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Selection of probiotic microorganisms with potential psychobiotic activity

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Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT; 5-hydroxytryptamine

A; Anxiety

AC; Ascending colon

ACC; Acceptance/Coping

Ach; Acetylcholine

AGG; Aggression

ADR; Adrenaline

CNS; Central nervous system

CON; Control/Perfectionism

CBB; CogState Brief Battery

D; Depression

DA; dopamine

DASS-42; Depression Anxiety Stress Scale

DC; Descending colon

DET; Detection performance

E; Epinephrine

ENS; Enteric nervous system

GABA; Gamma-aminobutyric acid

GLU; Glutamate

HOP; Hopelessness

IDN; Identification Test

ISLT: International Shopping List Test

LEIDS-r; Leiden Index of Depression Sensitivity-Revised Test

MSG: Monosodium glutamate

OCL; One card Learning Test

ONB; One Back Test;

RAV; Risk aversion

RCT; randomized controlled trial

RUM; Rumination

S; Stress

SCFA; Short-Chain Fatty Acid

SEC; Social-Emotional Cognition Test

SHIME: Simulator of the Human Intestinal Microbial Ecosystem

TC: Transverse colon

TRP; Tryptophan

NA; Noradrenaline

NE; Norepinephrine

Chapter 1

Overview

1.1 Overview

The gut way to the mental health

Recent years have witnessed the rise of microorganisms inhabiting our gut as a hot topic of research in biology. Variations in the composition of these microorganisms that compose the microbiota in the gastrointestinal tract can affect normal physiology and contribute to the onset of diseases ranging from inflammation to obesity. Now a growing body of literature is slowly unrevealing that the gut microbiota effect can extend beyond the gastrointestinal tract to communicates with the CNS—through vagus nerve, neuro-endocrine and immune pathways—and thereby influences mood and behavior. Dysfunction of these pathways are implicated in stress-related conditions. To this end, we proposed that the gut microbiota, by modulating one of these pathways, plays an influential role in the pathophysiology of stress and cognitive impairment related to chronic exposure to stress. In this regard, we focused on the neural pathway as one of the mechanisms in which the gut microbiota mediates the production of active neurotransmitters that pass from the gut to its target organs, including the brain.

Among the neurotransmitters investigated, GABA is the main inhibitory neurotransmitter in the CNS able to regulate several physiological and psychological functions that will be plenty described in **Chapter 3** and **Chapter 5**. The balance between GABA and his precursor glutamate is essentially important to maintain a stable nervous function, and in this respect we think that understanding the relationship between microbial composition and GABA-producing neurotransmitters can highlight the potential therapeutic role of some microbes which can redirect the microbiome activity towards the neurotransmitters homeostasis in the gut.

In this thesis, we wanted to verify this hypothesis using several approaches. We started in **Chapter 2** by introducing the concept of psychobiotic and the pathways involved in the microbiota-gut-brain axis followed by recent preclinical and clinical interventions investigating the effect of the probiotics on mental health. Further, we selected in **Chapter 3** candidate psychobiotic strains based on their ability to produce neurotransmitters. These results, let us to proceed in **Chapter 4** toward the optimization of the probiotic production in lab and industrial scaling. Afterwards, the stability of the probiotic strains in combination with other active ingredients were assessed to ensure that safety, efficacy, and quality were verified. In **Chapter 5** the psychobiotic activity of the final product was investigate in two in vitro model: the static *in vitro* batch fermentation and the continuous system fermentation, the SHIME. The study aimed to observe the interaction of the dietary supplement with the microbiota to: I) assess the effect of the final product on the composition of the microbiota and II) evaluate the capacity of the strains to higher the concentration of neurotransmitters. The promising findings achieved in this study support the possibility of therapeutic targeting of the gut microbiota in stress-related disorders and encouraged to move forward. Finally, in **Chapter 6**, we attempted to translate the candidate psychobiotic formula to an interventional pilot study in a cohort of healthy volunteers with mild-moderate level of stress. Highlighting the challenge in the translatability of the evidence from *in vitro* to clinical study.

1.1 Sommario

The gut way to the mental health

Gli ultimi anni hanno assistito ad una rivalutazione di microrganismi che abitano il nostro intestino come una principale branca di ricerca nella neurobiologia. Sempre più prove, stanno lentamente svelando che l'effetto del microbiota intestinale può estendersi oltre il tratto gastrointestinale e riuscire a comunicare con il SNC- attraverso diverse vie che coinvolgono il nervo vago, le vie neuroendocrine e immunitarie - e quindi influenzare l'umore e il comportamento. La disfunzione di questi percorsi può essere implicato nello sviluppo di condizioni psicologiche legate allo stress. Abbiamo proposto che il microbiota intestinale, modulando uno di questi percorsi, svolga un ruolo influente nella fisiopatologia dello stress e del deterioramento cognitivo correlato all'esposizione cronica allo stress. A questo proposito, ci siamo concentrati sul pathway neurale come uno dei meccanismi attraverso il quale il microbiota intestinale, mediando la produzione di neurotrasmettitori, riesce a influenzare l'umore le funzioni cerebrali. Tra i neurotrasmettitori studiati, il GABA è il principale neurotrasmettitore inibitore del SNC in grado di regolare diverse funzioni fisiologiche e psicologiche. La produzione di GABA da parte di alcuni porbiotici sarà ampiamente descritto nel **Capitolo 3** e nel **Capitolo 5**. L'equilibrio tra GABA e il suo precursore glutammato è essenziale per il corretto mantenimento del sistema nervoso, e a questo proposito pensiamo che la comprensione della relazione tra composizione microbica e neurotrasmettitori, quali GABA, possa evidenziare il potenziale ruolo terapeutico di alcuni microrganismi nel reindirizzare l'attività del microbioma verso l'omeostasi dei neurotrasmettitori nell'intestino. In questa tesi si è voluto verificare questa ipotesi utilizzando diversi approcci. Abbiamo iniziato nel **Capitolo 2** introducendo il concetto di "psicobiotico" e i percorsi coinvolti nell'asse microbiota-intestino-cervello seguiti da recenti interventi preclinici e clinici che studiano l'effetto dei probiotici sulla salute mentale. Inoltre, abbiamo selezionato nel **Capitolo 3** ceppi psicobiotici candidati in base alla loro capacità di produrre neurotrasmettitori. Questi risultati ci consentono di procedere nel **Capitolo 4** verso l'ottimizzazione della produzione di probiotici in laboratorio e su scala industriale. Successivamente, è stata valutata la stabilità dei ceppi probiotici in combinazione con altri principi attivi per garantire che la sicurezza, l'efficacia e la qualità fossero verificate. Nel **Capitolo 5** l'attività psicobiotica del prodotto finale è stata studiata in due modelli *in vitro*: la fermentazione batch statica *in vitro* e la fermentazione a sistema continuo, lo SHIME. Lo studio mirava ad osservare l'interazione dell'integratore alimentare con il microbiota per: I) valutare l'effetto del prodotto finale sulla composizione del microbiota e II) valutare la capacità dei ceppi di aumentare la concentrazione di neurotrasmettitori. I promettenti risultati raggiunti in questo studio supportano la possibilità di un targeting terapeutico del microbiota intestinale nei disturbi legati allo stress. Infine, nel **Capitolo 6**, abbiamo tentato di validare l'effetto del prodotto formulato con i psicobiotici in uno studio pilota interventistico, reclutando una coorte di volontari sani con un livello di stress lieve-moderato, ed evidenziando la sfida nella traducibilità delle prove dallo studio *in vitro* a quello clinico.

Chapter 2

Psychobiotics, gut microbiota and fermented foods can help preserving mental health

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Literature review

Abstract

Background: Psychobiotics include a novel class of probiotic microorganisms that convey benefit upon the host's mental health via the dynamic microbiota-gut-brain crosstalk. Research is bolstering the concept that gut bacteria are involved in the transmission of information between the gut and the brain, engaging neural, immune, and endocrine pathways. Factors such as diet, stress, and aging can shape the microbiota composition in a process that may also influence the onset and development of mental diseases.

Aim: This review aims to provide an outline of the link between the microbiota and brain function focusing on preclinical and clinical evidence of the potential application of psychobiotics in the context of the cognitive process and performance. The occurrence of metabolic precursors of neurotransmitters in foods that can be converted by the gut microbiota and play a role in the gut-brain axis is discussed.

Conclusion: The understanding of the molecular mechanisms by which communication occurs is still in its infancy; however clinical studies have shown that dietary interventions based on psychobiotics might be a novel nutritional approach targeting gut microbiota for managing cognitive performance and preventing memory decline across the lifespan.

Keywords: cognitive performance, fermented foods, memory, gut-brain axis, gut microbiome, probiotics.

2.1 Introduction

The gut microbiota is emerging as the gatekeeper of host physiology, playing critical roles in human health. Furthermore, mounting evidence supports its role in influencing brain physiology, human behavior and response to stress (Cryan et al., 2019). Probiotic supplementations or probiotic based foods are recommended as an effective dietary strategy in determining host health by modulating the composition and functionality of gut microbiota community (Järbrink-Sehgal & Andreasson, 2020). Moreover, the potential neurotropic activity of some probiotic strains has led to the emerging concept of "psychobiotic" to describe live microorganisms that when ingested in an appropriate amount yield a health benefit in patients suffering from psychological distress via "microbiota-gut-brain axis" (Dinan, Stanton, & Cryan, 2013).

Recently Sarkar and colleagues expanded the concept of psychobiotics to include also prebiotics – the fiber that acts as food for the probiotics and enhance their growth (Sarkar et al., 2016). Many mechanisms are involved in this bidirectional pathway, including neuroimmune and neuroendocrine activation and the production of microbial metabolites (Liu, Feenstra, Heringa, & Huang, 2020). The link between gut microbiota and the brain has raised a plethora of novel questions, some of which regarding the potential application of probiotic strains for affecting cognitive performance and overall brain function in humans. Therefore, the present review provides an overview of the gut-microbiota brain axis and the key pathways involved in message transmission from gut microbiota to the brain. We deal with the role of psychobiotic microorganisms in boosting the attention, learning and memory performance as influenced by diet, stress, aging, sleep disturbance, incorporating recent preclinical and clinical studies. Finally, we discuss the potential use of fermented foods as a delivery vehicle for probiotics in the management of mental health. Mounting evidence indicate psychobiotics can be a nutritional supplement able to improve mental well-being in addition to the physical health.

2.2 The microbiota-gut brain axis

The gut microbiota is composed of trillion of bacteria residing within the gastrointestinal tract (GIT) co-evolved with the human host over thousands of years leading to a symbiotic relationship (Strandwitz, 2018; Valdes, Walter, Segal, & Spector, 2018) . The composition of the human microbiota includes two major bacterial phyla belonging to Firmicutes and Bacteroidetes and other less populous phyla, including Proteobacteria, Actinobacteria,

Fusobacteria and Verrucomicrobia (Rinninella et al., 2019). Although there are high inter-individual variations in the gut microbiota composition, the overall functionality is conserved, being responsible for fundamental physiological activities, including not only intestinal motility, immune regulation, vitamin synthesis, digestion and energy metabolism (Rinninella et al., 2019). More recently a number of other functions in connection with other body organs were ascribed to the gut microbiota suggesting that it may be considered an organ itself (Anwar et al., 2020). The communication between the brain and the gut (or 'second brain', term coined by Michael Gershon in 1998 to refer to the network of nerve surrounding the gut) for the transfer of neural signals informing the brain of the metabolic and physiologic state of the body, has been a subject of investigation for decades (Tan et al., 2020; Wei, Keller, & Li, 2020). Several studies have included gut microbes as a mediator within this context, suggesting that 'microbiota-gut-brain axis' is a more appropriate model (Martin, Osadchiy, Kalani, & Mayer, 2018). The microbiota-gut-brain axis is the entwined communication between the microbiota, the gut and the brain that can occur through multiple systems comprising the central nervous system (CNS), the gut microbiota, the enteric nervous system (ENS), endocrine and immune (including cytokine) pathways (Long-Smith et al., 2020) (**Figure 2.1**). Gut microbiota can be involved in a top-down manner when CNS signals significantly alter the relative abundance of bacteria or in a bottom-up manner, delivering signal to CNS and influencing the brain function (Karl et al., 2018).

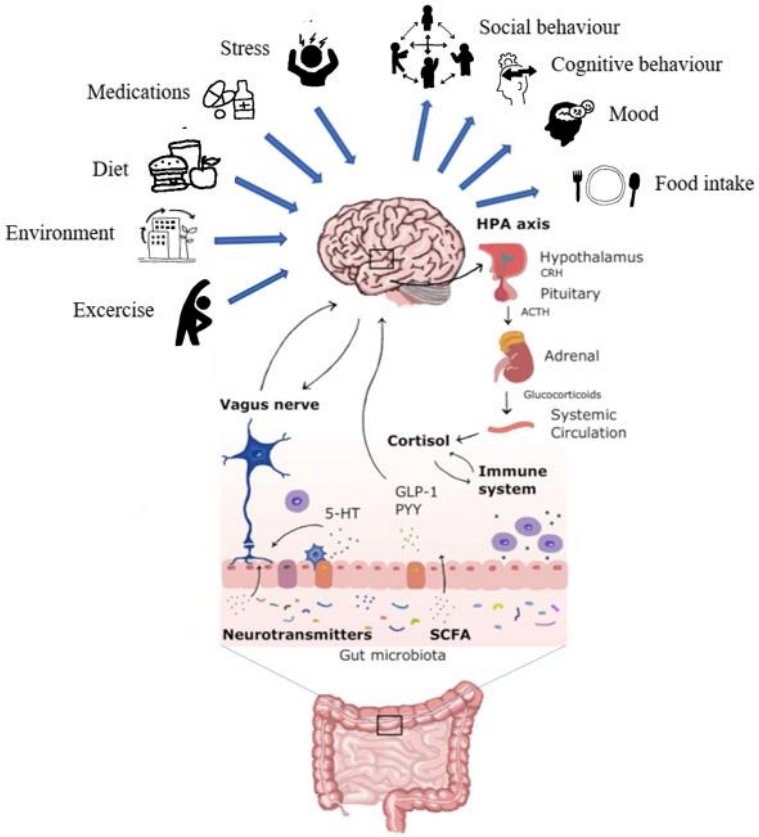

Figure 2.1 Schematic outline of the pathway involved in the bidirectional communication between the gut microbiota and the brain. *Gut microbiota can modulate the gut-brain axis through multiple pathways, that include neural pathway such as vagus nerve; neuroactive pathway, involving microbes able to synthesize neurotransmitters (i.e., gamma-aminobutyric acid, noradrenaline, acetylcholine, dopamine) and neuroactive metabolites (short-chain fatty acids can also modulate brain and behavior); the immune pathway, encompassing the production of cytokines; the hypothalamic-pituitary adrenal (HPA) axis, a key regulator of cortisol production through negative feedback, involved in several mechanisms of adaption to stressors. Cortisol can affect immune cells and cytokine secretion both locally and systemically but can also alter gut permeability and change gut composition under conditions of stress. The brain recruits the same mechanisms to influence the gut microbiota composition. The enterochromaffin cells shown in orange embedded in the intestinal epithelium, are enteroendocrine cells that modulate neuron signaling in the enteric nervous system via the secretion of the neurotransmitter 5-HT. The goblet cells (one represented in violet in the intestinal epithelium) are specialized epithelial cells secreting mucus and creating a protective mucus layer, whose secretion is influenced by the SCFAs. Common factors known to impinge on microbiota-gut brain activity are also shown, including stress, medications, diet, environment, exercise, as well as the various behaviors known to be affected by microbiota gut-brain axis perturbation, including social and cognitive behaviors, mood, and food intake. Abbreviations: CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone; 5-HT, serotonin; EC, enterochromaffin cells; GLP-1, glucagon-like peptide-1, PYY, peptide YY; SCFA, short-chain fatty acid.*

Neuroactive compounds

Gut microbes can regulate the expression of central neurotransmitter and related receptors, but also synthesize a wide range of metabolites such as neurotransmitters and short-chain fatty acids (SCFAs) (Cryan et al., 2019; Martin et al., 2018). A neuroactive substance is a chemical compound synthesized by a neuron which influence the properties of other neurons and muscle

cells. Many neuroactive compounds have significant roles as neurotransmitters, neuromodulators, and neurohormones (Zieger, Candiani, Garbarino, Croce, & Schubert, 2018). Most of the common neurotransmitters in the human brain such as Gamma-aminobutyric acid (GABA), acetylcholine, serotonin and other monoamines can be produced by gut microbes. *In vitro* studies showed strains of *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* species can produce serotonin; strains of *Bacillus* and *Serratia* species can produce dopamine; *Escherichia*, *Bacillus*, and *Saccharomyces* species can produce noradrenaline; the *Lactobacillus* species can produce acetylcholine; *Lactobacillus* and *Bifidobacterium* species can produce GABA, the main inhibitory neurotransmitter in the CNS (Strandwitz, 2018).

Recently, GABA- producing lactobacilli have raised considerable attention due to the potential therapeutic applications in metabolic as well as behavioral outcomes in mice models (Patterson et al., 2019). These findings suggest that psychobiotics can influence the brain function through the regulation of the neurotransmitter concentration (Del Toro-Barbosa, Hurtado-Romero, Garcia-Amezquita, & García-Cayuela, 2020). In ***Table 2.1*** are represented many of these host and microbial-derived neuroactive molecules that are also important signaling molecules in the intestinal interface. For example, 95% of the body's serotonin is produced by enterochromaffin cells of the GI tract, and it is associated with regulation of GI secretion and motility as well as regulation of cognition and mood in brain pathways (Mittal et al., 2017; Yaghoubfar et al., 2020).

Table 2.1 *Representative list of neurotransmitters produced from bacteria within the human gut, precursors, and their regulatory function.*

Neuroactive compound	Precursors	Genus	Regulatory function	Ref.
GABA	Glutamate	*Lactobacillus, Bifidobacterium Bacteroides*	Stress responsiveness Anxiety and depressive-like behaviour Mood	Altaib et al. 2021; *Microorganisms* Otaru et al. 2021; *Frontiers in Microbiology* Yunes et al. 2016; *Anaerobe*
Acetylcholine	Choline	*Lactobacillus, Bacillus*	Encoding of new memories Influences synaptic transmission Induces synaptic plasticity	Özogul 2011; *International Journal of Food Science and Technology* Wiedeman et al. 2018; *Nutrients*
Dopamine	Tyrosine	*Escherichia, Bacillus, Lactococcus, Lactobacillus, Streptococcus, Enterococcus*	Reward-motivated behavior Motivational decision-making	Shishov et al. 2009; *Applied Biochemistry and Microbiology* Villageliú and Lyte 2018; *PLoS ONE*
Serotonin	Tryptophan	*Streptococcus, Escherichia, Enterococcus, Lactococcus, Lactobacillus*	Modulation of intestinal secretion and motility Brain development Regulation of mood Stress reactivity	Özogul 2011; *International Journal of Food Science and Technology* Shishov et al. 2009; *Applied Biochemistry and Microbiology*

SCFA

Other bacterial metabolites with neuroactive properties are the short- chain fatty acids (SCFAs). Acetate, butyrate and propionate are the main SCFAs produced from bacterial fermentation of dietary fiber in the colon in a ratio 3:1:1, and all three metabolites are detectable with an average concentration of 17.0 pmol/mg of tissue for butyrate and 18.8 pmol/mg of tissue for propionate in the human brain (Bachmann, Colombo, & Berüter, 1979). SCFAs can modulate several physiological processes (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019) **(Figure 2.2).**

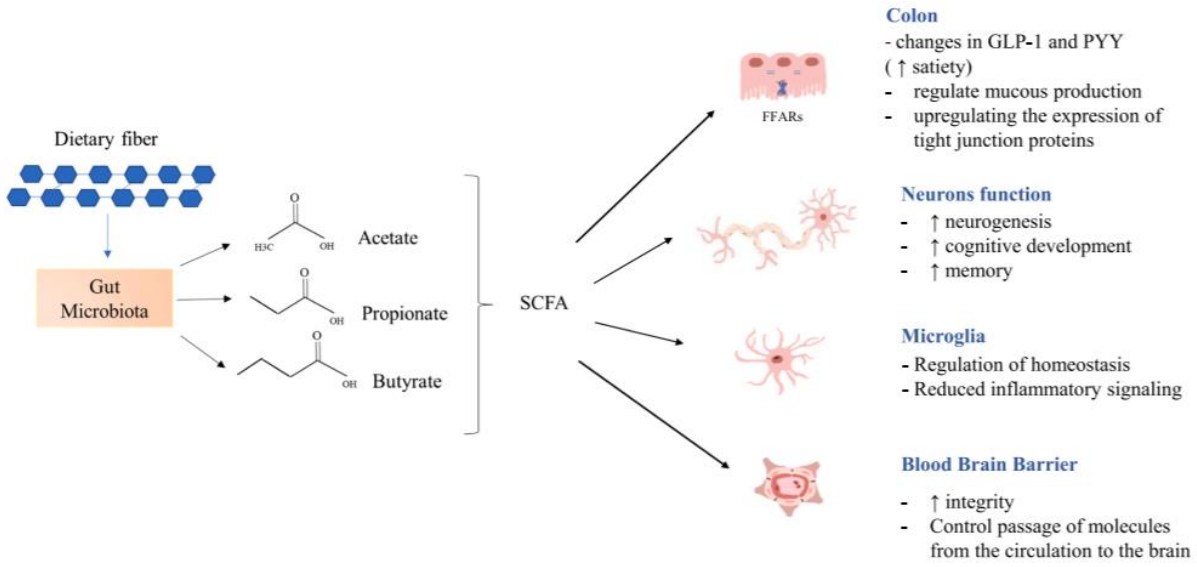

Figure 2.2 Microbiota-gut-brain pathway through which SCFAs might modulate brain function. *Fermentation of complex carbohydrates via interaction of fermentable dietary fiber, probiotics, and prebiotics contribute to increasing the concentration of short-chain fatty acid (SCFAs). SCFAs can act locally in the gut by influencing intestinal mucosal immunity and enhancing barrier integrity by upregulating the expression of thigh junction proteins. They can also modulate processes associate with mood, memory, and neural function, promoting indirect signaling to the brain via the systemic circulation or vagal pathways by inducing the secretion of gut hormones such as glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) from the enteroendocrine L cells. Through interaction with receptors on colonocytes, as free fatty acid receptors (FFARs) the SCFAs can directly activate vagal afferents. Peripherally, SCFAs influence neuroinflammation by affecting microglia, thereby potentially influencing emotion, cognition, and pathophysiology of mental disorders. Finally, the SCFAs can cross the blood–brain barrier (BBB) via transporters located on the endothelial cells and influence BBB integrity.*

Kimura-Todani et al.(2020), investigated the anxiolytic effects of acetate in mice, using special diets containing acylated starches that can reach the colon without being absorbed in the upper gastrointestinal tract (Kimura-Todani et al., 2020). This study showed decrease in anxiety- like behaviors on mice significantly associated with the cecal acetate contents when evaluated by marble-burying (MB) and plus-maze tests. Butyrate is the principal energy source for colonocytes and it can influence the gut-brain axis by upregulating the expression of tight junction proteins, whereby enhancing intestinal barrier integrity (Heyck & Ibarra, 2019).

SCFAs can also regulate mucous production in the gastrointestinal tract, decreasing the interaction between the epithelial cells and luminal microorganisms and toxic agents (van de Wouw et al., 2018). Via the endocrine pathway, SCFAs interaction with their receptors on colonocytes (FFARs) promotes indirect signaling to the brain via systemic circulation or vagal pathways by inducing the secretion of gut hormone, such as glucagon like peptide 1 (GLP1) ad peptide YY (PYY) from the enteroendocrine L cells, which can influence learning, memory, and mood (Dalile et al., 2019). Peripherally, butyrate may also act as an epigenetic modulator promoting brain-derived neurotrophic factor (BDNF) transcription linked to memory consolidation, especially in the hippocampus (van de Wouw et al., 2018). The decrement of this neurotrophic factor in neuroinflammation and aging, suggests that the maintenance of its adequate concentrations could potentially help to preclude or delay the onset of cognitive impairment (Silva, Bernardi, & Frozza, 2020). In a preclinical study, administration of SCFA to germ-free mice (GF), such as propionic acid, has demonstrated to regulate microglia homeostasis, the tissue macrophages of the brain, crucial for host defense and proper brain development (Erny et al., 2015). These findings suggest that host bacteria product or metabolites, such as SCFA are key molecules that vitally regulate microglia maturation and function, and morphology (Generoso, Giridharan, Lee, & Macedo, 2021).

Vagus nerve

Despite the exact molecular signaling has not been elucidated yet, it is clear these microbial products can influence the CNS affecting epithelial cells of the gut barrier, enteroendocrine cells to release hormones, and dendritic cells to modulate immune and microglia function, which play a fundamental role in ageing and neurological impairments (Ochoa-Repáraz, Ramelow, & Kasper, 2020). The vagus nerve is the principal component of the parasympathetic division of the autonomic nervous system. As mixed nerve is composed of 80% afferent nerve fibers (input) that can transfer the gut information to the SNC and 20% efferent nerve fibers (output) that project to the smooth muscle of the gut (Bonaz, Bazin, & Pellissier, 2018).

It is a main route for the signaling between the gut and the caudal nucleus of the solitary tract (cNST), located in the brainstem and involved in brain circuits dedicated to reward and pleasure (Tan et al., 2020). Indeed, the necessity of an intact vagal nerve for information transfer regarding intestinal microbial status has been also demonstrated in the landmark preclinical study of Bravo and coworkers (Bravo et al., 2011). They found that probiotic modulation of the gut microbiota with *L. rhamnosus* (JB-1) attenuated stress-induced corticosterone levels and anxiety-like behaviours, while damaging the integrity of the vagus nerve, by means of

vagotomy, abolished the effects observed in these studies. Despite the importance of vagal pathways, it must be noted that vagus nerve does not project into the lumen and its activation is partly mediated from the secretion of chemical signals produced by enteroendocrine cells (EECs) (Foster, Rinaman, & Cryan, 2017). These cells, which account for 1 % of the total intestinal epithelial cells, can detect signals from microbiota (such as SCFAs) through toll-like receptors (TLR) and regulate GI motility, secretion, food intake and interaction with receptor expressed on the afferent fibers of the vagal nerve. However, vagal afferents can be also directly activated from SCFAs via FFARs, thereby signaling to the brain (van de Wouw et al., 2018).

HPA and immune system

Commensal microbiota is also closely connected to the function and development of the hypothalamus-pituitary-adrenal (HPA) axis, the neuroendocrine core of stress system that coordinates the response of the organism to stressor of any type, turning in the release of corticotropin-releasing hormone (ACTH) form pituitary and cortisol from the adrenal gland (Frankiensztajn, Elliott, & Koren, 2020).

In dysbiosis condition, pathogenic live bacteria or bacterial components such as endotoxins, lipopolysaccharide (LPS) can induce paracellular permeability, pass the compromised intestinal epithelium leading to endotoxemia with low-grade immune reaction, and chronical releasing of the proinflammatory cytokine (IL-1, IL-6 and TNF- α), using humoral or vagal pathways to convey a message to the brain (Schoultz & Keita, 2020). The signal reaching the brain arises a neural cascade , leading to *de novo* synthesis of central proinflammatory cytokines. Importantly, stressors also induce the release of local inflammatory mediators which in turn can activate vagal afferents driving central effects express receptors for cytokines and induce central immune activation (Bonaz et al., 2018; Zheng et al., 2017).

Considering that inflammatory cytokines can influence the onset and progression of several neurodegenerative mood disorders, and the IL-1β rs16944 SNP is related to high cytokine levels and potentially affects mood disorders, a recent clinical study has examined the combined effect of IL-1β polymorphism and probiotic administration in mood disorder phenotypes in the Italian population, suggesting through their results the potential genetic association studies for psychobiotic-personalized therapy (Gualtieri et al., 2020).

Another study also found changes in the balance of HPA-axis-related receptors in the brain of stressed mice, which could be normalized through the intake of *B. breve* CCFM1025 (Tian et al., 2020). In this preclinical study, it was shown that chronic stress impaired the negative

feedback of corticosterone in the HPA axis, by down-regulating the glucocorticoid receptors (NR3C1), leading to glucocorticoids resistance, also coincident with a high level of inflammation.

Interestingly, *B. breve* CCFM1025 intervention normalized the stress-induced expression of brain *Nr3c1* in mice, as well as serum proinflammatory cytokine measures and in turn led to the alleviation of depression-related behaviors (Tian et al., 2020). Evidence for a link between microbiota and HPA axis have been also gathered from experiments carried out in microbiota depleted models, either through treatment with antibiotics or use of germ-free (GF) mice, demonstrating that stress response and certain altered brain developments could be partially resolved/improved when new born animals were reconstituted with a diverse and intact flora (Cryan et al., 2019).

Gut microbiota and cognitive impairment

Cognitive impairment is also associated to age, thus prevalent among the elderly. Treating the increasing worldwide cognitive impairment has important implications for dementia prevention. Aging is associated with decrease in microbial diversity that is important for health maintenance in the elderly (Kong, Deng, Li, & Zhao, 2019). Furthermore, increased inflammatory status, along with a reduction in the capacity to cope with stress, point at the possible contribution of the gut microbiota to an 'inflamm-ageing' process (Cryan & Dinan, 2012).

Studies in a model of middle-aged rats have been instrumental in showing the probiotic impact on cognitive decline through modulation of the microbiota-gut-brain axis. Some research groups have demonstrated a neuroprotective role of some probiotics such as *B. lactis*, *L. casei*, *B. bifidum*, and *L. acidophilus*, on aging-related cognitive dysfunction in clinical and preclinical studies (Akbari et al., 2016). Yang and colleagues expanded on this work and investigated the effects and underlying mechanism of the combination of these strains, showing that recovery from dysbiosis and inflammation led to the improvement of cognitive dysfunction in senile mice (Yang, Yu, Xue, Li, & Du, 2020). The study of Corpuz *et al*., had also taken into consideration that diet supplementation of *L. paracasei* K71 could prevent age-dependent cognitive decline in the SAMP8 mice model by upregulation of BDNF expression in the hippocampus (Corpuz et al., 2018). In another pre-clinical study, *L. johnsonii* BS15 was assessed for its psychoactive effect against memory dysfunction in mice induced by

psychological stress, by modulating intestinal inflammation and permeability (Hesong Wang et al., 2021)

Sleep is shown as an important factor that affects a person's psychological state, and whose deprivation is correlated with the risk of memory loss and decline in attention performance. A possible role of *Lactobacillus* and *Bifidobacterium* in sleep and stress response of the patients has resulted by a negative correlation between *Lactobacillus* counts and sleep and that between *Bifidobacterium* counts and serum cortisol levels (Aizawa et al., 2019). The study of the positive effect of probiotics on the improvement of psychological well-being by ameliorating aspects of sleep quality has been also recently shown (Marotta et al., 2019).

Only recently, the work of Hulme and colleagues have supported the existence of microbiome-mitochondria cross-talk within the host, giving the first mechanistic description of two novel gut microbiome-derived carnitine mimics, inhibiting the function of the mitochondria in cells of the CNS. They identified 3-methyl-4-(trimethylammonio) butanoate and 4-(trimethylammonio) pentanoate, as structural analogs of carnitine that are produced by anaerobic commensal bacteria from the Lachnospiraceae family and are present in both gut and white matter of the murine brain. These microbial metabolites inhibiting the crucial mediation of carnitine in the fatty acid oxidation (FAO) prevent the cell brain function to meet their high energy requirements, leading to neurological conditions. These findings, indicate that mental conditions, where mitochondrial dysfunction has been noted, as well as disturbance in the gut microbiome, should increase the emphasis on the potential role of microbiome as therapeutic target for mental diseases (Hulme et al., 2020).

