MODULATION OF VITAMIN D STATUS BY GUT MICROBIOTA: IMPACT ON DEPRESSION AND ANXIETY-RELATED BEHAVIOR IN ADULT C57BL/6J MICE

by

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Finally, this is for anyone struggling with his or her mental health. **You are not alone**.

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LIST OF ABBREVIATIONS

Abbreviation	Description
25D	25-Hydroxyvitamin D_3
VD	Vitamin D
CTR	Control
AB	Antibiotics
VAB	Vitamin D + Antibiotics
FOS	Fructooligosaccharides (Fiber)
VF	Vitamin D + Fiber
VFAB	Vitamin D + Fiber + Antibiotics
FST	Forced Swim Test
TST	Tail Suspension Test
OFT	Open Field Test
COVID-19	Coronavirus Disease 2019
US	United States
1,25D	1,25-Dihydroxyvitamin D_3
UVB	Ultraviolet-B
BDNF	Brain-Derived Neurotrophic Factor
HPA	Hypothalamic-Pituitary-Adrenal Axis
SPT	Sucrose Preference Test
EZM	Elevated Zero Maze

EPM	Elevated Plus Maze
LDBT	Light-Dark Box Test
GABA	Gamma-Aminobutyric acid
OVX	Ovariectomized
BDI	Beck Depression Inventory
MDD	Major Depressive Disorder
IBD	Inflammatory Bowel Disease
5-HT	5-Hydroxytryptamine
CNS	Central Serotonergic Neurons
5-HTP	5-Hydroxytryptophan
TPH2	Tryptophan Hydroxylase Type 2
ASD	Autism Spectrum Disorder
ADHD	Attention Deficit Hyperactivity Disorder
5-HIAA	5-Hydroxyindoleacetic Acid
NADH	Nicotinamide Adenine Dinucleotide
mRNA	Messenger RNA
SAD	Seasonal Affective Disorder
SCFAs	Short-Chain Fatty Acids
TPH1	Tryptophan Hydroxylase Type 1
CRF	Comparative Research Facility
IACUC	Institutional Animal Care and Use Committee

ELISA	Enzyme-Linked Immunosorbent Assay
ANOVA	Analysis of Variance
SEM	Standard Error of the Mean
UCMS	Unpredictable Chronic Mild Stress

I. LITERATURE REVIEW

Introduction

Depression and anxiety disorders are among the most common mental health disorders that affect U.S. adults today.[1,2]As a result of the COVID-19 pandemic, symptoms of both disorders were exacerbated globally.[3] Individuals struggling with mental illness are at an increased risk of developing a physical health condition such as cardiovascular disease, diabetes, and stroke.[4-6] Conversely, individuals living with a chronic medical condition are at higher risk of experiencing symptoms of depression, compared to their healthy peers.[7] The debilitating symptoms of depressive disorders include feelings of hopelessness, anhedonia, insomnia and/or oversleeping, appetite/weight changes, and suicidal ideation.[8] Anxiety disorders are characterized by symptoms such as increased sense of nervousness, persistent worrying, fatigue, sleep disturbances, trouble breathing, and chest pain.[9] The use of psychotropic medications, a common treatment option for these disorders, continue to rise among US adults aged 18 and over.[10] Risk of harmful side effects and the inconsistent efficacy of these medications has resulted in a growing number of individuals turning to complementary and alternative medicine, which has prompted rigorous evaluation.[11,12] Some common treatments include St. John's wort, omega-3 fatty acids, physical activity, and light therapy. In addition to these remedies, vitamin D (VD) status and gut microbiota have been linked to depressive and anxiety disorders.

Vitamin D Metabolism

Vitamin D is comprised of a group of compounds that include ergocalciferol and cholecalciferol, as well as its metabolites 25-hydroxyvitamin D_3 (25D) and 1,25-

dihydroxyvitamin D_3 (1,25D). In humans, the primary source of VD is synthesized in the body after solar ultraviolet-B (UVB) exposure converts 7-dehydrocholesterol to pre-VD_3, which is then converted to cholecalciferol (VD_3). Within enterocytes, VD can be absorbed into chylomicrons and circulate to hepatocytes or bind to circulating VD-binding protein and distributed to extrahepatic tissues. In the liver, VD is converted to 25-hydroxyvitamin D_3 (25D) by 25-hydroxylase. In the kidneys, 25D is further hydroxylated to its biologically active form 1,25-dihydroxyvitamin D_3 (1,25D) by the enzyme 1α-hydroxylase. In addition to sunlight, animal (cholecalciferol) and plant-based (ergocalciferol) sources of VD include fatty fish, liver, UV-treated mushrooms, and fortified foods.[13] Populations at risk of VD deficiency include older adults, obese individuals, individuals with darker skin, weight-loss surgical patients, and those with limited sunlight exposure. Additionally, individuals battling malabsorption syndrome caused by celiac disease, Crohn's disease, as well as liver and kidney disease are at an increased risk of impaired VD absorption. Apart from the well-known functions of VD for bone health, ongoing research suggests that inadequate levels of 25D may play a role in impaired immune function, cancer, inflammation, and poor cognition.[14,15] Further, VD deficiency has been associated with depression and anxiety-related symptoms, while results regarding the benefits of VD supplementation to alleviate symptoms in the clinical setting have been mixed.[16-20] Exact mechanisms are not clearly understood; however, it has been proposed that VD regulates brain-derived neurotrophic factor (BDNF) levels in the hippocampus, reduces inflammatory cytokines, reduces oxidative stress, and restores calcium and neurotransmitter imbalance to improve mood.[21,22]

Role of Gut Microbiota

The *gut-brain axis* describes the biochemical signals that foster communication between the microbes in the gut and the brain.[23] Although some medical historians argue this connection has long been recognized, growing research has pointed to several signaling mechanisms through which this complex relationship exists. These pathways involve the autonomic nervous system (i.e., the vagus nerve), gut hormones, tryptophan metabolism, microbial metabolites, intestinal permeability, immune system, and hypothalamic-pituitary-adrenal (HPA) axis.[23,24] Each individual has a unique microbial ecosystem, which suggests that the "perfect" gut microbiota community may not exist. However, a disruption in the balance of gut microbiota, or gut dysbiosis, has been shown to increase the risk of gastrointestinal diseases, including inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis) and colorectal cancer.[25,26] Previous research has also linked gut dysbiosis to neuropsychiatric disorders such as schizophrenia, major depressive disorder, and anxiety, although mechanistic links remain unclear.[27,28] Chronic stress, a common protocol that is also implemented to manifest depression and anxiety-like behavior in rodent models, has been shown to disrupt several of the mechanisms related to the gut-brain axis previously mentioned.[29] Additionally, prolonged use of antibiotics and poor diet have been shown to alter the composition of the gut, ultimately resulting in *leaky gut syndrome*.[30-32] This condition is characterized by intestinal hyperpermeability (as a result of loose tight junction proteins in the gut), which can cause undigested food particles, toxins, and digestive waste to leak out of the intestines and into the bloodstream, leading to further health complications.

Behavioral Testing

The efficacy of antidepressants is commonly assessed in rodents using the Forced Swim Test (FST), Tail Suspension Test (TST), and Sucrose Preference Test (SPT). The FST (also known as the Porsolt test) was originally reported in rats and is conducted over two consecutive days.[33,34] Rats are placed in an inescapable Plexiglas tank filled with water for approximately 15 minutes on day 1. Rats are placed in the water for 5 minutes and video recorded on day 2. In mice, this test is conducted over the course of one day and for 6 minutes.[35] Behavioral analysis includes scoring the amount of time the animal spends immobile, climbing, and swimming. Similar to the FST, the TST measures immobility time as a way to evaluate responsiveness to stress and behavioral despair.[33] This behavioral test involves suspending rodents by the tail and is only appropriate for mice because adult rats are too heavy. Anhedonia, or the inability to feel pleasure, is assessed by the SPT. This reward-based test involves presenting both a solution of sucrose dissolved in water and plain water over a period of time. Intake is measured and sucrose preference is then calculated. Rodents have a natural preference for sweetened foods and liquids. Thus, reduced preference for the sweetened solution indicates anhedonia, a common symptom of depression also seen in humans.[8]

The Open Field Test (OFT), Elevated Zero/Plus Maze (EZM/EPM), and Light-Dark Box Test (LDBT) are among the behavioral tests used in rodents to assess anxiety-related behavior.[33] The OFT, commonly used to examine the effectiveness of potential novel anxiolytics, involves a square-shaped box where rodents are allowed to roam freely for 10 minutes. Less anxious rodents will spend more time in the center of the field, whereas rodents perceived as more anxious will spend more time alongside the outer

edge of the field (thigmotaxis).[36] Crossing, rearing, and grooming behavior can also be recorded in the OFT.[37] Crossing describes the number of grid line crossings during the test period and can be used to measure locomotor activity. Rearing behavior describes the posture in which an animal stands on its hind paws in an upright position, with the intention to explore. Similar to the OFT, anxious rodents will spend less time exploring in the open arms of the EZM/EPM, compared to the enclosed areas. Moreover, rodents who are perceived as more anxious will spend more time in the dark portions in the LDBT, compared to less anxious rodents. Biological factors (i.e., mouse strain, age, sex, body weight) and external factors (i.e., handling, social interaction, diet manipulation, stressors, drug dosage, experimental designs) have been shown to affect behavior in rodents, which has called into question the validity of these behavioral tests.[38] As previously mentioned, unpredictable chronic mild stress is commonly utilized in rodents to induce depression and other behavioral changes.[29] Rodents are subjected to several different stressors for a number of weeks in order to assess the efficacy of said treatment. Examples of stressors in this model include damp bedding, cage tilting, food and water deprivation, restraint, inverted light/dark cycle, and tail pinch. In addition to these stressors, surgical procedures (i.e., ovariectomy and middle cerebral artery occlusion) and the administration of corticosterone are other methods researchers utilize in order to worsen symptoms and mirror human disease.[39-41]

Inadequate levels of VD are closely linked to complications from metabolic diseases like those seen in gut dysbiosis; however, whether gut dysbiosis and mood disorders are related to inadequate VD levels remains to be investigated. The review below serves to examine the literature regarding the association among VD, depression-

and anxiety-related behavior in animal and human models, as well as the potential underlying mechanisms that mediate this link.

Impact of vitamin D status on depressive- and anxiety-related behavior

Our extensive review has found that most studies fail to establish a link between VD deficiency and depression- and anxiety-related behaviors in animal models.[42-45] However, between two different mouse strains, C57BL/6J and BALB/c, Groves et al.[42] found that VD deficient BALB/c male mice spent significantly more time immobile during the FST, indicating an increase in depressive-like behavior. In a familiar environment, VD deficient BALB/c mice exhibited a significant reduction in locomotion during the OFT, compared to their respective controls; although, a similar effect was not seen in C57BL/6J mice.[42] It was shown that VD deficient BALB/c mice, but not C57BL/6J mice, spent significantly more time on the open arms of the EPM compared to their respective controls, indicating less anxious behavior.[42] In addition, no significant difference was reported in the LDBT test between the control and VD deficient fed groups of either mouse strain.[42] Discrepancies in the data may be explained, in part, by different methodological approaches, such as rodent type, strain, and sex. Thus, it is critical to include multiple behavioral tests for measurement of anxiety and/or depression, as well as establish standard testing procedures and analysis criteria when conducting these tests.

