

## Serum cortisol mediates the relationship between fecal *Ruminococcus* and brain N-acetylaspartate in the young pig

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### ABSTRACT

A dynamic relationship between the gut microbiota and brain is pivotal in neonatal development. Dysbiosis of the microbiome may result in altered neurodevelopment; however, it is unclear which specific members of microbiota are most influential and what factors might mediate the relationship between the gut and the brain. Twenty-four vaginally-derived male piglets were subjected to magnetic resonance spectroscopy at 30 d of age. Ascending colon contents, feces, and blood were collected and analyzed for volatile fatty acids, microbiota relative abundance by 16S rRNA, and serum metabolites, respectively. A mediation analysis was performed to assess the mediatory effect of serum biomarkers on the relationship between microbiota and neurometabolites. Results indicated fecal *Ruminococcus* and *Butyricimonas* predicted brain N-acetylaspartate (NAA). Analysis of serum biomarkers indicated *Ruminococcus* independently predicted serum serotonin and cortisol. A 3-step mediation indicated: i) *Ruminococcus* negatively predicted NAA, ii) *Ruminococcus* negatively predicted cortisol, and iii) a significant indirect effect (i.e., the effect of fecal *Ruminococcus* through cortisol on NAA) was observed and the direct effect became insignificant. Thus, serum cortisol fully mediated the relationship between fecal *Ruminococcus* and brain NAA. Using magnetic resonance spectroscopy, this study used a statistical mediation analysis and provides a novel perspective into the potential underlying mechanisms through which the microbiota may shape brain development. This is the first study to link *Ruminococcus*, cortisol, and NAA *in vivo*, and these findings are substantiated by previous literature indicating these factors may be influential in the etiology of neurodevelopmental disorders.

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## Introduction

The term microbiota-gut-brain axis is used to describe the bi-directional communication between the gut microbiota and the brain. Early in life the microbial community of the gut is rapidly evolving in parallel with brain development, providing a window during which highly dynamic changes in the brain could be susceptible to microbial alterations. Throughout this period, myelination, growth and expansion of neurons, and changes in neuro-metabolites are occurring, and the trajectory of these processes may be influenced by microbial dysbiosis.<sup>1,2</sup> In fact, other findings from animal studies have suggested an influence of the microbiota on brain metabolites,<sup>3</sup> behavior,<sup>4</sup> and neurogenesis.<sup>5</sup> Although the underlying mechanisms for these interactions are widely unknown, it has been suggested that bacterial

metabolites could mediate the communication between the gut and the brain.<sup>6</sup>

Early-life changes in the structure and function of the brain commonly result in neurodevelopmental disorders, and there is increasing evidence linking the microbiome to psychiatric and neurodevelopmental disorders, including autism spectrum disorder (ASD). While the microbiome is suggested to play a role in the etiopathogenesis of neurodevelopmental disorders, it is unclear which gut bacteria might be most influential in the observed comorbid brain differences. Current literature is limited in the characterization of specific microbiota, which might modulate particular aspects of brain development. Accordingly, studies using a multimodal, interdisciplinary approach are necessary to understand the complex communication between the gut, the brain, and mediating factors. Use

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of magnetic resonance imaging (MRI) offers a novel, non-invasive approach to answering fundamental questions relating to the gut-brain-axis. To our knowledge, only 2 studies have used this technique, these studies showed that dietary probiotics alter brain function in adult women<sup>7</sup> and brain neurometabolites in mice.<sup>3</sup> By elucidating the effects of specific members of microbiota on measures of brain health using MRI, researchers can better understand the underlying mechanisms through which gut microbiota modulate brain development.

