g-aminobutyric acid-producing bacteria:screening, probiotic potential, and impact on gut microbiota under a simulated human colon

Rojaalsadat Mousavi

Interdisciplinary School of Health Sciences

Faculty of Health Sciences

University of Ottawa, Ottawa, ON, Canada

A thesis submitted in partial fulfillment

of the requirements for the

M.Sc. degree in Interdisciplinary Health Sciences

University of Ottawa

ABSTRACT

This study aimed to isolate and characterize *in-vitro* and under simulated colonic conditions, probiotic candidates isolated from food environment producing γ-aminobutyric acid (GABA), a major inhibitory neuromediator of the enteric nervous system with a potential role in modulating the immune system in many health disorders. Several lactic acid bacteria were isolated and detected for the presence of the *gadB* gene using PCR and GAD enzymatic assay. The most active strains with high and fast production kinetics were identified,characterized, and included *Streptococcus thermophilus*, *Lactiplantibacillus plantarum*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*. The biological safety (i.e., sensitivity to antibiotics and the presence of virulence factors) and probiotic potential (i.e., resistance to gastrointestinal conditions and whole-genome sequencing) of identified bioactive strains was also confirmed *in vitro*. The growth, GABA production, and competitiveness of selected probiotic candidates (*B. animalis*, *S. thermophilus*, and *L. bulgaricus*) were investigated in the presence of human gut microbiota *ex vivo* in a model of a proximal colon mimicking physiological and microbiological conditions of the human large intestine. Supplementation with GABA-producing probiotic candidates did not affect the overall gut microbiota diversity over 48 h of treatment. However, we observed modulation of the microbiome composition, especially change of *Bacteroides* population, a key gut microbe associated with anti-depressive and anti-inflammatory activities. The level of microbiota-generated butyrate within 12 h of treatment was significantly increased compared to control. Results from this study demonstrated the probiotic potential of tested GABA-producing bacteria and their impact on gut microbiota structure and metabolism, suggesting their suitability for gut health-promoting application.

Keywords: lactic acid bacteria, γ-aminobutyric acid, probiotic potential, gut microbiota.

RÉSUMÉ

Cette étude visait à isoler et à caractériser *in vitro* et dans des conditions coliques simulées, des candidats probiotiques isolés de l'environnement alimentaire produisant de l'acide γ-aminobutyrique (GABA), unimportant neuromédiateur inhibiteur du système nerveux entérique qui pourrait jouer un rôle dans la modulation du système immunitaire dans de nombreux troubles de santé. Plusieurs bactéries lactiques ont été isolées et détectées pour la présence du gène *gadB* à l'aide de la PCR et du test enzymatique GAD. Les souches les plus actives avec une cinétique de production élevée et rapide ont été identifiées, caractérisées et comprennent *Streptococcus thermophilus*, *Lactiplantibacillusplantarum*, et *Lactobacillusdelbrueckii* subsp.*bulgaricus*. La sécurité biologique (c'est-à-dire la sensibilité aux antibiotiques et la présence de facteurs de virulence) et le potentiel probiotique (c'est-à-dire la résistance aux conditions gastro-intestinales et le séquençage du génome entier) des souches bioactives identifiées ont également été confirmés *in vitro*. La croissance, la production de GABA et la compétitivité des candidats probiotiques sélectionnés (*B. animalis*, *S. thermophilus* et *L. bulgaricus*) ont été étudiées en présence de microbiote intestinal humain *ex vivo* dans un modèle de côlon proximal simulant les conditions physiologiques et microbiologiques du colon humain. La supplémentation avec des candidats probiotiques producteurs de GABA n'a pas affecté la diversité globale du microbiote intestinal sur 48 heures de traitement. Cependant, nous avons observé une modulation de la composition du microbiome, en particulier un changement de la population de *Bacteroides*, un microbe intestinal clé associé à des activités antidépressives et anti-inflammatoires. Le niveau de butyrate généré par le microbiote dans les 12 heures suivant le traitement a été significativement augmenté par rapport au contrôle. Les résultats de cette étude ont démontré le potentiel probiotique des bactéries productrices de GABA testées et leur impact sur la structure et le métabolisme du microbiote intestinal, suggérant leur potentiel d'application pour favoriser la santé intestinale.

Mots-clés: bactéries lactiques, acide γ-aminobutyrique, potentiel probiotique, microbioteintestinal.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor, Dr. Riadh Hammami. This work could not happen without his continuous support and guidance as well as his valuable advice throughout the project. His feedback and guidance helped me for the entire research time and during the writing of my thesis. Also, many thanks go to my TAC committee members.

I was pleased to be working with smart and hardworking Postdoctoral scientist, Walid Mottawea, who I would like to thank for the time, energy, and support he provided to me through the entire project. His patience and determination helped me achieve all necessary research skills regarding the laboratory work. I also would like to express my gratitude to the other members of the lab namely Ayesha and Yasmina for through the comradery of this journey.

Thanks to theNutrition and Mental health Master's Scholarship from the University of Ottawa which I received. This financial support was extremely helpful to complete my study.

Finally, my special thanks go to my wonderful parents for their unstoppable love and support as well as my lovely brothers, Reza and Morteza who always have supported me in this path.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Recent scientific studies have reported that intestinal microbiota, with its structure and metabolism, may be a major contributory factor to the neurobiological and physiological function of the nervous system(Carabotti et al., 2015; Q. Ma et al., 2019). Gut microbiota dysbiosis has been linked to many brain functions and behavioral disorders. This link between gut microbiota and mental disorders has been confirmed via animal models, where germ free (GF) mice have developed anti-depression and anti-anxiety phenotypes as compared to specific pathogen free (SPF) mice as a result of hormonal imbalance in the hypothalamic-pituitary-adrenal (HPA) axis (Huo et al., 2017; Zheng et al., 2016).

Psychobiotics are beneficial bacteria (probiotics) or support for such bacteria (prebiotics) that can positively modulate microbiota–gut–brain interactions. Microbiota–brain communication channels through which psychobiotics exert effects include the enteric nervous system and the immune system (Sarkar et al., 2016). Besides, communication between the gut microbes and the brain can occur via multiple routes that include the vagus nerve, gut-secreted neuropeptides, sensory nerves, cytokines, tryptophan, and SCFAs (Kimura et al., 2011). Currently, there is some physiological evidence to support a psychological role of probiotics, such as decreasing serum cortisol and corticosterone levels related to stress responses (Logan & Katzman, 2005). For instance, a probiotic cocktail of *L. helveticus* and *B. longum* reduced anxiety-like behavior in animals, demonstrated positive psychological effects, and decreased serum cortisol levels in humans (Messaoudi et al., 2011). Although molecular mechanisms related to these outcomes are not known, it has been postulated that they may be due to damping of the effects of pro-inflammatory cytokines and oxidative stress, coupled with changes in nutritional status (Logan & Katzman, 2005). Despite some studies, the mechanisms by which these probiotics are able to exert these beneficial effects on the digestive tract and modulate the intestinal microbiota are far from being elucidated and remain very hypothetical. A possible mechanism by which probiotic bacteria modulate the intestinal microbiota and mediate communication with the brain, is through the secretion of modulatory substances, such as short-chain fatty acids (SCFAs)(Kimura et al., 2011). For instance, butyrate, one of the major SCFAs produced in the colon, has proven neuroactive functions including changes in histone deacetylation and brain-derived neurotrophic factor (BDNF) expression in brain (Schroeder et al., 2007).

Commensal gut bacteria also produce a range of neurotransmitters including GABA, acetylcholine, dopamine, and serotonin (Sarkar et al., 2016), with many intestinal physiological functions (Y. Li et al., 2012). The GABA receptor has been shown to regulate motility in the small intestine and colon by modulating the release of acetylcholine from enteric neurons (Auteri et al., 2015). GABA has been identified as an essential growth factor that solely can induce the growth of unculturable gut microorganisms (Strandwitz et al., 2019). The relative abundance of *Bacteroides*, a major γ-aminobutyric acid (GABA) producing members of gut microbiota, was negatively correlated with depression-associated brain signatures (Strandwitz et al., 2019), indicating a significant role of microbiota-derived GABA in brain functionality. *In vivo* GABA production by intestinal bacteria may impact the microbiota–gut–brain axis, and could have potential health benefits (Bienenstock et al., 2010). As it is a metabolic product of the gut commensal bacteria, and consumed probiotics, gut microbiota composition and diversity would control the enteric GABA levels(Carabotti et al., 2015). This neuroactive metabolite can, in turn,modulate the gut microbiota structure in various kinds of stress(Galland, 2014).GABA is produced mainly from the irreversible conversion of L-glutamate by the enzyme glutamate decarboxylase, a pyridoxal 5′-phosphate-dependent enzyme. A potential way of increasing GABA levels in the gut could be by harnessing the production ability of the intestinal microbiota or ingested probiotic bacteria that could use dietary glutamate to generate GABA in the gastrointestinal tract (Barrett et al., 2012). Particularly, several lactic acid bacteria (LABs; e.g., strains belonging to *Lactobacillus*, *Lactococcus*, and *Streptococcus*) are an important source for glutamate decarboxylase(H. Li & Cao, 2010), and thus can be regarded as promising GABA-producing candidates with psychobiotic effects(Yunes et al., 2020), especially as they are “generally-recognized-as-safe” and commonly found in fermented foods.

This study aimed to evaluate the GABA production capacity of LABs isolated from various starter cultures and fermented foods, characterize their probiotic features, and assess their capacity to grow, synthesize GABA,and modulate the human gut microbiotaunder a simulated colonic environment.Our resultsprovidethe first evidence of the capacity of LAB from the food environment to grow and produce GABAin the colon physiological conditions and to positively modulate the composition and metabolismof the gut microbiota, important probiotic features for the futuredevelopment of GABA-enriched foodsforgut health-promoting applications.

CHAPTER 1: LITERATURE REVIEW

1. Microbial γ-aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA) is a non-protein amino acid thatacts as chemical messengers for carrying information from one cell to another. One of GABA's essential physiological functions is neurotransmission by regulating brain signals(Dhakal et al., 2012). In fact, GABA can improve some physiological functions in the gut, maintain the function of the central nervous system (CNS)(Carabotti et al., 2015; Q. Ma et al., 2019), reduce pain by interacting with the pain receptors(Benke, 2020; C. Li et al., 2019)while being associated with anxiety and depression behaviors by changing the GABA receptors expression(Strandwitz et al., 2019).Over the past few decades, GABA has been established as the major neurotransmitter inhibiting neural excitability and one of the most studied neuroactive compounds in the brain (Smart & Stephenson, 2019). Evidence is accumulating on the prevalence of GABA-sensitive neuronal cells, the pivotal role of GABA in the host physiology, and the impact of itssignaling dysfunction on a wide range of diseases (Smart & Stephenson, 2019). Therefore, due to its physiological role and impact on various physical health aspects, multiple functional foods containing high GABA concentrations have been developed(Diana et al., 2014).

Several probiotic bacteria can produce GABA via the α-decarboxylation reaction of L-glutamic acid. The reaction catalyzes by the glutamate decarboxylase(GAD) enzyme with pyridoxal 5′ phosphate as a cofactor (PLP,the active form of vitamin B6)(Tavakoli et al., 2015). The commonGABA-producing microorganisms that have been employed as probiotics belong tothe genera *Bifidobacterium*and lactic acid bacteria (LAB), which are at its core *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Pediococcus*, and *Enterococcus*(Diana et al., 2014). Various parameters strongly affect the expression of each strain's *gad* genesand their GABA production (K. W. Lee et al., 2017).

1.1. GABA biosynthesis pathways

There are two microbial pathways to the biosynthesis of GABA, including the Glutamic acid decarboxylation pathway (GAD) and putrescine (Puu) pathways(H. Li & Cao, 2010). Most microorganisms can produce GABA using theGlutamic acid decarboxylase pathway, as shown in Figure1.1(Cui et al., 2020). GABA can synthesize from L-glutamate, the excitatory neurotransmitter,by anintracellular glutamate decarboxylase (Gad) enzyme with pyridoxal-5′-

phosphate (PLP), the active form of vitamin B6, as a cofactor (Wallace & Milev, 2017). GAD pathway performs by Glu/GABA antiporter's action, which is encoded by a *gadC* gene to convert monosodium glutamate (MSG) to GABA. The process conducts with the activity of the GAD enzyme and binds to PLP. *gadB* gene plays an important role in GAD and GABA production(Q. Wu et al., 2017).

The Puu pathway is not the usualroute to produce GABA in *Lactobacillus* or *Bifidobacterium* strains, but some strainssuch as *Escherichia coli* can use this pathway to produce GABA (Figure 1.2). Briefly, the first stage of GABA synthesis involves an antiporter, which is encoded by a *PuuP* gene to transfer Puu into the cell. Then, the Puu route proceeds with two different paths.One of them carries out with various enzymes including γ-glutamate-putrescine-synthetase, γ-Glutamyl-oxidase, γ-glutamyl-γ-butyraldehyde dehydrogenase, and γ-Glu-GABA hydrolase, which are codified with *PuuA*, *PuuB*, *PuuC*, and *PuuD* genes, respectively,to obtain GABA eventually.On the other hand, putrescine (Puu) convertsto GABA by an activity of a Puu-amino-transferase and a γ-aminobutyraldehyde- dehydrogenase, which are encoded by *YgjG*and *YdcW* genes, respectively(Q. Wu et al., 2017).

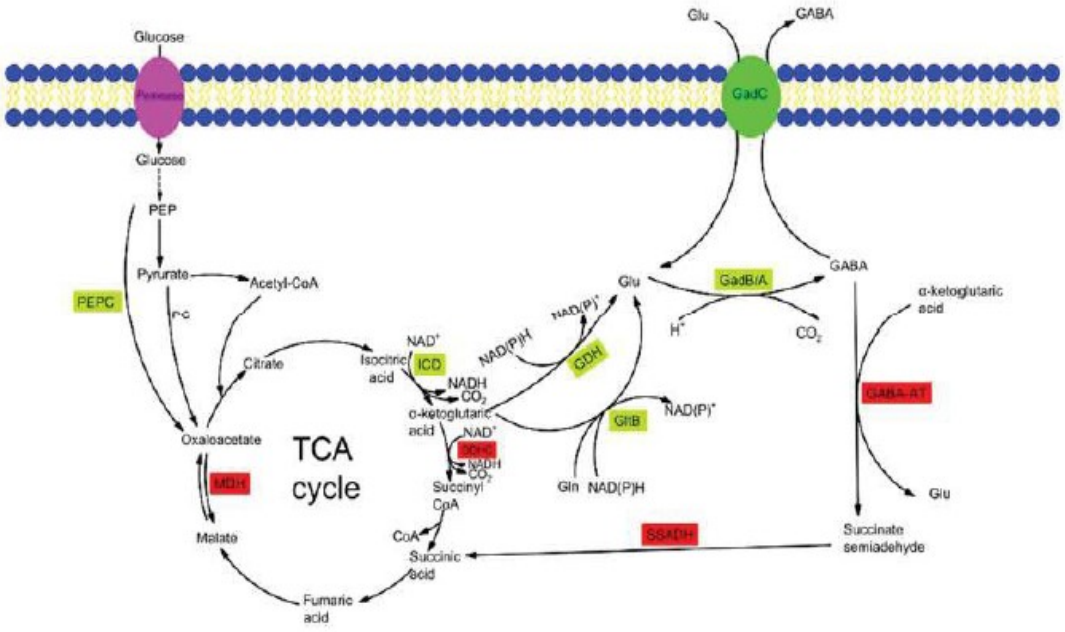


Figure 1.1. The biosynthetic pathway of GABA by microbes **(Cui et al., 2020)**.

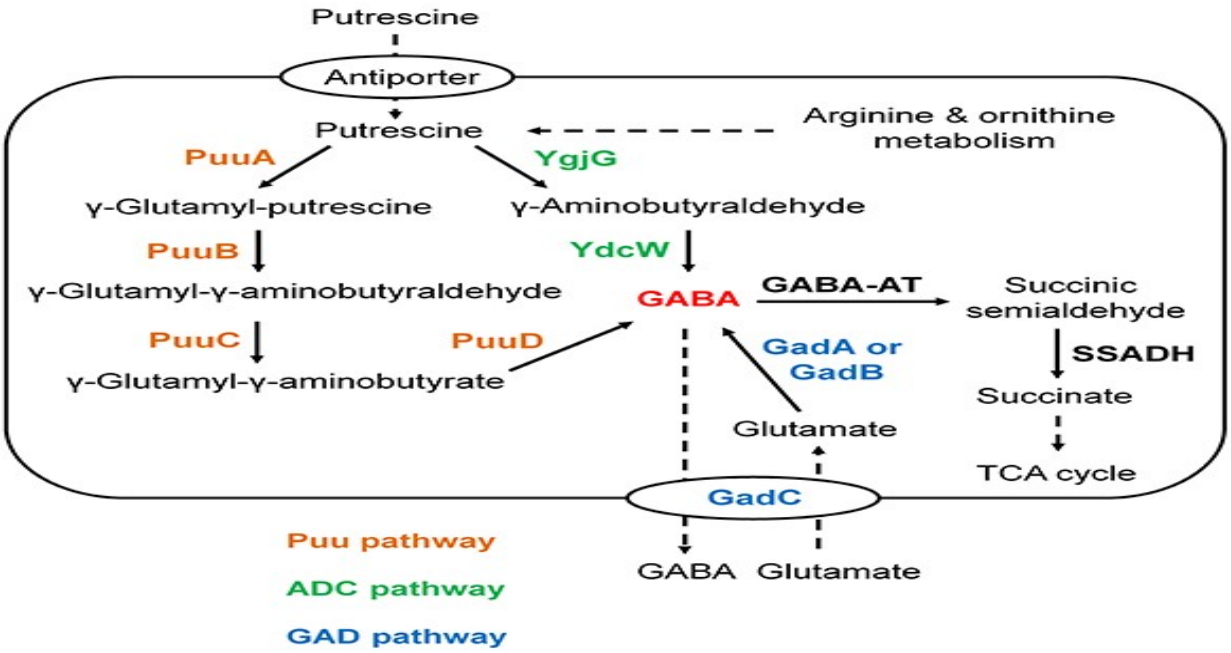


Figure 1.2. General GABA production in bacteria from GAD pathway and putrescine degradation pathways (Puu pathway and ADC pathway) **(Q. Wu et al., 2017)**.

1.2. The glutamate decarboxylase enzyme

Many strains of LAB and *Bifidobacteria*are known for their capability to produce GABA through the glutamic acid decarboxylase (GAD) enzyme, which is encoded by the gad operon (H. Li & Cao, 2010; Q. Wu et al., 2017). GAD, acid resistance (AR) system, is a pyridoxal enzyme that catalyzes glutamate's decarboxylation to yield GABA and CO_2(Ueno et al., 2013). This system includes the two main components Glu/GABA antiporter (*gadC*) and GAD enzyme, with two functionally identical GAD-encoding genes (*gadA* and *gadB*) (Q. Wu et al., 2017). The gad operon organization was found highly variable among LAB species(Yogeswara et al., 2020).The presence of fully active forms of the two elements of the GAD system(GadC and GadA/B) are associated with high microbial GABA production(Yogeswara et al., 2020). GAD system also consumes protons to protect the cell from acid stress and maintains the cellular pH equilibrium under acidic conditions during glutamate decarboxylation to regulate gad gene expression, activate the GAD enzyme, and synthesize GABA (Ogier & Serror, 2008).Genome-sequencing was performed to identify the presence of the GAD system, which is encoded by gad operon among various LABs and *Bifidobacteria* species to be efficient for GABA synthesis (Lim et al., 2017). Therefore, these strains could be considered an important microbial source for GAD-encoding genes (Ogier & Serror, 2008).

1.3. Factors affecting GABA synthesis

Various factors have been identified to impactthe growth, fermentation, and GABA synthesis of probiotics bacteria andcreate the optimal fermentation conditions for GABA-producing bacteria(Alkasir et al., 2017). The most common and essential parameters include pH, temperature, fermentation time, co-factors as enzymes, and culture mediacomposition for different media additives (Dhakal et al., 2012).

The biosynthesis of GABA is regulated by pH during the production process(Komatsuzaki et al., 2005). For instance, the bacterial growth of lactic acid bacteria is associated with acidification due to organic acids' production. The later decrease of pH activates the enzyme GAD, which converts glutamic acid to GABA as an acid resistance mechanism. Therefore, the initial pH could be adjusted timely to keep the optimal effective pH for producing maximum amounts of GABA yield (Dhakal et al., 2012). The incubation temperature also plays an important role in GABA biosynthesis during the fermentation process(Kim et al., 2009). The highest GABA synthesis in LAB strains is directly correlated with the cell density and GAD activation, which is dependent on the temperature(Yang et al., 2006). The optimum temperature for GABA production in most LAB strains was 37°C, at which MSG was converted into GABA completely(Dhakal et al., 2012). Other studies reported an optimum GABA metabolism at a fermenting temperature range of 35-40°C (Lim et al., 2017).

The cultivation time factor plays an effective role in the fermentation process to reach the highest GABA production level. For example, the highest GABA amount was reached at 30-35h when the fermentation process was performed at pH 5.7 and 37°C (Lim et al., 2017). Likewise, the variation of time intervals in addition to MSG and PLP coenzyme was demonstrated to improve GABA production yield significantly during the fermentation process(Dhakal et al., 2012).The nutritional content of medium composition and culture conditions of the fermentation process impacts the amount of GABA production by bacteria(Dhakal et al., 2012). Nitrogen and carbon source, MSG and ammonium sulfate, PLP are used as nutritive sources, substrate, and coenzyme supplementation respectively in the culture medium for enhancing GAD activity and increasing GABA yields during the fermentation by probiotic strains (Komatsuzaki et al., 2008). For example, Pyridoxal 5'phosphate (PLP), the active form of vitamin B6, is a significant cofactor of the glutamate decarboxylase enzyme involved in GABA production(Komatsuzaki et al., 2008).Indeed, PLP addition in the late stationary phase of bacterial growth can stimulate GAD enzyme activity and increase GABA production(Dhakal et al., 2012).

2. Physiological functions of GABA

2.1. Antihypertensive properties

As a potential antihypertensive dietary component, GABA plays an important role in the human diet, reducing blood pressure in spontaneously hypertensive humans(Yoshimura et al., 2010). Indeed, GABA as an effective blood pressure-lowering can regulate the sodium intake in hypertensive people's diet (Mayer et al., 2014). GABA receptor activation can decrease blood pressure and increase the heart rate.In addition, GABA as inhibitory neurotransmitter against the central norepinephrine can keep normal blood pressure(P. Ma et al., 2015).Therefore, consumption of GABA-enriched fermented functional food using probiotics bacteria has increased worldwide.

2.2. GABA as a growth factor in the gut

LAB strains can modulate the composition and function of the gut microbiota and gastrointestinal (GI) tract by modifying short chains fatty acid (SCFA) production such as butyrate and neuroactive metabolites such as GABA, as well as promoting complex bacterial community and diversity to produce a high amount of GABA through communication with gut resident bacteria(Yan et al., 2016). Of particular interest, the production of GABA by *Bacteroides fragilis* KL1758, a human gut resident,was reported essential for the growth of the commensal KLE1738 (uncultured bacterium being in the "most wanted list" of the National Institutes of Health), which depend on GABA as its only carbon source(Strandwitz et al., 2019).

2.3. Influence on central and peripheralnervous systems

GABA is found at high levels in the brain and plays a fundamental role in inhibitory neurotransmission in several of its routes within the central and enteric, and peripheral nervous systems (Diana et al., 2014). A recent study has demonstrated that GABA-producing bacteria can modulate the host GABAergic systems, as shown in Figure 1.3 (Foster & McVey Neufeld, 2013). GABA-producing bacteria can reduce pain, anxiety, and depression by having GABA receptors and altering both GABAA and GABAB receptor expression in specific brain areas(Mazzoli & Pessione, 2016). The GABA-producing bacteria modulate the gut microbiome and could interact with the brain through GABAergic signaling via vagal afferent neurons (Pokusaeva et al., 2017). The GABAergic system finds in the central nervous system and involves GABA receptors, neurons, and enzymes that regulate the immune system to release inflammatory cytokines (Bhat et al., 2010)and attenuate pain(Pokusaeva et al., 2017). The contribution of the GABAergic system in the pathogenesis of mood disorders is now well-recognized(Northoff & Sibille, 2014)(Romeo et al., 2018). Probiotics bacteria, *Bifidobacteria* and

L. rhamnosus, can alter the GABA receptors mRNA expression in the brain, which is associated with serum cortisol reduction, depressive and anxiety-like behaviors decrease in mice, respectively(Holzer & Farzi, 2014). Indeed, depression and anxiety can be related to the disruption of physiological pathways among the gut-brain axis and alteration of amino acid production such as the Glu/GABA system(Dhakal et al., 2012).The gut microbiota can influence brain function and improve mental health disorders through communication and bidirectional signaling(Foster & McVey Neufeld, 2013). The fecal abundance of GABA-producing bacteria from glutamate was reported negatively correlated to functional connectivity betweenthe left dorsolateral prefrontal cortex and the default mode network in individuals with depression, suggesting a potential influence of microbial-derived GABA on the host brain(Strandwitz et al., 2019).Likewise, decreased glutamate catabolism potential was observed in depressed individuals, linking microbial glutamate pathways to depression(Valles-Colomer et al., 2019).Hence, the modulation of gut microbiota with psychobiotic bacteria could be an effective strategy for treating mental health disorders such as anxiety, depression, social behavior, cognition, and pain (Kennedy et al., 2017).

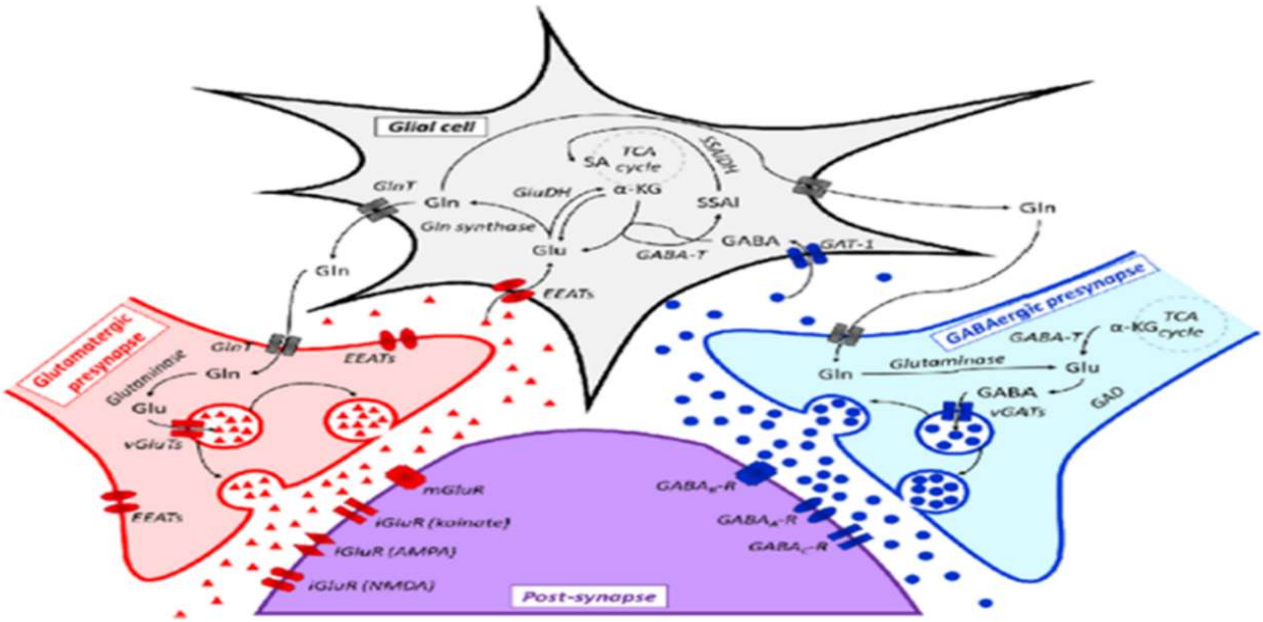


Figure 1.3. Scheme of the GABAergic neurotransmission in the CNS **(Mazzoli & Pessione, 2016)**.

3. GABA-producing microorganisms

3.1. Overview

Multiple bacterial species have been reported to produce GABA, including *Bifidobacteria* and some LAB such as *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Enterococcus*(Diana et al., 2014) with a concentration range of 15 - 99.9 mg/kg. For instance, *L. paracasei, L. delbrueckii subsp. bulgaricus, L. lactis, L. plantarum,* and *L. brevis* have been reported for the highest GABA production (99.9, 63.0, 36.0, 16.0, and 15.0 mg/kg, respectively) (Siragusa et al., 2007). These GABA-producing bacteria were isolated from various sources, mainly the food environment(Sahab et al., 2020), as summarized in Table 1.1.

