ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

BIOLOGIA CELLULARE E MOLECOLARE

Ciclo 38

Settore Concorsuale: 05/I2 - MICROBIOLOGIA

Settore Scientifico Disciplinare: BIO/19 - MICROBIOLOGIA

LEVILACTOBACILLUS BREVIS CRAI: A NOVEL GABA-PRODUCING STRAIN FOR NUTRACEUTICAL APPLICATIONS

Presentata da: Asia Pizzi

Coordinatore Dottorato

Anna Maria Porcelli

Supervisore

Beatrice Vitali

Co-supervisore

Francesca Patrignani

Esame finale anno 2026

Funded by the European Union - Next Generation EU, Mission 4, Component 2, Investment 3.3 (Ministerial Decree 117/2023) CUP J33C22001600009

Abstract

Gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid that acts as a major inhibitory neurotransmitter in the central nervous system and plays multiple physiological roles, including mediation of gut–brain axis signalling. Although the precise pathways linking intestinal GABA production to central effects remain incompletely resolved, vagal and enteric nervous system routes have been proposed. GABA is used clinically to alleviate stress and anxiety, yet conventional production methods are costly and environmentally demanding; some lactic acid bacteria (LAB) synthesize GABA spontaneously during fermentation, offering a potentially sustainable production route. The primary objective of this thesis was to isolate and characterize a microbial strain with high GABA-producing efficiency and probiotic potential. Only after confirming its functional properties, including GABA biosynthesis and probiotic traits, the strain was employed in fermentation trials using agrifood byproducts as substrates. This strategy enabled the development of a GABA-enriched nutraceutical while promoting the valorisation of nutrient-rich industrial waste streams. Additionally, an *in vitro* fermentation gut model was employed to evaluate the impact and efficacy of the probiotic strain and the GABA enriched prototype on intestinal functionality, particularly in relation to disorders such as irritable bowel syndrome (IBS), where reduced GABA levels are associated with symptom severity and depression. Thirteen LAB isolates were obtained from organic tomato and identified by 16S rRNA sequencing, PCR screening was targeted to *gadB*, the gene encoding for glutamate decarboxylase. Strains tested positive were cultivated under optimized conditions (4% monosodium glutamate - MSG, initial pH ≈5.0, 10% inoculum, 48 h at 32°C) and assayed for GABA production by HPLC. The best producer was *Levilactobacillus brevis* CRAI (GABA 179 mM and 75% yield); whole genome sequencing showed the absence of virulence and antibiotic resistance genes. Genomic analysis revealed two GABA related genes, *gadA* within the *gad* operon, and *gadB* outside the operon; transcriptional analysis showed that MSG supplementation induced a ~52fold and ~4-fold upregulation of *gadA* and *gadB,* respectively, correlating with enhanced GABA synthesis. *L. brevis* CRAI was functionally characterized and exhibited potent antimicrobial activity against enteropathogens, anti-inflammatory effect, and efficient adhesion to intestinal Caco2 cells. Fermentation trials on several fruit and vegetable byproducts identified tomato pomace as the most supportive matrix for *L. brevis* CRAI growth and GABA production. Central composite design (CCD) optimization (2% MSG, 1% yeast extract, matrix pH 5.08) led to the production of 93 mM GABA with 79% conversion in the laboratory matrix. Volatile profiling identified organic acids and alcohols conferring fruity

aromatic notes to the fermented product. Scale-up using a standardized dehydrated tomato substrate supplied by our industry partner DEPOFARMA under the previously determined optimal conditions, yielded 80 mM GABA with a 67% conversion efficiency. The fermented product was freeze-dried to develop the GABA-enriched nutraceutical prototype. Functional testing in an *in vitro* fermentation gut model simulating healthy and dysbiosis conditions (irritable bowel syndrome, IBS) compared the *L. brevis* CRAI, the GABA-enriched prototype, and the mixture composed by *L. brevis* CRAI + MSG + dehydrated tomato. All treatments increased the production of short chain fatty acids (acetate, propionate, butyrate), metabolites implicated in intestinal homeostasis with anti-inflammatory property. The mixture supported de novo GABA production, while the prototype maintained stable GABA levels over time, an outcome of potential relevance for IBS management. Microbiome analyses confirmed baseline dysbiosis in IBS samples (reduced alpha diversity and elevated *Ruminococcus*); treatments partially mitigated dysbiosis by increasing beneficial taxa, such as *Collinsella,* and preserving *Lachnospiraceae* and *Oscillospiraceae*, and the prototype notably increased *Bifidobacteriaceae* in IBS conditions. Regulatory assessment determined that the probiotic strain and the related prototype do not qualify as novel food given the documented use of *L. brevis* and its fermented products prior to 1997. The *L. brevis* CRAI strain was patented for its GABA producing capacity and probiotic properties (Patent No. 31.D1066.12.IT.4). Overall, this work demonstrates that *L. brevis* CRAI is a safe and effective GABA producer with strong probiotic properties; biotechnological fermentation on a valorised tomato matrix enables scalable, cost and resource efficient GABA production. The viable strain and the resulting GABA-enriched nutraceutical prototype modulate gut microbial composition and metabolite profiles *in vitro* in ways that support the translational potential of the novel probiotic for targeting gut–brain axis dysfunction in IBS.

Chapter 1

1. GABA and the gut-brain axis

1.1. γ-aminobutyric acid and its role in the central nervous system

Gamma-aminobutyric acid (GABA) was initially discovered in plants [1] and later in the mammalian brain by Eugene Roberts and Sam Frankel in the 1950 [2]. GABA is a non-protein amino acid and its biosynthesis pathway, known as the "GABA shunt," begins with the production of glutamate from α-ketoglutarate through a transamination reaction catalysed by glutamate dehydrogenase. Subsequently, glutamate undergoes decarboxylation to form GABA by glutamate decarboxylase (GAD) enzyme. This step is irreversible and involves the consumption of a proton and the release of CO_2 [3]. GAD genes play a crucial role in the brain, their expression is regulated at transcriptional and post-translational levels, and they play a key role in maintaining the balance between glutamate and GABA [4]. GABA is predominantly located in the brain, where it explains its function as one of the most important inhibitory neurotransmitters. Upon its synthesis in the neurons, GABA is stored in membrane-bound vesicles at the terminal part of the axon, that is known to be the vesicular compartment. Each vesicle contains thousands of GABA molecules (Figure 1) and upon release, these vesicles fuse with the neuronal membrane and GABA is released via exocytosis. At this point after being released, GABA diffuses across the synaptic cleft to bind to receptors located in the post-synaptic neurons. GABA exerts the inhibition of neural activity by binding to specific receptors, this binding is responsible for the opening of ion channels, allowing potassium ions to flow out of the cell and chloride ions to flow in [5]. This ion exchange results in a negative shift in the transmembrane potential, causing hyperpolarization and a reduction in neuronal excitability (Figure 1). GABA interacts with various receptor subtypes, some of which are in the endocrine system and peripheral tissues, where GABA also plays a role in oxidative metabolism. The GABA receptors are classified into types A, B, and C, which are located on the post-synaptic membrane. GABA-A and GABA-C receptors are ligand-gated ion channels, while GABA-B receptors are coupled with G proteins. GABA-A receptors mediate fast synaptic responses, whereas GABA-B receptors are involved in slower synaptic transmission, while the role of GABA-C receptors remains less well defined [6]. GABA that does not bind to receptors is either degraded by enzymes or actively reabsorbed into the presynaptic terminal by GABA transporters (GAT1 or GAT3). This reuptake is the first mechanism that stops synaptic

inhibitory neurotransmission and thus plays a crucial role in maintaining the balance between excitation and inhibition in the nervous system [7].

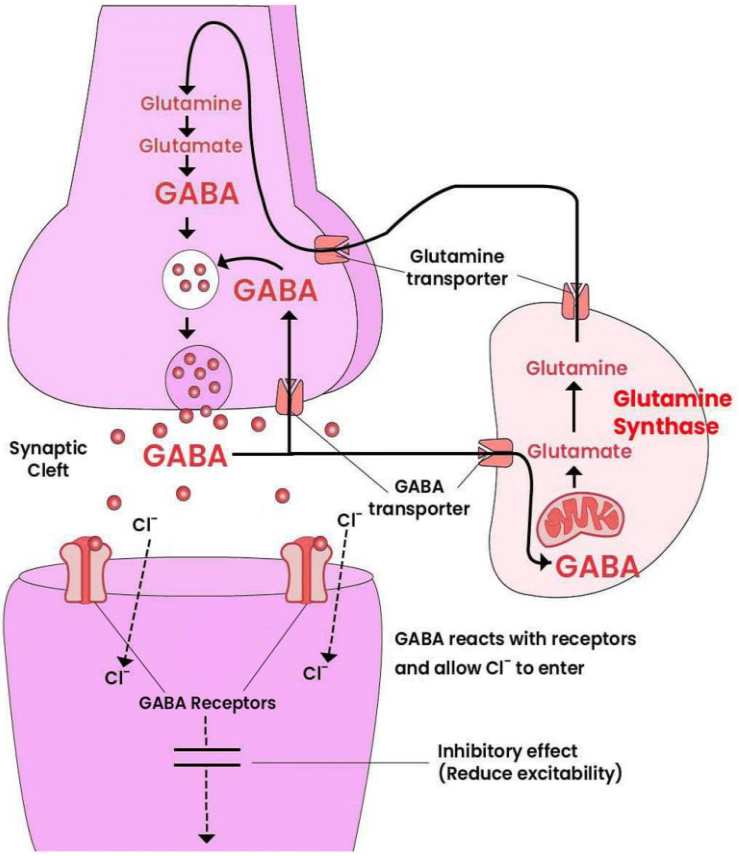


Figure 1. Mechanism of action of GABA in the neurons [7].

1.2. Neuromicrobiology: the gut microbiota as a modulator of neurotransmission

In recent years, the gut microbiome has emerged as a key player in human health. Increasingly, it is being studied in its entirety, including microbial structure, metabolite production, mobile genetic elements, and the diverse microbial communities that coexist and interact within the same ecological niche [8]. This shift in focus has led to a redefinition of the microbiome's role, which is no longer considered limited to metabolic functions such as digesting nutrients, producing short-chain fatty acids (SCFAs), or maintaining intestinal barrier integrity. Instead, it is now recognized as a central regulator of human physiology and health [9]. A growing body of evidence supports the emergence of a new interdisciplinary field known as neuromicrobiology, which investigates the complex bidirectional interactions between the gut microbiome and central nervous system (CNS) [10]. This line of research highlights the importance of neurometabolic communication pathways and reveals how microbial

populations can influence brain function and behaviour [11]. Understanding these mechanisms holds significant implications for both mental and neurological health. Indeed, numerous studies have demonstrated that alterations in the gut microbiome, commonly referred to as dysbiosis, are associated with various psychiatric and neurological disorders, including depression, anxiety, autism spectrum disorders, and neurodegenerative diseases [12]. Central to this discussion is the concept of the gut-brain axis (GBA), a bidirectional communication system linking the gastrointestinal tract (GIT) and the brain. This system plays a pivotal role in several physiological processes, including brain development, cognitive function, emotional regulation, mental state, and overall cerebral activity. Communication along the GBA occurs through multiple, interconnected pathways involving the nervous system (particularly the vagus nerve and the enteric nervous system, ENS), the immune system, and the endocrine system. The activity of the GBA can be modulated in two primary directions: top-down communication, where the brain influences the gut microbiota; bottom-up communication, where the gut microbiota sends signals to the brain [13]. In top-down regulation, the central nervous system modulates the composition and function of the gut microbiome primarily via the hypothalamic–pituitary–adrenal (HPA) axis. Activation of the HPA axis leads to the release of cortisol, a glucocorticoid hormone that acts on immune cells both locally (in the gut) and systemically. Cortisol also affects intestinal barrier function, modulating permeability, and consequently, microbiota composition [14]. Conversely, in bottom-up signalling, the gut microbiota can influence brain function through several mechanisms: activation of immune responses, leading to the production of cytokines; microbial-derived neuroactive metabolites, including neurotransmitters or their precursors; direct stimulation of the vagus nerve and the ENS. One key example involves tryptophan, an essential amino acid and precursor of serotonin. Both host metabolism and microbiota contribute to systemic tryptophan levels, and it is well known that certain microbial species can produce or modulate tryptophan, which in turn can activate the vagus nerve, allowing signals to reach the brain [15]. Recent studies have provided robust evidence for the ability of gut microbiota to produce neuroactive compounds, this highlights the critical role of the neuroendocrine system in mediating microbial–neural interactions [16], reinforcing the notion that the gut microbiome is an integral component of the gut-brain axis and a potential target for therapeutic intervention in neurological and psychiatric disorders.

It has been widely demonstrated that the gut microbiota plays a crucial role in the synthesis of several neurotransmitters, including GABA, serotonin, dopamine, norepinephrine, and other

microbial-derived neuroactive metabolites that exert significant influence on brain function [12]. These bioactive compounds are not exclusively synthesized by the CNS, rather, they are also produced by enteroendocrine cells (EECs), a specialized subset of intestinal epithelial cells capable of producing neurotransmitters in response to intestinal peptides and signals from the gut microbiota itself [17]. Although EECs comprise only approximately 1% of the intestinal epithelial cell population, they play a fundamental role in the gut-brain axis [18]. EECs detect the presence of luminal nutrients, such as carbohydrates, triglycerides, and proteins, and respond by secreting a variety of peptides and neurotransmitters that regulate gut motility, intestinal secretion, and food intake.

Dopamine is a key neuromodulator that plays a crucial role not only in CNS but also in the peripheral nervous system (Dopamine receptors: from structure to function). It is involved in fundamental processes such as learning and memory, pain modulation, and behavioral regulation. Dysregulation of dopaminergic signaling is a hallmark of several neurological and neuropsychiatric disorders, including Parkinson's disease (PD), schizophrenia, and autism spectrum disorders [19]. In addition to its central functions, dopamine exerts important regulatory actions within the gastrointestinal tract. Through the enteric nervous system, dopamine contributes to the maintenance of intestinal mucosal integrity and regulates gut motility, secretion, absorption, and other essential physiological processes, underscoring its role in gut–brain communication. Dopamine biosynthesis occurs via the phenylalanine–tyrosine–L-DOPA–dopamine pathway, which is active both in the brain and in peripheral tissues, including the intestine. Phenylalanine, an essential dietary amino acid, is converted into tyrosine primarily in the liver and subsequently released into circulation. Tyrosine is then converted into L-DOPA by tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis and a widely used marker of dopaminergic function. Both tyrosine and L-DOPA can cross the blood–brain barrier; however, L-DOPA is rapidly decarboxylated to dopamine in the small intestine due to its limited stability [19]. Probiotic-based modulation of the gut microbiota has been shown to exert neuroprotective effects in experimental models of Parkinson's disease. In particular, treatment of Parkinsonian mice with *Clostridium*-based probiotics increases the relative abundance of bacterial taxa such as *Akkermansia*, *Enterorhabdus*, and *Helicobacter*, thereby preventing the loss of tyrosine hydroxylase (TH) expression in dopaminergic neurons. This microbial shift is associated with an improvement in motor impairments and synaptic dysfunctions that are characteristic of Parkinson's disease [20]. Beyond its indirect effects on host neurobiology, the gut microbiota is also capable of directly contributing to dopamine

biosynthesis. Evidence indicates that berberine administration enhances intestinal L-DOPA and dopamine production by activating bacterial tyrosine hydroxylase and DOPA decarboxylase in dopamine-producing species, including *Enterococcus faecalis* and *Enterococcus faecium*. This microbiota-driven activation leads to increased dopamine availability in the brain and contributes to the amelioration of Parkinsonian phenotypes [21]. In addition to influencing dopamine synthesis, the gut microbiota plays a role in dopamine metabolism and signaling regulation. Elevated fecal levels of *Bacteroides uniformis* have been significantly correlated with increased expression of dopamine transporters in the brain, which are responsible for dopamine reuptake and synaptic clearance, suggesting that microbial composition can modulate synaptic dopamine homeostasis [22]. The gut microbiota also influences reward-related behaviors, including feeding, drinking, and addictive behaviors, through its impact on dopaminergic receptor signaling. In a model of compulsive alcohol-seeking behavior, an imbalance in striatal dopamine receptor expression has been observed, characterized by increased dopamine D1 receptor expression relative to dopamine D2 receptors in the dorsal striatum. Notably, D2 receptor mRNA levels were positively correlated with a reduced relative abundance of Firmicutes, whereas D1 receptor expression was negatively associated with the genera *Veillonella* and *Gemella*, members of the Ruminococcaceae family. Together, these findings further support a mechanistic link between gut microbial composition and dopaminergic signalling pathways underlying compulsive and addictive behaviours [23]

Norepinephrine exhibits structural similarity to epinephrine and is synthesized during states of arousal; it is also implicated in behavioural regulation and cognitive processes, including memory formation, learning, and attentional mechanisms. Furthermore, it participates in inflammatory pathways and modulates autonomic nervous system responses [24]. Norepinephrine shares functional attributes with autoinducer 3, a quorum-sensing molecule that enhances virulence and motility in Escherichia coli. Specific bacterial species, such as Bacillus mycoides, Bacillus subtilis, E. coli K12, Proteus vulgaris, and Serratia marcescens, produce norepinephrine at concentrations ranging from 0.045 to 2.13 mM, with evidence indicating its utilization in quorum-sensing processes [25]. Although direct modulation of norepinephrine by the human gut microbiota in vivo has not been definitively established, accumulating data suggest its involvement in host biosynthesis and catabolism. For instance, a recent investigation in germ-free mice revealed diminished norepinephrine levels in the cecal lumen and tissues compared to conventionally raised controls; these deficits were ameliorated following administration of a consortium comprising 46 Clostridium species [26]. This work highlights

the microbiota's capacity to influence luminal norepinephrine concentrations, raising questions about whether bacteria directly synthesize the catecholamine or indirectly regulate its host-derived production.

Serotonin, also referred to as 5-hydroxytryptamine (5-HT), plays a pivotal role in behavioral regulation and gastrointestinal physiology, exhibiting a close association with the intestinal microbiota that carries profound implications for the gut-brain axis. This biogenic amine contributes to the orchestration of diverse physiological processes, encompassing gastrointestinal secretion and peristalsis, respiratory control, vasoconstriction, behavioural modulation, and neural signalling [27]. The homeostasis of 5-HT levels is governed by the enzymes tryptophan hydroxylase 1 (TPH1) and TPH2 [28]. Although ENS neurons synthesize 5-HT, over 90% of bodily 5-HT is generated peripherally in the gastrointestinal tract by enterochromaffin cells (ECCs) [29]. Although various bacterial strains have been documented to synthesize serotonin, such biosynthetic capacity has not been observed within the resident gut microbiota. In germ-free models, circulating and colonic serotonin concentrations are markedly diminished relative to conventionally housed counterparts, a deficit that can be rectified through microbial recolonization or introduction of a spore-forming bacterial consortium [30]. Multiple investigations have substantiated these observations [31], providing robust evidence that host 5-HT production is modulated by microbial influences rather than direct bacterial synthesis. Instead, perturbations in serotonin homeostasis are primarily driven by microbial-derived small molecules, such as short-chain fatty acids (SCFAs) or secondary bile acids—that activate enterochromaffin cells to upregulate TPH expression and thereby enhance serotonin biosynthesis. Reigstad et al. demonstrated that sodium acetate at concentrations of 10–50 mM substantially elevated TPH1 mRNA levels in a human enterochromaffin cell line (BON cells). Similarly, butyrate at 0.5 and 1.0 mM induced over threefold increases in TPH1 expression; however, at 2.0 mM, butyrate elicited no significant change. Conversely, higher butyrate doses (8.0 and 16.0 mM) profoundly downregulated TPH1 expression by 13.5- and 15.7-fold, respectively, falling below baseline levels in untreated cells. These results underscore the stimulatory effect of gut microbiota-derived SCFAs on enterochromaffin cells, thereby promoting 5-HT production [32]. Dysregulation of 5-HT signaling is also implicated in inflammatory bowel disease (IBD). In cohorts with ulcerative colitis (UC) and Crohn’s disease (CD), colonic biopsies reveal a substantial proliferation of serotonin-immunoreactive cells [33]. A contemporary analysis posits that certain neurotransmitters may function as metabolic substrates for gut bacteria, with elevated 5-HT

potentially fostering microbial growth. Excessive 5-HT may reinforce gut epithelial barrier integrity by reducing permeability, whereas deficient levels suppress occludin expression, compromising the mucosal barrier and predisposing to heightened permeability and "leaky gut" syndrome [34].

Histamine is synthesized from the amino acid L-histidine via oxidative decarboxylation mediated by histidine decarboxylase, an enzyme present in numerous mammalian cell types [35]. These enzymes are predominantly expressed in mast cells and basophils, though they can also be detected in lymph nodes, the thymus, and gastrointestinal chromaffin cells [36]. Histamine serves as a key modulator of immune responses, including those involved in allergies and inflammation; it additionally influences gastrointestinal tract motility, enhances intestinal mucosal permeability, and affects mucosal ion secretion. Histamine metabolism primarily relies on histamine-N-methyltransferase (HNMT) and diamine oxidase (DAO) [35]. Furthermore, histamine is produced by neurons in the posterior hypothalamus, with axons projecting throughout the brain to function as a neurotransmitter. In addition to host cells, histamine can be generated by certain microbial strains, such as *Escherichia coli* and *Morganella morganii*. Notably, some bacteria regulate histamine production by expressing histidine decarboxylase (HDC), which converts histidine to histamine [37]. Recent proposals suggest that histamine receptor agonists used in allergy treatment may also alleviate visceral hypersensitivity, immune activation, and symptoms in patients with irritable bowel syndrome (IBS) [38]. Certain investigations indicate that histamine-intolerant individuals exhibit reduced α-diversity in the gut microbiota, along with altered abundances of *Proteus* and *Bifidobacterium* species. Histamine engages its G protein-coupled receptors, which feature seven transmembrane domains, including H1, H2, H3, and H4; these are expressed on both presynaptic and postsynaptic neuronal membranes. Research has identified H1 and H4 as the primary histamine receptors implicated in gastrointestinal processes, whereas H2 is linked to gastric acid secretion [39].

IBS patients who respond favorably to H1 antagonists differ from non-responders, indicating potential hyperstimulation of the histaminergic system in IBS [40]. Emerging evidence shows that histamine enhances sensitivity of mouse dorsal root ganglion neurons and human rectal submucosal neurons to TRPV1 via activation of the H1 receptor (HRH1). Moreover, supernatant from IBS biopsy tissue similarly potentiated dorsal root ganglion neuron sensitization in mice through HRH1. Given their upregulated expression in the colonic mucosa

of colitis patients, H1 and H4 receptors may play a significant role in colitis pathogenesis and visceral hypersensitivity.

The findings described above collectively indicate a strong association between a healthy gut microbiome architecture and balanced neurotransmitter levels in the host. Consistent with this relationship, substantial evidence demonstrates that alterations in gut microbial communities markedly influence neurotransmitter synthesis, as observed in both germ-free and antibiotic-treated mouse models. Multiple studies have reported significant changes in fecal and serum concentrations of neurotransmitters, including γ-aminobutyric acid (GABA), serotonin, and acetylcholine, as well as their metabolic precursors such as tryptophan and choline, in the absence of microbial colonization (i.e., germ-free mice) [41]. Similarly, antibiotic-induced depletion of the gut microbiota results in altered levels of neurotransmitters and their precursors in both intestinal contents and systemic circulation [42]. Notably, shifts in intestinal microbial abundance have also been shown to modulate the expression of neurotransmitter receptors in the brain [41].

Importantly, alterations in dietary patterns and environmental conditions can drive changes in gut microbial composition, thereby affecting neurotransmitter biosynthesis. For instance, to cope with limited food availability, certain animals such as voles engage in coprophagic behavior to fulfill their nutritional requirements. However, voles deprived of fecal consumption exhibit reduced intestinal microbiome alpha diversity, altered relative abundance of key bacterial taxa (including Firmicutes and Bacteroidetes), decreased cecal concentrations of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate, and reduced levels of tyrosine hydroxylase and neurotransmitters, including dopamine and serotonin, in the hippocampus and hypothalamus [43]. Additionally, other environmental factors, including high-fat or carbohydrate-rich diets and temperature fluctuations, significantly influence gut microbial composition and contribute to changes in neurotransmitter levels in feces, blood, and the central nervous system [44,45].

This multilayered system, encompassing enteroendocrine signalling, immune modulation, and microbial activity, is essential for maintaining gut microbiota homeostasis and overall physiological balance. Looking forward, probiotic-based vagal nerve stimulation represents a promising therapeutic avenue for neurological and psychiatric conditions, including depression, anxiety, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS).

The potential to modulate brain function through targeted microbiome interventions opens new perspectives in the field of psychobiotics and microbiota-driven neuromodulation [13].

1.3. Gut microbiota: composition, function and the impact on dysbiosis

The gut microbiota represents a highly complex and diverse community of microorganisms residing within the human GIT. Among the various microbial niches found in the human body, the gut harbours both the highest number and the greatest diversity of microbial species. This ecosystem comprises approximately 10^{13} microbial cells, and minimal estimates suggest that an individual harbors between 2,200 and 3,600 distinct bacterial species over an eight-month period [46]. The gut microbiota is a dynamic system that evolves throughout the human lifespan, indeed the relationship between the host and its gut microbiota is mutualistic and commensal, contributing significantly to host physiology and health. Several intrinsic and extrinsic factors influence the composition and function of this microbial community, including host genetics, diet, age, mode of delivery at birth, and exposure to antibiotics [47]. The human gut microbiota plays a crucial role in host health, and diet is one of the most significant modulators of its composition and function. The microbial metagenome, in the early stage of life, is enriched in genes responsible for the digestion of oligosaccharide present in breast milk, and solid foods are introduced during weaning, there is a notable shift toward microbial genes involved in the metabolism of complex polysaccharides and vitamins [48].

Aging is another critical factor that has an impact on gut microbiota composition. Initial colonization begins at birth and is strongly influenced by the mode of delivery: vaginal or caesarean. Immediately after birth, the gut is colonized by oxygen-tolerant bacteria, primarily belonging to the phylum *Proteobacteria*, at this point these bacteria reduce oxygen levels in the gut environment, creating favourable conditions for the expansion of strict anaerobes such as *Firmicutes* and *Bacteriodetes* [49]. In preadolescents, the gut microbiome is characterized by functional profiles potentially involved in developmental processes, including the biosynthesis of folate and vitamin B12. At this stage, the abundance of *Bifidobacteria* and *Clostridium* genera belonging to *Actinobacteria* and *Firmicutes* phylum is significantly higher compared to that observed in adults. In contrast, the gut microbiota of the elderly is often marked by a decline in *Firmicutes* and *Bacteriodetes*. This shift may be attributed to reduced dietary diversity and a rise in systemic inflammatory factors commonly associated with aging [50].

Different regions of the gastrointestinal tract are colonized by distinct microbial populations, in fact the colon contains the highest density and diversity of microbial species, while the stomach and small intestine are colonized by fewer and more specialized microbes. Notably, approximately 99% of the bacterial population in the gut consists of anaerobic organisms, adapted to the low-oxygen environment of the distal gut [51]. The gut microbiota performs a wide range of essential functions within the human body including the fermentation of non-digestible carbohydrates such as cellulose, hemicellulose, pectin, oligosaccharides, and lignin into short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate. These metabolites are predominantly produced by anaerobic microorganisms, particularly members of the *Firmicutes* and *Bacteroidetes* phyla [52]. In addition to its role in energy metabolism, the gut microbiota exerts beneficial effects on the host because contributes to the synthesis of essential micronutrients, including biotin, thiamine, cobalamin, and riboflavin, these microbial-derived vitamins play a key role in maintaining metabolic homeostasis and supporting overall host health [53]. Gut microbiota contributes to host defense by occupying mucosal surfaces, thereby preventing the colonization of pathogenic microorganisms through two mechanisms: the production of antimicrobial compounds on one hand and the strengthen of the mucosal barrier on the other hand; in this way, it plays a critical role in the development and modulation of the immune system [54]. Beyond its local functions within the gastrointestinal tract, the microbiota exerts effects also on distant organs, in fact acts as an endocrine-like organ because modulates satiety signalling, influences hormonal secretion, and has been shown to impact mood and behaviour. Importantly, the gut microbiota plays a central role in the gut-brain axis, affecting neural, endocrine, and immune pathways that influence host neurological function [55].

A complex symbiotic relationship exists between the human body and its gut microbiota, and disruption of this balance can have profound effects on both. The disruption of this equilibrium leads to a state known as dysbiosis, characterized by an altered composition of the microbial community, which has been linked to the onset and progression of various diseases. Indeed, the presence or absence of specific bacterial species has been associated with a range of pathological conditions [56]. Gut microbiota dysbiosis has implications not only in gastrointestinal disorders, but also in systemic diseases. The gastrointestinal tract dysbiosis is strongly associated with conditions such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and colorectal cancer (CRC). Studies conducted on IBD mouse models have shown a gut microbiota enriched in *Bacteroides* species such as *Sutterella* when compared to

healthy controls. Other taxa, including *Peptostreptococcaceae* and *Enterobacteriaceae*, have been identified in patients with ulcerative colitis, while Crohn's disease (CD) is characterized by reduction in microbial diversity while genera such as *Faecalibacterium* and *Methanobrevibacter* increase [57]. Intestinal microbiota dysbiosis also affects the gut–brain axis, with growing evidence supporting its involvement in various neurological and psychiatric disorders. These include stress-related conditions such as anxiety and depression, as well as neurodevelopmental and neurodegenerative diseases such as Alzheimer's and Parkinson's disease. For instance, an increased abundance of pro-inflammatory bacterial species, including *Enterobacteriaceae* and *Desulfovibrio*, has been observed in patients with anxiety and depressive disorders and recently has been also reported a reduction in short-chain fatty acid (SCFA)-producing bacteria, such as *Faecalibacterium* [58]. Recent studies in patient affected by Alzheimer's disease (AD), have highlighted the role of the gut microbiome in amyloid deposition. The analyses of faecal sample from individuals with Alzheimer's disease (AD), revealed a marked decrease in microbial diversity, with a reduced presence of *Firmicutes* and *Bifidobacteria*, alongside an increased abundance of *Bacteroides*. These microbial alterations underline the strong connection between gut dysbiosis and the pathophysiology of neurodegenerative disorders [59].

Given the growing recognition of the gut microbiome's pivotal role in maintaining systemic health and its association with a range of inflammatory and neurodegenerative diseases, several therapeutic strategies have increasingly focused on the use of probiotics. Probiotics are defined as live microorganisms which, when administered in adequate amounts, confer health benefits to the host [60]. Their influence on human health can be exerted directly and indirectly, through the modulation of the gut microbiota composition and, consequently, its metabolic output, including the production of bioactive compounds. Probiotics are non-pathogenic microbial strains that exert a range of beneficial effects, including immune system support, modulation of blood pressure and cholesterol levels, and potential preventive roles in cancer and gastrointestinal disorders [61]. These microorganisms can be introduced through dietary supplements or via fermented foods, which naturally contain microbial populations, in fact the consumption of such fermented products is thus considered a viable strategy to promote gut and overall health [62].

1.3.1. Irritable Bowel Syndrome: a functional gastrointestinal disorder from gut-brain axis to microbial imbalance

Irritable Bowel Syndrome (IBS) is a chronic functional gastrointestinal disorder characterized by recurrent abdominal pain and altered bowel habits in the absence of identifiable organic pathology. The condition significantly impairs quality of life. IBS predominantly affects women and is frequently associated with other pathologies, contributing to a substantial socioeconomic burden [63]. The global prevalence of IBS varies considerably across countries, likely due to differences in dietary habits, cultural factors, and diagnostic criteria. While the exact ethology remains unclear, increasing evidence implicates disruptions in the microbiota-gut-brain axis as central to IBS pathogenesis [64].

Alterations in gut microbiota composition, known as dysbiosis, appear to trigger immune responses that exacerbate gastrointestinal symptoms. This has led to the conceptualization of IBS as both a microbiome disorder and a gut-brain axis dysfunction. However, the directionality of the gut-brain interaction remains under debate. It remains unclear whether alterations in the central nervous system led to imbalances in the gut microbiota, or whether changes in the microbiota are the primary event, influencing the brain through pathways such as the vagus nerve. There is also evidence of clinical overlap between IBS and Inflammatory Bowel Disease (IBD), despite distinct biomarkers being used to differentiate the two. Patients exhibiting features of both conditions tend to present with persistent abdominal pain and diarrhea, and are more susceptible to post-infectious IBS, typically following enteric infections [65]. From a symptomatic perspective, abdominal pain and disrupted bowel function, including diarrhea, constipation, or alternating patterns, are the hallmarks of IBS. These symptoms are closely linked to visceral hypersensitivity, abnormal gastrointestinal motility, and dysregulation of the enteric nervous system. In IBS, significant alterations have been observed in key functions of the large intestine, which are closely associated with changes in microbial diversity, immune responses, barrier integrity, and gut-brain signalling. On the microbial level, significant alterations in fecal microbiota composition have been documented in IBS patients. Notably, reduced abundance of beneficial taxa such as *Bifidobacterium*, *Lactobacillus*, *Collinsella*, and *Escherichia coli* has been observed, alongside an increase in potentially pro-inflammatory or opportunistic genera like *Enterobacteriaceae*, anaerobic bacteria, and *Ruminococcus gnavus*. These microbial imbalances may contribute not only to gastrointestinal symptoms, but also to

broader neurological and behavioural changes. In conclusion there is a decrease in microbial diversity, an increase of pathogenic species and a decrease of probiotic species [66].

In healthy individuals, the presence of inflammatory cells within the lamina propria that colonize the mucosa, alongside the preservation of epithelial integrity, reflects a mutually beneficial relationship between the host and its microbiota. In irritable bowel syndrome (IBS), however, a low-grade mucosal inflammation is observed, which contributes to visceral hypersensitivity as well as epithelial and neuromuscular dysfunction, ultimately leading to altered gut motility [67]. The immune system expresses a series of pattern recognition receptors capable of distinguishing pathogenic microbes from harmless commensals. Among these, toll-like receptors (TLRs) are found on immune cells, enteroendocrine cells, and neurons of the enteric nervous system (ENS). These receptors are known to promote epithelial cell proliferation and enhance barrier function by upregulating the expression and assembly of tight junction proteins and the zonula occludens. Recent studies have demonstrated that in IBS, there is an upregulation and increased activation of TLRs, along with enhanced epithelial permeability as indicated by altered expression of tight junction components. This increased permeability is thought to contribute to the chronic inflammatory state [68]. This inflammatory state may further influence the CNS through the circulation of pro-inflammatory cytokines. In addition to TLRs, muscularis macrophages, immune cells distributed within the various layers of the intestinal wall, also play a key role. These macrophages are involved in the regulation of gut motility through the secretion of cytokines and other inflammatory mediators [69]. Altogether, these findings suggest that immune system activation is one of the factors that contribute to the pathogenesis of IBS. Moreover, psychological factors have been identified as significant contributors to immune activation, further highlighting the complex interplay between the gut and brain in functional gastrointestinal disorders [70].

Furthermore, elevated baseline levels of anxiety and depression are significantly associated with the development and severity of IBS symptoms, further supporting the gut-brain connection. Neuroimaging studies have shown that rectal stimulation activates multiple brain regions, including the anterior cingulate cortex, prefrontal cortex, insula, thalamus, and cerebellum. These activations are more pronounced in IBS patients, indicating a heightened pain processing response [71].

Diet plays a crucial role in the manifestation and modulation of symptoms in IBS, indeed between 64% and 89% of the patients report that specific foods trigger symptoms such as

abdominal pain and bloating. Fermentable Oligosaccharides, Disaccharides, Monosaccharides And Polyols (FODMAPs), that are fermentable carbohydrates found in many common foods, have been identified as key dietary contributors. A low-FODMAP diet has shown significant efficacy in reducing symptoms and improving quality of life in up to 70% of patients [72]. However, this diet requires professional supervision due to the risk of nutritional deficiencies and excessive dietary restriction, in addition to the fact that it has been associated with a reduction in beneficial gut bacteria, including Bifidobacteria. In IBS patients, high-FODMAP intake increases gas production and microbial imbalance, while healthy individuals appear unaffected, probably because the individual microbiota composition seems to influence response to the diet [73]. More research is needed to compare its effectiveness with other dietary strategies. Variability in FODMAP content across foods and regions complicates dietary standardization.

During visceral inflammation, cytokines and other neurotransmitters mediate pain signals to the central pathways in the spinal cord where increased nociceptive activity and central sensitization are translated into the experience of pain. GABA, particularly through GABA-B receptors, plays a role in modulating visceral pain and gastrointestinal (GI) functions like motility and sensation. GABA-B receptor agonists act as spinal anti-nociceptive agents, may also act on peripheral receptors. They can modulate responses of vagal afferents innervating the GI tract. GABA-B receptor agonists, such as baclofen, act as anti-nociceptive agents by inhibiting substance P release and modulating vagal responses, but their clinical use is limited because of the CNS side effects. GABA analogs like gabapentin and pregabalin have shown promising results in the treatment of visceral hypersensitivity in preclinical models [74]. The gut microbiota, especially certain *Lactobacillus* and *Bifidobacterium* strains can produce GABA, representing a potential therapeutic approach for alleviating IBS symptoms. However, the clinical impact of this strategy remains under investigation [75]. New therapeutic targets for IBS may include GABA-B modulators and other endogenous systems like cannabinoid, opioid, and neurokinin receptors.

1.4. The complexity of GABA's role in gut-brain axis

Recent studies highlight the correlation between the gut microbiota and the brain, supporting the hypothesis of the existence of the microbiota-gut-brain axis [76–78], and disruption of this axis has been implicated in the pathophysiology of neurodegenerative diseases [79]. The bidirectional communication between the GIT, including the resident microbiota, and the brain

defines the microbiota-gut-brain axis, although the exact mechanisms underlying this interaction remain poorly understood [80]. One of the method used to demonstrate the interaction between the gut and the brain, has relied on germ-free animal models, in fact, Sudo N. et al (2004) showed that the germ-free mice exhibit altered behaviour, including an increased stress response, this impairment can be reversed upon reintroducing a healthy microbiota [81]. Other studies conducted on mice suggest that fecal microbiota transplantation from aged mice to younger ones results in cognitive impairments and in a reduced expression of neurotrophic factors in brain, these cognitive deficits are completely reversed when the authors reintroduce the healthy microbiota in the mice [82]. Beyond germ-free animal models used to investigate the relationship between the central nervous system and the gut microbiota, clinical follow-up trials have also been conducted in humans. Indeed, recent studies have shown that diet-induced modulation of the gut microbiota is associated with effects on the central nervous system, influencing not only cognitive functions but also stress- and anxiety-related outcomes [44,45]. These studies confirm and establish the correlation between gut microbiome and the nervous system, but still the exact mechanisms of this correlation remain unclear. There are some hypotheses about the pathways involved, such as the hypothalamic-pituitary-adrenal axis, vagal nerve stimulation [83], or the ability of gut microbiota to modulate neurotransmitters through their biosynthesis (Figure 2) [84]. Another key player in this interaction is the enteric nervous system (ENS) that is a complex network of neurons and glial cells embedded in the walls of the gastrointestinal (GI) tract. The ENS reach the gut microbiota through 100 to 500 million neurons, these neurons are located in the myenteric and submucosal plexuses along the GI tract, from the oesophagus to the anus [85]. The ENS is composed of independent sensory and motor neurons that regulate intestinal muscle activity, gut wall motility, fluid secretion, mucosal blood flow, and barrier function. In the frame of this complexity of interactions the vagus nerve serves as a bridge that connects the brain and the ENS [83]. The afferent fibres of the ENS are distributed throughout the intestinal wall but do not traverse the epithelial layer, meaning they are not in direct contact with the microbiota residing in the gut [86]. However, they can indirectly sense signals from the microbiota, leading to changes in electrical activity in the brain regions through the vagus nerve [86]. Compounds and metabolites from the microbiota are detected by other cells in the epithelium, such as endocrine cells, which interact with the vagal afferents, creating a gut chemiosensing system [87]. Notably, GABA receptors are widely distributed along the ENS, in fact GABA plays a role in regulating intestinal motility through both GABA-A ionotropic and GABA-B metabotropic receptor activation [88]. It is well established that gut-resident bacteria are capable to produce GABA, indeed studies have shown

that germ-free mice exhibit significantly reduced peripheral GABA levels compared to the healthy colonized control [84]. Moreover, the ability of the microbiota to mediate improvements through GABA production has been widely demonstrated. Such as in the study of Janik R. et al., 2016 the author treats the BALB/c mice with strains belonging to the *Lactobacillus* genus that rises GABA levels in the hippocampus and prefrontal cortex, and this increase result in a reduction of behaviours associated to anxiety and depression symptoms [89]. Furthermore, it has been seen that certain microorganisms are also capable of modulating the expression of GABA receptors in the brain, resulting in reduced anxiety and depressive symptoms [90].

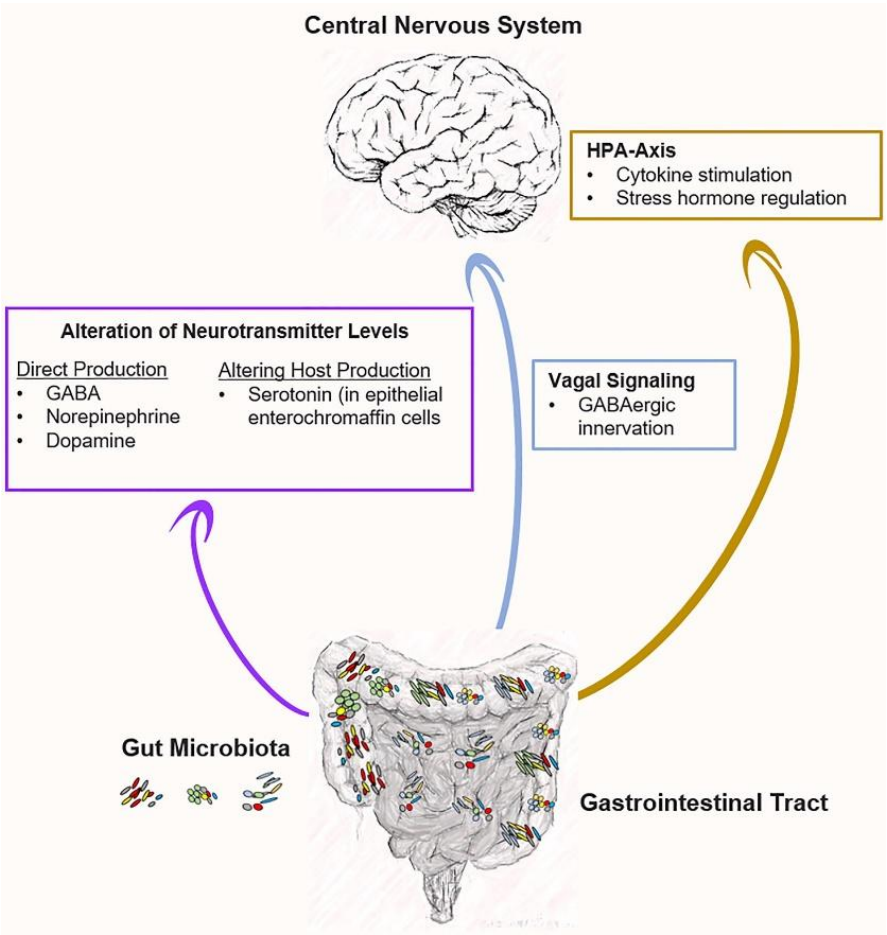


Figure 2. Pathways of gut microbiota-to-brain communication. The gut microbiota interacts with the central nervous system through multiple routes: (1) the direct synthesis or modulation of host neurotransmitter metabolism by microbiota that alter neurotransmitter levels. (2) The signaling through the vagus nerve, and (3) the activation of the hypothalamic–pituitary–adrenal (HPA) axis. While these mechanisms are commonly associated with microbiota–brain communication, similar processes are also involved in more localized signaling between the microbiota and the enteric nervous system [84].