When studying the underlying mechanisms of host-microbe interactions in humans, researchers must account for genetic and environmental variation, including diet and supplement use (i.e., probiotics and prebiotics). The use of gnotobiotic and germ-free rodent models has significantly advanced mechanistic microbiome-gut-brain axis research; however, rodent models are limited by physiologic and metabolic differences when compared with humans. Pigs and humans exhibit strikingly similar physiology, immune system function, dietary patterns, and disease progression, thereby elevating the pig as optimal translational model for assessing the interactions between diet, microbiota and host physiology.<sup>8</sup> Moreover, the piglet is a well-established translational model for gastrointestinal<sup>9</sup> and brain development,<sup>10</sup> and its use allows for tightly controlled dietary, genetic, and environmental influences. The objective of this exploratory study was to apply an interdisciplinary approach using the piglet model to: 1) identify predictive relationships between microbiota and magnetic resonance spectroscopy-derived neurometabolites, 2) identify predictive relationships between microbiota and intermediary biomarkers, and 3) characterize how the identified biomarkers mediate the relationship between microbiota and brain metabolites. To our knowledge, this is the first time this multimodal approach has been applied to elucidate possible mechanisms between the microbiome-gut-brain axis.

## Methods and materials

All animal care and experimental procedures were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

## Animals and housing

Twenty-four vaginally-derived intact male Yorkshire piglets from the University of Illinois Imported Swine Research Laboratory were obtained 48 h after birth, to allow for adequate colostrum consumption, and were subsequently artificially reared over a 30 d trial period. Piglets were provided one of 2 experimental diets, over the course of the 30 d trial, a detailed description of dietary treatment was described previously,<sup>11,12</sup> and diet was not significant in any of the outcomes assessed herein. A detailed description of animal husbandry was described previously,<sup>11,12</sup> and details of the non-invasive neuroimaging procedures performed at 30 d of age are described below. At 31 d of age, piglets were anesthetized by intramuscular injection at 0.03 mL/kg bodyweight using a telazol:ketamine:xylazine solution [50.0 mg tiletamine plus 50.0 mg zolazepam reconstituted with 2.50 mL ketamine (100 g/L) and 2.50 mL xylazine (100 g/L); Fort Dodge Animal Health]. Upon verifying anesthetic induction, piglets were euthanized via intracardiac administration of sodium pentobarbital (86.0 mg/kg bodyweight; Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA). Immediately following intracardiac injection, piglet heads were removed and trunk blood was collected. Whole blood was centrifuged and serum was collected and stored at  $-80^{\circ}\text{C}$  for future analysis. Ascending colon (AC) (the proximal one third of the colon) contents and feces were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for volatile fatty acids and microbiota analyses.

## Magnetic resonance imaging

Piglets were subjected to MRI procedures at 30 d-of-age, as described previously.<sup>11</sup> All scans were acquired using a Siemens MAGNETOM Trio 3T Imager using a Siemens 32-channel head coil. Magnetic resonance spectroscopy (MRS) was used to non-invasively quantify metabolites both in the hippocampi and intervening tissue, as described previously.<sup>13,14</sup> In short, an MRS spin-echo chemical shift sequence was used to assess a voxel which centered over the left and right dorsal hippocampi with a size of 12 mm  $\times$  25 mm  $\times$  12 mm. Sequence parameters used for MRS data acquisition are as follows: TR = 3000 ms; TE = 30 ms; signal averages = 128 (water-suppressed scan) and 8 (non-water suppressed scan); vector size = 1024 point. Water-suppressed and non-water-suppressed data were acquired in institutional units, and all MRS data were analyzed using LC Model (version 6.3), using methods described previously.<sup>13,14</sup>

Only neurometabolites for which a single data point was obtained for all piglets on the study were analyzed. Thus, neurometabolites in this analysis include: creatine + phosphocreatine (Cr+Pcr), glycerophosphocholine + phosphocholine (GPC+PCh), myo-inositol (mI), n-acetylaspartate (NAA), n-acetylaspartate + n-acetylaspartylglutamate (NAA+NAAG).

### **Volatile fatty acid quantification**

Concentrations of volatile fatty acids (VFA) in AC contents were determined as described previously.<sup>15</sup> The VFA concentrations were quantified using a Hewlett-Packard 5890A Series gas chromatograph and a glass column (180 cm 3 4 mm i.d.) packed with 10% SP-1200/1% H<sub>3</sub>PO<sub>4</sub> on 80/100 mesh Chromosorb WAW (Superlco). Volatile fatty acid production was calculated as VFA concentrations of substrate-containing tubes minus the VFA content of blank tubes divided by substrate weight expressed on a dry matter basis.

