# POTENTIAL OF LACTIC ACID BACTERIA TO PRODUCE FUNCTIONAL FERMENTED WHEY WITH PUTATIVE ANTIHYPERTENSIVE PROPERTIES : ENRICHMENT OF ANGIOTENSIN–I CONVERTING ENZYME (ACE)–INHIBITORY PEPTIDES AND GAMMA–AMINO BUTYRIC ACID (GABA)

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# ABSTRACT

## POTENTIAL OF LACTIC ACID BACTERIA TO PRODUCE FUNCTIONAL FERMENTED WHEY WITH PUTATIVE ANTIHYPERTENSIVE PROPERTIES : ENRICHMENT OF ANGIOTENSIN–I CONVERTING ENZYME (ACE)–INHIBITORY PEPTIDES AND GAMMA–AMINO BUTYRIC ACID (GABA)

This thesis focused on evaluation of the lactic acid bacteria (LAB) collection for their potential antihypertensive abilities and production of fermented whey bases for development of fermented whey beverages. For this aim, over 400 LAB were screened for their proteolytic activities in different skim milk media. Among the LAB, 39 (13%) were determined as highly proteolytic. Strains were selected to produce fermented milks, 11 LAB out of 34 with highest ACE inhibitor activities were utilized to generate fermented whey bases with co–cultures of Lactobacillus brevis D9 and Lactococcus lactis ssp. lactis C24. Proteolytic activities and ACE inhibitor activities in fermented milks were detected in the range of 4.11-1.8 mg/L Leucine and 49–86% activity, respectively. In co–culture fermented whey bases, ACE inhibitor activities found in the range of 80–97%. Proteolytic activities of 13 selected LAB were also determined during fermentation by HPLC and SDS–PAGE with evaluating the degradation profiles of whey proteins. GABA production capability of 33 strains among 300 LAB were observed in modified broth media after 48 h incubation at 37 or 42 °C using thin layer chromatography (TLC). In order to develop the fermented whey beverage base, total of 10 species of Lactobacillus e.g. *Lactiplantibacillus plantarum, Latilactobacillus curvatus* and *Levilactobacillus brevis* were selected according to their GABA concentrations yielded e.g. 50–367 mg/L into the modified broth media. Accordingly, GABA concentrations in fermented whey bases were quantified in the range of 11–21mg/L after 24h at 37 C.

Keywords: GABA, ACE inhibitor activity, Whey, Fermentation, antihypertensive effect

# ÖZET

## LAKTİK ASİT BAKTERİLERİNİN VARSAYILAN ANTİHİPERTANSİF ÖZELLİKLERE SAHİP FONKSİYONEL FERMENTE PEYNİR ALTI SUYU ÜRETME POTANSYELİ: ANJİYOTENSİN–I DÖNÜŞTÜRÜCÜ ENZİM (ACE)–İNHİBİTÖR PEPTİDLER VE GAMA–AMİNO BÜTİRİK ASİT (GABA) İLE ZENGİNLEŞTİRMESİ

Bu tez Laktik Asit Bakterileri koleksiyonunun (LAB) antihipertansif yetenek potensiyallerinin değerlendirilmesine ve fermente peynir altı suyu içecek bazlarının geliştirişmesine odaklanmıştır. Bu amaçla, 400 adetten fazla LAB proteolitik aktiviteleri açısından farklı yağsız süt ortamlarında taranmıştır. LAB'lar arasında 39 adedi yüksek proteolitik olarak tespit edilmiştir. Fermente sütleri üretmek için suşlar seçilmiştir, 34 en yüksek ACE inhibitor aktiviteli LAB'dan 13 tanesi *Lactobacillus brevis D9 and Lactococcus lactis ssp. lactis* C24 ile ikili kültürlü fermente peynir altı suyu bazı oluşturmak üzere kullanılmıştır. Fermente sütlerdeki proteolitik aktivite ve ACE inhibitor aktivite of 4.11-1.8 mg/L Lösin ve %49–86 aktivite olarak sırasıyla tespit edilmiştir. İkili kültür fermente peynier altı suyu bazlarında ACE inhibitör aktiviteler %80-97 aralığında bulunmuştur. Seçilen 11 LAB'ın fermentasyon surasındaki proteolitik aktiviteleri HPLC ve SDS-PAGE analizi ile peynir altı suyu proteinlerinin degredasyon profilleri ile değerlendirilmiştir. 300 LAB arasından modifiye sıvı besiyerinde 37 veya 42 C'de 48 saat inkübasyon sonrası 33 suş GABA üretim kapasitesi gösterdiği ince tabaka kromatografisi (TLC) ile gözlemlenmiştir. Fermente whey içeceği bazı feliştirmek için 10 LAB türü, örneğin *Lactiplantibacillus plantarum, Latilactobacillus curvatus* and *Levilactobacillus brevis,* modifiye sıvı besiyerinde 50–367 mg/L konsantrasyonlarında verim göstermesi nedeniyle seçilmiştir. Buna göre, fermente peynir altı suyu bazlarında 24 saat 37 °C sonrası GABA konsantrasyonları 11–21mg/L aralığında hesaplanmıştır.

Anahtar kelimeler: GABA, ACE inhibitör activity, peynir altı suyu, fermentasyon, antihypertensive etki

This thesis is dedicated to my beloved parents,
and my precious little pet Boncuk

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin Converting Enzyme |
| AHA | American Heart Association |
| ARB | Angiotensin Receptor Blockers |
| ASH | American Society of Hypertension |
| BLAST | Basic Local Alignment Search Tool |
| BP | Blood Pressure |
| CAD | Coronary Artery Disease |
| CCB | Calcium Channel Blockers |
| CNS | Central Nervous System |
| CV | Cardiovascular |
| CVD | Ccardiovascular Diseases |
| EDA | European Dairy Association |
| EFSA | European Food Safety Authority |
| ESC | European Society of Cardiology |
| ESH | European Society of Hypertension |
| FAO | Food and Agriculture Organization |
| FF | Fermented Foods |
| FUFOSE | The European Commission Concerted Action on Functional Food Science |
| GABA | Gamma–Amino Butyric Acid |
| GBD | Global Burden of Disease |
| ISAPP | The International Scientific Association for Probiotics and Prebiotics |
| LAB | Lactic Acid Bacteria |
| QPS | Qualified Presumption of Safety |
| SDGs | Sustainable Development Goals |
| SSADH | Succinic Semialdehyde Dehydrogenase |
| T2D | Type 2 Diabetes |
| Ca | Calcium |
| Cu | Cupper |
| Fe | Iron |

| | |
|---|---|
| K | Potassium |
| Mg | Magnesium |
| Mn | Manganese |
| Na | Sodium |
| P | Phosphorus |
| USA | United States of America |
| UN | United Nations |
| WHO | World Health Organization |
| WPC | Whey Protein Concentrate |
| WPI | Whey Protein Isolates |
| TAM | Testing and Analysis Access and Management department |

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# CHAPTER 1

# INTRODUCTION

## 1.1. Overall Introduction

A High blood pressure is named as hypertension, it is one of the leading death causes associated with cardiovascular diseases (CVD). It is also known as “silent killer” since its damage on the body and its destruction on the cardiovascular systems does not show any early symptoms and remain unknown until it is too late. Due to high prevalence of hypertension patients all around the world, The World Health Organization (WHO) sees it as a global health problem and supports countries for decline in hypertension patient number. Although the main strategy was known as medical treatment, in addition to or before pharmacological approach, dietary limitations also play a role in the reducing hypertension. Associated organizations such as American Heart Association (AHA), American Society of Hypertension (ASH), International Society of Hypertension (ISH) and WHO releases global guidelines on the reducing blood pressure level and recommend regular dietary changes. Besides well–known approaches such as reduction of salt intake, fat intake, carbohydrate intake and focusing on Mediterranean diet style, also functional foods gained importance due to their numerous health benefits.

On the other hand, nowadays, improper diet style and lack of physical activity due to global lifestyle changes considered to be closely linked with a range of disease conditions such as hypertension. Therefore, functional foods having health benefits gained importance due to the consumer preference as a natural way to decrease chronical diseases (Shahidi 2004). Although, fermentation of food has been long used for preservation and enhancing the flavor of food, it has been focused as a technique to increase nutritional value of food and to generate health promoting effects, recently. Based on the help of microorganisms, this ancient technique has produced functional foods.

Fermented foods (FFs) can show antidiabetic effect, decrease lactose content, help in obesity, increase digestibility of proteins, and reduce allergenicity of proteins, produce vitamins or other compounds having antioxidative effect, modulate gut-brain axis by modulating gut microbiota. FFs can also show antihypertensive effects as another health promoting effect of FFs (Shah et al., 2023). Fermentation can lead to secretion of compounds having health benefits such as gamma–aminobutyric acid (GABA) and formation of bioactive peptides having important inhibitor role in the renin–angiotensin system, inhibiting the angiotensin–converting enzyme (ACE).

Fermented milks (FM), cheese, fish, legumes, vegetables, alcoholic and non–alcoholic beverages have been widely produced and consumed Since milk is highly perishable, fermentation method has been used for the conversation and fermented milks become one of the most common FFs with over than 400 different type of commercial and traditional products (Shah et al., 2023). In addition, several commercial fermented milk products with antihypertensive peptides such as Ameal (Calpis Co., Tokyo, Japan), Calpis (Calpis Co.), and Evolus (Valio, Helsinki, Finland) have been in the market (Beltrán–Barrientos, et.al., 2016). Commercial products with antihypertensive properties and the wide range of fermented milk types indicates potential use of dairy for the generation of fermented functional food products having antihypertensive effects.

In global age, the world is under the stress and risk due to the global warming, poverty and hunger as a result of dramatically increased global population. Sustainable Development Goals (SDGs) including 17 SDGs and 169 targets for the 2030 Agenda of United Nations have been determined in 2015. The aim of this agenda was to provide social, economic and environmentally sustainable development and to stop hunger and poverty (UN 2023). It can be said that the sustainability is one of the keys for better global growth; therefore, sustainable food production has gained great importance.

Cheese is widely consumed and according to the European Dairy Association (EDA), cheese production between 2022–2023 was 10.740 million tonnes (EDA 2023). This results in huge amount of whey by product as a wastewater in the production process of cheese. Since disposal of whey leads loss of energy and harmful impact in the environment and leads the loss of nutritionally valuable product. Therefore, conversion of whey waste to valuable products in chemical and food industry becomes one of the main strategies for the sustainability of whey (Zandona, Blažić, and Režek Jambrak 2021).

The aim of this thesis was to produce fermented whey bases showing antihypertensive properties due to their GABA and ACE inhibitory peptide content. For this purpose, the abilities GABA production their proteolytic activities and GABA production potential of over than 400 lactic acid bacteria (LAB) culture collection of Molecular Food Microbiology Laboratory of Food Engineering Department, İzmir Institute of Technology were screened. LAB with putative antihypertensive properties were used in the development of fermented whey enriched with ACE inhibitory peptides and GABA. In addition, in silico analyses of selected LAB for their gad operon in their genome was evaluated in the scope of this thesis.

## 1.2. Hypertension

While the heart pumps the circulating blood against the arteries, pressure of measure of the force is defined as blood pressure (BP). During the heart beats, the pressure is the highest since it pumps the blood; this is called as systolic blood pressure (SBP). On the other hand, while the heart rest between the beats the pressure of the blood is the lowest, and this is called as diastolic blood pressure (DBP). High blood pressure is called as hypertension, and it is diagnosis if the SBP or DBP is greater than or equal specific limits. Previously, the limit for diagnosis of hypertension where SBP $\geq$ 140 mmHg or DBP is $\geq$ 90 mmHg (Chobanian et al. 2003). Figure 1.2. shows the biological and environmental factors can be effective on the development of hypertension. In addition to genetic reasons, several factors (Figure 1.2.) play role in the increase of BP such as lifestyle, diet, and psychological stress and mainly physiology changes in biological mechanisms (Mancia(Chairperson) et al. 2023).

### 1.2.1. Hypertension Associated Diseases

Hypertension is also known as “silent killer” since its damage on the body and its destruction on the cardiovascular systems does not show any early symptoms and remain

unknown until it is too late. It is the major risk factor one of the four major noncommunicable diseases: cardiovascular diseases (CVDs), diabetes, chronic respiratory diseases, and cancer (Budreviciute et.al., 2020). Increased body weight, blood pressure, glucose and fat level are major risk factors for these noncommunicable diseases. Furthermore, according to Global Burden of Disease (GBD) Study 2019 elevated BP is the primary risk factor for global mortality (up to 10.7 million deaths, causing 19% of global deaths) and premature death (Mills, Stefanescu, and He 2020). Ishaemic heart diseases, ischaemic and haemorrhagic stroke was the major reasons for these deaths. Dramatically, meta–analyses showed that arise in BP from the point of 115/75 mmHg was associated with CVD death (Forouzanfar et al. 2017). Furthermore, there was a twofold difference in the death rates from ischaemic heart disease and other CVD causes, as well as a more than twofold difference in the rate of stroke fatalities, when there was a difference in usual SBP of 20 mmHg or usual DBP of 10 mmHg (Lewington et al. 2002).

Sustainable development is the 3rd goal of SDGs in the 2030 Agenda for Sustainable Development, and the target 3.4 is titled as “Noncommunicable diseases and mental health”. In this target, the premature death rate associated with NCDs is aimed to lower by one third. Reducing high BP is significant for the lowering NCDs rates and premature deaths .

### 1.2.2. Hypertension and Its Prevalence

According to National Health and Nutrition Examination Survey, the recognition against high blood pressure was 51% before 1980s whereas it reached to 70%, and the treatment of this disease increased from 10% to 36% (Chobanian et al. 2003). According to WHO, most adults (46%) with hypertension does not know they have hypertension and only %21 of the patients with hypertension control their disease; therefore, global targets aim to reduce the prevalence of hypertension by 33% between 2010 and 2030 for non-communicable diseases (WHO 2023).

A comprehensive study explored more than 150 countries and included almost 9 million people between 2015–2016. It was found that the prevalence of silent killer disease was 29% and it was increasing with the age ( 63.1% in older patients above 60

years old), This study also concluded that the prevalence of this disease was higher in among non–Hispanic black race than non–Hispanic black, Asian or Hispanic (40.3% vs 27.8%, 25% or 27.8, respectively). Hypertension prevalence was stayed unchanged from 1999 to 2016. After the hypertension was taken under control, increasing values in prevalence was obtained in between 1999 to 2010 but later no change was recorded through 2016 (Fryar et al. 2015). Also, hypertension rates between women and men changes with the age. According to the Chinese Hypertension Guidelines, 24.5% of men had hypertension whereas this rate was 21.9% in women. Importantly, the prevalence of this disease was two–fold more in obese population compared with non–obese population (44.5% vs. 15.4%) (Reckelhoff 2018; J. G. Wang and Liu 2018). There is 34.5% of men and 33.4% of women as hypertension patient in the United States of America (USA), values are similar in Japan. Moreover, comprehensive research concluded that the rate of hypertension was higher in men compared with women (40% vs. 33%) among women based on the analysis results of 250,741 individuals from 13 countries (Guo et al. 2011; Reckelhoff 2018;).Whereas the data on the prevalence of hypertension shows why it's the leading cause form global deaths, the control rates much lower than needed.

### 1.2.3. Guidelines on Hypertension

In 2003, the seventh report of the Joint National Committee (JNC) defined levels associated with BP; SBP/DBP below 120/80 mm Hg was labeled as normal, 120–139/80–89 mmHg was named as prehypertension and the levels higher than these numbers were classified as hypertension; stage 1 and stage 2 hypertension were named for BP in the range of 140–159/90–99 mmHg and 160–179/100–109 mmHg, respectively(Chobanian et al. 2003). For medical treatment, ≥ 140/90 mm Hg and ≥ 130/80 mm Hg was defined for adults without other risks and with diabetes or CKD, respectively (Chobanian et al. 2003). In the following 8th report of JNC, the definitions about hypertension stages remained unchanged . In JNC 8, the threshold for medical approach was stated as ≥150 mmHg SBP or 90 mmHg DBP for population older than 60 years old, ≥140 mmHg SBP or 90 mmHg in adults younger than 60 years (James et al. 2014; Hernandez-Vila 2015).

In contrast to previous JNC reports and guidelines, classification of hypertension and the levels of blood pressure changed due to excessive and comprehensive research. In 2017, according to the American College of Cardiology (ACC) and AHA guidelines, normal BP was defined as BP below 120/80 mmHg, evaluated BP was classified in the range of 120–129/<80 mmHg DBP. Hypertension was defined when SBP or DBP is equal or higher than 130/80 mmHg; it was classified as stage 1 and hypertension in the range of 130–139 mmHg or 80–89 mm Hg and stage 2 when the pressure was ≥140 mm Hg or ≥90 mmHg pressure (W. P. Whelton 2017; Flack and Adekola 2020).
According to Canada Guidelines, blood pressure of individuals having mean awake SBP ≥135 mmHg or DBP ≥85 mmHg or 24–hour SBP≥130 mm Hg or DBP ≥80 mm considered high (Rabi et al. 2020).

In Europe, European Society of Hypertension (ESH) and European Society of Cardiology (ESC) give recommendations on high BP. According to the previous guideline of ESH/ESC published in 2018, it is called as hypertension when the SBP or DBP ≥ 140 mmHg or 90 mmHg, respectively. BP below 120/80 mmHg was named as optimal, the range between 120–129/80–84 mmHg named as normal. In the guideline, 130–139 and 85–89 mmHg were classified as high normal. Grade 1, grade 2 or grade 3 were defined if BP is in the range of 140–159/90–99, 160–179/100–110 and above 180/110 mmHg, respectively (Whelton and Williams 2018).

2018 ESC/ESH guideline states that individuals with high normal BP can have medical treatment if they have CVD risk. ESC/ESH suggests lifestyle change first for the patients with grade 1 BP without any organ damage or with low–moderate risk. The first aim of the recommendations is the reduction in SBP and DBP below 140mmHg/90 mmHg in all patients and 130mmHg/80mmHg in most of the patients, then and it targets SBP in the range of f 120–129 mmHg in adults younger than 65 years old. Guideline recommends 130–139 mmHg SBP in older adults. Also, the range of DBP in 70–79mmHg is intended. If the BP of adults are in the range of 130–139/85–89 mmHg and they have low or no CVD risk, only lifestyle change was recommended. However, if patients have high CVD risk such as stroke or coronary artery disease (CAD), the medical treatment could be considered even if the SBP is in the range of 130–140 mmHg (P. K. Whelton and Williams 2018). In Grade 1 type, patients are only treated medically after no alleviation is accomplished after 3–6 months lifestyle change; also, drug treatment is considered for patients with high CVD risk before waiting the positive effect of lifestyle advice (P. K. Whelton and Williams 2018). ESH/ESC guideline suggest at least 30

minutes exercise per 5–7 day in a week, quitting smoking, acceptable body mass index below 30 kg/m2, limits alcohol consumption, reduction of salt intake up to 5 g per day. Also, guideline recommends healthy diet consisting unsaturated fatty acids, fresh vegetables and fruits, reduced consumption of red meat and high fat dairy products (P. K. Whelton and Williams 2018)

In 2023, EHS renewed the guideline and more personalized antihypertensive treatment is stated. The recommendations about children, adolescents and young adults were mentioned for the first time, the classification of hypertension did not differ from the previous guideline (Mancia(Chairperson) et al. 2023).

The guideline of The Japanese Society of Hypertension defines 120–80 mm Hg as normal and evaluate 130–140 mmHg SBP and <80 mmHg as high normal blood pressure, 130–139 and 80–89 mmHg as elevated; the hypertension range starts above 140–90mm Hg blood pressure (Umemura et al. 2019).

In 2015, constructed and published the first report on hypertension based on international guidelines. In the first report, the classification was same with the 2018 ESH/ESC guideline. The second report was published in 2019, it used same categorization with JNC 7 report; the levels higher than 140 and/or 90 mmHg was classified as hypertension; stage 1 and stage 2 hypertension were named for BP in the range of 140–159/90–99 mmHg and 160–179/100–109 mmHg, respectively (Aydogdu et.al.,2019). Similarly, according to this report, patients with elevated BP. Same with ACC/AHA guideline, the Turkish report also demonstrated diagnosis based on different measurements of BP such as clinic or at home measurements, the mean of measurement during 24h or only daytime (Aydoğdu 2019).

In the Turkish report, the change in lifestyle in all stage of treatments is recommended. These suggestions can be listed as lowering overweight of the body up to 5–10%, reduction in salt intake (limited to 5 or 6 grams per day), limitation of tobacco use and alcohol consumption, Mediterranean type of diet style, physical activity and stress management . The threshold for pharmacological treatment was determined as ≥ 140 mmHg SBP or ≥ 90 mmHg DBP in adults younger than 80 years old, and ≥ 150 mmHg SBP for older adults (≥ 80). On the other hand, these treatments targeted reduction of SBP to the range of 120–130 SBP for adults (<65) and 130–140 mmHg for elders (≥ 65). The Turkish report aimed to maintain DBP in the range of 70–80 mmHg (aydoğdu 2019; Böcek Aker, Doğaner and Aydoğan 2020).

Since, there are different classifications and thresholds for treatments in various countries. WHO published a new guideline on hypertension as the first global guideline in the last two decades (WHO, 2021). WHO established the preparation of this guideline by concluding the scope and 11 PICO (population, intervention, comparison, outcome) questions of the guideline in Guideline Development Group meeting in 2019. According to WHO, the treatment aim of BP of all patients with no comorbidity and with CVD risk should be lower than 140/90 mmHg and 130 mmHg, respectively. Also, WHO recommends lover level than 130 mmHg for patients having high risks such as diabetes. Threshold for the medical interventions were demonstrated above these levels. Although there are 5 major drug classes, 2021 WHO guideline recommends 4 major drug classes: thiazide and thiazide–like agents (diuretic), ACEs/ARBs, long acting dihydropyridine CCBs. In older patients (≥65), also beta blockers are suggested (Moick, Sommer, and Gartlehner 2023). Similar to ESH/ESC, WHO also suggested lifestyle change as nonpharmacological method in all cases and the lowering salt consumption than 5 g per day is one of the major suggestions (Moick, Sommer, and Gartlehner 2023).

### 1.2.4. Pharmacological and Non–Pharmacological Antihypertensive Approaches

Lifestyle change is the major recommended approach, the change is suggested in both elevated and high BP conditions (Moick, Sommer, and Gartlehner 2023). In 997, National Heart, Lung, and Blood Institute (NHLBI) explored the association between dietary patters and BP under a RCT names as “Dietary Approaches to Stop Hypertension” (DASH) (Sacks, Moore, and Nerikaranja 1999). Based on DASH, recently known dietary recommendations such as has been made and the outcome of this RCT were published in the first JNC report. The study demonstrated the effect of high intake of low–fat dairy food, and fruits and vegetables for the treatment of high BP National Academy of Sciences also stated that at least 3 to 4 servings of dairy should be taken for the consumption of 1000 mg –1200 mg Ca2+ intake (Miller et al. 2000).

As a pharmacological approach, there is five main drug classes to reduce high BP: ACE inhibitors, angiotensin receptor blockers (ARBs), beta–blockers, calcium channel

blockers (CCBs), and diuretics (thiazides and thiazide–like diuretics such as chlortalidone and indapamide). According to evaluation in ESH/ESC 2018 version, ACE inhibitor or ARB with a CCB and/or a thiazide/thiazide–like diuretic treatments was suggested based on the findings of meta–analyses, randomized controlled trials (RCTs) (P. K. Whelton and Williams 2018).

Several RCT named such as PROGRESS (Williams, 2008), ADVANCE (A. Patel 2007)and HYVET (Beckett et al. 2008) investigated the effect of ACE inhibitors on hypertension patients having risk factors such as diabetes, stroke; 9, 5.6 and 15 mmHg reduction in SBP was observed as outcome leading reduction in stroke, vascular events and CV events, respectively. (A. Patel 2007; P. K. Whelton and Williams 2018; Beckett et al. 2008). A meta-analysis about the impact of ACE inhibitors and ARBs on hypertension mobility concluded that while ARBs have any effect, ACE inhibitor medical treatment significantly lowered mortality of hypertension (10%) (van Vark et al. 2012). Another meta–analysis focused on 55 RCTs to compare the effectiveness of antihypertensive drug classes; diuretics and beta–blockers lead decrease about 12/5 mmHg and 10.5/7 mmHg in 48898 and 18724 patients, respectively (Thomopoulos, Parati, and Zanchetti 2015). On the other hand, without any other drug combinations, ACE inhibitors revealed lower reduction in SBP/DBP levels, but Stroke and CV event was reduced up to 20% and 17, respectively (Thomopoulos, Parati, and Zanchetti 2015).A recent international cohort study focused on the efficiency of Renin–Angiotensin system inhibitors: ACE inhibitors and ARBs by evaluating the data of 3 million patients from 8 databases (Chen et al. 2021).

ACE inhibitors/ARBs are the most commonly used medical drug classes in hypertension patients without comorbidities (Abdelkader et al. 2023). Although ACE inhibitors can be used with or without the combinations of other drug classes, Lisinopril, an ACE inhibitor drug is the most used antihypertensive drug globally (Chen et al. 2021; Q. Gu et al. 2012; Abdelkader et al. 2023).

On the other hand, one of the major obstacles in the control of hypertension is medical adherence. Since silent killer does not show any symptoms, medication adherence level, which is the regular intake of antihypertensive drugs regularly, is not high (Poulter et.al., 2020). For example, almost half of the patients quit taking their drugs after 1 year (Vrijens et al. 2008).Therefore, attribution of low nonadherence rate to high the hypertension prevalence could be one of the reasons that why hypertension levels same even though the awareness increased with time. Major research focusing on

hypertension in 1990 to 2019 based on data from 104 million participants revealed that while 59% women were diagnosed, 47% of them were treated and 23% of these patients had hypertension level under control, 49% men were identified, 38 % of them were treated and 18% of these hypertension level under control (B. Zhou et al. 2021). Over than 70% of patients were treated and 50% of them had their BP under control in South Korea, Canada, and Iceland as the highest levels. (B. Zhou et al. 2021).

### 1.2.5. The Renin-Angiotensin System (RAS)

The renin-angiotensin system (RAS) is the major regulation mechanism of BP in human, RAS is mainly regulated by two enzymes, renin and ACE. In the body, angiotensinogen is degraded into Angiotensin-I form by the action of renin enzyme which is secreted from kidney. This decapeptide angiotensin-I is converted into Angiotensin-II (1-7) peptide because of ACE enzyme activity. This Angiotensin-II form. Angiotensin-II binds with AT-1 receptors and leads vasoconstriction and increase in BP. The drug classes of ARB reduce BP by preventing this binding. On the other hand, Angiotensin II also related with AT-2 receptor which involves in the vasodilation. In addition, ACE also degrade a vasodilator peptide bradykinin into its inactive form and increase in BP (Aluko 2015).

## 1.3. Gamma–Amino Butyric Acid (GABA)

GABA was first observed as a compound in plants and microbes in 1883, then its presence was determined, in potato tubers in 1949 (Thompson, Pollard, and Steward 1953). It is a four–carbon containing non–protein amino acids which means it could not be incorporated translationally into protein structure, it is called as gamma (γ–) due to the position of amino group in gamma–carbon instead of alpha–carbon position (Sarasa et al. 2020). It is an abundant compound found in microorganism such as LAB (Alizadeh Behbahani et al. 2020; Haifeng Li et al. 2023), yeast (S. Li et al. 2022) and mold such as

Rhizopus ssp. (AOKI et al. 2003; Zareian et al. 2020), Aspergillus ssp. (Wan-Mohtar et al. 2019; Zareian et al. 2020), Tetragenococcus halophilus (Sassi et al. 2022), mammalians (Tillakaratne, Medina-Kauwe, and Gibson 1995), plants (Z. C. Wu et al. 2016)and other living organisms such as honeybee (Barbara et.al., 2005).

The irreversible alpha–decarboxylation action of Glutamate decarboxylase (GAD; EC 4.1.1.15) on L–glutamic acid leads to production of GABA in microorganisms and this reaction is catalyzed by pyridoxal–5'–phosphate (PLP) as cofactor(Kanwal, Rastogi, and Incharoensakdi 2014; Yogeswara, Maneerat, and Haltrich 2020). While this enzyme is found in plants, microorganism, plants have other enzymes such as diamine oxidase (DAO, E.C. 1.4.3.6) and 4–aminobutyraldehyde dehydrogenase (ABALDH) and substrates such as γ–aminobutyraldehyde to produce GABA (Shelp et al. 2012; R. Yang, Guo, and Gu 2013). under stress conditions, e.g., cold, heat, wound or osmotic stress. On the other hand, GAD is the key enzyme in both mammalians and microorganisms including LAB (Haixing Li and Cao 2010).

### 1.3.1. The Role of GABA in Plants and Microbes

While GABA is present in almost all organisms, it has various and different roles in them. Its function in plants is mainly associated with abiotic and biotic stress (Bouché and Fromm 2004). When plant is triggered by a stress, GABA can be involved in tricarboxylic acid cycle, it can act as a protectant metabolite against osmotic pressure, has a function in regulation of pH at low pH conditions and it accumulates in the cytosols of plants (Bouché and Fromm 2004).Fast calcium accumulation or acidification in cytosol promotes GAD activity and leads to high amount of conversation of glutamate into GABA (Bown and Shelp 2016). For instance, up to 2700% and 2000% increase in GABA content in soybean leaves can occur due to physical and cold stress (Kinnersley and Turano 2000). Also, recent findings indicate its role in signaling mechanism of plants (Wu et al. 2020).

In microbes, amino acid dependent routines (Figure 2.2) such as arginine deaminase system (ADI), lysine, branch chain amino acids and glutamic acid is effective for acid tolerance; ADI and GAD pathways are used most frequently (Guan and Liu 2020;

Q. Wang et al. 2018; H. Yang, He, and Wu 2021). The tolerance to the acidic stress conditions is crucial for both pathogenic and probiotic microorganism; they need to tolerate low pH of food products and gut during digestion due to its organic and gastric acids, respectively (Guan and Liu 2020). pH homeostasis of inside of the cell is necessary against tolerance to acidic environment and LAB can consume and reduce H+ concentration of cell or it can transport H+ outside of the cell consumption of H inside of the cell or transporting H+ from inside of cell (Yang and Wu 2021). If the pH is higher than (>4.5) outside of the cell and cause mild stress (Feehily and Karatzas 2013), in the inside of the cell, glutamine can be transformed into glutamate, a substrate for GAD enzyme and leads formation of $NH_3$ which uses elevated H+ concentration inside of the cell to produce NH4. In addition, if the acidic stress is high (pH lower than 4.5, e.g, 3.5) (Feehily and Karatzas 2013), glutamate can be either inside of the cell or transport from outside of the cell based on the action of GAD, this mechanism uses $H^+$ of cell and convert it to both CO2 and GABA (Feehily and Karatzas 2013; H. Yang, He, and Wu 2021).

Even though the role of GABA about the protection of the cell under acidic conditions is known and its production mechanism was also found upregulated in *Escherichia coli* (*E. coli*), during anaerobic and high pH conditions. Although the mechanism is not clear, insufficient oxygen level could also be associated in GAD mechanism for the survival of the cells (Blankenhorn, Phillips, and Slonczewski 1999; Feehily and Karatzas 2013).

GABA can also maintain the cells under oxidative stress in low pH medium, activation of genes associated with the GAD and ADI mechanisms were found in *E. coli* O157:H7 under these conditions(Bearson, Lee, and Casey 2009) . Also, concluded that catabolic metabolism of glutamate due to the action of GAD and GABA shunt pathways could protect against oxidation stress (Feehily and Karatzas 2013) Oxidative stress also leads the production of GAD in Saccharomyces cerevisiae and E. coli (Coleman et al. 2001; Capitani et al. 2003). Since oxidations is also associated with the pH, it is suggested that antioxidative effect of GABA also could be a result of its role on the increase of intracellular pH. In addition to this, GABA shunt is activated by promoted expression of SSADH due to the oxidative stress triggered by H2O2. SSADH action on GABA results in the formation of NADH or NADPH (Coleman et al. 2001; Feehily and Karatzas 2013).

