A novel probiotic for use in sleep disorders: preparation, *in vitro* and *in vivo* analysis of *Lactobacillus brevis* KS1 cells.

McGill

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December 2023

A thesis submitted to McGill University in fulfillment of the Experimental Medicine Masters of Science

To my father, whose love and leadership were indispensable in accomplishing this master's degree

To my brother and sister. For their guidance and encouragement.

To Philippine, whose unabating support maintained me in high spirits.

Para Yayo, Por las llamadas en las que discutíamos de ciencia que me mantenían motivado.

Lastly,

I dedicate this thesis to my mother, whose artful approach to science permeates through me, and spearheaded this thesis.

Thank you for instilling in me the love for Science.

Abstract

The gut microbiota – the trillions of bacteria that reside within the gastrointestinal (GI) tract – has generated significant attention for its impacts not only on GI health, but also on pivotal pathways of the enteric and central nervous system via the microbiome gut - brain axis [1]. In light of this, microbiota-based interventions may represent an effective therapeutic strategy for alleviating symptoms of both somatic and neurological pathologies. Although microbial agents have made scarce progress as standalone prophylactics, their documented impact on a myriad of diseases holds promise. Specifically, γ-aminobutyric acid (GABA)-producing lactic acid bacteria could serve as a preventative therapeutic for various ailments with both gut, and brain etiologies [2]. For instance, sleep disorders, often comorbid with GI diseases, are affected by changes in GABAergic neurotransmission disbalance [3]. Enhancing GI GABA levels through the supplementation of GABA-yielding bacteria could herald a localized production of the neurotransmitter. This, in turn, may lead to the restoration of GABA level homeostasis in individuals experiencing sleep disorders. In this study, fifteen bacterial strains isolated from cheese were screened for their GABA productivity. A strain of *Lactobacillus brevis* originating from a healthy nonagenarian gut, later referred to as *Lactobacillus brevis* KS1 had a 68.5% conversion rate of glutamate to GABA, or 14.13 g/L at pH 5.0, a maximum within the assay. Upon growth of this strain under physiological conditions in a duodenal *in vitro* simulated media, *L. brevis* KS1 maintained its high GABA productivity with a peak GABA yield of 9.44 g/L. Additionally, the effects of this lactic acid bacteria on two key sleep quality and efficiency biomarkers in *Drosophila melanogaster* were investigated. *Lactobacillus brevis* KS1 significantly reduced wakefulness after sleep onset time (WASO) and sleep onset latency time (SL) in comparison to controls. Moreover, *L. brevis* KS1 displayed comparable effects on nighttime sleep patterns as potent sleep-inducing benzodiazepine drug alprazolam (XANAX). In conclusion, these findings position *Lactobacillus brevis* KS1 as a promising alternative for individuals grappling with sleep disorders, circumventing the adverse effects associated with current medications. A comprehensive overview of its potential as a next-generation preventative sleep therapy will be presented.

Keywords: Lactic acid bacteria, GABA, gut-brain axis, neurotransmitter, sleep disorder, enteric nervous system, *Drosophila melanogaster*.

Résumé

Le microbiote intestinal - les billions de bactéries qui résident dans le tractus gastro-intestinal (GI) - a suscité beaucoup d'attention en raison de son impact non seulement sur la santé GI, mais aussi sur les voies essentielles du système nerveux central et entérique par l'intermédiaire de l'axe microbiome-intestin-cerveau [1]. À la lumière de cela, les interventions basées sur le microbiote peuvent représenter une stratégie thérapeutique efficace pour soulager les symptômes des pathologies somatiques et neurologiques. Bien que les agents microbiens aient fait peu de progrès en tant que prophylaxie autonome, leur impact documenté sur une myriade de maladies reste prometteur. Plus précisément, les bactéries lactiques productrices d'acide γ-aminobutyrique (GABA) pourraient servir de thérapie préventive pour diverses pathologies dont l'étiologie est à la fois intestinale et cérébrale [2]. Par exemple, les troubles du sommeil, souvent comorbides avec les maladies GI, sont affectés par des changements dans le déséquilibre de la neurotransmission GABAergique [3]. L'augmentation des niveaux de GABA dans l'intestin par la supplémentation de bactéries produisant du GABA pourrait annoncer une production localisée du neurotransmetteur. Cela pourrait contribuer à restaurer l'homéostasie du niveau de GABA chez les personnes souffrant de troubles du sommeil. Dans cette étude, quinze souches bactériennes isolées de trois fromages ont été examinées pour leur productivité en GABA. Une souche de *Lactobacillus brevis* provenant d'un intestin sain de nonagénaire, appelée par la suite *Lactobacillus brevis* KS1, présentait un taux de conversion du glutamate en GABA de 68,5 %, soit 14,13 g/L à un pH de 5,0, un maximum dans le cadre de l'essai. Lors de la croissance de cette souche dans un milieu duodénal simulé in vitro, *L. brevis* KS1 a maintenu sa productivité élevée en GABA avec un rendement maximal en GABA de 9,44 g/L. En outre, les effets de cette bactérie lactique sur deux biomarqueurs clés de la qualité et de l'efficacité du sommeil chez la Drosophile ont été étudiés. *Lactobacillus brevis* KS1 a significativement réduit le temps d'éveil après l'endormissement (WASO) et le temps de latence d'endormissement (SL) par rapport aux contrôles. De plus, *L. brevis* KS1 a montré des effets comparables sur les structures du

sommeil nocturne à ceux de l'alprazolam (XANAX), une benzodiazépine puissante qui induit le sommeil. En conclusion, ces résultats positionnent *Lactobacillus brevis* KS1 comme une alternative prometteuse pour les personnes aux prises avec des troubles du sommeil, en contournant les effets indésirables associés aux médicaments actuels. Une vue d'ensemble de son potentiel en tant que thérapie préventive du sommeil de nouvelle génération sera présentée.

Mots-clés : Bactéries lactiques, GABA, axe intestin-cerveau, neurotransmetteur, troubles du sommeil, système nerveux entérique, *Drosophila melanogaster*.

Acknowledgements

I would like to extend a sincere thank you to the distinguished members of this master's evaluating committee:

Dr. Thomas Ming Swi Chang, Dr. Rongtuan Lin, Dr. Veronique Bohbot, and Dr. Shirin Abbasi Nejad Enger.

Special thank you to Dr. Prakash for his support, assistance and constructive criticism throughout this master's.

To my fellow colleagues at the Biomedical Technology and Cell Therapy Research Laboratory. Rahul, Ahmed, Waqar, Jacqueline, Merry, Paromita and Sabrina.

This work is supported by the Natural Sciences and Engineering Research Council (NSERC) Canada discovery grant awarded to Prakash S. Boyajian JL is funded by the Fonds de Recherche du Québec – Santé (FRQS, grant 335999-2024). Schaly S is funded by the Canadian Graduate Scholarship-Doctoral from the Natural Sciences and Engineering Research Council (NSERC, 569661-2022). Arora K is funded by NSERC, Canada. The funders had no role in study design, data collection and analysis, interpretation of the data or writing of the manuscript.

Contribution of Authors

- I (Karan Arora) performed all experimentation in the data chapters.
- Design of the study was done by Dr. Satya Prakash and I (Karan Arora).
- Conceptualization of the thesis was done by Dr. Satya Prakash and I (Karan Arora).
- Conceptualization of figures was done by Dr. Satya Prakash, Rahul Thareja and I (Karan Arora).
- I (Karan Arora) prepared figures 1, 2, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 using GraphPad Prism or Biorender.
- Rahul Thareja prepared figures 3 and 4.

- I (Karan Arora) collected the data and performed data analysis for chapters 3, 4 and 5.
- I (Karan Arora) performed the writing of all sections of this manuscript-based thesis.
- I (Karan Arora) was responsible for formatting of this manuscript-based thesis.
- Jacqueline Boyajian and Sabrina Schaly provided assistance in microbiological and cell culture techniques.
- Rahul Thareja provided assistance in bioinformatical analysis and Drosophila food preparation techniques.
- Dr. Satya Prakash (mainly) and I (Karan Arora) contributed to funding acquisition.
- Dr. Satya Prakash provided crucial feedback and project oversight for the writing of this manuscript-based thesis.

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List of Abbreviations

5-HT: Serotonin
ATP: Adenosine triphosphate
BBB: Blood-brain barrier
BSH: Bile salt hydrolases
BZD: Benzodiazepine
CFU: Colony-forming unit
CNS: Central nervous system
DAM: Drosophila activity monitoring
EEC: Enteroendocrine cells
EEG: Electroencephalography
ENS: Enteric nervous system
GABA: γ-aminobutyric acid
GAD: Glutamic acid decarboxylase
GBA: Gut-brain axis
GI: Gastrointestinal
GRAS: Generally recognized as safe
HGP: Human genome project
HMO: Human milk oligosaccharides
HPA: Hypothalamic-pituitary-adrenal
LAB: Lactic acid bacteria
MDD: Major depressive disorder
MRS: De Man-Rogosa Sharpe
MSG: Monosodium glutamate
NTS: Nucleus tractus solitarii
REM: Rapid eye-movement
SCFA: Short-chain fatty-acids
SL: Sleep latency
TFLA: Triphala
TLC: Thin-layer chromatography
WASO: Wakefulness after sleep onset

Chapter 1: Introduction

1.1 Rationale for the studies

One of the human's most primal and vital characteristics has become a seldomly affordable luxury: sleep. Approximately 40% of Canadians experience acute symptoms and 10% must battle the enduring challenges of prolonged periods of inadequate sleep known as chronic insomnia [4]. This could be due to a plethora of contraindications. One of them being the omnipresence of medications that negatively impact sleep patterns, such as antibiotics, caffeine, and oral contraceptives. The widespread prevalence of insomnia has led to a surge in the use of sleeping drugs such as barbiturates and benzodiazepines (BZD), despite the well-documented adverse effects they may engender. Indeed, BZD users have reported REM-sleep rebound and daytime drowsiness as part of their day-to-day underlying struggles. Seeking alternatives, insomniacs turn to natural remedies, but their efficacy proves vastly inferior to that of BZDs. This thesis postulates a middle-ground treatment and/or a preventative therapeutic approach for managing insomnia.

1.2 Research hypothesis and specific objectives

This study hypothesizes that a bacterium derived from a healthy human's gut will efficiently produce GABA under physiological conditions of pH and temperature. Furthermore, oral treatment of the bacteria cells will posit beneficial effects on a Drosophila model of sleep. In order to test the hypothesis, the following specific research objectives are outlined:

1. To screen for bacteria that produce GABA under small-intestinal pH and temperature.
2. To identify the maximum GABA producing bacteria under small-intestinal pH and temperature.
3. To analyze *in vitro* the bacterium GABA production and viability in a simulated small intestinal environment.

4. To investigate the preclinical efficacy of orally delivered GABA producing bacteria on sleep in *Drosophila melanogaster*.

1.3 Novelty

No study has yet performed a quantitative comparative analysis of GABA production from a gut-isolated LAB versus its non-human counterparts, both at the producing enzyme's optimal pH and at physiological pH. Subsequently, this study is the first to characterize a gut-sourced LAB's GABA production in its hypothesized habitat: a simulated duodenal microenvironment. Lastly, although the neurotransmitter GABA has previously been investigated for its effect on sleep in the Drosophila *in vivo* model [2,3], this thesis uniquely evaluates the influence of GABA-producing bacteria administration on sleep architecture in Drosophila, and compares these effects to a marketed sleep-inducing medication.

Chapter 2: Background and review of literature

2.1 Introduction to the gut microbiota

2.1.1 Microbiome, gut microbiota and lactic acid bacteria: our microbial ecosystem

From the year 1990 to 2003, a 3-billion-dollar global study involving 20 different universities, changed the outlook of a myriad of professions. The Human Genome Project's (HGP) geneticists succeeded – for the first time in history – in sequencing the entire genetic makeup of the human organism. When HGP researchers first embarked in this journey, they estimated the human genome to have approximately 100,000 to 120,000 genes that coded for proteins. However, much to their astonishment, they would uncover only 20,500 unique protein-encoding genes [5,6], a genetic complexity comparable to that of the roundworm or fruit fly[7]. All this additional DNA, which they had initially posited to be non-functional or "junk" DNA, ended-up being a substantial revelation. As bioinformaticians uncovered the data resulting from the study, the large majority of the remaining protein-encoding genes belonged to the trillions of bacteria living on and inside us [8,9]. Once the entirety of this "junk" DNA was compiled, the human genome project researchers ultimately uncovered 360 times more bacterial genes than human genes, equating to a total of 8 million bacterially-derived protein-coding genes [10]. This microbial ecosystem is referred to as the microbiome.

Bacteria making up our oral and gut microbiota have developed a mutualistic relationship with the host by aiding in digestion of hitherto indigestible fibers such as inulin, oligosaccharides, and resistant starches [11]. On the other hand, gut-inhabiting microorganisms hydrolyze these ingested carbohydrate and protein sources to meet their own metabolic requirements. This symbiotic relationship is what has evolutionarily entrenched them in their host's gastrointestinal (GI) tract. When in homeostasis, the gut microbiota can posit a plethora of other beneficial assist the host by metabolizing fibers that would be indigestible otherwise, training the host's adaptive immune system through its interaction with regulatory T cells and dendritic cells [11], and its production of thousands of metabolites that cross the gut epithelial barrier, entering the bloodstream. Promoting the balance of beneficial bacteria in the human gut through anthropometric measures is

quintessential to the hosts' digestive, respiratory, hepatic, and immune health. Notwithstanding, when in a state of disbalance, the effect on the host can be equally detrimental. A plethora of ailments have been associated to a dysbiosed (dysregulated) gut microbiota, in large part due to the chronic state of inflammation that can incur following the adherence of pathogens and pathobionts to the gut lining (Figure 1).

This ecological balance is unique from individual to individual. Similar to the human fingerprint, each human has their unparalleled microbial signature, which, in turn, has become as vital as our genetic self.

2.1.2 Impact of gut bacteria on human health

Considering the gut microbiota is the main portals of entry for food components, commensal but also pathogenic bacteria, as well as viruses, it is primordial to maintain its homeostasis by promoting proper dietary habits for the host and for its symbionts.

As mentioned previously, gut bacteria have developed a symbiotic relationship with the host by assisting in digestion of non-digestible fibers. These include oligosaccharides such as fructo-oligosaccharide found in garlic and onions, nonstarch polysaccharides such as pectin commonly found in oranges and other citruses, and resistant starches [12,13]. Without this secondary metabolism resulting from intestinal bacteria, these indigestible fibers would greatly accelerate transit time of ingested food, and cause bloating, gas and abdominal discomfort symptoms to the host [13-15]. Additionally, micronutrient uptake would be hindered, and the full nutritional values of meals – independent of diet quality – would be insufficient. Another key undigestible compound metabolized by gut bacteria are human-milk-oligosaccharides (HMO). As is well known, human breast milk has evolutionarily been tailored to feed infants with a highly nutritive formula. A subset of oligosaccharides is omnipresent in all breast milk: HMOs. These complex sugars' only purpose is to feed specific bacteria in the infant's fragile, relatively-sterile gut, and thus, promote the seeding and subsequent proliferation of beneficial gut bacteria genera such as Bifidobacteria [16].

Gut microbiota agents also play a key role in protein and fat metabolism. Whether the amount of macronutrient ingested exceeds the rate of digestion, or harbors an inordinate structural complexity for primary digestion, gut commensals insure adequate absorption of the metabolized peptides and fatty acids. Cell membrane proteases hydrolyze the remaining undigested peptides, and the resulting amino acids can then be catabolized by the host into a plethora of intracellular processes. In fact, microbial metabolism of foodborne macronutrients is not the sole direct benefit to human health. Bacterial by-products resulting from carbohydrate and protein metabolism also posit equally beneficial effects to the host. Notably, tryptophan catabolism by commensal bacteria results in the production of the neurotransmitter serotonin and the powerful neuroplastic bioactive kynurenine [17,18]. One of the most touted by-product of gut bacteria are the short chain fatty acids (SCFA) acetate, butyrate and propionate. These metabolites primarily produced by large-intestinal

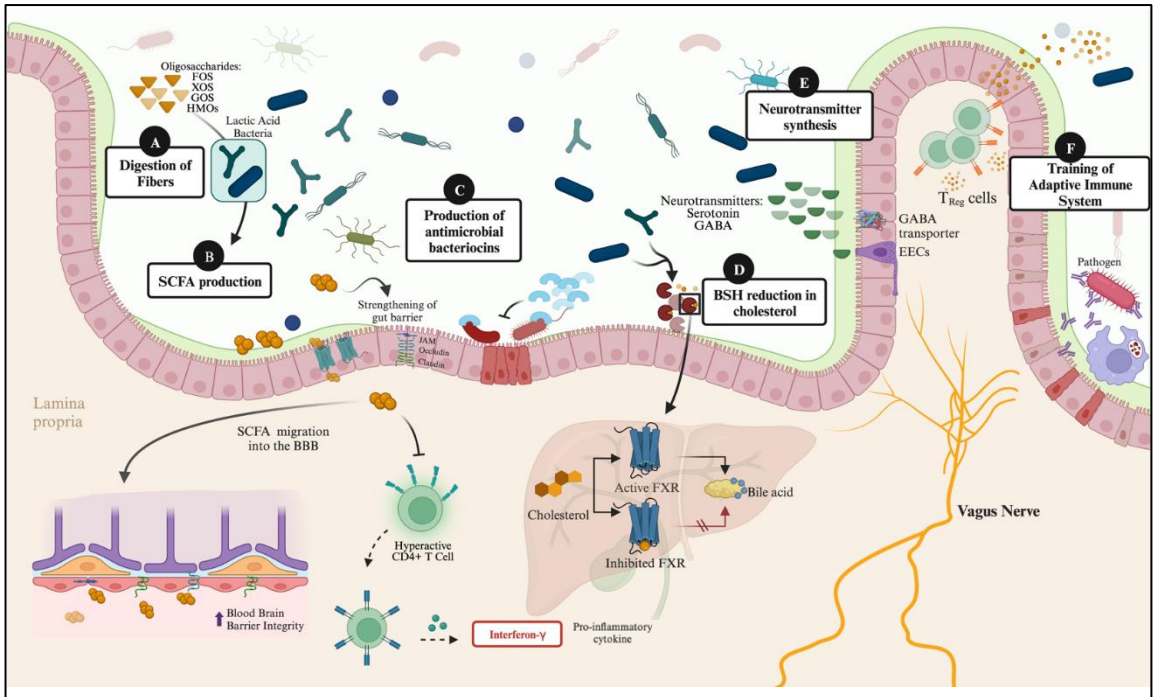

Figure 1: Gut microbiome and human health axis. Gut microbiome impacts human health through A. Digestion of indigestible fibers. B. Production of SCFA. C. Production of bacteriocins. D. Reduction in cholesterol. E. Serotonin, noradrenaline and GABA synthesis. F. Immunomodulation.

bacteria following ingestion of indigestible fibers induce myriad benefits on host immunity, energy supply, neuro-immunoendocrine health [19], cardiovascular health, and metabolic health [20]. Indeed, bacterial production of SCFA reduced recruitment of

neutrophils in intestinal crypts of inflammatory bowel disease patients [21]. Additionally, after administration of the SCFA butyrate, $CD4^+$ T cells were downregulated in the host [22], resulting in reduced production of Th1 cytokines such as interferon gamma and tumor-necrosis factor alpha, and thus, conferring an anti-inflammatory effect on the host [23]. Although exact mechanisms by which SCFA affect the host's brain health remain uncertain, a substantial body of evidence suggest that these gut microbiota-derived metabolites play a pivotal role on neurogenesis, microglia development and blood-brain barrier (BBB) integrity. Intraperitoneal injection of isotopic acetate in mice exhibited SCFA's ability to cross the BBB, and subsequently, to assist in production of various neuroactive compounds such as glutamine, glutamate and γ-amino butyric acid (GABA) [24]. Moreover, recolonization of germ-free mice with SCFA-producing bacteria resulted in an improved brain barrier integrity via the upregulation of tight junction claudins and occludin proteins [25]. Ultimately, but most notably, SCFA are prominent in literature for their function as an energy source for colon cells. SCFA represent 70-80% of colonocyte's energy supply [26]. Consequently, a reduction in luminal SCFA levels has been associated to a higher prevalence of colorectal cancer [26-28].

Other metabolic products of gut bacteria that have garnered significant academic attention include the production of bile salt hydrolases, antimicrobial bacteriocins, and the neurotransmitters serotonin and GABA. Bile salt hydrolases are bacterial enzymes that play a key role in lipid metabolism and more specifically, the assimilation of cholesterol via the hydrolysis of the amide bond of primary bile acids. Bile acids are synthesized in the liver from cholesterol via the farsenoid X receptor (FXR) [29]. Bacterially produced BSH indirectly promotes FXR signaling by cleaving its primary antagonist, tauro-beta-muricholic acid, and thus, promotes cholesterol breakdown and formation of primary bile acids in the liver [30,31]. The mechanisms by which gut bacteria produce neurotransmitters, and especially GABA will be introduced with greater depth in section 2.4.

2.2 Introduction to the gut-brain axis

2.2.1 Introduction to the enteric nervous system and the vagus nerve

Though initially the GI tract and nervous system may appear distinct organ systems, their metabolic and pathophysiologic interconnection has become academically undeniable. The GI tract supports the second largest nervous system in the body: the enteric nervous system (ENS) [32]. With the ability to coordinate GI motility, nociception, muscle contraction, pH regulation, enzyme secretion, mucus regeneration, an many more gut functions, the ENS orchestrates the frolic dance that is digestion with complete independence to the central nervous system (CNS) [33]. The ENS is composed of primarily afferent neurons receiving signals of GI chemical and mechanical stimuli. This is done by enteroendocrine cells (EEC) signaling through the production of peptide hormones such as glucagon-like peptide 1 (insulin secretion), ghrelin (appetite) and cholecystokinin (gastric emptying) [34]. EECs also play a role in GI neurotransmitter production. In fact, up to 95% of serotonin production occurs in the gut by a subset of EEC, enterochromaffin cells [35]. Gut serotonin is primarily produced to ensure adequate GI motility, however, other functions such as hepatic regeneration, and gut-brain communication have been hypothesized [36-38].

Various neurological disorders such as autism spectrum disorder (ASD), Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS) have gut comorbidities[33]. Conversely, conditions such as irritable bowel syndrome, Crohn's disease, and functional dyspepsia are frequently accompanied with high incidences of mood disorders such as depression, anxiety, and hypersensitivity [39]. These correlations substantiate the hypothesis that the ENS and CNS are intricately connected. One of the major highways between the GI tract and the CNS is the vagus nerve. This primary nerve in the parasympathetic nervous system is composed of 80% afferent, and 20% efferent innervations [40]. It is the main afferent axon joining the abdominal cavity to the CNS, sensing and communicating heart rate, respiration, digestion, and immune signals to the brain. The meandering nerve begins in the brain stem medulla oblongata, innervates a large majority of visceral organs, and culminates at the large intestine. For this reason, a myriad of recent research endeavours have undertaken the journey of elucidating the mechanisms

by which the vagus nerve could act as a communication channel between gut bacteria and the brain [41].

