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EFFECT OF PREBIOTICS ON NEUROACTIVE COMPOUND PRODUCTION BY LACTIC ACID BACTERIA

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ABSTRACT

Gamma-amino butyric acid (GABA) is a nonprotein amino acid, that plays an important role as an inhibitory neurotransmitter. It is also associated with anti-hypertensive, diuretic, tranquilizing and antidiabetic effects. Synthetic GABA supplementation, to circumvent the above-mentioned disorders, has shown significant side effects like drowsiness, dizziness, and addiction. Thus, the focus has now been shifted to natural GABA supplementation, using microorganisms, especially Lactic Acid Bacteria, to develop, a new form of therapeutic agent called the pyschobiotics. In this study, we were able to isolate two GABA producing organisms- IB-1 and IB-3 from Idli Batter, that remain unidentified. IB-1 produced 520 µg/mlof GABA, whereas IB-3 produced 1120 µg/ml of GABA. A study was also carried out to determine the effect of prebiotics on enhancement of GABA production. The findings report that the enhancement of GABA production, by addition of prebiotics is a function of the metabolic activity and the preference for a specific prebiotic/sugar source of the organism. GABA producing isolate IB-1, was the only isolate that reported increased GABA production in the presence of prebiotics.

KEYWORDS: Probiotics, Neuroactive Compound, GABA, Prebiotic.

INTRODUCTION

Gamma-amino butyric acid (GABA) is a nonprotein amino acid, that plays an important role as an inhibitory neurotransmitter. It is naturally found in plants, animals, and microorganisms. It is a primary neurotransmitter in the central nervous system (CNS) that is responsible for reducing neuronal excitability*(Rashmi et al., 2018)*. GABA regulates factors such as

emotional states, cognition and memory, circadian rhythms, and neural development by regulating the responsiveness, excitability, and synchronization of cortical neuronal activity*(Tette et al., 2022)*.Low GABA levels have been associated with several psychiatric and neurological disorders, such as anxiety, depression, insomnia and epilepsy*(Rashmi et al., 2018)*. Modulation of GABA signaling has been the main area of therapeutic research*(Tette et al., 2022).*

Synthetic GABA supplementation, to circumvent the above-mentioned disorders, has shown significant side effects like drowsiness, dizziness, and addiction. Thus, the focus has now been shifted to natural GABA supplementation. Microorganisms have been harnessed as a mode to produce natural GABA and various traditional food have been developed through microbial fermentation that have GABA content.*(Rashmi et al., 2018).*

An enzyme called glutamate decarboxylase (GAD) uses L-glutamate as a substrate to produce GABA in Lactic Acid Bacteria (LAB). The GAD enzyme is a part of system, including the enzyme, substrate, and a glutamate/GABA antiporter GadC. This system is a component of the amino acid-dependent acid resistance (AR) system, which uses GABA production to maintain the intracellular pH homeostasis.*(Kanklai et al., 2020).*

The gut microbiota is a dynamic entity that changes with respect to its composition and activity, in response to host factors, such as age and genetics, and to changing environmental factors, such as diet and drugs *(Morais et al., 2021)*. This phenomenon is termed as gut dysbiosis. Gut dysbiosis disrupts the symbiosis maintained by Gut Brain Axis and has been linked to a variety of mental health conditions such as obesity, Irritable Bowel Syndrome (IBS), schizophrenia, Parkinson's disease and Major Depressive Disorder (MDD) *(Yong et al., 2020).* To restore this malfunctioned axis, probiotics influencing the gut brain axis have been proposed. Probiotics are microorganism, mostly lactic acid bacteria, that, when consumed, help the host's physiology. Psychobiotics is a new class of probiotics with applications in psychiatry. Such "mind-altering" probiotics may work by producing a variety of biologically active compounds, such as peptides and mediators associated with mammalian neurotransmission. Several molecules with neuroactive functions, including gamma-aminobutyric acid (GABA), serotonin, catecholamines, and acetylcholine, have been reported to be derived from bacteria in the human gut *(Wall et al., 2014).*

Prebiotics are a "non-digestible food element that beneficially impacts the host by selectively boosting the growth and/or activity of one or a limited number of bacteria in the colon, and so enhances host health,"*(Gibson & Roberfroid, 1995).* A limited number of studies have been carried out to determine the effect of prebiotics on GABA production. This study expects to open an investigation into this undiscovered territory.

