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The Effects of *Alistipes*-Produced GABA on the Murine Gut-Brain Serotonergic System and Major Depressive Disorder: A Research Protocol

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Abstract

Introduction: Inspired by the link established between gut dysbiosis and Major Depressive Disorder (MDD), this research protocol aims to clarify the role of *Alitsipes* bacteria in the context of this disorder. Gut dysbiosis has been implicated in MDD, but the specific role of the *Alistipes* genus remains unclear. *Alistipes*, being an underrepresented constituent of gut microbiome-related literature, were chosen as a potential link to MDD via the hypothesized pathway of *Alistipes*-released GABA, subsequent production of serotonin from enterochromaffin (EC) cells, and eventual vagus nerve stimulation.

Methods: Four groups of 60 male adult gnotobiotic mice (strain C57BL/6) will be inoculated with different levels of *Alistipes* bacteria *(none, low, baseline, high).* Experiment 1 aims to understand the localization and quantity of *Alistipes*-derived GABA using PET/MRI. Subsequently, either bicuculline (a $GABA_A$ receptor antagonist), CGP56433A (a $GABA_B$ receptor antagonist), both in conjunction, or neither will be administered to understand how serotonin output varies based on GABAergic activity; PET/MRI will be used again for serotonin visualization. Lastly, an *in-vivo* whole-cell patch clamp experiment will help quantitatively assess vagus nerve stimulation resulting from serotonin secretion.

Results: We hypothesize that GABA in the GI tract will be localized at EC cells, and that inhibiting $GABA_B$ receptors will produce the highest serotonin output and subsequently the greatest vagus nerve stimulation.

Discussion: Current literature postulates that higher $GABA_B$ activity correlates with worse MDD symptoms, supporting the therapeutic potential of antagonizing this receptor's activity. The serotonin output-related excitatory $GABA_A$ receptor responses are well known, calling for the exploration of the optimal combination of *Alistipes* concentration to support $GABA_A$ activity and proper dosage to block $GABA_B$ receptors.

Conclusion: This study may have clinical implications for MDD therapies seeing that Selective Serotonin Reuptake Inhibitor treatments are ineffective for many patients; approaching this problem by drawing clearer conclusions about how GABA in the gut affects serotonergic activity in the brain and other nervous system components may help devise more novel treatments. If the proposed pathway holds true, a probiotic to help regulate the *Alistipes* level in the gut may serve as a potential therapeutic direction to take for such treatments.

Keywords: major depressive disorder; *alistipes*; GABA; enterochromaffin cells; 5-HT; vagus nerve

Introduction

Major Depressive Disorder (MDD) currently affects 280 million people worldwide [1]. In fact, the World Health Organization has predicted that by the year 2030, MDD will have the highest disease burden among all neuropsychological conditions [1,2]. Unfortunately for those battling this disorder, symptoms such as decreased enjoyment of pleasurable activities, low self-esteem, fatigue, or suicidal ideations can be an extreme hindrance to everyday life [2]. Additionally, the widely-used pharmaceutical and therapeutic treatments for depression are currently only between 50-60% effective for most patients [3]. For this reason, better elucidating the physiology underlying MDD is critical for developing therapies with higher rates of success.

The Gut-Brain Axis in MDD

It has been established that the bidirectional relationship between the brain and gastrointestinal (GI) tract plays a role in the pathology of MDD [4]. The enteric nervous system, also referred to as the "little brain," has the highest concentration of neurons in the body second only to the brain [4]. For this reason, the gut and brain are in constant communication and heavily influence one another's function [4]. Kumar et al. (2023) have established that inflammation

of the gut due to the dysbiosis of commensal microbiota is associated with MDD; in fact, roughly 25.8% of patients with inflammatory bowel disease (IBD) also suffer from depression [4]. IBD is characterized by an immune response of the patient to elements of their own GI tract, creating an environment rich in inflammatory cytokines such as interleukin (IL)-1 and IL-1ß [5]. The dangers of long exposure to these cytokines are interference with the body's serotonergic systems, inhibition of neurogenesis in the brain, and the loss of blood-brain barrier (BBB) integrity [4,6], all of which may enhance the pathobiology of MDD.

