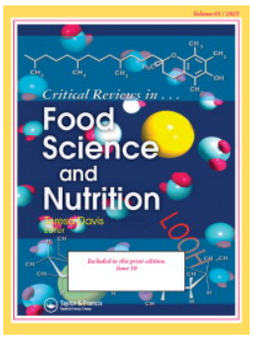

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REVIEW

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Psychobiotics and the gut-brain axis: advances in metabolite quantification and their implications for mental health

Vincent Owusu Kyei-Baffour, Akshay Kumar Vijaya, Aurelijus Burokas and Eric Banan-Mwine Daliri

Department of Biological Models, Institute of Biochemistry, Life Sciences Center, Vilnius University, Vilnius, Lithuania

ABSTRACT

Psychobiotics are live microorganisms that, when administered in adequate amounts, confer mental health benefits to the host. Several clinical studies have demonstrated significant mental health benefits from psychobiotic administration, making them an emerging topic in food science. Certain strains of *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Escherichia*, and *Enterococcus* species are known for their ability to modulate the gut-brain axis and provide mental health benefits. Proposed action mechanisms include the production of neuroactive compounds or their precursors, which may cross the blood-brain barrier, or transported by their extracellular vesicles. However, there is a lack of in vivo evidence directly confirming these mechanisms, although indirect evidence from recent studies suggest potential pathways for further investigation. To advance our understanding, it is crucial to study these mechanisms within the host, with accurate quantification of neuroactive compounds and/or their precursors being key in such studies. Current quantification methods, however, face challenges, such as low sensitivity for detecting trace metabolites and limited specificity due to interference from other compounds, impacting the reliability of measurements. This review discusses the emerging field of psychobiotics, their potential action mechanisms, neuroactive compound estimation techniques, and perspectives for improvement in quantifying neuroactive compounds and/or precursors within the host.

KEYWORDS

Microbiota-gut-brain axis; probiotics; neuroactive compounds; extracellular vesicles; cognitive wellness; LC-MS

Introduction

Microorganisms, including the human gut-inhabiting microconsortium, are capable of communication and collective behaviors. This extends not only to interactions within microbial social systems but also to dialogues between these microorganisms and the human host (Cai et al. 2023; Cryan et al. 2019; Oleskin and Shenderov 2019). Among these microorganisms, those that confer mental health benefits when administered in adequate amounts are called psychobiotics (Cryan and Dinan 2012). These psychobiotics interact with commensal gut bacteria, potentially influencing host health (Dinan, Stanton, and Cryan 2013). A well-established means of this interaction involves the recognition of bacterial components, such as lipopolysaccharides in gram-negative bacteria and peptidoglycan in gram-positive bacteria, by transmembrane pattern recognition receptors on enteric glial cells, which can affect the structural integrity and functioning of epithelial cells (Montagnani et al. 2023). Additionally, it is becoming apparent that communication may also occur through extracellular vesicles (EVs), which carry a variety of components, including neuroactive compounds capable of modulating host signaling pathways (Iyaswamy et al. 2023; Molina-Tijeras, Gálvez, and Rodríguez-Cabezas 2019). Evidence suggests that the gut microbiota plays a significant role in behavior regulation and immune system modulation, both of which are linked to symptoms of depression (Burokas et al. 2015). Consequently, dysbiosis in the gut microbiota is often observed in conditions like depression and autism spectrum disorder, where changes in gut permeability are also noted (Cruz-Pereira et al. 2020; Teskey et al. 2021; Zhu, Zhao, Zhang, et al. 2023). For example, oral administration of the human commensal Bacteroides fragilis has been shown to correct gut permeability and restore gut microbial balance, leading to improvements in autism spectrum disorder in mouse models (Hsiao et al. 2013).

Several mechanisms have been proposed through which psychobiotics exert mental health benefits, including activation of the vagus nerve, stimulation of endocrine cells, immune signaling, and the transportation of their EVs and metabolites from circulation to the brain (Ahmed et al. 2022; Burokas et al. 2015; Cryan et al. 2019). Regarding the proposed route of transportation of gut-derived neuroactive compounds and EVs from circulation to the brain, previous discussions have primarily focused on the mental health outcomes following psychobiotic administration. But, one critical question remains: how much of these gut-derived neuroactive compounds are required to reach the brain and produce a mental health benefit? To answer this, accurate quantification of psychobiotic metabolites within the host is necessary. Many of these compounds are small molecules, making them ideal

CONTACT Eric Banan-Mwine Daliri eric.daliri@gmc.vu.lt; Aurelijus Burokas aurelijus.burokas@gmc.vu.lt

for analysis using hyphenated mass spectrometry (MS) techniques, including liquid chromatography-MS (LC-MS).

This review aims to explore how neuroactive compounds produced by psychobiotics are quantified, tracked in circulation, and monitored in the brain in both preclinical and clinical studies. It also provides updated evidence of psychobiotics' mental health benefits and critically evaluates current estimation techniques for these compounds.

Psychobiotics and their neuroactive compounds

The ability of psychobiotics to produce or stimulate the production of some neuroactive compounds and positively impact mental health distinguishes them from conventional probiotics. Due to this potential, psychobiotics exhibit a wide range of applications, spanning from alleviating mood and stress to serving as adjuvants in therapeutic treatments for various neurodevelopmental and neurodegenerative disorders. The common psychobiotic bacteria belong to the genus Lactobacilli, Streptococci, Bifidobacteria, Escherichia, and Enterococci (Sharma et al. 2021).

Lactobacilli

Several *Lactobacillus* species, including *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus johnsonii*, and *Lactobacillus fermentum*, are frequently used as probiotics (Cheng et al. 2019; Gao et al. 2023). These strains have been shown to modulate the microbiota-gut-brain axis, with evidence suggesting their ability to stimulate the production of some neuroactive compounds essential for the proper functioning of the central and peripheral nervous systems (Gao et al. 2023). For instance, *L. plantarum* PS128 has been reported to improve dopamine metabolism and norepinephrine production in the striatum and prefrontal cortex of Wistar rats, reducing tic-like behaviors and pre-pulse inhibition deficits (Cheng et al. 2019; Liao et al. 2019). The mechanism involves increasing dopamine transporter and β-arrestin expression while reducing phosphorylation of dopamine and cAMP regulated phosphoprotein (DARPP-32), and extracellular regulated protein kinase (ERK). Also, PS128 regulates peripheral serotonin levels and reshapes cecal microbiota composition, contributing to its effects on dopamine and norepinephrine metabolism (Liao et al. 2019).

Furthermore, *L. rhamnosus* has been shown to suppress gamma amino butyric acid (GABA) receptor overexpression in depressive disorder patients by activating GABA signaling pathways through vagal afferents (Bravo et al. 2011; Strandwitz et al. 2019; Tette, Kwofie, and Wilson 2022; Yunes et al. 2016). Similarly, other studies (Gao et al. 2023; Jeong et al. 2021; Megur et al. 2023; Partrick et al. 2021) have highlighted that certain *Lactobacillus* strains possess genes encoding a complete biosynthetic pathway for tryptophan production, a precursor for serotonin synthesis, as demonstrated both in vitro and ex vivo using HT-22 cells (Jeong et al. 2021).

While these findings underscore the potential of *Lactobacillus* strains as psychobiotics, critical evaluation reveals several gaps in current knowledge. Despite promising preclinical results, many of such studies lack robust clinical evidence confirming these effects in human populations. The specific mechanisms by which these compounds traverse the gut-brain axis and exert their effects also require further investigations. For example, the extent to which microbial-derived dopamine or serotonin precursors influence neurotransmitter levels in the brain remains unclear, particularly given the complexity of the (blood brain barrier) BBB. Moreover, differences in gut microbiota composition, probiotic viability, and metabolite bioavailability across individuals pose significant challenges to standardizing these therapies. Although several studies report *Lactobacillus* could modulate neurotransmitter pathways, quantification of the effective concentrations of these microbial metabolites in physiological settings is often missing. Moving forward, human studies employing advanced techniques including metabolomics, neuroimaging, and gut microbiota profiling are essential to translate these promising findings into clinically relevant interventions.

Bifidobacterium

Another widely studied probiotic genus, *Bifidobacterium*, comprises gram-positive anaerobic bacteria that inhabit the gastrointestinal tract. Notable species include *Bifidobacterium adolescentis*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium lactis*, and *Bifidobacterium breve* (Rajanala, Kumar, and Chamallamudi 2021). Research has revealed their potential in modulating the microbiota-gut-brain axis, primarily through serotonergic pathways. For instance, Engevik et al. (2021) demonstrated that *B. dentium* modulates the host serotonergic system *via* multiple mechanisms. Specifically, *B. dentium* colonizes the intestinal mucus layer and delivers metabolites, including acetate, which stimulates enterochromaffin cells to release serotonin and increases the expression of serotonin receptors in both the gut and brain. In a similar sense, *B. breve* HNXY26M4 has shown promise in mitigating cognitive deficits and alleviating neuroinflammation and synaptic dysfunction in Alzheimer's disease mouse models (Zhu, Zhao, Wang, et al. 2023). Beyond individual strains, genome analyses of over 1,000 publicly available *Bifidobacterium* strains have identified *B. adolescentis* as a model GABA producer within the human gastrointestinal tract (Duranti et al. 2020). This finding is significant given the role of GABA in modulating the gut-brain axis. When Groningen rats were fed *B. adolescentis*, measurable GABA production was observed, providing functional evidence of its capability to influence gut-brain communication (Duranti et al. 2020).

