

Effect of Food and Probiotic Supplement Interactions on GABA Formation in Microbiota: *In Vitro* Gastrointestinal System Approach

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Abstract Gamma-aminobutyric acid (GABA) is a naturally occurring bioactive compound found in plants, microorganisms, animals, and humans. Specifically, it functions as a neurotransmitter in the central nervous system. Therefore, GABA-enriched functional foods are widely preferred by consumers. This study aimed to investigate the effects of adding various probiotic strains to selected foods on GABA formation during *in vitro* digestion. In the study, various probiotic supplements were added to banana, kiwi, avocado, pineapple, strawberry, tomato, onion, walnut, hazelnut, peanut, and pumpkin seed samples, and the formation of γ -aminobutyric acid (GABA), one of the neurotransmitters, was examined in *in vitro* digestion. *In vitro* pre-digestion GABA values were determined as 14.04 mg/100 g in banana, 13.42 mg/100 g in kiwi, 14.10 mg/100 g in avocado, 15.25 mg/100 g in pineapple, 15.74 mg/100 g in strawberry, 19.34 mg/100 g in tomato, 17.74 mg/100 g in onion, 14.98 mg/100 g in walnut, 18.28 mg/100 g in hazelnut, 17.10 mg/100 g in peanut, and 16.54 mg/100 g in pumpkin seed. *In vitro* digestions performed by adding probiotic supplements to these foods showed that the highest increase in the fruit and vegetable group was 243%, 248% in tomatoes and strawberries, respectively, and 238% in pumpkin seeds in the nut group, with the addition of the probiotic supplement 10. The study's findings showed that probiotic supplements containing combination of different bacterial strains were effective in increasing GABA production. These results are important for optimizing the interaction of probiotics with nutrients and GABA production.

Keywords: gamma-aminobutyric acid, probiotic, prebiotic, *in vitro* digestion

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1. Introduction

Gamma-aminobutyric acid (GABA) is a neurotransmitter that acts on the central nervous system and is critical to physiological and neurological functions [1]. GABA has important functions in stress management, sleep regulation, muscle relaxation, and cognitive functions. These functions of GABA have been effective in its use in the production of functional foods and supplements [2]. Deficiency of GABA has been linked to anxiety, depression, and neurodegenerative diseases [3].

GABA is naturally found in some foods, and fermented products, some vegetables, fruits, grains, and nuts are rich sources of GABA [4]. Glutamate found in food serves as a precursor to GABA and is converted to GABA by glutamic acid decarboxylase (GAD). This transformation contributes to the palatability of glutamate-rich foods [5]. In addition, foods containing GABA also have prebiotic properties (Figure 1).

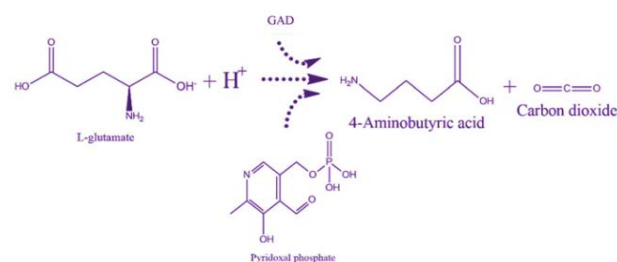


Figure 1. GABA Formation [4]

Prebiotics are defined as “a substrate that is selectively utilized by host microorganisms and confers health benefits” [6]. Major prebiotics include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and some resistant starches [7].

Prebiotics stimulate the growth of beneficial gut microbiota, particularly species of *Bifidobacterium* and *Lactobacillus*. Increased beneficial bacteria induce the formation of short-chain fatty acids (SCFA) and the

neurotransmitter, GABA, and limit the proliferation of pathogenic bacteria by lowering intestinal pH [8,9].

It has been stated in the literature that regular consumption of probiotics containing *Lactobacillus* and *Bifidobacterium* strains can reduce anxiety and stress levels and regulate neurological health [10].

Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts. Probiotics confer protection against a wide range of diseases, primarily through modulation of the gut microbiota [11]. Species of *Lactobacillus* and *Bifidobacterium*, among the most studied probiotics, have been shown to enhance GABA production [9]. Psychobiotics refer to specific probiotic strains that influence brain function and mood by modulating the gut microbiota [12]. By influencing neurotransmitter synthesis and signaling via the gut–brain axis, these probiotics may contribute to the mitigation of psychiatric symptoms such as depression, anxiety, and stress. However, more clinical studies are needed to clarify the underlying mechanisms.

GABA produced in the gut may affect brain function and behavior via the microbiota–gut–brain axis. These findings have underscored the potential role of probiotics as a therapeutic strategy for anxiety and depression [13].

In vitro models simulating the digestive system facilitate the evaluation of the GABA production capacity of probiotics when ingested with particular foods [14]. Thus, the GABA production potential of probiotics plays a significant role both in modulating gastrointestinal functions and in influencing the gut–brain axis [13]. The study demonstrates that certain foods influence the metabolic activity of probiotics and that the GABA production potential varies between animal- and plant-based foods [14].

Compatible combinations of specific prebiotics and probiotic strains can enhance the proliferation and functionality of beneficial gut microbiota [15]. Some studies reported the use of a single strain of LAB for GABA synthesis, while some studies reported GABA synthesis by using different bacterial strains together [16]. Formulations are available in multiple formats, such as capsules, powders, and fortified foods, thereby providing consumers with versatile options [17].

In recent years, research on the synthesis and neurological functions of GABA has expanded; however, further investigations and a deeper understanding of the underlying mechanisms remain necessary. A significant proportion of studies investigating GABA (gamma-aminobutyric acid) synthesis concentrate on the optimization of environmental and biological factors, particularly pH, temperature, and the selection of specific bacterial strains, in order to enhance production efficiency [18].