Table 1.1. Gamma-amino butyric acid-producing bacteria and its source of isolation(Sarasa et al., 2020)

Microorganisms	Isolation Source	References
Lacticaseibacillus paracasei, Streptococcus thermophilus, Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum	Cheese	(Franciosi et al., 2015)
Levilactobacillus brevis	Kimchi	(Lim et al., 2017)
Lactococcus lactis sp *lactis*	Cheese	(Lacroix et al., 2013)
Streptococcus thermophilus	Fish	(Linares et al., 2016)
Lentilactobacillus buchneri, Lactobacillus sp.	Cheese	(Ibrahim, 2016)
Lacticaseibacillus paracasei, Lactobacillus delbrueckii subsp. bulgaricus, Lactococcus lactis, Lactiplantibacillus plantarum, Levilactobacillus brevis	Cheese	(Siragusa et al., 2007)
Levilactobacillus brevis, Lactiplantibacillus plantarum	Egyptian dairy products	(Gomaa, 2015)
Lactobacillus helveticus	Koumiss	(Sun et al., 2015)
Enterococcus faecium	Traditional fermented food samples	(Lim et al., 2016)
Lactobacillus otakinesis, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum	Azorean cheese	(Ribeiro et al., 2018)
Limosilactobacillus fermentum	Fermented Thai foods	(Woraharn et al., 2014)
Levilactobacillus brevis	Fish intestine	(Ko et al., 2013)
Lactic acid bacteria	Kimchi, Jot-gal (traditional fermented foods)	(B.-J. Lee et al., 2010)
Lactiplantibacillus plantarum	Dadih	(Harnentis et al., 2019)
Levilactobacillus brevis	Paocai	(Huang et al., 2007)
Lactiplantibacillus plantarum	Kimchi	(Park et al., 2014)

Indeed, fermented food products are a valuable source of LABs with a high GABA production capacity (Dhakal et al., 2012). Still, GABA production in LABs varies considerably among isolated strains (Quílez & Diana, 2017).GABA biosynthesis acts as a communication mechanism among microbes such as LABs and their host (Siragusa et al., 2007). LABs strains can modify the mRNA expression of GABA receptors, which are associated with physiological functions of gut and brain activity (Lyte, 2016). Some studies have also shown that the LABs strains can alter the GABAergic signaling by interacting with receptors(Mazzoli & Pessione,

2016), increasing cytokine expression, and decreasing neuroinflammation (Muralidhara et al., 2015). Similarly, some strains of *Bifidobacteria* (*B. dentium, B. breve, B. animalis, B. longum*) can produce GABA from MSG (Diana et al., 2014), which can modulate the brain-gut axis(Sarkar et al., 2016). Indeed, some studies have shown that *Bifidobacteria* increase the level of GABA production in the gut. For example, *B. dentium* ATCC 27678 was shown to produce about 3000 µg/ml of GABA in the gut environment (Pokusaeva et al., 2017). *Bifidobacteria* were previously reported to reduce depression symptoms by altering neural functions and increasing GABA receptor expression (O'Hara & Shanahan, 2006). Likewise, *Bifidobacteria* can decrease gut inflammation by producing GABA as an inhibitory inflammatory neurotransmitter and alter gene expression related to the inflammatory improvement in the gut (Mayer et al., 2014). Some studies have indicated that the GABAergic signaling via the microbiota-gut-brain axis is linked to glutamate decarboxylation by *Bifidobacteria*, resulting in a significant pain reduction (Pokusaeva et al., 2017). GABA also has various functions in microbial systems. Maintaining a neutral pH under acidic conditions (defense against low pH environment) is one of the critical roles of GABA associated with glutamate-antiporter reactions (Sarasa et al., 2020). GAD enzyme can regulate the pH of the bacterial environment by consuming intracellular protons during the decarboxylation reaction (Sarasa et al., 2020). For example, acid stress induces an up-regulation of the *gadB*gene in *Bacillus megaterium* G18, demonstrating its involvement in an intracellular pH homeostasis mechanism under acidic stress(Goswami et al., 2018). Besides conferring resistance to acidic pH,GABA is functionally involved in the spore germination of *B. megaterium*. In addition, Dagorn et al., (2013)reported that GABA can affect the cytotoxicity of various strains of *Pseudomonas fluorescens*,alter their lipopolysaccharide (LPS) structure and bacterial surface properties. Indeed, GABA can affect the adhesion potential of *P. fluorescens*in addition to theirexpression of virulence factors as well as biosurfactants.Likewise, GABA was found to induce the enzymatic pathway in *P. aeruginosa* through intracellular polyamine levels(Chou et al., 2008).

3.2. Why using GABA-producing bacteria

Various kinds of fruits, edible plants, roots, and botanicals contain substantial amounts of neurotransmitters, including GABA that can be synthesized through metabolic processes (primary products), ecological interactions (secondary products), or microbial food fermentation (Naila et al., 2010). Still, dietary glutamate is most abundant in food products than GABA, rendering glutamate conversion to GABA more cost-effective. Also, dietary GABA found in edible plants is likely to vary with plant parts, morphological and chemotype differences,

adaptation to the environment and geographical location, seasons of growth and harvest, weather and climate changes, contaminants, and agricultural practices and nourishment process (Briguglio et al., 2018). Furthermore, we need to consider consumers' preferences and reluctance to certain foods (i.e.,appropriate taste, quality, texture, allergenicity).Besides its long safe use history, the utilizationof commensal microbes to produce GABA by food fermentation is an effective strategy to reduce production costs and decrease the environmental impact of the biosynthetic process (Boonstra et al., 2015; W. Zhao et al., 2014).In addition to the lack of scientific evidence to substantiate the related health claims, dietary GABA did obtain GRAS status only for use as a flavoring substance (FEMA #4288) (Waddell et al., 2007).Synthetic GABA supplements may also contain potentially harmful by-products beyond the active form of GABA. Therefore, theFood and Drug Administration (FDA) issued warnings on the potentially harmful by-products and toxicity of synthetic GABA and encouraged consumers to use safe and natural sources of GABA instead of widely available online GABA food supplements (Boonstra et al., 2015).Besides, GABA was found a growth co-factor for many beneficial gut microbes, including the *Bacteroidetes* population that could be promoted by the presence of competitive GABA-producing strains that could adhere to epithelial cells and continuously release GABA in the intestinal environment (Strandwitz et al., 2019)).Many studies reported that consuming foods fermented using GABA-producing probiotics could improve mental health disorders such as social anxiety, cognition, and pain sensitivity,mainly through interaction with gut microbiota (Appleton, 2018; Zhu et al., 2020).

4. Potential of GABA-producing bacteria as psychobiotics

4.1. Definitions

According to the last definition provided by the International Scientific Association for Probiotics and Prebiotics (ISAPP), probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”(Hill et al., 2014). Health Canada has approved the following probiotics for which non-strain-specific claims might be made: *Bifidobacterium* (*adolescentis*, *animalis*, *bifidum*, *breve,* and *longum*) and *Lactobacillus* (*acidophilus*, *casei*, *fermentum*, *gasseri*, *johnsonii*, *paracasei*, *plantarum*, *rhamnosus,* and *salivarius*), when delivered in food at a level of 1×10^9 colony forming units (CFU) per serving(Hill et al., 2014).In addition, psychobiotic microbes are defined as a novel class of health-promoting probiotics, which are potential candidates to treat psychiatric diseases (Barrett et al., 2012) and to enhance mental health through modulation of the gut microbiota and restoring intestinal dysbiosis (Del Toro-Barbosa et al., 2020; D. Johnson et al., 2021; Sarkar et al., 2016).

4.2. Probiotic features

In order to exert their beneficial effects, probiotic microorganisms need to be non-pathogenic and recognized as safe while being able to survive and grow under the physiological conditions of the digestive tract(Fijan, 2014). Therefore, several aspects, including safety, functional and technological properties, are considered when selecting probiotic candidates (Lyte, 2011).These evaluation tests include acid and bile salt tolerance, antibiotics resistance/susceptibility pattern, adhesion to intestinal epithelial cells, freedom from virulence factors, survival ability in the GIT, stabilization and modulation of gut microbiota, and viability during processing and storage (Ogier & Serror, 2008).For instance, intrinsic resistance/sensitivity to antibiotics is an important selection criterion to characterize the strains isolated as potential probiotics to be safe for use in different functional products (Wu et al., 2018). Likewise, survival of potential probiotic bacteria in the acidic condition during gastric transit and growth in bile presence are critical functional properties (Siragusa et al., 2007).

4.3. Psychobiotic mechanisms

The significant role of probiotics in immune modulation, neurotransmitter biosynthesis, gut homeostasis through restoring microbial balance and composition implies a complex bidirectional interplaywith the microbiota–gut–brain axis(Halloran & Underwood, 2019; D. Johnson et al., 2021; Rogers et al., 2016). Still, the associated action mechanisms by which psychobiotics exert beneficial health effects are not clearly defined (Del Toro-Barbosa et al., 2020; Sarkar et al., 2016). For instance, psychobiotics are capable of changing gut microbial composition and activity and stimulate the gut epithelial cells to produce various neuroactive compounds in the gut that influence metabolic activity, psychological and physiological function, brain's biochemistry and behavior, as shown in Figure 1.4(Yong et al., 2020). Psychobiotics can mediate anti-depressive effects by enhancing neurotransmitter production (Yong et al., 2020). For example,GABA-producing *L. plantarum* 90skand *B. adolescentis* 150have been associated with decreased depressive-like behavior in mice presumed by modulating the GABAergic signaling via GABA receptors of host enteric neurons(Yunes et al., 2020).A previous study has shown similar effects for *L. rhamnosus* JB-1 on depression and anxiety behaviors through alteration of GABA receptor expression level(Bravo et al., 2011).Furthermore, GABA-producing bacteria may impact the gut-brain axis by modulatingENS signaling and regulating the neural excitation–inhibition leveling(Mazzoli & Pessione, 2016).Psychobiotics bacteria could also modulate the brain indirectly through the vagal pathway and neuroendocrine systems, alter neurotransmission in the CNS and expression of inhibitory GABA receptors in the brain

(Carabotti et al., 2015; Mohajeri et al., 2018), reduce depression-associated inflammatory cytokines (e.g., IL- 1β, IL-6, TNFα) (Suneson et al., 2021; Yong et al., 2020).

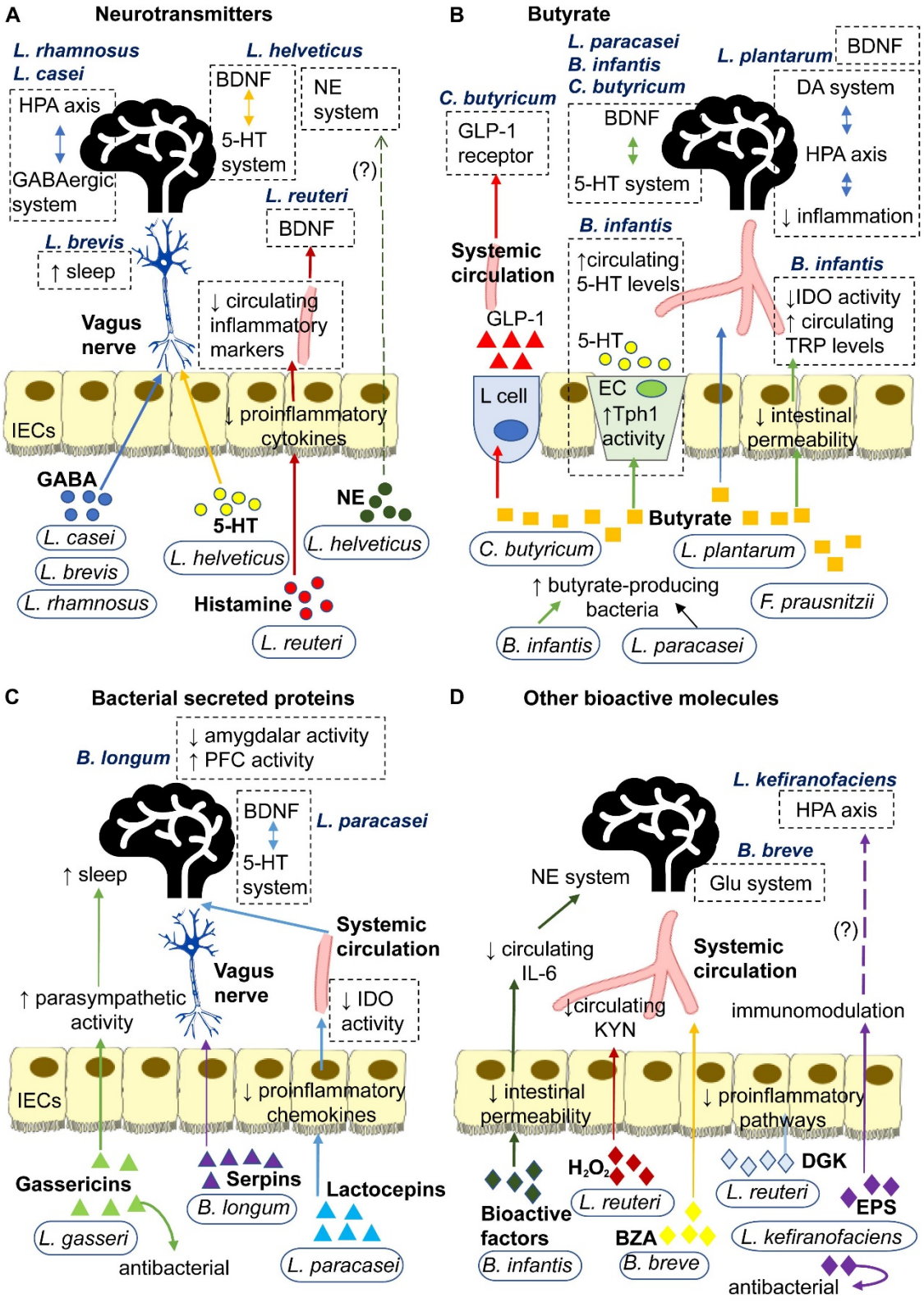


Figure1.4. Signaling mechanisms underlying antidepressive effects of psychobiotics mediated through secretion of neurotransmitters(**A**),butyrate(**B**), secreted proteins(**C**),and other bioactive molecules(**D**)(Yong et al., 2020).

4.4. Survival in the gut environment

A probiotic strain must survive the transit through the gastrointestinal tract, where bacterial cells are challengedby acid, bile, pancreatic enzymes, and competition from other microorganisms(Fernandez et al., 2014).Survival of several probiotic strains have been investigated *in vivo* and *in vitro,* with the survival rate beingstrain-dependent(Fernandez et al., 2014).For instance, while *L. johnsonii* La1 showed a 76% survival rate*in vitro*, only 0.1% of *L. rhamnosus* GG cells survived(Mainville et al., 2005). More generally, the survival rate of *Bifidobacteria* and *Lactobacilli* during transit in the human intestine has been estimated between 20 and 40% depending on the straintested(Bezkorovainy, 2001).

5. Interplay of GABA and their producing bacteria with gut microbiota

5.1. The human gut microbiota

The human intestinal microbiota is a highly complex mixture of microbial organisms (approximately 10^{11} per g feces) that play an important role in host nutrition, metabolism, and physiology. This microbiota “organ” is the central bioreactor of the gastrointestinal (GI) tract that carries out vital processes for normal digestive functions of the host and plays an important albeit not yet fully understood role in the maturation of human immunity and defense against pathogens(Verberkmoes et al., 2009). The microbiota acts as a barrier against the proliferation of exogenous pathogens by preventing their invasion with a highly dynamic modality, exerting metabolic functions, and stimulating the maturation of the immune system(Putignani et al., 2014). Commensal microbiota inhibits pathogen growth by producing and releasing organic acids, volatile fatty acids, antibiotic compounds, bacteriocins, and/or host immune response stimulating factors. The GI microbiota, predominated by bacteria, comprising at least 80 genera and around 1800 phylotypes(Rajilić-Stojanović et al., 2009),is incredibly diverse and dynamic, and each human possesses a unique community fingerprint of bacteria, archaea, and eukarya (Rajilić-Stojanović et al., 2007). The gut microbiota co-evolves with its human host from birth to adulthood. Several external factors, such as delivery mode, maternal and infant diet, antibiotic exposure, or other environmental stimuli, can affect microbiota composition(Guzman-Rodriguez et al., 2018). Perturbations in the composition of gut microbial communities, also known as dysbiosis, have been associated with several intestinal and extra-intestinal disorders(Carding et al., 2015). The causes and consequences of temporal and inter-individual variation in microbiota

community composition remain to be resolved and may be key to developing innovative strategies to improve human health and reduce gut-associated chronic diseases(K. V.-A. Johnson, 2020). Strategies to enhance microbial-host symbiosis in healthy individuals and re-establish symbiosis in diseased states are of current interest, with diet/dietary components receiving increasing attention, not only by those in the scientific community also by the food industry and consumers(McDonald, 2017).

5.2. Impact of GABA-producing bacteria on gut microbiota

Although GABA's multiple physiological roles in the gut environment, it seems that this neurochemical is produced as quorum sensing signaling to other bacteria(Quillin et al., 2021). Multiple neurotransmitters were found to facilitate the growth and cellular aggregation of microbes, thus confirming the communication between inter and intra-species(Burton et al., 2002).A variety of GABA-producing bacteria have been found that modulate human gut microbiota and promote the growth of key genera in the gut, such as *Bacteroides* ssp., which can produce large GABA quantities in the gut (Strandwitz et al., 2019). Multiple studies have demonstrated the role of microbial community structure in the gut and its impact on protein and biogenic amino acid synthesis, carbohydrate and neuroactive metabolism, and SCFAs production (Parkar et al., 2019; Sudo, 2019). Moreover, the abundance of fecal *Bacteroides* was reported to be associated with an altered GABA-mediated response and negatively correlated with brain signatures in depression state (Patterson et al., 2019; Valles-Colomer et al., 2019).

5.3. *Ex vivo* fermentation models simulating human colonic microbiota

Gut fermentation models are simulated systems mimicking most of the spatio-temporal, nutritional, and physiological conditions encountered in the human intestine (Macfarlane & Macfarlane, 2007). So far, various *ex vivo* models have been developed with theprimary objective to maintain stable, reproducible, and complex microbial communities in a well-controlled ecosystem (McDonald et al., 2013; Van den Abbeele et al., 2010). These models employ either single or multi-stage bioreactors inoculated, depending on the application, with fresh human feces or defined microbial communities. Multiple parameters are closely monitored, including pH, temperature, culture medium, and transit time (McDonald, 2017). Particularly, continuous culture fermentation models (Figure 1.5) have been established as insightful tools to understand complex microbial physiology and ecology in a controlled environment (Fernandez et al., 2016; Le Lay et al., 2015).

These*exvivo*fermentation models offer several advantages: no ethical constraints, continuous culture standardization, creation of stable phase, high reproducibility, monitor of

microbial composition and metabolic activity, control of various fermentation factors, maintenance of colonic situation, dynamic and unlimited sampling, and the absence of confounding factors (Venema & van den Abbeele, 2013). Hence, the *exvivo* proximal colon continuous fermentation model was shown representative of the *in vivo* condition of the gastrointestinal tract (GIT) in terms of the microbial community, composition, and metabolic activity.

Conversely, *ex vivo* models have certain limitations, such as potential oversimplification of the corresponding *in vivo* scenario, mainly due to the absence of epithelial mucosal barrier and interactions with the host immune system (Boureau, L. Hartmann, T. Karjalaine, 2000) and differences in the host parameters (e.g., diet, environment, age, and genetics) (McDonald, 2017)as well as the low number of donors and biological replicates which is due to the time and the high-cost constraints, short period of microbial treatment(Mottawea et al., 2020).Therefore, it is essential to properly validate *ex vivo* models to ensure highly accurate intestinal gut models closely related to *in vivo* systems.

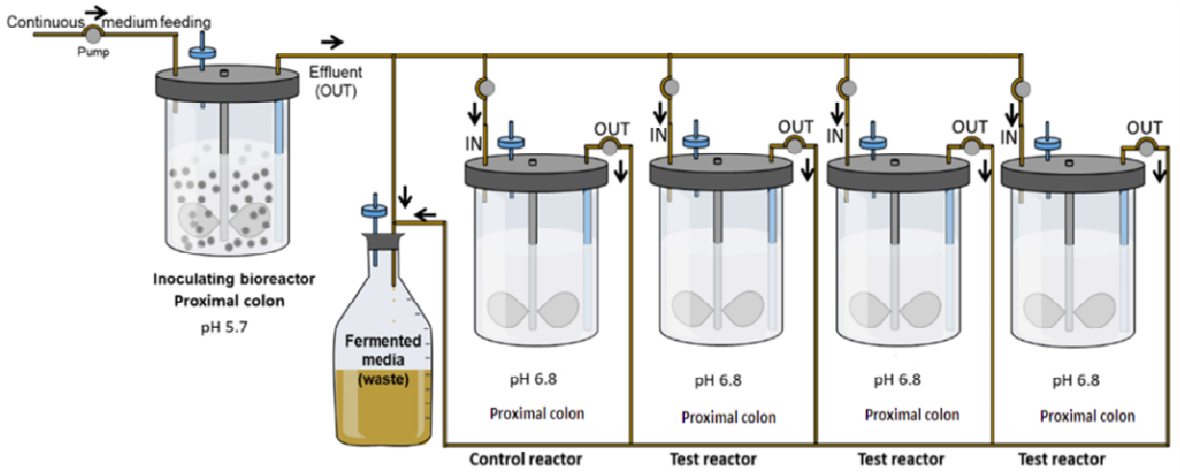


Figure1.5. Setup and design of a double-stage fermentation model used to culture human colonic gut microbiota (based on a PolyFermS model) **(Zihler Berner et al., 2013)**.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

Commensal gut bacteria produce a variety of inhibitory neurotransmitters such as γ-aminobutyric acid (GABA) which, beyond interacting with intestinal physiology, can influence the gut-brainaxis (Foster & McVey Neufeld, 2013).GABA-producing bacteria have been identified among the colon microbiota with a potential role in modulating gut microbiota functionality and promoting gut health. In this project, we hypothesize that GABA production is a probiotic mechanism through which beneficial bacteria could modulate the gut microbiota.The present study aimed to explore the capacity of lactic acid bacteria (LAB) strains to produce GABA and modulate the human gut microbiota.

The specific aims of the study were:

- **Aim 1.** Screening and identification of GABA-producing strains isolated from food environment*in-vitro*. Bacterialsamplesfrom various starter cultures and fermented foods were tested for the presence of the *gadB* gene,and active candidates were isolated and phylogenetically identified (*Chapter 3*).

- **Aim 2.** Characterization of the probiotic potential(i.e., sensitivity to antibiotics, resistance to gastrointestinal conditions, whole-genome sequence) of the isolated GABA-producing strains (from Aim 1) and investigation of their capacity to grow and synthesize GABA in pre-fermented MacFarlane broth that mimics colon physiological conditions, including microbiota metabolome (*Chapter 3*).

- **Aim 3.**Investigation of the growth, GABA production, and competitiveness of selected probiotic candidates from Aims 1-2 (*Bifidobacterium animalis*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*) in the presence of human gut microbiota *ex vivo* in a model of a proximal colon mimicking physiological and microbiological conditions of the human large intestine (*Chapter 4*).

CHAPTER 3: SCREENING, CHARACTERIZATION, AND GROWTH OF Γ-AMINOBUTYRIC ACID-PRODUCING PROBIOTIC CANDIDATES FROM FOOD ORIGIN UNDER SIMULATED COLONIC CONDITIONS

Rojaalsadat Mousavi[1], Walid Mottawea[1, 2], Hebatoallah Hassan[1], Ahmed Gomaa[1,3], Marie-Claude Audet[1], and Riadh Hammami[1,4,*]

[1] School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

[2] Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

[3] National Research Center, Nutrition and Food Science Department, Cairo 12622, Egypt.

[4] Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

* Corresponding author: riadh.hammami@uottawa.ca

(Submitted for publication in *Journal of Applied Microbiology*)

Abstract

Aims: This study aims to isolate probiotic bacteria candidates from various starter cultures and fermented foods and characterize their ability to produce γ-aminobutyric acid (GABA). GABA is a major inhibitory neuromediator of the central and enteric nervous systems with a role in several health disorders.

Methods and Results: Several lactic acid bacteria were isolated and screened for the presence of the glutamate decarboxylase (*gadB)* gene using PCR and GAD enzymatic assay. The most active strains with high and fast GABA production kinetics were identified andcharacterized, and included *Streptococcus thermophilus*, *Lactiplantibacillus plantarum*, and *Lactobacillus delbrueckii* subsp*. bulgaricus*. The most potent strains were able to grow and produce GABA in MRS broth and in pre-fermented Macfarlane broth, a medium mimicking the nutrient and metabolome composition encountered in the colon. The biological safety (i.e., sensitivity to antibiotics and presence of virulence factors) and probiotic potential (i.e., resistance to gastrointestinal conditions and whole-genome sequencing) of the identified bioactive strains was also confirmed *in vitro*.

Conclusions: Several probiotic candidates have shown potential to grow under simulated colonic conditions.

Significance and Impact of Study: Findings from this study provide evidence of the suitability of the isolated GABA-producing probiotic candidates for the development of health-oriented functional food products.

Keywords: lactic acid bacteria, γ-aminobutyric acid, probiotic potential, simulated colonic conditions

1. Introduction

Gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid that exhibits numerous and important physiological functions, such as anti-hypertensive and antidepressant activities (Ngo & Vo, 2019). GABA is widely found in microorganisms, plants, and animals(Dhakal et al., 2012). Indeed, GABA is a key metabolic product of commensal gut bacteria and probiotics (Strandwitz, 2018). Several microbes, including species of the *Lactobacillus* and *Bifidobacterium* genera, have been recognized to have the capability to produce GABA and alter the composition and diversity of the gastrointestinal microbiota (Mazzoli & Pessione, 2016; Strandwitz, 2018), which are promising features for potential use as psychobiotics(Sarkar et al., 2016). Of particular interest, Lactic acid bacteria (LAB) are an important source for glutamate decarboxylase (GAD), the enzyme converting L-glutamate to GABA) (Linares et al., 2017), while being “generally-recognized-as-safe” and commonly found in fermented foods. Molecular characterization of the GAD system and gene expression have been reported in many GABA-producing LAB strains (Lin, 2013). Microbial GABA is synthesized from L-glutamate via the GAD enzyme with pyridoxal phosphate, the active form of vitamin B6, as a cofactor (Cui et al., 2020). Synthesis of GABA is performed via the effective acid resistance system, including glutamate and two GAD isoforms (H. Li et al., 2010). The GAD enzyme is encoded by the gad operon consisting of *gadA* and *gadB*, and can convert glutamate to GABA while decreasing intracellular pH and consuming intracellular proton (Tavakoli et al., 2015). The expression of acid resistance and glutamate import into the cell is carried out by the antiporter*gadC*(Q. Wu et al., 2017). Some studies have shown that the high capacity of GABA production is associated with the GAD system's efficiency, which varies according to the fermentation process (Tavakoli et al., 2015).

GABA-producing bacteria have been identified as potential probiotic candidates and are forefront strategy for producing GABA-enriched functional foods (Lacroix et al., 2013). Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Probiotics bacteria have been recognized as an effective nutritional strategy with many beneficial effects on the gastrointestinal tract, including interaction with host cells and modulation of the gut microbiota (Fliss et al., 2011). Several criteria have to be investigated to elucidate the probiotic potential of microbes, including their health benefits, efficacy, and safety (Ogier & Serror, 2008). In the present study, we screened GABA-producing bacteria from commercial food products, characterized their probiotic potential, and provided some evidence of their capacity to grow and synthesize GABA in Macfarlane

broth, which mimics the composition of medium encountered the human colonic environment. Although previous studies have reported on the high GABA production capacity in LAB strains (Diana et al., 2014; Yunes et al., 2016), the present study is the first to investigate the survival and production of GABA-producing probiotic candidates in pre-fermented MacFarlane broth that mimic colon physiological conditions, including microbiota metabolome.

2. Materials and methods

2.1. Bacterial strains, media, and culture conditions

Several commercial starter cultures and fermented food products were purchased from a local food market, assessed for GABA production, and summarized in Table 1. *Bifidobacterium dentium* ATCC 27678, a GABA producing bacterium, was used as a positive control. All isolated bacteria were routinely cultured overnight, in Lactobacilli MRS medium (VWR Avantor, Canada), at 37°C under anaerobic conditions, a gas mixture of 5% CO_2, 5% H_2, and 90% N_2 (Whitley Anaerobe Systems A35, Don Whitley Scientific, UK). For the preparation of solid media, 1% (w/v) of bacteriological agar (VWR) was added to the liquid media, de Man-Rogosa-Sharpe (MRS) or M17 broth (Sigma-Aldrich), depending on the strain. The purified isolates were maintained in 20% glycerol stocks at −80°C until further use.

2.2. Growth conditions

2.2.1. Growthkinetics in mMRS broth

To measure the growth kinetics of isolated bacteria, the modified DeMan-Rogosa-Sharpe (mMRS) broth media, supplemented with 0.5% w/v monosodium glutamate (MSG; which acts as an inducer for GABA biosynthesis) and 0.01% w/v pyridoxal-5′-phosphate (PLP; the active form of vitamin B6 playing as a cofactor) was used. The selected bioactive strains were inoculated to mMRS with a final cell density of 10^6 CFU/ml and incubated at 37°C for 24 h. The growth kinetics and GABA production were determined in the mMRS medium during a 48-h incubation at 37°C using 1% of overnight inoculum of the bacterial strains. Samples were taken aseptically, every 4 h during the first 12 h and every 6 h during the last 36 h. Hence, growth kinetics were measured at 0, 4, 8, 12, 18, 24, 30, 36, 42, and 48 h of incubation. The experiment was performed in triplicates for each strain. 100 µl of each sample were transferred to a microtiter plate to determine the bacterial growth at OD 600 nm using a microplate reader (Tecan Spark GmbH, Austria). Colony counts (Log CFU/mL) were obtained following 10-fold serial dilutions and pour plate method. Simultaneously, the pH of samples was measured using a Benchtop pH Meter (Fisherbrand™ accumet™ AE150, Thermo Fisher Scientific, Canada). The remaining supernatants were centrifuged, filtered by size of 0.22 µm pore disks for taking

the cell-free culture supernatants (CFS), and then kept at -80 °C for GAD enzymatic activity and GABA quantification. Cell debris was suspended in phosphate-buffered saline (PBS) (pH 7.3) for GAD activity assay.

2.2.2. Comparative growth in mMRS and Macfarlane broths

GABA-producing bacteria were grown in mMRS broth supplemented and Macfarlane broth supplemented with 0.5% w/v MSG and 0.01% w/v PLP. The Macfarlane broth composition was previously described by (Le Lay et al., 2015) with some modification. The fresh culture media were inoculated with 1% overnight subculture and then incubated under anaerobic conditions at 37°C for 24 h. Samples were taken at 0, 24, and 48 h of incubation and analyzed for the viable bacterial counts (CFU/mL), pH, and GAD activity, as described below.

2.2.3. Pre-Fermented Macfarlane broth

Pre-Fermented broth medium is a Macfarlane broth media containing fecal microbial sample which was immobilized by gel beads, a solution containing 2.5 % gellan gum, 0.25 % xanthan gum, and 0.2 % sodium citrate (v/w), after the stabilization phase of fermentation in an inoculum reactor as described by(Mottawea et al., 2020). This medium, as a fermented outflow, was recovered from the fermentation system, which was fed continuously with fresh Macfarlane broth by replacing the fermented broth and set up at 80 rpm, 37 °C, pH 6.7, and sterile CO_2 sparger to maintain anaerobic conditions and stabilize the fecal microbiota, then centrifuged at 14,000 × g for 10 min, and filtered by 0.45 µm pore size for taking the supernatants for further experiment.