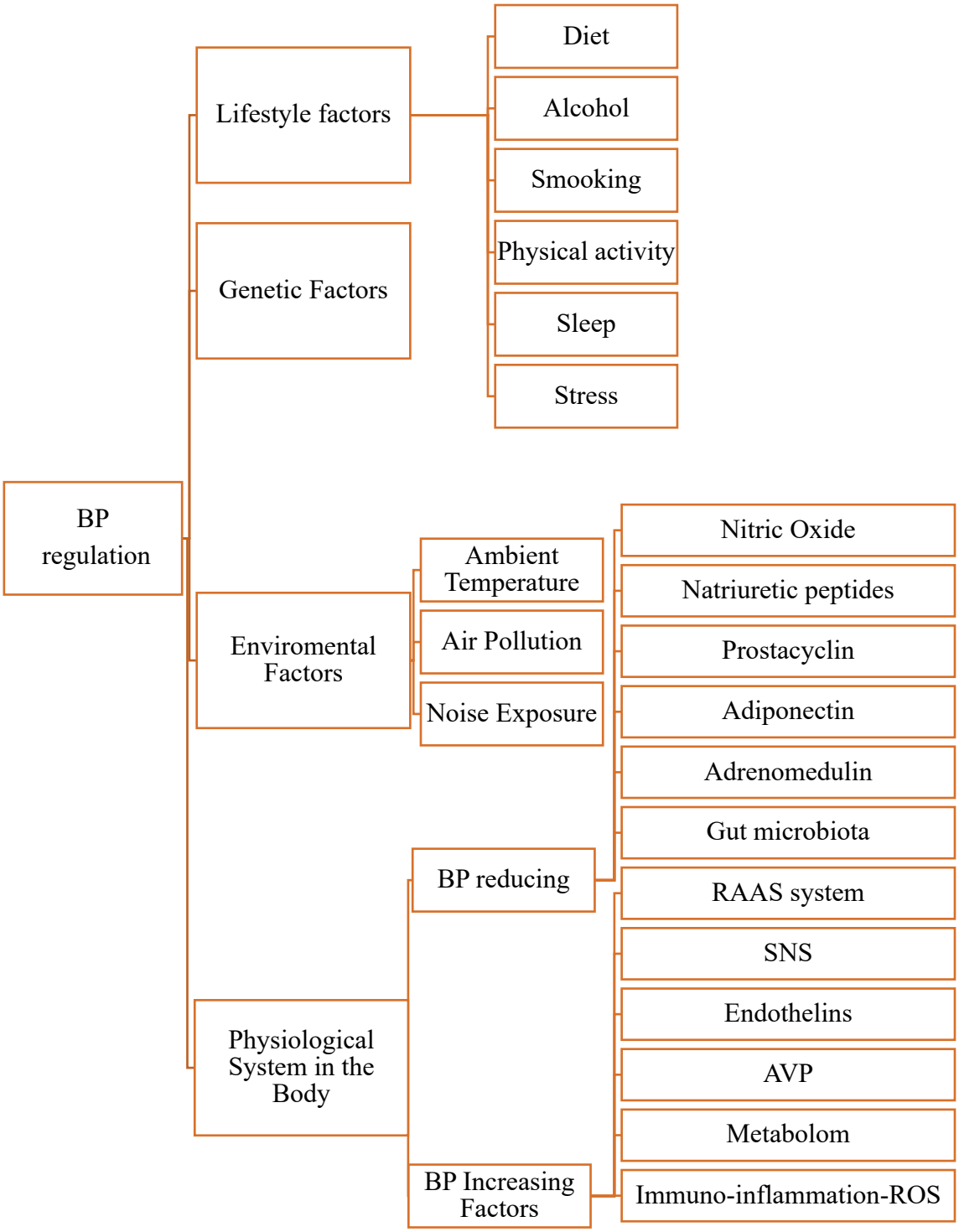


Figure 1.2. The biological and enviromental factors effecting the development of hypertension (modified from Mancia Chairperson et.al., 2018).

### 1.3.2. The Role of GABA in Humans

When it is found in the mammalian brain, GABA showed its potential involvement of in neural control and its neurotransmitter role in mammalians (Galindo 1969; Roberts 1986). Now, GABA is known as the major neurotransmitter, transferring the information between neurons, in mammalian central nervous system (CNS) for rapid inhibitory synaptic transmission and up to 50% of synapses uses GABA as neurotransmitter

GABA has 3 different types of receptors: GABA–A, GABA–B, GABA–C they also found in the peripheral system, endocrine, and several non–neural tissues and join the oxidative metabolism (Sarasa et al. 2020). These GABA receptors involved in different mechanism. While the function of GABA–C as receptor is not enlightened yet, the physiological involvement of GABA–A and B is well characterized. Fast and slow synaptic transmission is arranged by receptor A and B, respectively. GABA–A is ligand–gated ion channels and involved in Seizure, threshold, anxiety, and panic while GABA–B receptors are G protein–coupled receptors and involved in pain, memory, and mood (Sarasa et al. 2020; Ghit et al. 2021).

GABA is stored in the vesicles of presynaptic neurons stores and secreted due to the action of $Ca^{2+}$dependent mechanism into synaptic area (Sarasa et al. 2020). GABA–A receptor is formed by various protein subunits and classified in Cys loop ligand–gated ion channel family, this channel formed by 13 amino acids around 2 Cys residues and produce a pore which is specific for the transportation of $Cl^-$ ion. The differentiation in the subunits producing GABA–A receptor provides selective inhibition of these receptors and leads various functions by these inhibitions (Rashmi et al. 2018.). On the other hand, GABA–B receptor prevent neurotransmissions due to the $K^+$ and $Ca^{2+}$ ion channels mechanism by G–coupled proteins; it acts on $Ca^{2+}$ ion and $K^+$ ion during presynaptic and postsynaptic phases, respectively (Rashmi et al. 2018.).

GABA binds with GABA receptors and unlocks ion channels causing the transportation of $K^+$ to the outside of the cell and $Cl^-$ to the inside of the cell (Sarasa et.al., 2020). The roles of these receptors are significant in healthy progress of the brain, the dysfunction of these receptors is involved in the development of the neurological

diseases. Therefore, recent studies have been focused on these receptors and GABA in the neural system (Yu et al. 2020).

Due to these actions, GABA receptors and GABA is a significant factor for the development or alleviation of anxiety, depression, sleep disorders, epilepsy, pain perception, and neurological diseases such as Alzheimer's disease, Parkinson's disease, Schizophrenia, and epilepsy (Ochoa-de la Paz et al. 2021).

Several RCTs showed the effect of GABA consumption on stress levels and insomnia (Yamatsu et al. 2015; Hinton and Johnston 2020). A systematic review on RCTs showed that 30 mg and 100 mg GABA had impact on autonomic and central stress biomarkers, respectively; 2.01–100 mg consumption of GABA can have positive effect on stress (Hepsomali et al. 2020). Interestingly, the beneficial concentration of GABA changed depending on its biosynthetic or natural form. For example, this anti–stress effect has been found in the range of 2.01 and 26.4 mg (Okada et al. 2000; Hinton and Johnston 2020). For sleep improvement, much higher biosynthetic GABA concentration (100 and 300 mg) has been suggested (Hepsomali et al. 2020). Its supplementation can also enhance immunity; consumption of 200 ml milk containing 100 mg GABA fermented by commercially GABA producer LAB also showed improvements on immunity biomarkers in healthy but stressed adults (Abdou et al. 2006). Moreover, GABA is also secreted by beta cells in pancreas. In pancreas, GABA binds into alpha cells and prevent production of glucagon hormone. It can also improve the stability and formation of insulin secreting beta cells. Thus, it also plays a role in glucose homeostasis in blood (Sarasa et al. 2020). High GABA consumption (2g per day for a week and 2g in 3 times per day for a week) increased insulin levels significantly in healthy adults (Li et al. 2015), In contrast, a 500 mg GABA supplementation after each meal did not significantly change glucose levels in prediabetic patients (aged in the range of 50–70) (de Bie et al. 2023).

Another health promoting effect of GABA is its hypotensive effect. First, its impact on BP were demonstrated on rabbits. Later, several studies in animals confirm antihypertensive effect of GABA (Yamakoshi et al. 2007; Liu et al. 2011; Zareian et al. 2020). This health promoting effect was also determined in human studies (Inoue et al. 2003; Pouliot-Mathieu et al. 2013), Several mechanisms about how GABA reduce BP was enlightened as a result of in vitro animal studies. It is stated that both GABA–A and B receptors have a role in CV system, the injection of their agonists into ventromedial nucleus decreases BP (De Wardener 2001). BP lowering impact of GABA on CNS was explained and found associated by the level of GABA in the hypothalamus, nitric oxide

synthase activity in the brain (De Wardener 2001). Also, activity of GABA on renal nerves were found related with its hypotensive effect in hypertensive rats. The effect of GABA on GABA–B receptor in kidney can reduce secretion of noradrenaline. GABA also lowers renin level in the plasma and reduce conversion of angiotensin 2 from angiotensin 1 by effecting renal sympathetic nerve activity, therefore it leads a reduction in BP (Hayakawa, Kimura, and Kamata 2002). Also, a previous study revealed that GABA can trigger perivascular nerve and leading a rise in perfusion pressure in the mesenteric arterial bed and noradrenaline production due to its effect on presynaptic GABA–B receptors in spontaneously hypertensive rats ((Hayakawa, Kimura, and Kamata 2002)).

A RCT investigated the hypotensive impact of milk fermented by commercial strains *Lacticaseibacillus (L.) casei* Shirota and *Lc. lactis* YIT 2027; it was concluded that consumption of 10–12 mg GABA containing 100 ml FM leaded to 17.4±4.3 mmHg/7.2±5.7 mmHg reduction in SBP/DBP of hypertension patients after 4 weeks consumption (starting BP values in the range of 140–159 mmHg/90–99 mmHg), respectively (Inoue et al. 2003). Also, this study eliminated the contribution of potential ACE inhibitory activity of FM by stating that FM had no ACE inhibitory activity (Inoue et al. 2003).

Another clinical trial was applied on borderline and prehypertension patient for 4 weeks, it investigated the contribution of GABA and ACE inhibitory peptides in potassium rich bread and found consumption of 120 g bread containing GABA and ACE inhibitory peptides (22.8mg/100 mg GABA and 7.23mg/g low molecular weight peptides) reduced SBP and DBP as 0.75 mmHg/2.12 mmHg, respectively (Becerra-Tomas et al. 2015).

Another study explored the impact of consumption of 150 g rice meal having low (2.7 mg/100g) and high GABA content (11.2 mg/100g) in for 8 weeks in mild hypertension patients, the study concluded that GABA enriched rice meal lowered morning BP (Nishimura et.al., 2016). Another study focused on a beverage having vinegar, bonito with or without GABA (70 mg/90 ml) and found that both beverages was effective on mildly hypertensive patients after daily consumption for 12 weeks (Tanaka et al. 2009).

A Double blinded RCT revealed that Chlorella tablets with GABA reduced; doses of the tables were adjusted to digest 10 mg, 20 mg and 30 mg GABA daily in High–Normal Blood and Borderline Hypertension patients. As a result, both SBP and DBP

reduced after 4 weeks compared to baseline and this effect continued another 4 weeks after the daily intake routine. Hypotensive effect of GABA was found higher in patients with borderline hypertension (140–159 mmHg/90–99 mmHg) (Shimada et al. 2009).

Although placebo controls were administrated as the same food product having no GABA, it could lead a question mark in fermented food products. For example, studies investigating FFs could use different microorganisms as a starter to produce a placebo FFs and resulted in another FF with changed properties. For example, GABA containing cheese (16 mg/50 g) and placebo cheese were obtained after the fermentation by both a starter culture together with GABA producer and only by a starter culture, respectively (Pouliot-Mathieu et al. 2013). Since both cheese samples reduced DBP in patients having elevated BP, this could be also attributed to the effect of other compounds such as bioactive peptides having ACE inhibitory activity produced as a result of different fermentation (Pouliot-Mathieu et al. 2013).

On the other hand, recent research explored whether the consumption of GABA cheese or low–fat milk had any antihypertensive properties in individuals having high–normal BP (Rancourt-Bouchard et al. 2020). While both low fit milk and regular fat cheese having GABA (10.1mg/50 g) did not found effective for the reduction of BP, cheese consumption also increased LDL–cholesterol level and its size, and triglycerides (Rancourt-Bouchard et al. 2020).

Here it can be argued that even though GABA cheese can be considered as it has an antihypertensive property. However, because of its high cholesterol content its' positive effect can turn into a non-desirable form, and it should have been taken into account while developing functional food products (Rancourt-Bouchard et al. 2020). Although other studies showed antihypertensive potential of GABA consumption after 4 weeks, the inefficiency in the antihypertensive effect of GABA cheese could be also related with the shorter trial period.

Although, dairy products rich in $Ca^{2+}$, vitamin D and $Mg^{2+}$, their efficiency as antihypertensive compounds are not high and reliable (Houston and Harper 2008). There is no effect of vitamin D (Jorde and Bønaa 2000; Miller et al. 2000), small or no contribution of Ca2+ intake from the reduction of high BP in the reduction of BP (1–3 mmHg) (Jorde and Bønaa 2000; Miller et al. 2000; S. C. Shah et al. 2020). Similarly, one meta–analyses revealed that 483 ± 216 mg Mg2+ intake could help 1.5 mmHg/0.9mmHg decrease in BP (Geleijnse, Kok, and Grobbee 2004) while the other meta–analyses showed no attribution of Mg2+ in the alleviation of BP (Dickinson et al. 2006).

Therefore, according to the outcomes obtained from in vivo human studies, it could be concluded that both the source of GABA and the targeted disease was important for the effect of GABA in the determination of GABA consumption concentration.

### 1.3.3. GABA Containing Food and Supplements

GABA Containing Commercial Foods and Products have been increasing by time pass. Nowadays, it is stated that over than 400 commercial products having enhanced GABA in the Japan market. Recently, the first CRISPR–edited food, GABA enhanced tomato became available commercially in Japan (Waltz 2021). Also, GABA containing products such as chocolate beverage powder, GABA chocolate, GABA tea, GABA rice has been frequently sold in Japan market.

The production of GABA containing foods started by the research of Prof. Tsushida and his team in 1980s. They produced Gabaron tea which was enhanced with GABA. Then, they also showed the antihypertensive effect of synthetic GABA on rats and humans and Gabaron tea was commercially sold due to their health benefit, they discovered that green tea had high GABA due to long anaerobic fermentation time (Rashmi et al. 2018.; Omori et al. 1987). Later, it was discovered that along with GABA tea (Gabaron tea), green, black and oolong tea also contained GABA. While Gabaron tea had the highest amount of GABA (97.51 μg/g), black tea (55.45 μg/g), Pu–erh raw tea (45 μg/g) also had GABA. On the other hand, the GABA concentration in green tea changed based on the growth area of the tea leaves (Syu et al. 2008). While a recent study resulted with similar findings, they also showed that white tea has highest GABA concentration among oolong, pu–erh, green and black teas (Horanni and Engelhardt, 2013).

Due to its excessive consumption prevalence and GABA content, tomato is one of the most significant foods (Burton-Freeman and Reimers 2011). In 11 market and 38 processing cultivars, the level of GABA in various tomato were detected in the range of 39.6–102.5 mg and 35.4–93.3 mg/in per 100 g of fresh weight, respectively (Gramazio, Takayama, and Ezura 2020).

Beside tomato, other fruits and vegetables such as pumpkin (3.71–15.53 mg/g), lychee (1.7–3.5 mg/g), mulberry (0.86–1.86 mg/g) musklemon (0.103–0.722 mg/g), orange (0.344 mg/ml) and eggplant (0.23–0.38 mg/g) have superior GABA concentration (Biais et.al., 2010; Kim et.al., 2010; Qi et.al., 2012; Mori et.al., 2013; Wu et.al., 2016). GABA concentrations in plants were explained in a recent review in detail (Gramazio, Takayama, and Ezura 2020)

Like tomato, rice is another popular choice as a food by global population (above one of third) and almost all produced rice (90%) is consumed by Asia (Roohinejad et al. 2011). Both glutamic acid and the enzyme necessary for the transformation of GABA is found in brown rice. Based on this information, GABA–rich white rice was generated by a Japanese firm Satake Co., Ltd.(Nishimura et al. 2016). Recently it was reported that germination time and glutamic acid concentration in brown rice was associated with the GABA level after germination period (Roohinejad et al. 2011). In addition, different rice types can be classified high, medium, and low due to their GABA level in the range of 0.08–0.11 mg/g, 0.04–0.07 mg/g and 0–0.03 mg/g, respectively (Roohinejad et al. 2011).

LAB produce organic acids e.g. lactic acid, citric acid and acetic acid during fermentation and reduce the pH of the medium, LAB cell can protect itself by activating GAD system under these conditions and transferring GABA to the outside of the cell. Therefore, fermentation is an excellent approach to produce GABA enriched foods.

Even if the starter culture does not produce GABA during sourdough bread fermentation, GABA may still exist in sourdough bread due to various legume flours used; e.g. flours of Italian originated kidney bean, pea, chickpea, grass pea had high GABA content leading to GABA rich sourdough bread production (300 mg/kg) even though starter culture only showed proteolytic activity (Curiel et al. 2015).

Beside legume flours, cereal and pseudo–cereal flours also contain GABA. Another research explored the GABA levels in the doughs before and after fermentation with or without LAB; doughs without of LAB addition already had GABA (3–78 mg/kg), these levels raised to 15–468 mg/kg in the chemically acidified dough. Doughs made with buckwheat and quinoa flours had the highest GABA content before incubation (Coda, Rizzello, and Gobbetti 2010). After incubation, oat, amaranth and chickpea doughs had the best GABA levels. On the other hand, fermentation by *Lb. plantarum* C48 and *Lc. lactis ssp. lactis* PU1 increased GABA content up to 643–415 mg/kg in buckwheat and quinoa, 816–1031mg/kg in amaranth and chickpea sourdoughs, respectively (Coda, Rizzello, and Gobbetti 2010).A different study listed GABA content in various

commercial and artisanal breads and compared with their trial sourdough bread (Pannerchelvan et al. 2023). While GABA concentrations in commercial and artisanal sourdough breads ~~was~~ have been determined as 1.57–3.95 mg/100 g and 2.01–8.84 mg/100 g, its level was improved in the trial bread as 24.2 ± 0.87 mg/100 g (Pannerchelvan et al. 2023).

Since GABA can be formed upon the action of LAB during fermentation, FFs can also be a source of GABA. The ripening time, microbiome, and necessary amino acids levels such as glutamic acid and glutamine required to produce the significant GABA production in cheese (Ayag, Dagdemir, and Hayaloglu 2022). An Italian, Sardinia originated artisanal cheese produced by sheep's milk had the highest GABA concentration (10,013 mg/kg) (Manca et.al., 2015). In Spain, a mixture of milks of cow, sheep and goat were used to produce blue cheese containing extremely high concentrations of GABA (4760 mg/kg) (Redruello et al. 2013; 2022) .

During the ripening, increased proteolysis leads to the formation of accessible glutamate to produce GABA. When the ripening period keeps longer, GABA generation could be suppressed due to a raise in pH values related with the formation of ammonia and amines in aged and highly aged cheeses. Also, GABA production could be negatively affected when ripening period or temperature increases (Redruello et al. 2013; 2022).Recently, 37 various cheese samples in different geographical areas of Europe were evaluated for their GABA concentrations; the study concluded that mature, artisanal, blue cheeses and aged hard cheeses produced from raw cow's milk and raw sheep's milk had the highest amount of GABA (6480.06 mg/kg and 3747.38 mg/kg), respectively . Among these cheese samples, GABA was detected in the range of 2.06–6480.06 mg/kg (Redruello et al. 2020). In comparison to the European cheese study mentioned above, 30 cheese samples of Turkish origin have been investigated and their GABA levels were detected in the range of 0.00–83.05mg/100g, 1.65–45.79 mg/100 g and 0.20–21.06 mg/100g in Beyaz, Kasar and Tulum cheeses (Ayag, Dagdemir, and Hayaloglu 2022).

Furthermore, studies on fermentation of milk have been applied for many years for GABA production (Nejati et al. 2013). Fermented milk was produced by the action of GABA producer *Lc. lactis* strain with *Lacticaseibacillus (L.) rhamnosus* or *Lacticaseibacillus (L.) paracasei* with the addition of 5 mmol monosodium glutamate (MSG). Three different *Lc. Lactis* strains in MRS broth produced GABA concentration was 222 ± 59.0 mg/L, 135 ± 12.1 mg/L and 140 ± 1.4 mg/L for MK L37, MK L81 and MK L84 strains after 24 h fermentation. On the other hand, GABA content were

determined in FMs produced by *Lc. lactis* MK L37 and MK L84 as 113.41 ± 22.34 and 249.31 ± 33.8, 55.70 ± 2.76 and 73.12 ± 9.88 when MSG concentrations were 5 mmol and 267 mmol, respectively (Galli et al. 2022). Also, co–culture addition could be a strategy to increase GABA level. When *Lc. lactis* MK L37 and MK L84 co–cultured with a *L. rhamnosus* and *L. paracasei* strain, the GABA concentration increased up to 185.81 ± 24.0 and 319.72 ± 27.15b in FMs containing 5 mmol MSG (Galli et al. 2022).

GABA concentration in FMs produced by fermentation of *Bacillus subtilis* HA and *L. plantarum* EJ2014. Milks with 3% MSG and 2.5% glucose were used for the *B. substilis* HA (5%) fermentation at 42 °C at 160 rpm shaking conditions for 1 day, then 1.5% MSG were added with *L. plantarum* (1%), then fermentation kept for 5 days at 30 °C for 5 days. The GABA concentration in FM were detected as 2.61 mg/mL (Lee et al. 2022).Another study investigated GABA levels in FMs produced by single stain fermentation (1% inoculum, log Colony Forming Units (CFU)/mL) using 38 LAB strain containing *L. paracasei, L. delbrueckii, Levilactobacillus (L.) brevis, Lactobacillus acidophilus, Lb. plantarum.* After incubation at 72 h at 30 °C, the GABA contents were detected in the range of between 3 and 63 mg/L; however, most of the stains produced <10mg/L GABA in FMs (Ramos and Poveda 2022).

Likewise, FMs were produced by single fermentation of 20 strains of *Lactobacillus* ssp. and *Lactococcus* ssp. (3% inoculation), and the GABA levels were detected in the range of 2–11.2 mg/L after 48 h fermentation at 30 °C. While positive control strain (*Lc. lactis* NBRC 12005) created 25.7 ± 2.26 mg/L GABA, 2 out of 3 *Lc. lactis* strains produced GABA above 100mg/L (86.0 ± 11.9 and 86.2 ± 7.7 mg/L, respectively). On the other hand, when fermentation conditions changed to 37 °C of incubation, $10^9$ CFU/mL of inoculum, 3 g/L of glutamate, and 100 μM of PLP, FM produced by *Lc. lactis* 571 achieved the highest production (1153.1 ± 13.5 mg/L) (Santos-Espinosa et al. 2020).

There are several commercial capsule brands of GABA such as Now GABA 500mg, Solgar GABA 500mg and Thorne GABA 500mg. Thorne is a product of PharmaGABA; it has GABA produced by *Lentilactobacillus* (*L.) hilgardii* strain which is used in Kimchi fermentation. It is approved by Food and Drug Administration (FDA), and it was applied for GRAS Status. One capsule of PharmaGABA contains 250mg GABA and for daily consumption 1-3 capsule is recommended. For the Solgar GABA, daily 2 capsule is recommended. (Oketch-Rabah et al. 2021).

Although there is no recommended daily dosage for its antihypertension effect, general amount is known as 750mg for capsules and maximum daily dosage is recommended in between 3000-5000mg by FDA. A LD50 study of GABA showed that 5000mg/kg dosage was not deadly for rats (Oketch-Rabah et al. 2021).

In 2021, a total of 644 and 119 products with GABA were listed in Dietary Supplement Label Database of USA and the Health Canada Licensed Natural Health Products Database, respectively. While GABA is flavoring agent or adjuvant based on the FDA's Substances Added to Food database, it is acknowledged as medicinal and food supplement in Canada and Europe, respectively (Oketch-Rabah et al. 2021). The European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition, and Allergies has published an opinion; such claim for the cognitive effects of GABA consumption have not been recognized by the Panel (EFSA 2006).

In another study, Japan traditional foods were investigated such as narezushi, konkazuke, and ishiru. 53 strains of LAB were isolated from these foods and analyzed by 16Sribosomal RNA sequence method. They were screened for ACE inhibitory activities and GABA activities. The results showed that 10 LAB (*Lactobacillus buchneri*, *Lb. brevis* and *W. Hellenica*) possessed GABA producing ability more than >500mg in 100ml) and IC50 were < 1mg protein/ml at pH 3 in skim milk media with whey. They concluded that isolates could be used for functional food production (Barla et al. 2016).

## 1.4. Whey

During cheese or casein production, liquid by-product is formed and named as whey. Based on its rich riboflavin content, whey has a unique yellowish-greenish color and has a yellowish-greenish color (Ryan and Walsh 2016; Tsermoula et al. 2021)). Whey is classified as sweet whey and acidic whey based on how casein is withdrawn from milk during manufacturing. It can be either produced due to proteolytic enzymes such as rennet or organic acid/microorganism addition. When rennet is added, caseins in milk transform to the curds due to the action of chymosin enzyme in rennet and the liquid form is named as sweet whey (Ryan and Walsh 2016; Zotta et al. 2020). On the other hand, cheese production can be formed by the action of microorganisms or caseins can be produced by

organic acids/mineral acids (Ryan and Walsh 2016). During the production of cottage cheese or Greek yoghurt, acid whey is formed and has pH in the range of 4.5-5 pH values (Ryan and Walsh 2016); during the manufacturing of Cheddar, Kashar, Gouda, Mozzarella, Swiss cheese, sweet whey is generated as a by-product and has pH in the range of 6.0-7.0 pH values (Risner et al. 2019; Zotta et al. 2020; Gutiérrez-Hernández et al. 2022).

According to FAO records, 11 million tons of whey is produced as a wastewater (FAO 2021; Lucakova et al. 2022). Therefore, discharge of this amount of whey becomes critical, due to high biochemical oxygen demand (27–60 g/L) and high chemical oxygen demand (50–70 g/L). Therefore, disposal of whey into aquatic sources generates a great risk for the life by decreasing the soluble oxygen level in water, leading risk for also both environmental and human life (Risner et al. 2019; Zotta et al. 2020). Besides, discharge of whey in soil without any treatment could harm plants due to its salts and high lactose and protein content. Also, acidic whey can be harmful to the soil because of its high acidic pH range (Ryan and Walsh 2016).

Since treatment of whey for disposal becomes costly in dairy industry, Transformation of whey as a valuable product or generation of high value-added products from whey has been focused recently (Arshad et al. 2023). Whey without any modification has been evaluated as nutrition source for pigs, sheep and cattle) or fertilizer for a long time. However, the issues mentioned previously and the amount of recent whey production limit the use of whey without any treatment for these purposes (Ryan and Walsh 2016)

While whey was disposed as a waste in 1960s, it converted into various products with the developing technology and science and gained importance. It was sold as whey powder (1 $/kg), whey protein concentrate (WPC) (3 $/kg), WPC with 90% protein (10 $/kg), whey protein fractions (15-600 $/kg) and enriched whey fractions (18-300 $/kg) in 1970s, 1980s, 2000s, 2010s and 2020s, respectively (Tsermoula et al. 2021). Isolated compounds have been used in different industries. Demineralized whey, lactose and purified protein fractions have been added into infant formulae; whey powder and demineralized whey, reduced lactose whey and WPC have been used in dairy industry and confectionery to improve various properties of food products. Moreover, pure protein fractions, whey protein isolates (WPI) and lactose have been used for nutraceutical, dietetic and pharmaceutical purposes, respectively (Tsermoula et al. 2021). Animal feed,

land spreading, food products along with WPC and WPI compose higher than 50% of whey utilization (Gutiérrez-Hernández et al. 2022).

On the other hand, whey is a valuable by product due to rich nutritional contents. Up to 85–90% of milk convert into sweet whey in cheese manufacturing and this whey contains almost 6.5% dry matter (Tsermoula et al. 2021).; whey contains approximately 45–50 g/L lactose, 6–8 g/L protein, 4–5 g/L lipid and 8–10% mineral in dried whey; it is a by-product rich in nutritional compounds (Zotta et al. 2020). The minerals in whey are mainly calcium, potassium, sodium and magnesium salts (of >50 % NaCl and KCI, calcium salts) (Ryan and Walsh 2016).

Currently, whey has been used without any treatment to produce polysaccharides such as exopolysaccharides (Encinas-Vazquez et al. 2023; M. Patel et al. 2021), microbial cellulose (Kolesovs and Semjonovs, 2020), other biopolymers such as polyhydroxyalkanoates (Zikmanis, Kolesovs, and Semjonovs 2020; Gottardo et al. 2022), bioethanol (Zou and Chang, 2022), volatile fatty acids such as butyric acids (Dessì et al. 2020; Vieira et al. 2023), bioenergy (Kassongo, Shahsavari, and Ball 2020), lactulose (Schmidt et al. 2020), organic acids such as D-lactic acid (Liu et.al., 2018), pigments (Mehri et.al., 2021) and single cell proteins (Gutiérrez-Hernández et al. 2022).

Also, in food industry, whey proteins have been used for fat-replacement in yoghurt (Torres et al. 2018; Hongjuan Li et al. 2021), ice-cream (Yilsay, Yilmaz, and Bayizit 2006), sausage (Kwon et al. 2021). Moreover, whey has been incorporated into food product in various studies. The effect of whey addition in bread-making process (J. Zhou, Liu, and Tang 2018) biscuits(Hassanzadeh-Rostami, Abbasi, and Faghih 2020) , sausage (Marti-Quijal et al. 2019) have been explored. Alcoholic beverages such as whey whiskey and spirit has been produced (Risner et al. 2019).

## 1.5. Functional Foods

While it is not clearly known that whether Hippocrates really said this, the significant of the concept of "*Let thy food be thy medicine and medicine be thy food*" has been increasing (Witkamp and van Norren 2018). Today, the trend of searching for foods with health benefits have been increased significantly in the global society. Although

there is no consensus description, the term of “functional foods” has been developed to describe the food product with positive effect on physical or mental health of consumer (Balthazar et.al., 2022). The European Commission Concerted Action on Functional Food Science (FUFOSE) defined functional foods as “a food that beneficially affects one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. It is consumed as part of a normal food pattern. It is not a pill, a capsule or any form of dietary supplement” (European Commission 2010). The strategies about developing functional foods have been explained in detail in the current literature (Granato et al. 2020).

Functional foods can be effective on reducing inflammation and pathogenic microorganisms, they can show antioxidative activities, improve immunity and support healthy gut and microbiota development by providing probiotics and prebiotics . Several approaches such as addition of probiotics, prebiotics, plant-based compounds or microbial metabolites with or without utilization of novel food processing techniques such as ultrasound, high pressure processing have been studies in the development of functional foods (Balthazar et al. 2022). Fermented food production is one of the major techniques for generating functional foods due to its microbiota and microbial metabolites. Recently the association between functional foods and FFs and their production technology have been explored (Sun, Shahrajabian, and Lin 2022; Tamang et al. 2020; Galimberti et al. 2021).

### 1.5.1. Fermented Foods

According to the oldest records, fermentation has been used since BC 13 000 (Sun, Shahrajabian, and Lin 2022). It is one of the oldest applications for food preservation due to the formed organic acids or other antimicrobial compounds, this biotechnological approach been widely used globally. The International Scientific Association for Probiotics and Prebiotics (ISAPP) concluded that fermentation is “foods made through desired microbial growth and enzymatic conversions of food components” (Marco et al. 2017). In 2019, ISAPP coordinated a meeting with experts from various

scientific areas and created first consensus documentation on FFs to review beneficial health properties and safety of FFs (Marco et al. 2017).

During FF production, microorganisms forming FF might not survive in food processing step. For example, baking step results in death of living microorganisms in sourdough bread production and wine production, a phase for removal of microbiota. Therefore, it is stated that a food does not need to contain live microorganism to be named as FF according to ISAAP consensus report (Marco et al. 2017).

Furthermore, ISAAP clarified that food product such as vinegar containing salad dressings cannot be called as FFs although it contains ingredients formed by fermentation. Similarly, a food product such as chemically acidified pickles or probiotic containing bars cannot be named as FF even with the addition of microorganism or chemical acidifications (Marco et al. 2017).

After fermentation, the shelf-life of the food, its appearance, texture, flavour, and aroma of food changes, phenolics, vitamins such as riboflavin, short chain fatty acids (SCFA) such as butyric acid, organic acids such as lactic acid, bioactive peptides, polymers such as exopolysaccharides and neurotransmitters such as GABA can be generated due to the microbial action on food components (Marco et al. 2017; Y. Wang et al. 2021).

FFs can enhance health of the consumers by attribution of these metabolites of microorganisms. Current RCTs show the beneficial impacts of FFs intake. For example, a cohort study concluded the health promoting effect on CVD, type 2 diabetes (T2D), management of weight preservation, inflammatory bowel diseases and immunological diseases (Wang et al. 2021; Marco et al. 2017).Moreover, FFs can also modify gut microbiota and alter gut-brain axis due to the its metabolites or microbial composition. Therefore, both the metabolites produced by LAB and also their adaptivity in gut microbiota gain importance.

## 1.6. Lactic Acid Bacteria (LAB) in Fermented Food (FF) Production

Although FFs can be formed by the action of yeasts, molds and/or LAB, LAB in FFs have been most studied microorganisms and they have been widely used in the

production of FFs in numerous studies (Şanlier et al. 2017). LAB are Gram (+), non-motile, non-spore forming microorganism, they convert glucose as a carbohydrate source. LAB classification involves *Streptococcus*, *Lactococcus*, *Aerococcus*, *Pediococcus*, *Tetragenococcus*, *Leuconostoc*, *Oenococcus*, *Enterococcus*, *Carnobacterium* and *Lactobacillus* genera. Before 2020, *Lactobacillus* genus 264 different species with various phenotypic, genotypic and ecological diversity. Therefore, they were recategorized based on their these properties. From 264 Lactobacillus species 24 novel genera was classified; therefore, the name of species changed. For example, *Lactobacillus plantarum* is named as *Lactiplantibacillus plantarum* (Zheng et al. 2020).