Other bacteria species and yeasts

In addition to Lactobacilli and Bifidobacteria, other bacteria species and yeasts have been shown to produce metabolites that influence proper brain function. These include Streptococci, Escherichia, and Enterococci (Sharma et al. 2021) as well as

some *Saccharomyces* and *Kluyveromyces* species. *Streptococcus thermophilus* EG007 is known to encode for various metabolites including GABA and mice fed with *S. thermophilus* exhibited improvements in behavior tests assessing short-term spatial and non-spatial memory. In a similar study, the effect of long-term administration of yoghurt fermented by *S. thermophilus 1131* on the spatial memory and 5-hydroxytryptophan (5-HT) concentrations in the cerebral cortex of mice improved significantly (Kawase and Furuse 2019). The ability of *Escherichia coli* Nissle (EcN) to modulate the gut-brain axis has also been evaluated. To identify the molecular responses induced by EcN that may contribute to its probiotic properties, Zyrek et al. (2007) employed polarized T84 cells and utilized techniques, such as DNA microarrays, quantitative RT-PCR, Western blotting and specific protein kinase C inhibitors. Their findings revealed that EcN modulates the expression and distribution of zonula occludens-2 proteins, demonstrating its ability to restore a disrupted epithelial barrier. In a separate study, EcN was shown to stimulate the production of human β-defensin 2, which protects the mucosal barrier by preventing adhesion and invasion by pathogenic commensals (Azad et al. 2018). Furthermore, both in vivo and in vitro studies suggest that EcN exhibits protective functions against pathogens, such as Salmonella, Shigella, Candida, and other invasive commensals, and may also contribute to epithelial restoration by modulating tight junction and zonula occludens proteins (Azad et al. 2018; Park et al. 2021). This restoration is crucial, as a compromised epithelial barrier is a key driver of inflammation, which has implications for mental health.

Scientific evidence supports the safety of using *Enterococcus faecium* as a probiotic and its consumption has been linked to reduced intestinal inflammation, enhanced memory, elevated butyrate concentrations, and promoting neurogenesis (Romo-Araiza et al. 2023). A symbiotic mixture with *E. faecium* as a probiotic was known to increase the levels of brain-derived neurotrophic factor and butyrate concentrations in Prague-Dawley male rats (Romo-Araiza et al. 2018). In a separate study by Kambe et al. (2020), male mice that were fed a diet supplemented with *Enterococcus faecalis* EC-12 exhibited reduced anxiety-like behaviors.

Akkermansia muciniphila, a gut bacterium known for its role in maintaining intestinal health, has also been associated with mental health benefits due to its ability to modulate the gut-brain axis. Its potential mechanism of action includes the regulation of serotonergic pathways *via* EVs, which influence the expression of genes involved in serotonin synthesis, transport, and receptor activity in the gut. Yaghoubfar et al. (2020, 2023) orally administered male C57BL/6J mice with 10^9 CFU/200 µL of viable *A. muciniphila* suspended in PBS and 10 µg/200 µL of *A. muciniphila* EVs daily for four weeks. *A. muciniphila* and its EVs may have a biological effect on the increasing of serotonin levels in the colon and hippocampus of mice. Both the bacterium and its EVs had significant effects on the mRNA expression of genes, involved in serotonin signaling/metabolism in the colon and hippocampus of mice (significantly modulated the mRNA expression of serotonergic system-related genes, including *Tph1*, *Mao*, *Htr3B*, *Htr4*, and *Htr7*).

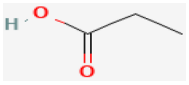
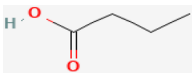
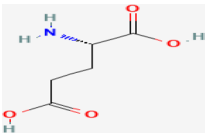
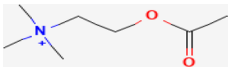
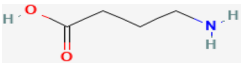
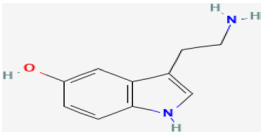
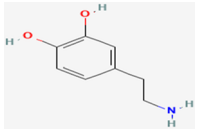
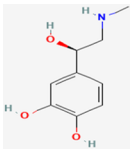
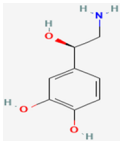
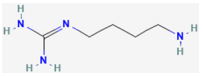
Although yeast has received less attention as psychobiotics compared to lactic acid bacteria, their ability to produce certain neuroactive compounds has been demonstrated. Sechi et al. (2014) reported that *Saccharomyces cerevisiae* JBCC-A74 could produce GABA at levels of 0.33 g/L, while Perpetuini et al. (2020) showed that *Kluyveromyces marxianus* K326 could produce 7.78 mg/L of GABA. To offer a concise overview, Table 1 presents information on microbial species and strains that confer mental health benefits when used as psychobiotics. The metabolites they produce have also been indicated.

Biosynthetic pathways of common neurotransmitters produced by psychobiotics

Gamma aminobutyric acid

Gamma-aminobutyric acid is an inhibitory neurotransmitter in the central and enteric nervous systems, with the potential to influence the peripheral nervous system *via* the gut-brain axis (Mousavi et al. 2022). Bacterial strains like *L. rhamnosus*, *B. adolescentis*, and *S. thermophilus* EG007, as well as yeast strains like *S. cerevisiae* SC125 and DL6–20, and *K. marxianus*, are known to produce GABA (Daliri et al. 2021; Duranti et al. 2020; Kim et al. 2022; Li, Zhang, et al. 2022; Perpetuini et al. 2020; Tette, Kwofie, and Wilson 2022; Zhang et al. 2020). Particularly in yeast, GABA has a role in cellular oxidative stress defense and provides succinate *via* GABA catabolism into the Krebs cycle during energy metabolism (Perpetuini et al. 2020). Psychobiotics can produce GABA using two separate mechanisms. One mechanism is known to involve an enzyme glutamate decarboxylase, which relies on pyridoxal phosphate and is encoded by the genes gadA or gadB (Liwinski et al. 2023; Yunes et al. 2016). This enzyme converts glutamate to GABA, generating carbon dioxide and using protons in the process. These psychobiotics can utilize extracellular glutamate or synthesize it from α-ketoglutarate, a tricarboxylic acid (TCA) cycle intermediate. This process involves the GABA shunt pathway, an alternative metabolic route that bypasses certain TCA cycle steps, converting α-ketoglutarate into glutamate and subsequently GABA, similar to the mechanism observed in the host (Figure 1) (Tette, Kwofie, and Wilson 2022). During the GABA shunt pathway, in both bacteria and the host, α-ketoglutarate is converted into L-glutamate by L-glutamate dehydrogenase. During this process, the activity of α-ketoglutarate dehydrogenase complex decreases which redirects metabolic flow from the TCA cycle to L-glutamate synthesis. α-Ketoglutarate is transaminated to glutamate by the enzyme glutamate dehydrogenase (GDH) using ammonia or amino donors. Glutamate, formed in the mitochondrial matrix, is exported into the cytoplasm *via* specialized transporters (Cui et al. 2020). In the cytoplasm, glutamate serves as the substrate for the enzyme glutamate decarboxylase (GAD), which catalyzes the decarboxylation of glutamate into GABA, releasing CO_2. This step requires pyridoxal phosphate (PLP) as a cofactor. GABA, once synthesized, can be transported back into the mitochondrial matrix *via* mitochondrial GABA transporters (Cui et al. 2020). Within the mitochondrial

Table 1. Metabolites involved in the gut-brain axis and the psychobiotics linked to their production or modulation

General metabolites	Specific metabolites	Chemical structure	Psychobiotics	Source
Short-chain fatty acids	Acetic acid		*Lactobacillus helveticus* R0052	De Oliveira et al. 2023
			Bifidobacterium dentium	Engevik et al. 2021
			Bifidobacterium breve HNXY26M4	Zhu, Zhao, Wang, et al. 2023
	Propionic acid		*Lactobacillus rhamnosus* GG	LeBlanc et al. 2017
			Lactobacillus gasseri PA 16/8	LeBlanc et al. 2017
			Lactobacillus agilis JCM 1048	Markowiak-Kopeć and Śliżewska 2020
	Butyric acid		*Bifidobacterium breve* CCFM1025	Zhu, Zhao, Wang, et al. 2023
			Escherichia coli Nissle	Ochoa-Sanchez et al. 2019
			Enterococcus faecium	Romo-Araiza et al. 2023
Neurotransmitters	Glutamate		*Corynebacterium glutamicum*	Nakayama 2021
			Brevibacterium lactofermentum	Baj et al. 2019
			Brevibacterium avium	Baj et al. 2019
	Acetylcholine		*Lactobacillus plantarum* PS128	Cheng et al. 2019
			Lactobacillus sporogenes	Sarkar, Mazumder, and Banerjee 2020
			Clostridium butyricum	Sarkar, Mazumder, and Banerjee 2020
	GABA		*Lactobacillus rhamnosus* (JB-1)	Tette, Kwofie, and Wilson 2022
			Bifidobacterium adolescentis	Duranti et al. 2020
			Streptococcus thermophilus EG007	Kim et al. 2022
			Saccharomyces cerevisiae JBCC-A74	Sechi et al. 2014
			Kluyveromyces marxianus K326	Perpetuini et al. 2020
	Serotonin		*Bifidobacterium dentium*	Engevik et al. 2021
			Lactobacillus plantarum PS128	Cheng et al. 2019
			Lactobacillus helveticus WHH1889	Gao et al. 2023
			*Streptococcus thermophilus*1131	Kawase and Furuse 2019
Neurohormones	Dopamine		*Lactobacillus plantarum* PS128	Cheng et al. 2019
			Bacillus licheniformis	Xu et al. 2022
			Serratia marcescens	Sittipo et al. 2022
	Epinephrine		*Bacillus subtilis*	Dicks 2022
			Escherichia coli K12	Dicks 2022
			Proteus vulgaris	Dicks 2022
			Serratia marcescens	Sittipo et al. 2022
	Norepinephrine		*Lactobacillus plantarum* PS128	Liao et al. 2019
			Bacillus mycoides	Giri and Sharma 2022
			Bacillus subtilis	Giri and Sharma 2022
			Proteus vulgaris	Giri and Sharma 2022
Polyamine	Agmatine		*Lactobacillus paracasei* TISTR 453	Elsanhoty 2014
			Lactobacillus casei DSMZ20011	Elsanhoty 2014
			Lactobacillus brevis	Elsanhoty 2014