2.3 Effect of psychobiotics on human cognitive process

The prospect of targeting microbiota to beneficially impact the brain function is appealing, and psychobiotics are emerging as a promising strategy in this field. To date, the literature on psychobiotics is mainly based on preclinical studies. However, pilot studies in healthy subjects demonstrated that specific probiotic strains could influence symptoms of stress-related gut-brain disorder and alter resting brain activity, cognitive performance and memory. The effects of probiotics and stress on cognition might share common pathways of action such as activation of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis.

Ten recent research articles were found where the influence of different gut microbiota intervention based on probiotics was evaluated on human brain structures/ function and cognition. Full details of these studies can be seen in **Table 2.2**.

Positive effects on cognition was observed in all apart from two studies (Kelly et al., 2017; Slykerman et al., 2018). These contrasting findings suggest that the psychobiotic performance can be species- and strain-dependent, which represents an important challenge in biotechnological selection of the strains and their further use in both preclinical and clinical testing. In all the study significant medical side effects were not observed. Five studies employed a single-species probiotic intervention (Chong et al., 2019; Kelly et al., 2017; Kobayashi, Kuhara, Oki, & Xiao, 2019; Lew et al., 2019; Huiying Wang, Braun, Murphy, & Enck, 2019). Five studies implemented multi-species probiotic interventions and included bacteria *Bifidobacteria, Lactococcus, Lactobacilli* or *Streptococcus* genera (Bagga et al., 2018; Inoue et al., 2018; Nobile & Puoci, 2021; Papalini et al., 2019; Slykerman et al., 2018).

Table 2.2 Clinical use of probiotics on mood and cognitive performance

Intervention	Study design	Duration of intervention	Outcomes	Ref.
(Ecologic®825) *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Bifidobacterium lactis* W51, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, *Bifidobacterium lactis* W52, *Lactobacillus plantarum* W62 and *Bifidobacterium bifidum* W23 (7.5×10^6 CFU/g). Daily doses containing 3 gr freeze dried powder	45 healthy adults in a double-blind, randomized study	4 weeks	Change in functional connectivity (using task-based fMRI) but no change in structural connectivity.	Bagga et al., 2018; *Gut Microbes*
Lactobacillus plantarum DR7 (1×10^9 CFU/day)	111 stressed adults, 18–30 years old in a double-blind, randomized, placebo-controlled study	12 weeks	Improved speed parameters for basic attention (probiotic: 2.70 ± 0.08; placebo: 2.75 ± 0.07; P = 0.034), social emotional cognition (probiotic: 3.43 ± 0.12; placebo: 3.59 ± 0.11; P30 years old benefited the younger adult's population via enhancing verbal learning and memory upon administration of DR7 compared to placebo over 12 weeks (probiotic: 27.08 ± 4.05; placebo: 24.70 ± 5.13; P = 0.066)	Chong H.X. et al., 2019; *Beneficial Microbes*
Bifidobacterium supplementation (1.25×10^{10} CFU *Bifidobacterium longum* subsp. *longum* BB536, *B. longum subsp. infantis* M−63, *Bifidobacterium breve* M−16 V and B. breve B-3)	38 healthy older adults, 66–78 years old in a double-blind, randomized, placebo-controlled trial	12 weeks	Probiotic had no significant effect on general cognitive function (MoCA-J scores). Low improvement in cognitive function regarding the flanker test, the change between groups of the correct response number was significantly different (0.35 ± 0.9 vs − 0.29 ± 1.1, P = 0.056)	Inoue et al., 2018; *Beneficial Microbes*
Lactobacillus rhamnosus (JB-1) (1×10^9 CFU)	29 healthy adults, 20–33 years in a double-blind, randomized, placebo-controlled, cross-over design	8 weeks (4 weeks treatment, no washout, 4 weeks switched treatment	No significant improvement of probiotic over placebo on cognitive	Kelly et al., 2017; *Brain, Behaviour, and Immunity*
Bifidobacterium breve A1 (>2.0×10^{10})	121 elderly subjects with memory complaints, 50–80 years old in a double-blind, randomized, placebo-controlled trial	12 weeks	Subjects with low RBANS total score at baseline (score < 41) have shown difference between the twogroups regarding the scores of the 'immediate memory' subscale (probiotic: 3.08 ± 13.10; placebo: −4.22 ± 11.62; P = 0.041). The total MMSE score in the *B. breve* A1 group significantly increased compared with the placebo group (probiotic: 1.93 ± 1.80; placebo: 0.65 ± 2.15; P = 0.027)	Kobayashi et al. 2019; *Beneficial Microbes*
Lactobacillus plantarum P8 (10log daily)	103 healthy stressed adults, mean age of 31.7 ± 11.1, in a double-blind, randomized, placebo-controlled trial	12 weeks	Stress reducing potential as compared to placebo (mean difference 2.94; P = 0.048) by DASS-42 questionnaire; speed for social emotion cognition (probiotic: 3.48 ± 0.04; placebo: 3.56 ± 0.04; P 22.17 ± 4,03; P = 0.079) in men	Lew et al., 2019; *Clinical Nutrition*

(continued on next page)

Intervention	Study design	Duration of intervention	Outcomes	Ref.
L. reuteri PBS072 and *B. breve* BB077 (2 × 10^9 CFU)	30 stressed students Proof of concept clinical trial	28 days	Short-Term Memory Test: improvement of correct answers in terms of the number of reached words, of almost 28% and a reduction of omitted words of 41%, during the study period ($p < 0.001$). Problem-Solving Flexibility: The neuropsychological test Wisconsin Card Sorting test (WCST) has been used to measure the ability to show cognitive flexibility in decision making processes. The latency between one answer and the following was significant with $p < 0.001$ and was reduced by 21% from the beginning of the trial. The Divided Attentional Performance Test: The test aim to measure the capacity to focus attention on two different irregular stimuli correct answers showed that correct answers significantly improved up to almost 12% with respect to the baseline ($p < 0.001$).	Nobile et,. 2021; Neuropsychobiology
(Ecologic®Barrier) *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and, *Lactococcus lactis* W58 (total cell count of 2.5 × 10^9 CFU/g, i.e. 5 × 10^9 CFU per day)	58 healthy adults, 18–40 years old in a double-blind, randomized, placebo-controlled trial	4 weeks	Pre vs Post stress working memory (probiotic 4.1, $p = 0.053$; placebo o < 1, $p = 0.36$) No effects in relative neutral situations	Papalini et al., 2019; *Neurobiology of Stress*
Lactobacillus rhamnosus HN001 (6 × 10^9CFU/day), *Bifidobacterium animalis* subsp.*lactis* HN019 (9 × 10^9 CFU/day)	474 children at risk of developing allergic disease in a double-blind, randomized, placebo-controlled trial	2 years. The effect on cognitive, behavioural and mood outcomes at age 11 years is a post-randomisation secondary outcome	Overall no significant differences in the neurocognitive outcomes between the treatment group	Slykerman et al., 2018; *Acta Pediatrica*
B. longum 1714TM (1x10^9 CFU/day) Zenflore	40 healthy adults during social stress, 31–33 years old, in a double-blind, randomized, parallel-group trial	4 weeks	Brain activity was measured using magnetoenecephalography.Correlation with enhanced vitality and reduced mental fatigue. Affects brain function through modulating neural oscillation which may be involved in the counter-regulation of negative emotions	Wang et al., 2019; *The American Journal of Gastroenterology*

Single-Species Probiotic Interventions

Randomized, double-blind, placebo-controlled trial with single species interventions ranged from 1 x 10^9 to 2 x 10^{10} cfu/dose/day for a duration of 4 or 12 weeks has been carried out to elucidate if single putative psychobiotic strain has psychotropic activity in humans and if a study conducted in healthy volunteers can replicate the beneficial effects on mental health observed in preclinical findings. *B. breve* A1 supplementation studied in elderly subjects with memory complaints, demonstrated beneficial effect on the cognitive impairment showing findings consistent with those reported in mice (Kobayashi et al., 2019).

Different results were observed in another clinical trial in which neither an effect of probiotic treatment on measures of sleep quality, anxiety and memory performance nor significant anti-inflammatory effects were found in humans to confirm the effect previously observed in animal models (Kelly et al., 2017). Consistent with the results reported in mice was the study of Lew et al. (2019) in which 12 weeks of intervention (daily; 2 × 10^{10}) with *Lactobacillus plantarum* P8 exhibited a stress-reducing potential together with improved cognition and memory by

contrasting inflammation (reduction of pro-inflammatory cytokines such as IFN- γ and TNF-α), and supporting the link between inflammation and brain pathophysiology. Furthermore, men outperformed women in verbal memory tasks, most probably attributed to the higher tendency of externalization symptoms in men (Lew et al., 2019). *L. plantarum* DR7 in stressed adults with the same duration of intervention showed improved cognitive and memory function, such as attention, emotional cognition and associate learning compared to the placebo group (Chong et al., 2019).

Multi-Species Probiotic Interventions

Some clinical studies have evaluated the state of inflammation, stress and cognitive outcomes in healthy subjects after the use of 'polybiotics', that means the use of more species and strains to have a synergic power effect of the probiotics (Bambury, Sandhu, Cryan, & Dinan, 2018). Bagga et al. (2018) showed that functional connectivity, under magnetic resonance imaging (MRI) setup, is sensitive to 4-week multi-strain probiotic administration. A significant observation in this study was a change in the probiotic-treated group of the middle and superior frontal gyrus network (MFGN) which plays a key role in orienting of spatial attention, decision-making and cognitive control (Bagga et al., 2018). Papalini et al. (2019) , have investigated the effect of a multispecies/strains probiotic preparation (Ecologic Barrier) on neurocognitive measures of emotion regulation and cognitive control using functional magnetic resonance imaging (fMRI) (Papalini et al., 2019). In a double-blind, randomized, placebo-controlled study on fifty-eight women participants was demonstrated that without stress induction, probiotics did not affect the brain. However, compared to placebo, the probiotics group did show a significant improvement in working memory performance under challenging situations inducted from physical and psychological acute stress conditions –i.e. socially evaluated cold pressor test and neutral and socially distant behavior adopted by the researcher - buffering against the effects of stress on cognition (Papalini et al., 2019). A significant improvement of cognitive functions, in terms of short-term memory, attention, and executive performance, as well as stress-related parameters were observed after 28 days of product intake of *L. reuteri* PBS072 and *B. breve* BB077 in a group of 30 stressed students (Nobile & Puoci, 2021). Therefore, the possibility that potential probiotic effects on cognition could exist only as a consequence of an increased stress condition at baseline should be taken into account (Inoue et al., 2018; Kobayashi et al., 2019).

2.4 Benefits of foods fermented by psychobiotics on mental health

Fermented foods are generally defined as foods and beverages made through enzymatic conversions of major and minor food components by microorganisms (Marco et al., 2017). Food is fermented primarily for preservation, but fermentation also induces changes in flavour, aroma and texture providing the product with desired characteristics (Aslam et al., 2020; Melini, Melini, Luziatelli, Ficca, & Ruzzi, 2019). Despite the ancient origins, it is only recently that the fermented foods and beverages are also considered as potential functional foods, due to additional health benefits beyond nutrition, such as increase digestibility, reduce risk of impaired glucose metabolism, reduction of blood pressure and total cholesterol, immunity boosting and maintenance of intestinal balance, by promoting intestinal barrier integrity (Marco et al., 2017). Furthermore, some probiotic lactic acid bacteria (LAB) may exert health-promoting activity even if inactivated, through microbial metabolites or components of bacteria cell walls released in the matrix (De Filippis, Pasolli, & Ercolini, 2020).

Many studies concur that the potential health effects of fermented foods can be attributed to the LAB of which they are potential source that can be delivered to the gut and become part of the gut microbiota (Pasolli et al., 2020). In this regards the use of metagenomics can be of importance for the identification of microbial genes and pathways leading to the production of metabolites associated with the health related activities (De Filippis, Parente, & Ercolini, 2018). As the variety of new types of fermented food have increased, some of them have been studied for their activity on the brain functionality including fermented milk, soybean, algae, and the potential biological effects of each fermented food are widely distinct. The specific effects could vary on the basis of the raw materials used, the method of fermentation or the

microorganisms involved. The most recent findings revealing the correlation between probiotics delivered through fermented foods and mental health are summarized in **Table 2.3**.

Table 2.3. Preclinical study and clinical study of fermented foods and mental health. *Abbreviations, BDNF: Brain derived neurotrophic factor, SOD: superoxide dismutase*

Fermented food	Probiotic involved	Sample	Time (treatment)	Outcomes	Ref.
GABA-rich fermented milk	*Lactobacillus brevis* DL-11	mice	1 month	Relieved anxiety and improved sleep quality, that may be associated with significant increases of SCFAs in the intestine and related to changes in the composition of the gut microbiota	Yu et al., 2020; *Microbiological research*
Black carrots	*Lactobacillus plantarum* FBLP	Type 2 diabetic rats with dementia	8 weeks	Better cognitive function by preventing hippocampal insulin resistance associated with lower amyloid-β deposition	Park et al., 2016; *Journal of functional foods*
Clinical outcomes					
Fermented food	**Probiotic involved**	**Sample**	**Time (treatment)**	**Outcomes**	**Ref.**
Yogurt	*Lactobacillus acidophilus* LA5 and *Bifidobacterium lactis* BB12 with a total of min 1 × 10^7 CFU	Petrochemical workers	6 weeks	Positive effect on general mental health	Mohammadi et al., 2016; *Nutritional Neuroscience*
Soybean	*Lactobacillus plantarum* C29 (DW2009)	Mild cognitive impairment individuals	12 weeks	Cognitive improvement associated with changes in serum BDNF level	Hwang et al., 2019; *Nutrients*
Saccharina japonica algae	*Lactobacillus brevis* BJ20	Healthy participants	4 weeks	Changes in memory ability via regulation of SOD antioxidant system	Jung Park et al., 2019; *European Journal of Integrative Medicine*
Milk drink containing lactononadecapeptide	*Lactobacillus helveticus* CM4	Healthy middle-aged adults	8 weeks	Improvement in attention and memory	Ohsawa et al., 2018; *International Journal of Food Sciences and Nutrition*

Potential probiotic strains are selected based on their established health benefits, how a strain can tolerate the harshness of the gastrointestinal tract, have safety aspects, and technological properties as withstand during manufacturing, processing, storage and transport of the food products, to guarantee a satisfactory level of viability at the time of consumption. Withstand the harshness of the gastrointestinal tract and adhesion ability to the intestines are classical criteria for potential probiotic bacteria to promote beneficial effect acting also as barrier to pathogens and toxins by competing for host cell binding sites and colonize the intestine. Compounds with prebiotic properties provide substrates for the metabolism of the probiotics. Examples of supplemented prebiotic formulation include carbohydrate-based compounds such as fructo-oligosaccharides (FOS) and inulin as well as polyphenols and polyunsaturated fatty acids. Williams et al (2016) demonstrated prebiotic fiber improved cognitive flexibility in animal models and could influence biochemical pathways involved in cognitive performance including brain-derived neurotropic factor. In the specific field of psychobiotics, several studies have reported the chemical changes such as enrichment in neuroactive peptides due to the fermentation process with a probiotic starter.

LAB species such as *S. thermophilus, L. brevis, L. paracasei, L. fitsaii, L. plantarum* and *B. adolescentis*, have shown the potential to increase the bioavailability of GABA in fermented food products, from which they are commonly isolated, such as koumiss, kimchi, zlatar cheese (Cui, Miao, Niyaphorn, & Qu, 2020). This is due mainly to the α-decarboxylation of l-glutamic

acid to GABA and CO2, catalyzed by glutamate decarboxylase (GAD), and the reaction requires pyridoxal- 5′-phosphate (PLP) as a cofactor (Otaru et al., 2021). Shekh *et al.*, identified and characterized *L. plantarum* strains isolated from various fruits and fermented foods which are safe and able to produce GABA, endorsing their potential as starter cultures in the manufacturing of functional fermented food products (Shekh, Dave, & Vyas, 2016). Glutamic acid decarboxylase has also been identified in yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* isolated from fermented products (Perpetuini, Tittarelli, Battistelli, Suzzi, & Tofalo, 2020; Zhang et al., 2020).

In one study the GABA production level achieved with the coculture *L. plantarum* and *S. cerevisiae* (2.42 g/L) had highest ability to produce GABA was higher than that obtained by fermentation with single culture *L. plantarum* or *S. cerevisiae* (1.45 g/L and 1.03 g/L, respectively) (Zhang et al., 2020). The coculture process with yeast, which excretes specific amino acids and small peptides and/or produces vitamins, in this study improved the microbial production of GABA by promoting the LAB growth. GABA acts as main inhibitor neurotransmitter, and it can promote relaxation and reduce anxiety (Banerjee et al., 2021). Indeed, in the last years, the food industry has shown interest in the utilization and mass production of GABA as a bioactive food compound (Hagi, Kobayashi, & Nomura, 2016; Sanchart, Rattanaporn, Haltrich, Phukpattaranont, & Maneerat, 2016). Novel functional beverages have been developed through fermentation by several strains of LAB of coconut water, mix cherry-kefir, and other residues from the food processing, offering sustainable solutions for the increasing commercial demand for GABA in food industry (Gharehyakheh, 2021; Hasegawa, Yamane, Funato, Yoshida, & Sambongi, 2018; Kantachote, Ratanaburee, Hayisama-ae, Sukhoom, & Nunkaew, 2017). Attempts have also been made to understand the effect of dietary GABA increased by incorporation of LAB in fermented food on the brain (Yu et al., 2020, Ratajczak et al., 2019). Yu and coworkers (2020) investigated the effect of milk fermented with the GABA-producing *L. brevis* DL1-11 (Yu et al., 2020). The effects were evaluated in mice, finding that the group treated with high-dose GABA fermented milk showed a significant increment of some SCFAs, such as butyric acid, and prolonged sleep duration. Furthermore, a change in the β diversity after administration of high-dose GABA fermented milk indicated that this treatment changed the composition of the gut microbiota in mice, towards potential SCFA-producing bacteria (Ratajczak et al., 2019).

L. brevis BJ20 fermented *Saccharina japonica* algae (FSJ) was evaluated on neurocognitive function in healthy participants, finding changes in memory ability and providing preliminary

evidence for the FSJ to influence brain activity via regulation of antioxidant activity of SOD (superoxide dismutase), which influences synaptic plasticity, learning and memory (Park, Kang, Jeong, Jeong, & Kim, 2016). In the study of Hwang et al. (2019), one hundred individuals with MCI were randomly assigned to take *L. plantarum* C29- fermented soybean (DW2009) (800 mg/day, n = 50) or placebo (800 mg/day, n = 50) for 12 weeks to investigate the effect on cognitive functions related to memory and attention (Hwang et al., 2019). Compared to placebo group, the DW2009 group showed a greater cognitive improvements associated with increased serum BDNF levels ($t = 2.83$, $p = 0.007$). (Hwang et al., 2019). This was preclinically anticipated with a cognitive enhancement by *L. plantarum* C29 in mice with TNBS-induced memory impairment (Lee, Jeong, Han, & Kim, 2018).

LAB are the most studied GABA producers, because they are usually employed as starters and they are able to generate higher concentration of GABA levels than other microbial groups. However, other microorganims such as the mould *Aspergillus oryzae* used to ferment rice-*koji* fermentation for brewing *sake*, can generate significant amounts of GABA. (Ando & Nakamura, 2016). Long consumption of black carrots fermented with *L. plantarum* (FBLP) and *Aspergillus oryzae* (FBAO) extract by type 2 diabetic rats with dementia, has demonstrated the efficacy of both on improving cognitive function by preventing insulin resistance and hippocampus amyloid- ß accumulation (Park et al., 2016). Indeed, the brain plays an important role in not only cognitive function but also energy and glucose metabolism (Coll & Yeo, 2013). In this respect, it is important to sustain brain insulin signaling to improve energy but also cognitive function. In a randomized, double-blind controlled study in healthy middle-aged adults a *L. helveticus*-fermented milk containing lactononadecapeptide showed improvement in attention and delayed memory score (Ohsawa, Nakamura, Uchida, Mizuno, & Yokogoshi, 2018). In a randomized, double-blind, placebo-controlled trial, the effects of a probiotic yogurt and multispecies probiotic capsule supplementation were investigated on petrochemical workers, who showed improved mental health parameters (Mohammadi et al., 2016). Overall, these results indicate that dietary habits and regular consumption of functional fermented foods may provide health benefits via gut microbiota-gut-brain axis.

2.5 Conclusions

Given the rapidly growing understanding of the microbiota role in the crosstalk between gut and brain, this axis an appealing target for improving the mental health. Psychobiotics, as a special class of probiotics, differ from conventional probiotics in their ability to produce or

stimulate the production of neurotransmitters, short-chain fatty acids, enteroendocrine hormones and anti-inflammatory cytokines. Therefore, sustaining cognitive function through nutritional supplementations based on psychobiotics is a fascinating concept underpinned now by mounting evidence. The beneficial effects of probiotics on cognitive outcomes can be explained both by competitive exclusion of deleterious gut pathogens, decreases in pro-inflammatory cytokines and communication with the CNS leading to changes in neurotransmitter levels or function. Major skepticism is warranted regarding a clinically relevant effect of such results of human emotions, cognition, and behavior, some of which could be underpowered for short-term analysis, confounders, differing sampling, and participants-selection-bias. Further studies should focus on the elucidation of therapeutic effect of psychobiotics in different subsets of subjects, to understand their mechanism of action, whether neurological responses require continued usage or are durable after cessation of treatment. Moreover, given the wide range of brain regions reported to change in response to probiotics, there is the possibility of psychological effects not captured by questionnaires used as tools to evaluate the mood and the cognitive performance. The potential use of fermented foods as source of psychobiotics or metabolic precursors of microbial production of neuroactive compounds is gaining popularity among consumers for its possible therapeutic and high marketing value. And especially, microorganism-based GABA production in fermented food has immense prospects. Finally, considering the effects of probiotics on the CNS functions as strain-specific, more clinical trials should be implemented in order to evaluate the synergic effect of psychobiotic strains as well as their possible coadministration with other psychotropics. This research hold the potential to yield novel microbial therapies that leverage the microbiota-gut-brain axis to shape behavior, emotion and cognition.

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Chapter 3

Selection of probiotic microorganisms for their potential psychobiotic activity

Abstract

Background: Probiotics are live microorganisms that upon ingestion in sufficient concentrations, confer health benefits to the host. There are ongoing studies on the identification of probiotic strains for novel potential application in the modulation and improvement of mood, stress response, and anxiety signs, by interacting in the gut-brain axis.

Aim: The goal of this work was to assess the psychobiotic properties of probiotic strains, by investigating their ability to produce neuroactive molecules such as GABA, serotonin, acetylcholine, and catecholamine. Through the production of neuroactive molecules, the strains should be able to modulate the connection between the colon and brain, in the also called gut-brain axis.

Materials and methods: We studied 13 LAB strains, belonging to the species *Levilactobacillus brevis, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Ligilactobacillus salivarius, Streptococcus thermophilus*, and characterized *in vitro* neuroactive properties of probiotic strains by using LC-MS/MS for the analysis of neuroactive compounds in the medium of growth supplemented with the precursors of the strains.

Results: These results support the evidence of *Levilactobacillus brevis* strains as a higher producer of GABA when optimal culture conditions were set up, while strains of *Lactiplantibacillus plantarum* showed lower ability to produce GABA than *Levilactobacillus brevis* but higher than other strains to produce acetylcholine. No production of other neurotransmitters has been observed.

Conclusion: Our findings add to the current body of evidence suggesting that some probiotics from the genus Lactobacillus may exert psychobiotic potential and introduce a more selective overview of potential candidates in the prevention or as a therapeutic adjuvant in the treatment of mental disorders

***Keywords**: neurotransmitters, microbiota-gut-brain axis, psychobiotic screening*

3.1 Introduction

Neuroactive molecules (NM) are a class of endogenous molecules that play an important role in transmitting signals from presynaptic neurons to postsynaptic neurons, binding receptors present on the membrane of the target cells. Based on their composition, neurotransmitters can be classified into indoleamines, which are mainly produced by the hydroxylation of aromatic amino acids in the cell body of neurons and include 5-hydroxytryptamine (5-HT) and tryptophan (TRP); catecholamines, a type of monoamine, derived from aromatic amino acids, e.g. (hydroxylation of tyrosine), including dopamine (DA), norepinephrine (NE) (noradrenaline; NA), epinephrine (E) (adrenaline; ADR); ACh synthesized from choline and acetyl-CoA under the catalysis of choline acetyltransferase; amino acids include gamma-aminobutyric acid (GABA), glutamate (Glu) (Brennenstuhl et al. 2019; Akyuz et al. 2021)

Abnormal alteration of neurotransmitters has been found to be closely associated with many mood-related diseases like depression, anxiety, and neurological diseases including Alzheimer's disease, Parkinsonism. Excessively increased glutamate, the excitatory amino acid, in the prefrontal cortex is closely related to the etiology of stress-induced physiological and behavioral impairments (Joffe et al. 2019). On the other hand, GABA, which is the main inhibitory neurotransmitter produced by the decarboxylation of glutamate can promote relaxation, reduce anxiety, enhance memory and improve sleep (Yu et al. 2020). Analysis of fecal GABA and glutamate levels from 77 participants demonstrated a negative correlation between both neurotransmitters, indicating that the microbiome was actively involved in converting the available glutamate in the gut to GABA (Altaib et al. 2021). Patients with major depressive disorder (MDD) have reduced synthesis of serotonin (5-hydroxytryptamine, 5-HT), harboring a microbiota distinct from that of healthy controls (Averina et al. 2020).

The gut microbiota in healthy adults consists of more than 1×10^{14} microbe cells and it is 90% represented by the phyla *Firmicutes* (including *Lactobacillus, Enterococcus, and Clostridium* genera) and *Bacteroidetes* (including *Bacteroides* genus), as well as *Actinobacteria* and *Proteobacteria* (Vaga et al. 2020). Preclinical and clinical studies have demonstrated that the gut microbiota affects the nervous system by mediating inflammation, the hypothalamic–pituitary–adrenal axis, and neurotransmitter regulation, affecting behavior, mood and cognitive outcomes. Neurotransmitters such as serotonin and gamma-aminobutyric acid (GABA), that are involved in biological pathway in bacteria, as survival in hostile condition or growth factor for some other bacteria. For example Strandwitz et al. (2019) described the ability of KLE1738, a Gram-positive bacterium of the *Ruminococcaceae* family, to use GABA as a growth-supporting nutrient, and a genome analysis supported a GABA-dependent metabolism.

Intestinal bacteria imbalance has been observed in diseases outside the digestive system. Several studies have demonstrated in depression an overabundance of potentially harmful and inflammatory bacteria belonging to the phylum of *Actinobacteria*, bacteria of *Bacteroides*

genus, and bacteria of the family *Enterobacteriaceae* combined with a decrease in beneficial bacteria such as *Faecalibacterium,* and *Firmicutes* in genera (Huang et al. 2019). The clinical study of Chen et al. (2019) showed a greater enrichment in *Escherichia–Shigella* and *Bacteroides* in a Chinese population with generally anxiety disorders (GAD), highlighting again the relationship between the presence of pathogens in the gut and anxiety. Altaib et al. (2021) analyzed the relationship between microbial composition and the levels of the fecal neurotransmitters, GABA and glutamate, founding a positive association between the abundance of *Bifidobacteriaceae, Enterococcaceae,* and *Streptococcaceae*, and fecal GABA concentrations.

Psychobiotics are a class of probiotics, that when ingested in appropriate amount are able to influence normal healthy brain function, through interaction with commensal gut bacteria. As probiotics they have to possess desirable functional properties in vitro and in vivo such as resistance and survival in gastric acidity and intestinal bile salts apart from other digestive enzymes, ability to adhere and colonize intestinal mucus layer and produce antimicrobial substances. Lactic acid bacteria (LAB) are the most commonly used probiotics and in recent years, numerous scientific investigations have been report to assess psychobiotic potential. Some of them focused on the isolation and characterization of these microorganisms for their psychobiotic potential through neurotransmitters production. To date, GABA-production has been mainly studied in LAB as well as in several *Bifidobacterium* spp. (Duranti et al. 2020) primarily for the development of probiotics and GABA-containing fermented food with health benefits on mood, cognition and sleep quality (Marotta et al. 2019; Wu et al. 2018; Wu et al. 2017). Recently, Kanklai et al. (2021) in a screening of 364 LAB strains isolated from Kimchi, discovered seven of them with GABA-producing capability and six of these strains were identified as *Levilactobacillus brevis*. *Lactiplantibacillus plantarum* has been demonstrated to produce serotonin by Özogul (2011) and to produce acetylcholine by Stanaszek, Snell, and Neill (1977). Acetylcholine has been detected also in a traditional sweet Japanese beverage (*koji* amazake) by lactic acid fermentation using *L. sakei* (Oguro et al. 2017). Other evaluation methods consist of preclinical and clinical studies, applied to observe the effect of the psychobiotics on microbiota composition or on behavior and mood disorders such as anxiety and stress (Del Toro-Barbosa et al. 2020).

Preclinical and clinical studies could facilitate the demonstration of these bidirectional communication, but their interpretation is hampered by a lack of dedicated reference able to answer the question of how to find such strains and which criteria to use for their selection.

The aim of this article is to extend our current knowledge about potential probiotics with beneficial effect on psychological well-being by targeting the microbiota composition. We investigate the ability of 13 lactic acid bacteria to produce neuro active compound, by using growth conditions that could induce the biological reaction. These strains are previously selected for their potential probiotic activity. These results may be used to compose a formula

with commercially available probiotic strains as food supplement formulation to influence cognitive outcomes in healthy subject by balancing the neurotransmitter-mediated response.

3.2 Materials and Methods

Bacterial Strains and reagents

11 probiotic strains selected for testing their psychobiotic activity belong to the culture collection of the PROGE FARM industry and was chosen according to the guidelines of FAO/WHO (2002). The strains *Streptococcus thermophilus* NCIMB 702392 and *Lactiplantibacillus plantarum* ATCC14917 were obtained respectively to NCIMB and DSMZ culture collection. De Man Rogosa and Sharpe (MRS) and M17 were purchased from Oxoid, while all the other chemicals including precursors, l-cysteine-HCl, pyridoxal – 5'- phosphate (PLP) were purchased from Sigma- Aldrich.

Screening of neurotransmitters -producing probiotics and cultivation conditions

13 *Lactobacillus* strains were inoculated and grown in De Man, Rogosa and Sharpe (MRS, Oxoid) broth at 37°C, while 2 *Streptococcus thermophilus* strains in M17 broth at 40°C. A fresh overnight culture of the strains were individually subcultured (1%, v/v) in MRS or M17 medium at their optimal temperature, supplemented with an amino-acid precursor of the neurotransmitter. In order to do that at the substrate was added 0.05% (w/v) l-cysteine-HCl, and for each modified substrate one precursor among monosodium glutamate (60 mM), for 16h and 48h, tyrosine (1,3 mM), choline (48mM) and tryptophan (39 mM) for 48h, respectively for inducing GABA, catecholamine, acetylcholine and serotonin production. The cofactor pyridoxal – 5'- phosphate was added in a concentration of 0.25 mM only in the growth media with tryptophan and tyrosine in order to facilitate the decarboxylation reaction. The growth of the strains was conducted under aerobiotic condition in order to permit the activity of the hydroxylase, enzyme limiting the reaction in the production of the catecholamine and serotonin. To observe the bacterial growth in the modified substrates, serial dilutions are carried out on the samples taken after 16 and 48 hours. Because the decarboxylation of glutamate plays a central role in acid stress resistance among lactic acid bacteria, measure of the pH in supernatant of MRS broth containing MSG at 24h and 48h, has given a first indication of the activity of GAD, the enzyme involved in the conversion of the glutamate in GABA. The KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database has been used via the KEGG WWW server (http://www.genome.ad.jp/kegg/) to predict the metabolic pathways that involve enzymatic reactions of chemical compounds used as precursors for the production of the neurotransmitters for each genus investigated.