While various LAB genera can be found in FFs, only some of them has been listed in the qualified presumption of safety (QPS) list. According to EFSA, there was no *Enterococcus* (E.) species and *Enterococcus faecium* has been rejected in QPS list in 2020 (Zheng et.al., 2020). In contrast, *L. casei* K9, *L. plantarum* CNCM I-3736 (DSM 11672), *Lactococcus lactis* NCIMB 30117, *Lentilactobacillus buchneri* CNCM I-4323 (NCIMB 40788), *L. hilgardii* CNCM I-4785, *L. brevis* WF-18, *Limosilactobacillus (L.) fermentum* K9-2 have been classified as QPS (Zheng et al. 2020).

During the manufacturing of FFs such as sourdough bread, cheese, kefir and kombucha, various LAB have grown in the food matrix. Yoghurt contains 2 specific LAB strain: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus (S.) thermophilus*. On the other hand, kefir microbiota consists of LAB, yeast, and acetic acid bacteria. While more than 80 microorganisms have been detected, several LAB species such as *Lentilactobacillus kefiri, L. casei, L. rhamnosus, L. paracasei, L. plantarum, Limosilactobacillus reuteri, Latilactobacillus sakei* have been identified frequently in kefirs (Yilmaz et al. 2022). Likewise, kombucha, fermented sparkling tea, have diverse variety of microorganisms and 30% of these microorganisms under LAB classification. In addition to *L. plantarum* and *L. rhamnosus*, other less common LAB such as *Pediococcus pentosaceus, Pediococcus acidiliactici, Lactobacillus mali, Lactobacillus nagelii* have been found in kombucha tea (Wang et al. 2022)paralel, microbial consortia of cheese show diversity due to different milk types, treatment of milk, production techniques such as traditional approaches, climate of environment (Yeluri Jonnala et al. 2018). Due to the significant effect of the origin of manufacturing cheese, several cheese types were classified under protected designation of origin (PDO). PDO Italian cheeses produced from whey (Mozzarella, Grana Padano, Parmigiano Reggiano) has been found rich in NSLAB; *Lactococcus (L.) lactis*, L. fermentum, *S. thermophilus*, Lb. delbrueckii,

and *L. helveticus* (De Filippis et al. 2014). In kaşar cheese, an old and traditional cheese of Turkey, the prevalence of *L. paracasei* has been found high in almost all samples whereas other species such as *L. rhamnosus, L. curvatus, L. plantarum, L. fermentum, E. casseliflavus, Lactococcus lactis ssp. lactis (L. lactis ssp. lactis), P. acidilactici and E. durans, P. lolii*, E. faecalis, E. faecium and *S. thermophilus* have been detected in only some kashar cheeses (Yuvaşen, Macit, and Dertli 2018). On the other hand, 19 Enterococcus species involving *E. durans, E. faecalis, E. faecium*, have been detected in various cheese types, especially in artisanal products (Dapkevicius et al. 2021).

### 1.6.1. GABA Containing Food and Supplements

During the fermentation, LAB in FFs generates several metabolites such as GABA, Conjugated linoleic acids (CLA), EPS, bacteriocins, due to their enzymatic metabolisms. Similarly, biologically active compounds can be transformed from substrates due to the lactic acid fermentation like polyphenols, SCFA and bioactive peptides (Abedin et al. 2023).

Unique peptide motifs in the food proteins can be cleaved due to action of enzymes in the food matrix. When these peptide fragments are formed from main protein, they can exhibit specific biological activities beside their nutritional values; therefore, these molecules are called as bioactive peptides (BP) (Ulug, Jahandideh, and Wu 2021). BPs can present various biological benefits such as antioxidant activity, immunomodulation, opoid properties, metal cheating such as mineral binding, antimicrobial activity, anticancer, antidiabetic, hypocholesterolemic, antidiabetic and antihypertensive effects (Akbarian et al. 2022). Antihypertensive effects of BPs can be generated based on several mechanisms such as renin inhibition, calcium channel blocking, endothelin-converting enzyme inhibition, acting as ARB on AT-II receptors, increasing NO production and ACE inhibition (Udenigwe and Mohan 2014). Likewise, major antihypertensive drug classes act on same mechanisms. Since most prescribed drugs are in the ACE inhibitor drug and these drugs side effects such as cough and bronchospasm, ACE inhibitor peptides gained importance.

## 1.6.2. GABA Regulation Mechanism in LAB

In LAB, *gad* genome regulates GAD system. GAD enzyme is transkripted by gad gene, and was detected in various different LAB species (Q. Wu and Shah 2017). Since nucleotide sequences of gene responsible for GAD encoding could show difference, several studies used different primers for the detection of gene and clarify that their LAB possessed the ability necessary to produce GABA. In LAB, *gadA* or *gadB* gene is required for GAD enzyme production. Another gene, *gadC* is responsible for the transportation of GABA and L-glutamic acid outside and inside of the cell. The regulation of this system is regulated by *gadR* gene (Cui et al. 2020).

Table 1.1. Various primers designed for the screening of *gad* gene in LAB

| ***Strain*** | **Forward-Reverse** | **Primer Design** | **Reference** |
|---|---|---|---|
| *L. delbrueckii ssp. bulgaricus* | FP | ATGGCAAAACACACGCATGAAA | Taherzadeh et.al., 2015 |
| | RP | TCAGTGCGTGAACCCGTATTC | |
| *S. thermophilus* | FP | CCTCGAGAAGCCGATCGCTTAGTTCG | Siragusa et.al., 2007 |
| | RP | TCATATTGACCGGTATAAGTGATGCCC | |
| | FP | GGTACATCTACAATTGGTTCTTCTGA | Somkuti et.al., 2012 |
| | RP | AAACCACCAGAAGCAGCRTCNACRTG | |
| | FP | ATGAATGAGAAGCTATTCAGAGAGAT | |
| | RP | TTAATGATGGAAGCCACTGCGGATG | |
| | FP | ATGAATGAGAAGCTATTCAGAGAG AT | Brasca et.al., 2016 |
| | RP | TTAATGATGGAAGCCACTGCGGATG | |
| | FP | ATGAATGAGAAGCTATTCAGAGAGATT | Hu et.al., 2020 |
| | RP | TTAATGATGGA AGCCACTGC | |
| *L. lactis ssp. lactis* | FP | ATGTTATACGGAAAAGAAAATC | Valenzuela et.al., 2019 |
| | RP | TTAGTGAGTAAAGCCATATG | |
| | FP | CGTTATGGATTTGATGGATATAAAGC | Nomura et.al., 2002 |
| | RP | ACTCTTCTTAAGAACAAGTTTAACAGC | |
| *E. avium* | FP | GGGCATATGAATCAGAAAAAATTATC | Lee et.al., 2017 |
| | RP | GGGCTCGAGATGTTTCAATGT GTG | |
| *L. paracasei ssp. paracasei* | FP | CCTCGAGAAGCCGATCGCTTAGTTCG | Alkay et.al., 2019 |
| | RP | TCATATTGACCGGTATAAGTGATGCCC | |
| *L. casei* | FP | CCTCGAGAAGCCGATCGCTTAGTTCG | |
| | RP | TCATATTGACCGGTATAAGTGATGCCC | |

Therefore, in LAB *gadA* or *gadB, gadC* and *gadR* genes forms gad operon in LAB, this gad operon is the system maintance GABA production and its transportation (Cui et al. 2020). While intact *gad* operon has been detected in various high GABA producer LAB species such as *L. brevis* and *L.lactis ssp. lactis*, some of them (such as *S. thermophilus*) only posses *gadB-gadC* in their genomes. On the other hand, several *gadB* or *gadC* genes were recorded in the geneom of various *Enteroccoccus* species (X. Gu et al. 2021).

# CHAPTER 2

# MATERIALS and METHODS

## 2.1. Materials

In addition to chemical materials, whey was used for fermentation media, and LAB isolates were used in this thesis.

### 2.1.1. Whey

Sweet whey as a raw material was obtained from local cheese producers (Eker Dairy Products, Bursa, Turkey) it was collected from the same production line of kashar cheese in the same facility. It was produced from heat treated milk (72˚C/ 1 minute). Whey samples were transferred from Bursa to İzmir at –18°C and preserved in dry ice. To prevent changes in the composition of whey, whey product of same line was collected and stored in 1.5 L bottle at –18˚C for further studies. In addition, Aliquots of whey and pasteurized whey were stored at –18˚C for further studies.

### 2.1.2. Microorganisms

*L. helveticus*, *L. rhamnosus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* coded with (NRLL B- 4526, NRRL B- 442 and *NRRL B- 548*, respectively) were obtained from Northern Regional Research Laboratory (NRLL) *Lc. lactis ssp. lactis* CECT-4432 were obtained from Spanish Type Culture Collection (CECT). These reference cultures were selected because their GABA production abilities were determined previously(Ozer et al. 2022) . Molecular Food Microbiology Laboratory of Food Engineering Department,

İzmir Institute of Technology provided all LAB strains used in thesis. The list of LAB used in this thesis were given in Appendix Figure B1, B2 and B3, respectively. In Figure B1, LAB isolated from artisanal yogurts were listed (Erkus et al. 2014; Bulut et al. 2005).

### 2.1.3. Chemicals

Agar and skim milk for microbial growth were purchased from AppliChem, (Darmstadt, Germany) and Biolife (Milan, Italy), respectively. MRS and M17 broth media were purchased from both Merck (Merk Millipore, Germany) and (Biolife, Milan, Italy).Applichem and Biolife, respectively. MSG was purchased from Alfa Aesar, (Ward Hill, MA, USA). All other chemicals used in the study were analytical grade unless otherwise mentioned and purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in HPLC analyses were HPLC grade purity.

## 2.2. Methods

Raw material sweet whey was characterized, LAB were screened for their GABA productivities and their proteolytic activities. Selected LAB were used to fermentation of skim milk and whey. ACE inhibitory activities in skim milk and whey were determined. GABA production in skim milk and whey was analysed.

### 2.2.1. Raw Material Analyses

Sweet whey as a raw material was obtained from local cheese producers (Eker Dairy Products, Bursa, Turkey) it was collected from the same production line of kashar cheese in the facility. It was produced from heat treated milk (72˚C/ 1 minute). Whey samples were transferred from Bursa to İzmir at –18°C and preserved in dry ice. To

prevent changes in the composition of whey, whey product of same line was collected and stored in 1.5 L bottle at –18˚C for further studies. In addition, Aliquots of whey and pasteurized whey were stored at –18˚C for further studies.

Mineral composition, protein, SCFA, organic acid, carbohydrate content, pH and IZTECH - Testing and Analysis Access and Management department (TAM), microbiological analysis was done in IZTECH Food Engineering Department. Other analyses were done in Ministry of Agriculture and Forestry Central Research Institute of Food and Feed Control (Bursa, Turkey).

### 2.2.1.1. Pasteurization of Whey and Microbiologic Evaluation

Whey was pasteurized at 85 °C for 30 min by autoclave. Microbiological Analysis of whey was applied before and after pasteurization step. Yeast, Mold, total aerobic counts were determined to show the effect of pasteurization. LAB counts of pasteurized and non–pasteurized whey samples were investigated to find out the initial LAB level of whey samples before fermentation. All microbiological analyses were duplicated with three parallels.

Whey sample (1 ml) were diluted in peptone water (0.1%) serially and dilution samples. For yeast and mold count, Potato Dextrose Agar (PDA) medium (acidified with tartaric acid) was used by spread plate methods and incubated at 25°C for 5–7 days. For total aerobic bacteria count, Plate Count Agar (PCA) medium was used by pour plate method and incubated at 37 °C for 48 h. For LAB count, M17 and MRS agar medium was used by pour plate technique and incubated at 37 °C for 48 h. After incubation, plates enumerated separately, calculated by multiplying with dilution factor (Ozer et al. 2022).

### 2.2.1.2. Mineral Composition Whey

Calcium (Ca), Cupper (Cu), Iron (Fe), Potassium (K), Magnesium (Mg), Manganese (Mn), Sodium (Na) and Phosphorus (P) mineral contents of whey and pasteurized whey samples were determined by Inductively Coupled Plasma Optical

Emission Spectroscopy ICP–OES (Agilent 5110) (Bakircioglu, Kurtulus, and Ucar 2011). After microwave digestion step, mineral standards and whey samples were analyzed with ICP–OES. Agilent's software was used to control the system. Operating conditions of the instrument are presented in Table 2.1. Analyses were carried out in TAM center of IZTECH.

Table 2.1. Instrumental conditions for ICP–OES

| Mineral | Wavelength for whey and pasteurized whey | Mineral | Wavelength for whey and pasteurized whey |
|---|---|---|---|
| Ca | 422,673 nm (317,933 nm for past.whey) | Mn | 257,610 nm |
| Cu | 327,395 nm | Na | 588,995 nm |
| Fe | 238,204 nm | P | 213,618 nm |
| K | 766,491 nm | Zn | 213,857 nm |
| Mg | 279,533 nm | | |

### 2.2.1.3. pH Measurement of Whey

After 24 h fermentation, pH values of the modified broth media and non–modified broth media inoculated with GABA producer isolates were determined by using pH meter (Hanna Instruments, HI2211).

### 2.2.1.4. Composition Analyses of Whey

Ash content of dry whey sample was determined according to (GMMAM, 1988). Dry matter of whey was determined according to the TS method (TS 1018). Apparent viscosity of whey samples was measured at room temperature (25ºC) was determined by using viscosimeter (Brookfield DV–II+Pro Viscometer). Results were recorded in centipoise. (AOAC, Method 978.18D). In addition, the density of whey was investigated by lactodensimeter (TS 1018). Lipid content of whey was determined by Gerber method

(FAO 1986). Kjeldahl method was applied for the detection of both total protein and non–protein nitrogen contents in whey; since it was whey the conversion factor was selected as 6.38 (AOAC, 2006). Total Acidity of whey was revealed by phenolphthalein indicator method (TS 1018). Exopolysaccharide (EPS) content of whey samples were detected based on the phenol–sulfuric acid method by spectrometer at 490 nm absorbance (DuBois et al., 1956). A standard curve of glucose (0.050, 0.075, 0.100, 0.125, 0.150, 0.200 mg/mL) was used to calculate EPS content in samples (see Figure A1 in Appx. A).

### 2.2.1.5. Organic Acid Profile of Whey

Lactic, acetic or formic acid content in unfermented whey analyzed based on the method with slight change (Donkor et al. 2007; Costa et al. 2016). 1 ml sample was diluted 5–fold, diluted solution was centrifuged at 5,000g for 20 min. The clear upper phase was , the supernatant was filtered via Minisart 0.2 μm membrane filter (Sartorius AG, Göttingen, Germany). Agilent module system equipped with an auto–sampler and Agilent UV detector set at 210 nm (Agilent, USA) was used for the chromatographic analysis. As a column, a BioRad Aminex HPX 87H cation exchange column (300 × 7,8 mm, Bio Rad Laboratories Hercules, CA, USA) were selected. For the chromatogphay, 4 mM $H_2SO_4$, was prepared and filtered to be used as mobile phase, injection volume increased as 50 μl, 0.6 ml/min flow was manintained at 65 °C. L. HPLC grade standards of were analyzed to form calibration curves. Standard solutions were diluted with ultrapure water in the range of 3 mg/ml – 0.1 mg/ml. Peaks in chromatograms were identified by their retention times. Analyses were carried out in TAM -IZTECH.

### 2.2.1.6. Sugar Profile of Whey

Carbohydrate content in fermented whey samples were quantified using a method modified slightly (Costa et al. 2016). Similarly organic acid content, same HPLC column, a BioRad Aminex HPX 87H cation exchange column (300 × 7,8 mm, Bio Rad

Laboratories Hercules, CA, USA) were selected for the analysis. 1 ml sample was diluted 10–fold and vortexed for homogenicity. . Then, it was passed through filter (Minisart 0.2 μm filter, Sartorius AG, Göttingen, Germany) in order to eliminate particules in the solution Minisart 0.2 μm membrane filter (Sartorius AG, Göttingen, Germany) before HPLC analysis. Chromatographic analysis of sugar content in whey were detedcted by Agilent module system equipped with an auto–sampler and Agilent RI detector (Agilent, USA). As stationary phase, 5 mM $H_2SO_4$ was prepared and filtered by membrane filter (0.2 μm, Sartorius) and its flow was maintained at 0.6 ml/min, analysis time was adjusted as 30 minutes, injection volume of diluted whey was 20 μl, chromatographic separation was obtained at 65 °C. Standards were prepared from HPLC grade Lactose (cat. 61345), galactose (cat. 09676), and glucose (cat. 46937) (Sigma–Aldrich). Standard sugars were prepared as concentrated stock solutions by ultrapure water, then they were diluted with ultrapure water in the range of 8 mg/ml – 0.01 mg/ml. Peaks were identified by their retention times. Analyses were carried out in TAM center of IZTECH.

### 2.2.1.7. Protein Profile of Whey

Degradation of serum albumin, α–lactalbumin, β–lactoglobulin A and β–lactoglobulin B was investigated by HPLC based on the modified method applied in (Ostertag et. al., 2021). Mobil phase was 80% Acetonitrile in ultra–pure water containing 0.05% TFA. The flow of phase was adjusted for 1 ml/min and the analysis were set for 50 minutees. 20 μl sample was used for injection.

An Agilent module system equipped with an auto–sampler and it UV detector (210 nm) (Agilent, USA) were used for chromatographic analysis. Protein standards were prepared in the mixture, the standard calibration range for serum albumin was 0,01–0,25 g/L, for α–lactalbumin 0,05–0,8 mg/ml was, β–lactoglobulin A was 0,1–2,5 mg/ml and for β–lactoglobulin B was 0,05–0,8 mg/ml. Analyses were studied in TAM center of IZTECH.

## 2.2.2. Proteolytic Activity of LAB

Two different method was used to determine LAB with high proteolytic activities in dairy medium. Both liquid medium and agar medium was used for screening.

### 2.2.2.1. Proteolytic Activity in Skim Milk Agar Media

For the determination of proteolytic activity, 10 µL LAB cultures were spotted on the surface of skim milk agar medium (prepared with 10% (w/v) skim milk powder (Biolife, Italy) and incubated at the 37 °C or 42 °C for 48h. Proteolytic activity was indicated as a clear zone around the colonies. Proteolytic activity was indicated by halos around the colonies and ranked as + (up to 2 mm radius), ++ (2 to 4 mm radius) and +++ (more than 4 mm radius) (Franciosi et al. 2009; Colombo et al. 2020).

### 2.2.2.2. Proteolytic Activity in Skim Milk Liquid Media

Rapid detection of proteolytic activity was determined (Fusieger et al. 2020). After cultivation, 40 µl LAB culture was inoculated into 160 µl skim milk broth (10% (w/v) with 0.01 g/1000 ml bromocresol purple) in the 96 well plates. Incubation was carried out at 37 °C or 42 °C and incubation time was selected as 24 h, yellowish color occurance was evaluated.

### 2.2.2.3. Fermentation of Skim Milk by High Proteolytic LAB isolates

Based on proteolytic activity results of section 2.2.1 and 2.2.2, selected LAB cultures were individually activated and used to ferment RSM (10%, w/v). After 2nd

cultivation in MRS broth or M17 broth, OD of cultures were adjusted to 1.00±0.02 at 600nm by spectrophotometer (Shimadzu, Japan), then 5% of LAB culture was used for prefermentation of reconstituted skim milk for at 37 °C or 42 °C for 24 h. Prefermented milk then was used to ferment skim milk at 42 °C for *S. thermophilus* and *L. delbrueckii ssp. bulgaricus species* and 37 °C for other LAB species, fermentation time was 24 h. Fermented milks were centrifuged at 10000 g for 20 min to remove particules and the supernatants stored at –18 °C for further analysis. Before centrifugation, fermented milks were also used for further analysis.

### 2.2.2.4. Degree of Hydrolysis in Fermented Milks

Proteolysis in fermented milks were monitored by measuring the release of free NH3 groups following the o–phthaldialdehyde (OPA) method with some modifications (Church et al. 1983; Donkor et al. 2007).. 4 mL from fermented milks was mixed with 8 mL of 0.75% trichloroacetic acid (TCA) and stored for 30 minutes .

The mixture were centrifuged at 5000 rpm at 4 for 10 minutes. Supernatants were stored –18 °C until analysis. The OPA reagent was prepared by combining 25 ml of sodium tetraborate buffer (100 mM; pH 9.3), 2.5 ml of sodium dodecyl sulphate (20%, w/w), 40 mg of OPA (dissolved in 1 ml of ethanol), and 100 µl of β–mercaptoethanol and diluting to 50 ml with water. 200 µL OPA reagent was mixed with 10 µL sample and absorbance of solution was measured by a spectrophotometer at 340 nm. A standard curve of L–leucine (0.05, 0.10 0.15, 0.25, 0.30, 0.40, 0.50, 0.60 mg/mL in distilled water) was used to calculate the degree of hydrolysis in fermented milks (see Figure A11 in Appx. A). Analyses were carried out in TAM center of IZTECH.

### 2.2.2.5. ACE inhibitory Activities in Fermented Milks

The substrate solution contained 2 mM–HHL in 100 mM sodium borate buffer with 0.3 M NaCl (pH 8.3) containing 25 mU/ml ACE and Vortex mixing for 2 min. The

assay was performed in eppendorf tubes; 250 ml HHL substrate solution was mixed with 50 µl inhibitor fermented sample and incubated for 10 min at 37 °C in a Block heater. 50 µl ACE solution was added and after vortex mixing for 30 s, the samples were further incubated at 37 °C for 30 min. The reaction was stopped by addition of 1 M HCl and volume of the reaction mixture increased with sodium tetraborate buffer (Wu et. al., 2002). For HPLC analysis, 50 µl injections were analysed on a Symmetry C18 column (5.9×150 mm, 5 µm, Waters) and HA and HHL were detected at 228 nm. The column was eluted (0.5 ml min−1) with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a 5–60% acetonitrile gradient for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 min. This was followed by isocratic elution for 4 min at the constant flow rate of 0.5 ml/min. External standard hippuric acid (HA) samples were prepared freshly (Church et al. 1983). Analyses were carried out in TAM -IZTECH.

### 2.2.2.6. pH Measurement

The pH values in fermented whey beverages were determined as mentioned in the section 2.2.2.3.

## Fermentation of Whey by Selected LAB

LAB isolates having high ACE inhibitory activities in fermented milks were evaluated for their ACE inhibitory activities in sweet whey. The fermentation procedure was applied to sweet whey as mentioned in section 2.2.2.3.

### 2.2.2.7. Degree of Hydrolysis in Fermented Whey

The Degree of hydrolysis in fermented whey beverages were determined as mentioned in the section 2.2.2.4

### 2.2.2.8. ACE inhibitory Activities in Fermented Whey

The ACE inhibitor activities in fermented whey beverages were determined as mentioned in the section 2.2.2.5.

### 2.2.2.9. Hydrolysis of Whey Proteins by SDS-PAGE

Whey proteins degradation was measured in 12 and 24–h–incubation samples by Tricine SDS–PAGE electrophoresis and performed as follows: fermented whey samples (35 µl) were suspended in 7 µl of sample buffer (6.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS and 5% β–mercaptoethanol) and heated at 95 °C for 5 min. The corresponding controls (non–fermented, heated RW), and the molecular weight (MW) marker (Protein marker, Benchmarker, Invitrogen) were loaded separately. Tricine SDS–PAGE were carried out on 15% (w/v) polyacrylamide gels on vertical slab electrophoresis cells (BIO RAD Mini PROTEAN® 3 System, Hercules, CA, USA) for 90 minutes at 100 V. Coomassie brilliant blue R250 was used for staining the gels. Then the gels were silver stained. Analyses were carried out in TAM center of IZTECH.

Before SDS-PAGE analyses, protein content in fermeted samples were quantified based on Bradford protein analysis method.After that, appropriate dilution of samples were used in the analysis for determination of protein degradation.

### 2.2.2.10. Hydrolysis of Whey Proteins by HPLC

The degradation of whey proteins in fermented whey bases were determined as mentioned in the section 2.2.1.7. Analyses were carried out in TAM center of IZTECH.

## 2.2.3. Co-Culture Fermentation of Whey by Selected LAB isolates

Fermented milks with high ACE inhibitor activities were evaluated for determination of the cultures for further studies. In the co–culture fermentation, *L. delbrueckii ssp. bulgaricus* species bTY6, bTY8, bTY 8b, bTY9b, bTY69, bTY71, bTY77a, bTY77b, bTY79, bTY80, bTY85 and were co–cultured with isolate D9 (previously identified as Lactobacillus ssp. and isolate C24 (previously identified as *L. lactis ssp. lactis* (Bulut et al. 2005). For this aim, all isolates were inoculated in whey (5%, v/v) after their optical density is adjusted to 1.00 ± 0.02. For prefermentation isolate D9 and C24 were inoculated at 37 °C, others were inoculated at 42 °C for 24 h.

### 2.2.3.1. Degree of Hydrolysis in Co-Culture Fermented Whey

The Degree of hydrolysis in fermented whey beverages were determined as mentioned in the section 2.2.2.3

### 2.2.3.2. ACE inhibitory Activities in Co-Culture Fermented Whey

The ACE inhibitor activities in fermented whey beverages were determined as mentioned in the section 2.2.2.4.

## 2.2.4. Screening of LAB GABA Production by Qualitative Methods

Two different methods were used to screen LAB isolates. pH indicator method based on the color change due to production of basic GABA substance and combined with the spectrophotometric approach. Chromatographic method based on spot match with the standard on the TLC plate.

### 2.2.4.1. pH Indication Method

GABA producer referance LAB strains (Ozer et al. 2022) were investigated to determine applicability of rapid colorimetric assay for GAD enzyme activity (Lacroix et al. 2013). For the assay, pellets of 2ml of activated cultures were collected by centrifuging at 6000 rpm for 5 minutes at 4‹C and washed twice. Pellets were resuspended, then resuspended in 500 µL of test solution and incubated for 4h at optimum growth temperature. Test solution was prepared by dissolving 1 g of L–glutamic acid, 300 µL of Triton X–100, 90 g of NaCl, and 0.05 g/L indicator (bromocresol green  in deionized sterile water. The change of color of solution to green or blue was considered as positive results as an indicator of low or high production. Afterwards, the test solution after reaction with culture pellet was centrifuged at 13,000 rpm for 2 min, and 100 µL of the clear supernatants were used for recording the absorption spectra in cuvette from 300 to 700 nm at 1 nm intervals. BCG shows peak at around 420 nm, GAD activity positive absorption spectra showed the appearance of a new peak at around 590–620 nm (Shiels, Murray, and Saha 2019). The spectrum of analysis were evaluated

### 2.2.4.2. TLC Method

Thin–layer chromatography (TLC) method was selected to rapid screening of GABA producing LAB strains. Each activated isolate was inoculated into modified broth

media, 10 ml of MRS or M17 broth with 1% (w/v) monosodium glutamate (MSG) and incubated at 37 °C or 42 °C for 48 h. Cultures were centrifuged (5000g, 4 °C, 10 min), and 1 µL of culture supernatant was spotted onto a TLC plate (Silica gel 60 F254; Merck Co., Germany). Separation was done with iso–butanol/acetic acid/water (4:1:1; v/v) (Ly et al., 2019). The plate was treated with 0.2% ninhydrin solution (w/v) to visualize the spots. Lactobacillus plantarum NRRL–B 4496 was used as a negative control ((Ly et al. 2019)).

### 2.2.5. Determination of LAB for GABA Production by Quantitative Method

Based on the TLC results, GABA producing LAB isolates used for further studiesGABA producer 33 LAB isolates were activated in 15 ml MRS or M17 broth media containing 1 % MSG and incubated at 37 °C for 24 h (approximately (108 CFU/ml for *L. brevis* isolates D9, C171. and C17.2, 109 CFU/ml for other isolates) and incubated at 37 °C for 24 h. After incubation, culture pellet was centrifuged at 5000 rpm for 10 min, and the clear supernatants were filter sterilized by Minisart 0.2 µm membrane filter (Sartorius AG, Göttingen, Germany) and stored at –18 °C HPLC analysis. Then, similarly to the previous analysis, for the standardization of the microbial density, optical density of each LAB isolate was adjusted to 1.00 ± 0.02 absorbance value at 600 nm. Isolates were inoculated to MSG containing broth media in the ratio of 5% percentage (108 CFU/ml for *L. brevis* isolates D9, C171. and C17.2, 109 CFU/ml for other isolates) and incubated at 37 °C for 24 h. After incubation, culture pellet was centrifuged at 5000 rpm for 10 min, and the clear supernatants were filter sterilized by Minisart 0.2 µm membrane filter (Sartorius AG, Göttingen, Germany) and stored at –18 °C HPLC analysis.

## 2.2.6. Enumeration of Microbial Growth in Modified Broth Media

Microbial growths of selected LAB isolates were determined by pour plate technique. After 24 h incubation in MRS or M17 broth media, 1 ml sample was taken and serially diluted with peptone water. Plates were allowed to incubate under anaerobic conditions with using anaerobic kit (Thermo Scientific™ Oxoid AnaeroGen, England) at 37°C for 48 hours and quantified as CFU/mL.

### 2.2.6.1. HPLC Analysis of GABA

HPLC method was used for quantification of GABA concentrations, for this purpose pre–column derivatization was selected. Phenylisothiocyanate method was applied as derivatization of GABA (Ozer et al. 2022). Briefly, after cenfriguation step, clear supernatant (1 ml) were added to 10 ml HCL (0.1 N) and mixed varigously. Then ultrasonic extraction step was applied, the supernatant-HCL mixutre was put into ultrasonic water bath for 20 minutes. Later on, the mixture was centrifuted (3000 × g for 10 min at 4°C). After this, 1 ml sample was added into 1 ml TCA solution (40%) for extraction, it was kept in 4°C. After 10 minute period, samples were was centrifuted (20000 × g for 10 min at 4°C), 25 µL of deproteinized supernatant taken and the liquid was removed by vacuum centrifugation by approximately 2 hours. After drying, coupling buffer (20 µL methanol:1M sodium acetate:triethylenamine; 2:2:1) was added and vacuum centrifuged for approximetly 1 h. The derivatization buffer (20 µL methanol: triethylenamine: deionized water: phenylisothiocyanate; 7:1:1:1) was added to the dried sample. After shaking rapidly, the sample was kept for 20 min at room temperature. The sample was vacuum centrifuged for approximetly 1 h, Then, in the last part, 1 mL of dilution buffer was added and filtered by a filter (0.22 µm diameter). Injection volume was adjusted as 80 µL for HPLC analysis.

Previously mentioned method was modified and development developed to produce peaks with high resolution and without any inference from other molecules by

selecting the best condition for the accurate chromatogram. GABA (Sigma) standards were prepared in 0.1 N HCl and they were injected into the HPLC column (Agilent 1100). Separation of GABA in 24 h old bacterial supernatants were performed using Pico.Tag Column (3.9 x 300 mm,4µm) by HPLC. A solution containing 2.5% acetonitrile, adjusted to pH 6.55 with acetic acid, was used as Buffer A. Buffer B was prepared by mixing acetonitrile: deionized water: methanol in a 9:8:3 (v/v/v) ratio. A 10 mg/L of disodium ethylenediaminetetraacetic acid ($Na_2EDTA$) was added to both buffers and the mixture was injected into the column with 1 mL/min flow rate at 254 nm at 45 °C. Identification was based on the retention time of GABA. Calibration curve for GABA were performed using pure standards at different concentrations (figure A17 and A18, Appendix A).

### 2.2.6.2. pH Measurements

The pH values in broth media after incubations were determined as mentioned in the section 2.2.1.3.

## 2.2.7. Fermentation Abilities of GABA Producing LAB in Dairy Models

A model milk system was prepared by reconstituted skim milk (10 %, w/v, Biolife, Italy) with yeast extract (RSM–YE), glucose (RSM–G) and both yeast extract and glucose (YE+G) or without any addition (RSM). RSM–YE was prepared by addition of 1% YE, RSM–G was prepared by addition of 1% GLU, RSM–YE–G was prepared by addition of 1% YE and 1%G. RSM was prepared by dispersing skim milk powder in distilled water with continuous stirring. Stirring was continued until all the powder dissolved. Model milks were heat treated at 110 °C for 10 minutes. After milks reached 30–37 °C. Each model was inoculated by 1% and 5% of GABA producing LAB (OD 600nm=1.00±0.002) and incubated at 37 °C for 24h. The pH values in fermented whey beverages were determined as mentioned in the section 2.2.1.3.

