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Gestational administration of *Bifidobacterium dentium* results in intergenerational modulation of inflammatory, metabolic, and social behavior

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ABSTRACT

Prenatal stress (PNS) profoundly impacts maternal and offspring health, with enduring effects including microbiome alterations, neuroinflammation, and behavioral disturbances such as reductions in social behavior. Converging lines of evidence from preclinical and clinical studies suggest that PNS disrupts tryptophan (Trp) metabolic pathways and reduces gut Bifidobacteria, a known beneficial bacterial genus that metabolizes Trp. Specifically, previous work from our lab demonstrated that human prenatal mood disorders in mothers are associated with reduced *Bifidobacterium dentium* in infants at 13 months. Given that *Bifidobacterium* has been positively associated with neurodevelopmental and other health benefits and is depleted by PNS, we hypothesized that supplementing PNS-exposed pregnant dams with *B. dentium* would ameliorate PNS-induced health deficits. We measured inflammatory outputs, Trp metabolite levels and enzymatic gene expression in dams and fetal offspring, and social behavior in adult offspring. We determined that *B. dentium* reduced maternal systemic inflammation and fetal offspring neuroinflammation, while modulating tryptophan metabolism and increasing kynurenic acid and indole-3-propionic acid intergenerationally. Additional health benefits were demonstrated by the abrogation of PNS-induced reductions in litter weight. Finally, offspring of the *B. dentium* cohort demonstrated increased sociability in males primarily and increased social novelty primarily in females. Together these data illustrate that *B. dentium* can orchestrate interrelated host immune, metabolic and behavioral outcomes during and after gestation for both dam and offspring and may be a candidate for prevention of the negative sequelae of stress.

1. Introduction

Maternal homeostasis and fetal development during gestation are highly susceptible to external effectors, such as immune activation, dietary insufficiency, and exposure to drugs, which can aberrantly shift normal progression (Doi et al., 2022; Solek et al., 2018). Prenatal stress (PNS) is a prime example of a disruptive and pernicious exposure that can detrimentally affect both maternal and offspring health. Specific offspring outcomes span from fetal insults including microglial dysregulation (Loayza et al., 2023) and aberrant neuroinflammation (Chen et al., 2020; Chen et al., 2022; Ünal et al., 2022; Diz-Chaves et al., 2012), to immune and social behavioral deficits that arise in later life (Gur et al., 2019; Gur et al., 2017; O'Connor et al., 2013; Jafari et al., 2017). Indeed, PNS is associated with elevated incidence of attention-deficit hyperactivity disorder and autism (Rai et al., 2013; Holingue et al., 2020; Gray et al., 2017; Buka et al., 2001; Goeden et al., 2016). While the short- and long-term effects of PNS are coming into focus, both how PNS effects are transmitted to the offspring is not well-elucidated.

Abbreviations: PNS, prenatal stress; I3LA, indole-3-lactic acid; I3PA, indole-3-propionic acid; I3AA, indole-3-acetic acid; KA, kynurenic acid; Trp, tryptophan.

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Additionally, abrogation of PNS is a significant topic, as PNS and its downstream ramifications are alterable and avoidable.

Research has identified tryptophan (Trp) metabolism as a key metabolic pathway in typical development (Lukić et al., 2019; Wei et al., 2021; Zhang et al., 2022). The abundance of Trp and its downstream metabolites such as kynurenic acid, 3-hydroxykynurenine, and the indoles may have distinctive effects on neurodevelopment at specific timepoints in steering growth (Forrest et al., 2013; Forrest et al., 2015; Gibney et al., 2014; Sathyasaikumar et al., 2010). Deviations imposed by PNS or other exposures upon baseline levels of the metabolites and the enzymatic machinery that controls their expression could have major consequences on development (Moura et al., 2022; Galley et al., 2021; O'Connor et al., 2021; Wang et al., 2022). Most host Trp is derived from dietary sources and metabolism of Trp into its three primary arms (indole, kynurenine, and serotonin) is mediated by a mix of host and microbial cells. The presence and absence of a microbiome or even individual microbes has been associated with Trp metabolite levels, gene expression, and downstream neurodevelopment (Clarke et al., 2013; Dehhaghi et al., 2019; Desbonnet et al., 2008). Psychological stress exposure, including prenatal stressors, have long been associated with alterations to microbiome community structure (Chen et al., 2022; Gur et al., 2017; Bailey et al., 2011; Allen et al., 2019; Jašarević et al., 2017). Thus, stressor-induced microbiome disruptions may be tied to Trp metabolic dysfunction, and ultimately physiological and neurodevelopmental deficits. Accordingly, identifying microbes that might either be stress-sensitive and/or Trp metabolizers might provide either therapeutic targets or opportunities to better explicate the mechanism by which PNS affects offspring health and development.

We previously identified that members of Bifidobacteria are sensitive to prenatal anxiety, stress, and depression, in a recent human study (Galley et al., 2023). One such member, *Bifidobacterium dentium*, had an especially strong association with mood disorders in multiple psychometric surveys. While *B. dentium* has not been tied directly to Trp metabolism, as many other Bifidobacteria have been (Tian et al., 2022; Fang et al., 2022; Meng et al., 2020), it is not without its own evidence of neuromodulatory and metabolic interventions. *B. dentium* can produce the neurotransmitter gamma-aminobutyric acid (GABA), reduce visceral hypersensitivity, increase serotonin production, and provide anxiolytic effects (Luck et al., 2021; Engevik et al., 2021; Pokusaeva et al., 2017). It also has anti-inflammatory properties, reducing NFκb phosphorylation and inflammatory cytokine levels, while stimulating anti-inflammatory IL10 release in a colonic inflammation model (Engevik et al., 2021). A bifidobacterial cocktail containing *B. dentium* increases microglial abundance and ramification and improves synapse function in germ-free mice (Luck et al., 2020). Together, these data emphasize that *B. dentium* has strong potential as a psychobiotic, the class of probiotics that provide mental health benefits.

B. dentium is stress-sensitive and reduced in subjects that have experienced PNS (Galley et al., 2023). Conversely, *B. dentium* beneficially modulates CNS development through reductions in synaptic density and increased neuronal activity, as well as elevations in neurotransmitters and reactive ameboid microglia. *B. dentium* treatment also influences behavior and abrogates gut inflammation (Luck et al., 2021; Engevik et al., 2021; Engevik et al., 2021; Luck et al., 2020). Thus, we hypothesized that treating PNS-exposed dams with *B. dentium* would result in abrogation of PNS-specific outcomes including inflammatory dysregulation in both offspring and dam, which we have previously associated with aberrant offspring neurodevelopment in our model (Chen et al., 2020; Chen et al., 2022). Members of *Bifidobacterium* also have noted Trp modulatory functionality and thus, we posited that *B. dentium* would also modulate Trp metabolic output. A connection between *B. dentium* and Trp metabolism has not yet been reported and would provide an important mechanistic step in further framing recent research that has shown that *B. dentium* can increase intestinal serotonin and brain serotonin receptors (Engevik et al., 2021). Altogether, these findings would indicate that *B. dentium* may have Trp-specific metabolic activity that can modulate neuroactive molecules such as indoles and serotonin.

To test this hypothesis, PNS-exposed dams were concomitantly administered *B. dentium*. Inflammation, tryptophan metabolism and behavioral outputs were then evaluated in the offspring. Indeed, *B. dentium* treatment had an anti-inflammatory effect on both fetus and dam, while exhibiting the ability to modulate plasma Trp metabolite levels intergenerationally and increase social behavior in offspring, despite the absence of a strong stress effect. These data indicate that *B. dentium* has beneficial neurodevelopmental and anti-inflammatory properties and may have efficacy as a gestational probiotic.

2. Materials and methods

2.1. Animals

All dams used in this study were female nulliparous 10-week-old C57Bl6/J mice ordered from Jackson Laboratories. Upon arrival, they were housed in an Ohio State University Lab Animal Resource vivarium for a minimum of 4 days prior to commencement of prenatal stress and breeding. Additionally, these mice were singly housed.

All animal procedures were reviewed and approved by the Ohio State University Institute for Animal Care and Use Committee (IACUC) under protocol #2014A00000048, most recently reviewed and approved on 9/6/2023. All experiments also followed Ohio State University research guidelines. ARRIVE guidelines were followed for the animal studies.

2.2. Prenatal stress model

The prenatal stress (PNS) model has been published and detailed in multiple studies (Chen et al., 2020; Chen et al., 2022; Gur et al., 2019; Gur et al., 2017; Galley et al., 2021). To briefly summarize, 10 week old nulliparous singly-housed females were ordered from Jackson Labs and allowed to acclimate for at least 5 days after arrival at the Ohio State University vivarium. After acclimation, females were weighed and then placed in a cage with a male breeder mouse. The female was paired with the male for up to 7 days. Each morning during the seven day breeding period, the female urogenital region was visually checked for the presence of a vaginal plug that would indicate copulation. In the event a plug was observed, the female mouse was removed from the male cage and placed back in its own original cage. If a plug was not observed, the female was re-weighed and returned to the male cage. The date of vaginal plug observation was considered E0.5. Female mice with plugs were left alone until E10.5 when they were re-weighed. Weight gain of $>$ 2 g from E0.5 to E10.5 were considered positive pregnancies and these dams were randomly assigned to one of four groups (No Stress + Vehicle (NSV), No Stress + *B. dentium* (NSP), Stress + Vehicle (SV), Stress + *B. dentium* (SP). Two experimental replicates were used for all non-metabolomics experiments with the following sample sizes for dams- NSV- 7; NSP- 6; SV-5; SP- 6. Two separate cohorts of dams were used for the metabolomics studies with the following sample sizes- NSV-6; NSP-4; SV-6; SP-5.