1.5. Lactic acid bacteria and their role in GABA production

Lactic acid bacteria (LAB), including various *Lactobacillus* species, are widely recognized as the primary microbial producers of gamma-aminobutyric acid (GABA). This capacity is due to the presence of the *gad* gene within their genomes, which encodes the GAD enzyme responsible for the conversion of glutamate into GABA. Several LAB species have been identified as GABA producers, including *Levilactobacillus brevis*, *Lentilactobacillus buchneri*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Lactobacillus helveticus*, *Lacticaseibacillus paracasei*, *Lactiplantibacillus plantarum*, and *Lactococcus lactis*, among others [91]. Fermented foods rich in L-glutamate serve as important sources of GABA-producing LAB; one of these is cheese that, due to its high casein content, can generate significant amounts of glutamate during fermentation. The ability to produce GABA varies significantly among LAB species, but as reported in multiple studies, strains belonging to *L. brevis* species have shown the highest capacities for GABA production compared to other strains [92–94], however also strains belonging to other *Lactobacillus* species have shown a notable GABA-producing ability [95]. In LAB, GABA biosynthesis occurs via the GAD pathway, which is composed by *gadC* gene that encode for a glutamate/GABA antiporter, that is responsible for the imports of glutamate, or its salt form monosodium glutamate (MSG), into the bacterial cell. The GAD enzyme, with pyridoxal 5′-phosphate (PLP) as a cofactor, catalyses the decarboxylation of glutamate into GABA, releasing CO_2. Subsequently, GABA is exported to the extracellular environment through the same antiporter. Notably, this decarboxylation reaction consumes one intracellular proton per cycle, thereby generating a proton motive force, which facilitates GABA export (Figure 3) [96]. GAD is an intracellular enzyme that plays a pivotal role in acid stress resistance, it reduces cytoplasmic proton concentration in the presence of L-glutamate, contributing to the so-called glutamate-dependent acid resistance (GDAR) system. This system is crucial for LAB survival in acidic environments, such as fermented foods, and for their colonization of the gastrointestinal tract [97]. The GDAR system comprises glutamate decarboxylase encoded by *gadB* (and in some strains belonging to *L. brevis* species also *gadA*) and the antiporter *gadC*. These genes are typically expressed during the stationary phase in nutrient-rich media, regardless of environmental pH. The organization of the *gad* operon in LAB is highly variable. It includes *gadR*, a transcriptional regulator that plays a fundamental role in the expression of *gadB/A* and *gadC;* in fact experimental deletion of *gadR* in *L. brevis* ATCC 367 has been shown to a significant reduction in the expression of these genes and consequently lower GABA production [98]. Not only *gadR* but all components of

the GAD operon are essential for both GABA production and acid resistance; in fact, it has been seen that deletion of *gadA*, *gadB*, or *gadC* results in complete loss of GABA biosynthesis and increase sensitivity to acidic conditions [99].

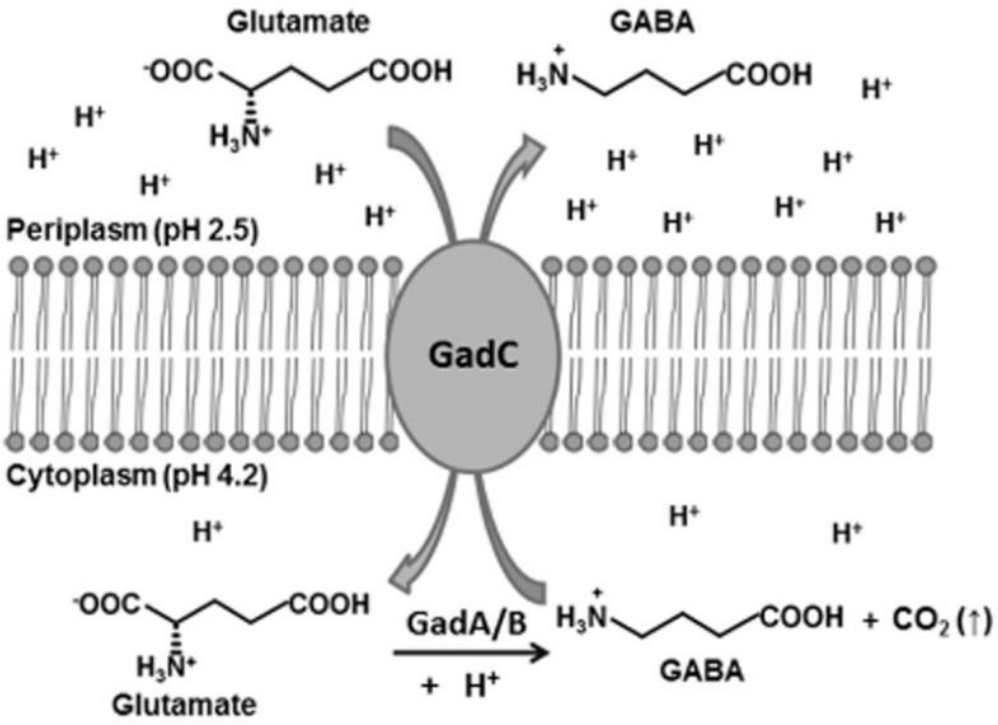


Figure 3. Glutamate-dependent acid resistance (GDAR) mechanism. Glutamate, which carries no net charge, is transported into the cell by the electrogenic L-glutamate/GABA antiporter GadC, a protein located in the inner membrane. Once inside the cell, glutamate is decarboxylated by GadA/B, a reaction that consumes one intracellular proton per cycle and supports the establishment of a proton motive force through the export of GABA, which has a net positive charge. Reproduced from De Biase D, et al 2012 [100].

Numerous lactic acid bacteria (LAB) species possess the ability to synthesize gamma-aminobutyric acid (GABA); however, production levels vary not only between species but also according to specific cultivation conditions. Key environmental factors such as pH, temperature, growth medium composition, and incubation time significantly influence GABA biosynthesis [101]. Among these, pH and temperature are particularly critical, as they directly modulate the activity of GAD, indeed shifts in pH can activate the GAD pathway as part of the cell's adaptive response to maintain intracellular homeostasis. Several studies have demonstrated that the initial pH of the fermentation medium has a substantial impact on GABA synthesis [94]. In general, LAB strains capable of producing GABA tend to perform better under acidic growth conditions [102,103]. Temperature is another determining factor in GABA

production, with the optimal working temperatures of GAD enzymes varying across species. For example, GAD from *Lactobacillus sakei* exhibits optimal activity at 55°C, while that of *Lactiplantibacillus plantarum* functions best at around 40°C. Overall, the optimal temperature range for GAD activity in LAB lies between 30°C and 60°C [101]. In addition to physical parameters, the nutrient composition of the culture medium plays a crucial role in enhancing GABA production, for example the supplementation of MSG, which serve as GABA precursors, has been shown to stimulate biosynthesis. Since many LAB strains are unable to generate enough glutamate endogenously to produce GABA, exogenous addition of MSG is often required. However, excessive concentrations of MSG may inhibit bacterial growth and consequently reduce GABA yield. The optimal MSG concentration for GABA synthesis is strain dependent, for instance, *L. plantarum* Taj-Apis362 achieved maximum GABA production at 400 mM MSG [104], in contrast, *L. brevis* CRL 1942 achieves maximum GABA production with a lower concentration of MSG, specifically at 270 mM [105]. Another critical factor is pyridoxal 5'-phosphate (PLP), which serves as a cofactor for GAD enzyme, indeed supplementation with PLP has been shown to enhance GAD activity, thereby increasing GABA synthesis. Additionally, the presence of nitrogen and carbon sources such as glucose and yeast extract can further support bacterial metabolism and promote GABA production [106], however the optimal concentrations of these nutrients also vary depending on the specific LAB strain employed [107].

2. Gut microbiota in focus: *in vitro* models and clinical perspectives

2.1. *In vitro* fermentation: model system ad methodology to study gut microbiota

A comprehensive understanding of how the gut microbiota contributes to human health and disease requires the disentanglement of complex microbial interactions. In this context, *in vitro* human gut microbiota (HGM) models have become essential tools in microbiome research, particularly in medical and nutritional sciences [108]. These models, when properly designed and applied, allow researchers to isolate and investigate microbe-microbe interactions, and also they are valuable for assessing the impact of environmental (abiotic) and biological (biotic) components of the exposome on microbial communities [109]. Importantly, *in vitro* fermentation systems offer an ethical and cost-effective alternative to animal testing, aligning with societal demands to reduce animal use in research. Moreover, they enable the preliminary evaluation and optimization of dietary compounds or pharmaceutical interventions prior to *in vivo* studies, thereby improving the efficiency and relevance of subsequent animal or human

trials [110]. A wide range of *in vitro* HGM model configurations exists, from simple batch cultures under anaerobic conditions to advanced, multi-stage, and continuously operated bioreactor systems.

The small and large intestines differ substantially in their physiological and microbial environments. The small intestine is the primary site for nutrient digestion and absorption by the host and is characterized by steep spatial and temporal gradients of both biotic and abiotic factors. These dynamic and heterogeneous conditions, such as fluctuating pH, oxygen levels, and rapid transit time (2–5 hours), represent a significant challenge for replicating this region in *in vitro* models [111]. Current models are predominantly used to simulate digestive and absorptive processes but often lack incorporation of microbial communities [112]. In contrast, the colon harbours a dense and diverse microbial community, and due to its functional relevance, most human gut microbiota (HGM) research has focused on modelling this region [113]. Culturing complex colonic microbial consortia remains challenging due to the labour-intensive process of isolating and maintaining fastidious anaerobes. Therefore, *in vitro* models capable of supporting stable and diverse microbial ecosystems are essential. These models rely on cross-feeding interactions among community members and require the inclusion of appropriate growth factors in fermentation media. Critically, the physicochemical conditions of *in vitro* systems must closely mimic the anaerobic environment of the donor's colon to ensure relevant outcomes [114]. The metabolic activity of HGM models can be evaluated through classical metabolite analysis, as well as advanced multi-omics approaches such as metatranscriptomics and metaproteomics. Chromatographic techniques are also commonly used to quantify fermentation end-products like short-chain fatty acids (SCFAs), which are indicative of microbial degradation of carbohydrates and proteins [115]. These metabolites serve as key markers for assessing the functional performance of the *in vitro* gut ecosystem.

The quality of the microbial inoculum is another critical factor influencing model outcomes. Several studies have demonstrated that fresh faecal samples are superior to frozen or cryopreserved ones in preserving the viability and compositional variability of the original microbiota [116]. *In vitro* HGM systems range from simple deep-well plates to highly sophisticated, fully controlled bioreactor setups that can operate in batch, semi-continuous, or continuous modes. These can be configured as single-stage or multi-stage systems depending on the experimental objectives [117]. Following inoculation, the microbial community must undergo a period of ecological adaptation to the artificial *in vitro* environment. The design and

operational parameters of the HGM model significantly influence community composition and metabolic output. Therefore, careful selection of culture media and experimental conditions is crucial to prevent the overgrowth of fast-replicating taxa at the expense of more sensitive species, which could compromise the ecological balance and functional integrity of the system [118].

Experiments based on batch fermentation models, also referred to static colonic fermentation systems, involve incubating human gut microbiota under strictly anaerobic conditions in a nutrient-rich medium that mimics the composition of colonic contents, typically for short durations [119]. These models can vary widely in scale, ranging from small-volume multi-well plates (2–5 mL) to larger bioreactors of 50 mL. Despite the technical complexity, these systems allow for tight control over critical environmental parameters, including pH, temperature, anaerobiosis, and mixing, which is essential to ensure reproducibility and relevance of the experimental data (Figure 4). Bioreactors used in such models generally operate at 37 °C, under continuous stirring, and maintain a stable pH (commonly pH 7) through automated regulation [120]. Batch fermentation models are widely employed, particularly in screening studies aimed at evaluating microbial growth dynamics or the bioconversion potential of specific substrates. For example, Amaretti et al. (2020) utilized a batch model to investigate the prebiotic effect of a long-chain dextran produced by *Weissella cibaria* on gut microbiota composition and their metabolite production [121]. However, batch systems present certain limitations, such as nutrient depletion, accumulation of fermentation by-products, pH reduction, and inhibition of microbial growth due to metabolite buildup. Consequently, the fermentation period in batch models is typically restricted to 24 hours. In contrast, dynamic colonic models offer a more advanced simulation of the human colon environment. These systems involve the continuous or semi-continuous input of fresh nutrients and the simultaneous removal of fermentation effluents, thereby maintaining a constant working volume.

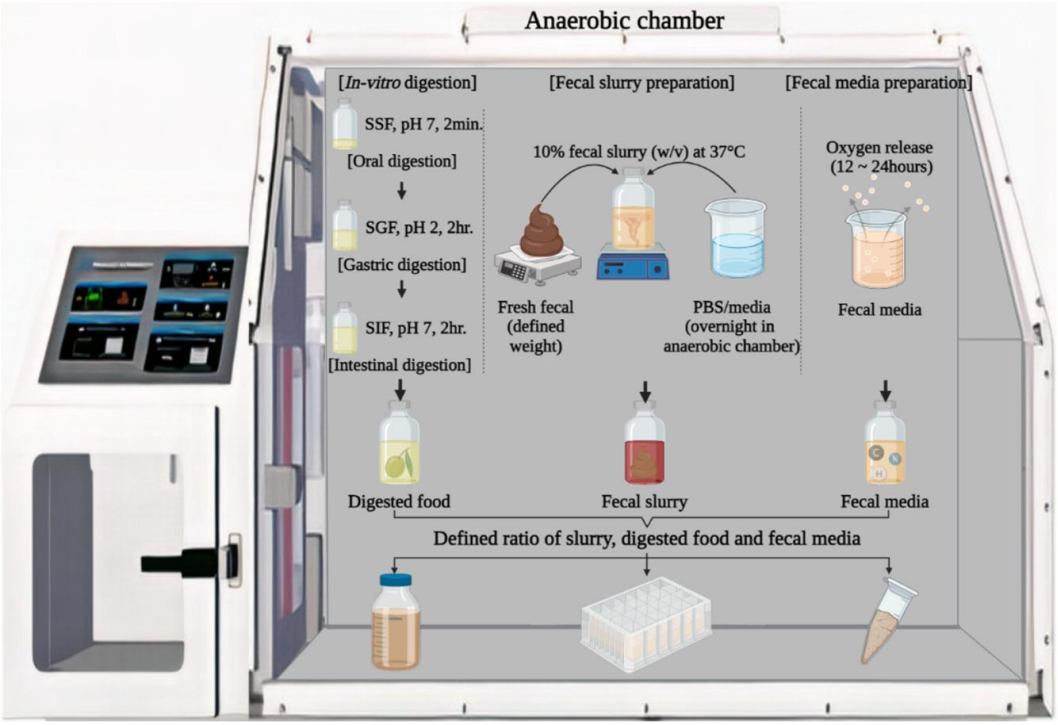


Figure 4. In static *in vitro* fermentation models commonly used in nutritional studies, a 5–10% anaerobic faecal slurry is employed as a microbial inoculum. The fermentation substrate typically consists of enzymatically digested food, prepared in combination with simulated digestive fluids (including PBS – phosphate-buffered saline; SGF – simulated gastric fluid; SIF – simulated intestinal fluid; and SSF – simulated salivary fluid), along with a growth medium that supports microbial proliferation at 37 °C. During fermentation, samples are incubated under agitation (e.g., on a shaker) to ensure homogeneous nutrient distribution and microbial contact. Sampling can be performed at various time points depending on the experimental design [122].

In models such as SHIME (Simulator of the Human Intestinal Microbial Ecosystem), fresh nutrients are introduced semi-continuously [123], whereas in systems like the Gibson model [124] or PolyFermS [125], nutrients are supplied continuously to the system, while, through the same flow, metabolites containing metabolic waste products and microorganisms are removed, thereby maintaining a constant and steady-state flow. These dynamic systems are designed to simulate the physiological process of digestion and microbial metabolism more accurately.

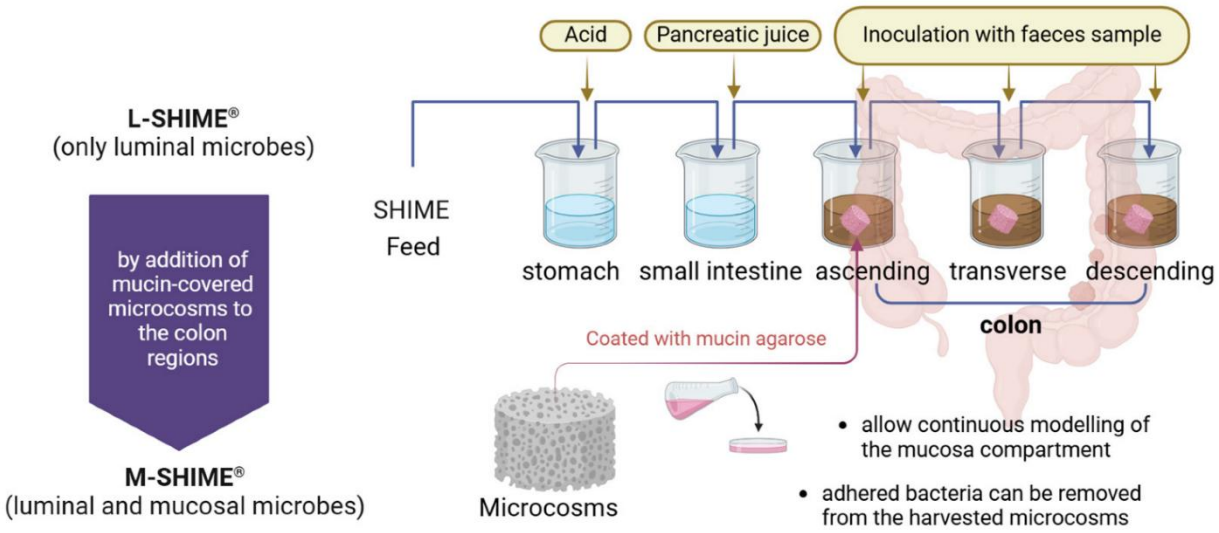


Figure 5. Schematic overview of the SHIME (Simulator of the Human Intestinal Microbial Ecosystem), a dynamic *in vitro* model that mimics the human gastrointestinal tract. The system consists of a series of interconnected vessels with dual outlets. Each vessel simulating different compartments of the GI tract, including the stomach, small intestine, and the three main regions of the colon. All vessels are maintained at 37 °C under anaerobic conditions, ensured by a daily flush of nitrogen gas introduced into the headspace of each reactor. The colon compartments are designed to host complex microbial communities. In its standard configuration, referred to as L-SHIME, the system models the luminal environment, which is predominantly colonized by planktonic microorganisms. In the M-SHIME configuration, an additional mucosal compartment is included, allowing for the colonization of mucin-covered microcosms, thereby enabling the study of both luminal and mucosa-associated microbiota [126].

The TIM-2 model further mimics metabolite absorption via an integrated dialysis system [127]. Dynamic models may be configured as either single-stage or multi-stage systems, depending on the study's objectives. Single-stage setups typically simulate only one section of the colon (e.g., the proximal colon) [128], whereas multi-stage systems replicate the three major regions of the colon, ascending, transverse, and descending, such as the pioneering three-stage Gibson model, thereby allowing for a more comprehensive reproduction of the spatially distinct environmental conditions present *in vivo* [129].

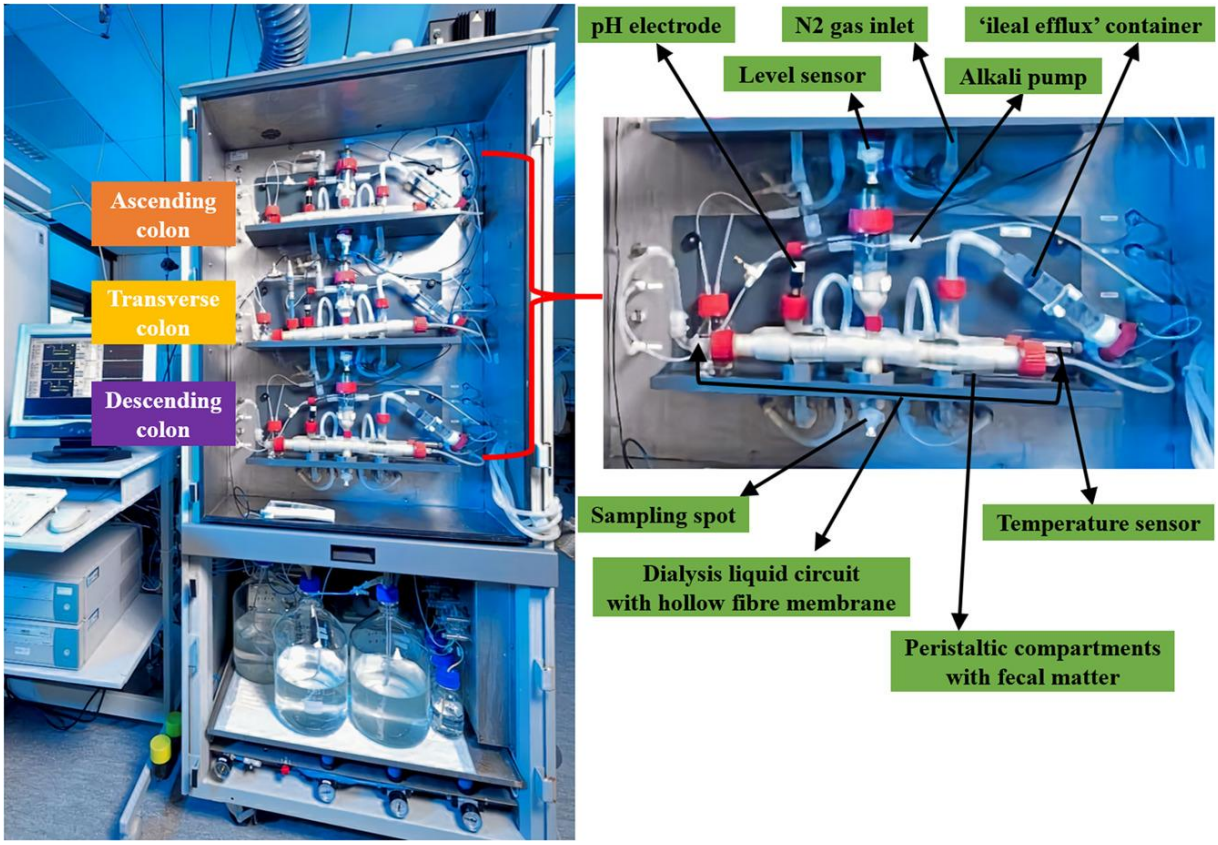


Figure 6. Real life image of TIM, (TNO) *in vitro* model specifically TIM-2 system; along with component of an individual colon compartment [130,131].

Despite their advantages, dynamic systems are sensitive to several variables, including pH control, anaerobic stability, and flow regulation. As a result, the microbial community typically requires a stabilization period of 7 to 14 days before experimental treatments can be applied, to ensure a steady-state microbial composition and function [132]. Ideally, and as is the case for all *in vitro* models, treatments should be applied to a microbiota community that has reached a stable state, in order to clearly distinguish treatment-induced effects from natural community fluctuations. However, this is often impractical due to the time required for 16S rRNA gene sequencing. As a result, model stability is commonly assessed using a day-to-day variation threshold, typically ranging from 10% to 20% [132].

Chapter 2

1. Microbial fermentation with lactic acid bacteria: valorising waste streams into GABA supplements

1.1. Lactic acid bacteria fermentation as a sustainable approach to agri-food by-product valorisation

Fermented foods have long been recognized not only for their improved preservation, safety, and sensory qualities but also for their remarkable health-promoting potential. Numerous studies have demonstrated that these benefits largely derive from the metabolic activity of microorganisms, primarily lactic acid bacteria (LAB) and yeasts, capable of transforming raw substrates into complex matrices rich in bioactive compounds [133,134]. Fermentation typically involves the microbial conversion of carbohydrates, proteins, and lipids naturally present in food matrices. The fermentation process leads to the generation of multiple bioactive molecules with documented health-promoting properties, such as pathogen inhibition, modulation of immune responses, anti-inflammatory and anti-allergenic activity, and enhanced bioavailability of vitamins and minerals. Furthermore, the biochemical transformations that occur during fermentation induce profound changes in the nutritional and functional profile of foods, often resulting in increased antioxidant capacity, improved digestibility, and reduced content of anti-nutritional compounds [135]. The production of specific microbial enzymes, for instance, facilitates the breakdown of complex polysaccharides and oligosaccharides, thereby improving nutrient accessibility and supporting the growth of beneficial gut microbiota [136]. Among the diverse metabolic pathways involved, four primary types of fermentation can be distinguished: alcoholic, acetic, alkaline, and lactic acid fermentation, depending on the microbial species and dominant biochemical conversions [137].

Alcoholic fermentation, which usually takes place in wine and beer, is driven by yeasts (i.e. *Saccharomyces cerevisiae*) that convert sugars into ethanol and CO_2, while acetic fermentation, mediated by *Acetobacter* spp., oxidizes ethanol to acetic acid, producing vinegar and kombucha. Alkaline fermentation, performed by *Bacillus* spp. and related bacteria, promotes proteolysis and generates amino acids and peptides, with an associated rise in pH and development of distinct umami and aromatic notes. Eventually, lactic acid fermentation, predominantly conducted by LAB, is the most common in food systems and can proceed via homofermentative or heterofermentative pathways, yielding products such as yogurt, kefir, sauerkraut, and kimchi [138]. Most LAB species used in food fermentation are considered

Generally Recognized as Safe (GRAS) or possess Qualified Presumption of Safety (QPS) status, ensuring their suitability for human consumption [133]. LAB encompass genera such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus*, which are recurrently identified in a wide variety of fermented products. These microorganisms synthesize a broad spectrum of metabolites, including organic acids, ethanol, antimicrobial peptides, bacteriocins, and short-chain fatty acids (SCFAs), that not only contribute to food safety and stability but also exert beneficial physiological effects on the host [133,137]. Beyond their technological significance, LAB and their metabolites are increasingly regarded as functional bioactive. LAB-fermented foods are known to contain viable and non-viable cells capable of exerting probiotic (living cells), postbiotic (metabolites), and paraprobiotic (dead cells) effects [139]. Their consumption has been associated with improved intestinal homeostasis, regulation of the immune system, enhanced resistance to pathogens, and production of SCFAs that act as key modulators of host metabolism [133].

Over the past decade, sustainability has become a central concern in the food sector, driven by increasing environmental pressures and the growing awareness of finite natural resources. According to the United Nations Environment Programme [140], approximately one-third of all food produced for human consumption, equivalent to nearly 1.3 billion tonnes per year, is wasted globally. The food industry, as a major contributor to this waste, faces an urgent need to implement innovative strategies for resource recovery and reuse, thereby reducing environmental impact and promoting the transition toward a circular economy [141]. For instance, fruits and vegetables are widely consumed both in their fresh form and as processed products, including juices, jams, pulps, syrups, and concentrates. However, these industrial transformations generate a considerable number of solid residues, such as peels, pomace, seeds, and pulps, that represent up to 50–60% of the original raw material [142]. Improper management or disposal of such by-products not only imposes significant economic and logistical burdens but also contributes to environmental pollution and the loss of valuable nutrients and bioactive compounds [143]. Although fruit and vegetable by-products can be rich in proteins, amino acids, fibres, pectins, and bioactive phytochemicals (polyphenols, carotenoids, flavonoids, anthocyanins, and phenolic acids), their effective reuse is hindered by technological barriers. In particular, the high proportion of insoluble fibres reduces the extractability and bioavailability of functional compounds, often compromising the sensory quality of derived food products in terms of texture, colour, and flavour [144]. Consequently, their valorisation through microbial fermentation has emerged as a sustainable and promising

strategy to recover these bioactive compounds and convert waste materials into high-value ingredients for use in the food, nutraceutical, and biotechnology sectors [145,146]. For instance, fermentation of brewer's spent grains with *Rhizopus oligosporus* has been shown to degrade insoluble fibres, enhance the accessibility of phenolic and protein fractions, and improve antioxidant activity as well as sensory quality in enriched bakery products [147]. Similarly, mixed microbial fermentation of grape pomace has been reported to double total polyphenol and anthocyanin content, intensify antioxidant and anti-inflammatory properties, and mitigate off flavours in fermented beverages. Blueberry pomace was fermented with the probiotic strain *Lactobacillus casei*. The fermented product exhibited markedly increased antioxidant capacity, modulation of the fecal microbiota, and elevated production of SCFAs in an *in vitro* gut fermentation model [148]. Similarly, tomato, melon, and carrot residues have been employed as fermentation substrates for several *Lactobacillus* species, including *L. plantarum*, *L. casei*, and *L. paracasei*. Extracts derived from these fermented materials showed significant antimicrobial activity against foodborne pathogens such as *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, highlighting their potential application as natural biopreservatives [149]. Furthermore, fermentation of tomato processing by-products, such as purees and juices, using *Pediococcus acidilactici* and *Lactobacillus rhamnosus* was reported to substantially increase total polyphenol content and antioxidant activity, further supporting the role of fermentation as a sustainable means to enhance the functional value of agro-industrial residues [150]. Collectively, these findings reinforce the concept that microbial fermentation represents a versatile and eco-efficient tool to upcycle plant-based by-products into nutritionally enriched, bioactive ingredients, bridging the gap between food sustainability and human health. In addition, Valle Vargas et al. (2024) [151] demonstrated that agro-industrial by-products such as sugarcane molasses and palm kernel cake can be effectively used as low-cost substrates for the cultivation of *Lactococcus lactis* A12 and other bacterial strains. The optimized formulation supported bacterial growth comparable to that obtained in commercial synthetic media, while reducing medium costs by nearly 87%, highlighting the potential of by-product-based media as a sustainable and economically viable alternative for microbial biomass production.

1.2. Fermentative production of GABA supplements using lactic acid bacteria on substrate-based media

GABA is the principal inhibitory neurotransmitter in the CNS, plays a crucial role in regulating a wide range of physiological processes. Its therapeutic efficacy has been demonstrated across multiple clinical areas. For instance, GABA has been shown to lower blood pressure, making it a promising agent in the management of hypertension, anxiety, and arrhythmias. It also contributes to liver function improvement, cardiac health, and insulin regulation, thus being investigated for potential use in the treatment of diabetes [152]. Due to its direct action on the CNS, GABA is additionally employed in the management of sleep disorders, depression, and epilepsy [152]. The expanding application of GABA as a nutraceutical and dietary supplement stems from the progressive elucidation of its diverse biological functions. GABA receptors have not only been identified in the CNS but also in the peripheral nervous system (PNS) and endocrine tissues, supporting its role as a potent analgesic and modulator of hormonal activity [153]. Historically, GABA was primarily obtained through chemical synthesis. While this method yields high-purity GABA, it is associated with high production costs, environmental concerns, and challenges related to process control. Moreover, chemically synthesized GABA is not suitable for direct incorporation into foods or use as a natural additive. Alternatively, GABA enrichment in plants has been explored, but the concentration of GABA is typically low, and purification processes remain technically demanding and inefficient. Biological synthesis offers a more sustainable alternative that could be the microbial transformation [154]. During fermentation, GABA is produced as a result of spontaneous enzymatic reactions, with lactic acid bacteria (LAB) playing a central role in this process.

Precision fermentation has emerged as a key trend within the framework of the fourth industrial revolution in the agri-food sector [155]. Advances in biotechnology and synthetic biology have enabled the genetic modification of microorganisms for the tailored biosynthesis of complex organic molecules [156]. However, the commercialization of genetically modified organisms (GMOs) for food applications continues to face considerable challenges. These are largely attributable to public scepticism, limited consumer understanding of GMO safety and risk profiles, and ongoing debates surrounding labelling and regulatory frameworks [154]. Precision fermentation is increasingly recognized as a transformative approach for the sustainable production of key food macronutrients such as proteins, lipids, and carbohydrates. Its potential is driven by growing global demands for environmentally responsible and resource-efficient

food systems. Importantly, these fermentation strategies are not limited to the production of bulk nutrients, they are also being applied to synthesize high-value bioactive compounds, such as GABA, which is of interest for its functional and nutraceutical properties. The successful integration of precision fermentation into modern food systems depends on several critical technological factors that span the entire value chain. These include: the selection of suitable fermentation substrates, the optimization of downstream processing techniques to efficiently isolate the target food component, the scale-up of microbial fermentation processes from laboratory to industrial scale, and the development of end products that meet consumer expectations in terms of quality, safety, and sensory appeal.

Given the growing market demand for natural and functional food ingredients, microbial fermentation has emerged as the preferred method of GABA production due to its cost-effectiveness, safety, and reduced environmental impact [154]. When it comes to GABA synthesis by LAB species, production is strongly strain-dependent and influenced by fermentation conditions. Factors such as pH, temperature, time, carbon and nitrogen source could be optimized in order to maximize GABA production [157]. Several studies have focused on the use of agro-industrial by-products as substrates for GABA production. For example, Thongruck K. et al. (2023) investigated the application of low-cost raw materials and by-products from the agri-food sector to enhance the biosynthesis of GABA by *Lactobacillus futsaii* CS3. In this study optimal culture conditions were determined using response surface methodology with a central composite design (CCD) and batch and fed-batch fermentation techniques were employed [158]. On going advancements in microbial biotechnology continue to improve and optimize fermentation processes to meet industrial needs, however, several knowledge gaps remain. These include limited understanding of GABA biosynthetic pathways in non-model organisms, the influence of environmental conditions on GABA yield, and the need to develop novel, large-scale biotechnological strategies capable of producing high yields at low cost and with minimal ecological footprint.

2. Regulatory landscape of GABA-based supplements

2.1. Regulatory framework governing the development and commercialization of dietary supplements

The growing trend of marketing food products containing concentrated nutrients in recent years, revealed the need for a unified regulatory framework within the European Union. For this reason, in 2002 the European Parliament and the Council adopted a directive aimed at

harmonizing the Member States' legislation on food supplements. This directive represents the primary regulatory framework for the marketing of GABA-based food supplements within the EU, including formulations obtained through controlled fermentation processes, such as the one formulated in this study based on tomato by-products fermented by *L. brevis* CRAI, and subsequently formulated in conventional dosage forms (e.g., capsules, tablets, or sachets). The directive defines food supplements as products intended to complement the regular diet, as they represent a concentrated source of nutrients or other substances that exert a nutritional or physiological effect, like GABA supplements [159]. According to the directive, food supplements must not pose any risk to human health and must be marketed with clear and appropriate labelling. The vitamins and minerals included are required to meet the established quality and safety standards, and the nutritional or physiological effects of each ingredient must be clearly defined. Additionally, to further safeguard costumers, a list of chemical substances authorized as sources of vitamins and minerals has been drawn up. Moreover, maximum intake levels must be defined, as excessive consumption of certain nutrients may lead to adverse health effects. The initial list of approved vitamins and minerals was later updated by Regulation (EC) No 1170/2009. In addition to vitamins and minerals, other substances with a nutritional or physiological effect, such as GABA, are subject to comparable quality and safety requirements, and their effects must be clearly defined in relation not only to the substance itself but also to the production process used to obtain it. In this context, GABA is generally considered safe at appropriate intake levels, its safety also depends on the purity of the final product. When obtained from fermentation of tomato by-products, careful control of potential impurities is essential.

In Italy, lactic acid bacteria have long been utilized in the dietary supplement sector within fermentation-based processes aimed at the production of functional ingredients. Over time, the term “lactic acid bacteria” was gradually replaced by “probiotic”. Microorganisms employed in food and dietary supplements are required to meet several essential criteria to ensure their safety and efficacy. These criteria, documented history of safe use, recognition as safe for human consumption, demonstrated intestinal activity, and ability to proliferate in the gut. To be classified as probiotics, these microorganisms must be accurately identified both at the species level, through 16S rRNA gene analysis, and at the strain level, through specific genetic identification methods, strain-level identification remains important for process traceability. Both criteria must be fulfilled for the probiotic designation to be valid. In the case of GABA supplements produced via fermentation, the safety assessment is closely linked to the

taxonomic identification of the production strain. In this context, *Lactobacillus brevis* CRAI has been identified at the strain level and belongs to a species included in the EFSA list of Qualified Presumption of Safety (QPS), thereby supporting its safe use in food-related applications. Regarding prebiotics, their definition is based on the FAO's *Technical Meeting on Prebiotics*, which describes them as a non-viable food component that confer a health benefit to the host by modulating the microbiota [160].

In 2015, Regulation (EU) 2015/2283 was introduced to govern the marketing of so-called "novel foods" within the European Union. The regulation emphasizes the importance of free circulation of safe and healthy food throughout the internal market, as this directly promotes European citizens' health and well-being. The same safety standards defined for traditional foods equally apply to novel foods. A food is considered "novel" under EU legislation, if it was not consumed to a significant degree within the EU prior to 15 May 1997, and if it falls into at least one of the following categories: foods with a new or intentionally modified molecular structure; foods derived from microorganisms, fungi, algae, mineral materials, plants, or animals; foods obtained from cell cultures; foods produced through novel production processes; or foods containing engineered nanomaterials. To gain authorization to market a novel food, applicants must submit a detailed application to the competent authorities. This document must include the name and address of the applicant, a description and identification of the novel food, and a detailed explanation of the production process, highlighting the waste valorisation approach, as well as the complete composition of the food, with particular emphasis on GABA purity and potential by-products, validated methods of analysis, and a proposed set of conditions for use and labelling. The labelling proposal must ensure that the consumer is not misled and, if necessary, should include a justification for any special indications presented on the product [161].

3. Biotechnological production of GABA using lactic acid bacteria: from strain isolation to nutraceutical development

In the current industrial context, the chemical synthesis of γ-aminobutyric acid (GABA) for use as a dietary supplement remains a costly process. The primary aim of this research is to reduce GABA production costs by optimizing a biotechnological approach that harnesses the natural ability of selected microorganisms to biosynthesize this bioactive compound. To achieve this, lactic acid bacteria (LAB) were isolated from a biologically derived tomato matrix, with the goal of identifying a novel strain exhibiting high GABA conversion efficiency. The new isolated

strain *Levilactobacillus brevis* CRAI was selected based on its high ability to produce GABA and then subjected to a comprehensive screening of its probiotic properties, including antagonistic activity against common enteropathogens, anti-inflammatory potential, and adhesion capacity to an intestinal epithelial cell line. Given the intended use of this strain within a GABA-enriched nutraceutical formulation, its complete genome was sequenced to ensure safety, with particular attention to the absence of genes associated with antibiotic resistance and virulence factors. Simultaneously, considering the large volume of nutrient-rich by-products generated by the agri-food industry, which pose both environmental and economic disposal challenges, this work also aims to contribute to the valorisation of agro-industrial waste. We sought to develop a cost-effective and eco-friendly production strategy by integrating the microbial ability to produce GABA with the sustainable use of food processing by-products as fermentation substrates. To this end, the selected LAB strain was cultivated in various agro-industrial matrices (e.g., tomato pulp, peach, apricot, grape, and apple pulp) to determine which substrate best supported microbial growth and GABA production. Following this selection, fermentation parameters were further optimized using a central composite design (CCD), testing key variables such as pH, yeast extract concentration, and matrix dilution. The predictive model was subsequently validated by verifying that the optimized conditions yielded maximal GABA conversion. Once chosen the best conditions, the industrial partner, Depofarma company provided a batch of lyophilized tomato extract (an industrial by-product), which was used as a substrate under the previously defined optimal conditions. The fermentation process was subsequently scaled up to assess GABA production at a larger volume. This step was necessary because, in larger-scale systems, the non-homogeneous distribution of nutrients may compromise consistent GABA synthesis. Once GABA production was quantified and confirmed at scale up, a GABA-enriched nutraceutical prototype was developed. Regulatory frameworks governing the commercialization of food supplements were assessed, with particular attention to determining whether the product would be classified as a novel food, which would require extensive safety and toxicity testing prior to market approval. Finally, the prototype was evaluated in *in vitro* gut models, that mimic conditions of eubiosis and dysbiosis, to assess its impact on gut microbiota composition and on the production of key microbial metabolites such as short-chain fatty acids (SCFAs), thereby exploring the potential health benefits associated with the final nutraceutical product.

3.1. From laboratory to market: safety, novel food evaluation, and patent protection of *L. brevis* CRAI

We addressed the regulatory and commercial aspects required for the potential market introduction of the probiotic strain *L. brevis* CRAI and the derived GABA-enriched product. These products are proposed as probiotic/nutraceutical dietary supplements. In the European Union, the commercialization of any food product must comply with strict safety and regulatory requirements to ensure consumer safety. A critical step in this process is determining whether the product qualifies as a "novel food." According to Regulation (EU) 2015/2283, a novel food is defined as any food that was not consumed to a significant degree within the EU before 15 May 1997 and falls into one or more specific categories, including foods with a new or intentionally modified molecular structure, or those produced using genetically modified organisms introduced after that date [161]. In our case, the new strain *Levilactobacillus brevis* CRAI, was employed to ferment a dehydrated tomato matrix, resulting in the biosynthesis of GABA. To assess whether our product falls under the novel food classification, we conducted a thorough literature review to verify the historical use of *L. brevis* in food prior to 1997. Multiple studies confirmed the presence and use of *L. brevis* in fermented foods such as vegetables, fruits, and cereals well before the regulatory cutoff date. For instance, *L. brevis* was identified in fermented maize dough [162], high-moisture corn silage [163], sourdoughs [164], and brined cucumbers [165], among others. These findings support the conclusion that *L. brevis* specie, has a documented history of safe use in food products prior to 1997, thereby exempting our formulation from novel food classification. An additional milestone in the project was the successful patenting of the *L. brevis* CRAI strain. This strain, originally isolated from tomatoes, was granted patent protection (Patent No. 31.D1066.12.IT.4) based on its unique functional properties. Specifically, *L. brevis* CRAI was recognized for its ability to produce high levels of GABA and for its probiotic activities, including inhibition of pathogenic microorganisms' growth and anti-inflammatory effects. The patent not only secures the intellectual property associated with the strain but also validates its biotechnological potential as a functional ingredient in nutraceutical applications. Taken together, these regulatory and intellectual property achievements strengthen the translational relevance of our findings and support the feasibility of introducing the probiotic strain *L. brevis* CRAI and the GABA-enriched prototype into the functional food market.

Chapter 3

1. Characterization of a GABA-producing *Levilactobacillus brevis* CRAI isolated from organic tomato: a new probiotic candidate

1.1. Material and methods

1.1.1. Isolation of lactic acid bacteria from tomatoes

Lactic acid bacteria (LAB) were isolated from different varieties of organic tomatoes harvested from agricultural areas in the Bologna region (Italy). The tomato pulp was first homogenized and then combined with de Man, Rogosa and Sharpe (MRS) broth (Difco, Detroit, MI, USA), enriched with 0.05% (w/v) L-cysteine (Merck, Milan, Italy). The resulting suspension was subjected to serial dilutions, which were subsequently plated on MRS agar. Plates were incubated at 37 °C for 72 hours under anaerobic conditions, by using anaerobic jars with GasPak™ systems (BD GasPak™ EZ Anaerobe Container System, Sparks, MD, USA). After incubation, bacteria whose colonies exhibiting different morphologies were examined under a light microscope. Colonies consisting of rod-shaped cells were selected for further analysis. These isolates were preserved in MRS broth supplemented with 10% (v/v) glycerol and stored at −80 °C. Prior to experimental use, the isolates were revived and cultivated in MRS broth supplemented with 0.05% L-cysteine, incubated at 37 °C under anaerobic conditions.

1.1.2. 16S rRNA gene sequencing and taxonomic identification of tomato-derived isolates

The pellet from each bacterial isolate was collected after overnight cultures by centrifugation at 10,000 × g for 10 minutes. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for "Pretreatment for Gram-positive bacteria" [166] The 16S ribosomal RNA (rRNA) gene was amplified using the universal primers 27F and 1492R [167]. PCR products were subsequently sequenced. The resulting sequences were compared against those available in the BLASTn database (https://blast.ncbi.nlm.nih.gov, accessed on 15 January 2024) to taxonomically identify the bacterial isolates based on sequence similarity.

1.1.3. Detection of *gadB* gene via PCR in lactic acid bacteria

Following genomic DNA extraction from the isolated LAB, DNA was used to assess the presence of the *gadB* gene, which encodes glutamate decarboxylase. Highly conserved regions of the *gadB* gene were used to design specific primers.

For *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* strains, the primers used were:
Forward 5′-CCTCGAGAAGCCGATCGCTTAGTTCG-3′;
Reverse 5′-TCATATTGACCGGTATAAGTGATGCCC-3′ [168].
For *Lactococcus lactis*, the primers were:
Forward:5′-CAACATGATCGCTGACCTTTGG-3′;
Reverse:5′-GCCATTCCACCAAGCATACAAG-3′ [169].