2.2.2 The bi-directional communication between the gut and the brain: The gut-brain axis

Prior to the discovery of the gut-brain axis (GBA) by Ebeid et al. in the late 1980s [42], a direct communication pathway between the gut and the brain could have been a concept considered science-fiction. Nonetheless, since then, over 8,000 individual studies exploring its potential mechanisms of action, therapeutic avenues, disease pathophysiology, and much more have burgeoned and bloomed from its field. The precise mechanisms for each pathway have not yet been fully elucidated. However, among the theories that have gained greater acceptance in literature, two main modalities have emerged in the realm of microbiota gut-brain communication.

The first one involves the immune system. Cell surface proteins and oligosaccharides such as toll-like receptor antigens and peptidoglycans on the bacterial cell envelope train the adaptive immune system to recognize self (safe), from non-self (pathogenic) bacteria [43]. However, this homeostatic relationship between the host's adaptive immune system and gut bacteria is quite delicate. Poor diet, antibiotics, overconsumption of alcohol or non-steroid anti-inflammatory drugs [44,45] can all substantially weaken the barrier between the gut and circulatory system. This is particularly detrimental since all ingested toxins, bacteria, virus, and many other pro-inflammatory agents have an easier access to systemic circulation and thus, the BBB. Although the BBB is a very selective barrier, the flooding of foreign bodies into the host's blood vessels will incur a chronic state of low-grade inflammation. This insidious condition has emerged as a prominent cause in the pathogenesis of many 21st century non-communicable diseases (or chronic diseases) such as Alzheimer's disease, Parkinson's disease, inflammatory bowel disease, type 1 and 2 diabetes, and obesity. [46-53]. Thus, working to ensure an adequate gut microbial balance that may competitively exclude pro-inflammatory pathogenic and pathobiont bacteria has been postulated as an indirect pathway for gut-brain impact.

The second proposed mode of action involves the gut microbiota' ability to produce metabolites. Examples of such metabolites are the neurotransmitters noradrenaline, dopamine, serotonin and GABA [54]. In fact, although both organisms are disparate karyotes, both gut bacteria and host share a similar endocrinological and neurotransmitter language i.e., endogenous microorganisms are capable of producing the same neurochemicals the human body uses to communicate [37]. Gut microbiota and human brain interact first through the ENS. Although the ENS has all the prerequisites to produce the neurotransmitters noradrenaline and dopamine, enteric neurons do not produce the enzyme capable of converting the produced noradrenaline into adrenaline. Host microbiota however, enzymatically convert noradrenaline into its active form [55].The mood stabilizer serotonin (5-HT) is another neurotransmitter whose production is gut microbiota-regulated. Bacteria can produce the serotonin precursor L-tryptophan, which is then metabolized into serotonin in enterochromaffin cells. They can also upregulate the activity of tryptophan hydroxylase-1 (TPH1), the rate-limiting enzyme for conversion of tryptophan to serotonin. Another

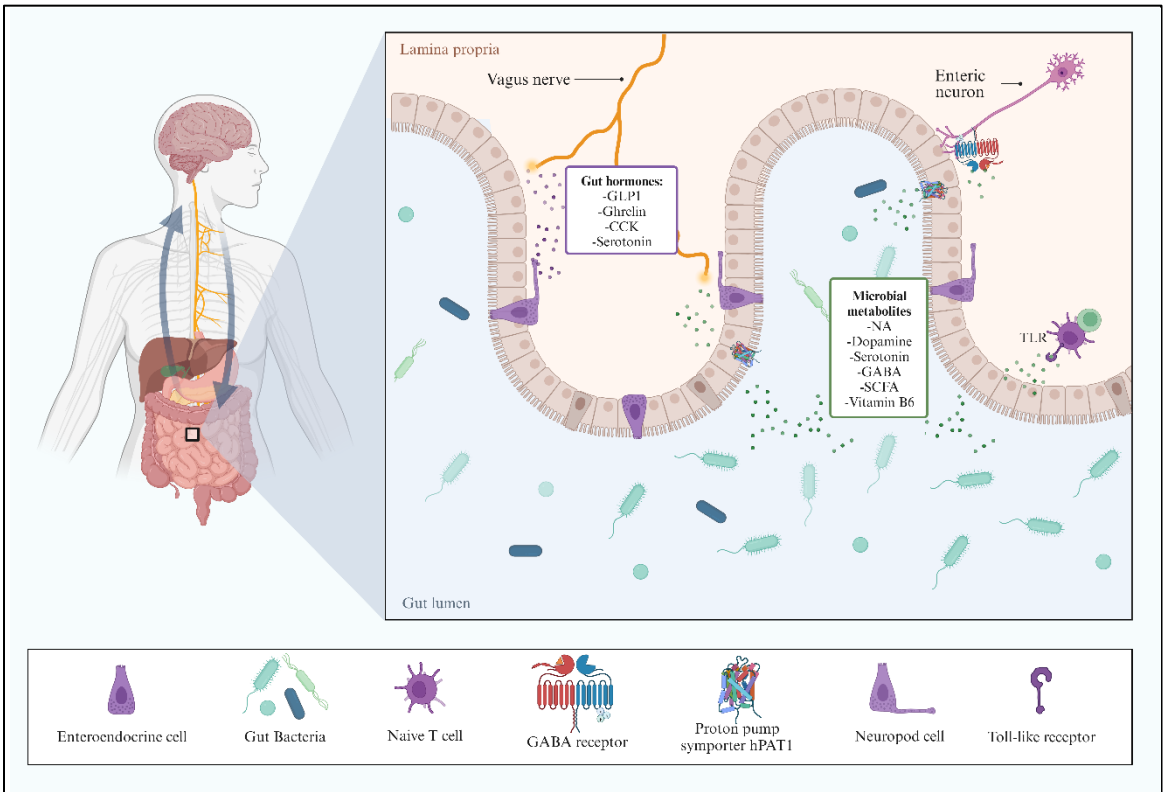

Figure 2: Mechanisms by which gut bacteria communicate with the brain via the gut-brain axis as described in section 2.2.2: Production of neurometabolites, interactions with toll-like receptors on T cells, interplay with vagus nerve.

noteworthy metabolic pathway by which beneficial bacteria can impact brain function is through the production of GABA. This neurotransmitter is a non-proteinic amino acid that is significantly involved in various somatic and neurological processes such as blood pressure regulation, mood regulation, relaxation, and sleep [56]. Being the main inhibitory neurotransmitter of the CNS, GABAergic transmission has often been at the forefront in tackling hyperarousal-related disorders through anxiolytic, and insomnia therapeutics [57-59]. Gut bacteria, and more specifically, the Bacteroides, Escherichia, and Lactobacilli genera have demonstrated the ability to produce GABA locally in the gut [60-62]. Additionally, bacteria such as *Bacteroides fragilis* and *Bifidobacterium longum* produce vitamin B_6, a key enzymatic co-factor in the catalysis of GABA in the brain [63]. Unphosphorylated vitamers of vitamin B_6 cross the blood brain-barrier [64,65], whose demand of vitamin B_6 is hundred times greater than the periphery [66]. Understanding the various mechanisms by which GI bacteria can influence central nervous system health and function is of paramount importance considering how interconnected both organ systems are. A specific focus of this thesis will be to investigate one particular pathway by which the gut may posit salubrious effects on the brain: the localized production of GABA by gut bacteria.

2.3 The somber reality of sleep disorders

2.3.1 Physiology of sleep and its most debilitating disorder: chronic insomnia

Two processes regulate sleep and wake periods: the chronobiological circadian cycle, and the homeostatic sleep drive. The former is an internal clock controlled by light and dark cycles, and the latter, often referred to as sleep debt, is generated by the progressive buildup of cerebral adenosine levels [67]. Adenosine is a by-product of the primary energy currency for all human cells: adenosine triphosphate (ATP). As neuronal cells undergo their day-to-day activities, ATP is consumed and adenosine builds up in the brain [68]. Neurons in the basal forebrain, a region responsible for wakefulness and arousal, is inhibited by adenosine [69,70]. Additionally, neurons responsible for sleepiness onset are disinhibited by adenosine agonists [71]. This interplay between activation of sleep-promoting brain regions and inhibition of wake-promoting brain regions is what orchestrates sleep every night. Human sleep begins in a state of drowsiness, once eyes are closed. Subsequently, neuron firing

rates experiences a decrescendo to approximately 8-13 Hz and light sleep begins [72]. After 1 to 5 minutes in light sleep, stage 2 (N2) sleep is initiated. At this stage, heart rate and body temperature drop, and neuronal firing is further decreased to 4-7 Hz [72] facilitating the transition to stages 3 and 4 (N3) deep sleep. During these latter stages, neural frequencies are dropped to its minima: 0.5 – 4 Hz, allowing the body to repair muscular injuries and assist the host's immune battles [73]. Following these 4 stages, which occur approximately during the first 90 minutes of sleep, the first episode of rapid-eye-movement (REM) sleep begins. Neuronal frequencies during REM-sleep resemble our awake-state, as it alternates between beta (12-35 Hz) and gamma (> 35Hz) action potential rates [71]. During this stage, memory consolidation, neurogenesis, learning retention, and dreaming takes place [74]. Sleep cycles occur in the following order: N1, N2, N3, N4, N2, REM, and can last approximately 100 - 110 minutes [75,76]. This cycle is then repeated until wakefulness, with REM stage duration increasing at every iteration [77].

In order for the brain to shift from one neuronal frequency to another, and thus, to orchestrate sleep, two things are required. Firstly, billions of neurons must be synchronized every night. Secondly, a sharp regulation of this same syntonization, granting the brain the ability to meander from one sleep stage to another. The complexity of sleep revolves around every night's unique frequency signature, since every day has its disparate level of physical activity, environmental factors, stress, and potential buildup of sleep debt.

Maintaining consistency in the organized chaos that is sleep architecture is bound to have fragility. Any alteration to this delicate homeostasis may lead to sleep disorders with varying severities. One in particular that has plagued human history since time immemorial is insomnia. In fact, sleep is a luxury, which many Canadians can't afford. Roughly 40% of Canadians must submit themselves to acute symptoms of insomnia, and a noteworthy 10% experience chronic insomnia [4,78]. Chronic insomnia is clinically diagnosed as a difficulty in maintaining sleep for a minimum of 3 times per week over 3 months [79]. Additionally, at least one daytime mood or fatigue affliction must be observed per week over the same time period for a complete assessment [79]. Although insomnia's pathophysiology remains incompletely understood, electroencephalography studies (EEG) have posited two key target biomarkers for sleep quality, which are now used by medical

practitioners to characterize the efficacy of their prescribed therapeutics [80-82]. These markers are (1) the time required for a person to fall asleep after lights are shut; sleep latency (SL), and (2) the time spent awake after having fallen asleep; wakefulness-after-sleep-onset (WASO) [81,83].

Considering the relatively high prevalence of insomnia and its unclear pathophysiology, several questions may serve as starting points for the development of novel therapeutic strategies. How does the brain regulate and synchronize these billions of neurons across distant brain regions? And most importantly, can this synchronization be assisted exogenously without the incumbent side-effects of current sleep-inducing drugs?

2.3.2 How GABA affects sleep circuitry in the brain

Synchronizing neurons at a given frequency allows for information to be transmitted with maximum efficiency, resulting in one continuous vector of equal brain states. As mentioned earlier, the first stage of initial non-REM sleep requires cortical oscillatory activity to decrease from 40 Hz (awake) to 8-13 Hz (light sleep) state. In order to reduce neuronal oscillatory frequency in sleep-regulating cortical networks, the brain coordinates GABA producing interneurons to fire synchronously. Once endogenous GABA binds to the surrounding neuron's GABAa receptor, an influx of chloride ions enters the cell and renders it dormant, as it is now hyperpolarized [84]. This is why GABA is referred to as the main inhibitory neurotransmitter of the brain, since it has the ability to inactivate brain regions at its disposal. Moreover, since the production of GABA occurs in synchrony at a given rate, the inhibition of particular brain regions can be tuned to that same frequency (Hz). That is the mechanism that coordinates neuronal frequencies and ultimately potentiates sleep.

Pharmacologists have grasped this mechanism and now target GABA receptors for their sleep-disorder related drugs. In fact, it was but a mere coincidence that the majority of current insomnia therapeutics were discovered. Benzodiazepines (BZD) and barbiturates were first explored as mood disorder drugs, but their sleep-promoting side-effects on humans were so prominent that the new class of therapeutic, hypnotics, emerged [85]. Albeit

their mechanism of action remained relatively undocumented, Mohler and Okada discovered in 1977 that BZD bound allosterically to GABAa receptors, thus correlating GABA to BZD administration, and ultimately, to sleep [86]. Mechanistically, BZD such as diazepam and alprazolam bind to α1 GABAa receptors' allosteric site and enhance the receptor's affinity for its neurotransmitter. Indeed, BZDs render GABAa receptors more reactive to GABA binding, and thus, allow for GABAergic inhibition of brain regions to occur at lower synaptic GABA concentrations. Administration of the sleep-inducing drug right before sleep onset thus reduces neuronal action potential firing rate at a physiologically lower level of brain circulating GABA. Nonetheless, natural healthy sleep requires the alternation of rapid eye movement (REM) sleep (35 Hz) and non-REM sleep (0.5 - 35 Hz). BZDs are potent in reducing neuronal frequency, yet are inefficient in regulating the non-REM – REM switching, due to their inability to increase neuronal frequency.

Considering how pervasive and entrenched insomnia and other sleep related deficiencies are in modern society, BZD hypnotic drugs have seen an unprecedented growth in usage rate [4]. Nevertheless, BZD's ubiquitousness is not proportional to its perfection. Therapeutic misuse from the patient, tolerance, rebound insomnia, poor adherence to treatment plan, and daytime sleepiness are all adverse effects well-known to the BZD user [87]. With little-to-no alternative, insomniacs resort to natural pathways such as herbal and psychosocial remedies, but their effectiveness is far from BZDs [88]. A middle-ground, therapeutic solution is what is postulated in this thesis.

2.4 GABA and lactic acid bacteria

2.4.1 Enzymatic production of GABA by lactic acid bacteria: its mechanism

Within the CNS, GABA is synthesized from its functionally-opposite biomolecule glutamate through the glutamate decarboxylase (GAD) enzyme. Interestingly, the same GABA-producing enzyme is produced by certain bacterial species. Particularly, lactic acid bacteria (LAB) have harbored the most attention in literature for their GABA productivity. LAB utilize GAD as an acid neutralization defense mechanism. Indeed, the bacterium

consumes an intracellular proton in order to convert extracellularly imported glutamate to GABA. The latter is then expelled from the cell via the GadC antiporter and a subsequent extracellular glutamate is internalized, which iterates the fore-mentioned reaction (see Equation 1 in Chapter 3) [89]. Several LAB have been purported to produce GABA, such as *Bacteroidetes fragilis* [60], *Lactobacillus rhamnosus* [1], *Bifidobacterium breve* [90], *Lactobacillus paracasei* [91,92], *Lactobacillus brevis* [2,93-97], and many more [98-106]. Notably, GABA-producing bacteria have been isolated from glutamate rich fermented foods such as cheese, kimchi, Chinese Pao Cai and fermented soybean [102,107-109].

Genes for GABA production in bacteria are optimally expressed under two conditions, at an acidic pH of approximately 4.5 – 5.5 [110], and when the bacterium is in the stationary growth phase (when exponential growth is halted, but metabolic activity remains prominent) [111]. This is where the brain-sourced GAD differs. Human brain GAD displays optimal enzyme kinetics at a pH of 6.8, which is coherent with its neuronal environment [112,113]. GABA production proficiency of a given bacterial strain depends on several factors. Firstly, the presence of its co-factor, vitamin B_6, greatly catalyzes the production of GABA through GAD. Secondly, optimal temperature for most LAB GAD is between 30 – 37 degrees Celsius [61]. Lastly, the concentration of surrounding glutamate is also a key factor for GABA production proficiency in LAB. Adding glutamate is not always beneficial to the enzyme however. In fact, LAB will experience increased osmotic pressure as a result of high surrounding glutamate concentrations, which will cause bacterial cells to burst, eradicating all metabolic activity of the bacteria [89].

In the gut, the full extent of the neurotransmitter's mechanism is yet to be elucidated. Few mechanistic studies posit a role in GI motility and intestinal fluid transport [114,115]. This is purported to occur through the interaction of microbial GABA with myenteric neurons across the gut epithelium, responsible for the postprandial peristaltic activity in the gut [116,117]. Moreover, oral supplementation of LAB that are known GABA-producers has shown promising results in academia for their analgesic, anxiolytic, and antidepressant effect in mice and human studies. For example, *Bifidobacterium breve* NCIMB8807 was genetically engineered to produce GABA, and when compared to its wild-type analogue, the mutated *B. breve* reduced sensitivity to visceral pain in rats [118]. Similarly,

supplementation of *Lactobacillus rhamnosus* JB-1 increased GABA, GABAa and GABAb receptor expression in the CNS of mice. This mechanism was found to be vagal-dependent, since, following vagotomy, beneficial effects on GABAergic activity were insubstantial [1]. In another study across the globe, a group of New-Zealand scientists lead by Dr. Slykerman investigated the effect of *L. rhamnosus* HN001 on postpartum depression in 423 women at 14-16 weeks gestation. Depression and anxiety-like symptoms were significantly reduced in the probiotic treatment group. The group postulated mechanism for the beneficial effects of the probiotic was GABA productivity [119]. Although this latter study involved human patients, a limitation encountered by research groups that hypothesize GABA-producing LAB as novel neurotherapeutics is their lack of translatability from mice to human trials. An example of such is the *L. rhamnosus* JB-1 follow-up clinical trial, which unfortunately did not engender significant effects on human mood, anxiety, stress or sleep scores [120].

Could the isolation of a GABA-producing LAB originating from the human gut serve as a solution to the translatability dilemma? A bacterium that has adapted to its human microenvironment and is capable of producing high levels of GABA under physiological conditions may help extrapolate animal studies to its anthropoid counterpart. The search and identification of GABA producing LAB, quantification of their metabolic proficiency under human small intestinal conditions, and effect on sleep quality and efficiency *in vivo* will be the primary objectives of this thesis.

Chapter 3: Isolation and identification of GABA producing lactic acid bacteria

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3.1 Abstract

Throughout the course of evolutionary development, bacteria residing within the GI tract have forged symbiotic associations with their respective hosts. In its essence, this mutualistic interaction between the gut and the gut microbiota originates from the latter's ability to digest complex fibers and produce myriad metabolites that can develop and maintain the host's immune system [1,2], promote anti-inflammatory responses [3,4], maintain the integrity of the gut epithelial barrier by producing bioactive compounds such as short-chain fatty acids [5,6], and promote the synthesis of neurotransmitters such as serotonin, noradrenaline, dopamine and gamma-aminobutyric acid (GABA) [7-10]. The enzyme accountable for the synthesis of GABA within human neuronal cells, glutamic acid decarboxylase (GAD) is the same enzyme present in gut bacteria. Consequently, the pursuit of a gut bacterium capable of producing GABA has garnered significant academic attention. Nonetheless, given the uncultivability of the majority of gut bacteria and their limited acknowledgement of safety by regulatory agencies, screening studies have been primarily done on lactic acid bacteria (LAB), including but not limited to Lactobacilli,

Lactococci, Streptococci, and Bifidobacteria. In this study, fifteen LAB strains were isolated from various cheeses and their GABA output capacity was qualified. Three strains of *Lactobacillus brevis* and two *Lactococcus lactis* were characterized as high GABA producers at GAD's optimal pH of 5.0. Furthermore, GABA-production ability was determined to be strain-specific, as different strains of *L. brevis* and *L. lactis* displayed disparate production profiles of GABA. Ultimately, when contrasting bacterial origins – by evaluating GABA productivity from a LAB of human gut origin versus its non-human-derived analogs – the *L. brevis* isolated from a healthy nonagenarian's stool exhibited a higher GABA production competence both at the enzyme's optimal pH of 5.0, and at a duodenal physiological pH of 7.0.

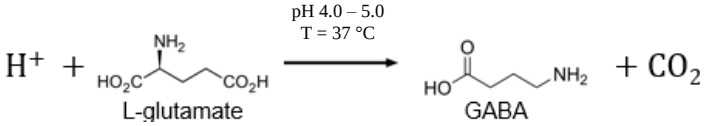
Keywords: Gamma-aminobutyric acid, lactic-acid bacteria, gut microbiota, fermented foods, microbial production, *Lactobacillus brevis*, *Lactococcus lactis*.

3.2 Introduction

The gastrointestinal (GI) tract harbors the largest microcosm of the human body. Coexistence of fungi, viruses, pathogenic, as well as beneficial bacteria, and gut eukaryotes make up what is known as the microbiome [11]. Of these favorable bacteria, over 10,000 different species work in consortium to engender myriad essential functions in the host, such as the digestion of undigestible oligosaccharides, sharpening of the adaptive immune system, and production of organic acids key in colonocyte energy catabolism [12]. Lactic acid bacteria (LAB) are a subset of the gut microbiota – the trillions of bacterial cells inhabiting the gut – which have colonized the human gut through deep-rooted host-microbial mutualism [13]. LAB are often part of probiotic supplementary agents as they are generally recognized as safe (GRAS) organisms that assist in fermentation of several fermented foods such as yogurt, kimchi, sauerkraut, sourdough bread, and cheese. On top of the other benefits mentioned above, some lactic-acid bacteria (LAB) have been characterized as psychobiotics i.e., bacteria that confer a beneficial effect on the host's nervous system [14]. Indeed, LAB play an important role in serotonin and γ-Aminobutyric acid (GABA) production in the gut. Although the exact function for the production of these neurotransmitters remains incompletely understood, the enzymatic pathways by which bacteria produce GABA and serotonin are identical to those seen in neuronal cells of the central nervous system (CNS) [15-17].

GABA is the primary inhibitory neurotransmitter of the human organism. A dysfunction in GABA receptor levels has been associated to several cognitive pathologies such as depression, anxiety, schizophrenia, and chronic insomnia. GABA can be biosynthesized by LAB via the glutamic acid decarboxylase (GAD) enzyme [18,19]. The latter is encoded by three genes, *gadA*, *gadB* and *gadC*. The former two are involved in the conversion of glutamic acid to GABA and the latter codes for an intracellular antiporter which internalizes one glutamic acid molecule and releases GABA extracellularly [19-21]. LAB produce GAD as an acid neutralization mechanism if their microenvironment renders too acidic. As hydrogen ions amass around the bacterial cell, the GadC transmembrane protein facilitates the entry of glutamate into the LAB's cytoplasm, which initiates the production of the GadA and GadB proteins. As per the following enzymatic reaction (Equation 1), an

intracellular hydrogen ion is consumed with the internalized glutamate to form GABA and carbon dioxide. The latter two are then transported into the bacteria's microenvironment and the process is iteratively performed until the cell's cytosolic pH is near-neutral. Consistent with the enzyme's function, its optimal pH ranges between 4.0 – 5.0 depending on the LAB.



Equation 1: Enzymatic reaction for GABA production in LAB.

Fermented foods are a common matrix in which GABA-producing LAB are isolated. Indeed, *L. rhamnosus, L. brevis, Lc. lactis, L. plantarum, L. paracasei, L. delbrueckii*, and many other prevalent LAB found in fermented foods have been characterized for their high proficiency in GABA production [22-37]. However, although several studies have followed up their excellent results with successful pre-clinical studies, their subsequent human clinical trials fail to correlate GABA productivity and any potential neuroactive benefits. At the source of these shortcomings could be the disparate environments in which GABA-producing bacteria are isolated. Several of these studies begin by searching for the LAB that maximally produce GABA at the enzyme's optimal condition, which is at a pH of 4.0 – 5.0 and a temperature of 37°C (for most LAB). However, the following study's research hypothesis posits that in order to maximize LAB GABA efficacy in humans, it is imperative to identify a bacterium capable of GABA production under physiological pH and temperature conditions. The search for said bacterium will be documented in this manuscript.