### **Pyrosequencing of bacterial 16S rRNA genes**

DNA extraction, 16S rRNA gene sequencing and sequence analysis were performed as described previously.<sup>12</sup> Briefly, DNA was isolated from AC contents and feces using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) in combination with the FastPrep-24 System (MP Biomedicals, Carlsbad, CA). DNA concentration was determined with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies). The V3 to V5 regions of the bacterial 16S rRNA genes were amplified for bacterial pyrosequencing with primers 341F and 907R (Klindworth et al 2013). Pyrosequencing was performed at Research Testing Laboratory (RTL; Lubbock, TX) using 454 Life Sciences Genome Sequencer FLX with Titanium series reagents and protocol (Roche Applied Science, Indianapolis, IN) (Dowd et al 2008, Handl et al 2011). Sequences processing and analysis were performed using a bioinformatic pipeline developed at RTL as described previously.<sup>12</sup> Candidate bacteria used in subsequent regression analysis were limited to only bacterial genera for which data was present for each individual piglet, thus resulting in 18 candidate genera for analysis.

### **Metabolomics**

Serum samples were prepared as described previously<sup>16</sup> by a commercial laboratory (Metabolon, Inc., Research

Triangle Park, NC). Briefly, sample extracts were divided into 5 fractions: 1) acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds; 2) acidic positive ion conditions, chromatographically optimized for more hydrophobic compounds; 3) basic negative ion optimized conditions using a separate dedicated C18 column; 4) negative ionizations following elution from HILIC column; and 5) reserved for backup. Next, non-targeted mass spectrometry analyses were performed, where extracts were subjected to UPLC-MS/MS using methods optimized for each sample fraction; detailed methods described previously.<sup>16</sup> Using a proprietary data analysis pipeline (Metabolon, Inc.), metabolites were characterized by ion features and compared with a reference library of chemical standards.<sup>17,18</sup> Ion peaks were quantified using area-under-the-curve counts. Raw area counts were normalized to account for variation from instrument inter-day tuning differences by the median value for each run-day. As such the median value for each run day was set to 1.0, thus allowing for metabolites of different raw peak curves to be similarly compared.

### **Statistical analysis**

#### **Stepwise regression for genera and diet predicting brain neurometabolites**

Statistical analysis was performed using SPSS Statistical Packages version 24 (SPSS, Inc., Chicago, IL, USA). First, a linear regression equation was created using the stepwise regression procedure in which candidate independent variables (i.e., relative abundances of AC bacterial genera and diet or fecal bacterial genera and diet) predicted the concentration of individual brain metabolites (i.e., derived from MRS); **Table 1**. Multicollinearity between independent variables was assessed using the variance inflation factor (VIF). Within each model, none of the independent variables exceeded VIF factors of 2, thus all candidate variables remained available for use in the final models. Upon construction of models, a Cook's D test was applied to each model to determine the influence of individual observations on the dependent variable; 3 models that met the criterion of a Cook's D value less than 0.5 and are reported herein. The Mallows' Cp value was also calculated and reported for each regression to assess goodness of fit. In the stepwise selection method, entry and stay  $\alpha$  levels were set at 0.05 and 0.10, respectively, for independent variables to be included and remain

**Table 1.** Candidate bacterial genera for inclusion in predictive models of MRS-derived metabolites.<sup>a</sup>

Ascending Colon & Fecal Genera (Candidate Independent Variables)		MRS-derived Metabolite (Dependent Variables)
<i>Butyricimonas</i>	<i>Clostridium XIVa</i>	Myo-Inositol (ml)
<i>Parabacteroides</i>	<i>Clostridium XIVb</i>	N-acetylaspartate (NAA)
<i>Prevotella</i>	<i>Clostridium IV</i>	Creatine+Phosphocreatine (CR+PCr)
<i>Alistipes</i>	Unclassified <i>Lachnospiraceae</i>	N-acetylaspartate+N-acetylaspartylglutamate (NAA+NAAG)
<i>Bacteroides</i>	Unclassified <i>Ruminococcaceae</i>	Glycerophosphocholine+Phosphocholine (GPC+PCh)
<i>Escherichia Shigella</i>	<i>Ruminococcus</i>	
<i>Lactobacillus</i>	<i>Erysipelotrichaceae</i>	
<i>Clostridium Sensu Stricto</i>	<i>Phascolarctobacterium</i>	
<i>Eubacterium</i>	<i>Blautia</i>	