Neurotransmitters analysis by LC-MS/MS

Cells were removed by centrifugation (4000 rpm, 4°C, 10 min), the supernatant was filtered with 0.22um RC filter for the detection of glutamate, GABA, acetylcholine, choline, tyrosine

and catecholamine and i-Phree SPE for the analysis of serotonin and tryptophan. 100ul of cell-free supernatants were eluted 1:1000 in acetonitrile 50% for detection of glutamate, GABA, tryptophan, serotonin, tyrosine, catecholamine, and in 90% acetonitrile for the detection of acetylcholine and choline. LC-MS/MS was run on an API 2000 triple quadrupole mass spectrometer (ABSciex, Carls- bad, CA). These compounds were separated on a TSKgel Amide-80 column (250mm x 2.0 mm, 5um, Tosoh Bioscence, Tokyo, Japan). The mobile phase consisting of acetonitrile 50% (Solvent A) and 0.1% formic acid in acetonitrile 50% (Solvent B) with the following elution profile (t in [min]/[%B]): (0.0/90), (4.0/70), (10.0/20), (13.0/20), (15.0/90) and (18.0/90). Detection of acetylcholine and choline was performed on the column Luna 3um HILIC Phenomenex by using the following phase B: Ammonium formate 10 mM and formic acid 0.125% in water, pH 3; phase A: 90% Acetonitrile and 10% of solvent B. Detection of tryptophan and serotonin was performed on phase A: 90% acetonitrile and 10% ammonium acetate (50mM) and phase B: 10% acetonitrile and 10%. Finally for the detection of tyrosine and catecholamine the mobile phase consisting of acetonitrile 50% (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). MS data was collected for 18 mins. The flow rate was set at 0.7 mL/min and the column temperature at 40°C. A calibration curve was built in the range 1ppm to 5ppb for all the compounds.

2.4 Data Analysis, Visualization, and Statistical Analysis

Data were analyzed and visualized using R software (v3.6.3) and SPSS. Statistical analysis was performed by one-way ANOVA, including Tukey's test to correct for multiple comparison. Significance level was set to $p < 0.05$.

3.3 Results

Selection of GABA-producing strains

Initial loads of probiotic microorganisms on all media used were around 10^8 CFU/ml. Increase of probiotic growth has been observed in the substrate enriched with monosodium glutamate, reaching 10^9 CFU/ml in the media after 48h. Our work interestingly indicates that on 12 strains tested, all showed at least a low activity of GABA production. *Levilactobacillus brevis* strains synthesize the highest amount of GABA reaching 77 mM and a conversion rate of 89%. *Lactiplantibacillus plantarum* strains showed a conversion rate of Glu to GABA of 2.48%, even if they used 71.63% of the Glu in the medium. GABA concentration was observed to be dependent on the substrate and the time. The amount of GABA produced within the same strain was less in the substrate with no addition of MSG, reaching the maximum concentration of 12,55 mM after 48 hours in *Levilactobacillus brevis* P30021. Furthermore, the concentration of GABA increased along the time, as it is observed in the Figure 3.1 when comparing the GABA concentration at 16 hours and 48 hours. The raise in GABA concentration was more evident in the substrate enriched in MSG. Data about the production of GABA are showed in ***Figure 3.1*** and ***3.2***

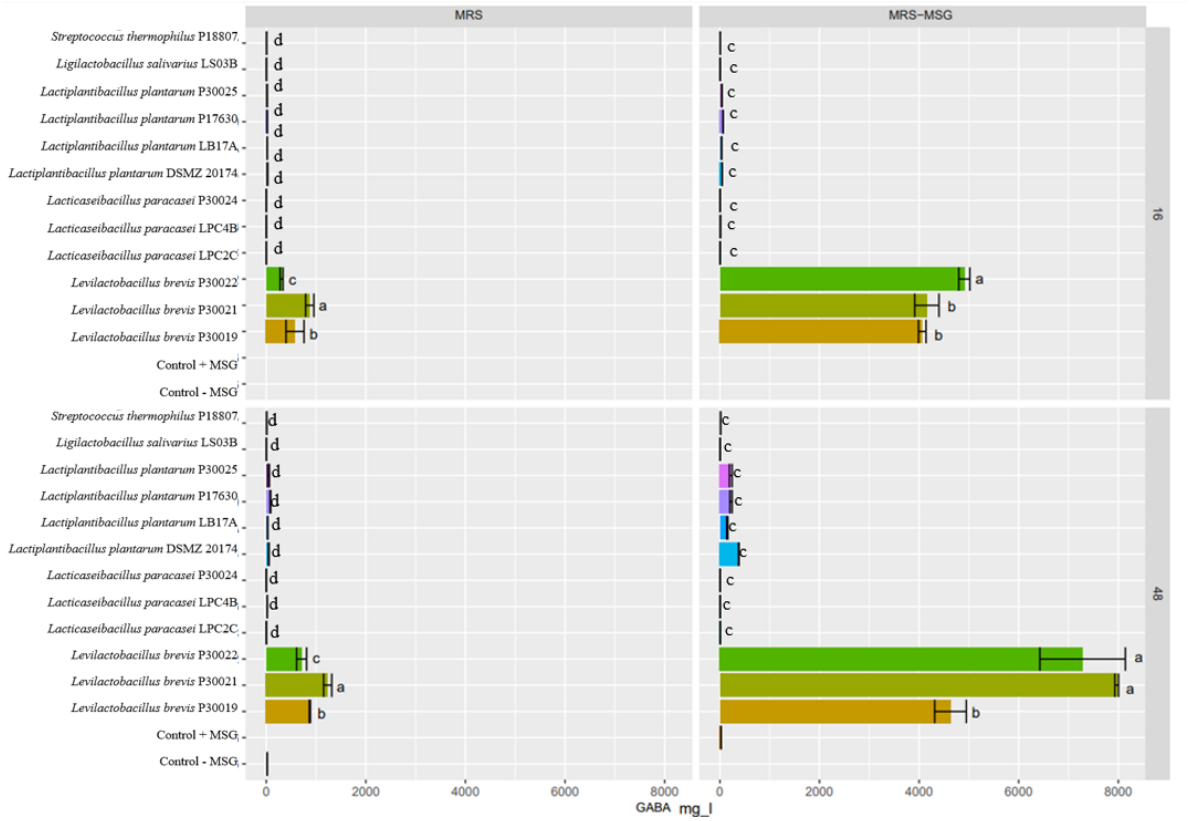

Figure 3.1. Concentration of GABA produced by the strains at 16 and 48 hours*. Concentration of GABA produced by the strains at 16 and 48 hours in MRS substrate and MRS enriched with MSG 1%. Data are mean ± standard deviation values of two determinations. Data in a column followed by the same letter are not significantly different (P < 0.05). Control with the addition of MSG (+MSG) and without the addition of MSG (-MSG) did not show production of GABA*

Effect of MSG on pH and cell growth

The presence of MSG is a key factor in producing GABA. Twelve probiotic strains were incubated in MRS broth and modified MRS (1% MSG) for 48 hours. The production of lactic acid in the LAB fermentation process led to the decrease of pH value. Indeed, as shown in **Table 3.1**, pH rapidly decrease from about 5.6 to 3.2-3.4 after 16 hours and increase again until 4.8-5.2 after 48 hours in the media of bacteria with highest GAD activity. After 48 h of incubation, the pH value of inoculated MRS broth with MSG was higher than that without the addition of MSG. *Levilactobacillus brevis* strains showed an increase in when inoculated in MRS+MSG. No difference of pH among the two media used were observed after 16 h. *Levilactobacillus brevis* strains were diluted and inoculated on MRS plates with 1% MSG, however there was no significant difference in the CFU growth on MRS plates and those growth on MSG addition (data no showed).

***Table 3.1**. pH value of MRS (blank) and in MRS containing 1% MSG after 16h and 48h from the inoculation of strains*

	16 h		**48 h**	
Strains	MRS+MSG	MRS	MRS+MSG	MRS
Streptococcus thermophilus P18807	$3,8 \pm 0,01^{a}$	$3,8 \pm 0,01^{a}$	$4,4 \pm 0,06^{b}$	$4,4 \pm 0,13^{a}$
Levilactobacillus brevis P30019	$3,3 \pm 0,03^{a}$	$3,5 \pm 0,06^{a}$	$5,2 \pm 0,02^{a}$	$4,4 \pm 0,01^{a}$
Levilactobacillus brevis P30022	$3,2 \pm 0,05^{a}$	$3,1 \pm 0,05^{a}$	$5,1 \pm 0,01^{a}$	$4,4 \pm 0,07^{a}$
Levilactobacillus brevis P30021	$3,2 \pm 0,01^{a}$	$3,5 \pm 0,01^{a}$	$5,0 \pm 0,13^{a}$	$4,1 \pm 0,43^{a}$
Lactiplantibacillus plantarum LB17A	$3,4 \pm 0,01^{a}$	$3,4 \pm 0,01^{a}$	$3,9 \pm 0,05^{b}$	$3,8 \pm 0,01^{a}$
Lactiplantibacillus plantarum P30025	$3,3 \pm 0,02^{a}$	$3,3 \pm 0,02^{a}$	$4,0 \pm 0,04^{b}$	$3,8 \pm 0,08^{a}$
Ligilactobacillus salivarius LS03B	$3,3 \pm 0,02^{a}$	$3,6 \pm 0,02^{a}$	$4, 0 \pm 0,03^{b}$	$3,8 \pm 0,10^{a}$
Lacticaseibacillus paracasei LPC4B	$3,3 \pm 0,02^{a}$	$3,5 \pm 0,02^{a}$	$4,0 \pm 0,10^{b}$	$3,7 \pm 0,14^{a}$
Lacticaseibacillus paracasei P30024	$3,2 \pm 0,01^{a}$	$3,5 \pm 0,01^{a}$	$3,9 \pm 0,01^{b}$	$3,8 \pm 0,08^{a}$
Lacticaseibacillus paracasei LPC2C	$3,4 \pm 0,03^{a}$	$3,5 \pm 0,03^{a}$	$4,0 \pm 0,16^{b}$	$3,8 \pm 0,18^{a}$
Lactiplantibacillus plantarum P17630	$3,4 \pm 0,06^{a}$	$3,4 \pm 0,06^{a}$	$4,0 \pm 0,05^{b}$	$3,8 \pm 0,01^{a}$
***Lactiplantibacillus plantarum DSMZ* 20174**	$3,3 \pm 0,04^{a}$	$3,4 \pm 0,04^{a}$	$4,0 \pm 0,02^{b}$	$3,8 \pm 0,03^{a}$

Note: The experimental values (means ± standard deviations for n = 2) that have no common superscript are significantly different ($p < 0.05$) according to the Tuckey test. Any two means not marked by the same superscript (for example a and b) are significantly different. Any two means marked by the same superscript (for example a and a or b and b) are not significantly different

Selection of probiotic producer of acetylcholine, catecholamine and serotonin and quantification

Lactiplantibacillus plantarum strains have showed better ability to produce acetylcholine (0,26-0,35 mM), which was also produced in lower amount also by *Levilactobacillus brevis* strains (0,02- 0,06 mM). Dopamine was discerned in *Levilactobacillus brevis* strains (range 10-20 uM), and not tyrosine for these strains was detected. NE (Norepinephrine) and E (Epinephrine) have not been produced by the strains. Neurotransmitters have not been detected in the substrate without inoculation. ***Figure 3.2*** summarizes the results obtained from 13 LAB strains.

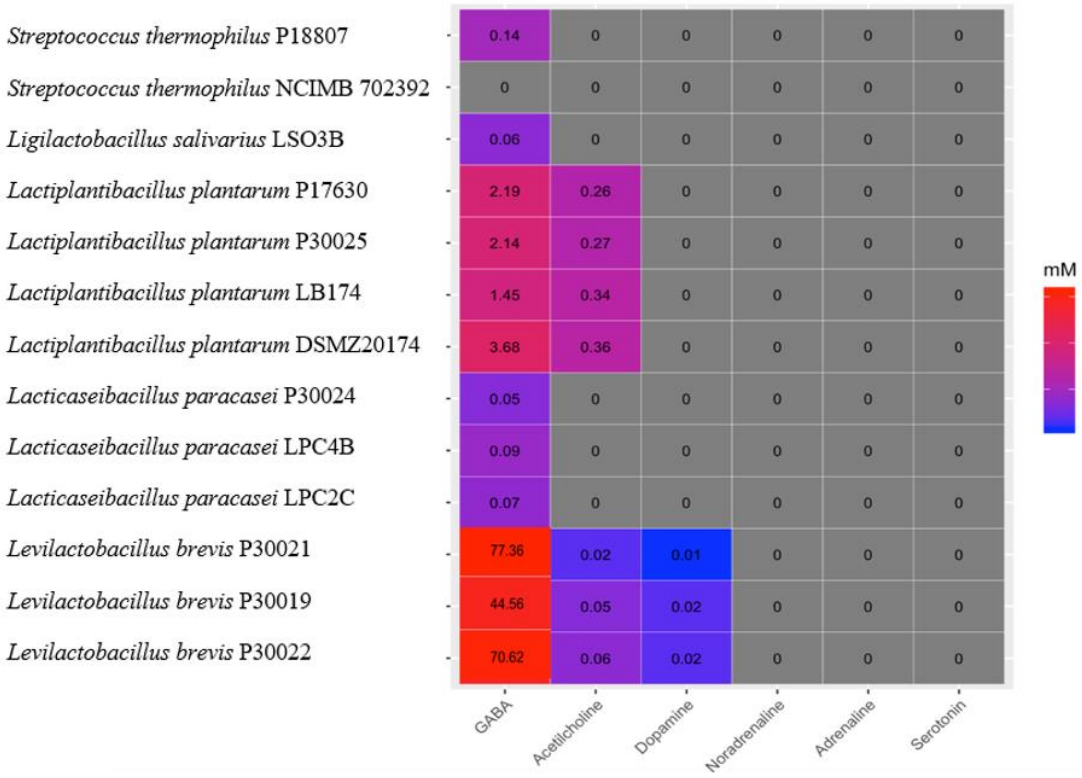

Figure 3.2 Heatmap of neurotransmitters concentration produced by 13 probiotics*. Bacteria are listed on the y-axis and neurotransmitters on the x-axis. The values of neurotransmitters produced by bacteria are expressed in mM and are reported on the gradient of each cell. Blue represents mM values less than 0.01; red presents neurotransmitters production greater than 7. The gradient bar on left shows intensity color depending on mM value. L. brevis strains have shown the highest production of GABA, and lower amount than GABA of acetylcholine and dopamine; Lactiplantibacillus plantarum strains have been able to produce more quantity of acetylcholine than Levilactobacillus brevis and lower of GABA.*

3.4 Discussion

Because of the people's awareness about their health and the increasing request of healthy food which consumption can ensure satisfaction and improvement of quality life, the objective was to use a good approach for screening 13 probiotic microorganisms in order to identify which one was able to produce neuroactive molecules useful to be beneficial to the mental health. Our findings suggest that neurotransmitters production yield depends on the used probiotic strains, as well as the growth condition, e.g. presence of the precursors, time. To our knowledge, this is the first report about a broad screening of probiotics to assess their ability to produce several neurotransmitters.

GABA is a major inhibitory neurotransmitter in the brain involved in the regulation of many psychological process, influencing anxiety, memory, mood, pain as well as hormone regulation and blood pressure (Sarasa et al. 2020). Decrease of GABA levels lead to cases of anxiety, depression and sleep disorder (Hepsomali et al. 2020).

Our results showed that all the strains tested had GABA- producing capability. These findings confirm studies reporting LAB spp. as the most predominant GABA-producing microorganisms (Cataldo et al. 2020; Wang et al. 2021; C. H. Wu et al. 2018; Zhang et al. 2020). In our study, *L. brevis* strains, heterofermentative LAB, showed the highest capacity to produce GABA, consistent with results reported previously (Banerjee et al. 2021; Patterson et al. 2019; C. H. Wu et al. 2018; Yu et al. 2020). Similar results about production of GABA by *Lactiplantibacillus plantarum* strains have also been observed (Yogeswara et al. 2021; Surachat et al. 2021; Zhang et al. 2020). Our results clearly reveal that the addition of monosodium glutamate (MSG) was the most significant factor influencing GABA yield, achieving with *Levilactobacillus brevis* P30021 a concentration of 7.96 g/L after 48h. This concentration was higher than what observed in the study of Lim et al. (2017) and Jin et al. (2021). However the findings of Phuengjayaem et al. (2021) found higher concentration of GABA produced by *L. brevis*. We supposed that the difference could be related to the different MSG concentration used in these two experiments, more than the specific strain. Furthermore the highest GABA production was observed at 72 h of cultivation and GABA, mainly produced during the stationary growth phase. Similar results achieved in GABA yield by *Lactiplantibacillus plantarum* has been observed by Yogeswara et al. (2021).

Further observations are needed to understand the optimization of culture condition to enhance GABA yield, such as the effect of precursor concentration, the temperature, the influence of carbohydrate catabolism on the neurotransmitter production, pH and the influence of the cofactor pyridoxal phosphate (Sahab et al. 2020; Santos-Espinosa et al. 2020). The biosynthesis of GABA by decarboxylation of L-glutamic acid is performed by the glutamic acid decarboxylase (GAD) system (which enzyme is encoded by gadA or gadB), the vitamin co-factor pyridoxal phosphate and glutamate/GABA antiporter GadC (Bagus et al. 2021). The different GABA yield between the species able to produce GABA is related to the different arrangement of genes in the GAD operon. Surachat et al. (2021) observed that many GABA-producing species encode only gadB, except for *Levilactobacillus brevis* strains in which were identified all GAD genes, including gadA, gadB, and gadC. However, as reported in literature, gadB contributed to the main GAD activity (Cui et al. 2020). This system, glutamate-dependent acid-resistance system (GDAR), that provides LAB strains a way to cope with acid stress, is crucial for successful colonization of the gastrointestinal tract (GIT) and survival in fermented food (Cui et al. 2020). It is not surprising that fermented food with high GABA content may result in the isolation of promising GABA-producing strains (Yogeswara, Maneerat, and Haltrich 2020). Changes in pH at 48 hours observed in the media with highest GABA- producer strains are comparable with those of Liu et al., (2021). Thus, suggest the pH measurement could be an easy first way to screen strains with GAD activity, but it need to be followed by a more accurate quantitative measurement, such as spectrometry approach. Our study set itself apart, from other study (Patterson et al. 2019; Q. Wu and Shah 2018) showing higher conversion of glutamate in GABA in aerobic conditions than anaerobic. Slight higher doses of glutamate at 48 hour confronted to 16 h, detected in *Streptococcus thermophilus* strain could be generated

by the conversion of the aminoacid glutamine and strains with this ability could be required to support bacteria able to produce GABA.

Acetylcholine (Ach) is a well-known neurotransmitter in the cholinergic nervous system, that is involved in cognitive function, particularly memory and learning (Decker and Duncan, 2020). It is synthesized mainly by choline acetyltransferase in the CNS (central nervous system) and carnitine acetyltransferase in the peripheral system. Part of the consumption of choline has been observed in *Lactiplantibacillus plantarum* to produce acetylcholine, confirming the work of Stanaszek, Snell et al. (1977). Row (1947) observed the effect of pantothenate (also known as vitamin B5) on acetylcholine, proving to be essential for acetylcholine formation and for growth. Due probably to the different methods of analysis the amount of acetylcholine produced by *Lactiplantibacillus plantarum* strains in our study is more than the amount reported in previous studies, reaching 52.5 mg/L (Horiuchi et al. 2003; Row 1947, Perez et al. 2020). Production of acetylcholine has also been observed in *Levilactobacillus brevis* strains reaching concentration that has also been observed by Row (1947) in *L. plantarum* strains. Other studies also demonstrated the presence of Ach in the bacteria *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* in concentration of 2.22, 0.39 and 55.7 pmol/10^{10} CFU respectively (Horiuchi et al. 2003). The ability of *Lactiplantibacillus plantarum* to produce acetylcholine has been investigated also in preclinical studies showing the effect of *L. plantarum* C29 to increase cognitive function in older mice with memory deficit induced by LPS- or scopolamine (Lee et al. 2018). These results have also been confirmed by another preclinical study in which a combined effect of *B. bifidum* and *L. plantarum* showed a protective effect on the damaged neurons in the hippocampus and improving Alzheimer disease-related impairment by increasing Ach and reducing plaques (Shamsipour et al. 2021). Further observations are needed to confirm these results.

Dopamine, is a catecholamine that mediate a variety of the CNS functions, modulating learning and motivation (Ranjbar-Slamloo Y et al. 2020). Dopamine biosynthesis is mediated by tyrosine decarboxylase (TDC), a pyridoxal 5-phosphate-dependent enzyme, mainly responsible for the decarboxylation of L-tyrosine and L-DOPA to tyramine and dopamine (Kessel et al. 2019; Zhu et al. 2016). Species of the genera *Lactobacillus* and *Enterococcus* have been reported to harbor this enzyme, including *L. brevis* (Moreno-Arribas and Lonvaud-funel 2001). However, unlike the findings of Moreno-Arribas et al. (1999) in which work they did not find production of dopamine by *L. brevis,* we provided the first demonstration of *Levilactobacillus brevis* strains to produce dopamine, reaching 3.3 µg/ml with *Levilactobacillus brevis* P30019. All the tyrosine added has been consumed by *Levilactobacillus brevis* strains but not all has been converted in dopamine, as probably it is mainly converted in tyrosine and CO_2 or histamine as reported to be produced by *L. plantarum* from Alan et al. (2018) and Yazgan et al. (2021). Unlike the previous study of Özogul (2011) that demonstrated the production of dopamine by *L. lactis, L. plantarum* and *S. thermophilus,* in our work *Lactiplantibacillus plantarum* species and *Streptococcus thermophilus* did not produce dopamine, maybe due to

the different method of cultivation of strains, and different substrate used as our strains grew individually in MRS/ and M17 enriched with tyrosine. Another study has investigated the neurochemical-producing potential *Enterococcus faecium* ML1082, showing highest level of dopamine production (133 μg/mL) (Villageliú and Lyte 2018). The psychobiotic effect of the strain *L. plantarum* PS128 has been assessed in a preclinical study with germ-free mice, in which chronic live *L. plantarum* PS128 ingestion induced increasing in the levels of both serotonin and dopamine in the striatum correlated with changes in emotional behaviors (W. H. Liu et al. 2016). Further screening test about the production of dopamine from lactic acid bacteria strains are warranted.

3.5 Conclusion

Understanding the right parameters could potentially result in an applicable and cost-effective neuroactive compound production for the food industry. Our results lead to the conclusion that using the combination of *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* strains in a formulation can offers a promising means of neurotransmitters production for industrial application. In addition, ongoing and future clinical studies are sorely needed to reveal the relative impact and causal contribution of mixed psychobiotic formulation to stress-related disorders in order to provide novel approaches for prevention and treatment of mental illness, including anxiety and depression.

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Chapter 4

Optimization and Evaluation of *Lactiplantibacillus plantarum* P30025 and *Levilactobacillus brevis* P30021 and their formulation in combination with active compounds.

Abstract

Background: Considering the impact of probiotics on the gut-brain axis increasingly appreciated, in the previous screening study, we selected neurotransmitters-producing bacteria (*Lactiplantibacillus plantarum* P30025 and *Levilactobacillus brevis* P30021) to formulate a dietary supplement with potential benefits on mental health. However, the beneficial effects of probiotics primarily depend on their viability, which demands technical optimization of biomass production, since processing and storage capacities are often strain-specific.

Aim: In this study, we aimed to optimize the production parameters for *Levilactobacillus brevis* and the viability of the selected strains in combination with other beneficial compounds to produce a dietary supplement with potential psychobiotic activity.

Materials and methods: Substrates that boosted biomass production in lab-scale fermentations were taken into consideration for the scale-up production. For the freeze-drying process, the composition of cryoprotective media was also relevant to allow optimal working conditions for maximum viability. The final yield and the stabilities of the probiotic strains were evaluated alone and in combination with active ingredients for the formulation of the final product.

Results: The strains showed good yield after the fermentation in the pilot study, resulting in easier scale-up production. Acticoa cocoa was chosen for the formulation, due to the better survival of the strains than in combination with propolis. Finally, vitamin D3 and zinc as a mineral have been included in the final product to contribute to the normal functioning of the nervous and immune system, while vitamin B6 enhances the psychobiotic activity of the strains by working as a cofactor in the production of GABA.

Conclusion: Our results indicate that the chosen time of fermentation, substrate and protectants allows a satisfying yield and exerts extensive protection on strains during the storage.

***Keywords**: Probiotic, pilot-fermentation, scale-up production, dietary supplement*

4.1 Introduction

The use of probiotics has been steadily increasing, mainly attributed to consumer awareness regarding the relationship between their digestive health and overall wellness. Application of probiotics as a new way to control and treat several illnesses by replenishing the gut microbiota, protecting against detrimental bacteria, and reaping a beneficial health effect is the basis of probiotic therapy (Milner et al. 2021). Probiotics are normally consumed through fermented foods and the global current probiotic market is mostly composed of nutritional supplements (Prajapati 2013). Probiotic bacteria, by definition, are living microorganisms that when administered in adequate amounts, confer a health benefit on the host (Morelli and Capurso 2012). Before a probiotic can benefit human health it must fulfill several criteria (FAO/WHO 2002). Firstly, a probiotic must have scientifically validated health properties and demonstrate safety. To this regard, probiotic microbes intended for human use may have a "generally recognized as safe" (GRAS) notification for specific intended use to the US Food and Drug Administration (FDA, 2019) or the "qualified presumption of safety" (QPS) status (European Food Safety Authority: EFSA, 2007). They are acid- and bile-tolerant, able to adhere to the intestinal tract as well as colonize it, and capable of protecting the host from noxious microorganisms (Binnendijk et al., 2013). Furthermore, it must exhibit high survival rates in downstream processes (such as centrifugation and freeze-drying) and during storage. A sufficient number of viable probiotic cells in a probiotic formulation is a prerequisite as being relevant for the effectiveness of the product. Accordingly, national public authorities in Canada and Italy suggest in their guidelines that the minimum number of live probiotic microorganisms imparting general health benefits should be at least 10^9 colony-forming units (CFUs) per day (Italian Ministry of Health) or per serving (Health Canada) (Hill et al. 2015). High survival through the upper gastrointestinal tract, adherence, and high viability at its site of action is also a requirement, together with high activity in the gut environment.

Time-saving and cost-effective methods to increase bacterial cell yield during the production progress are necessary to efficiently meet the growing demands of customers. Several parameters should be taken into consideration, such as cost-intensive fermentation that can negatively affect the scale-up of biomass production; adjustment of growth factors (e.g., substrates, pH, incubation time) to optimize biomass production since processing capacities are often strain-specific. Furthermore, the viability and activity of probiotics during storage are critical criteria for both the manufacturer and the customer. Storage conditions can affect the survival of bacterial cells and even influence the functionality of the probiotic such as stress resistance. Functional compound can be added to probiotics to improve the delivery of the probiotics or enhance their activity (Kobyliak et al. 2018). Several studies have also shown probiotic strains coupled with vitamins to be used for several beneficial effect (Ghaderi et al. 2019; Nayak et al. 2007; Odintsova et al. 2021).

In a previous study, two *Lactobacillus* strains (*Levilactobacillus brevis*, *Lactiplantibacillus plantarum*) were selected for their capacity to produce GABA and acetylcholine. These strains were previously tested by the company PROGE FARM for their applicability as a probiotic supplement, by testing their probiotic potential (unpublished data). The current study determined an industrial procedure to produce those probiotic strains, taking into account factors regarding biomass production, survival during lyophilization, and accelerated stability of storage in combination with other bioactive products were evaluated. A schematic representation of the production process for the industrial fermentation is represented in ***Fig 4.1***.

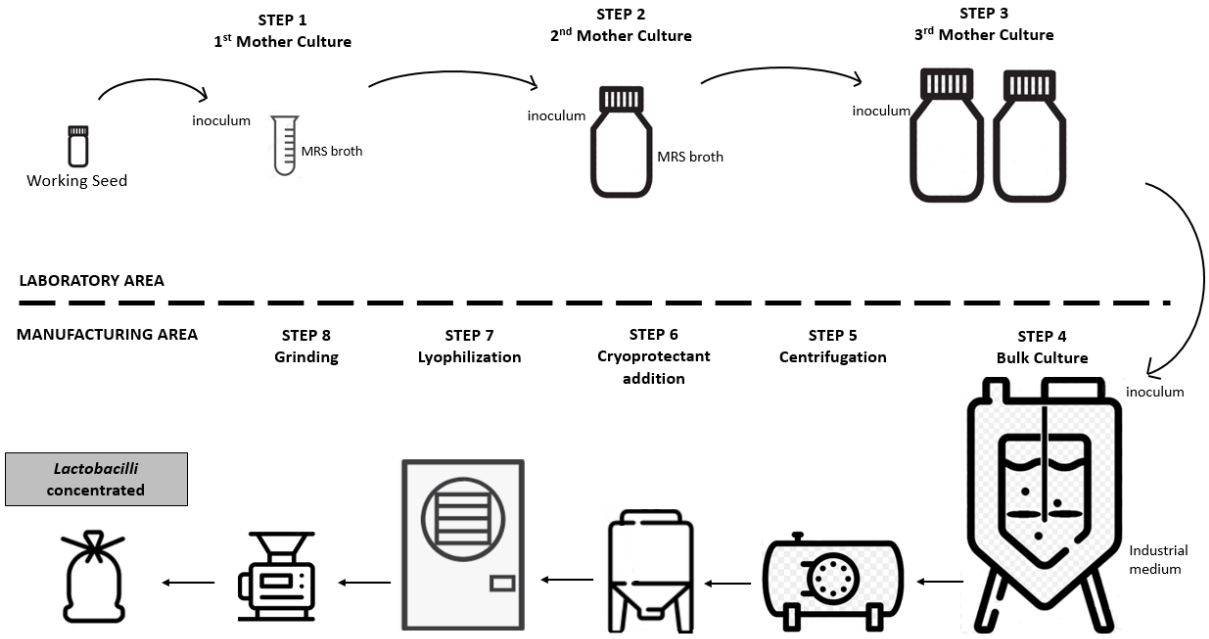

Figure 4.1. Overview of the industrial fermentation process

4.2 Materials and Methods

Microorganisms

Lactiplantibacillus plantarum P30025 and *Levilactobacillus brevis* P30021 belong to the culture collection of the PROGE FARM industry and were chosen according to the guidelines of FAO/WHO (2002). The strain was stored at −80 °C in 10% (v/v) glycerol.

