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Dietary Oligosaccharides Modulate Bifidobacterial Production of the Neurotransmitter Gamma-Aminobutyric Acid

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**DIETARY OLIGOSACCHARIDES MODULATE BIFIDOBACTERIAL
PRODUCTION OF THE NEUROTRANSMITTER γ -AMINOBUTYRIC ACID**

A Thesis Presented

by

MICHELLE R. ROZYCKI

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

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Department of Food Science

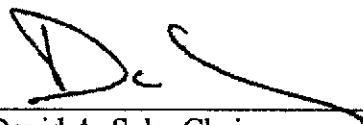
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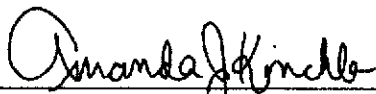
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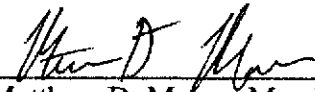
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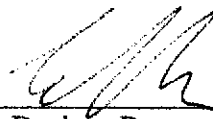
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ABSTRACT

DIETARY OLIGOSACCHARIDES MODULATE BIFIDOBACTERIAL PRODUCTION OF THE NEUROTRANSMITTER γ -AMINOBUTYRIC ACID

SEPTEMBER 2020

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Bifidobacteria are the predominant members of the infant gut, colonize adults to a lesser extent, and are recognized as beneficial microbes. Various bifidobacterial species produce γ -aminobutyric acid (GABA), the chief inhibitory neurotransmitter in the mammalian central nervous system. It is postulated that in order to produce GABA, the bifidobacterial genome must contain the *gadB* and *gadC* genes which encode a glutamate decarboxylase and a glutamate/GABA antiporter, respectively. Once exported by GadC, GABA is absorbed and transported systemically throughout the host. We hypothesize that specific dietary oligosaccharides will modulate bifidobacterial production of GABA due to varying intracellular concentrations of glutamate. To test this, 33 bifidobacterial strains were screened for GABA production via reverse phase HPLC. Interestingly, 10 strains contained both *gadB* and *gadC* genes, but only 8 strains produced detectable GABA *in vitro*. To further elucidate the extrinsic factors influencing GABA production, strains were subjected to different dietary components. Specifically, lactose and the dietary

oligosaccharide FOS were evaluated for the ability to promote biosynthesis of intracellular glutamate and thus potentially GABA. Understanding the relationship between diet, bifidobacterial physiology, and GABA production may inform dietary interventions to modulate this neurotransmitter *in vivo*.

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CHAPTER 1

INTRODUCTION

Bifidobacteria are the predominant members of the infant gut microbiome and are essential for establishing a stable early microbial community. Bifidobacterial colonization of the infant gut is mediated by the fermentation of human milk oligosaccharides (HMOs)¹⁻⁵. Although less abundant in adults, bifidobacteria are an important member of the adult gut microbiome as they ferment host-indigestible oligosaccharides. Host consumption of oligosaccharides, including fructooligosaccharides (FOS), galactooligosaccharides (GOS), and arabinoxylan oligosaccharides (AXOS) have been demonstrated to promote the growth of bifidobacteria *in vivo* and *in vitro*⁶⁻¹¹. Analysis of the *Bifidobacterium* pan-genome revealed 13.7% of *Bifidobacterium*-specific clusters of orthologous genes (BifCOGs) belonged to the carbohydrate metabolism functional family of proteins, which include glycosyl hydrolases (GH), glycosyl transferases (GT) and carbohydrate esterases (CE). Carbohydrate-active enzyme family GH13, enzymes characterized by their ability to degrade plant oligosaccharides, are commonly found in bifidobacteria genomes¹².

The importance of oligosaccharides extends further than increasing bifidobacterial populations, as there is a correlation between consumption of oligosaccharides and exertion of antidepressant and anxiolytic effects on the host¹³⁻¹⁶. Male mice exposed to chronic stress that consumed a combination of FOS+GOS had reduced levels of stress-induced corticosterone, as well as modified short-chain fatty acid (SCFA) concentrations in the gut¹³. GOS was supplemented in a human intervention study with female adolescents over a 4-week period. Reductions in anxiety were reported and microbial analysis of stool

samples revealed an increased abundance of *Bifidobacterium* spp., compared to the placebo group¹⁴. Physiological effects of plant oligosaccharide metabolism could be induced by a variety of bioactive metabolites, including γ -aminobutyric acid (GABA), produced by gut microbiota that interact with the gut-brain axis (GBA). The GBA is a bidirectional connection between the central and enteric nervous system. It has been suggested that molecules produced by gut microbiota, like GABA, have the potential to interact with the GBA, sending signals directly to the brain^{17,18}.

GABA is a bioactive non-protein amino acid and the primary inhibitory neurotransmitter of the central nervous system (CNS). It primarily interacts with GABA_A and GABA_B receptors in the host to induce hyperpolarization, which inhibit action potentials¹⁹⁻²¹. If GABA production decreases and GABA receptors interact with other neuroactive compounds, neurological disorders can arise. GABA pathophysiology has been implicated in anxiety disorders, depression and autism spectrum disorder²²⁻²⁶. GABA is primarily synthesized from glutamate by a glutamate decarboxylase (GAD, EC 4.1.1.15) in animals and bacteria. Gad1 and Gad2 are responsible for GABA synthesis in the brain²⁷ while GadA and/or GadB synthesize GABA in bacteria. Along with a glutamate decarboxylase, bacteria also possess a glutamate/GABA antiporter (GadC) that selectively transports glutamate into the cell and exports GABA²⁸⁻³¹. Some pathogenic bacteria, including *Escherichia coli* and *Listeria monocytogenes*, have adapted *gad* operons as acid resistance (AR) systems, protecting the organism from the acidic environment of the digestive system³²⁻³⁵. *E. coli* possess multiple *gad* genes, as a part of an acid resistance system. They possess *gadA*, *gadB*, *gadC* and *gadE*, the primary regular of the glutamate-mediated acid resistance system³³. GABA-producing AR systems are not exclusive to

pathogens; strains of *Lactobacillus* spp. possess Gad-mediated AR systems as well^{29,36,37}. *Bifidobacterium* spp. production of GABA is not part of an AR system, as they only possess *gadB* and *gadC* genes in their *gad* operon³⁰.