MATERIAL AND METHODS

1. Isolation of Lactic acid Bacteria from Food samples

For the isolation of Lactic acid bacteria, four food samples were chosen : Raw cow milk, fermented mung beans and fermented toor dal and idli batter using methods as described by*(Dhameliya et al., 2020; Iyer*, 20180724; Taye et al., 2021*). The isolates obtained were then subjected to Gram staining and Catalase test.

2. Screening for GABA production

The isolates and the standard GABA producer -*L. bulgaricus NCDC 318 (Sharafi & Nateghi, 2020)* are grown in MRS broth supplemented with 1% Monosodium Glutamate (MSG) and incubated for 48 h, under anaerobic conditions, using a desiccator. After incubation, the broth is centrifuged at 1500 rpm for 20 mins. The GABA produced is then determined by the Glutamate Decarboxylase Assay*(Lacroix et al., 2013).*

3. Quantification of GABA by colorimetric analysis

Using the Standard Gamma-aminobutyric acid (Loba Chemie), solutions of known concentrations of GABA were made, their O.D reading were recorded using a colorimeter (ELICO CL63) at 570 nm and used to develop a Standard curve. The standard curve is then used to derive the concentration of the unknown*(Iyer*, 20180724).*

4. Study the effect of prebiotics on GABA production

Three prebiotics were used in this study- Inulin, Mannitol, Pectin in the concentrations –1%, 1.5%, 2%. The method provided by *(Śliżewska & Chlebicz-Wójcik, 2020)* was followed, with few modifications. GABA production was determined by Glutamate Decarboxylase Assay *(Lacroix et al., 2013).*

5. Characterization of the GABA produced by High Performance Thin Layer Chromatography (HPTLC)

This step was outsourced to Guru Nanak Institute for Research and Development (GNIRD),

at Guru Nanak Khalsa College, Matunga West, Mumbai-400019. HPTLC characterization was carried out as per the method described by *(Saravana Babu et al.,2011)*for the GABA produced by GABA producing isolate IB-3.

6. Identification of the isolates by MALDI-TOF

This step was outsourced to SRL Diagnostics, Mahim West, Mumbai -400016. 48 hrs old culture streaked on Sterile MRS agar of the two GABA producing isolates – IB-1 and IB-3 were sent to the laboratory for identification.

RESULTS AND DISCUSSION

1. Isolation of Lactic Acid Bacteria from Food samples

Using Catalase test and Gram staining as the criteria for preliminary screening, 11 isolates were obtained, with desired properties – Catalase negative and Gram-positive nature.

Characteristics of the isolates obtained are as mentioned in *Table 1*

Table 1: Characteristics of the isolates obtained.

Source	Isolate Name	Catalase test	Gram nature
Raw Cow Milk	M1 (maintained on M17 Agar)	Negative	Gram positive Cocci in chains
Fermented Mung beans	MB1	Negative	Gram positive rods
	MB2	Negative	Gram positive short rods
	MB3	Negative	Gram positive short rods
	MB4	Negative	Gram positive slender rods
	MB5	Negative	Gram positive cocci
Idli tter	IB 1	Negative	Gram positive rods
	IB 2	Negative	Gram positive rods
	IB 3	Negative	Gram positive rods
	IB 4	Negative	Gram positive short rods

Note: Catalase test was run in the presence of a positive control = *Staphylococcus aureus*
Key = Catalase negative: absence of bubbles within 30 secs.

2. Screening of GABA production

In this study, from the 11 isolates upon screening, two GABA producing isolates were obtained – IB-1 and IB-3, from Idli Batter. *(M.-J. Kim & Kim, 2012), (Kanklai et al., 2020)* and *(Edalatian Dovom et al., 2023)* carried out similar screening tests in their respective studies. *(M.-J. Kim & Kim, 2012)* obtained 68 GABA producing Lactic Acid Bacteria upon screening a total of 230 isolates obtained from 20 Kimchi samples, a traditional fermented