Media composition and fermentation lab-scale

The lab-scale fermentation was performed from AAT Srl- R&D Laboratory. The cultivation medium used consisted of yeast extract; dextrose; proteose peptone; dipotassium phosphate; sodium acetate; Tween 80. The initial pH of the medium was adjusted to the value of 6.5. The inoculum concentration for the lab-scale fermentation was 1%. *Levilactobacillus brevis* P30021

strain was grown in a Sartorius fermenter (Biostat A Plus model) for 24 h at 37°C at pH 5.5, controlled by NaOH 5 M. The fermentation was conducted in a final operative volume of 4 Liters. Six-time points from T0 were sampled (T1.5, T3, T4.5, T6, T7.5, T24 hours). At the end of the fermentation, the biomass was centrifuged, the supernatant discarded and the pellet was resuspended in 100 ml of a cryo-preservative solution composed of maltodextrin + sucrose. The pellet was completely resuspended in the solution by careful agitation to dissolve any clump and the resulting cells suspension was submitted to decimal count on plate.

Lyophilization

The bacterial suspension was placed in the freeze-drying tray pre-treated with ethanol. The tray was placed at -80°C for 18h. The freeze-dryer BenchTop Pro with Omnitronics was used to freeze-dry the resuspended fermentation product. The automatic freeze-drying mode was set at -46°C condenser temperature and 225mT for the vacuum system. The product was freeze-dried for 18h. Lyophilized product was recovered with sterile spatulas in disposable sterile bag and reduced to powder by means of a pestle. 1 gr of the freeze-dried product was used for viable counts, performed following the "ISTISAN 2008/36 Reports, Traditional microbiological methods and molecular methods for the analysis of food supplements based on probiotics for human use". This document specifies for the *Lactobacillus* genus, the use of MRS agar plates and incubation for 72h at 37°C under anaerobic conditions.

Inoculum preparation

For the fermentation performed in a 2200 L fermenter, 1 cryogenic vial from working cell bank (containing 1 ml) was taken and used to inoculate 15 ml Greiner tube containing 10 ml MRS broth (Difco Laboratories, MI, USA). The stock culture of *Levilactobacillus brevis* P30021 was sub-cultured in fermentation medium overnight at 37°C under microaerophilic conditions to obtain an initial biomass concentration of approximately 10^7 CFU mL^{-1}. Afterward, three successive pre-cultures were prepared as represented in ***Table 4.1***

Table 4.1 *Inoculation and volumes for each pre-cultures in preparation of the inoculation in batch fermentation.*

Culture I				
Substrate	*Inoculation*	*Volumes*	*Time*	*Log (CFU/ml)*
MRS broth mod.	1%	30 ml	23h	6,4
Culture II				
Substrate	*Inoculation*	*Volumes*	*Time*	*Log (CFU/ml)*
MRS broth mod.	4%	600ml	23h	9,3

(Continued on the next page)

Culture III				
Substrate	*Inoculation*	*Volumes*	*Time*	*Log (CFU/ml)*
Yeast peptone	2,72%	1,8L	24,5h	9
Yeast extract				
Dextrose				
Sodium acetate trihydrate				
Polysorbate 80				

Batch fermentation of strains

The scale-up of the fermentation 2200L batch fermenter was carried out by PROGE FARM. The batch fermenter was sterilized before starting the fermentation to ensure the aseptic process throughout the processing cycle. The growth media comprising yeast extract, peptone yeast, sodium acetate trihydrate, dextrose, magnesium, manganese, polysorbate, sodium hydroxide were sanitized in situ at 95°C for 60 min before inoculation. Various operating parameters such as pH, temperature, pressure were monitored through the use of probes. The temperature was set to 37°C, the pH to 6.80. During the fermentation, the medium was stirred with an agitator. The fermentation lasted 42h. After centrifugation, cryoprotective agents such as sucrose and polysorbate were added to the biomass in an amount equal to 20% to preserve the vitality of the microorganism as much as possible during the freeze-drying process.

Analytical determination

The number of viable cells was determined as colony forming units (CFU). Serial decimal dilutions of each sample were plated in duplicate onto MRS agar (Merck) plates and incubated at 37 °C for 48 h before enumeration. Fermentation samples (50 mL) were collected also for analysis of mono-septic status the total aerobic microbial count (TYMC) were performed in Sabouraud Dextrose Agar and incubated for 5-7 days at 20-25°C and total yeast and mould count (TAMC) assays by standard streak plating on Sugar Free Agar (SFA) for 3-5 days at 30-35°C.

Formulation of the multi-strain probiotic product and stability analysis

The resultant *Levilactobacillus brevis* concentrates harvested by centrifugation were processed into a dried product intermediate and then grounded into fine powder more feasible to be used in a dietary supplement. The same flow chart reported in **fig. 4.1** for the industrial production was also followed for *Lactiplantibacillus plantarum* with some changes in the substrate composition and time of fermentation. Here, it has been chosen to report the steps for the optimization of the production of *Levilactobacillus brevis* because it was exploratory even for

the company, letting us to start from the lab scale fermentation to the industrial fermentation, while, on the other side, the company has already a strong know-how in the industrial production of *Lactiplantibacillus plantarum*. The viability of the probiotic strains chosen for the final product were assessed on the final probiotic powder, alone and in combination with cacao (ACTICOA) and commercial propolis (B Natural). The composition for each is reported in ***Table 4.2*** Studies of stability were performed in accelerated to increment the speed of degradation or physic changes. In this regard, the single strains and the combination of them with cacao were counted after storage at 37°C after 1 and 2 weeks. T0 is the beginning of the stability study and corresponds to the moment in which the samples are placed in the climatic chambers or the thermostats. The packaging method for the probiotic product was guided by an industry partners requirement, Salix S.r.l. The probiotic product was filled into plastic bags (Low-density polyethylene, 70 mM), sealed, and then the final formulation containing probiotics, vitamins, zinc, and cacao was packaged in 14 sticks (2 gr each) for each box.

Table 4.2 *Composition of probiotics with cacao and propolis*

Ingredients	**Formulation**	**Quantity**
Cacao	*Lactiplantibacillus plantarum/Levilactobacillus brevis*	100 mg
	Maize starch	1850 mg
	Cacao	50 mg
Propolis 6%	*Lactiplantibacillus plantarum/Levilactobacillus brevis*	100 mg
	Maize starch	1800 mg
	Propolis 6%	100 mg

Statistical Analysis

Bacterial count of the mix of the strains with propolis and cacao were performed in duplicates, after 7 and 14 days. The graphs were made by using R. Statistical significances of comparisons were assessed using two-way analysis of variance (ANOVA) with the statistics software GraphPad Prism version 8.0.0, San Diego, California USA

4.3 Results

Pilot fermentation

The first evaluation of the biomass production from *Levilactobacillus brevis* was carried out in a lab-scale fermenter. The fermentation plot from the raw data of viable counts per each time

point is represented in ***Figure 4.2*** The biomass information regarding the resuspended pellet, and the freeze-dried powder of *Levilactobacillus brevis* P30021 are reported in ***Table 4.3.***The data indicated a 64% survival of biomass following washing and resuspension (2.2 x 10^{10} CFU/ml) and 34% survival following lyophilization (6.7 x 10^{10} CFU/g).

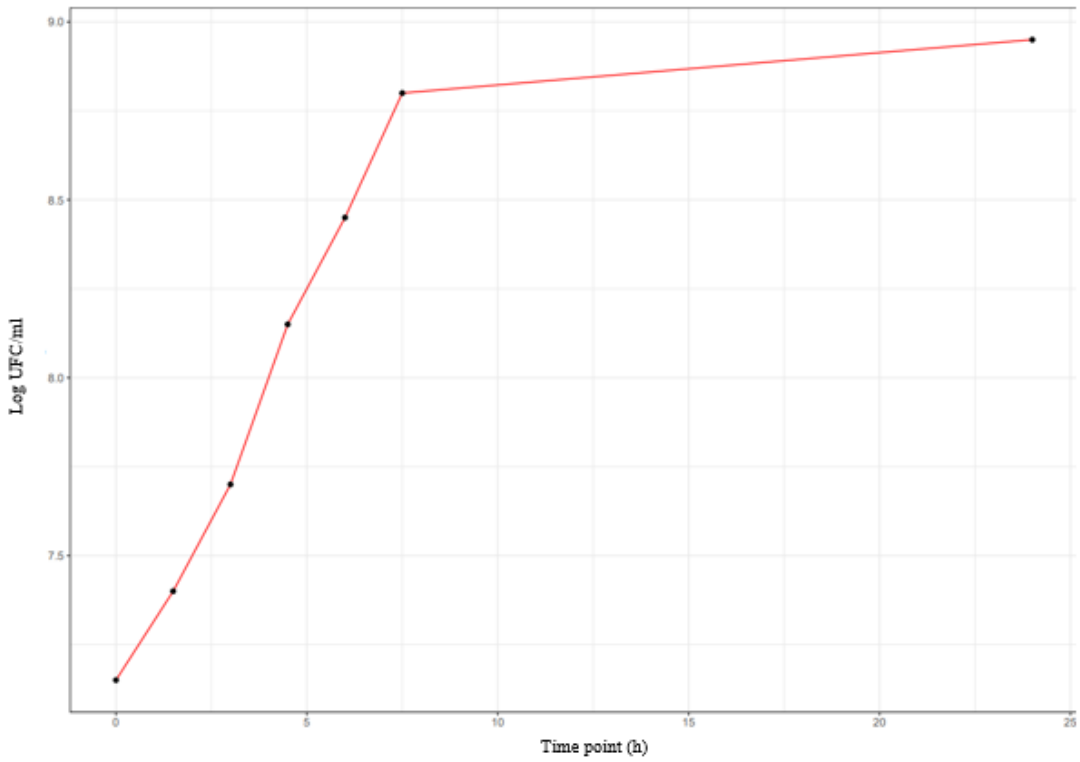

Figure 4.2 *Growth curve of strain Levilactobacillus brevis P30021 in the lab-scale fermenter*

Material	**Weight (g)**	**Dilution**		**Log10 CFUs**
Resuspended biomass	Wet 9,87	8	-9	10,3
	Resuspended 105,9	212	30	
Lyophilized powder	17,8	-9	-10	10,8
		66	8	

Table 4.3 *Biomass production information*

Batch fermenter- scale-up results

Bacterial growth began after 12h as indicated by the lowering of the pH from 6.25 to 5.5 and the subsequent tight fluctuation due to the continuous maintenance of the pH through titration of an alkaline solution, Na_4OH. From the final volume of 2017 L in the biofermenter, after centrifugation were obtained 60.40 Kg of biomass. At the end of the freeze-drying and grinding process, a good trend in the vitality of the strain was noted, obtaining a quantity of ground equal

to 10.5 log (CFU/ml). In ***Table 4.4*** the concentration in Log_{10} CFU/ml from the end of the fermentation to the ground powder of *Levilactobacillus brevis* obtained after lyophilization.

Table 4.4 *The concentration of Levilactobacillus brevis from the end of the batch fermentation to the final fine powder*

Samples	**Log10 CFU/ml Lactobacillus**
Fermentation product	9
Biomass	11
Lyophilized product	11
Grounded powder	11,8

Effect of the cacao, propolis, on the stability over the time of the probiotic strains

Probiotics combined with cacao and propolis were incubated at 37 °C for 2 weeks. The average net reductions in viable cell count during this period were 1.7 log and 2 log in *Levilactobacillus brevis* for cacao and propolis, respectively; 3.5 log and 3.1 log in *Lactiplantibacillus plantarum*. Control samples with only *Levilactobacillus brevis* showed 1.7 and 2.7 log reduction in the cell population during the respective period of stability assessment for cacao and propolis respectively, while control samples composed of only *Lactiplantibacillus plantarum* showed 3.03 and 3.1 log reduction (***Fig. 4.3***). Test of significance indicated that there were significant changes over the time *Levilactobacillus brevis* viability in combination with both ingredients (p<0.02) and *Lactiplantibacillus plantarum* with cacao (p<0.02) and propolis (p<0.001). No significant differences were observed in *Levilactobacillus brevis* between the added ingredients and their respective control after 2 weeks, as shown in ***Table 4.5***

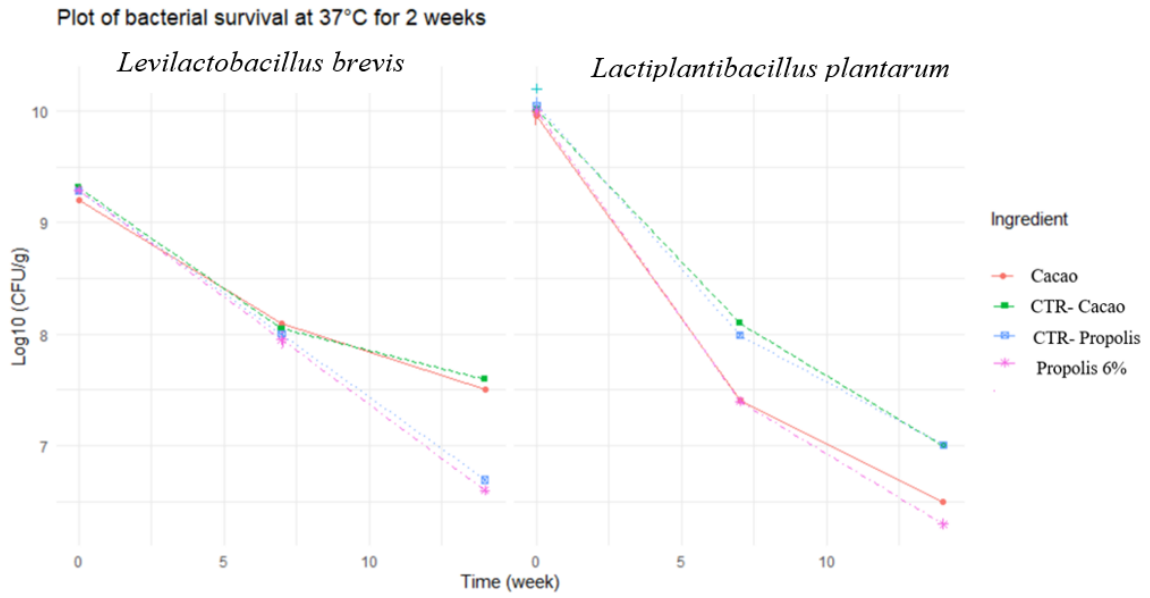

Figure 4.3 *Levilactobacillus brevis and Lactiplantibacillus plantarum count, incubated at 37°C, with cacao and propolis at times 0, 7, and 14 weeks. The control is the probiotic stability over time without the addition of cacao or propolis*

Table 4.5 *Bacterial count Mean. Diff on the different formulations and their controls*

	***Levilactobacillus brevis* P30021**			***Lactiplantibacillus plantarum* P30025**		
	T0	**T7**	**T14**	**T0**	**T7**	**T14**
Cacao vs Propolis	-0.09	0.15	0.9***	- 0.05	0.01	0.2***
Control cacao vs control propolis	0.03	0.05	0.9***	0	0.11	-0.01
Cacao vs control	-0.12**	0.05	-0.095	-0.06	-0.7*	-0.5***
Propolis vs-control	0	-0.05	-0.095	-0.05	-0.6*	-0.7*

4.4 Discussion

This study aimed to development at an industrial scale approach to produce probiotics that can be used in an oral dietary supplement and to evaluate the effect of two combinations of compounds on the survival rate of bacteria and subsequent storage. In this chapter, the optimization of the biomass production was looked over *Levilactobacillus brevis* P30021 as the main strain for the production of GABA in the final product and the first time of scale-up production of this strain for the company. The optimization of the strain production was accomplished in two consecutive steps: first the growth performance of the *Levilactobacillus brevis* P30021 strain and survival following lyophilization was evaluated by pilot-scale fermentation, and then a scale-up fermentation was performed in a 2000L fermenter system. In the pilot fermentation, the strain was able to increase the concentration in viable cells by about 2.0 Log in the fermentation medium, with a progressive increase for 8 hours before reaching the stationary phase. It is not excluded that the enrichment of the growth medium, providing modification of the recipe to respond to the specific metabolic requirement of *Levilactobacillus brevis*, could allow increasing the production of biomass. Further improvements could be envisaged for a cryo-preservative medium to increase the survival of strain *Levilactobacillus brevis* P30021. These preliminary data were fundamental to proceed with the scale-up in which some changes were made regarding the substrate (i.e. yeast peptone was used instead of proteose peptone) and cryoprotectant (i.e. sucrose and polysorbate instead of maltodextrin and sucrose), resulting in a highest final yield of 11 log (CFU/ml). This results can be considered an efficient production process compared to other studies (Wang et al. 2020)

Final biomass concentration and biomass yield could be further improved through the application of more complicated feeding strategies. For example, appropriate sensors in connection with the feeding pump may be used to accurately control glucose level in the culture for increase cell mass. According to Kuhan et *al*., (2021) addition of lactose may also increase biomass, as in his study emerged to be the most favored carbon source for the strain *Levilactobacillus brevis* C23. Time effective statistical approach, response surface methodology (RSM) or the conventional “one factor at a time” approach can be used to screen significantly contributing factors able to optimize cultivation medium (Manzoor et al. 2017; Selvamani et al. 2021). Cost-effective preparation procedure has been also investigated by employing the BIOLOG® technology to define the specific carbon source preferences of the probiotic *Lactobacillus* strains (Ren, Zentek, and Vahjen 2019). The BIOLOG® system is based on the reduction of a redox dye, which indicates during bacterial growth the utilization of substrates, directly proportional to metabolic activity (Stefanowicz 2006).

The assessment of the stability of the product also represents a fundamental requirement for the registration of new probiotic dietary supplements, to ensure that safety, efficacy, and quality are adequately verified before being placed on the market. In this regard, we performed the accelerated stability that is normally used to evaluate the effect of short excursions outside the

storage conditions indicated on the label, which may also occur during shipment. Because of the intensive physical stress due to the probiotics sensitivity to the temperature, the formulated product with cacao showed a decrease of the bacterial count over two weeks reaching a final count not lower than 6 log, which is generally accepted as the minimum concentration required in a probiotic product (Kechagia et al. 2013). However, long-term analysis to investigate the stability of the final formulation under recommended storage conditions for the shelf-life is carried out every 3 months the first year, and every 6 months in the second year by PROGE FARM personnel.

For the development of the finished product, scientific criteria were pursued, relating to the choice of nutrients and the relative dosages in line with the pre-established physiological objective and the group of the population for which the supplement is intended, and technical criteria through the preventive evaluation of the factors which could influence the stability of the hypothesized formulation and consequently the consistency with the quantities of the probiotics that are intended to be reported on the label, according to the International Probiotics Association (IPA). Both for the ingredients used and for the overall composition of the product, European and national standards have been applied.

4.5 Conclusion

In summary, two neurotransmitter-producing probiotic strains (*Levilactobacillus brevis* and *Lactiplantibacillus plantarum*) were used for the formulation of the final product. Optimization of biomass production of *Levilactobacillus brevis* P30021 was explored as well as the viability of the probiotic strains in combination with cacao and propolis. Furthermore, while formulating a dietary supplement composed of live probiotic bacteria, we performed some tests to make sure that the concentration of the microorganisms is adequate at the point of consumption to deliver the expected health benefits. This study showed optimal routines for lab-scale and large-scale production, processing, and storage of probiotic strains to increase the technical production of probiotics and ensure its beneficial effect.

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Chapter 5

Effect of supplementation containing probiotics on the modulation of neuroactive compounds in healthy human microbiota using the Simulator of the Human Intestinal Microbiota Ecosystem

Abstract

Aim: The present work aims to evaluate the influence of a dietary supplementation containing probiotic strains on the production of neuroactive compounds in different parts of the colon in a short-term experiment.

Materials and methods: A probiotic formulation made with *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025 have been added to batch culture of human microbiota reproducing the distal colon composition to be investigated for neurotransmitters production. The Simulator of the Human Intestinal Microbiota (SHIME) in vitro model has been performed to simulate colonic fermentation with a healthy adult intestinal microbiota. Samples of the colonic contents were evaluated chemically for the production of neurotransmitters by LC-MS and SCFA by GC, and microbiota composition was determined by 16S sequencing

Results: After treatment, an increment in GABA and a decrement in glutamate were observed *in vitro* batch fermentation. Production of GABA after the treatment with probiotics was confirmed in the short-term experiment in the SHIME®. No differences in short-chain fatty acids were observed between the control and the formulation with probiotic strains. After 72 hours of fermentation in the SHIME. The analysis of the 16S rRNA showed different microbiota composition among the three different part of the colon, with higher abundance of *Veillonellaceae and Lactobacillaceae* in the AC. Differences were also observed between the control and the treatments, exhibiting in the three vessel under treatment with probiotic relative abundance of *Bacteroides* and lower abundance of *Enterobacteriaceae* when compared to placebo

Conclusion: With regards to the results obtained from this probiotic formulation and its potential application, research in this area opens the way toward the possibility of the future use of neuroactive molecule-producing probiotics as therapeutic agents for the treatment of neurogastroenteric and/or psychiatric disorders.

***Keywords**: Gut microbiota, neurotransmitters, short-chain fatty acids, Levilactobacillus brevis , Lactiplantibacillus plantarum*

5.1 Introduction

The high neuroactive potential of microbe-derived metabolites has gained traction over the last few years, with metagenomic-based studies suggesting an important role of metabolites produced by the gut to modulate neural signaling within the enteric nervous system and consequently influence brain function and host behavior (Bonaz, et al. 2018). Among the neuroactive compounds produced by the gut microbes, L-Glutamate (Glu) and γ-aminobutyric acid (GABA) are mainly known for their role as the main neurotransmitters in the mammalian CNS, with excitatory and inhibitory roles, respectively(Cryan et al., 2010).

Probiotics can regulate the gut microbiota composition of their consumers, resulting in modulation of immuno-system, improvement of the nutritional value of food, nutrient bioavailability, and brain activity (Da Cruz Rodrigues et al., 2020; Song, Kim, & Paik, 2021; Terpou et al., 2019). Within brain activity, Lactobacilli and Bifidobacteria are reported as the main producers of GABA among the bacteria (Duranti et al., 2020). Many strains of *L.brevis, L.plantarum, L. buchneri, L. casei, L. paracasei*, as well as for strains belonging to *B. longum, B. dentium, B. infantis, B. adolescentis* can be considered as delivery vehicles of GABA by food or intake of dietary supplements. (Cataldo, et al., 2020; Cui, et al. 2020; Duranti et al., 2020; Kanklai, et al. 2021; Wu et al. 2017; Yunes et al., 2016).

However, the health effects are specific to each host and depend on the strain selected and more efforts are warranted into developing different strategies to study the effects of psychobiotics on gut microbial community and functionality, such as *in vitro* models that include static batch fermentation and continuous systems, animal models and human clinical/observational study.

In vitro strategies to enable the cultivation of human gut microbial community derived from fecal samples under simulated physiological conditions as in our study by exposing gut microbes to probiotics, vitamins, and zinc, provides essential information getting a step closer to gut microbiota modulation via diet. There are essentially two types of *in vitro* fermentation models, each one has its limitations and advantages/disadvantages (Pérez-Burillo et al., 2021).

The batch fermentation enables many samples to be studied at once, and the experiment can and it can be performed within a short period, usually, 24-48 h, as the waste product that cannot be controlled will accumulate potentially affecting the bacterial growth. The cost experiments are lower than the continuous systems since they do not require certain equipment such as pH controlling systems or pumps.

Continuous systems are closer to physiological conditions than batch fermentation, can be kept stable for longer periods of time, and mimic the conditions of the different portions of the colon in an automatized manner. The controls of parameters such as pH, temperature, and medium flow rate in three vessels mimicking the environment in the proximal, transverse, and distal colon allow the investigators to achieve a steady microbial composition. A widely used

continuous system is the Human Intestinal Microbial Ecosystem (SHIME), which also includes previous compartments to mimic gastric and intestinal digestion (Pérez-Burillo et al., 2021; Verhoeckx et al., 2015).

Both *in vitro* approaches complement each other. The *in vitro* fermentation was used for initial screening to give clues on how to select the dietary supplements to drive microbial metabolism toward the synthesis of neuroactive metabolites such as GABA and acetylcholine, before scaling up to continuous systems for the selected ones.

On the other side in vivo experiments are ideal and more representative study to evaluate the administration of probiotics with the highest physiological relevance although the cost, time, social and ethical issues may be a limiting factor (Allen et al., 2016; Chong et al., 2019; Gibson et al., 2011).

Therefore, the present study aimed to characterize GABA-enhancing dietary supplements for their potential psychobiotic activity. The commercial dietary supplement has been evaluated as vehicles for psychobiotic delivery on healthy human microbiota by using *in vitro* batch fermentation for a first screening and the SHIME® for investigating the occurrence of SCFA and neurotransmitters, with the selected one.

5.2 Materials and Methods

Chemicals

All chemicals used were purchased from Sigma–Aldrich (St. Louis, MO) unless stated otherwise. The tested compounds were: GABA, Acetylcholine, choline, glutamate, acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, and isobutyric acid.

Dietary supplement

The commercial dietary supplement GABAflor was furnished by PROGE FARM, Novara (IT), and used for the SHIME experiments. Each sachet (2 g) contained 9 log colony forming units/sticks of the micro-organisms strains *Lactiplantibacillus plantarum* P30025 and *Levilactobacillus brevis* P30021, vitamins B6, vitamin D, zinc, cacao Acticoa, and fructose.

In vitro fermentation with human faecal microbiota

Fresh fecal samples were collected from two adults, 26 and 30 years old, with a body mass index (BMI) between 18.5 and 25. All donors were in good health and with no history of gastrointestinal disorders or antibiotic treatment for at least 6 months before this study. Faecal slurries were processed within 2 h after defecation following the method described by Koper et al. (Koper, 2019). Batch-culture fermentation vessels (20 mL working volume) were sterilized and filled with 12,3 mL autoclaved basal nutrient medium, consisting of 5.22 g L K_2HPO_4,16.32 g L−1 KH_2PO_4, 2.0 g L^{-1} $NaHCO_3$, 2.0 g L−1 yeast extract, 2.0 g L^{-1} special peptone, 1.0 g L^{-1} mucin, 0.5 g L^{-1} L-cysteine HCl, 2.0 mL L^{-1} Tween 80, 20g/L monosodium glutamate and

810mg/L choline. Before the addition of 2 mL fecal slurries, vessels were flushed with N_2/CO_2 (80/20, v/v) gases to create an anaerobic condition. Substrates, containing the dietary supplementations were inoculated with fecal slurries at 37 °C with mild shaking. Samples were taken at 24, and 48 h after inoculation, and then immediately centrifuged (12 000 g, 5 min) and filtered (0.20 µm regenerated cellulose filter). The supernatant and residue were separately collected and frozen at −20 °C before analysis.

Description of the Simulator of the Human Intestinal Microbial Ecosystem model (SHIME)

The fermenter setup simulating the human gastrointestinal tract was derived from the SHIME. To optimally address the research questions and simulate a complete microbial fermentation, the SHIME setup was adapted to a TwinSHIME configuration. Each arm of the TwinSHIME consisted of a succession of four fermenters simulating the different regions of the gastrointestinal tract with and without dietary supplementation. The first fermenter mimicked the upper gastrointestinal tract with the subsequent simulation of a gastric and small intestinal phase. The three subsequent colonic fermenters simulated the ascending (AC), operating at pH 5.6-5.9, transverse colon (TC) operating at pH 6.15-6.4, and descending colon (DC) at pH 6.6-6.9. Frozen fecal microbiota already stabilized and adapted to the specific environment of each colon condition, in terms of pH range, retention time, and available carbon sources were used to inoculate the TWINSHIME setup. The donator was a nonsmoking adult, with no previous history of antibiotic and probiotic use for at least 6 months. The fed-batch cultures were maintained at 37°C using a water bath under agitation, closed, and kept under anaerobic conditions by flushing the headspace once a day with nitrogen (10 min). Every 8h, 140 ml fresh liquid feed (pH 2) entered in the stomach vessel for each donor with stable feed composition (1.2 g L^{-1} arabinogalactan, 2.0 g L^{-1} pectin, 0.5 g L^{-1} xylan, 0.4 g L^{-1} glucose, 3.0 g L^{-1} yeast extract, 1.0 g L^{-1} special peptone, 3.0 g L^{-1} mucin, 0.5 g L-cysteine-HCl, and 4.0 g L^{-1} starch). After 90 min, 60 ml of pancreatic juice (12.6 g L NaHCO3; 6 g L-1 Oxgall, BD Biosciences, The Netherlands; 0.9 g L-1 pancreatic from porcine ≥ 3 * USP) was added. After 90 min of the small intestinal phase, the total volume was transferred to the proximal colon connected in series to the transverse colon and the distal colon. The vessel volumes, pH, and retention times were kept constant at all times.

Experimental protocol

The experiment design consisted of 1 week, including 1 day with the standard feed, 4 days with monosodium glutamate (MSG), and choline supplementation where 10g/L of MSG and 0.2 g/L of choline were added to the feed. Afterward, the treatment was administered three consecutive days in one arm of the SHIME and the other used as control (**Figure 1**). Treatments: mix of the probiotic strains *Lactiplantibacillus plantarum* P30025 and *Levilactobacillus brevis* P30021 (10^9 CFU/ gr), vitamins B6 (2,8 mg, 200% VNR), vitamin D (0.005 mg), zinc (7,50 mg), cacao Acticoa (50 mg). The composition of the modified feed in represented in ***Table 1***.

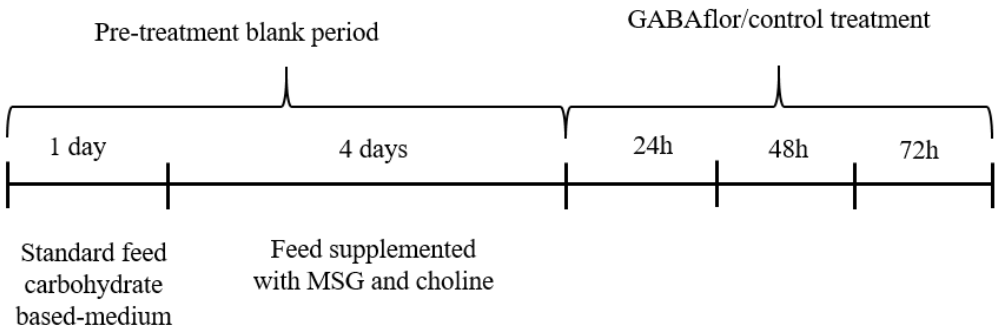

Figure 5.1 Schematic representation of the experimental design used in the short-term SHIME® run

Table 5.1. Ingredients Employed for Each Liter of the Modified Feed Used in the SHIME reactor

Ingredient	**Quantity**
Arabinogalactan	1.2 g L^{-1}
Pectin	2.0 g L^{-1}
Xylan	0.5 g L^{-1}
Glucose	0.4 g L^{-1}
Yeast extract	3.0 g L^{-1}
Special peptone	1.0 g L^{-1}
Mucin	3.0 g L^{-1}
L-cysteine-HCl	0.5 g L^{-1}
Starch	4.0 g L^{-1}
MSG	10 g L^{-1}
Choline	0.2 g L^{-1}

Samples collection from TWINSHIME

Samples from the AC, TC, and DC were collected during the fermentation at the moment of the supplementation, at 6, 12, 24, 36, 48 h. and immediately centrifuged at 9000*g* at 4°C, after which the supernatant was filtered using a 0.2 µm RC filter (Phenomenex, Torrance, CA). The pellet was stored at -80°C and the supernatant at -20°C until further analysis.