## 2.2.8. Fermentation of RSM by GABA Producing Lactobacillus Species

The highest GABA producer strains C18 and C22 were activated in M17 broth by transferring successively on 2 occasions. Incubations were at 37 °C for 24h. After OD adjustment to 1.00 absorbance at 600nm, 5% of culture C18 and C22 were individually transferred into 5 ml pasteurized 10% (w/v) RSM. Prefermented milks were inoculated individually (5%, v/v) to 50 mL bottles of pasteurized RSM. After incubation at 37 °C for 24 h, fermented milk samples were centrifuged at 10000g at 4 °C for 20 minutes. Clear supernatants were stored at –18 °C for investigation of GABA production.

## 2.2.9. Fermentation of Whey by GABA Producing *Lactobacillus* Species

Sweet whey was collected during kashar cheese production in Eker San. Tic. Ltd. (Bursa, Turkey) and immediately transferred to the Lab. and the mixture was pasteurized at 85 °C for 30 min. GABA producer Lactobacillus strains were cultivated two times. After OD adjustment (1.00 ±0.002 absorbance at 600nm), 5% of culture used for prefermentation and incubated at for 24 h, 5% of prefermented whey was used to fermentation of whey at 37 °C for 24h. For the fermentation of milk, RSM (10%, w/v) was used. After incubation at 37 °C for 24 h, fermented milk samples were centrifuged at 10000g at 4 °C for 20 minutes. Clear supernatants were stored at –18 °C for investigation of GABA production and ACE inhibitory activities.

### 2.2.9.1. pH Measurements

After 24 h fermentation, pH values of the modified broth media and non–modified broth media inoculated with GABA producer isolates were determined by using pH meter (Hanna Instruments, HI2211).

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### 2.2.9.2. Enumeration of LAB in Fermented Whey and Milk

Viability of GABA producing LAB isolates were determined by pour plate technique using MRS or M17 agar medium. For the enumeration, 1 ml fermented beverage was added to the peptone water at a ratio of 1:10. Plates were incubated under anaerobic conditions with using anaerobic kit (Thermo Scientific™ Oxoid AnaeroGen, England) at 37°C for 48 hours and the colonies were numbered. The number of live cells was expressed as colony forming units per ml (CFU/ml).

### 2.2.9.3. HPLC Analysis of GABA

GABA concentrations in fermented milk and fermented whey were quantified based on the study written in section 2.2.7.1.

### 2.2.9.4. HPLC Analysis of ACE inhibitory Activities

ACE inhibitor activities in fermented whey beverages were quantified based on the study written in section 2.2.2.5.

### 2.2.10. 16S rRNA identification of GABA Producing LAB

Genomic DNA of LAB cultures were isolated to investigate their *gad* gene in their genomes and for the identification of GABA producing isolated due to 16s RNA identification. For this purpose, genomic DNA of all LAB isolates (up to 300 LAB culture) used in this thesis were isolated. Genomic DNA was isolated using the following previously published method (Bulut et al. 2005).

Aquatayf Biotechnology Laboratories (Arı Teknokent, ITU, İstanbul, Turkey) applied PCR prosedure and DNA isolation of several LAB, Medsantek Laboratories (İstanbul, Turkey). Analyzed PRC products and applied sequence analysis. To differentiate bacterial species, Forward and Reverse sequences were compared by Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov) algorithm at National Center for Biotechnology Information (NCBI) and aligned in BioEdit sequence alignment editor v7.2.5 (June, 2021).

# CHAPTER 3

# RESULTS AND DISCUSSIONS

## 3.1. Characterization of Whey

Total aerobic bacteria results are found as $1x10^3$. No growth was seen in yeast and mold analyses. Lactic acid bacteria amount was determined as $2.1x10^5$CFU/ml in M17 Agar and $3x10^3$ CFU/ml in MRS agar, respectively. After pasteurization step, there were no selected bacterial growth in any agar medium as expected.

### 3.1.1. Characterization of Whey

As a result of the change in the cheese production steps, based on its pH value, whey was classified as sweet and acidic whey. The pH value of whey was expected to be higher since it was a by-product of kashar cheese, e.g. it was recorded as 6.67. Similarly, many studies showed that cheddar cheese which is like kashar has pH values of sweet whey, 6.38 ± 0.05 (Liaw et al., 2011), 6.21 ± 0.47 – 6.60 ± 0.16 (Carunchia–Whetstine et al., 2003) and 6.5 ± 0.05 (Smith et al., 2016).

### 3.1.2. Composition of Whey

The standard curves (see Figure A1 in Appx. A)-were used to calculate major Ca, K, Mg, Na, P) and minor (Cu, Fe, Mn, Zn) minerals in whey. Based on the calibrations, the mineral profiles of whey samples were calculated. The results were found parallel to the studies about sweet whey.In the literature, Ca, Mg, Na, P values were represented as 500 mg/L, 70 mg/L, 560 mg/L, 1450 mg/L, respectively (Brazinha et al. 2013).

In general, Ca, Mg, Na, K, and P major minerals were investigated. For sweet whey, Ca, Mg, Na, K and P was found as 36,5 mg/100g, 6,5 mg/100g, 45,5 mg/100g, 123 mg/100g and 43,0 mg/100g, respectively (Wong, LaCroix, and McDonough 1978); and Fe, Zn, Cu and Mn was recorded as 8,9 mg/100g, 11 mg/100g 3,5 mg/100g and 0,8 mg/100g, respectively (Wong, LaCroix, and McDonough 1978). In addition, recent studies determined mineral content of sweet whey as Ca 59 mg/100g ± 0,0003, K 0,12 ± 0,004 mg/100g, Mg, 0,017 ± 0,001 mg/100g, Na 0,31 ± 0,004 mg/100g and Ca 400 mg/L, K 1350 mg/L, Mg 80 mg/L, Na 42 mg/L (Smith, Metzger, and Drake 2016; Nishanthi, Chandrapala, and Vasiljevic 2017) and Ca 34 ± 8mg/100g, Mg 8,3 ± 0,6 mg/100g, Na 38 ± 4 mg/100g, K 114 ± 7 mg/100g, P 43 ± 4 mg/100g (Talebi et al. 2019). These results were found parallel with whey sample results. The value of phosphorus was found lower in whey sample. However, the phosphorus results observed in a wide range (32–96 mg/100ml) . In the whey, ash content was found %0.37. In the literature, the percentage of ash content of whey was found higher as 0.65%± 0,04 (Nishanthi, Chandrapala, and Vasiljevic 2017). Total dry matter was determined as 5.34% in the liquid whey. According to sweet whey studies in the literature, dry matter of whey was recorded as 6.77% ± 0.15–6.33% ± 076 (Carunchia Whetstine et al. 2003), 6.4–7.1% (Gallardo-Escamilla, Kelly, and Delahunty 2005) and 6.30 ± 0.34% (Campbell, Miracle, and Drake 2011). Also, mozzarella whey samples had higher content such as 6.50 ± 0.10% and 6.21 ± 0.05 % (Liaw et al., 2011). Low dry matter content of whey leaded to low ash content as expected.

The viscosity and density of whey was found as 11.2 cP and 1.0214 g/ml (Table 9). Total lipid content of whey was determined as 0.25% and it was rich in fat content (Table 9). Previous studies about whey showed generally lower results for sweet whey such as 0.13 ± 0.04 – 0.19 ± 0.03% (Carunchia–Whetstine et al. 2003), 0.38–0.47% (Gallardo–Escamilla et al., 2005), 0.21 ± 0.03% (Campbell et al., 2011) and for cheddar and mozzarella whey: 0.18 ± 0.04% and 0.21 ± 0.05 % (Liaw et al., 2011). Total protein content of sweet whey from kashar production was determined as 0.66% (Table 9). Cheddar and mozzarella cheese by product sweet whey also had similar protein content as 0.68–0.91 % (Gallardo-Escamilla, Kelly, and Delahunty 2005), 0.87 ± 0.02% (Campbell, Miracle, and Drake 2011). However, another study showed higher results for cheddar 1.40±0.20 % and 1.30±0.08% protein (Liaw et al. 2011).

Total acidity of whey was calculated as 0.07%. Compared with previous studies, it was found as at least 2–fold lower. Higher results were reported in several studies as

0.15±0.03– %0,22 ± 0,05, 0.29±0.01%, 0.53±0.1 and 0.15 ± 0.03%, respectively (Carunchia Whetstine et al. 2003; Yasmin et al. 2013; Smith, Metzger, and Drake 2016). EPS content of whey was determined as 259.14 mg/L spectrophotometer. It was calculated by using standard calibration curve of different glucose concentrations(See Appendix Figure A1). The EPS concentration in whey could not be found in previous studies. Since the potential of EPS production of LAB is going to be investigated in fermented whey beverage, EPS content of raw material was determined.

Lactose concentration in kashar whey were determined as 3.6 g/L. β-Lactoglobulin-A, lactalbumin, serum albumin, β-lactoglobulin-B were quantified as 1,517 mg/L, 0.543 mg/L, 0.074 mg/L and 0.675 mg/L, respectively.

## 3.2. Proteolytic Activity of LAB

### 3.2.1. Screening of Proteolytic Activity in Skim Milk Liquid Media

Based on the colour change of bromocresol purple in acidic pH, all isolates showed growth in RSM broth media at 37 after 24 h fermentation. Figure 3.1. represents the colour from bluish to yellowish change in liquid media via increased acidity. However, since the selectivity of this method was not high, agar screening method was applied as the second screening method.

### 3.2.2. Screening of Proteolytic Activity in Skim Milk Agar Media

Agar method was used in many studies, and it was based on the measurement of halo zone diameter (van den Berg et al. 1993; Fusieger et al. 2020). In addition, well-diffusion method has investigated extracellular protease activity (Ebadi Nezhad et al. 2020). However, the volume of inoculum on the plate could be effective on the halo zone size. Also, the halo zone in the well-diffusion method can be disturbed by the colony

formation if non- filtered supernatant of the isolate was used. In Figure 3.1., growth of LAB colony and zone formation was represented.

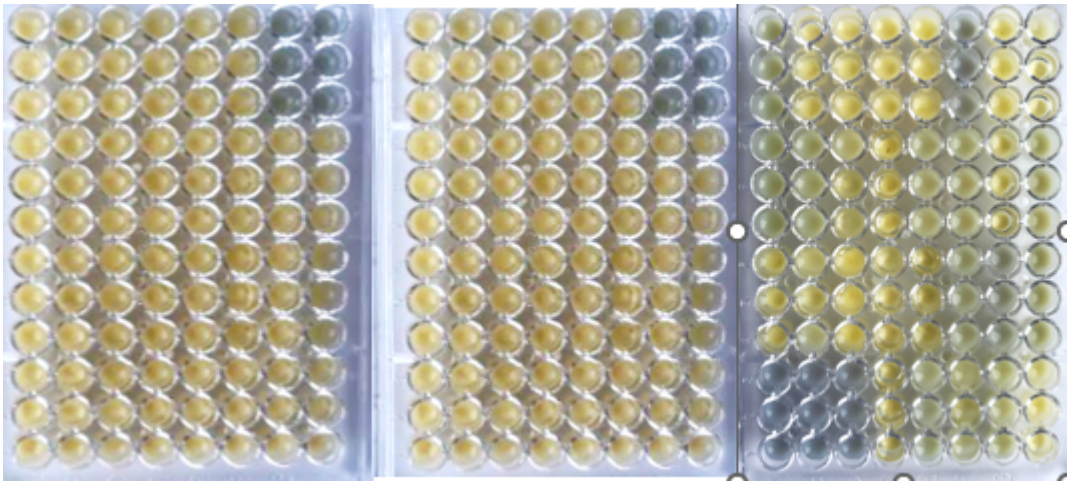

Figure 3.1. The results of broth screening of CTY (left) and C (right) series after 24 h incubation at 42ºC.

Therefore, another successful method (Okuklu 2014) was used for the rapid screening of proteolytic activity of LAB isolates. The results of isolates indicated that measuring the activity by zone diameter is not very applicable. Pipetting can be effective on the measurement of diameter. As Figure 15 shows, isolates can have significant differences between their zone diameters based on spot inoculation and colony formation. Therefore, the zone diameter could not be selected for categorizing LAB for their proteolytic activity.

Proteolysis is considered as one of the most important biochemical processes involved in manufacturing of many fermented dairy products for its role on organoleptic effect (El–Ghaish et al., 2010). Moreover, the contribution of the extracellular proteinases of LAB is required to produce ACE inhibitory and antioxidative activities in the fermented dairy products (Pihlanto et al., 2001; El–Ghaish et al., 2010). As observed by other studies, the results of analyses show that cheese isolates, especially Enterococcus species can have high proteolytic activity among LAB isolates (El-Ghaish et al. 2010; Ebadi Nezhad et al. 2020). However, technological properties such as proteolytic activity are strain–dependent characteristics. Therefore, each strain should be investigated specifically.

Isolates were classified based on their colony size; colony size were determined by calculating the differences between colony size and colony plus zone size. Proteolytic activity were categorised as low(<3mm), medium (3–4.5 mm) and high ( >4.5mm). Also, zone clearance was classified based on transparency; lowest clarity (+), low clarity (+), medium (++), high clarity (+++) ((El-Ghaish et al. 2010)). As a result, these clarities were numbered from 1 to 3; 3 explaining the highest zone clearance. For example, bTY8b isolate had zone size above 4.5 mm and had a high zone clarity, therefore this isolate was classified as "3.high". In general, cheese isolate results showed that Enterococcus and Lactococcus ssp. containing A, B, C series growth well in the SMA agar but, most of the zone clearance of their isolates were find in the range of 1 and 2. cTY and c isolates exhibited both strong and weak proteolytic activities in SMA agar. However, *L. delbrueckii ssp. bulgaricus* isolates (bTY and b series) exhibited generally strong proteolytic activity. Figure 3.2. represent zone formation and zone clearance indicating the proteolytic activity of isolates. From cheese isolates, 77% and 8% of cheese isolate exhibited medium and high activities. On the other hand, 54% and 6% of yoghurt isolates showed medium and high activities. However, when results were detailed in 3. medium class, 13% of yoghurt isolates and 2% of cheese isolates were classified in this class. 25.5% of yoghurt isolates and 17.7% of cheese isolates were classified in 2 medium class based on zone clearance and size. In addition to the screened isolates, unidentified LAB isolates were also screened.

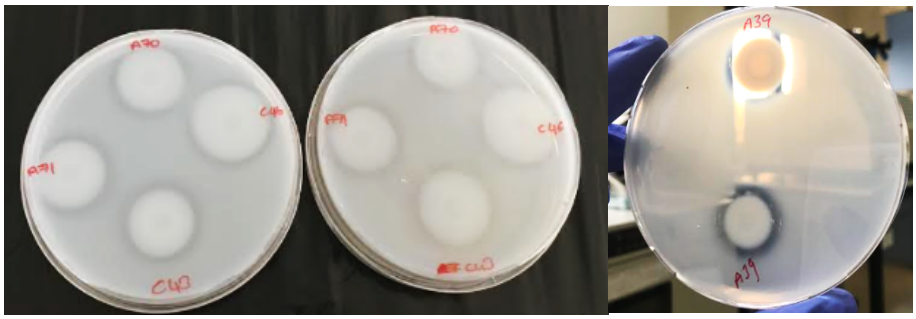


Figure 3.2. Screening of isolates by SMA assay. C43, C46, A70, A71 isolates with + zone clearance (left) and A39 isolate with +++ zone clearance (right).

Table 3.1. represents the yoghurt and cheese-based LAB. According to the results, *L. delbrueckii ssp. bulgaricus* species were found with the best proteolytic activities.

Therefore, *L. delbrueckii ssp. bulgaricus* species having high proteolytic activity on SMA media were selected for further studies.

Over than 100 LAB were isolated from human breast milk, goat milk and green olives. Breast milk isolates hardly grown in SMA medium and almost none of them showed proteolytic activity in SMA. Likewise, results were similar in goat milk isolates. However, 3 olive isolates exhibit great proteolytic activity potential in SMA. Since they were plant-based LAB and whey was the substrate, they were not used in the further analyses.

Table 3.1. Classification of Isolates for Their Proteolytic Activities (S represent *S. thermophilus*, Lb: *L. delbrueckii ssp. bulgaricus*, E: *Enterococcu*s species, Lc: *Lactococcus* species, Lb: *Lactobacillus* species,

| Classification | Yoghurt Isolates | Cheese Isolates | ***S.*** | *Lb.* | *E.* | *Lc.* | *Lb.* |
|---|---|---|---|---|---|---|---|
| 1.low | 14 | 12 | 10 | 4 | 0 | 8 | 4 |
| 2.low | 18 | 3 | 4 | 14 | 2 | 0 | 1 |
| 3.low | 3 | 0 | 0 | 3 | 0 | 0 | 0 |
| 1 .medium | 25 | 77 | 13 | 12 | 40 | 29 | 10 |
| 2. medium | 41 | 24 | 18 | 23 | 12 | 9 | 2 |
| 3. medium | 21 | 3 | 3 | 18 | 1 | 1 | 0 |
| 1.high | 3 | 9 | 1 | 2 | 4 | 5 | 0 |
| 2.high | 12 | 3 | 4 | 8 | 0 | 1 | 2 |
| 3.high | 3 | 0 | 0 | 3 | 0 | 1 | 0 |
| Low | 45 | 15 | 24 | 21 | 2 | 8 | 5 |
| Medium | 87 | 104 | 34 | 53 | 53 | 39 | 12 |
| High | 18 | 12 | 5 | 13 | 4 | 6 | 2 |
| Nd | 10 | 4 | 9 | 7 | 1 | 1 | 2 |
| Total number of isolates | 161 | 135 | 72 | 95 | 60 | 54 | 21 |

### 3.2.3. Fermentation of Skim Milk by Selected LAB isolates

Based on Table 3.2. 34 LAB were selected to evaluate proteolytic and ACE inhibitor activities reconstituted skim milk in fermentation by OPA method and by HPLC respectively. Out of 34 LAB isolates, 3 stain were *L. lactis ssp. lactis (*B15, C24, C47*),* 4 strains were *S. thermophilus* (UN5, cTY9, cTY23, cTY25*),* other LAB isolates were *L. delbrueckii ssp. bulgaricus* (b and bTY coded). The high prevalence of proteolytic activity in *L. delbrueckii ssp. bulgaricus* was expected due to their actions in yoghurt production.

Table 3.2. LAB isolates used for the fermentation of skim milk, (OD=1.00±0.02 adjusted LAB, inoculation rate: 5%, fermentation conditions were 24 h at 42 °C, UN5, cTY9, cTY23, cTY25: *S. thermophilus* strains, B15, C24*: L. lactis ssp. lactis* strains, others: *L. delbrueckii ssp. bulgaricus* strains

| Classification of LAB isolates due to their proteolytic activities | | | | | | | |
|---|---|---|---|---|---|---|---|
| No | 3th high | No | 2nd high | No | 3th medium | No | 3th medium |
| 1 | bTY8b | 4 | b30 | 15 | bTY6 | 30 | bTY85 |
| 2 | bTY69 | 5 | b33b | 16 | bTY8 | 31 | b71 |
| 3 | bTY71 | 6 | b53 | 17 | bTY9b | 32 | B15* |
| | | 7 | b79 | 18 | bTY11 | 33 | UN5* |
| | | 8 | b76 | 19 | bTY14a | 34 | cTY9* |
| | | 9 | bTY5 | 20 | bTY30 | | |
| | | 10 | bTY27a | 21 | bTY41 | | |
| | | 11 | C24* | 22 | bTY45 | | |
| | | 12 | UIB2* | 23 | bTY68 | | |
| | | 13 | cTY23* | 24 | bTY70 | | |
| | | 14 | cTY25* | 25 | bTY73 | | |
| | | | | 26 | bTY77a | | |
| | | | | 27 | bTY77b | | |
| | | | | 28 | bTY79 | | |
| | | | | 29 | bTY80 | | |

### 3.2.3.1. Determination of Proteolytic Activities in Fermented Milks

Degree of proteolysis was evaluated based on the absorbance values and standard L–leucine calibration curve (See App A, Figure A12). The figures showing proteolytic activities were given in Figure 3.3. and Figure 3.4.. High absorbance values and L–leucine content indicates high proteolytic activities in the food matrix. Figure 3.3. represents proteolysis based on absorbance values obtained in OPA analyses. Figure 3.4. shows proteolytic activities in terms of L-leucine. Based on Figure 3.4., it could be concluded that the low proteolytic activities were detected in *S. thermophilues* strains (UN5, cTY9, cTY23, cTY25), Low proteolytic activities were found parallel with curd formation, as expected.

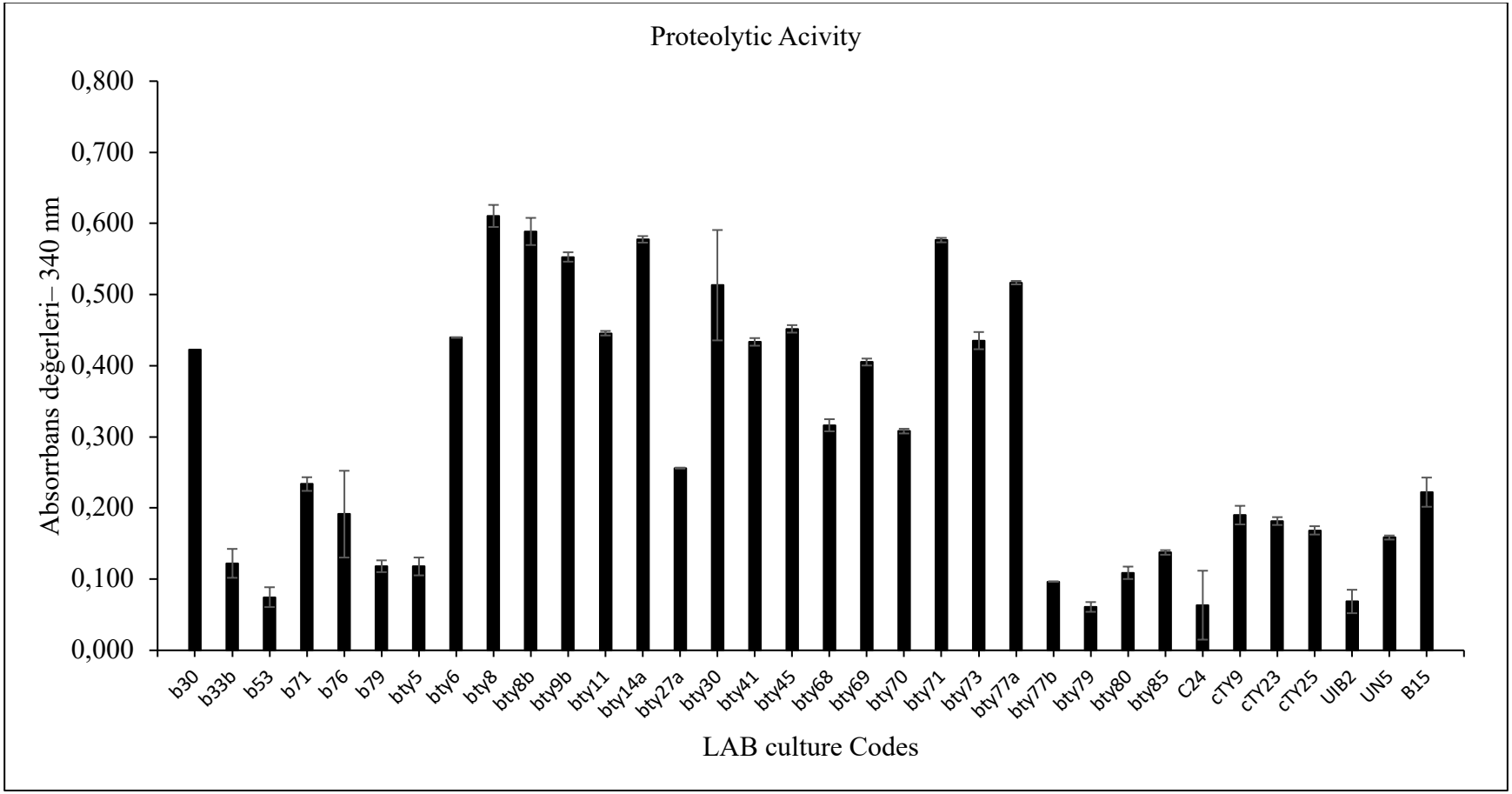


Figure 3.3. Proteolytic activities of strains grown in RSM (10%, w/v) over absorbance 340 nm. *L. delbrueckii ssp. bulgaricus* and*. S. thermophilus* species were incubated at 42°C, *L. lactis ssp. lactis* (B15 and C24) strains were incubated at 37°C for 24 hours. Proteolytic activities of fermented milks were determined by OPA method and evaluated according to absorbance values. The mean values given with the standard deviation are the result of at least 4 parallel measurements of two independent repetitions of experiments.

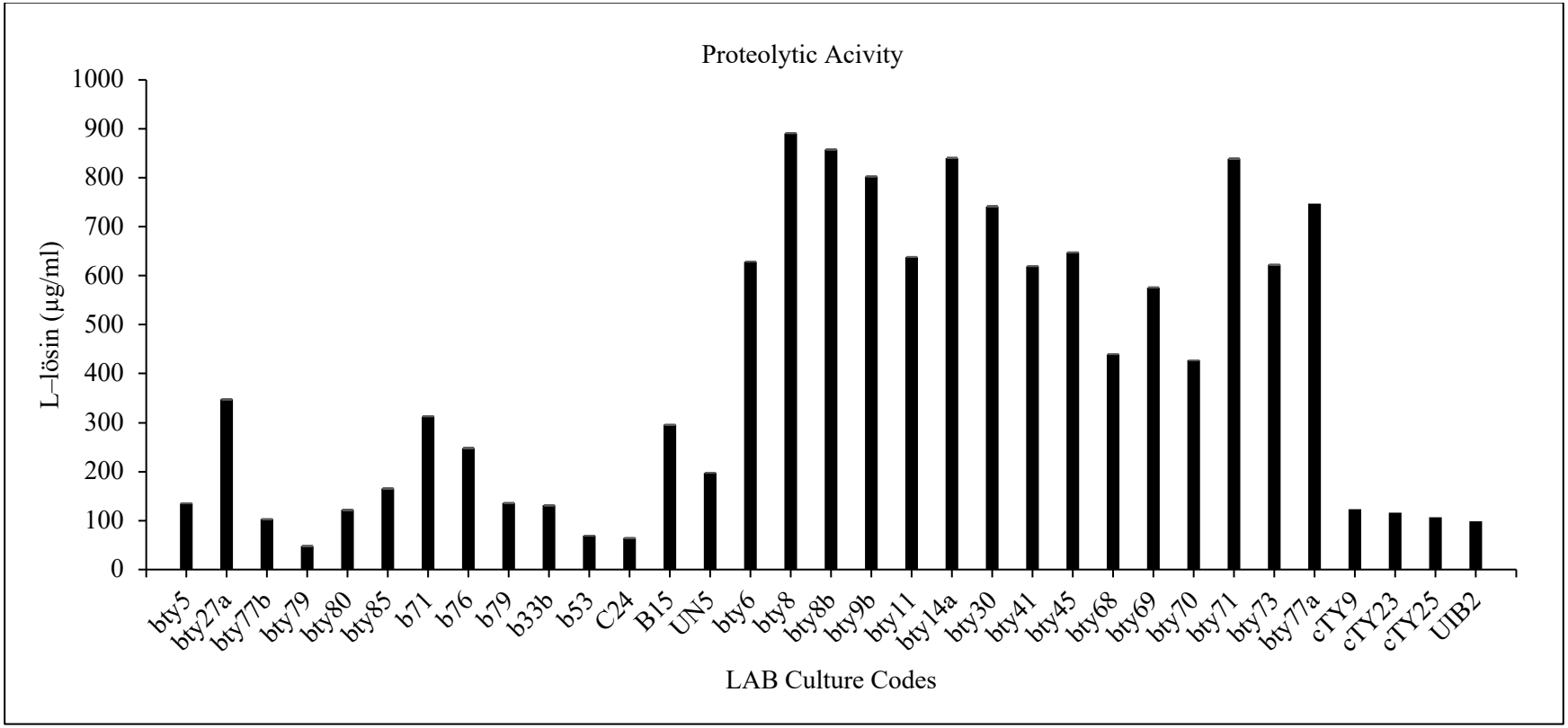


Figure 3.4. Proteolytic activities of strains grown in RSM (10%, w/v) over absorbance 340nm. *L. delbrueckii ssp. bulgaricus* and. *S. thermophilus (UN5,* cTY9, cTY23, cTY25) species were incubated at 42°C, *L. lactis ssp. lactis* (B15 and C24) strains were incubated at 37°C for 24 hours. Proteolytic activities of fermented milks were determined by OPA method and evaluated according to calibration curve of L–leucin. The mean values given with the standard deviations are the result of at least 4 parallel measurements of two independent repetitions of experiments.

### 3.2.3.2. Determination of ACE Inhibitor Activities in Fermented Milks

Fermented milks were produced by using these 34 strains and 14 of the strains was analysed for their ACE inhibitor activity (Figure 3.5, Table 3.4). Since these isolates were selected based on high proteolytic activities, it was expected that they could show both high proteolytic and ACE inhibitor activities. Results were obtained by two replicates.

Although *S .thermophilus* strains were found promising based on SMA agar analysis, they did not exhibit any inhibitor activity.. Out of 28 strains, only bTY8 resulted with ACE inhbiion below 50% and two other strain exhibit <60% inhibitor activity in skim milk. Therefore, these strains were eliminated for co–culture studies. Strains with high activities were investigated for co–culture studies

In the literature, most of the studies have focused on the ACE inhibitor activity of the final product (Donkor et al. 2007).On the contrary, limited number of research aimed to explore screening of ACE inhibitor activities of LAB fermented products.

Another study screened 25 LAB containing several reference cultures and based on the results they obtained, they researched the fermentation characteristics and ACE inhibitory activity after fermentation using *Lc. lactis ssp. lactis* ATCC 19435, *Lb. acidophilus* ATCC 4356, *Leu. mesenteroides* 358 and 356, *Lb. acidophilus* ATCC 4356, their combinations and modification of fermentation conditions. Single culture fermentation resulted with high ACE inhibitor activity (57–68%) (Pihlanto, Virtanen, and Korhonen 2010). Even if the strains show high ACE inhibitor activity in the form of single cultures, interestingly they may possess lower activity in their co-culture combinations.

Another study investigated single culture fermentation of milk by 2 of *L. lactis ssp. lactis*, 2 of *L. lactis* ssp. *cremoris,* 7 of *L. helveticus*, *L. acidophilus* and *S. thermophilus*. The ACE inhibition changed in a broad range (1–40%). Also, fermentation by *L. helveticus* showed that ACE inhibition was strain dependent changing from 11 to 34%. *L. lactis*, notably the *cremoris* ssp. species generated the maximum level inhibitor activities in fermented milks (25 -33%,), it was quite surprising that *L. lactis*, which is generally not considered highly proteolytic.

Another research reported how ACE inhibition, pH values and peptide content changed during shelf-life period. They concluded that approximately pH values in

fermented milks were reduced 0.3 and 0.4 point in milks produced by L. helveticus and . *lactis* ssp. *cremoris*, respectively. This reveled the metabolic activity in this species at 5 °C. After cold storage, the milks having low inhibitory activity (≤ 20) was not altered by storage. Interestingly, the ACE inhibitor activity was reduced in the milk fermented with *L. helveticus* strain 4080, dramatically. The best proteolytic strains represented the high ACE–inhibitory activity, the inhibition percentage increased upto ≥)40% level (Nielsen et al. 2009).

The ACE inhibitor activity found maximum (100%) at dried crude peptide fragments, these peptides were generated by the action of *L. helveticus,* in whey medium. On the other hand, the ACE activity with the freeze–dried crude peptides were in the range of 100% for *L. brevis* to 89.5% for *Lb. casei* and 63.9% for *L. paracasei* .