Presence of *gad* genes and production of GABA by *Bifidobacterium* spp. are not inherent to the genus. We identified 6 species of bifidobacteria (*Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium dentium*, *Bifidobacterium merycicum*, *Bifidobacterium moukalabense*, *Bifidobacterium ruminantium*) through NCBI/BLASTp that possess both *gadB* and *gadC* genes. Previous publications report GABA-producing bifidobacteria which include *B. adolescentis*, *B. angulatum*, *B. dentium* and *Bifidobacterium longum subsp. infantis*^{30,38,39}. Moreover, strains of *B. adolescentis*, *B. angulatum*, and *B. dentium* ferment FOS and GOS *in vitro*^{6,10,40,41}. Fermented oligosaccharides are cleaved into monosaccharides (including glucose and galactose) that can be metabolized in the fructose-6-phosphate phosphoketolase pathway (F6PPK). Intermediates of the pathway are proposed to be involved in the biosynthesis of various amino acids, including glutamate⁴². We hypothesize that dietary oligosaccharides will modulate bifidobacterial production of GABA due to varying intracellular concentrations of glutamate. Understanding the relationship between diet, bifidobacterial physiology, and GABA production may inform dietary interventions to modulate this neurotransmitter *in vivo*.

CHAPTER 2

METHODS & MATERIALS

2.1 Bacterial Propagation

37 strains of *Bifidobacterium spp.* and *Lactobacillus spp.* were screened for their ability to produce GABA. A list of strains can be found in Table 3.1. Bacteria was routinely propagated in De Mann Rogosa Sharpe (MRS, BD Difco, FisherScientific, Hampshire, NH) medium supplemented with 0.05% L-cysteine hydrochloride (Acros Organics, UK) at 37°C under anaerobic conditions (Coy Laboratory Products, Grass Lake, MI) for 24-48 hours. A set of experiments for observation of GABA production were conducted as follows: i) overnight cultures were inoculated into MRS medium supplemented with 3% (wt/v, 30 mg/ml) monosodium glutamate (MSG, Sigma-Aldrich, St. Louis, MO) for 72 hours. ii) overnight cultures of GABA producers were inoculated into MRS medium supplemented with 1%, 2%, and 3% MSG (wt/v, 10 mg/ml, 20 mg/ml, 30 mg/ml). iii) overnight cultures were inoculated into modified MRS (mMRS, without acetate and glucose) medium with 1% (wt/v) MSG and either lactose (FisherScientific) or fructooligosaccharides from chicory (Sigma-Aldrich) at 2% (wt/v). 1 ml of vortexed culture was bead beat at 5.5 m/sec for three 30 s intervals, cooled in ice in between, to release intracellular glutamate. Each experiment was evaluated in biological triplicates that were centrifuged at 10,000 rpm for 1 min and cell-free supernatant was stored at -20°C until used for HPLC analysis.

2.2 Quantification of bacterial produced GABA

Glutamate and GABA standards prepared in MRS and cell-free supernatants were derivatized using dansyl chloride according to the procedure outlined in Le Vo et al.²⁸ to produce dansyl glutamate and dansyl GABA. The dansyl derivatization method was adapted because dansyl amino acids can be quantified using a UV detector. To a 2 ml microcentrifuge tube the following were added: 100 ul of sample cell-free supernatant or standard, 200 ul of 1 M sodium bicarbonate buffer (pH 9.5), 100 ul of 80 mg/ml dansyl chloride in acetonitrile and 600 ul of double distilled water. The tube was vortexed and then incubated for 40 min at 80°C. Following incubation, 100 ul of diluted acetic acid (20 ul/1 ml dd water) was added to the microcentrifuge tube to stop the reaction. The derivatized solution was vortexed then centrifuged at 10,000 rpm for 5 min.

Supernatant from the derivatization process was filtered through a 0.2 µm Minisart RC 4 filter (Sartorius, FisherScientific) and analyzed using an Agilent 1260 Infinity HPLC (Agilent Technologies, Santa Clara, CA) system equipped with a UV detector. Separation was carried out using a Waters Corp XBridge BEH C18 Column (130 Å, 5 µm, 4.6 x 250 mm) with a XBridge BEH C18 VanGuard Cartridge (130 Å, 5 µm, 3.9 x 5 mm) and holder at 30°C with the detector temperature maintained at 30°C. As described by Wu and Shah⁴³ two mobile phases, A (30 mM ammonium acetate in HPLC water, Fisher Chemical) and B (100% acetonitrile, Optima, FisherScientific) were used for separation. The column was eluted with a modified linear gradient of 6 to 10% B over 0 to 5 min, 10 to 18% B over 5 to 7 min, 18 to 22% B over 7 to 15 min, 22 to 26% B over 15 to 18.5 min, 26 to 28.5% B over 18.5 to 22.5 min, 28.5 to 30% B over 22.5 to 24 min, 30 to 32% B over 24 to 27.5 min,

32 to 40% B over 27.5 to 30 min, 40 to 55% B over 30 to 40 min, 55 to 50% B over 40 to 45 min, 50 to 6% B over 45 to 48 min and held at 6% B for 2 min at a flow rate of 1 ml/min for a total of 50 mins. Each sample was injected twice with an injection volume of 20 ul and detected at an absorbance of 275 nm. Glutamate eluted from the column first at 14.5 mins and GABA eluted second at 25 mins. Glutamate and GABA standard curves were both prepared as 10 mM, 50 mM, 100 mM and 150 mM solutions.