Short-Chain Fatty Acid (SCFA) determination

Shimadzu GC-2014 (Kyoto, Japan) equipped with a flame ionization detector, a capillary fatty acid-free Stabil wax-DA column (1µm × 0.32 mm× 30 m T_{max} 240°C, Restek, USA) and a split injector was used to determine short-chain fatty acid (SCFA) composition in each sample. Thawed supernatant and calibration standards were mixed in a ratio of 2:1 with an internal standard containing 0.45 mg/ml 2-ethylbutyric acid in 0.3 M HCl and 0.9 M oxalic acid. Subsequently, the solutions were centrifuged for 4 min at 20000*g*. The injection volume of the supernatant was 0.5 µl. Nitrogen was used as a carrier gas, with a flow rate of 2.51 mL/min. The makeup gasses were nitrogen, hydrogen, and air with respective flow rates of 40 ml/min, 30mL/min, and 400 mL/min. The temperature was initially held at 100°C. After injection, the temperature was increased first to 180°C and then to 240°C, and both temperatures were held for 2 min. The samples were compared to 6 calibration standard solutions containing acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, and isobutyric acid. The results were processed using Chromeleon Edition 7 (Thermo Scientific, San Jose, CA) and normalized by control samples fermented without the dietary supplementation

GABA and Acetylcholine analysis

The samples were centrifuged for 10 minutes at 3000 rpm at 4°C with an Eppendorf centrifuge, and the supernatant was microfiltered through a 0.45 µm syringe filter Phenex (Phenomenex, Aschaffenburg, Germany). The samples were diluted in 50% ACN and 5 µL injected on a SeQuant® ZIC HILIC 3.5 µm, 4.6 x 150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 x 2.1 mm (Merck KGaS, 64271, Darmstadt, Germany). The analysis was carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The flow rate was set at 0.7 mL/min and the column temperature at 40°C. The mobile phases consisted of 0.1% formic acid (solvent A), acetonitrile with 0.1% formic acid (solvent B) with the following elution profile (t in [min]/[%B]): (0.0/90), (4.0/70), (10.0/20), (13.0/20), (15.0/90) and (18.0/90). MS data were collected for 18 mins. Data were processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan). The content of compounds was calculated by comparing the peak area with the standards. Each sample was analyzed in duplicate and the concentrations are expressed in µM.

Quantification of lactic acid bacteria

During the treatment period with the dietary supplements, samples were collected from the fermenters corresponding to the three-part of the colon to quantify the lactic acid bacteria. One mL of samples from each fermenter was suspended in 9 mL of sterile peptone water. Serial dilutions were carried and plated in MRS agar (Sigma-Aldrich®, Netherlands). The plates were incubated in anaerobiosis at 37 ◦C for 72 h.

Microbiological analysis employing 16S rRNA gene sequencing

Samples of the SHIME experiment have been stored at -80 °C before the analysis. Genomic DNA isolation, bacterial 16 s rRNA gene (V3-V4) PCR amplification, and library preparation were performed by University Federico II of Naples (Italy). The supernatant was centrifuged (12,000 x g, 2 min), and the pellet was used for DNA extraction by using the QIAamp BiOstic Bacteremia DNA kit (Mo Bio Laboratories, Inc., Carlsbad, CA). PCR mixtures were initially heated to 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and completed at 72 °C for 5 min. The PCR products were run on a 1.5% agarose gel. DNA was quantified and pooled in equal concentrations using Qubit Fluorometric Quantitation (ThermoFisher Scientific, Waltham, US) and qualified using DNA size profiling on a Fragment Analyzer (Agilent Technologies, Santa Clara, US). The V3-V4 region of the 16S rRNA gene was amplified by using the primers S-D-Bact-0341F5-CCTACGGGNGGCWGCAG and S-D-Bact-0785R5-GACTACHVGGGTATCTAATCC and protocol previously described (Quast et al., 2013). Library multiplexing, pooling, and sequencing were carried out according to the Illumina 16S metagenomic sequencing library preparation protocol on a MiSeq platform and using the MiSeq Reagent kit v2, yielding 2x250-bp

Statistical analysis

Comparison of normally distributed data of the different control and treatment weeks on SCFA were performed with a Student's T-test for pairwise comparisons, while the function pairwise.wilcox.test was used to calculate pairwise comparisons between group levels with non-parametric distribution. Differences were significant if $p < 0.05$, using SPSS Statistics version 20 (IBM Corporation, Armonk, New York, USA). Data were visualized using R software (v3.6.3). Gaba, glutamate, choline, and acetylcholine data detected in vitro batch fermentation were tested for normality using the Shapiro-Wilk test. If normally distributed, differences between groups and the different fermentation time points were evaluated using one-way ANOVA (Tukey's tests, $p < 0.05$), and with the Kruskal-Wallis test as a non-parametric alternative to one-way ANOVA. The correlation coefficient between GABA and glutamate concentrations was estimated statistically produced using *corrplot* (v0.84) and *rstatix* (v0.4.0) packages in R software (v3.6.3). Spearman's pairwise correlations were computed between ASV and other quantitative variables such as GABA, Glutamate, Choline, and Acetylcholine (the "corr. test" function in the psych package) and visualized by using the ComplexHeatmap package.

5.3 Results

In vitro batch fermentation of the probiotic dietary supplementation

Change in the concentration of GABA and glutamate was observed during fermentation (***Figure 5.2*** **and** ***Figure 5.3*****)**. The glutamate was directly accessible for microbes and its concentration decreased with incubation time. Conversely, the concentration of GABA increased. The correlation coefficient between GABA and glutamate concentrations was estimated as −0.84 after 48 hours, which indicates a negative correlation between both neurotransmitters, further highlighting the nature of glutamate as a primary precursor of GABA. No significant increment in the concentration of acetylcholine was detected. 5 dietary supplements were tested, containing the same probiotic strains and the same amount, by using two biological replicates. Differences in production of GABA were observed along the time between control and intervention with GABAflor, showing higher amount of GABA after 48h.

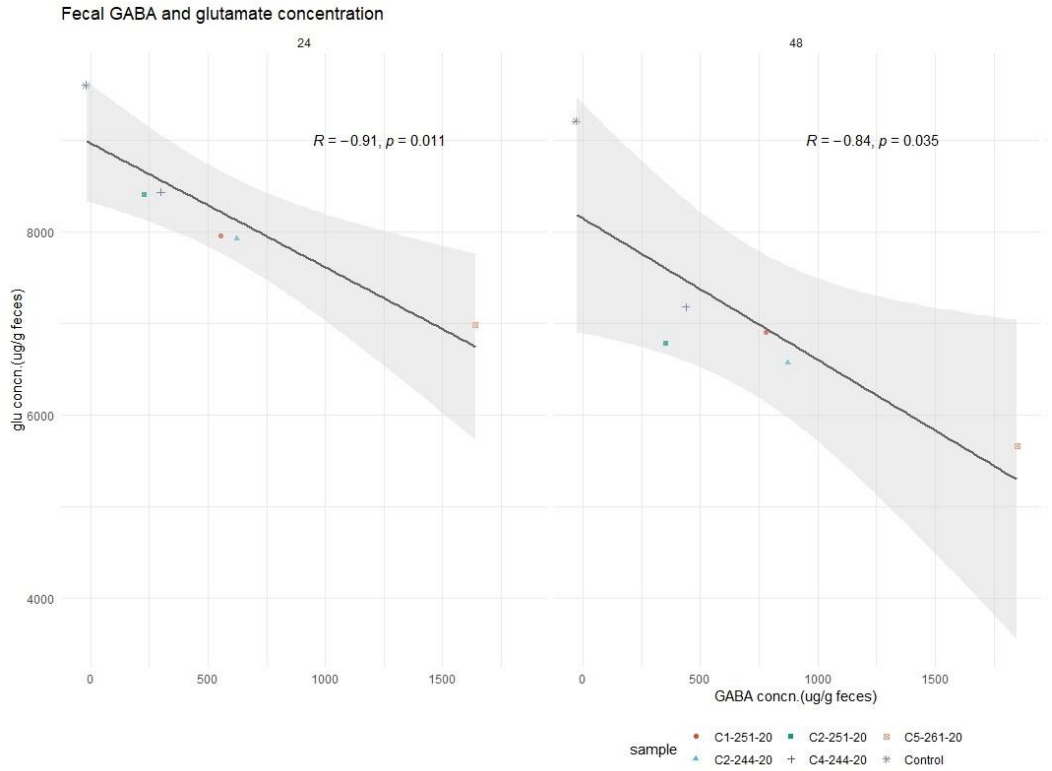

Figure 5.2 Fecal GABA and glutamate concentrations. *GABA and glutamate contents were analyzed after the in vitro batch fermentation of the fecal samples of two healthy human participants with 5 probiotic sticks and without (control).a) GABA and Glu content after 24h of in vitro batch fermentation; b) GABA and Glu content after 48h of fermentation. Regression curve is displayed on the figure with a deep gray line, showing a negative correlation between fecal GABA and glutamate concentrations. Confidence interval is expressed in a light gray color. The correlation coefficient (R) and p-value of the regression curve are also shown on the plot*

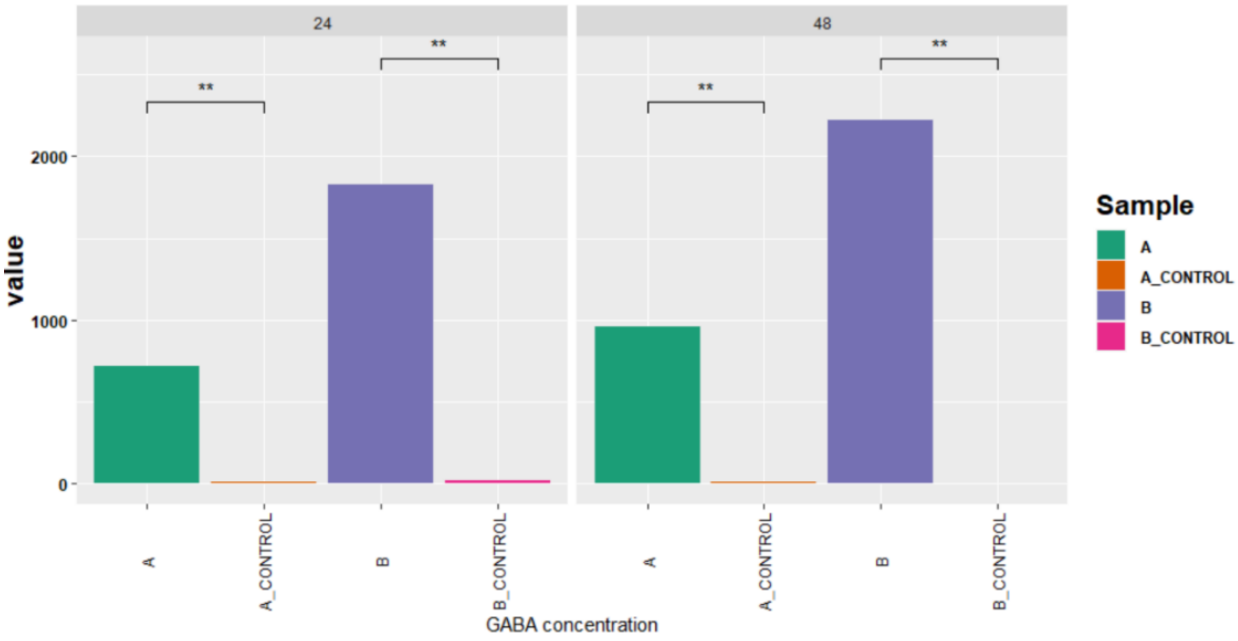

Figure 5.3 Concentration of GABA (µg/g feces) in vitro batch fermentation*. Concentration of GABA was analyzed in the samples at time 24 and 48 hours, for two biological replicates (donor A and donor B)*

Lactic acid bacteria count in SHIME experiment

No notable changes occurred in *Lactobacillus* spp. population amidst the three-vessel (AC, TC, DC) with daily administration of dietary supplementation and in three colon vessels of control (***Table 5.2***).

Table 5.2 Average Plate Count Measurements (± SEM). *The average is expressed in Log CFU mL-1 in MRS agar for Lactobacillus spp., SHIME Compartments and Periods*

Time		**Ascending colon**	**Transverse colon**	**Descending colon**
0	GABAflor	7,31 ± 0,02	7,23 ± 0,01	7,19 ± 0,05
	Control	7,30 ± 0,01	7,24 ± 0,00	7,26 ± 0,02
24	GABAflor	6,99 ± 0,04	7,16 ± 0,00	7,16 ± 0,05
	Control	7,16 ± 0,00	7,23 ± 0,01	7,17 ± 0,03
48	GABAflor	6,98 ± 0,03	7,06 ± 0,04	7,08 ± 0,02
	Control	7,05 ± 0,02	7,09 ± 0,01	7,00 ± 0,03
72	GABAflor	7,15 ± 0,02	7,01 ± 0,04	7,03 ± 0,03
	Control	6,83 ± 0,02	7,19 ± 0,02	7,07 ± 0,03

Effect of the dietary supplementation on SCFA production during colonic fermentation in SHIME

The short-chain fatty acids (SCFAs) detected consisted mainly of acetate, propionate, and butyrate. They were analyzed to determine whether there was a change in fermentation leading to a different production of SCFA. ***Figure 5.4*** shows that the total production of SCFA was higher in the DC and lowest in the AC, according to Van den Abbeele et al. (Abbeele et al., 2010). Overall, the SCFAs concentrations in all parts of the colon remained similar in the

control and in the vessels which dietary supplementation was added, showing rates of reproducibility along the times.

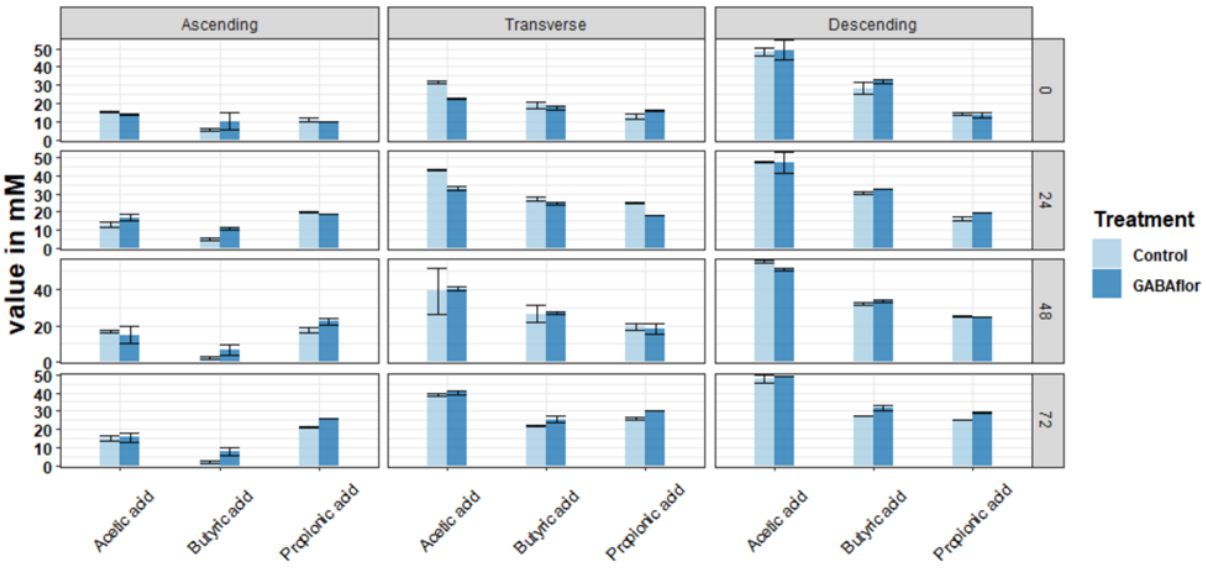

Figure 5.4. SCFA profile (mM) analyzed in SHIME. *The SCFA profile was analyzed for the ascending, transverse, and descending colon, after control and probiotics supplementation at time 0, after 24, 48, and 72h*

Neurotransmitters production in control and dietary supplementation in SHIME

To highlight the change of the neurotransmitters, the concentration at each colon tract and each point were reported in ***Figure 5.5***. The data in ***Fig 5.5*** production of GABA has been observed in the AC, TC, and DC (***Fig. 5.6***), while consumption of glutamate is mainly observed in the AC and TC, with an almost undetectable concentration in the DC. A small amount of acetylcholine has been observed to be produced in AC, TC, and DC after 48 h and 72h (***Figure 5.7***). Acetylcholine and GABA were produced chiefly in the AC.

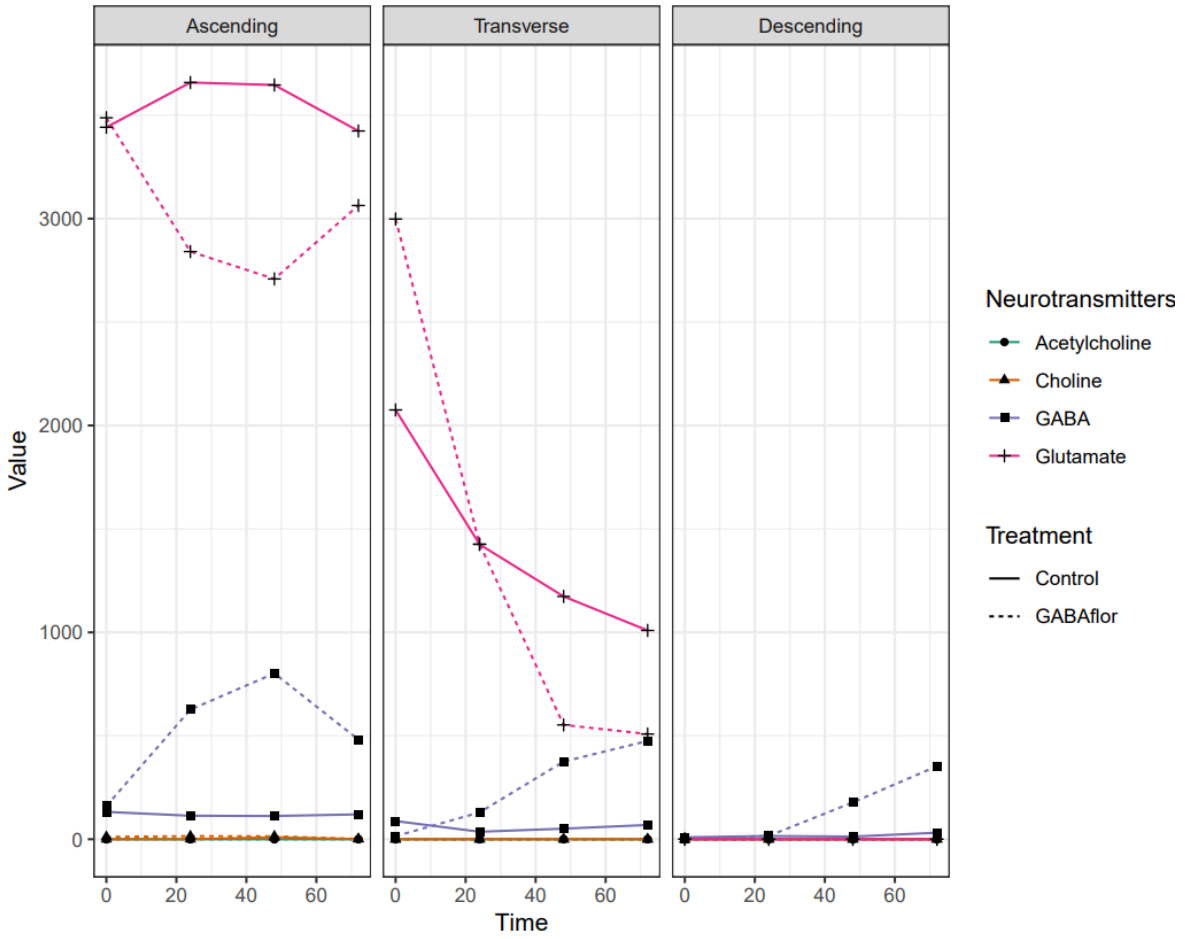

Figure 5.5. Neurotransmitters profile (mg/L) for the ascending, transverse, descending colon after a control and probiotics supplementation*. Each data point represents the GABA, Glutamate, acetylcholine, and choline profile at times 0, 24, 48, and 72 hours*

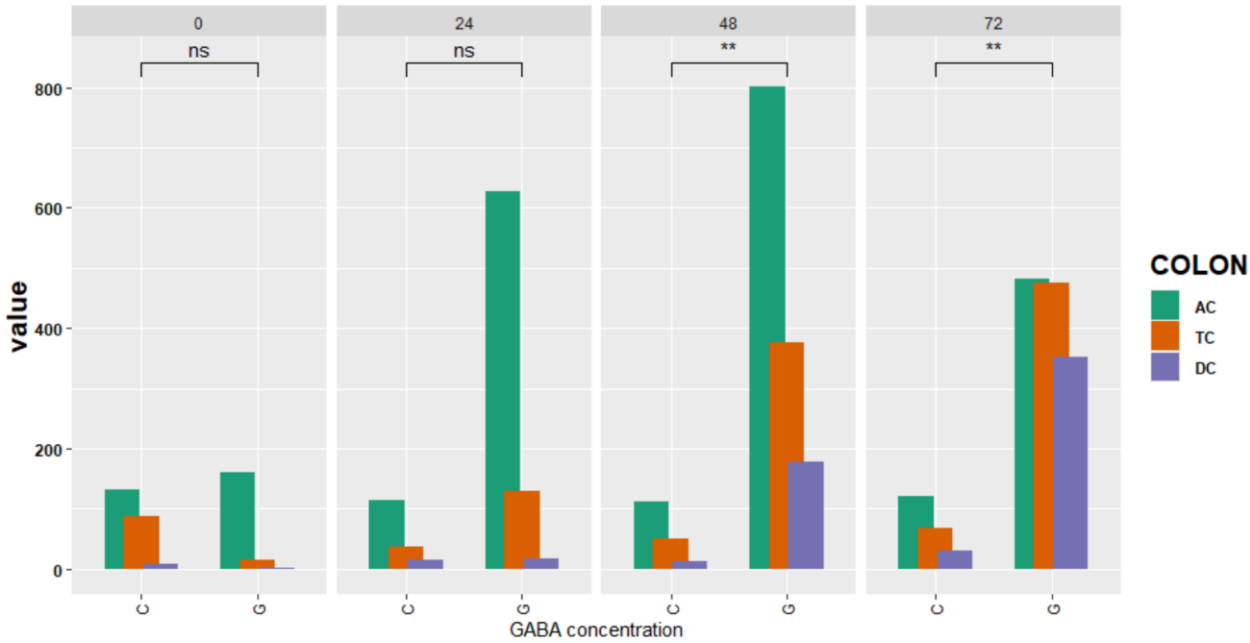

Figure 5.6 Change in GABA concentration between the control (C) and treatment with the probiotic supplementation (G) in the ascending (AC), transverse (TC), and descending colon (DC). *The samples were taken at time 0, 24, 48, and 72h from the SHIME*

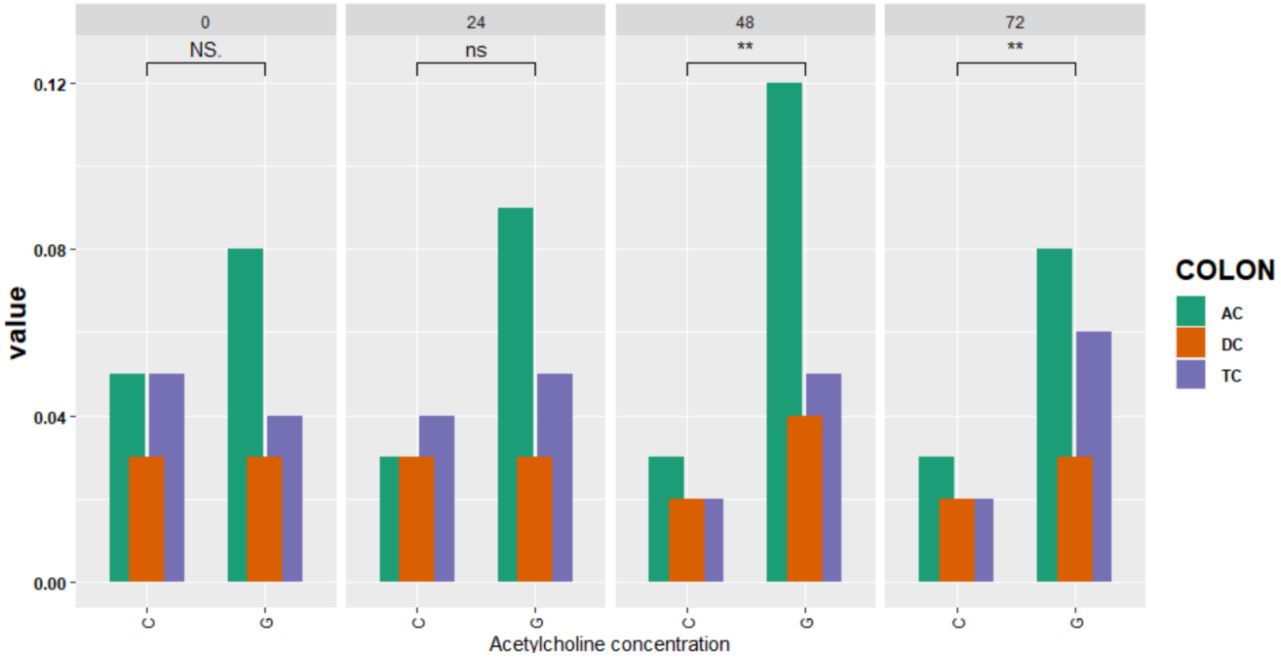

Figure 5.7 Change in acetylcholine concentration between the control (C) and treatment with the probiotic supplementation (G) in the ascending (AC), transverse (TC), and descending colon (DC). *The samples were taken at times 0, 24, 48, and 72h from the SHIME*

Changes in the gut microbiota during the treatment with probiotic formula

To identify the differences in microbial composition between samples, a heatmap was generated indicating the relative abundance of members of the microbiota in the samples including the treatment GABAflor, and the control in all colon regions at times 0, 24, 48, and 72 hours **(*Fig. 5.8*)**. Overall, different tracts of the colon in the SHIME system showed diversity in microbial composition. *Veillonellaceae and Lactobacillaceae* family, belonging to the phylum *Firmicutes,* and *Enterobacteriaceae* family, phylum *Proteobacteria*, were higher in abundance in the AC than in TC and DC. However, AC treated with the probiotic formula harbored a lower relative abundance of *Enterobacteriaceae* compared to the control while genus *Pediococcus* and *Bacteroides* showed more abundance in the treatment with the probiotic intervention at T3 and T7 than the control. Higher presence of *Lactobacillaceae* then in the control was also observed in DC at T5. TC shared more features with DC, showing to be predominantly dominated by *Firmicutes*. In DC *Lachnospiraceae* family, were principally abundant. The main differences in TC and DC between the control and the probiotics addition were the higher relative abundance of the genus *Blautia* and the species *B.ovatus* in the treatment with probiotics at T5 and T7. In DC, *Coprococcus*, *Dorea* and *R.gnavus* were more abundant in the control. Principal-component analysis did show clustering of the subjects according to the treatment received (see ***Fig. 5.9***), highlighting that *Bacteroides* were the relatively dominant microbial genera in the arm of the SHIME undergone to the probiotic treatment. Genera were excluded from the heatmap analysis if their relative abundance was $< 0.1\%$ on average.

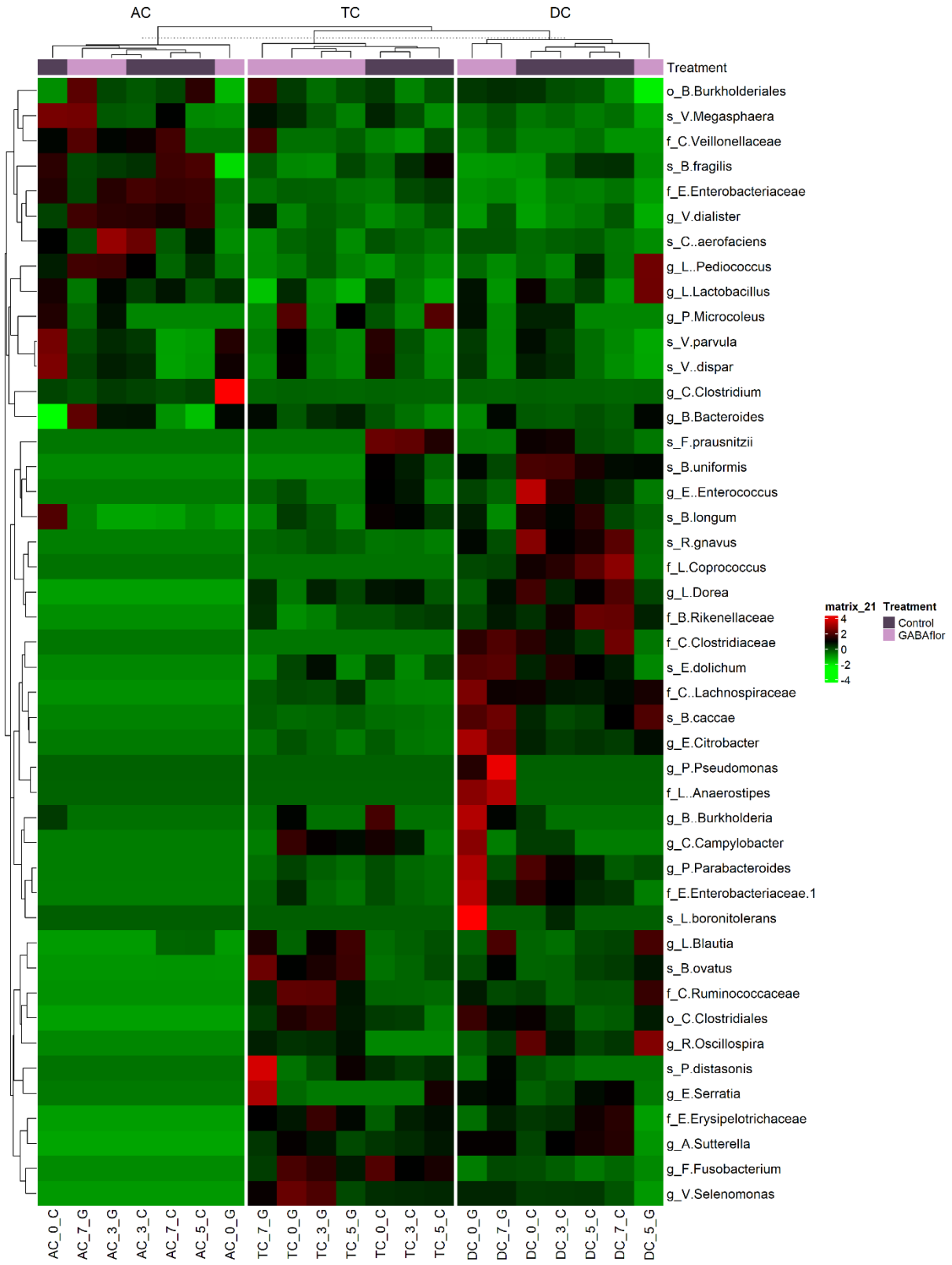

Figure 5.8. Heatmap with the relative abundance of 16S rRNA sequencing. *The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and light green indicating low abundance. The heatmap is splitted by columns in ascending colon (AC), transverse colon (TC), descending colon (DC). Each column bar on the top of the heatmap is divided per colored according to the Treatment: GABAflor (dietary supplement plus precursors of the*

neurotransmitters) and control (only the precursors were added). Samples collected at time 0 (T0), 24 (T3), 48 (T5) and 72h (T7) were clustered according to their similarities.

