Milk Fat Globule Membrane Enhancement of Neurotransmitter Synthesis from Lactic Acid Bacteria

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Abstract

Neurotransmitters like serotonin, dopamine, and gamma-amino butyric acid (GABA), are communication molecules known to improve mood and mental health. They can be produced in the gut and send signals to the brain via the gut-brain axis. This bidirectional link involves major body systems that influence physiological responses to the environment. To complement the host's neurotransmitter production, probiotic lactic acid bacteria (LAB) also produce neurotransmitters. These bacteria are present in dairy foods along with the milk fat globule membrane (MFGM) which is a bioactive component of milk that influences brain and gut health. We hypothesized that MFGM can enhance neurotransmitter production from LAB. Therefore, the objectives of this work were to characterize neurotransmitter production of selected LAB and determine the influence of MFGM on production. Four LAB strains (*Pediococcus acidilactici, Lactobacillus helveticus, Limosilactobacillus reuteri, and Lacticaseibacillus rhamnosus*) were selected based on screening of probiotic characteristics and/or possession of neurotransmitter synthesis genes. All strains were cultured overnight before incubation with amino acid precursors (L-Trp, L-Phe, or L-Glu) for each neurotransmitter and MFGM. For serotonin and dopamine, cell-free supernatants were partially purified using a C_{18} column followed by vacuum evaporation and water resuspension. Serotonin was measured using Ehrlich's reagent at A_{625}. Dopamine was quantified using ferric chloride and potassium hexacyanoferrate at A_{735}. For GABA, cell-free supernatants were concentrated using vacuum evaporation before mixing with GABase solution to measure absorbance at 340nm. Serotonin and dopamine were both produced by all strains, while GABA was not detected by any strain. Serotonin production was enhanced up to 5-fold with MFGM and dopamine was unchanged by MFGM. These findings lay the foundation to further explore how dairy foods containing probiotics influence human health.

Introduction

Neurotransmitters impact brain sensations and gastrointestinal tract (GIT) function through bidirectional communication known as the gut-brain axis. This communication between the central nervous system (CNS) and the enteric nervous system of the GIT via neurotransmitters impacts many body systems and reactions, including diseases and overall health. Responses to nutrients, medications, microbiota, and emotions all impact neurotransmitter levels and therefore body communication (Mittal et al. 2017). The gut-brain axis helps maintain gut homeostasis, proper digestion, normal brain function, and emotional behaviors (Suganya and Koo 2020). Furthermore, this communication allows brain signals to directly influence the gut physiology and immune response while the gut sensory signals travel through the vagus nerve (the largest cranial nerve responsible for brain and organ communication) to the CNS (Suganya and Koo 2020). This is important for communication between the endocrine, immune, and nervous systems with the gut microbiota (Yong et al. 2020). However, neurological disorders cause changes in the gut-brain axis relationship which may result in gut-brain disorders and dysbiosis (Suganya and Koo 2020). Gut dysbiosis can also be a cause for neurological disorders as beneficial bacteria presence is reduced (Yong et al. 2020). When the gut microbiota is in dysbiosis this impacts the CNS, the gut barrier integrity, and the gut-brain axis (Suganya and Koo 2020). Probiotic bacteria can influence the gut-brain axis by improving the gut microbiota which will decrease inflammation and restore homeostasis to then improve cognitive disorders and disruptions like anxiety and depression, and improve any cognitive impairment (Yong et al. 2020; Suganya and Koo 2020). Therefore, ingestion of probiotics can be beneficial for improving the gut-brain relationship.

Neurotransmitters are an essential part of the gut-brain axis. Neurotransmitters are chemical signals between neural synapses that modulate many systems in the body including immune response, digestion, nervous system reactions, and muscle movements. They also have a significant impact on the GIT enteric nervous system (Mittal et al. 2017). These signals are important for communication throughout the body to maintain homeostasis. Three neurotransmitters of high interest are serotonin, dopamine, and gamma-amino butyric acid (GABA), as they have a critical impact on both physiology and emotions.

Serotonin is important in the stress response and mood regulation regions of the brain, including aggression and impulsiveness (Daut and Fonken 2019; Danilovich et al. 2021). It impacts satiety, hunger, eating disorders, and psychiatric disorders, which can contribute to obesity control and reducing inflammation because serotonin receptors can be found on immune cells (Kałużna-Czaplińska et al. 2019; Danilovich et al. 2021). 95% of serotonin is produced by enterochromaffin cells and enteric nerves within the GIT. This is important as many serotonin receptors reside in the GIT and relay various responses to mitigate gut distress including regulation of pathogen colonization, metabolism control, and irritable bowel syndrome (Mittal et al. 2017; Danilovich et al. 2021).