2.3 Minimum inhibitory concentrations (MICs) of MSG and GABA

MSG (99%>) and GABA (99%>) were both obtained from Sigma-Aldrich in powder form and kept at room temperature until use. The *in vitro* antibacterial activities of both compounds were evaluated against *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918, both GABA producers. Preliminary MICs of MSG and GABA against the strains above were determined by twofold serial microdilutions in MRS medium, adapted from the procedure outlined in Gunes et al.⁴⁴. Preliminary results revealed a more narrow range of concentrations were required to elucidate accurate MICs for MSG and GABA. The solutions of MSG in MRS ranged from per ml 101.5 mg, 93.0 mg, 84.6 mg, 80.3 mg, 76.1 mg, 71.9 mg, 67.6 mg, 59.2 mg, 50.7 mg, 33.8 mg and 16.9 mg concentrations. The solutions of GABA in MRS ranged from per ml 123.7 mg, 103.1 mg, 87.7 mg, 82.5 mg, 77.3 mg, 72.2 mg, 67.0 mg, 61.9 mg, 41.2 mg, 20.6 mg and 10.3 mg concentrations. Solutions were inoculated with overnight bacterial cultures and distributed into 96-well microtiter plates. Initial OD_{600nm} absorbance values were read in an automated PowerWave HT microplate spectrophotometer (BioTek Instruments, Inc. Winooski, VT) placed in anaerobic chamber before incubation. Plates were incubated in anaerobic conditions at

37°C for 48 hours and final OD_{600nm} absorbance readings were taken in the microplate reader.

2.4 Primer design and PCR for detection of GABA-production related genes

Bifidobacterium-specific glutamate decarboxylase (*gadB*) oligonucleotide primers were constructed using *gadB* nucleotide sequences from *B. adolescentis*, *B. angulatum*, and *B. dentium* strains. Sequences were collected using the NCBI/BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and aligned using clustalX. Primers were synthesized by Invitrogen (ThermoFisher). The primers used were Gad2_F (TGCTGGGAGAAGTTCTGCAACTA) and Gad2_R (GATGGCSACSACGCCGATGGTGTT), 5'-3'. PCR was performed with DreamTaq Green Master Mix (2X, ThermoFisher) with DNA concentrations ranging between 10-20 ng/ul per 12.5 ul reactions in a thermocycler (Veriti, Applied Biosystems, Foster City, CA). The cycling conditions for PCR amplifications of *gadB* genes were as follows: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 7 mins. PCR products were run with a 1 kb DNA ladder in a 2% agarose gel at 60 volts for 60 to 90 mins and the presence of PCR product was visualized in the GelDoc-It Imaging System (UVP, Analytik Jena, Germany).

2.5 Statistical analysis

Metabolite concentrations were subjected to two-way analysis of variance (ANOVA) and Tukey's significant difference test for multiple comparisons of strains. Bacterial growth values were subjected to one-way ANOVA and Tukey's significant difference test for multiple comparisons of strains within a treatment.

CHAPTER 3

RESULTS

3.1 *Bifidobacterium* GABA production is strain-dependent and glutamate concentration-dependent.

Production of GABA in MRS supplemented with 3% glutamate (wt/v) after 72 hours of fermentation was quantified by HPLC in cell-free supernatants. Among the *Lactobacillus spp.* tested (Table 3.1), only *Lactobacillus brevis* NRRL-B4527_T produced a detectable concentration of GABA (76.08±12.28 mM) and served as a positive control for the screening purposes until a GABA-producing bifidobacteria was identified.

Table 3.1 *Bifidobacterium* and *Lactobacillus* strains screened in this study^a

<i>Lactobacillus spp.</i> & <i>Bifidobacterium</i>	Strain	Contains <i>gadB</i>	Contains <i>gadC</i>	Produces GABA
<i>L. brevis</i>	NRRL-B4527 _T	+	+	+
<i>L. johnsonii</i>	ATCC 33200	-	-	-
<i>L. pentosus</i>	NRRL-B227	-	-	-
<i>L. plantarum</i>	ATCC 14917 _T	+	+	-
	ATCC BAA-793	-	-	-
<i>L. reuteri</i>	NRRL-B14121	-	-	-
<i>B. adolescentis</i>	JCM 1251	+	+	+
	JCM 1275 _T	-	-	-
	JCM 15918	+	+	+
	JCM 7045	+	+	+
	JCM 7046	-	-	-
<i>B. angulatum</i>	JCM 1252	+	+	+
	JCM 7096 _T	+	+	+
<i>B. animalis subsp. lactis</i>	UMA 905039	-	-	-
<i>B. bifidum</i>	JCM 1254	-	-	-
<i>B. breve</i>	JCM 7019	-	-	-
<i>B. callitrichos</i>	JCM 17296 _T	-	-	-
<i>B. catenulatum subsp. kashiwanohense</i>	JCM 15439 _T	-	-	-
<i>B. dentium</i>	JCM 1195 _T	+	+	+
	JCM 7135	+	+	+