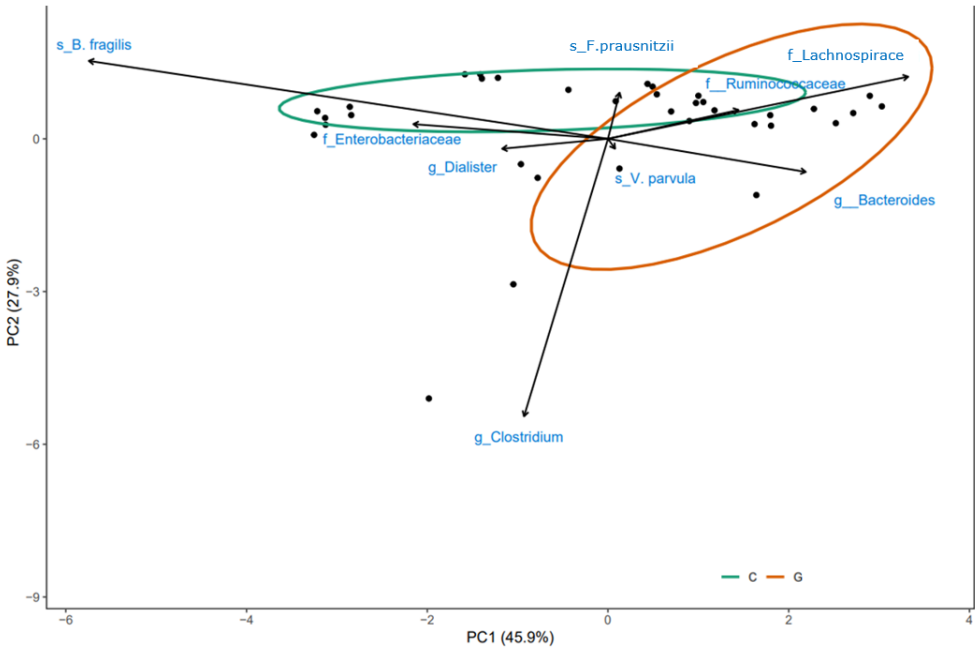

Figure 5.9. PCA showing the effect of the treatment with the probiotic supplementation (G) and the control on the overall microbial composition.

5.4 Discussion

In this study, the capability of a probiotic formula to influence neurotransmitters concentration was evaluated *in vitro* batch fermentation in three colon compartments by using the reliable SHIME model. To the best of our knowledge, our study is the first using SHIME *in vitro* model to assess the psychobiotic capacity of a probiotic formulation. Evidence of the positive effects attributed to probiotics are continuously expanding, and the modulation of the gut microbiota and its metabolism – using for instance probiotics – to improve the mental host's health via the 'gut-brain axis', is becoming increasingly important (Wieërs et al. 2019).

Change in neurotransmitters concentration during the fermentation *in vitro* and SHIME has been observed mostly in GABA, probably due to the wide function of these molecules, including communication between bacteria, pH homeostasis, and the generation of metabolic energy (i.e., proton motive force)(Otaru et al., 2021). Previous studies have shown that GABA, the major inhibitory neurotransmitter in the brain, can be produced by some bacteria as a strategy to harvest carbon and nitrogen sources under nutrients-limiting conditions or as an acid resistance mechanism, producing CO_2 and consuming a proton (Shaibe et al, 1985; Yogeswara, et al. 2020). Two distinct pathways are involved: in the first case, GABA is an intermediate for

the generation of succinate via the GABA shunt pathway in the latter case is the final molecule produced via the glutamate decarboxylase (GAD) system, converting glutamate to GABA (Strandwitz et al., 2019). An alternative ATP-generating pathway for GABA consumption was described for the environmental anaerobe *Clostridium aminobutyricum*. KLE1738 (Strandwitz et al., 2019).

Several studies suggest that genes encoding GAD could be present in a significant proportion of human gut microbiota, including Lactobacilli even if this metabolic ability is more likely strain- rather than genus-related (Otaru et al., 2021; Strandwitz et al., 2019). According to two different works (Bianchi et al., 2014; Da Cruz Rodrigues et al., 2020) the pH of the ascending colon is between 5.6 and 5.9, a range that favors the growth of saccharolytic bacteria (such as *Lactobacillus* spp.) in this part of the colon due to the greater quantity of carbohydrates available in this compartment. This could explain a higher concentration of GABA in the ascendant colon then in transverse and descendent colon.

During the treatment with the probiotic supplement in the SHIME, microbial fermentation of MSG resulted in a highest consumption in the AC and in TC. MSG and choline were added to the colon medium, in both arms (control and treatment), as precursors of GABA and acetylcholine. Comparable to the study of Peng et al., (2018), no significant changes in microbiota composition and metabolites production has been observed related to the only addition of MSG and its consumption by gut microbiota in the control. However, our work expand on the study of Peng et al. (2018), and highlights the important role of specific strains in the gut able to convert MSG in GABA. To the best of our knowledge this is the first study investigating the ability of an industrial formulation based on probiotics to influence the production of neurotransmitters *in vitro* batch fermentation, followed by the further assessment in SHIME.

We also highlighted that the gut microbiota composition varied among AC, TC, DC. Nevertheless, a certain variation trend of some specific intestinal bacteria genera were observed during the experiment in the treatment arm. An analysis at the genus level indicated that the relative abundance of *Bacteroides* increased slightly in the vessels where the probiotic formulation was added, whereas *Enterobacteriaceae* family higher in the control. With regards to the *Bacteroides*, Strandwitz et al., (2019) have indicated this genus as a GABA producing that may exert great influence on consumption of glutamate and survival of other species using GABA as growth factors. They also identified 102 potential consumers in the gut of this neurotransmitter, belonging mainly to the *Pseudomonas*, *Acinetobacter* or *Mycobacterium* genera. Some studies observed mucin degradation in *R. gnavus* and *Dorea* (Crost, et al. 2016, Saito et al. 2018) that were instead more abundant in the control then in then in the intervention with probiotic.

Even though the composition of the microbiota is strongly affected by inter-individual variation, Firmicutes and Bacteroidetes contributed the two predominant phyla in the samples,

which is consistent with previous studies that found that these two phyla compose the majority of human intestinal microorganism phyla (Marchesi et al., 2009). Members of the microbiota from phylum Bacteroidetes are represented by a variety of species. Otaru et al., (2021) screened several *Bacteroides* strains observing intra-species variation between *B. faecis* PB-SZSJC and DSM 2151 to produce GABA in presence of the same enriched glutamate substrate (YCFA) and that the highest GABA-producers (>35 mM) were *B. ovatus* DSM 1896, *B. fragilis* PB-SZSJC, and *B.faecis* PB-SZSJC whereas the lowest GABA levels was observed in *Bacteroides caccae* DSM 19024 (< 1 mM) . The only prominent variation detected between *Bacteroides* strains lies in the genomic distance between the gadB/gslA operon and the gadC/K+ channel-encoding gene cluster, potentially affecting gene co-regulation due to proximity.

In contrast, phylum Firmicutes is composed of more than 200 different genera such as *Lactobacillus, Bacillus, Clostridium, Enterococcus,* and *Ruminicoccus*. *Clostridium* and *Ruminococcus* were the main genera observed in this experiment, while Peng et al., (2018) described the Firmicutes represented 95% by *Clostridium* genera, which are known for their abilities to metabolize fiber and produce butyrate, a short-chain fatty acid. SCFAs were reported to be the major source of nutrition for colonic epithelial cells, especially butyrate. Within Lachnospiraceae observed in the treatment with probiotics, *Blautia* as butyrate producer, represent the genera most involved in the control of gut inflammatory processes, atherosclerosis, and maturation of the immune system (Vacca, 2020).

The results obtained from the plate count agar of *Lactobacillus* sp. did not show differences between the control and treatment and were similar to the results obtained in other SHIME experiments by Sivieri et *al*. (2013), after one week of treatment with *L. acidophilus* CRL 1014. The count we observed in the vessel mimicking the ascending, transverse, and descending colon were also consistent with those reported previously by Luiza et al., (2016).

Understanding the relationship between microbial composition and the level of fecal neurotransmitters GABA and glutamate can highlight the key role of some microbes able to redirect the microbiome activity towards GABA or glutamate production. In relation to this we also investigate negative correlation between GABA and glutamate *in vitro* batch fermentation that were consistent with those reported previously by Altaib et al., (2021).

SCFAs are the main carbon flow, produced by gut bacteria in the large colon. They represent the functionality of the gut microbiota community and are speculated to have a mediational role in the microbiota–gut–brain axis crosstalk. The metabolic abilities of intestinal microorganisms is able to transform complex nutrients, such as cellulose, pectin, hemicellulose, lignin and mucins into simple sugars that are fermented to form short-chain fatty acids. The concentration and ratio of resulting SCFAs did not showed change along the time, demonstrating stability of system.

Overall, the psychobiotic effect showed *in vitro* by the formulation of *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* might be hypothesized even after a relatively short intake

period. In both experiment, *in vitro* batch fermentation and in SHIME, the addition of MSG with the probiotic supplement prompted the production of GABA, no observed where only the precursors where included.

However, some limitations were also observed, such as the need to consider additional biological replicates in the SHIME experiment. An intrinsic limitation of the SHIME model system is that it does not model the intestinal absorption, so the neuroactive compounds produced are not constantly removed as can happen *in vivo* and an overestimation of their production is expected. Nevertheless, long-term studies with longer intake and wash out periods are needed to confirm such hypothesis. Despite the limitations, we could conclude that the dynamic view of microbiota and microbial psychotropic metabolites demonstrated by short-term treatment with *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* opened new perspectives on the interaction of probiotics with microbiota and metabolites. The results indicate that the probiotic formula has a positive impact on the modulation on the concentration of neuroactive compounds by determining a decrease in the concentration of glutamate, increase in GABA, and stimulating the growth of *Bacteroides* with potential benefits to mental health and promoting a healthy microbiota. Finally, a clinical trial is essential to confirm the results found in the dynamic colonic fermenter (SHIME®).

5.5 Conclusion

Microbial modulation of the gut microbiota is a safe and effective means to improve mental health outcomes via microbiota-gut-brain axis. The data presented herein reveals a potential role of a formulation based on probiotics, vitamins and zinc to modulate metabolic- and neuroactive-microbial metabolite in *in vitro* batch fermentation and SHIME. In these two experiments we highlighted the possibility to produce GABA only when to precursors were added the formulation made with psychobiotic investigated. This suggests the important role of the supplementation with GABA-producing probiotics to increase endogenous GABA concentration in the intestine. This study also highlights the importance of assessing the neuroactive potential and composition of the gut microbiota, emphasizing the imperative role performed by certain microbes for the production or consumption of specific neurotransmitters, such as GABA-producing *Lactobacillus*. The finding of this study may aid the development of potential probiotics to improve microbial neurotransmitters modulation, which can support the maintenance of the mental conditions and psychiatric health of the host. Further substantiation of these exploratory *in vitro* need to be confirmed with replicates and moved towards the clinic to confirm the potential mechanism of action observed in this study on mental outcomes such as cognitive performance and mood. Future experiments should focus on other potential mechanisms of action of these probiotics involved in the gut-brain axis. Furthermore, the potential for other neuro-active compounds-producing strains to have a causative effect on the outcomes should be explored.

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Chapter 6

Investigation of the mood and cognitive effect of a dietary supplement containing neurotransmitters-producing probiotic strains and vitamins: A randomized crossover double-blind, placebo-controlled study

Abstract

Background: Preclinical and clinical studies have identified certain probiotics as psychobiotics able to influence stress-related behavior, sleep, and cognitive outcomes. However, it is still unclear which strains have psychotropic activity in humans and the metabolic pathways involved. In our previous study *in vitro*, *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025 have been shown to produce GABA and acetylcholine, consequently, we examined if these promising findings could have an effect on cognitive behavior in human volunteers.

Aim: To investigate the effects of probiotics in the alleviation of stress in healthy adults, and their effect on several cognitive performance domains, along with our focus to identify and justify strain specificity on selected health benefits with a precisely targeted population. Furthermore, we wanted to investigate if the treatment could have influenced the production of GABA, glutamate, acetylcholine, and choline.

Methods: This 12-week randomized, double-blind, placebo-controlled, cross-over study investigated the effects of a probiotic formulation (*Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025) on psychological, memory, and cognition parameters in 22 (Probiotic= 22, Placebo=21) adults with a mean age of 26±10.1 years old. All subjects fulfilled the criteria of mild-moderate stress upon diagnosis using the DASS-42 questionnaire.

Results: There was no overall effect of probiotic treatment on subjective stress measures measured by DASS-42. The intervention of the probiotic formula has increased the speed for social-emotional cognition compared to baseline (p=0.0106, 95% CI=0.01 – 0.08), and on working memory measured by one card learning (p=0.046); while no significant effects were observed on visual learning and memory, basic attention, and verbal learning. Compared to participants who received the placebo, who received the 4-week multispecies probiotics did not show a reduced cognitive reactivity to sad mood, depression, and stress. No relevant correlations were observed between the neurotransmitters, scores of LEIDS, DASS-42, and cognitive tests.

Conclusion: Probiotic did not exert a benefit on mood measured by self-reported measures. However, relative to placebo, the probiotics group did show a significant stress-related increase in working memory performance after the intervention. These findings highlight the challenges, associated with the assessment of the psychotropic effect of probiotic strains in healthy human participants. Further interventional studies investigating the effect of these psychobiotics in populations with stressed-related disorders are required for a longer period of intervention and a larger sample size.

***Keywords*:** Stress, Cognition, Memory, *Levilactobacillus brevis* , *Lactiplantibacillus plantarum*, Gut-brain-axis, psychobiotics

6.1 Introduction

Stress is an emotional and physiological reaction to major life events and demanding circumstances. A successful stress response can be useful to stay alert and focused, while repeated exposure to stressful conditions can easily tip over the stress from being an important motivational mechanism into a maladaptive and inconvenient response that can have a detrimental effect on both physical and mental health (Morgado and Cerqueira 2018). Social stress is also known to induce depressive-like behaviors and to have well-documented effects on cognitive processes, such as working memory (WM) (Lukasik et al. 2019). Nevertheless, there are large interindividual differences in outcomes after stressful experiences (Leeuwen et al. 2018). It is thought that this adaptive response may be dysfunctional in vulnerable individuals, potentially contributing to the risk for psychopathology like the Major Depressive Disorders (MDD). The current pharmacological treatment approaches like the use of monoaminergic-based drugs inhibitors (SSRI) provide a significant therapeutic benefit, but some patients are considered treatment-resistant and the majority of them experience recurrence after interrupting the treatment, as well as undesired side effects that make the adherence of patients to these medicines is relatively low (Leeuwen et al. 2018; Lukasik et al. 2019). Thus, various efforts regarding feasible and natural approaches are initiated to tackle these conditions that can lead to psychiatric disturbances.

An increasing number of studies continue to highlight the importance of connections among the brain, and gut, including the intestinal microbiota. Evidence for a link between microbiota and stress has been gathered mostly from experiments carried out in the microbiota-depleted model, either through treatment with a combination of antibiotics or use germ-free (GF) lines (Luo et al. 2018; Sarkar et al. 2016; Luczynski et al. 2016) . Developing a novel therapeutic method targeting the gut microbiota for the treatment of mood disorders, especially derived from outside stressors, is coming out as an appealing strategy to moderate their impact on the quality of life and prevent cognitive impairment.

In this scenario, a novel class of probiotic microorganisms, called psychobiotics, is emerging as able to convey benefit upon the host's mental health via the bidirectional dynamic microbiota-gut-brain crosstalk. In Europe, probiotic bacteria with positive effects on mental health are not yet marketed with a recognized and specific health claim from EFSA (Gibson et al. 2011). Therefore, studies such as this can be pioneering in the production of basic information to be used for the development of innovative formulations, contributing not only to the advancement of knowledge and innovation in the sector but hopefully also to lay the foundations for the development of new nutraceutical products, with potential commercial impact. Evidence of probiotics' effect on cognitive functioning and mood was gleaned chiefly by preclinical studies and few encouraging proof-of-principle studies in healthy human volunteers have demonstrated that specific probiotic strains can influence symptoms of the stress-related gut-brain disorder and alter resting brain activity, cognitive performance, and

memory (Liu, 2019). However evidences on clinical studies are still at the beginning and some of them portray conflicting results, or difficulty in moving promising preclinical studies to healthy human participants (Kelly et al. 2017). The candidate psychobiotic used in this study displayed to produce neurotransmitters on their growth media enriched with precursors, *in vitro* batch fermentation, and in the Simulator of Human Intestinal Microbial Ecosystem (SHIME).

Many unanswered questions remain regarding the use of psychobiotics in clinical studies, with much work required to test optimal dosing, strain, timing in the application, and specific effect on mental health. So far several studies have also demonstrated the synergistic impact of combined vitamins or mineral and probiotic administration to affect hormonal, inflammatory, and oxidative stress parameters, but no study on humans have investigated this synergistic effect on human neural function and behavior, particularly those of relevance to stress-related disorders. Vitamins and minerals like zinc also claim effects on the normal functioning of the nervous functioning and to the normal psychological function (Pang et al. 2021; Singh et al. 2020; Steinert et al. 2020). The B vitamins collectively play a critical role at all levels of brain function as co-enzymes and precursors of enzymatic processes, for example, the vitamin B6 in the production of GABA, the main inhibitory neurotransmitters with anxiolytic effect (Young et al. 2019). Thus, the aim of this study is to investigate whether the intake of a psychobiotics, vitamin B6, D, and zinc could boost human cognitive performance and manage the psychological response to stress, via the microbiota-gut-brain axis.

6.2 Materials and Methods

Screening

Healthy study participants were recruited via advertisement, direct contact, and mailing list, from and around the Wageningen area. 107 volunteers responded to the advertisement and direct contact; Participants were screened based on the inclusion and exclusion criteria, after receiving the informed consent file from the participants. Inclusion criteria included men or women between the age of 18-60 years old, a BMI between the range of 18.5-25 kg/m2, and a mild/moderate stress score in the DASS-42 questionnaire. For the complete list of inclusion and exclusion criteria see ***Supplementary material (Appendix D).*** Participants received the DASS-42 questionnaire and a medical information questionnaire as screening tests to assess their eligibility. Based on the DASS-42 stress score and the medical questionnaire the decision to include or exclude the participants was made in collaboration with a medical specialist based on eligibility. The outline of the visit procedure is represented in ***Table 6.1*** and the flowchart of the study design in ***Figure 6.1***.

Table 6.1*.Outline of visit procedures*

Procedure	**Visit 1** screen	**Visit 2** Baseline 1	**Intervention 1**	**Visit 3** Intervention 1	**Visit 4** Baseline 2	**Intervention 2**	**Visit 5** Intervention 2
Informed consent	x						
Demographic data	x						
Medical questionnaires	x						
DASS-42 (Depression Anxiety Stress Scales (15 minutes)	x			x	x		
Inclusion/exclusion	x						
LEIDS-r (Leiden Index of Depression Sensitivity-revised) (10 minutes)		x		x	x		
Cognitive assessment (CogState Battery) Practice (45 minutes)		x		x	x		
Cognitive assessment (CogState Battery) (45 minutes)		x		x	x		
Well-being questionnaires		x		x	x		
Dispense study substance and give instructions		x			x		
Intervention probiotic/placebo			x			x	
Periodic reminder to ensure compliance with product consumption			x			x	
Take note of the menstrual cycle		x		x	x		x
Record the number of supplements taken				x			x
Registration of adverse events		x		x	x		x

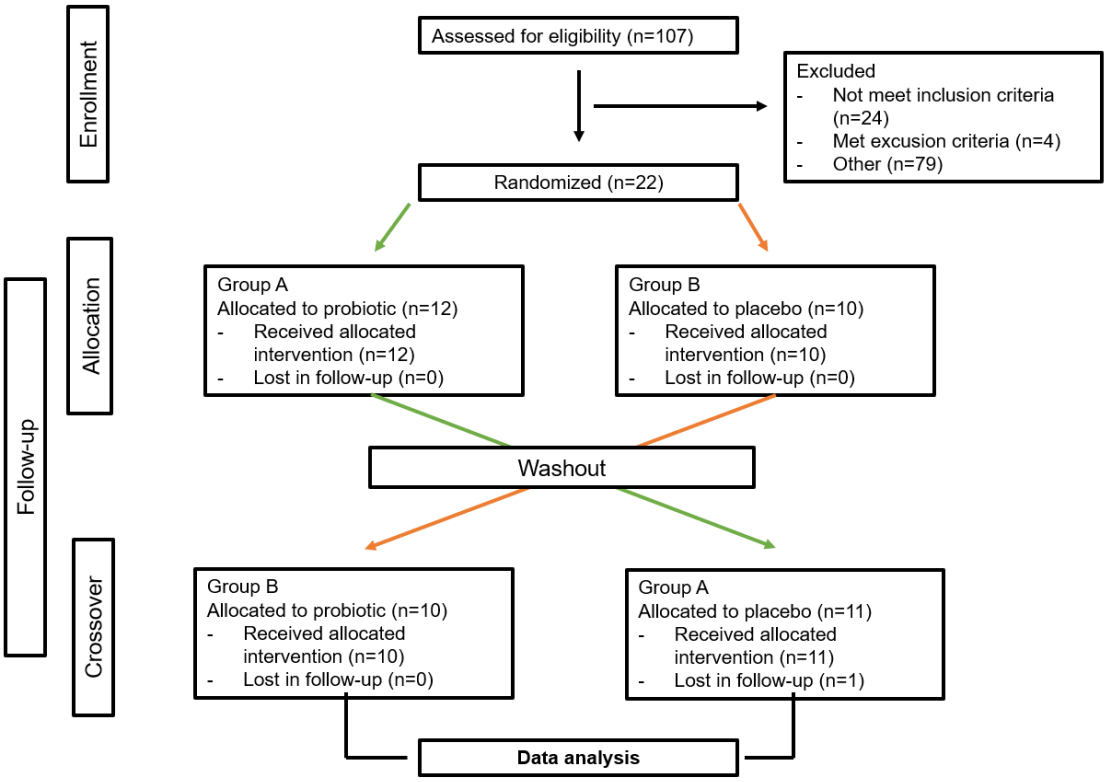

Figure 6.1. *Study design*

Randomization and masking

Randomization was done by a researcher who had no contact with the participants, in this way the study was double-blinded. A random sequence was randomly assigned to a confidential treatment number linked to the two treatment arms (first probiotic product and second placebo or vice versa). The allocation sequence was concealed from the researchers and details of the allocated group were given on code containing the sequential number which was placed on the product. The products of identical appearance were labelled with the treatment number by another independent researcher. The Independent study coordinator dispensed either placebo or probiotic sticks according to a computer-generated randomized sequence.

Blinding

All participants, study coordinators, and researchers were blinded throughout the entire study. The study was unblinded along with the entire study and available in the case of an emergency or at the end of the study when all statistical analyses were completed.

Study Protocol

The study was conducted as a randomized double-blind placebo-controlled cross-over intervention trial, to test the effect of a supplement consisting of probiotics, vitamins, and zinc on cognitive functioning in adult women and men. The crossover design allow to study the differences in treatments yielding to a more efficient comparison of treatments than a parallel design as the subjects are on their own controls and the within-patient variation is less than

between-patient variation (Solito et al. 2021). A washout of 4 weeks has considered enough to do not have carry-out effects. Furthermore, no habituation effects which could interfere with the outcomes were expected since the testing software is designed to limit learning effects.

Recruited individuals were randomized into two groups. See ***Table 6.1*** and ***Figure 6.1*** for the overview of the cross-over study design.

A. Probiotic supplementation – Placebo supplementation (PRO-PLA; $n = 12$)

B. Placebo supplementation – Probiotic supplementation (PLA-PRO; $n = 10$)

One group received supplementation with probiotic sachets containing > $2x10^9$ CFU/day *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025 and the other group a placebo (PLC) formulation, containing the same excipients without the probiotic strains, for 4 weeks. Probiotic (PRB) formulation was a 1:1 mixture of the 2 strains.

Ethical Statement

The study protocol was approved by the Medical Ethics Review Committee of Brabant (Tilburg, The Netherlands; Protocol number: NL76751.028.21). The study was conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was obtained from all participants at the screening visit before any study procedures were conducted. The consent form is in ***Appendix*** included in the PIF (Participant Information Form). Participants were free to withdraw from the study at any time.

Dietary intervention: Probiotics and placebo products

The supplement under investigation was GABAflor, produced by the company Proge Farm in Italy. GABAflor is a stick of powder that consists of: *Lactiplantibacillus plantarum* P30025 and *Levilactibacillus brevis* P30021 (Both 10^9 CFU/stick) which both have been shown to be beneficial for gut health, vitamin B6 included as pyridoxine hydrochloride (2,8 mg/stick), vitamin D3 as cholecalciferol (5 ug/stick), and zinc (7,5 mg). As already described in the introduction and rationale paragraph, both vitamins are associated with improving cognitive functioning. Lastly, the mineral zinc (zinc gluconate 64.655mg/stick) is included which is claimed to contribute to normal cognitive functioning. In order to give the supplement a pleasant taste both fructose and cacao powder are added. One stick per day was consumed for 4 weeks. The powder sticks could be dissolved in milk or any other liquid to make consumption easier.

The placebo was not able to be distinguished by package, color, taste, or smell in order to maintain treatment allocation concealed from the participants. The placebo had the same taste since it also consisted of cacao powder and fructose. See ***Supplementary material*** for further summaries.

Sample size

The appropriate sample size was estimated by a priori analysis for a clinical research study using G*Power software (Kang 2021). G*Power is a tool to compute statistical power analyses for many different tests and can also be used to compute effect sizes and to graphically display the results of power analyses. The primary objective of the study is to evaluate verbal learning and memory, processing speed, visual attention, learning, and working memory. With a power of 0.95 for a one-way ANOVA, a minimum sample size of 36 was required to demonstrate an effect size of 0.25 at alpha 0.05 and a power of 0.95. Allowing for a 20% drop-out, as seen in similar studies, at least 44 participants should be recruited, divided between 22 males and 22 females. This ensured enough power in case some of the assumptions were not met or in case of some incomplete results (i.e., missing data). A p-value of <0.05 was considered significant.

Primary outcome: Cognitive function assessment

All subjects were assessed for memory and cognitive functions using the computerized CogState Brief Battery (CBB) (Mielke et al. 2015). Administration of the CogState battery test was conducted in a personal laptop, installed with the CogState ClinicalTrials software. All subjects first underwent one initial practice prior to the actual test battery. The study coordinator was available to help the subjects understand the tasks during sessions. From the CogState test software, several tests were chosen. These tests were chosen because they have been shown to indicate a significant improvement in other two studies evaluating the effect of probiotics on mood (Lew et al., 2019; Chong et al.,2019). Outcome parameters of the tests include a number of correct and incorrect responses, response speed, and accuracy of performance.

The tests were administered in this order:

- *International Shopping List Test (ISLT)* - a list of items is read to the participants by the researcher. This list consists of 16 items which can be bought in the supermarket. The words are read at a rate of 1 word per 2 seconds. Immediately after the researcher is done reading the list, subjects are asked to recall as many words as possible in 1 minute. In the meantime, the researcher keeps track of which words have been said. Mistakes, duplicates and right answers are all noted and based on this, a score is calculated by the CogState software. The task is stopped once the time runs out or when the subject confirms that no more items can be recalled. This process is repeated a total of 3 times. After recording the trial 3 times, the task is stopped.

- *Detection (DET) task* – The subject is asked to press a key as soon as a playing card, that is displayed on the screen, turns over. The outcome of this task is speed and accuracy of each response. The outcome variable is measured in milliseconds for correct responses which is normalised using a logarithmic base 10 (Log 10 transformation).

- *Identification (IDN) task* – The subjects are asked to press the "yes" key when the card that has flipped over is red. If the card is not red, they have to press the "no" key. The task ends as soon as the subject has given 30 correct answers. The correct outcome is recorded in milliseconds and normalised using a logarithmic base 10 (log 10 transformation).
- *One Card Learning (OCL) task* – The subjects have to press "yes" when the card has appeared before and "no" if it has not. The task ends when 42 trials have been recorded. The outcome variable is the proportion of correct responses (accuracy) normalised with the help of an arcsine root transformation.
- *One Back (ONB) task* – a task that assesses working memory by asking the subject whether the presented card is the same as the previous presented card. The subject can either press "no" or "yes". The primary outcome variable of this task is accuracy. The task ends upon recording 30 trials.
- *Social Emotional Cognition (SEC) task* - 4 pictures of faces are presented on the screen. By clicking the odd-one-out, the task measures emotional recognition. The task end upon recording 30 trials. The outcome measures are accuracy and reaction speed.

A composition of each battery and their respective outcomes are listed in ***Table 2***.

Table 6.2 *Test information and cognitive domain assessed*

CogState Test (TCode)	Cognitive Domain	Test Description	Primary outcome and Interpretation
Detection Test (DET)	**Psychomotor function** Has the card been turned over?	Detecting and responding to a cue of a card turning over as quickly as possible	Speed of performance; mean of the log10 transformed reaction times for correct responses (**lmn**) **Lower score = better performance**
One Back Test (ONB)	**Working memory** Is this card the same as the previous card?	Remembering the previous card and identifying whether the presented card is the same	
Identification Test (IDN)	**Attention** Is the card red?	Identifying the color of a card and clicking the right button in accordance with it	
International Shopping List Test (ISLT)	**Verbal learning** Tell me the items on the shopping list	Remember and recall a list of 16 shopping items read to the participants in 3 trials	Number of correct responses made when remembering the word list on three consecutive trials (**cor**) **Higher score = better performance**
One Card Learning Test (OCL)	**Visual learning** Have you seen this card before?	Identifying whether the presented card was seen before	Accuracy of performance; arcsine square root proportion correct (**acc**) **Higher score = better performance**
Social-Emotional Cognition Test (SECT)	**Emotional Cognition** Which picture is different?	Identifying which of the four presented faces is the odd-one-out	

Secondary outcome: Questionnaires measuring Mood-related aspects

Leiden Index of Depression Sensitivity-Revised Test (LEIDS-r):

The secondary outcome of our study was the difference in the score at the Leiden Index of Depression Sensitivity-Revised test (LEIDS-r) between and within the subjects belonging to the experimental group and the control group. The LEIDS-R is a self-report questionnaire that tests cognitive reactivity to sad mood, which is an index of cognitive vulnerability to depression. LEIDS-r scores have been found in multiple longitudinal studies to predict depression incidence and to correlate with depression risk factors, such as depression history, genetic markers of depression. It consists of 34 items describing different situations. Before answering the items, participants are asked to take a few minutes to imagine their feelings and thoughts when they experience a sad mood. They then rate how much each item applies to themselves on a 5-point scale ranging from 0 (*not at all*) to 4 (*very strongly*). Of note, the experimenter emphasizes that each item describes a situation happening on *a day that is not good, but you don't feel depressed*. The LEIDS-R consists of 6 subscales: Hopelessness/Suicidality (5 items), Acceptance/Coping (6 items), Aggression (5 items), Control/Perfectionism (6 items), Risk aversion (6 items), and Rumination (6 items). The total score for each subscale is obtained by adding the scores from the corresponding item. The range of the total score for the Aggression and Hopelessness/Suicidality subscales is from 0 to 20. The range of the total score for the other three scales is from 0 to 24. The higher the total subscale score, the higher the vulnerability to the assessed dimension.