3.3 Materials and methods

3.3.1 Isolation of lactic acid bacteria from a cheese sample

An Italian unpasteurized sheep milk Pecorino Romano (8-month maturation), Québécois unpasteurized sheep milk cheese (4-6-month maturation), and a Spanish pasteurized sheep milk Manchego (2-month maturation) were selected as candidate cheeses for the isolation of GABA-producing LAB, since their medium-to-long maturation times would engender

a more conspicuous LAB growth than younger cheeses, while ensuring enough viability for subsequent plating and isolation. One gram of each cheese and 99 mL of peptone water was added to a Stomacher 400 circulator for homogenization at 230 rpm for 2 minutes. Once the cycle completed, 1 mL of the homogenized cheese sample was serially diluted to the dilutions 10E-3, 10E-5 and 10E-7. The three dilutions were then plated onto MRS agar by streaking 1 mL of sample onto 15 mL of pre-made solidified MRS agar petri dishes. All three cheeses were plated in duplicates onto lactobacilli selective media: MRS Agar (Fisher Scientific), and onto mesophilic selective media: Elliker (Sigma-Aldrich). The 36 plates were then incubated at 37 degrees Celsius in the case of MRS Agar and 30 degrees Celsius for Elliker plates.

3.3.2 Identification of lactic acid bacteria from a cheese sample

After 48 hours of growth, individual colonies were scraped using a sterilized inoculation loop and rehydrated into 100 mL of novel MRS broth media for its identification. After 24h rehydration, characterization of the isolated bacterium's genus and species was done using the API 50 CH test kit from Biomérieux. The bacterial pellet formed at the bottom of the rehydrated 100 mL bottle was mixed using a vortexer until the solution is opaque. 50 mL of sample was then transferred into a 50 mL falcon centrifuge tube and centrifuged at 2000 rpm for 15 minutes. Then, discard supernatant and resuspend cell pellet with 10 mL physiological saline water (0.85%) and recentrifuged at the pre-mentioned settings. Once the final centrifugation cycle finished, the cell pellet was resuspended in 2 mL saline and vortexed until homogeneous. As per the API 50 CH identification kit procedure, a McFarland turbidity standard of 2 was obtained by gently adding droplets of this resuspended solution into a new 5 mL saline water solution [38]. 100 microliters of this novel solution were added to the ready-made API 50 CH reagent mixture and all cupola were filled to half. 150 microliters of autoclaved paraffin oil were then added in order to maintain anaerobiosis within the reacting cupola and the API 50 CH plate was incubated at 37 degrees Celsius for 48h (Supplementary figure S1). This process was done for all 15 colonies identified from the three cheeses. Each cupola represents a carbohydrate to be metabolized, and depending on the carbohydrate metabolic profile of the isolated bacteria,

the kit would provide a confidence % for its speculated genus and species. Analysis of carbohydrate metabolic profile was performed using the API 50 CH software APIWeb from Biomérieux.

3.3.3 Qualification of GABA productivity using a pH enzymatic method

In order to mass screen for GABA producers, an adapted version of Yang et al. colorimetric method was utilized. In fact, instead of colorimetry, the inherent alkalizing property of the GAD enzyme was utilized for qualification of GABA production. *Lactobacillus delbrueckii, Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus brevis*, and *Lactococcus lactis* isolated from the cheese samples were cultured overnight and centrifuged at 4000g for 15 minutes. Their cell pellets were washed using 50 mL of physiological saline water (0.85%), homogenized, and re-centrifuged at the fore-mentioned settings. Supernatant was then discarded and 5mL of a 200 mM monosodium glutamate (MSG), as well as 0.1 mM Tween 80 solution was added to the cell pellet. The following solution was then incubated in a 37 degrees Celsius water bath and iCinac pH monitoring probes were added to the test sample. pH values were measured every 4 minutes and the test was stopped after 25 hours. If a substantial pH increase over time was observed in the assay, the tested bacterium would qualify as a GABA producer.

3.3.4 Isolation of lactic acid bacteria from a human sample

For the isolation of LAB in human stool samples, a similar method to Demirok et al. [39] was utilized. 1 gram from a stool single-use vial (Thermo Scientific) was added to 99 mL peptone water (0.1% peptone and 0.85% NaCl; 3M) immediately. Then, the solution was homogenized using a Stomacher 400 circulator. The homogenous solution is then serially diluted at 10E-7, 10E-9 and 10E-11 colony forming units (CFU), and 1 mL of sample was streak-plated in duplicate onto Lactobacilli selective MRS agar and Lactococcal selective Elliker agar. Anaerobic incubation for 48h at 37 degrees Celsius and 30 degrees Celsius for both medias respectively was performed. Finally, similar to above, the API 50 CH test

was used to identify the individual colonies. The process was performed iteratively until either candidate LAB, *L. brevis* or *Lc. lactis,* was identified.

3.3.5 Identification of enzymatic pathway for GABA production using thin-layer chromatography

In order to confirm the production of GABA from MSG by LAB, an adapted version of the thin-layer chromatography (TLC) method of Tanamool et al. was utilized [40,41]. Two controls: (1) 1% GABA and (2) 1% MSG, as well as three test samples: (3) supernatant of *L. brevis* in MRS, (4) *L. helveticus* and (5) supernatant of *L. brevis* in 1% MSG were spotted onto aluminum TLC plates (Sigma-Aldrich). Culture supernatant was obtained by centrifuging the overnight *L. brevis*/*L. helveticus* culture at 4000g for 10 minutes (samples 3 and 4). Subsequently, 1% MSG solution was added to the cell pellet at a 1:1 volume: weight ratio, and left to incubate for 12h, prior to retrieval of supernatant (sample 5). Two microliters of each sample were spotted onto the TLC plate and placed in an acetic acid: n-butanol: distilled water (4:1:1) mobile phase. After successful migration of the mobile phase, and marking of maximum travelled distance, the plate was sprayed with 1% ninhydrin solution and added to an oven pre-heated at 80 degrees Celsius. 5 minutes later, the plate was retrieved from the oven and the red hue spots were marked using a graphite pencil. Rf values of each sample was then calculated and noted.

3.4 Results and discussion

3.4.1 LAB isolation from the GABA-rich food source: cheese

Although our main hypothesis for optimized GABA production in humans revolved around the isolation of a human-sourced LAB, a screening method for rapid identification of GABA producing LAB had to be established. To achieve this, it was necessary to initially isolate as many LAB as possible from a classical source of lactic-acid producing bacteria: cheese. By performing the method 3.3.1 iteratively, fifteen different colonies were isolated, resulting in 8 unique bacterial species: see Table 1.

Code	Incubation conditions	Taxon Identification	Confidence (%)
A31	37 °C Anaerobiosis	*Lactobacillus delbruecki ssp. Lactis*	95.8%
A32	37 °C Anaerobiosis	*Lactobacillus acidophilus*	95.7%
A33	37 °C Anaerobiosis	*Lactobacillus rhamnosus*	99.7%
A31M	30 °C Anaerobiosis	*Lactococcus lactis ssp. Lactis*	97.9%
B51	37 °C Anaerobiosis	*Lactobacillus paracasei*	99.6%
B52	37 °C Anaerobiosis	*Lactobacillus paracasei*	99.6%
B53	37 °C Anaerobiosis	*Lactobacillus brevis*	99.7%
B54	37 °C Anaerobiosis	*Lactobacillus brevis*	99.7%
B55	37 °C Anaerobiosis	*Lactobacillus paracasei*	99.6%
B56	37 °C Anaerobiosis	*Lactobacillus paracasei*	99.6%
B57	37 °C Anaerobiosis	*Lactobacillus paracasei*	99.6%
B31M	30 °C Anaerobiosis	*Lactococcus lactis ssp. lactis*	64.2%
B32M	30 °C Anaerobiosis	*Lactococcus lactis ssp. lactis*	92.3%
C31	37 °C Anaerobiosis	*Lactobacillus paracasei*	91.2%
C31T	40 °C Anaerobiosis	*Streptococcus salivarius ssp. thermophilus*	50.2%

Table 1: Isolated LAB strains from cheese. Results of API CH50 Taxon Identification Kit.

3.4.2 Screening for GABA production and qualification of proficiency from isolated LAB

There were several methods that had been used in literature for the screening of GABA production, but the one that worked best for our application, was an adapted version of the simple and cost-efficient method of [42], in which GABA productivity was qualitatively determined using a colorimetry. For our intents and purposes, the method was adapted from a colorimetric, yes/no method to a semi-quantitative method. We utilized the inherent

property of the GABA producing enzyme – GAD – whereby its activity is directly proportional to the deacidification (thus alkalinization) rate of its environment. Indeed, the rate of pH rise in the solution containing LAB and 2% monosodium glutamate (MSG) is directly proportional to the enzyme's activity and thus, GABA production. A positive hit to our screening (GABA producing LAB) would see its solution's pH increase over time, as the reaction is shifting towards the products side (see equation 1), decreasing the

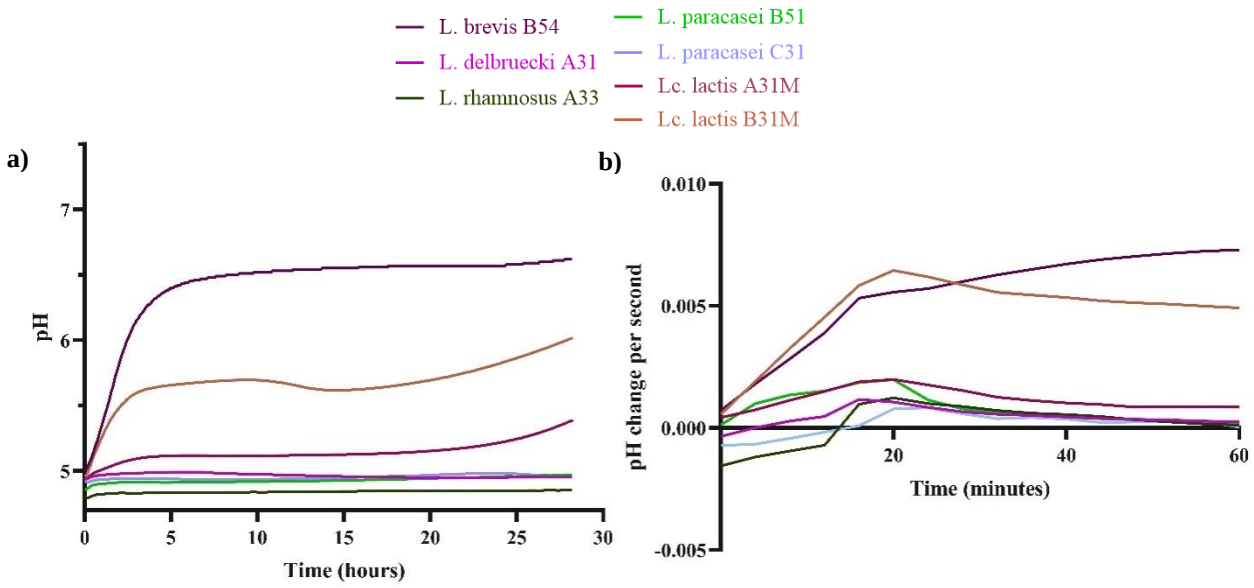


Figure 3: Two strains of *Lc. lactis* and one strain of *L. brevis* are identified as potential GABA producing bacteria. a) Qualitative GABA producers rise the solutions pH as a by-product of GAD enzymatic activity when subjected to MSG b) Rate of GABA production illustrated by pH change per second.

concentration of both the hydrogen ions and the glutamate and increasing the concentration of GABA in the sample. The same can be said for our negative hits. If the pH does not change over time, we can infer that the glutamate decarboxylase enzyme was not active, and therefore, those LAB are not GABA producers. Although this doesn't result in exact quantitative measurements of GABA yield, we can then extrapolate which strains should be targeted in humans for maximum localized GABA production. GABA productivity was determined using the iCinac fermentation pH monitor, collecting the solution's pH every 4 minutes. Out of the 15 bacterial strains isolated from the cheeses, two species in particular were considered good producers of GABA: *Lactobacillus brevis* and *Lactococcus lactis* (Figure 3).

Out of the previously isolated and identified strains, only the two strains of *Lc. lactis* and the strain *of L. brevis* were selected for further experiments. However, considering the high prevalence of *L. brevis* and *Lc. lactis* as GABA producers both in this experiment and in literature, the strain specificity of GABA production should be investigated. This would identify if *L. brevis* and *Lc. lactis* strains could then be isolated from a human source and should be expected to exhibit similar properties.

3.4.3 Determination of strain specificity for GABA production

Isolated *L. brevis* and *L. lactis* were then compared to two *L. brevis* and one *L. lactis* in the laboratory's strain bank to determine if their GABA production followed a similar GAD enzymatic profile. As can be seen in Figure 4, different bacterial strains within the same species produce GABA with different proficiencies.

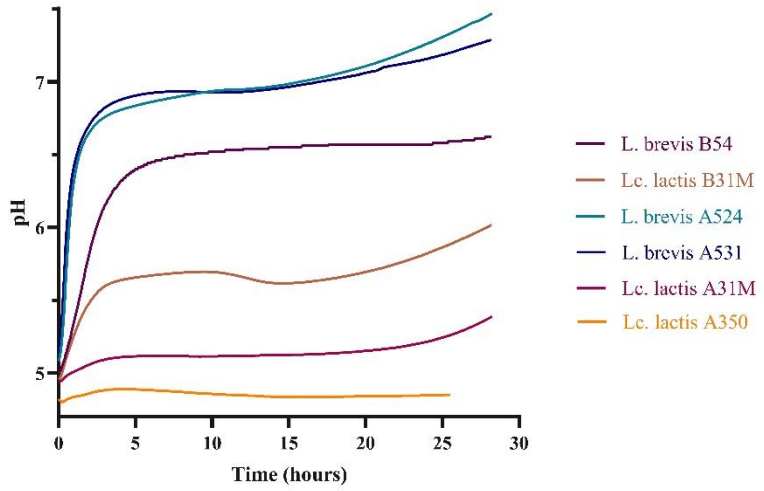


Figure 4: Production of GABA is a strain specific property. *L. brevis* B54, A524 and A531 have different GABA production profiles. *Lc. lactis* B31M, A31M and A350 also have disparate GABA proficiencies.

This finding suggests that some *L. brevis or Lc. lactis* strains' enzymatic activity may behave differently under a unique set of environmental conditions. It is coherent with the function of their GAD enzyme. LAB promote the production of GAD in order to maintain a viable cytoplasmic pH of 7.0 [43]. If said GAD-generating bacteria is isolated from a slightly acidic matrix such as cheese (ranging at pHs of 5.1 – 6.5) [44], due to evolutionary

pressure, it should have improved its GAD activity (thus GABA productivity) at low pHs. For our purpose, a particular *L. brevis or Lc. lactis* may be better suited for production under gut physiological conditions instead of GAD's optimal low pH, high temperature conditions [45-47].

3.4.4 Qualification of GABA productivity at optimal and physiological pH of a human-sourced *Lactobacillus brevis* compared to its non-human analogues

In order to test the hypothesis that a gut-derived high GABA producing species produces the neurotransmitter at an improved rate versus its non-human counterparts, a *L. brevis* or *Lc. lactis* from a human donor had to be isolated. To maximize our chances of isolating a LAB, a healthy individual with a regular diet was selected (See supplementary material S2 for consent form). After plating the stool sample onto Lactobacillus or Lactococcal selective agar media, three species were isolated and identified using the API 50 CH kit: Pediococcus parvulus, *Lactobacillus paracasei*, and one of our candidates: *Lactobacillus brevis*. This is no coincidence, as *L. brevis* is found in a myriad of food such as cheese, sourdough bread, pickles, yogurt and cocoa [48], and the selected nonagenarian is a regular consumer of the first two.

A comparison of the GAD activity of the human isolate L brevis with three non-human derived isolates L brevis was then undertaken. The reaction occurred in a 2% MSG solution at the enzyme's documented optimal pH of 5.0 (Figure 5a). Negative control was performed by a previously isolated non-GABA producing bacterium, L. acidophilus. In contrast to the other three L brevis isolates, the human isolate sample exhibited an increase in pH that was sustained at near-neutral pHs. Additionally, its peak rate of alkalinization (GABA productivity) was 55% greater than the second most prolific *L. brevis* (Figure 5b).

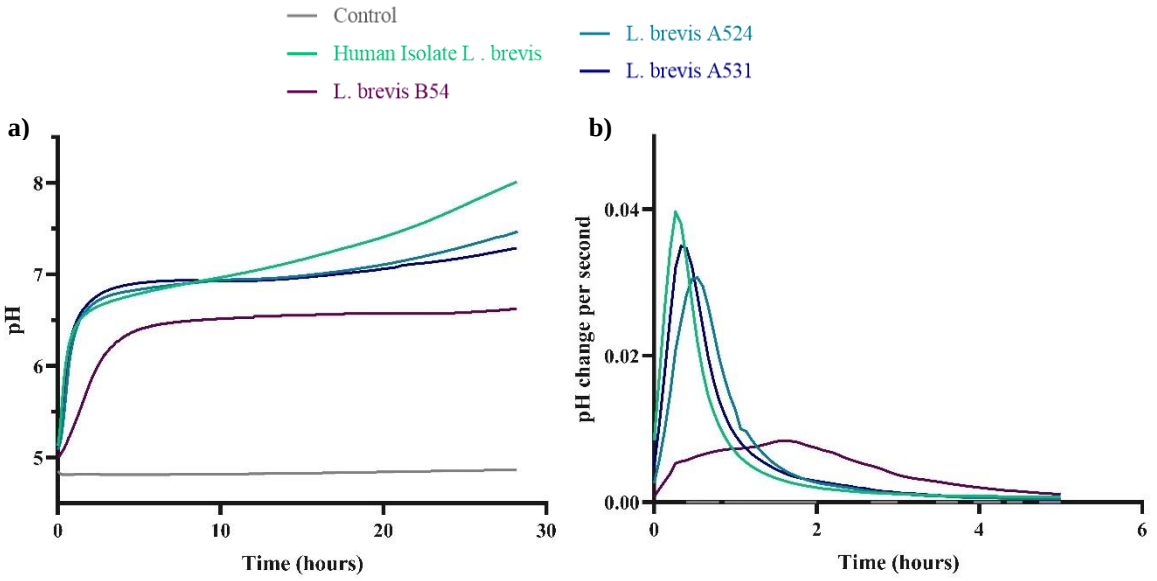


Figure 5: A human GI-isolated *L. brevis* is isolated and identified as a potentially potent GABA producer. a) Qualitative GABA producers see a rise in pH due to GAD activity after incubation with 2% MSG b) Rate of GABA production exhibited by pH change per second.

A considerable alkalinisation rate was also observed at a pH of 7.8, positing a potential secondary favorable pH for GABA production in the human isolate LAB (Figure 6a). This

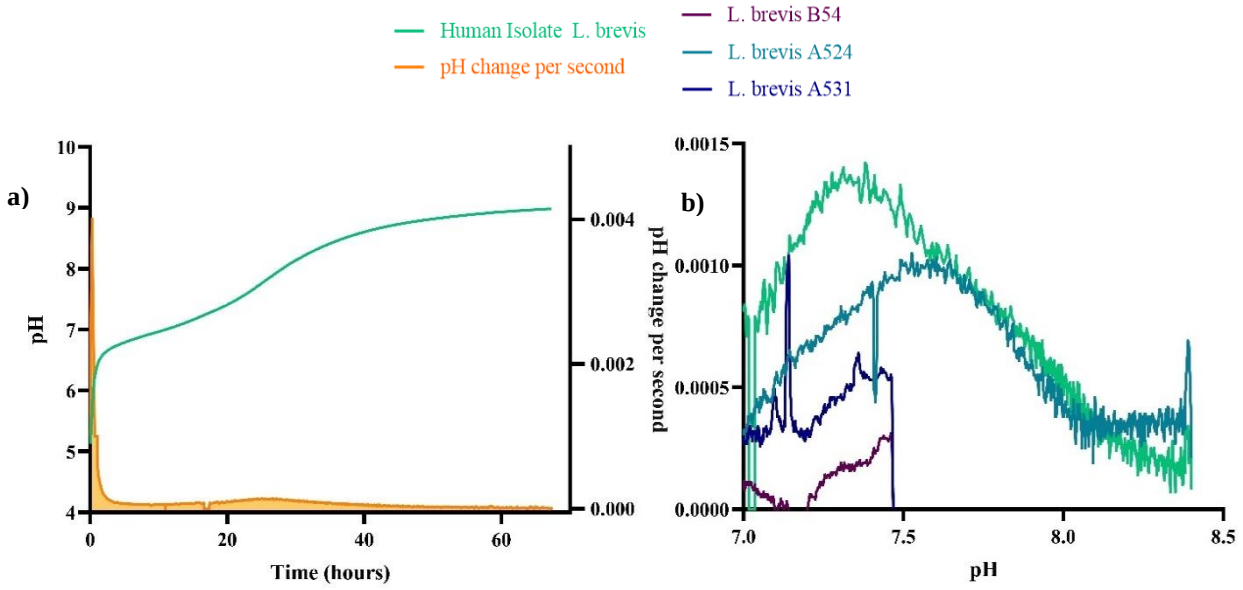


Figure 6: A human GI-isolated *L. brevis* exhibits a potential secondary peak of GABA production at pH 7.4. a) Human isolate *L. brevis* qualitative GABA production as a pH increase from GAD activity versus time b) rate of GABA production at near-neutral pH of the human isolate *L. brevis* compared to other *L. brevis* B54, A524 and A531.

secondary local maximum for GABA productivity was not as pronounced in other *L. brevis* (Figure 6b). Indeed, from pHs 7.0 – 7.4, not only did the human *L. brevis* have a higher rate of GABA production than its analogues, but this rate was sustained at even higher pHs, and thus, even lower concentrations of hydrogen ions.

Prior to confirming this study's primary hypothesis, GABA productivity has to be confirmed, and quantified. Although quantification is to be determined in the subsequent paper, confirming the presence of GABA following GAD activity was performed using thin-layer chromatography (TLC).

3.4.5 Confirmation of GABA production from *Lactobacillus brevis* from human isolate using thin-layer chromatography

Following the method of [49], GABA was confirmed to be present substantially in the control sample, the *L. brevis* in an MSG sample, and in the *L. brevis* in MSG and MRS sample, with an approximate Rf values of 0.42 (Supplementary Figure S3). However, a slight identification of GABA was observed on the weak producer line (*L. helveticus*), indicating that not only was *L. brevis* a producer of GABA, but it has a higher proficiency as well. Finally, the MRS and MSG sample proves that the GABA TLC signal is not due to any other component in the bacterium's initial growth media, as those spots are washed away during centrifugation and rehydration in MSG. Ultimately, these results demonstrate that GABA production is a strain-specific feature, whereby human stool-isolated bacterial strains may have adapted to their less acidic microenvironment and tailored their metabolic proficiency consequently.