<i>B. longum subsp. infantis</i>	UMA 272	-	-	-
	UMA 298	-	-	-
	UMA 299	-	-	-
	UMA 300	-	-	-
	UMA 301	-	-	-
	UMA 302	-	-	-
<i>B. longum subsp. longum</i>	ATCC 15707	-	-	-
	CECT 7894	-	-	-
<i>B. longum subsp. suillum</i>	JCM 19995 _T	-	-	-
<i>B. longum subsp. suis</i>	UMA 391	-	-	-
	UMA 399	-	-	-
<i>B. merycicum</i>	JCM 8219 _T	+	+	-
	JCM 8220	-	-	-
<i>B. moukalabense</i>	JCM 18751 _T	+	+	+
<i>B. ruminantium</i>	JCM 8221	-	-	-
	JCM 8222 _T	+	+	-
<i>B. scardovii</i>	JCM 12489 _T	-	-	-

^a NRRL- Agricultural Research Service Culture Collection; ATCC- American Type Culture Collection; JCM- the Japanese Collection of Microorganism; University of Massachusetts Amherst Culture Collection

_TDenotes type strain

Following *Lactobacillus spp.* screening, 31 strains of *Bifidobacterium spp.* were screened for GABA production (Table 3.1). 8 strains of bifidobacteria belonging to 4 species (3 *B. adolescentis*, 2 *B. angulatum*, 2 *B. dentium* and 1 *B. moukalabense*) produced GABA. Each strain produced a different concentration of GABA, regardless of species. *B. adolescentis* strains had absolute GABA concentrations ranging from 6.70 ± 1.98 mM (*B. adolescentis* JCM 1251) to 38.65 ± 11.94 mM (*B. adolescentis* JCM 15918), *B. angulatum* strains had absolute GABA concentrations ranging from 24.38 ± 0.71 mM (*B. angulatum* JCM 7096) to 90.98 ± 4.61 mM (*B. angulatum* JCM 1252) and *B. dentium* strains had absolute GABA concentrations ranging from 44.68 ± 7.70 mM (*B. dentium* JCM 7135) to

54.47±4.58 mM (*B. dentium* JCM 1195). *B. moukalabense* JCM 18751 produced 92.63±8.53 mM of GABA (Table 3.2).

Table 3.2 GABA-producing *Lactobacillus* spp. and *Bifidobacterium* spp.

Species	Strain	GABA concentration (mM)
<i>L. brevis</i>	NRRL-B4527 _T	76.08±12.28
<i>B. adolescentis</i>	JCM 1251	6.70±1.98
	JCM 15918	38.65±11.94
	JCM 7045	25.63±2.78
<i>B. angulatum</i>	JCM 1252	90.98±4.61
	JCM 7096 _T	24.38±0.71
<i>B. dentium</i>	JCM 1195 _T	54.47±4.58
	JCM 7135	44.68±7.70
<i>B. moukalabense</i>	JCM 18751 _T	92.63±8.53

Table 3.2 contains the absolute values of average final GABA concentrations of biological triplicates with the standard error of the mean.

To produce GABA, bifidobacteria must possess a *gadB* gene that encodes for a glutamate decarboxylase. PCR was performed with bifidobacteria-specific *gadB* primers on GABA-producing bifidobacteria. All 8 GABA-producing bifidobacteria were PCR positive for *gadB* (Table 3.3). Additional strains that did not produce GABA were also tested for the presence of *gadB*. *B. adolescentis* JCM 1275, the species type strain, was tested via PCR because three strains of *B. adolescentis* were *gadB* positive and produced GABA. *B. adolescentis* JCM 1275 did not produce GABA and was PCR negative for *gadB*. *B. merycicum* JCM 8219 and *B. ruminantium* JCM 8222 were identified through BLASTp to possess *gadB*, however these strains did not produce GABA. PCR was performed on *B. merycicum* JCM 8219 and *B. ruminantium* JCM 8222 to confirm the presence of *gadB* in their genome, however the results are to be determined.

Table 3.3 *gadB* PCR screened bifidobacteria

<i>Bifidobacterium</i> spp.	Strain	<i>gadB</i> PCR	Produces GABA
<i>B. adolescentis</i>	JCM 1251	+	+
	JCM 1275 _T	-	-
	JCM 15918	+	+
	JCM 7045	+	+
<i>B. angulatum</i>	JCM 1252	+	+
	JCM 7096 _T	+	+
<i>B. dentium</i>	JCM 1195 _T	+	+
	JCM 7135	+	+
<i>B. merycicum</i>	JCM 8219 _T	TBD	-
<i>B. moukalabense</i>	JCM 18751 _T	+	+
<i>B. ruminantium</i>	JCM 8222 _T	TBD	-

Table 3.3 contains the results of bifidobacteria-specific *gadB* PCR. TBD is to be determined. PCR was performed on *B. merycicum* and *B. ruminantium*, but results were not identified via gel electrophoresis because of Covid-related closures.