Mice in the Stress groups were placed in a well-ventilated 50-mL conical tube for 2 h every morning from 9am to 11am, during gestational days E10.5 to E16.5. Following the end of this stressor period, the mice were removed from the tubes. Non-stress mice were undisturbed during this time. Mice in the *B. dentium* groups were gavaged with 3.0–9.0 x 10^8 cfu / 150 uL saline immediately following the stressor. Mice in the Vehicle groups were gavaged with 150 uL of 0.9 % saline.

2.3. Timepoints

All experiments were performed with one of two endpoints- E17.5 and PND77 + . For the E17.5 timepoint, dams were euthanized at E17.5 and maternal ileal and spleen tissues and plasma as well as fetal brain and liver tissues and plasma were collected. For the PND77 + timepoint,

pregnant dams continued to birth, and the offspring were eventually weaned. The adult offspring were analyzed for social behavior at PND77 + and then euthanized the week following. Samples were labeled with identifiers non-specific to their treatment and stress group and all downstream analyses were thus blinded until end analysis.

2.4. Bacterial culturing

Bifidobacterium dentium 27678 was ordered from ATCC and utilized in this study. To grow, streaks from frozen stocks were made on tryptic soy agar plates with 5 % added defibrinated horse blood. These plates were placed in an anaerobic jar at 37degC for 48 h. Next, individual colonies were picked and transferred to Modified Reinforced Clostridial broth (ATCC Medium 2107) and grown for 24 h in anaerobic conditions. The tubes were then spun at 3000 g for 5 min to collect the bacteria and then resuspended in 0.9 % saline.

2.5. Offspring behavior

Two behavior types were measured- social behavior and anxiety-like behavior. Prior to social behavior, test mice were transferred to the behavior suite and allowed to acclimate for one hour. Social behavior (sociability and social approach) were analyzed using the three chamber box social behavior test paradigm and captured via Noldus EthoVision (Wageningen, Netherlands) (Chen et al., 2022). Briefly, mice were acclimated to and allowed to explore the entirety of the three chamber box, which is comprised of a small middle chamber and two larger side chambers (sides A and B). During acclimation, the side boxes each had a smaller empty cage within. Acclimation continued for five total minutes. Next, the test mouse was placed back in the middle section while one side of the cage had an orange bottle top placed within and the other side had a conspecific 'social' mouse placed within. The placement of object or social mouse was randomly determined prior to the study for each test mouse. During this sociability phase, the test mouse was allowed to explore for ten total minutes. After this phase, the novel object was removed from its cage and a new 'social' mouse was placed in that cage. The test mouse was then allowed to explore the entirety of the three-chamber box for ten more minutes for social novelty analysis. Following this phase, all mice were returned to their home cage and the box was cleaned out. Total time spent in each side as well as entrances into each side were measured with the EthoVision software. The Sociability index is calculated by finding the difference between time spent in the social box and the object box, dividing by the total time spent in both boxes, and standardizing by acclimation preference to the social box side. Similarly, the Social Novelty index is calculated by finding the difference between time spent in the novel mouse box and the familiar mouse box, dividing by total time, and standardizing by acclimation preference for the novel mouse side.

Light-dark test was performed for anxiety-like behavior test. Prior to light–dark testing, test mice were moved to the behavior suite for a one hour acclimation. Square boxes with a light open half and an enclosed dark half were used with Fusion Software (Omnitech, Columbus, Ohio). Mice were placed in the lower right-hand corner, in the light-half of the box and the software immediately began recording mouse movement throughout the box. Mice were left alone to explore the entire box for five total minutes. Upon completion of the run, mice were returned to their home cage and the box was cleaned prior to addition of the next mouse. Total distance traveled, time spent in the light or dark side, as well as entries into either side of the box were measured.

Social behavior tests were run first, and mice were given one full day off undisturbed before light–dark analysis was performed. Sample sizes utilized 1 female and 1 male mouse per litter. NSV: 6F, 3M; NSP: 2F, 5M; SV: 5F, 5M; SP: 6F, 5M. Attrition was due to uneven sex ratios in litters and utilization in other experiments prior to adulthood.

2.6. Host PCR

Host-targeted qPCR was performed as previously published (Chen et al., 2020; Galley et al., 2021). Briefly, RNA was isolated from mouse tissues utilizing the Trizol protocol for RNA. Isolated RNA was dissolved in nuclease-free water and then quantitated on a Nanodrop One (ThermoFisher). 2 ug of RNA for each sample was converted to complementary DNA (cDNA) using the High Capacity Reverse Transcription Kit (ThermoFisher). cDNA was then analyzed on the QuantStudio5 PCR system with the TaqMan Fast Advanced Master Mix that allowed for Fast Protocol performance. Ct values were then analyzed with the delta-delta-Ct method in order to ascertain fold changes compared to controls. All primer sets are listed in Table 1. Maternal qPCR n = 3–7. For offspring, one male and one female were used per litter and data was collapsed. Offspring qPCR n = 8–14.

2.7. Bacterial PCR

DNA was isolated from fecal and gut tissue samples using the Qiagen (Germantown, Maryland) PowerFecal Pro DNA Kit per manufacturer's instructions. Primer sets were ordered from IDT (Coralville, Iowa) and are listed in Table 2. PCR thermoprofiles and protocol were performed as previously published (Galley et al., 2021). PCRs were performed on a QuantStudio5 Real-Time PCR System (ThermoFisher). Data is presented as the delta of the universal 16S Ct and the unknown (*Bifidobacterium* genera) 16S Ct. Sample sizes- NSV- 7, NSP- 6, SV- 5, SP-4.

2.8. Metabolomics

2.8.1. Reagents and chemicals

Serotonin, Indole, Tryptophan, Kynurenine, Kynurenic acid, Quinolinic acid, 3-hydroxy-DL-Kynurenine, Indole-3-lactic acid (I3LA), Indole-3-acetic acid (I3AA) and indole-3-propionic (I3PA) acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stable isotopes internal standard L-Tryptophan ($^{13}C_{11}$, 99 %; $^{15}N_2$, 99 %) was obtained from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). LC-MS grade formic acid (FA), acetonitrile (CAN) and water - purification system (deionized water) were gotten from ThermoFisher Scientific (Waltham, MA USA). All the chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

2.8.2. Metabolomic instrumentation

All analyses were performed on a Thermo Scientific TSQ Quantiva LC-MS/MS with Dionex UltiMate 3000 RS UHPLC (ThermoScientific, Waltham, MA USA). The high performance liquid chromatography (HPLC) separation of serotonin was carried out on a Accucore C18 column (100 × 2.1 mm, 2.6 μm, ThermoFisher, Waltham, USA). Mobile phases consisted of water containing 0.1 % FA and 10 mM ammonium formate (mobile phase A) and acetonitrile containing 0.1 % FA (mobile phase B). The flow rate was 0.2 mL/min and the column temperature was 40 °C. The gradient: 5 % to 10 % B from 0-1 min, 10 % to 100 %B, 1–9 min. The sample injection volume was 5.0 μL. Between injections, the autosampler syringe was washed with 10 % methanol/water (v/v).

The MS analysis was operated in positive and mode with the

Table 1
Gene expression assays.

Gene	TaqMan Assay ID
IL6	Mm00446190_m1
CCL2	Mm00441242_m1
TNFα	Mm00443258_m1
TDO	Mm00451269_m1
IDO	Mm00492590_m1
TPH1	Mm01202614_m1
TPH2	Mm00557715_m1

Table 2
Bacterial PCR Primer targets.

Target	Forward Primer	Reverse Primer	Ref
Universal 16S	CGGTGAATACGTTCYCGG	GGWTACCTTGTTACGACTT	(Wirthgen et al., 2018)
Bifidobacterium Genus 16S	CTCCTGGAAACGGGTGG	GGTGTTCTTCCCGATATCTACA	(Dostal et al., 2017)

following settings: ion-spray voltage 3800 kV and −3600 KV, vaporizer temperature 300 °C, sheath gas 28 Arb, aux gas 2.5 Arb, sweep gas 3 Arb; Cycle time 0.6 sec. Data acquisition was achieved in the multiple reaction monitoring (MRM) scanning mode. The mass transitions, collision energy (CE) and RF less were displayed in Table 3.

2.8.3. Preparation of solutions

Stock solutions of Serotonin, Indole, Tryptophan, Kynurenine, kynurenic acid, Quinolinic acid, 3-hydroxy-DL-Kynurenine, I3LA, I3AA, and I3PA were prepared in 50 % methanol/water (v/v) at a concentration of 1.0 mg/mL. The stock solution of L-Tryptophan ($^{13}C_{11}$, $^{15}N_2$) was further diluted to yield an internal standard (IS) working solution at 500 ng/mL in 50 % MeOH. All solutions were stored at − 80 °C until analysis.

2.8.4. Preparation of calibration standards and samples

The calibration standards at concentrations of 1.0, 5.0, 25.0, 125.0, 250.0, 500 and 1000 ng/mL were prepared by serial dilution of the stock solution (1000 ng/mL) with internal standard solution. For plasma samples, 50 μL of sample was mixed with 200 μl MeOH, the mixture was vortexed for 1 min and the mixture was allowed to stand for 30 min at − 4 °C and then, was centrifuged at 14,000 × g for 10 min. The supernatant was dried under vacuum and then reconstituted with 50 μL of 50 % of MeOH with 5 min sonication. For stool samples, 500 uL of 80 % of MeOH was added to weighted samples, vortexed for 1 min and sonicated for 10 min at 4 °C. Samples were then centrifuged at 20,000 rcf and 4 °C for 10 min. The supernatant was spin dried. The dried samples were reconstituted by 2.5 times of weight of 50 % of MeOH containing 500 ng/mL of internal standard. For example, 25 uL of reconstituted solution will be added to 10 mg of stool. 5.0 μL was injected for LC-MS/MS analysis. Sample sizes for metabolomics-maternal- NSV-6; NSP-4; SV-6; SP-5. Fetal- whole litters pooled- n = 4–6.