2.2.4. Growth and GAD enzymatic activityin simulated colonic conditions

To determine the capability of the identified active strains to adapt to the simulated colonic condition, the different parameters described above were analyzed in the Pre-fermented Macfarlane broth media for one week. In brief, pre-fermented Macfarlane broth media was inoculated with the overnight culture at the final cell density of 10^6 CFU/ml and incubated under anaerobic conditions at 37°C for 24 h. Samples were taken after 24 h incubation to determine the CFU, pH, and enzyme activityas described above. Simultaneously, fresh Pre-fermented Macfarlane broth media was inoculated with overnight culture for redoing the test for the next 24 h. These experiments were performed daily for a week and in triplicate.

2.3. Bacterial enumeration

Bacterial counts were determined by plating ten-fold serial dilutions of culture on selective media using the drop plate method. All bacteria were counted using MRS broth medium and incubated at 37 °C for 48h anaerobically.

2.4. GADactivity and GABA quantification assays

2.4.1. Colorimetric microplate assay method for detection of GAD activity

The study used the bromocresol green-based colorimetric method for detecting GAD activity in a 96-well microplate format as previously described (Yu et al., 2011). In brief, a vortexed overnight culture of strains (10^6 CFU/mL) was transferred to the microplate for measuring OD 600nm. Each bacterial overnight culture with the same concentration (OD_{600} 0.6–0.8) was centrifuged at 14,000 × g at room temperature for 5 min. Bacterial cells were suspended in 5 mM PBS buffer (pH 7.3) and disrupted by sonication using a Qsonica Sonicator Q125 (Thermo Fisher Scientific, Canada). Cell debris was removed by centrifuging at 14,000 × *g* in 4°C for 30 min, and supernatants were taken as crude extracts for the GAD activity assay. The microplate was prepared with the reaction medium containing acetate buffer (20 mM, pH 4.8), 0.07 mM bromocresol green, 10 mM pyridoxal 5-phosphate (PLP), 5 µL glutamate, and 10 µL cell extract. The standard reaction medium mentioned above without cell extracts, PLP, and glutamate served as a negative control. After sample preparation, the microplate was placed in the microplate reader as the reaction proceeded and shaken for 10 s to mix thoroughly. The absorbance change at 620 nm was a function of time and recorded by a microplate reader (Tecan Spark GmbH, Austria) at 37°C. The absorbance was obtained every 60 s for 10 min. The measurement was done in triplicates.

2.4.2. GABA quantification using GC-FID

The production of GABA was determined by FID-GC (Lacroix et al., 2013). Briefly, samples were centrifuged for 5000g for 30 min, and then supernatants were collected. Derivatization of samples was performed using EZ:faast kit (Phenomenex, Torrance, CA, USA) according to the manufacturer's manual with norvaline (200 nmol/L) as internal standard. The derivatized samples were then analyzed by FID-GC (Nexis GC-2030, using a flame ionization detector (Shimadzu Corporation). A 1-µL volume of the derivatized sample was injected into a ZB-MultiResidual column with diameters of dimension is 30-meter x 0.25 mm x 0.25 µm (Phenomenex) using autosampler mode. The injection was performed in split mode with a split ratio of 1:15 at 300 °C using helium as the carrier gas with 1.0 mL/min flow; and a make-up gas flow of 23 mL/min; hydrogen flow of 30 mL/min; airflow of 300 mL/min; The temperature of the

oven program at 80°C with a 2-min hold and increased at a rate of 10°C/min to 320°C with a final hold of 2 min. For quantification purposes, the calibration curve was created for pure GABA (Sigma) in a range of 500 nmol/L to 64 µmol/L. Measurements were performed in triplicates.

2.5. Safety Evaluation and in vitro probiotic potential

2.5.1. Antibiotic susceptibility test

Isolated strains were tested for susceptibility to multiple antibiotics from different classes: streptomycin, gentamycin, vancomycin, ampicillin (dissolved in water), erythromycin, and chloramphenicol (dissolved in ethanol and water), tetracycline (dissolved in ethanol), ciprofloxacin (dissolved in 0.1 M HCl and water). Tests were performed in microplates according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2018) and the European Food Safety Authority (EFSA 2018) guidelines as described by (Hanchi et al., 2014). briefly, the microtiter plate with an equal volume of final cell density of overnight culture of each bacterial strain and MRS broth was prepared, and then serial dilution with different concentrations of stock solution of each antibiotic was added. The microplates were incubated at 37 °C for 24 h, and the minimum inhibitory concentration (MIC) as the lowest concentration of each antibiotic that inhibits the growth of each bacteria was reported at OD 600 nm using amicroplate reader (Tecan Spark GmbH, Austria). The level of susceptibility to antibiotics was expressed as resistant or sensitive according to the breaking points adopted from CLSI 2018 and EFSA 2018.

2.5.2. Presence of genes encoding virulence factors

Total DNA of two *Enterococcus faecium* strains isolated from this study was extracted and assessed for the presence of six virulence genes by PCR, using primers for various common virulence genes,including cell wall degrading bacteriocin (*EntLysA*), enterococcal surface protein (*esp*), aggregation substance (*asa*), Hyaluronidase (*hyl*), cytolysin operon (*cyl A*), and gelatinase (*GelE*) as previously described (Hanchi et al., 2014). *Enterococcus faecalis*ATCC 27275was used as a positive control. PCR was carried out in a 50 µL reaction mixture with template DNA, $MgCl_2$, deoxynucleoside triphosphate (dNTP), Taq polymerase, and each specific primer to virulence genes, and performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Canada). Primers and their PCR reaction conditions were used as previously described (Hanchi et al., 2014) and are provided in Table S1. PCR products were detected by gel electrophoresis in 1% agarose gel (Sigma, Oakville, ON, Canada) and analyzed with UV transillumination using a gel imaging system (Life Science Research, Bio-RadLaboratories, Canada).

2.5.3. Resistance to Simulated Gastric and Intestinal Fluids

2.5.3.1. Microbial Viability in Simulated Gastric Juice

The tolerance of selected isolates to simulated gastric and intestinal fluids was examined as per the method described elsewhere (Siragusa et al., 2007). Briefly, simulated gastric juice recommended by the U.S. Pharmacopeia (USP) was prepared by suspending 0.32% (w/v) pepsin in 0.084 M hydrochloric acid, 0.03 M sodium chloride with pH adjusted to 2.5 to the initial volume and filtered through a filter of 0.45 µm pore size before autoclaving as previously described (Vecchione et al., 2018) with some modifications. Briefly, the strains were grown in a MRS broth under anaerobic conditions at 37 °C for 18 h. Then, the overnight cultures were standardized at an OD_{600nm} = 1.8-2. Two hundred milliliters of filter-sterilized juice (2%) were placed in a 250 mL sterilized flask, inoculated with bacterial suspension at the rate of 2% (v/v), and incubated at 37°C for 150 min. The pH during digestion decreased according to the profile suggested by Marteau et al. (1991) for yogurt digestion in which gastric pH dropped gradually from 4.5 to 1.5 in 2.5 h (Marteau et al., 1991). The initial pH of gastric juice was adjusted at 4.5 prior to adding bacterial suspension and incubated at 37°C for 30 min. Then the pH decreased to 2.4, 1.7, 1.6, and 1.5 at 30 min intervals using 0.5 M HCl solution. Samples were aseptically withdrawn at zero time and at 30 min intervals during 2.5 h of incubation to determine the viable cell counts by plating serial dilution samples (made in 0.15% sterile peptone water) using the drop-plate method described by Herigstad et al. (2001) on MRS agar medium incubated at 37°C for 48 h. Results were expressed as CFU per milliliter (CFU/mL) (Herigstad et al., 2001). Results were expressed as a percentage of the survival of the bacteria after exposure to simulated gastric conditions.

2.5.3.2. Microbial Viability in Simulated Intestinal Fluid

The simulated intestine fluid was prepared according to the United States Pharmacopeial Convention (2004). Briefly, 0.68% of monobasic potassium phosphate, 19% (v/v) of sodium hydroxide 2 N, pancreatin 1% (w/v), 0.3% (w/v) porcine bile extract (Sigma Aldrich, Co., St. Louis, MO, USA), and 0.25% L-cysteine (Nikolic et al., 2012), digestion was done according to the profile suggested by Marteau (1991). Two hundred milliliters of filter-sterilized intestinal juice were placed in a 250 ml sterilized flask, inoculated with bacterial suspension at the rate of 2% (v/v), and incubated at 37°C for 180 min. The pH was adjusted to 7.2 by injecting 0.2 M sodium hydroxide solution. During 3 h of incubation, samples were aseptically withdrawn at initial time and hourly to determine the viable cell counts by the drop-plate method as described above.

2.6. Genomic analyses

2.6.1. Design of specific primers for gadB gene and its detection using PCR

The primers were designed using the Primer-BLAST tool according to the nucleotide sequence of the *gadB* gene of strains with known taxonomic information at the National Centre for Biotechnology Information (NCBI) databank. The potential matches of selective specific primers were performed with RDP Release 11 - Sequence Analysis Tools. The specific forward and reverse *gad* gene primers were used for PCR reaction are shown in Table 2. The PCR amplification was performed with different annealing temperatures with a gradient from 50-55°c for optimizing the results.All amplifications were performed in a T100™ Thermal Cycler (Bio-Rad Laboratories, Canada). After optimizing the respective annealing temperature, the bacterial strains were screened for the gad gene's presence in all the commercial starter culture samples by performing a PCR reaction.

2.6.2. Identification of GABA-producers by 16S rDNA sequencing

Total DNA of active strains isolated with having *gadB* gene was extracted using Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA) according to the manufacturer's instructions with some modifications, and DNA concentration was determined using NanoQuant Plate (Tecan Group Ltd. Männedorf, Switzerland) for PCR amplification. For each sample, the 16S region was amplified using universal primers Bact 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), 1391R (5'-GACGGGCGGTGTGTRCA-3') (Morrison et al., 2012) in a T100™ Thermal Cycler (Bio-Rad Laboratories, Canada). The total volume of each reaction mixture was 50 µl as described by (Hanchi et al., 2014). The thermal cycle program consisted of an initial denaturation and polymerase activation at 95 °C for 2 min, amplification for 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 1 min. Amplified products were verified by detecting single bands following electrophoresis (110 V for 45min) on 1% agarose gels and visualized by ethidium bromide staining using gel imaging system (Life Science Research, Bio-Rad Laboratories, Canada). The PCR amplicons were then purified using QIAquick PCR Purification Kit (QIAGEN, Canada), and sequencing ofamplified 16S rDNA gene from isolated strains was performed at the Ottawa Hospital Research Institute (OHRI) Sequencing Facility. The resulting sequence data were aligned and analyzed using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ribosomal Database Project (RDP Release 11 - Sequence Analysis Tools) (http://www.lcsciences.com/documents/sample_data/16S_sequencing/src/html/top1.html).

2.6.3. Whole-genome sequencing of selected bioactive strains

A total of 3 strainshaving high GAD activity, namely *Streptococcus Thermophilus* ST16, *Lactobacillus delbrueckii subsp. Bulgaricus* ST7, and *Bifidobacterium animalis* ST20, were selected for Whole-genome sequencing (WGS). These bacteria were grown in MRS broth under anaerobic conditions for 24 h at 37°C. The whole genomic DNA extraction of the bacterial samples was performed using NucleoSpin® Microbial DNA Kit (MP Biomedical, USA) according to the manufacturer's instructions with some modifications. The concentrations of the extracted DNA bacterial samples were quantified using a Qubit dsDNA BR Kit (NanoQuant Tecan Spark, Switzerland), and the concentration of each sample was set to yielding 30 ng/µl of high-quality DNA for PCR reaction to an average fragment library size of ~ 630 bases. DNA library for microbial WGS was prepared using the Nextera™ DNA Flex Library Preparation Kit (Illumina, San Diego, CA) following the manufacturer's recommended procedures. The sequencing library was sequencedon an Illumina MiSeq instrument using the MiSeq reagent kit with a paired-end 250 bases format. The PCR amplicons were > 150 bp. Sample bead purification was cleaned up amplicons >500 bp.Designed primers were used to cover the amplicon target region (50 bp per end reads). Base-calling was performed on the raw WGS data, the fastq data were generated, and the velvet assembled genomes were reported. The genome annotation was performed by the Rapid Annotation using Subsystem Technology (RAST) (https://rast.nmpdr.org/), an annotation server optimized for strains isolated. The DNA sequences obtained have been deposited in the GenBank database under accession numbers ST7 sp. 6666666.655309, ST16 sp. 6666666.655310, and ST20 sp. 6666666.655312.

2.7. Statistical analysis

Data were expressed as the mean ± standard error (SE). All experiments were conducted in biological and technical triplicates. Repeated measures 2-way ANOVA test with Tukey's was used to compare multiple groups using GraphPad Prism software version 8 (GraphPad Prism Inc., San Diego, CA, USA). A p-value at $p < 0.05$ or less was considered as the level of significance for some experiments. Significant differences were indicated in the figures by different P values (*$p < 0.05$, **$p < 0.01$).

3. Results

3.1. Detection of gadB genes and isolation of potent LABs species

A total of 30 microbial cultures were assessed for the presenceof the *gadB* gene, with only 14 samples being positive (Table 3.1). The potentially bioactive bacteria were then isolated

from the samples using selective culture media, and their identity was confirmed by 16SrRNA sequencing (Table 3.1). Of 14 isolated LABs, we identified 5 strains of *Streptococcus thermophilus* (isolated on M17 agar plates) and 4 of *Lactiplantibacillus plantarum* (isolated on MRS agar plates). Among isolated bioactive strains, we have also found non-expected strains that were undeclared or contaminants, such as *Enterococcus faecium*, a common contaminant of dairy food products, and *Bifidobacterium animalis* in sample 20.

3.2. GAD activity

GAD activity of the isolated strains was determined by rapid colorimetric assay using bromocresol green. As shown in Figure 3.1, GAD enzymatic activity of cell extracts was maximal for strains *S. thermophilus*ST16, *S. thermophilus* ST18, *S. thermophilus* ST8, *B. animalis* ST20, *L. acidophilus* LP16-2, and *L. plantarum*LP9 compared to species lacking the *gadB* gene (negative controls: *S. thermophilus* ST23 and *L. plantarum* LP27). GC-FID analyses confirmed the highest GABA production capacity of *S. thermophilus* ST16 (1641.5 ± 154.15 µmol/L), *S. thermophilus* ST8 (1724.5±48.08 µmol/L). In a lesser extent, *B. animalis* ST20, *L. acidophilus* LP16-2, and *E. faecium* ST3 respectively produced 947.5 ± 70.71, 918.0 ± 121.42, and 907.83 ± 55.15 µmol/L of GABA.

3.3. Growth and enzymatic activity in a simulated colonic environment

The growth kinetics, acidification profile, and GAD activity of the isolated strains were monitored in the mMRS broth, as shown in Figure 3.2. All strains had important growth, being maximal for *S. thermophilus* and *L. acidophilus*. The pH also decreased below 5 for all strains suggesting a significant acid production. Of particular interest, the strain *S. thermophilus* ST16 presented the highest growth, almost 10 logs CFU/mL after 24 h, and had a maximal GAD enzymatic activity (18-36 h) during the assay (Figure 3.2). The growth, pH, and enzyme activity of the GABA-producing strains were also investigated in the mMacfarlane medium, which mimics the physiological conditions in the colonic environment. All tested strains had growth, acidification profiles, and enzymatic activities relatively maximal in mMacfarlane medium close to those obtained in the mMRS broth (Figure 3.3). Finally, the capacity of our selected bacteria to grow and produce their metabolites was assessed in a microbiota pre-fermented mMacfarlane broth, which contains all metabolites produced by the gut microbiota, including short-chain fatty acids that could inhibit the growth of several microbes in the colonic environment. As shown in Figure 3.4, the growth, acidification capacity, and GAD enzymatic activity were monitored for seven consecutive days. The capacity of the selected bacteria to adapt to the pre-fermented media was confirmed, with a maximal growth obtained after 4 days.

The strain *S. thermophilus* ST16 also had the highest growth and GAD activity compared to the other tested strains. All these results confirmed the capacity of the selected bacteria to grow and survive in simulated human colonic conditions.

3.4.*In vitro* probiotic potential

3.4.1. Resistance to antibiotics of isolated strains

The minimal inhibitory concentration (MIC) of each antibiotic was detected by microdilution methods. The breakpoints of the antibiotics ampicillin, gentamicin, vancomycin, streptomycin, erythromycin, ciprofloxacin, tetracycline, and chloramphenicol were determined following CLSI 2018 and EFSA 2018 (Hanchi et al., 2014). All the strains were sensitive to streptomycin (aminoglycoside class of antibiotics) (Table 3.3). *S. thermophilus* strains were resistant to gentamycin, erythromycin, and tetracycline at the highest amount of 50, 6.25, and 12.5 µg/mL, respectively.*L. plantarum* strains were susceptible to gentamycin, streptomycin, tetracycline, andchloramphenicolbut resistant to ampicillin, erythromycin, vancomycin, and ciprofloxacin. The highest concentration value for ampicillin, vancomycin, erythromycin, and ciprofloxacin among isolated *L. acidophilus* (LP16-2) was measured at 1.562, 3.125, 1.562, and 6.25 µg/mL. The *B. dentium* and *B. animalis* ST20 were shown to be resistant to the vancomycin 3.125 µg/mL, erythromycin 1.562 µg/mL (Table 3.3). Two isolated *Enterococcus faecium*(ST2, ST3) were observed to resist to ampicillin, vancomycin, erythromycin, and ciprofloxacin with a MIC value of 12.5, 12.5, 3.125, and 1.562 µg/mL, respectively. Finally, *L. delbrueckii* subsp. *bulgaricus* was found to be resistant to vancomycin at 3.125 µg/mL, erythromycin at 1.562 µg/mL, tetracycline at 6.25 µg/mL, and ciprofloxacin at 6.25 µg/mL (Table 3.3).

3.4.2. Virulence Factors

The two isolated GABA-producing strains, *E. faecium* ST2 and *E. faecium* ST3, were screened for the presence of several determined virulence factors and compared to the positive control *Enterococcus faecalis* ATCC 27275. Six virulence genes involving *EntLysA*, *esp*, *asa*, *hyl*, *cyl A*, and *GelE* were tested for isolated strains in this study. None of the virulence genes (*EntLysA*, *esp*, *asa*, *hyl*, *cyl A*, *GelE*) were detected for the strains tested. The control strain *E. faecalis* ATCC 27275 was positive for the presence of enterolysin A (*EntLysA*), aggregation substance (*asa*), entrococcal surface protein (*esp*), and gelatinase (*gelE*) as described in previous studies (Hanchi et al., 2014; Kiruthiga et al., 2020) in respective product size of 345, 410, 560, and 250 bp. This strain was also negative for hyaluronidase (*hyl*), cytolysin (*cylA*).

3.4.3. Whole-genome sequencing

The whole-genome sequences of 3 isolated representative GABA-producing strains (*S. Thermophilus* ST16, *L. delbrueckii* subsp*. Bulgaricus* ST7, and *B. animalis* ST20) were determined and compared with some related closest LABs sequenced genomes available in the GenBank database. The genome sequence length, average G+C content, rRNA gene operons, and assembled contigs are summarized in Table 3.4. Genomic sequences of all active strains were examined to identify genes encoding *gadB* and *gadC* involved in GABA biosynthesis. The sequence alignments were compared to *gadB* belonging to *Streptococcus* and *Lactobacillus* genera possessing the GAD locus identified by BLAST software, as described previously (Duranti et al., 2020). The *gadC* was coded for glutamate/GABA antiporter and identified as a whole operon with *gts* (glutamyl-tRNA synthetase) for strains sequenced, contributing to GABA accumulation, similar to other whole genomes available in the GenBank for GAD detection. Moreover, we have surveyed the *pdxJ*, a gene involved in the synthesis of pyridoxal 5′-phosphate (PLP), a metabolically active form of vitamin B6 that plays a critical cofactor during glutamate bioconversion to GABA. Besides, no detected genes were associated with resistance to the common antibiotics proposed by CLSI guidelines and EFSA standards, confirming the safety of these probiotic candidates. Also, no virulence factor genes were identified in the sequenced genomes.

3.5.4. Survival in gastrointestinalfluids

The most bioactive strains were tested for their viability in gastric juice and intestinal fluid.All the selected LAB strains showed resistance to gastric juice at 2.4 pH and intestinal conditions and survived within the gastrointestinal environment. The viable cell counts of the LABs and *Bifidobacteria* strains were calculated as follows: *S. thermophilus* ST8 and ST 16*, L. acidophilus* LP16-2*,* and *L. plantarum*LP6 and LP9*,* had the highest survival rates, and the *L. plantarum* LP6 had the highest survival rate until pH 1.5 in gastric conditions (Figure 3.5). On the other hand, *B. animalis* ST20 survived only at pH 4.5 and 2.4 in the simulated gastric juice. After 3 h of exposure to the intestinal fluid, all bacterial strainsexhibited high survival rates after 3 h incubation at 37°C.

4. Discussion

This study aimed to screen the ability of LABs isolated from different commercial starter cultures and food products to produce GABA and to survive gastrointestinal conditions. Evidence is accumulating on a pivotal role for GABA in host physiology and on the impact that dysfunction in its signaling may have on a wide range of diseases (Smart & Stephenson, 2019).

For instance, the relative abundance of *Bacteroides*, a major GABA-producing member of commensal gut microbiota, was negatively correlated with depression-associated brain signatures (Strandwitz et al., 2019), indicating a potential role for microbiota-derived GABA in brain functionality. Importantly, GABA has been identified as an essential growth factor that can induce the growth of unculturable gut bacteria (Strandwitz et al., 2019), raising the possibility that*in vivo* GABA production by commensal and probiotic bacteria could exert mental health benefits through its actions on the gut microbiota (Bienenstock et al., 2010). In this study, we identified several *Lactobacilli* and *Bifidobacteria* strains with a high capacity to produce GABA, consistently with previous studies that described the GAD activity of GABA-producing LABs (Lacroix et al., 2013; Patterson et al., 2019). Whether these GABA-producing candidates interact with commensal gut bacteria and exert health benefits on the host, however, has yet to be determined.

During the isolation procedure, non-declared strains were identified, including *E. faecium, L. delbrueckii subsp. bulgaricus, L. acidophilus,* and *B. animalis*, suggesting bacterial contamination of the commercial products.A total of 14 LAB strains isolated from various commercial starter cultures and food products were identified as positive for the presence of the *gadB* gene (Richard & Foster, 2004) and found to produce GABA, with 5 *S. thermophilus* and 4*L. plantarum* strains being the most active isolates. In addition, these strains presented with a high growth and acidification capacity, which is likely due to the production of organic acids (mainly lactic acid). Our tested strains also survived in both the mMacfarlane and pre-fermented Macfarlane broths (the latter mimicking colon physiological conditions, including microbiota metabolome), suggesting their suitability as probiotics with high GABA production capacity. Indeed, beside metabolite production (GABA in our case), survival under simulated colonic conditions is an important criterion when investigating the probiotic potential of microbial candidates (Le Lay et al., 2015). Several studies illustrated the role of the bacterial GAD system in the pH tolerance of acidic environments. For example, *E. coli* contains about 12 metabolic stress-related genes, including the GAD system, which form an acid fitness island (Hommais et al., 2004; Mates et al., 2007). The GAD system maintains a neutral pH of the cytoplasm via the decarboxylation of glutamate to GABA and inversion of the membrane potential (Hommais et al., 2004; Mates et al., 2007). Our findings thus confirmed that our isolated strains could adapt to a colonic environment and are good GABA-producing candidates for developing functional food products.

The safety evaluations and *in vitro* probiotic potential of the selected strains were assessed by antibiotic susceptibility, detection of virulence-encoding genes, and survival in simulated gastric juice and intestinal fluid assays. Our isolated strains exhibited a species-dependent susceptibility to several antibiotics, being sensitive to critically important antimicrobials as previously observed for LAB strains (Hanchi et al., 2014). LABs (except Enterococci) are generally recognized as safe and are widely used as probiotics and fermentive cultures for various foods, supported by a long history of safe usage (Hammami et al., 2019). Many Lactobacilli have been reported as intrinsically resistant to antibiotics despite their safety status, such as vancomycin, gentamicin, kanamycin, streptomycin, and ciprofloxacin but susceptible to penicillin and β-lactams (Campedelli et al., 2018). In addition, *Bifidobacteria* strains tested in the current study were resistant to vancomycin, according to previous reports (Kheadr et al., 2007). Besides, the two non-expected isolated strains *E. faecium* ST2 and *E. faecium* ST3, in our study did not exhibit any virulence gene, thus confirming their safety. Indeed, the absence of virulence genes is an important criterion for Enterococci's probiotic selection (Hanchi et al., 2018).

Resistance to gastrointestinal conditions is also an essential criterion for probiotic assessment (Lone et al., 2021). All bacterial isolates in the current study were resistant to the gastric juice at pH 2.4, while only *L. plantarum* LP9 survived until pH 1.5, and *S. thermophilus* ST8, *L. plantarum* LP6, and *L. acidophilus* LP16-2 survived until pH 1.6. Remarkably, all tested strains exhibited very high survival rates in the pancreatic fluid. Resistance to gastrointestinal stresses was previously reported for several LABspecies while being species-dependent (Vecchione et al., 2018). Our WGS results confirmed that our tested strains (*L. delbrueckii* subsp. *bulgaricus* ST7, *S.thermophilus* ST16, *B. animalis* ST20) exhibited high GAD enzymatic activity, reflective of their capacity to produce GABA, while lacking plasmids, toxins, adhesion toxins, and transposable elements, indicating that their resistance profiles are chromosomic mediated. All safety and stability features of the tested GABA-producing strains position them as promising probiotic candidates for extending their application to the functional food industry(Lacroix et al., 2013; Siragusa et al., 2007).

The present study provides evidence that several LABs from food origin have the capacity to produce GABA and present essential probiotic features for their application as functional food products. Fourteen strains were isolated, identified, and tested for their GABA production and GAD enzymatic activity. The selected strains had fast product kinetics, did not harbor any virulence factors, were sensitive to antibiotics and presented with a high survival rate

in simulated gastrointestinal conditions. Our findings also confirm the capacity of these probiotic candidates to grow, survive, and produce their active metabolites, such as GABA, under human colonic physiological conditions that included microbiota metabolome. Although additional studies are still needed to fully characterize these bacteria, the establishment of these essential probiotic properties in our candidates offers a new opportunity to use these GABA-producing bacteria in the development of health-oriented functional food products.

Acknowledgments

This study was supported by a grant from the Canadian Tri-Agency (i.e., CIHR, NSERC, and SSHRC) Institutional Program [NFRFE-2018-00295] to MCA and RH and an NSERC-Discovery grant RGPIN-2018-06059 to RH. RM was supported by the Nutrition and Mental Health Master's Scholarship, University of Ottawa.

Author contributions

Conceptualization: R.M., W.M., and R.H.; methodology, R.M., W.M., and R.H.; validation, R.M., W.M., and R.H.; formal analysis, R.M, W.M, H.H., and A.G.; investigation, R.M., W.M., H.H; resources, R.H.; writing—original draft preparation, R.M.; writing—review and editing, W.M., H.H., M-C.A., R.H; visualization, R.M., W.M, H.H., and R.H.; supervision, R.H.; funding acquisition, R.H. and M-C.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Table 3.1. The list of Bacterial strains isolated from commercial food products.