Seeing that broad-spectrum antibiotics target a large portion of the commensal microbiota, the resulting dysbiosis likely reduces the beneficial effects of the gut microbiome [7]. For instance, this may involve an alteration in the secretion of key enzymes and anti-inflammatory molecules necessary for other metabolic pathways and overall brain health [7]. This potential mechanism further suggests the gut microbiome as a meaningful target for MDD treatment [7].

Alistipes and GABA in MDD Progression

A particular genus of bacteria found in the gut microbiome is *Alistipes*, which has been under recent investigation regarding its contribution to the development of inflammation and MDD [8]. The 12 species of bacteria within the Alistipes genus are gram-negative, catalase-negative, rod-shaped, and anaerobic in nature [8]. Parker et al. (2020) has noted an increase in *Alistipes* bacteria concentration in the GI tract with cases of anxiety and depression, yet decreases with many other conditions that are neuropsychiatric in nature [8]. *Alistipes* are responsible for indole production, meaning they are able to hydrolyze tryptophan into the intermediate compound indole, which in turn gets transformed into serotonin [4,8]. Seeing that serotonin deficiency directly correlates with MDD development, the influence of *Alistipes* on the tryptophan-to-serotonin pathway is a reason that we suspect an increase in abundance of *Alistipes* may occur [4]. However, due to the inability of serotonin to cross the blood-brain barrier (BBB) [9], it must act via the peripheral nervous system connected to the gut in order to appropriately affect the brain; this mechanism will be further discussed shortly. Another pathway that has been associated with the presence of *Alistipes* bacteria involves their secretion of glutamate decarboxylase (see Figure 1), an enzyme which catalyzes the transformation of glutamate into γ-aminobutyric acid (GABA) [10,11], an inhibitory neurotransmitter [10,11]. Deficiency of GABA has been shown to correlate with MDD, therefore it is also possible that the presence of *Alistipes* in the GI tract may be beneficial to increase production of GABA and decrease MDD severity [9]. Thus far, the exact role of *Alistipes* in the pathology of MDD is unclear, and the extent of research that has addressed it is limited.

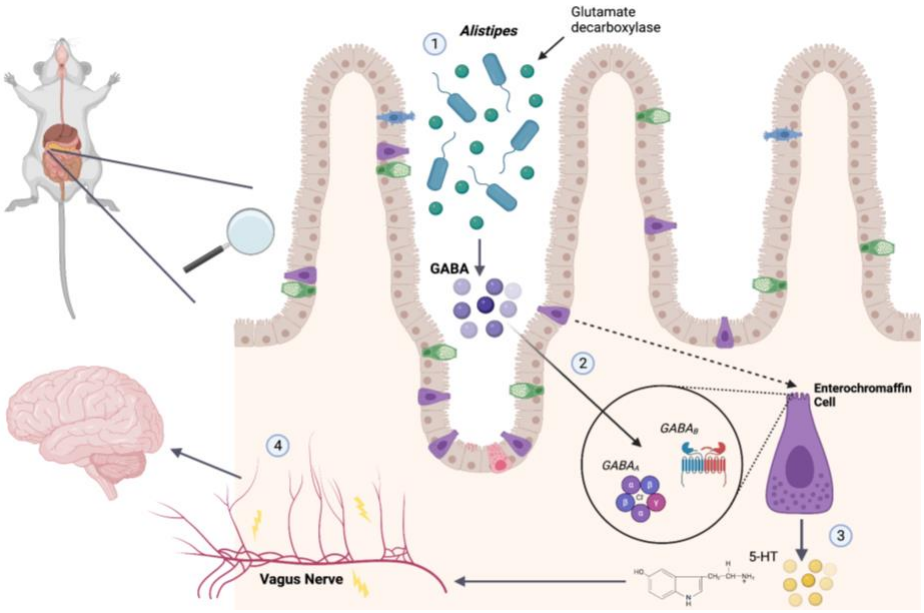

Figure 1. Hypothesized pathway of GABA due to Alistipes presence in the gut; (1) Alistipes bacteria secrete glutamate decarboxylase, which catalyzes the formation of GABA; (2) GABA acts on $GABA_A$ and $GABA_B$ receptors on the surface of EC cells; (3) EC cells secrete 5-HT (serotonin), which is created via the intermediate compound known as indole; (4) Serotonin acts on the vagus nerve, which plays a role in decreasing the symptoms of MDD. Pilot studies conducted on the amelioration of depressive-like behaviour through vagus nerve stimulation showed promising results of a 40% response rate and 17% full recovery or remission rates 10 weeks post-surgical implantation. Figure created with BioRender (https://www.biorender.com/).