food in Korea. *(Kanklai et al., 2020)*obtained 36 GABA producing Lactic acid bacteria upon screening 127 isolates obtained from various fermented Thai foods. *(Edalatian Dovom et al., 2023)* obtained 41 GABA producing Lactic Acid Bacteria, with GAD (Glutamic acid decarboxylase) enzyme activity ranging from strong to poor, upon screening 50 isolates obtained from Traditional Iranian dairy products. These findings suggest that fermented foods and dairy products are a good source of GABA producing Lactic acid Bacteria. GABA production by an organism is owed to the presence of GAD (Glutamic acid Decarboxylase gene) enzyme encoded by the *gad* genes *(Siragusa et al., 2007)*. Thus, the isolates IB-1 and IB-3 have the presence of *gad* genes and their molecular characterization can be carried out. GABA production plays an important role in acid conditions *(Karatzas et al., 2012)*. Due to the activation of the Glu/GABA antiporter, low extracellular pH causes a subsequent reduction in intracellular pH. Due to the biotransformation of a protonated Glu into GABA, which is then transferred to the extracellular media to reduce acid stress, the intracellular media become acidic, which causes proton consumption *(Lacroix et al., 2013)*. The Glutamate decarboxylase assay carried out to screen for GABA production, induces production due to the acidic pH of the reagent. The GABA produced and exported then raises the pH, thus changing the colour of the pH indicator, Bromocresol green. Thus, IB-1 and IB-3 showed moderate activity, as interpretated from the colour change of the GAD reagent*(Lacroix et al., 2013)*.

3. Quantification of GABA production

The GABA produced by the isolates obtained from this study, IB-1(= 520 µg/ml) and IB-3 (= 1120 µg/ml) was seen to be improved as compared to the GABA production reported by a similar study carried out by *(Iyer*, 20180724)*, the isolate obtained produced 0.00348 µg/ml of GABA. *(Kanklai et al., 2020)*reported GABA production of 4 isolates, F019A(=1680 µg/ml), F0324 (=1730 µg/ml), F064A(= 2850 µg/ml) and F087A (=1040 µg/ml). IB-3 showed comparable production, with isolate F0874, however *(Kanklai et al., 2020)*, supplemented 2% MSG for the production of GABA whereas the present study supplemented 1% MSG for the induction of GABA and supplemented 0.1g of Glutamic acid while detection of production. Thus, the higher production could be attributed to higher substrate concentration in the former study. The HPTLC results obtained for IB-3 as seen in *Figure 2*, report that the isolate produced 30,613.24 µg/ml of GABA, thus suggesting that IB-3 has high GAD activity and showed improved production than the isolates obtained by *(Kanklai et al., 2020)*. *L.bulgaricus NCDC 318*, showed improved GABA production (= 1620 µg/ml) as

compared to the findings reported by *(Sharafi & Nateghi, 2020)*, where in production of *L.bulgaricus* was noted to be 36.07 µg/ml. This suggests that *L. bulgaricus* readily used 1% MSG as a substrate as compared to 50mM Glutamic acid, to produce GABA. IB-3 showed higher GABA production of 27,287.12 µg/ml than the 6 lactic acid bacteria - *L. delbrueckii ssp. bulgaricus* (= 36.07 µg/ml), *Lactococcus. lactis ssp. Lactis* (741.14 µg/ml), *L. casei* (53.82 µg/ml), *L. rhamnosus* (51.08 µg/ml) , *L. brevis* (5960.0 µg/ml) ,and *St. thermophilus* (116.4 µg/ml), upon supplementation of 1% Glutamic acid as compared to the supplementation of 50mM Glutamic acid by *(Sharafi & Nateghi, 2020)*.

