABA production found in this experiment, *Lb. sakei* and *Lc. lactis subsp. cremoris* were used for the fermentation of soymilk in the further experiments.

8.4 GABA production of LABs in soymilk

8.4.1 Fermentation time 24 h

The initial test was the fermentation of soymilk for 24 h. Here, two media were used, soymilk and soymilk with 2% glucose added. Both were fermented with *Lb. sakei* and *Lc. lactis subsp. lactis*. After 24 h, the soymilk was separated as seen in Figure 8.8. The viscosity was not noticeable different from water while the curds were clumped together quite firm. Also, the soymilk had a spoiled smell. The soymilk was tested for pH, TA, GABA production and CFU. Fermentation in MRS was done to observe any noticeable differences between media.

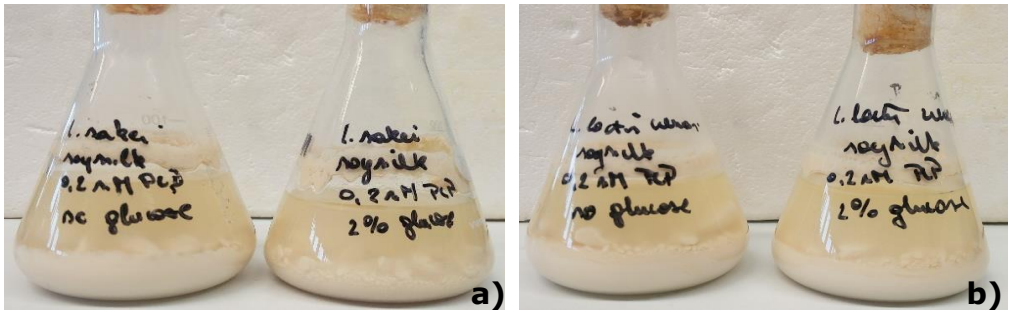


Figure 8.8: Separation of the soymilk fermented with a) Lb. sakei and b) Lc. lactis subsp. cremoris with and without the addition of 2% glucose

The initial free Glu content in soymilk was measured to be 36.5 ± 8.41 mg/L. After 24 h, no Glu peak was observed in fermented soymilk indicating that all Glu was used. The Glu peak in MRS was too high to be measured so no conversion rate was known. GABA production was the highest in MRS broth. *Lb. sakei* produced 103.8 ± 3.34 mg/L which is significantly higher than in soymilk. *Lc. lactis subsp. cremoris* also produced a lower amount in soymilk without glucose compared to MRS being 75.51 ± 4.54 mg/L. The conversion rate of Glu to GABA in fermented soymilk by both strains was calculated in Table 8.4.

Table 8.4: GABA production in soymilk after 24 h fermentation with Lb. sakei and Lc. lactis subsp. cremoris expressed as mg GABA/L soymilk (N=2)

Strain	Medium	Initial Glu (mg/L)	GABA (mg/L)	Conversion rate (%)
Lb. sakei	Soymilk - no glucose	36.5 ± 8.4	66.1 ± 8.4	44.4
Lb. sakei	Soymilk - 2% glucose	36.5 ± 8.4	63.7 ± 19.6	46.0
Lb. sakei	MRS – 200 mM MSG	NM	103.8 ± 3.4	NM
Lc. lactis subsp. cremoris	Soymilk - no glucose	36.5 ± 8.4	75.5 ± 4.5	38.8
Lc. lactis subsp. cremoris	Soymilk - 2% glucose	36.5 ± 8.4	60.7 ± 14.2	48.3
Lc. lactis subsp. cremoris	MRS - 200 mM MSG	NM	87.4 ± 4.6	NM

NM = Not Measured

Figure 8.9 shows a comparison between the four tested parameters of both strains: TA, pH, viable count and GABA production. Firstly, it was observed that the GABA production by both strains in soymilk was significantly lower than in MRS. Secondly, no difference was noticed in GABA production between strains, even when glucose was present. However, *Lb. sakei* clearly produced more GABA than *Lc. lactis subsp. cremoris* in MRS. Finally, the use of glucose to accelerate the fermentation also did not show the desired effect. Fermentation with *Lb. sakei* resulted in 66.1 ± 8.4 mg/L GABA without glucose, and 63.7 ± 19.6 mg/L when 2% of glucose was added. A similar trend was observed for *Lc. lactis subps. cremoris* with 75.5 mg/L ± 4.5 mg/L without glucose and 60.7 ± 14.2 mg/L with glucose. Thus, 2% glucose does not seem to enhance GABA production.