Adverse offspring health outcomes, beyond poor skeletal growth and bone health, have been linked to inadequate VD levels during pregnancy, including poor neurodevelopment.[46] A study conducted by Burne et al.[44] found that Sprague–Dawley

rats who were conceived and born to VD deficient dams made significantly more arm changes and spent more time in the center of the EPM, compared to rats that were conceived, born and weaned from VD deficient dams, as well as compared to rats that were deficient in VD from conception to 10 weeks of age and the control group. Although VD deficiency did not affect the amount of time spent in the open and closed arms of the EPM or immobility during the FST, these results suggest that postnatal restoration of VD status could have a subtle, yet positive, impact on behavioral changes compared to prolonged VD deficiency throughout adulthood. These researchers previously investigated prenatal VD deficiency in Sprague-Dawley rats and found that it had an acute impact on brain development.[47] This included reduced levels of glutamate decarboxylase (an enzyme involved in GABA synthesis), glial cell line-derived neurotrophic factor, nerve growth factor, as well as structural brain abnormalities. Similarly, Yates et al.[48] found that male offspring from Sprague–Dawley dams fed a VD deficient for five weeks failed to demonstrate a significant difference in sucrose preference, compared to the offspring from dams fed a VD sufficient diet. In contrast to these findings, Fu et al.[49] reported a significant reduction in sucrose preference at postnatal 14 weeks, indicating increased depression-related behavior, in offspring born to VD deficient ICR dams (albino mice of Swiss origin) compared to those born to dams fed a control diet. Assessment of anxiety-like behavior occurred during postnatal 15 and 16 weeks and found that VD deficient offspring spent significantly more time in the center of the OFT, which suggests a decrease in anxiety-related behavior.[49] However, the latency of the first entry into the open arms of the EPM and the number of open arm crossings was significantly decreased in VD deficient mice, indicating an increase in

anxiety-related behavior.[49] It should be noted that in the study by Yates et al.[48], all offspring were fed a control diet at weaning, while some offspring in Fu et al.[49] continued a diet that was deficient in VD. While early life nutrition and its impact on mental health disorders continue to be investigated, these studies suggest that early adequate nutrition may recover or improve cognitive and behavioral alterations seen in conception to early life VD-deficient offspring.

Though the mechanisms behind the association between VD deficiency and poor mental health outcomes remain unclear, previous studies suggest VD supplementation may provide additional benefits in mediating the symptoms related to depression and anxiety.[39,40,50,51] In a post-stroke depression model, C57BL/6 mice injected with vehicle solution (saline) exhibited significantly longer immobility times during the FST, compared to groups given VD.[40] Additionally, changes in depressive-related behavior has been shown to be dose-dependent, with the greatest efficacy observed at 25 μg/kg of VD.[40] Compared to control-treated mice, Xu et al.[40] also demonstrated that treatment with VD improved depression-related behavior in post-stroke stressed mice as measured by the SPT. In another example, long-term ovariectomized (OVX) Wistar rats (treated with saline) exposed to chronic stress for 28 days exhibited a significant increase in immobility time during the FST and reduction in the number of rearings and crossings during the OFT, compared to non-stressed and stressed sham operated rats.[39] Supplementation of VD (5 mg/kg) significantly improved the aforementioned outcomes in OVX stressed rats, compared to OVX and sham stressed rats treated with saline.[39] In support of these observations, administration of VD demonstrated protective effects against corticosterone-induced depressive-like behavior in adult female and male Swiss

mice, as indicated by a reduction in immobility time during the TST.[50,52] Interestingly, VD treated mice mirrored immobility times seen in mice treated with fluoxetine, a commonly administered antidepressant.[39,50,52] However, whether VD has an additive or synergistic effect to current available antidepressant treatments remains underexplored. With regard to anxiety disorders, Fedotova et al.[51] demonstrated that OVX Wistar rats treated with VD (1.0 mg/kg and 2.5 mg/kg) exhibited a significant increase in both the time spent and number of entries into the open arms of the EPM, which indicates a decrease in anxiety-related behavior, compared to the control group. These observations were consistent with the LDBT test, in which a dose-dependent increase in the time spent and number of entries in the brightly lit area was reported in VD-treated OVX rats, compared to vehicle-treated rats.[51]

In the clinical setting, multiple observational studies have demonstrated an association between VD deficiency and poor mental health outcomes. Compared to obese participants without depression, Kamalzadeh et al.[53] reported significantly lower mean serum 25D levels in obese participants with depression. However, it should be noted that these two groups were not age or sex matched. In a second study from Northwestern China, 60% of patients with rheumatoid arthritis experienced symptoms of depression and anxiety, as indicated by the Hamilton Depression Scale and Hamilton Anxiety Scale.[54] Compared to non-depressed patients, patients struggling with depression had significantly lower mean serum 25D levels.[54] In older adults, a population predisposed to both mental illness and VD insufficiency, Ceolin et al.[55] utilized the Geriatric Depression Scale and detected a positive association between serum VD deficiency and symptoms of depression. Limitations to these studies include small sample sizes, sampling from one

hospital/facility, drug-VD interactions, and unmeasured possible confounding factors (physical activity, smoking, diet). Additionally, depression and anxiety are multifactorial disorders, which can make studying mood in a clinical setting especially difficult.

Several human intervention studies have investigated the potential anti-depressant and anti-anxiety effects of VD. Gaughran et al.[56] reported no benefit to VD supplementation when compared to the placebo group on the total Positive and Negative Syndrome Scale score at 6 months in adults with early psychosis. However, VD supplementation significantly improved serum VD levels in participants, many of whom were VD deficient, particularly among minority participants. Additionally, Zhu et al.[16] found that daily VD supplementation (1,600 IU) for 6 months improved symptoms of anxiety (but not depression) in VD deficient participants, as indicated by the Hamilton Anxiety Rating Scale-14. These studies beg the question of whether VD supplementation can alleviate symptoms of mental illness in patients already severely deficient in VD, compared to VD insufficient and sufficient patients. Limitations to these studies include a small sample size, short treatment duration, and type of dosing (bolus vs. daily dose). Sharifi et al.[57] found that 3 months after receiving a single injection of VD (300,000 IU), Beck Depression Inventory (BDI) scores significantly improved in mild to moderate ulcerative colitis participants. Moreover, subgroup analysis showed that participants with sufficient VD levels at baseline benefited more from supplementation compared to VD deficient participants, as indicated by lower BDI scores.[57] Similar to the previous study referenced, further research is needed in order to determine whether a daily dose of VD (compared to bolus dosing), especially over a longer period of time, is more effective for participants who are VD deficient at baseline.

Although observational studies have revealed an association between mood disorders and inadequate VD levels, most studies have failed to demonstrate that a VD deficient diet leads to depression and anxiety-related behavior in rodents **(Table 1).** Interestingly, one study noted a significant difference in immobility time in the FST between two different strains of mice.[42] Interpretation of the FST may be problematic because immobility could mean that rodents have learned they would be rescued, particularly for rats on day 2 of the FST. Additionally, certain strains of rodents may contain behavioral traits associated with resilience and thus may not be ideal for this topic of research.[58] Yet, several studies have shown that VD supplementation improves depression-related behavior in rodents, as evidenced by a decrease in immobility in the FST and TST, as well as a decrease in anhedonia in the SPT. Two studies reference above [39,40] highlight the important link between chronic stress protocols and depression, which may be the reason behind why a VD-deficient diet alone does not induce behavioral changes. Further, rodents who undergo surgery (including a sham operation) or corticosterone treatment and chronic stress exhibit symptoms related to depression and anxiety, compared to unstressed rodents. When considering why VD deficient-fed rodents largely fail to display depression and anxiety-like behavior (compared to human subjects), it is important to consider that VD deficiency in combination with different factors (i.e., daily life stressors, chronic illness, trauma, poor diet, lack of exercise) may contribute to low psychological resilience and ultimately lead to behavioral distress we see in humans.

It is also critical to keep in mind that there are several study limitations and factors that may directly affect VD absorption, such as age, body mass index, and

underlying chronic diseases. These confounding factors could be why we are observing mixed results in clinical studies when VD supplementation was utilized to improve mood. Based on preliminary observations from animal studies, failure to consistently improve symptoms of depression and/or anxiety in VD deficient participants following supplementation suggests that sufficient VD status early in life, or prior to a mental illness diagnosis, is crucial. As researchers continue to investigate the important functions of VD (apart from stimulating calcium and phosphorus absorption) and with VD deficiency on the rise, perhaps a higher dose of VD than what is currently recommended, in order to exert a protective effect against mental illness, may be necessary.

Table 1. Summary of behavioral responses to vitamin D (VD) deficiency, VD supplementation, and gut dysbiosis in rodents.

Behavior	VD Deficiency	VD Supplementation	Gut Dysbiosis
Depression	Not affected	Positive	Negative
Anxiety	Not affected	Mixed (depends on test)	Mixed (depends on test)

Gut microbiota: The potential mediator between vitamin D and mental health disorders

The *gut-brain axis* constitutes a critical connection between cognition, mood, and peripheral intestinal function. Clinical studies have demonstrated a link between microbial community disruptions and mood disorders in Crohn's disease, post-acute stroke, and chronic pain patients.[59-61] The utilization of gnotobiotic animal models have allowed researchers to further investigate the association between gut microbiota and mental health disorders. Zheng et al.[62] observed a significant increase in immobility during the FST in germ-free mice that underwent a fecal transplant from patients

diagnosed with major depressive disorder (MDD), compared to mice with microbiota from healthy patients. Additionally, mice with microbiota from MDD patients also spent significantly less time in the center of the OFT, indicating an increase in anxiety-like behavior.[62] Although the depression-like phenotype was not observed in microbiome-depleted (via antibiotics) rats given microbiota from MDD patients, Kelly et al.[63] also reported an elevation in anxiety-like behavior, as evidenced by the OFT. In support of this gut microbiome hypothesis, several studies have reported that transfer of fecal microbiota from chronically stressed rodents to unstressed germ-free or antibiotic-treated mice, as well as chronic stress and antibiotic treatment themselves, were strongly associated with disruptions in mood.[62,64-66]

Fecal microbiota transplantations allow us to better understand and explain the comorbid relationship between gastrointestinal disorders and mental illness seen in the clinical setting. In a study by Jang et al.[67], it was found that fecal transplantation from IBD patients with depression produced more severe IBD-like colitis in C57BL/6J mice, compared to fecal from IBD patients without depression. Mice containing fecal from IBD-depressed patients demonstrated both depression and anxiety-like behaviors (as evidenced by the TST, FST, and EPM), while mice containing fecal from IBD patients without depression exhibited anxiety-like behavior only.[67] This corroborates what we know about anxiety and depression. That is, persistent states of anxiety can increase the risk of developing depression. Perhaps if the anxious mice containing fecal from IBD patients without depression were then exposed to unpredictable chronic mild stress, depression-like behavior would develop. Interestingly, fecal from healthy volunteers given to mice that previously received fecal from depressed IBD patients significantly

alleviated depression and colitis.[67] This suggests that, in addition to the well-recognized role of gut microbiota in inflammatory bowel disease, the gut microbial community may be crucial in treating mood disorders.