Dopamine receptors are found on intestinal nerve endings and in the mucosal layer (Mittal et al. 2017). Anticipation and the reward response is regulated by dopamine level. Stimulation from these receptors in the gut and activation of the reward pathway in the brain leads to the feeling of pleasure (Mittal et al. 2017; Webber et al. 2021). An example of this is caloric intake control; when dopamine is imparied, this can cause excess calorie intake and obesity, versus the anticipation and reward pathways associated with dopamine release following nutrient stimuli better control food intake (de Araujo et al. 2012). Dopamine greatly effects

cognitive motivation and effort, as dopamine levels decrease so does motivation (McGuigan et al. 2019). An example of this is Parkinson's Disease which is an autonomic motivational disorder where dopamine is low thus impairing motivation (McGuigan et al. 2019).

GABA is the main inhibitory neurotransmitter. Dysfunctions of the GABA signaling pathway include mental illnesses like anxiety and depression (Mittal et al. 2017). GABA can cross the blood-brain-barrier and inhibit certain stress receptors in the brain; it is most notably responsible for promoting relaxation and neural development through inhibition of stress, depression, sleepiness, neural degeneration, and organ damage (Ngo and Vo 2019; Yong et al. 2020). This is important to prevent neurodegenerative diseases, neural cell death, and improve cognitive function and memory (Ngo and Vo 2019). GABA signaling in the GIT contributes greatly to gut motility and inflammation as well (Mittal et al. 2017).

Bacteria, specifically probiotics, present in the intestine play a role in the gut-brain relationship. Probiotics are live microorganisms that provide a benefit to the host when given or ingested in an adequate amount (Suganya and Koo 2020). Probiotic bacteria possess functional properties including adhesion to gut mucosa, maintenance of the gut epithelial barrier, alteration of the gut ecology, and modulation of the immune system via the intestine (Rocha-Mendoza et al. 2020). Probiotics can survive the harsh GIT environments and be viable in the intestine where their beneficial functions are active. Probiotics present in foods can influence the gut-brain axis as responses to nutrient absorption occur. Many fermented, or cultured, foods contain probiotic bacteria including dairy products like yogurt, kefir, and buttermilk. Certain lactic acid bacteria (LAB) strains from dairy products have also demonstrated probiotic characteristics and the ability to produce neuroactive compounds, which will contribute greatly to gut-brain communication (Oleskin et al. 2014). An increased presence of neuroactive compounds in the

gut can interact with the CNS and impact human physiology. Since many of these LAB are nutritionally fastidious, they grow well in complete nutrient sources like dairy foods. Therefore, it is significant to use bioactive dairy component supplementation to further understand the impacts on neurotransmitter production.

It is known that fermented dairy products contain bacterial metabolites that may serve as bioactive food components. However, dairy products alone also contain significant compounds like the milk fat globule membrane (MFGM) which is made up of various proteins, lipids, and glycoproteins. The MFGM is naturally found in mammalian milk and is a phospholipid and protein tri-layer membrane that surrounds a triacyl glyceride dense globule. The tri-layer membrane contains essential phospholipids needed for infant development including choline, sphingomyelin, gangliosides, cholesterol, and the precursor to polysialic acid; these components contribute to neural cell adhesion, dendritic cell development and protection, establishment of synapse connections, metabolism, and microbial colonization in the gut (Berding et al. 2016; Brink and Lönnerdal 2020). The MFGM plays a significant role in establishing gut microbiota, immune system responses, metabolic disease prevention, along with neural development and cognitive abilities (Brink and Lönnerdal 2020). The MFGM is a bioactive food component that can work in tandem with LAB common in dairy products to influence the gut-brain axis.

The strains of LAB chosen for this research are part of the Parker endowed chair bacterial collection in the Food Science and Technology Department at The Ohio State University and were isolated from dairy foods. Our preliminary studies in combination with our previous research suggests that four strains (*Pediococcus acidilactici* OSU-PECh 3A (3A), *Lactobacillus helveticus* OSU-PECh 4A (4A), *Limosilactobacillus reuteri* OSU-PECh 48 (48), *Lacicaseibacillus rhamnosus* OSU-PECh 69 (69)) possess potential probiotic characteristics

including intestinal cell adhesion, viability through high acid and salt environments, may produce antimicrobial compounds, and has enhanced growth with MFGM supplementation (García-Cano et al. 2019; Rocha-Mendoza et al. 2020). After bioinformatic analysis, strains 3A and 4A also showed genes for production of serotonin, dopamine, and GABA. The linkage of the MFGM and probiotics to beneficial neurotransmitter production will strengthen the bridge between dairy products and health.