Note. <sup>a</sup>The same genera were included in as candidate variables for both ascending colon and fecal microbiota (candidate independent variables) presented as a relative abundance, MRS-derived metabolites (candidate dependent variables) presented in parts per million.

in the final model. Only linear relationships were tested and unstandardized regression coefficients ( $\beta$ )  $\pm$  SE are presented. This initial regression approach was used to characterize fecal microbiota which may serve as predictive fecal biomarkers for brain neurometabolites.

#### Stepwise regression for individual genus and diet predicting serum serotonin, serum cortisol, and ascending colon volatile fatty acids

From the initial stepwise regressions, the following candidate fecal genera were identified as significant predictors of one or more neurometabolites and were used in subsequent regression analyses to identify possible mediating variables: *Bacteroides*, *Clostridium XIVb*, *Ruminococcus*, and *Butyricimonas*. A linear regression equation was created using the stepwise regression procedure in which candidate independent variables (i.e., individual ascending colon or fecal bacterial genus and diet) predicted one of the dependent variables (i.e., serum serotonin, serum cortisol, or ascending colon volatile fatty acids). It is known that volatile fatty acids and microbiota are related, thus all volatile fatty acids (i.e., acetate, butyrate, propionate, valerate, isobutyrate, and isovalerate) were assessed as possible mediators in this analysis. Previous research indicates serum serotonin and serum cortisol relate to microbiota, thus in an effort to limit the metabolites analyzed in as possible mediators, only serotonin and cortisol were analyzed herein.<sup>1,2</sup> All selection criteria described above were applied to the regression described herein, and only models that met all specified criteria are presented.

#### Mediation analysis

A mediation analysis was performed using the *Process* macro designed for SPSS.<sup>19</sup> The mediation model was

used to characterize the relationship between relative abundances of fecal *Ruminococcus*, serum cortisol, and a MRS-derived brain metabolite (NAA) using a 3-step process. In the context of the following mediation, we tested whether the relationship between the fecal *Ruminococcus* and the MRS-derived brain NAA was mediated by serum cortisol. For a mediation to occur, there must have been a significant indirect effect,<sup>20</sup> meaning the effect of the independent variable (fecal *Ruminococcus*) through the mediator (serum cortisol) on the dependent variable (spectroscopy-derived NAA) must be significant.

The following 3-step framework was used to determine the mediation:

1. Path A: A regression model was used to describe the relationship between the independent variable (*Ruminococcus*) and the dependent variable (NAA).
2. Path B: A regression model was used to describe the relationship between the independent variable (*Ruminococcus*) and the mediator (cortisol).
3. In the third step, the *Process* macro was used and a bootstrapping method applied to estimate the mediation effects. The bootstrapping used 5000 samples with replacement from the data set to estimate the distribution for the direct and the indirect effects. The direct effect indicates the relationship between the independent variable (*Ruminococcus*) and the dependent variable (NAA) (Path A'). The indirect effect describes the relationship from the independent variable (*Ruminococcus*) through the mediator (cortisol) to the dependent variable (NAA) (Path B  $\times$  C).

A significant mediation occurred when the indirect effect did not include zero within the 95% bias-corrected confidence interval, and if the direct



mediation effect did include zero within the 95% bias-corrected confidence interval.<sup>20</sup> Unstandardized regression coefficients ( $\beta$ )  $\pm$  SE and P-values for each regression relationship are presented herein.

## Results

### Piglet characteristics

One piglet was determined to be an outlier (i.e.,  $>3$  SD from the mean) in all analyses and was therefore excluded from the data set, thus 23 subjects were used to generate the results presented herein. Piglet growth assessment was described previously.<sup>12</sup>

### Ascending colon eubacterium predicts brain NAA

Diet and 18 ascending colon genera (candidate independent variables) were entered into a stepwise regression procedure to generate individual linear models for 5 neurometabolites (dependent variables) (Table 1). Using a stepwise regression approach to identify candidate independent variables that predict brain NAA concentrations, *Eubacterium* ( $\beta_1 = -0.412 \pm 0.119$ ;  $R^2_{\text{model}} = 0.364$ ;  $P_{\text{model}} = 0.002$ ) entered the model first; diet and all other candidate genera did not meet criteria to enter the model. Predictive models for brain mI, GPC+PCh, NAA+NAAG, and Cr+PCr did not meet criteria set forth above and were therefore excluded.