1.1.4. LAB cultivation and Quantitative evaluation of GABA using High-Performance Liquid Chromatography (HPLC)

To maximize GABA yield, LAB strains positive for the *gadB* gene were cultured under optimized fermentation conditions, as described in literature [170–174]. Specifically, the strains were grown in MRS broth supplemented with 0.05% L-cysteine at 32 °C for 48 hours under anaerobic conditions. The medium was further enriched with increasing concentrations of monosodium L-glutamate (MSG; ThermoFisher, Kandel, Germany) as follows: 0 mM (0%); 59.1 mM (1%); 118.2 mM (2%); 177.3 mM (3%); 236.5 mM (4%). The initial pH of the medium was adjusted to 5.0, and the starting inoculum was standardized to $1x10^7$ CFU/mL. After 48 hours of incubation, cultures were centrifuged at 5,000 × g for 10 minutes at 4 °C to separate cells from the supernatants. The supernatants were collected and then filtered through 0.22 µm membrane filters and used for GABA quantification. GABA was derivatized by mixing 1.75 mL of 1 M borate buffer (pH 9), 750 µL of methanol, 1 mL of sample, and 20 µL of diethyl ethoxymethylenemalonate (DEEMM). The mixture was subjected to ultrasonic treatment for 30 minutes [175] to complete the derivatization reaction. Derivatized GABA was subsequently analysed using an Agilent Infinity 1290 HPLC-DAD system (Agilent Technologies, Waldbronn, Germany), following the binary gradient method described by Gómez-Alonso et al. (2007) [176]. The mobile phases consisted of phase A, 25 mM acetate buffer (pH 5.8) containing 0.02% sodium azide; and phase B, an 80:20 (v/v) mixture of acetonitrile and methanol. The flow rate was set to 0.9 mL/min. The protocol reported in Gómez-Alonso et al. (2007) was followed with minor modifications, the injection volume was reduced to 1 µL, and

the total chromatographic run time was shortened to 40 minutes (instead of 80 minutes), including a 5-minute isocratic phase with eluent A to allow for column re-equilibration between samples. Quantification of GABA was based on the peak area corresponding to the GABA derivative, compared with a standard calibration curve. The calibration curve was generated using commercial GABA (Merck Millipore, Darmstadt, Germany), derivatized and analysed under the same conditions. The concentration range of the calibration standards was from 0.96 to 29.09 mM, with a correlation coefficient of $R^2 = 0.999$.

1.1.5. Genomic characterization of *L. brevis* CRAI: sequencing and data analysis

The genomic DNA of *Levilactobacillus brevis* CRAI, previously extracted as described, was quantified using the Qubit dsDNA BR assay kit with the Qubit 2.0 fluorometer (Life Technologies, Monza, Italy). Whole-genome sequencing (WGS) was then performed using Oxford Nanopore technology. Briefly, the genomic library was prepared using the SQK-LSK109 and EXP-NBD196 kits (Oxford Nanopore Technologies, Oxford, UK), and the sequencing was carried out on an R9.4.1 flow cell (MIN106D, Oxford Nanopore Technologies, Oxford, UK) using the GridION X5 platform (Oxford Nanopore Technologies, Oxford, UK). *De novo* genome assembly was performed using Canu assembler version 2.2 [177] and genome annotation was carried out with Bakta version 1.4.1 [178] resulting a genome coverage of 39.47×. The *L. brevis* CRAI genome was screened for the presence of genes related to antibiotic resistance and virulence using three different tools: AMR Finder Plus (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/, version 4.0.15, updated on 15 September 2024), ResFinder (version 4.6.0, https://cge.food.dtu.dk/services/ResFinder/, accessed on 15 September 2024; identity threshold: 90.0%, minimum length: 60.0%) and the Resistance Gene Identifier (RGI version 6.0.3) using the Comprehensive Antibiotic Resistance Database (CARD, version 3.3.0; https://card.mcmaster.ca/, accessed on 15 September 2024). Amino acid sequences of GAD system proteins from *L. brevis* ATCC367, *L. brevis* CRL2013, *L. brevis* NCL912, *L. brevis* CD0817, and *L. brevis* YSJ3 were retrieved from the NCBI database. These sequences were then aligned using BLASTp (https://blast.ncbi.nlm.nih.gov, accessed on 15 June 2025) to assess sequence similarity.

1.1.6. RT-PCR-based expression analysis of *gadA* and *gadB* genes

As described in Section 6.1.4., *L. brevis* CRAI was cultured under optimized conditions, with the addition of 0% and 4% (0 and 236.53 mM) monosodium glutamate (MSG) to the medium. Total RNA was extracted after 48 hours of incubation using the RNeasy kit (Qiagen, Hilden, Germany), following the "Enzymatic Lysis, Proteinase K Digestion and Mechanical Disruption of Bacteria" protocol [179]. 1 µg of extracted RNA was used for reverse transcription using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). The resulting cDNA was then used as a template for quantitative Real-Time RT-PCR analysis, performed with the LightCycler 2.0 instrument (Roche, Mannheim, Germany) and the LightCycler® FastStart DNA Master SYBR Green I reagent (Roche, Mannheim, Germany). Amplicon specificity was verified at the end of each reaction through first-derivative melting curve analysis, and the integrity of the PCR products was confirmed by agarose gel electrophoresis. Relative expression levels of the *gadA* gene (primers: 5′-CAGGTTACAAGACGATCATGC-3′ and 5′-ATACTTAGCCAGCTCGGACTC-3′ [180]) and the *gadB* gene (primers: Core F and Core R) were normalized using the 16S rRNA gene as reference (primers: HDA1 5′-ACTCCTACGGGAGGAGGCAGCAGT-3′ and HDA2 5′-GTATTACCGCGGCTGCTGGCAC-3′ [181] based on the crossing point (CT) values. The fold change in the expression of *gadA* and *gadB* was calculated using the 2^−ΔΔCT method [182]

1.1.7. Evaluation of antagonistic activity of *L. brevis* CRAI toward pathogenic bacteria

L. brevis CRAI was cultured overnight, and the cell-free supernatant (CFS) was obtained by centrifugation of the culture at 5000×g for 10 minutes at 4 °C. The resulting supernatant was then filtered through a 0.22 µm pore-size membrane filter. The pH of the CFS was measured using a digital pH meter. The antimicrobial activity of the CFS was evaluated against the following enteropathogenic strains: *Escherichia coli* ETEC H10407; *Salmonella choleraesuis* serovar Typhimurium, both cultured twice in Nutrient Broth (NB; Difco, Detroit, MI, USA); and *Yersinia enterocolitica*, cultured twice in Trypticase Soy Broth (TSB; Difco, Detroit, MI, USA) prior to each experiment. For the assay, 100 µL of *L. brevis* CRAI CFS was distributed into the wells of flat-bottom 96-well microtiter plates, followed by inoculation with 100 µL of each pathogen suspension ($1x10^6$ CFU/mL). As a growth control, 100 µL of sterile MRS medium was added to 100 µL of the same pathogen suspension in separate wells. The plates were incubated at 37 °C for 24 hours. Pathogen growth was monitored by measuring absorbance at 600 nm using an EnSpire Multimode Plate Reader (PerkinElmer Inc., Waltham,

MA, USA). Residual growth in the presence of the CFS was calculated as a percentage of the absorbance measured in the corresponding growth control wells. Each experiment was conducted in duplicate and repeated at least six times to ensure reproducibility. To further assess the antagonistic potential of *L. brevis* CRAI cells against the same intestinal pathogens, the overlay assay was performed following the protocol described by Siroli et al. (2017) [183]. Briefly, 5 µL of an overnight culture of *L. brevis* CRAI (approximately $1x10^8$ CFU/mL) was spotted onto the surface of MRS agar plates (1.5% agar) and incubated under anaerobic conditions at 37 °C for 24 hours. After incubation, 0.1 mL (corresponding to $1x10^8$ CFU/mL) of an overnight culture of each pathogen was inoculated into 10 mL of soft agar (0.7% agar) prepared with NB for *E. coli* ETEC H10407 and *S. choleraesuis*, or with TSB for *Y. enterocolitica*. The soft agar medium inoculated with pathogens was gently poured over the surface of the MRS plates previously seeded with *L. brevis* CRAI. Plates were incubated at 37 °C for an additional 24 hours, after which inhibition zones were examined. Growth inhibition halos were measured by determining the radius from the edge of the inhibition zone toward the center of the *L. brevis* CRAI spot in four different directions. The mean radius was calculated and used to classify the level of antagonistic activity as follows: +: inhibition radius between 14–18 mm; ++: inhibition radius greater than 18 mm.

1.1.8. Evaluation of anti-inflammatory activity of *L. brevis* CRAI in LPS-induced RAW 264.7 cells

The murine macrophage cell line RAW 264.7 was cultured in Dulbecco's Modified Eagle Medium (DMEM) (EuroClone, Pero, Italy), supplemented with 10% fetal bovine serum (Gibco Life Technologies Corporation, Segrate, MI, Italy) and 1% L-glutamine (Merck, Saint Louis, MO, USA). For each experiment, cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The presence of nitrites in the culture medium was assessed using the Griess Reagent Nitrite Measurement Kit (Cell Signaling Technology, Inc., Trask Lane, Danvers, MA, USA) [184]. The Griess assay provides an indirect method for the detection of nitric oxide (NO) by quantifying nitrite, nitrate, and nitrosating species. For each assay, RAW 264.7 cells were seeded in 24-well plates at a density of 1×10^5 cells/well and cultured in complete medium for 48 hours. The inflammatory response was induced by stimulation with bacterial lipopolysaccharide (LPS from *E. coli*, Enzo Life Sciences, Serotype TLRGRADE) at a final concentration of 1 µg/mL. Selected wells were treated either with viable *L. brevis* CRAI cells or with its corresponding cell-free supernatant (CFS), using a ratio of 1:100 (eukaryotic cell:

bacterial cells), and incubated for 24 hours. After the incubation period, 100 µL of culture medium were mixed with 100 µL of Griess reagent in a 96-well plate. Absorbance was then measured at 550 nm using a spectrophotometer (EnSpire Multimode Plate Reader, PerkinElmer Inc., Waltham, MA, USA). The level of inflammation was calculated as a percentage relative to the absorbance of the positive control, consisting of RAW 264.7 cells stimulated with LPS alone (defined as 100% inflammation). Each experiment was performed in two independent trials, with a minimum of three technical replicates per trial.

1.1.9. *In vitro* adhesion capacity of *L. brevis* CRAI to intestinal epithelial Caco-2 cell line

To evaluate the adhesion ability of the *L. brevis* CRAI strain, the human epithelial colorectal adenocarcinoma Caco-2 cell line was used, as it exhibits morphological and functional characteristics similar to those of intestinal colonocytes, the protocol used was described by Giordani B. et al 2018 [185]. Glass coverslips were used as support for the seeding of Caco-2 cells at a density of 5×10^4 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) (EuroClone, Pero, Italy), supplemented with 10% fetal bovine serum and 1% L-glutamine. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 hours. At approximately 70% confluence, the cell monolayers were washed twice with sterile phosphate-buffered saline (PBS) to remove non-adherent cells or residual medium components. An overnight culture of *L. brevis* CRAI was centrifuged and washed twice in PBS, then resuspended in complete medium to reach a final concentration of $1x10^8$ CFU/mL. For the bacterial treatment, Caco-2 cells were exposed to the bacterial suspension at a ratio of 1:100 (epithelial cell: bacteria cells) and incubated for 3 hours at 37 °C in a 5% CO_2 atmosphere. Following incubation, non-adherent bacteria were removed by multiple washes with PBS. The cell monolayers were subsequently fixed and stained with Giemsa solution. Bacterial adhesion was assessed by light-microscopy (1000×). The number of *L. brevis* CRAI cells attached to 200 randomly selected Caco-2 cells was counted, and the mean number of adherent bacteria per cell was calculated as a measure of adhesion capability.

1.1.10. 16S rRNA sequences and *L. brevis* CRAI genome accession numbers

The 16S rRNA gene sequences of the isolated strains have been submitted to the GenBank database and assigned the following accession numbers: PV620908 for *Lactococcus lactis* CRAC; PV620909 for *Levilactobacillus brevis* CRAI; PV620910 for *Levilactobacillus brevis*

CRAR; PV620911 for *Levilactobacillus brevis* DRBA2; PV620912 for *Levilactobacillus brevis* DVBA2; PV620913 for *Lactococcus lactis* IBA; PV620914 for *Lactococcus lactis* IBB; PV620915 for *Lactococcus lactis* PBMC; PV620916 for *Lactococcus lactis* PBMD; PV620917 for *Lactiplantibacillus plantarum* PRA; PV620918 for *Lactiplantibacillus plantarum* DRBA1; PV620919 for *Lactiplantibacillus plantarum* DVBA1; and PV620920 for *Levilactobacillus brevis* DVB22. Additionally, the whole genome sequence of *L. brevis* CRAI has been deposited in the NCBI GenBank repository under accession number SAMN48404542. The *L. brevis* CRAI strain itself is preserved in the DSMZ microbial culture collection with the accession number DSM 35345.

1.1.11. Statistical and data analysis

Data obtained from HPLC quantification of GABA are reported as the mean values of two independent experiments, accompanied by their respective standard deviations (SD). Both antimicrobial and anti-inflammatory activities are expressed as mean ± SD derived from two independent experimental replicates. Statistical significance was assessed using one-way analysis of variance (ANOVA) with subsequent Bonferroni *post hoc* correction for multiple comparisons. Differences were considered significant at a threshold of $p < 0.05$. All statistical analyses were conducted using GraphPad Prism software, version 9.5.1 for the Windows operating system.

1.2. Results

1.2.1. Isolation and taxonomic profiling of LAB from tomato and molecular detection of *gadB* gene

A total of six different tomato varieties were used as the source for bacterial isolation. In total, 29 distinct colonies were detected on MRS agar plates incubated under selective anaerobic conditions. To perform a preliminary screening and select colonies of interest, cell morphology was examined under light microscopy, only those exhibiting a rod-shaped (bacillary) morphology were selected, reducing the number of isolates from 29 to 23 for further analyses. The selected isolates were subjected to taxonomic classification by sequencing the 16S rRNA gene. Sequence alignment and species identification were carried out using the BLASTn tool against the NCBI nucleotide database. Based on these analysis, 13 isolates were identified as lactic acid bacteria (LAB). Specifically, five isolates were assigned to the species *Levilactobacillus brevis* (DRBA2, DVBA2, DVBA22, CRAI, CRAR), three to

Lactiplantibacillus plantarum (DRBA1, DVBA1, PRA), and five to *Lactococcus lactis* (CRAC, IBA, IBB, PBMD, PBMC), as detailed in Table 1. Following taxonomic identification, the isolates were screened for the presence of the *gadB* gene to identify strains with potential γ-aminobutyric acid (GABA) biosynthetic capacity. PCR amplification was performed using specific primers targeting *gadB*, and the gene was successfully detected in all 13 LAB isolates (Table 1).

Table 1. Characterisation of LAB strains isolated form different varieties of tomatoes based on 16S rRNA sequence analysis and PCR detection of the *gadB* gene.

Tomato variety	**Species**	**Strain**	***gadB* gene**
Grape Red	*Levilactobacillus brevis*	DRBA2	+
	Lactiplantibacillus plantarum	DRBA1	+
Grape Green	*Lactiplantibacillus plantarum*	DVBA1	+
	Levilactobacillus brevis	DVBA2	+
	Levilactobacillus brevis	DVBA22	+
Cherry Red	*Levilactobacillus brevis*	CRAI	+
	Levilactobacillus brevis	CRAR	+
	Lactococcus lactis	CRAC	+
Plum	*Lactococcus lactis*	IBA	+
	Lactococcus lactis	IBB	+
Beefsteak	*Lactococcus lactis*	PBMD	+
	Lactococcus lactis	PBMC	+
Roma	*Lactiplantibacillus plantarum*	PRA	+

1.2.2. Screening of LAB for GABA production via HPLC and Selection of *L. brevis* CRAI

All 13 isolates tested positive for the presence of the *gadB* gene based on PCR results, consequently, all strains were subjected to GABA production analysis. For quantification, the isolates were cultured under conditions reported in the literature as optimal for GABA synthesis, specifically the supplementation of the growth medium with 236.53 mM (equivalent to 4%) MSG. GABA concentration was determined using HPLC. As shown in Table 2, all isolates

could produce quantifiable amounts of GABA, although the production levels varied significantly among strains, ranging from 0.73 mM to 179.15 mM. Notably, strains belonging to the species *L. brevis* exhibited the highest GABA production, with CRAI and CRAR strains reaching concentrations of 179.15 mM and 165.24 mM, respectively. In contrast, lower GABA levels were observed in strains belonging to *L. plantarum* and *L. lactis* species. Furthermore, *L. brevis* CRAI and CRAR displayed the highest glutamate-to-GABA conversion efficiencies, calculated at 75.75% and 69.88%, respectively (Table 2). These findings highlight *L. brevis* CRAI as the most efficient GABA producer among the tested isolates, making it a promising candidate for further investigation in GABA-enriched functional food applications.

Table 2. GABA production measured by HPLC and conversion rate in LAB strains cultured with 236.53 mM MSG.

Species	**strain**	**GABA mM**	**Conversion yield %**
Levilactobacillus brevis	CRAI	179.15	75.75
	CRAR	165.24	69.88
	DVBA2	7.10	3.00
	DVBA22	4.71	1.99
	DRBA2	0.73	0.31
Lactiplantibacillus plantarum	DRBA1	2.91	1.23
	DVBA1	2.25	0.95
	PRA	3.32	1.40
Lactococcus lactis	CRAC	8.24	3.49
	IBA	36.21	15.31
	IBB	1.99	0.84
	PBMD	0.97	0.41
	PBMC	34.59	14.63

1.2.3. Effect of MSG concentrations on GABA production by *L. brevis* CRAI

Levilactobacillus brevis CRAI strain was selected based on its superior GABA production capacity and subsequently evaluated for GABA biosynthesis under optimal culture conditions. The media was supplemented with different concentrations of MSG, ranging from 0% to 4%. GABA production was shown in Table 3 and seemed to be dependent on the concentration of MSG in the growth medium. Notably, the strain produced 6.94 ± 0.48 mM of GABA even in the absence of MSG. However, a significant increase in GABA levels was observed when 236.5 mM (4%) MSG was added, reaching a maximum production of 179.15 ± 15.14 mM (Table 3). The net GABA yield ranged between approximately 60% and 73%, with the highest value observed at 4% MSG.

Table 3. Quantitative analysis of GABA production by *L. brevis* CRAI in the presence of different MSG concentrations. Results are shown as mean ± SD from two independent experiments.

Strain	**MSG mM (%)**	**GABA mM**	**Net GABA yield %**
Levilactobacillus brevis CRAI	0	6.94±0.48	
	59.1 (1%)	48.32±5.98	70.0±10.1
	118.2 (2%)	77.66±16.46	59.8±13.9
	177.3 (3%)	116.29±13.96	61.6±7.8
	236.5 (4%)	179.15±15.14	72.8±6.4

1.2.4. Comprehensive genome analysis of *L. brevis* CRAI

The genome sequence of *L. brevis* CRAI has been deposited in GenBank under the accession number SAMN48404542. This strain possesses a circular chromosome comprising 2,550,312 base pairs, with an average GC content of 45.9%. Annotation data reveal the presence of two distinct genes encoding glutamate decarboxylase, namely *gadA* and *gadB*, which produce two isoforms of the GAD enzyme [181]. These genes share 64% sequence identity and are in separate loci on the chromosome. The *gadA* gene is part of a gene cluster that includes multiple functionally related genes. Upstream of *gadA* lies *gadR*, a transcriptional regulator. Downstream, the cluster includes *gadC*, encoding a glutamate/γ-aminobutyric acid (GABA) antiporter, followed by *gadA* itself, and *gadX*, which encodes a glutamyl-tRNA ligase. In contrast, the *gadB* gene is not part of this operon-like structure and is located approximately 1.7

Mb away from the *gadA* locus on the chromosome (Figure 7). The *gadA* gene of *L. brevis* CRAI exhibits a high degree of amino acid sequence identity with GABA-related proteins from other *L. brevis* strains described in the literature (Figure 7). In particular, the *gadA* sequence of *L. brevis* CRAI closely resembles that of *L. brevis* ATCC 367 and *L. brevis* CRL2013. However, the organization of the *gad* gene cluster varies among these strains. In *L. brevis* CRAI, as well as in *L. brevis* NCL912, *L. brevis* ATCC 367, and *L. brevis* CD0817, the *gadA* gene is located within the *gad* operon. In contrast, in *L. brevis* CRL2013 and YSJ3, the *gadB* gene is incorporated into the operon structure [181,186,187]. Despite this difference, the amino acid sequence of *gadB* of these strains shows 99% identity with that of *L. brevis* CRAI. Moreover, *L. brevis* YSJ3 displays a high degree of sequence conservation across all GAD cluster proteins when compared to *L. brevis* CRAI. On the other hand, *L. brevis* NCL912 and CD0817 show lower amino acid identity for the GAD-related proteins. Specifically, the *gadA*-encoded proteins in both *L. brevis* NCL912 and *L. brevis* CD0817 share only 91% identity with the corresponding protein in *L. brevis* CRAI.

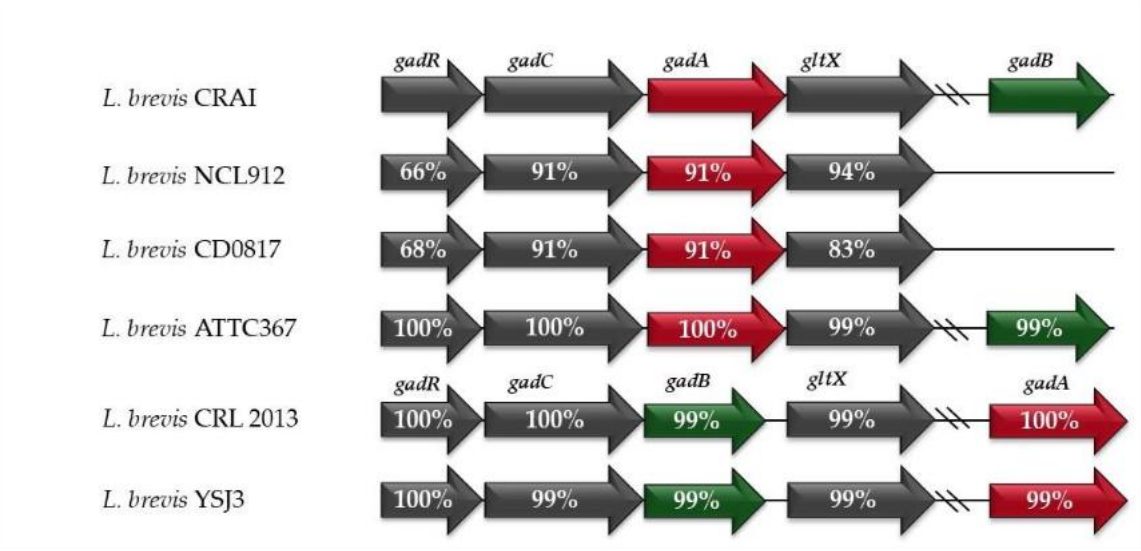


Figure 7. Gene organization of the GAD operon in *Levilactobacillus brevis* CRAI and comparison with other *L. brevis* strains.

The diagram illustrates the arrangement and orientation of genes within the operon. Amino acid sequence identity percentages between each gene product and the corresponding *L. brevis* CRAI protein are indicated within the arrows. The operon includes: *gadR* (a transcriptional regulator), *gadC* (encoding the glutamate/GABA antiporter), *gadA* and *gadB* (coding for glutamate decarboxylases A and B, respectively), and *gltX* (encoding the glutamate-tRNA ligase). Arrows represent the direction of transcription for each gene.

Further genome annotation of *L. brevis* CRAI revealed the presence of genes involved in riboflavin biosynthesis. Specifically, the *rib* operon comprises the genes *ribG*, *ribB*, *ribA*, *ribE*, and *ribH*, which collectively participate in the biosynthetic pathway for riboflavin. At the terminal region of the operon, a regulatory element belonging to the RFN family was also identified [188]. Additionally, genes associated with potential health-promoting functions were detected. Among them, the *bsh* gene, encoding bile salt hydrolase (BSH), was identified. This enzyme catalyses the hydrolysis of the amide bond in conjugated bile salts such as cholylglycine, contributing to cholesterol reduction through its deconjugation activity [189]. The safety profile of the strain was further evaluated using multiple bioinformatics tools, including AMRFinderPlus, ResFinder, and the Comprehensive Antibiotic Resistance Database (CARD). Both AMRFinderPlus and ResFinder did not detect any genes linked to antibiotic resistance or virulence factors. However, CARD analysis identified the presence of *vanT* and *nimA* genes potentially associated with resistance mechanisms. The *vanT* gene encodes an alanine racemase enzyme, while *nimA* encodes a protein belonging to the pyridoxamine 5′-phosphate oxidase superfamily, which is involved in vitamin B6 metabolism. However, neither of these genes is associated with antibiotic resistance. Whole-genome analysis of *L. brevis* CRAI also revealed the presence of two plasmids. According to annotation performed with the RAST platform, these plasmids carry genes implicated in potassium homeostasis and bile salt hydrolase activity. Importantly, no virulence factors or antibiotic resistance genes were identified on either plasmid.

1.2.5. Expression profile of *gadA* and *gadB* genes in *L. brevis* CRAI

Total mRNA was extracted from *L. brevis* CRAI to evaluate the expression levels of the *gadA* and *gadB* genes under varying concentrations of MSG. This analysis, conducted using RT-PCR, aimed to assess the impact of MSG concentration on GABA biosynthesis. Cultures grew under optimal conditions such as initial inoculum of $1x10^7$ CFU/mL, pH adjusted to 5.0, incubation at 32 °C for 48 hours. MSG was supplemented at a concentration of 236.53 mM (4%), and gene expression in this condition was compared to that observed in a control culture with no MSG supplementation (0%). The results indicate a significant induction of gene expression in response to MSG. Specifically, the presence of 4% MSG led to a 52.52-fold increase in *gadA* mRNA levels and a 4.44-fold increase in *gadB* mRNA levels, relative to the baseline condition (figure 8).

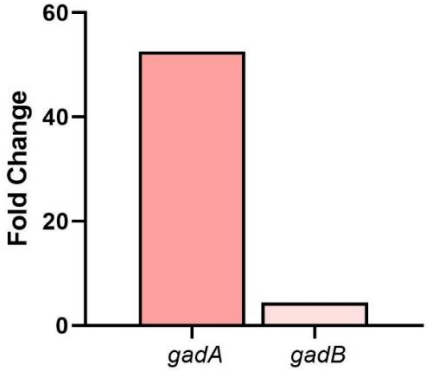


Figure 8. Relative expression levels of *gadA* and *gadB* in *L. brevis* CRAI after 48 h of growth in MRS medium supplemented with 4% MSG. Gene expression was quantified by RT-qPCR and normalized to the reference gene (16S). Results are expressed as fold change relative to the 0% MSG condition.

1.2.6. Inhibitory effects of *L. brevis* CRAI on the growth of enteropathogenic bacteria

The probiotic potential of *L. brevis* CRAI was further evaluated considering the growing scientific interest in beneficial bacteria capable of exerting multifunctional roles, including the modulation of the gut–brain axis through the production of bioactive compounds. In this context, the strain's ability to counteract gastrointestinal infections was investigated, thereby highlighting its health-promoting properties [189]. To assess this, three representative enteropathogenic bacteria were selected: enterotoxigenic *Escherichia coli* (ETEC), *Salmonella choleraesuis* serovar Typhimurium, and *Yersinia enterocolitica*. Both the cell-free supernatant (CFS) and viable cells of *L. brevis* CRAI were tested for antimicrobial activity. The CFS was used to evaluate the inhibition of planktonic growth, while the antimicrobial action of viable cells was assessed through an overlay assay. The results demonstrated a strong inhibitory effect of the CRAI-derived CFS on pathogen growth. Specifically, planktonic growth was reduced by 97.24 ± 1.53% for *E. coli* ETEC, 97.53 ± 0.74% for *S. choleraesuis*, and 99.12 ± 0.67% for *Y. enterocolitica*, compared to the untreated control (Table 4). Viable cells of *L. brevis* CRAI also exhibited antimicrobial activity, forming inhibition zones around 16 mm against *E. coli* ETEC and *S. choleraesuis*, and approximately 20 mm against *Y. enterocolitica* (Table 4).

Table 4. Inhibitory effect of *Lactobacillus brevis* CRAI cell-free supernatant (CFS) and viable cells against enteric pathogens. The tested enteropathogens included *Escherichia coli* ETEC, *Salmonella enterica* serovar Typhimurium (*S. choleraesuis*), and *Yersinia enterocolitica*. Inhibitory activity of the CFS was quantified by measuring the reduction in optical density at 600 nm (OD600) in treated cultures compared to the untreated control, set as 0% inhibition ±SD. Antimicrobial activity exerted by viable cells of *L. brevis* CRAI was evaluated through

the measurement of inhibition zones and categorized as follows: +, inhibition radius between 14 and 18 mm; ++, inhibition radius greater than 18 mm.

***L. brevis* CRAI**	***E. coli* ETEC**	***S. choleraesius* typhimurium**	***Y. enterocolitica***
CFS (%)	97.24±1.53	97.53±0.74	99.12±0.67
Viable cells (mm)	+	+	++

1.2.7. Characterization of the anti-inflammatory potential of *L. brevis* CRAI

Inflammation is often associated with dysbiosis, a condition characterized by an imbalance in the gut microbiota, which is frequently implicated as an indirect contributor to the inflammatory process. Based on this, we aimed to investigate the anti-inflammatory potential of *L. brevis* CRAI, with the broader goal of restoring intestinal homeostasis through probiotic supplementation [188]. In the context of inflammation, the murine macrophage cell line RAW 264.7 is commonly employed to assess inflammatory responses. In our study, we evaluated inflammation by quantifying nitric oxide (NO), a key pro-inflammatory mediator. Lipopolysaccharide (LPS) was used to stimulate the release of cytokines and mediators such as NO. To determine NO production by macrophages, we employed the Griess assay. RAW 264.7 cells were stimulated with LPS and treated either with the cell-free supernatant or the bacterial cells of *L. brevis* CRAI. NO levels were then quantified and compared to control conditions. As illustrated in Figure 9, LPS-stimulated RAW 264.7 cells showed a significant reduction in NO production when treated with *L. brevis* CRAI. Specifically, treatment with the bacterial supernatant resulted in a 16.50 ± 1.24% reduction, while treatment with bacterial cells led to a 32.33 ± 2.69% decrease in NO levels, compared to the LPS-stimulated control.

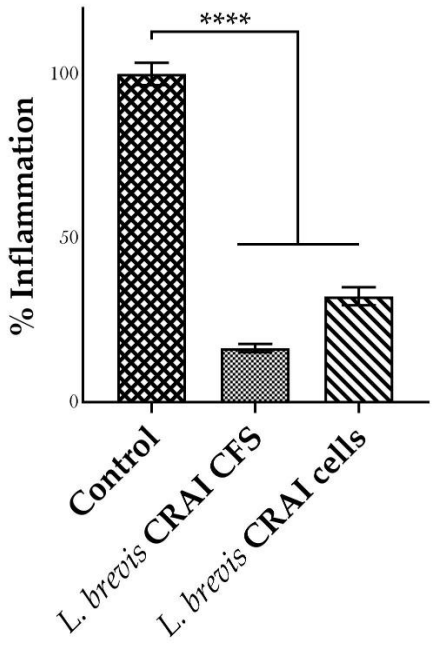


Figure 9. Anti-inflammatory effects of *L. brevis* CRAI CFS and viable cells. RAW264.7 macrophage cells were stimulated with LPS and subsequently treated with either the CFS or viable cells of *L. brevis* CRAI. Nitric oxide (NO) production is presented as a percentage relative to the untreated control group (set at 100%). Statistical significance was indicated by **** ($p < 0.0001$).

1.2.8. Evaluation of *L. brevis* CRAI adhesion to human intestinal epithelial cell line Caco-2

The human intestinal epithelial cell line Caco-2 was employed as an *in vitro* model to assess the adhesion capacity of *L. brevis* CRAI bacterial cells. A quantitative approach based on the enumeration of adherent viable cells was utilized. The results demonstrated that *L. brevis* CRAI could adhere to Caco-2 cells, with a ratio of viable adherent *L. brevis* cells to Caco-2 cells calculated at (3.06 ± 0.71):1.

1.3. Discussion

GABA is one of the most important inhibitory neurotransmitters in the central nervous system (CNS), playing a key role in various physiological processes, including the regulation of the sleep–wake cycle, motor function, and vascular tone. Beyond its neurological function, GABA also contributes to brain metabolism by providing energy and enhancing resistance to hypoxia and other stress conditions [190]. Due to its analgesic, antihypertensive, and antidepressant properties, GABA is widely used in the food and pharmaceutical industries [191]. It is well

established that lactobacilli residing in the gastrointestinal tract can synthesize GABA, and this metabolic activity is associated with beneficial effects on host health [97]. The first goal of the project was to identify a bacterial strain capable of efficient GABA production while also exhibiting multiple functional properties. To achieve this, six different tomato varieties were used as the source for the isolation of LAB. The isolated strains were then taxonomically characterized by 16S rRNA gene sequencing, leading to the identification of 13 distinct LAB strains belonging to the species *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, and *Lactococcus lactis*. The ability of LAB strains to produce GABA is due to the presence of the *gad* gene in their genome, which encodes the key enzymes involved in this biosynthetic pathway [186]. Specifically, *gadA* and *gadB* genes encode for two homologous forms of the glutamate decarboxylase (GAD) enzyme, and LAB strains may carry one or both genes [170]. In this study, PCR analysis was conducted to detect the presence of the *gadB* gene, which is considered the most conserved and widespread among LAB [192], with the objective of selecting potential GABA-producing strains. The PCR results confirmed the presence of the *gadB* gene in all thirteen isolates, thereby supporting their potential for GABA biosynthesis.

GABA production is influenced by multiple factors, indeed Wu CH et al. (2018) [193] demonstrated that the GABA synthesis efficiency of *L. brevis* RK03 varies according to fermentation parameters such as temperature, pH, and glutamate concentration. Based on this, fermentations were conducted under optimized conditions to evaluate the capacity of the isolated LAB strains to convert glutamate into GABA. The GABA synthesis pathway, commonly referred to as glutamate-dependent, has also been associated with the ability of bacterial strains to survive under acidic conditions [194]. In fact, the regulation of the pH in the culture medium is a key factor influencing GABA production. Another important variable is the availability of MSG, which serves as the precursor for GABA synthesis. However, excessively high concentrations of MSG (ranging from 6% to 15%) have been shown to inhibit GABA production [195]. Therefore, the LAB strains were cultured under optimal conditions, based on data reported in previous studies [170,172–174]. GABA production was then quantified via HPLC, allowing us to identify the strains with the highest glutamate to GABA conversion capacity. All tested LAB strains demonstrated the ability to produce GABA, though with variable efficiencies. Among them, the strain identified as the most efficient GABA producer was *L. brevis* CRAI, which starting from an initial MSG concentration of 236.53 mM (4%), achieved a conversion yield of 75.75% and a final GABA concentration of 179.15 mM. These results are consistent with recent findings indicating that, among lactic acid bacteria, *L.*

brevis is the species with the highest efficiency in GABA biosynthesis [196–198]. As highlighted by Li H. (2010) [195], GABA production can be enhanced by optimizing various culture parameters. For instance, the initial concentration of glutamate in the medium significantly affects both the growth of *L. brevis* NCL912 and its ability to synthesize GABA. In line with this, the present study tested different MSG concentrations, and GABA production was then quantified. The results showed in Table 3 demonstrate that the highest GABA yield was obtained with 236.53 mM (4%) of MSG. Since GABA biosynthesis is modulated by numerous factors, comparing production levels across different studies is challenging due to variation in experimental protocols. Nevertheless, the culture conditions employed in this study closely resemble those used by Villegas J. et al. (2016) in which *L. brevis* CRL1942 achieved GABA concentration of 255 mM in the presence of 4.5% MSG. This level of productivity is comparable to that observed in our experiments, however, the probiotic properties of *L. brevis* CRL1942 were not characterized in that study [105].

L. brevis CRAI had been selected as the best GABA producer, which prompted us to undertake a deeper characterization by sequencing its genome, with the aim of predicting its functional traits and elucidating the organization of genes involved in GABA biosynthesis. Genome annotation revealed that *L. brevis* CRAI harbours both *gadA* and *gadB* genes. The *gadA* gene is embedded within the *gad* operon, whereas *gadB* is located separately. A similar gene arrangement is observed in *L. brevis* ATCC367, where *gadA* resides in the operon, distantly from *gadB* [199]. This arrangement differs from most *L. brevis* strains, in which the gadCB operon configuration is more commonly observed [200]. Despite this divergent genomic placement, the gadA and gadB proteins in various *L. brevis* isolates exhibit a high degree of amino acid sequence identity, reflecting high degree of sequence conservation. In addition, genomic analysis revealed the presence of genes potentially linked to probiotic and functional activities, including those involved in bile salt hydrolysis and riboflavin biosynthesis. Notably, the gene encoding the choloylglycine hydrolase (BSH) enzyme, a bile salt hydrolase, was detected; this enzyme catalyses the deconjugation of bile salts. Such enzymatic activity is associated with bacterial tolerance to bile salts, as it allows liberated amino acids to serve as carbon and nitrogen sources, thereby aiding detoxification of the intestinal environment and possibly exerting hypocholesterolemic effects [200]. It has been proposed that BSH produced by the gut microbiota and beneficial microbes may influence host–microbiota crosstalk by modulating bile acid profiles [201]. Many LAB strains are capable of producing a diverse array of metabolites, including water-soluble B vitamins (such as riboflavin and folate) and low-

calorie sugars like exopolysaccharides, which have potential applications in the food and dairy sectors [202]. The biosynthesis of riboflavin from guanosine triphosphate (GTP) and ribulose 5-phosphate requires the activity of five enzymes encoded by *ribG*, *ribB*, *ribA*, *ribE*, and *ribH* genes, as described by Perkins et al. (1999) [203]. Interestingly, the arrangement of these genes within the operon does not reflect the sequential order of enzymatic steps in the riboflavin biosynthetic pathway. In the genome of *L. brevis* CRAI, we identified the complete operon along with the upstream regulatory region RFN, which controls transcription of the biosynthetic genes. The capacity of a probiotic strain to synthesize riboflavin is especially relevant, given that it is an essential metabolite that humans cannot biosynthesize. In situ production of riboflavin by beneficial gut bacteria may confer direct advantage to the host, while also supporting the stability of commensal populations like bifidobacteria [204]. Hence, the combination of bile salt stress tolerance with the ability to produce B vitamins comprises a functional trait set that strengthens the probiotic potential of the strain. To further characterize the safety profile of *L. brevis* CRAI, various bioinformatic approaches were employed. Using the RGI (Resistance Gene Identifier) software integrated within the CARD database, two putative antimicrobial resistance (AMR) genes were detected, *vanT* and *nimA*, both showing low sequence identity (32% and 48%, respectively). These same genes have been found in *L. brevis* CRL 2013, where *vanT*, a member of the racemase family, was deemed essential for LAB growth and not associated with the *vanG* cluster known to confer vancomycin resistance. In the same study, *nimA* was identified as encoding an enzyme implicated in vitamin B6 metabolism [205]. The genome sequencing also revealed the presence of two natural plasmid related sequences, previously observed in other *L. brevis* strains [206,207]. Functional annotation of the plasmid-borne genes disclosed involvement in potassium homeostasis and bile salt hydrolysis, whereas no virulence factors or antibiotic resistance genes were identified. Based on these results, *L. brevis* CRAI can be regarded as genetically safe, because of the absence of known virulence determinants or antimicrobial resistance genes.

The genomic analysis of *L. brevis* CRAI revealed, as already mentioned, the presence of two genes, *gadA* and *gadB*, implicated in the biosynthesis of GABA. To assess whether the differences observed in GABA production could be associated with variations in gene expression, we evaluated the transcriptional levels of these genes under two culture conditions, with and without the addition of MSG at 236.53 mM (4%). Our results demonstrated a significant upregulation of *gadA* and *gadB* mRNA, with increases of approximately 52.5-fold and 4.4-fold, respectively, in the presence of 4% MSG compared to the absence of the precursor.

These findings indicate that the expression of both genes is induced by extracellular glutamate. Notably, the *gadA* gene exhibited a considerably stronger induction, which is likely attributable to its location within an operon preceded by a regulatory element, referred to as *gadR.* Previous studies in *L. lactis* strains have shown that the transcriptional regulator *gadR* activates downstream gene expression in response to glutamate availability in the growth medium. Similarly, disruption of the *gadR* gene in *L. brevis* ATCC367 significantly impaired GABA synthesis due to diminished expression of *gadC* and *gadA* [208]. Based on these observations, it can be postulated that the regulatory sequence *gadR*, positioned upstream of the operon, is activated by glutamate presence, thereby strongly promoting the transcription of *gadA*.

LAB thanks to their notable functional traits, are regarded as promising probiotic candidates. The assessment of probiotic features typically includes their antagonistic activity against pathogenic microorganisms, anti-inflammatory effects, and the ability to adhere to epithelial cells [209]. In this part of the project, we evaluated the inhibitory capacity of *L. brevis* CRAI against *Escherichia coli* ETEC, *Salmonella choleraesuis*, and *Yersinia enterocolitica*. Initially, we demonstrated that CFS of *L. brevis* CRAI significantly suppressed the planktonic growth of these enteropathogens. Subsequently, the effect of viable *L. brevis* CRAI cells on the viability of the pathogens was assessed. Both bacterial CFS and viable cells exhibited antimicrobial activity against the tested enteropathogens, with a more pronounced effect observed against *Y. enterocolitica*. This antimicrobial effect is likely attributable to the production of organic acids, hydrogen peroxide, bacteriocins, and other bioactive compounds, consistent with observations reported for other *L. brevis* strains [210].

Among the diverse probiotic traits exhibited by functional strains, anti-inflammatory activity is of particular interest [51]. Macrophages, which play a central role in the inflammatory response, detect foreign stimuli through specific receptors and subsequently trigger inflammation. However, excessive production of pro-inflammatory mediators, such as nitric oxide (NO), can lead to hyperactivation of macrophages, potentially resulting in chronic inflammatory conditions including inflammatory bowel disease and arthritis [211]. Within this framework, the present study investigated the modulatory effects of *L. brevis* CRAI on the inflammatory response of RAW264.7 macrophages. Our results demonstrated a marked attenuation of lipopolysaccharide (LPS)-induced inflammation, with inflammation levels reduced to 16% and 32% upon treatment with CFS and viable *L. brevis* CRAI cells, respectively. Supporting these findings, previous research reported that *L. brevis* KU15147 suppresses nitric oxide and

prostaglandin E2 production by downregulating inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, without exerting cytotoxic effects [212]. Thus, our data further supports the anti-inflammatory potential of *L. brevis* CRAI.

The ability to adhere to and colonize the intestinal epithelium is widely recognized as a desirable characteristic for probiotic bacteria [213]. Liu et al. (2022) have demonstrated that adhesion efficiencies of various *L. brevis* strains to Caco-2 cells range from 1.98% to 8.53% [198]. Consistent with these observations, our results indicate that *L. brevis* CRAI exhibits a considerable adherence capacity to the Caco-2 cell line, with an approximate ratio of three bacterial cells per epithelial cell.

Chapter 4

1. Optimizing GABA-enriched fermentations using tomato and peach pomace with *L. brevis* CRAI: a circular bioeconomy perspective

1.1. Material and methods

1.1.1. Microbial isolates and experimental reagents

The *Levilactobacillus brevis* CRAI strain, preserved at the Department of Pharmacy and Biotechnology (FABIT) of the Alma Mater Studiorum – University of Bologna, was selected based on its demonstrated ability to synthesize GABA [214]. The strain was maintained at –80°C in glycerol stocks and, prior to experimental use, was first plated onto MRS (Difco, Detroit, MI, USA) agar 1,5%, enriched with 0.05% (w/v) L-cysteine (Merck, Milan, Italy) incubated at 37°C for 24 hours in anaerobiosis to ensure optimal revival and metabolic activity. Monosodium glutamate (MSG), supplied by SIGMA, was prepared by dissolving the compound in distilled water to a final concentration of 467 g/L. The solution was then sterilized through filtration using a 0.2 μm pore-size membrane to ensure sterility.