Addition of 2% glucose did not increase GABA production whereas an increase of lactic acid production for *Lb. sakei* was observed. Addition of 2% glucose enhanced lactic acid production by 21.7% while for *Lc. lactis subps. cremoris* an increase of 15.5% was observed. These increases also led to a decrease of the pH in both cases. In MRS, much more lactic acid was formed, around 1.60% lactic acid for both strains. Here, the pH was still around 4.0 because of the buffering capacity of the medium. The colony count was also higher in MRS although this was not the case with *Lc. lactis subsp. cremoris* in soymilk with 2% glucose.

Both strains showed a good survival in the soymilk with 9 log CFU/ml and 9.9 log CFU/ml for *Lb. sakei* and *Lc. lactis subps. cremoris,* respectively. This corresponds with an observation by Champagne *et al.* (2009) showing that *Lc. lactis* grows well on a soy environment.

Interestingly, both strains converted around half of the Glu to GABA in both soymilk media. A first reason could be the lower Glu concentration of 0.284 mM in contrast to 200 mM in MRS, so a more efficient conversion was performed. Also, MRS is a perfect medium which provides the necessary environment for proliferation. Therefore, less Glu is used in the GAD system because the pH is partially buffered by MRS. In soymilk, however, the buffering capacity is much lower (Lutchman *et al.*, 2006).

It should be noted that this conversion rate is most likely overestimated. As reported by Kovalenko et al. (2006), 7.22% of the proteins dry weight consists of Glu. So during proteolysis, extra Glu can be provided to use in the GAD system therefore increasing GABA yield while the initial Glu concentration remains the same.

Because the consistency of the soymilk was nowhere near usable after 24 h fermentation, the course of parameters was analysed as a function of time.