### Specific fecal bacterial genera predict brain myo-Inositol (mI), N-acetylaspartate (NAA), and creatine+phosphocreatine (Cr+PCr)

Diet and 18 fecal genera (candidate independent variables) were entered into a stepwise regression procedure to generate individual linear models for 5 neurometabolites (dependent variables) (Table 1). Using a stepwise regression approach to identify

candidate independent variables that predict brain mI concentration (Table 2), *Bacteroides* ( $\beta_1 = 0.039 \pm 0.011$ ;  $P_{\beta_1} = 0.001$ ;  $P_{\text{model}} = 0.002$ ) entered the model first and *Clostridium* XIVb ( $\beta_2 = 0.127 \pm 0.054$ ;  $P_{\beta_2} = 0.031$ ;  $P_{\text{model}} = 0.001$ ) entered the model second; diet and all other candidate genera did not meet criteria to enter the model. When identifying candidate variables that predict brain NAA (Table 2), *Ruminococcus* ( $\beta_1 = -0.160 \pm 0.045$ ;  $P_{\beta_1} = 0.002$ ;  $P_{\text{model}} = 0.002$ ) entered the model first and *Butyrivimonas* ( $\beta_2 = 0.155 \pm 0.060$ ;  $P_{\beta_2} = 0.017$ ;  $P_{\text{model}} = 0.001$ ) entered the model second; diet and all other candidate genera did not meet criteria to enter the model. Lastly, a linear model for brain Cr+PCr (Table 2) indicated that *Bacteroides* ( $\beta_1 = 0.010 \pm 0.004$ ;  $P_{\beta_1} = 0.013$ ;  $P_{\text{model}} = 0.013$ ) entered the model, and diet and all other candidate genera did not meet criteria to enter the model. Predictive models for brain GPC+PCh and NAA+NAAG did not meet criteria set forth above and were therefore excluded.

### Identification of candidate mediating variables for individual genus and MRS metabolite relationships

From the initial stepwise regressions, the following candidate genera were identified as predictors of one or more brain metabolites: ascending colon *Eubacterium*, fecal *Bacteroides*, fecal *Clostridium* XIVb, fecal *Ruminococcus*, and fecal *Butyrivimonas*. Candidate mediating variables in this analysis included: serum serotonin, serum cortisol, and ascending colon volatile fatty acids. Individual stepwise regressions indicated fecal *Bacteroides* was predictive of: serum serotonin ( $\beta_1 = 0.016 \pm 0.007$ ;  $P_{\text{model}} = 0.038$ ; Table 3), ascending colon valerate ( $\beta_1 = -0.174 \pm 0.048$ ;  $P_{\text{model}} = 0.002$ ; Table 4), and total branched-chain fatty acids ( $\beta_1 = -0.317 \pm 0.103$ ;  $P_{\text{model}} = 0.006$ ; Table 4); diet did not meet criteria to enter any of the described models. Similarly, stepwise linear models indicated fecal

**Table 2.** Fecal genera predict MRS-derived brain metabolites.<sup>a</sup>

Dependent Variable	Step	Variable Entered	No. of variables included	Partial R <sup>2</sup>	Model R <sup>2</sup>	C(p)	F-value	P-value
myo-Inositol (mI) <sup>b</sup>	1	<i>Bacteroides</i>	1	0.373	0.373	-9.145	12.51	0.002
	2	<i>Clostridium</i> XIVb	2	0.134	0.507	-9.245	10.28	0.001
N-acetylaspartate (NAA) <sup>c</sup>	1	<i>Ruminococcus</i>	1	0.367	0.367	9.530	12.17	0.002
	2	<i>Butyrivimonas</i>	2	0.160	0.527	4.331	11.13	0.001
Creatine + Phosphocreatine (Cr+PCr) <sup>d</sup>	1	<i>Bacteroides</i>	1	0.261	0.261	22.47	7.424	0.013

Note. <sup>a</sup>Fecal microbiota (independent variable) presented as a relative abundance, MRS-derived neurometabolite (dependent variable) presented in parts per million, diet and other candidate genera did not meet criterion for inclusion in the model, N = 23.