Sample	Products	Declared bacterial content	Isolated bacterial strains	Strain ID	Detection of GAD gene
1	Probiotic capsules	*Lactobacillus delbrueckii subsp. bulgaricus*	-	-	-
2	Probiotic mix: Lactobacillus and Bifidobacterium	*L. delbrueckii subsp. bulgaricus, L. acidophilus, L. casei, L. rhamnosus, B. infantis, B. bifidum, B. longum, B. breve, B. adolescentis, S. thermophilus*	*Enterococcus faecium*	ST2	+
3	Bulgarian Yogurt Starter Culture	*L. delbrueckii subsp. bulgaricus, S. thermophilus, B. infantis, B. bifidum, B. longum, B. breve, B. adolescentis*	*E. faecium*	ST3	+
4	Cheese Thermophilic starter	*S. thermophilus, L. helveticus*	*S. thermophilus*	ST4	+
5	Propionic Bacteria	*Propionibacterium freudenreichii subsp. shermanii*	-	-	-
6	Industrial Foamy Kefir	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, L. plantarumL. rhamnosus*	*L. plantarum*	LP6	+
7	Yogurt Starter: Bifidobacterium and Acidophilus	*B. sp., S. thermophilus, L. acidophilus*	*L. delbrueckii subsp. bulgaricus*	ST7	+
8	Yogurt Culture	*L. casei, B. longum, L. delbrueckii subsp. bulgaricus, S. thermophilus, L. acidophilus*	*S. thermophilus*	ST8	+
9	Digestive Care starter culture	*L. plantarum*	*L. plantarum*	LP9	+
10	Brevibacterium Linens	*Brevibacterium Linens*	-	-	-
11	Kefir Starter Culture	*L. lactis, L. lactis subsp. lactis biovar diacetylactis, L. lactis subsp. cremoris, Leuconostoc mesenteroides cremoris, L. kefiranofaciens*	-	-	-
12	Industrial mesophillic starter	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, Leuconostoc mesenteroides*	-	-	-
13	Industrial starter culture Flora	-	-	-	-
14	Probiotic capsules	-	*L. plantarum*	LP14	+
15	Cheese starter culture	-	-		-
16	Cheese starter culture	-	*S. thermophilus* *L. plantarum* *L.acidophilus*	ST16 LP16-1 LP16-2	+ + +
17	Cheese starter culture	*L. lactis, L. lactis subsp. cremoris*	-	-	-
18	Cheese starter culture	*S. thermophilus, L. delbrueckii subsp. bulgaricus*	*S. thermophilus*	ST18	+
19	Cheese starter culture	*L. lactis, L. lactis subsp. cremoris*	-	-	-
20	Yogurt Culture	*S. thermophilus, L. delbrueckii subsp. bulgaricus, L. lactis, L. acidophilus, B. longum*	*B. animalis*	ST20	+
21	Starter protective culture	-	*S. thermophilus*	ST21	+
22	Cheese starter culture	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, Leuconostoc mesenteroides*	-	-	-
23	Cheese starter culture	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar*	*S. thermophilus*	ST23	-

		diacetylactis, S. thermophilus			
24	Cheese starter culture	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, L. delbrueckii subsp. bulgaricus*	-	-	-
25	Starter protective culture	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, L. helveticus*	-	-	-
26	Cheese starter culture Flav	-	-	-	-
27	Starter protective culture	-	*L. plantarum*	LP27	-
28	Starter protective culture	*Brevibacterium Linens*	-	-	-
29	Starter protective culture	*L. lactis, L. lactis subsp. cremoris, L. lactis subsp. lactis biovar diacetylactis, Leuconostoc spp.*	-	-	-
30	ATCC reference strain	*B. dentium* ATCC 27678	-	-	+

Table 3.2. Designed specific primers used for the detection of microbial*gadB* gene

No	Specificity	Primer Name	Primer type	Sequence (5'-3')	GC content (%)	Melting Temp (°c)	Annealing temp gradient (°c)
1	*Bifidobacterium dentium*	GADB-F	Forward	TGGGACTTCCGCGTCAAGC	63.18	62.88	50-55°c
		GADB-R	Reverse	CGCAGGAACATGTAGTACTGC	52.38	59.07	
2	*Streptococcus thermophilus*	GADST-F	Forward	GTTTTTCTTGCCATCGGCGT	50	60.04	50-55°c
		GADST-R	Reverse	CCCCGGAGAAAACACTGAGG	60	60.32	
3	*Lactiplantibacillus plantarum*	GADLp-F	Forward	CGGGTACCTCTACGATTGGC	60	59.97	50-55°c
		GADLp-R	Reverse	TGATGCCCATGATACCGACG	55	59.97	
4	*Lactobacillus lactis* subsp *lactis*	GADLL-F	Forward	TGGCAATTGTGACCCATCGT	50	60.25	50-55°c
		GADLL-R	Reverse	TTTCAATCAATACCTCAGGCCA	40.91	57.69	
5	*Lactobacillus delbrueckii*	GADLD-F	Forward	CGGCTGGCTATCAAGTTTGC	55	59.9	50-55°c
		GADLD-R	Reverse	AACGACCTTATCGAGTGCGG	55	60.18	
6	*Lactobacillus lactis* subsp *cremoris*	GADLLc-F	Forward	TTCATAGCCATTCCCCCAAGC	52.38	60.41	50-55°c
		GADLLc-R	Reverse	GATGAAATGATAGATGAAGGGAACG	40	57.87	
7	*Levilactobacillus brevis*	GADLB-F	Forward	CCTCGACGATTGGTTCCTCC	60	60.18	50-55°c
		GADLB-R	Reverse	GTGTAAAATCCGCCGGAAGC	55	59.9	
8	*Lactococcus lactis subsp. diacetylactis*	GADLD'-F	Forward	CTCGCATTCCAAAGGTCAGC	55	59.55	50-55°c
		GADLD'-R	Reverse	ATTGAACCTCGAGTGGCCTA	50	58.43	
9	*Lactobacillus delbrueckii subsp. bulgaricus*	GADLb-F	Forward	ACTTCCGGTTGGCTAACGTC	55	60.32	50-55°c
		GADLb-R	Reverse	ATCATTTTGGGTCCCTTCCCG	52.38	60.34	

Table 3.3. Minimum inhibitory concentration of various antibiotics against isolated strains.

Strains		Antibiotics (µg/mL)															
		Gentamicin		Ampicillin		Streptomycin		Vancomycin		Tetracycline		Chloramphenicol		Erythromycin		Ciprofloxacin	
		MIC	I*	MIC	I*	MIC	I*	MIC	I*	MIC	I*	MIC	I*	MIC	I*	MIC	I*
S. thermophilus	Breakpoints**	32		0.25-8		64		1-4		2-8		4-16		0.25-2		4	
	ST4	50	*R*	3.125	*S*	50	*S*	1.562	*S*	12.5	*R*	3.125	*S*	6.25	*R*	3.125	*S*
	ST8	50	*R*	3.125	*S*	50	*S*	1.562	*S*	12.5	*R*	3.125	*S*	6.25	*R*	3.125	*S*
	ST16	50	*R*	3.125	*S*	50	*S*	1.562	*S*	12.5	*R*	3.125	*S*	6.25	*R*	3.125	*S*
	ST18	50	*R*	3.125	*S*	50	*S*	1.562	*S*	12.5	*R*	3.125	*S*	6.25	*R*	3.125	*S*
	ST21	50	*R*	3.125	*S*	50	*S*	1.562	*S*	12.5	*R*	3.125	*S*	6.25	*R*	3.125	*S*
	ST23	50	*R*	3.125	*S*	50	*S*	1.562	*S*	12.5	*R*	3.125	*S*	6.25	*R*	3.125	*S*
L. plantarum	Breakpoints**	16		0.125-2		2-1024		2-256		32		4-8		0.125-1		1	
	LP6	12.5	*S*	3.125	*R*	12.5	*S*	25	*R*	25	*S*	3.125	*S*	6.25	*R*	6.25	*R*
	LP9	12.5	*S*	3.125	*R*	12.5	*S*	25	*R*	25	*S*	3.125	*S*	6.25	*R*	6.25	*R*
	LP14	12.5	*S*	3.125	*R*	12.5	*S*	25	*R*	25	*S*	3.125	*S*	6.25	*R*	6.25	*R*
	LP16-1	12.5	*S*	3.125	*R*	12.5	*S*	25	*R*	25	*S*	3.125	*S*	6.25	*R*	6.25	*R*
	LP27	12.5	*S*	3.125	*R*	12.5	*S*	25	*R*	25	*S*	3.125	*S*	6.25	*R*	6.25	*R*
L. acidophilus	Breakpoints**	16		1		16		2		4		4		1		1	
	LP16-2	12.5	*S*	1.562	*R*	12.5	*S*	3.125	*R*	3.125	*S*	3.125	*S*	1.562	*R*	6.25	*R*
Bifidobacteria	Breakpoints**	64		2		128		2		8		4		1		2	
	B. dentium	50	*S*	1.562	*S*	50	*S*	3.125	*R*	6.25	*S*	3.125	*S*	1.562	*R*	3.125	*S*
	ST20	25	*S*	1.562	*S*	100	*S*	3.125	*R*	6.25	*S*	3.125	*S*	1.562	*R*	6. 25	*S*
L. delbrueckii	Breakpoints**	16		2		16		2		4		4		1		1	
	ST7	12.5	*S*	1.562	*S*	12.5	*S*	3.125	*R*	6.25	*R*	3.125	*S*	1.562	*R*	6.25	*R*
E. faecium	Breakpoints**	500		8-16		1000		4-32		4-16		8-32		0.5-8		1-4	
	ST2	25	*S*	12.5	*R*	100	*S*	12.5	*R*	3.125	*S*	6.25	*S*	3.125	*R*	1.562	*R*
	ST3	25	*S*	12.5	*R*	100	*S*	12.5	*R*	3.125	*S*	6.25	*S*	3.125	*R*	1.562	*R*

I*: MIC interpretation (R: resistant; S: sensitive); ** Breakpoints adopted from CLSI and EFSA 2018

Table 3.4. General genomic features of selected bioactive strains

Strain ID	ST7	ST16	ST20
BLAST identity	*Lactobacillus delbrueckii subsp. bulgaricus*	*Streptococcus thermophilus*	*Bifidobacterium animalis*
Biological origin	Food environment	Food environment	Food environment
Genome	ST7 sp.	ST16 sp.	ST20 sp.
Domain	Bacteria	Bacteria	Bacteria
Taxonomy	Bacteria; ST7 sp.	Bacteria; ST16 sp.	Bacteria; ST20 sp.
Genome length (bp)	1,913,256	1,812,085	4,982,268
GC Content%	49.8	38.9	50.1
N50	39756	66727	13730
L50	14	9	85
Number of Contigs (with PEGs)	259	125	2012
Number of Subsystems	189	216	270
Number of Coding Sequences	2091	2119	6151
Number of RNAs	77	56	101
Closest neighbour	*Lactobacillus delbrueckii subsp. bulgaricus* ATCC 11842	*Streptococcus thermophilus* CNRZ1066	*Lactobacillus brevis* ATCC 367
Identified gad gene	gadB	gadB	gadB
% Similarity compare RAST Database and BLAST	%99.3	%99.8	%98.6
Accession number	ST7 sp. (6666666.655309)	ST16 sp. (6666666.655310)	ST20 sp. (6666666.655312)

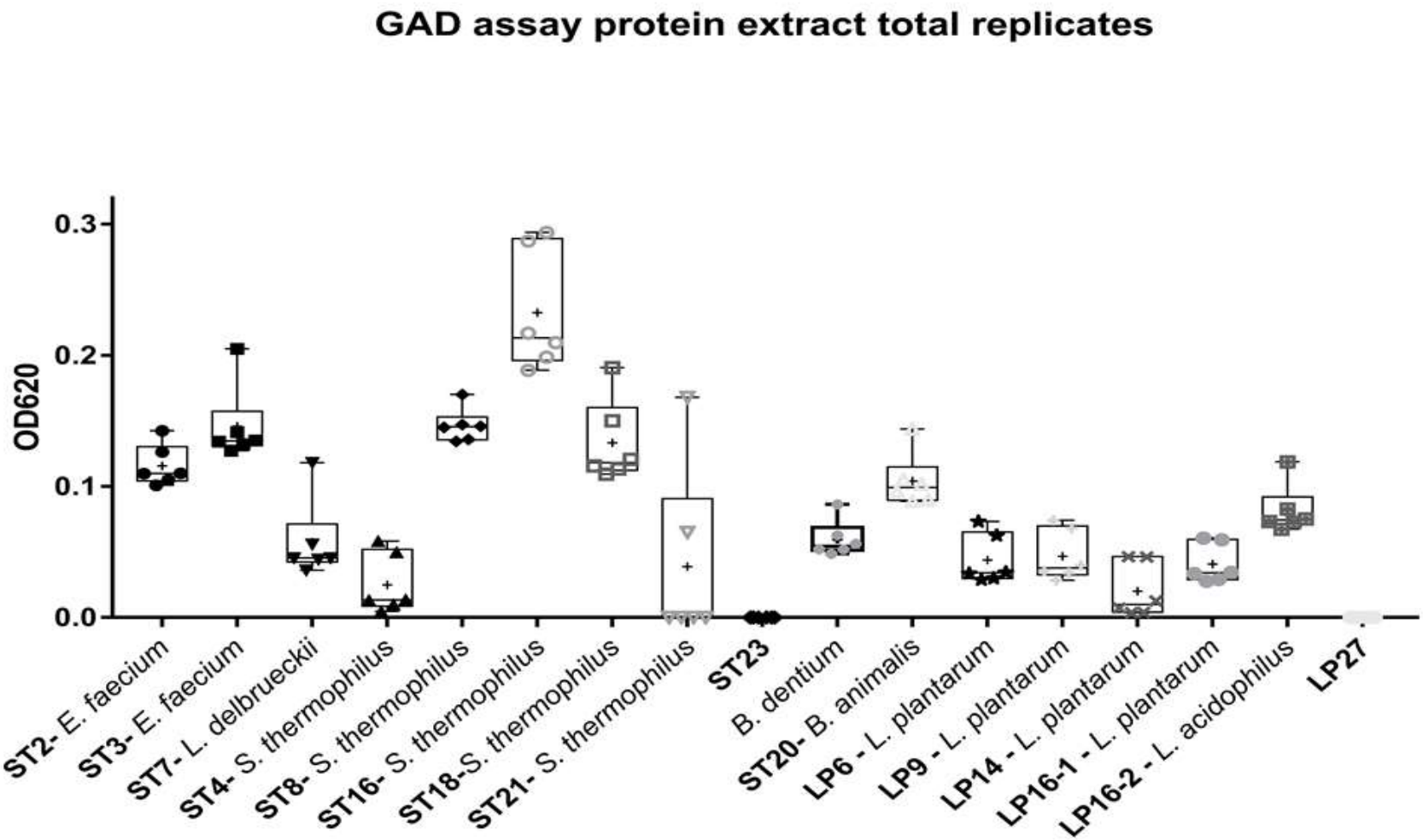


Figure3.1. GAD-catalyzed reaction of cell extracts of isolated bacterial strains compared to the positive and negative controls.

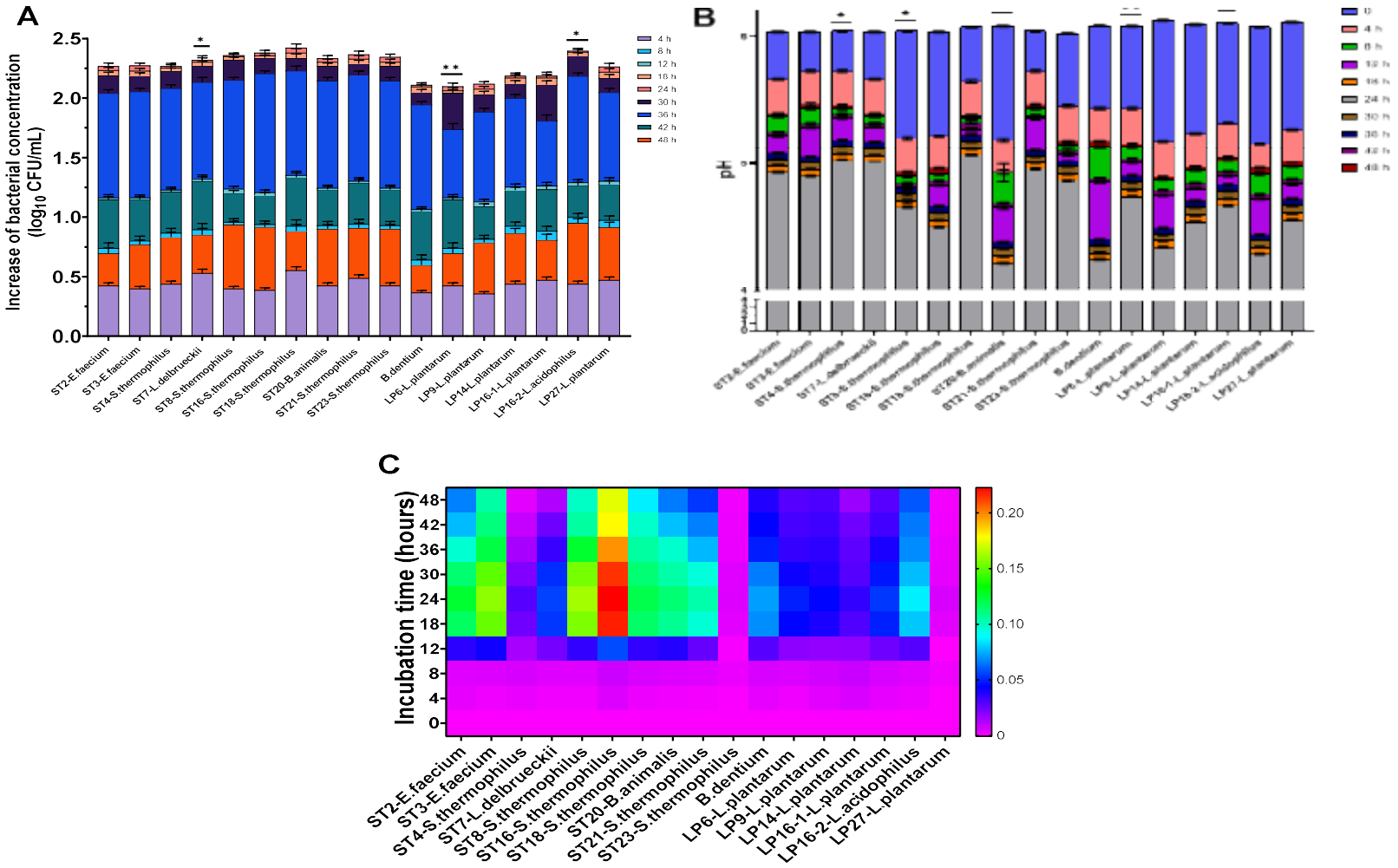


Figure3.2. Kinetics of growth (A), acidification (B), and GAD activity (C) of isolated lactic acid bacteria in mMRS broth (during anaerobic incubation at 37°C for 48 h). The initial cell density was Log 10^6 CFU/mL. Data are the average of analysis in triplicate.Statistical comparisons were conducted among different tested strains at the same time and among different time points within each tested strain using repeated measures 2-way ANOVA test with Tukey's multiple comparisons test. Significant differences were calculated for all obtained data with various tested strains and different time points(*p < 0.05, **p < 0.01).

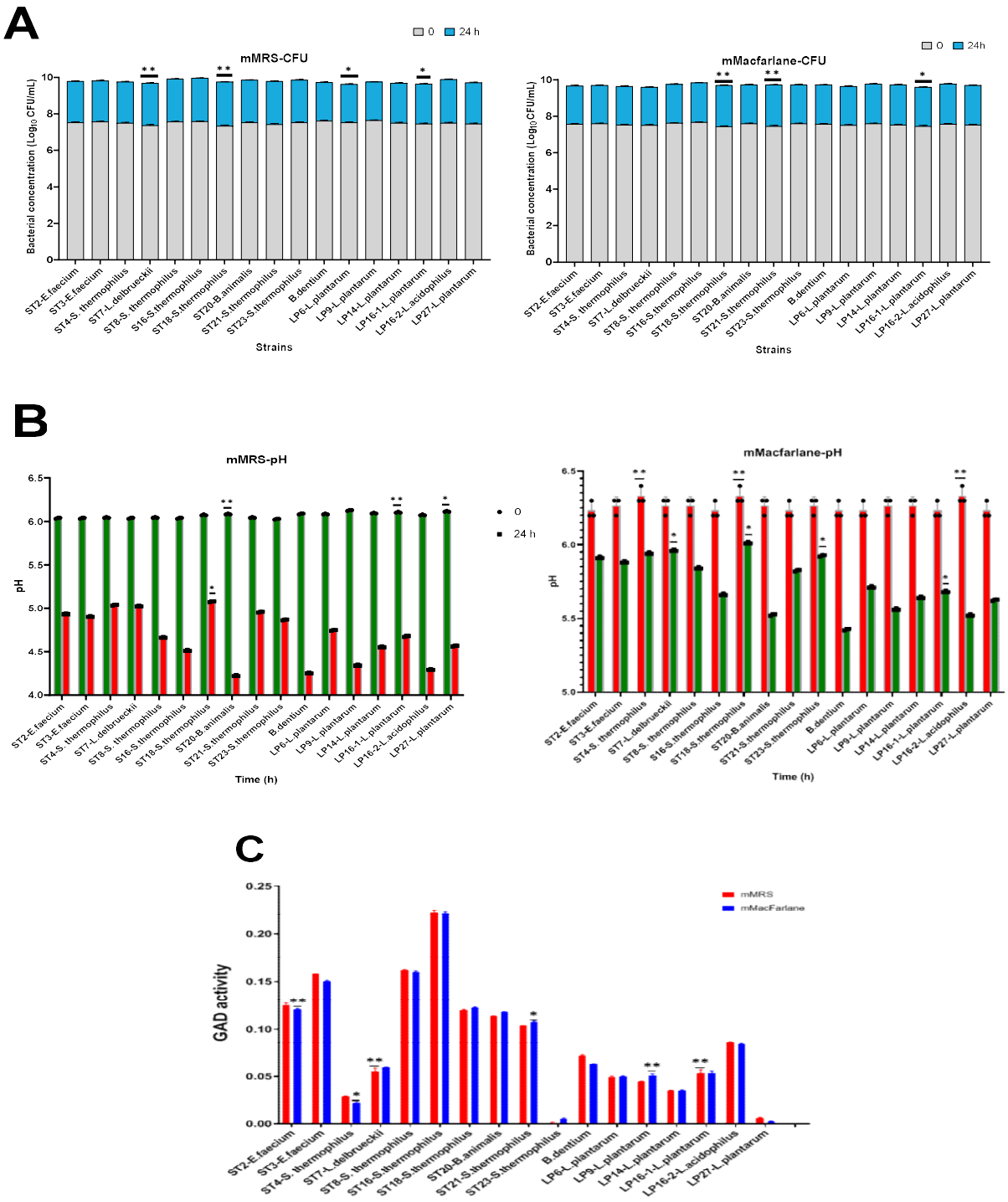


Figure 3.3. Kinetics of growth (A), acidification (B), and GAD activity (C) of the GABA-producing strains in the mMRS and mMacfarlane broths during anaerobic incubation at 37°C for 48 h. The initial cell density was Log 10^6 CFU/mL. Data are the average of analysis in triplicate.Statistical comparisons were conducted among different tested strains at the same time and among different time points within each tested strain using repeated measures 2-way ANOVA test with Tukey's multiple comparisons test. Significant differences were calculated for all obtained data with various tested strains and different time points (*p < 0.05, **p < 0.01).

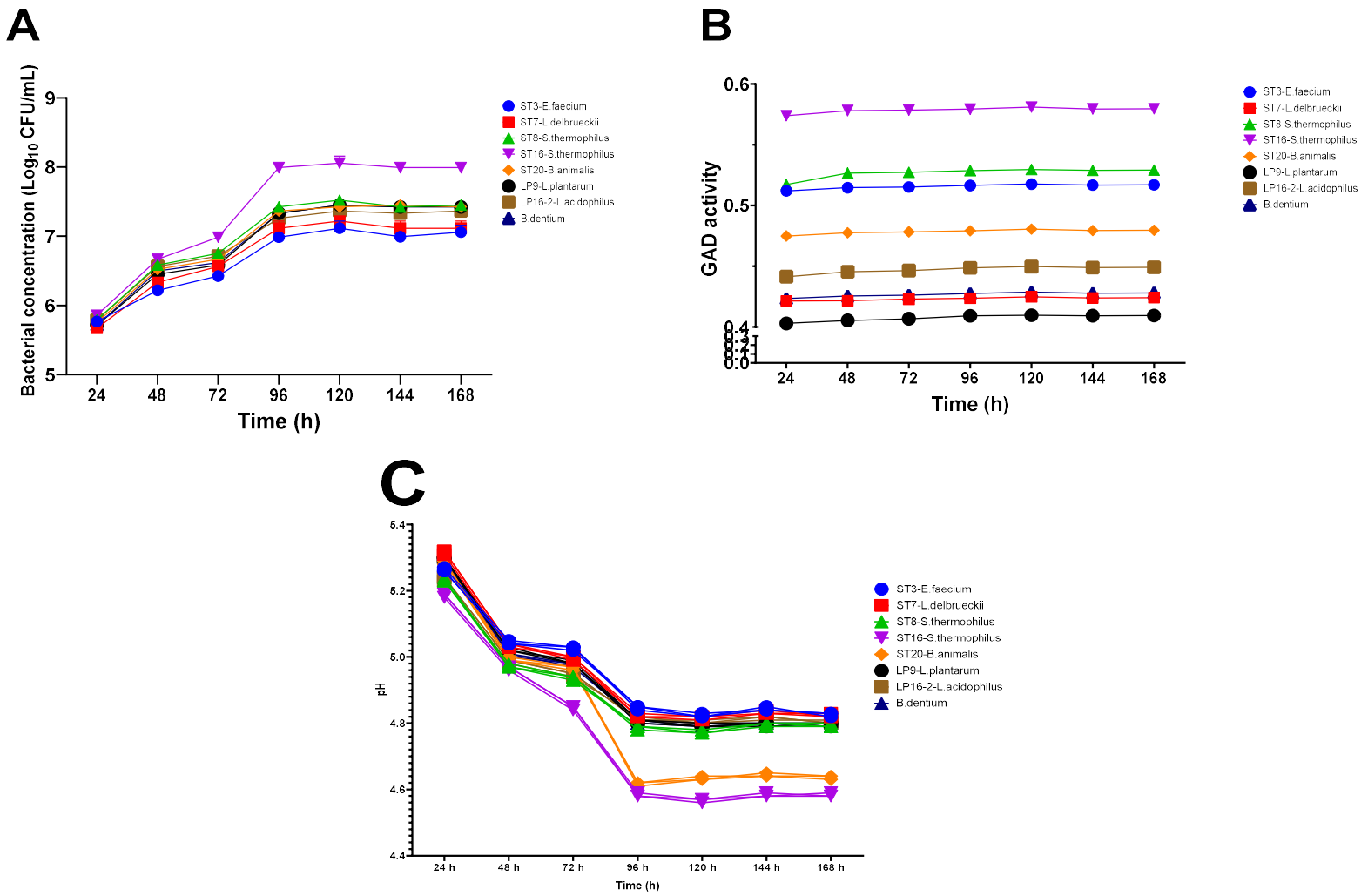
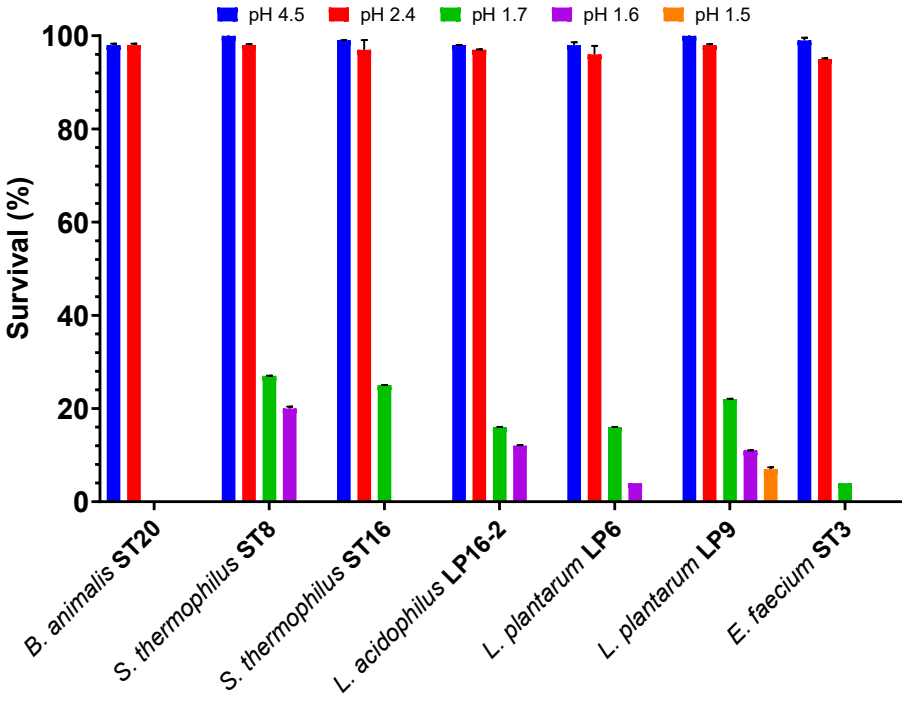


Figure 3.4. Kinetics of growth (A), GAD activity (B), and acidification (C) of isolated GABA-producing strains in the pre-fermented Macfarlane broth for seven consecutive days. The initial cell density was Log 106 CFU/mL. Data are the average of analysis in triplicate.Statistical comparisons were conducted among different tested strains at the same time and among different time points

within each tested strain using repeated measures 2-way ANOVA test with Tukey's multiple comparisons test. Significant differences were calculated for all obtained data with various tested strains and different time points (*$p < 0.05$, **$p < 0.01$).

(A)



(B)

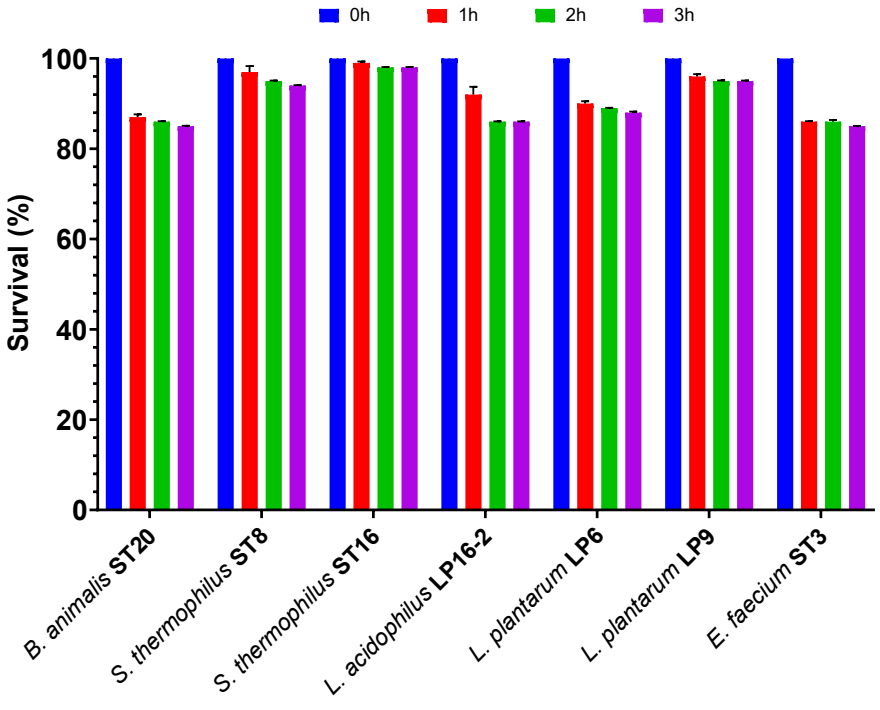


Figure 3.5. Survival of active isolated strains in the simulated gastric (A) and intestinal conditions (B).

Table 3.S1: PCR primers and products used for the detection of virulence genes of *E. faecium* and *E. faecalis* strains.