matrix, GABA undergoes transamination catalyzed by GABA transaminase, resulting in the formation of succinic semialdehyde (Tette, Kwofie, and Wilson 2022). This intermediate is further oxidized by succinic semialdehyde dehydrogenase to produce succinate. Succinate then reenters the TCA cycle, serving as a substrate for succinate dehydrogenase, a key enzyme in both energy production and metabolic homeostasis (Tette, Kwofie, and Wilson 2022). This entire process is summarized in Figure 1. Alternatively, there is a process where a set of enzymes during polyamine metabolism can transform arginine, ornithine, and agmatine into putrescine, then into GABA (Liwinski et al. 2023; Mousavi et al. 2022). During this process, two pathways can convert putrescine into GABA. In the first pathway, putrescine is converted into γ-glutamyl-putrescine by γ-glutamat-putrescine synthetase. This intermediate is then oxidized by γ-glutamyl oxidase to form γ-glutamyl-γ-aminobutyraldehyde, which is subsequently transformed into γ-glutamyl-γ-aminobutyric acid by γ-glutamyl-γ-aminobutyraldehyde dehydrogenase. Finally, γ-glutamyl-GABA hydrolase

synthesizes GABA from γ-glutamyl-γ-aminobutyric acid. In the second pathway, putrescine is converted into γ-glutamyl-γ-aminobutyraldehyde by putrescine-amino transferase and then to GABA by γ-aminobutyraldehyde dehydrogenase (Figure 2).

Serotonin

Serotonin, also called 5-HT, is an indolamine (Yu et al. 2023). This indoleamine is vital in the regulation of mood, hence impairment in its synthesis could result in many disorders, including depression and anxiety (Danilovich, Alberto, and Juárez Tomás 2021). It has been shown that some Lactobacilli and Saccharomyces are capable of producing serotonin and melatonin in culture media (Gallardo-Fernández et al. 2022). In bacteria, serotonin biosynthesis occurs through a pathway similar to that of animals (Gonçalves et al. 2022). In this pathway, serotonin synthesis begins with the essential amino acid L-tryptophan, which is obtained from dietary sources. The process occurs primarily in two steps; first, L-tryptophan is hydroxylated to 5-HT by the enzyme tryptophan hydroxylase (TrpH). This rate-limiting step requires molecular oxygen and tetrahydrobiopterin (BH4) as cofactors. Tryptophan hydroxylase exists in two isoforms: TPH1, predominantly found in peripheral tissues, and TPH2, mainly expressed in serotonergic neurons in the brain (Gonçalves et al. 2022). In the second step, 5-HT is rapidly decarboxylated into 5-HT by the enzyme aromatic L-amino acid decarboxylase (AAAD). This reaction also requires vitamin B6 as a cofactor. The newly synthesized 5-HT can then be stored in vesicles or metabolized further, depending on the cell type (Figure 3). While information on bacterial tryptophan decarboxylase (TrpD) and TrpH genes is scarce, there's evidence indicating that bacteria can produce serotonin through various aromatic amino-acid hydroxylases and AAAD

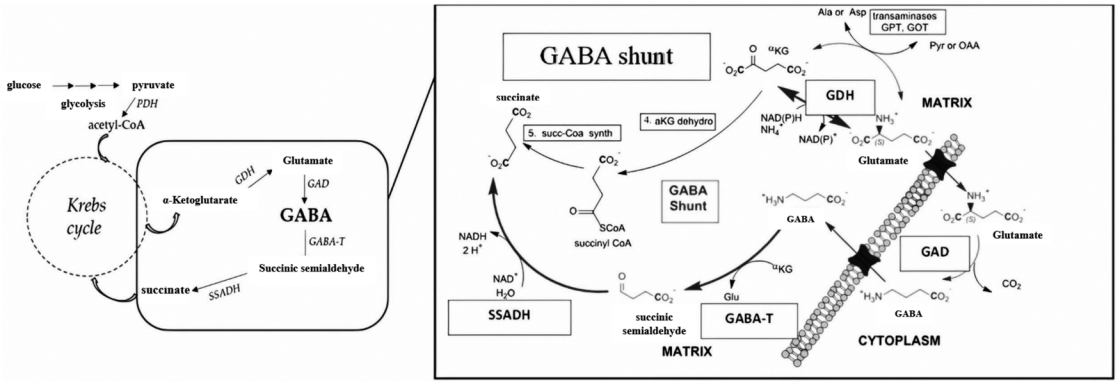


Figure 1. The GABA shunt pathwayfor the biosynthesis of GABA. The GABA biosynthesis pathway in bacteriademonstrates a metabolic shift from energy production to glutamate and GABAsynthesis. This process starts with α-ketoglutarate, an intermediate from glycolysis andthe Krebs cycle, which is converted into glutamate by glutamate dehydrogenase(GDH). Subsequently, glutamate is transported from the mitochondria matrix into thecytoplasm where decarboxylase (GAD) catalyzes the decarboxylation of glutamate toform γ-aminobutyric acid. GABA may then undergo further metabolism via the action ofGABA transaminase (GABA-T), producing succinic semialdehyde after its transportback into the matrix. Succinic semialdehyde is then oxidized by succinic semialdehydedehydrogenase (SSADH) to form succinate, which re-enters the Krebs cycle toregenerate energy.

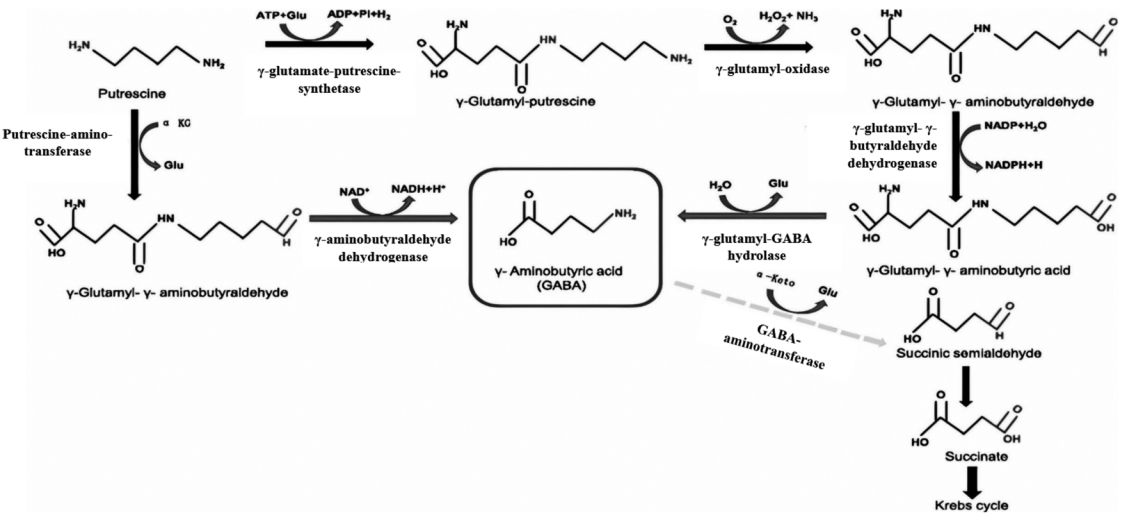


Figure 2. Polyamine metabolism as alternate pathway for microbial biosynthesis of GABA. Putrescine is metabolized into GABA through two pathways. The first involves the convertion of putrescine to γ-glutamyl-putrescine by γ-glutamat-putrescine-synthetase. The γ-glutamyl-putrescine is oxidized to γ-glutamyl- γ -aminobutyraldehyde by γ-glutamyl-oxidase, which is thenconverted to γ-glutamyl- γ -aminobutyric acid by γ-glutamyl- γ -aminobutyraldehyde dehydrogenase. GABA is then synthesized from γ-glutamyl- γ -aminobutyric acid by the enzyme γ-glutamyl GABA hydrolase. The second pathway converts putrescine into γ-glutamyl-γ-aminobutyraldehyde by putrescine-amino transferase, which is then transformed into GABA by γ-aminobutyraldehyde dehydrogenase.

besides TrpH/TrpD (Gonçalves et al. 2022; Wang et al. 2023). Nevertheless, the co-occurrence of aromatic amin-acid hydroxylases and AAAD genes within the same bacterial strain is exceedingly uncommon. Özoğul et al. (2012) demonstrated the *de novo* synthesis of serotonin through the combination of lactic acid bacteria, *L. plantarum* PS128, and food-borne pathogens. Engevik et al. (2021) again demonstrated that *B. dentium* could enhance the production of 5-HT from enterochromaffin cells in the gut as discussed earlier.