2.9. ELISA

The CCL2 DuoSet ELISA kits from R&D Systems (Minneapolis, Minnesota) was used for this study per manufacturer instructions with no deviations. In short, maternal and offspring plasma were assayed in duplicate and a four-parameter logistic curve was produced from standard absorbances to ascertain protein levels. Sample sizes- NSV-6; NSP-5; SV-5; SP-6.

2.10. Statistical analysis

In fetal analysis, a single male and female was used per litter to account for potential litter effects. For adult behavioral analysis, attempts were made for equal representation of males and females in each cohort. Two-way ANOVA was used to determine statistical significance of Stress, *B. dentium* and Interactions. The two-way ANOVA performed all analysis concomitantly. Significance was set at $p < 0.05$ and trends were

Table 3
Metabolomic data acquisition outputs.

Compound	Retention Time (min)	RT Window (min)	Polarity	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	Min Dwell Time (ms)	RF Lens (V)
Indole	7.5	15	Positive	118.06	65	35.2	21.233	131
Indole	7.5	15	Positive	118.06	89.071	42.36	21.233	131
Indole	7.5	15	Positive	118.06	91.071	23.74	21.233	131
Quinolinic Acid	7.5	15	Positive	168.029	78	24	21.233	51
Quinolinic Acid	7.5	15	Positive	168.029	106.071	16.04	21.233	51
Quinolinic Acid	7.5	15	Positive	168.029	150.071	10.86	21.233	51
Indole-3-AA	7.5	15	Positive	176.07	77	44.93	21.233	54
Indole-3-AA	7.5	15	Positive	176.07	103.054	33.05	21.233	54
Indole-3-AA	7.5	15	Positive	176.07	130.125	17.55	21.233	54
serotonin	7.5	15	Positive	177.1	115.083	29.09	21.233	39
serotonin	7.5	15	Positive	177.1	131.083	19.41	21.233	39
serotonin	7.5	15	Positive	177.1	160.065	12.03	21.233	39
Ky acid	7.5	15	Positive	190.049	130.125	16.71	21.233	59
Ky acid	7.5	15	Positive	190.049	144.125	20.63	21.233	59
Ky acid	7.5	15	Positive	190.049	172.054	11.57	21.233	59
Indole-3-PA	7.5	15	Positive	190.08	130.215	17.76	21.233	58
Indole-3-PA	7.5	15	Positive	190.08	144.125	21.13	21.233	58
Indole-3-PA	7.5	15	Positive	190.08	172.125	11.4	21.233	58
Try	7.5	15	Positive	205.09	118.054	27.07	21.233	47
Try	7.5	15	Positive	205.09	146.125	18.9	21.233	47
Try	7.5	15	Positive	205.09	188.054	10.69	21.233	47
Indole-3-LA	7.5	15	Positive	206.08	118.071	24.33	21.233	58
Indole-3-LA	7.5	15	Positive	206.08	130.083	31.37	21.233	58
Indole-3-LA	7.5	15	Positive	206.08	160.125	12.12	21.233	58
Kynurenine	7.5	15	Positive	209.09	94.125	15.61	21.233	49
Kynurenine	7.5	15	Positive	209.09	146.125	20.08	21.233	49
Kynurenine	7.5	15	Positive	209.09	192.071	9.84	21.233	49
Instd	7.5	15	Positive	218.3	127.083	28.04	21.233	49
Instd	7.5	15	Positive	218.3	156.196	18.35	21.233	49
Instd	7.5	15	Positive	218.3	200.155	11.61	21.233	49
3-oh-Kn	7.5	15	Positive	225.08	110.054	18.06	21.233	51
3-oh-Kn	7.5	15	Positive	225.08	162.125	20.21	21.233	51
3-oh-Kn	7.5	15	Positive	225.08	208.054	9.42	21.233	51
QA-Ng	7.5	15	Negative	166.029	78.07	15	21.233	51
QA-Ng	7.5	15	Negative	166.029	122.1	8	21.233	51

also mentioned and set at 0.05–0.10. Additionally, post-hoc Fisher LSD test was used for group comparisons. Analysis was performed in GraphPad version 7 (San Diego, CA). 1.5xIQR was used for outlier removal.

3. Results

3.1. Treatment with Bifidobacterium dentium results in elevated maternal gut Bifidobacterium abundance at least 24 h after last gavage

First, we utilized PCR to target total *Bifidobacterium* in order to determine if PNS-exposure affected bifidobacterial abundance. We did not detect any changes in *Bifidobacterium* levels as a function of PNS (Supp. Fig. S1A). Next, we analyzed if the *B. dentium* gavage increased bifidobacterial levels to ascertain if *B. dentium* gavage-induced increase may be lasting, measurable, and thus could influence host physiology. We hypothesized that *B. dentium* would increase bifidobacterial abundance for two reasons: *B. dentium* would be measured as a part of the overall bifidobacteria, and bifidobacterial probiotics can alter the microbiome and increase bifidobacterial levels(Sun et al., 2020). Indeed, *B. dentium* gavage was associated with increased *Bifidobacterium* 16S in maternal colonic stool at E17.5 (Supp. Fig. 1A) ($F(1,19) = 5.165$, *B. dentium* Factor- $p = 0.0349$), but not in maternal colonic tissue. This indicates that gavage does increase overall *Bifidobacterium* levels in the maternal gut.

3.2. B. dentium abolishes PNS-induced reductions in litter weight gain

To ensure that the restraint stressor had reproducible outcomes and affects dam physiology, we first examined how PNS exposure affected gestational weight gain since past results have highlighted that reductions in gestational weight gain are a hallmark of our PNS restraint model (Chen et al., 2022; Chen et al., 2024). PNS-exposed dams exhibited reduced weight gain over the course of gestational dates E10.5-E17.5 when standardized by overall litter size (Fig. 1A) ($F(1,20) = 17.27$, Stress Factor- $p = 0.0005$; $F(1,20) = 3.993$, Interaction Factor- $p = 0.0595$). Next, we found that PNS exposure showed a trend towards an increase in corticosterone in maternal plasma at E17.5 (Fig. 1B) ($F(1,16) = 3.779$, Stress Factor- $p = 0.0697$).

We administered *B. dentium* to mice to examine if the microbe improves these outputs. *B. dentium* treatment partially rescued the PNS-induced reduction in weight gain over gestation. While SV dams had significantly reduced weight gain compared to NSV and NSP dams (NSV vs. SV- $p = 0.0002$; NSP vs. SV- $p = 0.0028$), SP mice showed a trend towards an increase compared to SV mice (SV vs. SP- $p = 0.0545$). We then tested corticosterone levels in the PNS-exposed dams treated with *B. dentium.* The microbe did not affect corticosterone levels in the dam (Fig. 1B). These data suggest that *B. dentium* administration is capable of partially abrogating stressor-induced aberrations to gestational growth. However, this, and other *B. dentium* effects to counter PNS-associated deficiencies are mediated in a corticosterone-independent manner.

3.3. Gestationally-administered B. dentium has anti-inflammatory function for both dam and offspring

We have previously established that PNS can affect immunity and inflammation in dams and offspring (Chen et al., 2020; Chen et al., 2022; Chen et al., 2024; Antonson et al., 2020). Additionally, probiotics, including members of the *Bifidobacterium* genus, have well-reported effects in immunomodulation (Xue et al., 2017; Achi et al., 2019; Pagnini et al., 2018). Thus, our next step was to examine how *B. dentium* might affect any immune disruptions induced by PNS, focusing on E17.5, which follows the seven-day stressor period. We have previously identified CCL2, a pro-inflammatory chemokine, as being implicated in the transmission of PNS effects from dam to offspring (Chen et al., 2020; Chen et al., 2024). Thus, we examined CCL2 levels as a function of *B. dentium* treatment separately in non-stressed and stressed dams. Maternal plasma CCL2 protein levels were reduced in dams treated with *B. dentium* (Fig. 2A) ($t = 3.086$, $p = 0.0130$), but this did not extend to stress-exposed dams (Fig. 2B), indicating that the stressor may abrogate *B. dentium* from modulating CCL2.

We have shown that PNS influences systemic and neuro-inflammation, with a focus on CCL2, IL6, and TNFα (Chen et al., 2020, 2024). Thus, we next focused on gene expression for these targets in maternal ileal tissue and fetal offspring brain and liver tissue. First, we analyzed if we could recapitulate our previous findings on PNS effects on inflammation. There was not a stress-effect for *Il6* expression in either the maternal ileal or fetal brain tissue (Fig. 3A-B), while *Il6* expression was significantly increased by PNS in the fetal liver (Fig. 3C) ($F(1,34) = 7.291$, $p = 0.0105$). Fetal liver *Il6* post-hoc analysis demonstrated that SV was significantly increased over NSV in the fetal liver. Stress did not have an effect on either *Ccl2* or *Tnfα* (Fig. 3D-I). In sum, PNS effects on inflammatory genes were limited, which may be due to a secondary effect from excess mouse handling due to oral gavage.