Table 3.3. pH values of fermented milks prepared with 1% inoculation of selected LAB isolates at the end of 24h fermentation (*(B15, C24, C47: *L. lactis ssp. lactis*, UN5, cTY9, cTY23, cTY25: *S. thermophilus*, b and bTY coded isolates: *L. delbrueckii ssp. bulgaricus)*

| Isolate Code | MRS broth pH | Fermented Milk pH | Isolate Code | MRS broth pH | Fermented Milk pH |
|---|---|---|---|---|---|
| UIB2 | 4,4 | 3,75 | b71 | 4,4 | 4,19 |
| bty 8b | 3,98 | 3,54 | b30 | 4,16 | 3,56 |
| bty 69 | 4,13 | 3,61 | b33b | 4 | 3,39 |
| bty 71 | 4,38 | 3,84 | b53 | 4,08 | 3,94 |
| bty6 | 3,08 | 3,71 | b79 | 4,05 | 3,44 |
| bty8 | 3,85 | 3,63 | b76 | 4,09 | 3,6 |
| bty9b | 3,98 | 3,73 | bty5 | 4,29 | 3,72 |
| bty11 | 4,14 | 3,55 | bty27a | 4,4 | 3,75 |
| bty14a | 3,85 | 3,68 | UN5* | 5,66 | 4,62 |
| bty30 | 3,83 | 3,55 | cty9 * | 5,69 | 5,71 |
| bty41 | 3,87 | 3,86 | cty23* | 5,64 | 5,05 |
| bty45 | 3,94 | 4,04 | cty25* | 5,8 | 4,55 |
| bty68 | 4,18 | 3,77 | B15* | 4,4 | 4,27 |
| bty70 | 4,34 | 3,67 | C24* | 4,2 | 4,33 |
| bty73 | 4,22 | 3,78 | | | |
| bty77a | 4,28 | 3,94 | | | |
| bty77b | 3,9 | 3,98 | | | |
| bty79 | 4,38 | 3,65 | | | |
| bty80 | 3,98 | 3,59 | | | |
| bty85 | 3,92 | 3,58 | | | |

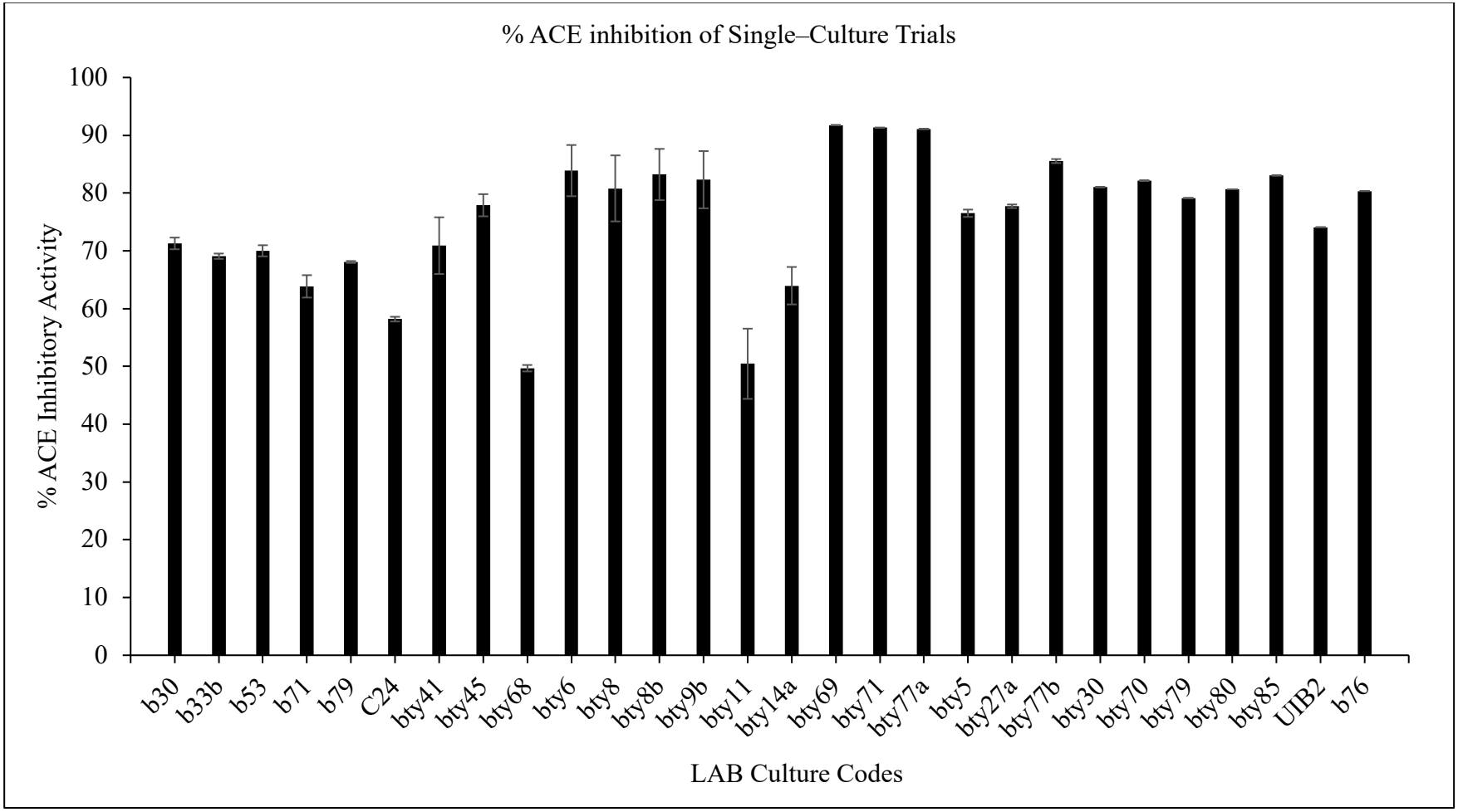


Figure. 3.5. ACE inhibitory activity results in fermented milks prepared by addition of % cell culture (OD600nm=1.00±0.02) in RSM (10% w/v) at the end of 24h fermentation

Tablo 3.4. ACE inhibitory activity results in fermented milks prepared by addition of 1% cell culture (OD600nm=1.00±0.02) in RSM (10% w/v) at the end of 24h fermentation.

| **Sample No** | **Code** | **Isolated Food** | **Species** | **%ACE inhibitor activity ±SD** |
|---|---|---|---|---|
| **1** | b30 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 62,2 ±0,0 |
| **2** | b33b | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 69,1 ±0,5 |
| **3** | b53 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 70,0 ±1,0 |
| **4** | b71 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 63,8 ±1,9 |
| **5** | b76 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 78,0 ±1,2 |
| **6** | b79 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 68,1 ±0,2 |
| **7** | bty5 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 76,5 ±0,6 |
| **8** | bty6 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 83,9 ±4,4 |
| **9** | bty8 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 80,8 ±5,7 |
| **10** | bty8b | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 83,2 ±4,4 |
| **11** | bty9b | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 82,3 ±5,0 |
| **12** | bty11 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 50,5 ±6,1 |
| **13** | bty14a | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 64,0 ±3,2 |
| **14** | bty27a | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 77,7 ±0,3 |
| **15** | bty30 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 62,2 ±0,5 |
| **16** | bty41 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 70,9 ±4,9 |
| **17** | bty45 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 77,9 ±1,9 |
| **18** | bty68 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 49,7 ±0,6 |
| **19** | bty69 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 79,5 ±2,8 |
| **20** | bty70 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 75,0 ±3,6 |
| **21** | bty71 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 75,7 ±6,4 |
| **22** | bty77a | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 75,7 ±6,3 |
| **23** | bty77b | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 85,5 ±0,3 |
| **24** | bty79 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 60,8 ±6,8 |
| **25** | bty80 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 69,2 ±4,1 |
| **26** | bty85 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 68,0 ±5,4 |
| **27** | C24 | Cheese | *L. lactis ssp. lactis* | 58,2 ±0,4 |
| **28** | CTY23 | Yoghurt | *S. thermophilus* | 0 ±0,6 |
| **29** | CTY25 | Yoghurt | *S. thermophilus* | 0 ±1,1 |
| **30** | CTY9 | Yoghurt | *S. thermophilus* | 3,6 ±0,0 |
| **31** | UIB2 | Yoghurt | *L. delbrueckii ssp. bulgaricus* | 74,1 ±0,0 |
| **32** | UN5 | Yoghurt | *S. thermophilus* | 0 –0,6 |

### 3.2.4. Co-Culture Fermentation of Whey by Selected LAB isolates

*L. delbrueckii ssp. bulgaricus* species showing ACE% inhibitor activities in fermented milks were used for co-culture fermentations with 2 different cheese LAB, strain D9 and C24.

### 3.2.4.1. Degree of Hydrolysis in Co-Culture Fermented Whey

Proteolysis in co-culture fermented whey was evaluated based on L-leucine determination. Figure 3.6. and 3.7 showed that-co–culture fermentation by *L. plantarum* D9 leads both higher proteolytic activities in terms of L–leucine and higher ACE % inhibitory activities in fermented whey beverage medium after 24 h fermentation at 42 °C compared to strain C24. According to results, strain C24 had higher proteolytic activity in single culture fermentation in whey, co-culture fermentation of *L. delbrueckii ssp. bulgaricus* and strain C24 resulted in low proteolytic activity. %

### 3.2.4.2. ACE Inhibitor Activities in Co-Culture Fermented Whey

In the literature, sustainability of whey via fermentation gained importance. Some studies investigated the ACE inhibitor activity after fermentation of WP instead of untreated whey (Daliri et al. 2018; Solieri et al. 2022). 2A recent study, spontaneously ferment whey and resulting in 60-70% ACE inhibition activity even after after 5 day incubation time (Mazorra-Manzano et al. 2020).When yogurt isolates were co-cultured *L. brevis* D9 in liqued whey, 70.4-90.7% inhbiton against ACE were recorded AB cheese isolate) On the otherhand, 62.1-84.1% inbibition was observed when fermentation was co-cultured by *L. lactis ssp. lactis* C24

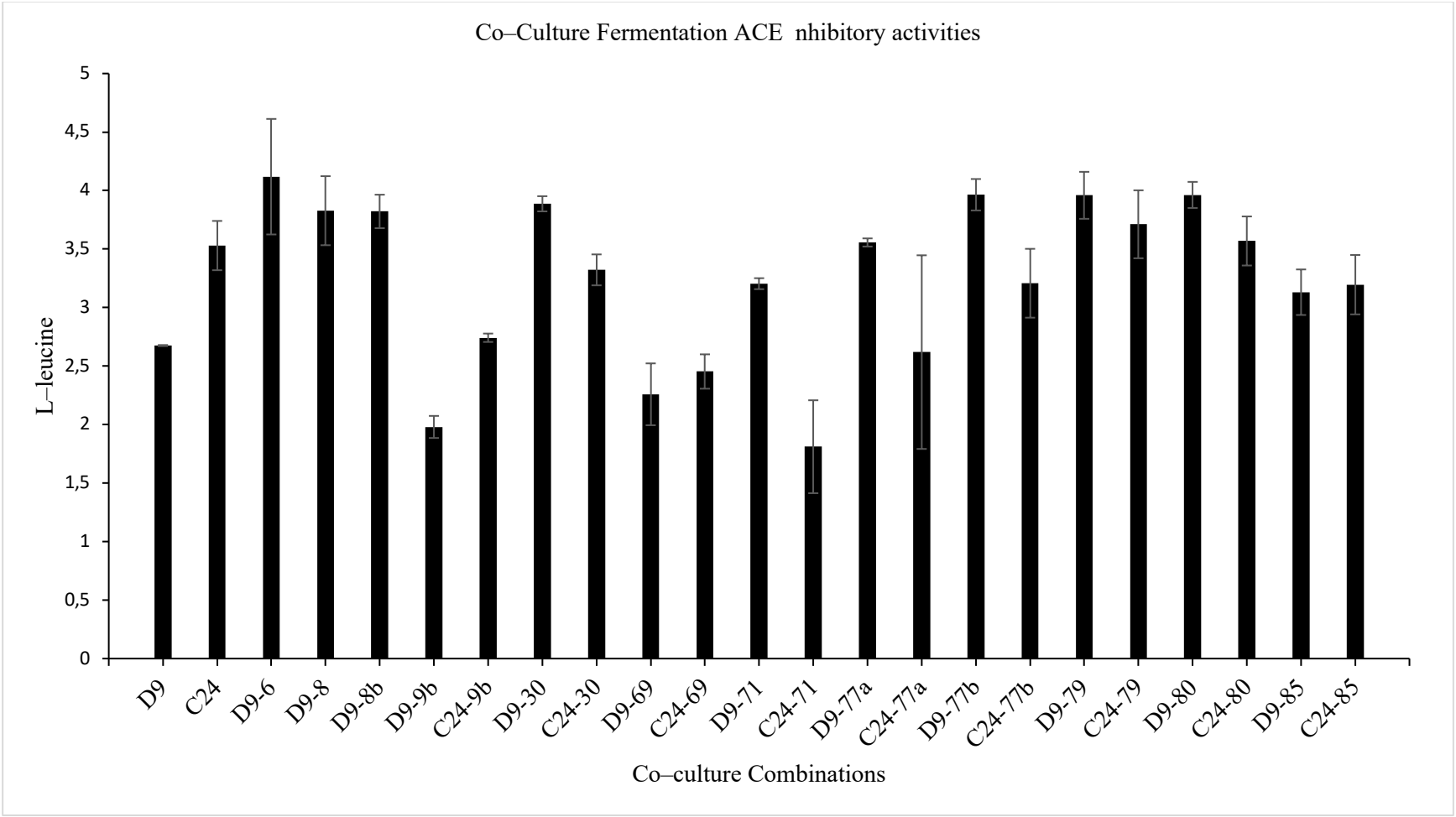


Figure 3.6. The proteolysis in fermented whey (containing 1% MSG, 1% YE and 1% glucose) using co–culture of *L. delbrueckii ssp. bulgaricus* isolates (bTY6, bTY8, bTY8b, bTY9b, bty30, bTY69, bTY77a, bTY77b, bTY79, bTY80 and bTY85), together with either D9 or C24, incubated at 42°C for 24 h.,

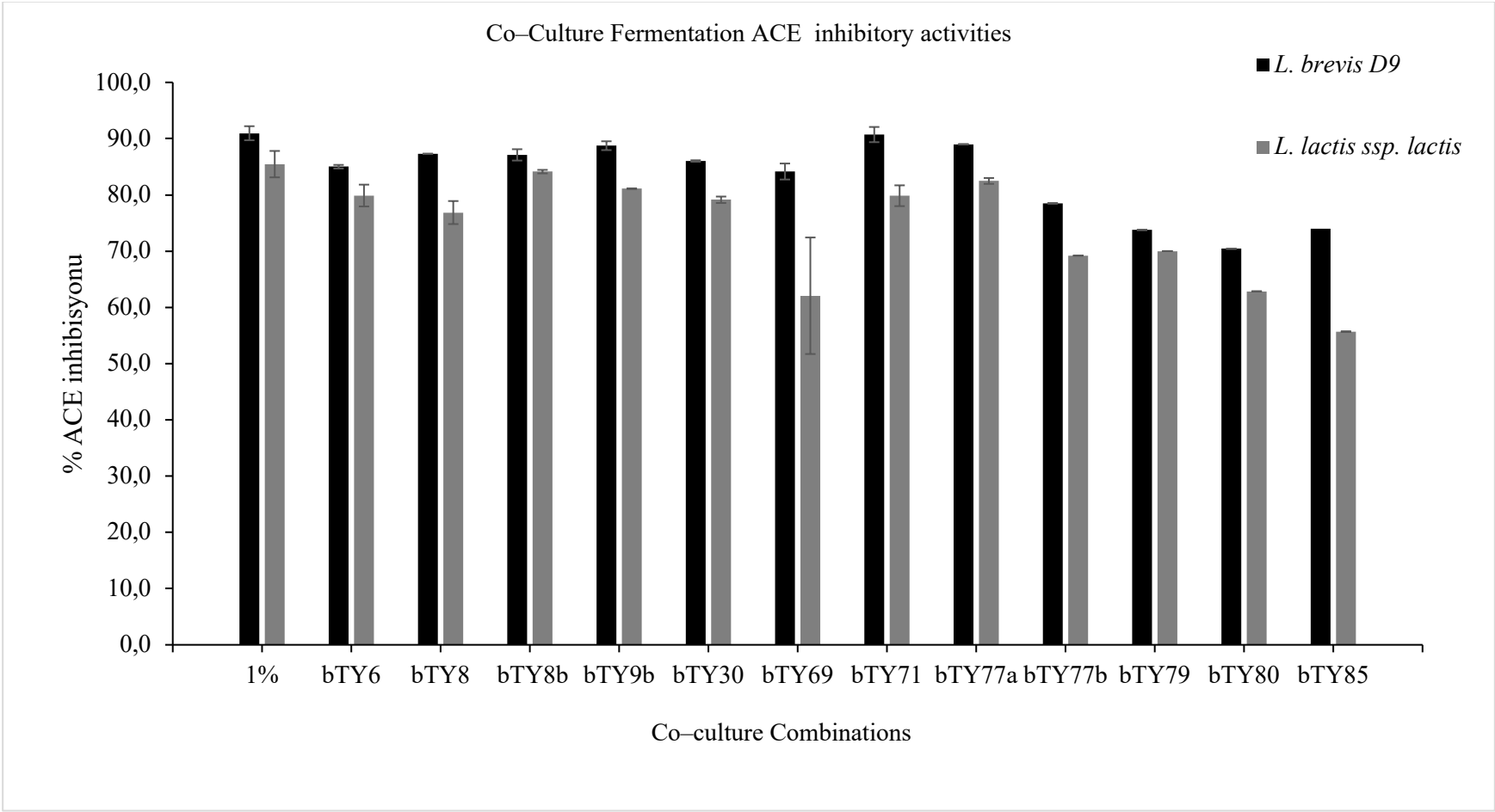


Figure 3.7. The % ACE inhibitory activities in fermented whey (containing 1% MSG, 1% YE and 1% glucose) using co–culture of *L. delbrueckii ssp. bulgaricus* isolates (bTY6, bTY8, bTY8b, bTY9b, bty30, bTY69, bTY77a, bTY77b, bTY79, bTY80 and bTY85), together with either D9 or C24, incubated at 42°C for 24 h.

### 3.2.4.3. Hydrolysis of Whey Proteins by SDS–PAGE

Although literature, SDS–PAGE was used for the evaluation of the degradation of proteins, silver staining was found to be necessary to observe degradation of proteins. Figure 3.8a showed that fermentation lead degradation of whey proteins.

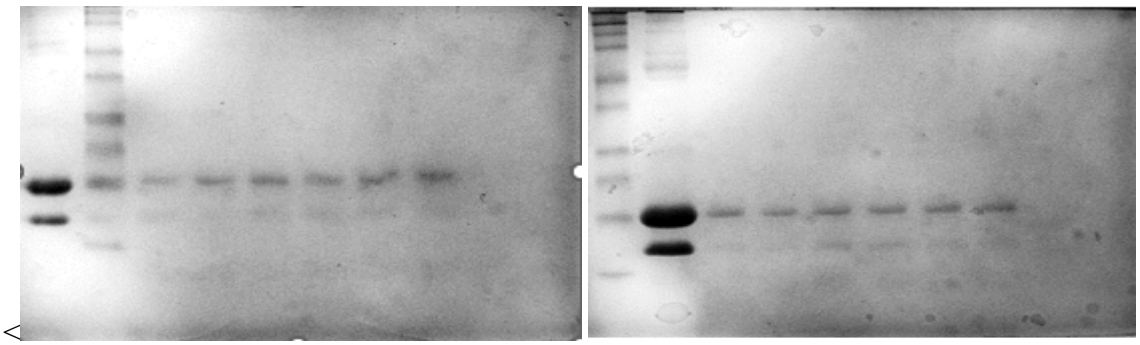

Figure 3.8. DS–PAGE results of fermented whey beverages with different single cultures; line 1; UFW, 2, Protein ladder, 3 Lb6, 4, Lb8, 5: Lb 8b, 6: Lb9, 7: Lb69, 7: Lb71 line 1: polypeptide ladder, line 2 protein ladder, line 3: bTY77a, line 4: 77b, line 5: bTY79, line 6: bTY80, line 7: bTY85, line 8: b76, respectively.

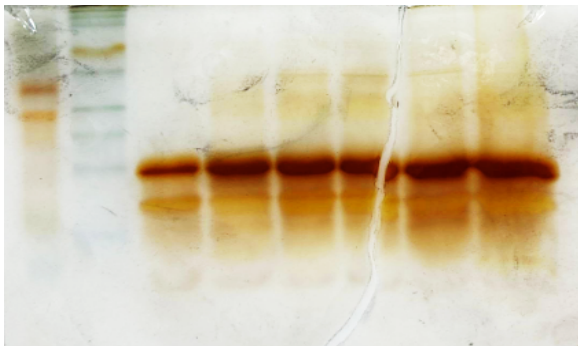

Figure 3.8. Silver–stained SDS–PAGE results of fermented whey beverages with different single cultures; line 1: polypeptide ladder, line 2 protein ladder, line 3: bTY77a, line 4: 77b, line 5: bTY79, line 6: bTY80, line 7: bTY85, line 8: b76, respectively.

The molecular weight of serum albumin, α–lactalbumin and β–lactoglobulin is approximately 66,4 kDa, 14,4 kDa and 18,4 kDa. respectively. Molecular weight of Based

on the silver–stained gel shown in Figure 3.8b, it can be evaluated that serum albumin and α–lactalbumin were degraded mostly, β–lactoglobulin was also degraded into the peptides. In contrast, a recent study reported that spontaneous fermentation exhibited limited proteolytic activity on β-lactoglobulin and α-lactalbumin even after 120 h fermentation of whey (Mazorra-Manzano et.al., 2020).

### 3.2.4.4. Hydrolysis of Whey Proteins by HPLC

SDS-PAGE analysis reveled that selected LAB exhibit high proteolytic activities on whey proteins. This degradation of whey proteins were also detected as reduction of peak areas in HPLC chromatograms revelaed that beta-lactogloblins were degredated by LAB, Another study also showed efficient proeolysis of whey by (Pescuma et al. 2011).

## 3.3. GABA Production Abilities of LAB Isolates

In the literature, several methods has been developed to quantify GABA concentration in the food matrix, human and animal tissue matrixes. However, for the screening of LAB for their GABA production abilities, two methods have been applied frequently.

### 3.2.1. Qualitative Findings by pH Indication

The assay relies upon the test solution, which detects glutamate decarboxylation as a result of the associated increase in the pH of the reagent to change from greenish yellow to green or blue (Figure 3.9) (Lacroix et al. 2013).

Reference LAB cultures, *L. plantarum* NRRL B– 4496, *L. rhamnosus* NRRL B–442, *Lactobacillus delbrueckii* subsp. *bulgaricus* NRRL B– 548, *Lactococcus lactis*

subsp. *lactis* CECT– 4432 which produced GABA in MSG containing MRS broth media (Ozer et al. 2022), investigated by pH indication method. At the end of 4 h incubation period, only one the LAB cultures showed clear color change. The pH values of reference cultures were found as 3.80 for *Lb. plantarum* NRRL B4496, 4.89 for *Lc. lactis ssp. lactis* CECT– 4432*,* 3.91 for *Lb. rhamnosus* NRRL B442 and 5.2 for *Lb. delbrueckii ssp. bulgaricus* NRRL B548. The results of pH indicator method showed unreliable results. The change of color from greenish yellow to blue was observed in *Lc. lactis ssp. lactis* CECT– 4432,; slight color change was detected in *Lb. delbrueckii ssp. bulgaricus* NRRL B548 (Figure 3.9). However, the color change of *Lb. rhamnosus*, which is known as a GABA producer (Ozer et al. 2022) remained greenish blue (Figure 3.9). Also, *Lb. plantarum* NRRL B4496 was used as a control LAB and the change of color was not determined clearly (data not shown). The results did not change when cultures were waited for 24 h. Therefore, this method was not chosen as an applicable method for the screening of 300 LAB isolates.

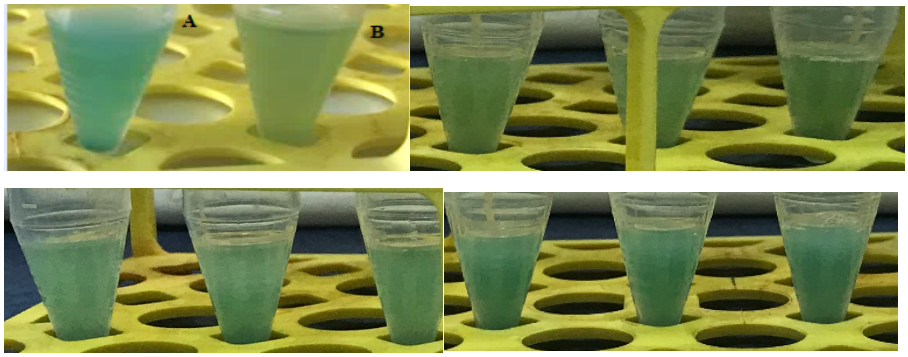


Figure 3.9. Screening of GABA production by pH indicator method, *Lc. lactis* subsp. *lactis* CECT– 4432 (A) and *Lb. plantarum* NRRL B4496 (B) Second analysis of *Lb. delbrueckii* subsp. *bulgaricus* NRRL B548, *Lc. lactis* subsp. *lactis* CECT– 4432 and *Lb. rhamnosus* NRRL B442, respectively.

### 3.3.1. Qualitative Findings by TLC

The TLC results of reference cultures were shown at Figure 22. *Lb. plantarum* NRRL B4496 was used as a negative control (Das and Goyal 2015) . Based on first TLC

results, both producers reference culture and negative control culture gave expected results.

Although n–butanol/acetic acid/water composition was stated as elution solvent (Das and Goyal 2015; Ly et al. 2019; Shiels, Murray, and Saha 2019)the iso–butanol was used in this trial. Since the separation of spots was dependent on elution solvent, iso–butanol usage could be the reason of low Rf value and weak separation of spots. Many researches (Seok et al. 2008; Villegas et al. 2016; Sanchart et al. 2017) has been conducted analyses of GABA production with TLC as a preliminary detection method successfully. Therefore, based on Figure 3.10, TLC screening method was chosen for rapid detection. Figure 3.10 and Figure 3.11 shows some of the GABA producer LAB.

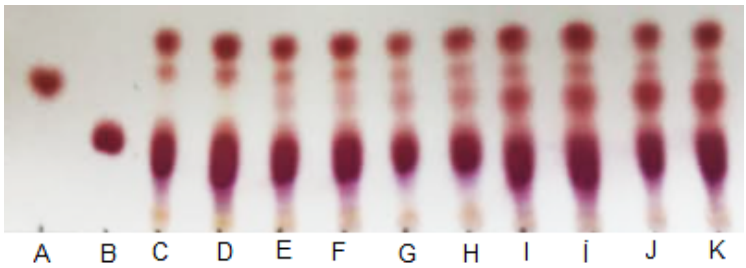


Figure 3.10. A: 10 mg/ml GABA, B: %1 MSG, C–D: %1 MSG containing broth (negative control), E–F: 2,5 mg/ml GABA containing broth, G–H: 5 mg/ml GABA containing broth, I–İ: 10 mg/ml GABA containing broth, J–K: 20 mg/ml GABA containing broth.

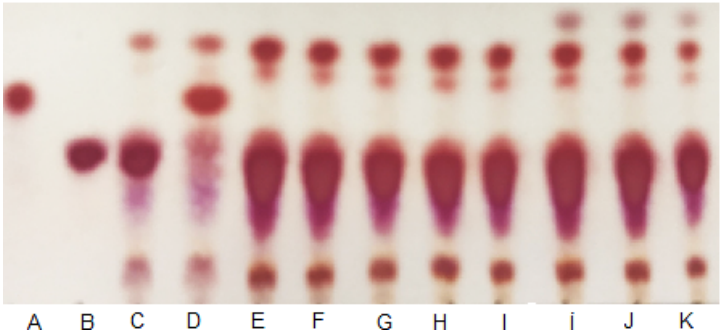


Figure 3.11. A: 10 mg/ml GABA, B: %1 MSG, C: %1 MSG containing broth (negative control), D: 10 mg/ml GABA and 1% MSG containing broth, E: A10, F: A11, G: A12, H: 13, I: A14, J: A17, K:A19 supernatants of cell cultures (0.5 µl).

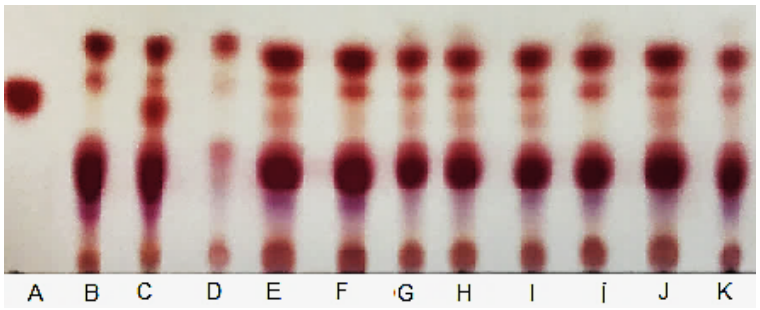


Figure 3.12. A: 10 mg/ml GABA, B: 1% MSG containing broth, C: 10 mg/ml GABA and 1% containing broth, D: D1, E: D2, F: D3, G: D4, H: D5, I: D6, İ: D7, J: D8, K: D9 supernatants of D series LAB isolates (0.5µl).

The results of TLC screening were listed in Table 3.15. Out of 135 cheese isolates, 33 of them were found to be GABA producers. GABA could not be detected in any of the *Enterococcus* species included in the B series, and 11 *L. lactis ssp. lactis* isolate, 3 E. *faecium i*solates, 1 *E. avium* isolate, 1 *L. curvatus* isolate were found. Unlike cheese isolates, no GABA spots were observed in any of the yogurt isolates as a result of TLC analysis. Based on the TLC analysis, 24,4 % of cheese isolates were evaluated as GABA producing LAB in total. In contrast, none of the LAB cultures isolated from yogurt produced GABA in MSG containing MRS or 17 broth media. Also, when yogurt isolates (Okuklu 2014) were screened by 5.0 pH adjusted MRS and M17 broth (containing 1% MSG), no GABA production were detected in TLC plates. Based on spots on the plates,According to the Table 3.5, it could be concluded that isolates C4, C22, C24 and C19.1 produced highest GABA among 33 LAB isolates.

### 3.3.2. Determination of Microbial Growth in Modified Broth Media

At the end of 24 h incubation at 1% MSG containing broth media, viable cell counts were determined in the range of 8.7–9.6 log CFU/ml (Figure 3.13). pH change in the broth media and MSG containing broth media showed that, MSG containing broth had slightly higher pH at the end of growth period (Figure 3.14).

Table 3.5. LAB isolates showing GABA Production Abilities in 1% MSG Containing MRS or M17 Broth

| No | Isolate Code | Species | No | Isolate Code | Species |
|---|---|---|---|---|---|
| **1** | D1 | *L. plantarum* | 18 | C22 | *L. lactis ssp. lactis* |
| **2** | D2 | *L. plantarum* | 19 | C24 | *L. lactis ssp. lactis* |
| **3** | D3 | *L. plantarum* | 20 | C30 | *E. faecium* |
| **4** | D4 | *L. plantarum* | 21 | C36 | *E. faecium* |
| **5** | D5 | *L. plantarum* | 22 | C39 | *E. durans* |
| **6** | D6 | *L. plantarum* | 23 | C40 | *E. durans* |
| **7** | D7 | *L. plantarum* | 24 | C46 | *E. avium* |
| **8** | C37 | *L. plantarum* | 25 | A30 | *L. lactis ssp. lactis* |
| **9** | D8 | *L. curvatus* | 26 | A35 | *L. lactis ssp. lactis* |
| **10** | C29 | *L. curvatus* | 27 | A37 | *L. lactis ssp. lactis* |
| **11** | D9 | *L. brevis* | 28 | A38 | *E. faecium* |
| **12** | C17.1 | *L. brevis* | 29 | A39 | *Enteroccus* |
| **13** | C17.2 | *L. brevis* | 30 | A40 | *L. lactis ssp. lactis* |
| **14** | C4 | *L. lactis ssp. lactis* | 31 | A42 | *E. faecium* |
| **15** | C11 | *L. lactis ssp. lactis* | 32 | A47 | *L. lactis ssp. lactis* |
| **16** | C18 | *L. lactis ssp. lactis* | 33 | A56 | *E. faecium* |
| **17** | C19.1 | *L. lactis ssp. lactis* | | | |

## 3.3.3. Quantitative Determination of GABA content in Broth Media

Previous results obtained from 24 h fermentation indicated that the production varied depending on the strain. Since the results are close to each other and the results are generally 48 hours in the literature, the optical densities of the bacteria were adjusted to be 1.00±0.02 at 600nm and the incubation time was increased to 48 hours and scanned again. HPLC analysis was performed with triplicate measurements. The results of HPLC analysis are shown in Table 3.6 for *Enterococci* and *Lactococci* species, analysis results are written as the average of at least three different replicates. Due to the wide range of measurements, isolates were classified as 10–50 mg/ml, 50–100mg/ml, 100–200 mg/ml and 200–400 mg/ml according to their production amount under these conditions. All isolates with a production capacity of 200–400 mg/ml were *L. lactis ssp. lactis* species and producing 100–200 mg/L GABA, except for C46 *Enterococcus* strain.

Out of all cocci isolates; 55% of them produced GABA between 10–50 mg/ml, 10% of them 50–100 mg/ml, 20% of them 100–200 mg/ml and 15% of them 200–400 mg/ml by the strain isolates.

Tablo 3.6. Classification of Lactococci and Enterococci isolates according to their production after 48 hours of incubation without shaking during at 37°C in M17 broth medium containing 1% MSG,

| GABA (mg/ml) | Isolate Code | GABA (mg/L) | Isolate Code |
|---|---|---|---|
| 10–50 mg/ml | A38 | 50–100 mg/l | C30 |
| | A39 | | C19.1 |
| | A42 | 100–200 gm/ml | C11 |
| | A56 | | C24 |
| | C36 | | C46 |
| | C39 | | A30 |
| | C40 | 200–400 mg/ml | A35 |
| | A37 | | C18 |
| | A40 | | C22 |
| | A47 | | |
| | C4 | | |

As reported in previous reporting periods, the highest GABA producer was again found to be C18 and C22 by the qualitative TLC method. The GABA production of these bacteria after 24 hours of incubation was also found to be the highest among other on the other hand, 10 of out of 13 *Lactobacillus* strains isolated from cheese samples were investigated in MRS medium containing 1% MSG as a substrate.