1.1.2. Preparation and screening of agro-industrial by-products for *L. brevis* CRAI fermentation

To investigate the fermentative capacity of *L. brevis* CRAI and its potential for GABA production, various agri-food by-products were selected as alternative substrates. Pomace materials derived from peach, apple, tomato, grape, and apricot were obtained by processing fresh fruits sourced from a local market. The fruits were manually juiced, and only the residual solid fraction (pomace) was retained for further use. The pomace samples were homogenized

and adjusted to pH 5.0 using either 5 M sodium hydroxide (NaOH) or 5 M hydrochloric acid (HCl), depending on the initial pH of the matrix. Each sample (35 g) was transferred into sterile 50 mL Falcon tubes and subjected to a short thermal pre-treatment at 75 °C for 10 minute to reduce native microbial load. Once cooled to room temperature, the samples were divided into two experimental groups: 1) substrate alone (control); 2) substrate inoculated with *L. brevis* CRAI. To prepare the inoculum, an overnight culture of *L. brevis* CRAI was harvested by centrifugation at 5000 × g for 10 minutes, followed by two washes with sterile physiological saline (0.9% NaCl) solution. The resulting pellet was resuspended in 10 mL of saline solution and added to the substrate at 10% (v/w), reaching an approximate final microbial concentration of $1x10^7$ CFU/mL. Fermentation trials were conducted at 32°C, and samples were collected at 0, 24, 48, and 72 hours to monitor microbial growth and pH variations. Microbial enumeration was performed by preparing serial decimal dilutions in sterile saline, followed by plating on MRS agar. Plates were incubated at 37 °C for 48 hours, and colony-forming units (CFU) were counted and expressed as CFU per gram of sample. For pH analysis, 1 mL of each fermented sample was analysed using a calibrated pH meter (Mettler Toledo). This allowed for monitoring the acidification profile of each fermentation over time.

1.1.3. Optimization of GABA biosynthesis by *L. brevis* CRAI in tomato and peach pomace substrates using Response Surface Methodology (RSM)

1.1.3.1. Experimental procedure

Tomato and peach pomace substrates were prepared following the previously described protocol. Prior to fermentation, the substrates were sterilized at 121 °C for 15 minutes. After cooling, MSG was added at a concentration of 2%, along with a bacterial inoculum corresponding to 10% (v/w). The Central Composite Design (CCD) allows for a substantial reduction in the number of experimental trials by selecting only a subset of all possible factor combinations. This approach, referred to a statistical methodology as *confounding* (Gacula, 1988), enables efficient and robust experimental planning. Although a standard CCD typically involves 17 runs, in our study the design was extended by incorporating five additional experimental points (runs 18 through 22) (Table 5). These supplementary runs were strategically included to improve model prediction accuracy, particularly at the lower and upper bounds of the three independent variables under investigation in accordance with Box et al. (1978) [215] and as recommended by Belletti et al. (2010) [216]. Samples were collected at the start (0 hour) and at the end (48 hour) of fermentation for microbial enumeration and pH

measurement, performed as described in Section 7.1.2. Remaining samples were stored at -20 °C for subsequent analyses, including GABA quantification and volatile compound profiling.

Table 5. Levels of independent variables used in the Central Composite Design*.

Run	Yeast extract (%)	pH	Matrice Dilution (%)
1	0.25	4	25
2	0.75	4	25
3	0.25	6	25
4	0.75	6	25
5	0.25	4	12,5
6	0.75	4	12,5
7	0.25	6	12,5
8	0.75	6	12,5
9	0.50	5	16,6
10	0.50	5	16,6
11	0	5	16,6
12	1	5	16,6
13	0.50	3	16,6
14	0.50	7	16,6
15	0.50	5	50
16	0.50	5	10
17	0.50	5	16,6
18	0	3	50
19	1	7	10
20	0	4	25
21	0.25	3	25
22	0.25	4	50

*The CCD was supplemented with five extra combinations (18, 19, 20, 21, and 22) to improve prediction accuracy at the extreme low and high levels of the three independent variables.

1.1.3.2. High-Performance Liquid Chromatography (HPLC) quantification for GABA production

Frozen samples were first thawed, then vortexed for 1 minute, followed by centrifugation at 15,000 rpm for 10 minutes. The resulting supernatant was collected and utilized for GABA quantification analysis. The determination of GABA concentration was performed according to the protocol described by Pizzi et al. (2025), with modifications applied to the initial extraction step of the compound [214]. For GABA quantification, the samples collected during the fermentation were defrosted then vortexed for 1 min and centrifugated at maximum speed for 10 minutes. The supernatant was collected and used for GABA quantification analysis.

1.1.3.3. Volatilome profile assessment using Solid Phase Microextraction (SPME) coupled with Gas Chromatography (GC)- Mass Spectrometry (MS)

The volatilome profiles of peach and tomato pomace substrates, analysed both prior to and following fermentation, were evaluated using solid-phase microextraction (SPME) coupled with gas chromatography–mass spectrometry (GC-MS), according to the methodology described by Siroli et al. [217]. Briefly, 3 grams of each sample were placed into sterile vials, and 6 µL of an internal standard solution (4-methyl-2-pentanol) was added to achieve a final concentration of 20 mg/kg. Samples were preheated at 45 °C for 10 minutes to facilitate equilibration of volatile compounds. Volatile compounds were extracted from the headspace using an 85 µm Carboxen/polydimethylsiloxane (Carboxen/PDMS) fiber (StableFlex, Supelco, Bellefonte, PA, USA) exposed for 30 minutes. The fiber was then desorbed for 10 minutes in the GC injector. Analytical separation was performed using an Agilent 7890A GC system coupled with an Agilent 5975C quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with a Chrompack CP-Wax 52 CB capillary column (Chrompack, São Paulo, Brazil), under the chromatographic conditions described by Siroli et al. [217]. Compound identification was achieved by matching the acquired mass spectra against reference libraries, including the NIST/EPA/NIH Mass Spectral Library (version 1.6, 2011) and the WILEY Library (6th edition, 1995, New York, NY, USA). The relative abundance of each identified compound was calculated as a percentage of the total area of all detected peaks within the chromatogram.

1.1.3.4. Development of the response surface model from CCD data

Data obtained from the Central Composite Design (CCD) were analysed using multiple regression analysis performed with Statistica 6.1 software (StatSoft Italy srl, Vigonza, Italy). The influence of yeast extract concentration, initial pH, and substrate dilution on GABA production after 48 hours of fermentation was evaluated. Model optimization was carried out using a backward stepwise elimination approach, progressively removing non-significant terms ($p > 0.05$) and incorporating them into the residual error. Consequently, only statistically significant main effects and quadratic terms ($p \leq 0.05$) were retained in the final predictive model. Model fit and adequacy were assessed through analysis of variance (ANOVA) and by calculating determination coefficients (R^2 and adjusted R^2), following the methodology described by Patrignani et al. (2016) [218].

1.1.4. Statistical analysis

Microbiological data were statistically analysed using two-way ANOVA followed by multiple comparison tests in Prism software. Results are presented as mean values ± standard deviation.

The Central Composite Design (CCD) was applied to model GABA production as a function of the selected independent variables. Data analysis and model fitting were carried out using Statistica for Windows (StatSoft, Tulsa, USA), which was employed to generate a second-order polynomial regression model based on the experimental data. The general form of the model is expressed as:

$$\gamma = \Sigma B_i \chi_i + \Sigma B_{ii} \chi_i^2 + \Sigma B_{ij} \chi_i \chi_j$$

where γ represents the response variable (i.e., GABA concentration), B_i, B_{ii}, and B_{ij} denote the coefficients for the linear, quadratic, and interaction effects respectively, and χ_i and χ_j are the coded values of the independent variables. Terms that did not reach statistical significance ($p > 0.05$) were excluded from the final model to improve predictive reliability and reduce overfitting. The fitted model enabled the quantitative assessment of the individual (linear), squared (quadratic), and combined (interaction) contributions of the variables to the response. To visualize the influence of these effects, three-dimensional response surface plots were generated, allowing for a clearer interpretation of the interactions among factors and their impact on GABA production.

1.2. Results

1.2.1. Evaluation of agri-industrial by-products as fermentation substrates for *L. brevis* CRAI

We investigated the growth capacity of *L. brevis* CRAI in five different by-product substrates: tomato, grape, peach, apricot, and apple pomace. As shown in Figure 10, tomato proved to be the most suitable substrate for supporting bacterial proliferation. In this matrix, an increase in viable cell counts was observed after just 24 hours of fermentation, reaching approximately 10^8 CFU/g, compared to the initial count at time 0. Bacterial growth significantly increases over the fermentation period, achieving 10^9 CFU/g after 72 hours. In peach pomace, a similar increase in viable cells was observed at 24 hours, with also reaching 10^8 CFU/g. However, in contrast to tomato, no further significant growth occurred beyond this time point, with cell concentrations remaining stable until 72 hours. In the remaining substrates, apple, apricot, and grape pomace, *L. brevis* CRAI exhibited limited proliferation. Viable cell counts showed only minimal increases over time, with no statistically significant differences from the initial levels during the entire 72-hour fermentation period. Supporting these microbial growth trends, Figure 11 presents pH measurements taken throughout the fermentation process. A marked decrease in pH was observed in both tomato and peach matrices, indicating active acidification driven by the metabolic activity of *L. brevis* CRAI. This drop in pH further validates the higher microbial activity in these two substrates compared to the others.

To further investigate the basis for the observed differences in bacterial performance, the macronutrient composition of the tested substrates was summarized in Table 6 [219–222]. As reported in Table 6, the protein content of tomato pomace was substantially higher than that of the other tested matrices, ranging from 10.5 to 25 g per 100 g of product, depending on the cultivar and source. This markedly elevated protein level is consistent with the well-documented nutrient profile of tomato-based substrates, which are rich in amino acids and peptides that support the metabolic requirements of lactic acid bacteria. In contrast, the protein content of the other by-products, such as apple, peach, and apricot pomaces, was significantly lower, typically between 0.7 and 5.6 g per 100 g, thereby offering a more limited nitrogen supply for microbial growth and enzyme synthesis. Surprisingly, grape pomace which exhibited relatively higher protein content than apple, apricot and apple pomace did not resulted as a promising substrate for the growth of *L. brevis* CRAI. Compared to tomato, all the other substrates presented higher amount of carbohydrates, especially grape which can play a role in

microbial growth. The overall composition, the presence of naturally present inhibitors and their ratios may restrict or promote the efficient LAB proliferation. GABA content was then measured in all fermented matrices; however, the levels detected were below the quantification limit of the HPLC method employed. Therefore, a series of optimization experiments aimed at enhancing GABA biosynthesis were carried out using a Central Composite Design (CCD) approach, applied exclusively to the matrices in which *L. brevis* CRAI was able to grow (tomato and peach pomace).

Table 6. Macronutrient composition (carbohydrates, proteins, and lipids) of tomato, peach, apricot, apple, and grape pomace. Values are reported per 100 grams of fresh weight (FW) for fruit pomaces and per 100 grams of dry weight (DW) for grape pomace. Where applicable, value ranges indicate variability among different cultivars or data reported in multiple studies.

	Tomato (g/100g)	Peach (g/100g)	Apricot (g/100g)	Apple (g/100g)	Grape (g/100g)
Total Carbohydrates	3.9-8.0	10.8-15.7	9.3-9.4	48.0-62.0	1.4-77.5
Proteins	10.5-25.03	0.7-1.5	1.1-4.9	2.9-5.6	5.4-14.2
Fats	3.6-5.9	0.2-0.8	0.2-3.1	1.2-3.9	8.2-11.9

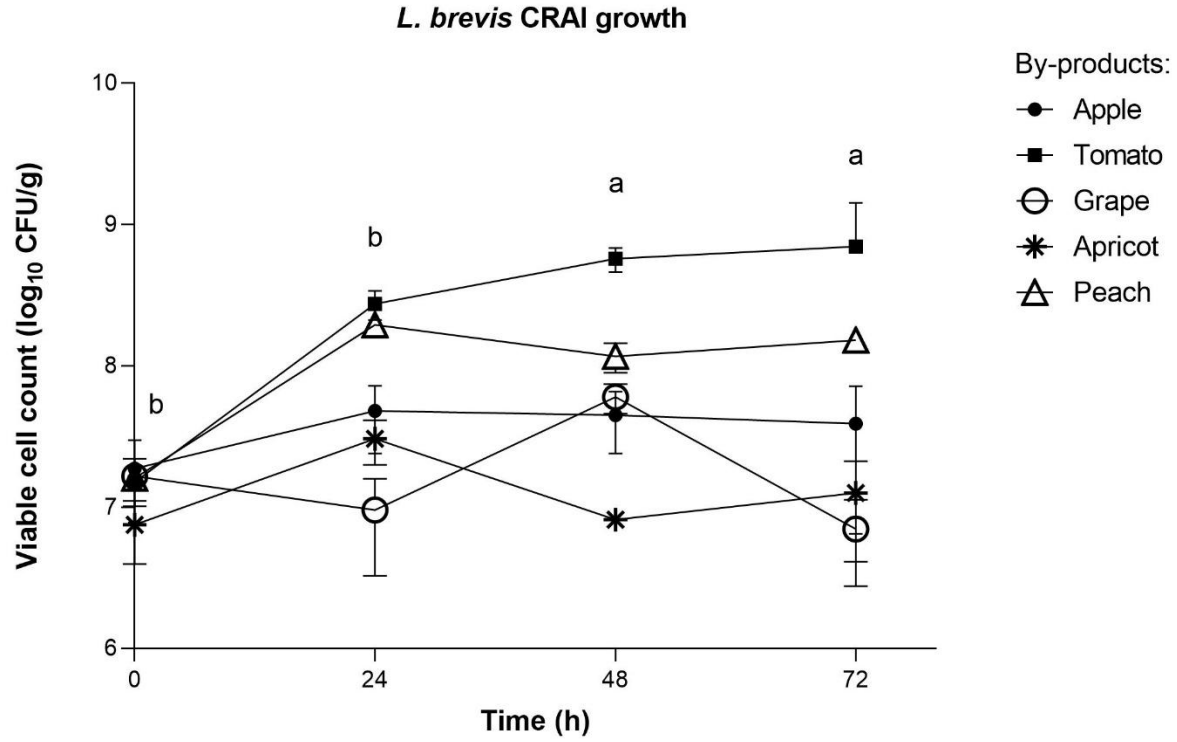


Figure 10. *L. brevis* CRAI cell load (log_{10} CFU/g) during fermentation in five different fruit-based substrates: apple, tomato, grape pomace, apricot, and peach. Cell concentrations were determined at 0, 24, 48, and 72 hours of fermentation at 32°C. Different lowercase letters indicate statistically significant differences among substrates at the same time point ($p < 0.05$).

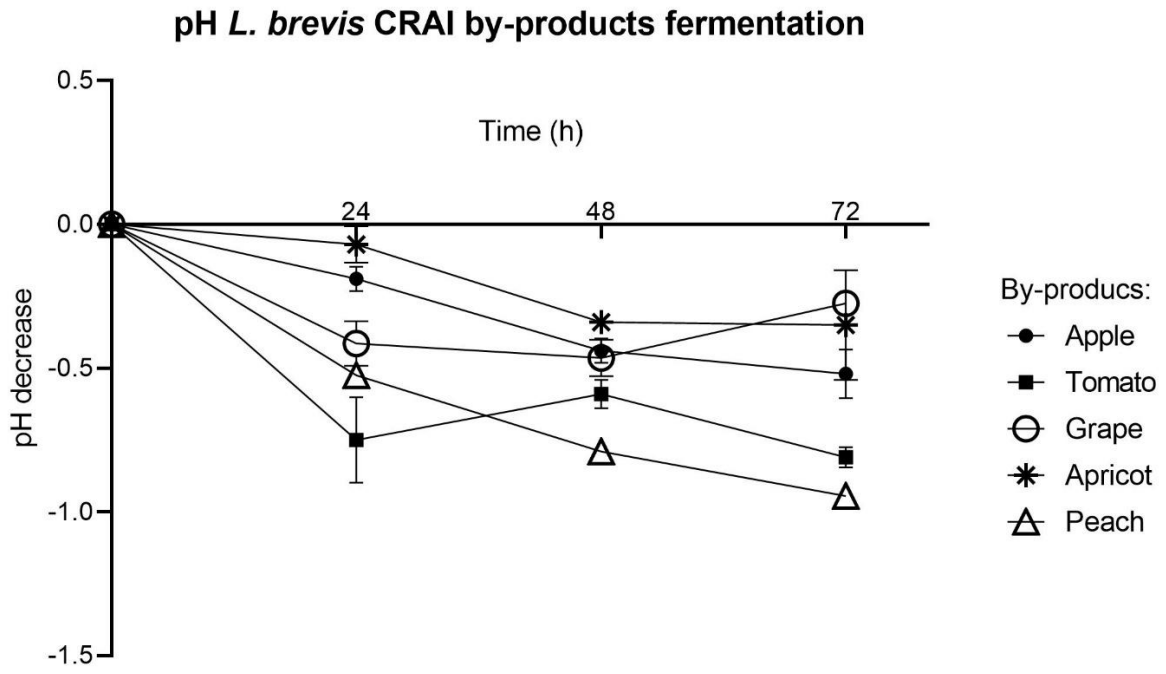


Figure 11. pH reduction in fruit-based substrates (peach, tomato, apple, grape pomace, and apricot) during fermentation with *L. brevis* CRAI over 72 hours at 32°C. pH decrease is expressed as the difference between the pH at each time point (24, 48, and 72 h) and the initial pH (0 h) for each substrate.

1.2.2. Optimization of fermentation parameters for GABA production by *L. brevis* CRAI

The effect of yeast extract concentration, initial pH, and matrix dilution on GABA production by *L. brevis* CRAI in tomato pomace was analysed using a central composite design (CCD). The design comprised 22 runs, including factorial, axial, and central points. Experimental, predicted, and residual values are reported in Table 7 wich presents the polynomial equation representing the linear, interaction, and quadratic effects of the independent variables on GABA production. Yeast extract was selected as the nitrogen source and was added at concentrations ranging from 0% to 1%, since higher levels could negatively affect the organoleptic properties of the final product.

Table 7. The predictive models for GABA production in tomato and peach pomace include only those variables that exhibited statistical significance ($P < 0.05$). The resulting best-fit equations are provided. YE: yeast extract, R: regression coefficient; F: *F*-value; SE: standard error of residuals.

Substrate	Equation	R	F	SE
Tomato pomace	$70.849\,[YE] + 149.857\,[pH] - 14.755\,[pH]^2 - 320.488$	0.781	9.36	682.37
Peach pomace	$94.834\,[pH] - 10.200\,[pH]^2 + 19.732\,[pH]\,[YE] - 190.654$	0.876	14.06	478.56

Backward stepwise regression retained yeast extract, pH, and pH^2 as significant predictors of GABA production ($p < 0.01$) in tomato pomace, while dilution and interaction terms were pooled into the residual error. ANOVA confirmed the adequacy of the model ($F = 9.36$; $p = 0.0006$), with an R^2 of 0.609 and an adjusted R^2 of 0.544, and a standard error of estimate of 26.1. Parameter estimates revealed that yeast extract ($\beta = 70.8 \pm 23.8$; $p = 0.008$) and pH ($\beta = 149.9 \pm 37.3$; $p = 0.0008$) exerted significant positive effects on GABA biosynthesis, while the quadratic effect of pH ($\beta = -14.8 \pm 3.8$; $p = 0.001$) described the concave relationship observed. The response surface derived from the regression equation predicted optimal conditions at pH ~5.1 with 1% yeast extract, yielding a maximum concentration of approximately 130.8 mM GABA (≈13.5 g/L) after 48 h fermentation. Also, in peach pomace some discrepancies were noted between observed and predicted concentrations, especially under extreme conditions. Backward stepwise regression retained pH, pH^2, the yeast extract × pH interaction, and the pH × dilution interaction as significant predictors of GABA production ($p < 0.05$), whereas other terms were pooled into the residual error. ANOVA confirmed the model adequacy ($F = 14.06$; $p < 0.001$), with $R^2 = 0.768$, adjusted $R^2 = 0.713$, and a standard error of estimate of 21.9. Parameter estimates indicated that pH ($\beta = 98.9 \pm 31.6$; $p = 0.006$) had a strong positive effect on GABA synthesis, counterbalanced by a significant quadratic term ($\beta = -10.2 \pm 3.3$; $p = 0.006$), which described the curvature of the response. The yeast extract × pH interaction ($\beta = 19.7 \pm 4.1$; $p < 0.001$) further emphasized the synergistic role of yeast extract under favourable pH conditions, while the pH × dilution interaction ($\beta = -0.24 \pm 0.10$; $p = 0.021$) suggested that substrate concentration modulated the pH effect. According to the response surface model, optimal conditions for GABA production in peach pomace were predicted around pH ~5.6 with

0.75% yeast extract, resulting in a maximum concentration of approximately 123 mM GABA (≈12.7 g/L) after 48 h fermentation (Figure 12).

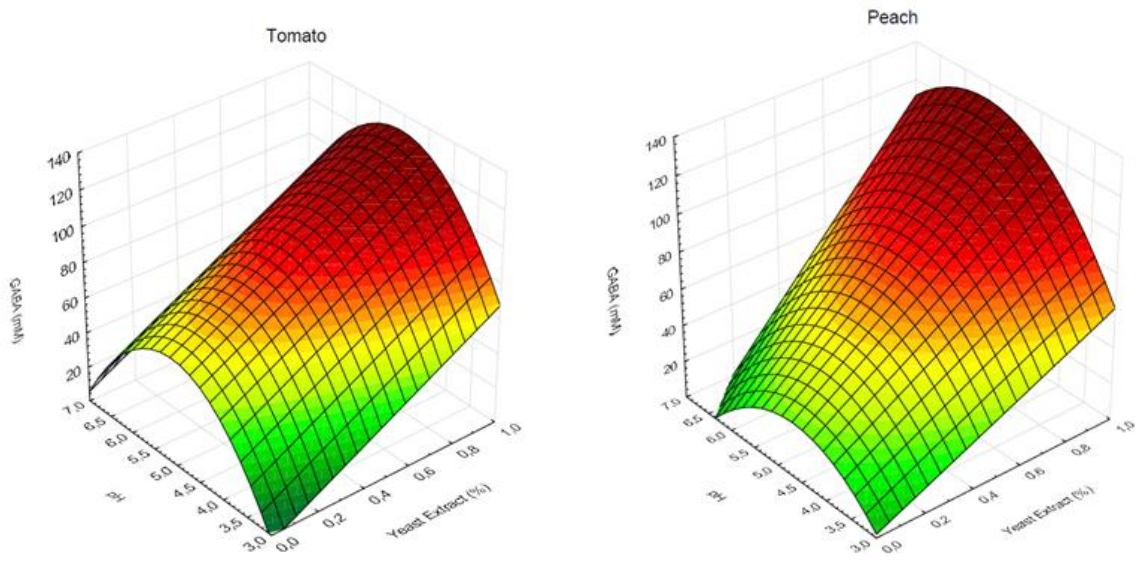


Figure 12. Response surface plots illustrating the effects of initial pH and yeast extract concentration (%) on GABA production (mM) by *L. brevis* CRAI in tomato (left) and peach (right) pomace after 48 hours of fermentation. The model indicates a significant positive linear effect of yeast extract concentration and a quadratic effect of initial pH on GABA yield. One of the three independent variables was not shown in the graphs, a constant value (the central points of the interval considered) was imposed on it.

Overall, the models highlight that the critical factors influencing GABA biosynthesis are both pH and yeast extract, while matrix dilution played a secondary role. Although the regression model for peach showed slightly higher predictive performance, the tomato model predicted higher GABA production.

To validate the model predictions, a confirmatory trial was performed under the optimized conditions. As shown in Table 8, GABA production reached 93.60 ± 5.39 mM in tomato pomace, corresponding to a glutamate conversion yield of 79.15 ± 4.56%. In contrast, the peach pomace supported significantly lower production, with 26.33 ± 1.90 mM GABA and a conversion yield of 22.27 ± 1.60%. Thus, the highest GABA concentration was achieved in the tomato substrate. Although the experimental results were broadly consistent with the predicted trends, the absolute values showed some discrepancies, likely reflecting the intrinsic complexity and variability of by-products, whose composition may differ slightly across trials. In addition

to GABA quantification, bacterial growth and pH changes were monitored throughout fermentation. After 48 h, *L. brevis* CRAI reached 10^8 CFU/mL in tomato pomace and $1x10^9$ CFU/mL in peach pomace (Table 8). Moreover, a slightly lower final pH was measured in peach pomace compared to the tomato one, indicating a more pronounced acidification of this matrix.

Table 8. Predicted values for matrix dilution, yeast extract concentration (%), and initial pH (pH_i), along with the corresponding GABA production (mM) and glutamate conversion rates (%) in tomato and peach matrices. The table also reports the viable cell counts (CFU/mL) of *L. brevis* CRAI and the final pH (pH_f) after 48 hours of fermentation in MRS-based medium.

	Matrice dilution (%)	Yest extract (%)	pH_i	pH_f	GABA (mM)	Yied (%)	CFU/mL
Tomato pomace	50	1.00	5.08	4.8	93.60 ± 5.39	79.15 ± 4.56	10^8
Peach pomace	50	0.75	5.75	4.4	26.33 ±1.90	22.27 ± 1.6	10^9

1.2.3. Volatile compound profiling of tomato and peach pulp fermented by *L. brevis* CRAI after 48 hours

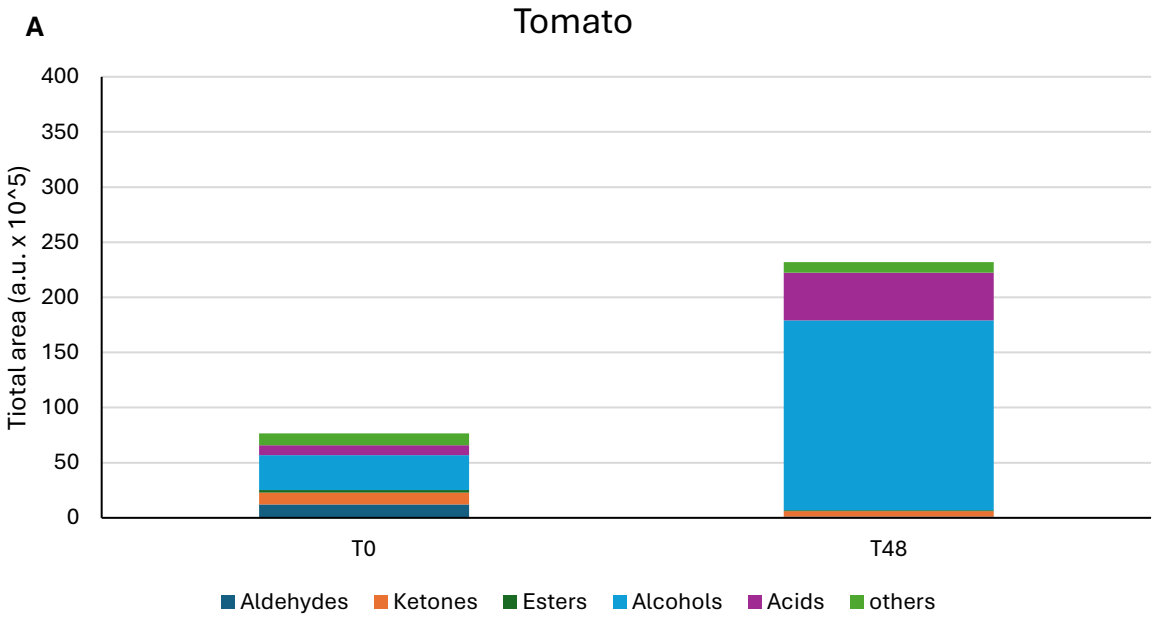
After 48 hours of fermentation, 52 volatile organic compounds (VOCs) were detected in the tomato pomace, while only 23 compounds were identified in the peach pomace. The VOCs found in both tomato and peach samples belong to the chemical classes of alcohols, acids, esters, ketones, aldehydes, and other compounds. As shown in Figure 13, the analysis of the volatile reveals a general increase in the total number of VOCs after fermentation, indeed in fermented tomato and peach pomace there is a rise of the total VOC area compared to the initial time point (T0).

In tomato pomace, the total area at T0 was 76.5×10^5 arbitrary units (a.u.), which increased to 231.8×10^5 a.u. after fermentation. From a qualitative point of view, the T0 sample contained several compounds, including methyl isobutyl ketone, 2-furanmethanol, (Z)-3-hexen-1-ol, phenylethyl alcohol, and acetic acid. Following fermentation, a substantial increase in the alcohol fraction was observed (from 31.7×10^5 to 172.2×10^5 a.u.), primarily due to the production of ethanol, phenylethyl alcohol, and benzyl alcohol. This trend was accompanied by a marked increase in acids, particularly acetic acid, which increased nearly tenfold (from

4.6×10⁵ to 41.0×10⁵ a.u.), reflecting the acidification typical of the heterofermentative metabolism of *L. brevis* species, to which the CRAI strain belongs. In contrast, aldehydes, especially 3-methylbutanal, decreased during fermentation. There is also a reduction of esters and ketones over time, thus contributing less to the final aroma profile.

In peach pulp, only 19 compounds were identified, but a similar fermentation trend was observed. Fermentation induced significant changes in the volatile composition: aldehydes such as 3-methylbutanal and benzaldehyde, which were abundant at T0, disappeared entirely after 48 hours, indicating their active conversion through microbial metabolism. At the same time, alcohols emerged as the most abundant class, increasing from 73.8×10⁵ to 211.1×10⁵ a.u., mainly due to the strong accumulation of ethanol (from 5.1×10⁵ to 143.0×10⁵ a.u.), 1-butanol, 3-methyl-1-butanol, 1-hexanol, and benzyl alcohol. Acids also played a key role at T48, with acetic acid reaching 104.0×10⁵ a.u., along with the formation of butanoic and 3-methylbutanoic acids, which were not detected at T0. Conversely, esters such as ethyl acetate decreased, while ketones (methyl isobutyl ketone and camphor) remained stable. Interestingly, compounds such as estragole increased from 7.1×10⁵ to 12.1×10⁵ a.u.

Volatile compounds are presented by chemical class in Table 9, whereas the individual identified compounds are listed in detail in Appendix 1.



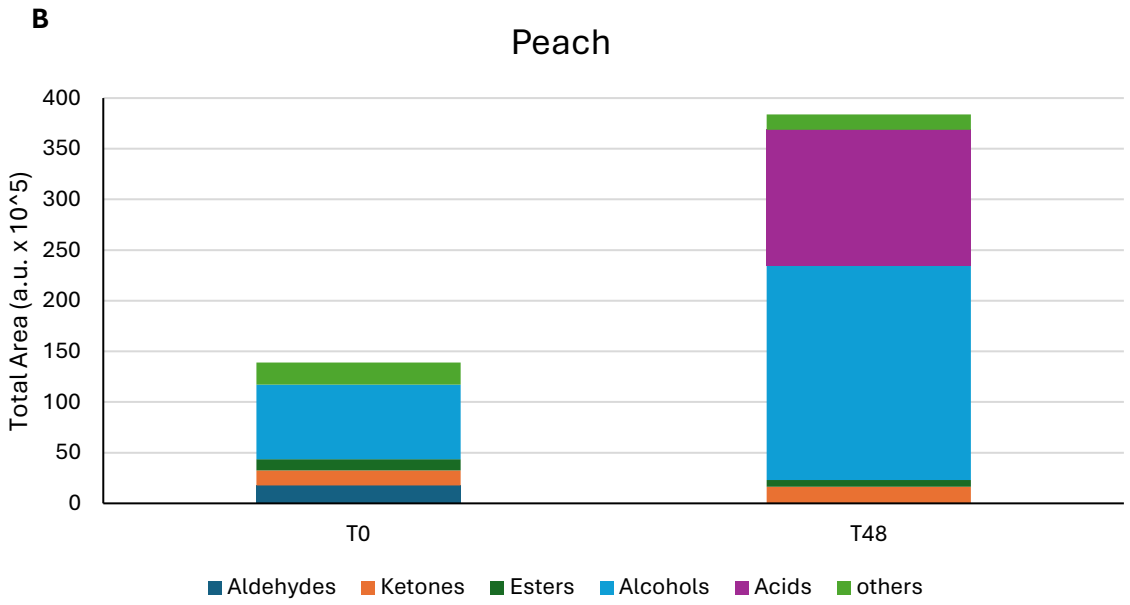


Figure 13. Volatile compound profiles generated during the 48-hour fermentation of two matrices: tomato pomace (A) and peach pomace (B). The graphs present the total signal areas of volatile compounds, categorized by chemical class (aldehydes, ketones, esters, alcohols, acids, and others). Data are expressed in arbitrary units and represent the mean of two biological replicates (n = 2).

Table 9. The table reports the peak area values obtained by GC–MS analysis of volatile compounds detected in fermented tomato and peach substrates at the initial time point (T0) and after 48 hours of fermentation (T48). Volatile compounds were grouped into chemical classes (aldehydes, ketones, esters, alcohols, acids, and other compounds), and for each class the total chromatographic peak area is presented.

	Peak Area Tomato		Peak Area Peach	
	T0	T48	T0	T48
Aldehydes	12.23267	1.045423	17.52375	0
Ketones	10.6813	4.850643	14.90346	16.53673
Esters	2.25547	1.119545	11.03815	6.60193
Alcohols	31.68772	172.1818	73.82776	211.1369
Acids	9.09648	43.22227	0	134.5068
Others	10.54041	9.37099	21.66544	14.92216

1.3. Discussion

According to the Food and Agriculture Organization (FAO), nearly one-third of all food produced globally for human consumption, approximately 1.3 billion tonnes per year, is either lost or wasted [223]. These losses have a significant environmental impact, particularly in terms of greenhouse gas emissions and pollution [224]. In response to this challenge, the European Circular Economy Action Plan [225] has emphasized the urgent need to minimize food waste by promoting strategies that retain food resources within the food chain for as long as possible. Among the various stages of the supply chain, the fruit and vegetable sector is the most critical, accounting for approximately 45% of total losses and waste [226]. These discarded food materials still contain valuable bioactive compounds, including dietary fibers, antioxidants, amino acids and micronutrients. The valorisation of such waste refers to the transformation of these by-products into new resources with added nutritional value and potential health and economic benefits [227]. In this context, microbial fermentation is emerging as a promising strategy to recover beneficial compounds from food waste. It can serve not only to make these nutrients available for microbial metabolism but also to generate new bioactive molecules, such as GABA [223]. In the present study, we investigated the potential for developing a GABA-enriched product by exploiting the ability of the *L. brevis* CRAI strain to biosynthesize GABA, using food industry by-products as substrates for fermentation. This approach aims to reduce production costs by optimizing the GABA fermentation process, while simultaneously adding value to agro-industrial waste, thus contributing to the development of a GABA-enriched nutraceutical.

We first evaluated the ability of the *L. brevis* CRAI strain to grow and ferment in various agro-food by-products, including peach, tomato, grape, apple, and apricot pomace all sourced from local farmers. The tomato and peach substrates were the most supportive of *L. brevis* CRAI growth, with tomato resulting best. An increase in cell counts was observed in peach and tomatoes pomace, in particular there is a significant increase of growth at 24 hours in the tomato substrate that reaches approximately 10^9 CFU/mL at 72 hours. In the peach pomace substrate, bacterial counts reached 10^8 CFU/mL at 24 hours and remained relatively stable thereafter. In contrast, no substantial or statistically significant growth was recorded in the other substrates throughout the fermentation process. The use of fruit and vegetable by-products for lactic fermentation is well supported in literature, primarily due to their high content of fermentable sugars, polyphenols, and essential nutrients. For instance, Bujna et al. (2018) employed apricot

juice for fermentation using both monocultures and mixed probiotic cultures, demonstrating an enhancement in the antioxidant properties of the final product [228]. Similarly, Simões et al. (2022) reported that fermentation of unripe tomato with a consortium of lactic acid bacteria (LAB) and yeasts led to an increase in ascorbic acid content and antioxidant capacity in the fermented product [229].

Further evidence of successful fermentation, particularly in the tomato and peach substrates, is provided by pH measurements. A marked decrease in pH was observed in both matrices, indicating increased acidification consistent with the metabolic activity of LAB. These results suggest that tomato and peach offer a more favourable environment for the fermentation of *L. brevis* CRAI, both in terms of microbial proliferation and acidification potential. We hypothesize that the enhanced growth observed in the tomato substrate, where *L. brevis* CRAI exhibited the highest growth performance, could be attributable to the fact that tomato represents the original isolation matrix for this strain [214]. This observation aligns with previous findings in which two *Bacillus megaterium* strains, originally isolated from saline soils, demonstrated a more pronounced expression of twelve plant growth-promoting (PGP) traits, including phytohormone production and nitrogen fixation, when cultivated in saline soils from which they were originally isolated, as opposed to non-saline conditions [230]. Similarly, twelve strains including *Pseudomonas, Acinetobacter, and Comamonas spp*., isolated from wheat fields under different tillage systems, maintained optimal growth and consistent phosphate, potassium, and zinc solubilization activity under pH, salinity, and temperature conditions that mirrored their native habitats [231].

Beyond its origin as an isolation source, the superior growth performance of *L. brevis* CRAI in tomato pomace, compared to other fruit- and vegetable-derived by-products, can be ascribed to the comprehensive nutritional profile of this substrate. Tomato-based matrices are widely recognized for their ability to support the proliferation of lactic acid bacteria (LAB), primarily due to the presence of key nutrients such as fermentable sugars, organic acids, pantothenic acid, amino acids, and proteins. Historically, tomato juice has served as one of the earliest substrates for LAB cultivation and continues to be included in selective media [232].

In relation to the compositional profiles of the tested substrates, tomato pomace contains the highest protein content among all matrices evaluated. While simple carbohydrates function as immediate energy sources, the availability of nitrogen, particularly in the form of amino acids, peptides, or proteins, is indispensable for the biosynthesis of cellular components, enzymatic

machinery, and overall metabolic activity in LAB. A substantial body of literature supports the notion that nitrogen-rich environments significantly enhance both the growth rate and metabolic performance of LAB strains. For example, *Lactobacillus helveticus* CRL 1062 exhibited increased growth and proteolytic activity when cultivated under nitrogen-enriched conditions, underscoring the essential role of amino acid availability in LAB metabolism [233]. Similarly, *Lactobacillus delbrueckii* subsp. *bulgaricus* demonstrated improved viability and biomass accumulation when supplemented with alternative nitrogen sources such as complex peptides or protein hydrolysates, reinforcing the concept that carbon availability alone is insufficient to sustain optimal microbial proliferation [234]. Further supporting this observation, a study conducted on *Lactobacillus plantarum* RPR42 revealed that the addition of protein-rich substrates to a sugar-based molasses medium resulted in a marked increase in biomass production, highlighting the critical importance of a balanced carbon-to-nitrogen (C/N) ratio in promoting LAB growth [235]. Interestingly, despite its relatively high sugar and protein content, grape pomace did not support the growth of *L. brevis* CRAI. This lack of proliferation may be attributed to the presence of inhibitory compounds, particularly polyphenols with known antimicrobial activity. These phenolic substances have been reported to interfere with microbial physiology by disrupting membrane integrity, inhibiting key enzymatic processes, and altering intracellular metabolic pathways [236–238]. Peach-based substrates, in contrast, have frequently been used in beverage formulation of probiotic products due to their capacity to support the growth of various functional activity of LAB strains [239,240]. On the other hand, Kalinowska et al. (2023) observed that some LAB strains were able to growth in apple pomace, while others do not [241], for instance, growth in apple, apricot and peach pomace, which are substrates that possess similar nutritional profiles, may be species and strain dependent. These findings suggest that the enhanced growth of *L. brevis* CRAI in tomato, followed by peach pomace, is likely driven by the complex nutrient composition of these substrates, which creates a more favourable environment for cellular metabolism, biosynthesis, and replication. Based on this observation, a targeted optimization strategy was subsequently developed to enhance GABA biosynthesis. To this end, a Central Composite Design (CCD) approach was employed, focusing exclusively on the matrices that supported the viable growth of *L. brevis* CRAI: tomato and peach pomace.

In addition to evaluating microbial growth, GABA levels were quantified in all fermented matrices. However, concentrations across all samples fell below the detection limit of the employed HPLC method. As a result, a targeted optimization strategy was implemented with

the goal of enhancing GABA biosynthesis. This was achieved by applying a Central Composite Design (CCD) approach, which was restricted to the matrices that supported viable growth of *L. brevis* CRAI: tomato and peach pomace. Through the modulation of key fermentation parameters, including the addition of different carbon and nitrogen sources, the CCD enabled systematic assessment of both individual effects and interactions among variables, ultimately facilitating the identification of optimal conditions for maximizing GABA production.

To enhance GABA production, previous studies have highlighted the importance of modulating growth conditions by supplementing the fermentation matrix with specific ingredients (e.g., monosodium glutamate, yeast extract, vitamins, or certain cofactors), which can stimulate microbial metabolism and increase GABA yield [94,208,242]. In the present study, the variables selected for the CCD-based optimization of GABA production included yeast extract concentration, matrix dilution, and initial pH. Due to the complexity of food by-products matrices and the potential presence of fermentation inhibitors, matrix dilution has been evaluated as a critical strategy to enhance microbial activity and optimize overall fermentation performance [243]. In the tomato-based matrix, the optimal conditions predicted by the response surface methodology were a pH of approximately 5.1 and 1% yeast extract, while optimal conditions for GABA production in peach pomace were predicted around pH ~5.6 with 0.75% yeast extract, furthermore, in both matrices the model highlight that matrix dilution did not affect GABA biosynthesis under the tested conditions. The model predicted condition was validated through confirmatory trial in which GABA yield conversion reached 79.15 ± 4.56% in tomato pomace, in contrast in peach pomace fermentation leads to a lower GABA conversion yield and production. The experimental results were aligned with the predicted trends, even if some deviations in the absolute values were observed. These differences are likely attributable to the inherent complexity and compositional variability of agro-industrial by-products, which may fluctuate slightly across trials. In comparison with the study conducted by Cho et al. (2012), which focused on the fermentation of tomato paste enriched in GABA using *L. brevis* B3 20, the yields obtained in our research were comparatively lower. This variation is likely attributable to differences in the composition of the fermentation media and the time of fermentation; we obtained a lower concentration of MSG (2%) and time of incubation (48h). In contrast, Cho et al 2012 utilized a medium supplemented with 3% MSG and 0.5% yeast extract, the fermentation was carried out for 72 hours at 30 °C, achieving a final GABA concentration of 143.4 mM [244]. More recent work by Nakatani et al. (2022) reported the production of GABA-enriched tomato juice fermented with *L. plantarum* KB1253 under

conditions of 20 °Brix and pH 4, which yielded 41.0 ± 1.1 mM GABA starting from ~ 0.6% of glutamate naturally present in the tomato juice [245], comparable to the concentrations observed in our peach-based substrate but still lower than those reached in tomato pomace. In addition to tomato-based substrates, various fruit-derived matrices have been explored for their potential to support GABA production through microbial fermentation. For example, Kanklai et al. (2020) obtained approximately 32 mM GABA in mulberry juice inoculated with 5% (w/v) of *L. brevis* F064A, supplemented with 2% MSG and incubated for 48 hours at 37 °C [196]. Similarly, Cataldo et al. (2020) reported significantly higher GABA levels, up to 262 mM, using *L. brevis* CRL 2013 in strawberry juice. However, the experimental conditions included extended fermentation times (up to 168 hours) and elevated MSG concentrations (up to 4.5%), in addition to supplementation with 1% yeast extract [246]. These comparisons underscore the relevance of the GABA concentrations achieved in our study, particularly in tomato pomace, even in the absence of high MSG supplementation or prolonged fermentation. Despite differences in microbial strains, processing conditions, and substrate formulations across studies, our findings support the suitability of tomato pomace as an effective and promising matrix for the development of GABA-enriched functional food products. Together with GABA quantification we also monitored bacterial growth and pH changes during fermentation and results showed that peach pomace supported slightly higher *L. brevis* CRAI proliferation with a stronger acidification compared to tomato pomace, although this did not correlate with increased GABA production. These findings suggest that the peach pomace matrix provides a more favourable environment for bacterial growth, potentially promoting greater organic acid production and a more pronounced pH decrease, although this occurs in parallel with reduced GABA biosynthesis. Beyond acid generation, fermentation processes can also modify other volatile compounds, which may influence the sensory and functional properties of the final product [247]. Fermentation process could improve the organoleptic and sensory characteristics of the final product. However, depending on the microorganism applied and time of fermentation, undesired volatile molecules can be produced and accumulated [248]. In this study the volatile organic compound (VOC) profiles of tomato and peach pomace fermented by *L. brevis* CRAI exhibited significant changes after 48 hours. Tomato fermentation yielded a greater diversity of VOCs (52 compounds) compared to peach (23 compounds). After fermentation both substrates showed increased total VOC abundances, with a marked rise in alcohols and acids, particularly ethanol and acetic acid, reflecting the metabolic activity of *L. brevis* CRAI. Fermentation significantly altered the volatile compound profiles of both tomato and peach pomaces, exhibiting unique changes that correspond to the intrinsic differences in

their compositions. The results suggest that both substrates evolve toward volatile profiles dominated by alcohols and acids during fermentation, consistent with observations reported by Liu et al. (2018) [247]. Tomato pomace preserves compounds associated with green and vegetal aromas while peach matrix exhibits a more pronounced fruity and floral character. Alcohols were identified as the predominant class of volatiles, aligning with multiple studies that describe their formation primarily through glucose metabolism or the breakdown of amino acids [249,250]. In particular, the tomato matrix exhibited a marked rise in alcohol content, with ethanol emerging as the main fermentation product which could derived by sugars via the phosphoketolase pathway [251], alternatively, ethanol formation can occur via the activity of alcohol dehydrogenase, which reduces acetaldehyde to ethanol. This mechanism was observed during tomato pomace fermentation, where an increase in alcohol levels was accompanied by a concurrent decrease in aldehyde concentrations. These findings are in line with the results of Liu et al. (2018), where *L. plantarum* and *L. casei* reduced the content of aldehyde of 25% during tomato juice fermentation ([247]. In peach fermentation, benzaldehyde was the only aldehyde detected initially and was completely depleted by the end of the process. This observation is consistent with previous studies reporting a decrease in benzaldehyde levels in fruits fermented with lactic acid bacteria [252]. In addition to an increase in alcohols, peach pomace exhibited a more pronounced rise in acids, notably acetic acid, which is a major fermentation metabolite. Acetic acid production is primarily driven by heterofermentative LAB such as *L. brevis* CRAI, which metabolize sugars through the phosphoketolase pathway to generate acetyl phosphate then converted to acetic acid via the action of acetokinase [251]. Although a slight reduction in ketones was observed in both tomato and peach substrates, this can be attributed to the common microbial conversion of unstable aldehydes and ketones into primary and secondary alcohols during monoculture fermentations, in line with previous findings [253]. In conclusion, aldehydes, ketones, and esters generally decreased, indicating their transformation or consumption during fermentation. These shifts in VOC composition highlight the dynamic biochemical modifications imparted by fermentation, which likely influence the sensory and functional properties of the final products [249,254].