Enterochromaffin Cells, Gaba, and the Vagus Nerve

Enterochromaffin (EC) cells of the GI tract have been shown to secrete 95% of serotonin in the body [9], which acts directly on the 5-HT receptors present on vagus nerve (VN) afferent fibers that communicate signals to the brain [11]. These VN afferents synapse onto regions of the brainstem known as the dorsal raphe nucleus and locus coeruleus, two regions crucial for serotonin output [12]. In a more clinical context, pilot studies have shown success in relieving MDD symptoms via vagus nerve stimulation by reducing suicidality in 56% of the patients and improving cognition, as well as motor and executive function 10 weeks into treatment [12,13]. GABA's two receptors, namely $GABA_A$ and $GABA_B$ (see Figure 1), are present on the surface of EC cells, therefore it is likely that GABA's presence in the gut has some effect on EC cell serotonin secretion [14]. A study conducted in canine models concluded that when acted upon by GABA agonists, $GABA_B$ receptors worked to inhibit serotonin release from EC cells, while $GABA_A$ receptors upregulated the production and secretion of serotonin [14, 15]. However, when a similar study was performed in a guinea pig model, $GABA_B$ receptors promoted the release of serotonin rather than inhibited it [15].

Conflicting findings such as these bring into question the precise role of GABA receptors in the context of serotonin release, which remains vague and underexplored to date. In this light, we aim to better understand how GABA production from *Alistipes* bacteria in the gut impacts the release of serotonin from EC cells in a murine model.

Methods

Animals and Handling

Adult male gnotobiotic mice of the strain C57BL/6 [16] will be purchased from Charles River Laboratories [16,17]. The use of gnotobiotic mice is crucial to ensure precise control of the microbiota [18]. The animals will also be individually housed in cages equipped with HEPA air filters to ensure germ-free status is maintained [18]. Although drinking water will be supplied *ad libitum*, our mice will be fed at regular times thrice per day with standard food pellets that have been autoclaved to avoid the ingestion of contaminants [19,20,21]. The temperature will be kept between 24-25˚C with normal light and dark cycles. Additionally, cages will be devoid of toys to eliminate confounding contributions of serotonin; this is also why the mice will be individually caged, however, cages will be in close proximity to one another. Physical handling of the animals will coincide with the Guide for the Care and Use of Laboratory Animals as established by the National Institute of Health [22].

Experiment 1: GABA localization in the GI tract with a controlled gut microbiome

Four groups of 60 adult mice will each be inoculated intraperitoneally with equal concentrations of the eight most common members of the gut microbiota: *Anaerotruncus colihominis, Bacteroides caccae, Bacteroides thetaiotaomicron, Clostridium symbiosum, Collinsella aerofaciens, Coprococcus comes, Providencia stuartii and Ruminococcus torques* in concentrations established by previous research [19]. Once a baseline microbiome has been established, the groups will each be inoculated in the same way with different concentrations of *Alistipes* bacteria of the species *putredinis*, which contributes largely to the secretion of glutamate decarboxylase [8]. Our negative control group will receive no *Alistipes* in addition to the regular gut microbiota. One group will be at baseline - mean abundance of 8.4%, another above baseline (12.4%), and the last group will be below baseline (4.4%) abundance [9]. After waiting 2 days to allow an adjustment to their artificially imposed microbiomes, the mice will be subjected to positron emission tomography (PET) and magnetic resonance imaging (MRI) using the Inveon microPET/microMRI device to determine the localization of GABA in the GI tract [23,24].