<sup>b</sup>Final model equation predicting brain mI, presented with unstandardized coefficients:  $Y = 7.180 + 0.039x_1 + 0.127x_2$ .

<sup>c</sup>Final model equation predicting brain NAA, presented with unstandardized coefficients:  $Y = 4.997 - 0.160x_1 + 0.155x_2$ .

<sup>d</sup>Final model equation predicting brain Cr+PCr, presented with unstandardized coefficients:  $Y = 3.778 + 0.010x_1$ .

**Table 3.** Fecal *Bacteroides* and *Ruminococcus* predict serum serotonin and cortisol.<sup>a</sup>

Dependent Variable	Step	Variable Entered	No. of variables included	Model R <sup>2</sup>	C(p)	F-value	P-value
Serotonin <sup>b</sup>	1	<i>Bacteroides</i>	1	0.189	2.631	4.885	0.038
Serotonin <sup>c</sup>	1	<i>Ruminococcus</i>	1	0.292	2.180	8.658	0.008
Cortisol <sup>d</sup>	1	<i>Ruminococcus</i>	1	0.360	1.205	11.81	0.002

Note. <sup>a</sup>Fecal microbiota (independent variable) presented as a relative abundance, serum metabolite (dependent variable) presented as scaled import measure, diet did not meet criterion for inclusion in the model, N = 23.

<sup>b</sup>Final model equation predicting serum serotonin, presented with unstandardized coefficients:  $Y = 0.642 + 0.016x_1$ .

<sup>c</sup>Final model equation predicting serum serotonin, presented with unstandardized coefficients:  $Y = 1.188 - 0.169x_1$ .

<sup>d</sup>Final model equation predicting serum cortisol, presented with unstandardized coefficients:  $Y = 1.138 - 0.109x_1$ .

*Ruminococcus* was predictive of serotonin ( $\beta_1 = -0.169 \pm 0.057$ ;  $P_{model} = 0.008$ ; Table 3) and cortisol ( $\beta_1 = -0.109 \pm 0.032$ ;  $P_{model} = 0.002$ ; Table 3); diet did not meet criteria to enter any of the described models. Individual regressions with ascending colon *Eubacterium* or fecal *Clostridium XIVb* and *Butyrivibrio* did not meet selection criteria for inclusion into stepwise predictive models of serum serotonin, serum cortisol, or volatile fatty acids, and were therefore excluded. Previous research indicates serum cortisol and serum serotonin influence the expression of one another, however there was no observed correlation ( $P = 0.148$ ) between cortisol and serotonin in this study.

### Serum cortisol fully mediates the relationship between fecal *Ruminococcus* and MRS-derived brain NAA

A formal mediation analysis was performed to assess the relationship between *Ruminococcus* and NAA through cortisol. The following regression framework was used to elucidate the characteristics of this relationship.

Higher *Ruminococcus* predicted decreased cortisol (Table 3), therefore cortisol was considered as a candidate mediating variable for the previously identified relationship between *Ruminococcus* and NAA (Table 2). Using the 3-step framework for a formal mediation analysis, higher *Ruminococcus* predicted decreased NAA concentrations ( $\beta = -0.160 \pm 0.045$ ;  $R^2_{model} = 0.367$ ;  $P_{model} = 0.002$ , Fig. 1 path A). Next, higher *Ruminococcus* predicted decreased cortisol concentrations ( $\beta = -0.109 \pm 0.032$ ;  $R^2_{model} = 0.360$ ;

$P_{model} = 0.002$ , Fig. 1 path B). Lastly, the formal mediation model, assessing the effects of serum cortisol concentrations on the relationship between *Ruminococcus* and NAA, was significant ( $R^2_{model} = 0.450$ ,  $P_{model} = 0.003$ ). The indirect pathway of the mediation was significant ([95%CI:  $-0.150 - -0.009$ ; Fig. 1 path B  $\times$  C] [ $\beta = 0.573 \pm 0.330$ ;  $P_\beta = 0.098$ ; Fig. 1 path C]), and the direct pathway of the mediation was not significant (95%CI:  $-0.238 - 0.012$ ;  $\beta = -0.113 \pm 0.060$ ;  $P_\beta = 0.075$ ; Fig. 1 path A'). Thus, cortisol concentrations fully mediated the relationship between fecal *Ruminococcus* and brain NAA concentrations.