Gene	Virulence factor	Primer name	Oligonucleotide sequence (5′ to 3′)	Product size (bp)	Annealing temperature gradient (°C)	Ref.
EntLysA	Enterolysin A	EntLysAfw	TGGGATCCTGTAGGTGGAGG	316	55°c	Liu et al., 2011
		EntLysArev	CCATGAACCTCCGAGGACCC			
asa1	Aggregation substance	ASA 11	GCACGCTATTACGAACTATGA	375	56°c	Galli et al., 1990
		ASA 12	TAAGAAAGAACATCACCACGA			
gelE	Gelatinase	GEL 11	TATGACAATGCTTTTTGGGAT	213	55°c	Su et al., 1991
		GEL 12	AGATGCACCCGAAATAATATA			
cylA	Cytolysin	CYT I	ACTCGGGGATTGATAGGC	688	54°c	Coque et al., 1995
		CYT IIb	GCTGCTAAAGCTGCGCTT			
esp	Enterococcal surface protein	ESP 14F	AGATTTCATCTTTGATTCTTGG	510	56°c	Willems et al., 2001
		ESP 12R	AATTGATTCTTTAGCATCTGG			
hyl	Hyaluronidase	HYL n1	ACAGAAGAGCTGCAGGAAATG	276	55°c	Rice et al., 2003
		HYL n2	GACTGACGTCCAAGTTTCCAA			

CHAPTER 4: SURVIVAL AND INTERPLAY OF Γ-AMINOBUTYRIC ACID-PRODUCING PROBIOTIC CANDIDATES WITH GUT MICROBIOTA IN A MODEL OF THE HUMAN COLON

Rojaalsadat Mousavi[1], Walid Mottawea[1, 2], Marie-Claude Audet[1] and Riadh Hammami[1, *]

[1] School of Nutrition Sciences, Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

[2] Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

* Corresponding author: riadh.hammami@uottawa.ca

Abstract

We have recently isolated and characterized *in-vitro* probiotic candidates producing γ-aminobutyric acid (GABA), a major inhibitory neuromediator of the enteric nervous system with a potential role in modulating the immune system in many health disorders. This study aims to investigate the growth, GABA production, and competitiveness of selected probiotic candidates (*Bifidobacterium animalis*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*) in the presence of human gut microbiota *ex vivo* in a model of a proximal colon mimicking physiological and microbiological conditions of the human large intestine. Supplementation with GABA-producing probiotic candidates did not affect the overall gut microbiota diversity over 48 h of treatment. However, we observed modulation of the microbiome composition, especially change of *Bacteroides* population, a key gut microbe associated with anti-depressive and anti-inflammatory activities. The level of microbiota-generated butyrate within 12 h of treatment was significantly increased compared to control. Results from this study demonstrated the probiotic potential of tested GABA-producing bacteria and their impact on gut microbiota structure and metabolism, suggesting their suitability for gut health-promoting application.

Keywords: GABA-producing bacteria; probiotics properties; gut microbiota; microbial metabolites; simulated human colon

1. Introduction

Gut microbiota dysbiosis has been linked to many brain functions and behavioral disorders(Carabotti et al., 2015; Q. Ma et al., 2019). This link between gut microbiota and mental disorders has been confirmed via animal models, where germ free (GF) mice have developed anti-depression and anti-anxiety phenotypes as compared to specific pathogen free (SPF) mice as a result of hormonal imbalance in the hypothalamic-pituitary-adrenal (HPA) axis (Huo et al., 2017; Zheng et al., 2016). Gut microbiota interacts with the host central nervous system directly through microbial metabolites such as neurotransmitters or short chain fatty acids (SCFAs)(Q. Ma et al., 2019), or indirectly via neuronal, immunological or endocrinal connections(Sarkar et al., 2016). Gut microbiota produces a variety of neurotransmitters including GABA, serotonin, and dopamine that contribute to gut-brain axis signaling (Sarkar et al., 2016; Yong et al., 2020). GABA has been identified as an essential growth factor that solely can induce the growth of unculturable gut microorganisms (Strandwitz et al., 2019). The relative abundance of *Bacteroides*, a major γ-aminobutyric acid (GABA) producing members of gut microbiota, was negatively correlated with depression associated brain signatures (Strandwitz et al., 2019) indicating a significant role of microbiota-derived GABA in brain functionality.

Probiotics represents the most common way to harness gut microbiota for a therapeutic benefit(Hemarajata & Versalovic, 2013). Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit to the host (Hill et al., 2014). Several beneficial effects have been associated with consumption of probiotics such as the improvement of digestion and intestinal transit (Verna & Lucak, 2010), prevention of food allergies (Matricardi, 2002), enhancement of the immune system (Maldonado Galdeano et al., 2019), fight against infectious and antibiotic-associated diarrhea (Agamennone et al., 2018), irritable bowel diseases (Dale et al., 2019), and more recently mental health disorders (Ansari et al., 2020). Probiotics alter gut environment by inducing the generation of a myriad of bioactive metabolites including short-chain fatty acids (SCFAs)and neurotransmitters (Yong et al., 2020). SCFAs, derived from intestinal microbial fermentation of indigestible fibers by anaerobic microbiota, have been known as main source of energy for epithelial cells of the colon that makes them crucial to gastrointestinal health and energy metabolism (van de Wouw et al., 2018). Several commensal lactic acid-producing bacteria were identified to synthesize and deliver neurotransmitters (i.e., GABA), including members of the *Bifidobacterium* and *Lactobacillus genera*(H. Li & Cao, 2010). GABA has been known as a chief inhibitory neurotransmitter in the central nervous system, which would be involved in regulating the

interaction between gut and brain (Foster & McVey Neufeld, 2013). GABA is synthesized from glutamic acid via the action of glutamic acid decarboxylase (GAD) and pyridoxal phosphate (PLP) as a co-factor (Dhakal et al., 2012).As it is a metabolic product of the gut commensal bacteria, and consumed probiotics, therefore, the gut microbiota composition and diversity would control the enteric GABA levels(Hemarajata & Versalovic, 2013; Yong et al., 2020). This neuroactive metabolite can in turn modulates the gut microbiota structure in various kinds of stress (Mayer et al., 2014).

There is a growing interest in identifying the high capability of LAB strains to produce GABA(Cui et al., 2020; Dhakal et al., 2012), however, the scientific evidence with relation to the capacity of these strains to grow, survive and produce GABA *invivo*, as well as their interaction with the colonic microbiota in the physiological colon conditions almost remains rare (Dinan & Cryan, 2017). Therefore, these strains could be screened *exvivo* for their abilities to modulate the gut microbiota composition and functionality and to maintain a certain microbial population of interest(Guzman-Rodriguez et al., 2018) before moving the promising candidates for *invivo* clinical trials. The present study aimed to evaluate the isolated GABA producing LAB, identified in chapter 3 for their capacity as potential probiotic candidates to survive, grow and produce GABA in an *exvivo* continuous fermentation model that mimics the physiological conditions of proximal colon. In addition, the impact of the isolated probiotic candidates and their metabolites on the gut microbiota composition and diversity was also examined.

2. Material and methods

2.1. Bacterial strains, media and culture conditions

Bacterial strains were isolated from commercial cultures as described in previous chapter 4, and pure overnight culture of all strains were cryopreserved in 20% glycerol at −80°C till use. All strains were cultured in Lactobacilli MRS broth (VWR Avantor, CANADA). Bacterial strains were incubated at 37°C for 24 h anaerobically (Whitley A35 Anaerobic Workstation, UK).

2.2. Bacterial enumeration by plate counts

Viable cell counts were determined using the drop plate method. Four 20 µl drops of each 8-fold serial dilution of overnight subculture with peptone water (0.15 %, pH 7.0) were plated in duplicate on selective media for enumerating bacterial colony counts. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp*. bulgaricus*were enumerated onM17 and MRS agar after aerobic incubation at 37°C for 24h respectively. *Bifidobacterium animalis* was counted

using MRS agar with 48h of anaerobic incubation at 37°C. Viable cell counts were demonstrated as log CFU/mL of fermentation culture medium.

2.3. human colonic fermentation model

2.3.1. Nutritive Culture Medium

Macfarlane broth is a complex nutritive medium mimicking the nutrients encountered in healthy adult large intestine (Macfarlane et al., 1998). The nutrient medium was described in (Mottawea et al., 2020). The medium was autoclaved for 15 min and a filter-sterilized mixture vitamins solution was added to the cooled medium before use.

2.3.2. Fecal sample collection and cell immobilization in gel beads

Fecal samples were obtained from two healthy adult donors (1 male and 1 female), with two different enterotypes (Mottawea et al., 2020)who had not exposed to antibiotic treatment or probiotic supplements for at least three months before donation. Collection of fecal samples was approved by The University of Ottawa Research Ethics Board and Integrity (Ethics file number: H-02-18-347; Approval date: 29/07/2019). The feces were processed to slurries by dilution in reduced peptone water (20%, w/v), homogenized and further immobilized in 1–2 mm gel beads consisting of gellan gum (2.5%, w/v), xanthan (0.25%, w/v) and sodium citrate (0.2%, w/v) under anaerobic conditions as described previously in details (Le Blay et al., 2012).

2.3.3. Experimental Setup and fermentation procedure

The continuous fermentation was carried out for 34 days using an *ex vivo* model of the human proximal colon (N*u*GUT Research Platform, University of Ottawa) as previously described in (Mottawea et al., 2020). The model consists of a two-stage design comprising an Inoculation Reactor (IR) (1L BioFlo® 120 vessel; Eppendorf, Mississauga, ON, Canada) with immobilized fecal microbiota used to continuously inoculate four second-stage DASGIP® bioreactors (Eppendorf, Mississauga, ON) operated in parallel and consisting of a control reactor (CR) (no treatment control) and three test reactors (TR1, TR2, TR3). Each reactor was set up to reproduce the physiological and microbiological conditions of the adult proximal colon (pH 5.7, stirring at 120 rpm, 37°C, and mean retention time of 8 h). Anaerobiosis was ensured through continuous headspace flushing of N_2 and CO_2 at a 0.9:0.1 ratio, and a constant pH of 5.7 was maintained by addition of 2.5 M NaOH. Fermentation was initiated by inoculating the IR containing 140 mL fresh sterile Macfarlane culture medium with 60 mL of immobilized gel beads. During the first 48 h, the colonic model was run in a batch mode to favor beads colonization, and subsequently switched to continuous mode for the rest of the experiment.

After stabilization of microbiota on continuous intestinal fermentation system for 2 weeks, the four bioreactors were set up and run without any treatment for 48 h to reach the stability of the microbial community. Once the stabilization is reached in all reactors, the bioreactors were subjected to probiotic treatment as follow: CR bioreactor: served as no treatment control; TR1 bioreactor: *Streptococcus thermophilus* ST16*;* TR2 bioreactor: *Bifidobacterium animalis* ST20*;* and TR3 bioreactor: a mixture of *Streptococcus thermophilus* ST16 and *Lactobacillus delbrueckii* subsp*. bulgaricus* ST7. Probiotic candidates were added once to the corresponding test reactor at final concentration of 10^9 CFU/mL each. Collected effluent samples (2 mL) were taken in duplicate and centrifuged at 14,000 × g for 5 min every 2h during the first 12h of treatment (0, 2h, 4h, 6h, 8h, 12h) and then at 24 and 48h from bioreactors. The collected samples were separated (centrifugation at 14 000 g, 5 min, 4 °C) to pellet used for metagenomic DNA extraction and supernatant used for short chain fatty acids (SCFA) analysis. Each fermentation experiment was conducted in duplicate for each fecal sample donor (a total of 4 biological replicates).

2.4. Microbiota diversity and microbial metabolism analyses

2.4.1. Genomic DNA extraction

The genomic DNA extraction was performed up to approximately 100 mg of wet weight microbial cell fermentation pellet using the FastDNA® Spin Kit (MP Biomedicals; Solon, OH, USA) and homogenized with Bead Mill-24 homogenizer (Fisher Scientific; Ottawa, ON, Canada) as described by (Mottawea et al., 2020). The initial DNA concentration (200 ng/µl) was quantified by Qubit Fluorometer using Qubit dsDNA BR Assay Kit (Qubit™ Flex Fluorometer, ThermoFisher Scientific, Wilmington, USA).

2.4.2. 16S Metagenomic Sequencing

The microbial composition and diversity of the fecal and effluent fermentation samples was assessed using 16S rRNA-based MiSeq (Illumina, CA, USA) with high-quality and throughput sequencing. The V3-V4 regions of the 16S rRNA gene were amplified using dual-barcoded primers, and the amplicon library for sequencing was constructed using Illumina standard protocol. The amplicon libraries were pooled in equimolar amounts and paired end sequenced with Illumina MiSeq platform (N*u*GUT Research Platform, University of Ottawa) using 600 bp MiSeq Reagent Kit v3 (Illumina; San Diego, CA, USA) as per standard protocol. Sequences were quality filtered, deblur denoised, and clustered into observed features based on 97%-similarity using the Greengenes database (v13.8) via QIIME 2.2020.8 software

(Caporaso et al., 2010). The observed features were rarefied into an equal number of 4,000 reads per sample using QIIME.

2.4.3. qPCR Analysis

quantitative PCR (qPCR) was performed following method as described by (Fernandez et al., 2016) on a Bio-rad CFX96 Real-Time PCR Detection System with the C1000 Touch TM Thermal Cycler (Biorad, Oakville, ON, Canada) in 96-well plates. Standard curves were produced for the qPCR relative quantification of the collected fermentation samples of bioreactors using the following strains: *S. thermophilus* ST16*, L. bulgaricus* ST7*,* and *B. animalis* ST20. Specific primers used in this study are summarized in Table 1. The qPCR reaction mixture (20 µL) contained 1× SYBR green PCR Master Mix (Bio-Rad; Mississauga, ON, Canada), 0.5µM of each forward and reverse qPCR specific primer (Millipore-Sigma, Cleveland, OH, United States), 6µL of DNase-free water (Invitrogen), and 25ng of extracted microbial DNA. qPCR quantification of each sample was performed in duplicate. The amplification program was set up for 98 °C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s followed by melting cycle of the PCR product from 65°C to 95°C. Cq values were extracted using Bio-Rad CFX Maestro software, and the relative abundance of each organism was determined as a ΔCq value (taxon Cq–universal 16S rRNA Cq) as described before (Mottawea et al, 2020) where the increase in ΔCq indicates a decrease in the relative abundance and the opposite is true.

2.4.4. Determination of SCFAs production using GC

The production of Short-chain fatty acids (SCFAs; butyrate, acetate, and propionate) in fermentation samples from all sub-reactors was determined using Gas Chromatography coupled to Flame Ionization Detector (GC-FID) (Shimadzu GC-2030) as previously described (Monk et al., 2015). In brief, 2ml of supernatants of collected effluent fermented samples were centrifuged twice at 14,000 ×g for 30 min at 4°C and filtered with 0.22µm membrane. 2-ethyl butyric acid was used as an internal standard and added to each sample at a concentration of 0.5 mM. The analysis was carried out using a capillary column Stabilwax-DA (60 m × 0.25 µm; Restek) at a flow rate of 0.4 ml/min with 2 µL were injected to the GC (Nexis GC-2030, Shimadzu, Japan). Helium was used as a carrier gas on the flame ionization detector (FID). The initial temperature of oven was 100°C and then was increased to 200°C at a rate of 10°C/min. Injector and detector temperatures were maintained at 200°C and 300°C respectively. The peaks were identified by comparing their retention times with Volatile acid standard mix from MilliporeSigma (Oakville,ON, Canada). The data collection was analyzed using Lab Solutions

software developed by Shimadzu Corporation, Japan. All samples were analyzed in duplicates and results expressed in mM.

2.5. Statistical analyses

Alpha diversity was estimated with observed features, Shannon entropy, Pielou_Evenness, and Faith_pd. Beta diversity among samples was calculated using Bray-Curtis distance and visualized using Principal Coordinate Analysis (PCoA). The contribution of different treatments to the diversity of gut microbiota community was assessed from the Bray-Curtis distance matrix using the permutational multivariate analysis of variance (PERMANOVA)-pairwise and 999 permutations (Bolyen et al., 2019). To identify differential taxa among different treatments, linear discriminant effect size analysis was conducted on the relative abundance of different taxa levels (Segata et al., 2011). Samples were labeled with the treatment type as the sample class, and the time points as the subclass. Taxa with $\log_{10}$ LDA score ≥ 2 and $p< 0.05$ were considered significant. When required Kruskal-Wallis test was applied for statistical analysis, and *P*-values were corrected using two-stage Benjamini, Krieger, and Yekutieli false discovery rate (FDR) procedure.

For SCFA contents, statistical analysis was performed using Prism software (GraphPad 8). Statistical comparisons were conducted among different treatments at the same time and among different time points within each treatment using repeated measures two-way ANOVA with Tukey's multiple comparisons test and Dunnett's multiple comparisons test for SCFA and qPCR results analysis, respectively. Significant differences were indicated in the figures by different *P* values.

3. Results

3.1. Gut microbiata diversity

Four diversity indices were calculated to compare the alpha-diversity of different treatments; observed features, Shannon entropy, Faith_PD and Pielou's eveness. Dual treatment with the combination of combination of *L. delbrueckii subsp. bulgaricus* and *S. thermophilus* (ST16ST7) strains exhibited significant increase in microbiota diversity as indicated by the increase in the number of observed features and Faith PD in comparison with no-treatment control and the increase in different indices as compared to other treatments (Figure4.1, $p<0.05$).Beta-diversity was calculated to identify which factor controls the microbiota diversity among different samples using Bray-Curtis distances and visualized using principal coordinate analysis (PCoA) across treatments, donor and biological replicates. Plots of PCoA

are shown in Figure4.2. The microbiota of different treatments was clustered by donor (PERMANOVA = 153.177, p =0.001; 999 permutations), and by biological replicate within each donor (PERMANOVA = 3.53, p =0.003; 999 permutations), and by treatment within each experiment replicate (PERMANOVA = 2.45, p =0.003; 999 permutations).

3.2. Effect of GABA-producing probiotic candidates on gut microbiota composition

We determined the change in gut microbiota composition over 48h post-treatment with *Bifidobacterium animalis*ST20, *S. thermophilus* ST16 and the combination of *Lactobacillus delbrueckii* subsp. *bulgaricus* ST7 and *S. thermophilus* ST16 as compared to the no-treatment control. In general, the developed microbiome in different sub-reactors was composed of the four major microbiota phyla; Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Figure4.3). Linear discriminant analysis (LDA) demonstrated the microbial taxa at different phylogenetic levels that are affected by the treatments as shown Figure4.4. The microbiota composition subjected to the combination of *L. bulgaricus* ST7 and *S.thermophilus* ST16 treatment was enriched in *Bacteroides* and *Lactobacillus* at the genus level, *Streptococaceae*, *Ruminococcaceae*, *Lactobacillaceae* and *Bacteroidaceae* at the family level, and *Lactobacillales* at the order level compared to un-treated microbiota (Figure4.4A; $p< 0.05$). The microbiota treated with *S. thermophilus* ST16 exhibited an increase in *Lactobacillus*, *Alistipes* and *Streptococcus*at the genus level, *Rikenellaceae* and *Erysipelotrichaceae* at the family level and *Lactobacillales* at the order level as compared to un-treated microbiota (Figure4.4B; $p< 0.05$). In contrast, *S. thermophilus* ST16 treatment was led to a major modification of microbiota with enrichment of Firmicutes at the phyla level and *Clostridiales* at the order level compared to the combination of *L.* bulgaricus ST7 and *S. thermophilus* ST16 treatment which didn't affect the microbiota at the phyla level with depletion of *Clostridiaceae* at the family level (Figure4.4C; $p< 0.05$).Microbiota subjected to the *Bifidobacterium animalisST20* treatment was also enriched in *Streptococaceae*and *Erysipelotrichaceae* at the family level and *Bacteroides, Lactobacillus*, and *Streptococcus* at the genus level and *Lactobacillales* at the order level compared to the control microbiota (Figure4.4D; $p< 0.05$).

3.3.Microbial Survival Analysis by qPCR

We monitored the levels of the probiotic candidates in their corresponding bioreactors via quantitative PCR and by using primers specific to the added strains. We were able to detect all the strains after 48 h of treatment in their corresponding sub-reactor. The relative abundance of all the strains weredecreased gradually over the 48 h and reached a significant reduction in their levels after 24 h and 48 h of the treatment in donor 1. For donor 2, we noticed the same

trend of reduction in their relative abundance over time, however, no significant difference was detected statistically (Figure4.5).

3.4. Effect of GABA-producing probiotic candidates on microbiota SCFAs generation.

We quantified the generation of SCFAs by the developed microbiota in response to the candidate probiotics treatment using GC. We detected the three major SCFAs generated by gut microbiota, including acetate, butyrate, and propionate. Inoculation of GABA-producing bacteria induced a significant increase in the three metabolites in both donors ($p < 0.05$; Figure4.6). For instance, the treatment with ST20 induced the most significant increase in butyrate and propionate compared to control but has a less inducing effect on acetate production (Figure4.6).

4. Discussion

Previous studies have demonstrated that probiotic bacteria can help maintain and restore gut microbiota homeostasis by modulating the beneficial bacteria populations and composition as well as their metabolic activities(Van den Abbeele et al., 2010). Hence, the present study assessed three active GABA-producing LAB strains as potential candidates for their ability to compete with the existing complex gut microbiota under simulated colon conditions and their impact on the microbiota structural and functional profiles (Zihler et al., 2010).We used an *ex-vivo* model which reproduced a stable microbial ensemble that mimics the colon microbiota(Mottawea et al., 2020). The identified features belonged to the four major phyla, Firmicutes, Bacteroidetes, Actinobacteria, and proteobacteria, which have been known as common phyla of the gut microbiota with a predominance of *Bacteroidetes* and *Firmicutes*(Mottawea et al., 2016). We developed two distinct communities from healthy donors. Both communities were different at the family level and demonstrated two distinct enterotypes that reflected the enterotypes of the two donors as presented before by our group (Mottawea et al., 2020). However, some variations between the donor stool microbiota and the one developed in the bioreactor were expected as a result of the variability of the microbiota at different anatomical locations of the gut, including the variabilities between the proximal colon adopted in our system and stool microbiota (Mottawea et al., 2019). Despite all the advantages of *exvivo* fermentation models such as stable and standardized culture, reproducible, and complex of microbial communities(Van den Abbeele et al., 2010)under controlled conditions (i.e., temperature, pH, growth medium, and transit time)that mimic the human colon environment (McDonald, 2017)still, we have to consider some limitations of the *exvivo* colon simulator models such as low number of biological replicates which is due to the time and the high-cost constraints, short period of microbial treatment, enrichment/depletion and lack of mucosal

environment (interaction between the gut microbiome and mucosal immune system)(Mottawea et al., 2020).

Interestingly, the combination of *L. delbrueckii subsp. bulgaricus* and *S. thermophilus* increased the microbiota diversity over 48 h of treatment. Recently, dietary GABA supplementation has been reported to increase gut microbiota diversity in *E. coli* infected piglet model (Chen et al., 2020). Also, administration of *Lactobacillus* cocktails has been reported to increase the microbiota diversity post-antibiotic treatment (Shi et al., 2017, 2018). Additionally, Han and his coauthors have reported that LPS-induced decrease in mice gut microbiota diversity was restored by a single strain of *S. thermophilus*(Han et al., 2020). Combining two *Lactobacillus* strains and *streptococcus thermophilus* has also improved the microbiota diversity in neonatal piglets (Veljović et al., 2017). However, treating the microbiota with a single GABA-producing strain did not alter the microbiota diversity assuming that the short time of the treatment limited the capacity of these strains to alter the microbiota diversity.

Population of *Bacteroidetes* as one of the major phyla identified in the generated dataset was increased by GABA-producers treatment. Administration of a mixture of four *Lactobacillus* strains isolated from fermented food have increased the levels of mice *Bacteroides* that were depleted by antibiotic consumption(Shi et al., 2018). Analyses of human fecal samples illustrated that *Bacteroides spp*. may produce large quantities of GABA by expressing GABA-producing pathways. Additionally, the relative abundance levels of faecal *Bacteroides* have been negatively associated with depression (Strandwitz et al., 2019). We found enrichment of *Lactobacillaceae* family after the addition of GABA-producing bacteria. Enhancement of *Lactobacillus* has been associated with decreasing the depression symptoms in patients with major depressive disorder (MDD) (Jiang et al., 2015). Similarly, the *Erysipelotrichaceae* family was significantly increased after GABA-producing bacteria treatment. The role of *Erysipelotrichaceae* family in health benefits is debatable. Some studies have reported correlation between abundance of *Erysipelotrichaceae* and disease phenotypes such as inflammatory bowel(IBD) disease (Umu et al., 2017); (Fehlbaum et al., 2018).In addition, the microbiota treated with GABA-producers were significantly depleted in *Clostridiales* and *Lachnospiraceae*, while the microbiota treated with the combination of *L. delbrueckii subsp. bulgaricus* and *S. thermophilus* strains was enriched by *Ruminococcaceae* family which has been known to have a beneficial effect on gut barrier functions and also introduced as adjuvants to immune checkpoint inhibitors (Jiang et al., 2015;(Lukić et al., 2019). Finally, an increased abundance was observed in the genus *Lactobacillus* and *Streptococcus*, which was expected

and may attributed to the test treatment that contains *S. thermophilus* and *L. delbrueckii subsp. bulgaricus.* Hence, administration of a single dose of these strains maintains a stable level of *Lactobacillaceae* and *Streptococcaceae* families over at least 48 h of treatment. This is also confirmed by the qPCR results.

SCFAs are saturated fatty acids with mainly acetate, butyrate and propionate are distributed in the intestine (Luo et al., 2021). The majority of the gut generated SCFAs are absorbed into the circulation with a minor part secreted in the feces (Luo et al., 2021). We detected high amounts of acetate and butyrate in our colon model, however, propionate concentration in the effluent samples was low. Usually, the ratio of acetate:propionate:butyrate is 60:20:20 (Luo et al., 2021; Parada Venegas et al., 2019) which is not the case in our results. This may be attributed to the high amount of indigestible fibers and the predominance of the phyla Firmicutes in our datasets compared to *Bacteroidetes*. However, the same base level is detected between different bioreactors before starting the intervention. A significant increase in SCFAs production especially butyrate was induced by the three tested probiotic formulas which was associated with increase of the butyrate-producing community. SCFAs have an essential role in intestinal barrier integrity and immune modulatory properties (Tan et al., 2014). This indicates that these strains have the potential as adjuvant biotherapeutics not only in mental health disorders but also in many other conditions characterized by depletion of SCFAs such as inflammatory bowel diseases (Mottawea et al., 2016; Parada Venegas et al., 2019), IBS(Luo et al., 2021) and colorectal cancer (Lavoie et al., 2020). Additionally, they may mitigate the extraintestinal mental symptoms associated with these disorders. For instance, a previous randomized clinical trial has been illustrated that butyrate-producers including *Lactobacillus* could decrease psychological symptoms associated with irritable bowel syndrome such as depression, anxiety (Jiang et al., 2015; Liśkiewicz et al., 2019).

5. Conclusion

We herein illustrate that GABA-generating probiotics have the capability to modulate the fecal microbiota and enhance the SCFAs production in the gut. This work also provides the evidence that such potential probiotics could be further exploited as functional food products and biotherapeutic regimens to mitigate human health disorders associated with gut microbiota dysbiosis. Hence, our obtained data provide the rational for future studies aim at investigating the potential symbiotic impact of GABA-producing bacteria on human microbiota composition and diversity, and their metabolism associated with diseases and disorders. Further research is still required to investigate the survival of GABA-producing bacteria in various parts of the gut,

and their ability to produce GABA as inhibitory neurotransmitter *in vivo* after oral consumption. We only tested the effects 3 food-isolated probiotics belonging to *Bifidobacteria, lactobacilli* and *streptococci* genera. Future work may investigate other human-originated GABA-generating gut bacteria and their impact on gut microbiota and disease status.

Availability of data and materials. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

This study was supported by a grant from the Canadian Tri-Agency (i.e., CIHR, NSERC, and SSHRC) Institutional Program [NFRFE-2018-00295] to MCA and RH and an NSERC-Discovery grant RGPIN-2018-06059 to RH. RM was supported by the Nutrition and Mental Health Master's Scholarship, University of Ottawa.