Research regarding the ability of VD to mitigate depression and anxiety through the gut microbiome remains limited. However, the impact of VD supplementation on gut microbiota in different disease states has been investigated in various studies. In one example, streptozotocin-induced diabetic Wistar male rats were fed a high-fat, high-sucrose diet and either given VD (500 IU/kg/day) or the antidiabetic drug metformin (200 mg/kg/day) for 8 weeks.[68] Supplementation with VD improved microbiome dysfunction as indicated by decreased pro-inflammatory markers and upregulation of tight junction protein expression.[68] Further, Zhang et al.[69] found that in a diet-induced rat model of nonalcoholic fatty liver disease, administration of VD (5 μg/kg twice per week) significantly increased the relative abundance of Lactobacillus and inhibited the high fat diet-induced increases seen in *Acetatifactor*, *Oscillibacter* and *Flavonifractor*. In a third study, VD deficient mice had significantly lower microbiota-dependent colonic T reg cells, compared to VD sufficient mice.[70] Fecal from both of these groups were transferred to germ-free mice and recipients of VD deficient mice had fewer abundance of *Bacteroides* and *Clostridium* species.[70] Additionally, VD deficient mice and recipients of VD deficient microbiota were both more susceptible to dextran sulfate sodium-induced colitis, compared to their VD sufficient counterparts.[70] Dietary resistant starch, a fermentable fiber known to positively impact gut microbiota, has also been shown to maintain serum 25D concentrations in Zucker diabetic obese rats.[71] In the clinical setting, intervention studies have demonstrated a therapeutic benefit to VD supplementation (or

status) on gut microbiota among IBD, ulcerative colitis, prediabetic, and healthy patients.[72,73] Although the underlying mechanisms are not entirely understood, mounting evidence has demonstrated the ability of VD to alter gut microbiome composition, improve intestinal epithelial barrier integrity, and regulate inflammatory responses.[72,73] Hence, strategy to restore or optimize VD levels through the microbiome may be beneficial in the treatment of depression and anxiety.

Role of vitamin D in serotonin synthesis: Impact on behavioral disorders

Serotonin is a neurotransmitter that specializes in the regulation of bodily functions including digestion, mood, sleep, and social cognition. The synthesis of 5-hydroxytryptamine (5-HT) begins when the central serotonergic neurons (CNS), located in the central nervous system, take up the amino acid tryptophan through active transport.[74] During this process, tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase type 2 (TPH2).[75] Once 5-HTP is produced, it's then decarboxylated by hydroxytryptophan decarboxylase to form serotonin. The availability of TPH2 can influence serotonin synthesis as its substrates are generally not saturated in certain physiological states.[74] The level of saturation is due to factors such as changes in free tryptophan in the plasma, modifications to tryptophan transporters, and altered compartmentalization of tryptophan.[74] Additionally, the lack of oxygen and cofactors may hinder the hydroxylation process.

Neuropsychiatric disorders, including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), bipolar disorder, schizophrenia, and impulsive behavior disorders, have been linked to low brain serotonin levels and polymorphisms on

the TPH2 gene.[76,77] In addition to increasing the susceptibility to these disorders, polymorphisms have been shown to affect social behaviors, as well as have been associated with aggression, depression, and anxiety.[76] To measure brain serotonin levels, the concentration of 5-hydroxyindoleacetic acid (5-HIAA) is used as a biomarker and has been linked behavioral distress.[76] This is due to altered tryptophan metabolism, which can be measured through low NADH levels. The presence of 1,25D has been shown to influence an up-regulation of TPH2 mRNA to synthesize more serotonin within the CNS.[78] Individuals with polymorphisms on the TPH2 gene, in addition to VD deficiency, may experience a disruption in serotonin synthesis. Further investigation is still warranted to investigate the underlying mechanism of how VD deficiency or insufficiency might affect anxiety or depression. However, as most serotonin production occurs in the gut, it is possible that VD status may alter gut microbiota composition, and thus indirectly regulate serotonin production.

Conclusion

As opposed to epidemiological research, the current body of literature fails to link VD deficiency to depressive and anxiety-like behavior in rodents. However, when rodents endure chronic unpredictable mild stress for several weeks and/or a surgical procedure to model mental despair, supplementation with VD has a positive influence on behavior. Research regarding the ability of VD supplementation to consistently improve depression and anxiety in human subjects is mixed. This may be due to limitations previously discussed. Both the imbalance and absence of gut microbiota have a negative influence on psychological disorders, particularly depression-related behavior in both

rodents and humans. Notably, most behavioral studies only include male rodents, which is important to consider when working to fill gaps in the literature. Although research regarding gut microbiota and mental health focuses primarily on the treatment with prebiotics and probiotics, further information regarding this topic is beyond the scope of this paper. Several studies have assessed the benefits of VD supplementation on the gut microbiome, particularly in different disease states. However, research regarding whether the anti-depressant and anti-anxiety effect of VD is mediated by gut microbiota in rodents remains limited. Further, current reports on serotonin synthesis and its impact on psychological disorders suggest VD may serve as a regulator.

II. INTRODUCTION

Vitamin D (VD) has long been studied for its role in bone health, specifically the regulation of calcium and phosphorus homeostasis. In recent decades, VD, a lipid-soluble pro-hormone, has been linked to inflammation, immune function, carcinogenesis, and cognitive function.[14,15] Seasonal affective disorder (SAD), a type of depression characterized by symptoms that include feelings of hopelessness, increased fatigue, appetite changes, and sleep disruptions, was one of the first mental health disorders linked to VD status.[79] Individuals diagnosed with SAD frequently have suboptimal levels of VD due to limited sun exposure related to seasonal changes and/or low dietary intake, however research has yet to demonstrate the efficacy of VD supplementation in relieving symptoms.[18,80,81] Other depressive and anxiety disorders have also been associated with low levels of VD; though, supplementation results have largely been inconclusive.[16,17,19,20] Discrepancies in the data may be explained by factors known to affect VD absorption such as body mass index, age, gastrointestinal disorders, liver disease, kidney disease, and differences in preclinical and clinical study designs. The mechanisms by which VD alleviates depression and anxiety-related symptoms are not clearly understood, although it has been proposed that VD plays a role in physiological functions like BDNF levels, mitigates inflammatory cytokines, reduces oxidative stress, and restores calcium and neurotransmitter imbalance.[21,22]

Along with VD, a substantial body of evidence suggests gut microbiota likely play a role in neuropsychiatric disorders.[23,63,82] The *gut-brain axis* illustrates the bidirectional relationship between the central and enteric nervous systems, largely through the vagus nerve.[23] This connection also involves gut hormones, tryptophan

metabolism, microbial metabolites (i.e., gamma-aminobutyric acid (GABA) and short-chain fatty acids (SCFAs)), intestinal permeability, the immune system, and the hypothalamic-pituitary-adrenal (HPA) axis.[23,24] The disruption of gut microbiota has been shown to increase the risk of developing chronic health conditions such as gastrointestinal disorders, obesity, and colon cancer.[25,26] An association between psychological disorders and gastrointestinal disease has also been observed, although the exact relationship (cause or consequence) remains unclear.[83,84] Factors such as "Western-style" diets, chronic stress, and the long-term use of antibiotics have been shown to hinder several of the mechanisms behind the gut-brain axis, resulting in *leaky gut syndrome*.[29-32] Impaired intestinal permeability can cause undigested food particles, toxins, and digestive waste to leak into the bloodstream and lead to disease. With respect to mental health disorders, both pre- and probiotic supplementation (also termed *psychobiotics*[85]) have been shown to improve symptoms in human and animal models; however, exact mechanisms have yet to be fully elucidated.[86-88]

Fructooligosaccharides (FOS) are made up of linear chains of fructose molecules (ranging from 2-60 units) with a terminal glucose unit, linked by β-(2,1) glycosidic bonds.[89] This non-digestible carbohydrate travels through the small intestine unabsorbed to the large intestine. In the large intestine, FOS remain structurally unchanged as humans lack the necessary digestive enzymes to hydrolyze most β-glycosidic bonds. Plant-derived sources of FOS include artichokes, garlic, asparagus, yacon, onions, leeks, and chicory.[89] In addition to acting as a low-calorie alternative sweetener, research indicates this soluble dietary fiber holds prebiotic properties, as it can be fermented by colonic microorganisms to directly support the growth of *Bifidobacterium* spp. and

protect against pathogenic flora.[90-92] Further, the production of microbial metabolites like SCFAs (i.e., acetate, propionate, and butyrate) have been shown to play an important role in immune regulation, oxidative stress, colon cancer, the mucosal barrier.[93,94] Decreased consumption of total dietary fiber and the negative shift in SCFA levels have both been associated with symptoms of depression among adults.[95,96]

Tryptophan, including L and D-tryptophan, is an essential amino acid found primarily in animal-based sources. In addition to protein synthesis, tryptophan serves as a precursor to a number of metabolites, including serotonin. Following absorption, microbial and dietary tryptophan is hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase type 1 (TPH1) in enterochromaffin cells of the intestinal epithelium (or TPH2 in enteric and central neurons). In turn, aromatic amino acid decarboxylase converts 5-HTP to produce 5-hydroxytryptamine, commonly known as serotonin. The vast majority (approximately 90 percent) of serotonin synthesis occurs in the gastrointestinal tract. Here, serotonin plays a role in a number of different physiological processes including gastric motility and permeability, endocrine function, metabolic homeostasis, and the inflammatory response.[97] In the central nervous system, serotonin is produced by neurons located in the raphe nuclei of the brainstem and acts as a neurotransmitter to regulate the sleep-wake cycle, body temperature, memory, mood, and learning.[98]

Although VD insufficiency has been shown to amplify complications from metabolic diseases similar to those seen in gut dysbiosis, whether VD metabolism is dependent on gut microbiota and if mental health disorders are related remains unclear. In this study, we hypothesized that depletion of gut microbiota (via antibiotics) would

disrupt host VD status, or enhance urinary excretion of 25D, and promote behaviors related to depression and anxiety in adult mice. Moreover, we hypothesized that shifting of microbial composition via FOS supplementation would “rescue” or restore VD loss and ameliorate symptoms of depression and anxiety.