### Discussion

This study uses multimodal methodology to provide a novel insight into the complex interactions between the microbiome-gut-brain axis in early development. Due to their strikingly similar physiology, immune system function, dietary patterns, and disease progression, pigs have been identified as an optimal translational model for assessing the interactions between diet, microbiota and host physiology.<sup>8</sup> The piglet is considered the best model for human gastrointestinal<sup>9</sup> and brain development<sup>10</sup> with proven clinical translatability. Recent studies have used gnotobiotic piglet models,<sup>21,22</sup> thereby establishing the piglet as a useful model for assessing the influence of specific microbiota throughout development. Using the biomedical piglet model, these findings provide a previously unidentified link between relative abundances of specific bacterial genera,

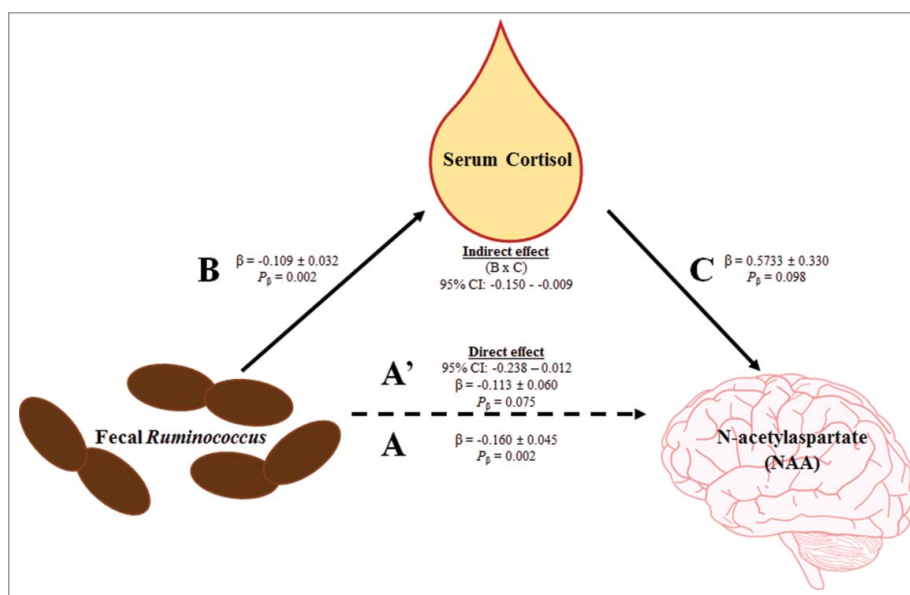
**Table 4.** Fecal *Bacteroides* predicts ascending colon valerate and total branched-chain fatty acid concentrations.<sup>a</sup>

Dependent Variable	Step	Variable Entered	No. of variables included	Model R <sup>2</sup>	C(p)	F-value	P-value
Valerate <sup>b</sup>	1	<i>Bacteroides</i>	1	0.393	1.354	12.97	0.002
Total branched-chain fatty acids <sup>c</sup>	1	<i>Bacteroides</i>	1	0.321	1.004	9.456	0.006

Note. <sup>a</sup>Fecal microbiota (independent variable) presented as a relative abundance, ascending total branched chain fatty acids (dependent variable) presented in  $\mu\text{mol/g}$  (DMB), diet did not meet criterion for inclusion in the model, N = 23.

<sup>b</sup>Final model equation predicting ascending colon valerate, presented with unstandardized coefficients:  $Y = 13.042 - 0.174x_1$ .

<sup>c</sup>Final model equation predicting ascending colon total branched-chain fatty acids, presented with unstandardized coefficients:  $Y = 29.822 - 0.317x_1$ .