With this study, we were able to select tomato pomace as the optimal fermentation substrate and thanks to the CCD approach we found the best conditions that maximize GABA production by *L. brevis* CRAI. The tomato pomace by-products and conditions can be applied for the formulation of a GABA-enriched nutraceutical prototype, with potential for large-scale production.

Chapter 5

1. Impact of GABA-enriched nutraceutical on gut microbiota and metabolite modulation in an *in vitro* fermentation model

1.1. Materials and methods

1.1.1. Bacterial Strain and freeze-drying process

Levilactobacillus brevis CRAI strain belongs to the Department of Pharmacy and Biotechnology (FABIT) of the Alma Mater Studiorum University of Bologna. The strain was freeze-dried using an optimized cryoprotective medium designed to enhance survival during the lyophilization process. Prior to lyophilization, the strain was cultivated in MRS broth in anaerobic conditions at 37 °C for 24 hours. Then the culture was centrifuged at 10,000 × g for 10 minutes. The pellet was washed with sterile saline solution (0.9% NaCl) and 10^{11} CFU of *L. brevis* CRAI were resuspended in 10 mL of lyophilization medium to obtain a final concentration of 10 CFU/mL. The lyophilization medium consisted of 10% (w/v) skim milk, 0.5% (w/v) yeast extract, and 0.5% (w/v) lactose. An ascorbic acid solution was prepared and filter-sterilized, it was then added to the autoclaved lyophilization medium to reach a final concentration of 0.1% (w/v). For freeze-dried samples, the cell suspensions were put at –20 °C overnight and then freeze-dried (Alpha 1-2 LSCbasic, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 0.4 mbar and −60 °C for 24 h; the freeze-dried CRAI were stored at +4–8 °C until use.

1.1.2. Development of GABA-enriched prototype fermentation process and scale-up

The formulation of a nutraceutical prototype enriched in GABA was developed by fermenting *L. brevis* CRAI in a tomato by-product substrate, under the optimal conditions defined in the previous chapter. The strain was inoculated in a dehydrated tomato powder (DTP) provided by the company DEPOFARMA. Fermentations were initially performed on a small scale and later scaled up. The dehydrated tomato powder was weighed and rehydrated with distilled water at a 1:10 (w/v) powder-to-water ratio. The resulting matrix was sterilized by autoclaving at 121 °C for 15 minutes and then cooled to room temperature. Optimal fermentation conditions identified in preliminary experiments were maintained: MSG was added to a final concentration of 2% (w/v), yeast extract to 1% (w/v), and the pH was adjusted to 5.08 using 5 M NaOH. The *L. brevis* CRAI strain, previously propagated, was harvested by centrifugation at 10,000 × g for 15 minutes, washed with sterile saline solution, resuspended, and inoculated into the tomato

matrix at a final concentration of $1x10^7$ CFU/mL. Fermentation was carried out at 32 °C for 72h, samples for cell count, GABA quantification, and pH measurement were collected at 48 and 72h. GABA quantification was performed as described in 7.1.3.2. section. At the end of the incubation period, fermentation was stopped, and the fermented product was then frozen at −80 °C and subsequently lyophilized for further use.

1.1.3. *In vitro* colonic fermentation

1.1.3.1. Composition of basal media

The basal medium was prepared by combining yeast extract 2 g/L, peptone water 2 g/L, KH_2PO_4 0.04 g/L, K_2HPO_4 0.04 g/L, $MgSO_4 \cdot 7H_2O$ 0.01 g/L, NaCl 0.1 g/L, $CaCl_2 \cdot 6H_2O$ 0.01 g/L, and $NaHCO_3$ 2 g/L. Additional components included Tween 80 (2 mL/L), hemin 0.05 g/L dissolved in a few drops of 1M NaOH, vitamin K 10 µL/L, L-cysteine HCl 0.5 g/L, and bile salts 0.5 g/L. A redox indicator, resazurin solution (0.025 g/100 mL), was added at 4 mL/L. The pH was adjusted to 7.0. The medium was then heated, allowed to cool, dispensed into Duran bottles, sterilized by autoclaving, and stored under appropriate conditions until further use.

1.1.3.2. Set up of the in vitro batch culture fermentation experiments

Faecal samples were obtained from eight participants: 2 healthy females (F) and 2 healthy males (M) (aged 18-65 years old), and 4 females (F) with irritable bowel syndrome (IBS) (aged 18-65 years old). The higher number of females' participants with gut dysbiosis reflected the greater prevalence of IBS among women in the general population. All donors have provided written informed consent and filled in a standard questionnaire to collect information regarding health status, drug use, clinical anamnesis, and lifestyle factors. The University of Roehampton Research Ethics Committee (L24/399) approved this study in accordance with the Declaration of Helsinki. All faecal samples were placed in an anaerobic jar (AnaeroJarTM 2.5 L, Oxoid Ltd.) including a Gas-Generating Kit (AnaeroGenTM, Oxoid Ltd.) in order to reproduce anaerobic conditions inside the chamber.

An *in vitro* dynamic gut model simulating colonic conditions [255] was used to assess the impact on human gut microbiota composition and metabolic profile of 3 different samples: i) *L. brevis* CRAI strain in the lyophilized form, ii) GABA-enriched prototype obtained by the fermentation of *L. brevis* CRAI in dehydrated tomato powder and iii) the mixture comprising of *L. brevis* CRAI, MSG and dehydrated tomato powder (DTP). Each sample was inoculated in a different vessel pre-filled with 45 mL of sterile basal medium heated to 60°C. An aliquot of 5

mL of fresh faecal slurry was diluted (1:10 w/v) in PBS, pH 7.4, and homogenized (Stomacher 400, Seward, UK) for 2 min at 240 paddle beats per min. Samples were added to anaerobic fermenters within 15 min of voiding. Batch cultures were run in four donors (n = 4) over a period of 24 h, and samples were collected from each vessel at 0, 4, 8 and 24 h for SCFAs, amino acid quantification and microbiome analysis.

1.1.4. Sample processing for SCFAs analysis trough Ultra-High-Performance Liquid Chromatography

Samples collected during the fermentation processes were centrifuged at 15,000 × g for 5 minutes. The resulting supernatants were used for the quantification of SCFAs and amino acids, while the pellets were resuspended in PBS containing 50% (v/v) glycerol (1:1 ratio) and stored at -20 for subsequent DNA extraction. For SCFA analysis, the supernatants were further filtered using Microcon 30 kDa centrifugal filter units with Ultracel-30 membranes (Merck, Darmstadt, Germany) by centrifugation at 19,000 × g for 30 minutes to remove any remaining particulates. 20 µL of the filtered samples were mixed with 20 µL of an internal standard solution (10 mM $^{13}C_2$ acetic acid, 10 mM $^{13}C_1$ propionic acid and 10 mM $^{13}C_2$ butyric acid) and 20 µL of LC-MS grade water. Derivatisation was achieved by addition of 20 µL of 200 mM 3-nitrophenylhydrazine hydrochloride prepared in 50% v/v acetonitrile in water, and 20 µL of 120 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) prepared in 6% (v/v) pyridine in ethanol solution. The plate was covered with an aluminium mat and agitated at 1400 rpm for 10 s before heating at 40°C for 30 min. The derivatization reaction was quenched by adding 150 µL 0.1% formic acid solution in LC-MS grade water, again agitating for 10 s at 1400 rpm. A total of 100 µL of the supernatant was transferred to a high-recovery LC-MS plate for UHPLC-MS analysis.

Chromatographic separation was carried out using a Waters Acquity UHPLC system equipped with a Waters CSH C18 analytical column (100 mm × 2.1 mm, 1.7 µm particle size, 130 Å pore size) [256]. A binary solvent system was employed, with mobile phase A consisting of LC-MS grade water containing 0.1% formic acid, and mobile phase B comprising LC-MS grade acetonitrile with 0.1% formic acid. The separation was performed using a gradient elution program initiated at 20% B, for 2 minutes, followed by a linear increase to 55% B over 6 minutes. At 8.1 minutes, the mobile phase composition was abruptly shifted to 99% B and maintained for 1.4 minutes. The system was then returned to 20% B for column re-equilibration from 9.5 to 10.5 minutes. During this sequence, the flow rate was maintained at 0.35 mL/min,

except between 8.1 and 9.1 minutes, during which it increased to 0.4 mL/min. The column temperature was maintained at 40°C throughout the run. Detection of SCFAs was performed using a Waters Xevo TQ-Sμ triple quadrupole mass spectrometer operated in negative electrospray ionization (ESI) mode. Multiple reaction monitoring (MRM) transitions were selected based on previously reported parameters [257]. Instrument settings included a capillary voltage of 2.4 kV, a cone temperature of 150°C, desolvation gas flow at 550 L/h, and a desolvation temperature of 350°C. Raw mass spectrometric data were converted using MSConvertGUI and subsequently processed using a custom in-house analytical pipeline developed previously and based on Behrends et al. 2011 [258].

1.1.5. LC-MS analysis for amino acid quantification

Sample collected, filtered and used for SCFAs analysis were also used for amino acid quantification, which were quantified using the AccQ Tag quantification kit according to manufacturer's instructions. Briefly, a volume of 10 μL of each sample was transferred to a 96-well plate, and proteins were precipitated using 40 μL of cold (-20°C) isopropanol (plus 1% formic acid (v/v)). After 20 minutes at room temperature (RT), samples were centrifuged (2000 rpm, 5 minutes, RT), and 10 μL of the supernatant were transferred to a new 96-well plate for derivatization. The derivatization reagent was mixed with 1 mL of reagent diluent and activated by heating to 55°C for 15 minutes. Samples in each well were mixed with 70 μL of borate buffer (pH 8.6) and 20 μL of AccQTag Ultra derivatizing reagent solution, then transferred to a high recovery well plate and diluted 1:10 with Optima grade water for analysis. The samples were then injected into UPLC-MS system for analysis. Pooled biological quality control samples (pbQCs) were prepared by combining equal aliquots from representative biological specimens. These pbQCs were processed using the same protocol as the study samples and injected at regular intervals, specifically after every eighth injection, to monitor instrument stability and analytical reproducibility throughout the run. Mass spectrometric analyses were conducted in positive ionization mode using a Waters Xevo TQ-Sμ triple quadrupole mass spectrometer coupled to an Acquity UPLC system (Waters, Milford, MA, USA). Chromatographic separation was achieved on an HSS T3 reversed-phase column (Waters) maintained at a controlled temperature. The mobile phase consisted of (A) LC-MS grade water with 0.1% formic acid (v/v) and (B) LC-MS grade acetonitrile containing 0.1% formic acid (v/v). The chromatographic gradient was initiated at 4% B and maintained for 0.5 minutes, followed by a linear increase to 10% B over 2.0 minutes. The gradient was then ramped to 28% B over the

next 2.5 minutes, and subsequently to 95% B, held for 1 minute. The system was then re-equilibrated to the initial conditions (4% B) over 1.3 minutes. The flow rate was kept constant at 0.6 mL/min throughout the run. MS data acquisition was performed using a capillary voltage of 3.5 kV and a sheath gas flow rate of 450 L/h. Multiple reaction monitoring (MRM) transitions were optimized based on previously validated conditions and applied for compound identification and quantification. Raw data were processed using a custom in-house workflow (based on [258]) designed to ensure consistency and accuracy across all analytical runs.

1.1.6. DNA extraction

Total DNA was extracted from the bacterial pellets obtained as described in paragraph 8.1.4. DNA extraction was carried out following a protocol incorporating chemical, mechanical, and thermal lysis [259]. Briefly, 0.5 g of zirconia glass beads were added to each pellet along with 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris–HCl pH 8, 50 mM EDTA, and 4% SDS). Samples were subjected to 3 rounds of mechanical disruption using a FastPrep instrument (Qiagen Tissuelyser LT 85600) at 5.5 movements/s for 1 minute, followed by placement on ice. Subsequently, samples were heated at 95 °C for 15 minutes, with mixing by inversion every 5 minutes. After incubation, tubes were centrifuged at 4 °C for 5 minutes at maximum speed. The supernatant was carefully transferred to a new tube, and 260 µL of 10 M ammonium acetate was added. Samples were vortexed and incubated on ice for 5 minutes, followed by centrifugation at 4 °C for 10 minutes at full speed. The resulting supernatant was transferred to a new eppendorf, and an equal volume of absolute ethanol (98%) was added. After vortexing, samples were incubated on ice for 30 minutes, then centrifuged at 4 °C for 15 minutes. The supernatant was discarded, and the pellet was washed (without resuspension) with 300 µL of 70% ethanol. Residual ethanol was removed, and pellets were allowed to air-dry completely. Dried pellets were resuspended in 100 µL of TE buffer and incubated on ice for 30 minutes. To remove RNA, 2 µL of RNase (10 mg/mL) was added, and the mixture was incubated at 37 °C for 15 minutes. For protein digestion, 15 µL of proteinase K and 200 µL of Buffer AL were added, followed by vortexing and incubation at 70 °C for 10 minutes. Subsequently, 200 µL of 98% ethanol was added and vortexed. From this point onward, the DNA purification protocol followed the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and quality were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

1.1.7. 16S rRNA gene amplification and sequencing

The hypervariable V3-V4 regions of the 16S rRNA gene were amplified using primers 341F and 785R, which included Illumina adapter overhang sequences, as previously reported [260]. Amplicons were purified using a magnetic bead-based cleanup system (Agencourt AMPure XP, Beckman Coulter, Brea, CA, USA). Indexed libraries were then prepared through limited-cycle PCR with Nextera technology, followed by an additional purification step. The libraries were pooled at an equimolar concentration of 4 nM, denatured, and diluted to a final concentration of 5 pM for sequencing. Sequencing was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using a 2 × 250 bp paired-end run.

1.1.8. Bioinformatics

Raw sequencing reads were processed using PANDASeq [261] for read merging, followed by quality filtering and length trimming within the QIIME 2 framework [262]. Amplicon sequence variants (ASVs) were inferred using the DADA2 algorithm [263], and taxonomic assignment was performed using the VSEARCH method [264,265]. Alpha diversity was assessed through multiple indices, including number of observed features, Shannon entropy, and Faith's phylogenetic diversity.

1.1.9. Statistical analysis

For SCFAs and amino acid analysis was used GraphPad Prism 8.0.2, amino acids were showed as mean of the different healthy (n=4) and IBS (n=4) donors.

For microbiome data, statistical analyses were conducted using RStudio 1.0.44 with R software version 3.3.21, employing packages such as stats, made4 and vegan (https://cran.r-project.org/web/packages/vegan/vegan.pdf, accessed on 10 september 2025). Kruskal–Wallis test, along with Wilcoxon tests (paired or unpaired as required), was employed for assessing differences in alpha diversity and relative abundances of taxa. To account for multiple comparisons, p values were adjusted using the false discovery rate (FDR) method. p values $\leq$ 0.05 were considered statistically significant.

1.2. Results

1.2.1. Development and scale-up of a GABA-enriched prototype by *L. brevis* CRAI fermentation in a dehydrated tomato powder

Previous experiments enabled the optimization of GABA production using a tomato pomace substrate. Through a Central Composite Design (CCD), optimal fermentation conditions were identified, including the addition of 1% yeast extract and acidification to an initial pH of 5.08. After confirming the strain efficiency under these conditions, we applied the same parameters to a matrix composed of dehydrated tomato powder supplied by the company DEPOFARMA. In this setup, we modified the dilution of the matrix, reducing it to 10% instead of the previously used 50%. This decision was based on prior results indicating that dilution level does not significantly influence GABA production, allowing us to minimize the dilution percentage without compromising yield. Fermentations were conducted initially on a small scale and subsequently scaled up, with GABA quantification performed at 48 and 72 hours, as shown in Table 10. In small-scale fermentations, GABA production reached 16.7 ± 4.3 mM after 48-hour, with a conversion yield of 14.2 ± 3.6%. The substrate supported microbial growth up to $1x10^9$ CFU/mL, final pH was 4.25. Extending the fermentation to 72 hours resulted in a substantial increase in GABA concentration, reaching 83.0 ± 5.5 mM, and a conversion yield of 70.3 ± 4.7%. Microbial growth remained stable at $1x10^9$ CFU/mL, and the final pH was 4.6. For large-scale fermentations, the working volume is scaled up to 1 liter. Considering the increased volume the process was conducted directly for 72 hours. Under these conditions, GABA production reached 80 ± 4.11 mM, with a conversion yield of 67.7 ± 3.48 %. Microbial growth was maintained at levels comparable to the small-scale setup, and the pH was similarly acidified to 4.6. These results demonstrate that the scaled-up process effectively replicated the performance observed in small-scale fermentations at 72 hours. Having confirmed that GABA production can occur on a large scale, we lyophilized the fermented product and used it as a prototype of a GABA-enriched nutraceutical.

Table 10. GABA production using dehydrated tomato powder (DTP) fermented with *L. brevis* CRAI in the presence of 2% (w/v) MSG. The table summarizes the fermentation conditions, including matrix dilution, yeast extract concentration (%), initial pH (pH_i) final pH (pH_f), and viable cell count at the end of fermentation. It also reports the resulting GABA concentrations and conversion yields. Fermentations were performed at small scale for 48 and 72 hours. In large-scale trials (1 L volume), the same conditions were applied, and fermentation was monitored up to 72 hours. Values are shown as mean ± SD of n=3.

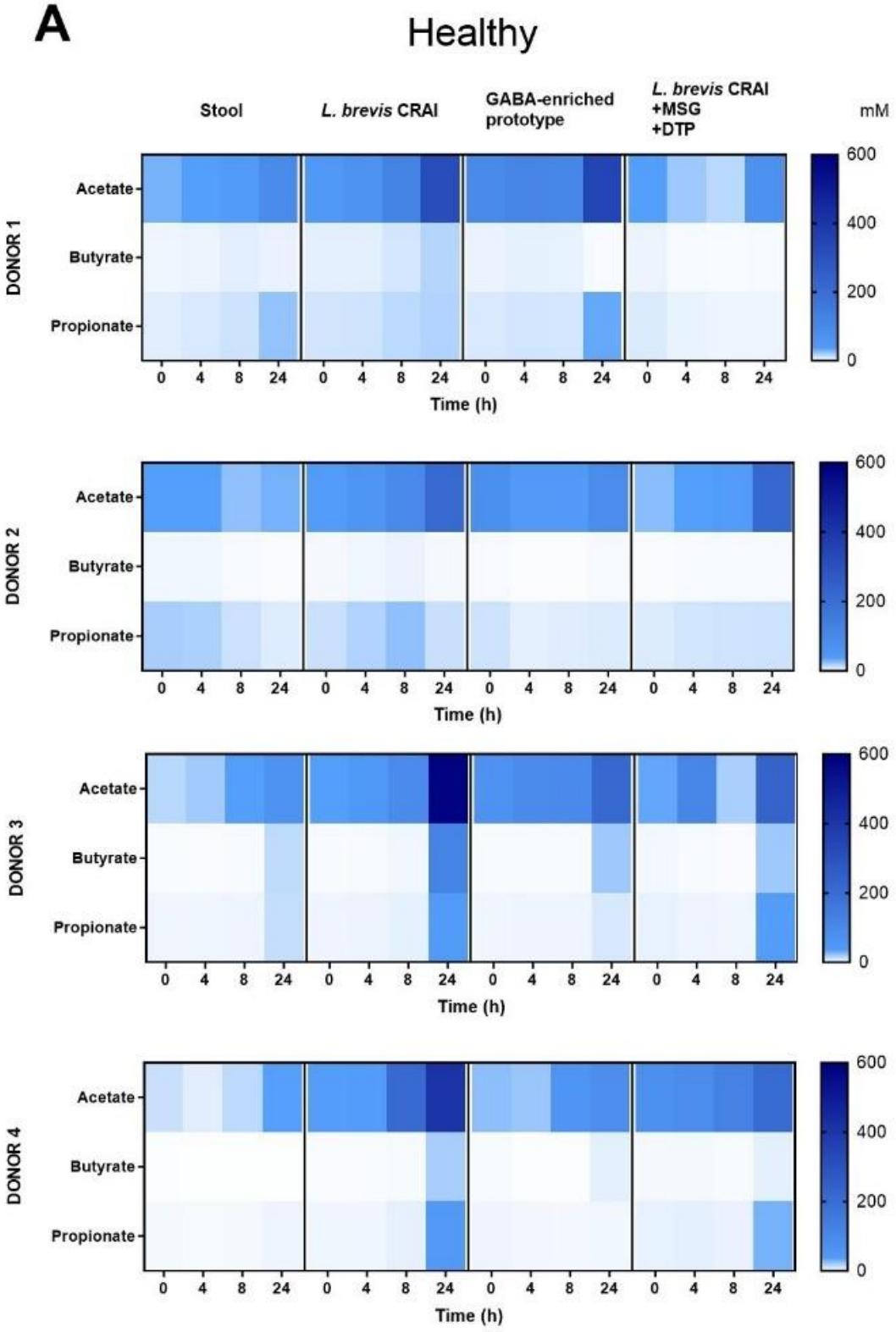
DTP	Matrice dilution (%)	Yest extract (%)	pHi	pHf	GABA (mM)	Yied (%)	CFU/mL
Small scale 48h	10	1.00	5.08	4.25	16.7±4.3	14.2±3.6	$1x10^9$
Small scale 72 h	10	1.00	5.08	4.6	83.0 ±5.5	70.3±4.7	$1x10^9$
Large scale 72h	10	1.00	5.08	4.6	80.0 ±4.11	67.7± 3.48	$1x10^9$

1.2.2. *In vitro* gut model fermentation, bacterial metabolite profile

An *in vitro* fermentation gut model was employed to simulate the physiological conditions of the distal colon. This system utilizes vessels, sealed bottles maintained under strictly controlled anaerobic conditions, temperature, and pH, to replicate the colonic environment. Within these fermenters, fecal slurry was inoculated, followed by the administration of various substrates. In this study, fecal samples were obtained from both healthy donors and individuals diagnosed with IBS. Three distinct treatments were evaluated: (i) viable cells of *L. brevis* CRAI, (ii) a GABA-enriched prototype, and (iii) a mixture comprising of *L. brevis* CRAI + MSG + dehydrated tomato powder (DTP). The objective was to investigate the impact of these treatments on gut microbiota composition, short-chain fatty acid (SCFA) production, and amino acid profiles over time, under both eubiotic and dysbiotic conditions. Fecal samples used in the gut model provide a rich and diverse microbial ecosystem along with a complex array of metabolic by-products, offering a non-invasive and representative means of assessing intestinal health. Comprehensive analysis of microbial community structure and metabolite production enables the evaluation of dietary and microbial interventions on intestinal homeostasis.

1.2.3. SCFAs profiling in an *in vitro* colonic fermentation model

Short-chain fatty acids are the main metabolic products of the gut microbiota. In this study, SCFAs were quantified from supernatants obtained through *in vitro* fecal fermentations as described in 8.2.2. Fermentation supernatants were collected at multiple time points, and SCFAs concentrations were measured via gas chromatography. As shown in Figure 14, the quantified SCFAs included acetate, butyrate, and propionate. Acetate production showed a consistent upward trend across all treatments and donors at the 24-hour time point. The highest concentration was observed in healthy DONOR 3 following treatment with *L. brevis* CRAI, the highest value among IBS donors was also recorded under the same treatment (Figure 14, panel A). This trend was evident in both healthy and IBS donors, suggesting a comparable acetate production pattern across groups. A significantly higher increase in butyrate at 24-hour compared to 0 hour was observed in DONOR 3 following treatment with *L. brevis* CRAI. We also observed among IBS donors an increased butyrate levels at 24 hours, when stool was treated with the three samples compared to the untreated stool. As shown in Figure 14, panel B, butyrate concentrations are low at 24 hours. Treatment with *L. brevis* CRAI or the GABA-enriched prototype led to markedly higher butyrate levels, for example, in DONOR 5. A similar trend was observed for propionate, with production increasing at 24 hours in nearly all donors, regardless of health status or treatment condition.


A
Healthy
Stool
L. brevis CRAI
GABA-enriched prototype
L. brevis CRAI +MSG +DTP
mM
600
400
200
0
DONOR 1
DONOR 2
DONOR 3
DONOR 4
Acetate
Butyrate
Propionate
0 4 8 24 0 4 8 24 0 4 8 24 0 4 8 24
Time (h)

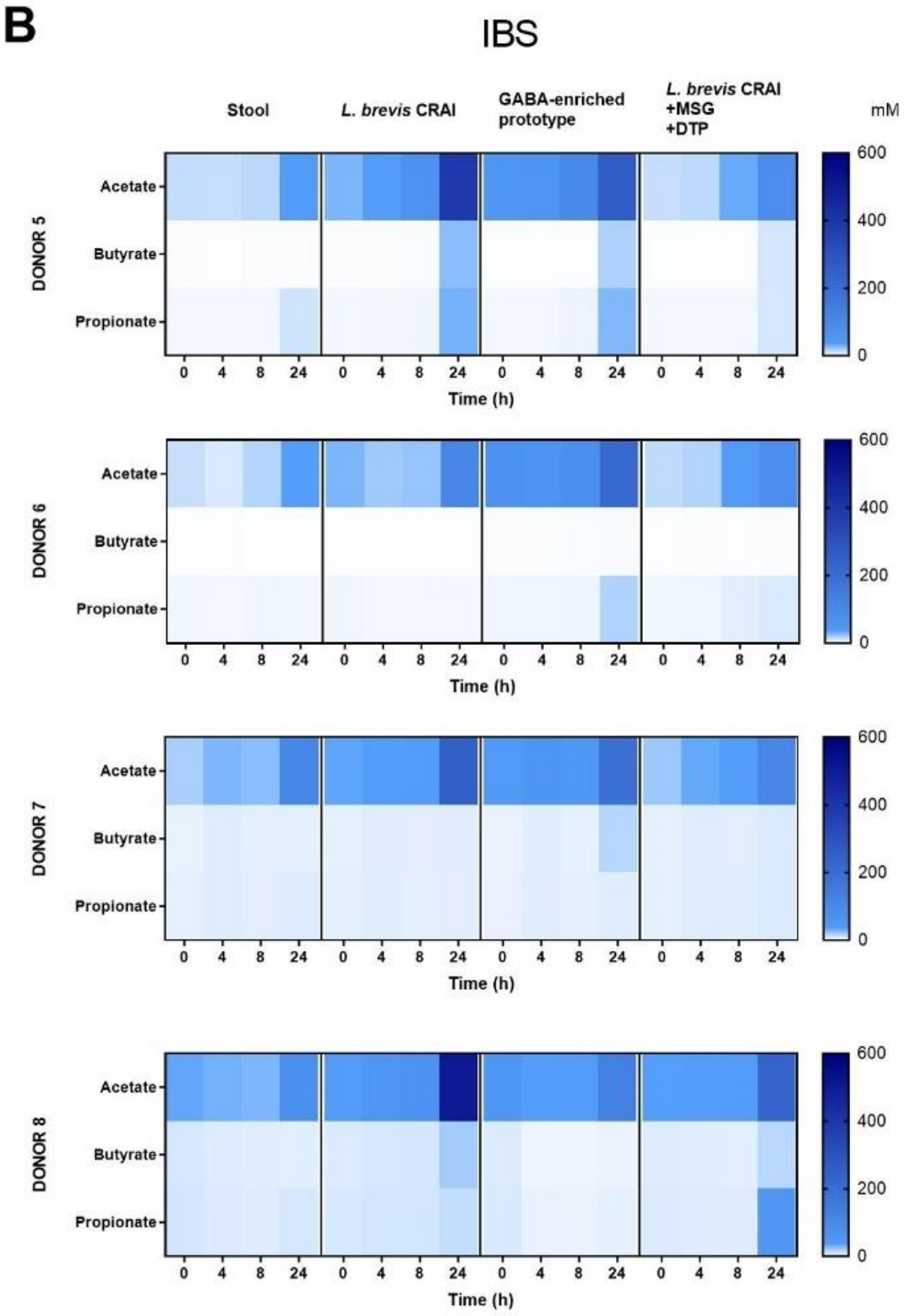
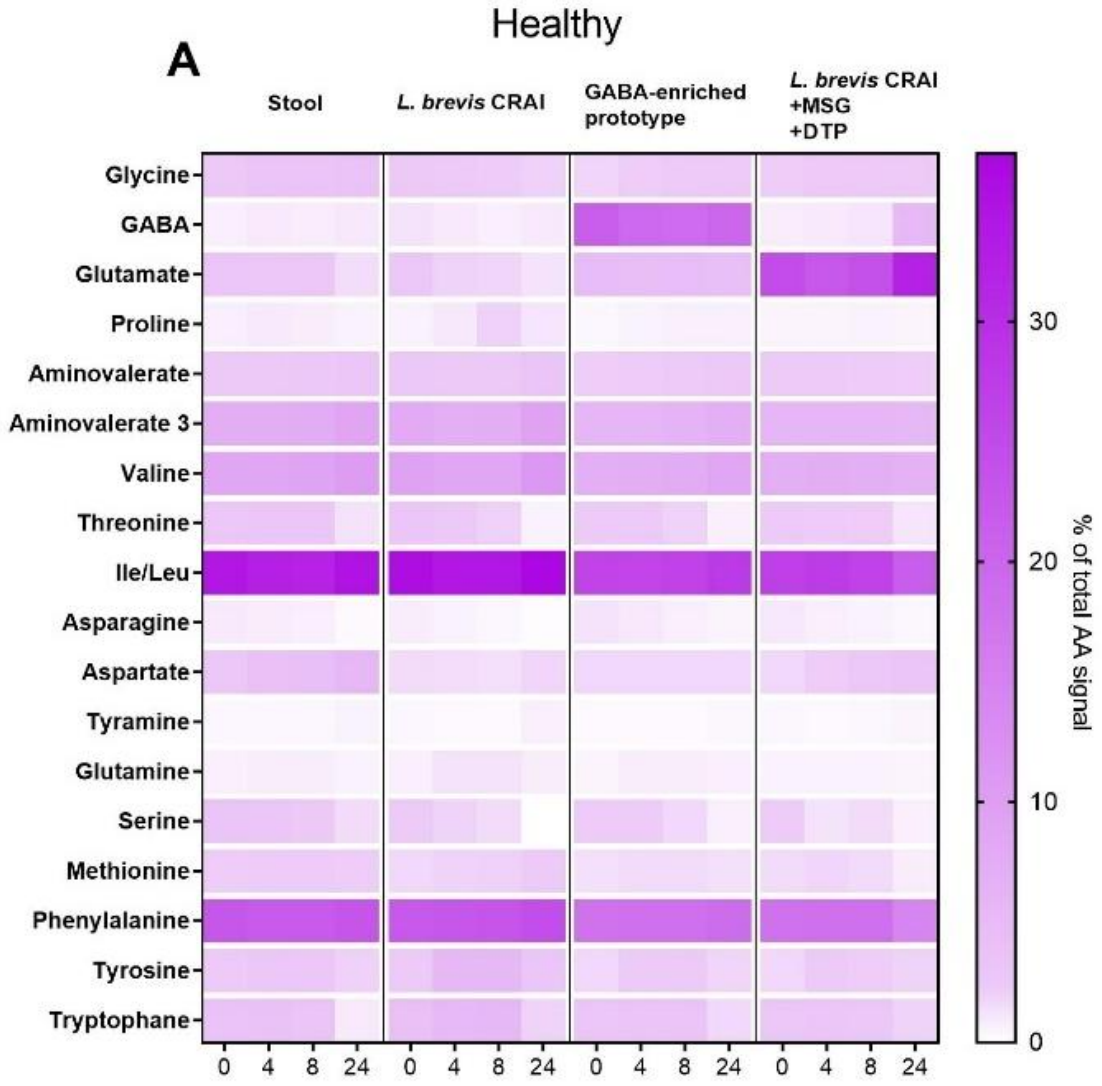


Figure 14. Quantification of short-chain fatty acids (SCFAs) acetate, propionate, and butyrate (mM) during *in vitro* gut fermentation. SCFAs concentrations were measured at 0, 4, 8, and 24 h of fermentation in an *in vitro* gut model inoculated with fecal microbiota from healthy donors (panel A) and donors with IBS (panel B). Each fermentation

was carried out in the presence of: (i) untreated stool samples, (ii) stool treated with *L. brevis* CRAI, (iii) GABA-enriched prototype, and (iv) *L. brevis* CRAI + MSG + DTP (dehydrated tomato powder).

1.2.4. Amino acid profiling and GABA quantification in an *in vitro* gut fermentation model

Certain gut microbiota strains can produce metabolites such as the previously discussed SCFAs, as well as other biologically relevant compounds like amino acids. In this study, selected amino acids produced during fermentation were quantified, with a particular focus on GABA. As shown in Figure 15, several amino acids including threonine, serine, tryptophan, and, to a lesser extent, asparagine, were consumed during fermentation at the 24h time point. This trend was consistently observed across all donors, both healthy and IBS, and under all treatment conditions. Conversely, amino acids such as proline and tyramine were produced over time in both healthy and IBS donors, specifically in fermentations treated with *L. brevis* CRAI. Other amino acids, including isoleucine/leucine, phenylalanine, valine, and aspartate, remained stable throughout the fermentation period. Notably, the relative abundance of GABA in untreated samples from healthy donors was higher than in those from IBS donors. This deficiency in IBS samples was compensated in all treated conditions, as GABA levels increased at 24 hours. As expected, GABA concentrations were the highest in fermentations supplemented with the GABA-enriched prototype, confirming that the system accurately detected the added compound in both healthy and IBS donors. High GABA levels were also observed in 24-hour samples treated with *L. brevis* CRAI + MSG + DTP, which exceeded those of treatment with *L. brevis* CRAI alone or the untreated conditions. In samples where GABA was externally added (GABA-enriched prototype treatment), glutamate, already present and not supplemented, was not consumed. In contrast, in fermentations treated with *L. brevis*, GABA production was accompanied by a corresponding decrease in glutamate levels, a trend that was more pronounced in IBS donors.


Healthy
A
Stool
L. brevis CRAI
GABA-enriched prototype
L. brevis CRAI +MSG +DTP
Glycine
GABA
Glutamate
Proline
Aminovalerate
Aminovalerate 3
Valine
Threonine
Ile/Leu
Asparagine
Aspartate
Tyramine
Glutamine
Serine
Methionine
Phenylalanine
Tyrosine
Tryptophane
0 4 8 24 0 4 8 24 0 4 8 24 0 4 8 24
30
20
10
0
% of total AA signal

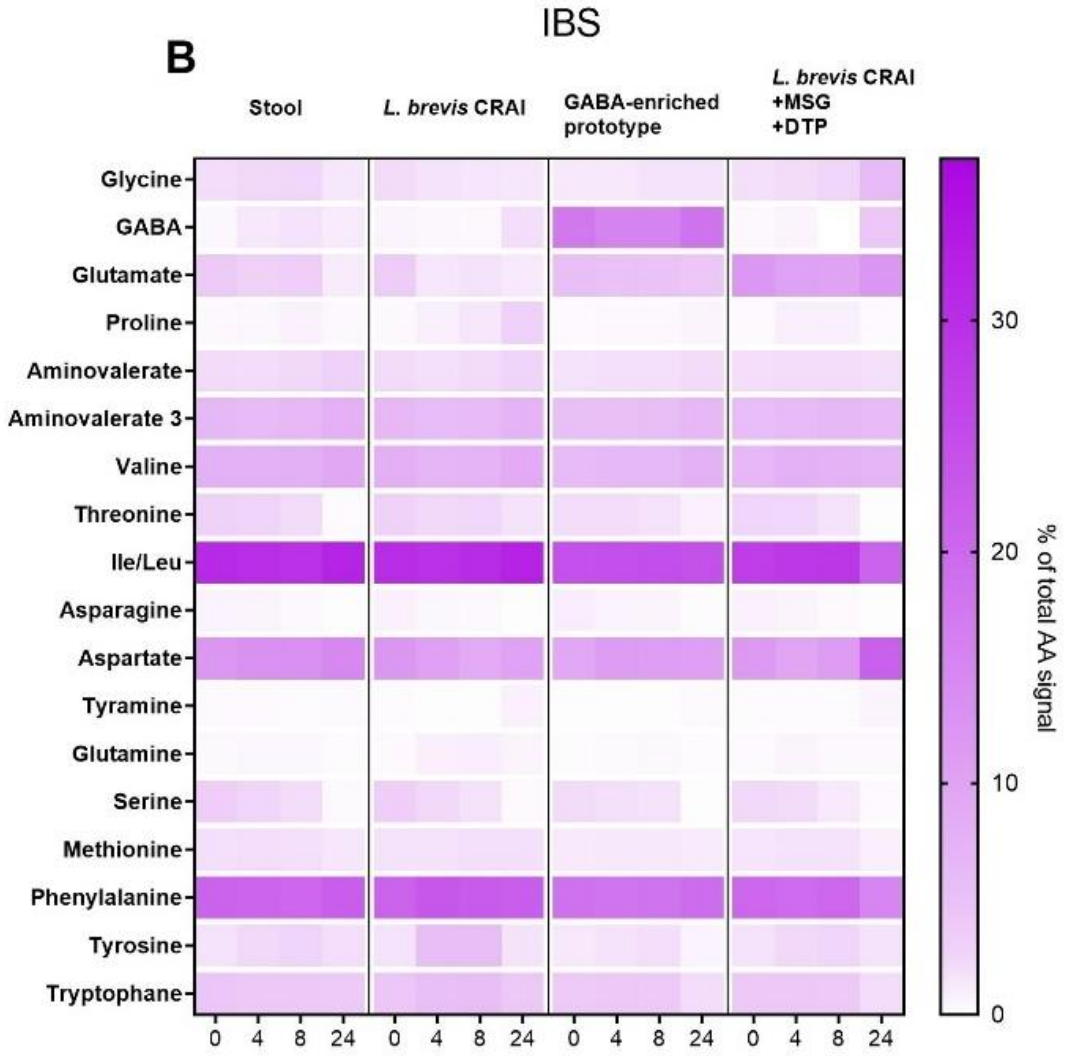
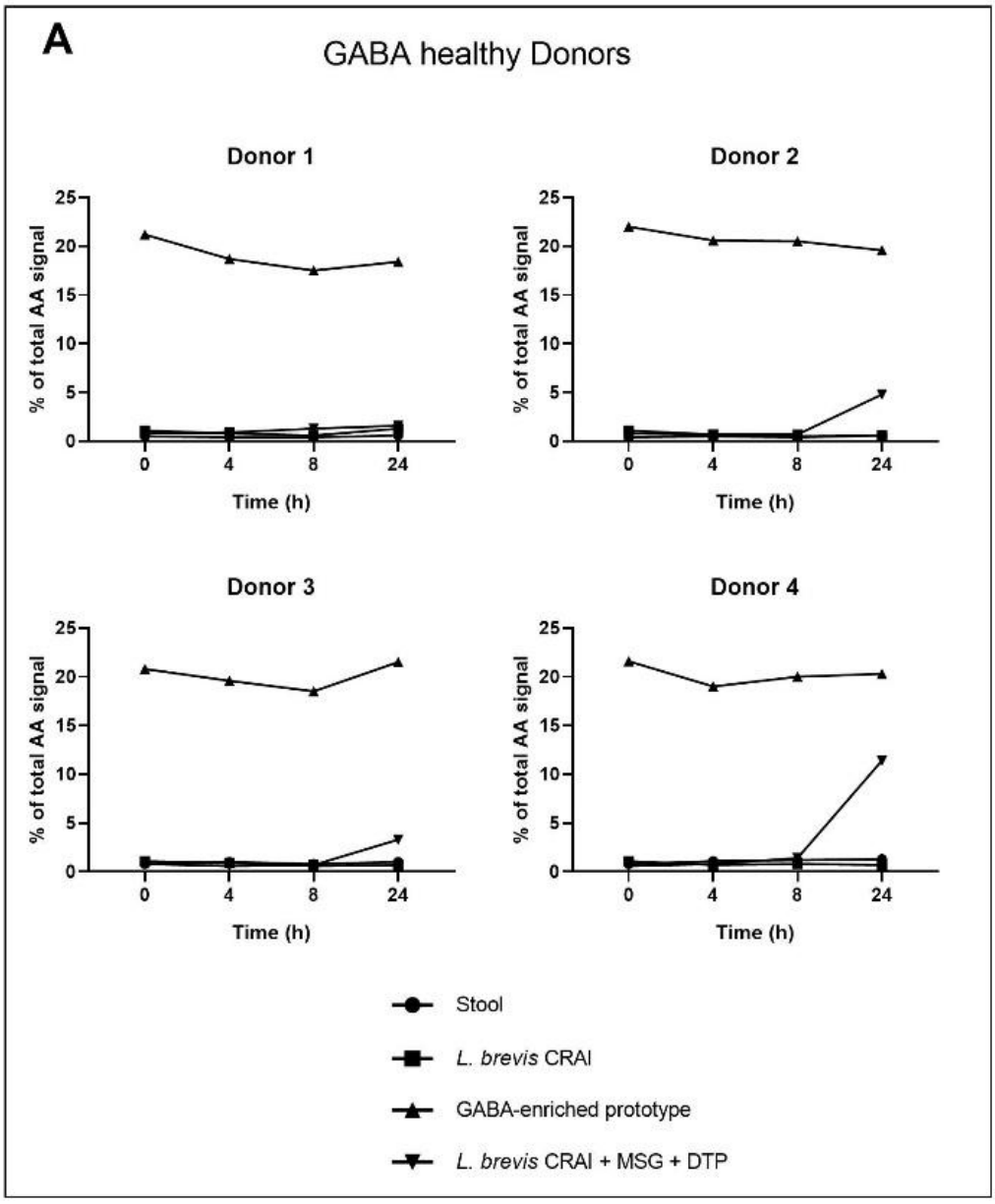


Figure 15. Relative abundance of amino acids quantified in *in vitro* gut fermentation models. Amino acid profiles were obtained from different time point (0, 4, 8, 24h) of *in vitro* fermentations inoculated with fecal microbiota from healthy donors (panel A) and donors with IBS (panel B). Each fermentation was performed under different conditions, including: (i) untreated stool samples, (ii) stool treated with *L. brevis* CRAI, (iii) a GABA-enriched prototype, and (iv) *L. brevis* CRAI + MSG + DTP. Amino acids are expressed as % of relative abundance of the total amino acids detected. Data represent the mean values and 4 different healthy donors (n=4) and 4 different IBS donors (n=4).