3.5 Conclusion

LAB production of GABA is no novel discovery. Myriad studies describe how to optimally produce GABA through microorganism fermentation. However, translatability of these findings from murine to human models are complex when administrating GABA producing LAB as therapeutic agents. The avenue of isolating a human-sourced LAB, which may have the host's GI tract as microenvironment, posits a novel approach that could aid in

result translatability. Indeed, duodenal pH conditions are quite different from murine to humans, ranging from a pH of 4.74 (fasted) and 4.87 (fed) to 5.5 (fasted) and 7.0 (fed) [50,51]. Considering the significant pH dependency of the GABA producing mechanism in LAB, attempting to isolate a gut-derived LAB that maintains its GABA proficiency at higher pH may be a step in the right direction for its use as an adjuvant therapeutic for ailments who's GABAergic signaling is deficient. Further studies quantitating GABA production, and determining the bacterium's effect *in vivo* will be required prior to confirming the hypothesis.

Supplementary Material

Supplementary material S1: Example of a API 50CH identification assay from Biomérieux

Supplementary material S2: Letter of consent for fecal sample analysis and gut bacterium isolation from nonagenarian

Supplementary material S3: TLC confirmation of GABA production from the human isolated *L. brevis*.

Author Contributions

KA and SP: conceptualization. KA: writing of original draft. KA: data curation and visualization. SP: funding acquisition, project administration, resources, and validation. KA, JB, SC, RT: scientific experiments. KA and RT: software and figures. All authors contributed to the article and approved the submitted version.

Funding

This work is supported by the Natural Sciences and Engineering Research Council (NSERC) Canada discovery grant awarded to Prakash S. Boyajian JL is funded by the Fonds de Recherche du Québec – Santé (FRQS, grant 335999-2024). Schaly S is funded by the Canadian Graduate Scholarship-Doctoral from the Natural Sciences and

Engineering Research Council (NSERC, 569661-2022). Arora K is funded by NSERC, Canada. The funders had no role in study design, data collection and analysis, interpretation of the data or writing of the manuscript.

Preface to the following chapter

The previous manuscript described the methods and outcomes stemming from the isolation and identification of *L. brevis* KS1, a gut-sourced GABA-producing bacterium. The subsequent logical progression to substantiate this thesis' hypothesis, stating that a human-derived bacterium will display an improved GABA proficiency under physiological conditions, involves quantifying both bacterial growth and GABA production of the strain in its primary host habitat, the duodenum.

In Chapter 4, the quantification of GABA production of *L. brevis* KS1 is determined at the GABA producing enzyme's optimal pH of 5.0 and at a duodenal-like pH of 7.0. This GABA production ability is then contrasted with prominent GABA-producing LAB mentioned in existing literature, such as *L. rhamnosus*, *L. helveticus* and *L. paracasei*, serving as a barometer to *L. brevis* KS1. Moreover, this comparison will also serve as a benchmark for evaluating the strain's potential as a therapeutic agent for brain disorders, given the documented success of the three comparative bacterial strains in mice and/or human models of depression and anxiety.

However, pH does not fully encompass a duodenal-like environment. Indeed, factors such as temperature, carbohydrate, proteinic, vitaminic, enzymatic, and bile acid levels are key determinants influencing a bacterial strain's metabolic proficiency. As a result, *L. brevis* KS1 will be incubated in a simulated gut environment to evaluate its GABA production under simulated physiological conditions. Upon quantifying its growth and GABA production in a simulated culture medium, whose pH, temperature and nutrients levels mimic the upper GI tract, this thesis' hypothesis will be further substantiated.

The following chapter provides corroborative data indicating that the human-isolated *L. brevis* KS1 could have potential in GABAergic neurotransmission-related disorders.

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Chapter 4: Quantification and *in vitro* analysis of GABA production from human-gut derived *Lactobacillus brevis* KS1 under small intestinal temperature, pH, and nutrient conditions

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4.1 Abstract

The gut microbial ecosystem residing the gastrointestinal (GI) tract has garnered considerable attention for its impact on not only GI health, but also on essential pathways of the enteric and central nervous systems [1]. Responsible for these beneficial, or detrimental effects on the brain, is the microbiota brain-gut bidirectional communication pathway, discovered in 1897 by the Nobel prize winner Ivan Pavlov [2,3]. Bearing these pointers in mind, alterations to the GI microenvironment presents itself as a potentially effective therapeutic strategy for mitigating symptoms associated with both somatic and neurological pathologies. However, despite the theoretical promise of microbial agents as therapeutics due to their documented impact on a multitude of diseases, its success from pre-clinical trials to clinical trials is seldom translational. One metabolic property of LAB that has garnered significant academic attention, but has ultimately proven insufficient as a therapeutic intervention is their production of γ-aminobutyric acid (GABA).

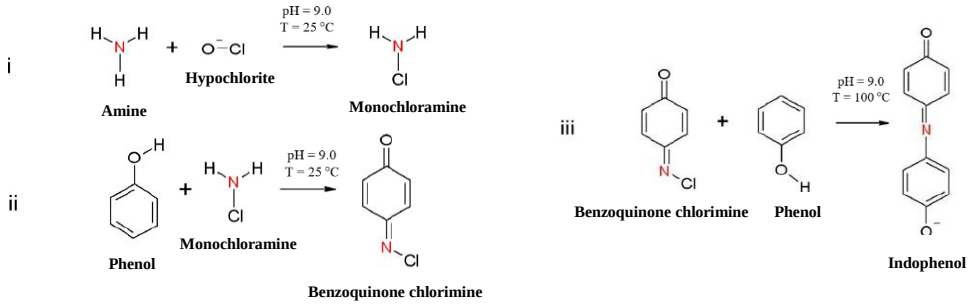
In light of this, the study's objective was to discern which lactic acid bacteria (LAB) could herald the highest potential as a psychotropic by proposing a paradigm shift compared to similar studies: LAB GABA proficiency had to be at a maximum under human GI conditions of pH, temperature and nutriment levels. The GABA production efficiency of well-documented GABA-producers, including *Lactobacillus rhamnosus*, *Lactobacillus helveticus*, *Lactobacillus paracasei*, and *Lactobacillus brevis*, was quantified and compared to that of the human-sourced *Lactobacillus brevis* KS1. This quantitative assay was conducted at the optimal pH for the catalytic reaction, as well as at physiologically-relevant pH levels of 5.0 and 7.0, respectively. The bacterium with the highest GABA productivity at pH 7.0 was then tested for its viability and metabolic proficiency under simulated duodenal-like conditions in an adapted culture media. *Lactobacillus brevis* KS1 of human origin, exhibited the highest GABA productivity at pH 7.0 with a 66.3% conversion rate of glutamate to GABA, equating to 13.67 g/L. Upon cultivation of this strain in the duodenal *in vitro* model media, the bacterium was capable of maintaining its high metabolic activity under physiological conditions resulting in an approximate yield of 9.44 g/L. Additionally, *L. brevis* KS1 also ensured its growth to a maximum of 498 million CFU/mL. This study will set the foundation for future studies aiming to determine whether the candidate *L. brevis* KSI high GABA productivity initially translates to GABAergic-related sleep disorders in animal and/or invertebrate models and ultimately, in humans.

Keywords: Gamma-aminobutyric acid, lactic-acid bacteria, gut microbiota, *Lactobacillus brevis*, gut-brain axis, simulated GI model, neurotransmitter production, vagus nerve.

4.2 Introduction

The neurotransmitter γ-amino butyric acid (GABA) will mark, in 2025, its 75th anniversary since its discovery by Eugene Roberts and Sam Frankel [4]. Interestingly, seven years following Roberts and Frankel's paper, the inhibitory function of GABA was resolved by a cohort of McGill University researchers from the Montreal Neurologic Institute [5]. Since these relatively recent papers, over 60 distinct GABAergic drugs have been developed which in turn facilitate the lives of individuals that live with anxiety, depression, panic disorders, insomnia, seizures, alcohol withdrawal, and muscle spasticity from cerebral palsy [6,7]. Although GABA is primarily produced by CNS presynaptic neurons from glutamate via the glutamic acid decarboxylase (GAD) enzyme, gut bacteria have also generated recent academic attention for their GABA-producing abilities (citations here). In fact, production of GABA by gut bacteria occurs through the same enzymatic pathway. GAD activity in bacteria is heavily pH and temperature dependent, as the microbial enzyme's primary function is to de-acidify the bacterium's acidic cytoplasmic space when in a low pH microenvironment. At pH 4.0 - 5.0, microbial GAD operates optimally [8]. Lactic acid bacteria (LAB) are among the most prominent bacteria that efficiently produce GABA at this pH. In the above-cited mass-screening quantitative research papers, a single LAB strain is typically identified as producing a prodigious amount of GABA. This LAB is then further investigated for beneficial effects on the host, whether they be gut or CNS-related. However, in this study, a shift in focus is suggested. In searching for a potent GABA producer, emphasis will be placed on the bacterium's ability to produce GABA under physiological conditions. As such, the medium in which GABA productivity will be quantitated shall simulate a fed-state small-intestinal polysaccharidic, proteinic, and vitaminic, and enzymatic level, while at a temperature of 37 degrees Celsius, and a pH of 7.0 [9,10].

A spectrophotometric method following the Berthelot colorimetric reaction of amines will be employed for the quantitation of GABA production. Berthelot's reaction consists of three steps (Equation 2). In the first step, GABA's amine group reacts with hypochlorite to form monochloramine at pH 9.0. Secondly, monochloramine reacts with a phenol to form benzoquinone chlorimine. Lastly, indophenol is formed by reacting the latter with a second phenol. This produces a dark blue dye from the originally green phenol. The more amine groups there are on the molecule of interest, and thus, the higher its concentration, the more red-light is absorbed by the blue dye, whose maximum absorption peak is at 630nm.

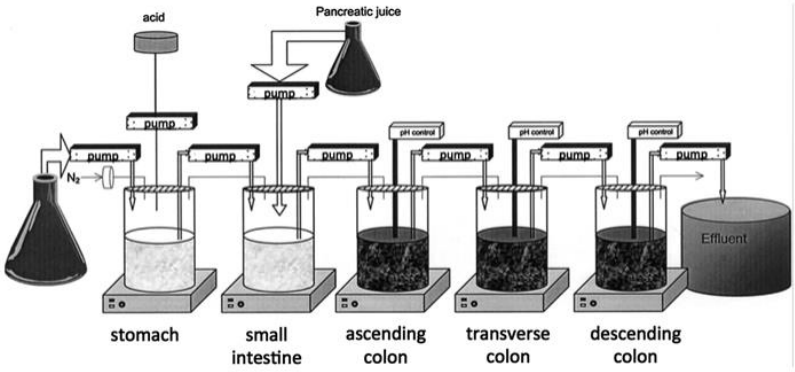


Equation 2: Chemical reaction for spectrophotometric quantification of GABA production by LAB. GABA quantity is proportional to the intensity of blue colour in the solution, which stems from Indophenol.

The media simulating GI conditions will be an adapted version of the well-documented GI model by Van der Wiele et al. More specifically, its small intestinal bioreactor culture media [11]. In human microbiota studies, the GI model from Van der Wiele is a validated medium for mimicking gut conditions to study bacteria growth and metabolic proficiency [11-14]. This template system seen in Figure 7a is composed of 5 interconnected jacketed-glass vessels (bioreactors) emulating pH, temperature, nutrient and enzymatic conditions of the stomach, small intestine, ascending colon, transverse colon, and descending colon respectively. These reactors replenish their media's nutritional values recurrently by being fed novel media, while discharging an equivalent volume of its digested media into the subsequent bioreactor. Food media is composed of complex carbohydrates, proteins, vitamins, minerals and mucins such as pectin, arabinogalactan, glucose, yeast extract, peptone and cysteine powder [11,13]. Additionally, pancreatic and bile liquids, containing ox gall and pancreatin in physiologically relevant concentrations, is added to the small intestinal vessel in order to simulate the process of digestion. Ultimately, the pHs of each

vessel is controlled at their respective physiological pHs, and anaerobiosis is ensured by saturating the vessel's headspace with nitrogen gas. As we intend to identify the bacterial strain with optimal GABA productivity under physiological conditions, incubation of the most productive strain will be assayed in an adapted version of Van der Wiele GI model, in our novel simulated duodenal model seen in Figure 7b.

a)



b)

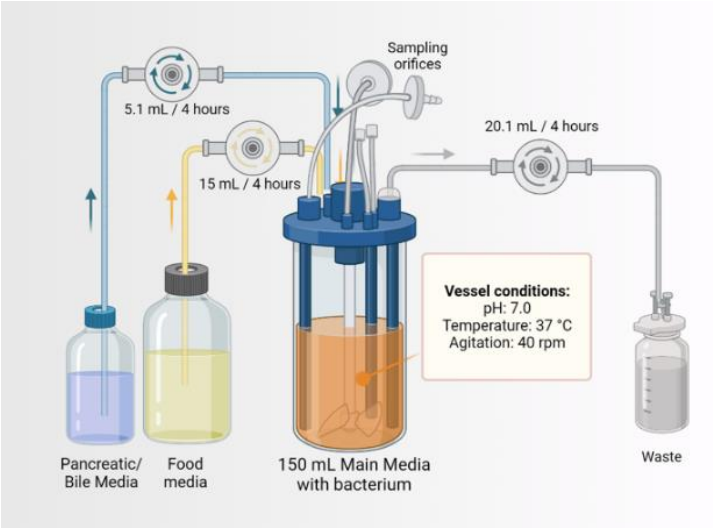


Figure 7: a) Schematic of classical GI model from Van der Wiele et al. b) adapted simulated GI model media for duodenal growth and metabolic activity.

4.3 Methodology

4.3.1 Quantification of GABA production using spectrophotometric method

Individual bacterial strains were grown in De Man–Rogosa–Sharpe (MRS) media at 37 degrees Celsius and under anaerobic conditions for 16h. Resulting bacterial culture is then centrifuged at 4000g for 15 minutes. Supernatant was discarded and 50 mL of physiological saline water (0.85%) was added to the cell pellets. After vortexing, the resuspended cell-pellet was centrifuged a second time at 4000g for 15 minutes. Following the removal of the supernatant, 5mL of a 200 mM monosodium glutamate (MSG) and 0.1 mM Tween 80 solution were added to the cell pellet. The following solution was then incubated in a 37 degrees Celsius incubator for subsequent GABA production quantification.

An adapted version of the Watchararparpaiboon et al. [15] quantitative GABA measuring method was used for this study. It is the most commonly used quantitative method after HPLC, and was selected due to its flexibility and speed for multiplexed assays. 1 mL of GABA-containing sample was placed in a test tube. 0.2 mL of borate buffer (0.2M sodium borate and 0.2M boric acid, normalized at a pH of 9.0 using sodium hydroxide 0.1N) was added to the sample and vortexed. 0.3 mL of 6% Folin & Ciocalteu's phenol reagent (Milipore Sigma) was then added to the solution of borate buffer and sample. The solution was then vortexed and placed in ice water for 1 minute. 0.15 ml of 7.5% sodium hypochlorite (Milipore Sigma) was then added to the cooled solution and vortexed. The totality was then added to boiling water for 12 minutes, and instantly put in ice water for 5 minutes. 1 mL of the solution was then added to cuvettes for absorbance reading at 630 nm against a blank containing the reagents, without sample. Standard curve was done in triplicate at concentrations 40, 50, 75, 100, 120 mM, equating to 4.1, 5.2, 7.7, 10.3 and 12.4 g/L. The resulting standard curve can be seen in Supplementary Figure S4.

4.3.2 *In vitro* quantification of GABA production by *L. brevis* KS1 in a simulated duodenal growth medium

500 mL of duodenum-mimicking media was prepared and separated into three 250 mL Pyrex bottles for this assay (150 mL each). Duodenal media was composed of the polysaccharides, proteins, vitamins and minerals found in table 2a [11,13,14].

Components of main media	Concentration (g/L)	Media composition (g/500 mL)
Pectin	4.0	2
Dextrins	3.0	1.5
Glucose	0.4	0.2
Yeast extract	3.0	1.5
Protease peptone	1.0	0.5
Mucin	4.0	2
Cysteine	0.5	0.25
$NaHCO_3$	0.4	0.2
NaCl	0.08	0.04
K_2HPO_4	0.04	0.02
KH_2PO_4	0.04	0.02
$CaCl_2$	0.008	0.004
$MgSO_4$ $7H_2O$)	0.008	0.004
Haemin	0.005	0.0025
Tween 80	1.0	0.5
Biotin	0.002	0.001
Menadione	0.001	0.0005
Pantothenate	0.01	0.005
Nicotinamide	0.005	0.0025
Vitamin B_{12}	0.0005	0.00025
Thiamin	0.004	0.002
Para-aminobenzoic acid	0.005	0.0025

Table 2a: Media composition of the adapted simulated GI model media for duodenal growth and metabolic activity.

The simulated duodenal media was fed every 4 hours with 3 mL of food media and 1 mL pancreatic solution per 30 mL of main media, which equated to 15 mL Food media and 5.1 mL pancreatic solution per 4h for 150 mL main media [16]. This served the purpose of replenishing key macromolecules, pancreatic enzymes and bile in the simulated media, mimicking the periodic ingestion of food. Its ingredients can be found in Table 2b and c.

Components of food media	Concentration (g/L)	Media composition (g/100 mL)
Arabinogalactan	1	0.1
Pectin	2	0.2
Xylan	1	0.1
Corn starch	3	0.3
Glucose	0.4	0.04
Yeast extract	3	0.3
Peptone	1	0.1
Mucin	4	0.4
Cystein powder	0.5	0.05

Table 2b: Media composition of the food solution in the adapted simulated duodenal model.

Components of pancreatic/bile media	Concentration (g/L)	Media composition (g/50 mL)
Sodium bicarbonate	12	0.6
Ox gall	6	0.3
Pancreatin	0.9	0.045

Table 2c: Media composition of the pancreatic enzyme and bile solution in the adapted simulated duodenal model.

To prepare the MSG-supplemented media, 1.5g of MSG was added to 150 mL of main media. Similarly, for the prebiotic fiber triphala-supplemented media, 0.5g of *Emblica officinalis*, *Terminalia bellirica* and *Terminalia chebula*, were weighed individually and mixed to form 1.5g of triphala (TFLA), which was added to 150 mL of the main media.

The three media were autoclaved at 120 degrees Celsius for 15 minutes for decontamination prior to inoculation with the GABA producing *Lactobacilli*.

The highest producing strain at pH 7.0 resulting from the assay in 4.3.1 was selected for incubation in the simulated duodenal media. The selected strain was grown, centrifuged and washed as previously stated, and 3 mL of the resulting resuspended cell pellet was inoculated into 150 mL of the simulated main media, MSG-supplemented media, and TFLA-supplemented media. Quantification of GABA production was performed at time points 4h, 8h, 12h, 24h, and 48h. Additionally, bacterial counts were measured at these time-points in order to characterize the growth and viability of our selected strain in a simulated duodenal environment.

4.4 Results and discussion

4.4.1 GABA production quantification of various *L. brevis*, including the human-sourced *L. brevis* KS1

Following our previous study and the qualification of GABA productivity, quantitation of GABA production from the human stool-derived *L. brevis* remained to be elucidated. In literature, *L. brevis* is the resounding frontrunner for GABA production proficiency. Indeed, as long as no optimization of the assay is performed, such as genetic engineering of the strain, and a minimum concentration of MSG of 10g/L is added to the bacterium, the highest GABA production is observed by an *L. brevis*. The *L. brevis* strain RK03 converted 650 mM L-glutamic acid to 62.52 g/L of GABA, resulting in a conversion rate of 93.3% after 88h of fermentation at a pH of 4.5 [17]. In our previous study, we identified 4 high-GABA producing *L. brevis*; three from our strain bank of non-human source, and one isolated from a healthy nonagenarian. To compare its GABA production ability to the industry, a similar assay as *L. brevis* RK03 was performed. However, we opted for an MSG concentration of 200 mM instead of an initial L-glutamic acid concentration 650 mM considering our lower relative bacterial growth. Indeed, the group using the *L. brevis* RK03 obtained consistently 8 billion CFU/mL for their brevis, whereas *L. brevis* KS1, in MRS, could only attain a maximum of 650 million CFU/mL. It has been demonstrated that a high

glutamic acid concentration relative to bacterial cell counts increases osmotic pressure in cells, which disturbs its metabolism and potentiates cell lysis [18]. Thus, an adapted concentration of 200mM was employed in this assay, and in order to remain consistent with the previous study, initial pH remained at 5.0 (Figure 8).

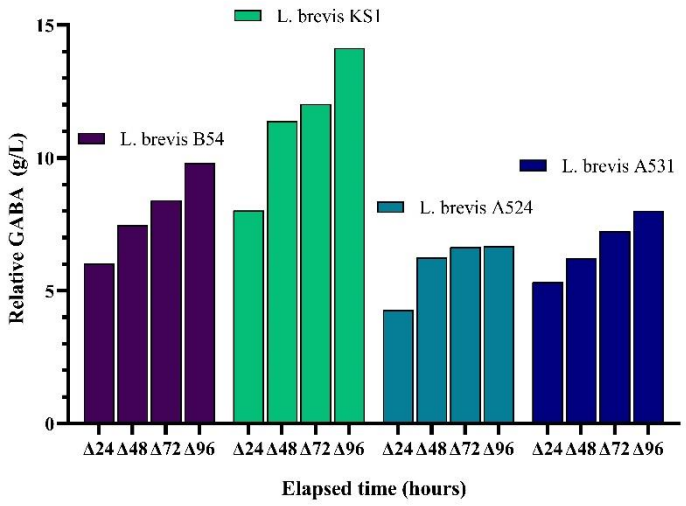


Figure 8: *L. brevis* KS1 produces more GABA than its intraspecific counterparts *L. brevis* B54, A524 and A531 at GAD's optimal pH of 5.0. Relative GABA concentration produced after 24, 48,72, and 96h by several *L. brevis*.

Following these parameters, maximum GABA was seen with *L. brevis* KS1 at pH 5.0, producing 14.13 g/L, equating to a 68.5% conversion rate after 96h. *L. brevis* B54 had the second-best production of GABA at 9.81 g/L and a conversion rate of 47.6%, followed by *L. brevis* A531 at 8.01 g/L and 38.8%, and ultimately, *L. brevis* A524 with a 6.68 g/L production at 32.4%. These results are coherent with our previous study, where *L. brevis* KS1 had the steepest rate of alkalization in our qualitative assay. Although the conversion rate from MSG to GABA by *L. brevis* KS1 is not as elevated as *L. brevis* RK03, its productivity and rate of conversion is superior to a large majority of other *L. brevis*, positing it as a high-producing strain within its species. In order to further substantiate which strain will be best suited for production under physiological conditions, a comparison of GABA output of *L. brevis* KS1 and other high GABA-yielding species found in literature had to be investigated.

4.4.2 GABA production quantification of human-gut derived *L. brevis* KS1 versus documented species in literature at GAD optimal pH

For reference, the high GABA producing *L. brevis* strains from our previous study were compared to other documented producers such as *L. rhamnosus* [19,20], *L. helveticus* [21], and *L. paracasei* [22,23] (Figure 9).

At GAD's optimal pH of 5.0, *L. rhamnosus* GABA production after the first 24h of the assay was 6.66 g/L, and reached a maximum of 12.93 g/L after 96h, resulting in a conversion rate of 62.7% *L. rhamnosus* has seen considerable academic interest, as several *L. rhamnosus* strains have shown both an apt proficiency in GABA production and, for some strains, an anti-depressant effect via the hypothalamic-pituitary-adrenal (HPA) axis [24]. Most notably, *L. rhamnosus* JB-1 increased GABAa and GABAb receptor levels in mice, and improved the murine model's abilities in a Forces Swim Test i.e., a marker for anti-depressant propensity [25]. However, it is important to note that the group of scientists conducting this study did not test for GABA production of their strain, thus, correlation between GABA productivity and mood disorders is uncertain.