The results in Figure 3.15 and Table 3.7 showed that D2, D5, D9, C17.1, C17.2 and C37 could be the culture candidates for whey beverage since they showed high GABA production in MRS broth. Therefore, highest GABA producers strains C18 and C22 were utilized in the skim milk fermentation, lactobacillus species were utilized in whey fermentation.

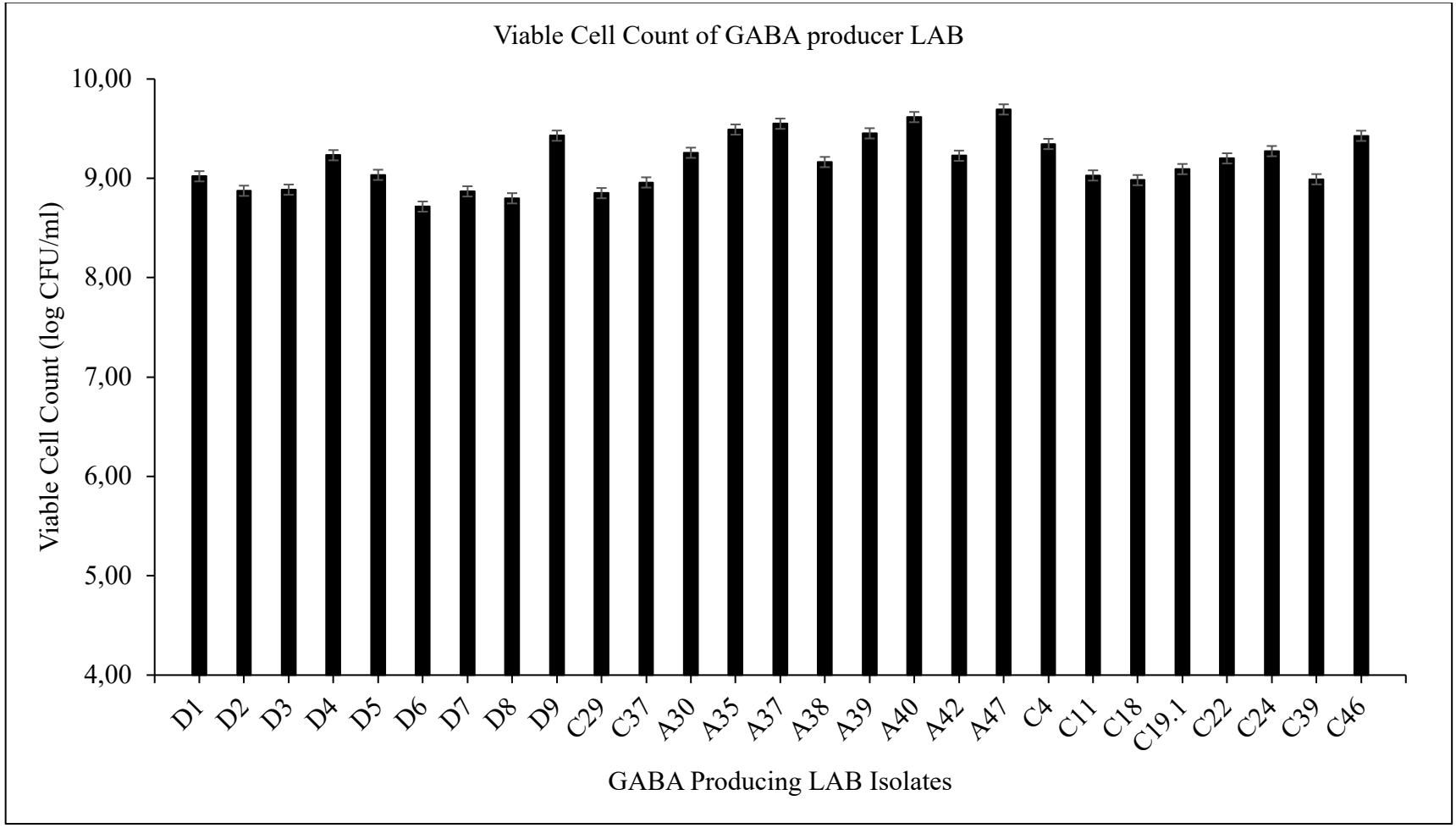


Figure 3.13. Growth (log CFU/ml) of GABA producer LAB strains in MRS or M17 broth medium (black lines) and 1% MSG containing MRS or M17 medium (grey line) after 24 h incubation at 37 °C, D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D3, D8, C29: *L. curvatus*, D9: *L. brevis,* A30–A47: *Enterococcus ssp.,* C4–C46: *Enterococcus ssp.*

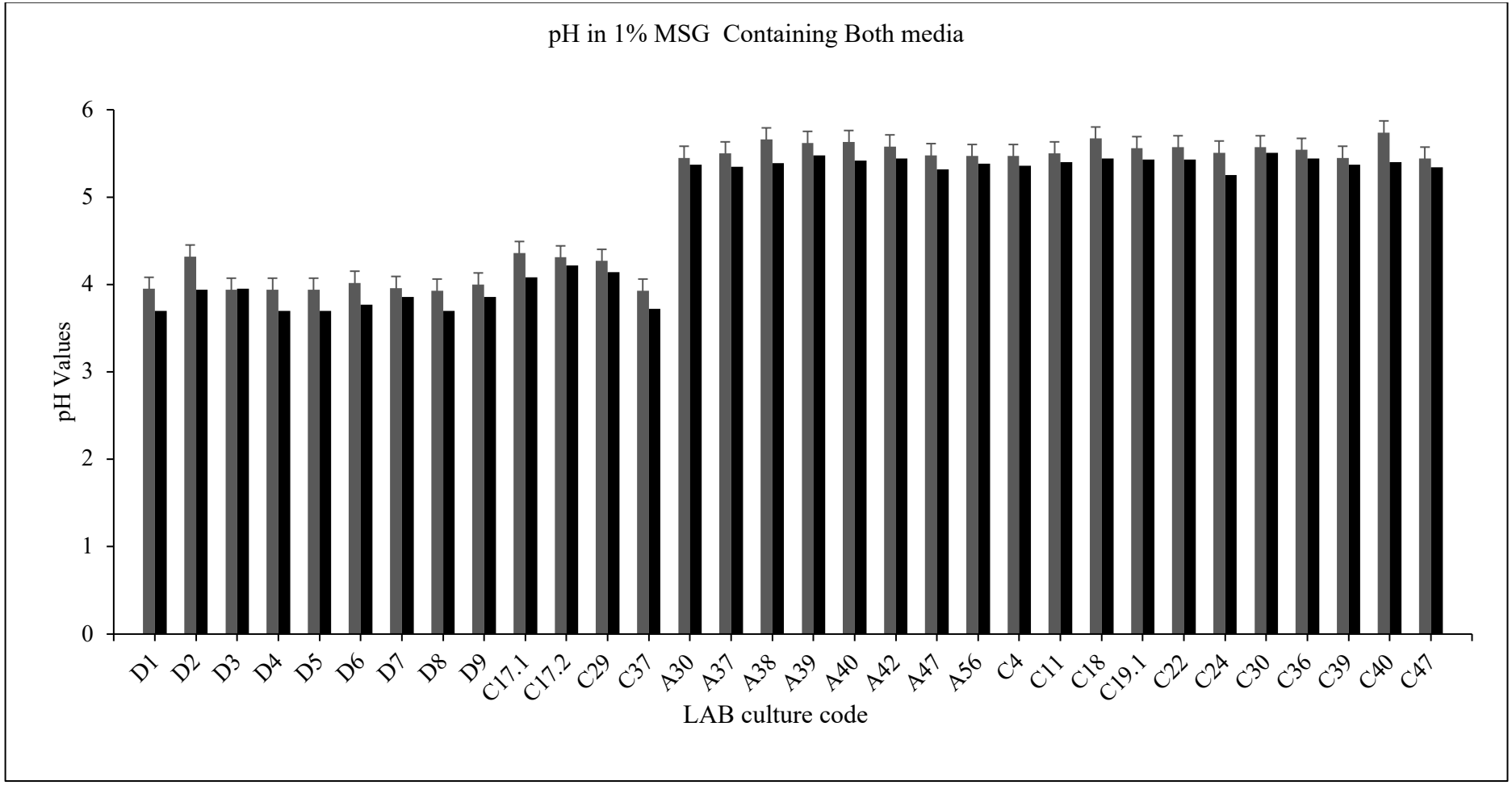


Figure 3.14. Acidification of GABA producer LAB strains in MRS or M17 broth medium (black lines) and 1% MSG containing MRS or M17 medium (grey line) after 24 h incubation at 37 °C, D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D3, D8, C29: *L. curvatus*, D9, C17–1, C17–2: *L. brevis,* A30–A47: *Enterococcus ssp.,* C4–C46: *Enterococcus ssp.*

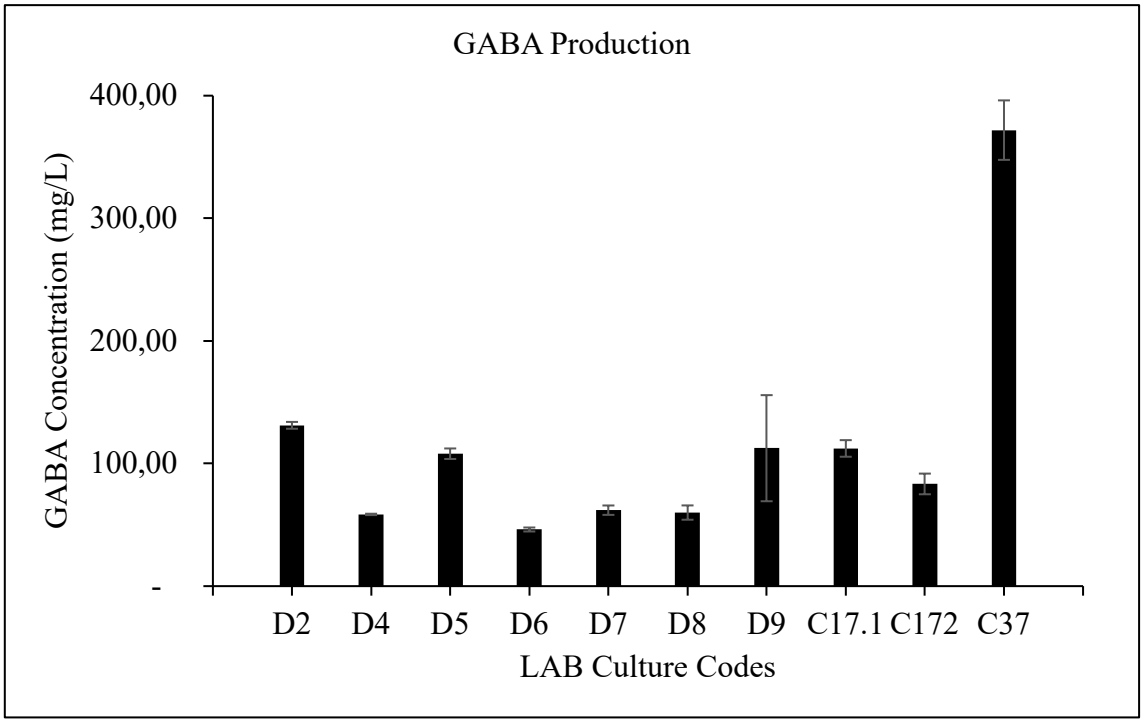


Figure 3.15. GABA producer LAB strains in MRS or M17 broth medium (black lines) and 1% MSG containing MRS or M17 medium (grey line) after 24 h incubation at 37 °C, D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D8: *L. curvatus*, D9, C17–1, C17–2: *L. brevis.*

Table 3.7. GABA Concentration (mg/L) in MRS broth (1% MSG) at 24 h, 37 °C, , D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D8: *L. curvatus*, D9, C17–1, C17–2: *L. brevis.*

| No | Code | Species | GABA (mg/L) ± SD | | |
|---|---|---|---|---|---|
| 1 | D2 | *L. plantarum* | 131,2 | ± | 2,8 |
| 2 | D4 | *L. plantarum* | 58,5 | ± | 0,6 |
| 3 | D5 | *L. plantarum* | 108,0 | ± | 4,3 |
| 4 | D6 | *L. plantarum* | 46,2 | ± | 1,6 |
| 5 | D7 | *L. plantarum* | 62,0 | ± | 3,8 |
| 6 | D8 | *L. curvatus* | 60,0 | ± | 5,8 |
| 7 | D9 | *L. plantarum* | 112,5 | ± | 43,3 |
| 8 | C17.1 | *L. brevis* | 112,3 | ± | 6,8 |
| 9 | C172 | *L.brevis* | 83,4 | ± | 8,4 |
| 10 | C37 | *L. plantarum* | 371,9 | ± | 24,2 |

### 3.3.4. Fermentation Abilities of GABA Producing LAB in Dairy Models

Since GABA producing LAB could not grow in skim milk media, several compounds were added to initiate their growth. The addition of glucose or yeast extract was necessary for their growth in this food matrix. While they were previously isolated from artisanal cheese samples, it is possible that these strains were found in cheese after ripening period, their growth in cheese could be a result of the action of other microorganisms; these *L. brevis, L. curvatus or L. plantarum* strains could be able to grow after lactose is conversed into glucose. In addition, several studies used glucose and yeast extract addition to increase GABA production (Wang et al. 2018; Park et al. 2021).

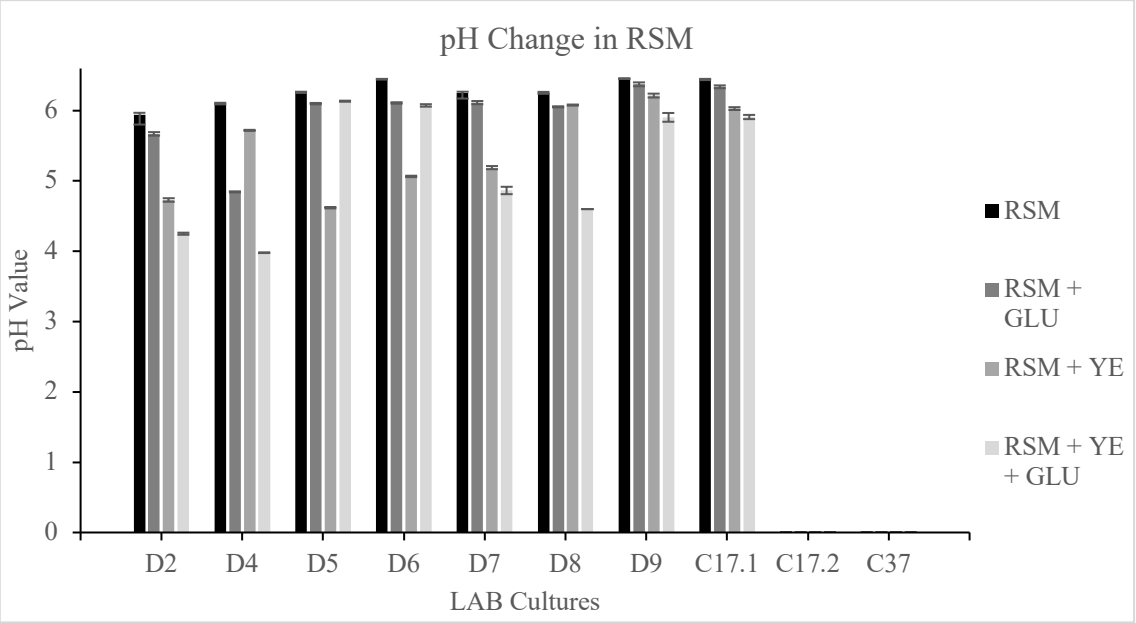


Figure 3.16a. pH change in FM samples after 24 h at 37 C, inoculum rate 1%

Although the increase in inoculum level was used as a strategy, it did not found effective without glucose or yeast extract (Figure 3.13.b). Only *L. plantarum* D2 had ability to reduce pH under 4.6 after 24h fermentation. Based on these, selected LAB strains were used in skim milk or whey media containing 1% yeast extract and glucose. Also 5% inoculum ratio was selected for further studies.

On the other hand, as observed by other studies, in the literature, desirable pH values for the GABA generation by *L. brevis* strains showed a broad range of spectrum; while strain NCL912 needs pH 5.0 as optimum value, it could be high as pH 6.8 for DPC6108 or low as pH 4.7 for strain for PM17 (Siragusa et al. 2007; S. Li et al. 2022). On the other hand, formation of GABA in *L. helveticus* can be optimum at pH range between 3.5– 4.6 pH values and long as much as 30h and 120h, respectively It was found that *Lb. rhamnosus* strains needed long time such as 120 h at pH4.7 for excessive GABA concentration. Interestingly, low pH (pH value 4.0) created best environmental factor for GABA production in 24 h for *Lc. lactis ssp. lactis* PU1 . Therefore, it can be concluded that pH value is important factor for the generation of GABA. While it is strain depended, it should be lower than 5.0 pH value in most cases. Even though LAB grow in food matrix in high numbers, it does not necessarily indicate GABA production if pH is not low enough to trigger action of GAD enzyme by causing a stress factor.

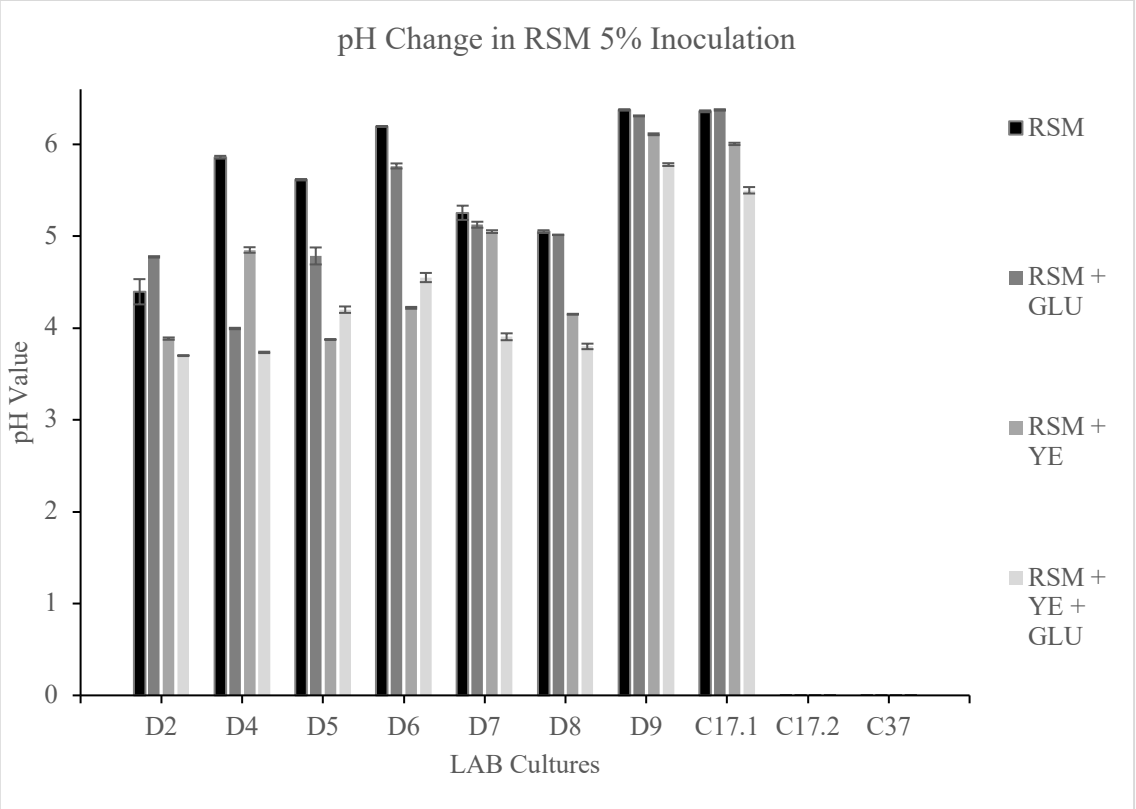


Figure 3.16b. pH change in FM samples after 24 h at 37 C, inoculum rate 5%

### 3.3.5. Fermentation of RSM by GABA Producing Lactobacillus Species

*Enterococcus* ssp. C18 and C24 isolates produced highest GABA concentration in M17 broth containing 1% MSG. Therefore, they were selected to ferment dairy medium. For this purpose,reconstituted skim milk was used. However, milk individually fermented by C18, C22 or milk fermented by co-cultures C18 and bTY8, C18 and bTY14a, C22 and bTY8, C22 and bTY14a did not contain GABA after 24 h incubation at 37 °C. Although co–cultures were prepared together with high proteolytic *L. delbrueckii ssp. bulgaricus*, their contribution on GABA production of C18 and C22 could not be observed. Since *Enterococcu*s ssp. are not in and their high producer isolates could not secrete GABA in dairy medium, the usage of *Enterococcus* species are not involved in further studies. This could be found associated with their low growth in RSM medium compared to MRS broth medium.

### 3.3.6. Fermentation of Whey by GABA Producing Lactobacillus Species

*L. brevis* and *L. plantarum* species are known for their non–proteolytic characteristics. The prevalence of cell envelope proteinases in LAB is not high; therefore, these LAB species are non-proteolytic because of their genomes. For example, *L. brevis* is one of these species, due to their natures their ability to growth and ferment the dairy matrix is not likely possible . On the other hand, due to their important metabolites such as GABA, strategies have been developed on how to utilize *L. brevis* as a starter microorganism with proteolytic properties . A study showed that while *L. brevis* has genes for GABA production and can produce GABA, it could not ferment milk after 24 h incubation and pH value was almost high as unfermented milk. However, the researchers co-cultured *L. brevis* strain with various *S. thermophilus* strains for both fermentation and GABA production in milk (Q. Wu, Law, and Shah 2015). In a current study, a *L. plantarum* Y7 has been focused to whether it could be a starter LAB for the generation of fermented milk with GABA; however, this stains could not transform GABA in different temperature conditions. On the other hand, thanks to the co–culture with *S.*

*thermophilus, L. plantarum* produced GABA in fermented milk. The findings of authors were similar to *L. reuteri* studies, although *L. plantarum* strains have ability to form GABA, their low proteolytic properties are the limiting factor for their application in dairy matrix (Kim et al. 2022) Another recent study showed *gad* gene in *L. brevis* strain however it did not produce any GABA in milk during 48 h fermentation and could not ferment lactose. When it was co–cultured with *S. thermophilus,* it produced approximately 0.2g/L GABA in milk medium (Xiao and Shah 2022).

In this thesis, GABA production was detected in the range of 5.51-20.97 mg/L. Based on the GABA concentration in broth media and growth off LAB in whey media, it could be said that food matrix was important factor for the GABA generation. Therefore, the matrix could be an effect for the production of this stress associated microbial compound, GABA. This could be an explanation for the lower GABA concentrations (mg/L) in fermented whey samples (Figure 3.16, Table 3.8).

In literature, *L. plantarum* fermented whey beverage generated 195.5 ppm GABA after optimization of fermentation process and 3 days long fermentation steps (Zarei et al. 2018). On the contrary, production of GABA were increased in dairy medium compared to broth media for various strains of both *L. plantarum and L. brevis* species . Recently, a study explored GABA production in fermented whey and also investigated their ACE inhibition activities. The authors found that *P. pentosaceus* ENM104 and *L. plantarum* SPS109 resulted in 3.91 ± 0.10 µg/ml GABA and 36.07 ± 3.94% ACE inhibition activity after 3 days fermentation of whey (Jitpakdee et al. 2022)

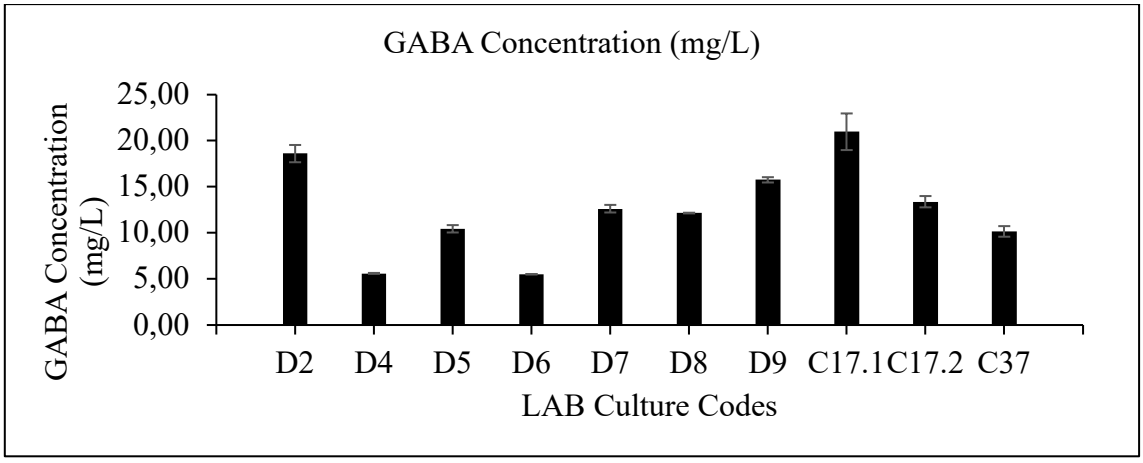


Figure 3.16. GABA producer LAB strains in MRS or M17 broth medium (black lines) and 1% MSG, 1% YE , 1%glucose containing whey, 24 h fermentation at 37 °C..

Table 3.8. GABA Concentration (mg/L) in Whey Containing 1% MSG, 1% YE, 1% Glucose at 24 h, 37 °C.

| No | **Code** | *Species* | GABA Concentration (mg/L) ± SD |
|---|---|---|---|
| 1 | **D2** | *L. plantarum* | 18,6 ± 0,9 |
| 2 | **D4** | *L. plantarum* | 5,6 ± 0,0 |
| 3 | **D5** | *L. plantarum* | 10,4 ± 0,4 |
| 4 | **D6** | *L. plantarum* | 5,5 ± 0,0 |
| 5 | **D7** | *L. plantarum* | 12,6 ± 0,4 |
| 6 | **D8** | *L. curvatus* | 12,2 ± 0,0 |
| 7 | **D9** | *L. plantarum* | 15,8 ± 0,3 |
| 8 | **C17.1** | *L. brevis* | 21,0 ± 2,0 |
| 9 | **C17.2** | *L.brevis* | 13,4 ± 0,6 |
| 10 | **C37** | *L. plantarum* | 10,2 ± 0,6 |

### 3.3.6.1. pH Values in Fermented Whey Beverage Bases

Figure 3.18 showed the pH values after whey fermentation, pH values were found higher in fermented whey base compared to fermented milks under the same conditions (data not shown). However, these values were found highly close to the range to trigger GABA production. On the other hand, the pH range in broth media was much lowe up to 3.3 pH values. This also indicates LAB could not reduce pH much lower by generating organic acids. Since these LAB isolates needed glucose to grow in skim milk and whey media, it indicates low lactic acid production in fermentation medium.

## 3.3.7. Enumeration of LAB in Fermented Whey and Milks

All GABA producing LAB had viable counts in the range of 8.6–9.6 log CFU/ml, except D6. Viable cell number of *L. plantarum D6* in fermented whey was detected as 7.5 log CFU/ml (Figure 3.19).

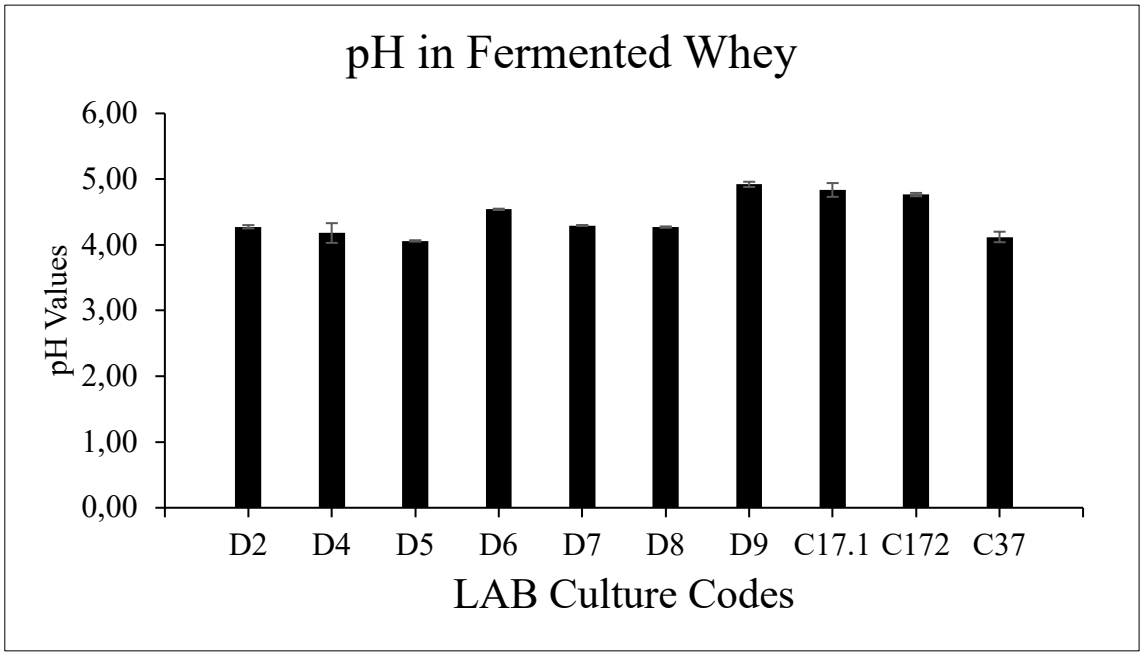


Figure 3.18. pH values in Fermented Whey Containing 1% MSG, 1% YE, 1% Glucose at 24 h, 37 °C; D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D8: *L. curvatus*, D9, C17–1, C17–2: *L. brevis.*

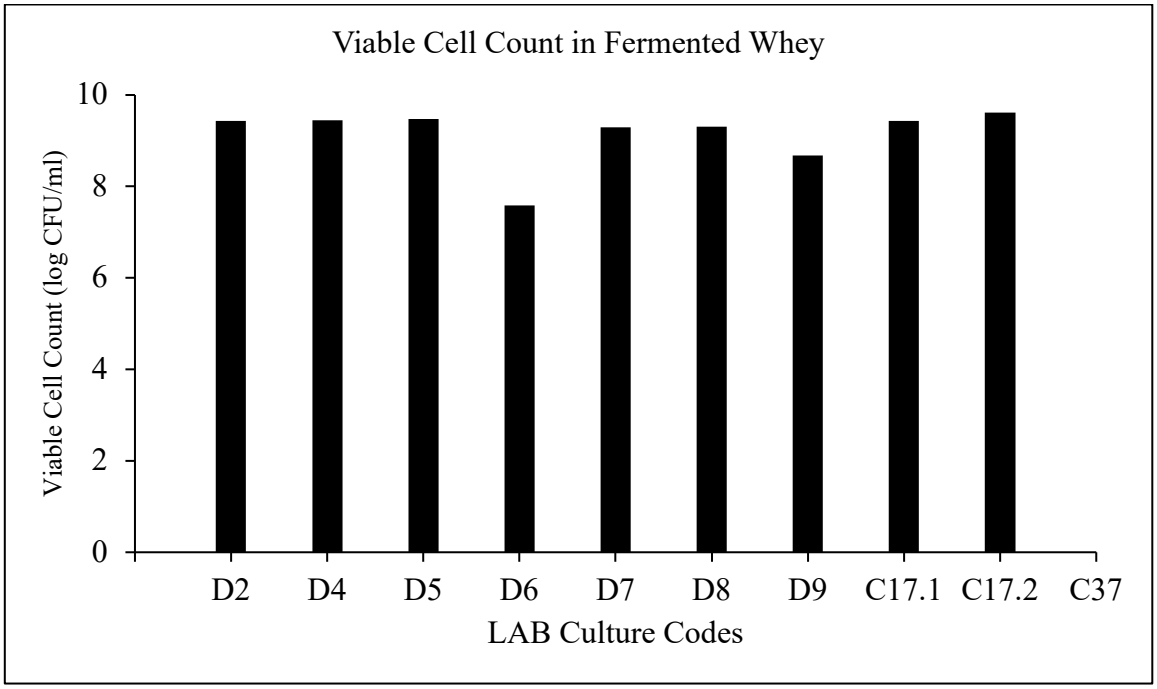


Figure 3.19. Growth (log CFU/ml) of GABA producer LAB strains in fermented whey containing 1% MSG, 1% YE and 1% glucose, after 24 h incubation at 37 °C D9, C17.1, C17.2D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D3, D8, C29: L. curvatus, D9, C17.1, C17.2: *L. brevis*.