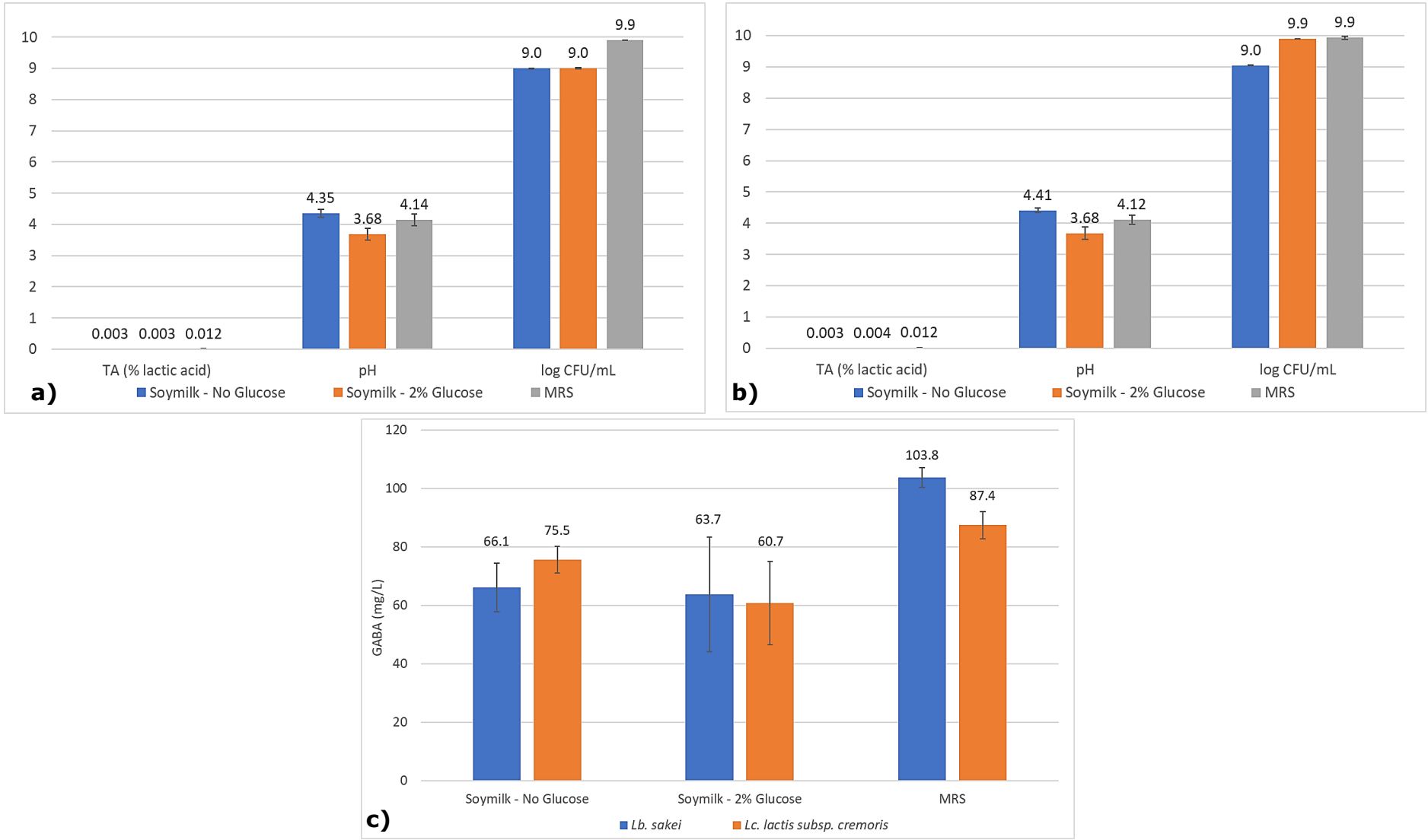


Figure 8.9: TA, pH and log CFU/ml for a) Lb. sakei and b) Lc. lactis subsp. cremoris; c) comparative GABA production after 24 h fermentation of soymilk (N=2)

8.4.2 Function of fermentation time

This experiment shows the development of soymilk as a function of the fermentation time. Only one series of the four is shown in Figure 8.10 because no visual difference was observed between series. The soymilk started to separate after 4 h and this was clearly seen after 8 h of fermentation. Up until 4 h of fermentation, the soymilk had a pleasant yoghurt-like smell. The viscosity increased with fermentation time up until 3 h, the viscosity was lost after 8 h of fermentation. The viscosity after 3 h could be compared with a drinking-yoghurt.

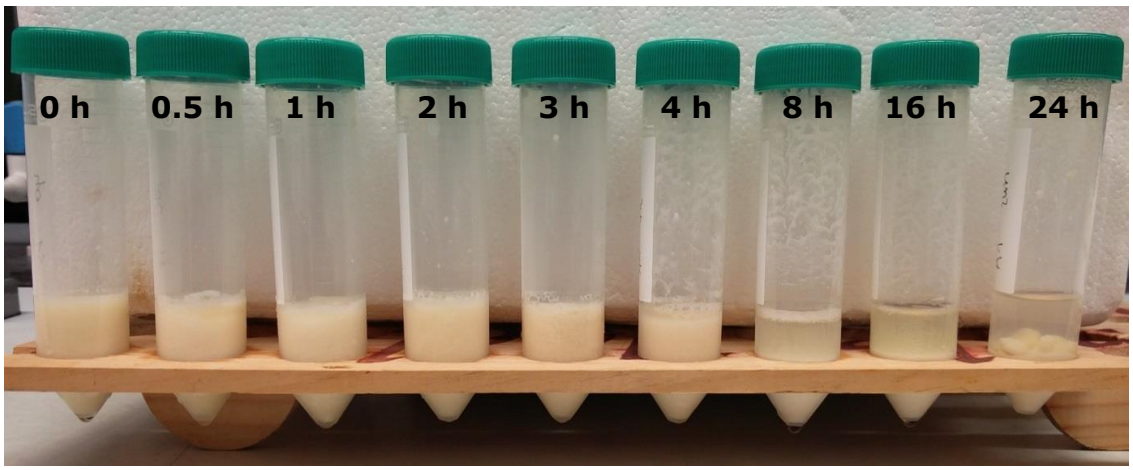


Figure 8.10: Development of soymilk as function of the fermentation time for Lb. sakei without glucose

Figure 8.11 shows the course of the parameters as a function of fermentation time for *Lb. sakei*. The TA increased slightly for soymilk with and without glucose up until 4h where the TA was the same, around 0.20% lactic acid. Then, the increase in TA was significantly more in soymilk with glucose where it reached a maximum of 0.47 ± 0.03 % lactic acid after 16 h. In MRS however, lactic acid was produced faster and in larger quantities where it sharply increased after 4 h.