Given that GABA is the metabolite of primary interest in this study and the *L. brevis* CRAI was selected for its ability to produce this amino acid, we focused our analysis on GABA production in different donors. As shown in Figure 16, the sample supplemented with the GABA-enriched prototype exhibited a higher relative abundance of GABA already at time point 0 hour compared to the other treatments, and this level remained stable over time. This trend was observed consistently in both healthy and IBS donors. Regarding GABA production in healthy

individuals, except for DONOR 1, the highest GABA levels at 24 hours was detected in the system treated with the mixture of *L. brevis* CRAI + MSG + DTP, with the most pronounced production for DONOR 4 (Figure 16, panel A). In contrast, GABA production among IBS donors did not follow a uniform pattern (Figure 16, panel B). For instance, in DONOR 6, a marked increase in GABA levels at 24h was observed in the sample treated with *L. brevis* CRAI, exceeding the levels detected in the untreated stool. Similarly, in DONOR 8, GABA levels increased at 24 hours when the system was treated with the *L. brevis* CRAI + MSG + DTP formulation.



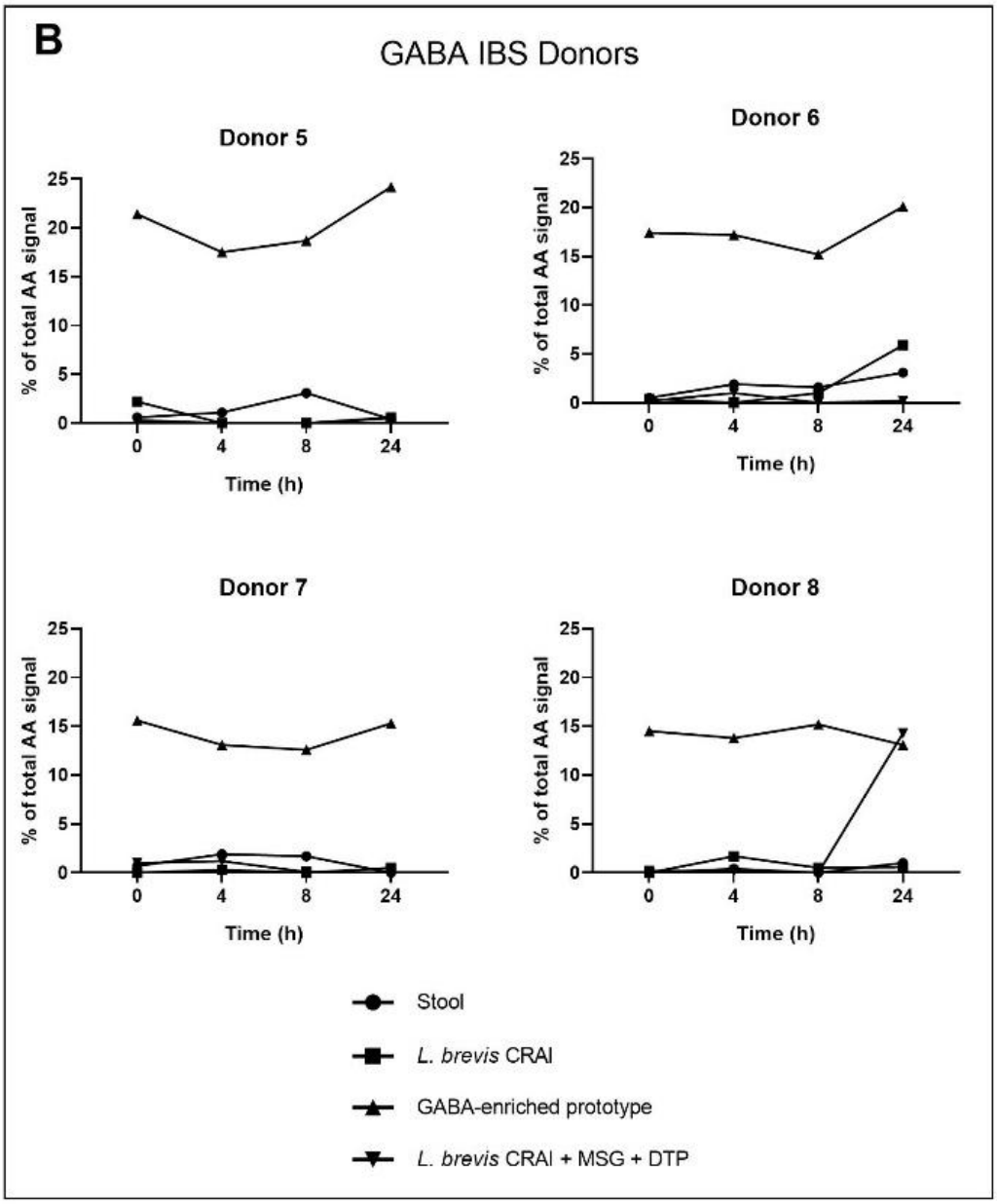


Figure 16. Relative abundance of GABA produced during *in vitro* gut fermentation. GABA levels were quantified at different time points (0, 4, 8, 24h) during fermentations using fecal inoculum from healthy donors (A) and IBS donors (B). Each fermentation was conducted under different treatment conditions: (i) untreated stool, (ii) stool treated with *L. brevis* CRAI, (iii) GABA-enriched prototype, and (iv) *L. brevis* CRAI + MSG + DTP. GABA concentrations are expressed as % of the total quantified amino acid.

1.2.5. Gut microbiome profile

Microbiome analysis was performed to assess the impact of different treatments on the microbial composition. First, the gut microbiome of IBS donors was compared to that of healthy donors. As shown in Figure 17, right panel, IBS donors were characterized by lower alpha diversity ($P = 0.03$), consistent with previous studies [266]. Taxonomically, the gut microbiome

of IBS donors was depleted in the genera *Collinsella*, *Fusicatenibacter*, and several members within the *Oscillospiraceae* family, while enriched in *Mediterraneibacter* ($P < 0.05$) (Figure 17, left panel).

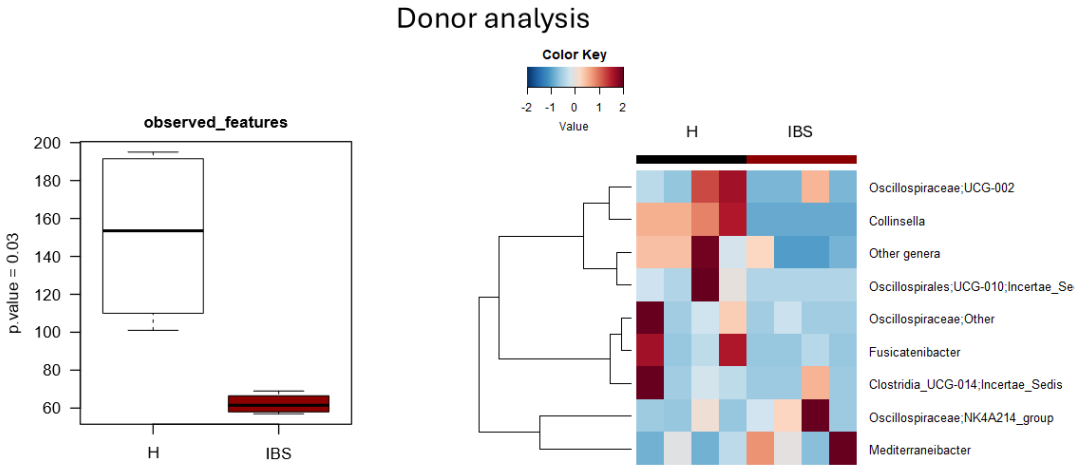
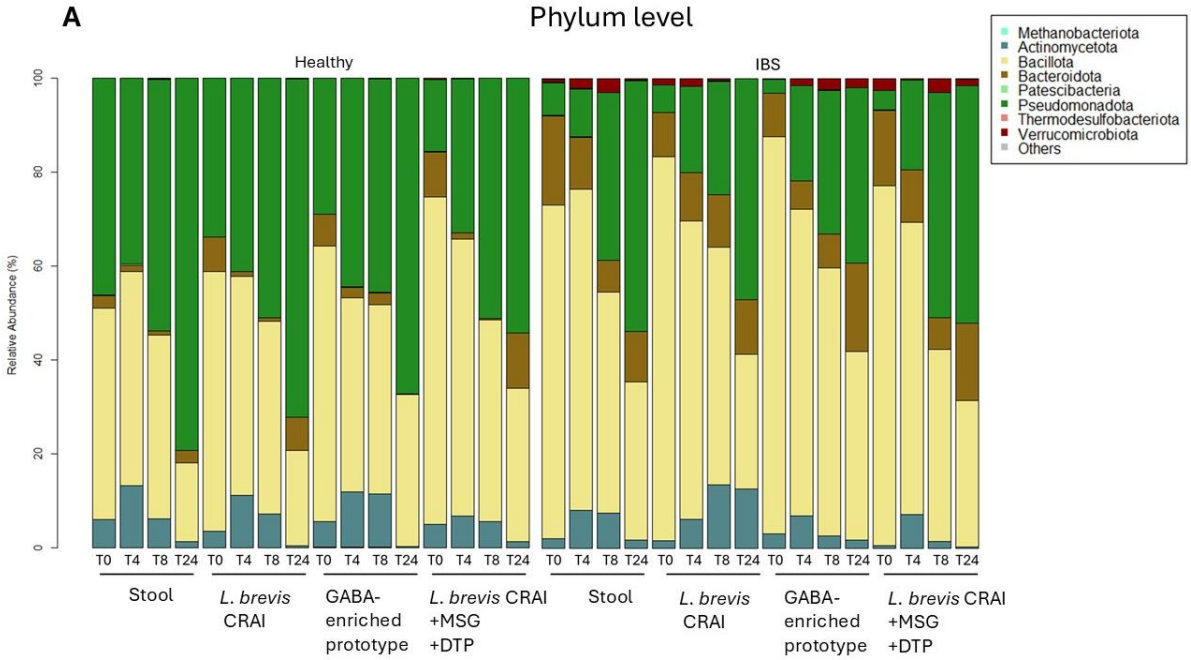


Figure 17. Gut microbiome of IBS donors compared to healthy donors. Boxplots showing the distribution of alpha diversity, estimated with the number of observed features, in the gut microbiome of IBS donors and healthy donors (right). Heatmap showing Ward-linkage clustering based on Pearson's correlation coefficients of the relative abundance of gut microbiota genera differentially represented between study groups ($P < 0.05$) (left).

Next, changes in fecal-derived microbial communities of IBS and healthy donors were monitored over time under *in vitro* gut fermentation conditions, the treatments tested on both donor groups were *L. brevis* CRAI, GABA-enriched prototype, and *L. brevis* CRAI combined with MSG and DTP (dehydrated tomato), collected at different time points throughout the fermentation process. Timepoint 24h was excluded from the analysis due to system saturation by members of the *Enterobacteriaceae* family (with relative abundance up to 50%). As shown in Figure 18, panel A, all samples, regardless of health status, treatment and timepoint, were dominated by the phyla Bacillota, Pseudomonadota, Bacteroidota and Actinomycetota. However, Bacillota tended to decrease over time, paralleled by an increase in Pseudomonadota and Bacteroidota. Furthermore, fecal-derived microbial communities of IBS donors were enriched in Verrucomicrobiota (mucus lovers) compared to the microbial communities of healthy donors. At the family level (Figure 18, panel B) the ecosystem was dominated by a few highly abundant families, including *Lachnospiraceae*, *Peptostreptococcaceae*, *Burkholderiaceae*, and *Enterobacteriaceae*, which constituted the core microbial community throughout the fermentation process. *Bifidobacteriaceae* and *Coriobacteriaceae* showed a progressive increase over time in fecal-derived microbial communities of IBS donors, which

exhibited lower baseline levels of these taxa compared to the microbial communities of healthy donors. Notably, a shift in the microbial community structure was observed at 4h in IBS donors treated with the combination of *L. brevis* CRAI, MSG and DTP. This intervention led to an enrichment of the *Bifidobacteriaceae* family, whose relative abundance increased from 0% to 4%. Conversely, *Lachnospiraceae*, *Ruminococcaceae*, and *Oscillospiraceae* (families that typically dominate adult gut microbial ecosystems) generally decreased across conditions, except in fecal-derived microbial communities of healthy donors treated with *L. brevis* and the GABA-enriched prototype at 8h, and in those of IBS donors across all treatments. *Peptostreptococcaceae* showed an upward trend at 4h and 8h in healthy donors across all treatments compared to baseline (0 hour); this increase was also observed in IBS donors, with higher relative abundance in treated groups. Similar results were observed at the genus level, with *Bifidobacterium* and *Collinsella* increasing at 4h in IBS donors treated with *L. brevis* CRAI + MSG + DTP. In contrast, *Roseburia* (*Lachnospiraceae*), *Oscillospiraceae* UCG-002 and UCG-005, and *Faecalibacterium* (*Ruminococcaceae*) generally declined in healthy donors, but not in IBS groups treated with *L. brevis* CRAI and the GABA-enriched prototype, the levels of which were maintained or slightly increased (Figure 18, panel C).



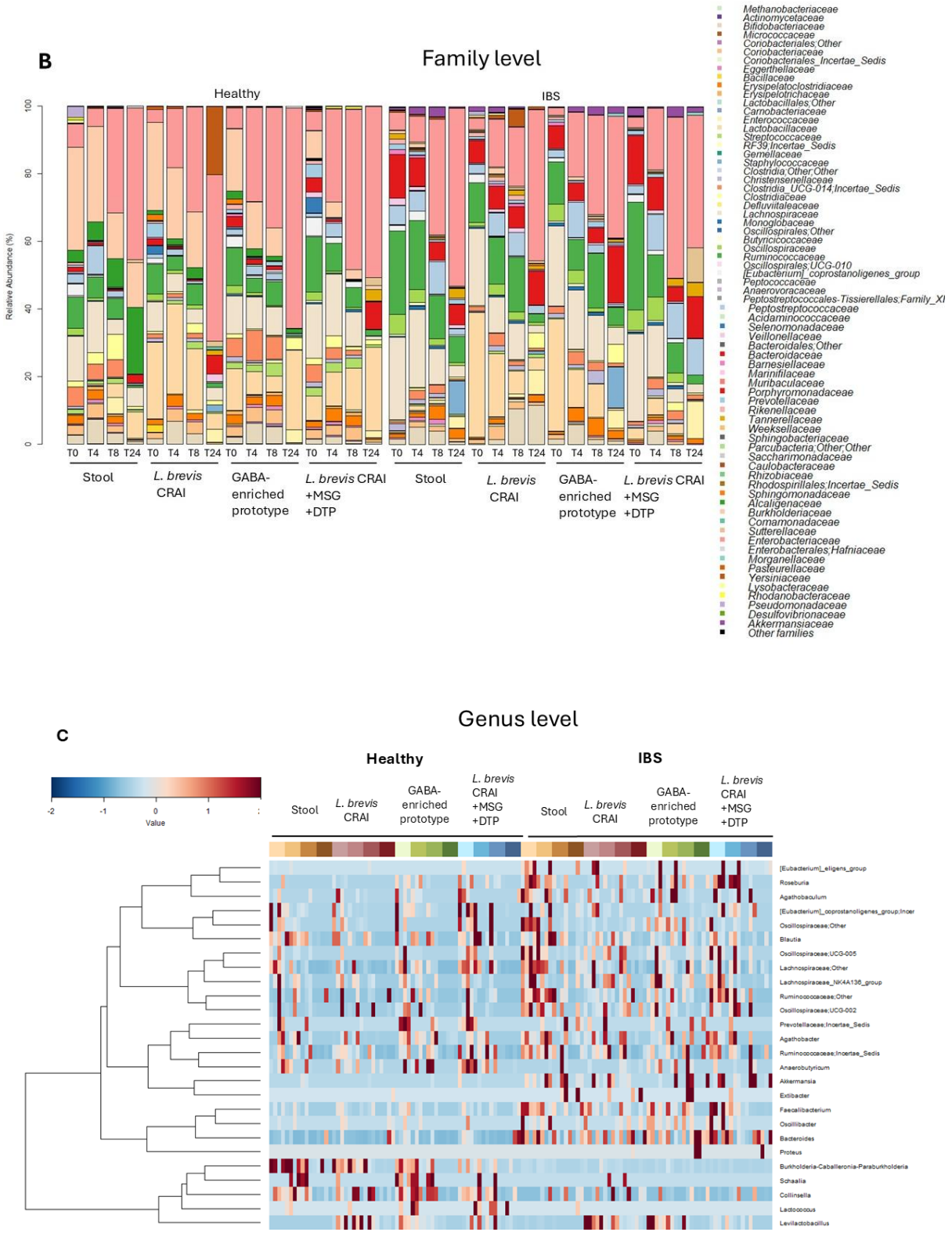


Figure 18. Fecal-derived microbial communities of IBS and healthy donors in 24-h fermentation experiments in the presence of stool (i.e., faecal slurry without any substrate addition), *L. brevis* CRAI, GABA-enriched prototype, *L. brevis* CRAI + MSG + DTP. Bar plots showing the major phyla (panel A) and families in faecal-derived microbial communities of 4 healthy donors and 4 IBS donors at 0, 4, 8 and 24 h of fermentation in the presence of

stool (i.e., faecal slurry without any substrate addition), *L. brevis* CRAI, GABA-enriched prototype, *L. brevis* CRAI + MSG + DTP. Heatmap showing Ward-linkage clustering based on Pearson's correlation coefficients of the relative abundance of genera differentially represented between study groups ($p < 0.05$), across different time points and donor types (panel C). Samples include stool (i.e., faecal slurry without substrate addition*), L. brevis* CRAI, GABA-enriched prototype, and *L. brevis* CRAI + MSG + DTP.

1.3. Discussion

Numerous GABA-based supplements are commercially available, several traditional and functional foods, including kimchi are commonly enriched with GABA [267,268]. The health-promoting properties of GABA have been extensively discussed in previous sections. Accordingly, the optimized GABA production from tomato pomace served as a foundational step for the development of a GABA-enriched food prototype intended for industrial-scale production. Building on this objective, a dehydrated tomato powder was selected as the fermentation substrate for *L. brevis* CRAI, under previously established optimal conditions for GABA biosynthesis. Experimental data confirmed that the substrate provided by DEPOFARMA supported bacterial growth at both 48 and 72 hours, with GABA production reaching its peak at 72 hours. The results clearly indicate that fermentation time is a critical parameter in optimizing GABA production. Extending the process to 72 hours resulted in a marked increase in yield, with conversion rates rising from 14% at 48 hours to 67% at 72 hours. This observation guided our decision to prolong the fermentation period, particularly in view of scale-up considerations, where time becomes a key factor in ensuring metabolic efficiency. The strain appears to require extended incubation to reach its full biosynthetic potential. This approach is consistent with findings from other bioprocess optimization studies. For instance, in the work by Xie et al. (2025), the authors demonstrated that extending fermentation time during the scale-up of cellulase production in *Trichoderma* sp. KMF006 significantly improved both yield and performance, highlighting the importance of temporal parameters in industrial bioreactor operations [269].

Subsequently, fermentation volumes were scaled up while maintaining the same operational parameters and extending the process to 72 hours. As expected, the transition from laboratory to industrial scale introduced several challenges. Fermentation scale-up is inherently complex and often relies on empirical adjustments, leading to inconsistencies in yield and conversion efficiency across scales [270]. In our case, both GABA concentration and conversion percentage were slightly reduced during the scale-up. This outcome is consistent with literature reports indicating that nutrient distribution becomes less homogeneous in larger bioreactors,

potentially affecting microbial performance and metabolite production. Takors et al. (2012) demonstrated that industrial-scale fermentations involving *Escherichia coli*, *Saccaromices cerevisiae*, and *Corynebacterium glutamicum* are particularly sensitive to mixing inefficiencies, which can compromise productivity and yield [271]. Similar findings have been reported in more recent studies; for example Hoang et al. (2023) demonstrated that compartmentalized bioreactors, characterized by zones of poor mixing, can significantly affect L-phenylalanine production by *E. coli* [272]. Likewise, Arauzo-Aguilera et al. (2023) and Junker (2004) underscored how variations in strain performance and reactor design, particularly in terms of nutrient and oxygen distribution, can lead to substantial differences in productivity under industrial-scale conditions [273,274]. The possibility of producing GABA-enriched foods using agro-industrial substrates has been explored in a lot of studies, for instance, Del Toro-Barbosa et al. (2025) developed a fermented whey beverage enriched with raspberry juice, in which a consortium of LAB produced 1.5 mM of GABA. This formulation was proposed as a psychobiotic dietary intervention, highlighting the dual benefit of valorizing food industry waste while promoting public health [275]. Similarly, Redruello et al. (2021) isolated GABA-producing *L. lactis* strains from camel's milk and employed them as starter cultures for GABA-enriched cheese. Their process achieved a GABA concentration of 4.43 mM (equivalent to 457 mg/kg), demonstrating the potential of microbial fermentation in dairy matrices for functional food innovation [276]. Overall, the outcomes of our study are consistent with previous reports demonstrating that microbial fermentation using agro-industrial substrates can yield substantial amounts of GABA under optimized conditions. Despite the expected challenges associated with scale-up, our results confirm the possibility of producing GABA-enriched food matrices on an industrial scale, aligning with recent literature that highlights both the biotechnological potential and the nutritional relevance of such functional formulations.

The prototype developed in this study was evaluated using an *in vitro* gut fermentation model to assess its impact on microbiota modulation and metabolite production. In addition to the prototype, we tested the strain responsible for GABA synthesis to determine whether its administration alone could confer health benefits. Furthermore, we investigated a formulation composed of *L. brevis* CRAI combined with MSG, as well as a substrate based on dehydrated tomato powder to enhance GABA production.

We utilized a static *in vitro* gut fermentation model to perform preliminary screening of the *L. brevis* CRAI, GABA-enriched prototype, *L. brevis* CRAI + MSG + DTP, assessing their effects on microbiome composition and metabolite profiles in fecal microbial communities from IBS

donors. The static *in vitro* gut model was selected for its proven utility in simulating colonic fermentation processes under controlled conditions. This system allows to test dietary supplements and microbial modulation prior to *in vivo* validation. Several studies have demonstrated its effectiveness in evaluating microbiota composition and associated metabolic products, including short-chain fatty acids and neurotransmitter precursors [121]. Its capacity to isolate specific variables, such as microbial community structure, nutrient availability, and fermentation kinetics, makes it a valid system to investigate microbiome-modulating strategies. We focused our investigation on fecal-derived microbial communities from donors with irritable bowel syndrome (IBS), a disorder characterized by both gastrointestinal and neuropsychological symptoms. IBS is commonly associated with gut dysbiosis, including reduced microbial diversity and altered abundance of key taxa involved in mucosal integrity and metabolite production [74]. In addition to its intestinal manifestations, IBS is frequently comorbid with anxiety and depression, suggesting a disruption of the gut–brain [277]. Emerging evidence indicates that patients with IBS exhibit reduced levels of GABA, a key inhibitory neurotransmitter involved in mood regulation and visceral sensitivity [74]. Given this context, we hypothesized that supplementation with a GABA-producing strain, *L. brevis* CRAI, and its GABA-enriched prototype could beneficially modulate the microbial ecosystem and enhance GABA biosynthesis. The inclusion of MSG and dehydrated tomato powder (DTP) in the administered mixture was intended to assess whether these components could replicate the effects of the prototype, by providing precursors and substrates for GABA synthesis.

Initially, we assessed the concentration of short-chain fatty acids (SCFAs), which are low-molecular-weight organic acids characterized by a carbon backbone of six atoms or fewer. These metabolites are predominantly generated through the anaerobic breakdown of non-digestible carbohydrates, such as dietary fibres and resistant starches, by the microbiota residing in the colon [278]. The majority of SCFAs are represented by acetate, propionate, and butyrate. SCFAs are absorbed by colonocytes via proton- or sodium-dependent transporters (MCTs and SMCTs). While some SCFAs are metabolized locally, others enter the portal circulation and fuel liver cells, except acetate, which bypasses hepatic oxidation. Only a small portion of SCFAs reaches systemic circulation [279]. SCFAs support gut health by exerting various local actions, including strengthening the intestinal barrier, enhancing mucus secretion, modulating inflammatory responses, and potentially lowering the risk of developing colorectal cancer [280]. Their physiological roles are exerted through multiple pathways, including the stimulation of G-protein-coupled receptors, interaction with free fatty acid receptors, and

regulation of gene expression via epigenetic mechanisms [278]. SCFAs influence systemic physiology by blocking histone deacetylase (HDAC) enzymes, which enhances lysine acetylation on histones across diverse cell types. This molecular signalling has been identified not only in the gut and immune-related tissues, but also in the peripheral and central nervous systems. These effects suggest a broader role for SCFAs beyond local intestinal functions [281]. Despite limited systemic circulation, SCFAs exert broad effects at systemic level, notably modulating immune responses through butyrate-induced Treg differentiation and inflammation control. They also influence gut health, energy metabolism, liver function, and neurobehavioral aspects like appetite and sleep [282]. Microbial metabolites can either improve or aggravate conditions such as inflammatory bowel disease [283].

SCFAs have been shown to alleviate stress-induced behavioral alterations, modulate intestinal permeability, and exert antidepressant and anxiolytic effects [284]. In our study, we detected high concentrations of acetate, followed by propionate and butyrate, with ratios consistent with those reported in the literature (acetate:propionate:butyrate = 60:20:20) [283,285].

SCFA production increased significantly at 24 hours across all donors with some differences across the different treatments, with levels exceeding those observed in untreated stool controls. This increase was particularly pronounced in samples from IBS donors. These findings are in line with previous studies, such as Mousavi et al. (2022), who used a dynamic gut fermentation model inoculated fecal samples obtained from two healthy adult donors. The authors demonstrated that co-administration of three GABA-producing strains (two *Lactobacillus* spp. and one *Streptococcus* spp.) led to elevated SCFA levels at 12 hours post-treatment compared to the untreated control [286]. In our study, we observed a general increase in SCFA levels as early as 8 hours across conditions, indicating a rapid onset of microbial metabolic activity. These findings suggest that the introduction of GABA-producing strains, such as *L. brevis* CRAI, may promote the enrichment of microbial communities involved in SCFA synthesis. SCFAs play a crucial role in maintaining intestinal barrier integrity, protecting against inflammation, and facilitating gut-brain communication [287]. Acetate has been shown to suppress appetite, reduce body weight, and lower serum cholesterol and triglyceride levels [288]. Propionate enhances epithelial barrier function and gut integrity, and influences glucose and lipid homeostasis [289], in addition to modulating gene expression through immunomodulatory pathways [287]. Among SCFAs, butyrate is considered the most functionally significant. It serves as a primary energy source for colonocytes, promotes tight junction expression, and supports barrier integrity, thereby preventing pathogen invasion [290].

Butyrate also exhibits anti-inflammatory and antitumor properties by inhibiting proinflammatory cytokine release and tumor cell proliferation [291,292]. Moreover, SCFAs are directly involved in neuroendocrine signalling along the microbiota–gut–brain axis, contributing to brain physiology and neuroprotection [293].

Our analyses revealed that untreated fecal-derived microbial communities from IBS donors exhibited reduced or absent SCFA production over time compared to treated samples. This observation is consistent with studies that demonstrate low intestinal SCFA concentrations in dysbiosis conditions [293–296]. The administration of probiotics capable of restoring SCFA levels may represent a promising strategy for managing these conditions and alleviating associated symptoms. Our findings suggest that *L. brevis* CRAI, the GABA-enriched formulation, and the mixture of all three components (*L. brevis* CRAI + MSG + dehydrated tomato powder) due to their ability to increase SCFA levels may serve as potential biotherapeutic adjuvants, not only for mental health disorders but also for conditions characterized by SCFA depletion, such as IBD, IBS, and colorectal cancer [297].

In addition to the quantification of SCFAs, we also investigated amino acid production during the fermentation period across all treatment conditions in both healthy individuals and those with IBS. Dietary proteins are primarily metabolized in the small intestine, with approximately 5–10% of ingested proteins escaping absorption and reaching the colon [298]. These proteins are catabolized into amino acids, which can enter systemic circulation and act as key intermediates in the complex host–microbiota interactions. It is well established that the gut microbiota contributes to the energetic metabolism of host-derived proteins and peptides, generating SCFAs and indoles, but also potentially toxic nitrogenous and sulfur metabolites, such as ammonia, amines, nitrates, nitrites, hydrogen sulphide, and phenolics [299]. In our study, amino acid profiling revealed notable trends, particularly a progressive depletion of tryptophan over time. This reduction was observed in all treatments among healthy subjects and was especially pronounced in IBS samples treated with the GABA-enriched prototype and *L. brevis* CRAI + MSG + DTP compared to untreated controls. Tryptophan is an essential amino acid that influences the production of several key metabolites, including indoles, kynurenine, and serotonin [300]. In particular, it is a precursor of serotonin, which is synthesized in the gut and plays a critical role in regulating motility, visceral sensitivity, and mucosal secretion. It also contributes to central nervous system physiology and neurobiological functions [301]. The gut microbiota has been shown to play a central role in tryptophan metabolism. Alterations in microbial metabolism of tryptophan have been linked to changes in mood and cognitive

function via the central nervous system, as well as to enteric nervous system activity, including secretion, motility of the GI tract, and sensory perception [302]. Probiotic strains belonging to the genera *Lactobacillus* and *Bifidobacterium* are known to positively modulate tryptophan metabolism. These microbes can either directly convert tryptophan into serotonin [303] or indirectly enhance colonic serotonin synthesis [304]. Our findings are in line with existing literature, supporting the hypothesis that the observed reduction in tryptophan, particularly in treated samples, may reflect its utilization for the synthesis of serotonin or other microbial-derived metabolites such as indoles. Zelante et al. (2013) demonstrated that certain probiotic strains are capable of degrading tryptophan into indole derivatives, which act as ligands for the aryl hydrocarbon receptor (AhR) [305]. AhR signalling is a key component of mucosal immunity, contributing to epithelial renewal, barrier integrity, and immune cell regulation [306]. Impaired microbial capacity to convert tryptophan into AhR ligands has been associated with gastrointestinal disorders characterized by disrupted AhR pathways [307]. Based on our data, the administration of the GABA-enriched prototype may help restore this metabolic pathway, particularly in IBS patients, but potentially also in healthy individuals.

The amino acid of particular interest in this study is GABA, which plays a crucial role in modulating neuronal excitability and contributes to the regulation of sleep and anxiety by counterbalancing excitatory processes triggered by stress and mood disorders [308]. GABA is involved not only in CNS signalling but also in GI tract regulation, facilitating communication along the gut–brain axis. It contributes to the homeostasis of the enteric nervous system influencing processes such as gastric acid secretion, gastric emptying, intestinal motility, and visceral pain perception [309]. GABA has been shown to regulate emotional stress responses, aiding the brain in processing and reacting to stress-related signals, thereby impacting GI symptoms. Emotional stress can alter gut motility, increase visceral hypersensitivity, and disrupt nervous system function, collectively exacerbating IBS symptoms such as abdominal pain, bloating, diarrhea, and constipation [310]. Notably, reduced GABA levels have been reported in IBS patients and are associated with comorbid anxiety and depressive symptoms [311]. Enhancing GABAergic activity may therefore help mitigate these symptoms by modulating excessive GI responses and alleviating abdominal discomfort and related manifestations [312]. Quantitative analysis of GABA in our *in vitro* model revealed that treatment with the GABA-enriched prototype maintained stable GABA levels up to 24 hours. Furthermore, when the system was supplemented with the components required for GABA biosynthesis (*L. brevis* CRAI+MSG+DTP), GABA was actively produced. A lower but detectable level of GABA was

also observed when only the GABA-producing strain *L. brevis* CRAI was administered. These findings are consistent with recent work by Casertano et al. (2024), in which 13 LAB strains belonging to *Levilactobacillus brevis*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Ligilactobacillus salivarius*, and *Streptococcus thermophilus* were tested in the SHIME system. That study demonstrated the ability of these strains to modulate microbiota composition, specifically increasing the relative abundance of taxa associated with anti-inflammatory effects, and to promote GABA production [313]. Additional evidence supports the role of GABA-producing probiotics in modulating CNS function. Long-term administration of *Lactobacillus rhamnosus* JB–1 has been shown to alter GABA receptor expression in the brain, resulting in reduced anxiety-like and depressive behaviours [314]. Similarly, oral supplementation with GABA-producing species such as *Bifidobacterium dentium* has demonstrated efficacy in reducing visceral hypersensitivity and abdominal pain in animal models [315]. Taken together, these findings suggest that the GABA-enriched prototype tested in our study holds promise as a potential therapeutic supplement for IBS, a condition characterized by GABA deficiency. Its ability to sustain GABA levels over time may contribute to symptom relief. Moreover, the mixture containing *L. brevis* CRAI + MSG + DTP also showed notable GABA production, indicating its potential as an alternative GABA-enhancing supplement.

Changes in microbial communities were monitored over time to assess the dynamics of the *in vitro* fermentation system. At 24 hours, a notable overgrowth of *Enterobacteriaceae* was observed, accompanied by a drop in alpha diversity. This trend is consistent with previous reports showing that *Enterobacteriaceae* are opportunistic taxa that proliferate under conditions of high nutrient availability and limited microbial competition, typical of static, low-density *in vitro* gut models [316]. Similar findings have also been reported in other *in vitro* studies, including those evaluating lignan metabolism [317]. Based on this, our analysis focused primarily on earlier time points, which better reflect physiologically relevant microbial interactions. For this reason, microbiome analyses in our study were limited to the first 8 hours of fermentation. First, we confirmed that the gut microbiome of IBS patients is dysbiotic, characterized by reduced alpha diversity and lower relative abundance of taxa that are typically represented in a healthy gut microbiome [318].

Across all samples, in healthy donors and IBS donors, in untreated and treated condition, the microbial ecosystem was consistently dominated by the phyla Bacillota, Pseudomonadota, Bacteroidota, and Actinomycetota. Notably, IBS-derived communities exhibited a distinct

enrichment in Verrucomicrobiota, a phylum associated with mucin degradation, potentially reflecting altered mucosal interactions typical of IBS pathology in line with litterarure [319]. At the family level, *Lachnospiraceae*, *Peptostreptococcaceae*, *Burkholderiaceae,* and *Enterobacteriaceae* formed the core microbiota across conditions. A progressive increase in *Bifidobacteriaceae* and *Coriobacteriaceae* was observed in IBS donors, particularly following treatment with *L. brevis* CRAI + MSG + DTP. This mixture induced a marked shift at 4 hours, elevating *Bifidobacteriaceae* from undetectable levels to 4%, indicating a potential prebiotic effect. This shift is likely linked to enhanced SCFAs production, especially acetate, which was notably elevated [320]. Additionally, our data showed that *Lachnospiraceae* maintained consistently high relative abundance across all samples. This is a favourable outcome, as members of this family are associated with several health-promoting activities in the gut and beyond, mainly mediated by SCFAs [321].

Conversely, families traditionally dominant in adult gut ecosystems, *Ruminococcaceae*, and *Oscillospiraceae*, showed a general decline across conditions. Exceptions were noted in healthy donors treated with the GABA-enriched prototype at 8 hours and in IBS donors across all treatments, where these families, were maintained or slightly increased. This preservation may suggest a stabilizing effect of *L. brevis* CRAI formulations on key SCFAs-producing taxa [322].

Interestingly, previous studies have reported a reduction in *Collinsella aerofaciens* in the fecal microbiota of IBS patients compared to healthy controls, with lower abundance of this genus being associated with increased symptom severity [323,324]. In our study, we observed a notable increase in *Collinsella* and *Bifidobacterium* (*Bifidobacteriaceae* family) at 4 hours in IBS donors treated with *L. brevis* CRAI + MSG+ DTP, suggesting that this formulation may help restore beneficial taxa typically depleted in IBS. Moreover, *Faecalibacterium*, a well-known butyrate producer belonging to the *Ruminococcaceae* family, is frequently found to be reduced in IBS and other intestinal and metabolic disorders [325,326]. Although in our study we saw a slight increase in *Faecalibacterium* (even though did not reach statistical significance) and its levels were maintained or slightly elevated in IBS donors treated with *L. brevis* CRAI and the GABA-enriched prototype. This is particularly relevant given the role of *Faecalibacterium* in producing butyrate [327], a key metabolite, and its positive association with improved stool consistency, as measured by the Bristol Stool Scale [328].

Overall, the treatments tested in this study appear to impact the fecal-derived microbial communities of both healthy and IBS donors. In particular, the treatments partially reversed

IBS-related dysbiosis, by increasing the proportion of healthy taxa in IBS donors. These findings suggest that the *L. brevis* CRAI strain and the GABA-enriched prototype may exert a stabilizing effect on the health-related taxa and on SCFA-producing microbial communities in dysbiotic environments, potentially contributing to both intestinal and neurological symptom relief in IBS. Further studies are needed to explore the functional consequences of these compositional shifts and validate their relevance *in vivo*.

Several limitations should be acknowledged. First, the study involved a limited number of donors, which may have contributed to substantial inter-individual variability in microbiota composition and treatment response. Second, the use of a closed *in vitro* fermentation system does not fully replicate the dynamic and absorptive processes of the human gastrointestinal tract. Consequently, the substrates in this model remain confined within the glass system or are metabolized without systemic uptake, unlike *in vivo* conditions. Despite these constraints, the findings offer valuable preliminary evidence supporting the potential of the *L. brevis* CRAI and the derived GABA-enriched nutraceutical to modulate the gut microbiota. Future studies should aim to translate these *in vitro* observations into *in vivo* contexts, including animal models and clinical trials, to further assess the efficacy and physiological relevance of the probiotic/nutraceutical under real-life conditions.

Conclusions

This study began with the screening of lactic acid bacteria isolated from tomato, aiming to identify strains with high GABA production potential. The isolates were taxonomically identified through 16S rRNA gene sequencing, initial selection was based on molecular screening for the presence of the *gadB* gene, which encodes glutamate decarboxylase, an enzyme responsible for the conversion of glutamate into GABA. Only strains that possess *gadB* gene were tested for GABA production. To enhance GABA biosynthesis, optimal fermentation conditions in MRS were established, including the addition of 4% MSG and acidification of the initial pH at 5. Quantification of GABA production revealed that the selected *L. brevis* CRAI strain was the most efficient producer with GABA production of 179 mM. This strain was further characterized for its functional properties, demonstrating strong inhibitory activity against pathogenic bacteria and notable anti-inflammatory potential, as well as a good adhesion capacity to intestinal epithelial cells. Whole-genome sequencing of *L. brevis* CRAI confirmed the absence of genes associated with virulence or antibiotic resistance, supporting its safety for human consumption. Genomic analysis revealed the presence of two genes involved in GABA production: *gadA*, located within an operon, and *gadB*, positioned downstream and outside the operon. Notably, the addition of MSG led to a significant increase in glutamate availability, which was associated with a 52-fold up-regulation of *gadA* mRNA expression, suggesting a key role of this operon in enhancing GABA synthesis. Following strain selection, a GABA-enriched prototype was developed by fermenting *L. brevis* CRAI in a tomato-based by-product. This approach aimed to valorise nutrient-rich waste streams. Among various fruit and vegetable substrates tested, tomato pomace, also the strain's original isolation source, proved most effective in supporting bacterial growth. Fermentation conditions were optimized using a central composite design (CCD), resulting in a GABA 93 mM and a production efficiency of 79%. To further characterize the sensory profile of the fermented product, the volatile aromatic compounds were analysed. The fermentation process led to the production of acids and alcohols, contributing fruity notes to the final product. These findings support the potential for consumer acceptability and sensory appeal of the GABA-enriched formulation. For industrial scalability and matrix standardization, the fermentation process was transferred to a dehydrated tomato substrate provided by partner company DEPOFARMA. Fermentations were first conducted at laboratory scale and subsequently scaled up, following the previously optimized conditions. The results confirmed that GABA production and yield remained consistent across volumes (GABA 80 mM and 67% conversion yield), demonstrating the robustness of the

process for industrial application. To evaluate its functional impact on the gut microbiota and associated metabolites, *L. brevis* CRAI, the GABA-enriched prototype, and a combination of *L. brevis* CRAI + MSG + dehydrated tomato were tested in an *in vitro* intestinal fermentation model simulating both healthy and dysbiosis (IBS) conditions. All treatments promoted the production of SCFAs, including acetate, propionate, and butyrate. The combination treatment supported de novo GABA production, while the GABA-enriched prototype maintained stable GABA levels over time, an important finding given the reported GABA deficiency in IBS patients and its association with symptom severity. Taxonomic analysis revealed that the treatments differentially supported an increase in the relative abundance of beneficial taxa such as *Collinsella*, *Lachnospiraceae*, and *Oscillospiraceae*. Notably, the mixture of *L. brevis* CRAI + MSG + tomato dehydrated significantly increased the abundance of *Bifidobacteriaceae* in IBS samples, where this family is typically depleted. After confirming the *in vitro* health-promoting properties of the *L. brevis* CRAI strain and the relative prototype, regulatory considerations were addressed. Based on historical evidence of food use prior to 1997, the strain and the GABA-enriched prototype were not classified as novel food. Furthermore, the *L. brevis* CRAI strain was patented (Patent No. 31.D1066.12.IT.4) for its GABA-producing capacity and its probiotic properties, including antimicrobial and anti-inflammatory activities.

In conclusion, this study demonstrates not only the safety and intestinal efficacy of the *L. brevis* CRAI strain and the derived GABA-enriched prototype, but also their potential as sustainable probiotic/nutraceutical products. By utilizing an agro-industrial by-product as a fermentation substrate, this approach offers an environmentally and economically advantageous strategy for the development of functional foods aimed at supporting gut health, particularly in individuals with dysbiosis-related conditions such as IBS. As this is a preliminary study, future perspectives include optimizing the formulation of probiotic/nutraceutical products and evaluating the efficacy of the bioactive molecules through animal models or clinical trials, to validate the potential health benefits and support the application in nutrition strategies.