In the present study, the human digestive system was simulated *in vitro* to investigate GABA formation in the large intestine following the digestion of various foods combined with probiotic supplements, either containing a single bacterial species or a mixture of different species. The primary objective of this study is to investigate the effects of various probiotic strains and commercial probiotic products on GABA synthesis in different foods, thereby contributing to the development of functional foods.

2. Material-Method

Table 1. Bacterial Contents and Amounts of Probiotic Supplements

PS Code	Bacterial Species	CFU
PS 1	<i>Lactobacillus acidophilus</i>	5×10 ⁹
PS 2	<i>Lactobacillus plantarum</i>	5×10 ⁹
PS 3	<i>Lactobacillus rhamnosus</i>	5×10 ⁹
PS 4	<i>Lactobacillus gasserii</i>	5×10 ⁹
PS 5	<i>Lactobacillus crispatus</i>	5×10 ⁹
PS 6	<i>Bifidobacterium lactis</i>	5×10 ⁹
	<i>Bifidobacterium Lactis</i>	1*10 ⁹
	<i>Lactobacillus Acidophilus</i>	0,85x10 ⁹
	<i>Lactobacillus Rhamnosus</i>	0,85x10 ⁹
	<i>Bifidobacterium Bifidum</i>	0,50x10 ⁹
	<i>Streptococcus Thermophilus</i>	0,50x10 ⁹
	<i>Lactobacillus Paracasei</i>	0,50x10 ⁹
	<i>Lactobacillus Gasserii</i>	0,40x10 ⁹
	<i>Lactobacillus Plantarum</i>	0,40x10 ⁹
	<i>Lactobacillus Reuteri</i>	0,40x10 ⁹
	<i>Lactobacillus Bulgaricus</i>	0,40x10 ⁹
	<i>Lactobacillus Salivarius</i>	0,30x10 ⁹
	<i>Saccharomyces Boulardii</i>	0,30x10 ⁹
	<i>Bifidobacterium Longum</i>	0,30x10 ⁹
PS 7	<i>Lactobacillus rhamnosus</i>	1x10 ⁹
	<i>Lactobacillus acidophilus</i>	1x10 ⁹
	<i>Lactobacillus gasserii</i>	1x10 ⁹
	<i>Lactobacillus reuteri</i>	1x10 ⁹
	<i>Lactobacillus plantarum</i>	1x10 ⁹
	<i>Lactobacillus fermentum</i>	1x10 ⁹
	<i>Bifidobacterium bifidum</i>	1x10 ⁹
	<i>Bifidobacterium longum</i>	1x10 ⁹
	<i>Bifidobacterium infantis</i>	1x10 ⁹
	<i>Lactobacillus delbrueckii subsp. Bulgaricus</i>	750x10 ⁶
	<i>Streptococcus thermophilus</i>	250x10 ⁶
	<i>Lactobacillus Acidophilus La-14</i>	1x10 ⁹
	<i>Lactobacillus Helveticus</i>	1x10 ⁹
	<i>Lactobacillus Rhamnosus Lr-32</i>	1x10 ⁹
<i>Bifidobacterium Longum BI-05</i>	1x10 ⁹	
<i>Bifidobacterium Infantis BI-26</i>	1x10 ⁹	
<i>Bifidobacterium Lactis BI-04</i>	1x10 ⁹	
<i>Bacillus Subtilis</i>	1x10 ⁹	
PS 8	<i>Bifidobacterium Bifidum Bb-06</i>	0,75x10 ⁹
	<i>Lactobacillus Plantarum Lp-115</i>	0,50x10 ⁹
	<i>Lactobacillus Bulgaricus Lb-87</i>	0,50x10 ⁹
	<i>Lactobacillus Reuteri IE1</i>	0,50x10 ⁹
	<i>Lactobacillus Paracasei Lpc-37</i>	0,25x10 ⁹
	<i>Lactobacillus Brevis Lbr-35</i>	0,25x10 ⁹
	<i>Streptococcus Thermophilus St-21</i>	0,25x10 ⁹
PS 9	<i>Bifidobacterium Lactis BI-04</i>	
	<i>Bifidobacterium Lactis BI-07</i>	
	<i>Lactobacillus Paracasei Lpc-37</i>	10x10 ⁹
	<i>Lactobacillus Acidophilus GLA14</i>	

PS: Probiotic Supplements, CFU: Colony Forming Unit

For sample selection, foods with potential for GABA synthesis and probiotic supplements containing various bacterial strains were chosen. The probiotic supplements were sourced from local pharmacies, while the food samples were obtained from local markets.

The foods used in this study were divided into two groups: the vegetable and fruit group, and the nut group. Samples from the fruit and vegetable group included banana, kiwi, avocado, pineapple, strawberry, tomato, and onion, while the nut group comprised walnut, hazelnut, peanut, and pumpkin seed.

Ten different probiotic supplements were supplied for added to foods, and their compositions are detailed in Table 1. Each food sample was individually treated with the probiotic supplements listed in Table 1.

2.1. Enzymes and Chemicals

Chicken Alpha-amylase (1.5 U/mg, from *Aspergillus* powder), lipase (100–500 U/mg protein), pancreatin (8 × USP), pepsin (≥ 250 U/mg, lyophilized powder), NaCl, CaCl₂·2H₂O, urea, uric acid, bovine serum albumin, KCl, mucin, NaHCO₃, and bile salts mixture were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used in the study.