Author contributions

Conceptualization: R.M., W.M., and R.H.; methodology, R.M., W.M., and R.H.; validation, R.M., W.M., and R.H.; formal analysis, R.M. and W.M.; investigation, R.M. and W.M.; resources, R.H.; writing—original draft preparation, R.M.; writing—review and editing, W.M., M-C.A., R.H; visualization, R.M., W.M, and R.H.; supervision, R.H.; funding acquisition, R.H. and M-C.A. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Table 4.1. Specific primers used for the qPCR

No	Specificity	Primer Name	Primer type	Sequence (5'-3')	GC content (%)	Melting Temp (°c)	Ref.
1	*Lactobacillus delbrueckii subsp. bulgaricus*	LdelbF	Forward	GGRTGATTTGTTGGACGCTAG	47.6	66.9	Byunet al., 2004
		LdelbR	Reverse	GCCGCCTTTCAAACTTGAATC	47.6	66. 7	
2	*Streptococcus thermophilus*	S. thermophilusF	Forward	TTATTTGAAAGGGGCAATTGCT	36.3	65.2	Stachelska et al., 2017
		S. thermophilusR	Reverse	GTGAACTTTCCACTCTCACAC	47.6	58.8	
3	*Bifidobacterium animalis*	IDB61F	Forward	GCATGTTGCCAGCGGGTGA	63.1	73.1	Kwon et al., 2005
		IDBC1R	Reverse	ATCCGAACTGAGACCGGTT	52.6	63.5	

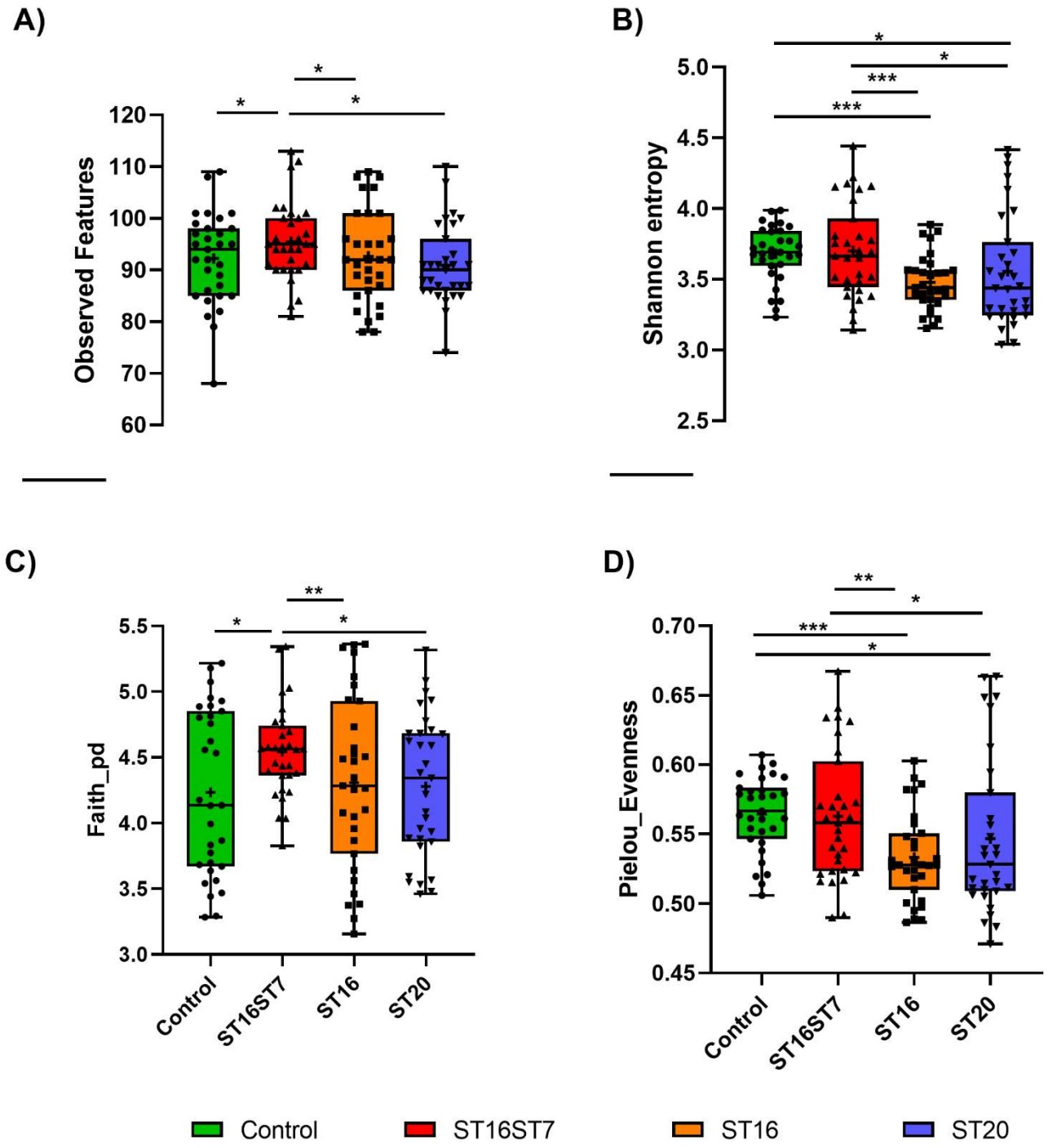


Figure 4.1. Observed features **(A)**, Shannon indices **(B)**, Faith's-PD **(C)**, and Pielou's evenness **(D)** estimates of the identified microbiota in each sample from bioreactors with either a no-treatment control, or treated with ST16-*S. thermophilus*, combination of ST7-*L. delbrueckii subsp. bulgaricus* and ST16-*S. thermophilus* or ST20-*B. animalis*. Data was analyzed using the Kruskal-Wallis test and Two-stage Benjamini, Krieger, and Yekutieli FDR procedure (**P*< 0.05, ***P*< 0.01, ****P*< 0.001).

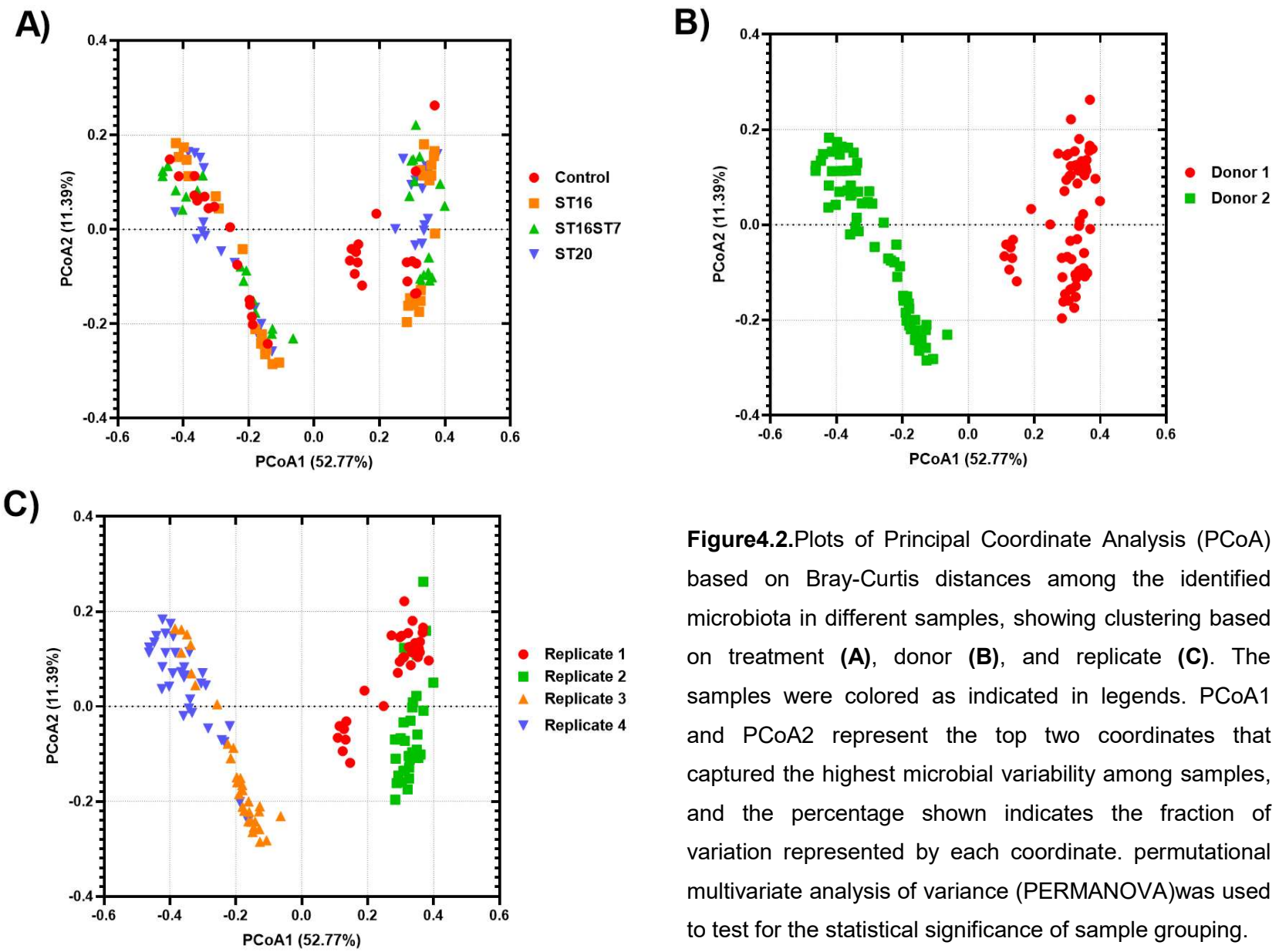


Figure4.2. Plots of Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances among the identified microbiota in different samples, showing clustering based on treatment **(A)**, donor **(B)**, and replicate **(C)**. The samples were colored as indicated in legends. PCoA1 and PCoA2 represent the top two coordinates that captured the highest microbial variability among samples, and the percentage shown indicates the fraction of variation represented by each coordinate. permutational multivariate analysis of variance (PERMANOVA)was used to test for the statistical significance of sample grouping.

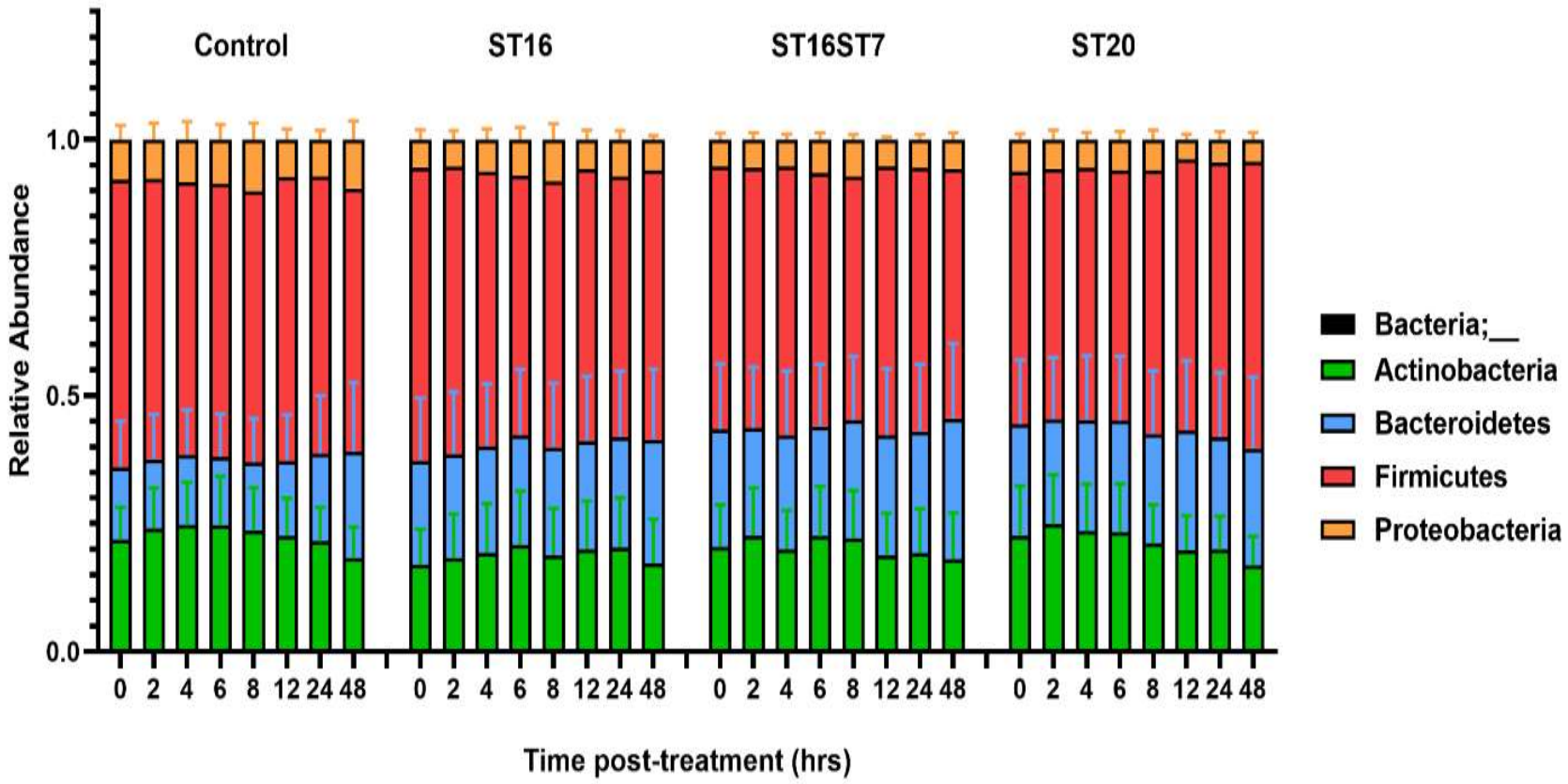


Figure4.3. The microbiota composition of gut microbiota was determined based on the treatment with active strains over 48 h from the two fecal sample of healthy donors. Four bacteria phyla were identified in the generated dataset including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria.

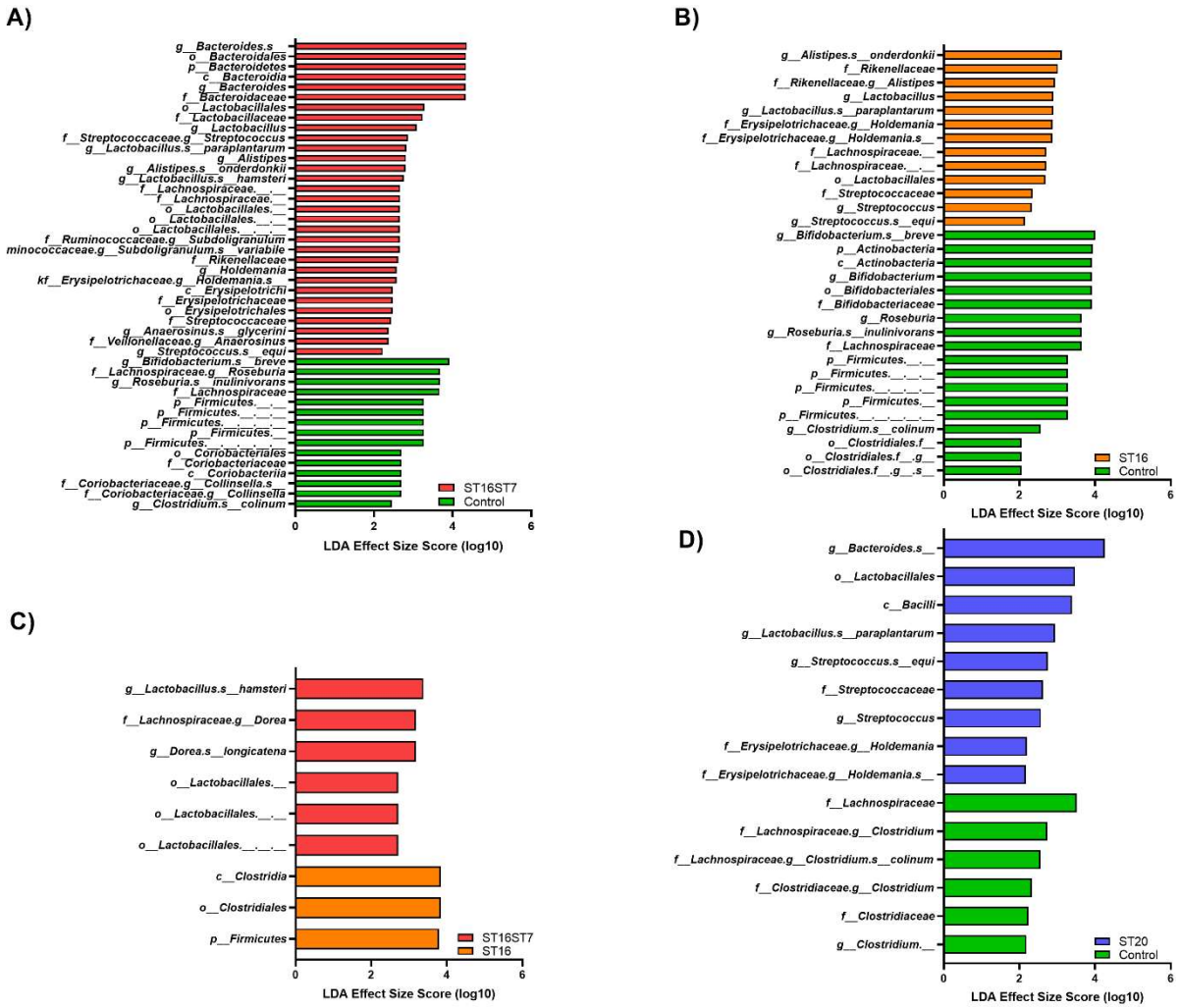


Figure4.4. The test probiotic candidates modulate gut microbiota composition. **(A–D)** Histograms of the linear discriminant analysis (LDA) scores showing microbial taxa that vary significantly in abundance between: **(A)** no-treatment control and test treatment of combination of *L. delbrueckii subsp. bulgaricus* and *S. thermophilus,***(B)** no-treatment control and test treatment of S. thermophilus **(C)** compare test treatment of *S. thermophilus* and combination of *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*, **(D)** no-treatment control and test treatment of *B. animalis*.

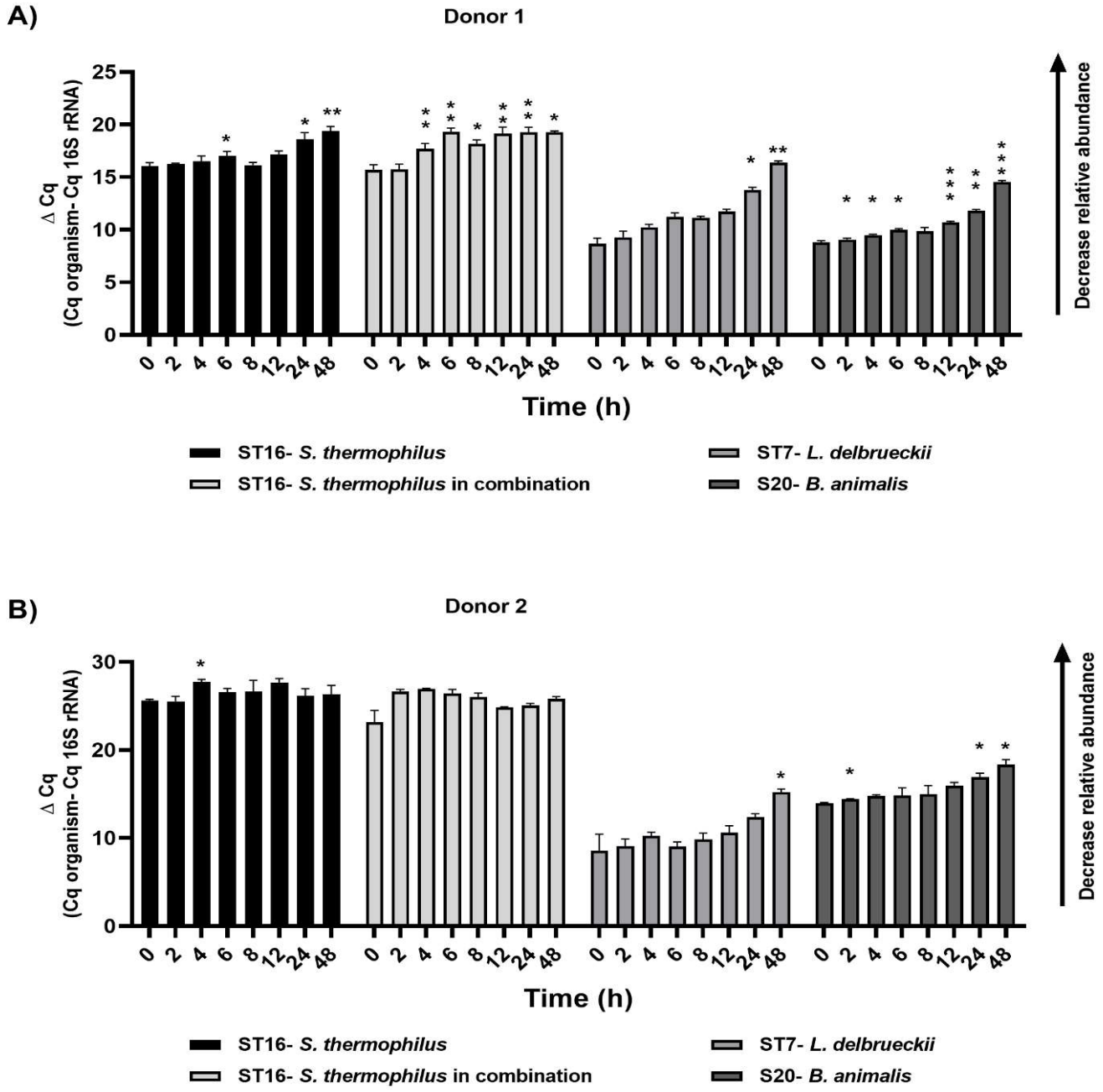


Figure4.5. Relative abundance of target species identified by qPCR as indicated by ΔCq: relative abundance of inoculated probiotic candidates in various bioreactors with donor 1 **(A)** and donor 2**(B)** microbiota over 48 h post-treatment. The increase in ΔCq indicates a decrease in the relative abundance. Repeated-measures ANOVA with Dunnett's multiple comparisons test was used for statistics. Significant differences were indicated for each time point compared to the zero time (*p< 0.05, **p< 0.01, ***p<0.001).

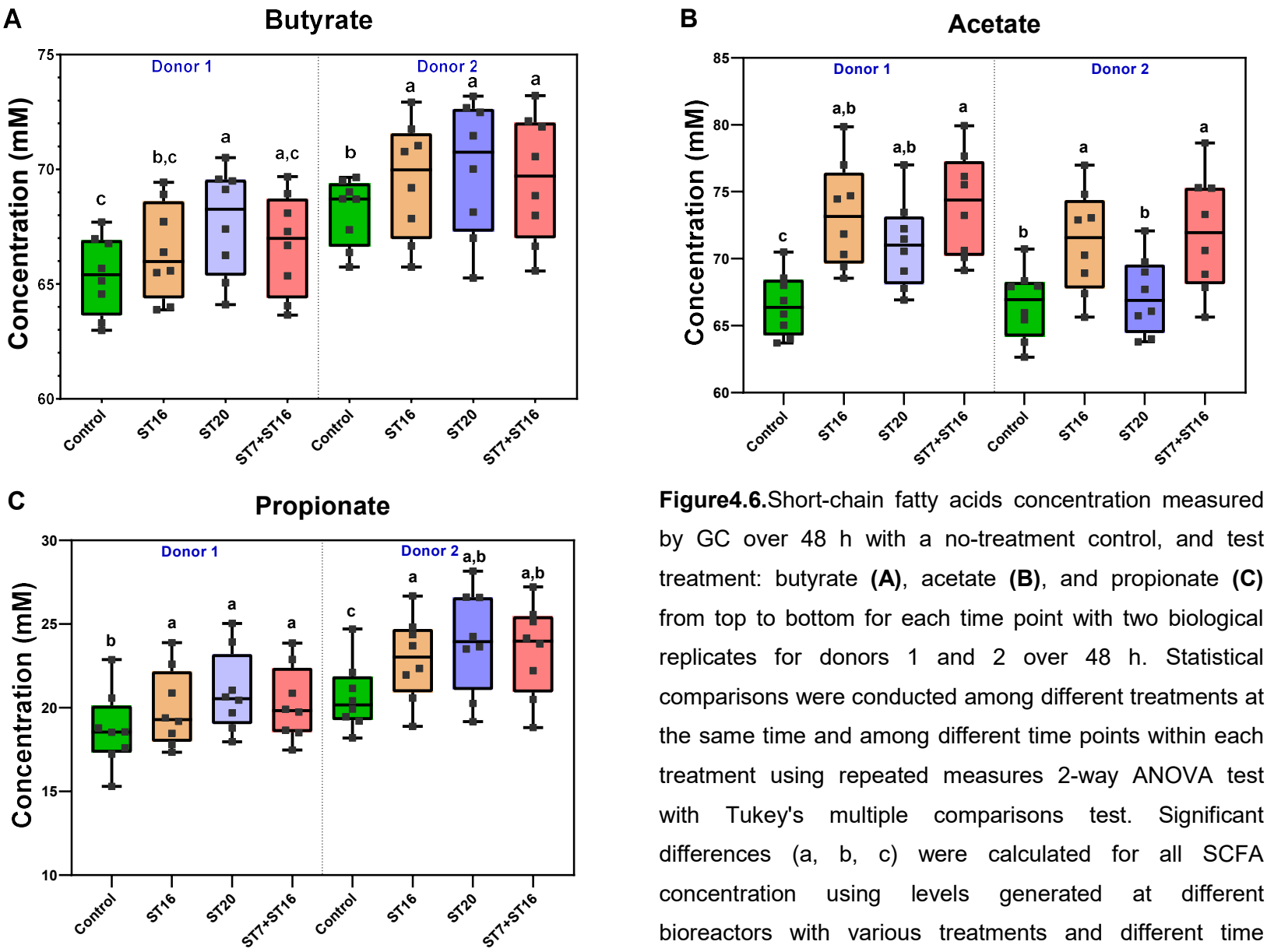


Figure4.6. Short-chain fatty acids concentration measured by GC over 48 h with a no-treatment control, and test treatment: butyrate **(A)**, acetate **(B)**, and propionate **(C)** from top to bottom for each time point with two biological replicates for donors 1 and 2 over 48 h. Statistical comparisons were conducted among different treatments at the same time and among different time points within each treatment using repeated measures 2-way ANOVA test with Tukey's multiple comparisons test. Significant differences (a, b, c) were calculated for all SCFA concentration using levels generated at different bioreactors with various treatments and different time

points.

CHAPTER 5: GENERAL CONCLUSION AND PERSPECTIVES

This study investigated the growth, GABA production, and competitiveness of probiotic candidates in the presence of human gut microbiota *exvivo* in a model of a colon mimicking physiological and microbiological conditions of a human large intestine.

In Chapter 3, we have identified several Lactobacilli and a *Bifidobacterium*that harbor *gadB* gene, encoding a glutamate decarboxylase that can convert L-glutamate to GABA, with high potential to produce GABA, consistently with previous studies that described the GAD activity of GABA producing LABs (Lacroix et al., 2013; Q. Wu et al., 2017).Of 30 LAB strains, 14 isolates were identified as positive for the presence of *gadB* gene and found to produce GABA, with five *S. thermophilus* and four *L. plantarum* being the most active isolates. These strains exhibitedhigher enzyme activity than*Bifidobacterium dentium* ATCC 27678 (positive control)(Pokusaeva et al., 2017). The *in vitro* probiotic properties of selected LAB strains were studied by evaluating sensitivity to antibiotics, the presence of virulence factors, and survival rate in simulated gastrointestinal fluids. Our isolated strains were sensitive to the most common antibiotics, as previously observed for LAB strains (Hanchi et al., 2014). Most of the tested *Lactobacilli* were

resistant to ampicillin, vancomycin, and ciprofloxacin compared to the previous studies that have reported an intrinsic resistance to antibiotics despite their safety status (Campedelli et al., 2018). In addition, *Bifidobacteria* strains tested in this study were resistant to vancomycin, in accordance with previous reports (Kheadr et al., 2007). The observed resistance to tetracycline, erythromycin, and gentamicin antibiotics was detected among *S. thermophilus* isolates in contrast with previous reports(Zhou et al., 2012). *S. thermophilus* and *L. delbrueckiisubsp. bulgaricus* are routinely used as fermenting yogurt starter cultures in the food industry, couldtransfer resistance genesto gut microbiota.Therefore, despite their GRAS status, LABs used as probiotics or functional foods should be free of major antibiotic resistance genes to minimize theirwidespread in the gut environment. Likewise, the absence of virulence factors is an essential criterion for *Enterococci*'s probiotic selection (Hanchi et al., 2018). The two non-expected isolated strains *E. faecium* ST2 and *E. faecium* ST3, were free of virulence gene, thus confirming their safety.Acid tolerance is also an important criterion for the probiotics' survival in acidic stomach conditions. All bacterial isolates were resistant under the gastric juice at pH 2.4, while only *L. plantarum* LP9 survived until pH 1.5. in addition, *S. thermophilus* ST8, *L. plantarum* LP6, and *L. acidophilus* LP16-2 were resistant in gastric juice until pH 1.6. Besides, the tested strains exhibited very high survival rates in the pancreatic fluid (mimicking small intestine physiological conditions) in accordance with previous reports (Vecchione et al., 2018). The genomic analyses for *L. delbrueckii subsp. bulgaricus* ST7*, S. thermophilus* ST16*,* and *B. animalis* ST20confirmedpresence of GADgenes, lack of plasmids, toxins, adhesion toxins, and transposable elements, thus indicating chromosomal-mediated resistance forisolated strains.Our tested strains also exhibited an important survival rate in pre-fermented Macfarlane broth that mimic colon physiological conditions, including microbiota metabolome (such as SCFA), indicating their suitability as potential GABA-producing candidates. Our isolated strains could adapt to the gut environment, a significant criterion for selecting potent probiotics having the ability to survive and produce their active metabolites under GIT.In summary, all safety and stability features of selected GABA-producing strains promote them as promising probiotic candidates for extending their application to the functional food industry (Lacroix et al., 2013; Siragusa et al., 2007).

Chapter 4 investigated the effect of isolated GABA-producing probiotic candidates (in chapter 3) on gut microbiota using an *ex vivo* continuous fermentation model mimicking the human adult proximal colon, as described in (Mottawea et al., 2020). The *ex vivo* proximal colon models as continuous fermentation models create a stable environment and mimic the colon's microbial

composition and physiological conditions,allowing microbial communities to reach a steadystate (Guzman-Rodriguez et al., 2018). These systems are physiologically relevant models and can monitor changes in the gut microbiota composition and functionality overtime of the fermentation process and react to perturbations/treatments, including probiotics (McDonald, 2017; Newton et al., 2019).The used model isbased on PolyFermS, whichwas successfully employed in previous studies on the impact of probiotics bacteria on gut microbiome and metabolome (Le Lay et al., 2015; Zihler et al., 2010; Zihler Berner et al., 2013). Our developedmicrobiota model was composed of the four major phyla, *Firmicutes, Bacteroidetes, Actinobacteria,*and *proteobacteria*, known as common phyla of the gut microbiota with a predominance of *Bacteroidetes* and *Firmicutes*(Mottawea et al., 2020).