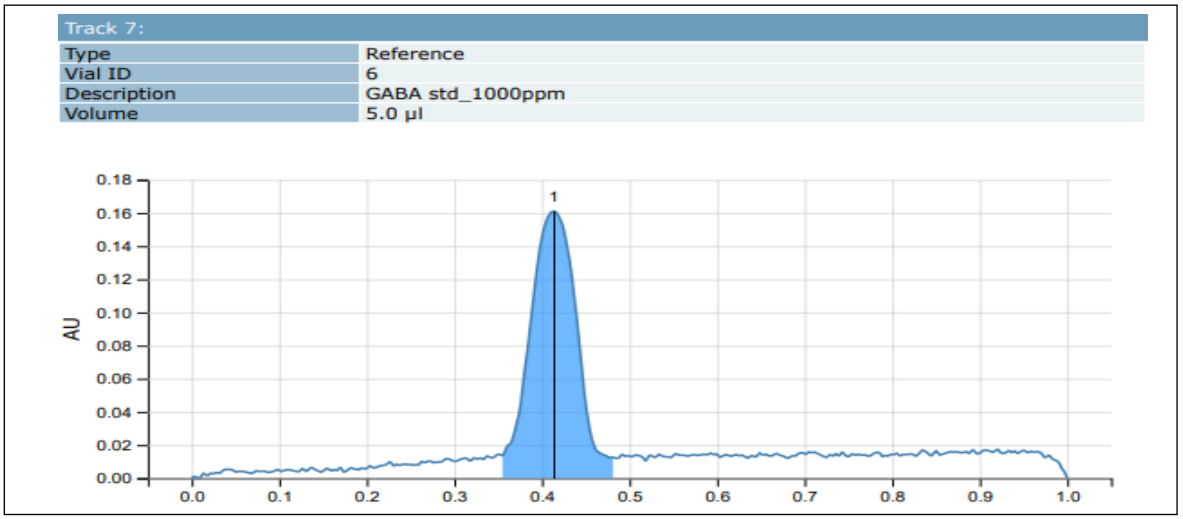
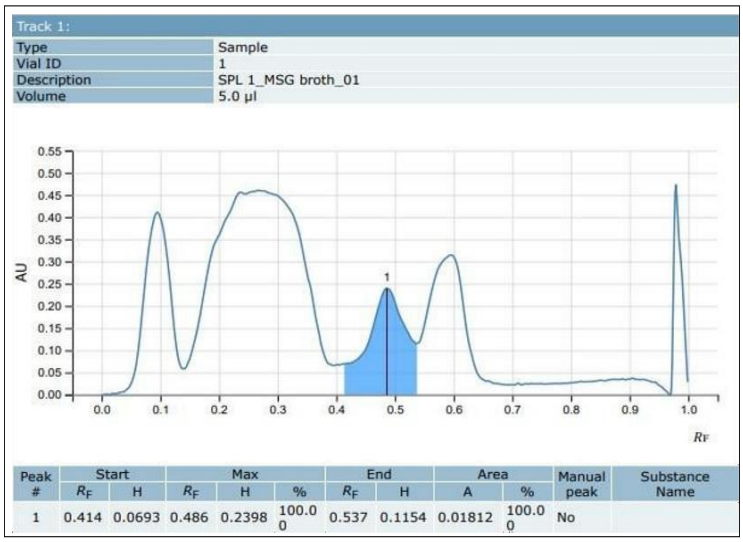
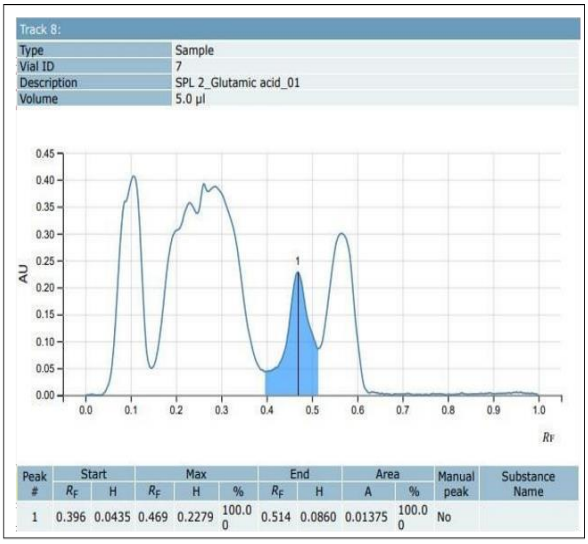


Figure 1: Chromatograph for GABA standard at 550 nm.



(a)



(b)

Figure 2: a) Chromatograph for GABA produced from MRS + 1% Monosodium Glutamate broth b) Chromatograph for GABA produced from MRS + 1% Glutamic acid broth.

4. Effect of Prebiotics on GABA production

The addition of prebiotics in some instances showed an increase in growth, however the increase in biomass did not correlate to a high GABA production, in all instances as seen in *Table 2*. Lactic acid bacteria have varied ability to utilize a prebiotic source and produce GABA, this could be because each Lactic acid bacteria strain has its preferred choice of prebiotics as substrates for fermentation depending on their respective genetic characteristics *(Mandadzhieva et al., 2011)*. Depending upon the substrate, there are specific transport systems for trisaccharides and tetrasaccharides, that lead to changes in metabolic capacity.