Catecholamines

Dopamine, norepinephrine, and epinephrine serve as both hormones in the endocrine system and neurotransmitters in the central and peripheral nervous systems (Skolnick and Greig 2019). Their primary functions include emotions, cognition, and regulation of information memorization (Mittal et al. 2017; Skolnick and Greig 2019). González-Arancibia et al. (2019) proposed that dysfunction in catecholamine neurotransmission may be associated with neurological and neuropsychiatric conditions, including depressive disorders. L-tyrosine is an amino acid and serves as a precursor for the synthesis of dihydroxyphenylalanine (L-DOPA) in the human body through a hydroxylation reaction catalyzed by tyrosine hydroxylase. This reaction marks the initial step in the biosynthesis of catecholamines (González-Arancibia et al. 2019). L-Phenylalanine can alternatively be converted to tyrosine through the action of phenylalanine hydroxylase (Delva and Stanwood 2021). During dopamine synthesis, L-DOPA undergoes decarboxylation by AAAD enzyme to form dopamine. Subsequently, dopamine is converted to norepinephrine by dopamine β-hydroxylase. Finally, norepinephrine is transformed into epinephrine by transferring a methyl group to norepinephrine through the action of phenylethanolamine N-methyltransferase (Figure 4) (Delva and Stanwood 2021; Skolnick and Greig 2019). *L. plantarum* PS128, *Bacillus licheniformis*, and *Serratia marcescens* are recognized for their ability to produce or stimulate the production of catecholamines including dopamine (Cheng et al. 2019; Sittipo et al. 2022; Xu et al. 2022). *Bacillus sp.* JPJ has been demonstrated to encode the tyrosine hydroxylase gene and could express it to convert L-tyrosine to L-DOPA in vitro, achieving a conversion efficiency of 99.4% at a pH of 8 (Jagtap and Chavan 2014). Additionally, Villageliú and Lyte (2018) demonstrated that various strains of *E. faecium* can transform L-DOPA into dopamine in vitro, with conversion efficiencies reaching 96.1%.

Proposed mode of action of bacteria and their neuroactive compounds on host brain function

The bidirectional communication of the gut microbiota and the brain is believed to involve various pathways, including activation of the vagus nerve, stimulation of endocrine cells (including enterochromaffin cells), immune-mediated signaling, and transportation of gut-derived neuroactive compounds from circulation to the brain (Ahmed et al. 2022; Burokas et al. 2015; Cryan et al. 2019).

The vagus nerve

The vagus nerve serves as a direct connection between the muscular and mucosal layers throughout the gastrointestinal tract and the brainstem (Needham, Kaddurah-Daouk, and

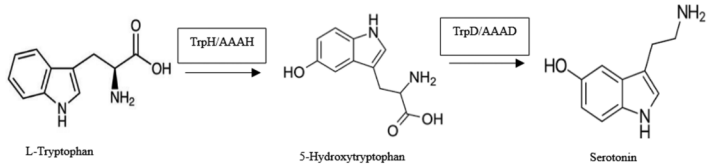


Figure 3. The proposed biosynthetic pathways of serotonin inpsychobiotics. Tryptophan is converted to 5-hydroxy tryptophan by tryptophanhydroxylase or an aromatic amino acid decarboxylase. The 5-hydroxytryptophan isthen converted into serotonin by tryptophan decarboxylase or an aromatic amino aciddecarboxylase. TrpH; Tryptophan hydroxylase, TrpD; Tryptophan decarboxylase, AAAD; Aromatic amino acid decarboxylase, AAAH; Aromatic amino acid hydroxylase. Gonçalves et al. (2022).

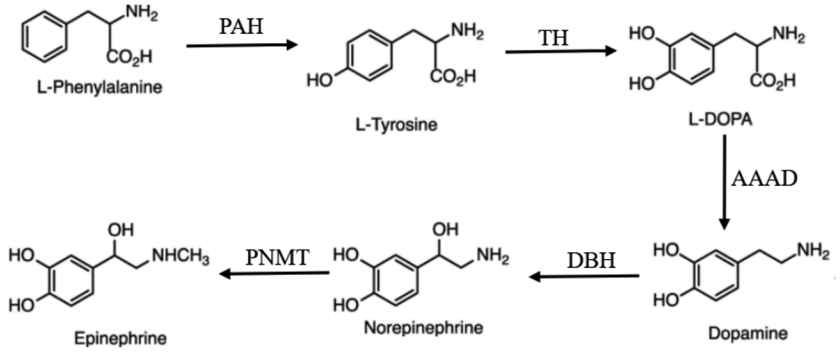


Figure 4. Biosynthetic pathway of catecholamines. L-phenylalaninecould be converted to tyrosin by phenylalanine hydroxylase or tyrosin available tyrosindirectly converted to L-DOPA by tyrosine hydroxylase. The L-DOPA produced by the action of an aromatic amino acid decarboxylase is converted to dopamine. Dopaminecould be converted to norepinephrine and subsequently to epinephrine by dopamine β-hydroxylase and phenylaethanolamine N-methyltransferase respectively. PAH: phenylalanine hydroxylase, TH: tyrosine hydroxylase, AAAD: aromatic amino aciddecarboxylase, DBH: dopamine β-hydroxylase, PNMT: phenylaethanolamine Nmethyltransferase. Huang et al. (2019).

Mazmanian 2020). It is a well-established signaling pathway that influences various behaviors, including anxiety-like and depressive-like behaviors (Needham, Kaddurah-Daouk, and Mazmanian 2020). It has been shown that chronic treatment with *L. rhamnosus* (JB-1) induces region-specific alterations in $GABA_{B1b}$ mRNA expression in the brain, reduces stress-induced corticosterone levels, and alleviates anxiety- and depression-related behaviors. However, these effects were absent in vagotomized mice, highlighting the vagus nerve as a key communication pathway between gut bacteria and the brain (Bravo et al. 2011). The role of the vagus nerve in mediating neuronal activation following oral treatment with *L. rhamnosus* (JB-1) was again explored by Bharwani et al. (2020) demonstrating that live *L. rhamnosus* (JB-1) but not the heat killed, induced vagal neuron activation in specific brain regions, evidenced by c-Fos expression.

Enterochromaffin cells

Enterochromaffin cells are specialized endocrine cells found in the lining of the gut, responsible for producing and secreting ~90% of the body's serotonin in response to persistent intestinal signals (Mawe and Hoffman 2013). Colonic enterochromaffin cells express receptors for and are responsive to various microbial metabolites, including microbe-associated molecular patterns, short-chain fatty acids, aromatic amino acid metabolites, and secondary bile acids (Needham, Kaddurah-Daouk, and Mazmanian 2020; Wei, Singh, and Ghoshal 2022). *L. rhamnosus* KY16 has been shown to alleviate depression by stimulating enterochromaffin cells to secrete 5-HTP in the gut, which enters the bloodstream and enhances 5-HT synthesis in the brain (Xie et al. 2024).

Immune cells mediation

Bacterial metabolites acting as microbe-associated molecular patterns, such as lipopolysaccharides, have been used to stimulate the immune system and shown to induce depressive-like symptoms in mice (Salvo-Romero, Stokes, and Gareau 2020). On the other hand, other gut metabolites, such as short-chain fatty acids are likely to mitigate chronic inflammation by activating G protein-coupled receptors and inhibiting histone deacetylase activity, thereby reducing systemic and neuroinflammation (Caspani and Swann 2019). Neuroinflammation is known to detrimentally affect neurons and other brain cells through the sustained production of pro-inflammatory cytokines and other mediators. Peptidoglycans and lipopolysaccharides derived from bacterial cell walls and outer membranes, respectively, activate central pattern-recognition receptors, stimulating the innate immune system and influencing behavior (MacCain and Tuomanen 2020). Specifically, the translocation of lipopolysaccharides into the brain is proposed to be regulated by propionate, a gut microbial metabolite known to modulate BBB permeability (Fock and Parnova 2023). Pro-inflammatory cytokines present in the gastrointestinal (GI) tract can also influence central stress circuitry by stimulating the vagus nerve and activating the hypothalamic-pituitary-adrenal (HPA) axis (Needham, Kaddurah-Daouk, and Mazmanian 2020).

Direct effect of bacterial neuroactive compounds

The human host encounters many bacterial metabolites in the gut, which can have local effects in the gastrointestinal environment or be absorbed, enter the systemic circulation, and reach distant organs. Bacterial neuroactive compounds, including GABA, serotonin, and dopamine precursors, are increasingly recognized for their influence on brain function (Wall et al. 2014). While the blood brain barrier is highly selective, certain neuroactive molecules may be transported *via* specific carrier-mediated processes and in cases of compromised blood brain barrier (Gyawali and Kang 2020). For example, tryptophan-derived metabolites may cross the blood brain barrier through neutral amino acid transporters (Gyawali and Kang 2020). Additionally, short-chain fatty acids may modulate the blood brain barrier permeability, indirectly facilitating neuroactive compound transport (O'Riordan et al. 2022). Emerging evidence suggests that EVs from gut bacteria, carrying neuroactive cargo, may serve as potential vehicles for direct intercellular communication, as discussed below in the psychobiotics and their EVs section.

Psychobiotics and their extracellular vesicles

Studies suggest that bacterial EVs may play crucial roles in mediating the effects exerted by psychobiotic bacteria on the brain (Morad et al. 2019). EVs derived from psychobiotic bacteria have been demonstrated to be absorbed from the gastrointestinal tract, penetrate the brain, and deliver their intracellular contents, thereby exerting beneficial multidirectional effects (Bleibel et al. 2023). Indeed, through the regulation of epigenetic factors, EVs from psychobiotics seem to enhance the expression of neurotrophic molecules, improve serotonergic neurotransmission, and potentially supply astrocytes with glycolytic enzymes to promote neuroprotective mechanisms (Bleibel et al. 2023). Aside bacteria, some eukaryotic yeast is known to produce EVs that may transport cargo to the brain. For instance, it has been demonstrated that EVs from *S. boulardii* CNCM I-745 and *S. cerevisiae* stimulated the production of IL-1β and IL-8 (Kulig et al. 2024; Nenciarini et al. 2024). Three primary mechanisms have been proposed to explain how EVs influence the brain. First, EVs absorbed from the gastrointestinal tract may cross the BBB, particularly in conditions where the BBB is compromised (Matsumoto et al. 2017). Second, EVs may utilize the vagal nerve as a transport route to the CNS. Finally, EVs could activate leukocyte trafficking, facilitating their delivery to the brain (Bleibel et al. 2023). These potential pathways are illustrated in Figure 5 below.