Interestingly, the combination of *L. delbrueckii* subsp*. bulgaricus* and *S. thermophilus* increased the microbiota diversity over 48 h of treatment. Recently, dietary GABA supplementation has been reported to enhance intestinal immunity in pathogenic enterotoxigenic *Escherichia coli* (ETEC) infected weaning piglets by altering gut microbiota and microbial metabolism and also promoting secretion of intestinal secretory immunoglobulin A (SIgA) (Y. Zhao et al., 2020).Likewise, a yogurt starter culture of *S. thermophilus* and *L. bulgaricus* was shown to modulate the immune response in a mouse model of colitis (Wasilewska et al., 2019). Besides, administration of *Lactobacillus* cocktails has been reported to increase the microbiota diversity post-antibiotic treatment in a mouse model,likely due to the SCFAs production, appropriate cell adhesion, and high survival rates in the gastrointestinal tract (Shi et al., 2017). Recently, Han and his coauthors (2020)have reported that LPS-induced decrease in mice gut microbiota diversity was restored by using a single strain of *S. thermophilus*, suggesting a potential application as a biologic therapy to alterthe expression of gut inflammatory factors.Combining two *Lactobacillus* strains and *S. thermophilus* has also improved the gut microbiota diversity in neonatal piglets (Veljović et al., 2017)while treating the microbiota with a single GABA-producing strain did not alter the microbiota diversity, assuming that the short time of the treatment limited the capacity of these strains to alter the microbiota diversity. Of particular interest, the population of *Bacteroidetes,* one of the major identified phyla, was enriched by the treatment using our isolated GABA-producers.Similar results were reported afteradministering a mixture of four *Lactobacillus* strains isolated from fermented food with increased levels of mice *Bacteroidetes* that were depleted by antibiotic consumption(Shi et al., 2018). Importantly, *Bacteroidetes* was recently inversely correlated with depression phenotype (Strandwitz et al., 2019). In addition, the same authors have reported that *Bacteroides* ssp. could produce GABA by expressing GABA-producing pathways (Strandwitz et al., 2019).The abundance of

fecal*Bacteroides* hasalso beennegatively correlated with intestinal inflammation (Flowers et al., 2017). Besides, we found enrichment of the *Lactobacillaceae* family after the addition of GABA-producing bacteria. Enhancement of *Lactobacillus* has been previously associated with decreasing the depression symptoms in patients with major depressive disorder (MDD) (Jiang et al., 2015). In addition, the microbiota, treated with the combination of *L. delbrueckii subsp. bulgaricus* and *S. thermophilus* strains were enriched by the *Ruminococcaceae* family, known to have a beneficial effect on gut barrier functions, and introduced as adjuvants to immune checkpoint inhibitors (Jiang et al., 2015; Lukić et al., 2019).

SCFAs are saturated fatty acids produced by commensal microbes through fiber fermentation, with acetate, butyrate, and propionate being the most dominant metabolites in the intestine (Luo et al., 2021). Most gut-generated SCFAs are absorbed into the circulation, while a minor part is excreted in the feces (Luo et al., 2021). We detected high amounts of acetate and butyrate in our colon model, while propionate concentration in the effluent samples was low. Furthermore, a significant increase in SCFAs production, especially butyrate, was induced by the three tested probiotic formulas,along with the butyrate-producing community. SCFAs produced in the colonic environment have an essential role in regulating gut epithelial barrier integrity in addition to their immune-modulatory properties (Parada Venegas et al., 2019). Our findings indicate that the tested strains promote butyrate-producing bacteria and, thus, have the potential as adjuvant biotherapeutics not only in mental health disorders but also in many other conditions characterized by depletion of SCFAs such as inflammatory bowel diseases (Mottawea et al., 2016) and irritable bowel syndrome (Luo et al., 2021).

Despite the relevant results presented here, several limitations of the study should be considered for further study using the colon model. The present study was performed with fecal slurry from two healthy donors and was not strictly homogenous in terms of age and several variables such as the composition of human gut microbial community and their metabolites activity (Gupta et al., 2017). Therefore, in order to confirm such results on a broader basis, the number of fecal samples from different donors should be high. The current *ex vivo* model was limited by the number of donors, biological replicates, and treatment time, which may affect some expected results according to previous findings (Mottawea et al., 2020). There was also the inter-individual variation of the gut microbiota combined with different responses to the same treatment due to differences in the host parameters (e.g., diet,

environment, age, and genetics), which was made it difficult to explore the changes in the gut microbiota composition and diversity (McDonald, 2017). The study used feces for gut microbiome studies which were not representative of all microbial communities found in the gastrointestinal tract (GIT), as microbial populations and metabolism vary along the length of the gut (Mottawea et al., 2020). The microbial communities were effectively grown under simulated colon conditions due to restrictions in substrate supply. Also, our model had lack feedback mechanisms by the host and did not combine with cell culture systems (e.g., epithelial and immune cells) to mimic host-microbe interactions (Poeker et al., 2018). In most cases, physiological parameters, which impact microbial metabolism, composition, and balance, used in the colon model were obtained from healthy adults, which was unclear whether these parameters would apply to patients with several disorders or diseases (Cani, 2018).

The present study provided evidence of several GABA-producing lactic acid bacteria from food origin with probiotic features extending their application as functional food products. Our tested strains had fast product kinetics, were free from virulence factors, sensitive to antibiotics, in addition to surviving simulated gastrointestinal conditions and producing GABA under simulated human colonic conditions. Although we should be careful about generalizing our findings to the overall capability of GABA-producing probiotics to modulate the gut microbiota and promote the production of SCFAs in the gut, this study provided evidence that such potential probiotics could be further exploited as functional food products and biotherapeutic regimens to mitigate human health disorders associated with gut microbiota dysbiosis. Hence, our obtained data provide the rationale for future studies investigating the potential symbiotic impact of GABA-producing bacteria on human microbiota composition and diversity and their metabolism associated with diseases and disorders. Also, the safety and the stability of our tested GABA-producing isolates under simulated colon conditions promote them aspromisingprobiotic candidates for developing health-beneficial functional food products. We only tested the effects of 3 food-isolated probiotic candidates belonging to Bifidobacteria, lactobacilli, and streptococci genera, and future work may investigate other human-originated GABA-generating gut bacteria and their impact on gut microbiota and disease status.

For future works, the next direction to take in this area of research would be to:

- Complete the probiotic characterization of identified candidates,such asassessing adhesion potential to human colonic epithelial cells.

- Study the survival of GABA-producing bacteria across the gut and their ability to produce GABA *in vivo* after oral consumption.
- Evaluate the effectiveness of the tested strains on patients with mental health disorders and elucidate the impact of our probiotic candidates on microbiota structure and their health benefits.

REFERENCES

Agamennone, V., Krul, C. A. M., Rijkers, G., & Kort, R. (2018). A practical guide for probiotics applied to the case of antibiotic-associated diarrhea in The Netherlands. *BMC Gastroenterology*, *18*(1), 103. https://doi.org/10.1186/s12876-018-0831-x

Alkasir, R., Li, J., Li, X., Jin, M., & Zhu, B. (2017). Human gut microbiota: The links with dementia development. *Protein & Cell*, *8*(2), 90–102. https://doi.org/10.1007/s13238-016-0338-6

Ansari, F., Pourjafar, H., Tabrizi, A., & Homayouni, A. (2020). The Effects of Probiotics and Prebiotics on Mental Disorders: A Review on Depression, Anxiety, Alzheimer, and Autism Spectrum Disorders. *Current Pharmaceutical Biotechnology*, *21*(7), 555–565. https://doi.org/10.2174/1389201021666200107113812

Appleton, J. (2018). The Gut-Brain Axis: Influence of Microbiota on Mood and Mental Health. *Integrative Medicine (Encinitas, Calif.)*, *17*(4), 28–32.

Auteri, M., Zizzo, M. G., & Serio, R. (2015). GABA and GABA receptors in the gastrointestinal tract: From motility to inflammation. *Pharmacological Research*, *93*, 11–21. https://doi.org/10.1016/j.phrs.2014.12.001

Barrett, E., Ross, R. P., O'Toole, P. W., Fitzgerald, G. F., & Stanton, C. (2012). γ-Aminobutyric acid production by culturable bacteria from the human intestine. *Journal of Applied Microbiology*, *113*(2), 411–417. https://doi.org/10.1111/j.1365-2672.2012.05344.x

Benke, D. (2020). GABAB Receptors and Pain. *Current Topics in Behavioral Neurosciences*. https://doi.org/10.1007/7854_2020_130

Bezkorovainy, A. (2001). Probiotics: Determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition*, *73*(2 Suppl), 399S-405S. https://doi.org/10.1093/ajcn/73.2.399s

Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R. W., & Steinman, L. (2010). Inhibitory role for GABA in autoimmune inflammation. *Proceedings of the National Academy of Sciences*, *107*(6), 2580–2585. https://doi.org/10.1073/pnas.0915139107

Bienenstock, J., Forsythe, P., Karimi, K., & Kunze, W. (2010). Neuroimmune aspects of food intake. *International Dairy Journal*, *20*(4), 253–258. https://doi.org/10.1016/j.idairyj.2009.12.002

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Boonstra, E., de Kleijn, R., Colzato, L. S., Alkemade, A., Forstmann, B. U., & Nieuwenhuis, S. (2015). Neurotransmitters as food supplements: The effects of GABA on brain and behavior. *Frontiers in Psychology*, *6*. https://doi.org/10.3389/fpsyg.2015.01520

Boureau, L. Hartmann, T. Karjalaine, H. (2000). Models to Study Colonisation and Colonisation Resistance. *Microbial Ecology in Health and Disease*, *12*(2), 247–258. https://doi.org/10.1080/08910600050216246

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 16050–16055. https://doi.org/10.1073/pnas.1102999108

Briguglio, M., Dell'Osso, B., Panzica, G., Malgaroli, A., Banfi, G., Zanaboni Dina, C., Galentino, R., & Porta, M. (2018). Dietary Neurotransmitters: A Narrative Review on Current Knowledge. *Nutrients*, *10*(5), E591. https://doi.org/10.3390/nu10050591

Burton, C. L., Chhabra, S. R., Swift, S., Baldwin, T. J., Withers, H., Hill, S. J., & Williams, P. (2002). The Growth Response of Escherichia coli to Neurotransmitters and Related Catecholamine Drugs Requires a Functional Enterobactin Biosynthesis and Uptake System. *Infection and Immunity*, *70*(11), 5913–5923. https://doi.org/10.1128/IAI.70.11.5913-5923.2002

Campedelli, I., Mathur, H., Salvetti, E., Clarke, S., Rea, M. C., Torriani, S., Ross, R. P., Hill, C., & O'Toole, P. W. (2018). Genus-Wide Assessment of Antibiotic Resistance in *Lactobacillus* spp. *Applied and Environmental Microbiology*, *85*(1), e01738-18, /aem/85/1/AEM.01738-18.atom. https://doi.org/10.1128/AEM.01738-18

Cani, P. D. (2018). Human gut microbiome: Hopes, threats and promises. *Gut*, *67*(9), 1716–1725. https://doi.org/10.1136/gutjnl-2018-316723

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: Interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology*, *28*(2), 203–209.

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., & Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microbial Ecology in Health and Disease*, *26*, 26191. https://doi.org/10.3402/mehd.v26.26191

Chen, S., Wu, X., Xia, Y., Wang, M., Liao, S., Li, F., Yin, J., Ren, W., Tan, B., & Yin, Y. (2020). Effects of dietary gamma-aminobutyric acid supplementation on amino acid profile, intestinal immunity, and microbiota in ETEC-challenged piglets. *Food & Function*, *11*(10), 9067–9074. https://doi.org/10.1039/D0FO01729A

Chou, H. T., Kwon, D.-H., Hegazy, M., & Lu, C.-D. (2008). Transcriptome analysis of agmatine and putrescine catabolism in Pseudomonas aeruginosa PAO1. *Journal of Bacteriology*, *190*(6), 1966–1975. https://doi.org/10.1128/JB.01804-07

Cui, Y., Miao, K., Niyaphorn, S., & Qu, X. (2020). Production of Gamma-Aminobutyric Acid from Lactic Acid Bacteria: A Systematic Review. *International Journal of Molecular Sciences*, *21*(3), 995. https://doi.org/10.3390/ijms21030995

Dagorn, A., Chapalain, A., Mijouin, L., Hillion, M., Duclairoir-Poc, C., Chevalier, S., Taupin, L., Orange, N., & Feuilloley, M. G. J. (2013). Effect of GABA, a Bacterial Metabolite, on Pseudomonas fluorescens Surface Properties and Cytotoxicity. *International Journal of Molecular Sciences*, *14*(6), 12186–12204. https://doi.org/10.3390/ijms140612186

Dale, H. F., Rasmussen, S. H., Asiller, Ö. Ö., & Lied, G. A. (2019). Probiotics in Irritable Bowel Syndrome: An Up-to-Date Systematic Review. *Nutrients*, *11*(9). https://doi.org/10.3390/nu11092048

Del Toro-Barbosa, M., Hurtado-Romero, A., Garcia-Amezquita, L. E., & García-Cayuela, T. (2020). Psychobiotics: Mechanisms of Action, Evaluation Methods and Effectiveness in Applications with Food Products. *Nutrients*, *12*(12), E3896. https://doi.org/10.3390/nu12123896

Dhakal, R., Bajpai, V. K., & Baek, K.-H. (2012). Production of gaba (γ - Aminobutyric acid) by microorganisms: A review. *Brazilian Journal of Microbiology: [Publication of the Brazilian Society for Microbiology]*, *43*(4), 1230–1241. https://doi.org/10.1590/S1517-83822012000400001

Diana, M., Quílez, J., & Rafecas, M. (2014). Gamma-aminobutyric acid as a bioactive compound in foods: A review. *Journal of Functional Foods*, *10*, 407–420. https://doi.org/10.1016/j.jff.2014.07.004

Dinan, T. G., & Cryan, J. F. (2017). Microbes, Immunity and Behavior: Psychoneuroimmunology Meets the Microbiome. *Neuropsychopharmacology*, *42*(1), 178–192. https://doi.org/10.1038/npp.2016.103

Duranti, S., Ruiz, L., Lugli, G. A., Tames, H., Milani, C., Mancabelli, L., Mancino, W., Longhi, G., Carnevali, L., Sgoifo, A., Margolles, A., Ventura, M., Ruas-Madiedo, P., & Turroni, F. (2020). Bifidobacterium adolescentis as a key member of the human gut microbiota in the production of GABA. *Scientific Reports*, *10*(1), 14112. https://doi.org/10.1038/s41598-020-70986-z

Fehlbaum, S., Prudence, K., Kieboom, J., Heerikhuisen, M., van den Broek, T., Schuren, F., Steinert, R., & Raederstorff, D. (2018). In Vitro Fermentation of Selected Prebiotics and Their Effects on the Composition and Activity of the Adult Gut Microbiota. *International Journal of Molecular Sciences*, *19*(10), 3097. https://doi.org/10.3390/ijms19103097

Fernandez, B., Hammami, R., Savard, P., Jean, J., & Fliss, I. (2014). Pediococcus acidilactici UL5 and Lactococcus lactis ATCC 11454 are able to survive and express their bacteriocin genes under simulated gastrointestinal conditions. *Journal of Applied Microbiology*, *116*(3), 677–688. https://doi.org/10.1111/jam.12391

Fernandez, B., Savard, P., & Fliss, I. (2016). Survival and Metabolic Activity of Pediocin Producer Pediococcus acidilactici UL5: Its Impact on Intestinal Microbiota and Listeria monocytogenes in a Model of the Human Terminal Ileum. *Microbial Ecology*, *72*(4), 931–942. https://doi.org/10.1007/s00248-015-0645-0

Fijan, S. (2014). Microorganisms with claimed probiotic properties: An overview of recent literature. *International Journal of Environmental Research and Public Health*, *11*(5), 4745–4767. https://doi.org/10.3390/ijerph110504745

Fliss, I., Hammami, R., & Le Lay, C. (2011). Biological control of human digestive microbiota using antimicrobial cultures and bacteriocins. In *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation.* (pp. 240–263). Woodhead Publ Ltd. http://scholar.google.com/scholar?cluster=7819682438621639613&hl=en&oi=scholarr

Flowers, S. A., Evans, S. J., Ward, K. M., McInnis, M. G., & Ellingrod, V. L. (2017). Interaction Between Atypical Antipsychotics and the Gut Microbiome in a Bipolar Disease Cohort. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *37*(3), 261–267. https://doi.org/10.1002/phar.1890

Foster, J. A., & McVey Neufeld, K.-A. (2013). Gut-brain axis: How the microbiome influences anxiety and depression. *Trends in Neurosciences*, *36*(5), 305–312. https://doi.org/10.1016/j.tins.2013.01.005

Franciosi, E., Carafa, I., Nardin, T., Schiavon, S., Poznanski, E., Cavazza, A., Larcher, R., & Tuohy, K. M. (2015). Biodiversity and *γ* -Aminobutyric Acid Production by Lactic Acid Bacteria Isolated from Traditional Alpine Raw Cow's Milk Cheeses. *BioMed Research International*, *2015*, 1–11. https://doi.org/10.1155/2015/625740

Galland, L. (2014). The Gut Microbiome and the Brain. *Journal of Medicinal Food*, *17*(12), 1261–1272. https://doi.org/10.1089/jmf.2014.7000

Gomaa, E. Z. (2015). Enhancement of γ-Amminobutyric Acid Production by Co-Culturing of Two Lactobacilli Strains. *Asian Journal of Biotechnology*, *7*(3), 108–118. https://doi.org/10.3923/ajbkr.2015.108.118

Goswami, G., Panda, D., Samanta, R., Boro, R. C., Modi, M. K., Bujarbaruah, K. M., & Barooah, M. (2018). Bacillus megaterium adapts to acid stress condition through a network of genes: Insight from a genome-wide transcriptome analysis. *Scientific Reports*, *8*(1), 16105. https://doi.org/10.1038/s41598-018-34221-0

Gupta, V. K., Paul, S., & Dutta, C. (2017). Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity. *Frontiers in Microbiology*, *0*. https://doi.org/10.3389/fmicb.2017.01162

Guzman-Rodriguez, M., McDonald, J. A. K., Hyde, R., Allen-Vercoe, E., Claud, E. C., Sheth, P. M., & Petrof, E. O. (2018). Using bioreactors to study the effects of drugs on the human microbiota. *Methods*, *149*, 31–41. https://doi.org/10.1016/j.ymeth.2018.08.003

Halloran, K., & Underwood, M. A. (2019). Probiotic mechanisms of action. *Early Human Development*, *135*, 58–65. https://doi.org/10.1016/j.earlhumdev.2019.05.010

Hammami, R., Fliss, I., & Corsetti, A. (2019). Editorial: Application of Protective Cultures and Bacteriocins for Food Biopreservation. *Frontiers in Microbiology*, *10*, 1561. https://doi.org/10.3389/fmicb.2019.01561

Han, F., Wu, G., Zhang, Y., Zheng, H., Han, S., Li, X., Cai, W., Liu, J., Zhang, W., Zhang, X., & Hu, D. (2020). Streptococcus thermophilus Attenuates Inflammation in Septic Mice Mediated by Gut Microbiota. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.598010

Hanchi, H., Hammami, R., Kourda, R., Hamida, J. B., & Fliss, I. (2014). Bacteriocinogenic properties and in vitro probiotic potential of enterococci from Tunisian dairy products. *Archives of Microbiology*, *196*(5), 331–344. https://doi.org/10.1007/s00203-014-0978-y

Hanchi, H., Mottawea, W., Sebei, K., & Hammami, R. (2018). The Genus Enterococcus: Between Probiotic Potential and Safety Concerns—An Update. *Frontiers in Microbiology*, *9*. https://doi.org/10.3389/fmicb.2018.01791

Harnentis, H., Nurmiati, N., Marlida, Y., Adzitey, F., & Huda, N. (2019). γ-Aminobutyric acid production by selected lactic acid bacteria isolate of an Indonesian indigenous fermented buffalo milk (dadih) origin. *Veterinary World*, *12*(8), 1352–1357. https://doi.org/10.14202/vetworld.2019.1352-1357

Hemarajata, P., & Versalovic, J. (2013). Effects of probiotics on gut microbiota: Mechanisms of intestinal immunomodulation and neuromodulation. *Therapeutic Advances in Gastroenterology*, *6*(1), 39–51. https://doi.org/10.1177/1756283X12459294

Herigstad, B., Hamilton, M., & Heersink, J. (2001). How to optimize the drop plate method for enumerating bacteria. *Journal of Microbiological Methods*, *44*(2), 121–129. https://doi.org/10.1016/S0167-7012(00)00241-4

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Holzer, P., & Farzi, A. (2014). Neuropeptides and the microbiota-gut-brain axis. *Advances in Experimental Medicine and Biology*, *817*, 195–219. https://doi.org/10.1007/978-1-4939-0897-4_9

Hommais, F., Krin, E., Coppée, J.-Y., Lacroix, C., Yeramian, E., Danchin, A., & Bertin, P. (2004). GadE (YhiE): A novel activator involved in the response to acid environment in Escherichia coli. *Microbiology*, *150*(1), 61–72. https://doi.org/10.1099/mic.0.26659-0

Huang, J., Mei, L., Wu, H., & Lin, D. (2007). Biosynthesis of γ-aminobutyric acid (GABA) using immobilized whole cells of Lactobacillus brevis. *World Journal of Microbiology and Biotechnology*, *23*(6), 865–871. https://doi.org/10.1007/s11274-006-9311-5

Huo, R., Zeng, B., Zeng, L., Cheng, K., Li, B., Luo, Y., Wang, H., Zhou, C., Fang, L., Li, W., Niu, R., Wei, H., & Xie, P. (2017). Microbiota Modulate Anxiety-Like Behavior and Endocrine Abnormalities in Hypothalamic-Pituitary-Adrenal Axis. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00489

Ibrahim, S. A. (2016). Lactic Acid Bacteria: Lactobacillus spp.: Other Species. In *Reference Module in Food Science* (p. B978008100596500857X). Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.00857-X

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., Wang, W., Tang, W., Tan, Z., Shi, J., Li, L., & Ruan, B. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain, Behavior, and Immunity*, *48*, 186–194. https://doi.org/10.1016/j.bbi.2015.03.016

Johnson, D., Thurairajasingam, S., Letchumanan, V., Chan, K.-G., & Lee, L.-H. (2021). Exploring the Role and Potential of Probiotics in the Field of Mental Health: Major Depressive Disorder. *Nutrients*, *13*(5), 1728. https://doi.org/10.3390/nu13051728

Johnson, K. V.-A. (2020). Gut microbiome composition and diversity are related to human personality traits. *Human Microbiome Journal*, *15*, 100069. https://doi.org/10.1016/j.humic.2019.100069

Kennedy, P. J., Cryan, J. F., Dinan, T. G., & Clarke, G. (2017). Kynurenine pathway metabolism and the microbiota-gut-brain axis. *Neuropharmacology*, *112*, 399–412. https://doi.org/10.1016/j.neuropharm.2016.07.002

Kheadr, E., Dabour, N., Le Lay, C., Lacroix, C., & Fliss, I. (2007). Antibiotic Susceptibility Profile of Bifidobacteria as Affected by Oxgall, Acid, and Hydrogen Peroxide Stress. *Antimicrobial Agents and Chemotherapy*, *51*(1), 169–174. https://doi.org/10.1128/AAC.00261-06

Kim, J. Y., Lee, M. Y., Ji, G. E., Lee, Y. S., & Hwang, K. T. (2009). Production of γ-aminobutyric acid in black raspberry juice during fermentation by Lactobacillus brevis GABA100. *International Journal of Food Microbiology*, *130*(1), 12–16. https://doi.org/10.1016/j.ijfoodmicro.2008.12.028

Kimura, I., Inoue, D., Maeda, T., Hara, T., Ichimura, A., Miyauchi, S., Kobayashi, M., Hirasawa, A., & Tsujimoto, G. (2011). Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *Proceedings of the National Academy of Sciences of the United States of America*, *108*(19), 8030–8035. https://doi.org/10.1073/pnas.1016088108

Kiruthiga, A., Padmavathy, K., Shabana, P., Naveenkumar, V., Gnanadesikan, S., & Malaiyan, J. (2020). Improved detection of esp, hyl, asa1, gelE, cylA virulence genes among clinical isolates of Enterococci. *BMC Research Notes*, *13*(1), 170. https://doi.org/10.1186/s13104-020-05018-0

Ko, C. Y., Lin, H.-T. V., & Tsai, G. J. (2013). Gamma-aminobutyric acid production in black soybean milk by Lactobacillus brevis FPA 3709 and the antidepressant effect of the fermented product on a forced swimming rat model. *Process Biochemistry*, *48*(4), 559–568. https://doi.org/10.1016/j.procbio.2013.02.021

Komatsuzaki, N., Nakamura, T., Kimura, T., & Shima, J. (2008). Characterization of Glutamate Decarboxylase from a High γ-Aminobutyric Acid (GABA)-Producer, *Lactobacillus paracasei*. *Bioscience, Biotechnology, and Biochemistry*, *72*(2), 278–285. https://doi.org/10.1271/bbb.70163

Komatsuzaki, N., Shima, J., Kawamoto, S., Momose, H., & Kimura, T. (2005). Production of γ-aminobutyric acid (GABA) by Lactobacillus paracasei isolated from traditional fermented foods. *Food Microbiology*, *22*(6), 497–504. https://doi.org/10.1016/j.fm.2005.01.002

Lacroix, N., St-Gelais, D., Champagne, C. P., & Vuillemard, J. C. (2013). Gamma-aminobutyric acid-producing abilities of lactococcal strains isolated from old-style cheese starters. *Dairy Science & Technology*, *93*(3), 315–327. https://doi.org/10.1007/s13594-013-0127-4

Lavoie, S., Chun, E., Bae, S., Brennan, C. A., Gallini Comeau, C. A., Lang, J. K., Michaud, M., Hoveyda, H. R., Fraser, G. L., Fuller, M. H., Layden, B. T., Glickman, J. N., & Garrett, W. S. (2020). Expression of Free Fatty Acid Receptor 2 by Dendritic Cells Prevents Their Expression of Interleukin 27 and Is Required for Maintenance of Mucosal Barrier and Immune Response Against Colorectal Tumors in Mice. *Gastroenterology*, *158*(5), 1359-1372.e9. https://doi.org/10.1053/j.gastro.2019.12.027

Le Blay, G., Hammami, R., Lacroix, C., & Fliss, I. (2012). Stability and Inhibitory Activity of Pediocin PA-1 Against Listeria sp. In Simulated Physiological Conditions of the Human Terminal Ileum. *Probiotics and Antimicrobial Proteins*, *4*(4), 250–258. https://doi.org/10.1007/s12602-012-9111-1

Le Lay, C., Fernandez, B., Hammami, R., Ouellette, M., & Fliss, I. (2015). On Lactococcus lactis UL719 competitivity and nisin (Nisaplin(®)) capacity to inhibit Clostridium difficile in a model of human colon. *Frontiers in Microbiology*, *6*, 1020. https://doi.org/10.3389/fmicb.2015.01020

Lee, B.-J., Kim, J.-S., Kang, Y. M., Lim, J.-H., Kim, Y.-M., Lee, M.-S., Jeong, M.-H., Ahn, C.-B., & Je, J.-Y. (2010). Antioxidant activity and γ-aminobutyric acid (GABA) content in sea tangle fermented by Lactobacillus brevis BJ20 isolated from traditional fermented foods. *Food Chemistry*, *122*(1), 271–276. https://doi.org/10.1016/j.foodchem.2010.02.071

Lee, K. W., Shim, J. M., Yao, Z., Kim, J. A., Kim, H.-J., & Kim, J. H. (2017). Characterization of a Glutamate Decarboxylase (GAD) from Enterococcus avium M5 Isolated from Jeotgal, a Korean Fermented Seafood. *Journal of Microbiology and Biotechnology*, *27*(7), 1216–1222. https://doi.org/10.4014/jmb.1701.01058

Li, C., Lei, Y., Tian, Y., Xu, S., Shen, X., Wu, H., Bao, S., & Wang, F. (2019). The etiological contribution of GABAergic plasticity to the pathogenesis of neuropathic pain. *Molecular Pain*, *15*, 1744806919847366. https://doi.org/10.1177/1744806919847366

Li, H., & Cao, Y. (2010). Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids*, *39*(5), 1107–1116. https://doi.org/10.1007/s00726-010-0582-7

Li, H., Qiu, T., Huang, G., & Cao, Y. (2010). Production of gamma-aminobutyric acid by Lactobacillus brevis NCL912 using fed-batch fermentation. *Microbial Cell Factories*, *9*(1), 85. https://doi.org/10.1186/1475-2859-9-85

Li, Y., Xiang, Y.-Y., Lu, W.-Y., Liu, C., & Li, J. (2012). A novel role of intestine epithelial GABAergic signaling in regulating intestinal fluid secretion. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *303*(4), G453-460. https://doi.org/10.1152/ajpgi.00497.2011

Lim, H. S., Cha, I.-T., Lee, H., & Seo, M.-J. (2016). Optimization of ��-Aminobutyric Acid Production by Enterococcus faecium JK29 Isolated from a Traditional Fermented Foods. *Microbiology and Biotechnology Letters*, *44*(1), 26–33. https://doi.org/10.4014/mbl.1512.12004

Lim, H. S., Cha, I.-T., Roh, S. W., Shin, H.-H., & Seo, M.-J. (2017). Enhanced Production of Gamma-Aminobutyric Acid by Optimizing Culture Conditions of Lactobacillus brevis HYE1 Isolated from Kimchi, a Korean Fermented Food. *Journal of Microbiology and Biotechnology*, *27*(3), 450–459. https://doi.org/10.4014/jmb.1610.10008

Lin, Q. (2013). Submerged fermentation of Lactobacillus rhamnosus YS9 for γ-aminobutyric acid (GABA) production. *Brazilian Journal of Microbiology*, *44*(1), 183–187. https://doi.org/10.1590/S1517-83822013000100028

Linares, D. M., Gómez, C., Renes, E., Fresno, J. M., Tornadijo, M. E., Ross, R. P., & Stanton, C. (2017). Lactic Acid Bacteria and Bifidobacteria with Potential to Design Natural Biofunctional Health-Promoting Dairy Foods. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00846

Linares, D. M., O'Callaghan, T. F., O'Connor, P. M., Ross, R. P., & Stanton, C. (2016). Streptococcus thermophilus APC151 Strain Is Suitable for the Manufacture of Naturally GABA-Enriched Bioactive Yogurt. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01876

Liśkiewicz, P., Pełka-Wysiecka, J., Kaczmarczyk, M., Łoniewski, I., Wroński, M., Bąba-Kubiś, A., Skonieczna-Żydecka, K., Marlicz, W., Misiak, B., & Samochowiec, J. (2019). Fecal Microbiota Analysis in Patients Going through a Depressive Episode during Treatment in a Psychiatric Hospital Setting. *Journal of Clinical Medicine*, *8*(2), 164. https://doi.org/10.3390/jcm8020164

Logan, A. C., & Katzman, M. (2005). Major depressive disorder: Probiotics may be an adjuvant therapy. *Medical Hypotheses*, *64*(3), 533–538. https://doi.org/10.1016/j.mehy.2004.08.019

Lone, A., Mottawea, W., Mehdi, Y., & Hammami, R. (2021). Bacteriocinogenic probiotics as an integrated alternative to antibiotics in chicken production—Why and how? *Critical Reviews in Food Science and Nutrition*, 1–17. https://doi.org/10.1080/10408398.2021.1932722

Lukić, I., Getselter, D., Ziv, O., Oron, O., Reuveni, E., Koren, O., & Elliott, E. (2019). Antidepressants affect gut microbiota and Ruminococcus flavefaciens is able to abolish their effects on depressive-like behavior. *Translational Psychiatry*, *9*(1), 133. https://doi.org/10.1038/s41398-019-0466-x

Luo, M., Zhuang, X., Tian, Z., & Xiong, L. (2021). Alterations in short-chain fatty acids and serotonin in irritable bowel syndrome: A systematic review and meta-analysis. *BMC Gastroenterology*, *21*(1), 1–13. https://doi.org/10.1186/s12876-020-01577-5

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. *BioEssays*, *33*(8), 574–581. https://doi.org/10.1002/bies.201100024

Lyte, M. (2016). Microbial Endocrinology. In *The Gut-Brain Axis* (pp. 89–108). Elsevier. https://doi.org/10.1016/B978-0-12-802304-4.00006-2

Ma, P., Li, T., Ji, F., Wang, H., & Pang, J. (2015). Effect of GABA on blood pressure and blood dynamics of anesthetic rats. *International Journal of Clinical and Experimental Medicine*, *8*(8), 14296–14302.