Hypothesis

The supplementation of MFGM in bacterial culture media will enhance the neurotransmitter production from *Pediococcus acidilactici* OSU-PECh 3A (3A), *Lactobacillus helveticus* OSU-PECh 4A (4A), *Limosilactobacillus reuteri* OSU-PECh 48 (48), *Lacicaseibacillus rhamnosus* OSU-PECh 69 (69) lactic acid bacteria strains.

Materials and Methods

Bacterial and medium preparations

The use of casein glucose broth (CGB) media was chosen due to its low glucose concentration which enables reduction of background interference during spectrophotometric measurements. CGB was prepared from 0.5% yeast extract (Sigma, St. Louis, MO), 2% bactotryptone (Fisher Scientific, Watham, MA), 1% glucose (Sigma), 0.005% manganese sulfate (Sigma), 0.2% ammonium citrate (Sigma), 0.2% disodium phosphate, 0.01% magnesium sulfate (Sigma), 0.1% Tween-80 (Sigma), and diH_2O to reach 500 mL and autoclaved to sterilize. To prepare CGB with 0.5% MFGM supplementation, various samples of Liprogen® ingredient were tested for protein and phospholipid composition before deciding which sample was best to use for supplementation. This was to ensure the presence of known MFGM proteins and phospholipids

in the ingredient. Liprogen® MFGM powder (JLS Foods International, Schaumburg, IL, USA) was added to sterile phosphate-buffered saline (PBS) solution to make a 10% MFGM solution. MFGM cannot be autoclaved because the temperature will denature the proteins, so the solution was batch pasteurized at 65°C for 45 min using a water bath to eliminate any contaminating bacteria. Two samples were incubated at 37°C overnight to confirm sterility by plating and media turbidity in a culture tube. 25-mL of the sterilized 10% MFGM-PBS solution was added to 500 mL of sterile CGB to yield a 0.5% MFGM-CGB media. 0.5% MFGM was chosen because according to Rocha-Mendoza et al. (2020) 0.5% milk phospholipid supplementation allows for the best bacterial growth adaptation. Preparation of bacteria involved thawing of frozen stocks followed by inoculation of 3 mL CGB with 20 µL frozen stock for all strains *P. acidilactici* (4A), *L. helveticus* (3A), *L. reuteri* (48), and *L. rhamnosus* (69); incubated at 37°C overnight. 100 µL of each strain's culture was then sub-cultured in 5mL of CGB for experimentation.

Sample Preparation for Serotonin and Dopamine Analysis

All strains were grown in CGB and sub-cultured at pH 7 with the addition of 0 mM, 3.3 mM, 6.6 mM, 13.2 mM, 19.8 mM, 26.4 mM, and 33 mM L-tryptophan (Sigma) from a 1mg/30mL stock solution for serotonin. Tryptophan is the precursor to serotonin, but it does not dissolve in water. Sodium hydroxide was used to help dissolve L-tryptophan which altered the pH, so when L-tryptophan was added to the culture tube, the pH was adjusted to 7 to optimize the bacterial growth conditions. Those same concentrations of L-phenylalanine (Sigma) were used from a 1mg/30mL stock solution for dopamine analysis. However, tyrosine is the precursor to dopamine, but tyrosine is ionizable, does not dissolve well in water, and required sodium hydroxide to aid solvation. When the sub-culture tube pH was adjusted after addition of tyrosine, the amino acid precipitated; therefore, L-phenylalanine was used instead, as phenylalanine can

form tyrosine. L-phenylalanine still required sodium hydroxide to dissolve in water; therefore, sub-culture tube media needed the pH adjusted to optimize growth conditions. The amino acid supplemented tubes were incubated overnight at 37°C. Samples were treated according to Kato et al. (2007) as follows: samples underwent centrifugation for 10 minutes at 8000rpm, 0.22 µm filtration with Nalgene™ syringe filters (Thermo Scientific, Waltham, MA) of the supernatant, addition of 0.2 g sodium tetraborate (Sigma) to 0.5 mL of sample and 0.5 mL of water, a second centrifugation for 10 minutes at 3000rpm, removal of supernatant for serotonin and dopamine isolation using C_{18} column (Waters, Milford, MA). The samples in the columns were washed with 10mL diH_2O followed by 1mL methanol for sample elution. Elutant was then evaporated using a vacufuge (Eppendorf, Framingham, MA) at room temperature until dry. It was decided for both dopamine and serotonin analysis to use C_{18} silica cartridges and evaporation to better isolate the compounds.