L. helveticus is another species that was analyzed due to its documented anxiolytic properties [26] and GABA production [12]. At a pH of 5.0, *L. helveticus* produced 7.71 g/L after

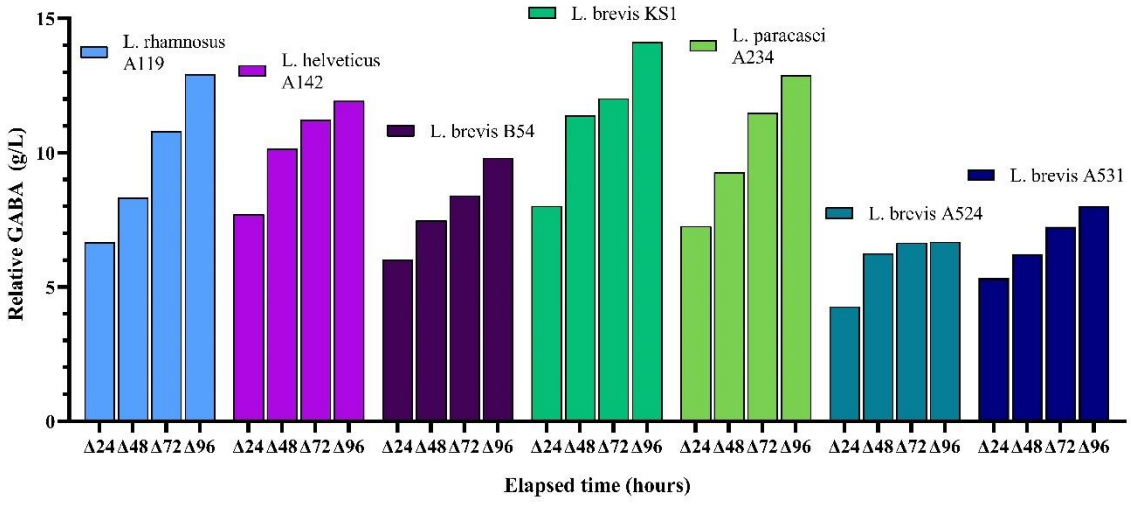


Figure 9: *L. brevis* KS1 produces the most GABA at GAD's optimal enzymatic pH of 5.0 when compared to potent GABA producers from literature. Relative GABA concentration produced after 24, 48,72, and 96h by several *L. brevis*, and documented potent producers. Maximum production of each strain from left to right: 12.93 g/L; 11.93 g/L; 9.81 g/L; 14.13 g/L; 12.89 g/L; 6.68g/L; 8.02g/L.

24h, and 11.93 g/L after 96h incubation in MSG, reaching a slightly lower conversion rate than *L. rhamnosus* of 57.9%.

The final species tested in conjunction with the fore-mentioned *L. brevis* strains was *L. paracasei*. The latter is a commonly isolated GABA-producing organism in cheeses, and has a high GABA anabolic activity, having shown the ability to produce 302 mM GABA at a 60.4% conversion rate [23]. Similarly, in our assay, *L. paracasei* reached a maximum GABA content after 96h of 12.89 g/L, equating to a 62.5% conversion rate. The most published strain of *L. paracasei* (520 research articles) whose strain holds the name of its microbiologist discoverer, *Lacticaseibacillus paracasei* Shirota, has been shown to alleviate mild depressive disorder following daily intake of 80 billion CFU for 12 weeks [27]. Additionally, administration *L. paracasei* Shirota fermented milk was also shown to improve hypertension in the elderly [28]; which is another condition alleviated by GABAergic agonists [29-31]. Although the mechanisms by which this *L. paracasei* strain affects mood disorders and hypertension have not yet been fully elucidated, the link between its beneficial effects and GABAergic transmission are well-characterized and should be considered. All in all, *L. rhamnosus*, *helveticus* and *paracasei* produce GABA at a higher rate than *L. brevis* A531, *L. brevis* A524 and *L. brevis* B54, but have comparable productivity than *L. brevis* KS1 at its metabolically optimal pH of 5.0.

4.4.3 GABA production of human-gut derived *L. brevis* KS1 versus documented species in literature physiological duodenal pH

GABA production proficiency at its optimal pH does not necessarily correlate with a higher probability of beneficial effect, as is demonstrated by the *L. rhamnosus* and *helveticus* studies. However, the strain's ability to produce GABA at physiological pH could lead to a higher likelihood of beneficial effects, as the produced neurometabolite's bioavailability in the duodenal luminal space would be increased. Indeed, in a fed state, the human duodenum harbors a neutral pH of 7.0, whereby in its fasted state, duodenal pH drops to approximately 5.7, due to periodic discharging of chyme and gastric juices from the stomach [10]. For our intents and purposes, as GABA is produced from its precursor glutamate, a common amino-acid found in meats, seafood, cheese, and nuts [32], the

proficiency of the bacterium to produce GABA in a GI fed state (pH of 7.0, temperature of 37 degrees Celsius) is of prime importance for its translatability to humans. Additionally, the duodenum was selected in this physiological model since it is the area in the GI tract that primarily harbors Lactobacilli, representing 6% of this subsection's total bacterial composition [33,34]. With this in mind, the same bacterial strains tested in the previous assay were now tested for their GABA production at pH 7.0. GABA production experienced a -12% decrease in *L. rhamnosus*, -6% drop for *L. helveticus*, -11% for *L. brevis* B54, -3% for *L. brevis* KS1, -35% for *L. paracasei*, -20% for *L. brevis* 524 and -0.1% decrease for *L. brevis* A531 (Figure 10).

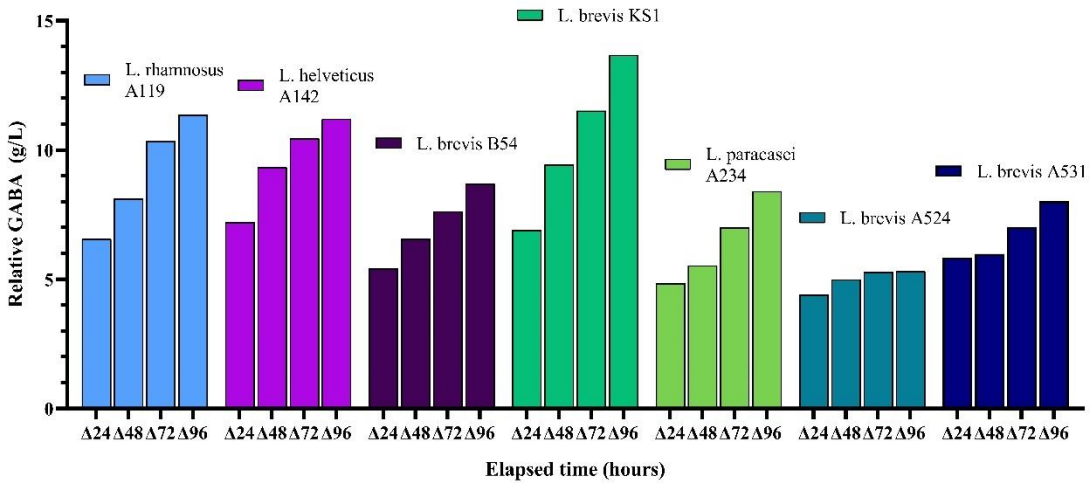


Figure 10*: L. brevis* KS1 produces the most GABA at a physiologically-consistent pH of 7.0 when compared to potent GABA producers from literature. Relative GABA concentration produced after 24, 48,72, and 96h by several *L. brevis*, and documented potent producers. Maximum production of each strain from left to right: 11.35 g/L; 11.21 g/L; 8.70 g/L; 13.67 g/L; 8.41 g/L; 5.32 g/L; 8.01 g/L.

Both *L. brevis* KS1 and *L. brevis* A531 showed favorable results for their potential duodenal production prowess, but considering the significantly lower GABA production yield in *L. brevis* A531 (38.8% at pH 5.0 and 38.9% at pH 7.0), *L. brevis* KS1 was the only strain selected for further examination in a simulated duodenal culture media.

4.4.3 Production of GABA by *L. brevis* KS1 in a simulated human GI model media

As the main portal of entry for pathogens, viruses and other extraneous compounds into the human body, the GI tractus must haven harsh conditions in order to shield the host from possible assailants. On the other hand, beneficial bacteria must also uphold its metabolic abilities in this same challenging environment to secure the necessary nutrients for their survival. As a result, GABA productivity of *L. brevis* KS1 in the human duodenum could not be confidently concluded unless its growth and GABA productivity were measured in a simulated intestinal ecosystem. That is, GABA production of our candidate bacteria must be determined under duodenal, fed-state pH, nutrient levels, and temperature. To do so, *L. brevis* KS1 was incubated in an adapted version of the well-documented simulated human intestinal microbial ecosystem media developed by Van der Wiele et al (Figure 7b). Moreover, the effect of MSG supplementation, and administration of the prebiotic fiber triphala on GABA production and bacterial growth was investigated in tandem. First and foremost, bacterial survivability and GABA proficiency was observed in an unaltered duodenal model medium without MSG supplementation (Figure 11). *L. brevis* KS1 was

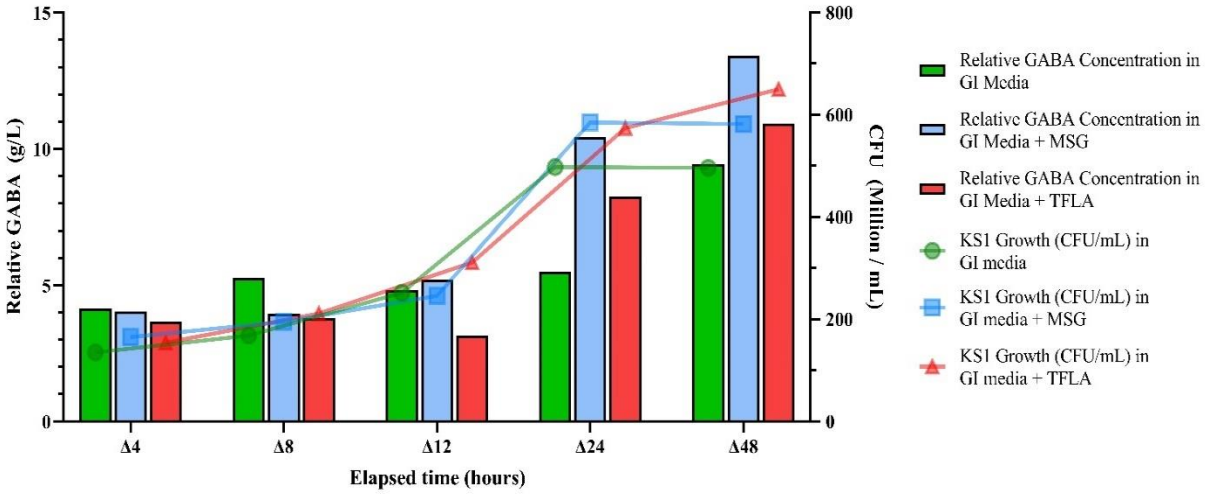


Figure 8: *L. brevis* KS1 displays bacterial growth, and maintains its relative GABA high production ability in a simulated GI environment over 48h. Relative GABA production concentration over time (columns; left axis) and bacterial cell count (symbol and line; right axis) of *L. brevis* KS1 in a simulated duodenal model media over 48 hours. Maximum GABA production of each media: Unsupplemented classical duodenal media 9.44 g/L; 1% MSG-supplemented 13.43 g/L; 1% TFLA-supplemented 10.93 g/L. Maximum growth of each media: Unsupplemented classical duodenal media 498 MCFU/mL; 1% MSG-supplemented 585 MCFU/mL; 1% TFLA-supplemented 650M CFU/mL.

capable of producing a maximum of 9.44 g/L of GABA in the unsupplemented simulated duodenal media, suggesting GABA could still be produced in the gut without co-

supplementation of MSG. Indeed, the feeding of proteolytic enzymes from the pancreatic/bile media bottle to the standard small intestinal solution resulted in the breaking down of main media complex proteins into amino acid i.e., glutamic acid, which could then act as precursors to the conversion of GABA. Nonetheless, in comparison to the GABA production levels in the MSG supplemented simulated duodenal media, 100 mM supplementation of MSG increased GABA yield by 42% (from 9.44 g/L to 13.43 g/L). Notably, survivability of *L. brevis* KS1 remained the same as the standard medium.

In the prebiotic triphala-supplemented simulated duodenal media, GABA production was similar to the traditional media (10.93 g/L). Conspicuously, viability of *L. brevis* KS1 experienced a 31% increase compared to the non-supplemented media after 48h. Prebiotics such as triphala polyphenols are fibers that can uniquely be digested by a subset of LAB, acting as growth substrates for the latter [14,35]. Accordingly, Triphala phenolic compounds are biotransformed by LAB to create phenolic acids, which in turn have antimicrobial properties, thus increasing LAB relative fitness against other bacterial competitors [36-39]. The increased cell count observed in the triphala-supplemented small intestinal medium can be attributed to this growth-promoting effect, as triphala polyphenols could have acted as an additional substrate to LAB growth, while inhibiting other bacterial growth in the medium.

All things considered, results from this research quantitatively confirm that the GABA producing ability is highest for *L. brevis* KS1 at pH 7.0, 37 degrees Celsius versus comparable strains within its species, and well-touted species from literature (*L. rhamnosus, L. helveticus, L. paracasei*). Moreover, its production capacity is maintained in an MSG supplemented simulated intestinal environment, going from 13.67 g/L at pH 7.0, 37 degrees Celsius, in a 200 mM MSG solution, to 13.43 g/L in the small intestinal media. A notable change to this assay compared to the one done in 4.4.2 which may explain higher than expected GABA productivity results. The simulated intestinal media was refreshed every 4h with food medium, consequently replenishing glutamate levels accessible to *L. brevis* KS1. Although this undeniably influenced the total amount of GABA produced by *L. brevis* KS1 in the simulated gut environment, the noteworthy observation that GABA production capability was maintained in a medium mimicking the

small intestine without extraneous MSG supplementation serves as a promising foundation moving forwards into *in vivo* and clinical studies.

4.5 Conclusion

Although there have been many quantitative studies detailing the proficient production of GABA by LAB, a paradigm shift is required if any translatability from *in vitro* to *in vivo* is the end goal. Similar studies have approached these quantitative *in vitro* studies with a set mechanism to maximize, which, although may result in accrued academic interest, results in a complex rabbit hole where metabolic proficiency becomes the ultimate finish line, but physiological effect becomes an afterthought. This is adequate for some studies, where GABA, as a supplement, is aimed to be maximally produced through bacterial fermentation. But in the scenarios where the LAB is to be clinically tested afterwards, it may not be the optimal course of action. In this study, several LAB's GAD activity was measured at the enzyme's optimal pH, and at physiologically-relevant pH in order to determine which bacteria strain could be best suited for GABA production in the host gut. Conspicuously, the human stool-isolated *Lactobacillus brevis* displayed the highest GABA productivity at pH 7.0. The latter's metabolic activity was then measured in a simulated media mimicking pH, temperature, and nutrient levels of the human duodenum in a fed-state. Both growth of the bacterium, and GABA productivity was observed in the simulated medium. As we continue on this trajectory, supplementing the GABA-producing LAB to animal models will be an interesting avenue to substantiate our purported paradigm shift.

Supplementary material

Supplementary Figure S4: Standard curve of GABA concentration (g/L) vs absorbance at OD630 nm

Author Contributions

KA and SP: conceptualization. KA: writing of original draft. KA: data curation and visualization. SP: funding acquisition, project administration, resources, and validation. KA, JB, SC, RT: scientific experiments. KA and RT: software and figures. All authors contributed to the article and approved the submitted version.

Funding

This work is supported by the Natural Sciences and Engineering Research Council (NSERC) Canada discovery grant awarded to Prakash S. Boyajian JL is funded by the Fonds de Recherche du Québec – Santé (FRQS, grant 335999-2024). Schaly S is funded by the Canadian Graduate Scholarship-Doctoral from the Natural Sciences and Engineering Research Council (NSERC, 569661-2022). Arora K is funded by NSERC, Canada. The funders had no role in study design, data collection and analysis, interpretation of the data or writing of the manuscript.

Preface to the following chapter

The previous chapter put forth *L. brevis* KS1 as a potent GABA-producing bacteria both at a pH of 5.0 and 7.0. Additionally, *L. brevis* KS1 displayed adequate growth and GABA production in a simulated duodenal media. One of the primary hypotheses put forth in the previous chapter discusses a paradigm shift for mass-screening of GABA-producing bacteria that are then selected for its potential impact on human health. In essence, scientists should seek bacteria that not only best produce GABA under optimal conditions, but also, under human gut-like conditions, as it is the primary habitat for GABA-producing bacteria. Thus, having identified *L. brevis* KS1 as a potent producer under both conditions, a logical progression to substantiate this paradigm shift would be to evaluate the bacterium's therapeutic potential for GABA-related ailments *in vivo*. To achieve this, it was essential to investigate the effect of *L. brevis* KS1 supplementation on *in vivo* models of anxiety, depression, or sleep disorders – conditions whose pathophysiology intertwine with GABAergic neurotransmission. Hence, oral treatment of the bacteria cells in a Drosophila model of sleep would serve as preclinical evidence prior to murine and/or clinical studies.

In Chapter 5, the effect of *L. brevis* KS1 administration on key biomarkers of insomnia, such as sleep latency and wakefulness-after-sleep-onset in Drosophila melanogaster will be examined and discussed. This study will build upon one of the previous chapter's main findings, whereby *L. brevis* KS1 can produce considerable amounts of GABA under a wide range of pH conditions. Indeed, the ability of *L. brevis* KS1 to produce GABA in a simulated physiological microenvironment increases the likelihood that supplementation of the bacterium could impact GABAergic neurotransmission in an invertebrate model such as Drosophila. The quality of Drosophila sleep will be compared with untreated Drosophila. Moreover, Drosophila under the supplementation of alprazolam – a potent hypnotic benzodiazepine – will serve as a positive control to our treatment plan.

The findings resulting from this study will serve as a foundation for the sleep-promoting effects of *L. brevis* KS1.

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Chapter 5: Novel *L. brevis* KS1 bacterial cells improved sleep quality and efficiency in *Drosophila melanogaster*

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5.1 Abstract

Given the proven role of GABA agonists in sleep onset and maintenance, and the myriad sleep-inducing therapeutics with a GABA receptor target, modulating this neurotransmitter's level has long been a target for the regulation of sleep. The widespread use of hypnotic and sedative drugs has urged the drug development market to produce benzodiazepine-like agents with minimal side-effects, and abuse potential. In fact, over the past three decades, there has been a decline in the prescription of sedatives and hypnotics in the United States [1]. This reduction may be due to the medical community's enhanced awareness of these drug's limitations in chronic insomnia situations whereby chronic use of sleep medication has been associated with a predisposition to depression and drug-dependence [2]. Aiding sleep patterns from within, through behavioral methods and healthy sleep hygiene practices such as: rising at consistent times each morning, selective use of one's sleeping environment, and limiting hyperarousal habits prior to sleep has become the preferred primary intervention pathways for alleviating milder version of insomnia [3].

Nonetheless, the latter's efficacy is seldom as potent as the fore-mentioned sleep-promoting drugs.

In this study, an alternative pathway for treating sleep disorders is investigated: the production of GABA by the gut microbiota. To investigate this, the sleep-promoting effect of a proficient GABA-producing lactic acid bacteria (LAB), *Lactobacillus brevis* KS1, was evaluated in *Drosophila melanogaster*. Upon supplementation with *L. brevis* KS1 flies exhibited a reduced sleep latency onset time, requiring 20 minutes (21.3%) less to fall asleep during nighttime. Additionally, their WASO decreased by 21%, whereby flies fed *L. brevis* KS1 spent 20 minutes and 30 seconds less time awake per night in comparison to the untreated fly group. This outcome was a result of an augmentation in nighttime sleep bout length from 72 minutes to 103 minutes long. In a subsequent sleep pattern assay comparing the effect of a low-dose (0.1%) benzodiazepine and high-dose (0.2%) benzodiazepine supplementation to *L. brevis* KS1- fed flies, the GABA-producing LAB exhibited equal or improved effects on sleep quality markers of sleep latency, sleep bout length and WASO. Most notably, the BZD-treated groups exhibited, on average, 8 minutes and 45 minutes more time awake during the nighttime, respectively, in contrast to the *L. brevis* KS1 fed flies. This implies a lower sleep quality in the BZD-treated group and in controls, compared to the group administered *L. brevis* KS1.

Keywords: Gamma-aminobutyric acid, lactic-acid bacteria, gut microbiota, *Lactobacillus brevis*, gut-brain axis, neurotransmitter production, vagus nerve, sleep disorders, insomnia, *Drosophila melanogaster*, benzodiazepine, sleep latency, WASO.

5.2 Introduction

γ-amino butyric acid (GABA), the primary inhibitory neurotransmitter in the brain, plays a crucial role in reducing neuronal excitability in various brain regions. A dysregulated GABAergic neurotransmission has been associated to ailments such as epilepsy, anxiety, insomnia, Huntington's disease, schizophrenia, depression and autism spectrum disorder. Myriad GABA receptor agonist drugs have been developed to tackle these neurological, mood and psychiatric disorders. However, these do not come without their fair share of debilitating side-effects, as well as a high risk of addiction due to its promoted neuroplasticity in dopaminergic rich brain regions [4,5]. Direct GABA supplementation has garnered recent academic attention as a plausible alternative prophylactic for GABAergic signal homeostasis, without the incumbent side-effects. Although various GABA pills can be found on the supplement market, supplemented GABA's ability to cross the blood-brain barrier remains without a consensus opinion. Another posited mechanism by which GABAergic signaling could be beneficially regulated is via the production of GABA by gut bacteria, and more specifically, from lactic acid bacteria (LAB). The primary difference between supplemented GABA and bacterially-produced GABA is the proposed mechanism of action for its effects. Direct supplementation of GABA has the benefit of high bioavailability, rendering it readily available to cross the blood-brain barrier into the central nervous system (CNS). On the other hand, LAB-produced GABA in the gut interacts with another nervous system to posit its beneficial effect on GABAergic neurotransmission: the enteric nervous system (ENS). As mentioned earlier however, GABA is often characterized as BBB-impermeable. This main disparity may be the determinative factor for each treatment's success.

Often referred to as the second brain due to its second most prominent neuronal density in humans, the ENS plays a fundamental role on GI tract motility, mucosal secretions, angiogenesis, and gut-brain communication. GABA produced in the gut can cross the intestinal epithelial barrier separating the gut luminal space and the ENS via GABA transporter proteins present in the duodenum, ileum and colon [6,7]. Subsequently, GABA can exert an inhibitory effect on enteric neurons by binding to its GABAa or GABAb receptors [7]. Upon binding, chloride ions influx into the neuronal cell results in

hyperpolarization of the neuron, inhibiting action potentials from that cell and rendering it dormant until its subsequent firing period [8]. The ENS interacts with the CNS primarily through the vagus nerve. This bi-directional communication between the GI tract and the central nervous system is denoted as the gut-brain axis (GBA), and serves as one of the many potential mechanisms of action for LAB-producing GABA to posit a beneficial effect on neurological disorders.