III. MATERIAL AND METHODS

Animal Design

Four-week-old male and female C57BL/6J mice were purchased in cohorts of 24 from The Jackson Laboratory (Bar Harbor, ME) and acclimated for two weeks. To prevent cross contamination of gut microbiota (as a result of coprophagy), all mice were individually housed. Appropriate environmental enrichment and nesting materials were provided to reduce possible despair caused by isolation. All mice were maintained under a 12-hour light/dark cycle, with a room temperature of ~22-25°C and 40-60% humidity in the Comparative Research Facility (CRF) at Texas State University. With the exception of being fasted overnight (16 hours) for euthanasia, food and water were provided *ad libitum.* Body weight, food, and water intake were measured twice weekly using a digital scale. All mice were handled at least three times per week throughout the duration of the study to minimize stress related to sample collection and behavioral testing. Behavioral testing was performed 7-weeks post dietary intervention. At 14-weeks of age, mice were deeply anesthetized with 3% isoflurane by an enclosed chamber using an isoflurane vaporizer. Blood was collected immediately via cardiac puncture, followed by euthanasia via cervical dislocation. Serum was isolated from whole blood via centrifugation at 1,500 x *g* for 15 minutes and stored at –80℃ for subsequent analysis. Tissues and cecal content were collected, weighed, and stored at -80°C. This experimental procedure was approved by the Institutional Animal Care and Use Committee (IACUC) at Texas State University (San Marcos, Texas), protocol #8007. An overview of our study design is displayed in **Figure 1**.

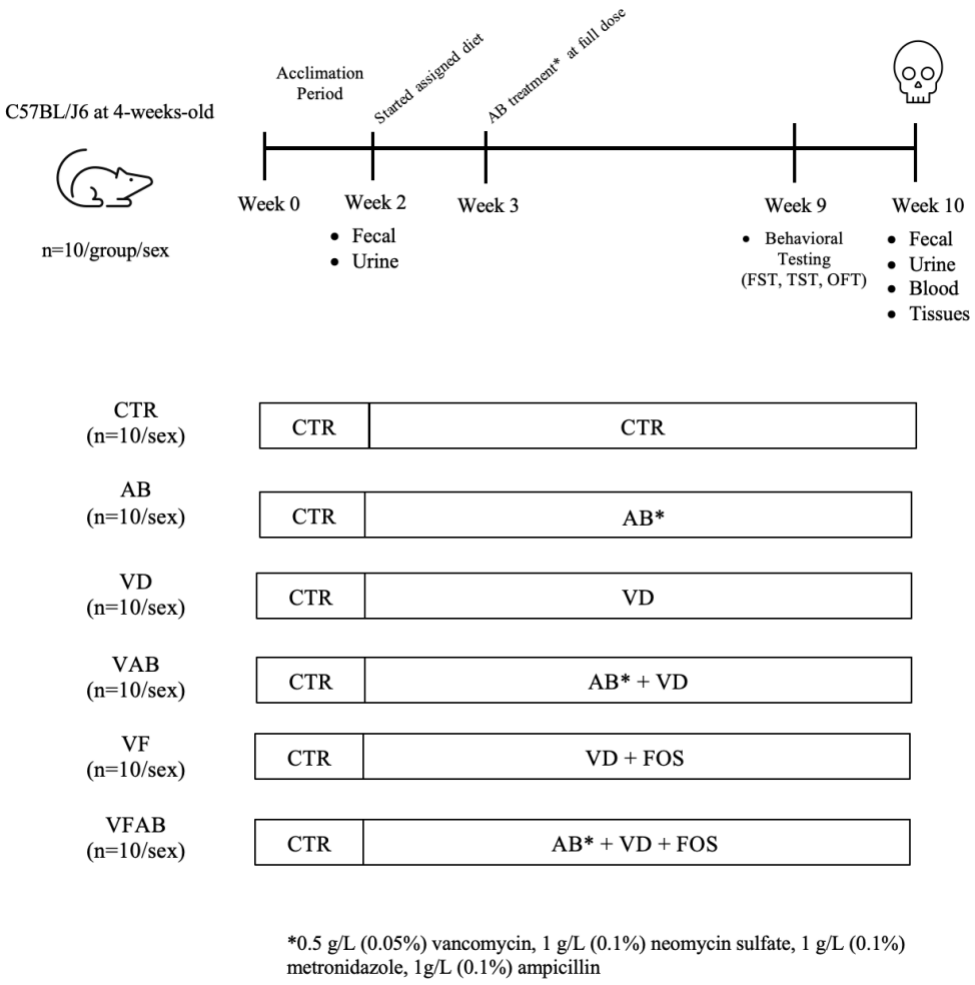

Figure 1. Study design and experimental groups. CTR, control; AB, antibiotics; VD, vitamin D_3; FOS, fructooligosaccharides; FST, forced swim test; TST, tail suspension test; OFT, open field test.

Urine and Fecal Sample Collection

Urine and fecal samples were collected at week 2 (prior to diet intervention) and week 10 (prior to euthanasia) and stored at -80°C for subsequent analysis. For urine collection, slight pressure was applied to the bladder. For fecal collection, most mice produced enough pellets following handling. If a fecal sample was not produced, the animal was placed in a clean cage (no bedding) for either ~15 minutes or when pellets

were produced, whichever came first. If 200 µL of urine and 3-4 pellets (per mouse) were not collected, the animal was returned to their home cage and the procedure was repeated after 1 hour. The entire process was repeated no more than 2 times per day and continued the next day, if necessary.

Treatment

Diet

During a two-week acclimation period, all mice were fed standard AIN-93G chow (CTR) purchased from Research Diets, Inc. (New Brunswick, NJ). Fructooligosaccharides (FOS) were obtained from BENEO (Orafti-P95; Germany) and vitamin D_3 (VD) was purchased from Sigma Aldrich (St. Louis, MO) and supplied to Research Diets, Inc. for diet customization. In order to prevent bacterial and fungi contamination that could interfere with microbiota analysis, all three diets were irradiated by the manufacturer. Further, each diet was given in pelleted form. Both the VD and VD supplemented with FOS (VF) diets were similar in composition to the CTR diet; however, the VD diet was supplemented with an additional 4000 IU of VD_3 per kilogram of food (bringing the final concentration to 5000 IU/kg of VD_3) and the VF diet was supplemented with both VD and 5% w/w FOS. All diets were kept at -20°C throughout the study to prevent spoilage. The composition of each diet is displayed in **Table 2**. At 6-weeks of age, all mice were

randomly assigned to receive one of the six treatments (per sex) that included the control diet (CTR), control diet treated with antibiotics (AB), control diet supplemented with

VD_3 (VD), VD treated with antibiotics (VAB), VD supplemented with 5% w/w FOS (VF), and VF diet treated with antibiotics (VFAB).

Table 2. Diet composition.

Class description	**Ingredients**	**CTR Diet***	**VD Diet***	**VD + FOS Diet***
		Grams (g)	**Grams (g)**	**Grams (g)**
Protein	Casein, Lactic, 30 Mesh	200.00 g	200.00 g	200.00 g
Protein	Cystine, L	3.00 g	3.00 g	3.00 g
Carbohydrate	Starch, Corn	397.49 g	397.49 g	397.49 g
Carbohydrate	Lodex 10	132.00 g	132.00 g	132.00 g
Carbohydrate	Sucrose, Fine Granulated	100.00 g	100.00 g	100.00 g
Fiber	Solka Floc, FCC200	50.00 g	50.00 g	50.00 g
Fat	Soybean Oil, USP	70.00 g	70.00 g	70.00 g
Mineral	Mineral Mix (S10022G)	35.00 g	35.00 g	35.00 g
Vitamin	Vitamin Mix (V10037)	10.00 g	10.00 g	10.00 g
Vitamin	Choline Bitartrate	2.50 g	2.50 g	2.50 g
Antioxidant	tert-Butylhydroquinone (tBHQ)	0.01 g	0.01 g	0.01 g
	Total:	1000.00 g	1000.00 g	1000.00 g
Others				
Vitamin	Vitamin D3 (in diet) (IU)	1000 IU	1000 IU	1000 IU
Vitamin	Vitamin D3 (supplemented) (IU/kg)		4000 IU	4000 IU
Fiber	Fructooligosaccharide (supplemented)	.	.	50 mg/g

CTR, control; VD, vitamin D_3; FOS, fructooligosaccharides

Antibiotic Cocktail

A combination of antibiotics, purchased from Sigma Aldrich (St. Louis, MO), were given to three treatment groups to deplete gut microbiota. The antibiotics were diluted in drinking water, which was provided *ad libitum,* and replaced each week. To prevent taste aversion, the antibiotic-water mixture was provided at 0.25x during the first 3 days of treatment and gradually increased to 0.5x for 4 days. All mice began drinking the full dose of the antibiotic cocktail by the following week. This regimen consisted of 0.5 g/L (0.05%) of vancomycin, 1 g/L (0.1%) of neomycin sulfate, 1 g/L (0.1%) of metronidazole, and 1g/L (0.1%) of ampicillin. This combination of antibiotics has been shown to successfully deplete gut bacteria in rodents.[99]

Behavioral Tests

During week 9 of the experimental period, mice (13 weeks of age) were moved to the behavioral suite of the CRF. All mice were allowed to have access to food and water throughout testing. One behavioral test was performed per day and approximately 48 hours elapsed between tests. Environmental factors, including lighting and room temperature, have been shown to influence behavior.[100] Therefore, lighting in the behavioral suite was dimmed and room temperature was consistent throughout testing. Waste was removed from the chambers between animals and disinfected thoroughly with a 70% ethanol solution. Following competition of each test, all mice were returned to their home cage and placed back in their housing room.

Forced Swim Test

The FST was used to measure depression-like behavior. Each mouse was placed in a Plexiglas cylinder (20 x 40 cm) filled with room temperature water (~25 ± 0.5°C) to a depth of ~30 cm (MazeEngineers, Skokie, IL) for a total of 6 minutes. The water in the FST chamber was changed consistently throughout testing to minimize contamination and influence on gut microbiota as a result of water ingestion. Further, this behavioral test was conducted in a randomized, blinded manner, over the course of one day. All mice were videotaped and later scored by an observer blinded to the treatment group. The last 4 minutes of the FST was scored for immobility (measured in seconds), which was defined as an absence of escape-related behavior (i.e., swimming and climbing).

Tail Suspension Test

In order to further determine the effect of VD on depression-like behavior, mice were suspended by the tail for 6 minutes with adhesive tape (15 cm length x 1.5 width) that was placed ~1 cm from the tip of the tail (MazeEngineers, Skokie, IL). To prevent tail climbing, a common behavior in C57BL/6J mice, a clear cylinder (4.15 cm length x 1.06 cm outside diameter) was placed around the base of each animal's tail.[101] This test was conducted in a randomized, blinded manner, over the course of one day. Each mouse was videotaped and later scored by an observer blinded to the treatment group, prior to decoding. The last 4 minutes of the TST was scored for immobility (measured in seconds), which was defined as an absence of escape-related behavior.

Open Field Test

The open field test (OFT) was used to assess anxiety-like behavior, more specifically the reaction to the stress of being in an open and novel environment.

Behaviors such as center entries, locomotion, rearing, and grooming were also recorded. Mice were placed in a clear Plexiglass computerized open-field chamber (16 x 16 x 16 inches in width, length, and height) and allowed to roam freely for a total of 10 minutes (Coulbourn Instruments, LLC., Allentown, PA). Animal activity was monitored and recorded by TRU SCAN software (Coulbourn Instruments, LLC., Allentown, PA). The chamber was cleared of any waste before the next mouse was tested. This behavioral test was conducted over the course of one day.