PET is a well-studied technique in murine models to deduce the localization of specific target molecules [24,25], and will be utilized in conjunction with MRI to deduce whether GABA aggregation can be observed at EC cells. The GABA-specific radiotracer [^{11}C] flumazenil [25,26] will be injected into the GI tract of all mice. Our MRI parameters will be adjusted according to the work of Frost et al. (2020), with a volume coil set to 300Hz, a field of view set to 6 x 3 x 2.5 cm, and a spatial resolution of 234 x 234 x 260 µm. Additionally, repetition time will be set at 0.8s, echo time will be set at 43.3 ms, and a RARE factor of 16 will be utilized [24,27].

Experiment 2: The Second Experiment in this Investigation Aims to Quantify the Secretion of Serotonin from Each Group of Mice with Varied Alistipes Concentrations

A serotonin transporter radiotracer [^{11}C]DASB will be injected intraperitoneally into each mouse to estimate the density of serotonin in the membrane of presynaptic neurons and determine serotonin levels in the EC cells before it is dispatched [26,28]. Baseline levels of serotonin production will be imaged using PET/MRI with conditions as previously described. Once this control has been established, antagonists will be administered following the Latin Square Design which allows a level of pseudo-randomization necessary to avoid any extraneous effects from order of antagonist administration (see Table 1) [29,30]. Therefore, in a randomized pattern, an equal number of mice in each group will be inoculated with the $GABA_B$ antagonist, for example, first as depicted by the table below.

Table 1. Latin square design for mice inoculation with GABA receptor antagonists

Subgroups of 5 Mice Within the No *Alistipes* (control) Group → / **Experiment Round Number ↓**	**1 (mice 1-15)**	**2 (mice 16-30)**	**3 (mice 31-45)**	**4 (mice 46-60)**
1	A	B	C	D
2	B	A	D	C
3	C	D	A	B
4	D	C	B	A

The Treatment Protocol legend: A: Bicucilline; B: CGP56433A; C: Bicuculline + CGP56433A; D: None

To eliminate a potential confounding variable, each experiment will be performed at the same time of day; the inoculations will occur at 9 AM. This will ensure that results may be as consistent as possible given that serotonin levels, like many other neurotransmitters, naturally cycle diurnally [31].

For the mice being intravenously administered the $GABA_A$ antagonist bicuculline (Treatment Protocol A) first: 10 minutes after administration at the concentration of 0.1 mg/kg, the serotonin radiotracers will be injected, and PET/MRI will be performed once again.

Subsequently, for the groups being administered the $GABA_B$ antagonist (Treatment Protocol B), benzyl[3[[1(3,4 dichlorophenyl) ethyl] amino] 2 hydroxypropyl] phosphinic acid (CGP56433A) [32] will be injected and PET/MRI scans taken. Finally, for Treatment Protocol C, bicuculline will be utilized in conjunction with CGP56433A to observe the effects of inhibiting both $GABA_A$ and $GABA_B$ receptors [28,33]. After injecting both antagonists, the PET/MRI procedure will be performed once more. After each treatment protocol, a day's wait time will be provided to ensure that the drug washes out the system of each mouse.

Table 2. An outline of the experimental conditions applied to each of the four groups of mice

Treatment Protocol and Purpose	**Blocking**	**Intravenous Treatment**	**Concentration of Antagonist**
Protocol D: Negative control	No	Saline solution	0.9% [27]
Protocol A: Investigating the effect on $GABA_A$	$GABA_B$	CGP56433A	3 mg/kg [28]
Protocol B: Investigating the effect on $GABA_B$	$GABA_A$	Bicuculline	3 mg/kg [28]
Protocol C: Investigating the effect of blocking both	$GABA_A$ & $GABA_B$	CGP56433A & Bicuculline	1.5 mg/kg of CGP56433A and 1.5 mg/kg of bicuculline [28]

Experiment 3: This Experiment Utilizes an in-vivo Whole-Cell Patch Clamp to Investigate the Impact of Serotonin on the Vagus Nerve and Innervation of Enteric EC Cells

The *in-vivo* whole-cell patch clamp technique will be used to examine the direct impact of enteric serotonin release on the vagus nerve innervating the EC cells. This technique has been employed both *in vivo* and *ex vivo* in the past to gauge nerve excitability and continues to stand as a reliable measure of localized impacts and downstream cascades of the neurotransmitters in our experiment [34].