Next, we analyzed the efficacy of B. dentium in reducing maternal or fetal inflammation in both non-stressed and stressed mice. *B. dentium* gavage reduced *Il6* expression in maternal ileal tissue (Fig. 3A) ($F(1,11) = 6.039$, *B. dentium* factor- $p = 0.0289$) and fetal brain tissue (Fig. 3B)($F(1,36) = 6.248$, *B. dentium* Factor- $p = 0.0171$, but it had no effect on the stressor-induced increase in fetal liver *Il6* expression. Maternal ileal *Ccl2* gene expression exhibited a trend towards a decrease by *B. dentium* (Fig. 3D) ($F(1,19) = 3.445$, *B. dentium* Factor- $p = 0.079$). Fetal brain *CCL2* was unchanged by either the microbe (Fig. 3E), while fetal liver *Ccl2* showed an interaction trend (Fig. 3F) ($F(1,34) = 3.621$, Interaction Factor-$p = 0.0656$), wherein SV was significantly reduced compared to NSV ($p < 0.05$) and significantly reduced compared to SP ($p < 0.05$). *Tnfα* levels were unaffected by *B. dentium* treatment in any of the tissues (Fig. 3G-I). In all, these data highlight that *B. dentium* can mediate inflammatory output in a narrowly focused manner, as *Il6* and *Ccl2* were affected but *Tnfα* was not.

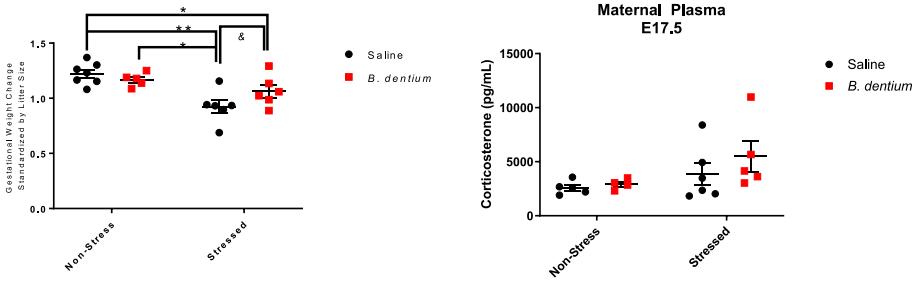

Fig. 1. Prenatal stress effects on gestational outcomes are partially abrogated by *Bifidobacterium dentium* administration. a. Litter weight gain standardized by litter size (E17.5 wt – E10.5 wt); n = 5–7. Two-way ANOVA Stress Factor- $F(1,20) = 17.27$, $p = 0.0005$; LSD post-hoc- NSV vs. SV- $p = 0.0002$; NSV vs. SP- $p = 0.0247$; NSP vs SV-$p = 0.0028$. b. Maternal plasma corticosterone levels at E17.5; n = 4–6. Two-way ANOVA Stress Factor- $F(1,16) = 3.779$, $p = 0.0697$. *- ANOVA $p < 0.05$; **- ANOVA $p < 0.0005$; &-ANOVA $p < 0.10$.

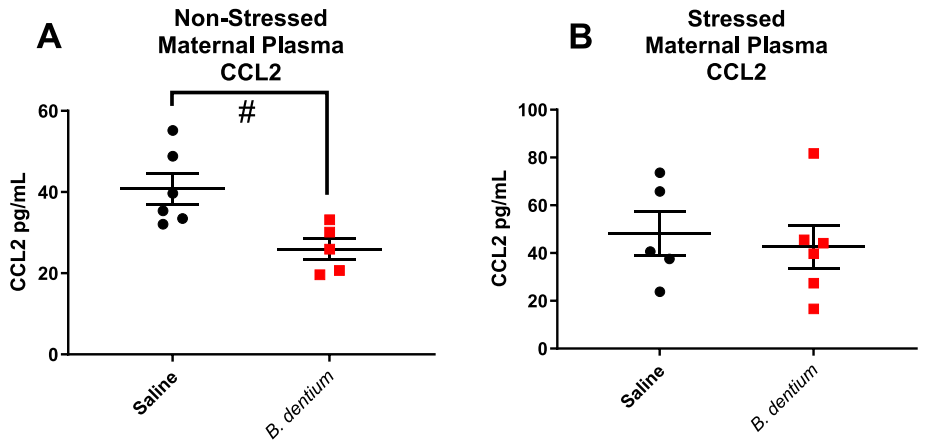

Fig. 2. *Bifidobacterium dentium* reduces CCL2 levels in non-stressed pregnant mice. a. Corticosterone ELISA of maternal plasma of non-stressed dams at E17.5, n = 5–6. T-test- t = 3.086, p = 0.0130. b. Corticosterone ELISA of maternal plasma of stressed dams at E17.5; n = 5–6. T-test- p = not significant. #-T-test p < 0.05.

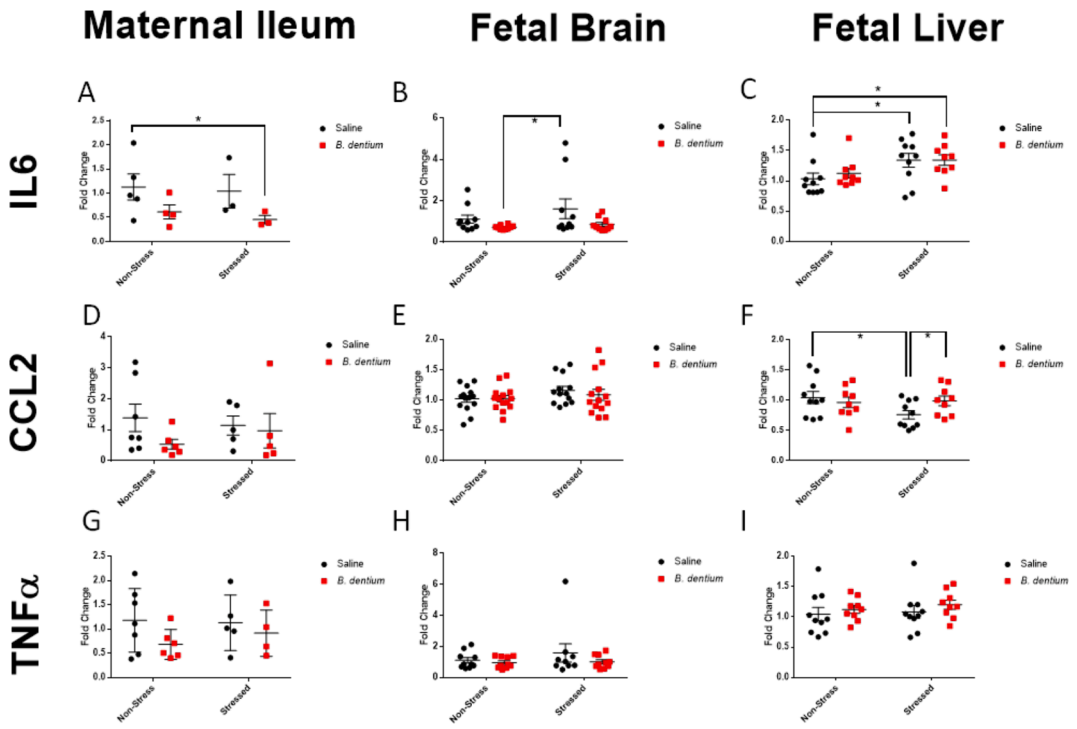

Fig. 3. *Bifidobacterium dentium* administration results in inflammatory reductions in both dam and fetus. qPCR of inflammatory targets in maternal ileum, fetal brain, and fetal liver samples, utilizing the ddCt method for fold change differences. Statistics are based on dCt. a. Maternal Ileum *Il6;* n = 3–5. Two-way ANOVA *B. dentium* Factor- F(1,11) = 6.309, p = 0.0289; LSD post-hoc- NSV vs. SP- p = 0.0477. b. Fetal brain *Il6;* n = 10. Two-way ANOVA *B. dentium* Factor- F(1,36) = 6.248, p = 0.0171; LSD post-hoc- NSP vs SV- p = 0.0156. c. Fetal liver *Il6;* n = 9–10. Two-way ANOVA Stress Factor- F(1,34) = 7.291, p = 0.0105; LSD post-hoc- NSV vs SP- p = 0.0190, NSV vs. SV- p = 0.0267. d. Maternal ileum *Ccl2;* n = 5–7. Two-way ANOVA *B.* dentium Factor- F(1,19) = 3.445, p = 0.079. e. Fetal brain *Ccl2;* n = 13–14. Two-way ANOVA, p = not significant. f. Fetal liver *Ccl2;* n = 9–10. Two-way ANOVA Interaction Factor- F(1,34) = 3.621, p = 0.0656; LSD post-hoc- NSV vs. SV- p = 0.0185; SV vs. SP- p = 0.0425. g. Maternal ileum *Tnfα*, n = 4–7. Two-way ANOVA, p = not significant. h. Fetal brain *Tnfα*, n = 9–10. Two-way ANOVA, p = not significant. i. Fetal liver *Tnfα*, n = 9–10. Two-way ANOVA, p = not significant. *- ANOVA p < 0.05.

3.4. Kynurenine- and serotonin- associated gene expression may be modulated by B. dentium gavage

PNS disrupts genes involved in the kynurenine metabolic pathway, including tryptophan 2,3-dioxygenase 2 (*Tdo2*) in the fetal brain (Galley et al., 2021), and can affect overall kynurenine levels in the dam (Notarangelo and Schwarcz, 2017). Since aberrant levels of kynurenine and its downstream metabolites have been associated with cognitive deficiency, poor neurodevelopment, and neurological disease (Forrest et al., 2015; Solvang et al., 2019; Pearson and Reynolds, 1992), we next examined if PNS disrupted the expression of these genes. However, we did not observe a stress effect on either *Tdo2* or *Ido1* expression, potentially due to secondary effects of gavage handling (Fig. 4A-D).