Absolute GABA concentrations post-fermentation of different starting glutamate concentrations were quantified by HPLC in cell-free supernatants from the 8 GABA producing bifidobacteria. The starting concentrations of glutamate were 1%, 2% or 3% (wt/v). For most of the tested bifidobacteria GABA production was not significantly different from one concentration of glutamate to another ($p > 0.05$). For *B. angulatum* JCM 1252 and *B. moukalabense* absolute GABA concentrations significantly increased ($p < 0.0001$) when the starting glutamate concentration increased from 1% to 2%, however when starting glutamate concentrations increased from 2% to 3% absolute GABA concentrations were not significantly different ($p > 0.05$). This indicates that maximum GABA production is dependent on the concentration of glutamate available, but there is a limit to how much GABA the organism can produce.

Figure 3.1 GABA production varies with starting glutamate concentrations

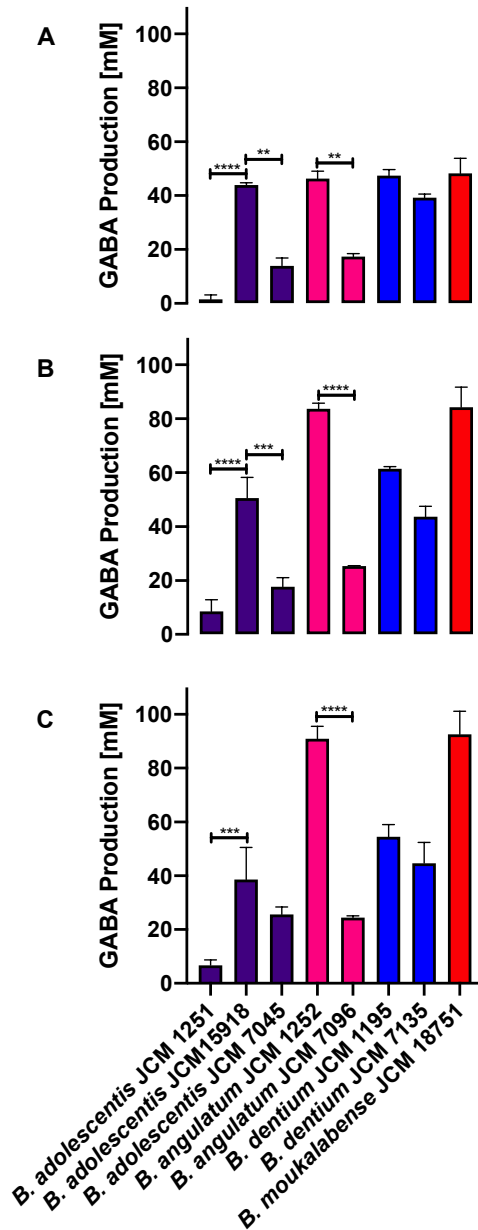


Figure 3.1 represents absolute GABA concentrations of *B. adolescentis* JCM 1251, *B. adolescentis* JCM 15918, *B. adolescentis* JCM 7045, *B. angulatum* JCM 1252, *B. angulatum* JCM 7096, *B. dentium* JCM 1195, *B. dentium* JCM 7135 and *B. moukalabense* JCM 18751 after fermentation in (A) 1% (wt/v, 60 mM), (B) 2% (118 mM), or (C) 3% MSG (177 mM). Colored bars represent different *Bifidobacterium* spp. (Purple: *B.*

adolescentis; Pink: *B. angulatum*; Blue: *B. dentium*; Red: *B. moukalabense*) and the average final GABA concentration of biological triplicates. Error bars show the standard error of the mean. Significant differences among the GABA production of strains on each glutamate concentration are computed using two-way ANOVA with significance at ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

GABA production was not consistent amongst *Bifidobacterium* spp. In 1% glutamate (wt/v) *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 7045 produced significantly less ($p < 0.01$) GABA (1.50 ± 1.69 mM and 14.00 ± 2.79 mM, respectively) than *B. adolescentis* JCM 15918 (43.95 ± 0.86 mM). This trend was also observed in the *B. angulatum* strains. The absolute concentration of GABA produced in 1% glutamate (wt/v) by *B. angulatum* JCM 1252 (46.36 ± 2.77 mM) was significantly more ($p < 0.01$) than *B. angulatum* JCM 7096 (17.33 ± 1.16 mM, Fig. 3.1A). For all glutamate concentrations (1%, 2% and 3% wt/v) *B. angulatum* JCM 1252 produced significantly more glutamate than *B. angulatum* JCM 7096 ($p < 0.01$, Fig. 3.1). These results indicate GABA production is strain-dependent, not species-dependent.

3.2 MICS of MSG and GABA

Table 3.4 MIC values of GABA and MSG

Strain	GABA MIC (mg/ml)	MSG MIC (mg/ml)
<i>B. adolescentis</i> JCM 1251	103.1	84.6
<i>B. adolescentis</i> JCM 15918	82.5	67.6

MICs were performed to determine the antibacterial effects of GABA and MSG on *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918. MIC values can be found in Table 3.4. Both compounds exerted antibacterial effects on the tested strains. GABA inhibited growth for *B. adolescentis* JCM 1251 at a concentration of 103.1 mg/ml (1 M)

and inhibited growth for *B. adolescentis* JCM 15918 at a concentration of 82.5 mg/ml (800 mM). MSG inhibited growth for *B. adolescentis* JCM 1251 at a concentration of 84.6 mg/ml (500 mM) and inhibited growth for *B. adolescentis* JCM 15918 at a concentration of 67.6 mg/ml (400 mM).