Chemical materials: GABA (analytical standard), sodium hydroxide (NaOH, $\geq 98\%$), and sodium acetate (CH₃COONa × 3H₂O, $\geq 99.0\%$) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, gradient grade, $\geq 99.9\%$) and sodium bicarbonate (NaHCO₃, $\geq 99.7\%$) were obtained from Honeywell (Charlotte, NC, USA). Dansyl chloride and sodium hydrogencarbonate were purchased from Sigma-Aldrich (BioReagent, $\geq 99\%$), methanol, ethanol, and acetone (analytical grade) were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Labconco System by Millipore (Bedford, MA, USA).

2.2. In Vitro Gastrointestinal Digestive System Method

Foods were homogenized individually, and each food–probiotic supplement combination was subjected to *in vitro* digestion. The *in vitro* digestive system simulated the conditions of the mouth, stomach, small intestine, and large intestine, each involving specific enzymatic digestion processes. In the control digestion group, food samples were subjected to *in vitro* digestion without the addition of probiotic supplements. GABA formation was determined from nutrients digested in the *in vitro* gastrointestinal model. The digestion procedure was a modified version of the method described by Lee et al. [19]. The simulated mouth, stomach, small intestine, and large intestine environments in the *in vitro* gastrointestinal system were prepared in 500 mL of distilled water using specific enzymes, as well as organic and inorganic compounds. The pH of each solution was adjusted to the appropriate value.

Preparation of solutions for the *in vitro* digestive system:

Oral phase; 1.7 mL of NaCl solution (175.3 g/L), 8 mL of urea solution (25 g/L), 15 g of uric acid, 280 mg of α -amylase, and 25 mg of mucin were dissolved in deionized water in a 500 mL erlenmeyer flask. The volume was then adjusted to 500 mL with deionized water, and the pH was set to 6.8 ± 0.2 .

Gastric phase; 6.5 mL of HCl solution (37 g/L), 18 mL of CaCl₂·H₂O solution (22 g/L), 1 g of bovine serum albumin, 2.5 g of pepsin, and 3 g of mucin were dissolved

in deionized water in a 500 mL erlenmeyer flask. The volume was then adjusted to 500 mL with deionized water, and the pH was set to 1.5 ± 0.02 .

Small intestine phase; 6.3 mL of KCl solution (89.6 g/L), 9 mL of CaCl₂·H₂O solution (22.2 g/L), 2 g of bovine serum albumin, 1 g of pancreatin, and 1.5 g of lipase were dissolved in deionized water in a 500 mL conical flask. The volume was then adjusted to 500 mL with deionized water, and the pH was set to 8.0 ± 0.2 .

Bile solution; 68.3 mL of NaHCO₃ solution (84.7 g/L), 10 mL of CaCl₂·H₂O solution (22.2 g/L), 1.8 g of bovine serum albumin, and 30 g of bile were dissolved in deionized water in a 500 mL Erlenmeyer flask. The volume was then adjusted to 500 mL with deionized water, and the pH was set to 7.0 ± 0.2 .

Large intestine phase; *Escherichia coli* was cultured using liquid agar, 2.5 g of *Luria-Bertani (LB) broth*, and 100 mL of purified water. *Lactocaseibacillus casei* was prepared with liquid agar, 100 mL of purified water, and 5.5 g of *Lactobacilli MRS broth*. Each agar preparation was sterilized by autoclaving at 121 °C for 15 minutes. Frozen stocks of *Escherichia coli* and *Lactocaseibacillus casei* stored at -80 °C were thawed to room temperature and then incubated at 37 °C. Subsequently, 1/100 aliquots of the *Escherichia coli* and *Lactocaseibacillus casei* stocks were inoculated into appropriately sterilized liquid agar. For activation, *Escherichia coli* and *Lactocaseibacillus casei* cultures were incubated at 37 °C for 12 hours. Following incubation, the colony counts of *Escherichia coli* and *Lactocaseibacillus casei* reached approximately $\log 10^8$ to 10^{10} CFU/mL. For large intestine digestion, 10 mL of *Escherichia coli* and *Lactocaseibacillus casei* cultures were added to the samples (following small intestine digestion) and incubated at 37 °C for 4 hours [19].

The *in vitro* digestion procedure was performed as follows:

Five grams of each sample were placed into 100 mL Erlenmeyer flasks, and *in vitro* digestion was carried out by sequentially adding simulated mouth, stomach, small intestine, and large intestine solutions.

In the oral phase, 5 mL of the prepared oral solution was added to the samples weighed into 100 mL beakers and mixed using a vortex mixer to ensure homogeneity. The mixture was then incubated in a shaking water bath at 37 °C for 5 minutes.

In the gastric phase, 12 mL of the prepared gastric solution was added to the mixture obtained from the oral phase, and incubated in a shaking water bath at 37 °C for 2 hours.

In the small intestine phase, 10 mL of the prepared small intestine solution and 5 mL of bile solution were added to the mixture obtained after the gastric phase and incubated in a shaking water bath at 37 °C for 2 hours.

In the large intestine phase, 10 mL of the large intestine solution was added to the indigestible fraction obtained after the small intestine phase and incubated at 37 °C for 4 hours. Following the completion of the digestion process, the final volume was adjusted to 50 mL with deionized water.

The samples were then centrifuged at 8000 rpm for 10 minutes, filtered through a 0.45 μ m membrane filter, and injected into the high-performance liquid chromatography (HPLC) system [19].

2.3. Determination of GABA Content

GABA amounts were determined using high-performance liquid chromatography (HPLC) based on a modified version of the method described by Pencheva et al. [20].