Members of Bifidobacteria are able to metabolize tryptophan (Tian et al., 2022; Fang et al., 2022), but as yet, it is unknown if *B. dentium* harbors these capabilities, though it is able to influence the abundance of aromatic amino acids (Luck et al., 2021). We sought to determine if *B. dentium* gavage could regulate the expression of either *Tdo2* or indole 2,3-dioxygenase 1 (*Ido1)*, both of which are involved in kynurenine synthesis from tryptophan, in maternal and fetal tissues at E17.5

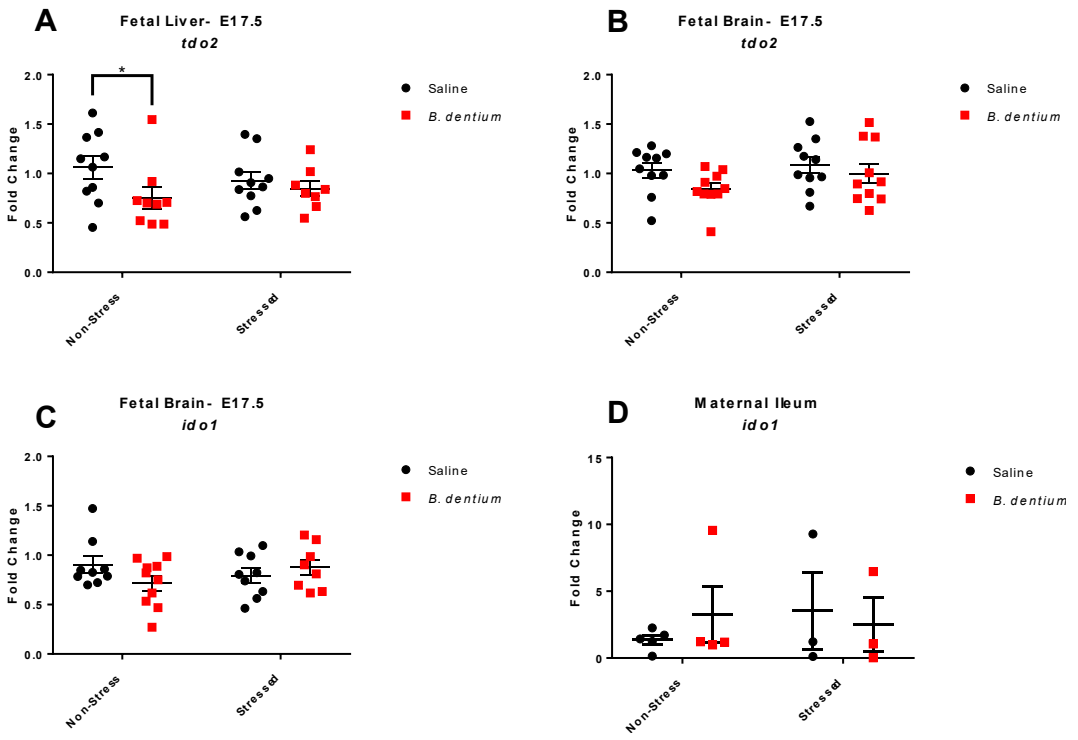

Fig. 4. Kynurenine metabolic genes exhibit a trend for down-regulation by *Bifidobacterium dentium*. qPCR of kynurenine targets in maternal ileum, fetal brain, and fetal liver samples, utilizing the ddCt method for fold change differences. Statistics are based on dCt. a. Fetal liver *Tdo2*; n = 8–10. Two-way ANOVA *B. dentium* Factor- F(1,33) = 3.968, p = 0.0547; LSD post-hoc- NSV vs. NSP- p = 0.0275. b. Fetal brain *Tdo2;* n = 9–10. Two-way ANOVA B. dentium Factor- F(1,34) = 3.993, p = 0.0537. c. Fetal brain *Ido1;* n = 8–10. Two-way ANOVA, p = not significant. d. Maternal ileum *do1;* n = 3–5. Two-way ANOVA, p = not significant.

following the completion of the PNS stressor exposure. *B. dentium* administration trended towards a significant reduction on *Tdo2* expression in fetal liver (Fig. 4A) (F(1,33) = 3.968, *B. dentium* Factor-p = 0.0547) and in the fetal brain (Fig. 4B) (F(1,34) = 3.993, *B. dentium* Factor- p = 0.0537). Further post-hoc analysis indicated that NSP offspring *Tdo2* expression was significantly reduced compared to NSV offspring in fetal liver only. *Ido1* expression was unchanged (Fig. 4C-D). However, these data do suggest that *B. dentium* is able to affect offspring systemic and neurological kynurenine metabolism.

Since kynurenine is only one arm of tryptophan metabolism, we also measured gene expression for the other arm, serotonin. PNS is associated with microbiota-dependent elevations in placental serotonin (Chen et al., 2020), though serotonin is reduced by stress in other studies that have utilized both pregnant and non-pregnant mice (Moura et al., 2022; O'Connor et al., 2021; Kiank et al., 2010). We targeted both *Tph1* (in fetal liver and maternal ileum) and *Tph2* (in fetal brain), as well as placental *Tph1* at E17.5. Placental *Tph1* was not affected by PNS (data not shown). While PNS did not affect fetal brain *Tph2* (Fig. 5A) or fetal liver *Tph1* (Fig. 5B), PNS exposure did exhibit a trend towards reducing *Tph1* in maternal ileum (Fig. 5C) (F(1,9) = 4.404, Stress Factor- p = 0.0653).

B. dentium has been shown to increase tryptophan hydroxylase 1 (*Tph1*) expression, which metabolizes serotonin from Trp, increasing serotonin in enteroid modelling (Engevik et al., 2021). Serotonin has a myriad of functions, and influences neurodevelopment, immunity, cognition, and overall mental health (Wu et al., 2019; Shajib et al., 2017; Hanswijk et al., 2020) and therefore, it would be important to examine how *B. dentium* might modulate it during prenatal stress, and particularly in a model of pregnancy. As with PNS, *B. dentium* did not affect placental *Tph2* (data not shown). Interestingly, we found that *B. dentium* abrogated *Tph2* expression in the fetal brain (Fig. 5A) (F(1,36) = 5.23, *B. dentium* Factor- p = 0.0282). The microbe had no effect on *Tph1* expression in fetal liver (Fig. 5B) or maternal ileum (Fig. 5C). In sum, these data highlight that *B. dentium* can affect serotonin synthesis from Trp but only through *Tph2*.

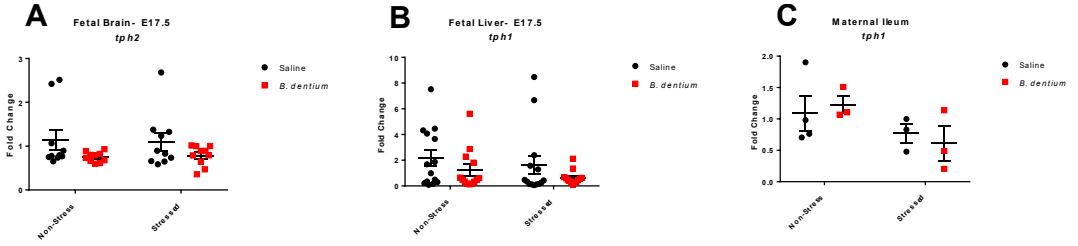

Fig. 5. Fetal brain serotonin metabolic genes are down-regulated by *Bifidobacterium dentium*. qPCR of serotonin targets in maternal ileum, fetal brain, and fetal liver samples, utilizing the ddCt method for fold change differences. Statistics are based on dCt. a. Fetal brain *Tph2*, n = 9–10. Two-way ANOVA *B. dentium* Factor- F(1,36) = 5.23, p = 0.0282. b. Fetal liver *Tph1*, n = 13–14. Two way ANOVA, p = not significant. c. Maternal ileum *Tph1*, n = 3–4. Two way ANOVA Stress Factor- F(1,9) = 4.404, p = 0.0653. *- ANOVA $p < 0.05$.

3.5. B. dentium modulates Trp-associated metabolites in both dam and offspring plasma

We next examined if effects on Trp metabolite gene expression might be extrapolated to actual metabolite levels. LC-MS/MS was performed on both maternal and fetal plasma to examine systemic levels of Trp-associated metabolites and how they might be transmitted from dam to offspring intergenerationally. We focused on Trp and each of the three major arms of Trp metabolism: serotonin, kynurenine (as well as kynurenic acid (KA)) and indole (I3LA, I3PA, I3AA) to better understand how PNS might shunt metabolism in different directions. Indole analysis was included as the bacterially-derived indoles have been implicated in anti-inflammatory activity(Meng et al., 2020; Ji et al., 2020; Scott et al., 2020; Sun et al., 2022). PNS exposure reduced maternal plasma Trp levels (Fig. 6A) ($F(1,17) = 4.839$, Stress Factor- p-0.0419), but this effect was not seen in the fetal plasma (Fig. 6B). Of note, maternal plasma kynurenine levels were unaffected by stress (Fig. 6C). Maternal or fetal plasma serotonin levels were unaffected by stress (Fig. 6E-F). Indole levels in the maternal plasma showed a trend towards a reduction due to stress (Fig. 6G) ($F(1,17) = 4.399$, Stress Factor-$p = 0.0512$).