Analysis of Urine and Serum 25D Concentrations

Both serum and urine concentrations of 25D were assessed by a commercially available ELISA kit (Crystal Chem, Inc., Elk Grove Village, IL) according to manufacturer's instructions.

Analysis of Urine Creatinine Concentrations

To normalize urine concentrations of 25D, creatinine concentrations were assessed by a commercially available colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI) according to manufacturer's instructions.

Analysis of Serum Serotonin

Concentrations of serotonin were assessed by a commercially available ELISA kit (Enzo Life Sciences, Inc., Farmingdale, New York) according to manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed using SigmaPlot version 14.5 (Inpixon, Palo Alto, CA). Treatment effects on average body weight, feed intake, and water intake were analyzed using Friedman's two-way repeated measures ANOVA on ranks, followed by protected Tukey's post hoc. Differences in serum 25D, urinary 25D, and serotonin concentrations were determined by two-way ANOVA, followed by protected Tukey's post hoc test or Dunn's Method for multiple comparison of unequal groups. Because data for serum 25D were not normally distributed, data were log-transformed prior to two-way ANOVA. Data from behavioral tests were analyzed by two-way ANOVA, followed by Fisher's LSD Method for multiple comparison. The independent variables for these analyses were sex and treatment groups. To further determine treatment effects within each sex, data were analyzed by one-way ANOVA. Kruskal-Wallis' one-way ANOVA on ranks was used for data that failed normality. Mice that were euthanized early due to health complications and/or abnormal behavior were excluded in the analyses. Statistical significance was indicated by $p < 0.05$. All results are expressed as mean ± SEM, unless otherwise indicated.

IV. RESULTS

Treatment effects on body weight, hydration status, and feed intake

Although relatively safe in rodents, initial exposure to an antibiotic regimen has been shown to lead to weight loss; thus, body weight, water, and feed intake were recorded each week to monitor health status and VD intake.[99,102] Body weight in male mice differed among treatment groups, X^2 (5, 100) = 53.449, p = < 0.001 **(Fig. 2A)**. Male mice supplemented with additional VD (i.e., VAB, VF, and VFAB) weighed significantly more than CTR and AB. Further, VF and VAB male mice weighed more than mice supplemented with VD alone. In female mice, a difference in body weight between treatments was observed, X^2 (5, 100) = 53.124, p = < 0.001 **(Fig. 2B)**. More specifically, female mice treated with the antibiotic regimen alone (i.e., AB) and in combination with VD supplementation (i.e., VAB) weighed more compared CTR, VD, VF, and VFAB-treated mice. However, body weight between female mice in AB and VAB treatment groups did not differ (p = 0.994).

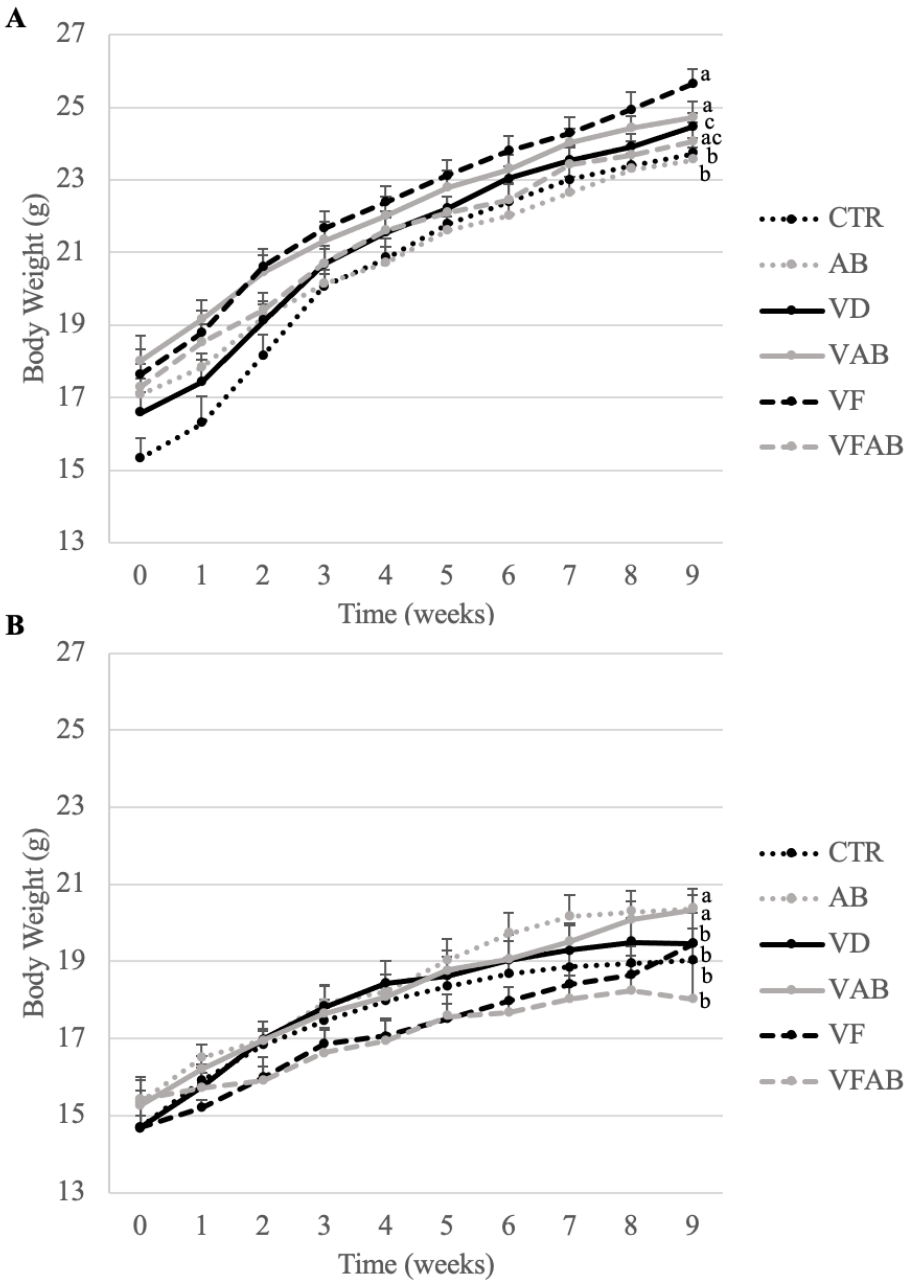

Figure 2. Effect of antibiotic treatment, vitamin D, and fructooligosaccharide supplementation on average body weight (grams) per week in adult male (A) and female (B) C57BL/6J mice. At week 2, mice were randomized to one of the six treatment groups. By the beginning of week 3, the cocktail of antibiotics was administered at full dose. Different letters indicate statistical differences between diets within each sex at $p < 0.05$. Data are expressed as mean ± SEM (n = 8 – 10/group/sex). CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

Antibiotics were administered through drinking water and provided *ad libitum*. Although this method is less stressful for mice (versus daily gavage or injection), intake measurements should be reviewed with caution, as water bottles were prone to leakage. In addition to weight loss, rodents treated with antibiotic water have been shown to become dehydrated. In this study, a significant difference in water intake among treatment groups in male mice was observed, X^2 (5, 80) = 134.728, p = < 0.001 **(Fig. 3C)**. More specifically, all groups treated with antibiotics exhibited a significant increase in water consumption compared to non-antibiotic treated male mice of the same diet. In addition to water intake, a significant difference in feed intake between treatment groups in male mice was also determined X^2 (5, 80) = 90.958, p = < 0.001 **(Fig. 3A)**. Compared to non-AB treated males of the same diet, male mice treated with the antibiotic regimen exhibited a significant decrease in feed intake. Within female mice, a significant difference in water intake among treatment groups was also observed, X^2 (5, 80) = 172.562, p = < 0.001 **(Fig. 3D)**. Similar to male mice, treatment with antibiotics significantly increased water intake. Differences in feed intake among treatment groups in female mice were also found, X^2 (5, 80) = 33.333, p = < 0.001 **(Fig. 3B)**. Specifically, feed intake was higher in VD and VF females compared to CTR and AB. Further, VD-treated female mice ate more than VAB.

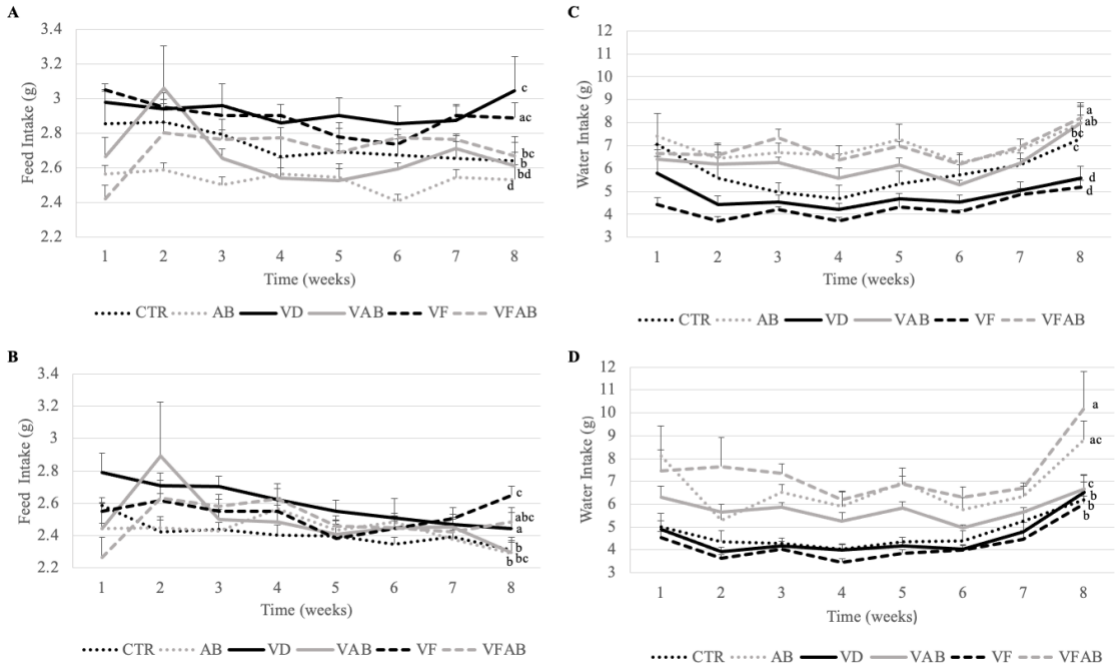

Figure 3. Effect of antibiotic treatment, vitamin D, and fructooligosaccharide supplementation on daily average feed and water intake (grams) per week in adult male (A and C, respectively) and female (B and D, respectively) C57BL/6J mice. At week 2, mice were randomized to one of the six treatment groups. By the beginning of week 3, the cocktail of antibiotics was administered at full dose. Different letters indicate statistical differences between diets within each sex at $p < 0.05$. Data are expressed as mean ± SEM (n = 8 – 10/group/sex). CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

Antibiotic-induced microbiome depletion alone did not affect serum 25D concentrations in adult mice

To determine if host VD metabolism is dependent on the gut microbiome, serum circulating 25D levels were measured following 8 weeks of treatment. Treatment ($p < 0.001$) and sex ($p < 0.001$) effects on serum levels of 25D were observed. When compared to non-VD supplemented groups (i.e., CTR and AB), higher levels of circulating 25D levels were observed in all VD-supplemented groups (i.e., VD, VAB, VF, and VFAB) **(Fig. 4)**, supporting the establishment of our animal model. However, we did not observe a difference in serum 25D levels between CTR and AB groups (p =

1.000), which suggests VD status was not impacted by the disruption in gut bacteria. This was consistent in mice supplemented with VD, in which AB treatment did not alter serum 25D concentrations (i.e., VD vs. VAB), though a decrease was observed in VF mice supplemented with AB regimen (VF vs. VFAB, $p = 0.003$). Additionally, except for CTR mice, female mice exhibited higher levels of serum 25D, compared to their male counterparts ($p < 0.001$).