## 3.8. HPLC Analysis of ACE inhibitor Activity in Fermented Whey Beverages

All of ten GABA producing LAB showed above 80% ACE inhibitory activities in fermented milks. This could be also associated with the effect of high inoculum rate (5%, v/v) (Figure 3.20, Table 3.9). While ACE inhibitor activities in various food matrices have been well-studied, limited number of research focused on whey. A screening study focused on 34 LAB for their antihypertensive activities in whey media after 24 h incubation and demonstrated that the ACE inhibition activities varied from 52.40% to 84.70% (Daliri et al. 2018). Another research determined that 3 *L. plantarum* strains represent in the range of 67–85% ACE inhibitor activity formation in whey media .(Luz et al. 2018). Another screening research focused on 25 strains of different LAB species and found that some of these strains did not form any inhibitor activity in milks, whereas others leaded 4-74% inhibitor activity after 44 h period (Pihlanto, Virtanen, and Korhonen 2010).

Table 3.9. GABA Concentration (mg/L) in Whey Containing 1% MSG, 1% YE, 1% Glucose at 24 h, 37 °C. D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D3, D8, C29: *L. curvatus*, D9, C17.1, C17.2: *L. brevis.*

| LAB strains | % ACE inhibition | ±SD |
|---|---|---|
| D2 | 79 | 0,4 |
| D4 | 81 | 0,0 |
| D5 | 82 | 0,1 |
| D6 | 90 | 0,1 |
| D7 | 83 | 0,7 |
| D8 | 81 | 0,2 |
| D9 | 97 | 0,1 |
| C17.1 | 98 | 0,1 |
| C17.2 | 94 | 1,8 |
| C37 | 92 | 0,2 |

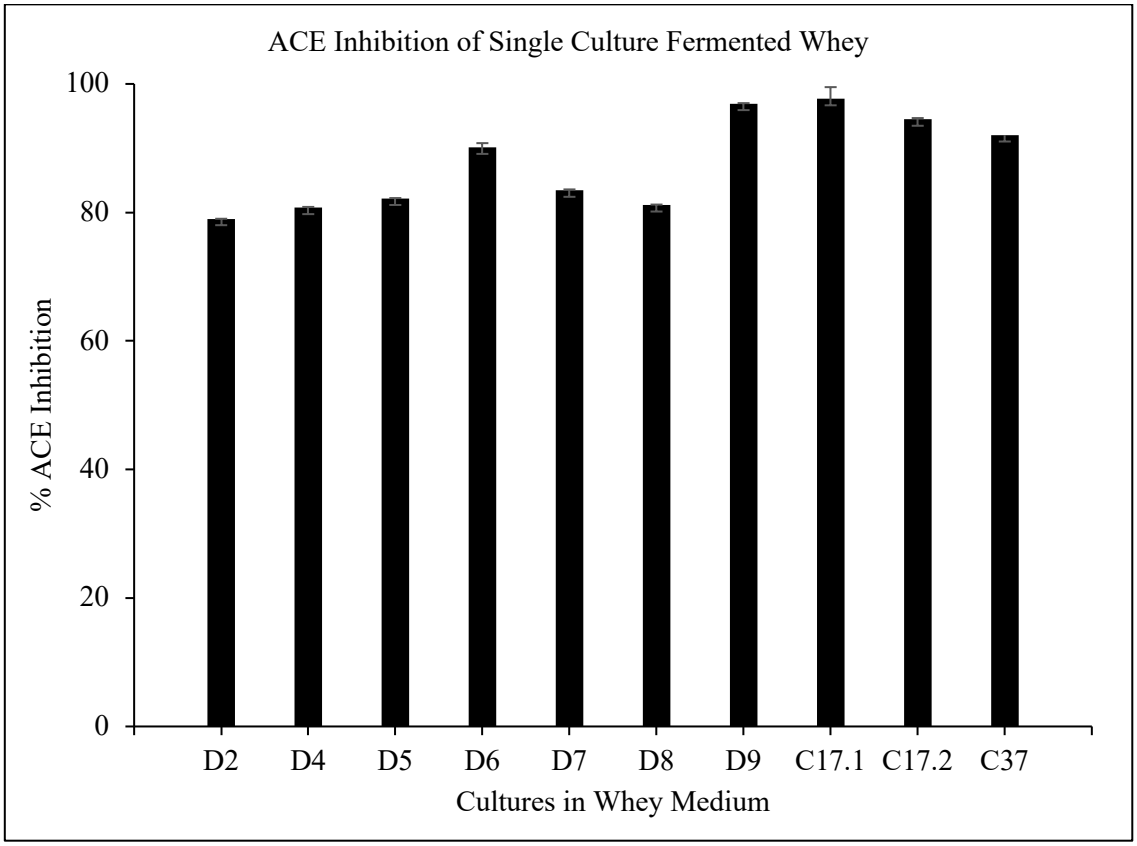


Figure 3.20. Growth (log CFU/ml) of GABA producer LAB strains in fermented whey containing 1% MSG, 1% YE and 1% glucose, after 24 h fermentation at 37 °C; D1, D2, D4, D5, D6, D7, C37: *L. plantarum*, D3, D8, C29: *L. curvatus*, D9, C17.1, C17.2: *L. brevis.*

## 3.9. 16S rRNA identification of GABA Producing LAB

Previously isolated GABA producers were identified by 16S rRNA method. D2 and D4 were previously identified as *L. casei* and D1, D3, D5, D6, D7, D8, D9 and C37, C17.1, C17.2 could only be identified as Lactobacillus ssp. (Bulut, 2005). In that study PCR–RAPL method and biochemical tests were used for the identification of LAB isolates. On the other hand, D1, D2, D4, D5, D6, D7, C37 were identified *as L. plantarum*; D9, C17.1 and C17.2 were identified as *L. brevis*; D3, D8 and C29 were identified as *L. curvatus* by 16s rRNA method. Blast results between reference cultures indicated the homological similarity between 95–100% (Table 3.10).

Table 3.10a. Identification of cheese isolates using BLAST tool

| | GABA producers | Isolate Code | Genetic identification (16s rRNA) | Reference LAB | 16s RNA Accession no | Query Covarege | Identity (%) |
|---|---|---|---|---|---|---|---|
| **1** | + | D1 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain CIP 103151* | NR_104573.1 | 100% | 98% |
| **2** | + | D2 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain CIP 103151* | NR_104573.1 | 97% | 98% |
| **3** | + | D3 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain CIP 103151* | NR_104573.1 | 96% | 98% |
| **4** | + | D4 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain JCM 1149* | NR_117813.1 | 99% | 98% |
| **5** | + | D5 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain CIP 103151* | NR_104573.1 | 97% | 98% |
| **6** | + | D6 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain JCM 1149* | NR_117813.1 | 97% | 98% |
| **7** | + | D7 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain JCM 1149* | NR_117813.1 | 97% | 98% |
| **8** | + | D8 | *Latilactobacillus curvatus* | *Latilactobacillus curvatus strainNBRC 15884* | NR_113334.1 | 98% | 97% |
| **9** | + | D9 | *Levilactobacillus brevis* | *Levilactobacillus brevis ATCC 14869 = DSM20054* | NR_116238.1 | 96% | 98% |
| **10** | – | C3 | *Latilactobacillus curvatus* | *Latilactobacillus curvatus strain NBRC 15884* | NR_113334.1 | 94% | 96% |
| **11** | – | C7 | *Latilactobacillus curvatus* | *Latilactobacillus curvatus strain NBRC 15884* | NR_113334.1 | 97% | 96% |
| **12** | – | C9 | *Latilactobacillus curvatus* | *Latilactobacillus curvatus strain NBRC 15884* | NR_113334.1 | 96 | 96 |
| **13** | + | C17–1 | *Levilactobacillus brevis* | *Levilactobacillus brevis ATCC 14869 = DSM 20054* | NR_116238.1 | 99% | 97% |
| **14** | + | C17–2 | *Levilactobacillus brevis* | *Levilactobacillus brevis strain ATCC 14869* | NR_044704.2 | 94% | 95% |
| **15** | + | C29 | *Latilactobacillus curvatus* | *Latilactobacillus curvatus strain NBRC 15884* | NR_113334.1 | 97% | 98% |
| **16** | + | C30 | *Enterococcus faecium* | *Enterococcus faecium strain DSM 20477* | NR_114742.1 | 99% | 97% |
| **18** | – | C32 | Lactococcus lactis | Lactococcus lactis strain NCDO 604 | NR_040955.1 | 99% | 97% |
| **19** | + | C37 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain CIP 103151* | NR_104573.1 | 97% | 97% |
| **20** | + | C39 | *Enterococcus durans* | *Enterococcus durans strain JCM 8725* | NR_113257.1 | 99% | 99% |
| **21** | + | C40 | *Enterococcus durans* | *Enterococcus durans strain JCM 8725* | NR_113257.1 | 99% | 95% |
| **22** | – | C42 | *Enterococcus durans* | *Enterococcus durans strain JCM 8725* | NR_113257.1 | 91% | 98% |
| **23** | – | C47 | *Lactiplantibacillus plantarum* | *Lactiplantibacillus plantarum strain CIP 103151* | NR_104573.1 | 96% | 98% |

Table 3.10b. Identification of cheese isolates using BLAST tool

| **PCR numbers** | **GABA producers** | **Isolate Code** | **Genetic identification (16s rRNA)** | **Reference LAB** | **Accession numbers for 16 s RNA** | **Query Covarege** | **Identity (%)** |
|---|---|---|---|---|---|---|---|
| **24** | + | A39 | *Enterococcus faecalis* | *Enterococcus faecalis strain NBRC 100481* | NR_113902.1 | 99% | 98% |
| **25** | + | A42 | *Enterococcus faecium* | *Enterococcus faecium strain NBRC 100486* | NR_113904.1 | 94% | 96% |
| **26** | – | A53 | *Enterococcus ssp.* | *Enterococcus faecalis strain NBRC 100481* | NR_113904.1 | 94% | 96% |
| **7** | + | A56 | *Enterococcus faecium* | *Enterococcus faecium strain NBRC 100486* | NR_113904.1 | 97% | 98% |
| **28** | – | A64 | *Enterococcus durans* | *Enterococcus duransstrain JCM 8725* | NR_113257.1 | 94% | 97% |
| **29** | – | A65 | *Enterococcus durans* | *Enterococcus durans strain 98D* | NR_036922.1 | 96% | 97% |
| **30** | – | A69 | *Enterococcus durans* | *Enterococcus durans strain JCM 8725* | NR_113257.1 | 61% | 97% |

# CHAPTER 4

# CONCLUSIONS

In this thesis, potential metabolites of LAB formed during milk and whey fermentation was investigated for their antihypertensive properties. Cheese isolates had ability to produce GABA in both broth media and whey media. On the other hand, yogurt isolates showed proteolytic activities in milk media and produce high ACE inhibitor activities in fermented milks. Although GABA producer strains exhibits high ACE inhibitor activity in whey media, their proteolytic activity in casein containing milk media were not found sufficient. While *L. delbrueckii ssp. bulgaricus* species were effective on milk-based proteins, cheese strains could also show proteolytic activities on other food proteins such as plant based proteins. Thus, the results obtained by screening LAB collection for their proteolytic activities could be useful in future plant-based fermentations. Similarly, it is concluded that LAB strains could generate high concentration of GABA, but the food matrix is also important for the productivity. Hence, it is worth to note that LAB isolates used in this thesis, need specific energy sources for their biomass growth and GABA production in selected food matrices. In addition, although fermented whey bases possessed lower GABA concentrations than clinical RCT studies, their ACE inhibitor activities were also enhanced during fermentation. Therefore, combining effect of both GABA and BP showing ACE inhibitor activity could be significant in further studies. In addition to GABA content in fermented whey bases, these microorganisms can adhere in the gut such a GABA producing LAB being as probiotic. Therefore, further studies could also focus on their adherence capacities *in vitro* cell culture models and their other potential probiotic properties. Since antihypertensive activity of food products could not be determined by *in vitro* cell culture studies. Further studies on spontaneously hypertensive rats or patients having elevated BP should be considered to claim the effectiveness of fermented whey.

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# APPENDICES

## APPENDIX A. The Standard Curves Used for The Spectrophotometric Methods

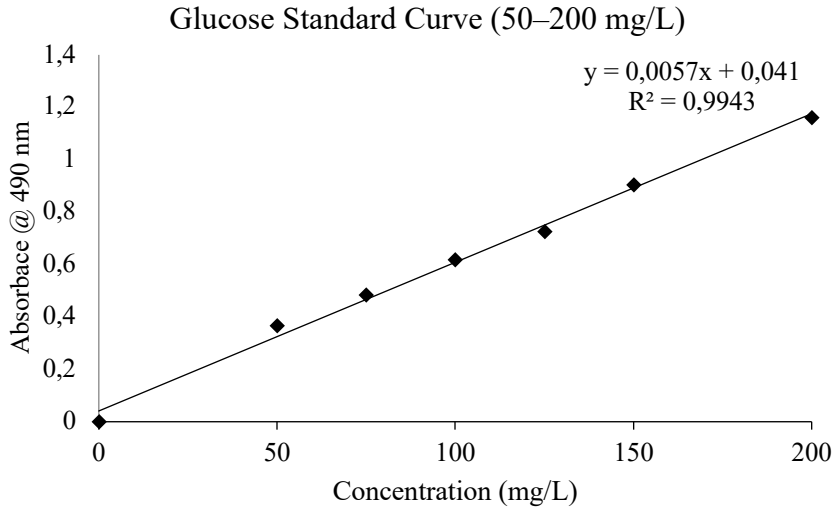


Figure A1. The standard curve prepared with glucose for determination of EPS content in whey. y = 0,0057x + 0,041 where y is absorbance value at 490 nm and x is the concentration of glucose, respectively.

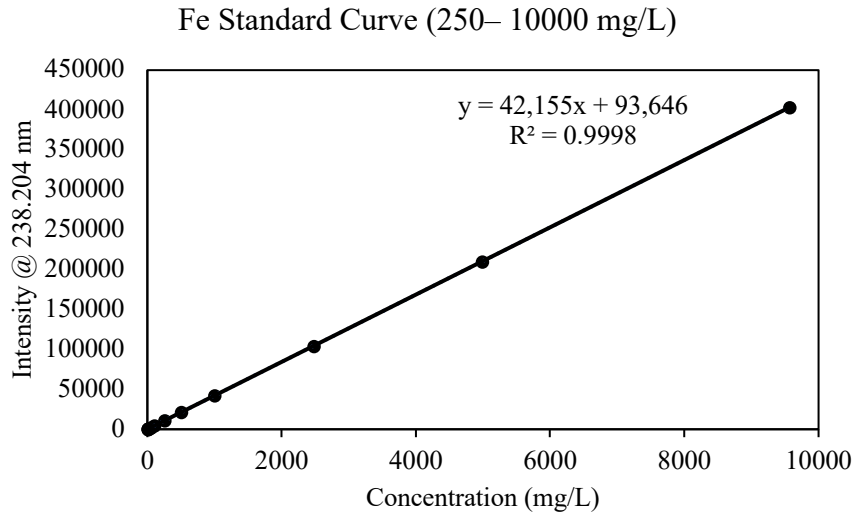

Figure A2. The standard curve prepared with mineral Fe for determination of Fe content in whey. y = 42,155x + 93,646 where y is absorbance value at 238.204 nm and x is the concentration of Fe, respectively.

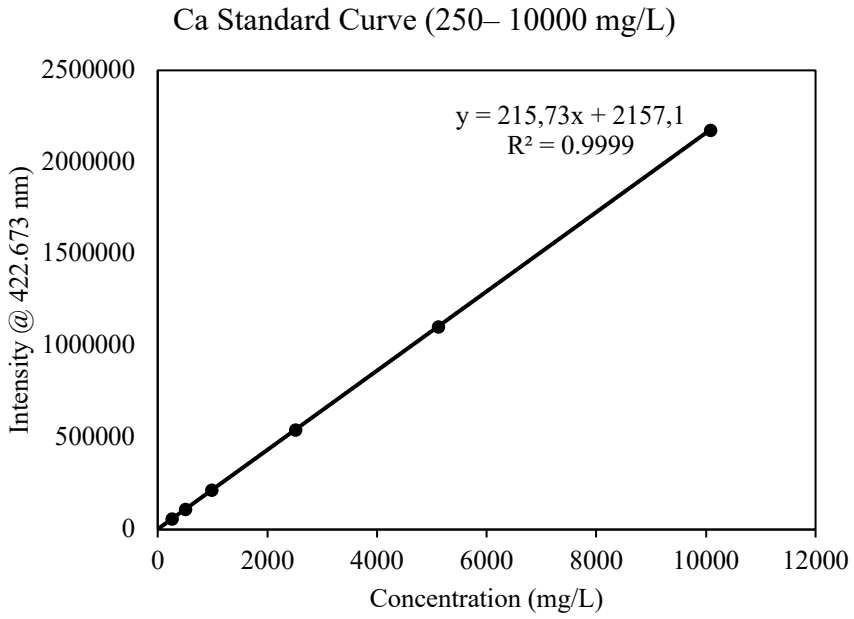


Figure A3. The standard curve prepared with mineral Ca for determination of Ca content in pasteurized and unpasteurized whey. y=215,73x + 2157,1 is absorbance value at 422.673 nm and x is the concentration of Ca, respectively.

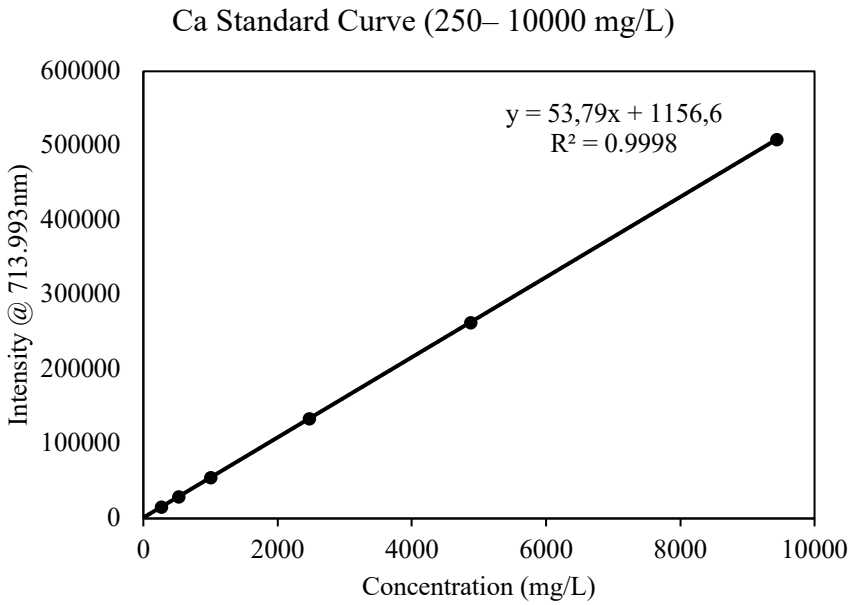


Figure A4. The standard curve prepared with mineral Ca for determination of Ca content in pasteurized whey. y = 53,79x + 1156,6is absorbance value at 713.993 nm and x is the concentration of Ca, respectively.

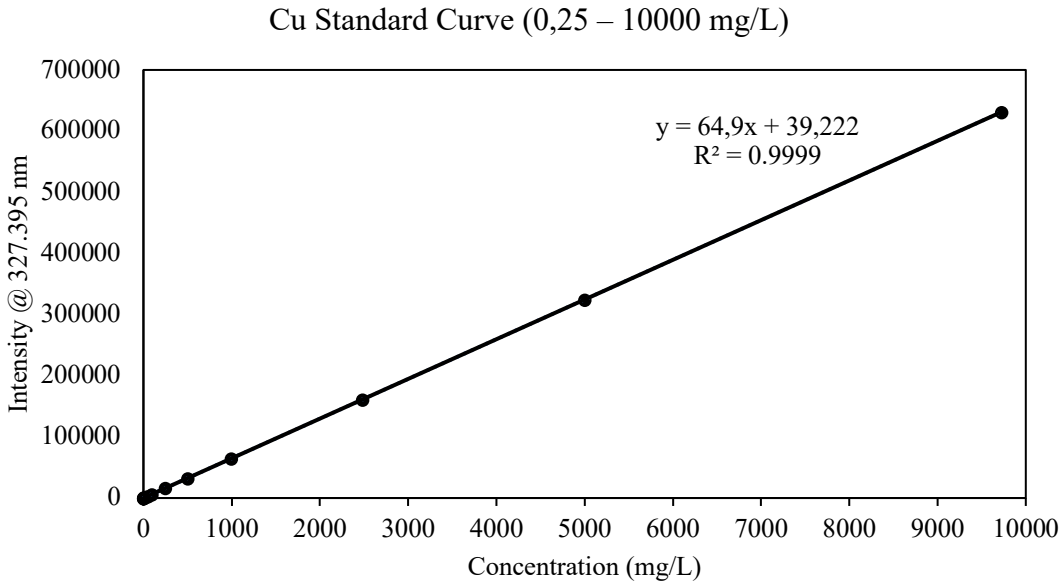


Figure A5. The standard curve prepared with mineral Cu for determination of Cu content in unpasteurized and unpasteurized whey. y = 64,9x + 39,222, y is absorbance value at 327.395 nm and x is the concentration of Cu, respectively.

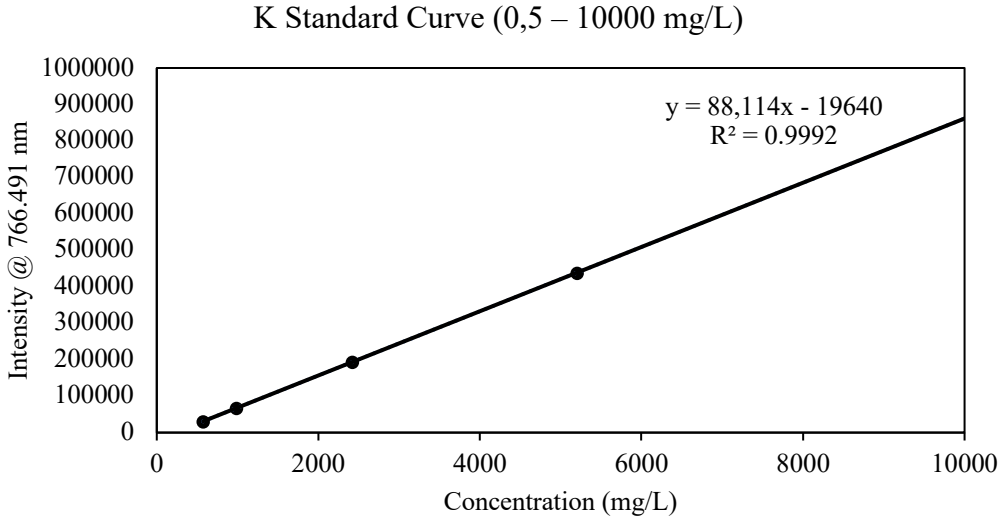


Figure A6. The standard curve prepared with mineral K for determination of K content in pasteurized and unpasteurized whey. y = 88,114x – 19640, y is absorbance value at 766.491 nm and x is the concentration of K, respectively.

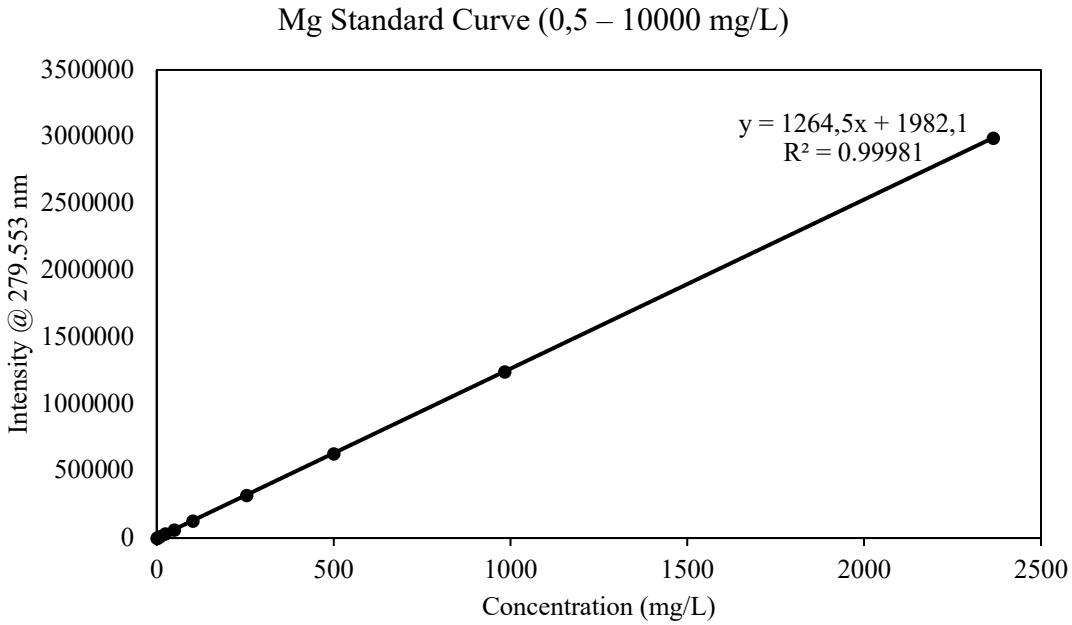


Figure A7. The standard curve prepared with mineral Mg for determination of Mg content in pasteurized and unpasteurized whey. y = 1264,5x + 1982,1, y is absorbance value at 729.553 nm and x is the concentration of Mg, respectively.

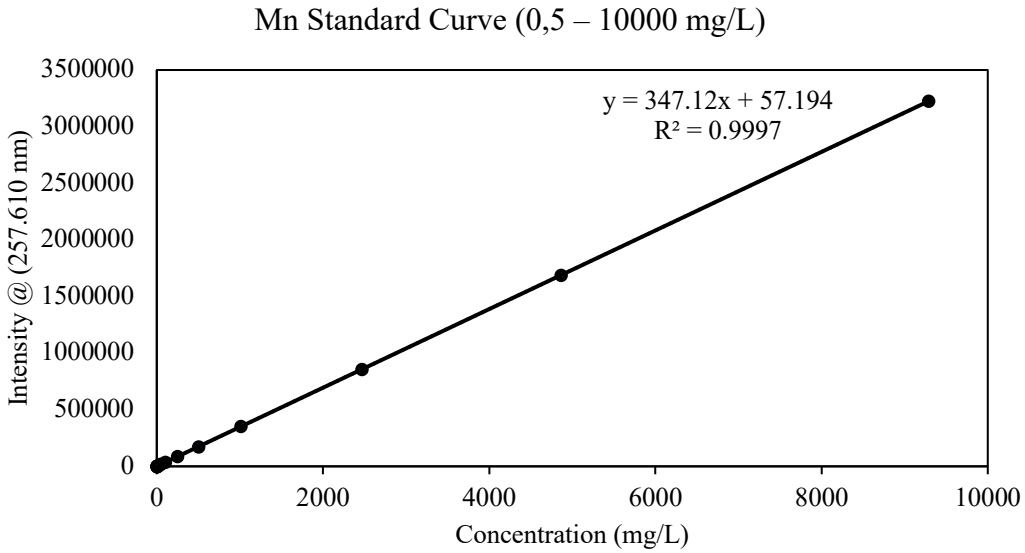


Figure A8. The standard curve prepared with mineral Mn for determination of Mn content in pasteurized and unpasteurized whey. y = 347.12x + 57.194y is absorbance value at 257.610 nm and x is the concentration of Mn, respectively.

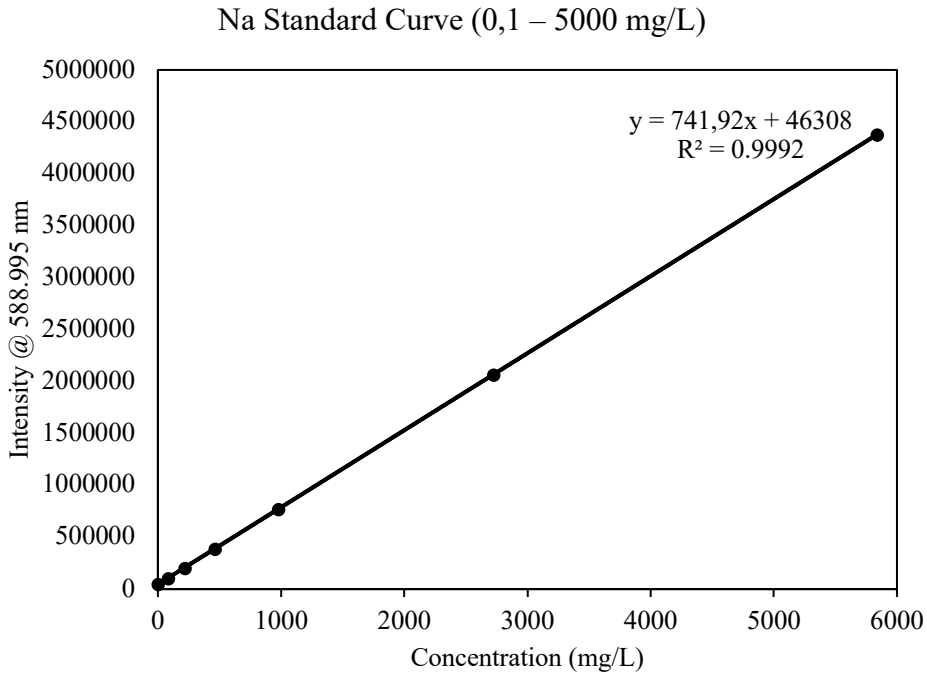


Figure A9. The standard curve prepared with mineral Na for determination of Na content in pasteurized and unpasteurized whey. y = 741,92x + 46308, y is absorbance value at 588.595 nm and x is the concentration of Na, respectively.

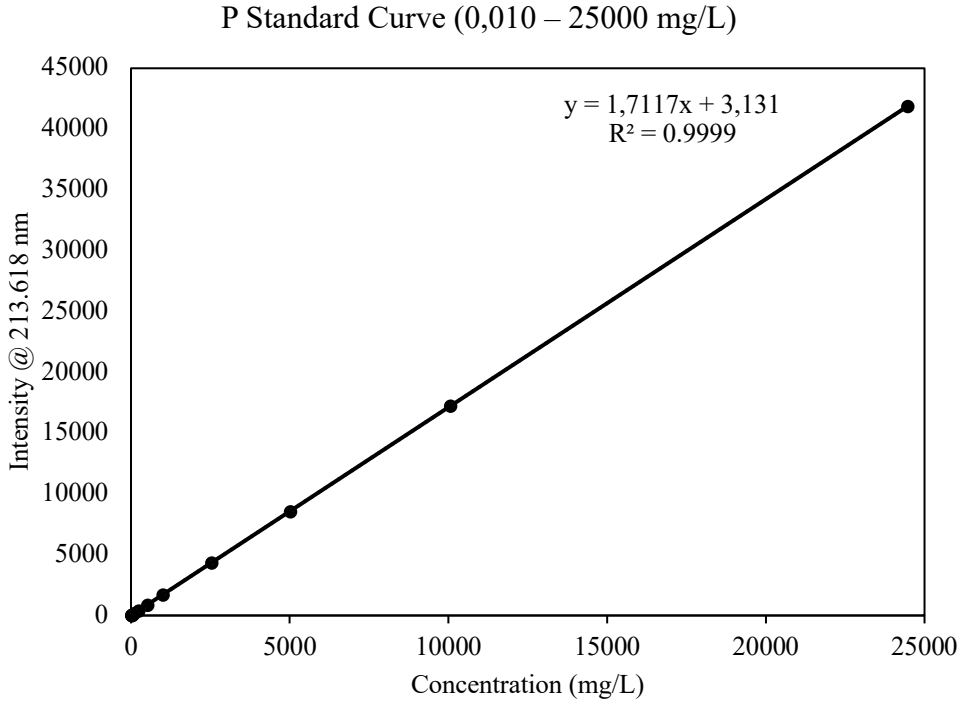


Figure A10. The standard curve prepared with mineral P for determination of P content in pasteurized and unpasteurized whey. y = 1,7117x + 3,131, y is absorbance value at 213.618 nm and x is the concentration of P, respectively.