Evidence of psychobiotics' impact on mental health

Psychobiotics can have significant impact on the brain, influencing behavior, mood, and cognition in both experimental and clinical settings. Following the initial work by Sudo et al. (2004), which demonstrated that commensal microbiota can influence the postnatal development of the hypothalamic–pituitary–adrenal stress response in mice, there has been a growing focus on the relationship between

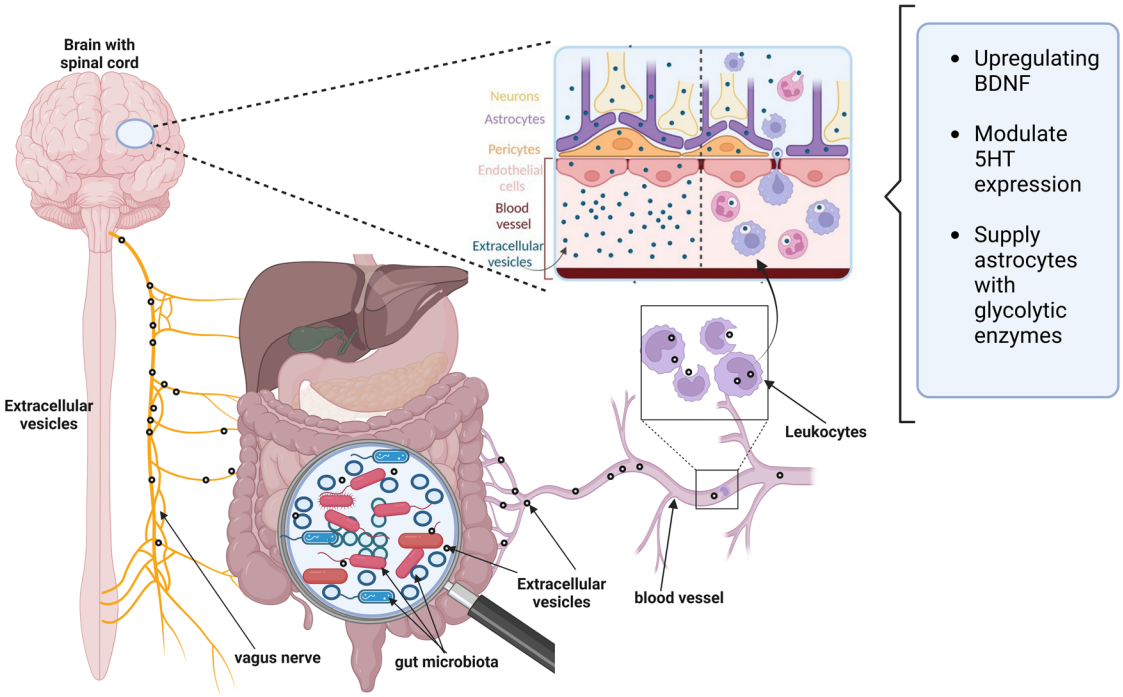


Figure 5. Proposed transportation channels of psychobiotic EVs tothe brain. EVs may cross a compromised blood-brain barrier, transported via the vagalnerve and also via activated leukocytes. In the brain, psychobiotic EVs produceantidepressant-like effects by upregulating brain-derived neurotrophic factors (BDNF), modulating 5-hydroxytryptophan expression, and possibly supplying astrocytes withglycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). 5-HT: 5-hydroxytryptophan, and SERT: 5-HT transporter.

psychobiotics and mental health (Butler et al. 2019). More recently, a strong relationship between the gut microbiota and central nervous system related disorders in humans, including Parkinson's disease, Alzheimer's disease, major depressive disorders, and autism spectrum disorder has been reported (Cai et al. 2023; Cryan et al. 2020; Doroszkiewicz, Groblewska, and Mroczko 2021; Megur et al. 2020). Again, Desbonnet et al. (2015) found that depleting the gut microbiota induced cognitive deficits, altered dynamics of tryptophan metabolic pathways, and significantly reduced expression of brain-derived neurotrophic factor, oxytocin, and vasopressin in the brains of adult mice. *L. johnsonii* BS15, in particular, is known to prevent stress related memory dysfunction in mice (Wang et al. 2021). In a similar study, Xu et al. (2023) showed that treatment with *L. rhamnosus* zz-1 mitigated depression-like behavioral disorders of depressed mice and reduced the expression of pro-inflammatory cytokines (IL-1β and TNF-α) in the hippocampus. Interestingly, even modulation of the gut microbiota by prebiotics has an anxiolytic and antidepressant-like effects and reverse the impact of chronic stress in mice (Burokas et al. 2017) and prevent the detrimental effects of high-fat diet on microglia functionality in aging mice (Vijaya et al. 2024). Most recent study has demonstrated that unusual probiotics like *Blautia wexlerae* can have positive effects on preventing food addiction in mouse model (Samulėnaitė et al. 2024). Other pre-clinical studies have also demonstrated the positive effects of psychobiotics on mental health (Chao et al. 2020; Fung, Olson, and Hsiao 2017; Liu, Walsh, and Sheehan 2019; Martin et al. 2018; Mayneris-Perxachs et al. 2022; Sen et al. 2022; Tian et al. 2020).

Some clinical studies have reported similar benefits in humans. Reiter et al. (2020) demonstrated that administering various species of *Bifidobacterium* and *Lactobacillus* (including *B. bifidum* W23, *B. lactis* W51 and W52, *L. acidophilus* W22, *L. casei* W56, *L. paracasei* W20, *L. plantarum* W62, *L. salivarius* W24, and *Lactococcus lactis* W19) alongside standard treatment, resulted in decreased IL-6 gene expression compared to control groups that received only the usual treatment. *L. plantarum* 299v have been found to enhance attention, processing speed, and verbal learning and memory in patients with major depressive disorder (Rudzki et al. 2019). Similarly, patients with major depressive disorder who received a four-week treatment with a probiotic cocktail comprising *S. thermophiles, B. breve, B. longus, B. infantis, L. acidophilus, L. plantarum, L. paracasei, and L. delbrueckii* exhibited significant improvement in depression symptoms (Schneider et al. 2023). Other clinical studies have linked psychobiotic administration with decreased mental health symptoms (Huang et al. 2022; Jamilian and Ghaderi 2021; Kumperscak et al. 2020; Reininghaus et al. 2020; Sepehrmanesh et al. 2021; Zhang et al. 2021). Table 2 offers a synopsis of these studies, detailing major outcomes, as well as sample and population characteristics.

Quantification techniques for gut-derived neuroactive compounds from psychobiotics

Insights into the microbiota-gut-brain axis require not only an understanding of the structure and function of the gut microbiota, made possible by next-generation sequencing-based approaches, but also the measurement of some neuroactive

Table 2. Clinical studies demonstrating the impact of psychobiotics on psychiatric symptoms and Central nervous system function

Study reference	Type of study	Psychobiotic intervention (strain/ dosage)	Demographic characteristics	Affection associated with the brain	Administration of psychobiotics	Results and observations
Schneider et al. (2023)	Placebo controlled randomized controlled trial (PC RCT)	Multiple species (*Streptococcus thermophile*; *Bifidobacterium brev*; *Bifidobacterium longus*; *Bifidobacterium infantis*; *Lactobacillus acidophilus*; *Lactobacillus plantarum*; *Lactobacillus paracasei*; *Lactobacillus delbrueckii*/9*10^{11} CFU in total)	Average age (years): 39.43; Proportion of females (%): 67; Study duration (weeks): 4	Major depressive disorder (International Classification of Diseases, 10th version)	Capsule and orally taken as a supplement with maltose	Significantly improved immediate recall in verbal learning memory test in the probiotic group. Hippocampus activation during working memory processing, revealing a remediated hippocampus function in the probiotic group.
Moloney et al. (2021)	Randomized placebo-controlled, repeated measures and cross-over intervention	Single strain (*Bifidobacterium longum* 1714/1*10^{9} CFU)	Average age (years): 20.7; Proportion of females (%): 0; Study duration (weeks): 8	Prolonged stress, cognitive performance, and mood in healthy male volunteers (Cambridge Neuropsychological Test Automated Battery)	Capsule form together with corn starch, magnesium stearate, hypromellose, and titanium dioxide and administered orally	While *B. longum* 1714 improved sleep quality and duration, it did not alleviate symptoms of chronic stress, depression, or any measure of cognitive assessment.
Sepehrmanesh et al. (2021)	Double blinded placebo controlled randomized controlled trial (DB PC RCT)	Multiple species (*Lactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Bifidobacterium bifidum*/2*10^{9} CFU each)	Average age (years): 9.3; Proportion of females (%): 17.6; Study duration (weeks): 8	Attention deficit and hyperactivity disorder in children (Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision)	Powdered probiotics in a sachet and to be taken orally	Decrease in total attention-deficit/ hyperactivity disorder rating scale and Hamilton Anxiety Rating Scale (HAM-A) in treated group than that of the placebo. A considerable decrease in high sensitivity C-reactive protein of serum as well as a substantial rise in plasma overall antioxidant volume in treated groups.
Jamilian and Ghaderi (2021)	Double blinded placebo controlled randomized controlled trial (DB PC RCT)	Multiple species (*Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*/2*10^{9} CFU each)	Average age (years): 46.4; Proportion of females (%): 0; Study duration (weeks): 12	Schizophrenia (Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision)	Capsule of probiotic and selenium co-supplement orally administered	A significant improvement in the general positive and negative syndrome scale score compared with the placebo. A significant elevation in total antioxidant capacity and total glutathione. A significant reduction in high-sensitivity C-reactive protein levels.
Zhang et al. (2021)	Double blinded placebo controlled randomized controlled trial (DB PC RCT)	Single strain (*Lacticaseibacillus paracasei* Shirota/1*10^{8} CFU)	Average age (years): 45.8; Proportion of females (%): 63.15; Study duration (weeks): 9	Major depressive disorder (Diagnostic and Statistical Manual of Mental Disorders, fifth edition)	Orally administered as a drink (100 ml)	The Beck Depression Index (BDI) and Hamilton Depression Rating Scale (HAM-D) scores were significantly decreased, and the degree of depression was significantly improved. The intervention group showed increased levels of beneficial Adlercreutzia, Megasphaera, and Veillonella. Interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) levels were significantly decreased in the treated group.