To measure the spatiotemporal summations of synaptic inputs to the VN, an intracellular recording from the nerves in the area where the PET scans in Experiment 2 revealed serotonergic presence will be conducted. This procedure will be carried out on each mouse in all four groups outlined in Table 2 without having to sacrifice them.

The depolarisation power of the serotonin released will be measured in terms of the amount of current passing across the cell membrane with this electrophysiological technique. This will help link GABA secretions from the *Alistipes* to vagus nerve stimulation.

Statistical Analysis

Analysis will be conducted using the PETsurfer package of FreeSurfer; minor translational shifting will be performed manually to achieve optimal alignment of the PET and MRI scans [24]. A set of one-way ANOVA will be run for Experiment 1 measuring the differences in the GABA-*Alistipes* relationship between groups, and two-way ANOVA will also be conducted between groups of Experiment 2. Bartlett's test will be utilized to assess normality, and homogeneity of variance will be assessed with a Shapiro-Wilk test [35,36,37]. As well, a Bonferroni correction will be performed for both the one-way and two-way ANOVA [37]; R-Studio will be employed for both these ANOVA tests [38].

Results

With regard to our first experiment, it is expected that PET scan results will indicate a strong signal for GABA localized in the GI tract, with signal strength proportional to *Alistipes* concentration. If no GABA is identified within the GI tract following Experiment 1, it would indicate that

GABA behaves in a way contradictory to our prediction and perhaps does not stay within the confines of the GI tract; if this result should be obtained, conducting a future study on what exactly happens to *Alistipes*-derived GABA would be beneficial.

Similarly, in our second experiment, we expect to see a positive correlation between the level of *Alistipes* administered and serotonin output from EC cells; this prediction is based on the assumption that Experiment 1 findings will be as expected. It is hypothesized that *Alistipes* in the gut produce GABA which will complex with its receptors on the surface of EC cells and stimulate the secretory pathway of serotonin, followed by subsequent stimulation of the VN. Although some findings in the past have conflicted with one another [12,14,15], we have based our predictions for Experiment 3 on the extensive work of Ghose et al. (2011). This study concludes that in murine models, greater inhibition of $GABA_B$ results in lower manifestation of depressive-like symptoms [39], therefore we suspect that administration of $GABA_B$ antagonists will result in greater serotonin secretion than administration of $GABA_A$ antagonists. However, what we are particularly interested in understanding is how the interactions between both bicuculline and CGP56433A will affect the concentration of serotonin release; it is not yet known if the opposing receptor activities vary in strength or even contribution.

As a consequence of altering the GABA receptor activity, we expect to see very little stimulation of the VN following bicuculline administration. Opposingly, when administering only CGP56433A, it is suspected that VN activity will be at its highest across all trials. If the concentration of GABA produced by the *Alistipes* is significant in abundance, and affects EC cells in the suspected manner, it may be possible to derive an MDD therapy on the basis of GABA receptor activity.

Discussion

This research protocol helps not only to clarify the GABA-ergic effect on EC cells and serotonergic release, but also acknowledges the importance of understanding gut dysbiosis in the context of MDD. Previous literature has delved into many of the proposed mechanisms in which the microbiota is deeply interconnected with the functions of the central nervous system, such as through the VN and gut-brain axis [11]. However, research into the specific effects of select gut microbial species still remains a rather unexplored yet important domain in order to develop more specific biomarkers and targeted treatments.