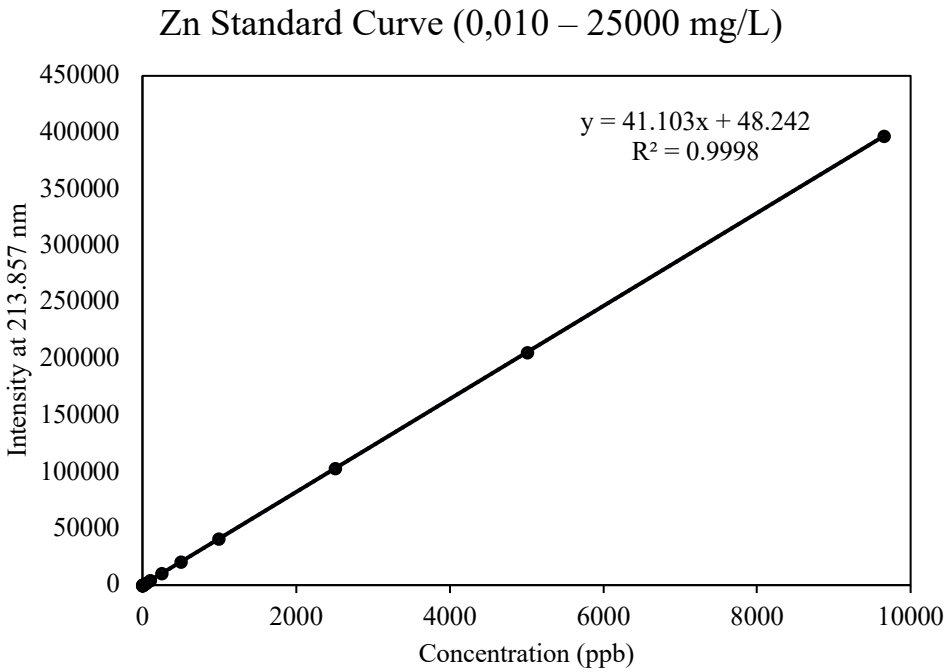


Figure A11. The standard curve prepared with mineral Zn for determination of Zn content in pasteurized and unpasteurized whey. y = 41.103x + 48.242, y is absorbance value at 213.618 nm and x is the concentration of Zn, respectively.

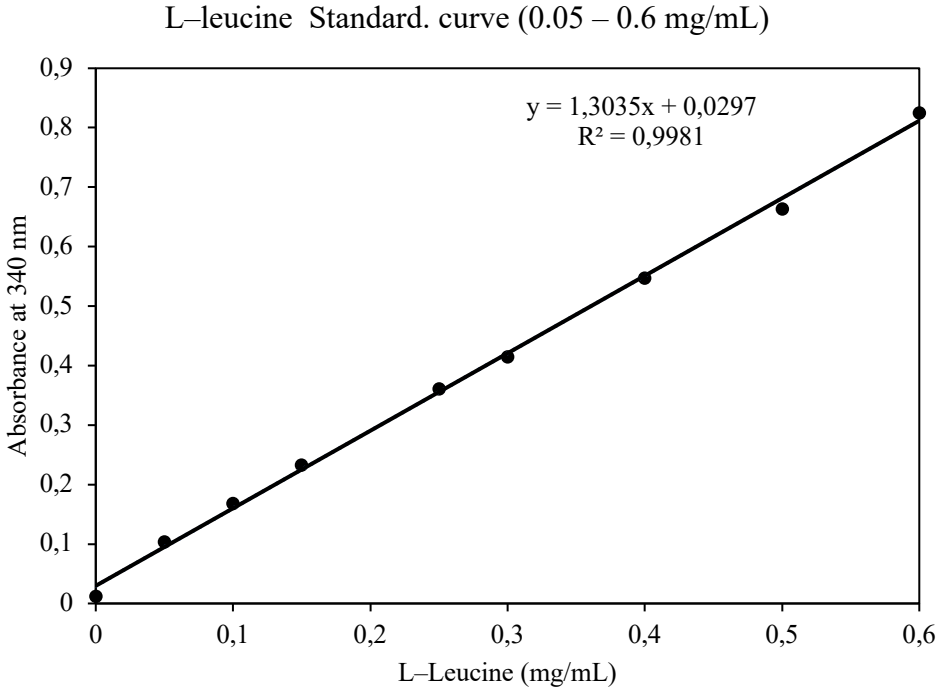


Figure A12. The standard curve prepared with L–leucine for determination of the degree of hydrolysis in terms of L–leucine in fermented milk and fermented whey beverages. content in whey. y = 1,3035x + 0,0297, where y is absorbance value at 340 nm and x is the concentration of L–leucine, respectively.

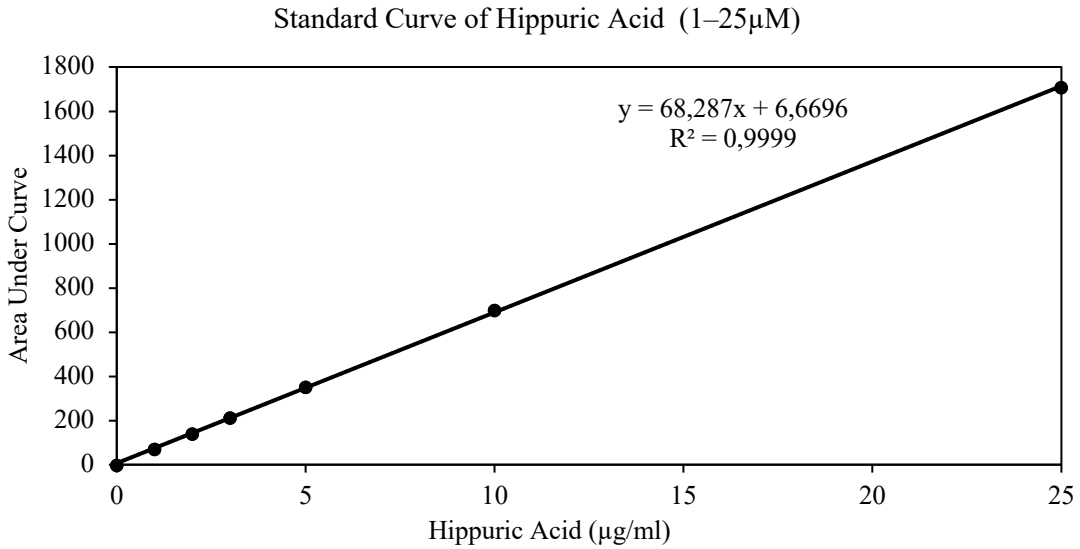


Figure A13. The standard curve of the ACE activity product, HA (1 – 25 µM) for determination of HA concentration in ACE enzymatic reaction mixture prepared by addition of sample from fermented milk and whey beverages, y = 68,287x + 6,6696, where y is the area under curve obtained by HPLC analysis at 228 nm and x is the concentration of HA, respectively.

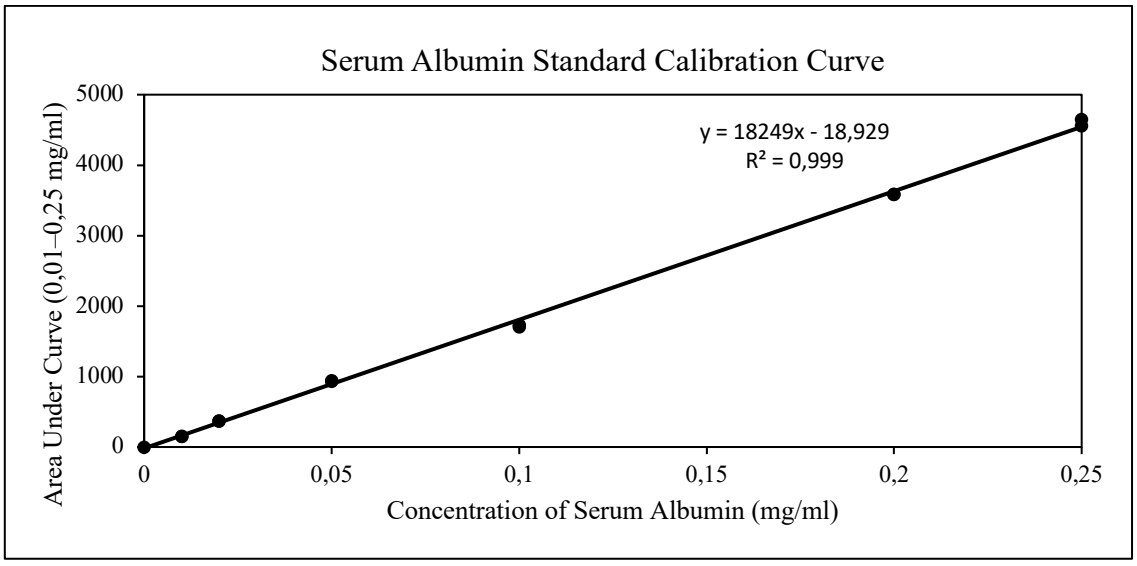


Figure A13. The standard curve of the serum albumin (0,01–0,25 mg/ml) for determination of whey raw material and fermented whey beverages obtained by HPLC analysis. Mobile phase 80% Acetonitrile in ultra–pure water containing 0.05 TFA, injection volume 20 µl, flow rate 1 ml/min, UV detection at 210 nm.

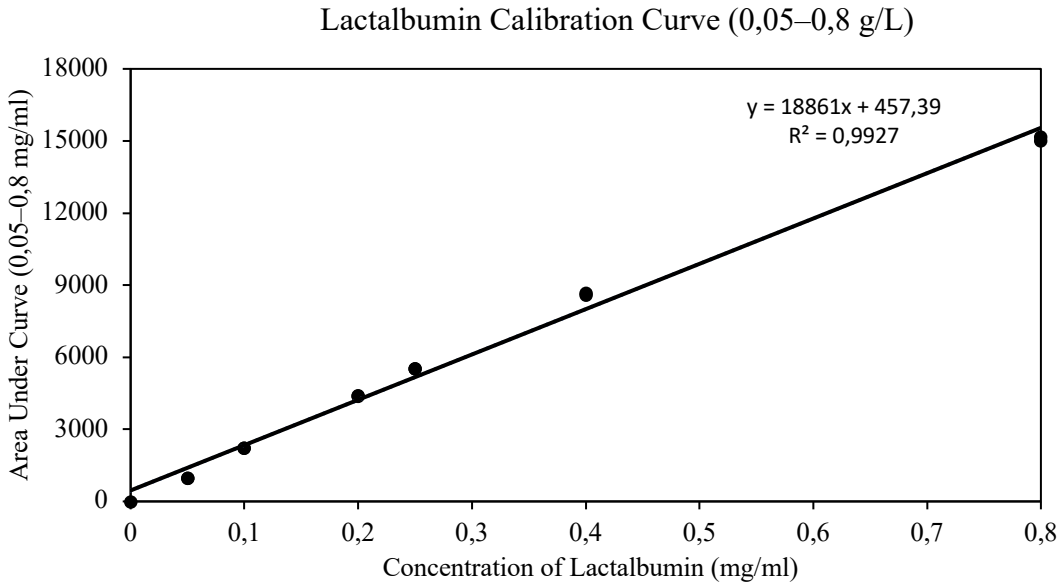


Figure A14. The standard curve of the α–lactalbumin (0,01–0,25 mg/ml) for determination of whey raw material and fermented whey beverages obtained by HPLC analysis. Mobile phase 80% Acetonitrilin ultra–pure water containing 0.05 TFA, injection volume 20 µl, flow rate 1 ml/min, UV detection at 210 nm.

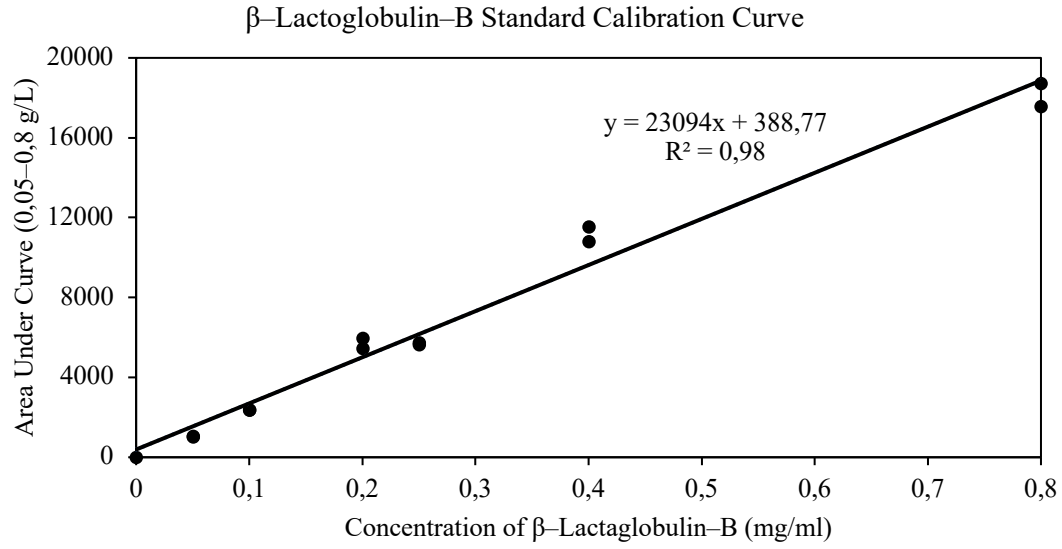


Figure A15. The standard curve of the β–Lactoglobulin B Standard Calibration Curve (0,05–0,8 g/L) for determination of whey raw material and fermented whey beverages obtained by HPLC analysis. Mobile phase 80% Acetonitrile in ultra–pure water containing 0.05 TFA, injection volume 20 µl, flow rate 1 ml/min, UV detection at 210 nm.

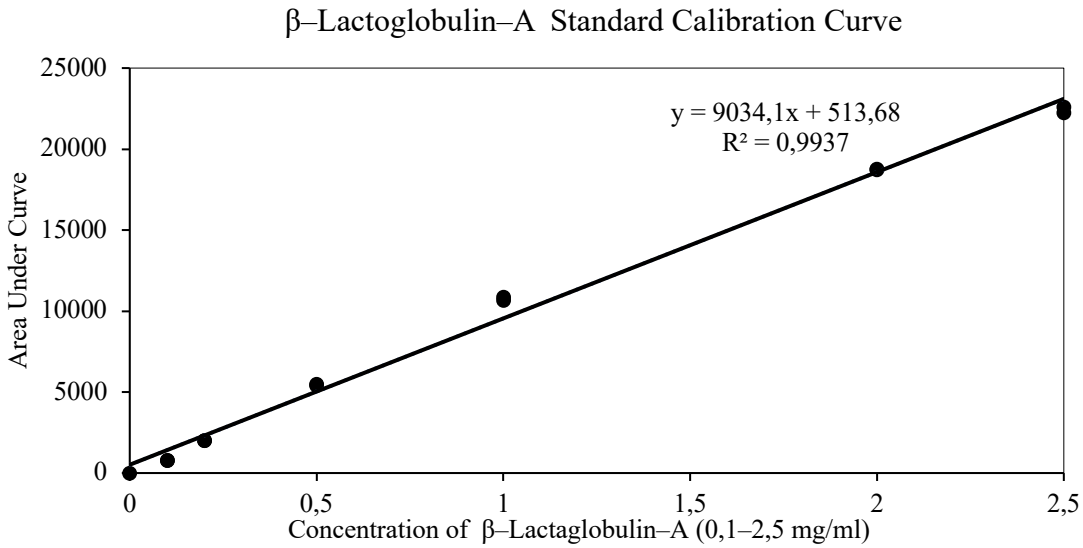


Figure A16. The standard curve of the β–Lactoglobulin A Standard Calibration Curve (0,1–1,25 mg/ml) for determination of whey raw material and fermented whey beverages obtained by HPLC analysis. Mobile phase 80% Acetonitrile in ultra–pure water containing 0.05 TFA, injection volume 20 µl, flow rate 1 ml/min, UV detection at 210 nm.

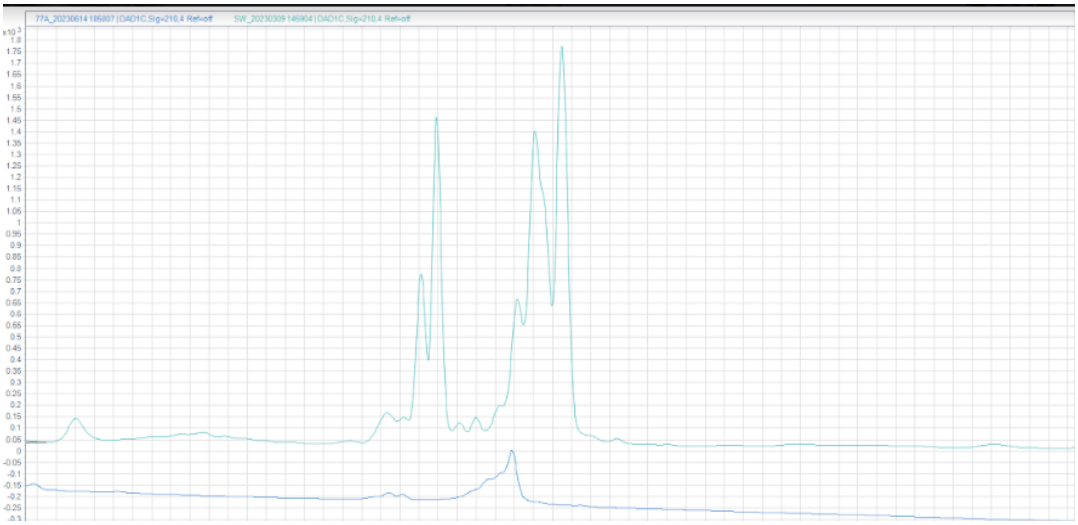

Figure A17. Proteolysis in whey proteins; the upper chromatogram belongs to unfermented whey sample, the below chromatogram belongs to whey sample fermented by *L. plantarum* D7

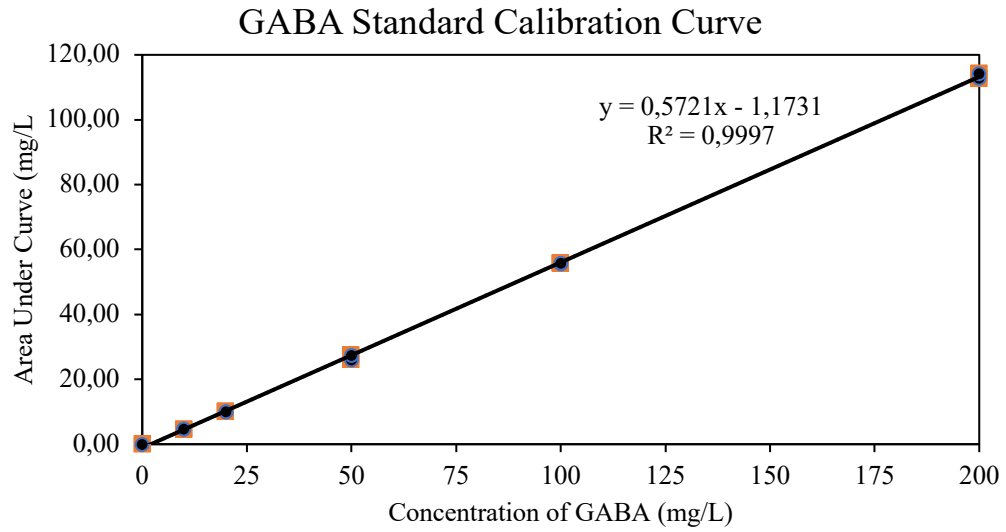


Figure A17. The standard curve of the GABA (50–100 mg/L) for determination of GABA in broth, fermented whey and milk beverages obtained by HPLC analysis. Mobile phase A: 2.5% acetonitrile, containing 10 mg/L $Na_2EDTA$, pH 6.55), mobile phase: B acetonitrile: deionized water: methanol (9:8:3, v/v/v) containing 10 mg/L $Na_2EDTA$, injection volume 80 µl, flow rate: 1 mL/min, UV detection at 254 nm.

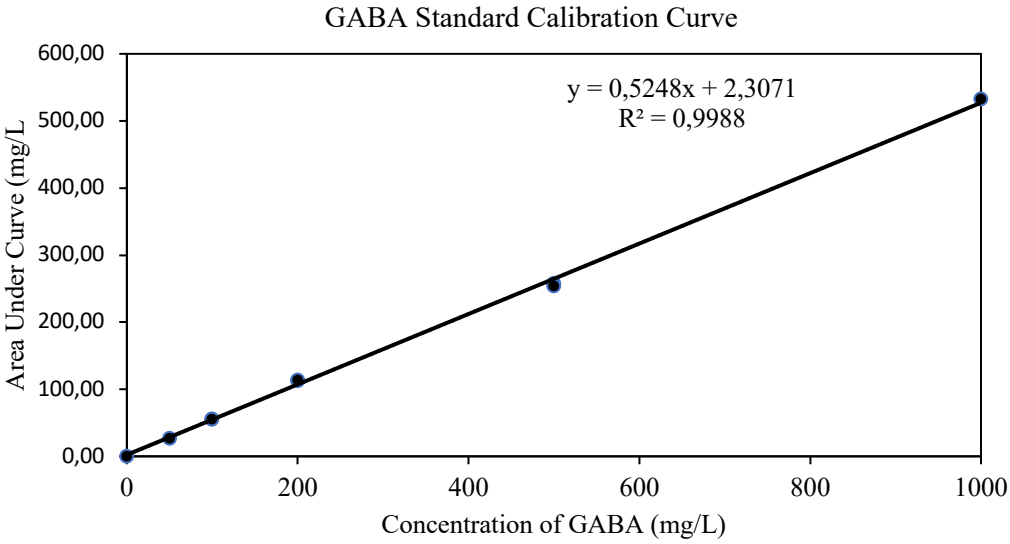


Figure A18. The standard curve of the GABA (50–100 mg/L) for determination of GABA in broth, fermented whey and milk beverages obtained by HPLC analysis. Mobile phase A: 2.5% acetonitrile, containing 10 mg/L $Na_2EDTA$, pH 6.55), mobile phase: B acetonitrile: deionized water: methanol (9:8:3, v/v/v) containing 10 mg/L $Na_2EDTA$, injection volume 80 µl, flow rate: 1 mL/min, UV detection at 254 nm.

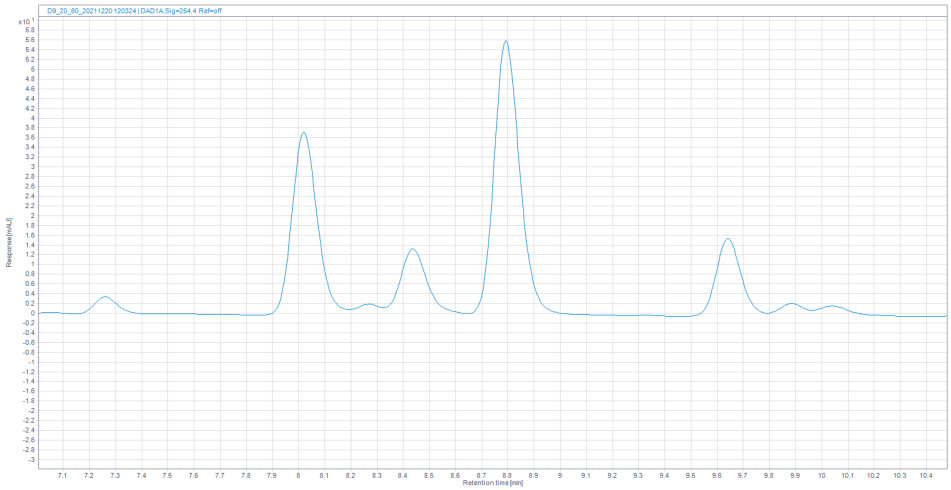

Figure A19. HPLC chromatogram of GABA production in modified broth *L. brevis* D9. The standard curve of the GABA (50–100 mg/L) for determination of GABA in broth, fermented whey and milk beverages obtained by HPLC analysis. Mobile phase A: 2.5% acetonitrile, containing 10 mg/L Na2EDTA, pH 6.55), mobile phase: B acetonitrile: deionized water: methanol (9:8:3, v/v/v) containing 10 mg/L Na2EDTA, injection volume 80 µl, flow rate: 1 mL/min, UV detection at 254 nm.

Figure A.20. Raw material sweet whey, obtained after kashar cheese production stored at -18 C

Figure A.21. Fermented whey sample. Co-culture fermentation; *L. plantarum* or *L. lactis ssp. lactis* C24 with selected *L. delbrueckii ssp. bulgaricus* species.

# APPENDIX B. The Data Tables of The Experimental Findings

Table B1. LAB Isolates from yoghurt samples

| *L. delbrueckii ssp. bulgaricus* | | | *Streptococcus thermophilus* | | | |
|---|---|---|---|---|---|---|
| **Code** | **Code** | **Code** | **Code** | **Code** | **Code** | **Code** |
| bTY5 | bTY43 | b30b | UZ8 | cTY8 | cTY61 | c66b |
| bTY5b | bTY45 | b33 | UZ12 | cTY9 | cTY62 | c71 |
| bTY6 | bTY68 | b33b | UZ16 | cTY10 | cTY63 | c74 |
| bTY7 | bTY69 | b34 | UZ18 | cTY12 | cTY63/2 | c77a |
| bTY8 | bTY70 | b44 | UN26 | cTY14 | cTY65 | c77b |
| bTY8b | bTY71 | b48 | UZ22 | cTY15 | cTY 67 | c78 |
| bTY9a | bTY73 | b49 | UZ32 | cTY17 | cTY69 | c79 |
| bTY9b | bTY77a | b51 | DT54 | cTY20 | cTY70 | c85 |
| bTY11 | bTY77b | b53 | DT62A | cTY21 | cTY71 | c90b |
| bTY14a | bTY79 | b54 | DT62B | cTY23 | cTY72 | c94 |
| bTY14b | bTY80 | b57 | DT6 | cTY24 | cTY75 | c94a |
| bTY16 | bTY83 | b62 | DT74 | cTY25 | cTY77 | c95–1 |
| bTY17 | bTY85 | b64 | UIN4B | cTY26 | cTY78 | c95–2 |
| bTY20 | bTY86 | b69 | UIN18 | cTY27 | cTY79 | c97–1 |
| bTY21 | bTY87 | b71 | UIN22 | cTY29 | cTY81 | c97–2 |
| bTY22a | bTY88 | b76 | UIN26 | cTY30 | cTY82 | UN5 |
| bTY22b | bTY90 | b77 | UIN42 | cTY31 | c29 | UN9 |
| bTY23 | bTY91 | b79 | UIIN4 | cTY32 | c38c | UN19 |
| bTY24 | bTY92 | GA12 | UIIN18 | cTY38 | c39a | UIN9 |
| bTY27a | b16 | UF6 | UIIN24 | cTY41 | c47 | UIB31 |
| bTY30 | b22 | UIB2 | UIIN26 | cTY44 | c50 | |
| bTY34 | b22b | UN26 | UIIN44 | cTY45 | c52 | |
| bTY36b | b24 | | | cTY47 | c60 | |
| bTY40 | b25 | | | cTY53 | c62 | |
| bTY41 | b26 | | | cTY55 | c65 | |
| bTY42 | b30 | | | cTY57 | c66a | |

Table B2. LAB isolates from artisanal cheese samples (A coded)

| Code | Species | Code | Species | Code | Species |
|---|---|---|---|---|---|
| **A1** | *L. lactis ssp. lactis* | **A27** | *L. lactis ssp. lactis* | **A57** | *E. faecium* |
| **A2** | *L. lactis ssp. lactis* | **A28** | *L. lactis ssp. lactis* | **A58** | *E. faecium* |
| **A3** | *L. lactis ssp. lactis* | **A31** | *E. faecium* | **A59** | *E. faecium* |
| **A5** | *L. lactis ssp. lactis* | **A32** | *E. faecium* | **A60** | *Enterococcus ssp.* |
| **A6** | *L. lactis ssp. lactis* | **A33** | *E. faecium* | **A61** | *Enterococcus ssp.* |
| **A7** | *L. lactis ssp. lactis* | **A34** | *E. faecium* | **A62** | *Enterococcus ssp.* |
| **A8** | *L. lactis ssp. lactis* | **A35** | *L. lactis ssp. lactis* | **A63** | *Enterococcus ssp.* |
| **A9** | *L. lactis ssp. lactis* | **A37** | *L. lactis ssp. lactis* | **A64** | *Enterococcus ssp.* |
| **A10** | *L. lactis ssp. lactis* | **A38** | *E. faecium* | **A65** | *Enterococcus ssp.* |
| **A11** | *L. lactis ssp. lactis* | **A39** | *Enterococcus ssp.* | **A66** | *E. faecium* |
| **A12** | *L. lactis ssp. lactis* | **A40** | *L. lactis ssp. lactis* | **A67** | *E. faecium* |
| **A13** | *L. lactis ssp. lactis* | **A41** | *E. faecium* | **A67b** | *E. faecium* |
| **A14** | *L. lactis ssp. lactis* | **A42** | *E. faecium* | **A68** | *E. faecium* |
| **A16** | *L. lactis ssp. lactis* | **A43** | *Enterococcus ssp.* | **A69** | *Enterococcus ssp.* |
| **A17** | *E. faecium* | **A44** | *L. lactis ssp. lactis* | **A70** | *E. faecium* |
| **A18** | *E. faecium* | **A45** | *L. lactis ssp. lactis* | **A71** | *E. faecium* |
| **A19** | *L. lactis ssp. lactis* | **A46** | *L. lactis ssp. lactis* | | |
| **A20** | *L. lactis ssp. lactis* | **A47** | *L. lactis ssp. lactis* | | |
| **A21** | *L. lactis ssp. lactis* | **A48** | *L. lactis ssp. lactis* | | |
| **A22** | *L. lactis ssp. lactis* | **A49** | *E. faecium* | | |
| **A23** | *L. lactis ssp. lactis* | **A50** | *E. faecium* | | |
| **A25** | *L. lactis ssp. lactis* | **A53** | *Enterococcus ssp.* | | |
| **A26** | *L. lactis ssp. lactis* | **A56** | *Enterococcus ssp.* | | |

Table B3. LAB isolates from artisanal cheese samples (B, C and D coded)

| Code | Species | Code | Species | Code | Species |
|---|---|---|---|---|---|
| **B8** | *L. lactis ssp. lactis* | **C1** | *L. lactis ssp. lactis* | **C34** | *L. lactis ssp. lactis* |
| **B10** | *L. lactis ssp. lactis* | **C3** | *Lactobacillus ssp.* | **C35** | *L. lactis ssp. lactis* |
| **B11** | *L. lactis ssp. lactis* | **C4** | *L. lactis ssp. lactis* | **C36** | *E. faecium* |
| **B15** | *L. lactis ssp. lactis* | **C5** | *Lb. paracasei ssp.paracasei* | **C37** | *Lactobacillusssp.* |
| **B16** | *E. durans* | **C7** | *Lactobacillus ssp.* | **C38** | *E .faecalis* |
| **B17** | *E. faecium* | **C8** | *Lb. paracasei ssp. paracasei* | **C39** | *Enterococcusssp.* |
| **B19** | *E. faecalis* | **C9** | *Lactobacillus ssp.* | **C40** | *Enterococcusssp.* |
| **B20** | *L. lactis ssp. lactis* | **C10** | *L. lactis ssp. lactis* | **C41** | *E. faecalis* |
| **B21** | *L. lactis ssp. lactis* | **C11** | *L. lactis ssp. lactis* | **C42** | *Enterococcus ssp.* |
| **B22** | *E. faecium* | **C12** | *Lb. paracasei ssp. paracasei* | **C43** | *E. faecium* |
| **B23** | *E. faecium* | **C15** | *L. lactis ssp. lactis* | **C46** | *E. avium* |
| **B24** | *E. faecium* | **C16** | *L. lactis ssp. lactis* | **C47** | *L. plantarum* |
| **B25** | *E. faecium* | **C17.1** | *L. brevis* | **D1** | *L. plamtarım* |
| **B26** | *E. faecium* | **C17.2** | *L. brevis* | **D2** | *L. plamtarım* |
| **B27** | *E. faecium* | **C18** | *L. lactis ssp. lactis* | **D3** | *L. curvatus* |
| **B28** | *E. faecium* | **C19.1** | *L. lactis ssp. lactis* | **D4** | *L. plamtarım* |
| **B29** | *E. faecium* | **C19.2** | *L. lactis ssp. lactis* | **D5** | *L. plamtarım* |
| **B30** | *E. faecalis* | **C22** | *L. lactis ssp. lactis* | **D6** | *L. plamtarım* |
| **B31** | *E. durans* | **C24** | *L. lactis ssp. lactis* | **D7** | *L. plamtarım* |
| **B32** | *E. faecalis* | **C27** | *Lb. casei* | **D8** | *L. curvatus* |
| **B33** | *E. avium* | **C28** | *L. lactis ssp. lactis* | **D9** | *L. brevis* |
| **B34** | *E. avium* | **C29** | *L. curvatus* | | |
| **B35** | *E. faecalis* | **C30** | *Enterococcus ssp.* | | |
| **B36** | *E. faecalis* | **C31** | *Enterococcus ssp.* | | |
| **B37** | *E. faecalis* | **C32** | *Lactococcus ssp.* | | |

# VITA

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**2013-2015 M.Sc.** **Department of Biotechnology**, Faculty of Science, Dokuz Eylül University, Izmir, Turkey
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**Thesis:** Antioxidant Activities in Foods
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## PROJECTS

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**2019-2022** **IZTECH-BAP –** Development of Fermented Whey Beverage Formulation and Determination of in-vitro Digestion, (Project No: İYTE0306), 01.04.2019-01.10.2022.

## PUBLICATIONS

Agirbasli, Z., & Harsa, S. Discovery, Characterization, and Databases of Enzymes from Sourdough. In Sourdough Innovations (pp. 161-198). CRC Press.

Agirbasli, Z., & Cavas, L. (2017). In silico evaluation of bioactive peptides from the green algae Caulerpa. Journal of Applied Phycology, 29, 1635-1646.