The pH dropped sharply until 8 h and reached 4.49 ± 0.26 in soymilk without glucose added whereas the pH was 4.21 ± 0.11 when glucose was added. For the latter, the pH dropped even further and reached to 3.68 ± 0.18 after 24 h, soymilk without glucose reached to 4.35 ± 0.13. The pH in MRS remained constant after 8 h, but much more lactic acid was produced. This can again be explained by its buffering capacity.

The low pH in soymilk with glucose had an impact on the growth with a decrease after 16 h. Although *Lb. sakei* grew faster with glucose than without, a higher viable count was obtained in soymilk without glucose. This was probably due to the lower pH in soymilk with glucose which inhibited growth. The growth in MRS increased throughout the fermentation without an observable decrease.

As for GABA production, quite some variations were observed between the two replicates. Therefore, significant differences cannot be seen. So, GABA production remained the same between the two soymilk samples. The production increased linear up until 16 h fermentation where it reached an optimum, 63.5 ± 26.9 mg/L without glucose added and 68.4 ± 16.6 mg/L with glucose. This maximum was not practical however, because at this point the soymilk was separated. So, for the consumer, the GABA content at 4 h should be considered the maximum. The GABA content after 4 h fermentation was 36.0 ± 2.3 mg/L without added glucose and 32.6 ± 16.3 mg/L with glucose. The highest GABA production was obtained in MRS after 24 h, 103.8 ± 3.4 mg/L.

Finally, the production remained constant in both soymilk samples, in MRS the production continued. This stagnation is most likely caused by the low pH which in turn caused cell decay after 16 h, especially in soymilk with 2% glucose. Again, the futile proton hypothesis could play a role in soymilk with 2% glucose. The pH dropped from 4.09 after 16 h to 3.68 after 24 h. Therefore, Glu was mostly protonated and counteracted the proton release from the cell. Subsequently, the intracellular pH dropped and GAD activity decreased. This slowed down the GABA production and caused the beginning of cell decay (Feehily & Karatzas, 2013). A similar result was obtained by Kook *et al.* (2010) also with *Lb. sakei*, where GABA production slowed down when a pH lower than 4.0 was reached.