2.4. Preparation of Extracts and Standards

One gram of sample was mixed with 15 mL of 75% ethanol and vortexed for 15 minutes. The resulting extract was filtered, and the supernatant was transferred into vials for derivatization and subsequent HPLC analysis. For the derivatization of GABA, the derivatization reagent was prepared by dissolving 5 mg of dansyl chloride in 10 mL of acetone. The GABA standard solution was prepared using ultrapure water.

For derivatization, 100 μ L of sample (GABA standard or extract) was brought to a final volume of 2 mL by adding 900 μ L of 0.1 M sodium bicarbonate buffer (pH 8.7) and 1000 μ L of dansyl chloride solution. The solution was homogenized by vortexing and incubated at 55 $^{\circ}$ C for 1 hour for derivatization. It was then cooled to room temperature and filtered through a 0.45 μ m membrane filter before HPLC analysis.

2.5. HPLC Conditions

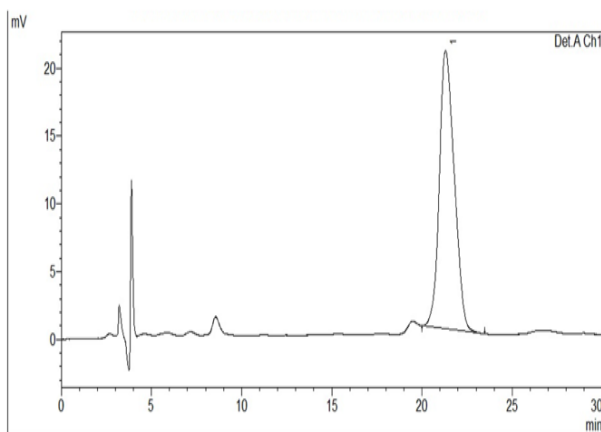


Figure 2. HPLC chromatogram of GABA

HPLC determination of GABA derivatives; HPLC analyses were conducted using a Nexera-i LC-2040C Plus UHPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV detector and a binary pump. The chromatographic column used was an ACE C18 column (100 \AA , 5 μ m, 4.6 \times 250 mm) maintained at 30 $^{\circ}$ C. The mobile phase consisted of 900 mL of 0.05 M sodium acetate buffer and 100 mL of methanol, adjusted to pH 8.0.

The flow rate was set isocratically at 1.0 mL/min, the injection volume was 10 μ L, and the total analysis time was 30 minutes. Detection of the derivatives was performed at a wavelength of 254 nm [20].

2.6. Statistical Analysis

In this study, GABA values of the samples were

analyzed in triplicate on the HPLC device.

Data analysis was performed using Minitab Statistical Software 22.1.0 program. Significant differences among the results were determined by analysis of variance (ANOVA) by Tukey's test and statistical significance level was accepted as $p < 0.05$.

3. Results and Discussion

In this study, the GABA levels of fruit-vegetable and nut groups were determined before and after *in vitro* digestion. Changes in GABA formation were examined during the *in vitro* gastrointestinal digestion of these food groups following the addition of probiotic supplements.

GABA levels before and after *in vitro* digestion in the fruit and vegetable group are presented in Table 2 and Figure 3. The pre-digestive GABA content in the fruit and vegetable group was determined as 14.04 ± 0.65 mg/100 g in banana, 13.42 ± 0.62 mg/100 g in kiwi, 14.10 ± 0.65 mg/100 g in avocado, 15.25 ± 0.71 mg/100 g in pineapple, 15.74 ± 0.73 mg/100 g in strawberry, 19.34 ± 0.90 mg/100 g in tomato, and 17.74 ± 0.82 mg/100 g in onion. After control digestion of the fruit and vegetable group without the addition of any supplements, GABA levels were determined to range from a minimum of 15.16 ± 0.75 mg/100 g in kiwi to a maximum of 21.87 ± 1.08 mg/100 g in tomato.

Significant increases in GABA production were observed following the addition of probiotic supplements to the fruit and vegetable group. Among the probiotic supplements added to the fruit and vegetable group, the highest increase in GABA levels was observed with the 10th probiotic supplement. With the addition of the 10th probiotic supplement, GABA levels increased to 38.31 ± 1.80 mg/100 g in banana, 62.25 ± 2.92 mg/100 g in kiwi, 45.95 ± 2.16 mg/100 g in avocado, 53.72 ± 2.52 mg/100 g in pineapple, 64.13 ± 3.53 mg/100 g in strawberry, 66.50 ± 2.70 mg/100 g in tomato, and 64.16 ± 3.01 mg/100 g in onion. These increases were statistically significant ($p < 0.05$).

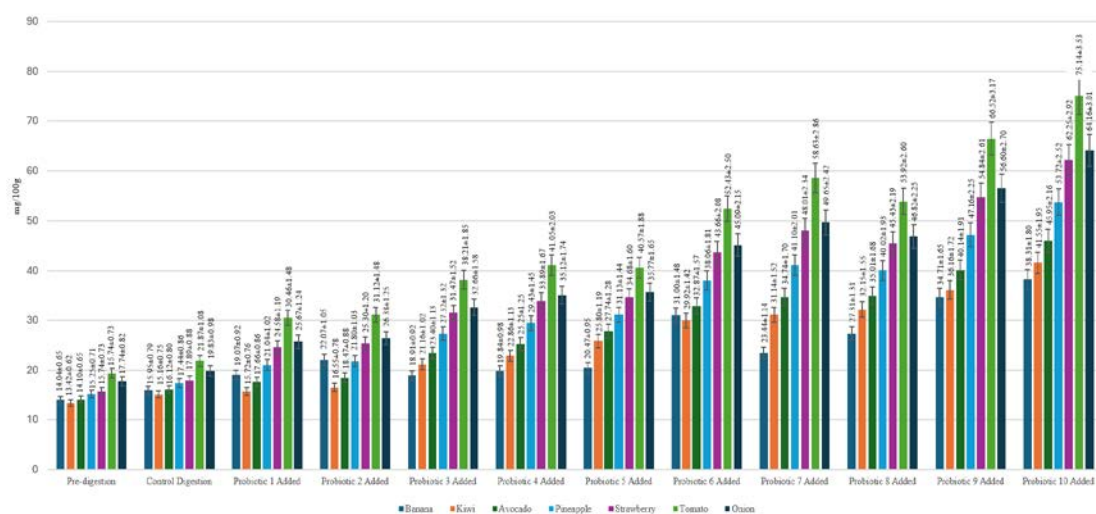
Glutamic acid (Glu), the precursor of GABA, plays a crucial role in GABA formation in plants. In the literature, glutamic acid content has been reported as 2.2 mg/100 g in strawberries, 13.6 mg/100 g in onions, and 53.58 mg/100 g in tomatoes, while GABA levels were reported as 4.4 mg/100 g in strawberries, 4.03–4.93 mg/100 g in onions, and 17.73–36.82 mg/100 g in tomatoes [20].