Ma, Q., Xing, C., Long, W., Wang, H. Y., Liu, Q., & Wang, R.-F. (2019). Impact of microbiota on central nervous system and neurological diseases: The gut-brain axis. *Journal of Neuroinflammation*, *16*(1), 53. https://doi.org/10.1186/s12974-019-1434-3

Macfarlane, G. T., & Macfarlane, S. (2007). Models for intestinal fermentation: Association between food components, delivery systems, bioavailability and functional interactions in the gut. *Current Opinion in Biotechnology*, *18*(2), 156–162. https://doi.org/10.1016/j.copbio.2007.01.011

Macfarlane, G. T., Macfarlane, S., & Gibson, G. R. (1998). Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon. *Microbial Ecology*, *35*(2), 180–187. https://doi.org/10.1007/s002489900072

Mainville, I., Arcand, Y., & Farnworth, E. R. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *International Journal of Food Microbiology*, *99*(3), 287–296. https://doi.org/10.1016/j.ijfoodmicro.2004.08.020

Maldonado Galdeano, C., Cazorla, S. I., Lemme Dumit, J. M., Vélez, E., & Perdigón, G. (2019). Beneficial Effects of Probiotic Consumption on the Immune System. *Annals of Nutrition and Metabolism*, *74*(2), 115–124. https://doi.org/10.1159/000496426

Marteau, P., Pochart, P., Mahé, S., Crine, L., Huneau, J. F., Tomé, D., & Rambaud, J. C. (1991). Gastric emptying but not oroceacal transit time differs between milk and yoghurt in lactose digesters. *Gastroenterology*, *100*, A535.

Mates, A. K., Sayed, A. K., & Foster, J. W. (2007). Products of the *Escherichia coli* Acid Fitness Island Attenuate Metabolite Stress at Extremely Low pH and Mediate a Cell Density-Dependent Acid Resistance. *Journal of Bacteriology*, *189*(7), 2759–2768. https://doi.org/10.1128/JB.01490-06

Matricardi, P. M. (2002). Probiotics against allergy: Data, doubts, and perspectives. *Allergy*, *57*(3), 185–187. https://doi.org/10.1034/j.1398-9995.2002.1a3299.x

Mayer, E. A., Knight, R., Mazmanian, S. K., Cryan, J. F., & Tillisch, K. (2014). Gut Microbes and the Brain: Paradigm Shift in Neuroscience. *Journal of Neuroscience*, *34*(46), 15490–15496. https://doi.org/10.1523/JNEUROSCI.3299-14.2014

Mazzoli, R., & Pessione, E. (2016). The Neuro-endocrinological Role of Microbial Glutamate and GABA Signaling. *Frontiers in Microbiology*, *7*, 1934. https://doi.org/10.3389/fmicb.2016.01934

McDonald, J. A. K. (2017). *In vitro* models of the human microbiota and microbiome. *Emerging Topics in Life Sciences*, *1*(4), 373–384. https://doi.org/10.1042/ETLS20170045

McDonald, J. A. K., Schroeter, K., Fuentes, S., Heikamp-Dejong, I., Khursigara, C. M., de Vos, W. M., & Allen-Vercoe, E. (2013). Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model. *Journal of Microbiological Methods*, *95*(2), 167–174. https://doi.org/10.1016/j.mimet.2013.08.008

Messaoudi, M., Lalonde, R., Violle, N., Javelot, H., Desor, D., Nejdi, A., Bisson, J.-F., Rougeot, C., Pichelin, M., Cazaubiel, M., & Cazaubiel, J.-M. (2011). Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. *The British Journal of Nutrition*, *105*(5), 755–764. https://doi.org/10.1017/S0007114510004319

Mohajeri, M. H., La Fata, G., Steinert, R. E., & Weber, P. (2018). Relationship between the gut microbiome and brain function. *Nutrition Reviews*, *76*(7), 481–496. https://doi.org/10.1093/nutrit/nuy009

Monk, J. M., Zhang, C. P., Wu, W., Zarepoor, L., Lu, J. T., Liu, R., Pauls, K. P., Wood, G. A., Tsao, R., Robinson, L. E., & Power, K. A. (2015). White and dark kidney beans reduce colonic mucosal damage and inflammation in response to dextran sodium sulfate. *The Journal of Nutritional Biochemistry*, *26*(7), 752–760. https://doi.org/10.1016/j.jnutbio.2015.02.003

Morrison, M. J., Frégeau-Reid, J. A., & Cober, E. R. (2012). Genotype and environment influence gamma aminobutyric acid concentration in short-season soybean. *Canadian Journal of Plant Science*, *92*(6), 1093–1100. https://doi.org/10.4141/cjps2011-271

Mottawea, W., Butcher, J., Li, J., Abujamel, T., Manoogian, J., Mack, D., & Stintzi, A. (2019). The mucosal–luminal interface: An ideal sample to study the mucosa-associated microbiota and the intestinal microbial biogeography. *Pediatric Research*, *85*(6), 895–903. https://doi.org/10.1038/s41390-019-0326-7

Mottawea, W., Chiang, C.-K., Mühlbauer, M., Starr, A. E., Butcher, J., Abujamel, T., Deeke, S. A., Brandel, A., Zhou, H., Shokralla, S., Hajibabaei, M., Singleton, R., Benchimol, E. I., Jobin, C., Mack, D. R., Figeys, D., & Stintzi, A. (2016). Altered intestinal microbiota-host mitochondria crosstalk in new onset Crohn's disease. *Nature Communications*, *7*, 13419. https://doi.org/10.1038/ncomms13419

Mottawea, W., Sultan, S., Landau, K., Bordenave, N., & Hammami, R. (2020). Evaluation of the Prebiotic Potential of a Commercial Synbiotic Food Ingredient on Gut Microbiota in an Ex Vivo Model of the Human Colon. *Nutrients*, *12*(9). https://doi.org/10.3390/nu12092669

Muralidhara, Divyashri, G., Krishna, G., & Prapulla, S. G. (2015). Probiotic attributes, antioxidant, anti-inflammatory and neuromodulatory effects of Enterococcus faecium CFR 3003: In vitro and in vivo evidence. *Journal of Medical Microbiology*, *64*(12), 1527–1540. https://doi.org/10.1099/jmm.0.000184

Naila, A., Flint, S., Fletcher, G., Bremer, P., & Meerdink, G. (2010). Control of biogenic amines in food—Existing and emerging approaches. *Journal of Food Science*, *75*(7), R139-150. https://doi.org/10.1111/j.1750-3841.2010.01774.x

Newton, R. U., Christophersen, C. T., Fairman, C. M., Hart, N. H., Taaffe, D. R., Broadhurst, D., Devine, A., Chee, R., Tang, C. I., Spry, N., & Galvão, D. A. (2019). Does exercise impact gut microbiota composition in men receiving androgen deprivation therapy for prostate cancer? A single-blinded, two-armed, randomised controlled trial. *BMJ Open*, *9*(4), e024872. https://doi.org/10.1136/bmjopen-2018-024872

Ngo, D.-H., & Vo, T. S. (2019). An Updated Review on Pharmaceutical Properties of Gamma-Aminobutyric Acid. *Molecules*, *24*(15), 2678. https://doi.org/10.3390/molecules24152678

Nikolic, M., López, P., Strahinic, I., Suárez, A., Kojic, M., Fernández-García, M., Topisirovic, L., Golic, N., & Ruas-Madiedo, P. (2012). Characterisation of the exopolysaccharide (EPS)-producing Lactobacillus paraplantarum BGCG11 and its non-EPS producing derivative strains as potential probiotics. *International Journal of Food Microbiology*, *158*(2), 155–162. https://doi.org/10.1016/j.ijfoodmicro.2012.07.015

Northoff, G., & Sibille, E. (2014). Why are cortical GABA neurons relevant to internal focus in depression? A cross-level model linking cellular, biochemical and neural network findings. *Molecular Psychiatry*, *19*(9), 966–977. https://doi.org/10.1038/mp.2014.68

Ogier, J.-C., & Serror, P. (2008). Safety assessment of dairy microorganisms: The Enterococcus genus. *International Journal of Food Microbiology*, *126*(3), 291–301. https://doi.org/10.1016/j.ijfoodmicro.2007.08.017

O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Reports*, *7*(7), 688–693. https://doi.org/10.1038/sj.embor.7400731

Parada Venegas, D., De la Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., Harmsen, H. J. M., Faber, K. N., & Hermoso, M. A. (2019). Short Chain Fatty Acids (SCFAs)-Mediated Gut Epithelial and Immune Regulation and Its Relevance for Inflammatory Bowel Diseases. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.00277

Park, S.-Y., Lee, J.-W., & Lim, S.-D. (2014). The probiotic characteristics and GABA production of Lactobacillus plantarum K154 isolated from kimchi. *Food Science and Biotechnology*, *23*(6), 1951–1957. https://doi.org/10.1007/s10068-014-0266-2

Parkar, S. G., Davis, P., Trower, T. M., Hedderley, D. I., Sutton, K. H., & Ingram, J. R. (2019). Factors affecting microbial metabolism in a human fecal fermentation model to evaluate prebiotics. *Journal of Nutrition & Intermediary Metabolism*, *15*, 55–57. https://doi.org/10.1016/j.jnim.2018.12.003

Patterson, E., Ryan, P. M., Wiley, N., Carafa, I., Sherwin, E., Moloney, G., Franciosi, E., Mandal, R., Wishart, D. S., Tuohy, K., Ross, R. P., Cryan, J. F., Dinan, T. G., & Stanton, C. (2019). Gamma-aminobutyric acid-producing lactobacilli positively affect metabolism and depressive-like behaviour in a mouse model of metabolic syndrome. *Scientific Reports*, *9*(1), 16323. https://doi.org/10.1038/s41598-019-51781-x

Poeker, S. A., Geirnaert, A., Berchtold, L., Greppi, A., Krych, L., Steinert, R. E., de Wouters, T., & Lacroix, C. (2018). Understanding the prebiotic potential of different dietary fibers using an in vitro continuous adult fermentation model (PolyFermS). *Scientific Reports*, *8*(1). https://doi.org/10.1038/s41598-018-22438-y

Pokusaeva, K., Johnson, C., Luk, B., Uribe, G., Fu, Y., Oezguen, N., Matsunami, R. K., Lugo, M., Major, A., Mori-Akiyama, Y., Hollister, E. B., Dann, S. M., Shi, X. Z., Engler, D. A., Savidge, T., & Versalovic, J. (2017). GABA-producing *Bifidobacterium dentium* modulates visceral sensitivity in the intestine. *Neurogastroenterology & Motility*, *29*(1), e12904. https://doi.org/10.1111/nmo.12904

Putignani, L., Del Chierico, F., Petrucca, A., Vernocchi, P., & Dallapiccola, B. (2014). The human gut microbiota: A dynamic interplay with the host from birth to senescence settled during childhood. *Pediatric Research*, *76*(1), 2–10. https://doi.org/10.1038/pr.2014.49

Quílez, J., & Diana, M. (2017). Gamma-Aminobutyric Acid-Enriched Fermented Foods. In *Fermented Foods in Health and Disease Prevention* (pp. 85–103). Elsevier. https://doi.org/10.1016/B978-0-12-802309-9.00005-4

Quillin, S. J., Tran, P., & Prindle, A. (2021). Potential Roles for Gamma-Aminobutyric Acid Signaling in Bacterial Communities. *Bioelectricity*, *3*(2), 120–125. https://doi.org/10.1089/bioe.2021.0012

Rajilić-Stojanović, M., Heilig, H. G. H. J., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., & de Vos, W. M. (2009). Development and application of the human intestinal tract chip, a phylogenetic microarray: Analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environmental Microbiology*, *11*(7), 1736–1751. https://doi.org/10.1111/j.1462-2920.2009.01900.x

Rajilić-Stojanović, M., Smidt, H., & de Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. *Environmental Microbiology*, *9*(9), 2125–2136. https://doi.org/10.1111/j.1462-2920.2007.01369.x

Ribeiro, S. C., Domingos-Lopes, M. F. P., Stanton, C., Ross, R. P., & Silva, C. C. (2018). Production of Y-aminobutyric acid (GABA) by *Lactobacillus otakiensis* and other *Lactobacillus* sp. Isolated from traditional Pico cheese. *International Journal of Dairy Technology*, *71*(4), 1012–1017. https://doi.org/10.1111/1471-0307.12527

Richard, H., & Foster, J. W. (2004). Escherichia coli Glutamate- and Arginine-Dependent Acid Resistance Systems Increase Internal pH and Reverse Transmembrane Potential. *Journal of Bacteriology*, *186*(18), 6032–6041. https://doi.org/10.1128/JB.186.18.6032-6041.2004

Rogers, G. B., Keating, D. J., Young, R. L., Wong, M.-L., Licinio, J., & Wesselingh, S. (2016). From gut dysbiosis to altered brain function and mental illness: Mechanisms and pathways. *Molecular Psychiatry*, *21*(6), 738–748. https://doi.org/10.1038/mp.2016.50

Romeo, B., Choucha, W., Fossati, P., & Rotge, J.-Y. (2018). Meta-analysis of central and peripheral γ-aminobutyric acid levels in patients with unipolar and bipolar depression. *Journal of Psychiatry & Neuroscience: JPN*, *43*(1), 58–66.

Sahab, N. R. M., Subroto, E., Balia, R. L., & Utama, G. L. (2020). γ-Aminobutyric acid found in fermented foods and beverages: Current trends. *Heliyon*, *6*(11), e05526. https://doi.org/10.1016/j.heliyon.2020.e05526

Sarasa, S. B., Mahendran, R., Muthusamy, G., Thankappan, B., Selta, D. R. F., & Angayarkanni, J. (2020). A Brief Review on the Non-protein Amino Acid, Gamma-amino Butyric Acid (GABA): Its Production and Role in Microbes. *Current Microbiology*, *77*(4), 534–544. https://doi.org/10.1007/s00284-019-01839-w

Sarkar, A., Lehto, S. M., Harty, S., Dinan, T. G., Cryan, J. F., & Burnet, P. W. J. (2016). Psychobiotics and the manipulation of bacteria-gut-brain signals. *Trends in Neurosciences*, *39*(11), 763–781. https://doi.org/10.1016/j.tins.2016.09.002

Schroeder, F. A., Lin, C. L., Crusio, W. E., & Akbarian, S. (2007). Antidepressant-like effects of the histone deacetylase inhibitor, sodium butyrate, in the mouse. *Biological Psychiatry*, *62*(1), 55–64. https://doi.org/10.1016/j.biopsych.2006.06.036

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shi, Y., Zhai, Q., Li, D., Mao, B., Liu, X., Zhao, J., Zhang, H., & Chen, W. (2017). Restoration of cefixime-induced gut microbiota changes by Lactobacillus cocktails and fructooligosaccharides in a mouse model. *Microbiological Research*, *200*, 14–24. https://doi.org/10.1016/j.micres.2017.04.001

Shi, Y., Zhao, X., Zhao, J., Zhang, H., Zhai, Q., Narbad, A., & Chen, W. (2018). A mixture of Lactobacillus species isolated from traditional fermented foods promote recovery from antibiotic-induced intestinal disruption in mice. *Journal of Applied Microbiology*, *124*(3), 842–854. https://doi.org/10.1111/jam.13687

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C. G., Coda, R., & Gobbetti, M. (2007). Synthesis of -Aminobutyric Acid by Lactic Acid Bacteria Isolated from a Variety of Italian Cheeses. *Applied and Environmental Microbiology*, *73*(22), 7283–7290. https://doi.org/10.1128/AEM.01064-07

Smart, T. G., & Stephenson, F. A. (2019). A half century of γ-aminobutyric acid. *Brain and Neuroscience Advances*, *3*, 239821281985824. https://doi.org/10.1177/2398212819858249

Strandwitz, P. (2018). Neurotransmitter modulation by the gut microbiota. *Brain Research*, *1693*, 128–133. https://doi.org/10.1016/j.brainres.2018.03.015

Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., McDonald, D., Dietrich, D., Ramadhar, T. R., Lekbua, A., Mroue, N., Liston, C., Stewart, E. J., Dubin, M. J., Zengler, K., Knight, R., Gilbert, J. A., Clardy, J., & Lewis, K. (2019). GABA-modulating bacteria of the human gut microbiota. *Nature Microbiology*, *4*(3), 396–403. https://doi.org/10.1038/s41564-018-0307-3

Sudo, N. (2019). Biogenic Amines: Signals Between Commensal Microbiota and Gut Physiology. *Frontiers in Endocrinology*, *10*, 504. https://doi.org/10.3389/fendo.2019.00504

Sun, Z., Liu, W., Song, Y., Xu, H., Yu, J., Bilige, M., Zhang, H., & Chen, Y. (2015). Population structure of Lactobacillus helveticus isolates from naturally fermented dairy products based on multilocus sequence typing. *Journal of Dairy Science*, *98*(5), 2962–2972. https://doi.org/10.3168/jds.2014-9133

Suneson, K., Lindahl, J., Chamli Hårsmar, S., Söderberg, G., & Lindqvist, D. (2021). Inflammatory Depression—Mechanisms and Non-Pharmacological Interventions. *International Journal of Molecular Sciences*, *22*(4), 1640. https://doi.org/10.3390/ijms22041640

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). Chapter Three—The Role of Short-Chain Fatty Acids in Health and Disease. In F. W. Alt (Ed.), *Advances in Immunology* (Vol. 121, pp. 91–119). Academic Press. https://doi.org/10.1016/B978-0-12-800100-4.00003-9

Tavakoli, Y., Esmaeili, A., & Rabbani, M. (2015). Identification and molecular cloning of glutamate decarboxylase gene from Lactobacillus casei. *Molecular Biology Research Communications*, *4*(3), 161–165.

Ueno, S., Katayama, T., Watanabe, T., Nakajima, K., Hayashi, M., Shigematsu, T., & Fujii, T. (2013). Enzymatic production of γ-aminobutyric acid in soybeans using high hydrostatic pressure and precursor feeding. *Bioscience, Biotechnology, and Biochemistry*, *77*(4), 706–713. https://doi.org/10.1271/bbb.120740

Umu, Ö. C. O., Rudi, K., & Diep, D. B. (2017). Modulation of the gut microbiota by prebiotic fibres and bacteriocins. *Microbial Ecology in Health and Disease*, *28*(1), 1348886. https://doi.org/10.1080/16512235.2017.1348886

Valles-Colomer, M., Falony, G., Darzi, Y., Tigchelaar, E. F., Wang, J., Tito, R. Y., Schiweck, C., Kurilshikov, A., Joossens, M., Wijmenga, C., Claes, S., Van Oudenhove, L., Zhernakova, A., Vieira-Silva, S., & Raes, J. (2019). The neuroactive potential of the human gut microbiota in quality of life and depression. *Nature Microbiology*. https://doi.org/10.1038/s41564-018-0337-x

van de Wouw, M., Boehme, M., Lyte, J. M., Wiley, N., Strain, C., O'Sullivan, O., Clarke, G., Stanton, C., Dinan, T. G., & Cryan, J. F. (2018). Short-chain fatty acids: Microbial metabolites that alleviate stress-induced brain-gut axis alterations: SCFAs alleviate stress-induced brain-gut axis alterations. *The Journal of Physiology*, *596*(20), 4923–4944. https://doi.org/10.1113/JP276431

Van den Abbeele, P., Grootaert, C., Marzorati, M., Possemiers, S., Verstraete, W., Gérard, P., Rabot, S., Bruneau, A., El Aidy, S., Derrien, M., Zoetendal, E., Kleerebezem, M., Smidt, H., & Van de Wiele, T. (2010). Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for Bacteroidetes and Clostridium cluster IX. *Applied and Environmental Microbiology*, *76*(15), 5237–5246. https://doi.org/10.1128/AEM.00759-10

Vecchione, A., Celandroni, F., Mazzantini, D., Senesi, S., Lupetti, A., & Ghelardi, E. (2018). Compositional Quality and Potential Gastrointestinal Behavior of Probiotic Products Commercialized in Italy. *Frontiers in Medicine*, *5*, 59. https://doi.org/10.3389/fmed.2018.00059

Veljović, K., Dinić, M., Lukić, J., Mihajlović, S., Tolinački, M., Živković, M., Begović, J., Mrvaljević, I., Golić, N., & Terzić-Vidojević, A. (2017). Promotion of Early Gut Colonization by Probiotic Intervention on Microbiota Diversity in Pregnant Sows. *Frontiers in Microbiology*, *8*, 2028. https://doi.org/10.3389/fmicb.2017.02028

Venema, K., & van den Abbeele, P. (2013). Experimental models of the gut microbiome. *Best Practice & Research Clinical Gastroenterology*, *27*(1), 115–126. https://doi.org/10.1016/j.bpg.2013.03.002

Verberkmoes, N. C., Russell, A. L., Shah, M., Godzik, A., Rosenquist, M., Halfvarson, J., Lefsrud, M. G., Apajalahti, J., Tysk, C., Hettich, R. L., & Jansson, J. K. (2009). Shotgun metaproteomics of the human distal gut microbiota. *The ISME Journal*, *3*(2), 179–189. https://doi.org/10.1038/ismej.2008.108

Verna, E. C., & Lucak, S. (2010). Use of probiotics in gastrointestinal disorders: What to recommend? *Therapeutic Advances in Gastroenterology*, *3*(5), 307–319. https://doi.org/10.1177/1756283X10373814

Waddell, W., Cohen, S., Feron, V., Goodman, J., Marnett, L., Portoghese, P., Rietjens, I., Smith, R., Adams, T., Gavin, C. L., McGowen, M., & Williams, M. C. (2007). GRAS Flavoring substances 23—The 23rd publication by the FEMA Expert Panel presents safety and usage data on 174 new generally recognized as safe flavoring ingredients. *Food Technology 61 (2007) 8*, *61*.

Wallace, C. J. K., & Milev, R. (2017). The effects of probiotics on depressive symptoms in humans: A systematic review. *Annals of General Psychiatry*, *16*(1). https://doi.org/10.1186/s12991-017-0138-2

Wasilewska, E., Zlotkowska, D., & Wroblewska, B. (2019). Yogurt starter cultures of Streptococcus thermophilus and Lactobacillus bulgaricus ameliorate symptoms and modulate the immune response in a mouse model of dextran sulfate sodium-induced colitis. *Journal of Dairy Science*, *102*(1), 37–53. https://doi.org/10.3168/jds.2018-14520

Woraharn, S., Lailerd, N., Sivamaruthi, B. S., Wangcharoen, W., Sirisattha, S., & Chaiyasut, C. (2014). Screening and kinetics of glutaminase and glutamate decarboxylase producing lactic acid bacteria from fermented Thai foods. *Food Science and Technology (Campinas)*, *34*(4), 793–799. https://doi.org/10.1590/1678-457X.6519

Wu, C.-H., Hsueh, Y.-H., Kuo, J.-M., & Liu, S.-J. (2018). Characterization of a Potential Probiotic Lactobacillus brevis RK03 and Efficient Production of γ-Aminobutyric Acid in Batch Fermentation. *International Journal of Molecular Sciences*, *19*(1), 143. https://doi.org/10.3390/ijms19010143

Wu, Q., Tun, H. M., Law, Y.-S., Khafipour, E., & Shah, N. P. (2017). Common Distribution of gad Operon in Lactobacillus brevis and its GadA Contributes to Efficient GABA Synthesis toward Cytosolic Near-Neutral pH. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00206

Yan, S., Huang, J., Chen, Z., Jiang, Z., Li, X., & Chen, Z. (2016). Metabolomics in gut microbiota: Applications and challenges. *Science Bulletin*, *61*(15), 1151–1153. https://doi.org/10.1007/s11434-016-1142-7

Yang, S.-Y., Lu, Z.-X., Lü, F.-X., Bie, X.-M., Sun, L.-J., & Zeng, X.-X. (2006). A SIMPLE METHOD FOR RAPID SCREENING OF BACTERIA WITH GLUTAMATE DECARBOXYLASE ACTIVITIES. *Journal of Rapid Methods and Automation in Microbiology*, *14*(3), 291–298. https://doi.org/10.1111/j.1745-4581.2006.00054.x

Yogeswara, I. B. A., Maneerat, S., & Haltrich, D. (2020). Glutamate Decarboxylase from Lactic Acid Bacteria-A Key Enzyme in GABA Synthesis. *Microorganisms*, *8*(12), E1923. https://doi.org/10.3390/microorganisms8121923

Yong, S. J., Tong, T., Chew, J., & Lim, W. L. (2020). Antidepressive Mechanisms of Probiotics and Their Therapeutic Potential. *Frontiers in Neuroscience*, *13*, 1361. https://doi.org/10.3389/fnins.2019.01361

Yoshimura, M., Toyoshi, T., Sano, A., Izumi, T., Fujii, T., Konishi, C., Inai, S., Matsukura, C., Fukuda, N., Ezura, H., & Obata, A. (2010). Antihypertensive effect of a gamma-aminobutyric acid rich tomato cultivar “DG03-9” in spontaneously hypertensive rats. *Journal of Agricultural and Food Chemistry*, *58*(1), 615–619. https://doi.org/10.1021/jf903008t

Yu, K., Hu, S., Huang, J., & Mei, L.-H. (2011). A high-throughput colorimetric assay to measure the activity of glutamate decarboxylase. *Enzyme and Microbial Technology*, *49*(3), 272–276. https://doi.org/10.1016/j.enzmictec.2011.06.007

Yunes, R. A., Poluektova, E. U., Dyachkova, M. S., Klimina, K. M., Kovtun, A. S., Averina, O. V., Orlova, V. S., & Danilenko, V. N. (2016). GABA production and structure of gadB / gadC genes in Lactobacillus and Bifidobacterium strains from human microbiota. *Anaerobe*, *42*, 197–204. https://doi.org/10.1016/j.anaerobe.2016.10.011

Yunes, R. A., Poluektova, E. U., Vasileva, E. V., Odorskaya, M. V., Marsova, M. V., Kovalev, G. I., & Danilenko, V. N. (2020). A Multi-strain Potential Probiotic Formulation of GABA-Producing Lactobacillus plantarum 90sk and Bifidobacterium adolescentis 150 with Antidepressant Effects. *Probiotics and Antimicrobial Proteins*, *12*(3), 973–979. https://doi.org/10.1007/s12602-019-09601-1

Zhao, W., Huang, J., Peng, C., Hu, S., Ke, P., Mei, L., & Yao, S. (2014). Permeabilizing Escherichia coli for whole cell biocatalyst with enhanced biotransformation ability from l-glutamate to GABA. *Journal of Molecular Catalysis B: Enzymatic*, *107*, 39–46. https://doi.org/10.1016/j.molcatb.2014.05.011

Zhao, Y., Wang, J., Wang, H., Huang, Y., Qi, M., Liao, S., Bin, P., & Yin, Y. (2020). Effects of GABA Supplementation on Intestinal SIgA Secretion and Gut Microbiota in the Healthy and ETEC-Infected Weanling Piglets. *Mediators of Inflammation*, *2020*, 1–17. https://doi.org/10.1155/2020/7368483

Zheng, P., Zeng, B., Zhou, C., Liu, M., Fang, Z., Xu, X., Zeng, L., Chen, J., Fan, S., Du, X., Zhang, X., Yang, D., Yang, Y., Meng, H., Li, W., Melgiri, N. D., Licinio, J., Wei, H., & Xie, P. (2016). Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Molecular Psychiatry*, *21*(6), 786–796. https://doi.org/10.1038/mp.2016.44

Zhou, N., Zhang, J. X., Fan, M. T., Wang, J., Guo, G., & Wei, X. Y. (2012). Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. *Journal of Dairy Science*, *95*(9), 4775–4783. https://doi.org/10.3168/jds.2011-5271

Zhu, S., Jiang, Y., Xu, K., Cui, M., Ye, W., Zhao, G., Jin, L., & Chen, X. (2020). The progress of gut microbiome research related to brain disorders. *Journal of Neuroinflammation*, *17*(1), 25. https://doi.org/10.1186/s12974-020-1705-z

Zihler, A., Gagnon, M., Chassard, C., Hegland, A., Stevens, M. J. A., Braegger, C. P., & Lacroix, C. (2010). Unexpected consequences of administering bacteriocinogenic probiotic strains for Salmonella populations, revealed by an in vitro colonic model of the child gut. *Microbiology*, *156*(11), 3342–3353. https://doi.org/10.1099/mic.0.042036-0

Zihler Berner, A., Fuentes, S., Dostal, A., Payne, A. N., Vazquez Gutierrez, P., Chassard, C., Grattepanche, F., de Vos, W. M., & Lacroix, C. (2013). Novel Polyfermentor Intestinal Model (PolyFermS) for Controlled Ecological Studies: Validation and Effect of pH. *PLoS ONE*, *8*(10), e77772. https://doi.org/10.1371/journal.pone.0077772