The effect of the GABA-producing *Lactobacillus brevis* KS1 on sleep behavior in *Drosophila melanogaster* will be delved into in this study. Drosophila exhibit similar fundamental sleep characteristics as mammals, whereby flies display sustained periods of complete immobility, increased arousal threshold for wakefulness, and operate with a circadian clock [9]. Additionally, flies, just like humans, are victim of sleep debt. After pronounced periods of sleep deprivation, it was demonstrated that flies enter a recovery sleep state in which their arousal threshold increases [10].

There are two ways fly sleep can be characterized: 1) by electroencephalographic (EEG) criteria, or 2) through behavioral indicators. In general, a bout of fly sleep is defined by a period of absolute immobility lasting 5 minutes [9,10]. Considering the inherent challenger in conducting EEG measurements in the minuscule fly, this study will use behavioral markers as indicators of sleep quality. This is done by measuring the fly's locomotor activity. Several studies have employed the Drosophila activity monitoring (DAM) system to examine sleep patterns of flies following administration of an intervention. DAM analysis functions by shining infrared lasers into a tray full of fly-containing Pyrex tubes [11]. A phototransistor on the other end receives the signal of the laser if no fly comes into contact with the laser. Each minute, the number of times a fly crosses the infrared light source is counted, and this data is extrapolated to infer the locomotor activity of the fly, and thus, its sleep patterns. This system has been used for drug development [12], circadian rhythm studies [13], the study of alpha-synuclein formation in a Parkinson's disease model and its effect on locomotor activity [14], and the effect of potential behavioral therapies on sleep efficiency and sleep latency in a Drosophila insomnia model [15].

Sleep quality is measured through two biomarkers, namely sleep latency and wakefulness-after-sleep-onset (WASO). Notably, while the diagnostic criteria for insomnia have lacked

a universally standardized definition, an increased sleep onset latency (SL) and WASO have remained standard benchmarks in the diagnostic paradigm [16,17]. The effect of *L. brevis* KS1 on Drosophila sleep patterns, and more precisely, on its SL, WASO, duration of sleep bout, and total sleep duration, will be delved into in this study. This will assist in determining whether or not *L. brevis* KS1, a proficient human gut-derived GABA producer, can impact sleep quality in an invertebrate model whose sleep characteristics strongly resemble those of mammals.

5.3 Methodology

5.3.1 Drosophila culture

Wildtype Canton-S *Drosophila melanogaster* were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington Indiana). Fly food was the standard, pre-weighed cornmeal-sucrose-yeast-agar media recommended by the Bloomington Drosophila Stock Center (Genesee Scientific), with added propionic acid for preservation. 17.57g of premade mixture was added to 50mL of 50 degrees Celsius water and mixed at a moderate speed. Volume of the solution was then topped off to 100 mL. After reaching boiling point for 10 seconds, the fly food mixture was left to cool while stirring manually. The beaker withholding the solution was covered with aluminum paper. At 70 degrees Celsius, 490 microliters of 99% propionic acid (Fisher Scientific) was added and homogenized manually. 50 mL of Drosophila food was then poured into fly conical square-based bottles (Genesee Scientific) and kept at room temperature for 2 hours covered with a cheese cloth to minimize condensation onto the food. Fly food stocks were made fresh for each assay. *L. brevis* KS1, alprazolam 0.1% and alprazolam 0.2% fly food followed the same preparation as mentioned above, but the LAB/BZD were added after cooling the boiling food solution to 40 degrees Celsius, prior to solidification of the media.

Drosophila were then transferred onto the assay's respective food vials and incubated in a controlled environment at 20 degrees Celsius and 50% relative humidity with a 12h:12h light: dark cycle. 3-5-day old flies were selected for the assays. Flies were transferred into fresh food bottles every week in order to replenish nutrient levels and maintain an adequate

fruit fly density. For transferring, the old vial is flipped and its orifice is conjoined with a new vial with fresh food. The bottom of the old vial (flipped) is then tapped until all flies are transferred and larvae/pupae remain in the old vial. Cotton was then used to cover each vial in order to maintain aerobiosis.

5.3.2 LAB culture

L. brevis KS1 was grown in De Man–Rogosa–Sharpe (MRS) media under anaerobic conditions at 37 degrees Celsius for 16h. Afterwards, the bacterial culture was centrifuged for 15 minutes at 4000g. The supernatant was discarded and 50 mL of physiological saline water (0.85%) was added to the pellets. A second centrifugation at 4000g for 15 minutes was performed after vortexing the resuspended cell pellet. Another 20 mL of physiological saline was then added to this final concentrated cell pellet and its optical density was measured in order to always add 0.5 Billion CFU/g of *L. brevis* KS1 to the fly food.

5.3.3 Sleep analysis of *Drosophila melanogaster* via the Drosophila activity monitoring (DAM) system.

Sleep architecture analysis was performed by the Drosophila activity monitoring (DAM) system by Trikinetics. The locomotor activity measurement apparatus DAM collects fly movement throughout the day in 1-minute bins. This data was then compiled using the VANESSA-Shiny application for the analysis of fly sleep quality and efficiency [18]. Fly food was prepared as described in the fore-mentioned procedure, but poured into a petri dish instead of bottles. DAM pyrex vials were then placed into the solidified fly food in order to cover one end of the 15 mm vials with feed. This end of the vial was covered with paraffin, while the other end was covered with cotton to ensure fly respiration. 5-day old flies post eclosion were then placed into a separate empty fly bottle and anesthetized using the FlyNap kit. After 2-3 minutes exposed to the anesthetizing gas, flies would be transferred into individual food vials, and viability of each fly was confirmed prior to placing the Pyrex vials into the activity monitor. Tubes were then centered into the monitor. Drosophila were left in the DAM system for 1 day in order to familiarize itself with its

novel environment, and assaying of locomotor activity began on day 2. Flies were kept on a 12:12 light: dark cycle at room temperature. Of the 32 vials, 16 vials were loaded with normal food, and the latter 16 vials contained *L. brevis* KS1 supplemented fly food. In a second assay, the first 16 vials had 0.1% alprazolam, whereby the final 16 had 0.2% alprazolam-supplemented fly food.

Vanessa DAM Sleep architecture software was used to arbitrarily analyze fly sleep date from the DAM system. Sleep latency, WASO, sleep fractions at night, and wake-time during day data were plotted on violin plots using the software. All data was downloaded in .csv files for our figures. All code for the apps can be found on GitHub (https://github.com/orijitghosh/VANESSADAM), and R was used for compilation of the code. Compared to other fly analysis software, VANESSA DAM allows for direct analysis of key sleep disorder-related markers, and an intuitive user interface generating a plethora of publication-quality plots.

5.4 Results and discussion

5.4.1 Sleep quality and efficiency is improved in Drosophila fed *Lactobacillus brevis* KS1 supplemented media.

In layman's nomenclature, the medical term sleep latency (SL) describes the time required for an individual to fall asleep once the lights go out. It is a measure of sleep quality, and when dysregulated, becomes a determinant symptom for insomnia. Following *Lactobacillus brevis* KS1 supplementation, sleep onset latency improved in *Drosophila melanogaster* (Figure 12a).

On average, flies subjected to the GABA-producing LAB medium required 21.3% less time to enter a dormant state, equating to a decrement of 20 minutes. This result correlates with a similar study whereby direct administration of GABA reduced sleep latency in flies

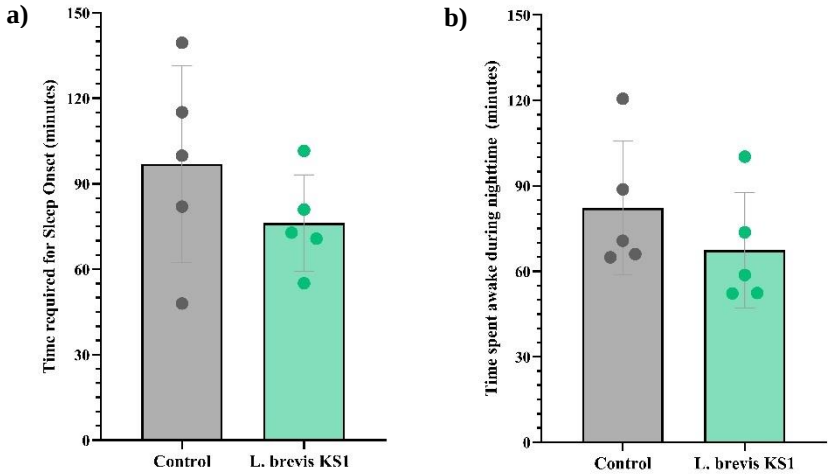

Figure 9: Effect of *L. brevis* KS1 on Sleep latency and WASO in *Drosophila melanogaster*. Nighttime analysis was conducted for 5 nights. a) Sleep latency of control and *L. brevis* KS1 administered flies. Flies fed *L. brevis* KS1 require 20 fewer minutes to fall asleep in comparison to untreated fruit flies. b) WASO. Flies fed *L. brevis* KS1 spend 14 fewer minutes awake during nighttime.

and increased sleep duration, overall enhancing sleep patterns in comparison to flies fed a normal diet [19]. An improved SL may serve as a determinant factor in mitigating insomnia. Indeed, although its diagnosticating symptom thresholds have long been subject to debate, the group of Lineberger et al. proposed an average SL of 20 minutes over 2 weeks as the biomarker that best discriminated primary insomniacs from normal sleepers [16]. Furthermore, a similar meta-analysis of the quantitative criteria for insomnia diagnosis by the group of Lichstein et al. posit a SL of 31 minutes or more occurring three times a week as a defining criterion for insomnia [20]. Thus, the fore-mentioned 21.3% decrease in SL may constitute the decisive factor in distinguishing between the expression of insomnia or experiencing satisfactory sleep.

Another commonly documented diagnostic parameter describing the severity of a patient's insomnia is WASO, i.e. minutes spent awake following sleep onset [16]. Flies fed the GABA-yielding *L. brevis* KS1 spent 17.9% less time awake during dark hours compared to its untreated analogue (Figure 12b). This equates a reduction in nighttime wakefulness of 14 minutes and 46 seconds on average over the 5-night period. Compared to control

Drosophila, *L. brevis* KS1 supplemented flies experienced an average increase in nighttime sleep bout duration (Figure 13b), rising from 72 minutes to 103 minutes. On the other hand, flies feeding off *L. brevis* KS1 supplemented food spent on average 34 minutes 25 seconds more time sleeping during daytime when compared to flies fed normal cornmeal diet. This could be due to the flies' tendency to feed themselves the most within the first four hours awake [21,22]. This extra ingestion of GABA-*L. brevis* KS1 may have resulted in the daytime short sleep periods experienced by the LAB-treated group.

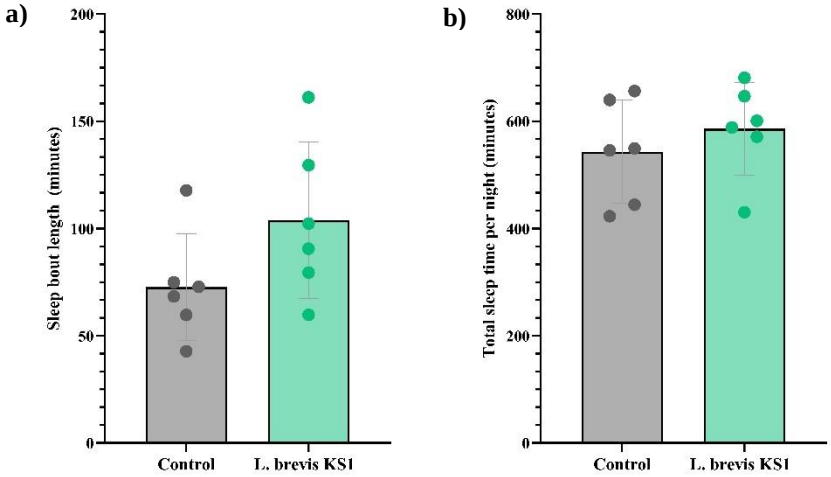

Figure 13: Effect of L. brevis KS1 on sleep bout length and total sleep time per night in *Drosophila melanogaster* over a 5-night period. a) Supplementation of *L. brevis* KS1 increases mean sleep bout duration from by 31 minutes. b) *L. brevis* KS1 fly group spends 43 more minutes asleep than untreated flies over a five-night period.

Healthy sleep involves the cyclic transition between rapid eye movement (REM) sleep and non-REM sleep. Endogenous GABA binds to GABAa receptors in the wakefulness-promoting brainstem and hypothalamus and regulates this oscillation [23]. As arousal-inducing brain regions are deactivated, slow-wave sleep (light and deep sleep) is maintained. Furthermore, GABA orchestrates the harmonic progression of neuronal frequencies, thereby regulating sleep stages. These two mechanisms also serve as a cornerstone for sleep-inducing drugs, referred to as hypnotics. Hypnotics such as barbiturates, benzodiazepines (BZD), and sedating anti-histamines have excellent efficacy. However, incumbent side-effects such as REM-sleep rebound, resulting in daytime drowsiness and memory impairment, irritability, and rebound anxiety [24] have been

extensively documented following their discovery. Additionally, several of today's most prominent drugs are documented to induce varying degrees of severity of insomnia. Examples of such therapeutics are antibiotics, caffeine, ephedrine, alcohol, oral contraceptives, corticotropin, cortisone, selective-serotonin reuptake inhibitors such as Prozac and Zoloft [25,26].

5.4.2 Flies supplemented *Lactobacillus brevis* KS1 had comparable sleep quality biomarker levels as benzodiazepine-supplemented flies.

In order to best ascertain the mechanism by which GABA-producing *L. brevis* KS1 acts on sleep, its effects were compared to the benzodiazepine alprazolam. Benzodiazepines allosterically bind to cerebral GABAa receptors and increase its affinity to GABA, thus, engendering a hyperpolarization of the subdued brain region ultimately resulting in GABAergic inhibition of the afflicted cortical area. Via this mechanism, BZDs decelerate neuronal action potential frequencies and proliferate their sleep-inducing effects by calming down the brain areas responsible for alertness and wakefulness.

In the locomotor activity assay, the mean onset of sleep for the 0.2% BZD-treated flies was reduced by 11 minutes compared to the *L. brevis* KS1 group, while the 0.1% BZD supplemented group experienced a 15-minute reduction in sleep onset [18] (Figure 14a). This posits the hypothesis that the sleep-inducing effect of BZD might not be dose-dependant, as the allosteric sites on initial sleep-inducing GABAa receptors may have already been saturated at a 0,1% concentration.

Considering BZD's ability to rapidly cross the blood brain barrier and thus, rapidly induce its effects on the fly's lateral ventral neurons – the brain region governing sleep onset latency – these results are consistent with the notion flies fed BZD-supplemented food

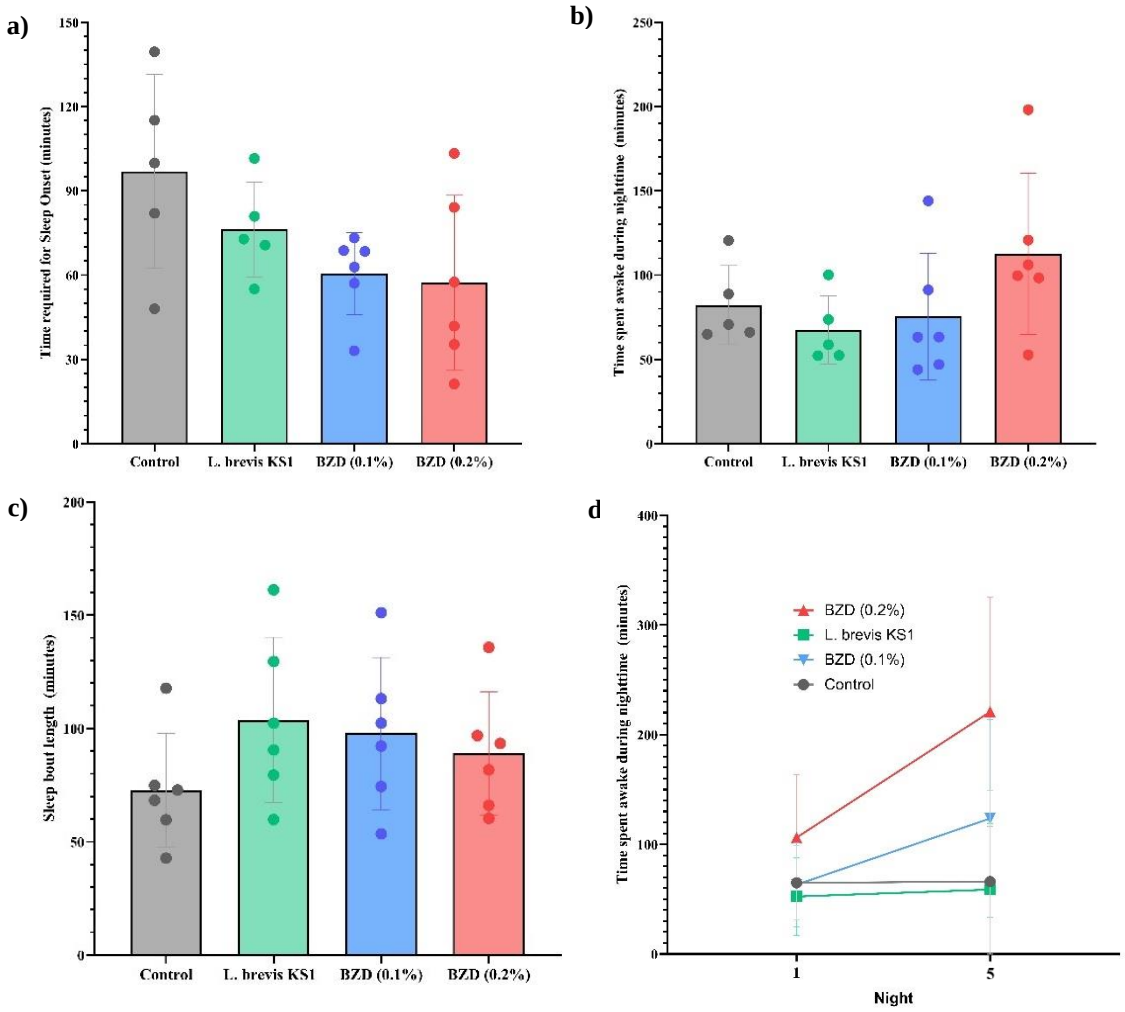

Figure 11: Comparison of effects of *L. brevis* KS1, control, low-dose (0.1%), and high-dose BZD (0.2%). a) sleep latency in flies supplemented *L. brevis* KS1, 0.1% alprazolam and 0.2% alprazolam. b) WASO. c) average length of sleep bout. d) comparison from night 1 to night 6 in WASO. *L. brevis* KS1 fed flies see a slight improvement in effects on a) sleep Latency, and c) sleep bout length as the sleep-promoting BZD alprazolam; and an amelioration in WASO as seen in b) and d)

required less time to fall asleep compared to control and *L. brevis* KS1 groups [27]. Although the effects of BZD on sleep latency in humans are also well documented with similar modes of actions, the detrimental repercussions that BZD can have on human sleep quality is a prominent side-effect. In flies however, a 0,1% BZD dose administration to Drosophila lead to a similar WASO of 75 minutes on average per night, compared to the 67 minutes of the *L. brevis* KS1 group. However, sleep quality was affected by a higher dose BZD. At 0,2% supplementation, flies spent 66% more time awake compared to the *L. brevis* KS1 group, and 37% more WASO compared to the Control group for a total of 112 minutes

awake after light had been turned off (Figure 14b). This was seen as a progressively worsening WASO, whereby on the first day of testing, WASO for the 0,2% BZD group was 106 minutes, whereas at the sixth day, the 16 assayed flies spent on average 198 minutes awake during dark time (Figure 14d).

The worsening of sleep quality for high dose BZD consumers can be due to a plethora of causes. Firstly, high-dose administration of BZD has been shown to reduce REM sleep times, and increase light-sleep non-REM times, leading to a lower quality sleep, and increased semi-wakeful periods. Secondly, benzodiazepines suppress deep sleep periods. Deep sleep is not only responsible for revitalization of muscles and memory building, it is the sleep state whose arousal threshold (stimuli required for one to be abruptly awoken) is the highest. By-passing such an important sleep period thus increases the likelihood of that an external stimulus awakens the fly, and thus, increases its WASO. Not only that, but by reducing the restorative properties of these two sleep cycles, its detrimental effects cumulate and result in progressively worsening sleep. Nonetheless, BZD have been shown to be proficient reducers of sleep latency, and promoters of sleep duration. The latter was also seen in Drosophila whereby BZD 0.1% and BZD 0.2% supplementation increased the mean length of sleep bout from 72 minutes to 98 minutes (34% increase) and 89 minutes (22%) increase respectively (Figure 14c).

L. brevis KS1 fed flies experienced a slightly improved sleep bout duration of 103 minutes. The increased amount of time spent awake during night time for the BZD supplemented group could therefore be due to an increased difficulty in re-falling asleep following the first few bouts. This could be due to the relatively low half-life of certain BZDs, ranging from 1.5 – 5h for rapid-onset BZDs [28]. Alprazolam has been documented to reduce REM sleep duration in adult men with Major Depressive Disorder (MDD) [29]. A persistent decrease in REM sleep from habitual use of BZD drugs leads to REM-sleep rebound on ensuing nights. This then disbalances REM and non-REM sleep equilibrium, and affects the body's memory consolidation, modulation of emotional reactivity and ability to overcome stressful stimuli [30,31]. Chronic lack of quality sleep has been shown to lead to daytime sleepiness, as well as potentially exacerbating mood disorders [32-34]. In this assay, flies fed 0.1% alprazolam spent an average of 87.6% of the night sleeping, which closely

resembled untreated flies with an 87.3% fraction (Supplementary Figure S5). On the other hand, high dose BZD-fed Drosophila had a reduced fraction of nighttime sleeping of 82.0%, nearly 9% less than the *L. brevis* KS1 treated group, which spent 90.7% of the night in a dormant state. Despite a shortened sleep onset time in flies treated with hypnotics, the resultant sleep was of lower quality than that of the *L. brevis* KS1 fed group. This was primarily due to the flies decreased mean time spent awake during nighttime, and increased fraction of sleeping flies at night.

Although the results of this study show promise, the precise mechanism by which *L. brevis* KS1 produced GABA influenced sleep architecture in *Drosophila melanogaster* remains undetermined. Microbially-produced GABA have the inability to passively cross the gut barrier from its luminal space to the ENS, but can actively cross through GABA/H+ symporters. After translocation from the luminal space into the extra-intestinal space, there are several modes of actions still up to debate. Some studies suggest that GABA enters the bloodstream and is capable of crossing the very selective blood-brain barrier in minute quantities, thus affecting GABAergic signaling [35,36]. Other studies support the hypothesis that GABA is not BBB permeable, and that microbially produced GABA interacts with GABA receptors on several immune cells, playing a role in immune cell activation and regulating GI inflammatory contingencies [37]. These GABA receptors can be found on members of the innate and adaptive immune system and initiate potent anti-inflammatory properties. Adequate GABAergic neurotransmission in the ENS leads to a less erratic inflammatory system, a marker often seen in a plethora of gut and brain comorbidities such as inflammatory bowel disease and anxiety/depression, or metabolic syndrome and sleep disorders [38,39].