The GABA level in the tomatoes used in this study was comparable to that reported in the aforementioned study, whereas higher GABA levels were observed in strawberries and onions. In this study, the highest GABA level within the fruit and vegetable group was detected in tomato. Similarly, previous research has indicated that tomatoes possess the highest GABA concentration among vegetables and fruits. Additionally, glutamic acid content in tomatoes increases gradually during fruit ripening, reaching its peak at full maturity [5].

Table 2. GABA levels before and after *in vitro* digestion in the fruit-vegetable group

Fruit-vegetable group	Banana (mg/100g)	Kiwi (mg/100g)	Avocado (mg/100g)	Pineapple (mg/100g)	Strawberry (mg/100g)	Tomato (mg/100g)	Onion (mg/100g)
Pre-digestion	14.04±0.65 ^e	13.42±0.62 ^f	14.10±0.65 ^f	15.25±0.71 ^f	15.74±0.73 ^f	19.34±0.90 ^e	17.74±0.82 ^e
Control Digestion	15.95±0.79 ^{fg}	15.16±0.75 ^f	16.12±0.80 ^f	17.44±0.86 ^{ef}	17.89±0.88 ^f	21.87±1.08 ^g	19.83±0.98 ^{fg}
Probiotic 1 Added	19.07±0.92 ^{ef}	15.72±0.76 ^f	17.66±0.86 ^f	21.04±1.02 ^e	24.58±1.19 ^e	30.46±1.48 ^f	25.67±1.24 ^{ef}
Probiotic 2 Added	22.07±1.05 ^{de}	16.55±0.78 ^f	18.47±0.88 ^f	21.80±1.03 ^e	25.30±1.20 ^e	31.12±1.48 ^{ef}	26.38±1.25 ^e
Probiotic 3 Added	18.91±0.92 ^{ef}	21.16±1.02 ^e	23.40±1.13 ^e	27.32±1.32 ^d	31.47±1.52 ^d	38.21±1.85 ^{de}	32.66±1.58 ^d
Probiotic 4 Added	19.84±0.98 ^{de}	22.86±1.13 ^{de}	25.25±1.25 ^{de}	29.43±1.45 ^d	33.89±1.67 ^d	41.05±2.03 ^d	35.12±1.74 ^d
Probiotic 5 Added	20.47±0.95 ^{de}	25.80±1.19 ^d	27.74±1.28 ^d	31.13±1.44 ^d	34.68±1.60 ^d	40.57±1.88 ^d	35.77±1.65 ^d
Probiotic 6 Added	31.00±1.48 ^b	29.92±1.42 ^c	32.87±1.57 ^c	38.06±1.81 ^c	43.66±2.08 ^c	52.43±2.50 ^c	45.09±2.15 ^c
Probiotic 7 Added	23.44±1.14 ^d	31.14±1.52 ^c	34.74±1.70 ^c	41.10±2.01 ^c	48.01±2.34 ^c	58.63±2.86 ^c	49.65±2.42 ^c
Probiotic 8 Added	27.31±1.31 ^c	32.15±1.55 ^c	35.01±1.68 ^c	40.02±1.93 ^c	45.43±2.19 ^c	53.92±2.60 ^c	46.82±2.25 ^c
Probiotic 9 Added	34.71±1.65 ^b	36.16±1.72 ^b	40.14±1.91 ^b	47.16±2.25 ^b	54.84±2.61 ^b	66.52±3.17 ^b	56.60±2.70 ^b
Probiotic 10 Added	38.31±1.80 ^a	41.55±1.95 ^a	45.95±2.16 ^a	53.72±2.52 ^a	62.25±2.92 ^a	75.14±3.53 ^a	64.16±3.01 ^a

Values are presented as means ± standard deviation (n = 3). Different letters within the same column indicate statistically significant differences between samples (ANOVA, p < 0.05; Tukey's test)

**Figure 3. GABA Formation in Fruit and Vegetable Groups in the *In Vitro* Gastrointestinal Tract****Table 3. Increase rates of GABA formation in the fruit and vegetable group during *in vitro* digestion with added probiotic supplements**

Fruit-Vegetable Group	Banana %	Kiwi %	Avocado %	Pineapple %	Strawberry %	Tomato %	Onion %
Probiotic 1 Added	20	4	10	21	38	39	30
Probiotic 2 Added	38	9	15	25	41	42	33
Probiotic 3 Added	19	40	45	57	76	75	65
Probiotic 4 Added	24	51	57	69	89	88	77
Probiotic 5 Added	28	70	72	78	94	85	80
Probiotic 6 Added	94	97	104	118	144	140	127
Probiotic 7 Added	47	105	115	135	168	168	150
Probiotic 8 Added	71	112	117	129	154	147	136
Probiotic 9 Added	118	138	149	170	206	204	185
Probiotic 10 Added	140	174	185	208	248	243	223

Table 3 shows the increase in GABA formation resulting from *in vitro* digestion with the addition of probiotic supplements to vegetables and fruits, with the highest GABA formation observed in strawberries and tomatoes.