Next, since *B. dentium* affected both kynurenine and serotonin gene expression, particularly in the fetal brain, we studied how *B. dentium* affects these levels concomitant with the stressor. *B. dentium* had no effect on Trp levels in either dam or fetus (Fig. 6A-B). Likewise, there was no *B. dentium* effect on maternal plasma kynurenine (Fig. 6C) while fetal plasma kynurenine displayed a slight trend towards an interaction effect with PNS (Fig. 6D) ($F(1,15) = 3.094$, Interaction Factor- p-0.0989). Specifically, fetal plasma kynurenine was increased in SP compared to SV ($p = 0.035$). *B. dentium* administration was associated with a reduction in abundance of maternal plasma serotonin (Fig. 6E) ($F(1,14)$-6.501, *B. dentium* Factor- $p = 0.0231$). Post-hoc analysis indicated that SP dam serotonin was significantly reduced compared to SV dams ($p = 0.009$). This pattern was not observed in the fetal plasma (Fig. 6F). Lastly, while *B. dentium* did not have an effect on maternal plasma indole (Fig. 6G), there was an interaction trend with PNS in the fetal plasma (Fig. 6H)($F(1,15) = 3.467$, Interaction Factor- $p = 0.0823$). Post-hoc analysis revealed trends for SV fetuses to have reduced indole compared to both NSV ($p = 0.0545$) and SP ($p = 0.056$) offspring, suggesting that stressor-induced reductions in indole in the fetus may be rescued by *B. dentium* gavage to the mother.

In our analysis of further downstream Trp metabolism, we found that KA exhibited a PNS effect in maternal plasma (Fig. 7A)($F(1,17) = 4.831$, Stress Factor- $p = 0.0421$), while no such effect was evident in fetal plasma (Fig. 7B). There were no PNS effects on l3PA (Fig. 7C-D), but PNS did reduce I3LA levels in the maternal plasma (Fig. 7E)(($F(1,17) = 7.076$, Stress Factor- $p = 0.0165$), while having no effect on fetal plasma levels (Fig. 7F). Lastly, there was no PNS effect on I3AA levels (Fig. 7G-H).

Next, we analyzed how B. dentium affected these downstream metabolites in both the presence and absence of PNS. *B. dentium* increased kynurenic acid levels in maternal plasma (Fig. 7A); $F(1,17) = 8.287$, *B. dentium* Factor- $p = 0.0104$). Post-hoc analysis indicated that the NSP group was significantly increased over NSV ($p = 0.0042$), SV ($p = 0.0029$), and SP ($p = 0.0143$) dams, suggesting that *B. dentium* can increase KA levels, but stressor exposure abolishes this metabolic shift. Fetal plasma exhibited a similar result, wherein *B. dentium* increased overall KA levels (Fig. 7B) ($F(1,14) = 13.39$, *B. dentium* Factor- $p = 0.0026$), as NSP was increased over all other groups ($p < 0.05$), further indicating that *B. dentium*-directed influence on kynurenine metabolism can be transmitted from dam to offspring. Likewise, *B. dentium* increased I3PA in both maternal plasma (Fig. 7C) ($F(1,17) = 6.808$, *B. dentium* Factor- $p = 0.0183$) (NSP vs. NSV, SV, SP- $p < 0.05$) and fetal plasma (Fig. 7D) ($F(1,14) = 12.82$, *B. dentium* Factor- $p = 0.003$) (NSP vs. NSV, SV, SP- $p < 0.05$). I3LA was not affected by *B. dentium* (Fig. 7E). However, post-hoc analysis of the aforementioned PNS effect demonstrated that while SV was significantly reduced compared to both NSV and NSP ($p < 0.05$), SP were not significantly altered compared to the non-stress groups. I3LA was unchanged by *B. dentium* in the fetal plasma (Fig. 7G). I3AA had a trend towards an interaction effect between PNS and *B. dentium* (Fig. 7H) ($F(1,17) = 4.36$, Interaction- $p = 0.0521$), as NSP was increased over NSV ($p = 0.0454$), but this was abolished by PNS exposure ($p = 0.0372$). Altogether, these metabolomic data strongly suggest that *B. dentium* is able to modulate and increase certain Trp metabolites, primarily KA and I3PA. However, these outputs can be ablated by PNS.

3.6. B. dentium gavage increases social behavior in a sex-specific manner

Shifts in social behavior in the adult offspring of dams exposed to PNS has been a key finding in past studies (Chen et al., 2020; Gur et al., 2019; Gur et al., 2017). We interrogated the effect of *B. dentium* on two social behavior metrics: sociability and social novelty. In the sociability

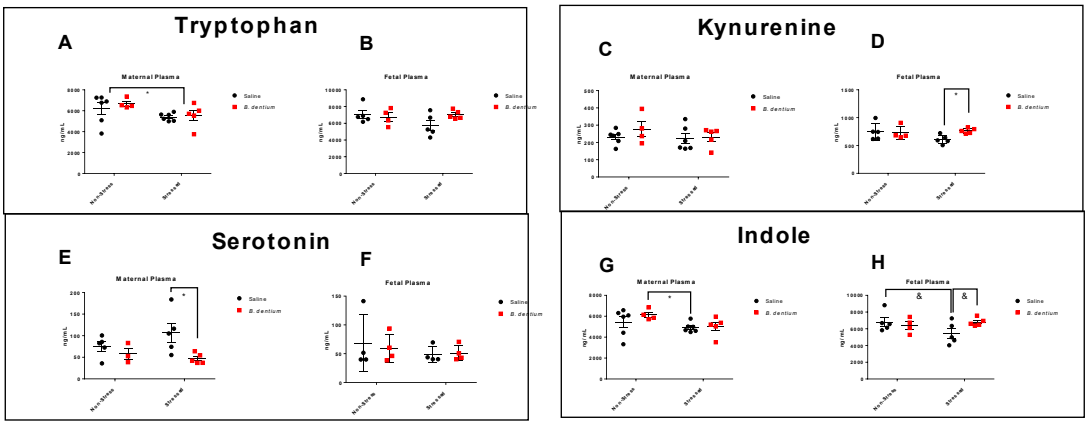

Fig. 6. Tryptophan-associated metabolites in maternal and fetal plasma are differentially affected by prenatal stress and/or *Bifidobacterium dentium* gavage. a. Maternal plasma tryptophan levels, n = 4–6. Two-way ANOVA Stress Factor- $F(1,17) = 4.839$, $p = 0.0419$. b. Fetal plasma tryptophan levels, n = 4–5. Two-way ANOVA- p = not significant. c. Maternal plasma kynurenine levels, n = 4–6. Two-way ANOVA- p = not significant. d. Fetal plasma kynurenine levels, n = 4–5. Two-way ANOVA Interaction Factor- $F(1,15) = 3.094$, $p = 0.0989$; LSD post-hoc- SV vs. SP- $p = 0.0355$. e. Maternal plasma serotonin levels, n = 3–5. Two-way ANOVA *B. dentium* Factor- $F(1,14) = 6.501$, $p = 0.0231$; LSD post-hoc- SV vs. SP- $p = 0.009$. f. fetal plasma serotonin levels, n = 4. Two-way ANOVA- p = not significant. g. maternal plasma indole levels, n = 4–6. Two-way ANOVA Stress Factor- $F(1,17) = 4.399$, $p = 0.0512$; LSD post-hoc- NSP vs. SV- $p = 0.0485$. h. fetal plasma indole levels, n = 4–5. Two-way ANOVA Interaction Factor- $F(1,15) = 3.467$, $p = 0.0823$; LSD post-hoc- NSV vs. SP- $p = 0.0545$; SV vs. SP- $p = 0.056$. *-ANOVA $p < 0.05$; &- ANOVA $p < 0.10$.

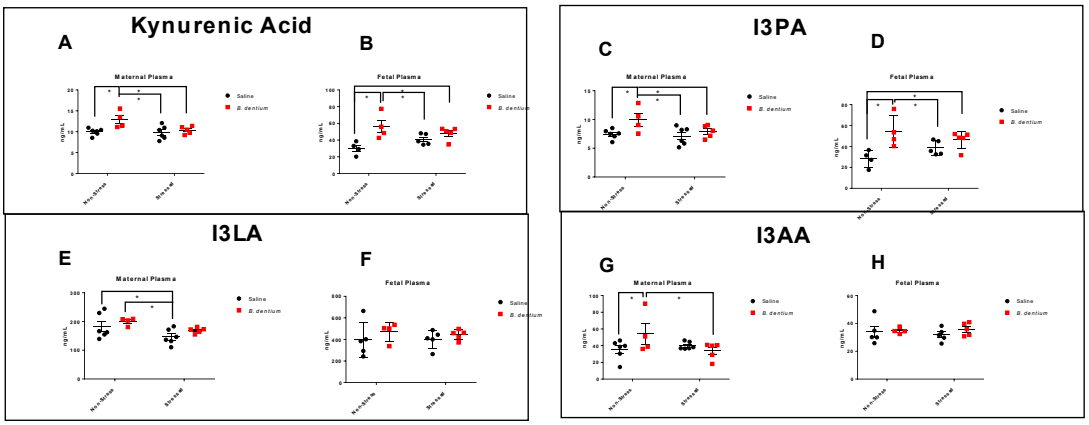

Fig. 7. Downstream tryptophan metabolites, including kynurenic acid, are elevated by *Bifidobacterium dentium*. a. maternal plasma kynurenic acid levels, n = 4–6. Two-way ANOVA Stress Factor- F(1,17) = 4.831, p = 0.0421; *B. dentium* Factor- F(1,17) = 8.287, p = 0.0104; LSD post-hoc- NSV vs. NSP- p = 0.0042; NSP vs. SV- p = 0.0029; NSP vs. SP- p = 0.0143. b. fetal plasma kynurenic acid levels, n = 4–5. Two-way ANOVA *B. dentium* Factor- F(1,14) = 13.39, p = 0.0026. LSD post-hoc- NSV vs. NSP- p = 0.0019; NSV vs. SP- p = 0.0144; NSP vs. SV- p = 0.0318. c. maternal plasma indole-3-propionic acid levels, n = 4–6. Two-way ANOVA *B. dentium* Factor- F(1,17) = 6.808, p = 0.0183; LSD post-hoc- NSV vs. NSP- p = 0.0143; NSP vs SV- p = 0.0069; NSP vs. SP- p = 0.0471. d. fetal plasma indole-3-propionic acid levels, n = 4–5. Two-way ANOVA *B. dentium* Factor- F(1,14) = 12.82, p = 0.003; LSD post-hoc- NSV vs. NSP- p = 0.0024; NSV vs. SP- p = 0.0167; NSP vs. SV- p = 0.0343. e. maternal plasma indole-3-lactic acid levels, n = 4–6. Two-way ANOVA Stress Factor- F(1,17) = 7.076, p = 0.0165; LSD post-hoc- NSV vs. SV- p = 0.0425; NSP vs. SV- p = 0.0098. f. fetal plasma indole-3-lactic acid levels, n = 4–5. Two-way ANOVA- p = not significant. g. maternal plasma indole-3-acetic acid levels, n = 4–6. Two-way ANOVA Interaction Factor, F(1,17) = 4.36, p = 0.0521; LSD post-hoc- NSV vs. NSP- p = 0.0454; NSP vs. SP- p = 0.0372. h. fetal plasma indole-3-acetic acid levels, n = 4–5. Two-way ANOVA, p = not significant. *- ANOVA p < 0.05.