Dr. John Bienenstock from the Brain-Body Institute and his research on psychobiotic LAB have explored another plausible mechanism generating substantial academic interest. LAB-produced GABA may impact cerebral GABA receptor levels via the vagus nerve. Indeed, *L. rhamnosus* JB-1 treatment upregulated GABA receptor expression in the murine amygdala and hippocampus – two brain regions connected to the ENS by the vagus nerve [40,41]. Subsequent to surgical excision of the vagus nerve, the observed GABAergic effect ceased to manifest, implying that the heightened expression of GABA receptors in the CNS

was contingent upon the integrity of the vagus nerve and, consequently, the enteric nervous system (ENS). This study represents the most proximate advancement in any study purporting the psychotropic therapeutic potential of LAB, particularly in establishing a mechanism of action.

Another noteworthy finding suggesting a potential translocation of microbial GABA into the brain is elucidated in a study from Jeong et al. Their research investigated the binding dynamics of *Lactobacillus brevis*-produced GABA on human GABAa receptors *in vitro* [19]. LAB-derived GABA activated GABAa receptors in a dose-dependent manner with an EC_{50} of 33.3 micromolar, indicating that LAB GABA has similar agonistic properties as physiological GABA (EC_{50} of 26-107 micromolar)[42]. This study and the previous *L. rhamnosus* JB-1 study support the hypothesis that Lactobacilli GABA could have beneficial effects onto GABA-related ailments via gut-brain reciprocal communication pathways.

While numerous quantitative studies have already greatly identified a plethora of proficient GABA-producing LAB, the human-sourced *Lactobacillus brevis* KS1 has shown an adept GABA productivity under physiologically relevant conditions, and now, an equal or better effect as BZDs on sleep induction and quality. Incorporating *L. brevis* KS1 as a preventative therapeutic, and/or as an adjuvant, could provide another tool to the medical practitioner's arsenal of sleep disorder interventions.

5.5 Conclusion

Individuals experiencing insomnia exhibit diminished levels of GABA in the brain compared to those with regular sleep patterns [43]. Although GABA's ability to cross the BBB remains a subject of academic debate, the myriad articles describing how proficient microbial-GABA can be in addressing disorders whose etiology stems from GABA neurotransmission offers a robust foundation for prospective therapeutic alternatives to the imperfect BZD. Several pathways by which GABA may affect sleep have been proposed in this article. Whether it be from interactions with the vagus nerve, GABA receptors in the ENS, or GI immune-inflammatory regulation, these three pathways are plausible modes of

action for *L. brevis* KS1-produced GABA to impact sleep architecture. Drosophila have an analogous nerve for the vagus nerve, known as the antennal nerve, as well as a functionally similar BBB to humans [44,45]. Given this, the significant effect of *L. brevis* KS1 administration on sleep onset latency, and wakefulness-after sleep onset – two pivotal diagnostic markers of sleep disorders – present LAB as a potentially interesting tool for the medical practitioner. Nonetheless, further studies on the strain's efficacy in humans, and elucidation on the microorganism's exact modalities of action are required prior to heralding *L. brevis* KS1 as standalone prophylactics.

Supplementary material

Supplementary Figure S5 a) Sleep fraction of benzodiazepine-treated flies. b) Sleep fraction of *L. brevis* KS1 vs untreated group

Author Contributions

KA and SP: conceptualization. KA: writing of original draft. KA: data curation and visualization. SP: funding acquisition, project administration, resources, and validation. KA, JB, SC, RT: scientific experiments. KA and RT: software and figures. All authors contributed to the article and approved the submitted version.

Funding

This work is supported by the Natural Sciences and Engineering Research Council (NSERC) Canada discovery grant awarded to Prakash S. Boyajian JL is funded by the Fonds de Recherche du Québec – Santé (FRQS, grant 335999-2024). Schaly S is funded by the Canadian Graduate Scholarship-Doctoral from the Natural Sciences and Engineering Research Council (NSERC, 569661-2022). Arora K is funded by NSERC, Canada. The funders had no role in study design, data collection and analysis, interpretation of the data or writing of the manuscript.

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Chapter 6: Discussion

6.1 The supplemented GABA translatability dilemma

There is no scarcity in research papers screening for potent GABA-producing Lactobacilli. Indeed, a total of 104 studies have isolated bacteria from a plethora of fermented foods, beverages, soil, etc., whereby most studies denoted an outstandingly prominent GABA production at GAD's optimal pH: 4.0 – 5.0. Of these identified GABA-producing bacteria few have seen follow-up studies investigating their effect on GABA-related ailments in murine models, and even fewer have been subsequently tested into humans. This could stem from a multitude of reasons.

Firstly, the vast majority of assays screening for GABA producers strive to maximize production of the neurotransmitter by any means. Whether it be incorporating an unorderly amount of its co-factor, Vitamin B_6, or its precursor, L-glutamate, a high excreted GABA concentration is often the ultimate objective of these studies.

Secondly, considering that the main premise of the GABA-producing enzyme is to de-acidify its environment, the low GI pH of the mouse model may exacerbate GABA productivity, a proficiency that may not translate to humans as the mean fed-state duodenal pH of humans is much less acidic, ranging from 6.0 - 7.0 [121,122].

Thirdly, the GI tract is a difficult microhabitat characterized by intense ecological competition. Indeed, ingested LAB must overcome the highly acidic pH of the stomach. Subsequently, they translocate into the small intestine, which already hosts trillions of microorganisms entrenched in lichen-like biofilms competing for identical resources. Ultimately, the LAB that survive such formidable challenge must maintain a viable cell-count in order to uphold its GABA proficiency. Understandably, many LAB may not have the resilience to live and tell the tale. When transitioning these LAB from mice to human studies, GABA yield sustenance is rare given the vastly disparate microenvironment in which the LAB is subjected to.

An example illustrating this lack of translatability can be seen in the following study. *L. rhamnosus* JB-1 was administered daily to healthy BALB/c mice for four weeks, and cerebral GABA levels were upregulated from baseline in anxiety, depression and memory-

related brain regions [1,123]. Although this study was ground-breaking in the field of potential gut-brain modulation, the murine results did not translate to healthy male humans [124]. This first-of-its-kind clinical trial tested a LAB's proficiency in modifying stress, inflammation and cognitive aptitude biomarkers, but ultimately did not result in significant changes in patient depression, anxiety, or sleep scores. A key difference between the mouse study and the human study was the choice of subjects. Treatment of *L. rhamnosus* JB-1 was administered to an anxious mouse model, whereas the probiotic was given to healthy male subjects in the clinical trial. This was not an oversight by the investigators, it is due to prerequisites set by regulatory bodies such as the Food and Drug Administration's for substantiating health claims on probiotics. Given that *L. rhamnosus* JB-1 was first characterized as a probiotic, and as such, falls within the category of a food supplement, the human study had to be conducted in a healthy population for a health claim to be considered [125]. As stated by the various medical researchers conducting the *L. rhamnosus* JB-1 study, this was a major limitation in their clinical study. Notably, the current most prominent class of sleep-inducing drug class, BZD, also exhibit minimal sleep-promoting effects on healthy individuals [126].

Nevertheless, the sequence of experiments led by Dr. John Bienenstock will spearhead many more of its kind, including this master's thesis. Another notable example is the study by Strandwitz et al, which utilized an innovative approach for the identification of GABA-proficient microorganisms. While searching for mechanisms to culture bacteria from the NIH's Most Wanted list of unculturable taxa, the group of Northeastern University scientists identified a *Bacteroides fragilis* strain capable of producing GABA [60]. Several other Bacteroides, Parabacteroides and Bifidobacteria were then identified as GABA producers, but bacteria from the Bacteroidetes phyla displayed the highest production of GABA at a pH of 6.0 – 7.0. Subsequently, the group collaborated with the Montreal Neurological Institute to analyze 23 stool samples of Major Depressive Disorder (MDD) patients. The objective was to inquire into the correlation between Bacteroidetes levels in the gut and neuronal activity in brain regions hyperactive in depressive individuals. Relative abundance of Bacteroidetes in the patient's stool was inversely proportional with functional connectivity in the default mode network, a brain region that plays a major role in the overthinking/negative dwelling symptom of depression [127]. Antidepressant drugs

with GABAergic mode of actions are known to alleviate this symptom. This correlated the Bacteroidetes production of GABA and the potential mitigation of a hallmark MDD symptom. In fact, this study was the first correlating GABA production by bacteria and its effect on human cerebral GABAergic signaling. However, a limitation of this investigation revolves around the biosafety of some *Bacteroidetes fragilis* strains. Indeed, although *B. fragilis* has proven beneficial effects on symptoms of depression, improved mucosal immunity, protection against colorectal cancer, and weight loss, the Government of Canada's Pathogen Safety identifies *B. fragilis* as an infectious substance [128] [129]. When compartmentalized within the colon, *B. fragilis* is harmless, but if the epithelial barrier separating the circulatory system to gut contents is compromised and *B. fragilis* enters the bloodstream, the resulting bacteremia can be lethal [130]. This toxicity is uncommonly found in Lactobacilli, considering their Generally Recognized as Safe (GRAS) label by regulatory agencies [131].

This thesis is a projection of both innovative studies, as well as their limitations. For clinical relevance, GABA production by bacteria should only be measured under physiologically relevant conditions of pH, carbohydrate, protein, vitamin, minerals, enzymes, and bile levels. This would maintain its metabolic activity and ultimately maximize the likelihood to engender a prophylactic effect on its host, not only in murine models, as was the case in the first study. The nonagenarian gut-derived *Lactobacillus brevis* KS1 emerged as a top candidate for GABA production at the enzyme's optimal acidic pH, but also under human gut-like conditions. Consistent with Strandwitz's healthy human stool-derived *B. fragilis* KLE1758, the source of the bacterium for *L. brevis* KS1 may play a determinant factor in explaining why this LAB in particular could maintain its elevated GABA productivity in a simulated duodenal medium.

6.2 Hypothesized modalities of action for GI GABA's effect on the brain

A considerable unknown when discussing the production of GABA by gut bacteria involves the mechanism by which excreted GABA can impact, let alone, reach the brain. In a study by Frost et al., a novel mechanism for direct translocation of bacterial metabolites was discovered. Indeed, labeled isotopic carbons originating from colonic bacterially-

produced acetate were identified in neuroglial cells, and successfully translocated into the hypothalamus [132]. Bacterial acetate readily crossed the BBB and its isotopic carbon was found in CNS glutamate, glutamine and GABA neurotransmitters. This upregulation in hypothalamic GABA lead to a supressed appetite in mice. This study suggested that bacterially-produced metabolites may directly impact GABAergic neurotransmission in the CNS by crossing the BBB.

Initially, microbial GABA was believed to follow a similar mechanism. Orally ingested GABA supplements was proven to enter systemic circulation with a peak bioavailability thirty minutes after ingestion [133]. Once in the bloodstream however, GABA has long been characterized as a non-BBB-permeable amino acid. This is attributed to its 50 kilodalton size, and due to the absence of GABA-specific active transport proteins facilitating its transport into the CNS. Few studies have hypothesized that minute amounts of GABA may cross the BBB, but all testing had been done on mice models. Until date, no human proof of GABA permeability into the BBB has been postulated. Although this may appear like a shortcoming, translocation of exogenous neurotransmitter into the highly fragile homeostatic environment that is the CNS might be more detrimental than beneficial.

Another mode of action through which GABA-yielding bacteria may impact cognitive function centers around the vagus nerve. Its myriad of afferents innervates all sections of our GI tract and ensure that any gut anomaly is alerted to the brain. Consequently, vagal nerve afferent density is at its maximum in the proximal duodenum, and at a minimum in the transverse colon. As mentioned in the introduction, the vagus nerve also oversees a plethora of indispensable bodily functions, such as the regulation of GI immunity, energy homeostasis and appetite control (satiety) [40]. An interesting study published last year revealed that oral supplementation of GABA induced a feeling of satiety in mice, evidenced by a reduced time require for refeeding [134]. Beneficial effects on the mice feeding habits was dependent on the vagus nerve. In fact, the postprandial satiety-inducing effects were completely nullified following surgical removal of subdiaphragmatic vagus nerve afferents, suggesting that GABA's effect on satiety is vagus nerve-dependent.

Numerous studies have demonstrated the vagal dependency of gut-brain beneficial effects through surgical vagotomy on mice. However, the precise pathways elucidating how

GABA interacts with the vagus nerve remain unknown. One proposed mechanism involves the active transport of GABA across the epithelial barrier. Indeed, GABA produced by LAB could cross the cell line separating the gut and vagal afferents – known as the gut

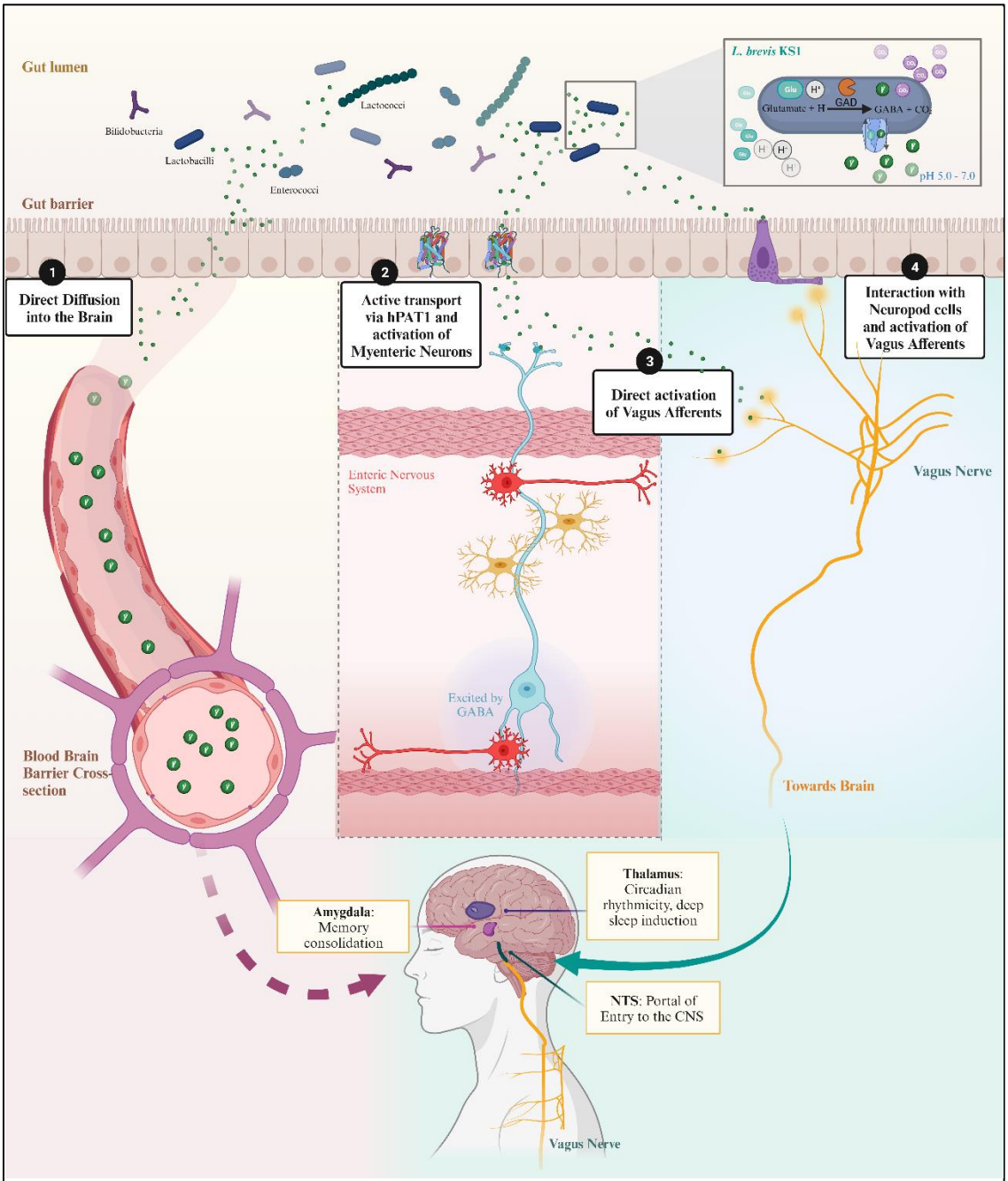


Figure 12: Four potential mechanisms of action for the novel *L. brevis* KS1 effect the brain and body's nervous systems and potentially, sleep disorders. 1) GABA may diffuse in extremely minute amounts across the BBB. 2) LAB GABA can cross the epithelial barrier via hPAT1 and active enteric neurons in the myenteric plexus, and/or 3) the vagus nerve. 4) GI GABA can interact with neuropod enteroendocrine cells, which directly communicates with the vagus nerve.

epithelial barrier – via a proton pump symporter hPAT1, which is unanimously expressed in the GI tract, but especially in the small intestine [135]. Once the neuroactive transcends the gut barrier, it can interact with GABA receptors on myenteric neurons of the enteric nervous system. However, once bound to GABA receptors on ENS neurons, GABA promotes the depolarization of the neuron i.e., the action potential of the neuron, by increasing the neurons membrane potential to its threshold potential [117,136]. This is counter intuitive, since the function of GABA in the CNS is mostly inhibitory, but in the ENS myenteric plexus, GABA promotes activity. Ensuring an adequate GABAergic neurotransmission in ENS fosters immune cell activation and a more stable GI immune system. Conversely, a dysregulated GI immune system may lead to a weakened intestinal epithelial barrier, perpetuating a chronic state of inflammation. This conditions has been attributed to various neurological disorders including Alzheimer's disease and Parkinson's disease [137].

Lastly, GABA is hypothesized to interact with a specialized type of gut endocrine cells, neuropod cells, which directly synapse with vagal afferents. Neuropods have glutaminergic and GABAergic neurotransmission capabilities [138], rendering them a potential target for mechanistic studies of GABA-producing bacteria. Ultimately, the complete mechanism by which GABA from *L. brevis* KS1 could reach the brain remains incompletely understood, but the fore-mentioned studies present compelling evidence presenting the vagus nerve and enteric nervous system as integral contributors to this process.

6.3 Filling the void: The need for alternative sleep disorder therapeutic strategies

Ailments affected by a dysregulated GABAergic neurotransmission are plentiful. Consistent with the previous paragraph's premise, rare are the CNS disorders that have clear mechanism of action, nor root causes for its debilitations. In this master's thesis, *Drosophila melanogaster* fed with *L. brevis* KS1 exhibited improvements in various biomarkers associated with one of these GABA-related processes: sleep. Current medication for sleep disorders is primarily dominated by BZD drugs. Comparatively to the BZD alprazolam, *L. brevis* KS1 displayed a slightly lower sleep latency time, a similar WASO to low-dose BZD, and an improved effect on sleep fraction percentage. In

literature, similar results have been observed. For example, *Lactobacillus fermentum* PS150 significantly decreased sleep onset latency time in mice. This was hypothesized to occur via the upregulation of adenosine receptors in the preoptic area of the hypothalamus. The aforementioned brain region is not only responsible for sleep onset, but is also intricately linked to the vagus nerve via the *nucleus tractus solitarii* (NTS). Consequently, an elevation in inhibitory adenosine receptors could set forth the bacterium's mode of action. In a human study, supplementation of the psychobiotic *Lactobacillus plantarum* JYLP-326 attenuated anxiety and insomnia symptoms in questionnaires of 60 test-anxious college students [139]. *Lactobacillus gasseri* CP2305 is another strain that has amassed significant academic attention for its improvement on sleep quality in adults. Following administration of the paraprobiotic bacterium for 12 weeks, EEG measurements revealed a significantly reduced sleep latency, WASO in the last 120 minutes of sleep, in the test group compared to controls. Additionally, adults fed *L. gasseri* CP2305 spent a higher ratio of their night in N3 deep sleep when compared to controls. These murine and human studies are consistent with this thesis' sleep-promoting results, and suggest that LAB may play an advantageous role in promoting sleep onset and quality. With this in mind, how do LAB compare BZDs, which are recognized for their potency in current treatment plans for sleep disorders? Is there an avenue for replacement?

As described with further detail in chapter 2, BZDs bind allosterically to the GABA receptor, increasing the receptor's affinity to GABA, and shifting the neurotransmitter's dose-response curve towards a lower required effective concentration. The primary issues with BZD therapy, involves the debilitating side-effects, and reduced sleep quality when chronically administered. In fact, BZDs are the most commonly prescribed medication for sedative, hypnotic, and anxiety-related disorders. Roughly 10% of Canadians have reported using BZDs once annually, and 1 in 10 of this subset are classified as chronic users [140]. In Quebec however, 76% of Quebecers who intake psychotropic drugs are chronic users for a minimum of 6 months, and 29.6% of chronic users have been consuming BZDs recurrently for 5 years [141]. Although these two statistical studies are dating, a narrative review detailing BZD consumption statistic from 1995 to 2016 showed that the usage rate of the psychotropic drug has remained relatively stable over the years [142]. These studies are particularly disconcerting considering that a 9.5% increase in antidepressant usage within

the 18-34 demographic was observed in the aftermath of the pandemic. This could snowball into a considerable portion of the population enduring chronic use of BZDs and its side-effects.

Acute BZD use can incur relatively benign side-effects such as daytime drowsiness and fatigue [87], but once BZD consumption becomes habitual, the adverse effects are extremely deleterious. A more chronic administration of BZD has been shown to increase non-melanoma cancer prevalence by 35% within an average of 7.6 years [143]. Moreover, during a 15-year longitudinal study, newly-prescribed BZD users increased the risk of dementia with a multivariable adjusted hazard ratio of 1.60, meaning that long-term users are 1.6 times more likely to develop the disease over non-consumers [144]. The latter study was performed in men and women with a mean age of 78.2 who were non-BZD consumers at the onset of the study. This outlines the danger of chronic prescription of BZD in the elderly demographic.

In cases of insomnia particularly, chronic use of BZD is frequently observed since the sleep-promoting effects of the drug tend to diminish after a few weeks use [145]. Although these psychotropic drugs are effective at reducing the time required to fall asleep, sleep quality is not improved. This is corroborated by a reduction in the BZD user's restorative REM sleep time [146]. Elderly are more prone to sleeping difficulties, and have also the shortest REM sleep time of all age groups. Considering this, the elderly population is more prone to chronic BZD use, a practice that has been highlighted above as particularly perilous.

It is important to emphasize that this does not reflect a deficiency in the medical practitioner's judgement, rather, it highlights the lack of medical alternatives to BZD as sleeping agents. Indeed, a survey of British general practitioners revealed that sleep hygiene recommendations were the most common first treatment given to patients with anxiety and insomnia. However, following the pressure imposed by the patient due to the inefficacy of the proposed treatment, BZDs were subsequently prescribed [147].

This lays the foundation for the envisioned use case of GABA-producing bacteria: as an adjuvant to BZDs, and/or as an alternative preventative therapeutic approach for sleep disorders. Consequently, a comprehensive investigation into the former's safety and

efficacy is imperative prior to asserting such health claims. Nonetheless, clinical practice is seeing a progressive distancing from BZD prescription as a first-line treatment for its associated disorders. Instead, prescription guidelines have started promoting psychosocial and non-pharmacological interventions as favored treatments for insomnia and anxiety [148]. Considering the limited amounts of non-drug treatments for the fore-mentioned disorders, a substantial gap in the market is still being filled by prescribed BZDs. Ultimately, investigating ulterior options, such as the GABA-producing *L. brevis* KS1, may serve to replenish this void and mitigate the market's dependence on BZDs.