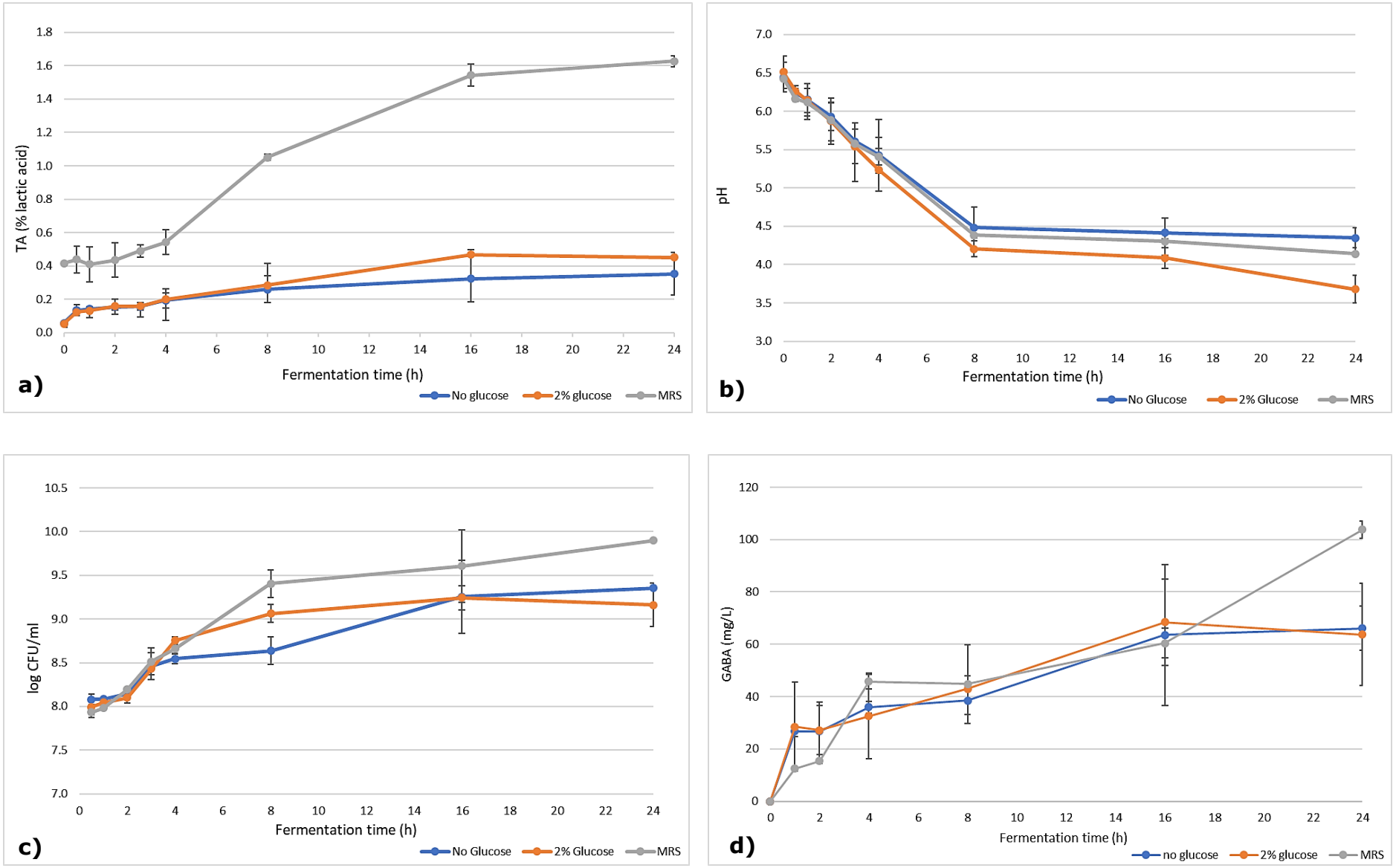


Figure 8.11: Course of a) TA, b) pH, c) log CFU/ml and d) GABA as a function of fermentation time for Lb. sakei in different media at 30°C (N=2)

Fairly similar results were obtained with *Lc. lactis subsp. cremoris* (Figure 8.12). The course of TA was near identical with fermentation by *Lb. sakei*. Lactic acid concentration was the same up until 8h, where it then increased faster in soymilk with glucose. After 24 h, 0.41 ± 0.07 % lactic acid was produced without glucose, and 0.48 ± 0.02 % with glucose. The higher concentration in the latter is due to the extra glucose, which extends the fermentation to lactic acid. In MRS, the TA reached 1.66 ± 0.03 % after 24 h, which is similar compared to *Lb. sakei*.

The pH decreased sharply up until 8h fermentation, a pH-value of 4.44 ± 0.06 was obtained without glucose and a slightly lower value of 4.18 ± 0.23 with glucose. Again, a further decrease was observed in soymilk with glucose with a final value of 3.68 ± 0.20. Without glucose, the pH remained constant and reached 0.41 ± 0.07 after 24 h of fermentation.

Interestingly, growth did not suffer from the low pH in soymilk with glucose whereas a decrease was observed in raw soymilk. However, GABA production remained constant after 16h with glucose added. This could also be explained by the futile proton hypothesis. Because the growth was not affected, other acid-resistance mechanisms could be in play. These include: conversion of arginine to ornithine (Gänzle *et al.*, 2007), conversion of agmatine to putrescine and decarboxylation of histidine (Lucas *et al.*, 2007). All these processes consume intracellular protons.

A maximum GABA content was obtained after 24 h in soymilk without glucose added of 75.5 ± 4.5 mg/L. For soymilk with added glucose, the maximum was at 16 h of fermentation which gave 65.1 ± 9.4 mg/L. Again, these maximum values are not practical because of the separation. For commercial uses, the maximum values should be taken after 4 h of fermentation. These were 33.6 ± 13.9 mg/L and 39.7 ± 2.1 mg/L for soymilk without and with 2% glucose respectively. The highest GABA content was again produced in MRS broth with 200 mM Glu and 0.2 mM PLP, being 87.4 ± 4.6 mg/L. This is 18.7% lower than the production by *Lb. sakei* under the same circumstances. This difference was already observed after the GABA screening of 17 strains (section 8.3), where *Lb. sakei* produced 6.67% more than *Lc. lactis subsp. cremoris* in MRS after 96 h. So, it is possible that *Lb. sakei* produces more GABA under these fermentation parameters than *Lc. lactis subsp. cremoris*. However, after 24 h in soymilk without glucose, *Lc. lactis subsp. cremoris* produced 14.2% more GABA than *Lb. sakei*.