test, which measures a test mouse's preference for a social mouse over an object, we demonstrated an interaction effect (PNS x *B. dentium*) on (Fig. 8A) (F(1,33) = 9.946, Stress Factor- p = 0.0034; Interaction Factor- F(1,33) = 5.565, p = 0.0244) the total number of entries the test mouse made to the social side of the box. Post-hoc analysis indicated that NSP is increased over NSV, SV, and SP (ps < 0.05). This result is sex-specific, as it was not present in females (Fig. 8B) but was in the males (Fig. 8C) (F (1,13) = 12.29, Stress Factor- p = 0.0035) (NSP vs. NSV/SV/SP- ps < 0.05). There was also an interaction effect of the probiotic with PNS on the sociability index (Fig. 8D) (F(1,33) = 4.468, Interaction Factor- p =

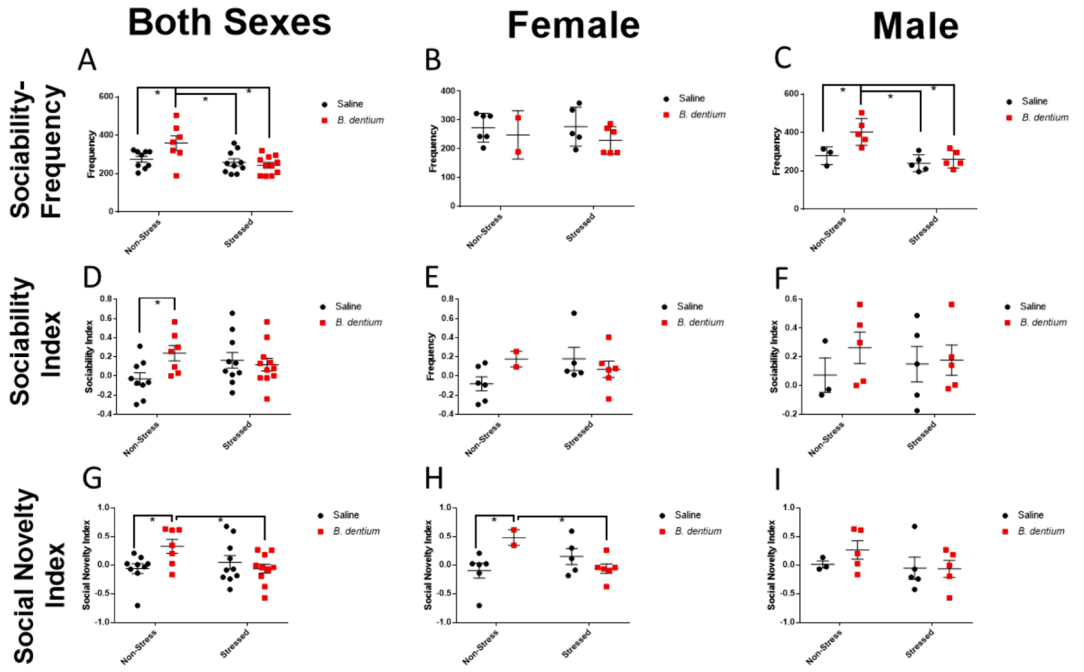

Fig. 8. *Bifidobacterium dentium* increases adult offspring social behavior in a sex-specific manner. (a.-c.) Sociability Social Box Frequency- a. Both sexes, n = 7–11. Two-way ANOVA Interaction Factor- F(1,33) = 5.565, p = 0.0244; Stress Factor- F(1,33) = 9.946, p = 0.0034; LSD post-hoc- NSV vs. NSP- p = 0.0121; NSP v SV- p = 0.0026; NSP v SP- p = 0.0006. b. females, n = 2–6. Two-way ANOVA- p = not significant. c. males, n = 3–5. Two-way ANOVA Stress Factor- F(1,13) = 12.29, p = 0.0035; *B. dentium* Factor- F(1,13) = 7.854, p = 0.0141; LSD post-hoc- NSV vs. NSP- p = 0.0067; NSV vs. SV- p = 0.0003; NSP vs. SP- p = 0.0009 (d.-f.) Sociability Index d. both sexes, n = 7–11. Two-way ANOVA Interaction Factor- F(1,33) = 4.468, p = 0.0422; LSD post-hoc- NSV vs. NSP- p = 0.0225. e. females, n = 2–6. Two-way ANOVA- p = not significant. f. males, n = 3–5. Two-way ANOVA- p = not significant. (g.-i.) Social Novelty Index g. both sexes, n = 7–11. Two-way ANOVA Interaction Factor- F(1,33) = 6.007, p = 0.0197; LSD post-hoc- NSV vs. NSP- p = 0.0166; NSP vs. SP- p = 0.0125. h. females, n = 2–6. Two-way ANOVA Interaction Factor- F(1,33) = 7.929, p = 0.0130; LSD post-hoc- NSV vs. NSP- p = 0.0209; NSP vs. SP- p = 0.029. i. males, n = 3–5. Two-way ANOVA- p = not significant. *- ANOVA p < 0.05.

0.0422), a measurement of the amount of time spent in the social side over the object side, wherein NSP offspring were significantly increased over NSV ($p = 0.0225$), but SV and SP had no change. Male and female offspring had no change by *B. dentium* when analyzed separately (Fig. 8E-F).

Next, we analyzed the social novelty index, which measures time spent by a test mouse with a 'novel' new mouse compared to a familiar mouse. In the combined sex analysis, there was an interaction effect (Fig. 8G) ($F(1,33) = 6.007$, Interaction Factor- $p = 0.0197$). NSP is increased over NSV ($p = 0.0166$), but this is abrogated by stress ($p = 0.0125$). We found that this is specific to females (Fig. 8H) ($F(1,33) = 7.929$, Interaction- $p = 0.0130$) and not observed in males (Fig. 8I). In sum, these data indicate that, as with the metabolomics outcomes, *B. dentium* has beneficial effects on social behavior that can be abolished by PNS.

4. Discussion-

In this study, we focused on probiotic functionality of *Bifidobacterium dentium* to better understand and treat PNS. Maternal stress, anxiety, and depression is associated with lower *B. dentium* abundance, and it has multiple reported beneficial effects upon inflammation, neurodevelopment, and metabolism (Galley et al., 2023; Luck et al., 2021; Engevik et al., 2021; Engevik et al., 2021; Luck et al., 2020). Importantly, many of these outcomes are also negatively impacted by exposure to PNS (Chen et al., 2020; Gur et al., 2019; Gur et al., 2017; Chen et al., 2024; Shang et al., 2021; Jašarević et al., 2015). Therefore, we administered *B. dentium* to dams that were being exposed to our prenatal restraint stress model and examined many of these read-outs in dams as well as offspring at fetal and adult timepoints. We discovered that *B. dentium* administration was associated with anti-inflammatory activity in the dam and fetal offspring, as well as the ability to modulate the downstream metabolism of the aromatic amino acid, Trp, intergenerationally. Lastly, we found that *B. dentium* treatment in dams resulted in elevations in social behavior in their adult offspring, suggesting further intergenerational effects of this probiotic. Many of these outcomes were evident despite the lack of a consistent stress effect, which could potentially stem from heavy animal handling from the oral gavage of the probiotic.

PNS effects on dam and offspring health include maternal immunosuppression and fetal neuroinflammation, mediated by the pro-inflammatory chemokine CCL2, with concomitant elevations in microglia abundance (Chen et al., 2020; Ünal et al., 2022; Chen et al., 2024). In addition, behavioral sequelae include deficits in offspring social behavior and increased anxiety-like behaviour (Gur et al., 2019; Gur et al., 2017). In addition, prenatal restraint stress affects Trp metabolic gene expression as well as the abundance of the metabolites (Galley et al., 2021; Notarangelo and Schwarcz, 2017; Ohta et al., 2017). In this study, PNS effects were not as widespread. While stress affected litter growth and trended towards an increase in maternal corticosterone, which are two typical hallmarks of psychological stress, effects on *CCL2* expression, Trp metabolic gene expression and behavioral outputs were limited. This could potentially be due to the heavy handling procedures the mice endure during oral gavage. Mouse handling can influence behavioral and metabolic outcomes (Ghosal et al., 2015). Thus, all mice having received oral gavage, be it either the microbe or saline control, may have resulted in all test mice having a basement stress effect. Previously reported effects from studies that did not incorporate gavaging, including stressor-induced increases to fetal liver and fetal brain CCL2 as well as reductions in social behavior, were not present in this study (Chen et al., 2020; Chen et al., 2024). Alternatives to oral administration of anaerobes such as *B. dentium* is difficult as time to administration from anaerobic culture must be short. Future studies may take advantage of lyophilized microbial cultures, but would require deeper analysis on potential effects on *B. dentium*'s probiotic efficacy (Bircher et al., 2018). Also, improved habituation to handlers may improve outcomes (Marcotte et al., 2021).