6.4 Limitations of this thesis' primary outcomes

This thesis effectively validated the hypothesis that a human-sourced LAB could sustain a proficient production of GABA under GAD-optimal conditions, and in a physiologically-relevant simulated setting. However, it is essential to acknowledge certain limitations of the studies. The enzymatic reaction for GABA anabolism requires L-glutamic acid to be within the vicinity of the bacterium. If it isn't co-supplemented, it may be challenging to ensure that a sufficient quantity is near *L. brevis* KS1 in order to produce GABA. There are two ways that this could be remediated. One adaptation could be to adapt the clinical subject's diet when supplementing the gut bacterium intervention. Yet, this approach may pose challenges when elucidating if the diet, or the bacterium is responsible for the potential sleep-inducing effects. Alternatively, the clinical trial intervention could be devoid of glutamate. This is a viable option since glutamine, the precursor to glutamate, is the most abundant free amino acid in the human body. The enzyme glutaminase then converts glutamine into L-glutamic acid, and is particularly active in the duodenal mucosa whereby it produces 53 nanomoles of glutamate per minute, per mg of protein [149].

Moreover, compared to its highly-touted beneficial effects in *in vitro* and mice models, the history of success for LAB as therapeutics is limited. This lack of translatability from pre-clinical to clinical trials can be due to many factors. One of them is the heterogeneity of microbiota ecosystems amongst individuals. These differences in bacterial relative abundances lead to unique microbial – and antimicrobial – signatures that limit the adherence of ingested LAB. Indeed, if a species of bacteria is not already colonizing the

gut of the patient, a supplemented LAB will have a very slim probability of adhering to the gut lining [150]. Additionally, gut bacteria as biotherapeutics require the bacteria to not only be metabolically active in the gut microenvironment, it requires for it to be alive during its transit. Many variables work against the livelihood of transient supplemented bacteria, such as the harsh acidic conditions, pathogenic bacteria, bile salts, and the production of antimicrobial peptides by other commensal bacteria. Moreover, Zmora et al described that there might be two different degrees of tolerability in individuals, from permissive to resistant, concluding that even if the gut bacterium is present in the resistant subtype, you still can not guarantee its colonization.

Lastly, despite the Drosophila model's similarities in CNS constitution, its BBB's permeability is vastly disparate. Indeed, the fruit fly have a vagus nerve analogue – the antennal nerve – as well as direct equivalents to astrocytes, pericytes, epithelial cells, and tight junctions in their BBB [151,152]. However, barriers are not uniquely judged on its constituents, but also by its ability selectively restrict diffusion. The BBB in Drosophila exhibits the unique capability to regulate its permeability to xenobiotic molecules with a circadian cycle dependence. Indeed, the group of Zhang et al. demonstrated that the anti-epileptic phenytoin crossed the invertebrate's BBB more effectively during nighttime [153]. This phenomenon could have promoted the translocation of GABA from *L. brevis* KS1 into the brain via direct diffusion, instead of the hypothesized vagus nerve mechanism.

Chapter 7: Contribution to knowledge and summary of key findings

Lactic acid Bacteria supplementation has a rich history in academia for their beneficial effects on several organ systems, particularly the GI system and central nervous system. One hypothesized mechanism by which the latter may occur is through the production of the calming neurotransmitter GABA. However, although several bacteria have been discovered as potent GABA producers, few experience a translatable effect on humans. Consequently, this results in lackluster clinical studies whose primary intervention targeted GABAergic neurotransmission.

This study aimed to provide a framework for the isolation and identification of a potent GABA producer that would maintain its proficiency in humans, and confer a beneficial effect on the host's GABAergic neurotransmission.

In order to initiate this challenging endeavor, it was imperative to isolate and identify a bacterium that exhibits high GABA productivity. To achieve this objective,

Article 1

1. Fifteen Lactic acid Bacteria strains were isolated from three cheeses with disparate maturation times, and their ability to produce GABA was qualitatively investigated.
2. Out of the fifteen strains, two strains of *Lactococcus lactis* and one strain of *Lactobacillus brevis* isolated from three different cheeses qualitatively displayed the ability to produce GABA.
3. GABA production ability was determined to be strain-specific, given that different strains of *Lactobacillus brevis* and *Lactococcus lactis* produced GABA at differing rates.
4. A strain of *Lactobacillus brevis* was then isolated from a healthy nonagenarian, and qualified as a prominent GABA producer at a pH of 5.0 and 7.0.
5. The human isolated strain had a greater rate of GABA production in comparison to the other *Lactobacillus brevis* strains originating from non-human samples.

Since the candidate human-sourced *Lactobacillus brevis* KS1 was now identified and qualitatively examined, a relative quantification of its GABA production ability was the subsequent objective. The results are the following:

Article 2

6. *Lactobacillus brevis* KS1 displayed a greater GABA yielding capacity at the enzyme's optimal pH of 5.0, as well as at a physiologically-relevant pH level of 7.0 when compared to other potent GABA-producing species found in academic papers such as *Lactobacillus rhamnosus, Lactobacillus helveticus,* and *Lactobacillus paracasei.*
7. At a pH of 5.0, *Lactobacillus brevis* KS1 produced a GABA concentration of 137 mM, or 14.13 g/L, resulting in a 68.5% conversion rate. On the other hand, *Lactobacillus rhamnosus* produced 12.93 g/L (62.7%), *Lactobacillus helveticus* produced 11.93 g/L (57.9%), and *Lactobacillus paracasei* produced 12.89 g/L (62.5%).
8. At a pH of 7.0, *Lactobacillus brevis* KS1 yielded a GABA concentration of 132.6 mM, or 13.67 g/L of GABA, which equates to a drop of 3.2% in conversion rate. Alternatively, *Lactobacillus rhamnosus* experienced a 12.2% decrease in GABA production compared to its yield at pH 5.0. *Lactobacillus helveticus*' GABA production diminished 6.1% and *Lactobacillus paracasei* saw a decrease of 34.8%.
9. *Lactobacillus brevis* KS1 displayed bacterial growth to 496 million CFU/mL and a GABA production of 9.44 g/L after 48h in a simulated small intestinal culture medium, mimicking the pH, temperature and nutriment levels of the duodenum.
10. GABA production of the bacterium rose to 13.43 g/L if the media was supplemented with MSG. *Lactobacillus brevis* KS1 experienced an improved growth to 650 million CFU/mL after 48h in the medium with the prebiotic triphala.

Following the demonstrated proficiency of the candidate *L. brevis* KS1 as a GABA producer under human GI-like *in vitro* conditions, its effects *in vivo,* in an invertebrate model, was the next step. Assessing the effects of this bacterium on sleep in a model with an analogous sleep architecture to humans would serve as a robust preliminary screening

prior to mice studies and eventually, human clinical studies. Administration of *L. brevis* KS1 to fruit flies culminated into the following results:

Article 3

11. *Lactobacillus brevis* KS1 fed to *Drosophila melanogaster* decreased the time required for the fly to fall asleep decreased from 96.91 minutes to 76.22 minutes, a 21.3% decrease.
12. Drosophila spent 29% less time awake during the night if they were administered *Lactobacillus brevis* KS1, an effect comparable to the potent sleep-inducing BZD alprazolam (XANAX).
13. Drosophila fed *Lactobacillus brevis* KS1 also experienced longer sleep duration, whereby their average bout duration went from 71.7 minutes in the untreated group to 97 minutes in the KS1 group.
14. Drosophila fed a low-dose of alprazolam spent an average of 87% of the night sleeping, which closely resembled untreated flies with an 86.6% fraction. *Lactobacillus brevis* KS1-fed Drosophila spent 90.2% of the night in a dormant state, significantly greater than the 0.2% alprazolam treated group, with a sleep fraction of 81.2%.

The aforementioned findings contribute to the growing body of research putting forth *L. brevis* KS1 as a plausible alternative to existing sleep disorder therapies. Future murine and human studies are required to ascertain its mechanism.

Chapter 8: Conclusion

Until date, there has been a void in pharmacological interventions for sleep disorders that are not accompanied with debilitating side-effects. Current drugs often exhibit limited efficacy across the wide range of the disorders' severity and may posit a considerable risk of dependence and addiction. With this in mind, microbiome-based therapies emerge as an interesting alternative to contemporary sleep disorder medications. In the experiments encompassing this thesis, we have adequately demonstrated that the human gut-derived *Lactobacillus brevis* KS1 had a higher rate of GABA production under duodenal-like conditions than its intraspecific and interspecific counterparts. Administration of this *L. brevis in vivo* to *Drosophila melanogaster* led to a reduction in sleep onset latency time (SL) and a decrease in nighttime wakefulness (WASO) when compared to the untreated fly group. Moreover, *L. brevis* KS1 effect on SL and sleep bout length was comparable to the potent sleep-inducing benzodiazepine alprazolam, whereby its effect on WASO and sleep fraction surpassed those of a high-dose benzodiazepine treatment. These results represent a positive progression towards the objective of categorizing L. brevis KS1 as a therapeutic alternative to BZDs.

However, a significant limitation of LAB as potential psychotropic therapeutics is the lack of mechanistic studies that detail their already well-documented effects. Additionally, the multifaceted nature of sleep disorders adds a layer of complexity to elucidating *L. brevis* KS1's mode of action. Consistent with fore-mentioned trends in literature, a future mechanistic study should examine the effect of *L. brevis* KS1 administration on murine models of sleep disorders prior and post-vagotomy. This future study will aim to uncover potential correlations between *L. brevis* KS1-generated GABA, the vagus nerve, and cerebral GABAergic neurotransmission. Regardless, until a definite mechanism of action is established for any purported GABA-producing LAB, their legitimacy as standalone interventions should be questioned.

Microbiome has emerged as one of the key tools to manage complex diseases such as neurological disorders. Recently, various microbiome-based formulations have been approached to potentially modify brain activity through the GBA. L. brevis KS1 appears to be one of the best working in GI conditions and has the potential to be used as probiotic

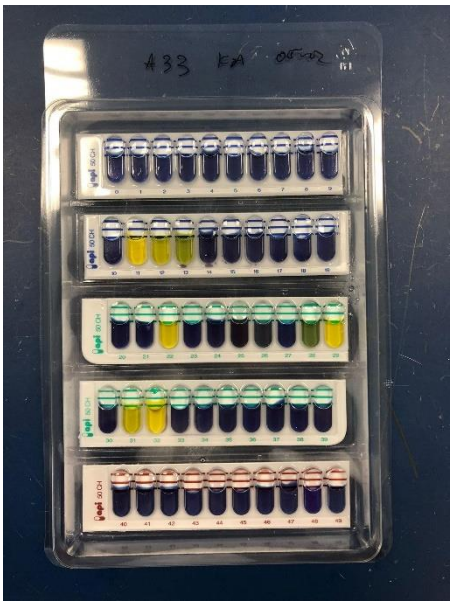
in the treatment and/or prevention of sleep disorders. However, further studies are required in humans and clinical subjects prior one can conclusively confirm these indications.

As long as science around this field remains robust and authoritative, steering away from extravagant claims, the realm of microbiota studies may retain its zeal. Considering that the genetic material of our microbiome outnumbers our human genetic content 10 to 1; that a direct line of communication exists between the gut and the brain, potentially regulating mood via an array of bioactive compounds; and that gut bacteria have been evolutionarily entrenched in their host for the better part of human existence, these microbial factories warrant further investigation in the field of medical research. As the author of the book '*The human superorganism'*, Dr. Rodney Dietert, eloquently poses the question:

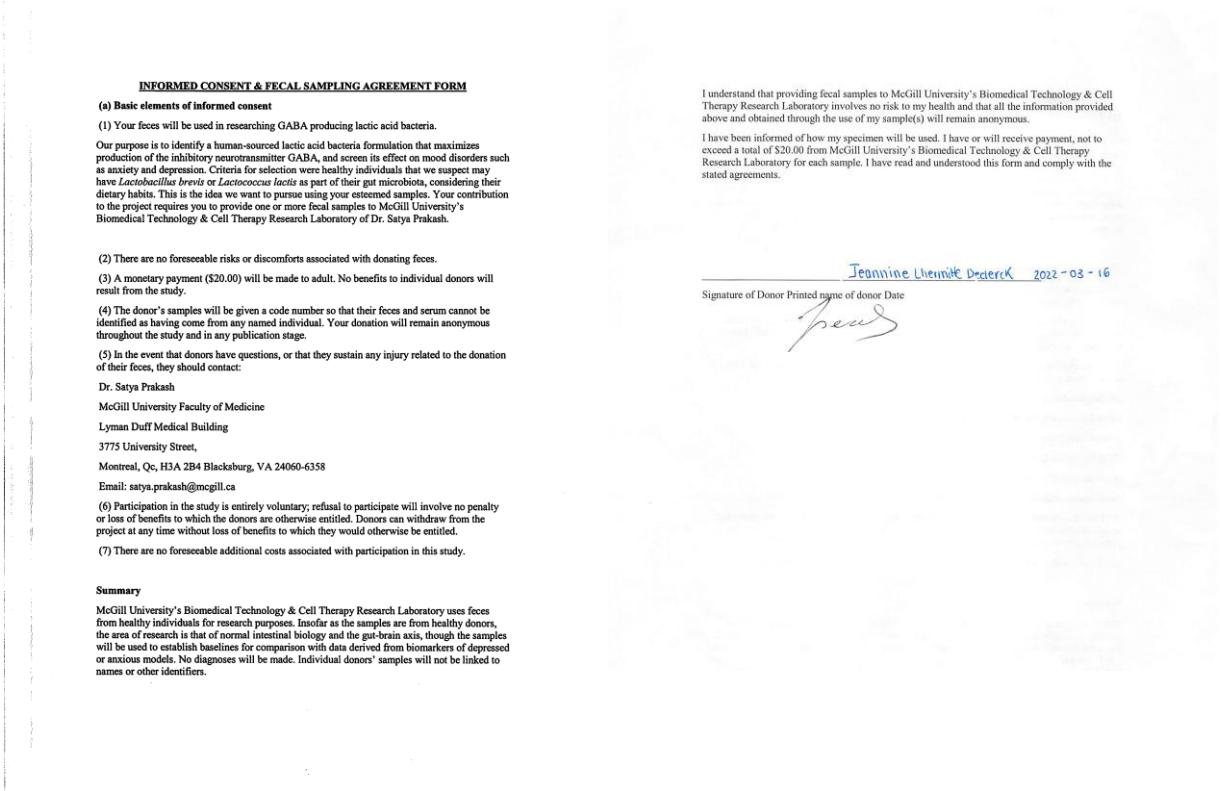
"*Who's in charge? Did humans acquire microbes to enable them to build a better human, or did microbes design a better human as a new and improved vessel for their subsequent generations?* […] *We are a microcosm of earth's species. They virtually encase us, existing both inside and outside of us.*

We are a hybrid: each one of us is a superorganism".

Supplementary Material



Supplementary Material S1: Supplementary material S1: Example of a API 50CH identification assay from Biomérieux



INFORMED CONSENT & FECAL SAMPLING AGREEMENT FORM

(a) Basic elements of informed consent

(1) Your feces will be used in researching GABA producing lactic acid bacteria.

Our purpose is to identify a human-sourced lactic acid bacteria formulation that maximizes production of the inhibitory neurotransmitter GABA, and screen its effect on mood disorders such as anxiety and depression. Criteria for selection were healthy individuals that we suspect may have *Lactobacillus brevis* or *Lactococcus lactis* as part of their gut microbiota, considering their dietary habits. This is the idea we want to pursue using your esteemed samples. Your contribution to the project requires you to provide one or more fecal samples to McGill University's Biomedical Technology & Cell Therapy Research Laboratory of Dr. Satya Prakash.

(2) There are no foreseeable risks or discomforts associated with donating feces.

(3) A monetary payment ($20.00) will be made to adult. No benefits to individual donors will result from the study.

(4) The donor's samples will be given a code number so that their feces and serum cannot be identified as having come from any named individual. Your donation will remain anonymous throughout the study and in any publication stage.

(5) In the event that donors have questions, or that they sustain any injury related to the donation of their feces, they should contact:

Dr. Satya Prakash

McGill University Faculty of Medicine

Lyman Duff Medical Building

3775 University Street,

Montreal, Qc, H3A 2B4 Blacksburg, VA 24060-6358

Email: satya.prakash@mcgill.ca

(6) Participation in the study is entirely voluntary; refusal to participate will involve no penalty or loss of benefits to which the donors are otherwise entitled. Donors can withdraw from the project at any time without loss of benefits to which they would otherwise be entitled.

(7) There are no foreseeable additional costs associated with participation in this study.

Summary

McGill University's Biomedical Technology & Cell Therapy Research Laboratory uses feces from healthy individuals for research purposes. Insofar as the samples are from healthy donors, the area of research is that of normal intestinal biology and the gut-brain axis, though the samples will be used to establish baselines for comparison with data derived from biomarkers of depressed or anxious models. No diagnoses will be made. Individual donors' samples will not be linked to names or other identifiers.

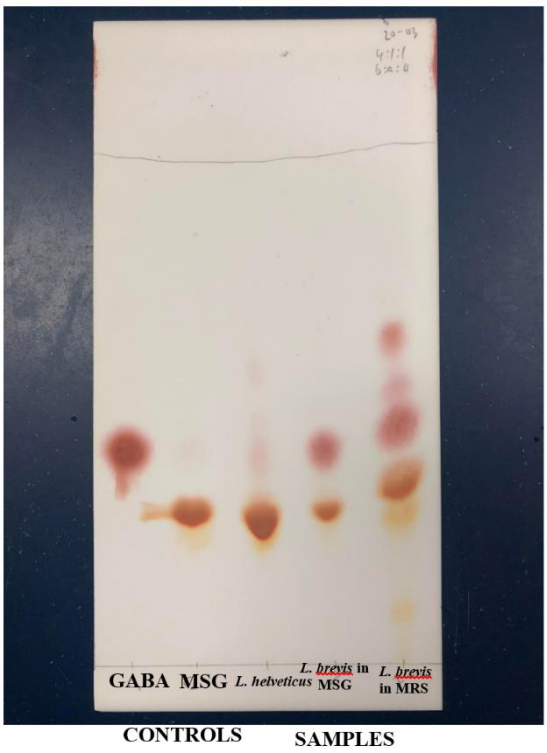
I understand that providing fecal samples to McGill University's Biomedical Technology & Cell Therapy Research Laboratory involves no risk to my health and that all the information provided above and obtained through the use of my sample(s) will remain anonymous.

I have been informed of how my specimen will be used. I have or will receive payment, not to exceed a total of $20.00 from McGill University's Biomedical Technology & Cell Therapy Research Laboratory for each sample. I have read and understood this form and comply with the stated agreements.

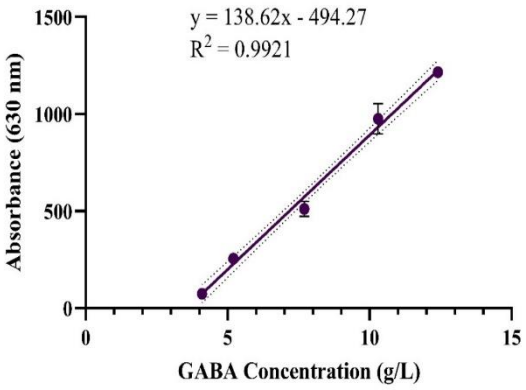
Jeannine Uwimana Dieterick 2022-03-16

Signature of Donor Printed name of donor Date

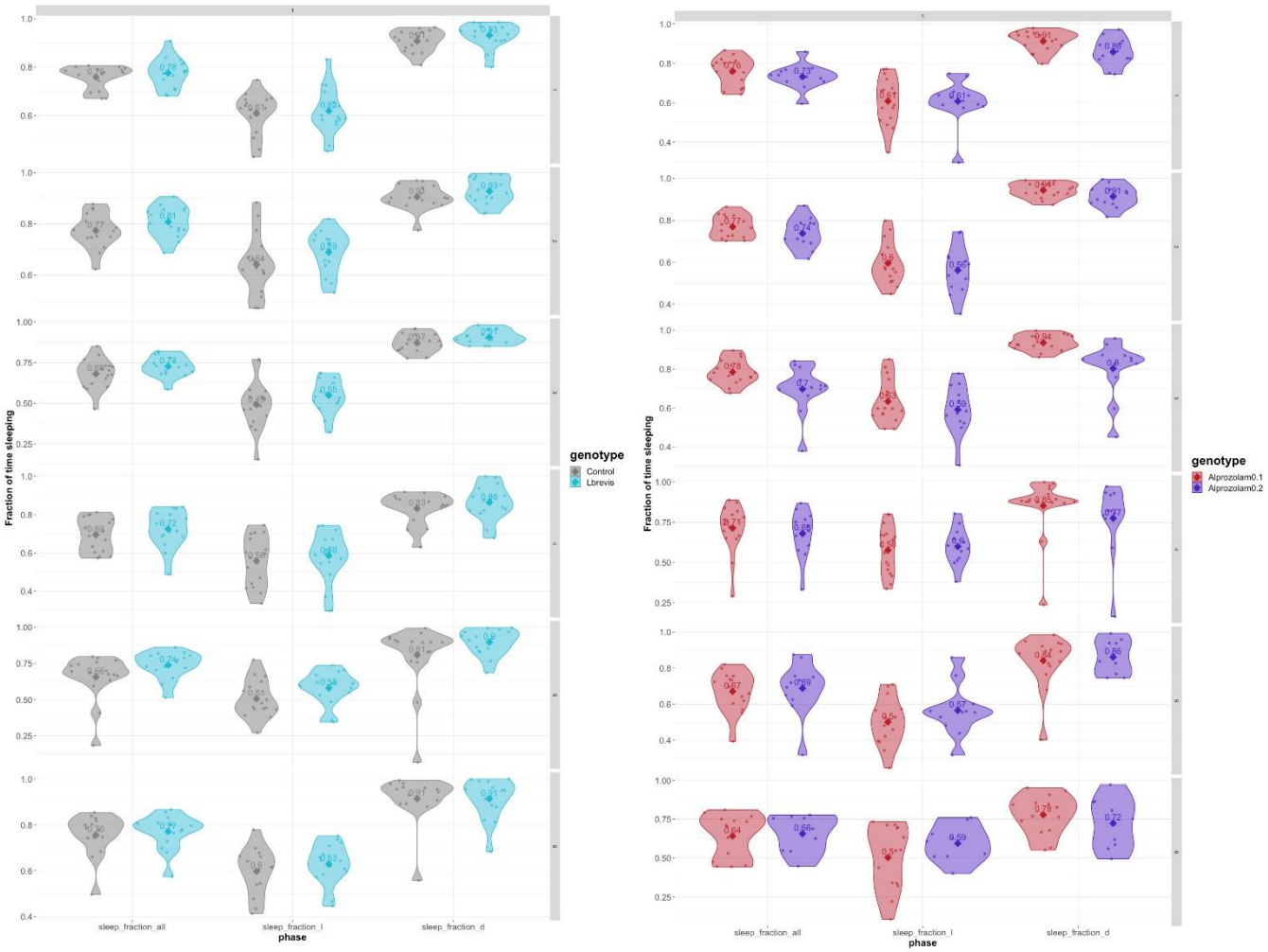
Supplementary material S2: Letter of consent for fecal sample analysis and gut bacterium isolation from nonagenarian



Supplementary material S3: TLC confirmation of GABA production from the human isolated L. brevis.



Supplementary Figure S4: Standard curve of GABA concentration



Supplementary Figure S6 a) Sleep fraction of Probiotic vs untreated group. b) Sleep fraction of Benzodiazepine-treated flies. Figure from the VANESSA-Shiny DAM MATLAB software

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