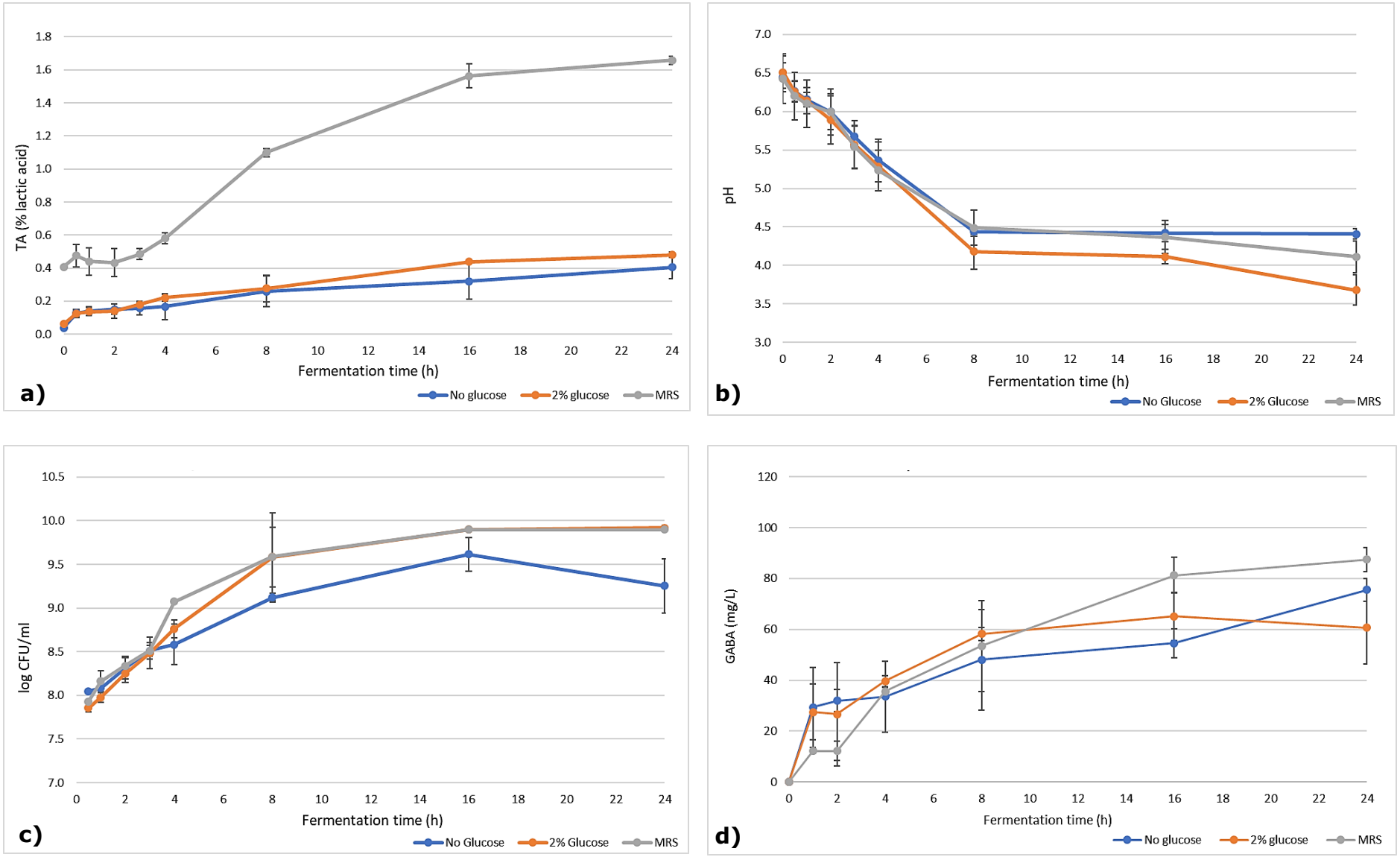


Figure 8.12: Course of a) TA, b) pH, c) log CFU/ml and d) GABA as a function of fermentation time for Lc. lactis subsp. cremoris in different media at 30°C (N=2)

For both strains, the concentration after 4 h could be enough to obtain beneficial effects in humans. To have blood-pressure lowering effects, 10-12 mg of GABA each day could be sufficient (Inoue *et al.,* 2003). So, one should drink 300-400 mL of fermented soymilk from this experiment each day. To lower cholesterol levels, 12 mg/L of GABA is needed. So, 250 mL of this fermented soymilk should be sufficient (Roohinejad *et al.*, 2010).