Compared to control digestion, GABA formation in strawberries increased by 38% with the first probiotic supplement and by 248% with the tenth probiotic supplement.

In tomatoes, GABA formation increased by 39% with probiotic supplement 1 and by 243% with probiotic supplement 10. These increases may be attributed to the

nutritional matrix of the fruits and vegetables and their glutamate content. In this study, the effects of probiotics on the *in vitro* digestive system within the fruit and vegetable group were evaluated, and the findings demonstrated a significant increase in GABA formation.

In the fruit and vegetable group, among the probiotic supplements, probiotic supplement 10 had the greatest effect on GABA formation, resulting in a minimum increase of 140% in bananas and a maximum increase of 248% in strawberries. This finding highlights that the

activity of the glutamate decarboxylase (GAD) enzyme in probiotic interactions with different foods may vary depending on the food matrix [18]. These increases in GABA formation via the GAD enzyme, resulting from the metabolic activities of probiotics, may be attributed to the fact that glutamate-rich foods provide a favorable environment for the metabolic activities of probiotic strains [21]. Similarly, the literature reports that *Lactobacillus* and *Bifidobacterium* strains possess a high capacity for GABA production [18]. This increase, which occurs particularly through the fermentation of fruits and vegetables with probiotics, highlights the potential for microbial GABA formation.

GABA values before and after *in vitro* digestion in the nut group are presented in Table 4 and Figure 4. The pre-digestion GABA content in the nut group was determined as 14.98 ± 0.70 mg/100 g in walnuts, 18.28 ± 0.85 mg/100 g in hazelnuts, 17.10 ± 0.79 mg/100 g in peanuts, and 16.54 ± 0.77 mg/100 g in pumpkin seeds. After control digestion of the nut group without any supplementation, GABA levels ranged from a minimum of 16.95 ± 0.84 mg/100 g in walnuts to a maximum of 21.79 ± 1.08 mg/100 g in hazelnuts. When GABA levels before and after *in vitro* digestion were evaluated in the nut group, an increase in GABA concentration was observed in all samples after digestion. GABA levels following digestion with the addition of probiotic supplements were higher

compared to the control digestion without supplementation. These differences in GABA levels were statistically significant (p < 0.05).

Post-digestion GABA levels were examined in the nut group after the addition of probiotic supplements. In walnuts, the pre-digestion GABA level was measured at 14.98 ± 0.70 mg/100 g, while this value increased to 16.95 ± 0.84 mg/100 g after control digestion. The highest GABA level, 44.77 ± 2.13 mg/100 g, was determined with the addition of probiotic supplement 9. In hazelnuts, the GABA level increased from 18.28 ± 0.85 mg/100 g before digestion to 21.79 ± 1.08 mg/100 g after control digestion. The highest GABA levels in hazelnuts supplemented with probiotics were 52.16 ± 2.48 mg/100 g and 51.71 ± 2.43 mg/100 g following the addition of probiotic supplements 9 and 10, respectively. The pre-digestion GABA level in peanuts was 17.10 ± 0.79 mg/100 g, and the highest increase was observed at 53.33 ± 2.50 mg/100 g following digestion with the addition of probiotic supplement 10. While the pre-digestion GABA level in pumpkin seeds was 16.54 ± 0.77 mg/100 g, the highest level after digestion with probiotic supplementation was 63.40 ± 2.98 mg/100 g following the addition of probiotic supplement 10. Statistically significant differences were observed between the GABA values of the nut group before digestion, after control digestion, and after digestion with probiotic supplementation (p < 0.05).

Table 4. GABA levels before and after *in vitro* digestion in the nut group

	Nut group	Walnut (mg/100g)	Hazelnut (mg/100g)	Peanut (mg/100g)	Pumpkin seeds (mg/100g)
Before Digestion	Pre-digestion	14.98±0.70 ^e	18.28±0.85 ^f	17.10±0.79 ^e	16.54±0.77 ^f
	Control Digestion	16.95±0.84 ^{fg}	21.79±1.08 ^{ef}	20.61±1.02 ^{fg}	18.74±0.93 ^f
	Probiotic 1 Added	19.27±0.93 ^f	30.29±1.47 ^{cd}	21.38±1.04 ^{fg}	25.19±1.22 ^e
	Probiotic 2 Added	24.97±1.18 ^{de}	27.57±1.31 ^{cd}	22.15±1.05 ^f	25.90±1.23 ^e
	Probiotic 3 Added	18.18±0.88 ^{fg}	27.74±1.34 ^{cd}	27.57±1.33 ^e	32.14±1.56 ^d
	Probiotic 4 Added	26.89±1.33 ^d	26.74±1.32 ^{de}	34.65±1.71 ^d	34.59±1.71 ^d
	Probiotic 5 Added	22.67±1.05 ^e	30.80±1.42 ^{cd}	24.44±1.13 ^{ef}	35.28±1.63 ^d
	Probiotic 6 Added	33.44±1.59 ^c	41.70±1.99 ^b	38.10±1.81 ^{cd}	44.49±2.12 ^c
	Probiotic 7 Added	22.97±1.12 ^e	32.25±1.57 ^c	40.93±2.00 ^c	48.98±2.39 ^c
	Probiotic 8 Added	36.63±1.76 ^{bc}	43.32±2.09 ^b	40.09±1.93 ^c	46.23±2.22 ^c
After Digestion	Probiotic 9 Added	44.77±2.13 ^a	52.16±2.48 ^a	46.89±2.23 ^b	55.89±2.66 ^b
	Probiotic 10 Added	39.83±1.87 ^b	51.71±2.43 ^a	53.33±2.50 ^a	63.40±2.98 ^a