Limitations

A challenge associated with performing these protocols in mice is the concern regarding limited transferability to humans. While mice represent a good mammalian model for understanding internal anatomy and biochemical processes, the tremendous complexity of the human body rarely allows for the identical translation of treatments that were originally adapted in mice [40]. Furthermore, this study investigates the effects of *Alistipes* on the pathology of MDD, but not the other way around; it may be beneficial to enhance this protocol in the future by including an experiment that draws conclusions about how *Alistipes* levels vary in response to MDD. It is also noted that extraneous factors such as presence of toys and social interactions with other mice will be limited to control for any unwanted fluctuations in serotonin levels; however, it is possible that this confounding variable may still present a challenge when quantifying serotonin output specifically from the proposed pathway. Additionally, with the exact half life of CGP56433A unknown, if the length of time between different experimental rounds is too short it is possible that remaining CGP56433A in the murine microbiomes will interfere with subsequent treatments. We will attempt to compensate for this by waiting a full day between experiments. Furthermore, the use of 60 mice per experimental group will aid us in accounting for the attrition rate throughout the course of the experiment.

Furthermore, the decision was made to halve the concentrations of each antagonist when using both in conjunction (during experiment 2) in order to maintain a consistent concentration of administered treatment, however, this may alter the strength of antagonist activity. Despite this, we chose to keep this condition because it is not yet known whether the simultaneous use of these antagonists will have harmful effects; if results show that this treatment causes no harm, it will be beneficial to play around with varying the concentrations of each drug in a future study to deduce an optimal treatment.

Lastly, the uncertainty surrounding the precise extent to which gut serotonin serves as a proxy for the effect of this neurotransmitter in the brain also serves as a possible challenge in this study.

Future Directions

Establishing a link between the *Alistipes* genus and the serotonergic pathway opens many future research avenues, such as devising a mechanism through which *Alistipes*-produced GABA may potentially contribute to MDD treatment or reveal important biomarkers to assess the severity of this mental health disorder. While the excitatory and inhibitory repercussions of having GABA receptors on EC cells are under ongoing investigation, streamlining this serotonergic pathway could potentially involve the use of $GABA_A$ or $GABA_B$ antagonists as discussed; with our proposed experiment in conjugation with previous studies, GABA receptor targeting presents a potential mechanism for MDD symptom alleviation [40].

Furthermore, the use of probiotics as an adjuvant to aid antidepressant efficacy against MDD deserves greater attention, as they are a relatively attainable and non-intimidating class of drug for many patients. In fact, probiotics are administered as live microorganisms and

function to preserve the integrity of our commensal microbiome [41,42,43]. If *Alistipes putredinis* is shown to stimulate the vagus nerve in a manner which reduces depressive symptoms, it may show promise as a probiotic for those diagnosed with or at risk of MDD.

Conclusion

With the alarmingly low roughly 50-60% effectiveness of present therapies and antidepressants such as SSRIs, which are often a first-of-line treatment [3], it is important to explore novel pathways, like the gut-brain axis, through which we can target and improve MDD treatment. While previous works accentuate the uncertainty surrounding the cascading effects of GABA binding to particular ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors, this research protocol aims to understand the linkage between GABA released by a specific microbe genus *Alistipes* and its effect on EC cells and MDD pathology. Looking at the effects on serotonin release from EC cells, we aim to establish a link between the composition of the gut microbiome and the alleviation of MDD symptoms through the route of vagus nerve stimulation. Overall, *Alistipes* should remain a prime area of future exploration in the context of gut-brain-immune communication in MDD and other neuropsychiatric conditions.

List of Abbreviations Used

CGP56433A: benzyl[3[[1(3,4 dichlorophenyl) ethyl] amino] 2 hydroxypropyl] phosphinic acid
EC: enterochromaffin cell
GABA: γ-aminobutyric acid
GI: gastrointestinal
IBD: inflammatory bowel disease
MDD: major depressive disorder
MRI: magnetic resonance imaging
PET: positron emission tomography
VN: vagus nerve

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study will acquire the review of the University of Toronto Ethics in Animal and Research Teaching committee. Due to the use of murine models, careful review of protocols will be taken and ensured to fall under the strict guidelines of ethical use of animal models in research.

Authors' Contributions

JNV: Made significant contributions to the designing of the study, incorporation of technical neuroscience techniques, drafted the manuscript, and continued to refine ideas. Agreement for accountability for all aspects of this manuscript was provided.
JCI: Contributed to the study design, drafting of the manuscript, creation of Figure 1, and continuous refinements to ideas and writing. This author is in agreement to take accountability for all components of this manuscript.

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