Optimization of L-Tryptophan Precursor for Serotonin Production

The dried sample from the sample preparation was then resuspended in 300 µL of water and 200 µL of sample was added to 500 µL Ehlich's reagent (Sigma) and held in a heat block at 50°C for 30 minutes according to Jin et al. (2008). Subsequently, 300 µL of 0.1 M HCl was used to stop the reaction and samples transferred to a 96-well plate (Thomas Scientific, Swedesboro, NJ) and read at 625 nm on using a Multiskan Go plate reader (Fisher Scientific). All samples were measured in triplicate to determine the optimal amino acid concentration for future experiments. Neurotransmitter quantification (average ± standard deviation) was determined using a standard curve -prepared from a stock solution of serotonin (Sigma).

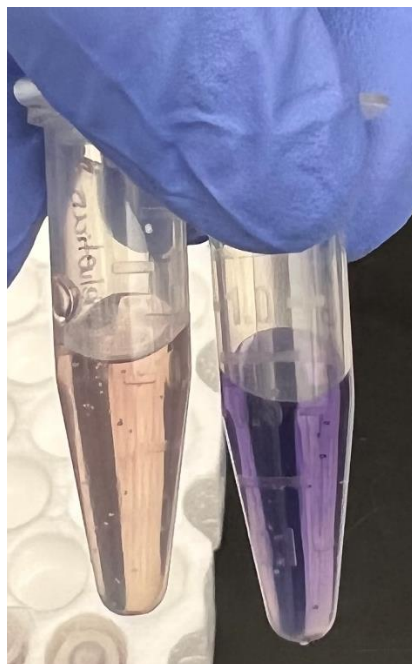

Figure 1: *Reaction of Ehrlich's reagent with water (left) and serotonin (right)*

Optimization of L-Phenylalanine Precursor for Dopamine Production

Following vacufuge treatment for sample preparation, the dried sample was resuspended in 300 µL of water and 250µL of sample was combined with 250µL of 0.03M ferric chloride (Sigma) and 250µL of 0.0081M potassium hexacyanoferrate (Sigma) according to Mahood and Hamzah (2010). Aqueous ferric chloride was prepared on the day of analysis, as it would precipitate out of solution when prepared prior. The sample mixture was incubated at room temperature for 15 minutes before being transferred to a 96 well plate and read on a spectrophotometer at 735nm. Samples were measured in triplicate. Neurotransmitter quantification (average ± standard deviation) was determined using a standard curve -prepared from stock a solution of dopamine (Sigma). The optimal amino acid concentration was determined for future experiments.

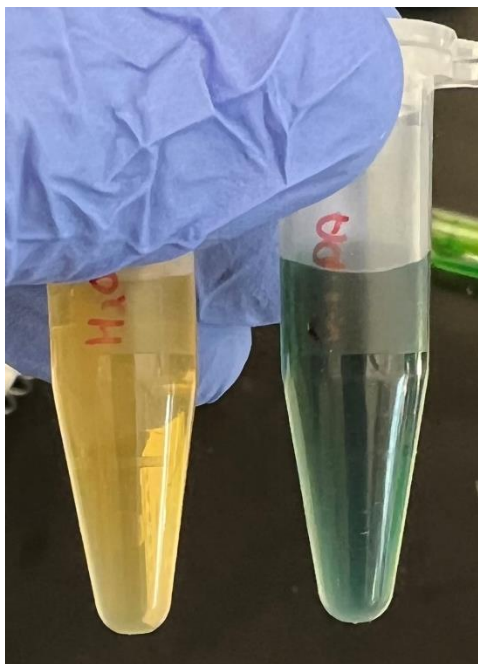

Figure 2: *Reaction of ferric chloride and potassium hexacyanoferrate with water (left) and dopamine (right)*

Gamma-aminobutyric acid

To determine if the bacteria possess glutamic acid decarboxylase (GAD) enzyme, a colorimetric assay was performed following Priya and Prigalya (2018). All strains were grown in CGB media followed by centrifugation and saline wash. The pellet was then resuspended in 0.5mL GAD reagent solution: 0.1g L-Glutamic acid (Sigma), 0.03mL Triton X-100 (Sigma), 9g of NaCl solution, 0.005g of bromocresol green (sigma) in RO water, and water to reach 100mL held at a pH of 4. The tubes were then incubated at 37°C for four hours. If the color changed from yellow to green or blue, it indicated a low or high enzyme activity respectfully.