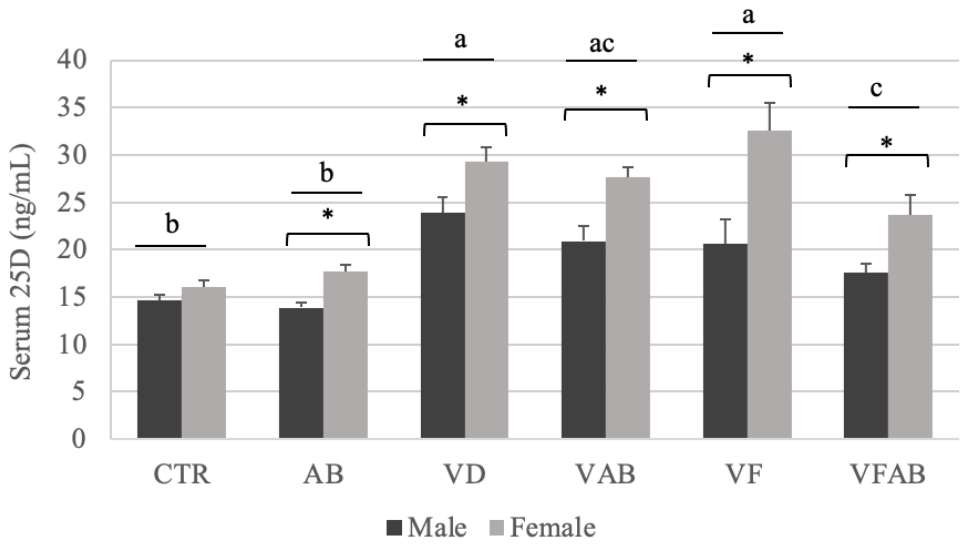

Figure 4. Circulating 25-hydroxycholecalciferol (25D) in adult male and female C57BL/6J mice. Different letters indicate statistical differences between diets at $p < 0.05$. Statistical differences between sex within each diet group are expressed as $*p < 0.05$. Data are expressed as mean ± SEM (n = 6 – 7/group/sex). CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

We further evaluated the effect of treatment on serum 25D levels within each sex. An overall difference in serum 25D levels was observed in male mice, $H(5) = 25.000$, $p = < 0.001$. Specifically, male mice supplemented with VD had higher serum 25D levels compared to male mice in the CTR ($p = 0.004$) and AB ($p < 0.001$) groups, respectively.

Additionally, male mice treated with VAB had higher of 25D levels compared to mice in the AB group ($p = 0.026$). Differences in serum 25D levels between treatment groups in female mice were also observed, H (5) = 28.985, $p = < 0.001$. Female mice treated with VD and VF had higher serum 25D levels, compared to CTR female mice.

Antibiotic-induced microbiome depletion alone did not affect urine 25D concentrations in adult mice

To further determine if VD status is directly affected by the excretion of 25D, and whether disruptions in the gut microbiome enhance urinary excretion of 25D, we examined changes of 25D in the urine. A two-way ANOVA analysis showed a treatment effect, F (5,52) = 3.503, $p = 0.008$, and sex effect, F (1,52) = 4.141, $p = 0.047$ **(Fig. 5)**. Overall, mice treated with VD and VFAB exhibited higher concentrations of 25D in the urine compared to the CTR group ($p = 0.041$ and $p = 0.009$, respectively). Consistent with serum 25D concentrations, we did not observe a difference between mice treated with CTR and AB ($p = 0.755$). Compared to females, male mice exhibited higher concentrations of 25D in their urine. Specifically, within the VD group, male mice excreted ~82% more 25D compared to female mice.

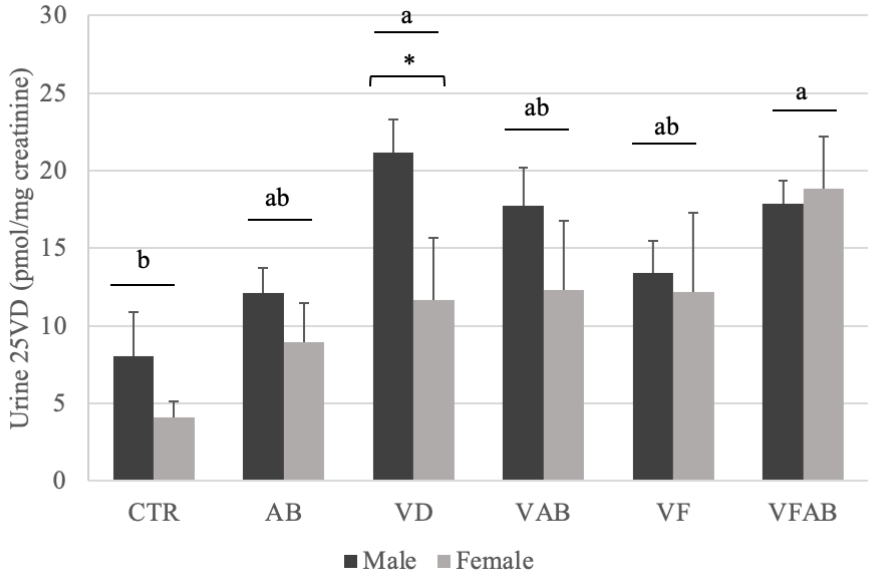

Figure 5. Normalized urine 25-hydroxycholecalciferol (25D) in adult male and female C57BL/6J mice. Different letters indicate statistical differences between diets at $p < 0.05$. Statistical differences between sex within each diet group are expressed as $*p < 0.05$. Data are expressed as mean ± SEM (n = 4 – 7/group/sex). CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

There was a difference in male urine 25D levels between treatment groups as determined by one-way ANOVA, F (5,27) = 4.740, $p = 0.003$. Male mice treated with VD had about a 1.6- fold significant increase in urine 25D concentrations, compared to males in CTR ($p = 0.004$). In female mice, there were no treatment differences in urine 25D levels ($p = 0.282$).

Effect of antibiotic administration, VD supplementation, and dietary fiber on serum serotonin concentrations

Treatment effects on serum serotonin are displayed in **Figure 6**. Neither the effects of diet and sex were significant, however, there was a significant interaction between treatment and sex, F (5,61) = 4.704, $p = 0.001$. While the antibiotic regimen did

not affect serum serotonin in AB (vs. CTR) or VAB (vs. VD) supplemented mice, the combination of antibiotics and VF (i.e., VFAB mice) suppressed circulating serotonin concentrations compared to mice treated with VAB and VF alone. Consistent with data from serum and 25D status, serum serotonin levels did not differ between CTR and AB-treated mice. Furthermore, male mice in the AB and VAB groups demonstrated higher levels of serum serotonin, compared to females within the same treatment group (p = 0.006 and p = 0.026). However, a reverse relationship was observed in female mice fed VF, in which serum serotonin was 64% significantly higher than males of the same treatment (p = 0.003).

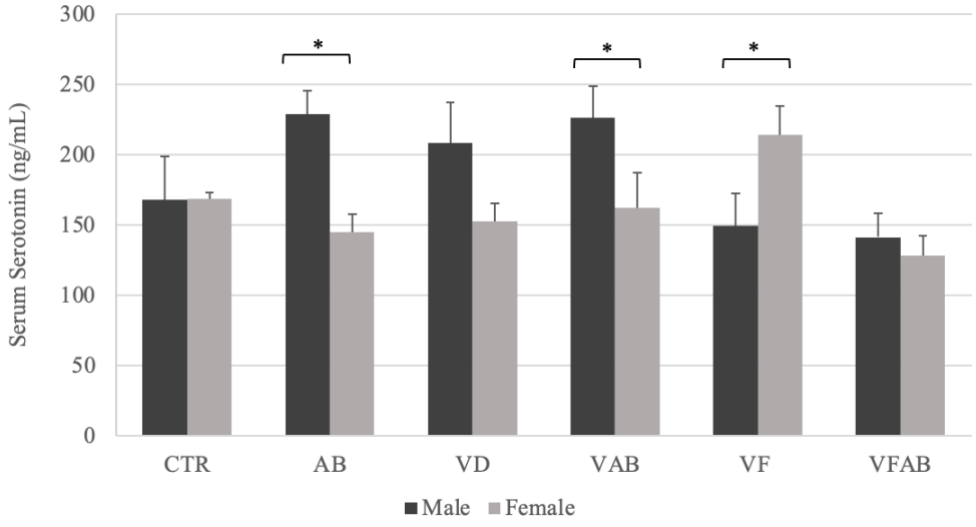

Figure 6. Circulating serotonin in adult male and female C57BL/6J mice. Different letters indicate statistical differences between diets at $p < 0.05$. Statistical differences between sex within each diet group are expressed as $*p < 0.05$. Data are expressed as mean ± SEM (n = 5 – 7/group/sex). CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

We further investigated treatment effects on serotonin within each sex. Serum serotonin levels differed between treatment groups in male mice, F (5,31) = 2.798, p =

0.034. However, Tukey's post-hoc test failed to reveal any differences between treatment groups within male mice. Serotonin levels between treatment groups in female mice also differed, $F(5,30) = 5.170$, $p = 0.002$. Specifically**,** serum serotonin levels in VF treated mice were higher than all other treatment groups in female mice.

Supplementation with VD and dietary fiber decreased anxiety- but not depression-related behavior

Forced Swim Test and Tail Suspension Test

The effects of VD and FOS supplementation, with and without antibiotic treatment, on depressive-related behavior in adult male and female mice is displayed in **Table 3**. A decrease in escape-related behavior, or an increase in immobility, is indicative of a depression-like state in rodents. We did not observe a treatment or sex effect in the time spent immobile during the FST ($p = 0.152$ and $p = 0.445$, respectively) or TST ($p = 0.882$ and $p = 0.321$). Further, an interaction between treatment and sex was not observed in either behavioral test ($p = 0.602$ and $p = 0.943$, respectively). Immobility times from the FST and TST were also combined (i.e., total immobility), however, differences among treatment ($p = 0.574$) and sex ($p = 0.275$) were not found.