(*Continued*)

Table 2. Continued.

Study reference	Type of study	Psychobiotic intervention (strain/ dosage)	Demographic characteristics	Affection associated with the brain	Administration of psychobiotics	Results and observations
Reiter et al. (2020)	Double blinded placebo controlled randomized controlled trial (DB PC RCT)	Multiple species (*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51 and W52, *Lactobacillus acidophilus* W22, *Lactobacillus casei* W56, *Lactobacillus paracasei* W20, *Lactobacillus plantarum* W62, *Lactobacillus Salivarius* W24, *Lactococcus lactis* W19/7.5*10^9 CFU in total)	Average age (years): 43; Proportion of females (%): 71.4; Study duration (weeks): 4	Major depressive disorder (Mini-International Neuropsychiatric Interview)	Orally administered as a drink	The intervention group showed decreasing IL-6 gene expression levels while the placebo group showed increasing gene expression levels of IL-6.
Kumperscak et al. (2020)	Randomized controlled trial (RCT)	Single strain (*Lactobacillus rhamnosuss* GG ATCC53103/1*10^{10} CFU each)	Average age (years): 11.4; Proportion of females (%): 33.3; Study duration (weeks): 13	Attention deficit and hyperactivity disorder (Diagnostic and Statistical Manual of Mental Disorders, fifth edition)	Orally administered as a capsule with hydroxypropyl methylcellulose, maltodextrins, and the coloring titanium dioxide as excipients	Significant improvement in the pediatric quality of life inventory child self-report total score after 3 months of treatment in the probiotic. Significant differences in the levels of serum cytokines between the groups after the 3-month treatment period.
Reininghaus et al. (2020)	Double blinded placebo controlled randomized controlled trial (DB PC RCT)	Multiple species (*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51 and W52, *Lactobacillus acidophilus* W22, *Lactobacillus casei* W56, *Lactobacillus paracasei* W20, *Lactobacillus plantarum* W62, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19/7.5*10^9 CFU in total)	Average age (years): 51.5; Proportion of females (%): 45; Study duration (weeks): 13	Bipolar disorder (Diagnostic and Statistical Manual of Mental Disorders, fifth edition	Orally administered as a powdered supplement and mixed with water together with biotin	KEGG-analysis showed elevated inflammation-regulatory and metabolic pathways in the intervention group.
Rudzki et al. (2019)	Double blinded placebo controlled randomized controlled trial (DB PC RCT)	Single strain (*Lactobacillus plantarum* 299 v/10*10^{10} CFU)	Average age (years): 39.9; Proportion of females (%): 76.7; Study duration (weeks): 8	Major depressive disorder (Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision)	Orally administered as a capsule with	Improvement in attention and perceptivity test and in California verbal learning test total recall in the intervention group compared with the placebo. Significant increase in 3HKYN:KYN ratio in the intervention group.
Allen et al. (2016)	Repeated measure placebo-controlled randomized trials	Single strain (*Bifidobacterium longum* 1714/1*10^9 CFU)	Average age (years): 18 to 40; Proportion of females (%): 0; Study duration (weeks): 4	Stress response, cognition, and brain activity patterns (Cambridge Neuropsychological Test Automated Battery)	Probiotic sticks and the content of sticks mixed with milk and taken orally	Reduction in daily reported stress, improvements in hippocampus-dependent visuospatial memory performance, and enhanced frontal midline electroencephalographic mobility.

compounds acting along this axis. Considering that a given threshold concentration of a neuroactive compound may be required for a measurable health effect, it is essential to develop potent metabolomic strategies for quantifying the levels of such bioactive compounds. More so, understanding how neuroactive compounds produced by psychobiotics in the gut are quantified, tracked in circulation, and monitored in the brain becomes imperative. These processes begin with the accurate quantification of the metabolites within the gut using analytical techniques including MS and LC. Once these

metabolites enter the bloodstream, it is essential to track their presence and concentration using blood sample analysis and specific biomarkers (Bar et al. 2020; Chen et al. 2023; Kunevičius et al. 2024). To determine the impact on brain function, it is key to again monitor how these metabolites are able to cross the BBB and their subsequent effects within the brain (Montagne et al. 2016). This comprehensive approach allows for a deeper understanding of the pathways involved and the potential therapeutic applications of psychobiotics and their metabolites in mental health.

Quantification techniques in the gut

Quantification of neuroactive compounds produced by psychobiotics in the gut begins with sample collection. Due to the increasing interest in studying the complex interactions between the gut microbiota and the host, fecal samples have become important biological matrix. They offer a direct means to probe the connection between intestinal bacteria and the physiology of the host (Karu et al. 2018). Accordingly, fecal samples from the host can be collected and analyzed using various analytical techniques, including MS, LC, nuclear magnetic resonance (NMR) spectroscopy, and enzyme-linked immunosorbent assay (ELISA). Fecal sample collection is a noninvasive approach providing a comprehensive profile of metabolites present in the large intestine (Karu et al. 2018). However, since metabolite composition can vary significantly along the gastrointestinal tract, complementary methods are necessary to capture a complete picture. Obtaining intestinal biopsies through endoscopic procedures allows for site-specific analysis of metabolites directly from the gut lining (Santoru et al. 2021). This invasive method provides high-resolution data on local metabolite concentrations but is limited by the procedure's complexity and the discomfort it causes to test models.

Analyzing biological samples of clinical interest presents many analytical challenges. One of the most critical steps is sample preparation, which is essential for accurately determining metabolites of interest (Kunevičius et al. 2024). Proper sample preparation helps to avoid error propagation in subsequent separation and detection steps and simplifies data analysis for acquiring reliable and interpretable data (Fiori et al. 2020; Kyei-Baffour et al. 2021; Malcangi et al. 2022). Sample preparation techniques like solid-liquid extraction, liquid-liquid extraction, and solid phase