We do report a number of stress-independent effects for *B. dentium*. Interestingly, *B. dentium* improved numerous outcomes in non-stressed animals, including social behavior, KA levels, and I3PA levels, which would indicate that some of the beneficial effects conferred by *B. dentium* upon dam and offspring are sensitive to PNS. An especial focus was placed on social behavior outcomes as these tests can measure behaviors that are considered murine analogs to autism spectrum disorder (ASD), which is associated with social deficits (Zhan et al., 2014). Thus, we sought to determine if *B. dentium* could improve social outcomes similarly to how *Lactobacillus reuteri* can in high-fat diet models (Buffington et al., 2016). The three-chamber test used in this study can accurately demonstrate social deficits in *Shank3*-deficient mice, an animal knockout model for ASD, and in general, has been widely characterized and accepted for social testing (Buffington et al., 2016; Wang et al., 2011; Rein et al., 2020). The three chamber test is not without its limitations, a topic covered in considerable depth by Jabarin et al. (Jabarin et al., 2022). Limitations include lack of standardization and the fact that a single social test cannot properly define social behavior in a mouse. We have attempted to address such shortcomings by following a consistent protocol that has been utilized for multiple publications (Chen et al., 2020; Gur et al., 2019; Chen et al., 2024), while also using advanced Noldus Ethovision software that can accurately follow and measure mouse behaviors.

We also report sex-specificity in the behavior outcomes. In the sociability index, the *B. dentium* effect in non-stressed mice was observed primarily in males, while the *B. dentium* effect in the social novelty index was dominant in females. Male and female rats have displayed unique outcomes in these two social behavior tests: a study by Gillette et al demonstrated that male rats had higher sociability scores and reduced activity compared to females, while in the social novelty test, females scored higher (Gillette et al., 2022). In seeking an understanding of why these specificities exist, we hypothesize that it is highly likely to be due to differential stress effects upon sex hormones including testosterone. Microglia are modulated by sex steroid hormones (Breach and Lenz, 2023) and experimentally manipulating microglia can perturb mouse behavior, dependent upon sex (VanRyzin et al., 2019; Kopec et al., 2018). Thus, a potential explanation for these types of sex-specific outcomes may be that prenatal stress or *B. dentium* are modulating sex hormones, which then affects microglial development, possibly in the fetal compartment. This then leads to behavioral shifts. Comparatively little is known about sex differences in Trp metabolism and as such, this must be a future research focal point.

That *B. dentium* protective effects can be ablated in the presence of stress suggests that the mechanism may be specific to *B. dentium*. We know that *B. dentium* is sensitive to stress in humans (Galley et al., 2023), and this is further evidence that *B. dentium* might be especially susceptible to psychological stress insult. Further investigation into the interplay between PNS hormones such as corticosterone, norepinephrine and epinephrine on the *B. dentium* transcriptome and metabolome is necessary for elucidation.

We report that *B. dentium* increases kynurenine and indole in the plasma of fetuses from stressed dams, while also increasing KA and I3PA in both offspring and dam in the absence of stress. Trp metabolic capabilities for *B. dentium* have not yet been reported, though *B. dentium* can increase serotonin levels and produce GABA (Luck et al., 2021; Engevik et al., 2021). Other Bifidobacteria can increase indoles and KA, including *B. longum, B. infantis* and *B. breve* (Desbonnet et al., 2008; Fang et al., 2022; Sakurai et al., 2019). Probiotic manipulation of KA could have considerable therapeutic benefits, as KA can inhibit excitotoxicity and is generally immunomodulatory (Kiank et al., 2010; Sapko et al., 2006; Salimi Elizei et al., 2017). KA has also been associated with neuropsychological disease, including schizophrenia and cognitive deficiencies (Huang et al., 2020; Pocivavsek et al., 2012). These contrasting relationships imply that there is a fine balance required for KA levels, a concept that has been explored previously (Wirthgen et al.,

2018). Kynurenine and Trp have similar balancing acts. High and low levels of kynurenine have been associated with mood and behavioral disorders (Solvang et al., 2019; Huang et al., 2020; Dostal et al., 2017; Colle et al., 2020), and both depleted and enhanced tryptophan diets have been tied to disease outcomes (Yusufu et al., 2021; Choi et al., 2020; Tsuji et al., 2013; Yin et al., 2021). In this study, *B. dentium* modulated *IL6* and CCL2 levels and improved social behavior indices, suggesting that probiotic administration did not raise KA or kynurenine to deleterious levels and only increases it within normal physiological bounds. *B. dentium* may be able to rescue or even improve the levels of these metabolites to developmentally and immunologically beneficial extents. However, more must be done to examine how the microbe works enzymatically, particularly if mechanisms differ in stressed and non-stressed microenvironments.

Indoles are tryptophan metabolites that are produced by the microbiome. I3PA has myriad protective effects, ranging from IL6 and TNFα neuroinflammation and cognition to gut barrier function and synaptic pruning (Sun et al., 2022; Zhao et al., 2019; Wang et al., 2023). Here we observed *B. dentium*-induced elevations in I3PA in both maternal and fetal plasma, which may provide an understanding of the mechanism by which *B. dentium* is providing therapeutic benefits to the host. *B. dentium* also rescues stressor-induced reductions in fetal plasma indole and kynurenine, while reducing *tdo2* gene expression in the fetus. *tdo2* is increased by immune stimuli (Manuelpillai et al., 2003) and its reduction by *B. dentium* may be part of the probiotic's anti-inflammatory toolset. Likewise, elevations in kynurenine and indole (Meng et al., 2020; Shen et al., 2022; Sorgdrager et al., 2019), which reduce inflammation, may be part of a general immunomodulatory functionality of *B. dentium*. We also report that *B. dentium* decreased *tph2* expression as well as stressor-induced elevations in maternal plasma serotonin levels. This runs counter to previous studies that showed a *B. dentium*-induced increase in serotonin. Our study does differ in that conventional mice were treated with the probiotic as opposed to germ-free mice that were mono-associated and this may also be due to a specific stress amelioration property of *B. dentium*.

The mechanism by which *B. dentium* is mediating these diverse outcomes for both dam and offspring is an open question. However, these data and other studies illustrate that *B. dentium* can influence serotonin, indoles and kynurenines, all three major arms of the Trp pathway, as well as modulate inflammation and social behavior. Given that typical PNS exposure hallmarks include neuroinflammation, microglial increases and morphological changes (Ünal et al., 2022);(Diz-Chaves et al., 2012; Chen et al., 2024), and eventually cognitive and behavioral deficits (Jafari et al., 2017; Shang et al., 2021; Walsh et al., 2019), identifying early intervention strategies is crucial and *B. dentium*'s therapeutic effects lend credence to its functionality as a potential gestational probiotic. That the probiotic is delivered to the dam and the metabolic effect is passed on to the fetal offspring at E17.5 is most intriguing as it indicates that the beneficial properties of a gestational probiotic can safely be conferred upon the fetal offspring. Additional focus must also be placed on mapping *B. dentium*'s metagenomic and metatranscriptomic functionality, in both *in vivo* and *in vitro* designs. *B. dentium* can produce gamma-amino butyric acid (GABA) and tyrosine and induce the production of acetate and serotonin (Luck et al., 2021; Engevik et al., 2021; Engevik et al., 2021). We have demonstrated that *B. dentium* can modulate KA and I3PA, all of which indicates that the microbe is closely associated with neuro-active molecules involved in neurodevelopment and behavior. *In vivo* omics-based investigations, including single-cell metagenomics, would highlight the specific enzymatic machinery being amplified by both *B. dentium* and the larger microbiota in the presence and absence of prenatal stress and with or without *B. dentium* treatment. Potential pathways include *B. dentium* inducing heretofore unreported indole production or other Trp metabolic pathways, or *B. dentium* utilizing Trp enzymatic homologs. Additionally, *in vitro B. dentium* monocultures in combination with Trp metabolites, stress hormones and/or neurotransmitters would allow us to home in on how the latter two may influence and abrogate or even silence *B. dentium* Trp metabolism. This would indicate that *B. dentium* may be sensitive to specific stress hormones, as *Escherichia coli* has been shown to be to serotonin, epinephrine and norepinephrine (Kumar et al., 2020; Bansal et al., 2007).

Future studies must delineate why select *B. dentium* effects can be abolished by the presence of a stressor by parsing the complex interplay between the beneficial metabolic and inflammatory properties of *B. dentium* and the ablative outputs of stressor exposure. Further, we hope that *B. dentium* may one day be a viable probiotic with clinical use. While Engevik et al have reported that *B. dentium* displays acid resistance even at a gut-proportionate pH3 (Engevik et al., 2021), more must be done on characterizing the viability of *B. dentium* within the intestinal tract, especially regarding lyophilization and potential encapsulation. At present however, the data herein provide compelling support for *B. dentium*'s efficacy in improving systemic and neuro-inflammation, Trp metabolism, and social behavior in both dams and offspring.

CRediT authorship contribution statement

Jeffrey D Galley: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Mackenzie K King:** Writing – review & editing, Validation, Investigation, Formal analysis. **Therese A Rajasekera:** Writing – review & editing, Investigation. **Anandi Batabyal:** Writing – review & editing, Investigation. **Samantha T Woodke:** Writing – review & editing, Investigation. **Tamar L Gur:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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