Table 3. Time (seconds) spent immobile during the forced swim test (FST) and tail suspension test (TST) in adult male (A) and female (B) C57BL/6J mice.

A

Diet	FST (sec)	TST (sec)	Total Immobility (sec)
CTR	150.11 ± 11.33	95.89 ± 16.18	246.00 ± 20.97
AB	177.20 ± 10.82	101.20 ± 12.74	278.40 ± 15.63
VD	161.70 ± 13.40	94.60 ± 15.44	256.30 ± 21.40
VAB	158.44 ± 17.10	98.11 ± 12.49	256.56 ± 16.18
VF	175.11 ± 19.08	94.22 ± 16.74	266.88 ± 21.49
VFAB	157.75 ± 15.49	84.44 ± 13.92	235.75 ± 18.27

B

Diet	FST (sec)	TST (sec)	Total Immobility (sec)
CTR	158.70 ± 9.82	114.00 ± 18.07	272.70 ± 22.16
AB	182.78 ± 10.83	90.44 ± 13.96	274.33 ± 20.11
VD	137.56 ± 21.72	109.00 ± 13.99	246.56 ± 26.80
VAB	185.67 ± 9.50	105.78 ± 12.82	291.44 ± 17.27
VF	182.11 ± 13.79	104.44 ± 14.78	286.56 ± 17.19
VFAB	170.60 ± 10.70	93.40 ± 12.57	264.00 ± 21.02

Data are expressed as the mean ± SEM (n = 8 – 10/group/sex) for the last 4 minutes of the observation period. CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

Open Field Test

The OFT is commonly used to assess the efficacy of anxiolytic agents. Rodents that exhibit less anxious-like behavior will spend more time in the center of the field, whereas rodents perceived as more anxious will spend more time alongside the outer edge of the field.[33] Behaviors such as the total time spent in the center of the field, center entries, rearing, grooming, and general locomotor activity are typically reported.[37]

Regarding the time (seconds) spent in the center of the OFT, we did not observe a treatment or sex effect. However, an interaction between treatment and sex, F (5,100) = 5.248, p = <0.001 was revealed **(Fig. 7)**. Male mice in the CTR and AB groups spent more time in the center of the OFT, compared to females of the same treatment group (p = 0.002 and p <0.001, respectively). Female mice in the VF group spent significantly more time in the center of the OFT, compared to the males that received the same treatment (p = 0.024). Differences between VD, VAB, or VFAB-treated mice, in relation to sex, were not observed.

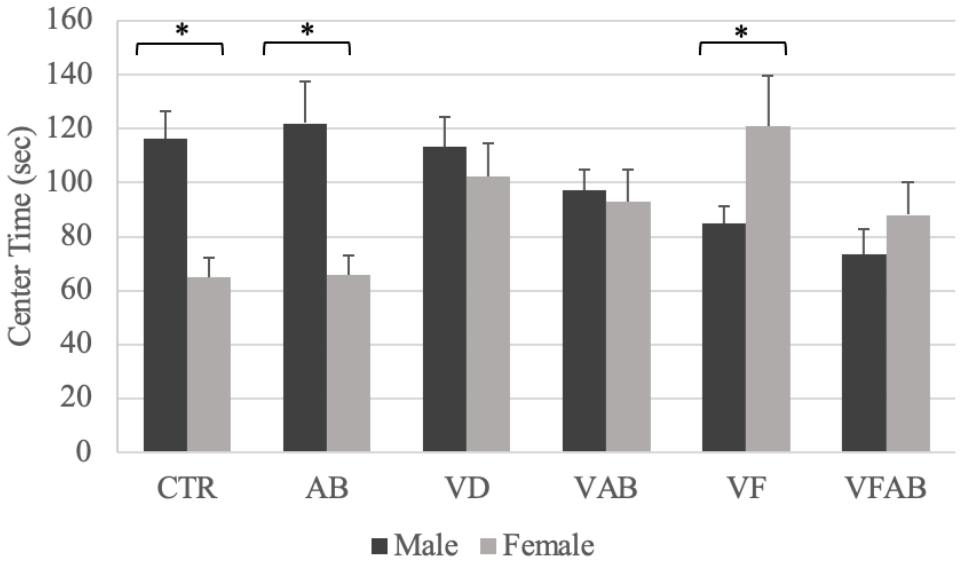

Figure 7. Time (seconds) spent in the center of the open field test in adult male and female C57BL/6J mice. Statistical differences between sex within each diet group are expressed as *p < 0.05. Data are expressed as the mean ± SEM (n = 8 – 10/group/sex) observed over a 10-minute period. CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

Treatment effects on center time within each sex were also evaluated. There was a difference in center time in the OFT between groups for both male, F (5,51) = 3.308, p =

0.012 and female mice, F (5, 49) = 3.256, $p = 0.013$. Specifically, male mice in the CTR and AB treatment groups spent significantly more time in the center of the field, compared to male mice in the VF and VFAB groups. Interestingly, we observed a decreasing trend ($p = 0.058$) in VF-treated male mice compared to males given VD. In contrast, female mice treated with VD and VF spent significantly more time in the center of the OFT, compared to the mice of the same sex in the CTR group. A decreasing trend in center time among VFAB and VF-treated female mice was also observed ($p = 0.054$). Overall, the AB regimen did not alter center time of the OFT across both sexes.

Treatment affected the number of entries into the center zone of the field, F (5, 100) = 3.578, $p = 0.005$ **(Fig. 8)**. Specifically, mice supplemented with VD made more center entries compared to CTR ($p = 0.006$) and AB ($p = 0.008$). VD-treated mice made 63.872 ± 3.241 entries, CTR-treated mice made 50.989 ± 3.241 entries, and AB-treated mice made 51.411 ± 3.241 entries into the center zone. Additionally, there was a difference in center entries between VD and VAB (54.167 ± 3.427 entries) treated mice, as well as VF (58.422 ± 3.241 entries) and VFAB (46.556 ± 3.241) groups. Taken together, administration of an antibiotic cocktail decreased the number of entries into the center of the OFT in mice supplemented with additional VD.

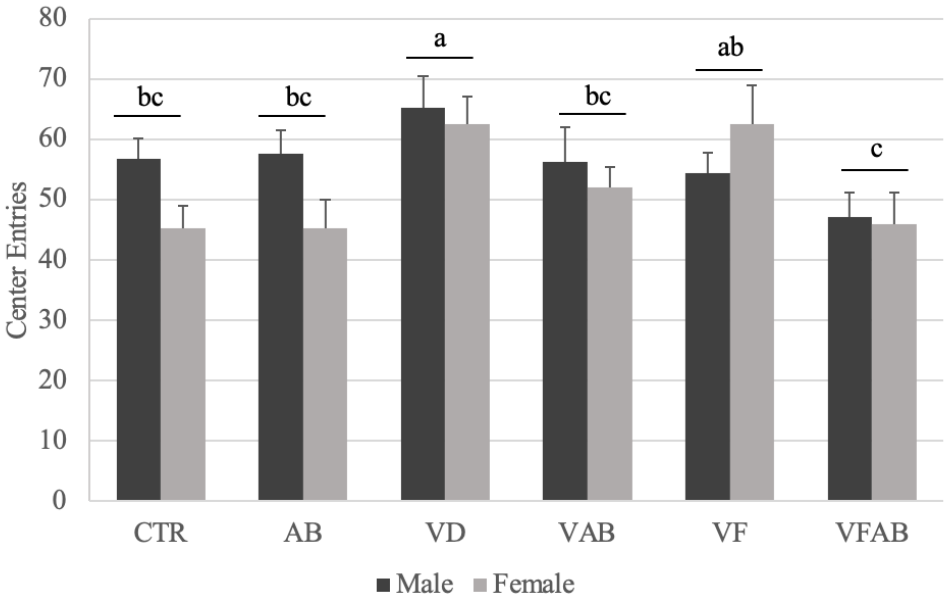

Figure 8. The number of entries into the center during the open field test in adult male and female C57BL/6J mice. Different letters indicate statistical differences between diets at $p < 0.05$. Data are expressed as the mean ± SEM (n = 8 – 10/group/sex) observed over a 10-minute period. CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

We compared treatment groups within each sex and found no difference in male mice, as assessed by one-way ANOVA ($p = 0.129$). However, there was a difference between treatment within female mice, $F(5,49) = 2.929$, $p = 0.022$. VD and VF-treated female mice made more entries into the center of the field compared to CTR. Moreover, VFAB-treated female mice made fewer entries into the center compared to VF mice of the same sex ($p = 0.019$).

Rearing behavior, in which rodents briefly stand on their hind legs, is an exploratory behavior in the OFT. There was a treatment effect, $F(5,100) = 2.888$, $p = 0.018$, and sex effect, $F(1,100) = 10.971$, $p = 0.001$ **(Fig. 9A)**. It was revealed that all groups, with the exception of VF, spent more time rearing compared to VFAB. Further, male mice spent more time rearing compared to females. There was also a significant sex

effect on stereotypy, F (1,100) = 58.872, p = < 0.001 **(Fig. 9B)**. Specifically, male mice spent more time in stereotypic behaviors than females.