Depression Anxiety Stress Scale (DASS-42)

DASS-42 is a 42 item self-report validated inventory comprising of three scales designed to measure the negative emotional states of depression, anxiety, and stress, where each of the three scales contained 14 items. DASS-42 has been widely used in both clinical and non-clinical settings. The depression scale assessed dysphoria, hopelessness, devaluation of life, self-deprecation, lack of interest/involvement, anhedonia, and inertia. The anxiety scale assessed autonomic arousal, skeletal muscle effects, situational anxiety, and subjective experience of anxious affect, while the stress scale assessed difficulty in relaxing, nervous arousal, and being easily upset/agitated, irritable/overreactive, and impatient. Subjects were assessed based on a 4- point Likert scale (0 ¼ did not apply to me at all, 1 ¼ applied to me to some degree or some of the time, 2 ¼ applied to me to a considerable degree or a good part of the time, 3 ¼ applied to me very much or most of the time). Scores for each subscale were categorized into five severity ranges, namely normal, mild, moderate, severe, and extremely severe. DASS-42 was used as a screening test and for assessment at intervals of 4-weeks (week4, 8, 12).

Fecal sample analysis

Faecal samples of the volunteers were collected at baseline and every four weeks (during every test session). For analysis, fecal samples were diluted 10 times with deionized water (w/v) and homogenized. After centrifugation at 2,000 RPM for 10 minutes, the supernatant was filtered through a 0.45 µm syringe filter Phenex (Phenomenex, Aschaffenburg, Germany). Samples were further diluted in 50% ACN and 5 µL injected on a SeQuant® ZIC HILIC 3.5 µm, 4.6 x 150 mm (Merck KGaS, 64271, Darmstadt, Germany) attached to a SeQuant® ZIC HILIC PEEK coated guard column 20 x 2.1 mm (Merck KGaS, 64271, Darmstadt, Germany). The analysis will be carried out with a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The flow rate set at 0.7 mL/min and the column temperature at 40 °C. The mobile phases consist of 0.1% formic acid (solvent A), acetonitrile with 0.1% formic acid (solvent B) with the following elution profile (t in [min]/[%B]): (0.0/90), (4.0/70), (10.0/20), (13.0/20), (15.0/90) and (18.0/90). MS data is collected for 18 mins. Data is processed with LabSolutions (Shimadzu Corporation, Kyoto, Japan).

Clinical and adherence monitoring

All the analyses were performed at the recruitment (T0), after the first 4 weeks of treatment (T1), at the beginning of the second phase (T2) and end of washout, and after the last 4 weeks of treatment (T3). Case report forms (CRF) were completed. Patients were asked to report any adverse reaction, antibiotic therapy or other drug administration that occurred. The adherence was monitored counting the returned sachets

Statistical Analysis

To characterize the relationship between treatments and cognitive function performance, as well as mood survey, linear mixed-effects analyses were performed using the lme4 (Version 1.1–17) package in R (Version 3.5.0). Mixed-effects analyses allowed for the modeling of variation in how individual participants reacted to each treatment. Treatment was included as a categorical fixed effect with three levels (baseline, probiotic, placebo) in models of all outcomes. Models of the DASS-42 and LEIDS-r measures included the fixed effect of group and interaction between treatments and group to identify differences in the outcomes between groups under a different sequence of treatment. Models of cognitive tasks included the fixed effect of the language. To account for potential moodiness or depression due to the female period, changes in DASS-42 and LEIDS-r were modeled over the course of the experiment period by including a fixed effect of gender and taking into account the female period calendar. Following current best practices to evaluate the significance of fixed effects of models fit with lme4, p-values were derived using Satterthwaite approximations for degrees of freedom with the lmerTest package (Version 3.0–1). Intercepts for the random effect of

participants for the effect of treatment and group (when applicable) were included in each model. We though assessed the effects of probiotics versus placebo (pre vs. post intervention).

6.3 Results

We recruited 22 individuals (M/F 7/15). The enrollment was from July 2021 to September 2021; the follow-up ended in December 2021. 21 completed the first and the second part of the study. No adverse events were reported in any part of the study. Baseline characteristics were similar between groups (***Table 6.3***). The compliance was high; only one patient returned 6 placebo sachets. The self-reported levels of product consumption did not differ significantly between groups (27±1.6 for the probiotic mix and 27±1.4 for placebo).

Table 6.3 *Participants characteristic*

	Treatment group A (N=12)	Treatment group B (N=10)	Overall (N=22)
Age			
Mean (SD)	27.1 (3.60)	28.7 (8.74)	27.8 (6.34)
Median [Min, Max]	27.5 [21.0, 33.0]	26.5 [21.0, 52.0]	27.0 [21.0, 52.0]
Weight			
Mean (SD)	60.2 (11.1)	64.2 (10.1)	62.0 (10.6)
Median [Min, Max]	61.5 [42.0, 75.0]	65.5 [50.0, 79.0]	64.5 [42.0, 79.0]
Height			
Mean (SD)	1.67 (0.119)	1.66 (0.0764)	1.66 (0.0997)
Median [Min, Max]	1.65 [1.50, 1.86]	1.66 [1.53, 1.77]	1.66 [1.50, 1.86]
BMI			
Mean (SD)	21.6 (2.19)	23.1 (2.10)	22.3 (2.25)
Median [Min, Max]	21.6 [16.8, 25.1]	23.8 [19.1, 25.3]	22.0 [16.8, 25.3]
Stress			
Mean (SD)	18.5 (2.75)	18.1 (2.88)	18.3 (2.75)
Median [Min, Max]	18.5 [15.0, 23.0]	17.0 [15.0, 25.0]	17.0 [15.0, 25.0]
Gender			
Female	7 (58.3%)	8 (80.0%)	15 (68.2%)
Male	5 (41.7%)	2 (20.0%)	7 (31.8%)

Cognitive assessment

The cognitive results are reported in ***Table 6.2***

Detection Test (DET)

There was no overall effect of treatment on the total speed (p= 0.88). The group allocation did not show to influence the treatment between subjects (p=0.70). There was no significant difference within-subject of the treatment (p= 0.92) The language also did not have an effect on the outcome (Language English p=0.367; Language Non English p=0.330).

One card back (ONB)

No significant differences were observed between treatments (p=0.48) on the total speed.

One card learning (OCL)

ANOVA of the linear mixed model showed the influence of the group on the outcome (p=0.01). The intervention with probiotics in the Group 2 showed better performance than the placebo (p=0.046).

Identification Test (IDN)

The treatment with probiotics did not show a significant difference in speed of performance measured as mean reaction times for correct responses in log10 (p=0.27).

Social-Emotional Cognition (SEC)

There was a significant increase in the accuracy of performance following treatment with probiotics (p=0.023) and not observed in placebo compared to baseline, however, the differences between placebo and probiotics were not significant.

International Shopping List Test (ISLT)

No significant changes have been observed in the treatment with probiotics (p=0.40) and placebo (p=0.81) in the number of correct responses made when remembering the word list.

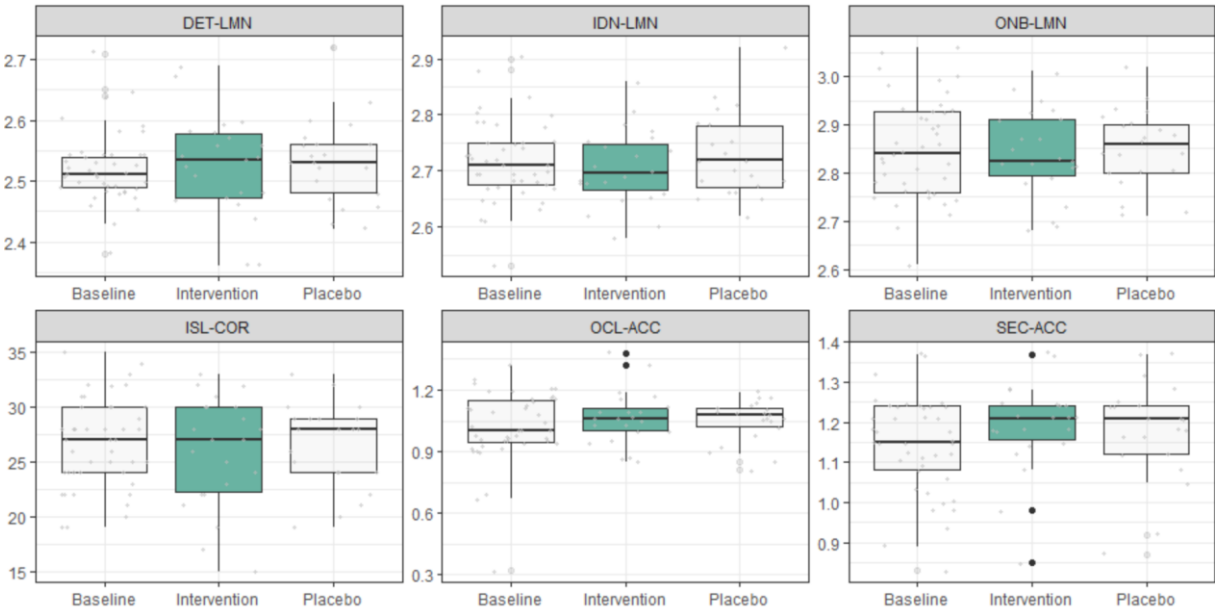

Figure 6.2 *Cognitive outcomes are represented by box plots with data represented as median with inter-quartile range. DET: Detection performance; IDN: Identification Test; OCL: One card Learning Test; ONB: One Back Test; SEC: Social-Emotional Cognition Test. Highlighted the data related to the intervention with probiotics*

Subjective stress measures

On basis of participant selection, statistical analysis performed on the DASS-42 total score revealed no main effect of treatment by group interaction (p= 0.16). Similarly, for Acceptance/Coping (ACC), Aggression (AGG), Control/Perfectionism (CON), Risk aversion (RAV), and Rumination (RUM) scores, no effect was observed for treatment on the group. Hopelessness (HOP) showed significant differences in the baseline

between Group A and Group B (p=0.043). No significant differences were observed between baselines of treatments in the DASS-42, showing no carry over effects in the Group 1. Mixed-effects model analyses were performed for DASS-42, LEIDS-R, and cognitive outcomes, taking into consideration interaction between the crossover arm and the treatment type, and also by both of them with the period. Thus, the two groups of participants (A and B) were comparable in terms of depression, anxiety, and stress scores at baseline and follow-up for the DASS-42.

LEIDS-r

To investigate the positive influence of the probiotic supplementation on cognitive reactivity to sad mood the Leiden index of depression sensitivity was used (LEIDS-r). No significant differences were observed between treatments. (***Fig. 6.3; Fig 6.4)***

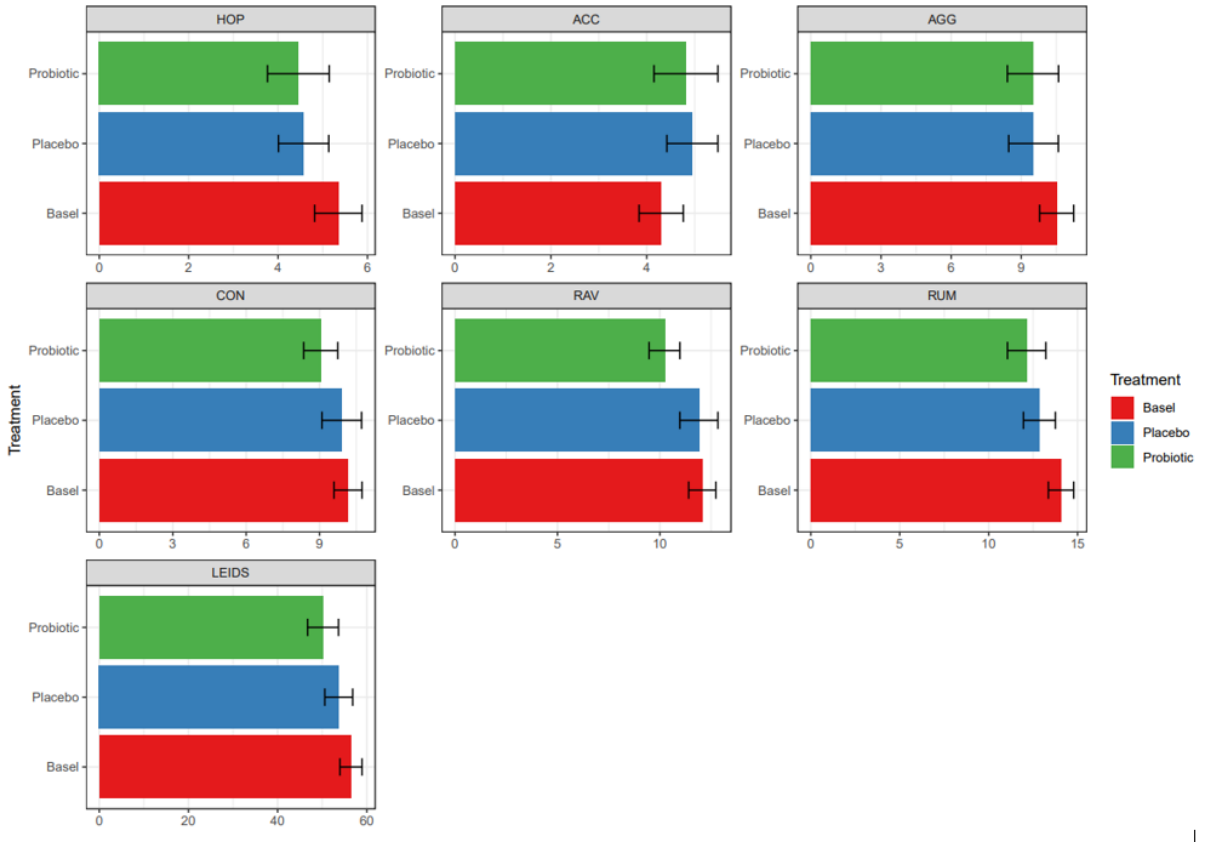

Figure 6.3 *Mood outcomes measured by LEIDS-r. Hopelessness (HOP), Acceptance/Coping (ACC), Aggression (AGG), Control/Perfectionism (CON), Risk aversion (RAV), and Rumination (RUM) and cognitive reactivity (indexed by the LEIDS-R) are represented by bar graphs for baseline in red, placebo in blue and probiotic mix in green*

DASS-42

Further exploitation of the probiotic supplementation on psychometric status came from self-reported measures using the DASS-42 questionnaire. Overall, the participants self-reported no changes after intervention. The interaction between the treatment with probiotics and gender

showed an increment for males in the score of DASS and depression not statistically significant (***Fig. 6.4)***.

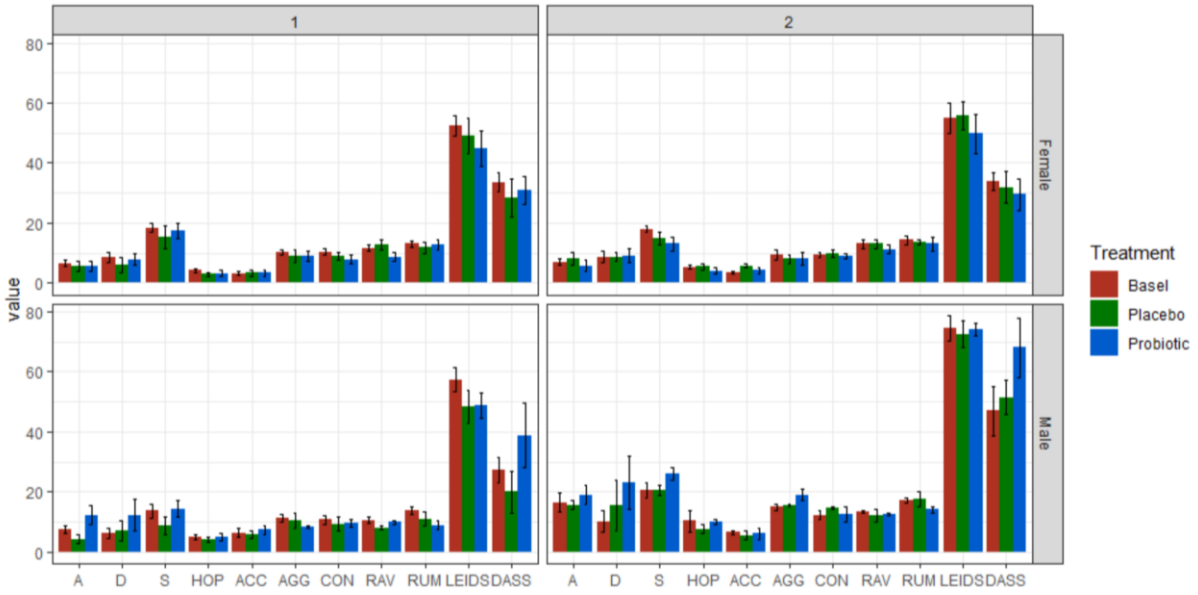

Figure 6.4 *Mood results measured by DASS-42 and LEIDS-r. Bar charts represent changes in self-reported scores measured by DASS-42 and LEIDS-R per group and gender. Anxiety (A), Depression (D), Stress (S), the total score (DASS), Hopelessness (HOP), Acceptance/Coping (ACC), Aggression (AGG), Control/Perfectionism (CON), Risk aversion (RAV), and Rumination (RUM) and the total score (LEIDS)*

Neurotransmitters analysis

To investigate the microbiome activity for GABA and acetylcholine production, fecal GABA, acetylcholine, choline and glutamate levels were evaluated in 22 participants. The analysis of the concentration of each neurotransmitter in the fecal sample didn't show any significant differences between the treatment with GABAflor and the placebo (data no reported). The correlation coefficient between GABA and glutamate concentrations was estimated as −0.23, which indicates a negative co-relation between both neurotransmitters. (**Fig 6.5**).

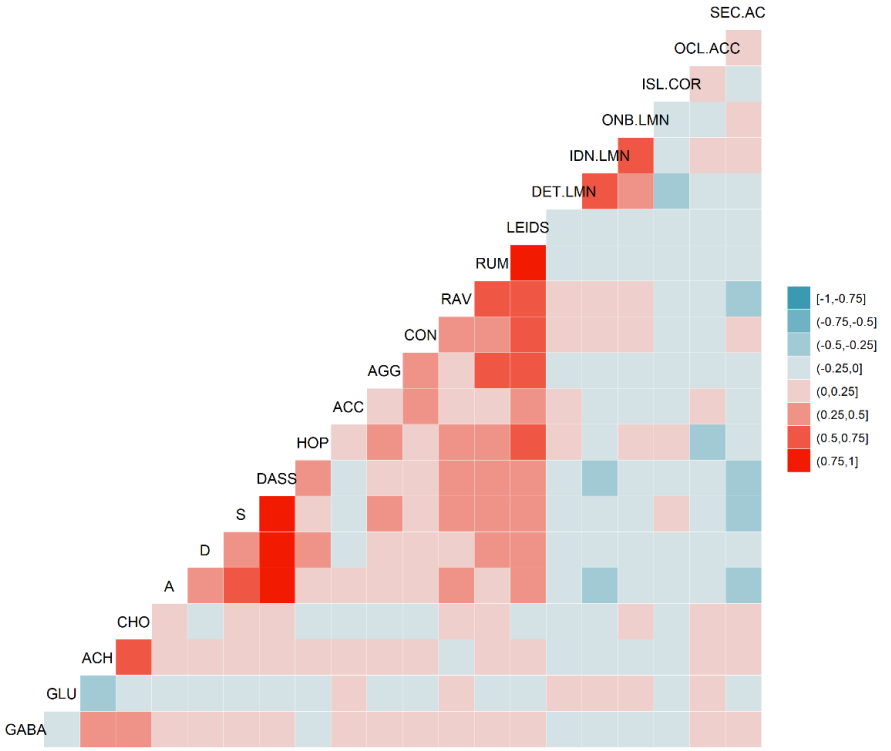

Figure 6.5 *Spearman's correlation between neurotransmitters, mood questionnaires (DASS-42 and LEIDS-r), and cognitive tasks. DET: Detection performance; IDN: Identification Test; OCL: One card Learning Test; ONB: One Back Test; SEC: Social-Emotional Cognition Test. Anxiety (A), Depression (D), Stress (S), the total score (DASS), Hopelessness (HOP), Acceptance/Coping (ACC), Aggression (AGG), Control/Perfectionism (CON), Risk aversion (RAV), and Rumination (RUM) and the total score (LEIDS). Red for positive correlation and blue for the negative correlation.*

6.4 Discussion

Probiotics have been long reported to exert an array of gut health benefits, with recent evidence on mental well-being, along the gut-brain axis. Clinical studies demonstrated the ability of a single probiotic strain or a mix of them to improve neurocognitive performance and psychological parameters following chronic stress (Chong et al. 2019; Lew et al. 2019; Wu et al. 2021). For our first aim , this study was designed to assess whether the neurocognitive effects of *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* in combination with some vitamins versus placebo would be seen on general cognitive control, and whether these effects on cognition (i.e. working memory) were visible by improving stress score. DASS-42 and LEIDS-r questionnaires are validated psychological instruments that correlated with psychological, clinical emotional, and behavioral measures. All subjects were recruited based on mild/moderate stress levels as assessed by DASS-42.

The information about the potential activity of the probiotic on mental health is conflicting. In contrast to the study of Lew et al. (2019), in this cross-over study, the administration of the probiotic formula did not yield significant changes in stress, anxiety levels as compared to

placebo or baseline after 4 weeks. However, our results are consistent with the work of Chong et al. (2019) who did not observe significant differences in the DASS-42 scores after 4 weeks but only after 12 weeks, highlighting the challenges to understanding the sufficient period of intake of candidate psychobiotics to exert an effect on mental well-being in stress-susceptible healthy humans by successfully alter microbiota composition. The information about the potential activity of the probiotic on mental health is conflicting.

Our results are in line with the previous research of Kelly et al. (2017) which employed a cross-over clinical study with a small sample size of healthy volunteers (n=29) and observed no effect of *L.rhamnosus* JB-1 on cognitive performance, as well as inflammatory response, stress-related behaviors and brain activity. Steenbergen *et al.* (2015) in a randomized placebo controlled study with 20 healthy participants did not find significant changes in mood or anxiety as measured by the Beck Depression Inventory or Beck Anxiety Inventory. However, in contrast to our work they observed a reduction on subscales of LEIDS for rumination and aggressive thoughts, suggesting an influence of probiotic mix intake on cognitive mechanisms associated with vulnerability to mood disorders

A more recent cross-over intervention study examined the effect of *B.logum* on measures of stress, cognitive performance, and mood in twenty male students during the university exam period used as a naturalistic chronic stress (Moloney et al. 2021). Moloney et al.(2021) observed stress and depression scores increased in both placebo and probiotic treated groups during the exam period, while overall sleep quality improved significantly in probiotic treatment compared to placebo. Similar to our results their study showed no efficacy in improving measures of visual memory, sustained attention and neither alleviate symptoms of chronic stress, depression (Moloney et al. 2021). Contrary to these findings, the same strain was also assessed in healthy adults undergoing an acute stressor, showing attenuation in psychological reaction along with enhanced frontline midline electroencephalographic mobility. These results suggested that the beneficial effect of strains could also be specific for the type of stress. Furthermore the quality of sleep here was not investigate but should be taken in consideration for further analysis to do not exclude their potential beneficial effect in this field as also demonstrated in previous studies for combination of probiotic strains (Marotta et al. 2019).

The cognitive outcomes were our primary outcome to investigate. Even if effects of probiotics and stress on cognition might share common pathways of action (Sarkar et al. 2016), it is still to clarify whether probiotics might affect cognitive performance independent or dependent of the detrimental effects of stress. Thus, we observed in our study that over the 12 week period of treatment, several cognitive tasks were constant and no significantly altered from baseline during placebo or probiotic interevent. Probiotic treatment enhanced social-emotional cognition from baseline (encoding, memory, and interpretation of social information) as also observed in the study of Lew et al. (2019) without showing significant difference in the interaction with the arm allocation. Conversely to the work of Lew et al. (2019), no interaction with gender was observed in our study. Treatment in working memory showed to be influenced by the group,

so we decided to focus in the group 2 in which was observed significant change related to the treatment with probiotic over placebo. As also previously suggested by Papalini *et al.* (2019) this results may be due to the protective effects of probiotics against further stress, associated to a certain level of optimization in cognitive control processes as it is working memory (Papalini et al. 2019).

Given the evidences of studies that reported reduction in levels of GABA synthetic enzymes during chronic stress, and that the concentration GABA/Glutamate is associated with microbiota composition, we decided to identify potential correlations between human fecal neurotransmitters and stress/depression (Altaib et al. 2021; Zhou et al. 2022). This was the first study to our knowledge with the aim to associate the concentration of GABA/Glutamate, acetylcholine and choline with mood scores measured by DASS-42 and LEIDS-r as well as outcomes of cognitive tests. However, we did not observe any trend of correlation between neurotransmitters concentration and mood or cognitive performance. Negative correlation was observed between GABA and glutamate, confirming the results of Altaib et al., (2021). A positive correlation observed between the mood questionnaire LEIDS-r and DASS-42, except for the subscale ACC (indexes of acceptance assessed by means of the LEIDS-R) . Indeed it is speculated that increasing acceptance might protect healthy individuals from developing depressive mood (Marotta et al. 2019).

Several meta-analysis suggested that utilizing probiotic may be a useful adjunctive treatment with greater benefits from in patients with certain co-morbidities, such as irritable bowel syndrome (Noonan et al. 2020), or in Major Depressive Disorder (MDD) in which the therapy tended to be more effective with time of psychobiotic supplementation and gender-influenced (Misera et al. 2021).

Limitation

Our study is not without limitation, these include the fact that our sample size was small, as we did not reach 44 participants. A bigger sample size could have helped to manage the placebo effect that we observed in some of the cognition and mood measures. In this regard, the study is still ongoing with the recruitment of other 22 participants. Our sample included more female than male participants, and future studies should employ gender-balanced samples. Furthermore, we did not investigate the sleep parameters and neither we examined brain imaging or EEG which has revealed to be a functional readout of efficacy in probiotic strains (Allen et al. 2016). The composition of the microbiota will be examined when all the 44 participants will conclude the study. Furthermore, we propose that further studies should control participant's diet habits during treatment. The potential of the psychobiotic as treatment stress-related disorders by targeting the microbiota holds much promise. However, our present data suggest that some caution is required regarding expectations of improving stress-related condition by targeting the microbiota in healthy participants. It is still to explore the role for specific strains in specific clinical settings and more research in this field are needed.

6.5 Conclusions

Stress and anxiety involve a broad spectrum of behavioral symptoms that are individual dependent with low medical success rates amid various reports of side effects. To our knowledge, this is the first RCT administering two probiotic strains able to produce neurotransmitters in vitro to healthy human. This 12 week randomized cross-over trial did not show that the probiotic formula reduced some stress and anxiety symptoms measured by DASS-42. Improvement in the cognitive ability were observed in social-emotion condition score when compared to the baseline. The pilot study demonstrated that 4 weeks of this intervention was safe, well-tolerated, but not efficacious in improving cognitive reactivity to mood. The only outcome significantly improved was the working memory in one arm of the intervention. The effect of this probiotic in larger test populations and broader period of intervention taking in account differences between men and women, age, microbiota composition and neurotransmitters analysis warrants further investigation. Efforts should aim to elucidate whether the perceived efficacy of probiotic therapy are maintained through continued treatment, or post cessation. Future studies that incorporate a more thorough screening to rule out the influence of comorbidity and include extensive background details (e.g., history of psychological treatment) will provide an important extension of our findings.

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Chapter 7

General discussion

Microbiota-targeting intervention

The thesis entitled 'selection of probiotic microorganisms with potential psychobiotic activity' describes different approaches we used to evaluate the psychobiotic potential of several bacterial strains. Evaluation methods more commonly applied to explore the potential of a strain, or formulation to modulate the gut microbiota composition, cognition, and mood disorders such as anxiety or stress are summarized in **Figure 1**. In our study we performed pre-clinical and clinical study to I) evaluate the ability of probiotic strains to produce neurotransmitters in substrates enriched in precursors, II) investigate the interaction between the gut microbiota and the selected psychobiotic strains, III) assess the translatability of the psychobiotic activity of neurotransmitters-producing probiotics in a clinical intervention on healthy humans.

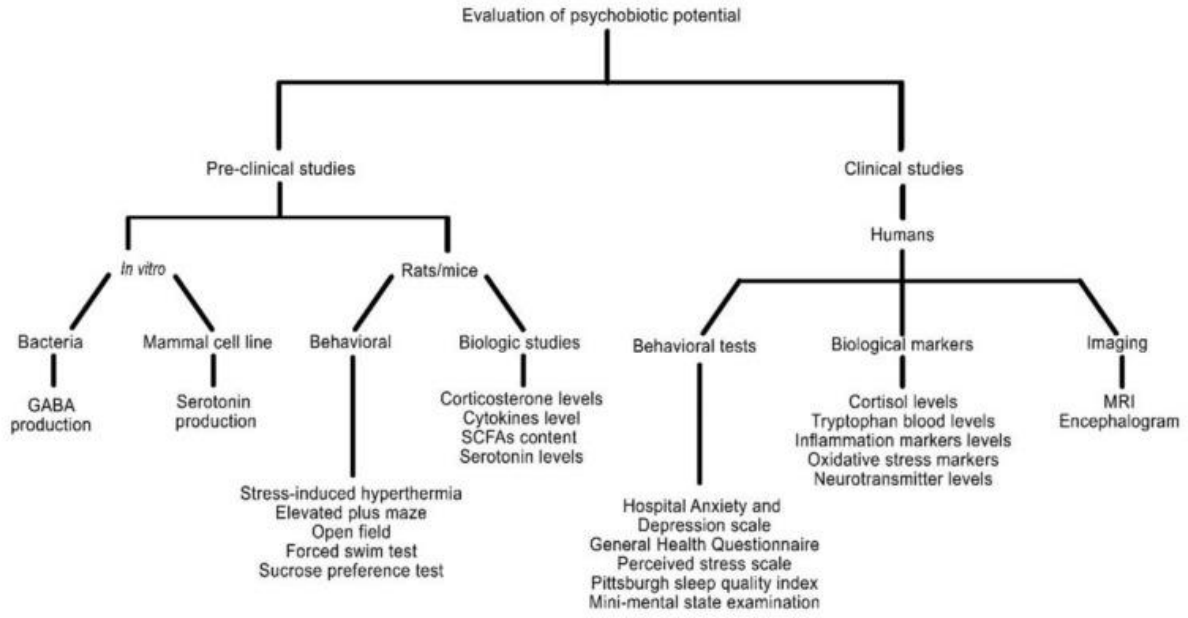

Figure 1 *Diagram of preclinical and clinical tests used to evaluate psychobiotic potential. GABA, gamma-aminobutyric acid; SCFAs, Short Chain Fatty Acids; MRI, magnetic resonance imaging. Figure from Del Toro-Barbosa et al., (2020)*

Starting by a detailed analyses in **Chapter 2** about the complex communication system that exists between the gut and the brain, it was assessed that the relationship between these two organs is bidirectional and the microbiota has a role of peacemaker in this interaction. Its function goes further than just the maintenance of homeostasis, by integrating both the cognitive and the emotional domains of the brain with peripheral gut functions like the enteric reflex, immune system stimulation, and enter-endocrine signaling (Del Toro-Barbosa et al. 2020). In this paragraph is also introduced the term of "Psychobiotic", coined for the first time by the psychiatric Dr. Ted Dinan and the neuroscientist Dr. John F. Cryan, to indicate a class of probiotics that when ingest in adequate amount are able to exert positive influence on the psychological status of the host, by influencing the gut brain signaling (Dinan et al., 2013). The

review ends with new evidences of the potential effect of fermented foods on mental health. The purposeful application of fermentation on food to enhance cognitive performance and improve blue mood is still an early stage, making necessary further validation in clinical studies

As we explored in **Chapter 3** the capacity of probiotics to produce GABA, acetylcholine, dopamine, noradrenaline, adrenaline, serotonin, following addition of glutamate, choline, tyrosine, tryptophane respectively to the substrate is species-specific, (in literature is reported to be strain-specific). We showed that all *Levilactobacillus brevis* strains had highest ability to convert glutamate in GABA among the probiotic strains tested while best performance to produce acetylcholine was found in *Lactiplantibacillus plantarum*. Slight ability to produce dopamine also was observed in *Levilactobacillus brevis*. Based on these evidences and literature research we presumed that GABA-producing *Lactobacillus* strains are great candidates to use as psychobiotics (Yunes et al., 2020). Thus, we selected *Levilactobacillus brevis* P30021 and *Lactiplantibacillus plantarum* P30025 as potential psychobiotic strains for the formulation of the final product and the further validation

In **Chapter 4** the process for the biomass production of *Levilactobacillus brevis* was observed firstly in a lab-scale fermentation. These results provided useful information about the cultivation conditions for growing *Levilactobacillus brevis* in batch fermenter in order to boost biomass to be used as industrial probiotic. Furthermore, viability and stability of *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* were evaluated in combination with cacao and propolis, leading to choice cacao as ingredient added in the final product.