Values are presented as means ± standard deviation (n = 3). Different letters within the same column indicate statistically significant differences between samples (ANOVA, p < 0.05; Tukey's test)

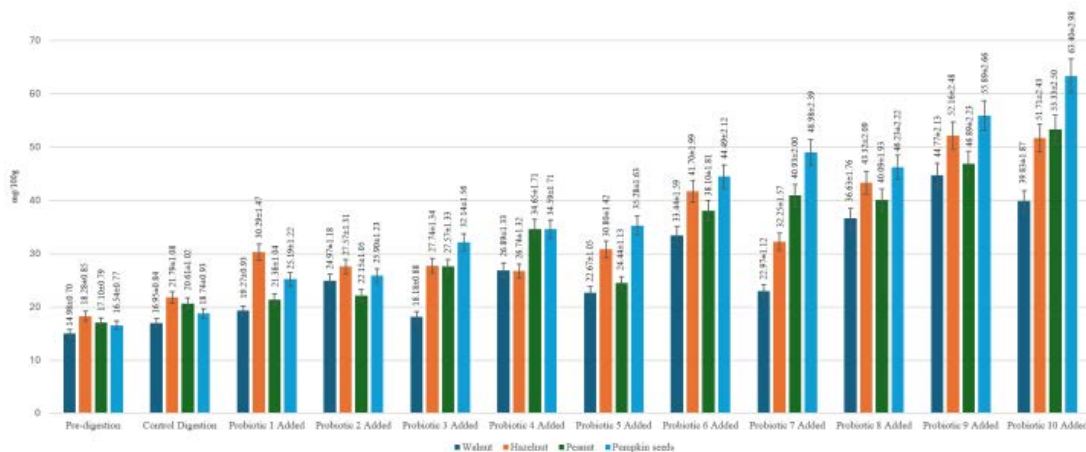


Figure 4. GABA Formation in Nut Group in the *In Vitro* Gastrointestinal Tract

Table 5 shows the increase in GABA formation resulting from *in vitro* digestion with the addition of probiotic supplements to nuts. When the probiotic supplement 1 was added, the highest GABA formation was observed in hazelnuts; with the probiotic supplement 2, it was observed in walnuts; and for *in vitro* digestions with other probiotic supplements, the highest GABA formation was observed in pumpkin seeds. Compared to control digestion, GABA formation in pumpkin seeds increased by 34% with the probiotic supplement 1 and by 238% with the probiotic supplement 10, representing the minimum and maximum increases, respectively. In hazelnuts, the probiotic supplement 4 resulted in a minimum increase of 23%, while the probiotic supplement 9 produced the maximum increase of 139%. In walnut, the probiotic supplement 3 resulted in a minimum increase of 7%, while the probiotic supplement 9 produced the maximum increase of 164%. In peanuts, the probiotic supplement 1 resulted in a minimum increase of 4%, while the probiotic supplement 10 yielded a maximum increase of 238%. In the nut group, the probiotic supplement 9 induced the highest increase in GABA formation among the supplements, with increases of 164% in walnuts and 139% in peanuts; meanwhile, the probiotic supplement 10 resulted in increases of 159% in hazelnuts and 238% in pumpkin seeds.

Table 5. Increase rates of GABA formation in the nut group during *in vitro* digestion with added probiotic supplements

Nut group	Walnuts	Hazelnuts	Peanuts	Pumpkin seeds
	%	%	%	%
Probiotic 1 Added	14	39	4	34
Probiotic 2 Added	47	27	7	38
Probiotic 3 Added	7	27	34	72
Probiotic 4 Added	59	23	68	85
Probiotic 5 Added	34	41	19	88
Probiotic 6 Added	97	91	85	137
Probiotic 7 Added	35	48	98	161
Probiotic 8 Added	116	99	94	147
Probiotic 9 Added	164	139	127	198
Probiotic 10 Added	135	137	159	238

In this study, the high GABA concentrations obtained by adding probiotic supplements 9 and 10 to the nut group are thought to be attributable to the type, quantity, and diversity of bacterial strains present in these formulations. A study has shown that the activity of probiotic supplements is influenced by the type and quantity of bacterial strains they contain [22]. The composition of food matrices can significantly influence the activity of probiotics and the production of GABA. However, it has been reported that the presence of antinutritional factors such as phytic acid and tannins in nuts may limit the activity of probiotics [23]. In a study investigating the phytic acid content of dried nuts, the levels in hazelnuts, peanuts, and walnuts were reported to be 0.95%, 0.58%, and 0.87%, respectively [24]. The physicochemical properties of the food matrix have an important effect on the metabolic activities of probiotics [25].

In this study, greater increases in GABA were observed in foods like walnuts and pumpkin seeds, whereas lower increases were detected in samples such as peanuts and

hazelnuts. These differences in GABA levels among nuts may be attributed to the chemical composition of the food matrix and the metabolic activity of probiotics within these environments. In protein-based foods, free amino acids released during digestion are believed to enhance the GABA production capacity of probiotics [25]. In addition, the environmental pH and substrates released during fermentation can also influence the potential for GABA formation. Among fermented foods, sauerkraut and fermented dairy products are known to contain high levels of GABA. For instance, the GABA content in miso soup has been reported to range from 50 to 300 mg/kg [1].