To measure GABA production, all strains were sub-cultured with 1% L-Glutamate (Sigma) from a stock concentration of 1mg/10mL and incubated at 37°C overnight. Samples were centrifuged and the supernatant was filtered using 0.22µm Nalgene™ syringe filters, followed by

concentration using a vacufuge at room temperature. Analysis was performed following Tsukatani et al. (2005): 90µL of the GABase enzyme solution –750mM sodium sulfate (Sigma), 10mM dithiothreitol (Sigma), 80mM tris-HCl buffer at pH: 9 (Sigma), 1.4mM NADP+ (Sigma), 2.0mM alpha-ketogluterate (Sigma), 3.33mg/10mL of GABase (containing GABA-T and SSDH enzymes) (Sigma), and water to reach 10mL—was added to each well on a 96 well plate. 10µL of sample was added to the GABase solution in the plate. As a blank, a GABase sample containing no GABase was used and added to the plate at 100µL volume. The plate was incubated at room temperature for one hour before being read on a spectrophotometer at 340nm. Samples were measured in duplicate. Neurotransmitter quantification (average ± standard deviation) was determined using a standard curve -prepared from a stock solution GABA (Sigma).

MFGM Supplementation

Following optimization of L-Tryptophan and L-Phenylalanine concentration for 4A, 3A, 48, and 69 bacterial strains to determine the concentration which provided the greatest neurotransmitter production without addition of MFGM, preparation of strains from frozen stocks was performed as previously mentioned. After initial incubation, all strains were sub-cultured with supplementation of optimal L-Tryptaphan concentration and 0.5% MFGM at pH 7 for serotonin analysis, optimal L-Phenylalanine concentration and 0.5% MFGM at pH 7 for dopamine analysis, and 1% L-Glutamine and 0.5% MFGM at pH 7 for GABA analysis. All samples underwent the same preparation and spectrophotometric assays for the respective neurotransmitter quantification as mentioned above to elucidate the impact of MFGM on neurotransmitter production.

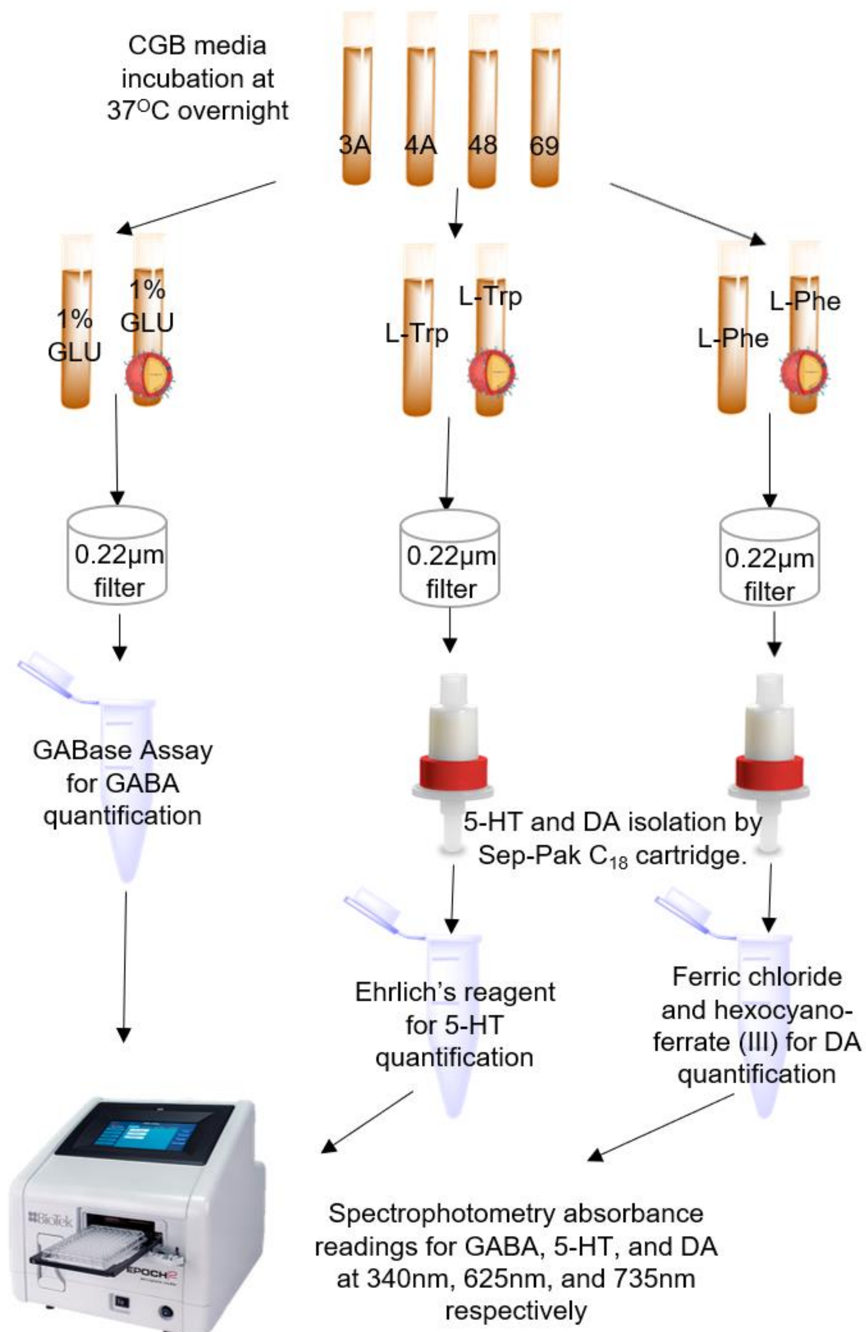

Figure 3: *Laboratory protocol for serotonin (5-HT), dopamine (DA), and gamma-amino butyric acid (GABA) characterization and quantification*