The interaction of the final product, GABAflor, with the gut microbiota was explored in **Chapter 5**, firstly *in vitro* batch fermentation and then in the SHIME. Production of GABA during both fermentations, was observed when the probiotic formulation and the precursor were added together; while only addition of the precursors did not induced production of GABA. The analyses of the microbiota performed in the SHIME showed differences in the microbiota composition along the three part of the colon and between treatment and control. The treatment demonstrate to induce higher abundance of *Bacteroides* that in literature is linked with higher concentration of GABA in the fecal samples (Altaib et al., 2021)

Finally in **Chapter 6** the beneficial effect on mental health of the probiotic formulation was investigated in a randomized cross-over double blind, placebo-controlled study, involving 22 healthy humans with mild/moderate level of stress. The product conveyed no beneficial effect on mood, while regarding the effect on the cognitive performance only the task related to the working memory showed significant improvement of the probiotic group over the placebo group. The analyses of neurotransmitters in the fecal samples did not revealed any correlation with the stress, anxiety and depression. However, the enrollment of further participants is still carrying out to achieve the power needed to evaluate the validity of the study. Furthermore, the microbiota composition of the participants will be analyzed when all the participants, required for the study, will conclude the study.

Reliable and beneficial changes in stress symptoms and cognitive impairment stress-related after manipulation of the gut microbiota are likely to be the most pragmatic way to give evidence of the microbiome role in the pathophysiology of mood disorders. In this context, our study can give a contribution to understand one of the way how some probiotics, through production of neuroactive compounds and interaction with gut microbiota, can influence the brain function. Considering the complication in translating promising results gained from *in vitro* study to *in vivo* clinical study, this work deliver a conving call to investigate further the effect of psychobiotics on humans together with the analysis of microbiota. Furthermore, stratification of patient groups by specific neurological phenotypes might help identify which individuals would benefit particularly from psychobiotics.

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Appendix

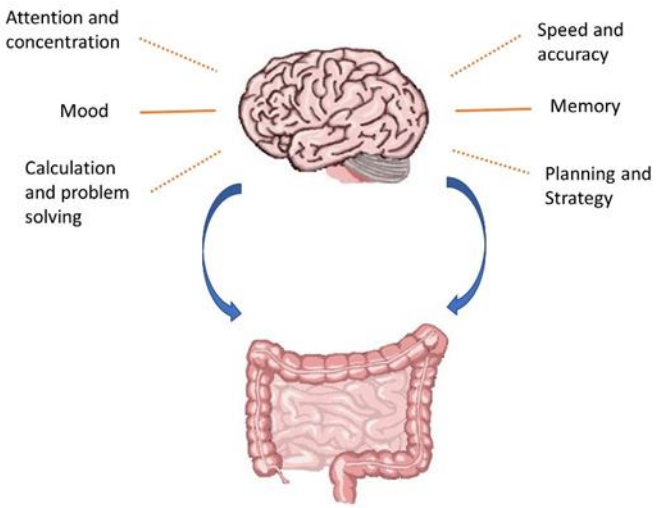
Supplementary materials

E3. Information research subjects (English version)

The Psychobiotics Study

Official title:

The effect of a dietary supplement containing probiotics, vitamins and zinc on cognition and mood: a randomized, double-blind, placebo-controlled crossover study in healthy people



Preface

Dear Sir / Madam,

With this information letter we would like to ask you if you would like to participate in medical scientific research. Participation is voluntary. The aim of the study is to determine the effectiveness of a food supplement containing probiotics and vitamins on cognitive performance and mood. Here you can read about the research, what it means for you, and what the advantages and disadvantages are. Participation in this study requires your written consent, therefore you will be given an explanation about the study. If you would like to participate, please complete and sign the form provided in Appendix C.

Ask your questions

You can determine your decision to participate with the information provided in this letter. You can also do the following:

- Ask questions to the researcher who is giving you this information.

- Talk to your partner, family or friends about this research.

- Ask an independent expert about this research: Dr. N. Muhsen, MD, MFPM nmuhsen@hotmail.com

- Read the information on www.rijksoverheid.nl/mensenonderzoek

1. General information

This research has been set up by the University Federico II of Naples and is carried out by the Agrotechnology and Food Sciences Department of Wageningen University, in the Human Nutrition facility located in Helix (building 124), Stippeneng 4, 6308 WE, Wageningen. 44 subjects are needed for this study. The Medical Ethical Review Committee (METC) Brabant has approved this investigation. General information about the assessment of research can be found at http://www.government.nl/topics/medical-research

2. What is the purpose of the study?

The aim of this study is to investigate whether taking a dietary supplement containing probiotics, vitamins B6, D and zinc daily can improve brain function and have positive effects on feelings of depression and anxiety in healthy volunteers with moderate stress levels.

3. What is the background of the research?

Researchers are beginning to understand how microorganisms in the gut can affect the brain through their ability to produce substances similar to neurotransmitters (the communication molecules of your brain). When your intestines are in balance, your brain is calm. But when pathogenic bacteria in the gut threaten your health, substances are produced in the gut that can cause anxiety in your brain. A new class of probiotic ("good") microorganisms has the potential to improve your mood. Despite the growing evidence that treatment with these particular probiotic strains appears to be an attractive strategy to reduce stress, anxiety and depression, research in this area is still limited and further research into the efficacy of these psychobiotics in mood disorders is needed. With this research we hope to gain more information about the effects of a dietary supplement (with these probiotics and a mix of zinc and vitamins) on mood and cognition in healthy volunteers with moderate stress levels. We analyze the effects of daily intake of the supplement on brain functioning through cognitive proficiency tests and mood questionnaires.

4. What participation means

How long will the investigation take?

Participation in the study takes approximately 12 weeks. In total we are looking for 44 participants. If you participate, it will cost you time for the selection examination and four testing sessions.

Are you suitable to participate?

We first want to know if you are suitable to participate. In order to participate in the study, it will be examined whether you meet the following criteria:

• 18-60 years old

• BMI within a healthy range (18.5-25 kg/m^2)

• Scores mild to moderate stress level using the DASS-42 (Depression Anxiety and Stress Scale)

• Available for the study visits

The participant is willing to maintain the usual diet (including caffeine and alcohol intake) and physical activity pattern throughout the study period. A potential subject who meets one of the following criteria will be excluded from this study:

- You have type 1 diabetes

- You are currently taking medication or dietary supplements that would interfere with the objectives of the study, pose a safety risk or interfere with the interpretation of the study results, such as: melatonin, vitamin E, multivitamins, vitamin B complex, ginkgo Bilbao, fish oil or other cognitive tonic nutritional or herbal supplements.

- You consume concentrated sources of probiotics (e.g. probiotic / prebiotic tablets, capsules, powders) daily, other than the research products provided.

- You are currently taking or in the 4 weeks before the first day of study that would interfere with the purpose of the study, such as psychoactive drugs (anxiolytics, sedatives, hypnotics, antipsychotics, antidepressants, anticonvulsants, centrally acting corticosteroids, opioid painkillers)

- You have an excessive intake of caffeine (> 500 mg per day, which is approximately the amount of caffeine in 5 cups of brewed coffee)

- You have an excessive alcohol consumption (corresponding to more than 21 units / week (for men) or more than 14 units / week (for women) for 2 weeks before the screening and during the intervention period

- You have recently (in the last 4 weeks prior to screening) or during the intervention period, taken antibiotics

- You have a history of dementia, stroke or other neurological conditions

- You are an employee of Wageningen University, division Human Nutrition

- You are currently or have participated in another clinical trial in the 3 months prior to the start of this trial

The treatment

For this study you will undergo two interventions of 4 weeks each and a period of 4 weeks in between during which you do not use a dietary supplement. You will be asked to take two different treatments for 28 days. The order in which you take the treatments will be random. You and the researcher do not know which treatment you will be currently taken. If it is important to your health, it can be looked up.

The compositions of the treatments are reported in the appendix D.

You can take the supplements at home. The testing and completion of the questionnaires takes place at the university in the "Helix" in Wageningen.

The selection survey will take twenty minutes and the test sessions will take almost one hour each.

Investigations and measurements

Before starting the study, you will fill a general information questionnaire and one to assess your stress level. If you meet the conditions to participate in the study, you will be asked to come to Wageningen University once every 4 weeks. In all sessions, you will be asked to complete questionnaires to assess levels of anxiety, stress, and depression, perform cognitive tests, and ask women to report their menstrual cycle. The examination therefore takes about four hours in total, divided over four sessions.

You should bring a fresh stool sample with you with each session. You will be given a container to store the collected sample The stool should be kept in the refrigerator and delivered to the study group no later than two hours later. A minimum of 40 grams of stool is required.

Given the Covid-19 situation, it would also be possible to send the questionnaires four times by e-mail.

Meeting	Session 1	Session 2	Session 3	Session 4
LEIDS-r (Leiden Index of Depression Sensitivity-revised) 8 minutes	x	x	x	x
DASS-42 (Depression Anxiety Stress Scale) 15 minutes		x	x	x
Cognitive test 27 minutes	x	x	x	x
Stool samples	x	x	x	x

5. What agreements do we make with you?

We want the investigation to run smoothly. That is why we make the following agreements with you:

• You take the dietary supplement in the way that the researcher has explained to you

• You do not participate in any other medical scientific research during this research

• You come to every appointment

• You contact the researcher in these situations:

- You are going to take other medicines. Even if these are homeopathic remedies, natural remedies, vitamins or medicines from the chemist.

- You are admitted to or treated in a hospital

- You suddenly have problems with your health

- You don't want to participate in the investigation anymore

- Your telephone number, address or e-mail address changes

6. Possible inconveniences

Cognitive tests and mood questionnaires

The cognitive tests and mood questionnaires will take approximately 55 minutes. This can be experienced as intensive, but does not entail any further inconveniences.

Follow nutritional guidelines

We ask that you do not consume foods containing probiotics,

as described in point 5

Collection of the stool

You will be asked to bring a stool sample with you for all sessions that will be collected shortly before the start of the tests. The stool should be fresh. This is described in more detail under point 4

7. Possible pros and cons

Probiotics are defined by the FAO / WHO as live microorganisms that, when administered in sufficient amounts, provide a health benefit to the host by promoting a healthy gut and immune system. In this particular study, participation may have a beneficial effect on your brain function and mood. The supplement also contains Vitamin B6 which contributes to the normal functioning of the nervous system and normal psychological function. In addition, vitamin B6 contributes to the reduction of tiredness and fatigue. Vitamin D3 in the supplement contributes to the normal absorption and utilization of calcium and phosphorus and to the normal functioning of the immune system; Zinc in the supplement contributes to normal cognitive function and to the protection of cells against oxidative stress: Acticoa cocoa is rich in polyphenols, also gives a pleasant taste.

You will also contribute to scientific research into the effect of a dietary supplement on the functioning of the brain. In addition, you will receive a participant fee of € 98 when you complete the research.

Disadvantages of participating in the study can be:

- Time it takes you

- Agreements that you must adhere to

All these matters are described under points 4, 5 and 6

8. When will the investigation end?

Your participation in the study will stop when:

- All visits as described under point 2 are over;

- You choose to stop; You decide whether you participate in the study. Participation is voluntary. If you participate, you can always change your mind and still stop, even during the study. You don't have to say why you are quitting. You must, however, report this immediately to the researcher. The data collected up to that point will be used for the research.

- The study doctor thinks it is better for you to stop.

- Wageningen University or Federico II University decides to stop the research

If you are interested in the outcome of the study, you can indicate this on the consent form (see Appendix B). After processing all the data, the researcher will inform you about the most important outcomes.

9. Using and retaining your data and stool sample

For this research, your personal data and a sample of your stool will be collected, used and stored. This concerns information such as your name, address, date of birth and information about your health. The collection, use and storage of your data and your stool is necessary in order to be able to answer the questions asked in this study and to be able to publish the results. We ask your permission for the use of your data and body material.

How do we protect your privacy?

To protect your privacy, your data and the stool sample are coded. Your name and other data will be omitted so that the collected data cannot be linked to you personally. Data can only be traced back to you with the key to the code. The key to the code remains safely stored in the local research institute (Wageningen University). The data and body material sent to the client only contain the code, but not your name or other data with which you can be identified. The data cannot be traced back to you in reports and publications on the research either.

Who can see your data?

Some individuals may have full access to your data at the study site. Also to the non-encrypted data. This is necessary to check whether the study is being carried out properly and reliably. Individuals who have access to your data for review include: the committee overseeing the safety of the study and national and international regulatory authorities inspecting healthcare. They will keep your information confidential. We ask for your permission for this access.

How long do we keep your data and body material?

We keep your data for 15 years. We do this at the research location.

Can you withdraw your consent to the use of your data?

You can withdraw your consent to the use of your personal data at any time. This applies to use in this research and to use in other research. The research data collected up to the moment you withdraw your consent will still be used in the research. But beware: are you withdrawing your consent, and have medical researchers already collected data for an investigation? Then they may still use this information.

Would you like to know more about your privacy?

- Would you like to know more about your rights with regard to the processing of personal data? Then look at www.autoriteitpersoonsgegevens.nl

- If you have any questions about your rights, please contact the person responsible for processing your personal data. For this research that is: Wageningen University. See Appendix A for contact details.

- If you have any complaints about the processing of your personal data, we recommend that you first discuss these with the university. You can also contact the Data Protection Officer or the Dutch Data Protection Authority. Contact details can be found in Appendix A.

10. Do you receive compensation if you participate in the study?

You will receive € 98 for participation in the full study. If you stop before the study has ended, you will not receive any reimbursement. We are obliged to declare this compensation as income to the tax authorities.

11. Insurance for test subjects

We do not expect you to run any additional health risks when participating in the study, but we cannot rule this out entirely. Therefore, insurance for test subjects is compulsory for everyone participating in this study. In the event that you incur damage by participating in the study, this will in most cases be covered by insurance. In Appendix B you will find more information about the insurance and the exceptions. It also states to whom you can report damage.

12. Inform the GP

Your doctor will be informed that you are participating in this study. This is for your safety. If you do not agree with this, you cannot participate in this study.

13. Do you have any questions?

If you have any questions, please contact the research team. If you have any questions about the study that you do not wish to discuss with the investigators, please contact the independent physician. He knows a lot about the investigation, but has nothing to do with the investigation. Are things unclear or are you not satisfied with the way things are going? We think it is important to hear this from you so that we can come to a solution together. If necessary, you can appeal to the independent complaints officer of Wageningen University. All contact details can be found in Appendix A: Contact details.

14. Signing of the consent form

When you have had sufficient reflection time, you will be asked to decide whether to participate in this study. If you give permission, you must confirm this in writing. By your written consent, you indicate that you have understood the information and agree to participate in the study. The consent form will be retained by the research team. You will receive a copy of this consent form.

Thanks for your time

15. Appendix to this information

Appendix A: Contact Information

Appendix B: Information about the insurance

Appendix C: Consent Form

Appendix D: Composition of the treatment

Appendix A. Contact details

Contact persons

Melania Casertano, MSc

Food Quality and Design, Wageningen University

Bornse Weilanden 9, Axis (building 118)

6708 WG Wageningen

Email: melania.casertano@wur.nl

Dr.Ir.Matthijs Dekker

Food Quality and Design, Wageningen University

Bornse Weilanden 9, Axis (building 118)

6708 WG Wageningen

Email: matthijs.dekker@wur.nl

Independent physician

Dr. N. Muhsen, Art researcher in Utrecht. During office hours, Dr. Muhsen can be reached on 06-1696 3517. If necessary, leave a message on the voicemail, so that he can call you back. You can also ask questions by email: nmuhsen@hotmail.com

Complaints Officer

Eveline Waterham

Department of Human Nutrition, Wageningen University

Helix building 124, Stippeneng 4

6708 WE Wageningen

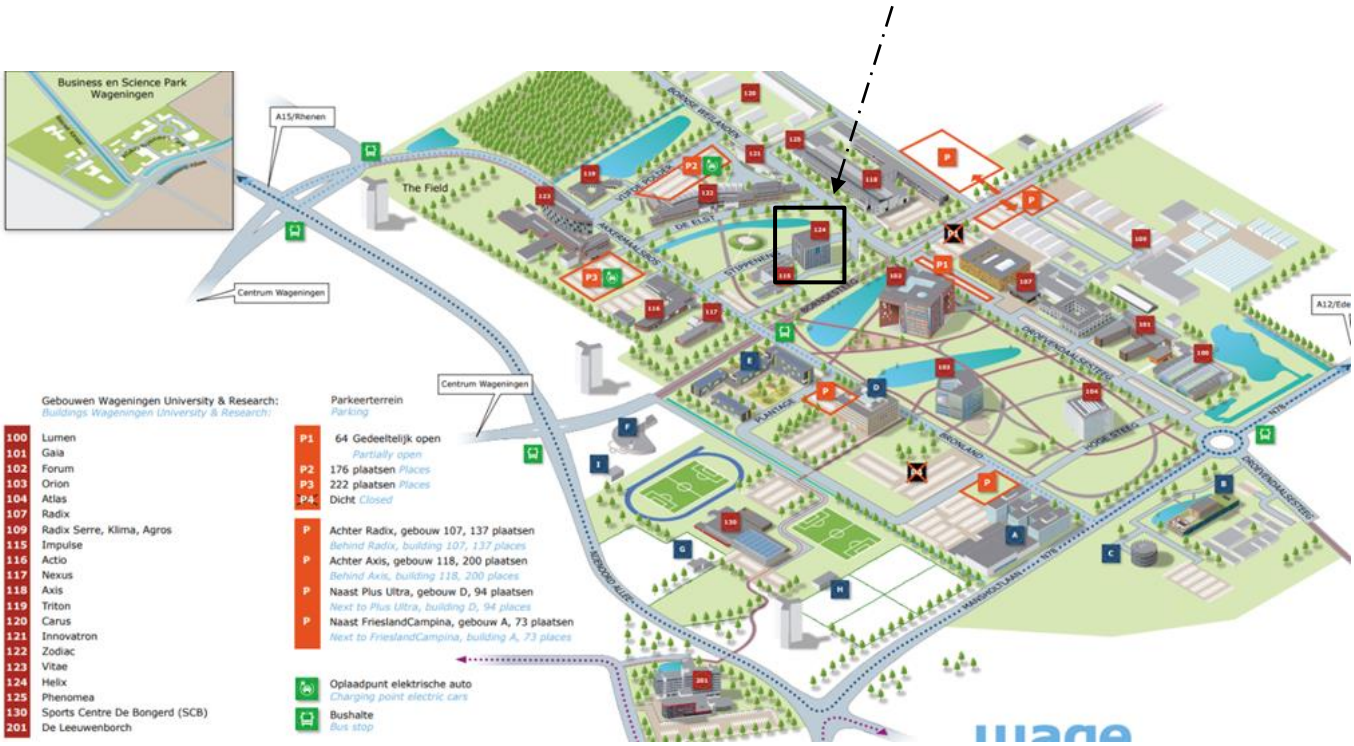
Eveline.Waterham@wur.nl

Study location

Wageningen University

Helix, building 124, Stippeneng 4

6708 WE, Wageningen



Appendix B. Information about the insurance

Wageningen University & Research Center has taken out insurance for everyone who takes part in this study. The insurance covers damage resulting from participation in the study. This applies to damage during the investigation. You must report damage to the insurer within those four years.

The insurance does not cover all damage. At the bottom of this text it is briefly stated which damage is not covered.

These provisions are contained in the Decree on compulsory insurance for medical scientific research involving humans. This decision can be found on www.cmo.nl, the website of the Central Committee on Human Research (see "Library" and then "Laws and regulations").

In case of damage, you can contact the insurer directly.

The insurer of the study is:

Name: HDI- Global SE, the Netherlands

Adress: Westblaak 14

3012 KL Rotterdam

Policy number: V-055-862-396-3 / V0100109572

The insurance offers cover up to a maximum amount of € 650,000 per participant, with a maximum of € 5,000,000 per study. A maximum of € 7,500,000 applies for all research at Wageningen UR per insurance year.

The insurance does not cover the following damage:

- damage due to a risk about which you have been informed in the written information. This does not apply if the risk is more serious than foreseen or if the risk was very unlikely;

- damage to your health that would also have occurred if you had not participated in the study;

- damage due to not (fully) following directions or instructions;

- damage to your offspring as a result of a negative effect of the research on you or your offspring;

- damage caused by an existing treatment method when researching existing treatment method

Appendix C. Subject Consent Form

- I have read the information brochure for participants. I was able to ask questions. My questions have been answered to my satisfaction. I had enough time to decide whether to participate.

- I know participation is voluntary. I know that I can decide for myself not to participate or to stop participating in the study at any time. I do not have to give a reason for this

- I give permission for my GP to be informed that I am participating in this study

- I know some people have access to my data. These people are mentioned in the information brochure.

- I consent to the collection and use of my data to answer the research question in this research

- I consent to my data being stored for up to 15 years after completing the study.

- I'll give the permission to keep my material after this research and to use it for research later, as stated in the information letter

- I want to participate in this study

Name contestant: _____

Signature of participant: _____

Date (day / month / year): ___ \ _____ \ ____

Place: _____

I would like to receive the results after the study

_____

I hereby declare that I have sufficiently informed this participant about this study.

If information becomes known during the study that may have an influence on the consent statement of the participant, I will inform the participant about this in good time

Name of researcher: _____

Signature of examiner_____

Date ____/____/____

Place: _____

The participant will receive a copy of the signed consent form.

APPENDIX D: Composition of the treatments

TREATMENT A

Ingredients	mg/stick
Fructose	1719,403
***Levilactobacillus brevis* P30021** ***Lactiplantibacillus plantarum* P30025**	Non less than 2 billion of live cells
Zinc	7,50
Silicon dioxide	60,00
Cocoa powder	50,00
Vitamin B6	2,8
Vitamin D	0,005

TREATMENT B

Ingredients	mg/stick
Fructose	1950,00
Cocoa powder	50,00
TOT	2000,000

Supplementary Table and figures Chapter 5

***Tabel S1**. Participant characteristic.*

Participant characteristic	Total sample (n=22)
Dutch	3
Not Dutch	19
Native English speaker	1
Not English speaker	18
Alcohol (units per week)	7.5±2.1
Smoker	4
Stress (measured with DASS-42)	Tot: 18,45±2,75
	Female (15): 18.80±3,12
	Male (7): 17,71±2,69

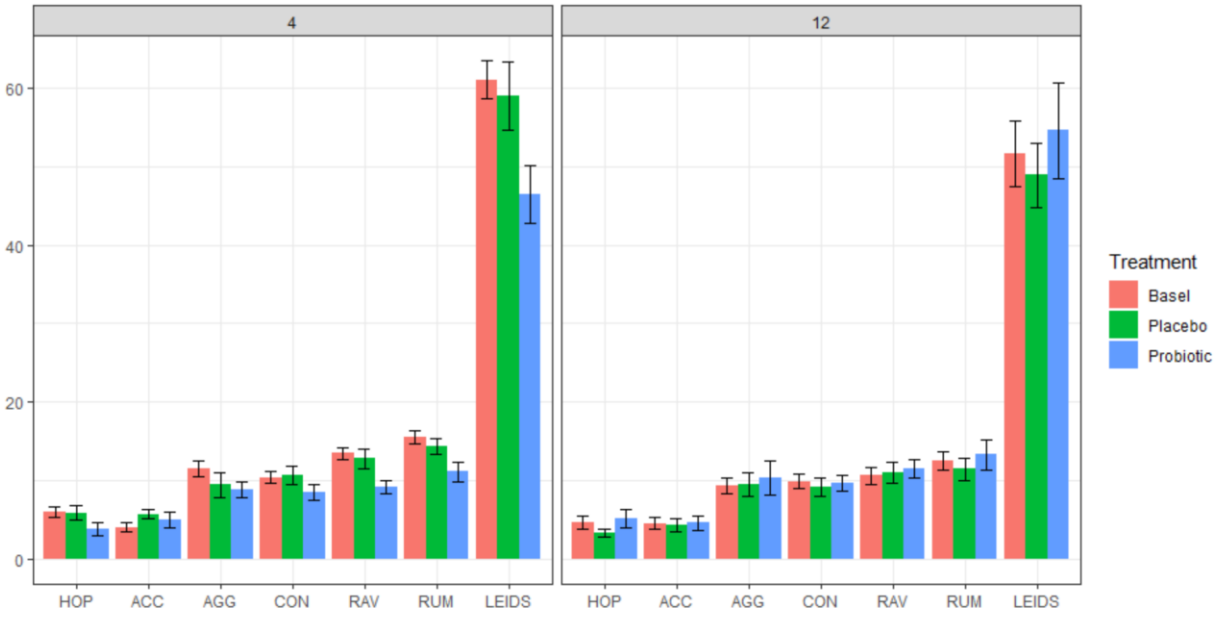


***Figure S1**. Bar graph representing the changes in LEIDS-r score between the intervention in week 4 and in week 12. Different colors represent the different conditions of intervention. Blue for probiotics intake, green for placebo, and red the baseline. Hopelessness (HOP), Acceptance/Coping (ACC), Aggression (AGG), Control/Perfectionism (CON), Risk aversion (RAV), and Rumination (RUM) and the total score (LEIDS)*

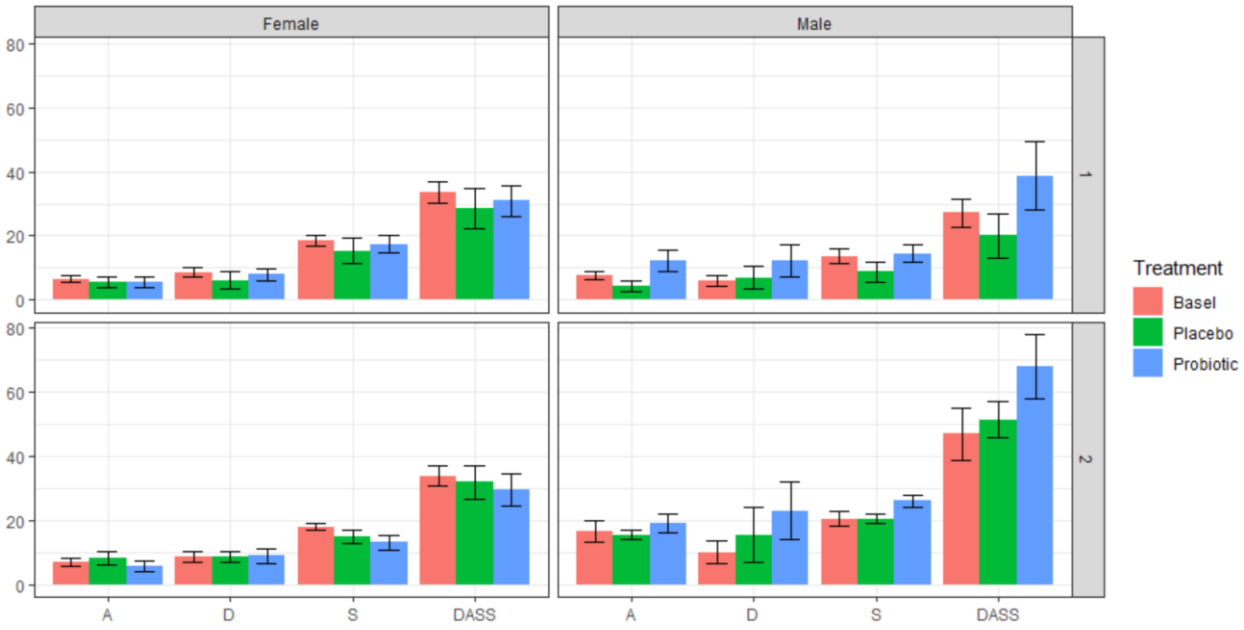


***Figure S2**. Bar graph representing the changes of the subscales of Anxiety (A), Depression (D), Stress (S) and the total score (DASS) performed by males and females in group A (1) and Group B (2)*

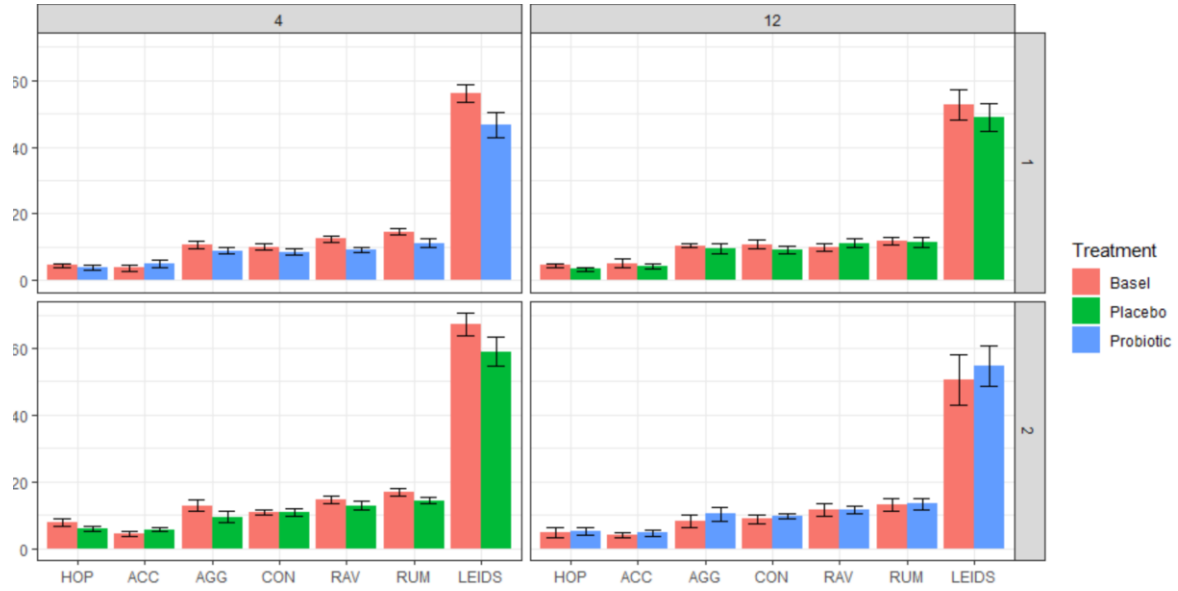


***Figure S3.** Differences in LEIDS-R score and its subscales at week 4, including baseline (in red) at time 0 and after 4 weeks of intervention with probiotic (blue) or a placebo (red), and week 12, including baseline after washout (week 8) and intervention with probiotic and placebo after 12 weeks.*

Appendix

Acknowledgment

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