The fermentation of soymilk to increase GABA production was not optimised, so this could be the start of further research. The effect of different concentrations of glucose or other sugars could be measured. Maybe new strains that produce a higher amount of GABA could be used and would need other fermentation parameters. Finally, the effect on rheological parameters could be considered as well.

8.4.3 Effect of PLP

To determine the effects of PLP on the soymilk fermentation, soymilk was fermented by *Lb. sakei* and *Lc. lactis subsp. cremoris* without the addition of 0.25 mM PLP. Figure 8.13 shows the effect of 0.25 mM PLP on TA, pH, colony forming units and GABA production after 4 h of fermentation.

The TA in all soymilk samples showed no significant difference when 0.25 mM PLP was added. In MRS, it seemed that more lactic acid was produced without the addition of PLP, but because of the high standard deviation this cannot be confirmed.

The same can be said about the pH, this appeared slightly lower in samples with 0.25 mM PLP but the high standard deviation covers that range.

However, the effect of PLP was clearly observed in growth after 4h. The growth in all samples where PLP was present, was higher than without PLP. For *Lb. sakei*, addition of PLP increased growth with 3.5% and 2.6% in soymilk without and with glucose respectively. No effect in growth was observed in MRS. For *Lc. lactis subsp. cremoris*, only a significant increase of 4.3% was observed in soymilk with 2% glucose. In MRS, growth was 1.8% higher with the addition of PLP. Indeed, addition of PLP helps the GAD system to shield the micro-organisms from the low acidity. Therefore, a higher viable count was observed (Komatsuzaki *et al.*, 2005).

Finally, GABA production benefited the most from the addition of PLP. *Lb. sakei* produced 342% and 1413% more GABA in soymilk without and with 2% glucose respectively. Similar results were found by Sa *et al.* (2015) with *Lb. sakei* A156 where the activity of GAD depends on the PLP concentration. A maximum activity was found with 0.1 mM PLP, higher concentrations did not affect GAD activity.

For *Lc. lactis subsp. cremoris* this increase was 158% and 633%. Lacroix *et al.* (2013) also found that addition of 50 µM PLP had an impact on GABA production by *Lactococcus* strains.

Finally, also more GABA was produced in MRS although this effect was lower compared to GABA production in soymilk. *Lb. sakei* produced 50.7% more GABA in MRS and *Lc. lactis subsp. cremoris* 56.4%. Indeed, MRS medium already results in a good GABA production because GAD is in a stable environment. Therefore, PLP will have a lower effect.

In further experiments, PLP should be added especially for *Lb. sakei* and *Lc. lactis subsp. cremoris*. However, when designing an optimisation experiment, the concentration of PLP should also be considered because it could have an effect on GABA production.