Statistical Analysis

A one-way ANOVA test was performed using SPSS and GraphPad Prism V9.5 to determine significance.

Results and Discussion

Serotonin

Serotonin was produced by all four LAB strains and production was significantly enhanced with MFGM supplementation. Strains 3A and 69 had the greatest fold increase in serotonin production with MFGM; 3A demonstrated a 3.5-fold increase and 69 showed a 5-fold increase. 4A had the greatest production of serotonin both with and without MFGM addition, but the lowest fold change. All strains produced serotonin between 0.03-0.2 mg/mL this is below the levels previously detected from a select dairy culture starter strain of *Lactobacillus helveticus* by Oleskin et al. (2014). This analysis saw enhanced serotonin production when compared to milk using a milk hydrolysate-based medium that was supplemented with hydrolysates of casein like the use of bactotryptone and L-tryptophan in CGB. These results demonstrate the bacterial use of milk components and the serotonin amino acid precursor to stimulate production of detectable amounts of serotonin as supported by figure 4. As serotonin is an important neurochemical produced in the gut, this can significantly impact the gut-brain access' ability to regulate mood and mental health. This supports the hypothesis that MFGM enhances the production of select neurotransmitters from these bacteria.

The use of Ehrlich's reagent to measure serotonin is significant for detection of indole compounds. Ehrlich's reagent contains p-dimethylaminobenzaldehyde (DMAB) which is used to indicate the presence of indoles through complexing reactions. As serotonin is an indole DMAB

will react with it Lamb et al. (2015); however, it may not be selective to detect only serotonin but other indoles present in the sample as well. Even so, there was still a significant increase in detection after the addition of MFGM.

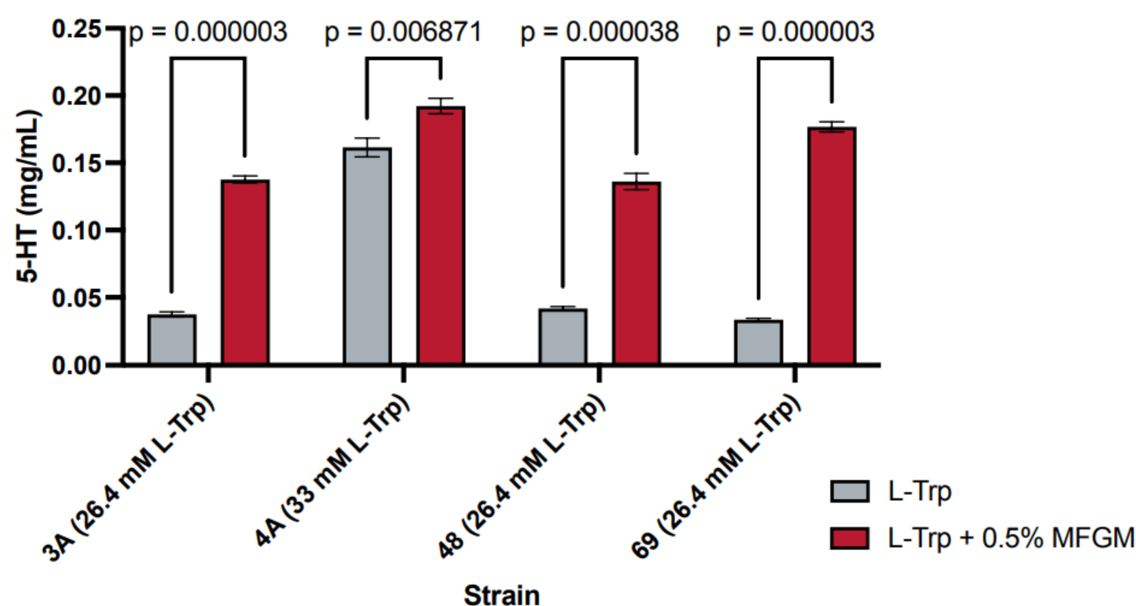

Figure 4: *5-HT production from each strain was significantly different between samples with and without MFGM supplementation.*

Dopamine

Dopamine was produced in a greater amount than serotonin with concentrations between 1-3 mg/mL. Production from 3A, 48, and 69 saw a significant decrease with the addition of MFGM to the growth medium. While 4A demonstrated little change in production with MFGM supplementation. Previous work done by Oleskin et al. (2014) measured catecholamines, including dopamine production, from select dairy starter culture strains and found that production increased with the use of a milk medium. This suggests that bacterial use of milk

components can stimulate the production of multiple classes of beneficial neurochemicals and could be a basis for future work to measure not only dopamine, but other catecholamines with MFGM supplementation.