The degree of polymerization of prebiotics like pectin and inulin, is quite higher than compared to mannitol and dextrose which is present in MRS broth, which causes the organism to spend extra energy to break them down to smaller monosaccharides*(Hussin et al., 2021)*. In the case of *L. bulgaricus NCDC 318*, the standard GABA producer, 1.5% and 2% pectin showed maximum growth, which did not translate to a high GABA production. Instead, production slightly higher than the control (=1.62 mg/ml), was found when supplemented with 1% Mannitol (=1.64 mg/ml). These findings do not align with those reported by *(J. Kim et al., 2022)*were in the presence of Mannitol, low GABA production was observed. In the case of IB-1, a higher growth at 1.5% Inulin, correlated with a high GABA production (=1.96 mg/ml), highest achieved amongst the isolates. A better production in the presence of inulin compared to other prebiotics was also seen in a study by*(Hussin et al., 2021)*.Increased production was also observed in the presence of Mannitol, which decreased with an increase in Mannitol concentration. A complete absence of GABA production was also noted, in the presence of High concentration of inulin and all concentrations of Pectin. In the case of IB-3, decreased growth in the presence of high concentrations pectin, showed low GABA production, as compared to the control (=1.12 mg/ml). The study carried out by *(Seo et al., 2013)* corroborate the fact that simple sugars are more readily utilizable and give good GABA production. A complete absence of GABA production was also noted, in the presence of low concentration of pectin and all concentrations of Mannitol and Inulin. The findings reported by *(J. Kim et al., 2022)*highlight the preferential utilization of simple sugars over prebiotics, with lack of GABA production in the presence of 2% inulin.

Table 2: A Table representing the consolidated results of the effect of prebiotics and control medium (Sterile MRS broth without prebiotics) on GABA production.

IsolateName	Concentration of GABA produced (µg/ml)									
	Control	**1% Inulin**	**1.5% Inulin**	**2% Inulin**	**1% Pectin**	**1.5% Pectin**	**2% Pectin**	**1% Mannitol**	**1.5% Mannitol**	**2% Mannitol**
L.bulgaricus NCDC 318	**1620**	1000	1100	1060	600	120	850	**1640**	920	1560
IB-1	520	900	**1960**	-	-	-	-	**1780**	1120	1040
IB-3	**1120**	-	-	-	-	900	1060	-	-	-

DISCUSSION

Fermented foods are a rich source of Lactic acid bacteria. Lactic acid bacteria are responsible for the characteristic flavour and aroma of these foods and contribute to their nutritional value. The ability of Lactic acid Bacteria to produce Gamma-aminobutyric acid (GABA) , a hypotensive, diuretic, soothing, anti-stress and anti-depressive, stimulant of immune cells, anti-diabetic and a regulator of the cardiovascular system, that also helps in the management of several psychiatric disorders is a desirable property. *(Devi et al., 2023).*

With increase in demand for natural supplementation for Gamma-aminobutyric acid using microorganisms and fermented foods, this study isolated two GABA producing isolates-IB-1 and IB-3, which remain unidentified from fermented food, that is Idli Batter and compared their GABA production to Standard GABA producer *L. bulgaricus NCDC 318*. GABA production was screened using Glutamate Acid Decarboxylase Assay and quantitated, with IB-1 producing 520 µg/ml, IB-3 producing 1120 µg/ml and *L. bulgaricus NCDC 318* producing 1620 µg/ml of GABA. High performance thin layer chromatography was also performed as a confirmatory test to confirm the presence of GABA. The GABA produced by IB-3 was characterized, with IB-3 producing 30,613.24 µg/ml of GABA upon using 1% MSG and 27,287.12 µg/ml of GABA upon using 1% Glutamic acid. The study also investigated the effect of prebiotics on the enhancement of GABA production. Positive effects of prebiotic inulin on GABA production were observed in the case of isolate IB-1. In the case of *L. bulgaricus NCDC 318*, 1.5% and 2% pectin showed maximum growth, which did not translate to a high GABA production and when supplemented with 1% Mannitol, high GABA production was observed. While in IB-3, decreased growth in the presence of high

concentrations of pectin showed GABA production. Thus, using the GABA producing isolate IB-I, a synbiotic having pyschobiotic effects can be developed. IB-3 and *L. bulgaricus NCDC 318*, could be harnessed to be used as a starter culture for generation of fermented foods with natural GABA supplementation.

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