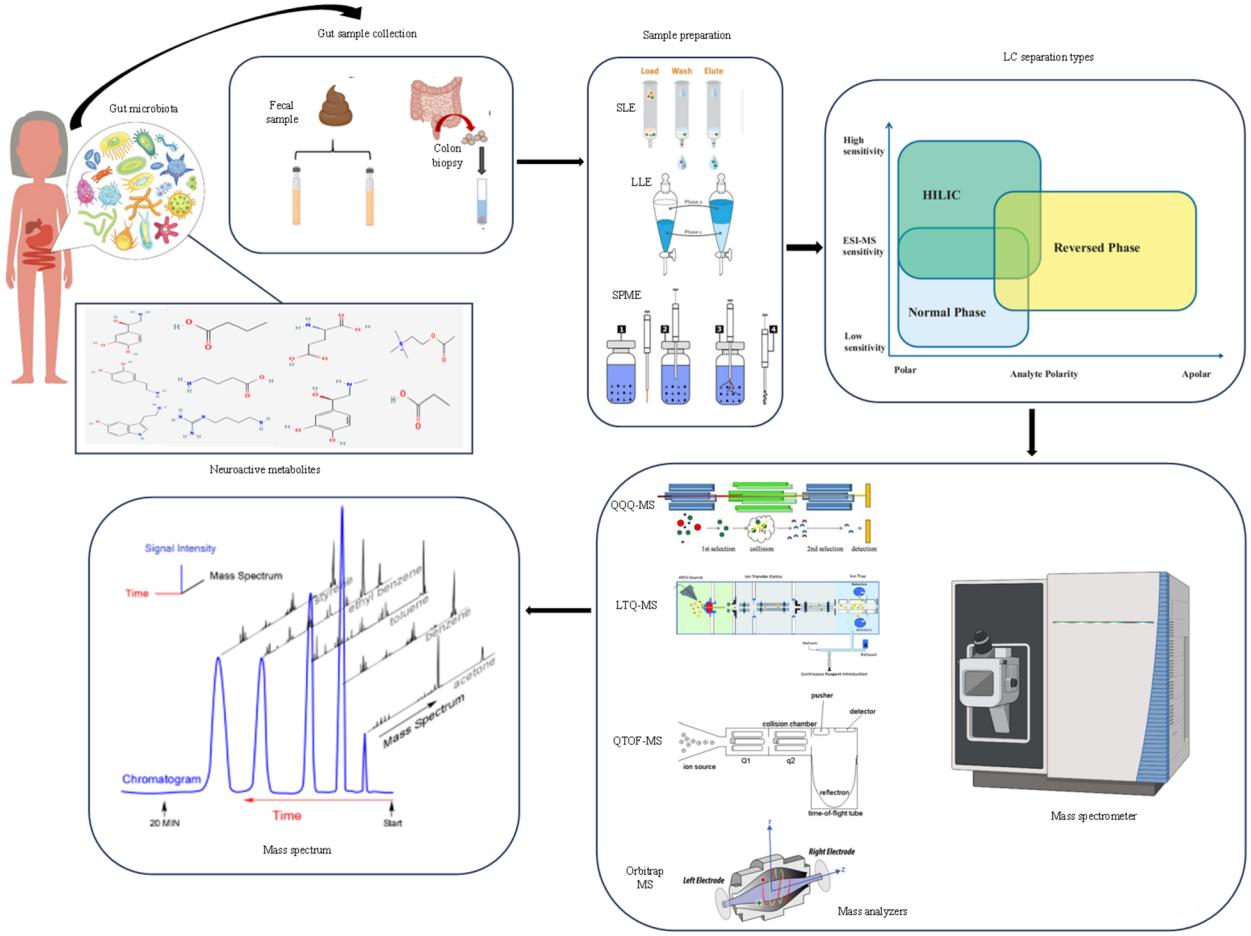


Figure 6. A schematic diagram for the determination andquantification of gut derived neuroactive metabolites. The gut microbiota, includingpsychobiotics, can produce neuroactive metabolites in the gut. Fecal or colon biopsysamples are collected and prepared using various extraction techniques such as SLE,LLE, and SPME. The compounds in these prepared samples are then separated usingreversed phase, normal phase, or hydrophilic interaction LC. These separatedcompounds are transferred to a MS, where they are detected and quantified usingvarious mass analyzers like triple quadrupole (QQQ), linear trap quadrupole (LTQ), quadrupole time of flight (QTOF), and orbitrap MS. The detected compounds arerepresented as peaks and mass spectra via electrical signals from a detector.

microextraction (SPME) are used to efficiently extract metabolites for analysis (Fiori et al. 2020). In solid-liquid extraction, water or aqueous buffers are added to fecal samples, and the liquid phase (fecal water) is then obtained by ultracentrifugation (Yen et al. 2018). Liquid-liquid extraction is frequently used on the fecal water or solution obtained by ultracentrifugation. Diethyl ether is a popular choice for liquid-liquid extraction due to its ability to efficiently extract a wide range of compounds that partition favorably in a water/ether system (Fiori et al. 2020). SPME on the other hand is widely used in chromatographic-based targeted metabolomics, particularly for analyzing fecal samples to determine volatile organic compounds, especially short chain fatty acids (Reyes-Garcés and Gionfriddo 2019). The enrichment capacity of SPME mainly depends on the adsorptive or absorptive coating materials. Fiber coatings containing carboxen have proven to be highly effective for capturing short chain fatty acids in headspace SPME mode (Reyes-Garcés and Gionfriddo 2019).

Table 3. Recent studies that have employed advance chromatography and mass spectrometry techniques to quantify gut-derived neuroactive compounds and the associated psychobiotics

Study	Quantitation method employed	Neuroactive compound(s) determined	Psychobiotic species associated with metabolite production
Luck et al. (2021)	Liquid chromatography coupled to hybrid triple-quadrupole/ linear ion trap MS system	GABA	*B. dentium* ATCC 27678
Eastwood et al. (2023)	High performance liquid chromatography coupled to triple-quadrupole MS with electrospray ion source	GABA, serotonin, tryptophan, and dopamine	*Lactococcus lactis* W58 and *Lactobacillus rhamnosus* W198
Ding et al. (2021)	Ultra high-performance liquid chromatography coupled to Q-exactive Orbitrap MS	Serotonin and dopamine	*Akkermansia muciniphila*
Casertano et al. (2024)	Liquid chromatography coupled to a triple quadrupole MS	GABA, acetylcholine and serotonin	*Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Ligilactobacillus salivarius*, *Streptococcus thermophilus*
Li, Li, et al. (2022)	Ultra-high performance liquid chromatography coupled to a triple quadrupole MS	Serotonin	Genetically engineered *Escherichia coli* Nissle 1917 (EcN-5-HT)
Zhao et al. (2022)	Ultra-high performance liquid chromatography coupled to Q-exactive Orbitrap MS	GABA	*Bifidobacterium*, *Lactobacillus*, and *Enterococcus faecalis*
Wang et al. (2020)	Ultra-high performance liquid chromatography coupled to a triple quadrupole MS	Glutamate, GABA, L-DOPA, serotonin	*Bifidobacterium infantis* Bi-26, *Lactobacillus rhamnosus* HN001, *Bifidobacterium lactis* BL-04, and *Lactobacillus paracasei* LPC-37

While no single analytical platform can capture all small metabolites in biological samples during quantification, MS-based metabolomics offer the most comprehensive approach to monitor metabolites associated with the gut microbiota and the brain. LC is mostly employed to separate complex mixtures of extracted samples and subsequent identified with MS (Daliri et al. 2017). This method is particularly effective for analyzing small molecular weight metabolites (Levi Mortera et al. 2016). Based on the polarity of the compounds, reversed-phase liquid chromatography (RPLC) is used for analyzing medium to low polarity components, while hydrophilic interaction chromatography is used for high polarity components (Jandera and Hájek 2020). After separation, mass analyzers including triple quadrupole (QQQ-MS), linear trap quadrupole (LTQ-MS), time-of-flight (TOF-MS), quadrupole time-of-flight (QTOF-MS), and Orbitrap-MS are employed for quantitative analysis (Levi Mortera et al. 2016; Valdés et al. 2023). Figure 6 summarizes these quantification techniques in a schematic diagram. Imaging mass spectrometry (IMS) techniques have also been developed and emerging as an attractive tool in metabolomics. The application of IMS does not require prior knowledge of the analyzed samples and offers high-throughput profiling of small molecular metabolites with accurate mass (Bourceau et al. 2023). This sets it apart from traditional imaging methodologies like radiochemistry and immunohistochemistry. IMS techniques including

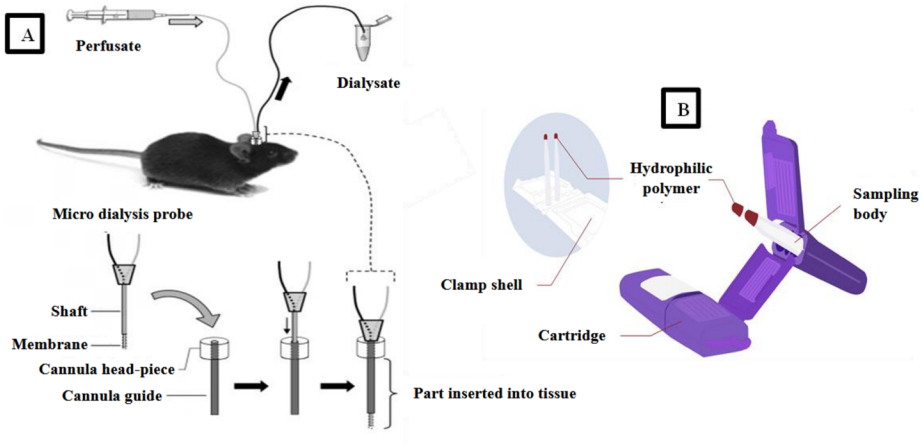


Figure 7. Sampling techniques for monitoring metabolites in the blood. (A) Microdialysis technique, showing the probe inserted into the tissues ofsubjects for real-time dialysate collection. (B) VAMS, displaying the hydrophilic polymertips for both cartridge and clamp shell casings.

matrix-assisted laser desorption/ionization IMS, desorption electrospray ionization IMS, and nano-structure-initiator IMS have been developed to analyze small molecular metabolites with neurological functions in complex tissues like the gut and brain (Bourceau et al. 2023; Cameron and Takáts 2018). Another detection technique, capillary electrophoresis mass spectrometry, combines capillary electrophoresis for separating metabolites based on their charge and size with MS detection. This method is highly efficient for analyzing charged small metabolites (Luan, Wang, and Cai 2019). Other techniques used in quantifying gut-derived metabolites include NMR spectroscopy and ELISA. NMR is a nondestructive method to quantify gut metabolites. Although less sensitive than MS, NMR does not require extensive sample preparation and can quantify metabolites directly even in complex biological mixtures. ELISA on the other hand is used for the targeted quantification of specific metabolites. This immunoassay technique relies on antibodies specific to the metabolite of interest, providing high specificity and allowing for the quantification of metabolites even at low concentrations (Cameron and Takáts 2018; Luan, Wang, and Cai 2019). Table 3 reviews various studies that have used these advanced analytical techniques to quantify gut-derived neuroactive compounds.

Monitoring metabolites into circulation and the brain

Volumetric absorptive microsampling (VAMS) is a newly developed method for minimally invasive single-drop blood collection (Bossi et al. 2024). This micro-sampling technique involves collecting blood *via* a prick, which is then absorbed into a hydrophilic polymer attached to a closable cartridge (Figure 7B). The VAMS device is left to dry at room temperature and can be stored for subsequent analytical studies, including metabolomic profiling of both animal models and humans (Volani et al. 2023). Integrating VAMS with conventional MS-based metabolomics is an attractive, less-invasive alternative to phlebotomy (venipuncture), offering the added potential for remote sample collection (Bossi et al. 2024). Phlebotomy however is the standard method for obtaining blood samples which involves drawing blood from a vein, typically in the arm or thigh (Bossi et al. 2024). On the other hand, micro-dialysis is another technique where a probe is inserted into the bloodstream to continuously sample metabolites in real-time (Lunte and Lunte 2021). This minimally invasive method enables dynamic monitoring of metabolite levels over time (Figure 7A). After blood sampling, metabolites or their biomarkers are detected and quantified using the aforementioned chromatography and mass spectrometry techniques.