Dopamine was analyzed using ferric chloride in combination with hexacyanoferrate (III). In the dopamine quantification assay, Fe (3+) from ferric chloride is reduced to Fe (2+) by dopamine. Interaction of Fe (2+) with hexacyanoferrate (III) results in a blue color (seen in figure 2) allowing for colorimetric analysis (Mahood and Hamzah 2010).

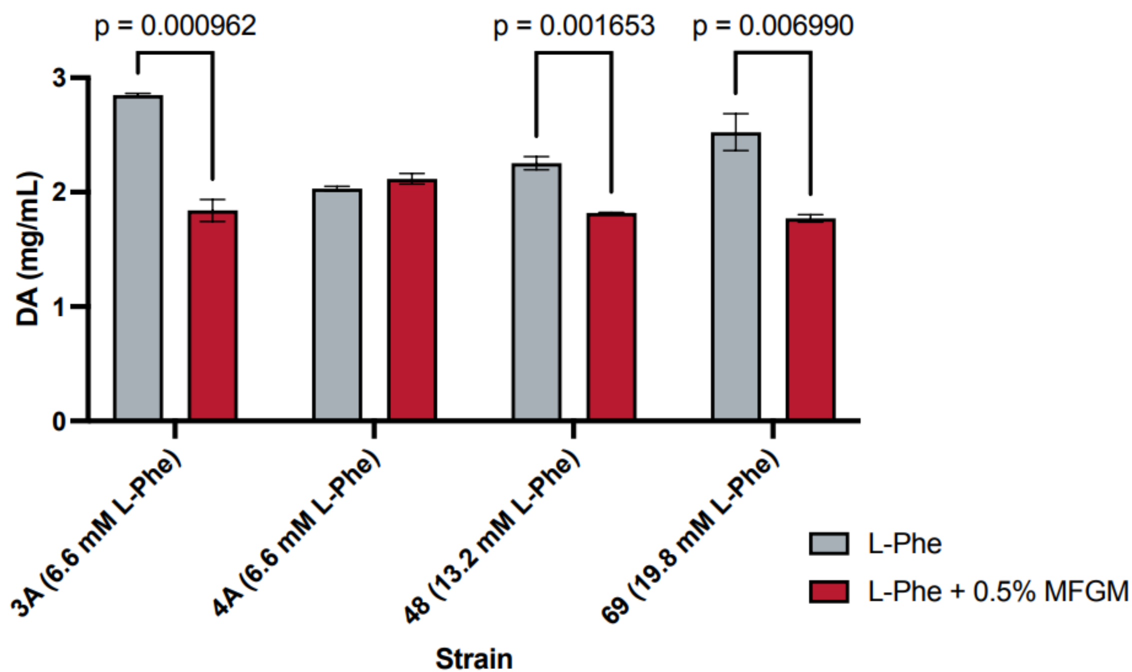

Figure 5: *DA production from 3A, 48, and 69 was significantly different between samples with and without MFGM supplementation.*

Gamma-aminobutyric acid

Results of the GAD assay revealed medium GAD activity from 3A, 4A, 48, and 69 strains as a green/blue color was observed, shown in figure 6.