In fermented dairy products, particularly yogurt and kefir, GABA production can be enhanced by probiotic bacteria [26]. The GABA concentration in sprouted brown rice has been reported to range between 3.96 and 17.87 mg/100 g [27]. Additionally, sprouting pseudocereals such as quinoa has been shown to significantly increase GABA content. Optimizing the combinations of food matrices and probiotic strains is crucial to maximize the potential for GABA formation in these foods. To increase the GABA production capacity in nuts, pre-treatment of these foods or modification of fermentation processes may be recommended [18].

In this study, varying amounts of GABA were detected in foods from the fruit-vegetable and nut groups. In the fruit-vegetable group, GABA levels ranged from 13.42 to 19.34 mg/100 g, with the highest concentration found in tomatoes. In the nut group, GABA levels ranged from 14.98 to 18.28 mg/100 g, with the highest amount detected in hazelnuts. Following *in vitro* digestion with the addition of probiotic supplements, GABA formation increased in both groups. Specifically, in the fruit-vegetable group, the highest increase was observed in tomatoes with the addition of probiotic supplement 10, reaching 75.14 mg/100 g after digestion. In the nut group, the greatest increase was found in pumpkin seeds with the probiotic supplement 10, reaching 63.40 mg/100 g after digestion. In this study, it was observed that vegetables, fruits, and nuts significantly influence GABA formation capacity, depending on their protein and amino acid profiles. These findings demonstrate that probiotic supplementation positively affects GABA production and holds potential for the development of GABA-enriched functional foods.

Findings from this study indicate that probiotics stimulate GABA formation in the gastrointestinal tract and enhance its bioaccessibility during *in vitro* digestion simulation. The superior effectiveness of probiotic supplement 10 in GABA production observed in this study may be attributed to the efficiency of the bacterial strains it contains in glutamate metabolism [28]. The varying capacities of probiotic strains to form GABA are attributed to their genetic and metabolic characteristics. For instance, probiotics such as *Lactobacillus plantarum* and *Lactobacillus brevis* are known to exhibit higher glutamate decarboxylase enzyme production capacities [29]. This study demonstrates that specific probiotic strains can enhance GABA production through interactions with various food matrices. Studies have reported that strains such as *Lactobacillus plantarum* and *Bifidobacterium lactis* possess the capacity to synthesize GABA [30,31]. Another study evaluated the potential for

GABA formation through food fermentation, observing high GABA production in fermentations conducted with yeast containing *Saccharomyces boulardii* [32]. Additionally, a study using a culture medium inoculated with *Streptococcus thermophilus* resulted in GABA-rich foods after fermentation [33].

In this study, the addition of probiotic supplement 7, containing *Akkermansia muciniphila*, to the food groups increased GABA formation in the *in vitro* gastrointestinal system. It has been demonstrated that high carbohydrate and dietary fiber content effectively supports enhanced GABA production by next-generation probiotics such as *Akkermansia muciniphila*. The combination of these probiotics with prebiotic foods, known for their beneficial effects on intestinal health, is thought to create a synergistic effect on GABA production [34]. However, the limited increase in GABA observed in some foods may be attributed to the low levels of free glutamate in their composition or the presence of components that inhibit probiotic activity. Additionally, this may be related to the adaptability of probiotics within such matrices [25]. In this study, increases in GABA production within the *in vitro* gastrointestinal system following supplement addition were observed to be lower in hazelnuts from the nut group and bananas from the fruit and vegetable group compared to other foods.

Studies have specifically focused on the potential of *Lactobacillus plantarum* to synthesize GABA across arious substrates and cultivation conditions. *L. plantarum* is a mesophilic bacterium with an optimal growth temperature of approximately 37°C. Research indicates that the optimal conditions for maximum GABA production typically fall within a temperature range of 30–40°C and a pH range of 5.0–6.0 [35]. The findings from various studies suggest that co-culture formulations involving *Lactobacillus plantarum* in combination with other bacterial species represent a promising approach to enhance fermentation quality and promote GABA synthesis [36].

In a review study examining the GABA production potential of *Lactobacillus plantarum*, it was reported that GABA yield varied depending on factors such as fermentation time, temperature, pH, and the composition of the culture medium, with production levels ranging from 82.12 to 115.86 mg/100 g [37].

In the present study, the fermentation time, pH, and temperature within the *in vitro* gastrointestinal (GI) system differed from the optimal conditions typically used in culture media for maximal GABA production. As a result, GABA formation in the GI tract is generally considered to be lower compared to the levels achieved under controlled culture conditions.

4. Conclusion

This study presents significant findings by utilizing an *in vitro* digestive system to evaluate the GABA production potential of probiotic supplements across different food matrices. It was observed that probiotics produced GABA at varying levels depending on the food matrix, with this effect being particularly pronounced in foods with high glutamate content. Furthermore, the

glutamate decarboxylase activity of the probiotic strains emerged as a key determinant of GABA production.

These results suggest that the beneficial effects of probiotic supplements on the gut-brain axis may be enhanced through their combination with functional foods. In particular, probiotics that promote the production of GABA, an inhibitory neurotransmitter in the central nervous system, hold potential for use in the treatment of anxiety, depression, and other neurological disorders. The findings indicate that foods supplemented with probiotics can increase GABA intake. However, further research is required to optimize the stability and bioavailability of probiotics within various food matrices.

Variations in intestinal microflora among individuals, as well as the viability of probiotics within the intestinal environment, may influence the clinical relevance of these findings. Nevertheless, these results demonstrate that probiotic supplementation represents a promising strategy in food technology and functional food development, not only for promoting intestinal health but also for enhancing the production of bioactive compounds.

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