1. Bibliography

1. Thompson JF, Pollard JK, Steward FC. Investigations of Nitrogen Compounds and Nitrogen Metabolism in Plants. Iii. γ-Aminobutyric Acid in Plants, With Special Reference to the Potato Tuber and a New Procedure for Isolating Amino Acids Other Than α-Amino Acids. Plant Physiol. 1953;28. https://doi.org/10.1104/pp.28.3.401

2. ROBERTS E, FRANKEL S. gamma-Aminobutyric acid in brain: its formation from glutamic acid. J Biol Chem. 1950;187. https://doi.org/10.1016/s0021-9258(19)50929-2

3. Zhu S, Noviello CM, Teng J, Walsh RM, Kim JJ, Hibbs RE. Structure of a human synaptic GABAA receptor. Nature. 2018;559. https://doi.org/10.1038/s41586-018-0255-3

4. Chen Y, Dong E, Grayson DR. Analysis of the GAD1 promoter: Trans-acting factors and DNA methylation converge on the 5′ untranslated region. Neuropharmacology. 2011;60. https://doi.org/10.1016/j.neuropharm.2010.09.017

5. Cellot G, Cherubini E. GABAergic signaling as therapeutic target for autism spectrum disorders. Front. Pediatr. 2014. https://doi.org/10.3389/fped.2014.00070

6. Leresche N, Lambert RC. GABA receptors and T-type Ca2+ channels crosstalk in thalamic networks. Neuropharmacology. 2018. https://doi.org/10.1016/j.neuropharm.2017.06.006

7. Sarasa SB, Mahendran R, Muthusamy G, Thankappan B, Selta DRF, Angayarkanni J. A Brief Review on the Non-protein Amino Acid, Gamma-amino Butyric Acid (GABA): Its Production and Role in Microbes. Curr. Microbiol. 2020. https://doi.org/10.1007/s00284-019-01839-w

8. De Vos WM, Tilg H, Van Hul M, Cani PD. Gut microbiome and health: mechanistic insights. Gut. 2022;71. https://doi.org/10.1136/gutjnl-2021-326789

9. Berg G, Rybakova D, Fischer D, Cernava T, Vergès MCC, Charles T, et al. Microbiome definition re-visited: old concepts and new challenges. Microbiome. 2020. https://doi.org/10.1186/s40168-020-00875-0

10. De La Fuente-Nunez C, Meneguetti BT, Franco OL, Lu TK. Neuromicrobiology: How Microbes Influence the Brain. ACS Chem. Neurosci. 2018. https://doi.org/10.1021/acschemneuro.7b00373

11. Agirman G, Hsiao EY. SnapShot: The microbiota-gut-brain axis. Cell. 2021;184. https://doi.org/10.1016/j.cell.2021.03.022

12. Cryan JF, O'Riordan KJ, Sandhu K, Peterson V, Dinan TG. The gut microbiome in neurological disorders. Lancet Neurol. 2020. https://doi.org/10.1016/S1474-4422(19)30356-4

13. Miri S, Yeo JD, Abubaker S, Hammami R. Neuromicrobiology, an emerging neurometabolic facet of the gut microbiome? Front. Microbiol. 2023. https://doi.org/10.3389/fmicb.2023.1098412

14. Cryan JF, Dinan TG. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 2012. https://doi.org/10.1038/nrn3346

15. Valles-Colomer M, Falony G, Darzi Y, Tigchelaar EF, Wang J, Tito RY, et al. The neuroactive potential of the human gut microbiota in quality of life and depression. Nat Microbiol. 2019;4. https://doi.org/10.1038/s41564-018-0337-x

16. Lai Y, Dhingra R, Zhang Z, Ball LM, Zylka MJ, Lu K. Toward Elucidating the Human Gut Microbiota-Brain Axis: Molecules, Biochemistry, and Implications for Health and Diseases. Biochemistry. 2022. https://doi.org/10.1021/acs.biochem.1c00656

17. Wu T, Rayner CK, Young RL, Horowitz M. Gut motility and enteroendocrine secretion. Curr. Opin. Pharmacol. 2013. https://doi.org/10.1016/j.coph.2013.09.002

18. Bellono NW, Bayrer JR, Leitch DB, Castro J, Zhang C, O'Donnell TA, et al. Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. Cell. 2017;170. https://doi.org/10.1016/j.cell.2017.05.034

19. Furness JB. The enteric nervous system and neurogastroenterology. Nat. Rev. Gastroenterol. Hepatol. 2012. https://doi.org/10.1038/nrgastro.2012.32

20. Bhattarai Y, Si J, Pu M, Ross OA, McLean PJ, Till L, et al. Role of gut microbiota in regulating gastrointestinal dysfunction and motor symptoms in a mouse model of Parkinson's disease. Gut Microbes. 2021;13. https://doi.org/10.1080/19490976.2020.1866974

21. Wang Y, Tong Q, Ma SR, Zhao ZX, Pan L Bin, Cong L, et al. Oral berberine improves brain dopa/dopamine levels to ameliorate Parkinson's disease by regulating gut microbiota. Signal Transduct Target Ther. 2021;6. https://doi.org/10.1038/s41392-020-00456-5

22. Hartstra A V., Schüppel V, Imangaliyev S, Schrantee A, Prodan A, Collard D, et al. Infusion of donor feces affects the gut–brain axis in humans with metabolic syndrome. Mol Metab. 2020;42. https://doi.org/10.1016/j.molmet.2020.101076

23. Jadhav KS, Peterson VL, Halfon O, Ahern G, Fouhy F, Stanton C, et al. Gut microbiome correlates with altered striatal dopamine receptor expression in a model of compulsive alcohol seeking. Neuropharmacology. 2018;141. https://doi.org/10.1016/j.neuropharm.2018.08.026

24. Borodovitsyna O, Flamini M, Chandler D. Noradrenergic Modulation of Cognition in Health and Disease. Neural Plast. 2017;2017. https://doi.org/10.1155/2017/6031478

25. Tsavkelova EA, Botvinko I V., Kudrin VS, Oleskin A V. Detection of neurotransmitter amines in microorganisms with the use of high-performance liquid chromatography. Doklady biochemistry : proceedings of the Academy of Sciences of the USSR, Biochemistry section / translated from Russian. 2000;372.

26. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, et al. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. Am J Physiol Gastrointest Liver Physiol. 2012;303. https://doi.org/10.1152/ajpgi.00341.2012

27. Berger M, Gray JA, Roth BL. The expanded biology of serotonin. Annu. Rev. Med. 2009. https://doi.org/10.1146/annurev.med.60.042307.110802

28. Israelyan N, Margolis KG. Serotonin as a link between the gut-brain-microbiome axis in autism spectrum disorders. Pharmacol. Res. 2018. https://doi.org/10.1016/j.phrs.2018.03.020

29. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015;161. https://doi.org/10.1016/j.cell.2015.02.047

30. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci U S A. 2009;106. https://doi.org/10.1073/pnas.0812874106

31. De Vadder F, Grasset E, Holm LM, Karsenty G, Macpherson AJ, Olofsson LE, et al. Gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks. Proc Natl Acad Sci U S A. 2018;115. https://doi.org/10.1073/pnas.1720017115

32. Reigstad CS, Salmonson CE, Rainey JF, Szurszewski JH, Linden DR, Sonnenburg JL, et al. Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. FASEB Journal. 2015;29. https://doi.org/10.1096/fj.14-259598

33. El-Salhy M, Danielsson Å, Stenling R, Grimelius L. Colonic endocrine cells in inflammatory bowel disease. J Intern Med. 1997;242. https://doi.org/10.1046/j.1365-2796.1997.00237.x

34. Kelly JR, Kennedy PJ, Cryan JF, Dinan TG, Clarke G, Hyland NP. Breaking down the barriers: The gut microbiome, intestinal permeability and stress-related psychiatric disorders. Front. Cell. Neurosci. 2015. https://doi.org/10.3389/fncel.2015.00392

35. Comas-Basté O, Latorre-Moratalla ML, Bernacchia R, Veciana-Nogués MT, Vidal-Carou MC. New approach for the diagnosis of histamine intolerance based on the determination of histamine and methylhistamine in urine. J Pharm Biomed Anal. 2017;145. https://doi.org/10.1016/j.jpba.2017.06.029

36. Ijzerman AP, Jacobson KA, Müller CE, Cronstein BN, Cunha RA. International Union of Basic and Clinical Pharmacology. CXII: Adenosine Receptors: A Further UpdateS. Pharmacol. Rev. 2022. https://doi.org/10.1124/pharmrev.121.000445

37. Mishima Y, Ishihara S. Molecular mechanisms of microbiota-mediated pathology in irritable bowel syndrome. Int. J. Mol. Sci. 2020. https://doi.org/10.3390/ijms21228664

38. Lobo B, Ramos L, Martínez C, Guilarte M, González-Castro AM, Alonso-Cotoner C, et al. Downregulation of mucosal mast cell activation and immune response in diarrhoea-irritable bowel syndrome by oral disodium cromoglycate: A pilot study. United European Gastroenterol J. 2017;5. https://doi.org/10.1177/2050640617691690

39. Botschuijver S, van Diest SA, van Thiel IAM, Saia RS, Strik AS, Yu Z, et al. Miltefosine treatment reduces visceral hypersensitivity in a rat model for irritable bowel syndrome via multiple mechanisms. Sci Rep. 2019;9. https://doi.org/10.1038/s41598-019-49096-y

40. Hattori T, Watanabe S, Kano M, Kanazawa M, Fukudo S. Differential responding of autonomic function to histamine H1 antagonism in irritable bowel syndrome. Neurogastroenterology and Motility. 2010;22. https://doi.org/10.1111/j.1365-2982.2010.01568.x

41. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterology and Motility. 2011;23. https://doi.org/10.1111/j.1365-2982.2010.01620.x

42. Fujisaka S, Avila-Pacheco J, Soto M, Kostic A, Dreyfuss JM, Pan H, et al. Diet, Genetics, and the Gut Microbiome Drive Dynamic Changes in Plasma Metabolites. Cell Rep. 2018;22. https://doi.org/10.1016/j.celrep.2018.02.060

43. Bo TB, Zhang XY, Kohl KD, Wen J, Tian SJ, Wang DH. Coprophagy prevention alters microbiome, metabolism, neurochemistry, and cognitive behavior in a small mammal. ISME Journal. 2020;14. https://doi.org/10.1038/s41396-020-0711-6

44. Hwang YH, Park S, Paik JW, Chae SW, Kim DH, Jeong DG, et al. Efficacy and safety of lactobacillus plantarum C29-fermented soybean (DW2009) in individuals with mild cognitive impairment: A 12-week, multi-center, randomized, double-blind, placebo-controlled clinical trial. Nutrients. 2019;11. https://doi.org/10.3390/nu11020305

45. Uemura M, Hayashi F, Ishioka K, Ihara K, Yasuda K, Okazaki K, et al. Obesity and mental health improvement following nutritional education focusing on gut microbiota composition in Japanese women: a randomised controlled trial. Eur J Nutr. 2019;58. https://doi.org/10.1007/s00394-018-1873-0

46. Rosenberg E. Diversity of bacteria within the human gut and its contribution to the functional unity of holobionts. NPJ Biofilms Microbiomes. 2024. https://doi.org/10.1038/s41522-024-00580-y

47. Di Vincenzo F, Del Gaudio A, Petito V, Lopetuso LR, Scaldaferri F. Gut microbiota, intestinal permeability, and systemic inflammation: a narrative review. Intern. Emerg. Med. 2024. https://doi.org/10.1007/s11739-023-03374-w

48. Zhang P. Influence of Foods and Nutrition on the Gut Microbiome and Implications for Intestinal Health. Int. J. Mol. Sci. 2022. https://doi.org/10.3390/ijms23179588

49. Voreades N, Kozil A, Weir TL. Diet and the development of the human intestinal microbiome. Front Microbiol. 2014;5. https://doi.org/10.3389/fmicb.2014.00494

50. Strasser B, Wolters M, Weyh C, Krüger K, Ticinesi A. The effects of lifestyle and diet on gut microbiota composition, inflammation and muscle performance in our aging society. Nutrients. 2021;13. https://doi.org/10.3390/nu13062045

51. Thursby E, Juge N. Introduction to the human gut microbiota. Biochemical Journal. 2017. https://doi.org/10.1042/BCJ20160510

52. Gomaa EZ. Human gut microbiota/microbiome in health and diseases: a review. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology. 2020. https://doi.org/10.1007/s10482-020-01474-7

53. Lin L, Zhang J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. BMC Immunol. 2017. https://doi.org/10.1186/s12865-016-0187-3

54. Adak A, Khan MR. An insight into gut microbiota and its functionalities. Cellular and Molecular Life Sciences. 2019. https://doi.org/10.1007/s00018-018-2943-4

55. Zheng P, Zeng B, Liu M, Chen J, Pan J, Han Y, et al. The gut microbiome from patients with schizophrenia modulates the glutamate-glutamine-GABA cycle and schizophrenia-relevant behaviors in mice. Sci Adv. 2019;5. https://doi.org/10.1126/sciadv.aau8317

56. Dixit K, Chaudhari D, Dhotre D, Shouche Y, Saroj S. Restoration of dysbiotic human gut microbiome for homeostasis. Life Sci. 2021. https://doi.org/10.1016/j.lfs.2021.119622

57. Bander Z Al, Nitert MD, Mousa A, Naderpoor N. The gut microbiota and inflammation: An overview. Int. J. Environ. Res. Public Health. 2020. https://doi.org/10.3390/ijerph17207618

58. Simpson CA, Diaz-Arteche C, Eliby D, Schwartz OS, Simmons JG, Cowan CSM. The gut microbiota in anxiety and depression – A systematic review. Clin. Psychol. Rev. 2021. https://doi.org/10.1016/j.cpr.2020.101943

59. Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson SC, et al. Gut microbiome alterations in Alzheimer's disease. Sci Rep. 2017;7. https://doi.org/10.1038/s41598-017-13601-y

60. Latif A, Shehzad A, Niazi S, Zahid A, Ashraf W, Iqbal MW, et al. Probiotics: mechanism of action, health benefits and their application in food industries. Front. Microbiol. 2023. https://doi.org/10.3389/fmicb.2023.1216674

61. Szydłowska A, Sionek B. Probiotics and Postbiotics as the Functional Food Components Affecting the Immune Response. Microorganisms. 2023. https://doi.org/10.3390/microorganisms11010104

62. Dahiya D, Nigam PS. Use of Characterized Microorganisms in Fermentation of Non-Dairy-Based Substrates to Produce Probiotic Food for Gut-Health and Nutrition. Fermentation. 2023. https://doi.org/10.3390/fermentation9010001

63. Roisinblit KC. Irritable Bowel Syndrome in Women. J Midwifery Womens Health. 2013;58. https://doi.org/10.1111/j.1542-2011.2012.00200.x

64. Kennedy PJ, Cryan JF, Dinan TG, Clarke G. Irritable bowel syndrome: A microbiome-gut-brain axis disorder? World J. Gastroenterol. 2014. https://doi.org/10.3748/wjg.v20.i39.14105

65. Klem F, Wadhwa A, Prokop LJ, Sundt WJ, Farrugia G, Camilleri M, et al. Prevalence, Risk Factors, and Outcomes of Irritable Bowel Syndrome After Infectious Enteritis: A Systematic Review and Meta-analysis. Gastroenterology. 2017;152. https://doi.org/10.1053/j.gastro.2016.12.039

66. Ringel Y, Ringel-Kulka T. The Intestinal Microbiota and Irritable Bowel Syndrome [Internet]. 2015. www.jcge.com

67. Wouters MM, Vicario M, Santos J. The role of mast cells in functional GI disorders. Gut. 2016;65. https://doi.org/10.1136/gutjnl-2015-309151

68. Piche T. Tight junctions and IBS - the link between epithelial permeability, low-grade inflammation, and symptom generation? Neurogastroenterology and Motility. 2014. https://doi.org/10.1111/nmo.12315

69. Mikkelsen HB. Interstitial cells of Cajal, macrophages and mast cells in the gut musculature: Morphology, distribution, spatial and possible functional interactions. J Cell Mol Med. 2010;14. https://doi.org/10.1111/j.1582-4934.2010.01025.x

70. Elsenbruch S. Abdominal pain in Irritable Bowel Syndrome: A review of putative psychological, neural and neuro-immune mechanisms. Brain Behav. Immun. 2011. https://doi.org/10.1016/j.bbi.2010.11.010

71. Nisticò V, Rossi RE, D'Arrigo AM, Priori A, Gambini O, Demartini B. Functional Neuroimaging in Irritable Bowel Syndrome: A Systematic Review Highlights Common Brain Alterations With Functional Movement Disorders. J. Neurogastroenterol. Motil. 2022. https://doi.org/10.5056/jnm21079

72. Volta U, Pinto-Sanchez MI, Boschetti E, Caio G, De Giorgio R, Verdu EF. Dietary triggers in irritable bowel syndrome: Is there a role for gluten? J Neurogastroenterol Motil. 2016;22. https://doi.org/10.5056/jnm16069

73. Staudacher HM, Lomer MCE, Anderson JL, Barrett JS, Muir JG, Irving PM, et al. Fermentable carbohydrate restriction reduces luminal bifidobacteria and gastrointestinal symptoms in patients with irritable bowel syndrome. Journal of Nutrition. 2012;142. https://doi.org/10.3945/jn.112.159285

74. Chen M, Ruan G, Chen L, Ying S, Li G, Xu F, et al. Neurotransmitter and Intestinal Interactions: Focus on the Microbiota-Gut-Brain Axis in Irritable Bowel Syndrome. Front. Endocrinol. (Lausanne). 2022. https://doi.org/10.3389/fendo.2022.817100

75. Laroute V, Beaufrand C, Gomes P, Nouaille S, Tondereau V, Daveran-Mingot ML, et al. Lactococcus lactis NCDO2118 exerts visceral antinociceptive properties in rat via GABA production in the gastrointestinal tract. Elife. 2022;11. https://doi.org/10.7554/eLife.77100

76. Valdes AM, Walter J, Segal E, Spector TD. Role of the gut microbiota in nutrition and health. BMJ (Online). 2018;361. https://doi.org/10.1136/bmj.k2179

77. Cenit MC, Sanz Y, Codoñer-Franch P. Influence of gut microbiota on neuropsychiatric disorders. World J. Gastroenterol. 2017. https://doi.org/10.3748/wjg.v23.i30.5486

78. Ohara TE, Hsiao EY. Microbiota–neuroepithelial signalling across the gut–brain axis. Nat. Rev. Microbiol. Nature Research; 2025. p. 371–84. https://doi.org/10.1038/s41579-024-01136-9

79. Quigley EMM. Microbiota-Brain-Gut Axis and Neurodegenerative Diseases. Curr. Neurol. Neurosci. Rep. 2017. https://doi.org/10.1007/s11910-017-0802-6

80. Schneider E, O'Riordan KJ, Clarke G, Cryan JF. Feeding gut microbes to nourish the brain: unravelling the diet–microbiota–gut–brain axis. Nat. Metab. Nature Research; 2024. p. 1454–78. https://doi.org/10.1038/s42255-024-01108-6

81. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. Journal of Physiology. 2004;558. https://doi.org/10.1113/jphysiol.2004.063388

82. Li Y, Ning L, Yin Y, Wang R, Zhang Z, Hao L, et al. Age-related shifts in gut microbiota contribute to cognitive decline in aged rats. Aging. 2020;12. https://doi.org/10.18632/aging.103093

83. Bonaz B, Bazin T, Pellissier S. The vagus nerve at the interface of the microbiota-gut-brain axis. Front. Neurosci. 2018. https://doi.org/10.3389/fnins.2018.00049

84. Strandwitz P. Neurotransmitter modulation by the gut microbiota. Brain Res. 2018. https://doi.org/10.1016/j.brainres.2018.03.015

85. Breit S, Kupferberg A, Rogler G, Hasler G. Vagus nerve as modulator of the brain-gut axis in psychiatric and inflammatory disorders. Front. Psychiatry. 2018. https://doi.org/10.3389/fpsyt.2018.00044

86. Wang F Bin, Powley TL. Vagal innervation of intestines: Afferent pathways mapped with new en bloc horseradish peroxidase adaptation. Cell Tissue Res. 2007;329. https://doi.org/10.1007/s00441-007-0413-7

87. Raybould HE. Gut chemosensing: Interactions between gut endocrine cells and visceral afferents. Auton. Neurosci. 2010. https://doi.org/10.1016/j.autneu.2009.07.007

88. Auteri M, Zizzo MG, Serio R. GABA and GABA receptors in the gastrointestinal tract: From motility to inflammation. Pharmacol. Res. Academic Press; 2015. p. 11–21. https://doi.org/10.1016/j.phrs.2014.12.001

89. Janik R, Thomason LAM, Stanisz AM, Forsythe P, Bienenstock J, Stanisz GJ. Magnetic resonance spectroscopy reveals oral Lactobacillus promotion of increases in brain GABA, N-acetyl aspartate and glutamate. Neuroimage. 2016;125. https://doi.org/10.1016/j.neuroimage.2015.11.018

90. Jiang H, Ling Z, Zhang Y, Mao H, Ma Z, Yin Y, et al. Altered fecal microbiota composition in patients with major depressive disorder. Brain Behav Immun. 2015;48. https://doi.org/10.1016/j.bbi.2015.03.016

91. Franciosi E, Carafa I, Nardin T, Schiavon S, Poznanski E, Cavazza A, et al. Biodiversity and γ -aminobutyric acid production by lactic acid bacteria isolated from traditional alpine raw cow's milk cheeses. Biomed Res Int. 2015;2015. https://doi.org/10.1155/2015/625740

92. Seo MJ, Nam Y Do, Lee SY, Park SL, Yi SH, Lim S Il. Expression and characterization of a glutamate decarboxylase from lactobacillus brevis 877G producing γ-aminobutyric acid. Biosci Biotechnol Biochem. 2013;77. https://doi.org/10.1271/bbb.120785

93. Li H, Qiu T, Huang G, Cao Y. Production of gamma-aminobutyric acid by Lactobacillus brevis NCL912 using fed-batch fermentation. Microb Cell Fact. 2010;9. https://doi.org/10.1186/1475-2859-9-85

94. Wang Q, Liu X, Fu J, Wang S, Chen Y, Chang K, et al. Substrate sustained release-based high efficacy biosynthesis of GABA by Lactobacillus brevis NCL912. Microb Cell Fact. 2018;17. https://doi.org/10.1186/s12934-018-0919-6

95. Zhuang K, Jiang Y, Feng X, Li L, Dang F, Zhang W, et al. Transcriptomic response to GABA-producing Lactobacillus plantarum CGMCC 1.2437T induced by L-MSG. PLoS One. Public Library of Science; 2018;13. https://doi.org/10.1371/journal.pone.0199021

96. Lee SJ, Jeon HS, Yoo JY, Kang YJ, Kim MJ, Kim TJ, et al. Characterization of a novel glutamate decarboxylase (GAD) from Latilactobacillus curvatus K285 isolated from Gat -Kimchi. Food Sci Biotechnol. 2022;31. https://doi.org/10.1007/s10068-021-01005-8

97. Dhakal R, Bajpai VK, Baek KH. Production of GABA (γ-aminobutyric acid) by microorganisms: A review. Brazilian Journal of Microbiology. 2012;43. https://doi.org/10.1590/S1517-83822012000400001

98. Gong L, Ren C, Xu Y. Deciphering the crucial roles of transcriptional regulator GadR on gamma-aminobutyric acid production and acid resistance in Lactobacillus brevis. Microb Cell Fact. BioMed Central Ltd.; 2019;18. https://doi.org/10.1186/s12934-019-1157-2

99. Lyu C, Zhao W, Peng C, Hu S, Fang H, Hua Y, et al. Exploring the contributions of two glutamate decarboxylase isozymes in Lactobacillus brevis to acid resistance and γ-aminobutyric acid production. Microb Cell Fact. 2018;17. https://doi.org/10.1186/s12934-018-1029-1

100. De Biase D, Pennacchietti E. Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: Function, distribution and biomedical implications of the gadBC operon. Mol. Microbiol. 2012. https://doi.org/10.1111/mmi.12020

101. Cui Y, Miao K, Niyaphorn S, Qu X. Production of gamma-aminobutyric acid from lactic acid bacteria: A systematic review. Int. J. Mol. Sci. MDPI AG; 2020. https://doi.org/10.3390/ijms21030995

102. Zhang Q, Zeng L, Tan X, Tang J, Xiang W. An efficient γ-aminobutyric acid (GABA) producing and nitrite reducing ability of lactobacillus plantarum BC114 isolated from Chinese paocai. Food Sci Technol Res. 2017;23. https://doi.org/10.3136/fstr.23.749

103. Yang SY, Lü FX, Lu ZX, Bie XM, Jiao Y, Sun LJ, et al. Production of γ-aminobutyric acid by Streptococcus salivarius subsp. thermophilus Y2 under submerged fermentation. Amino Acids. 2008;34. https://doi.org/10.1007/s00726-007-0544-x

104. Tajabadi N, Ebrahimpour A, Baradaran A, Rahim RA, Mahyudin NA, Manap MYA, et al. Optimization of γ-aminobutyric acid production by Lactobacillus plantarum Taj-Apis362 from honeybees. Molecules. 2015;20. https://doi.org/10.3390/molecules20046654

105. Villegas JM, Brown L, Savoy de Giori G, Hebert EM. Optimization of batch culture conditions for GABA production by Lactobacillus brevis CRL 1942, isolated from quinoa sourdough. LWT. 2016;67. https://doi.org/10.1016/j.lwt.2015.11.027

106. Hasegawa M, Yamane D, Funato K, Yoshida A, Sambongi Y. Gamma-aminobutyric acid fermentation with date residue by a lactic acid bacterium, Lactobacillus brevis. J Biosci Bioeng. 2018;125. https://doi.org/10.1016/j.jbiosc.2017.10.003

107. Duchen MR. Contributions of mitochondria to animal physiology: From homeostatic sensor to calcium signalling and cell death. Journal of Physiology. 1999. https://doi.org/10.1111/j.1469-7793.1999.001aa.x

108. Veintimilla-Gozalbo E, Asensio-Grau A, Calvo-Lerma J, Heredia A, Andrés A. In vitro simulation of human colonic fermentation: A practical approach towards models' design and analytical tools. Applied Sciences (Switzerland). 2021. https://doi.org/10.3390/app11178135

109. Pham VT, Mohajeri MH. The application of in vitro human intestinal models on the screening and development of pre-And probiotics. Benef Microbes. 2018;9. https://doi.org/10.3920/BM2017.0164

110. Roupar D, Coelho MC, Gonçalves DA, Silva SP, Coelho E, Silva S, et al. Evaluation of Microbial-Fructo-Oligosaccharides Metabolism by Human Gut Microbiota Fermentation as Compared to Commercial Inulin-Derived Oligosaccharides. Foods. 2022;11. https://doi.org/10.3390/foods11070954

111. Hewes SA, Wilson RL, Estes MK, Shroyer NF, Blutt SE, Grande-Allen KJ. In Vitro Models of the Small Intestine: Engineering Challenges and Engineering Solutions. Tissue Eng. Part B Rev. 2020. https://doi.org/10.1089/ten.teb.2019.0334

112. Fernandez B, Savard P, Fliss I. Survival and Metabolic Activity of Pediocin Producer Pediococcus acidilactici UL5: Its Impact on Intestinal Microbiota and Listeria monocytogenes in a Model of the Human Terminal Ileum. Microb Ecol. 2016;72. https://doi.org/10.1007/s00248-015-0645-0

113. Vemuri R, Shankar EM, Chieppa M, Eri R, Kavanagh K. Beyond just bacteria: Functional biomes in the gut ecosystem including virome, mycobiome, archaeome and helminths. Microorganisms. 2020. https://doi.org/10.3390/microorganisms8040483

114. Hoek MJA van, Merks RMH. Emergence of microbial diversity due to cross-feeding interactions in a spatial model of gut microbial metabolism. BMC Syst Biol. 2017;11. https://doi.org/10.1186/s12918-017-0430-4

115. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environ. Microbiol. 2017. https://doi.org/10.1111/1462-2920.13589

116. Aguirre M, Eck A, Koenen ME, Savelkoul PHM, Budding AE, Venema K. Evaluation of an optimal preparation of human standardized fecal inocula for in vitro fermentation studies. J Microbiol Methods. 2015;117. https://doi.org/10.1016/j.mimet.2015.07.019

117. Sanabria J, Egan S, Masuda R, Lee AJ, Gibson GR, Nicholson JK, et al. Overview of the Nomenclature and Network of Contributors to the Development of Bioreactors for Human Gut Simulation Using Bibliometric Tools: A Fragmented Landscape. J. Agric. Food Chem. 2022. https://doi.org/10.1021/acs.jafc.2c03597

118. Clavel T, Horz HP, Segata N, Vehreschild M. Next steps after 15 stimulating years of human gut microbiome research. Microb. Biotechnol. 2022. https://doi.org/10.1111/1751-7915.13970

119. Li L, Abou-Samra E, Ning Z, Zhang X, Mayne J, Wang J, et al. An in vitro model maintaining taxon-specific functional activities of the gut microbiome. Nat Commun. 2019;10. https://doi.org/10.1038/s41467-019-12087-8

120. Bircher L, Schwab C, Geirnaert A, Lacroix C. Cryopreservation of artificial gut microbiota produced with in vitro fermentation technology. Microb Biotechnol. 2018;11. https://doi.org/10.1111/1751-7915.12844

121. Amaretti A, Bottari B, Morreale F, Savo Sardaro ML, Angelino D, Raimondi S, et al. Potential prebiotic effect of a long-chain dextran produced by Weissella cibaria: an in vitro evaluation. Int J Food Sci Nutr. 2020;71. https://doi.org/10.1080/09637486.2019.1711026

122. Singh V, Hwang N, Ko G, Tatsuya U. Effects of digested Cheonggukjang on human microbiota assessed by in vitro fecal fermentation. Journal of Microbiology. 2021;59. https://doi.org/10.1007/s12275-021-0525-x

123. Molly K, Vande Woestyne M, Verstraete W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. Appl Microbiol Biotechnol. 1993;39. https://doi.org/10.1007/BF00228615

124. Gibson GR, Cummings JH, Macfarlane GT. Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Appl Environ Microbiol. 1988;54. https://doi.org/10.1128/aem.54.11.2750-2755.1988

125. Zihler Berner A, Fuentes S, Dostal A, Payne AN, Vazquez Gutierrez P, Chassard C, et al. Novel Polyfermentor intestinal model (PolyFermS) for controlled ecological studies: validation and effect of pH. PLoS One. 2013;8. https://doi.org/10.1371/journal.pone.0077772

126. Zhu W, Zhang X, Wang D, Yao Q, Ma GL, Fan X. Simulator of the Human Intestinal Microbial Ecosystem (SHIME®): Current Developments, Applications, and Future Prospects. Pharmaceuticals. Multidisciplinary Digital Publishing Institute (MDPI); 2024. https://doi.org/10.3390/ph17121639

127. Minekus M, Smeets-Peeters M, Havenaar R, Bernalier A, Fonty G, Marol-Bonnin S, et al. A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. Appl Microbiol Biotechnol. 1999;53. https://doi.org/10.1007/s002530051622

128. Marzorati M, Van den Abbeele P, Bubeck SS, Bayne T, Krishnan K, Young A, et al. Bacillus subtilis HU58 and bacillus coagulans SC208 probiotics reduced the effects of antibiotic-induced gut

microbiome dysbiosis in an M-SHIME® model. Microorganisms. 2020;8. https://doi.org/10.3390/microorganisms8071028

129. Neves Casarotti S, Fernanda Borgonovi T, de Mello Tieghi T, Sivieri K, Lúcia Barretto Penna A. Probiotic low-fat fermented goat milk with passion fruit by-product: In vitro effect on obese individuals' microbiota and on metabolites production. Food Research International. 2020;136. https://doi.org/10.1016/j.foodres.2020.109453

130. Van Der Werf MJ, Venema K. Bifidobacteria: Genetic modification and the study of their role in the colon. J Agric Food Chem. 2001;49. https://doi.org/10.1021/jf000952o

131. Singh V, Son HW, Lee GD, Lee S, Unno T, Shin JH. Role, relevance, and possibilities of in vitro fermentation models in human dietary, and gut-microbial studies. Biotechnol. Bioeng. 2022. https://doi.org/10.1002/bit.28206

132. Isenring J, Bircher L, Geirnaert A, Lacroix C. In vitro human gut microbiota fermentation models: opportunities, challenges, and pitfalls. Microbiome Research Reports. 2023. https://doi.org/10.20517/mrr.2022.15

133. Castellone V, Bancalari E, Rubert J, Gatti M, Neviani E, Bottari B. Eating fermented: Health benefits of lab-fermented foods. Foods. 2021. https://doi.org/10.3390/foods10112639

134. Pant A, Xin Ni PL, Chua CK, Tan UX. Valorisation of vegetable food waste utilising three-dimensional food printing. Virtual Phys Prototyp. 2023;18. https://doi.org/10.1080/17452759.2022.2146593

135. Thirunathan P, Manickavasagan A. Processing methods for reducing alpha-galactosides in pulses. Crit. Rev. Food Sci. Nutr. 2019. https://doi.org/10.1080/10408398.2018.1490886

136. Adebo OA, Njobeh PB, Adebiyi JA, Gbashi S, Phoku JZ, Kayitesi E. Fermented Pulse-Based Food Products in Developing Nations as Functional Foods and Ingredients. Functional Food - Improve Health through Adequate Food. InTech; 2017. https://doi.org/10.5772/intechopen.69170

137. Mannaa M, Han G, Seo YS, Park I. Evolution of food fermentation processes and the use of multi-omics in deciphering the roles of the microbiota. Foods. 2021. https://doi.org/10.3390/foods10112861

138. Marcelli A, Osimani A, Aquilanti L. Vegetable By-Products from Industrial Processing: From Waste to Functional Ingredient Through Fermentation. Foods. Multidisciplinary Digital Publishing Institute (MDPI); 2025. https://doi.org/10.3390/foods14152704

139. Cuevas-González PF, Liceaga AM, Aguilar-Toalá JE. Postbiotics and paraprobiotics: From concepts to applications. Food Research International. 2020. https://doi.org/10.1016/j.foodres.2020.109502

140. UNEP. UNEP Food Waste Index Report 2021 | UNEP - UN Environment Programme. Unep. 2021.

141. Del Rio Osorio LL, Flórez-López E, Grande-Tovar CD. The potential of selected agri-food loss and waste to contribute to a circular economy: Applications in the food, cosmetic and pharmaceutical industries. Molecules. 2021. https://doi.org/10.3390/molecules26020515

142. Sadh PK, Duhan S, Duhan JS. Agro-industrial wastes and their utilization using solid state fermentation: a review. Bioresour. Bioprocess. 2018. https://doi.org/10.1186/s40643-017-0187-z

143. Bharat Helkar P, Sahoo A. Review: Food Industry By-Products used as a Functional Food Ingredients. Int J Waste Resour. 2016;6. https://doi.org/10.4172/2252-5211.1000248

144. Bilal M, Niu D, Wang Z. Novel enzyme-fermentation process for bioconversion of restaurant food waste into isomaltooligosaccharide-and L-lactic acid-enriched animal feed. Front Sustain Food Syst. 2024;8. https://doi.org/10.3389/fsufs.2024.1326304

145. Sagar NA, Pareek S, Sharma S, Yahia EM, Lobo MG. Fruit and Vegetable Waste: Bioactive Compounds, Their Extraction, and Possible Utilization. Compr Rev Food Sci Food Saf. 2018;17. https://doi.org/10.1111/1541-4337.12330

146. Zhu Y, Luan Y, Zhao Y, Liu J, Duan Z, Ruan R. Current Technologies and Uses for Fruit and Vegetable Wastes in a Sustainable System: A Review. Foods. 2023. https://doi.org/10.3390/foods12101949

147. Xu Y, Leong ZN, Zhang W, Jin X, Kong JW, Chan GCT, et al. Impact of Brewers' Spent Grain-Containing Biscuit on Postprandial Glycaemic Response in Individuals with Metabolic Syndrome: A Crossover Randomised Controlled Trial. Nutrients. 2024;16. https://doi.org/10.3390/nu16060909

148. Cheng Y, Wu T, Chu X, Tang S, Cao W, Liang F, et al. Fermented blueberry pomace with antioxidant properties improves fecal microbiota community structure and short chain fatty acids production in an in vitro mode. LWT. 2020;125. https://doi.org/10.1016/j.lwt.2020.109260

149. Ricci A, Bernini V, Maoloni A, Cirlini M, Galaverna G, Neviani E, et al. Vegetable by-product lacto-fermentation as a new source of antimicrobial compounds. Microorganisms. 2019;7. https://doi.org/10.3390/microorganisms7120607

150. Ricci A, Marrella M, Hadj Saadoun J, Bernini V, Godani F, Dameno F, et al. Development of lactic acid-fermented tomato products. Microorganisms. 2020;8. https://doi.org/10.3390/microorganisms8081192

151. Valle Vargas MF, Villamil Diaz LM, Ruiz Pardo RY, Quintanilla Carvajal MX. Design of an agro-industrial by-products-based media for the production of probiotic bacteria for fish nutrition. Sci Rep. Nature Research; 2024;14. https://doi.org/10.1038/s41598-024-68783-z

152. Savage K, Firth J, Stough C, Sarris J. GABA-modulating phytomedicines for anxiety: A systematic review of preclinical and clinical evidence. Phytotherapy Research. 2018. https://doi.org/10.1002/ptr.5940

153. Sakashita M, Nakamura U, Horie N, Yokoyama Y, Kim M, Fujita S. Oral Supplementation Using Gamma-Aminobutyric Acid and Whey Protein Improves Whole Body Fat-Free Mass in Men After Resistance Training. J Clin Med Res. 2019;11. https://doi.org/10.14740/jocmr3817

154. Milon RB, Hu P, Zhang X, Hu X, Ren L. Recent advances in the biosynthesis and industrial biotechnology of Gamma-amino butyric acid. Bioresour. Bioprocess. 2024. https://doi.org/10.1186/s40643-024-00747-7

155. Hassoun A, Bekhit AED, Jambrak AR, Regenstein JM, Chemat F, Morton JD, et al. The fourth industrial revolution in the food industry—part II: Emerging food trends. Crit. Rev. Food Sci. Nutr. 2024. https://doi.org/10.1080/10408398.2022.2106472

156. Tubb C, Seba T. Rethinking Food and Agriculture 2020-2030: The Second Domestication of Plants and Animals, the Disruption of the Cow, and the Collapse of Industrial Livestock Farming. Industrial Biotechnology. 2021;17. https://doi.org/10.1089/ind.2021.29240.ctu

157. Luo H, Liu Z, Xie F, Bilal M, Liu L, Yang R, et al. Microbial production of gamma-aminobutyric acid: applications, state-of-the-art achievements, and future perspectives. Crit. Rev. Biotechnol. 2021. https://doi.org/10.1080/07388551.2020.1869688

158. Thongruck K, Maneerat S. Enhanced Production of Gamma-Aminobutyric Acid (GABA) from Lactobacillus futsaii CS3 Using Agri-Food Industries By-Products Under Batch and Fed-Batch Fermentation. Indian J Microbiol. 2023;63. https://doi.org/10.1007/s12088-023-01101-9

159. Gazzetta Ufficiale UE. Direttiva 2002/46/CE del Parlamento Europeo e del Consiglio del 10 giugno 2002 per il ravvicinamento delle legislazioni degli Stati membri relative agli integratori alimentari. Gazzetta ufficiale delle Comunità europee. 2002;183/51-183/57.

160. Pineiro M, Asp NG, Reid G, Macfarlane S, Morelli L, Brunser O, et al. FAO Technical meeting on prebiotics. J Clin Gastroenterol. 2008;42 Suppl 3 Pt 2. https://doi.org/10.1097/mcg.0b013e31817f184e

161. REGOLAMENTO (UE) 2015/ 2283 DEL PARLAMENTO EUROPEO E DEL CONSIGLIO - del 25 nove mbre 2015 - relativo ai nuovi alimenti e che modifica il regolamento (UE) n. 1169/ 2011 del Parlamento europeo e del Consiglio e abroga il regolamento (CE) n. 258/ 97 del Parlamento europeo e del Consiglio e il regolamento (CE) n. 1852/ 2001 della Commissione.

162. Hounhouigan DJ, Nout MJR, Nago CM, Houben JH, Rombouts FM. Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of maw , a fermented maize dough from Benin. Int. J. Food Microbiol. 1993.

163. Hesser JM, Hartman PA, Saul RA. Lactobacilli in ensiled high-moisture corn. Appl Microbiol. 1967;15. https://doi.org/10.1128/aem.15.1.49-54.1967

164. Antonia Martinez-Anaya M, Llin ML, Macias MP. Regulation of acetic acid production by homo- and heterofermentative laetobaeilli in whole-wheat sour-doughs. Z Lebensm Unters Forsch. 1994.

165. Etchells JL, Costilow RN, Anderson2 TE, Bell TA. Pure Culture Fermentation of Brined Cucumbers' Natural fermentations of brined cucumbers have been studied extensively, and their microbiology found to [Internet]. Appl. Microbiol. Dow Chemical Co; 1964. https://journals.asm.org/journal/am

166. Parolin C, Marangoni A, Laghi L, Foschi C, Palomino RAÑ, Calonghi N, et al. Isolation of vaginal lactobacilli and characterization of anti-candida activity. PLoS One. Public Library of Science; 2015;10. https://doi.org/10.1371/journal.pone.0131220

167. Lane D. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. Nucleic Acid Technique in Bacterial Systematics. 1991.