3.3 Bifidobacterial GABA production varies with carbohydrate source

Screening for GABA production utilized glucose in MRS as a carbohydrate source. To investigate if carbohydrate source modulated GABA production cell-free supernatants of 8 GABA-producing bifidobacteria grown in different carbohydrates sources were quantified by HPLC. All organisms were grown with a starting concentration of glutamate at 1% (wt/v). Carbohydrate sources investigated were glucose (2% wt/v), lactose (2% wt/v) or FOS (2% wt/v).

Glucose-mediated GABA production had the lowest absolute GABA concentrations of all tested sugars (1.50 ± 1.69 mM to 48.22 ± 5.62 mM). *B. adolescentis* JCM 1251, *B. adolescentis* JCM 7045, *B. angulatum* JCM 7096, and *B. dentium* JCM 7135 had glutamate present in the media post-fermentation, however the sum of remaining glutamate and GABA produced did not equal the starting glutamate concentration of 60 mM (1% wt/v). For example, *B. angulatum* JCM 7096 had 6.94 ± 0.83 mM of glutamate remaining and produced 17.33 ± 1.16 mM of GABA. For all tested bifidobacteria, glutamate to GABA conversion was not 1:1 post-glucose fermentation (Fig. 3.2A), indicating glutamate is being consumed by those organisms for purposes other than GABA production. In general, absolute GABA concentrations were lower during glucose fermentation, which could be due to bifidobacterial preference for other sugars.

Figure 3.2 Absolute glutamate and GABA concentrations following fermentation of different carbohydrate sources

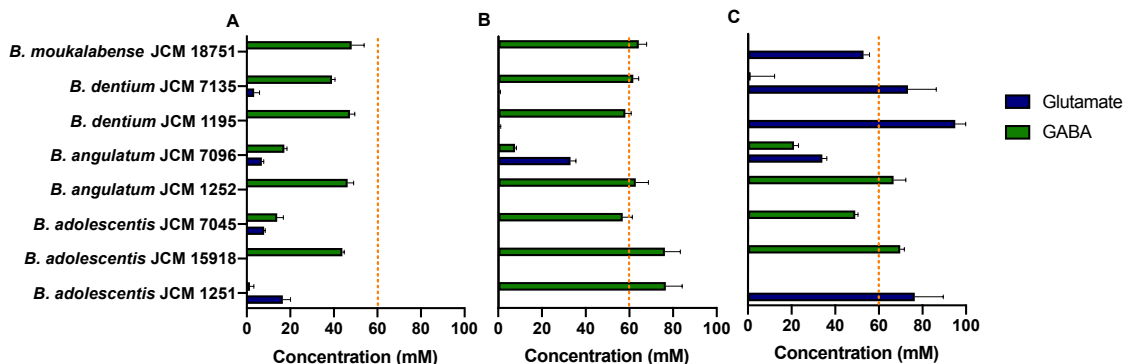


Figure 3.2 represents the absolute glutamate and GABA concentrations of *B. adolescentis* JCM 1251, *B. adolescentis* JCM 15918, *B. adolescentis* JCM 7045, *B. angulatum* JCM 1252, *B. angulatum* JCM 7096, *B. dentium* JCM 1195, *B. dentium* JCM 7135 and *B. moukalabense* JCM 18751 after fermentation on various sole carbon sources. Shown are GABA and glutamate concentrations after fermentation of (A) glucose (B) lactose or (C) FOS. Dotted lines represent the starting concentration of glutamate (1% wt/v, 60 mM) before fermentation. Bars represent the average final glutamate or GABA concentration of biological triplicates and error bars show the standard error of the mean.

Absolute GABA concentrations during lactose fermentation were significantly different ($p < 0.05$) from GABA concentrations during glucose fermentation for all tested strains except *B. angulatum* JCM 7096 and *B. dentium* JCM 1195. Almost every strain, except for *B. angulatum* JCM 7096, utilized all 60 mM of glutamate to produce 60 mM of GABA (Fig. 3.2B) during lactose fermentation. *B. angulatum* JCM 7096 had post-fermentation glutamate and GABA concentrations (33.12 ± 2.43 mM glutamate and 7.71 ± 0.61 mM GABA) that did not amount to the starting concentration of glutamate (60 mM, 1% wt/v). *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918 both produced

an excess of GABA (76.77 ± 7.41 mM and 76.31 ± 7.10 mM, respectively) while consuming all provided glutamate. This indicates intracellular production of glutamate was increased during lactose fermentation, which allowed GABA concentrations to exceed 60 mM. Based on these results, lactose might be utilized as the primary carbohydrate source for maximum GABA production.

Previously published *B. adolescentis*, *B. angulatum* and *B. dentium* strains had demonstrated growth on plant oligosaccharides, including FOS_{6,9,40,41}. To modulate GABA production with plant oligosaccharides, all 8 GABA-producing bifidobacteria strains were propagated with 2% (wt/v) FOS and 1% (wt/v) glutamate for 72 hours. Growth was variable on FOS (Fig. 3.3), especially among *B. adolescentis* strains. *B. adolescentis* JCM 7045 grew significantly more on FOS than *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918 ($p < 0.001$).