Monitoring gut-derived metabolites in the brain is necessary to understand their potential impact on neurological functions and behavior. The process of monitoring involves assessing the ability of gut-derived metabolites to cross the BBB and their subsequent effects within the brain. Studying the ability of metabolites to cross the BBB involves using both in vitro and in vivo models (Chaulagain et al. 2023; Harris et al. 2023). Conventional in vitro models consist of isolated primary brain endothelial cells or immortalized cells as a monolayer in two-dimensional BBB models (Chaulagain et al. 2023). The development of tight junction proteins using endothelial cells and astrocytes also led to co-culture transwell models. This was followed by transwell co-culture models incorporating three different cell types, with a greater emphasis on trans-endothelial electrical resistance as a measure of barrier integrity. The emergence of dynamic and 3D models, such as organoids or barriers on chips using microfluidics further advanced the field. Modern models however have refined microfluidic design and emphasized vasculogenesis to more accurately mimic the BBB (Schreiner et al. 2022). Nonetheless, in vivo animal models have also been used to study how neuroactive compounds may transverse the BBB. The most common in vivo approach for monitoring metabolite permeation across the BBB is intravenous injection. In this method, the metabolite is administered to the animal intravenously, and its concentration in the brain is measured at different times (Harris et al. 2023). This approach keeps all physiological and metabolic systems intact, providing the most realistic assessment of what actually enters the brain.

Among the techniques for investigating neurotransmitter distribution in the brain, MS, either coupled with LC or using direct methods like matrix-assisted laser desorption/ionization (MALDI) stands out (Andersson, Andren, and Caprioli 2010). However, these techniques have some drawbacks. While LC-MS does not provide information on the spatial distribution of compounds in brain tissues, MALDI requires a matrix with a proton donor for the sample, which assists in analyte desorption but also hinders the analysis of small-mass compounds due to ion interference from the matrix (Shariatgorji et al. 2016). In this context, an MS imaging technique that allows mapping the spatial distribution of compounds in samples and do not require a matrix-assisted desorption will be a better alternative (Maciel, Martins, et al. 2022). Desorption electrospray ionization (DESI) MS imaging technique employs a pneumatically-assisted electrospray system and is focused on the brain tissue section surface, making a liquid film that dissolves

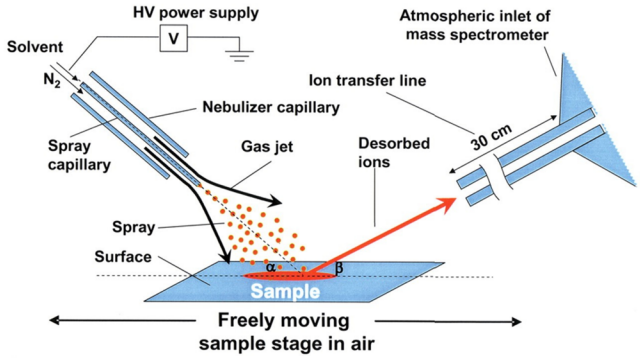


Figure 8. Schematic diagram of DESI ion source. A pneumaticallyassistedelectrospray needle produces charged solvent droplets directed onto thesurface to be analyzed, creating a thin solvent film that dissolves the surfacecomponents. New charged droplets release secondary microdroplets containinganalytes from the surface. These secondary droplets are then evaporated by astandard electrospray mechanism as they travel to the mass spectrometer inlet.

analytes from the sample surface. When further primary droplets impact, secondary droplets containing the analyte ions are ejected and ionized through an electrospray-like mechanism. These ions are then sampled by the mass spectrometer using an extended inlet (sniffer). For imaging applications, the sample position relative to the DESI sprayer assembly is adjusted in continuous horizontal movements, while spectra are acquired for each pixel of the resulting image (Figure 8) (Kumar 2023). This makes DESI useful for analyzing low molecular weight compounds, such as neurotransmitters in brain tissues. DESI mass spectrometry technique has been employed to analyze the spatial distribution and concentrations of neurotransmitters in mouse brain tissues (Maciel, Pereira, et al. 2022; Shariatgorji et al. 2021).

Genome sequence and cell-based assays used in selecting psychobiotic candidates

The mental health benefits of psychobiotics are strain-specific, relying on their ability to produce gut-derived neuroactive compounds like GABA, 5HT, or dopamine (Royo et al. 2023; Torres-Maravilla et al. 2022). This was demonstrated by *B. adolescentis* IPLA60004 ability to convert glutamate to GABA in vitro, while *B. adolescentis* LGM10502, could not (Royo et al. 2023). The selection process for capable strains depends on genome sequencing and cell-based assays to identify strains with relevant functional genes and metabolic activity.

Whole-genome sequencing (WGS) has transformed psychobiotic research by enabling the identification of genes encoding enzymes important for neuroactive compound synthesis, offering comprehensive insights into bacterial genetic makeup (Torres-Maravilla et al. 2022). For instance, WGS has identified the glutamate decarboxylase gene, essential for GABA production, in *Lactobacillus* and *Bifidobacterium* species (Gao et al. 2019; Torres-Maravilla et al. 2022). Comparative genomics highlights strain-specific metabolic pathways and regulatory mechanisms, aiding in selecting strains with enhanced neuroactive potential (Yousuf et al. 2024), while advances in next-generation sequencing (NGS) technologies, like Illumina and PacBio, have also enabled high-throughput and precise gene identification (Hu et al. 2021). Metagenomic approaches also, complement WGS by assessing the functionality of microbial communities in the gut, providing a broader context for psychobiotic potential (Karcher et al. 2021; Torres-Maravilla et al. 2022). Cell-based assays and in vitro models have become crucial for validating the functional activity of genes identified through sequencing (Royo et al. 2023). Reporter gene assays for instance, where promoter activity of neuroactive compound genes is linked to luminescent or fluorescent markers, provide rapid and quantitative measurements of gene expression (Li et al. 2017). Polymerase chain reaction (PCR) is another sensitive and specific molecular technique widely used in screening probiotic candidates (Ruiz-Moyano et al. 2008). It allows for the detection of specific genes associated with the production of neuroactive compounds or their precursors. By using gene-specific primers, PCR can amplify target sequences to confirm the presence or absence of key biosynthetic genes. Advanced PCR variants, such as quantitative PCR (qPCR), can also quantify gene expression levels under different conditions, providing insights into the biosynthetic potential of a specific strain (VanGuilder, Vrana, and Freeman 2008).

Conclusions and future prospects

Preclinical and clinical studies support the role of psychobiotics and their metabolites in regulating brain functions through various mechanisms. However, several limitations and gaps in the current literature need to be addressed to advance this field. For instance, while detection of GABA in stool samples after probiotic administration (Duranti et al. 2020) suggests potential psychobiotic activity, this evidence alone is insufficient to confirm direct effects on brain functions. Similarly, findings from in vitro studies demonstrating the ability of certain bacteria to synthesize neuroactive compounds do not guarantee that these compounds will be produced in vivo, as the physiological environments differ significantly. One widely proposed mechanism is the production of neuroactive compounds that may travel to the brain to confer mental health benefits. However, the concentration of these compounds produced in vitro and in vivo varies widely, and the threshold levels required to exert measurable effects in the host remain unclear. Many bacterial strains can produce neuroactive compounds at low concentrations that may have no meaningful impact, underscoring the need to establish effective dosing thresholds. Furthermore, the question of whether microbial-derived tyrosine and tryptophan cross the BBB to contribute to catecholamine synthesis in the brain remains unresolved. Additionally, further studies are required to confirm whether microbial membrane vesicles, such as EVs, indeed transport neuroactive compounds synthesized by gut microbes to the brain. Advances in quantification techniques, such as tracking metabolites from the gut into circulation and monitoring their presence in the brain, are crucial. These methodologies, when applied in tandem, can provide comprehensive insights into the availability, transport, and functional impact of gut-derived neuroactive compounds on brain health.

While cell, tissue, and animal models have provided valuable insights, translating these findings into robust clinical evidence remains a significant challenge. Applying advanced quantification, tracking, and monitoring techniques in human studies will be critical to bridging this gap. Such efforts can help establish the clinical relevance of gut microbial metabolites and their role in modulating brain function and mental health outcomes. Addressing these gaps will be essential for developing targeted therapies and interventions aimed at leveraging the microbiota-gut-brain axis to enhance mental health and neurological well-being.

Author contributions

Vincent Owusu Kyei-Baffour: conceptualization, writing–original draft, writing–review and editing, visualization. Akshay Kumar Vijaya: writing–review and editing, visualization. Aurelijus Burokas: writing–review and editing, supervision. Eric Banan-Mwine Daliri: conceptualization, writing–review and editing, supervision, funding acquisition.

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ORCID

Vincent Owusu Kyei-Baffour http://orcid.org/0000-0001-9107-0693
Akshay Kumar Vijaya http://orcid.org/0009-0000-5106-7983
Aurelijus Burokas http://orcid.org/0000-0002-0364-3496
Eric Banan-Mwine Daliri http://orcid.org/0000-0003-1039-0287

Data availability statement

No data was used for the research described in the article.

Abbreviations

5-HT	5-hydroxytryptophan
AAAD	aromatic amino acid decarboxylases
BBB	blood brain barrier
EcN	*Escherichia coli* Nissle
ELISA	enzyme-linked immunosorbent assay
EVs	extracellular vesicles
GABA	gamma-aminobutyric acid
IMS	imaging mass spectrometry
LC	liquid chromatography
L-DOPA	dihydroxyphenylalanine
MS	mass spectrometry
NMR	nuclear magnetic resonance
SPME	solid phase microextraction
TCA	tricarboxylic acid
TrpD	tryptophan decarboxylase
TrpH	tryptophan hydroxylase
VAMS	volumetric absorptive micro-sampling

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