168. Kim SH, Shin BH, Kim YH, Nam SW, Jeon SJ. Cloning and expression of a full-length glutamate decarboxylase gene from Lactobacillus brevis BH2. Biotechnology and Bioprocess Engineering. 2007;12. https://doi.org/10.1007/BF02931089

169. Nomura M, Nakajima I, Fujita Y, Kobayashi M, Kimoto H, Suzuki I, et al. Lactococcus lactis contains only one glutamate decarboxylase gene. Microbiology (N Y). 1999;145. https://doi.org/10.1099/13500872-145-6-1375

170. Yogeswara IBA, Maneerat S, Haltrich D. Glutamate decarboxylase from lactic acid bacteria—a key enzyme in Gaba synthesis. Microorganisms. MDPI AG; 2020;8:1–24. https://doi.org/10.3390/microorganisms8121923

171. Li H, Cao Y. Lactic acid bacterial cell factories for gamma-aminobutyric acid. Amino Acids. 2010. p. 1107–16. https://doi.org/10.1007/s00726-010-0582-7

172. Phuengjayaem S, Kuncharoen N, Booncharoen A, Ongpipattanakul B, Tanasupawat S. Genome analysis and optimization of γ-aminobutyric acid (GABA) production by lactic acid bacteria from plant materials. Journal of General and Applied Microbiology. Microbiology Research Foundation; 2021;67:150–61. https://doi.org/10.2323/jgam.2020.10.002

173. Pannerchelvan S, Rios-Solis L, Faizal Wong FW, Zaidan UH, Wasoh H, Mohamed MS, et al. Strategies for improvement of gamma-aminobutyric acid (GABA) biosynthesis via lactic acid bacteria (LAB) fermentation. Food Funct. 2023. https://doi.org/10.1039/d2fo03936b

174. Edalatian Dovom MR, Habibi Najafi MB, Rahnama Vosough P, Norouzi N, Ebadi Nezhad SJ, Mayo B. Screening of lactic acid bacteria strains isolated from Iranian traditional dairy products for GABA production and optimization by response surface methodology. Sci Rep. 2023;13. https://doi.org/10.1038/s41598-023-27658-5

175. Cejudo-Bastante MJ, Sonni F, Chinnici F, Versari A, Perez-Coello MS, Riponi C. Fermentation of sulphite-free white musts with added lysozyme and oenological tannins: Nitrogen consumption and biogenic amines composition of final wines. LWT. Academic Press; 2010;43:1501–7. https://doi.org/10.1016/j.lwt.2010.02.011

176. Gómez-Alonso S, Hermosín-Gutiérrez I, García-Romero E. Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. J Agric Food Chem. 2007;55. https://doi.org/10.1021/jf062820m

177. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: Scalable and accurate long-read assembly via adaptive κ-mer weighting and repeat separation. Genome Res. 2017;27. https://doi.org/10.1101/gr.215087.116

178. Schwengers O, Jelonek L, Dieckmann MA, Beyvers S, Blom J, Goesmann A. Bakta: Rapid and standardized annotation of bacterial genomes via alignment-free sequence identification. Microb Genom. 2021;7. https://doi.org/10.1099/MGEN.0.000685

179. Morse SMJ, Shaw G, Larner SF. Concurrent mRNA and protein extraction from the same experimental sample using a commercially available column-based RNA preparation kit. Biotechniques. 2006;40. https://doi.org/10.2144/000112100

180. Wu Q, Tun HM, Law YS, Khafipour E, Shah NP. Common distribution of gad operon in Lactobacillus brevis and its GadA contributes to efficient GABA synthesis toward cytosolic near-neutral pH. Front Microbiol. Frontiers Research Foundation; 2017;8. https://doi.org/10.3389/fmicb.2017.00206

181. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, et al. Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl Environ Microbiol. 2000;66. https://doi.org/10.1128/AEM.66.1.297-303.2000

182. Zhang JD, Ruschhaupt M, Biczok R. ddCt method for qRT – PCR data analysis. Bioconductor. 2015;

183. Siroli L, Patrignani F, Serrazanetti DI, Parolin C, Palomino RA ñahui, Vitali B, et al. Determination of antibacterial and technological properties of vaginal lactobacilli for their potential application in dairy products. Front Microbiol. 2017;8. https://doi.org/10.3389/fmicb.2017.00166

184. Corazza E, Pizzi A, Parolin C, Giordani B, Abruzzo A, Bigucci F, et al. Orange Peel Lactiplantibacillus plantarum: Development of A Mucoadhesive Nasal Spray with Antimicrobial and Anti-inflammatory Activity. Pharmaceutics. Multidisciplinary Digital Publishing Institute (MDPI); 2024;16. https://doi.org/10.3390/pharmaceutics16111470

185. Giordani B, Melgoza LM, Parolin C, Foschi C, Marangoni A, Abruzzo A, et al. Vaginal Bifidobacterium breve for preventing urogenital infections: Development of delayed release mucoadhesive oral tablets. Int J Pharm. 2018;550. https://doi.org/10.1016/j.ijpharm.2018.09.003

186. Gao D, Chang K, Ding G, Wu H, Chen Y, Jia M, et al. Genomic insights into a robust gamma-aminobutyric acid-producer Lactobacillus brevis CD0817. AMB Express. Springer Verlag; 2019;9. https://doi.org/10.1186/s13568-019-0799-0

187. Banerjee S, Poore M, Gerdes S, Nedveck D, Lauridsen L, Kristensen HT, et al. Transcriptomics reveal different metabolic strategies for acid resistance and gamma-aminobutyric acid (GABA) production in select Levilactobacillus brevis strains. Microb Cell Fact. 2021;20. https://doi.org/10.1186/s12934-021-01658-4

188. Cao F, Jin L, Gao Y, Ding Y, Wen H, Qian Z, et al. Artificial-enzymes-armed Bifidobacterium longum probiotics for alleviating intestinal inflammation and microbiota dysbiosis. Nat Nanotechnol. 2023;18. https://doi.org/10.1038/s41565-023-01346-x

189. FAO. Guidelines for the Evaluation of Probiotics in Food. Report of Joint FAO/WHO. Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food Canada: London Ontario. 2002;

190. Petroff OAC. GABA and glutamate in the human brain. Neuroscientist. 2002. p. 562–73. https://doi.org/10.1177/1073858402238515

191. Adeghate E, Ponery AS. GABA in the endocrine pancreas: Cellular localization and function in normal and diabetic rats. Tissue Cell. 2002;34. https://doi.org/10.1054/tice.2002.0217

192. Bajic SS, Djokic J, Dinic M, Veljovic K, Golic N, Mihajlovic S, et al. GABA-producing natural dairy isolate from artisanal zlatar cheese attenuates gut inflammation and strengthens gut epithelial barrier in vitro. Front Microbiol. 2019;10. https://doi.org/10.3389/fmicb.2019.00527

193. Wu CH, Hsueh YH, Kuo JM, Liu SJ. Characterization of a potential probiotic lactobacillus brevis RK03 and efficient production of γ-aminobutyric acid in batch fermentation. Int J Mol Sci. MDPI AG; 2018;19. https://doi.org/10.3390/ijms19010143

194. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol. 2014;11. https://doi.org/10.1038/nrgastro.2014.66

195. Li H, Qiu T, Gao D, Cao Y. Medium optimization for production of gamma-aminobutyric acid by Lactobacillus brevis NCL912. Amino Acids. 2010;38. https://doi.org/10.1007/s00726-009-0355-3

196. Kanklai J, Somwong TC, Rungsirivanich P, Thongwai N. Screening of gaba-producing lactic acid bacteria from thai fermented foods and probiotic potential of levilactobacillus brevis f064a for gaba-fermented mulberry juice production. Microorganisms. 2021;9. https://doi.org/10.3390/microorganisms9010033

197. Cataldo PG, Villegas JM, Savoy de Giori G, Saavedra L, Hebert EM. Enhancement of γ-aminobutyric acid (GABA) production by Lactobacillus brevis CRL 2013 based on carbohydrate fermentation. Int J Food Microbiol. 2020;333. https://doi.org/10.1016/j.ijfoodmicro.2020.108792

198. Liu C, Xue WJ, Ding H, An C, Ma SJ, Liu Y. Probiotic Potential of Lactobacillus Strains Isolated From Fermented Vegetables in Shaanxi, China. Front Microbiol. 2022;12. https://doi.org/10.3389/fmicb.2021.774903

199. Li H, Li W, Liu X, Cao Y. gadA gene locus in Lactobacillus brevis NCL912 and its expression during fed-batch fermentation. FEMS Microbiol. Lett. 2013. https://doi.org/10.1111/1574-6968.12301

200. Horáčková Š, Plocková M, Demnerová K. Importance of microbial defence systems to bile salts and mechanisms of serum cholesterol reduction. Biotechnol. Adv. 2018. https://doi.org/10.1016/j.biotechadv.2017.12.005

201. Dong Z, Yang S, Tang C, Li D, Kan Y, Yao L. New insights into microbial bile salt hydrolases: from physiological roles to potential applications. Front. Microbiol. Frontiers Media SA; 2025. https://doi.org/10.3389/fmicb.2025.1513541

202. Thakur K, Tomar SK, De S. Lactic acid bacteria as a cell factory for riboflavin production. Microb. Biotechnol. 2016. https://doi.org/10.1111/1751-7915.12335

203. Perkins JB, Sloma A, Hermann T, Theriault K, Zachgo E, Erdenberger T, et al. Genetic engineering of Bacillus subtilis for the commercial production of riboflavin [Internet]. J. Ind. Microbiol. Biotechnol. 1999. http://www.stockton-press.co.uk/jim

204. Thakur K, Tomar SK. Invitro study of riboflavin producing lactobacilli as potential probiotic. LWT. 2016;68. https://doi.org/10.1016/j.lwt.2015.12.059

205. Cataldo PG, Urquiza Martínez MP, Villena J, Kitazawa H, Saavedra L, Hebert EM. Comprehensive characterization of γ-aminobutyric acid (GABA) production by Levilactobacillus brevis CRL 2013: insights from physiology, genomics, and proteomics. Front Microbiol. Frontiers Media SA; 2024;15. https://doi.org/10.3389/fmicb.2024.1408624

206. Bergsveinson J, Baecker N, Pittet V, Ziola B. Role of plasmids in Lactobacillus brevis BSO 464 hop tolerance and beer spoilage. Appl Environ Microbiol. 2015;81. https://doi.org/10.1128/AEM.02870-14

207. Jeon J-H, Kim JS, Kim Z-H, Jung JY. Complete genome sequence of Levilactobacillus brevis NSMJ23, makgeolli isolate with antimicrobial activity . Microbiol Resour Announc. 2024;13. https://doi.org/10.1128/mra.01060-23

208. Cataldo PG, Urquiza Martínez MP, Villena J, Kitazawa H, Saavedra L, Hebert EM. Comprehensive characterization of γ-aminobutyric acid (GABA) production by Levilactobacillus brevis CRL 2013: insights from physiology, genomics, and proteomics. Front Microbiol. Frontiers Media SA; 2024;15. https://doi.org/10.3389/fmicb.2024.1408624

209. Ramos CL, Thorsen L, Schwan RF, Jespersen L. Strain-specific probiotics properties of Lactobacillus fermentum, Lactobacillus plantarum and Lactobacillus brevis isolates from Brazilian food products. Food Microbiol. 2013;36. https://doi.org/10.1016/j.fm.2013.03.010

210. Jang HJ, Lee NK, Paik HD. Probiotic characterization of Lactobacillus brevis KU15153 showing antimicrobial and antioxidant effect isolated from kimchi. Food Sci Biotechnol. 2019;28. https://doi.org/10.1007/s10068-019-00576-x

211. Stewart AG, Beart PM. Inflammation: Maladies, models, mechanisms and molecules. Br. J. Pharmacol. 2016. https://doi.org/10.1111/bph.13389

212. Hyun JH, Yu HS, Woo IK, Lee GW, Lee NK, Paik HD. Anti-inflammatory activities of Levilactobacillus brevis KU15147 in RAW 264.7 cells stimulated with lipopolysaccharide on attenuating NF-κB, AP-1, and MAPK signaling pathways. Food Sci Biotechnol. 2023;32. https://doi.org/10.1007/s10068-023-01318-w

213. Nami Y, Bakhshayesh RV, Jalaly HM, Lotfi H, Eslami S, Hejazi MA. Probiotic properties of enterococcus isolated from artisanal dairy products. Front Microbiol. 2019;10. https://doi.org/10.3389/fmicb.2019.00300

214. Pizzi A, Parolin C, Gottardi D, Ricci A, Parpinello GP, Lanciotti R, et al. A Novel GABA-Producing Levilactobacillus brevis Strain Isolated from Organic Tomato as a Promising Probiotic. Biomolecules. Multidisciplinary Digital Publishing Institute (MDPI); 2025;15. https://doi.org/10.3390/biom15070979

215. Parameswaran R, Box GEP, Hunter WG, Hunter JS. Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building. Journal of Marketing Research. 1979;16. https://doi.org/10.2307/3150696

216. Belletti N, Kamdem SS, Tabanelli G, Lanciotti R, Gardini F. Modeling of combined effects of citral, linalool and β-pinene used against Saccharomyces cerevisiae in citrus-based beverages subjected to a mild heat treatment. Int J Food Microbiol. 2010;136. https://doi.org/10.1016/j.ijfoodmicro.2009.10.030

217. Siroli L, Giordani B, Rossi S, Gottardi D, McMahon H, Augustyniak A, et al. Antioxidant and Functional Features of Pre-Fermented Ingredients Obtained by the Fermentation of Milling By-Products. Fermentation. 2022;8. https://doi.org/10.3390/fermentation8120722

218. Patrignani F, Siroli L, Gardini F, Lanciotti R. Contribution of two different packaging material to microbial contamination of peaches: Implications in their microbiological quality. Front Microbiol. 2016;7. https://doi.org/10.3389/fmicb.2016.00938

219. Ali MY, Sina AAI, Khandker SS, Neesa L, Tanvir EM, Kabir A, et al. Nutritional composition and bioactive compounds in tomatoes and their impact on human health and disease: A review. Foods. 2021;10. https://doi.org/10.3390/foods10010045

220. Terenzi C, Bermudez G, Medri F, Davani L, Tumiatti V, Andrisano V, et al. Phenolic and Antioxidant Characterization of Fruit By-Products for Their Nutraceuticals and Dietary Supplements Valorization under a Circular Bio-Economy Approach. Antioxidants. Multidisciplinary Digital Publishing Institute (MDPI); 2024;13. https://doi.org/10.3390/antiox13050604

221. Serra S, Anthony B, Masia A, Giovannini D, Musacchi S. Determination of biochemical composition in peach (prunus persica l. batsch) accessions characterized by different flesh color and textural typologies. Foods. 2020;9. https://doi.org/10.3390/foods9101452

222. Guo R, Fan R, Hu J, Zhang X, Han L, Wang M, et al. Valorization of apple pomace: Structural and rheological characterization of low-Methoxyl Pectins extracted with green agents of citric acid/sodium citrate. Food Chem X [Internet]. Elsevier; 2024 [cited 2025 Oct 27];24:102010. https://doi.org/10.1016/J.FOCHX.2024.102010

223. Hadj Saadoun J, Bertani G, Levante A, Vezzosi F, Ricci A, Bernini V, et al. Fermentation of agri-food waste: A promising route for the production of aroma compounds. Foods. 2021;10. https://doi.org/10.3390/foods10040707

224. Sánchez M, Laca A, Laca A, Díaz M. Value-Added Products from Fruit and Vegetable Wastes: A Review. Clean (Weinh). 2021. https://doi.org/10.1002/clen.202000376

225. Corrado S, Caldeira C, Eriksson M, Hanssen OJ, Hauser HE, van Holsteijn F, et al. Food waste accounting methodologies: Challenges, opportunities, and further advancements. Glob Food Sec [Internet]. Elsevier; 2019 [cited 2025 Oct 27];20:93–100. https://doi.org/10.1016/J.GFS.2019.01.002

226. Fidelis M, De Moura C, Kabbas T, Pap N, Mattila P, Mäkinen S, et al. Fruit seeds as sources of bioactive compounds: Sustainable production of high value-added ingredients from by-products within circular economy. Molecules. MDPI AG; 2019. https://doi.org/10.3390/molecules24213854

227. Rao M, Bast A, de Boer A. Valorized food processing by-products in the eu: Finding the balance between safety, nutrition, and sustainability. Sustainability (Switzerland). 2021;13. https://doi.org/10.3390/su13084428

228. Bujna E, Farkas NA, Tran AM, Dam MS, Nguyen QD. Lactic acid fermentation of apricot juice by mono- and mixed cultures of probiotic Lactobacillus and Bifidobacterium strains. Food Sci Biotechnol. 2018;27. https://doi.org/10.1007/s10068-017-0269-x

229. Simões S, Santos R, Bento-Silva A, Santos M V., Mota M, Duarte N, et al. Improving nutritional quality of unripe tomato through fermentation by a consortium of yeast and lactic acid bacteria. J Sci Food Agric. 2022;102. https://doi.org/10.1002/jsfa.11476

230. Ait Bessai S, Cruz J, Carril P, Melo J, Santana MM, Mouazen AM, et al. The Plant Growth-Promoting Potential of Halotolerant Bacteria Is Not Phylogenetically Determined: Evidence from Two Bacillus megaterium Strains Isolated from Saline Soils Used to Grow Wheat. Microorganisms. 2023;11. https://doi.org/10.3390/microorganisms11071687

231. Yaghoubi Khanghahi M, Strafella S, Allegretta I, Crecchio C. Isolation of Bacteria with Potential Plant-Promoting Traits and Optimization of Their Growth Conditions. Curr Microbiol. 2021;78. https://doi.org/10.1007/s00284-020-02303-w

232. Siragusa S, De Angelis M, Calasso M, Campanella D, Minervini F, Di Cagno R, et al. Fermentation and proteome profiles of Lactobacillus plantarum strains during growth under food-like conditions. J Proteomics. 2014;96. https://doi.org/10.1016/j.jprot.2013.11.003

233. Hebert EM, Raya RR, De Giori GS. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of Lactobacillus helveticus CRL 1062. Appl Environ Microbiol. 2000;66. https://doi.org/10.1128/AEM.66.12.5316-5321.2000

234. Ayivi RD, Ibrahim SA, Krastanov A, Somani A, Siddiqui SA. The impact of alternative nitrogen sources on the growth and viability of Lactobacillus delbrueckii ssp. bulgaricus. J Dairy Sci. 2022;105. https://doi.org/10.3168/jds.2022-21971

235. Papizadeh M, Rohani M, Hosseini SN, Shojaosadati SA, Nahrevanian H, Talebi M, et al. Screening for efficient nitrogen sources for overproduction of the biomass of the functionally probiotic L. plantarum strain RPR42 in a cane molasses-based medium. AMB Express. 2020;10. https://doi.org/10.1186/s13568-020-00976-x

236. Liu Z, de Souza TSP, Wu H, Holland B, Dunshea FR, Barrow CJ, et al. Development of Phenolic-Rich Functional Foods by Lactic Fermentation of Grape Marc: A Review. Food Reviews International. 2024. https://doi.org/10.1080/87559129.2023.2230278

237. Lopes J da C, Madureira J, Margaça FMA, Cabo Verde S. Grape Pomace: A Review of Its Bioactive Phenolic Compounds, Health Benefits, and Applications. Molecules. Multidisciplinary Digital Publishing Institute (MDPI); 2025. https://doi.org/10.3390/molecules30020362

238. García-Ruiz A, Cueva C, González-Rompinelli EM, Yuste M, Torres M, Martín-álvarez PJ, et al. Antimicrobial phenolic extracts able to inhibit lactic acid bacteria growth and wine malolactic fermentation. Food Control. 2012;28. https://doi.org/10.1016/j.foodcont.2012.05.002

239. Pakbin B, Razavi SH, Mahmoudi R, Gajarbeygi P. Producing Probiotic Peach Juice. Biotechnology and Health Sciences. 2014;1. https://doi.org/10.17795/bhs-24683

240. Ossowski S, Rybak K, Pobiega K, Sękul J, Domżalska Z, Gregorek K, et al. Antioxidant Activity and Microbial Quality of Freeze-Dried, Lactic Acid Fermented Peach Products. Molecules. Multidisciplinary Digital Publishing Institute (MDPI); 2025;30. https://doi.org/10.3390/molecules30112360

241. Kalinowska M, Gołębiewska E, Zawadzka M, Choińska R, Koronkiewicz K, Piasecka-Jóźwiak K, et al. Sustainable extraction of bioactive compound from apple pomace through lactic acid bacteria (LAB) fermentation. Sci Rep. 2023;13. https://doi.org/10.1038/s41598-023-46584-0

242. Park SJ, Kim DH, Kang HJ, Shin M, Yang SY, Yang J, et al. Enhanced production of γ-aminobutyric acid (GABA) using Lactobacillus plantarum EJ2014 with simple medium composition. LWT. 2021;137. https://doi.org/10.1016/j.lwt.2020.110443

243. Thuy CX, Pham VT, Nguyen TTNH, Nguyen TTN, Ton NTA, Tuu TT, et al. Effect of Fermentation Conditions (Dilution Ratio, Medium pH, Total Soluble Solids, and Saccharomyces cerevisiae Yeast Ratio) on the Ability to Ferment Cider from Tamarillo (Solanum betaceum) Fruit. J Food Process Preserv. 2024;2024. https://doi.org/10.1155/2024/8841207

244. Cho S-C, Kim D-H, Park C-S, Koh J-H, Pyun Y-R, Kook M-C. Production of GABA-rich Tomato Paste by Lactobacillus sp. Fermentation. The Korean Journal of Food And Nutrition. 2012;25. https://doi.org/10.9799/ksfan.2012.25.1.026

245. Nakatani Y, Fukaya T, Kishino S, Ogawa J. Production of GABA-enriched tomato juice by Lactiplantibacillus plantarum KB1253. J Biosci Bioeng. 2022;134. https://doi.org/10.1016/j.jbiosc.2022.08.008

246. Cataldo PG, Villena J, Elean M, Savoy de Giori G, Saavedra L, Hebert EM. Immunomodulatory Properties of a γ-Aminobutyric Acid-Enriched Strawberry Juice Produced by Levilactobacillus brevis CRL 2013. Front Microbiol. 2020;11. https://doi.org/10.3389/fmicb.2020.610016

247. Liu Y, Chen H, Chen W, Zhong Q, Zhang G, Chen W. Beneficial effects of tomato juice fermented by lactobacillus plantarum and lactobacillus casei: Antioxidation, antimicrobial effect, and volatile profiles. Molecules. 2018;23. https://doi.org/10.3390/molecules23092366

248. Rajendran S, Silcock P, Bremer P. Flavour Volatiles of Fermented Vegetable and Fruit Substrates: A Review. Molecules. 2023. https://doi.org/10.3390/molecules28073236

249. Pang Z, Hao J, Li W, Du B, Guo C, Li X, et al. Investigation into spatial profile of microbial community dynamics and flavor metabolites during the bioaugmented solid-state fermentation of Baijiu. Food Biosci. 2023;56. https://doi.org/10.1016/j.fbio.2023.103292

250. Chen C, Zhao S, Hao G, Yu H, Tian H, Zhao G. Role of lactic acid bacteria on the yogurt flavour: A review. Int. J. Food Prop. 2017. https://doi.org/10.1080/10942912.2017.1295988

251. Bamforth CW. Food, Fermentation and Micro-organisms. Food, Fermentation and Micro-organisms. 2007. https://doi.org/10.1002/9780470995273

252. Pan X, Zhang S, Xu X, Lao F, Wu J. Volatile and non-volatile profiles in jujube pulp co-fermented with lactic acid bacteria. LWT. 2022;154. https://doi.org/10.1016/j.lwt.2021.112772

253. Hadj Saadoun J, Calani L, Cirlini M, Bernini V, Neviani E, Del Rio D, et al. Effect of fermentation with single and co-culture of lactic acid bacteria on okara: Evaluation of bioactive compounds and volatile profiles. Food Funct. Royal Society of Chemistry; 2021;12:3033–43. https://doi.org/10.1039/d0fo02916e

254. Sun J, Song X, Zhang X, Mushtaq A, Da M, Chen F. Fermentation performances, bioactivity, and metabolic profiles of fermentated tomato juice by lactic acid bacteria isolated from Chinese traditional fermented foods. Food Biosci [Internet]. Elsevier; 2025 [cited 2025 Oct 27];66:106216. https://doi.org/10.1016/J.FBIO.2025.106216

255. Obayiuwana OA, Behrends V, Calle-Patino Y, Barone M, Turroni S, Brigidi P, et al. Cooking, Digestion, and In Vitro Colonic Fermentation of Nigerian Wholegrains Affect Phenolic Acid Metabolism and Gut Microbiota Composition. Int J Mol Sci. 2023;24. https://doi.org/10.3390/ijms241814111

256. Song HE, Lee HY, Kim SJ, Back SH, Yoo HJ. A facile profiling method of short chain fatty acids using liquid chromatography-mass spectrometry. Metabolites. 2019;9. https://doi.org/10.3390/metabo9090173

257. Valdivia-Garcia MA, Chappell KE, Camuzeaux S, Olmo-García L, van der Sluis VH, Radhakrishnan ST, et al. Improved quantitation of short-chain carboxylic acids in human biofluids using 3-nitrophenylhydrazine derivatization and liquid chromatography with tandem mass spectrometry (LC-MS/MS). J Pharm Biomed Anal. 2022;221. https://doi.org/10.1016/j.jpba.2022.115060

258. Behrends V, Tredwell GD, Bundy JG. A software complement to AMDIS for processing GC-MS metabolomic data. Anal Biochem. 2011;415. https://doi.org/10.1016/j.ab.2011.04.009

259. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36. https://doi.org/10.2144/04365st04

260. D'Amico F, Rinaldi M, Pascale R, Fabbrini M, Morelli MC, Siniscalchi A, et al. Gut microbiome dynamics and Enterobacterales infection in liver transplant recipients: A prospective observational study. JHEP Reports [Internet]. Elsevier; 2024 [cited 2025 Oct 27];6:101039. https://doi.org/10.1016/J.JHEPR.2024.101039

261. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: Paired-end assembler for illumina sequences. BMC Bioinformatics. 2012;13. https://doi.org/10.1186/1471-2105-13-31

262. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 2019. https://doi.org/10.1038/s41587-019-0209-9

263. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13. https://doi.org/10.1038/nmeth.3869

264. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: A versatile open source tool for metagenomics. PeerJ. 2016;2016. https://doi.org/10.7717/peerj.2584

265. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;41. https://doi.org/10.1093/nar/gks1219

266. Wu B-Y, Xu P, Cheng L, Wang Q-Q, Qiu H-Y, Yan X-J, et al. Mucosa-Associated Microbiota Dysbiosis in the Terminal Ileum Correlates With Bowel Symptoms in Diarrhea-Predominant Irritable Bowel Syndrome. Clin Transl Gastroenterol [Internet]. 2024;15:e00658. https://doi.org/10.14309/ctg.0000000000000658

267. Xuan J, Han X, Che J, Zhuo J, Xu J, Lu J, et al. Production of γ–aminobutyric acid–enriched sourdough bread using an isolated Pediococcus pentosaceus strain JC30. Heliyon [Internet]. Elsevier; 2024 [cited 2025 Oct 27];10:e31236. https://doi.org/10.1016/J.HELIYON.2024.E31236

268. Hou D, Tang J, Feng Q, Niu Z, Shen Q, Wang L, et al. Gamma-aminobutyric acid (GABA): a comprehensive review of dietary sources, enrichment technologies, processing effects, health benefits, and its applications. Crit. Rev. Food Sci. Nutr. 2024. https://doi.org/10.1080/10408398.2023.2204373

269. Myeong S, Lee YY, Yun J. Optimization and Bioreactor Scale-Up of Cellulase Production in Trichoderma sp. KMF006 for Higher Yield and Performance. Int J Mol Sci. Multidisciplinary Digital Publishing Institute (MDPI); 2025;26. https://doi.org/10.3390/ijms26083731

270. Jamshidzadeh M, Griesz AU, Jensen JW, Krühne U, Woodley JM, Gernaey K V., et al. CFD-guided scaling of Pseudomonas putida fermentation. Biochem Eng J [Internet]. Elsevier; 2025 [cited 2025 Oct 27];213:109549. https://doi.org/10.1016/J.BEJ.2024.109549

271. Takors R. Scale-up of microbial processes: Impacts, tools and open questions. J Biotechnol. 2012;160. https://doi.org/10.1016/j.jbiotec.2011.12.010

272. Hoang MD, Polte I, Frantzmann L, von den Eichen N, Heins AL, Weuster-Botz D. Impact of mixing insufficiencies on L-phenylalanine production with an Escherichia coli reporter strain in a novel two-compartment bioreactor. Microb Cell Fact. 2023;22. https://doi.org/10.1186/s12934-023-02165-4

273. Arauzo-Aguilera K, Buscajoni L, Koch K, Thompson G, Robinson C, Berkemeyer M. Yields and product comparison between Escherichia coli BL21 and W3110 in industrially relevant conditions: anti-c-Met scFv as a case study. Microb Cell Fact. 2023;22. https://doi.org/10.1186/s12934-023-02111-4

274. Junker BH. Scale-up methodologies for Escherichia coli and yeast fermentation processes. J. Biosci. Bioeng. 2004. https://doi.org/10.1016/S1389-1723(04)70218-2

275. Del Toro-Barbosa M, Uribe-Velázquez T, Hurtado-Romero A, Rosales-De la Cruz MF, Carrillo-Nieves D, Garcia-Amezquita LE, et al. Evaluation of GABA-Producing Fermented Whey Formulations:

From Strain Selection to Raspberry-Enriched Beverages with Psychobiotic Potential. Foods. Multidisciplinary Digital Publishing Institute (MDPI); 2025;14. https://doi.org/10.3390/foods14162762

276. Redruello B, Saidi Y, Sampedro L, Ladero V, Del Rio B, Alvarez MA. Gaba-producing lactococcus lactis strains isolated from camel's milk as starters for the production of gaba-enriched cheese. Foods. 2021;10. https://doi.org/10.3390/foods10030633

277. Fatimi H El, El-Mansoury B, Tinakoua A, Ndayambaje M, Zaid Y, Khalki H, et al. Gut microbiota in anxiety and depression: mechanisms, drug interactions, and therapeutic implications. Pharmacological Reports. Springer Science and Business Media Deutschland GmbH; 2025. https://doi.org/10.1007/s43440-025-00759-w

278. Silva YP, Bernardi A, Frozza RL. The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Front. Endocrinol. (Lausanne). 2020. https://doi.org/10.3389/fendo.2020.00025

279. Vijay N, Morris M. Role of Monocarboxylate Transporters in Drug Delivery to the Brain. Curr Pharm Des. 2014;20. https://doi.org/10.2174/13816128113199990462

280. O'Keefe SJD. Diet, microorganisms and their metabolites, and colon cancer. Nat. Rev. Gastroenterol. Hepatol. 2016. https://doi.org/10.1038/nrgastro.2016.165

281. Stilling RM, van de Wouw M, Clarke G, Stanton C, Dinan TG, Cryan JF. The neuropharmacology of butyrate: The bread and butter of the microbiota-gut-brain axis? Neurochem. Int. 2016. https://doi.org/10.1016/j.neuint.2016.06.011

282. Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nat. Neurosci. 2017. https://doi.org/10.1038/nn.4476

283. Luo M, Zhuang X, Tian Z, Xiong L. Alterations in short-chain fatty acids and serotonin in irritable bowel syndrome: a systematic review and meta-analysis. BMC Gastroenterol. 2021;21. https://doi.org/10.1186/s12876-020-01577-5

284. van de Wouw M, Boehme M, Lyte JM, Wiley N, Strain C, O'Sullivan O, et al. Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain–gut axis alterations. Journal of Physiology. 2018;596. https://doi.org/10.1113/JP276431

285. Venegas DP, De La Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, et al. Short chain fatty acids (SCFAs)mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. Front. Immunol. 2019. https://doi.org/10.3389/fimmu.2019.00277

286. Mousavi R, Mottawea W, Audet MC, Hammami R. Survival and Interplay of γ-Aminobutyric Acid-Producing Psychobiotic Candidates with the Gut Microbiota in a Continuous Model of the Human Colon. Biology (Basel). 2022;11. https://doi.org/10.3390/biology11091311

287. Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L. The Role of Short-Chain Fatty Acids in Health and Disease. Adv Immunol. 2014. https://doi.org/10.1016/B978-0-12-800100-4.00003-9

288. Liu L, Fu C, Li F. Acetate affects the process of lipid metabolism in rabbit liver, skeletal muscle and adipose tissue. Animals. 2019;9. https://doi.org/10.3390/ani9100799

289. Langfeld LQ, Du K, Bereswill S, Heimesaat MM. A review of the antimicrobial and immune-modulatory properties of the gut microbiota-derived short chain fatty acid propionate - What is new? Eur. J. Microbiol. Immunol. (Bp). 2021. https://doi.org/10.1556/1886.2021.00005

290. Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front. Microbiol. 2016. https://doi.org/10.3389/fmicb.2016.00979

291. Liu P, Wang Y, Yang G, Zhang Q, Meng L, Xin Y, et al. The role of short-chain fatty acids in intestinal barrier function, inflammation, oxidative stress, and colonic carcinogenesis. Pharmacol. Res. 2021. https://doi.org/10.1016/j.phrs.2021.105420

292. Gheorghe AS, Negru Șerban M, Preda M, Mihăilă RI, Komporaly IA, Dumitrescu EA, et al. Biochemical and Metabolical Pathways Associated with Microbiota-Derived Butyrate in Colorectal Cancer and Omega-3 Fatty Acids Implications: A Narrative Review. Nutrients. MDPI; 2022. https://doi.org/10.3390/nu14061152

293. O'Riordan KJ, Collins MK, Moloney GM, Knox EG, Aburto MR, Fülling C, et al. Short chain fatty acids: Microbial metabolites for gut-brain axis signalling. Mol. Cell. Endocrinol. 2022. https://doi.org/10.1016/j.mce.2022.111572

294. Sun M, Wu W, Liu Z, Cong Y. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. J. Gastroenterol. 2017. https://doi.org/10.1007/s00535-016-1242-9

295. Blaak EE, Canfora EE, Theis S, Frost G, Groen AK, Mithieux G, et al. Short chain fatty acids in human gut and metabolic health. Benef. Microbes. 2020. https://doi.org/10.3920/BM2020.0057

296. Kong Y, Jiang B, Luo X. Gut microbiota influences Alzheimer's disease pathogenesis by regulating acetate in Drosophila model. Future Microbiol. 2018;13. https://doi.org/10.2217/fmb-2018-0185

297. Lavoie S, Chun E, Bae S, Brennan CA, Gallini Comeau CA, Lang JK, et al. Expression of Free Fatty Acid Receptor 2 by Dendritic Cells Prevents Their Expression of Interleukin 27 and Is Required for Maintenance of Mucosal Barrier and Immune Response Against Colorectal Tumors in Mice. Gastroenterology. 2020;158. https://doi.org/10.1053/j.gastro.2019.12.027

298. Evenepoel P, Claus D, Geypens B, Hiele M, Geboes K, Rutgeerts P, et al. Amount and fate of egg protein escaping assimilation in the small intestine of humans. Am J Physiol Gastrointest Liver Physiol. 1999;277. https://doi.org/10.1152/ajpgi.1999.277.5.g935

299. Ratzke C, Gore J. Modifying and reacting to the environmental pH can drive bacterial interactions. PLoS Biol. 2018;16. https://doi.org/10.1371/journal.pbio.2004248

300. Roager HM, Licht TR. Microbial tryptophan catabolites in health and disease. Nat. Commun. 2018. https://doi.org/10.1038/s41467-018-05470-4

301. Golubeva A V., Joyce SA, Moloney G, Burokas A, Sherwin E, Arboleya S, et al. Microbiota-related Changes in Bile Acid & Tryptophan Metabolism are Associated with Gastrointestinal Dysfunction in a Mouse Model of Autism. EBioMedicine. 2017;24. https://doi.org/10.1016/j.ebiom.2017.09.020

302. Tiwari S, Paramanik V. Role of Probiotics in Depression: Connecting Dots of Gut-Brain-Axis Through Hypothalamic-Pituitary Adrenal Axis and Tryptophan/Kynurenic Pathway involving Indoleamine-2,3-dioxygenase. Mol. Neurobiol. Springer; 2025. p. 7230–41. https://doi.org/10.1007/s12035-025-04708-9

303. O'Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behavioural Brain Research. 2015. https://doi.org/10.1016/j.bbr.2014.07.027

304. Hara T, Mihara T, Ishibashi M, Kumagai T, Joh T. Heat-killed Lactobacillus casei subsp. casei 327 promotes colonic serotonin synthesis in mice. J Funct Foods. 2018;47. https://doi.org/10.1016/j.jff.2018.05.050

305. Zelante T, Iannitti RG, Cunha C, DeLuca A, Giovannini G, Pieraccini G, et al. Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22. Immunity. 2013;39. https://doi.org/10.1016/j.immuni.2013.08.003

306. Agus A, Planchais J, Sokol H. Gut Microbiota Regulation of Tryptophan Metabolism in Health and Disease. Cell Host Microbe. 2018. https://doi.org/10.1016/j.chom.2018.05.003

307. Liu JR, Miao H, Deng DQ, Vaziri ND, Li P, Zhao YY. Gut microbiota-derived tryptophan metabolism mediates renal fibrosis by aryl hydrocarbon receptor signaling activation. Cellular and Molecular Life Sciences. 2021. https://doi.org/10.1007/s00018-020-03645-1

308. Bicakci AO, Sarkar M, Chang YH, Kahl E, Ragazzi L, Moldes-anaya A, et al. Anxiolytic-like Effects of the Positive GABAB Receptor Modulator GS39783 Correlate with Mice's Individual Basal Anxiety and Stress Reactivity. Pharmaceuticals. 2022;15. https://doi.org/10.3390/ph15020233

309. Azarfarin M, Moradikor N, Matin S, Dadkhah M. Association Between Stress, Neuroinflammation, and Irritable Bowel Syndrome: The Positive Effects of Probiotic Therapy. Cell Biochem. Funct. John Wiley and Sons Ltd; 2024. https://doi.org/10.1002/cbf.70009

310. Bravo JA, Forsythe P, Chew M V., Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A. 2011;108. https://doi.org/10.1073/pnas.1102999108

311. Potts RA, Tiffany CM, Pakpour N, Lokken KL, Tiffany CR, Cheung K, et al. Mast cells and histamine alter intestinal permeability during malaria parasite infection. Immunobiology. 2016;221. https://doi.org/10.1016/j.imbio.2015.11.003

312. Aggarwal S, Ahuja V, Paul J. Dysregulation of GABAergic signalling contributes in the pathogenesis of diarrheapredominant irritable bowel syndrome. J Neurogastroenterol Motil. 2018;24. https://doi.org/10.5056/jnm17100

313. Casertano M, Fryganas C, Valentino V, Troise AD, Vitaglione P, Fogliano V, et al. Gut production of GABA by a probiotic formula: an in vitro study. Benef Microbes. 2024;15. https://doi.org/10.1163/18762891-20230025

314. Jiang C, Chen Y, Sun T. From the gut to the brain, mechanisms and clinical applications of γ-aminobutyric acid (GABA) on the treatment of anxiety and insomnia. Front. Neurosci. Frontiers Media SA; 2025. https://doi.org/10.3389/fnins.2025.1570173

315. Pokusaeva K, Johnson C, Luk B, Uribe G, Fu Y, Oezguen N, et al. GABA-producing Bifidobacterium dentium modulates visceral sensitivity in the intestine. Neurogastroenterology and Motility. 2017;29. https://doi.org/10.1111/nmo.12904

316. Poppe J, Vieira-Silva S, Raes J, Verbeke K, Falony G. Systematic optimization of fermentation conditions for in vitro fermentations with fecal inocula. Front Microbiol. 2023;14. https://doi.org/10.3389/fmicb.2023.1198903

317. Corona G, Kreimes A, Barone M, Turroni S, Brigidi P, Keleszade E, et al. Impact of lignans in oilseed mix on gut microbiome composition and enterolignan production in younger healthy and premenopausal women: An in vitro pilot study. Microb Cell Fact. 2020;19. https://doi.org/10.1186/s12934-020-01341-0

318. Khalighi Sikaroudi M, Soltani S, Ghoreishy SM, Ebrahimi Z, Shidfar F, Dehnad A. Effects of a low FODMAP diet on the symptom management of patients with irritable bowel syndrome: a systematic umbrella review with the meta-analysis of clinical trials. Food Funct. Royal Society of Chemistry; 2024. p. 5195–208. https://doi.org/10.1039/d3fo03717g

319. Gryaznova M, Smirnova Y, Burakova I, Morozova P, Lagutina S, Chizhkov P, et al. Fecal Microbiota Characteristics in Constipation-Predominant and Mixed-Type Irritable Bowel Syndrome. Microorganisms. Multidisciplinary Digital Publishing Institute (MDPI); 2024;12. https://doi.org/10.3390/microorganisms12071414

320. Tsukuda N, Yahagi K, Hara T, Watanabe Y, Matsumoto H, Mori H, et al. Key bacterial taxa and metabolic pathways affecting gut short-chain fatty acid profiles in early life. ISME Journal. 2021;15. https://doi.org/10.1038/s41396-021-00937-7

321. Yang J, Ren H, Cao J, Fu J, Wang J, Su Z, et al. Gut commensal Lachnospiraceae bacteria contribute to anti-colitis effects of Lactiplantibacillus plantarum exopolysaccharides. Int J Biol Macromol [Internet]. Elsevier; 2025 [cited 2025 Oct 27];309:142815. https://doi.org/10.1016/J.IJBIOMAC.2025.142815

322. Fusco W, Lorenzo MB, Cintoni M, Porcari S, Rinninella E, Kaitsas F, et al. Short-Chain Fatty-Acid-Producing Bacteria: Key Components of the Human Gut Microbiota. Nutrients. 2023. https://doi.org/10.3390/nu15092211

323. Malinen E, Krogius-Kurikka L, Lyra A, Nikkilä J, Jääskeläinen A, Rinttilä T, et al. Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome. World J Gastroenterol. 2010;16. https://doi.org/10.3748/wjg.v16.i36.4532

324. Rizzello F, Ricci C, Scandella M, Cavazza E, Giovanardi E, Valerii MC, et al. Dietary geraniol ameliorates intestinal dysbiosis and relieves symptoms in irritable bowel syndrome patients: A pilot study. BMC Complement Altern Med. 2018;18. https://doi.org/10.1186/s12906-018-2403-6

325. Ricci C, Rizzello F, Valerii MC, Spisni E, Gionchetti P, Turroni S, et al. Geraniol Treatment for Irritable Bowel Syndrome: A Double-Blind Randomized Clinical Trial. Nutrients. 2022;14. https://doi.org/10.3390/nu14194208

326. Liu HN, Wu H, Chen YZ, Chen YJ, Shen XZ, Liu TT. Altered molecular signature of intestinal microbiota in irritable bowel syndrome patients compared with healthy controls: A systematic review and meta-analysis. Digestive and Liver Disease. 2017. https://doi.org/10.1016/j.dld.2017.01.142

327. Turroni S, Brigidi P, Cavalli A, Candela M. Microbiota-Host Transgenomic Metabolism, Bioactive Molecules from the Inside. J. Med. Chem. 2018. https://doi.org/10.1021/acs.jmedchem.7b00244

328. Tigchelaar EF, Bonder MJ, Jankipersadsing SA, Fu J, Wijmenga C, Zhernakova A. Gut microbiota composition associated with stool consistency. Gut. 2016. https://doi.org/10.1136/gutjnl-2015-310328

Appendix 1

Table A1. reports the individual volatile compounds identified in the tomato substrate by GC–MS analysis at the initial time point (T0) and after 48 hours of fermentation (T48). For each compound, the corresponding chromatographic peak area is presented as detected by GC-MS.

	Peak Area Tomato	
	T0	T48
Butanal, 3-methyl-	4,30E+05	0,00E+00
Benzaldehyde	3,84E+05	0,00E+00
Benzaldehyde, 2-methyl-	0,00E+00	1,05E+05
2-Octenal, 2-butyl-	4,09E+05	0,00E+00
Methyl Isobutyl Ketone	6,81E+05	4,21E+05
3-Penten-2-one, 4-methyl-	8,32E+04	0,00E+00
5-Hepten-2-one, 6-methyl-	1,30E+05	2,13E+04
2-Buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	5,81E+04	4,27E+04
5,9-Undecadien-2-one, 6,10-dimethyl-	1,16E+05	0,00E+00
Ethanol	0,00E+00	1,03E+07
1-Butanol	1,14E+05	1,10E+06
1-Butanol, 2-methyl-	4,25E+05	1,34E+06
2-Penten-1-ol, (Z)-	4,05E+04	0,00E+00
Cyclobutanemethanol, .alpha.-methyl-	0,00E+00	1,30E+04
1-Pentanol, 3-methyl-	0,00E+00	2,17E+05
1-Hexanol	4,32E+05	3,78E+05
4-Penten-1-ol, 3-methyl-	2,81E+04	0,00E+00
3-Hexen-1-ol, (Z)-	5,33E+05	4,14E+05
5-Hepten-2-ol, 6-methyl-	2,85E+05	1,29E+06
2-Nonen-1-ol	1,27E+04	0,00E+00
1,6-Octadien-3-ol, 3,7-dimethyl-	1,40E+05	1,91E+05
1,3-Benzenediol, 4-ethyl-	1,26E+04	1,91E+04
2-Furanmethanol	5,84E+05	3,30E+05
5-Octen-2-ol, 5-methyl-	0,00E+00	3,87E+03
Benzyl alcohol	0,00E+00	1,81E+05
Phenylethyl Alcohol	4,85E+05	1,34E+06
5,9-Undecadien-2-ol, 6,10-dimethyl-	7,60E+04	9,35E+04
Bicyclo[3.1.1]hept-2-ene-2-methanol, 6,6-dimethyl-	0,00E+00	1,71E+04
Acetic acid	4,56E+05	4,10E+06
Propanoic acid, 2-methyl-	6,29E+04	2,75E+04
Butanoic acid	2,89E+05	1,46E+05
Pentanoic acid, 3-methyl-	7,13E+04	0,00E+00
Octanoic acid	3,04E+04	5,18E+04
Acetic acid, 2-phenylethyl ester	0,00E+00	1,71E+04
Acetic acid, methoxy-, 2-phenylethyl ester	1,07E+05	9,48E+04

Hexadecanoic acid, methyl ester	5,85E+04	0,00E+00
9,12-Octadecadienoic acid, methyl ester, (E,E)-	5,97E+04	0,00E+00
Phenol, 2-methoxy-	3,30E+05	2,92E+05
Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate	2,13E+04	4,64E+04
Phenol, 2,4-bis(1,1-dimethylethyl)-	1,14E+05	0,00E+00
Benzyl nitrile	7,59E+04	7,26E+04
2-Isobutylthiazole	1,80E+05	1,18E+05
Dodecane	5,35E+04	1,29E+05
Pyrazine, 2,5-dimethyl-	1,34E+05	1,01E+05
Pyrazine, 2-ethyl-3,5-dimethyl-	6,66E+04	2,87E+04
cis-3-Hexenyllactate	0,00E+00	1,24E+04
Benzoic acid, 2-(2-methylpropyl)oxy-, methyl ester	0,00E+00	1,18E+04
Benzofuran, 2,3-dihydro-	0,00E+00	4,33E+04
(+)-2-Bornanone	2,38E+04	4,12E+04
.alpha.-Terpineol	3,48E+04	1,50E+04
trans-Isoeugenol	2,11E+04	2,67E+04

Table A2. presents the detailed list of volatile compounds identified in the peach substrate by GC–MS analysis at T0 and T48. For each compound, the detected chromatographic peak area is reported.

	Peak Area Peach	
	T0	T48
Ethyl Acetate	1,10E+06	6,60E+05
Butanal, 3-methyl-	1,10E+06	0,00E+00
Benzaldehyde	6,49E+05	0,00E+00
Methyl Isobutyl Ketone	8,00E+05	9,19E+05
Camphor	6,91E+05	7,34E+05
Ethanol	5,09E+05	1,43E+07
1-Butanol	0,00E+00	1,07E+06
1-Butanol, 3-methyl-	0,00E+00	9,49E+05
1-Hexanol	0,00E+00	8,08E+05
2-Hexen-1-ol, (Z)-	1,27E+06	8,37E+05
1,6-Octadien-3-ol, 3,7-dimethyl-	4,92E+06	1,68E+06
1-Nonanol	6,84E+05	7,15E+05
Benzyl alcohol	0,00E+00	5,84E+05
Phenylethyl Alcohol	0,00E+00	1,27E+05
Acetic acid	0,00E+00	1,04E+07
Butanoic acid	0,00E+00	6,47E+05
Butanoic acid, 3-methyl-	0,00E+00	2,36E+06
Cyclohexane, 2-butyl-1,1,3-trimethyl-	1,46E+06	2,85E+05
Estragole	7,09E+05	1,21E+06