Figure 3.3 Bacterial growth after fermentation of fructooligosaccharides

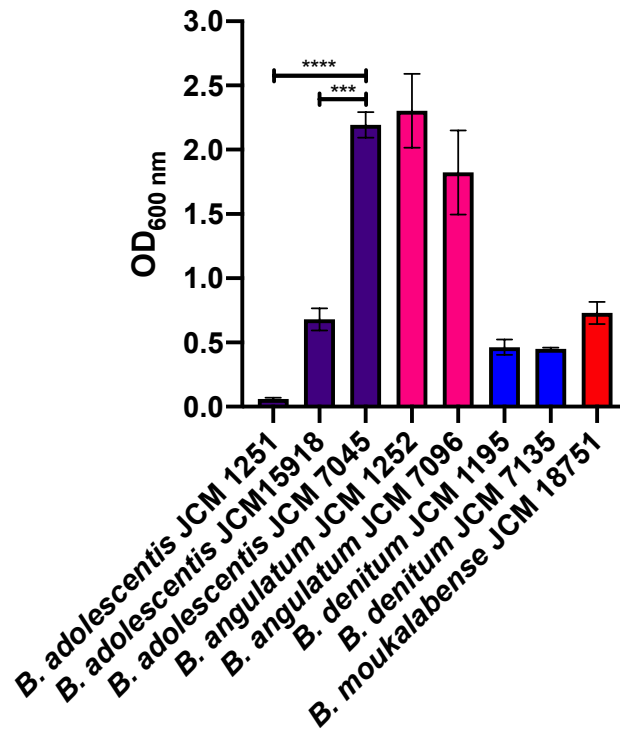


Figure 3.3 represents the growth of *B. adolescentis* JCM 1251, *B. adolescentis* JCM 15918, *B. adolescentis* JCM 7045, *B. angulatum* JCM 1252, *B. angulatum* JCM 7096, *B. dentium* JCM 1195, *B. dentium* JCM 7135 and *B. moukalabense* JCM 18751 on FOS as a sole carbon source. Colored bars represent different *Bifidobacterium* spp. (Purple: *B. adolescentis*; Pink: *B. angulatum*; Blue: *B. dentium*; Red: *B. moukalabense*) and the average final OD₆₀₀ growth value of biological triplicates. Error bars show the standard error of the mean. Significant differences among the growth profiles of strains on FOS are computed using one-way ANOVA with significance at ***p<0.001 and ****p<0.0001.

Regardless of growth all tested strains produced GABA or glutamate, except *B. moukalabense* who grew on FOS (OD_{600nm} 0.73±0.09), but did not produce GABA. Glutamate concentrations increased in *B. dentium* JCM 1195, *B. dentium* JCM 7135 and *B. adolescentis* JCM 1251, however these organisms did not produce detectable concentrations of GABA (Fig. 3.2C). *B. adolescentis* JCM 15918 and *B. angulatum* JCM 1252 both consumed all present glutamate and produced excess concentrations of GABA (69.91±1.78 mM and 66.76±5.64 mM, respectively), indicating they were able to utilize FOS and increase intracellular glutamate production. In total, only 4 strains produced GABA on FOS, including *B. adolescentis* JCM 15918, *B. angulatum* JCM 1252, *B. adolescentis* 7045 (49.22±1.23 mM GABA), and *B. angulatum* JCM 7096 (21.23±1.95 mM GABA). Although *B. moukalabense* produced the highest absolute GABA concentrations during glucose and lactose fermentation, during FOS fermentation it did not produce detectable GABA and had decreased glutamate concentration (47.86±2.63 mM) from the starting concentration of 60 mM (1% wt/v). Not only can these organisms grow

on FOS, but these results also indicate that FOS modulates GABA and glutamate production.

CHAPTER 4

DISCUSSION & CONCLUSION

4.1 Discussion

Bifidobacteria are important members of the gut microbiome as they ferment host-indigestible oligosaccharides and produce beneficial metabolites, including SCFAs and amino acids^{42,45,46}. GABA, a non-protein amino acid and the primary inhibitory neurotransmitter of the CNS, is produced by some *Bifidobacterium* spp. To produce GABA, the bifidobacterial chromosome must encode a glutamate decarboxylase, which synthesizes GABA from glutamate. Previously published GABA-producing bifidobacteria include strains of *B. adolescentis*, *B. angulatum*, *B. dentium*, and *B. longum* subsp. *infantis*^{30,38,39}, however additional species were identified through BLASTp of a *gadB* amino acid sequence from *B. adolescentis* BBMN23⁴⁷. The additional bifidobacteria we identified through BLASTp were strains of *B. merycicum*, *B. moukalabense* and *B. ruminantium*.

In this study, 33 strains of bifidobacteria were screened for the ability to produce GABA. 20 strains were from species that i) had published GABA-producing strains or ii) had strains identified through BLASTp of *gadB*. We have identified 8 GABA-producing strains through our screening conditions (Table 3.2). Strains of *B. merycicum* and *B. ruminantium* used in this study were identified to encode *gadB* through BLASTp, however neither of these species had GABA-producing strains. Although glutamate was present in the growth media and the genomes of these strains encoded *gadB*, GABA was not produced. Under these experimental conditions, we cannot determine a direct relationship between GABA production and the presence of *gadB* in the genome.

We determined that presence of *gadB* in the genome is strain-dependent, not species-dependent. This became apparent when we analyzed *gadB* through BLASTp. A *B. longum* subsp. *infantis* strain had been published to produce GABA₃₉ but did not appear in the BLASTp results. We screened six strains of *B. longum* subsp. *infantis* for GABA production, all which failed to produce detectable levels of GABA, and did not have *gadB* in their genomes. This point is further validated by the results of the five *B. adolescentis* strains screened for GABA production. A multitude of *B. adolescentis* strains were identified through BLASTp of *gadB*, but only three of our tested strains produced GABA. *B. adolescentis* JCM 1275_T, the type strain, did not produce GABA. *Bifidobacterium*-specific *gadB* PCR was performed on all of the *B. adolescentis* strains, and *gadB* was confirmed in only the GABA-producing *B. adolescentis* strains (JCM 1251, JCM 15918 and JCM 7045).