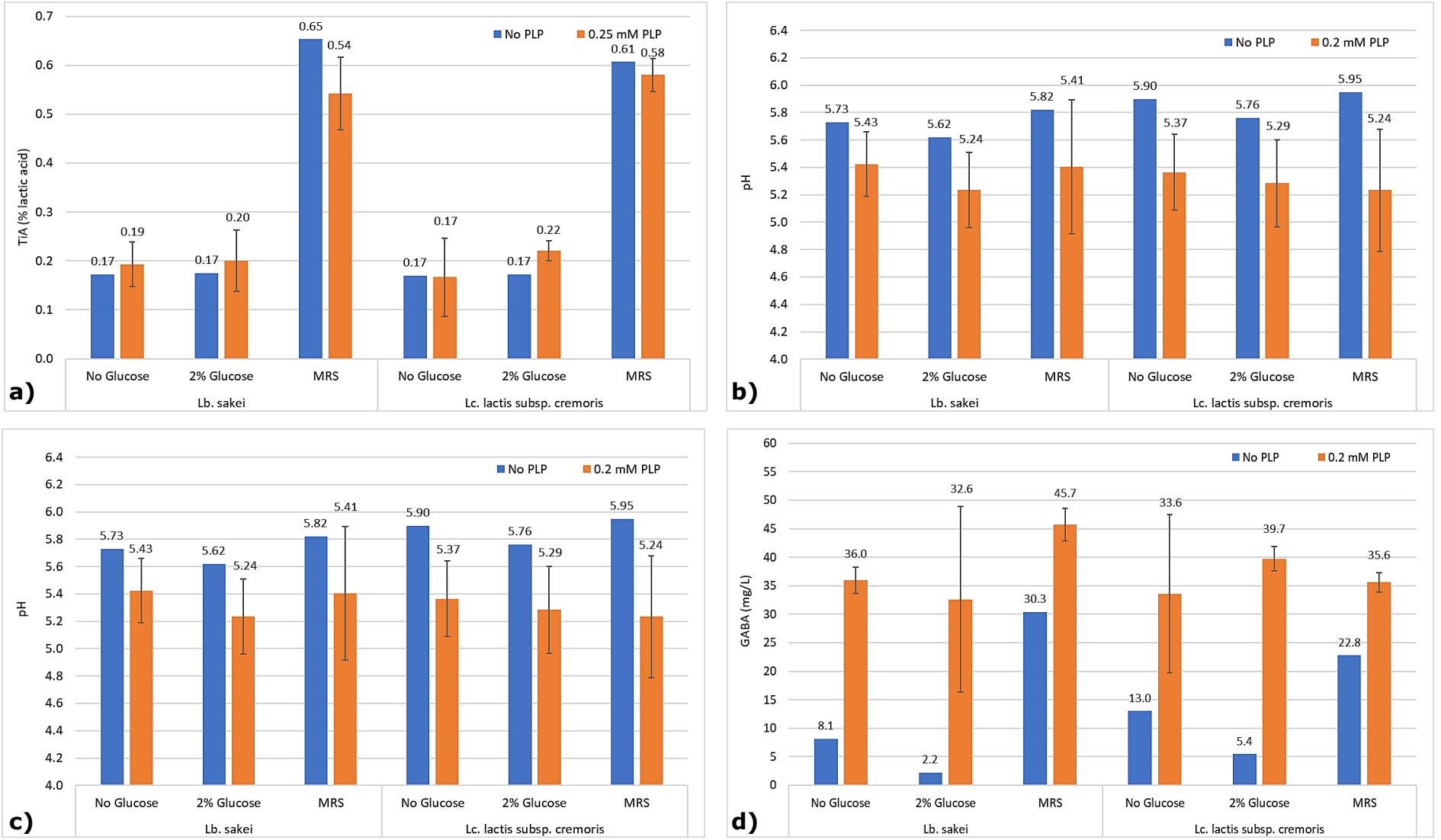


Figure 8.13: Effect of 0.25 mM PLP on a) TA, b) pH, c) log CFU/ml and d) GABA in different media fermented by Lb. sakei and Lc. lactis subsp. cremoris for 4 h at 30°C (N=2)

CONCLUSIONS AND FUTURE PERSPECTIVES

Various experiments are already (being) performed to increase GABA content in a multitude of foods and beverages. Of these, fermentation is considered to be the most promising in terms of cost-efficiency so there is a need of high-GABA producing strains, in this case LABs. Therefore, 17 LABs were screened for their ability to produce GABA and were implemented to ferment soymilk.

Extraction of GAD was unsuccessful, either the micro-organisms did not produce the enzyme or the extraction itself failed. The latter is most likely because GAD should be present in micro-organisms from Glu-rich fermented food. If the aim is to purify the enzyme, the extraction method needs to be looked in to. Additional control experiments such as SDS-PAGE could be performed to verify the enzyme was extracted at all.

GABA production by the micro-organisms and analysis in the medium worked best. The trial tests with *Lb. plantarum* and *Lb. brevis* showed that they were able to produce GABA in the adjusted MRS medium. Moreover, addition of PLP increased significantly GABA production by *Lb. plantarum* whereas no increase was found for *Lb. brevis*. The fermentation in NB, where no pH drop was observed and therefore no GABA was produced, showed that the acidic environment is crucial for GABA synthesis.

In the adjusted MRS medium, two LAB strains, *Lb. sakei* and *Lc. lactis subsp. cremoris*, produced significantly more GABA than the others. However, extremely low conversion rates were found. To solve this, the fermentation parameters need to be optimised for each strain to produce a maximal amount of GABA. Additionally, strains should be grown in MRS containing MSG before inoculation to activate the production of GAD.

The fermentation in soymilk showed promising results, especially after 4 h of fermentation. The fermented soymilk had a pleasant smell and the viscosity of a drinking-yogurt. GABA production was still low, but conversion rates were increased. With the produced GABA content, the blood-pressure lowering effect can possibly be obtained when patients drink around 300 mL of the fermented soymilk each day. Additionally, cholesterol could also be lowered with the obtained concentration. However, the uptake of GABA in humans through food needs to be studied more thoroughly.

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