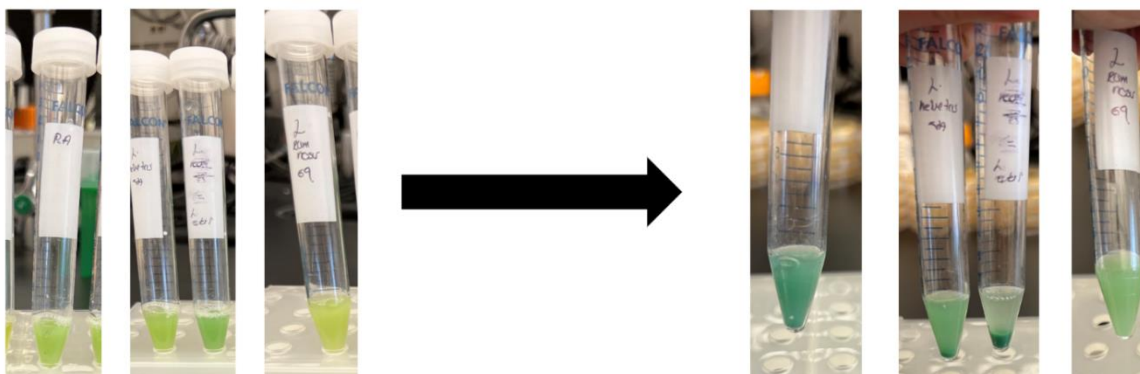

Figure 6: *GAD assay results show medium enzyme activity for all strains.*

GABA was not detected by 3A, 4A, 48, or 69; figure 7 reveals quantification results were below the range of the standard curve. The GABase reagent contained glutamate aminotransferase (GABA-T) and succinic semialdehyde dehydrogenase (SSDH). When GABA is present with α-ketoglutarate, contained in the GABase assay solution, GABA-T will convert these molecules to succinic semialdehyde and glutamate. Succinic semialdehyde in the presence of NADP+ and water, present in the GABase assay solution, will be converted to succinate, NADPH, and H+ via SSDH. The conversion of NADP+ to NADPH causes an absorbance change in the ultraviolet light wavelength region that can then be quantified according to Tsukatani et al. (2005). This method was insufficient to detect GABA by 3A, 4A, 48, and 69 even though the GAD assay results revealed that the bacteria possessed the necessary enzyme for production of GABA.

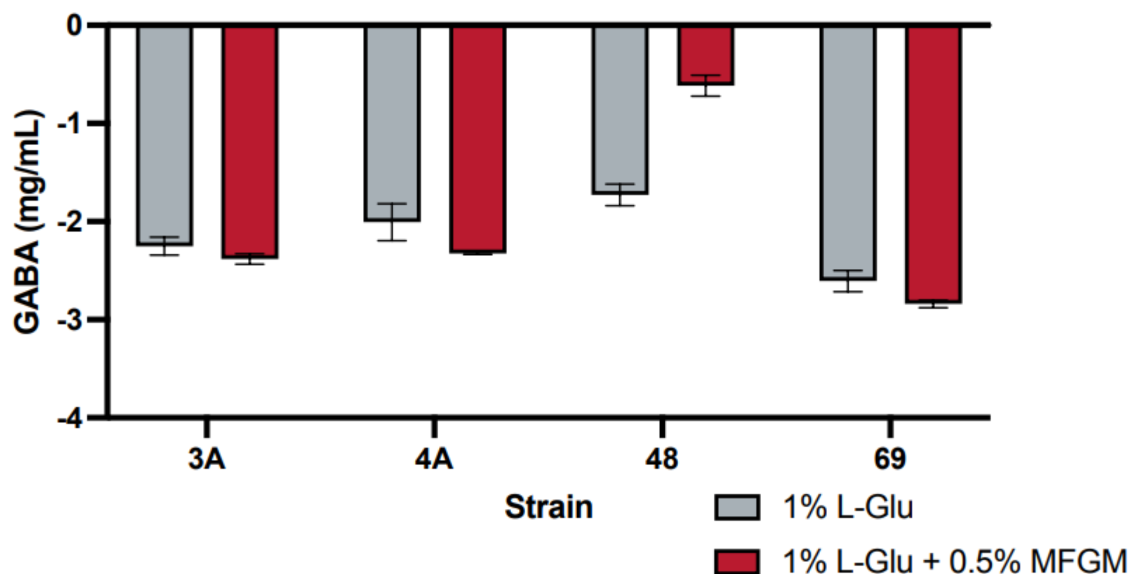

Figure 7: *GABA production was not detected both with and without MFGM supplementation.*

Conclusion

The hypothesis of this work was that MFGM enhances the production of select neurotransmitters from *Pediococcus acidilactici* OSU-PECh 3A, *Lactobacillus helveticus* OSU-PECh 4A, *Limosilactobacillus reuteri* OSU-PECh 48, *Lacicaseibacillus rhamnosus* OSU-PECh 69 lactic acid bacteria strains. The findings complement this with regards to serotonin. Serotonin production was significantly enhanced in all strains up to 5-fold with 0.5% MFGM supplementation. Where *Lactobacillus helveticus* OSU-PECh 4A produced the greatest amount of serotonin with and without MFGM and *Lacicaseibacillus rhamnosus* OSU-PECh 69 had the most significant production increase with MFGM. However, even though dopamine was detected in the greatest amounts relative to the other neurotransmitters amongst all strains, the production decreases with MFGM supplementation. And GABA was not detected from any strain using these methodologies. These results lay the foundation for future research to further

understand how probiotics and MFGM can work in dairy foods as functional ingredients. This can also aid in the understanding of the gut-brain axis and its influence on health.

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