The primary growth media for bifidobacteria is MRS with 2% glucose (wt/v) as the sole carbohydrate source. To investigate if carbohydrate source modulated GABA production GABA-producing bifidobacteria were propagated in mMRS with lactose (2% wt/v) as the sole carbon source and supplemented MSG (1% wt/v). Lactose-mediated GABA production was significantly higher ($p < 0.05$) than glucose-mediated GABA production for most of the GABA-producing bifidobacteria (excluding *B. angulatum* JCM 7096 and *B. dentium* JCM 1195). This phenomenon could be caused by a metabolic preference of lactose over glucose by the bifidobacteria⁴⁸⁻⁵⁰. The metabolic preference of lactose could also explain the absolute GABA concentrations of *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918 exceeding 60 mM (the starting concentration of glutamate). Based on these results, we can conclude the metabolism of lactose by *B.*

adolescentis JCM 1251 and *B. adolescentis* JCM 15918 increased intracellular glutamate concentrations, which led to an increase in GABA concentrations past the provided concentration of glutamate. Glutamate is the primary excitatory neurotransmitter of the central nervous system and precursor to GABA. It functions as oxidative fuel for the intestine, as it can be synthesized into alpha-ketoglutarate and utilized in the tricarboxylic acid (TCA) cycle⁵¹.

Host-indigestible oligosaccharides are common carbohydrate sources in the small intestine^{5,11,52,53}. In order to metabolize oligosaccharides, bifidobacteria have adapted various enzymes that cleave oligosaccharides into monosaccharides, which can be further metabolized for energy¹². To investigate the relationship between oligosaccharide consumption and GABA production GABA-producing bifidobacteria were propagated in mMRS with FOS (2% wt/v) as the sole carbon source and supplemented MSG (1% wt/v). Only one GABA producer, *B. adolescentis* JCM 1251, failed to grow on FOS, indicating a shift in physiology. As hypothesized, fermentation of FOS modulated glutamate and GABA production. Absolute concentrations of glutamate increased for *B. adolescentis* JCM 1251, *B. dentium* JCM 1195 and *B. dentium* JCM 7135, but no detectable GABA was measured for these organisms. *B. adolescentis* JCM 15918, *B. adolescentis* JCM 7045 and *B. angulatum* JCM 1252 produced significantly more ($p < 0.01$) GABA during fermentation of FOS compared to fermentation of glucose. Absolute GABA concentrations of *B. adolescentis* JCM 15918 and *B. angulatum* JCM 1252 exceeded 60 mM (the starting concentration of glutamate), indicating FOS metabolism increased intracellular glutamate concentrations. *B. angulatum* JCM 7096 did not produce a significantly different concentration of GABA during FOS fermentation compared to glucose and lactose

fermentation. These results indicate FOS fermentation induced a shift in glutamate production and altered expression of *gadB*. *B. moukalabense* grew on FOS, but did not produce GABA during FOS fermentation. Absolute glutamate concentrations decreased following FOS fermentation, indicating *B. moukalabense* utilized glutamate for other metabolic processes. Glutamate has other metabolic fates and can be synthesized into glutamine or proline⁴² if it is not irreversibly decarboxylated by GadB.

One phenomenon that was consistent across all carbohydrate sources was the production of GABA in the presence of glutamate. In our study GABA was not produced by bifidobacteria unless extracellular glutamate was present. This phenomenon was observed with glutamate production as well; excess glutamate was only observed if glutamate was already present in the extracellular environment. When *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918 fermented lactose supplemented with MSG (1% wt/v, 60 mM), GABA concentrations exceeded 60 mM, indicating intracellular glutamate production had been induced by lactose fermentation. This was not observed when *B. adolescentis* JCM 1251 and *B. adolescentis* JCM 15918 fermented lactose without added glutamate. Neither glutamate nor GABA were detected following fermentation of lactose alone. We observed this in all tested organisms fermented in all carbohydrate sources. It is unclear why this phenomenon is occurring, but glutamate is clearly exerting a physiological effect on the bifidobacteria.

4.2 Conclusion

Among the 33 bifidobacterial strains tested, only 8 strains, *B. adolescentis* JCM 1251, *B. adolescentis* JCM 15918, *B. adolescentis* JCM 7045, *B. angulatum* JCM 1252, *B. angulatum* JCM 7096, *B. dentium* JCM 1195, *B. dentium* JCM 7135, and *B. moukalabense* JCM 18751, utilized glutamate to produce GABA. Absolute GABA and glutamate concentrations were modulated during fermentation of glucose, lactose, and FOS as sole carbohydrate sources. Glucose fermentation resulted in the lowest absolute GABA concentrations while lactose fermentation resulted in the highest GABA concentrations. FOS fermentation modulated GABA and glutamate concentrations. This indicates a shift in bifidobacterial metabolism and *gadB* regulation. Glutamate's influence on bifidobacterial metabolism should be further investigated, as it increased intracellular glutamate concentrations only when glutamate was present in the external environment. Observing gene expression during fermentation of different carbohydrate sources and glutamate could elucidate the influence these compounds have on bifidobacterial physiology. The primers developed for this study will be used to detect and quantify *gadB* in microbiome samples, allowing us to investigate the relationship between *gadB* expression and GABA production in the adult microbiome. Bifidobacterial GABA production *in vivo* may be influenced by host consumption of plant oligosaccharides, which can be further investigated through dietary interventions of humans and animal.

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