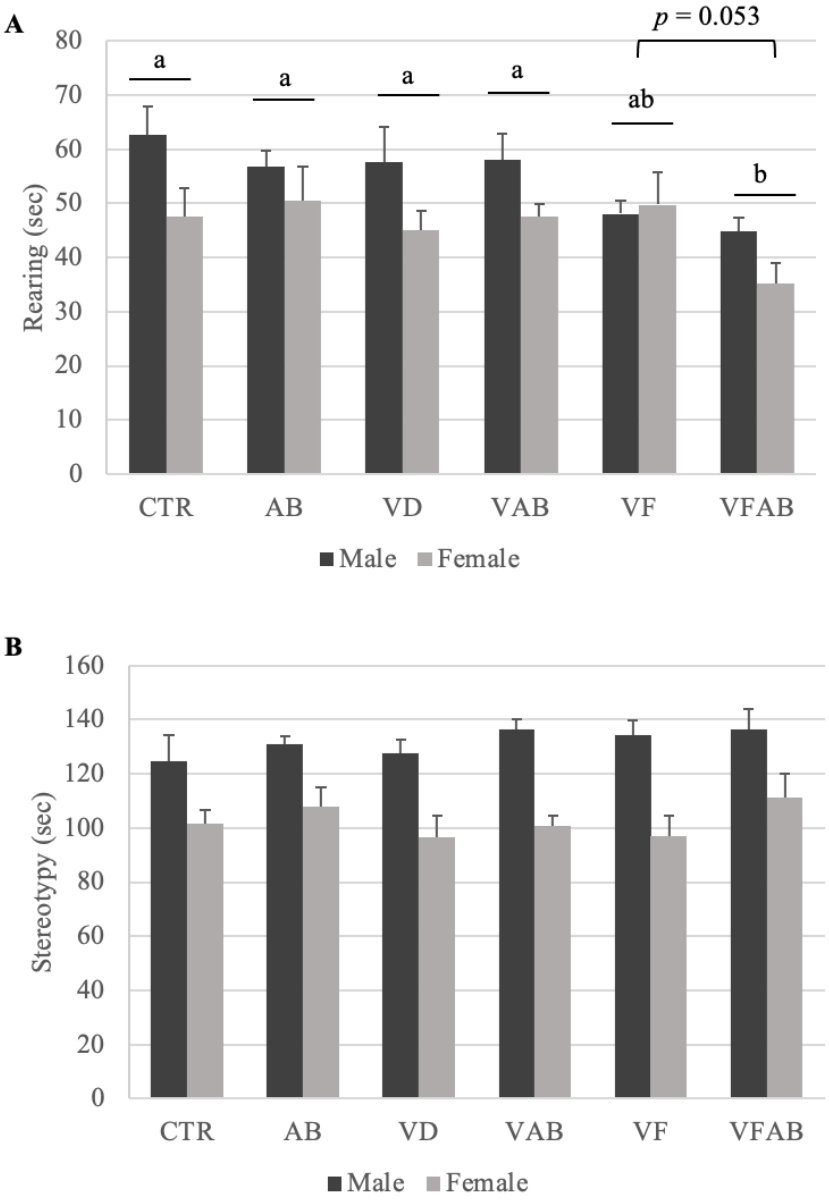

Figure 9. Time (seconds) spent rearing (A) and in stereotypic behavior (B) during the open field test in adult male and female C57BL/6J mice. Different letters indicate statistical differences between diets at $p < 0.05$. Data are expressed as the mean ± SEM (n = 8 – 10/group/sex) observed over a 10-minute period. CTR, control; AB, antibiotics; VD, vitamin D_3; VAB, vitamin D_3 + antibiotics; VF, vitamin D_3 + fiber; VFAB, vitamin D_3 + antibiotics + fiber.

V. DISCUSSION

The present study was designed to determine the role of gut microbiota in regulating VD metabolism and behaviors related to depression and anxiety. In contrast to our hypothesis that depletion of gut microbiota would disrupt VD metabolism, our results suggest that VD status, as indicated by serum concentrations of circulating 25D, is not impacted by antibiotic-induced microbiota depletion. Further, the AB regimen did not have an impact on depression and anxiety-like behavior, as evidenced by immobility time in the FST and TST, as well as center time in the OFT (respectively). However, VF female mice exhibited a decrease in anxiety-like behavior, as indicated by increased center time of the OFT, compared to male mice of the same treatment. Similar observations were detected in circulating serum 25D and serotonin levels between sex in this treatment group.

In our study, regardless of host microbiome status, the additional 4000 IU/kg of VD_3 raised serum 25D levels. However, the normalization of intake by body weight is necessary, and will be calculated, in order to determine whether VD at this dose is an effective supplementation regimen for this animal model. In contrast with our circulating 25D finding, Bora et al.[103] demonstrated the effect of microbes on 25D and 1,25D metabolism using germ-free mice. Microbial transplantation in these mice restored 25D and 1,25D levels, as well as calcium homeostasis. Study design (i.e., antibiotic cocktail vs. germ-free mice) likely plays a role in the difference of results, as some microbes and microorganisms are still present in antibiotic-treated rodent models.[104] Additionally, mode of antibiotic delivery (i.e., gavage vs. drinking water) and antibiotic mixture have been shown to impact host microbiota, which can influence study results.[99,105]

Ongoing research suggests that dietary fibers play a crucial role in the gut microbiome to improve overall health, including mood and cognition.[106,107] In our study, female mice supplemented with FOS had significantly higher serum 25D levels, regardless of host microbiome status (i.e., VF and VFAB-treated groups), compared to males of the same treatment. Although 25D levels between female mice fed VD and VF did not differ significantly, this begs the question whether FOS supplementation benefits females more than males. Some evidence highlights the potential sex difference in microbial fermentation and composition.[108,109] However, what effect that has on VD metabolism, if any, remains unclear.

Consistent with serum 25D results, urine concentrations of 25D did not differ between CTR and AB treatment groups, which further suggests that the disruption of gut microbiota does not augment VD loss. As expected, supplementation with additional VD led to greater concentrations of 25D in the urine. Although we expected FOS supplementation to prevent loss of 25D through the urine, we did not observe a difference between VAB and VFAB groups. Conversely, supplementation with dietary resistant starch, a fermentable fiber, has been shown to maintain kidney health and circumvent excessive excretion of 25D in the urine using a chronic disease animal model, specifically the Zucker diabetic obese rat.[71] In our study, though the difference was not significant, mice treated with VF did excrete about 22% less 25D than mice given VD alone, suggesting dietary fiber may maintain 25D levels in an optimal microbial environment. Interestingly, males treated with VD excreted significantly more 25D compared to females within the same treatment group. This is reflected in circulating serum 25D, as female VD-treated mice had higher 25D concentrations compared to VD-treated males.

Preliminary data from our lab suggests that male mice in this experiment, compared to the opposite sex, had higher visceral fat content, which may have contributed to lower serum and higher urinary concentrations of 25D, although further statistical analyses are needed.

The majority of serotonin in the body is synthesized in the gut from tryptophan. Moreover, microbial species have been shown to stimulate TPH1 and the release of serotonin from enterochromaffin cells through the production of SCFAs.[110] In the central nervous system, research suggests VD may play a role in the regulation of serotonin, specifically TPH2, to improve symptoms of depression.[76-78] Although we expected to find lower circulating serotonin levels in AB treated mice (relative to CTR), that was not the case. Additionally, VF and VAB groups had higher serotonin levels, compared to VFAB. When comparing treatment and sex interactions, male mice treated with AB and VAB had higher serotonin levels than females of the same treatment. Conversely, female mice fed VF had significantly higher serotonin, compared to both males in the same treatment and female mice of all other groups. Although VF female mice exhibited a decrease in anxiety-like behavior, as evidenced by the OFT, hippocampal serotonin is a more indicative measurement associated with mood disorders.

Independently, VD and the gut microbiome have been shown to play a role in brain physiology, including mental health disorders. Despite the strong association between VD insufficiency and mental health disorders in humans, the current body of literature largely fails to link depressive and anxious behavior in rodents fed a VD deficient diet.[42-45] However, when rodents endure chronic stress for several weeks and/or a surgical procedure to induce emotional distress, VD supplementation has been shown to

mitigate behavior related to depression and anxiety.[39-41,50-52] In our study, differences in depressive-like behavior (i.e., immobility) were not observed among treatment groups in the FST and TST. These findings are consistent with previous studies that did not include an unpredictable chronic mild stress (UCMS) protocol. In addition to inducing depression-like behavior, chronic stress has repeatedly been shown to agitate bacteria in the gut.[62,64-66] Our initial intention of this study was to focus on VD metabolism and its relation to the gut microbiome, thus, we ruled out incorporating an UCMS protocol to prevent a negative influence on the microbiome of mice treated with VD or VF (without antibiotics).

Regarding anxiety-like behavior (i.e., center time) in the OFT, a significant interaction between treatment and sex was shown. Compared to female mice within the same treatment, CTR and AB treated males spent significantly more time in the center of the field. Conversely, females treated with VF spent significantly more time in the center, compared to their male counterparts. This observation mirrored circulating serum 25D levels between female and male mice fed VF. Within male mice, CTR and AB treatment significantly increased center time, compared to VF and VFAB. Supplementation with VD alone also increased center time in males, compared to males given VFAB. In contrast, female mice fed VD and VF spent more time in the center than females in the CTR and AB groups. Although statistically non-significant, VF females spent more time in the center of the field compared to VD and VFAB, which suggests supplementation with FOS in an undisrupted microbiome may contribute to the alleviation of anxiety-like behavior in females. Further, female mice treated with VD and VF made more entries into the center of the field compared to CTR, AB, and VFAB mice.

Strengths and Limitations

The strengths of this study include the use of both male *and* female mice, as biological sex has been shown to impact VD metabolism and gut bacteria, as well as influence behavior. All mice were repeatedly handled throughout the study, minimizing any stress associated with handling during sample collection and behavioral testing. Behavioral testing was conducted by the same (blinded to treatment) individual. This is an important factor to consider, as different experimenters can influence behavior in rodents, largely through the olfactory system.[111] Further, both the FST and TST were scored for immobility by an observer blinded to treatment, prior to decoding for analyses. Our study also had several limitations. That is, several factors (i.e., rodent strain, age, body weight) have been shown to influence behavioral testing results, while C57BL/6J mice are less likely to exhibit changes in behavior.[38,112] Our extensive literature review highlights the importance of exposing rodents to an UCMS protocol in order to induce behavioral despair. This protocol has been revised specifically for C57BL/6J mice, which have been shown to be resilient to depression-like behavior.[112]

VI. CONCLUSION

VD insufficiency and mental illness continue to contribute to the overall global burden of disease. As researchers continue to investigate the role of the gut microbiome in host health, it's becoming increasingly clear that gut bacteria serve as a key player in mental health disorders, including depression and anxiety. In this current study, we hypothesized that antibiotic-depletion of gut microbiota would disrupt host VD status, or enhance urinary excretion of 25D, and ultimately lead to behaviors related to depression and anxiety in adult male and female mice. Between CTR and AB-treated mice, we found no difference in serum or urinary 25D concentrations. While supplementation with FOS prevented loss of 25D in an optimal microbiome environment (i.e., VD vs. VF), this difference was not significant. Female mice supplemented with FOS, with and without antibiotic treatment, had higher circulating serum 25D compared to their male counterparts. This leads us to believe that sex differences in the gut microbiome, including the efficacy of dietary fiber supplementation, warrants further investigation. With respect to behavior, immobility time (i.e., depression-related behavior) in the FST and TST did not differ across all groups. However, a significant interaction between treatment and sex in center time (i.e., anxiety-related behavior) in the OFT was revealed. Specifically, VF-treated females experienced a significant decrease in anxiety-like behavior (i.e., increased center time), compared to their male counterparts. A similar observation was detected between sex in circulating serum 25D and serotonin levels of mice fed VF. Including an UCMS protocol, particularly in rodent strains that have been shown to be resilient to behavioral changes like the C57BL/6J mouse, is a critical component to mirroring human disease and has been shown to influence study results.

Thus, future researchers with the goal of examining behavior should design a study that accounts for these factors.

Future Research

Results from the current experiment will expand to determine whether consumption of VD, with and without antibiotic treatment, has an impact on the composition of the gut microbiome. Previous research regarding the effect of gut microbiota on VD metabolism remains limited, however, several preclinical studies have found that supplementation with VD has an impact on the gut microbiome.[68-70] Moreover, VD deficiency through the manipulation of diet, as well as *Cyp27b1* and VD receptor $(VDR)^{-/-}$ mice, has been shown to increase the growth of *Bacteroidetes* and *Proteobacteria*.[113] Further, our lab will assess changes in mRNA expression of *VDR*, *Cyp27b1*, and *Cyp24a1*, both locally (colon) and in the kidney. We will also determine whether the environment of the gut has an impact on the intestinal serotonergic system by measuring mRNA expression and protein levels of TPH1 and serotonin transporter (SERT).

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