**Figure 1.** Serum cortisol fully mediates the relationship between fecal *Ruminococcus* and MRS-derived brain N-acetylaspartate. First, fecal *Ruminococcus* negatively predicted MRS-derived brain NAA concentrations (path A). Second, fecal *Ruminococcus* negatively predicted serum cortisol concentrations (path B). Third, mediation effects were assessed for serum cortisol concentrations. The indirect effect (i.e., the effect of fecal *Ruminococcus* through serum cortisol on MRS-derived brain NAA; path B  $\times$  C) was significant, and the direct effect (path A') was not significant. Thus, serum cortisol fully mediates the relationship between fecal *Ruminococcus* and MRS-derived brain NAA. Abbreviation: N-acetylaspartate (NAA).

neurometabolites and serum metabolites. In doing so, these results may identify a novel mechanism through which the microbiome may influence neurodevelopment. However, it should be noted that the proposed mechanism described herein should be further substantiated through empirical studies, thus this finding is reported as an exploratory analysis and should be considered as hypothesis generating evidence for future work. In the following paragraphs, we will discuss: i) predictive models between fecal microbiota and brain metabolites, ii) predictive models between fecal microbiota and serum/colonic biomarkers, and iii) a mediation model through which serum biomarkers mediate the relationship between fecal microbiota and brain metabolites.

### Fecal microbiota predict neurometabolites

The first objective of this analysis was to identify novel relationships between fecal microbiota and neurometabolites. Our results indicated brain mI, a marker for glial cells and pre-myelinating events in the developing brain,<sup>23</sup> was positively predicted by both *Bacteroides* and *Clostridium* XIVb. Additionally, brain Cr+PCr, an indicator of energy metabolism,<sup>24</sup> was positively predicted by *Bacteroides*. Lastly, these analyses indicated brain NAA, a marker of neuronal

health,<sup>24</sup> was negatively predicted by *Ruminococcus* but positively predicted by *Butyricimonas*. Concentrations of these brain metabolites change over the course of development<sup>23</sup> and our research suggests gut colonization of microbiota may play a pivotal role in this phenomenon. Dysbiosis in the gut microbiota is known to alter behavior in both humans and animal models,<sup>4,25,26</sup> and changes in neurometabolites may underlie these behavioral phenotypes. Interestingly, altered brain Cr+PCr,<sup>27-29</sup> NAA,<sup>30</sup> and mI<sup>27,31</sup> have been reported in subjects diagnosed with ASD, yet no studies have identified specific links between gut microbes and these metabolites. Mice supplemented with a daily dose of *Lactobacillus rhamnosus* for 2 weeks showed an increase in brain NAA quantified by MRS, thus providing evidence that brain metabolite concentrations can be modulated by microbial shifts in the gut.<sup>3</sup> Although *Lactobacillus* was not identified as a predictive bacteria for neurometabolites in this study, we assessed the interaction of *Lactobacillus* and brain NAA and the correlation was not significant, data not shown.

### Fecal microbiota predict serum/colonic biomarkers

The next step of the analysis framework was to identify possible mediatory biomarkers that relate to the

previously identified fecal bacterial genera. This analysis showed that *Bacteroides* positively predicted serum serotonin, whereas *Ruminococcus* negatively predicted both serum serotonin and cortisol. These findings have not previously been reported in the literature. However, correlations between operational taxonomic units that belong to *Ruminococcus* and daily cortisol levels in depressed patients have been reported.<sup>32</sup> Previous research indicates both serotonin and cortisol release can be influenced by bacterial strains.<sup>25</sup> In a probiotic study, supplementation of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 decreased urinary cortisol levels in healthy human volunteers, further indicating bacterial strains can influence cortisol levels.<sup>33</sup> Although *Lactobacillus* was not identified as a predictive bacteria for neurometabolites in this study, we assessed the interaction of *Lactobacillus* and serum cortisol and the correlation was not significant, data not shown. Interestingly, alterations in serum serotonin and cortisol, as well as fecal *Bacteroides* and *Ruminococcus* levels, have been described in patients with ASD.<sup>6</sup>

### Serum cortisol as a mediator between fecal ruminococcus and brain NAA

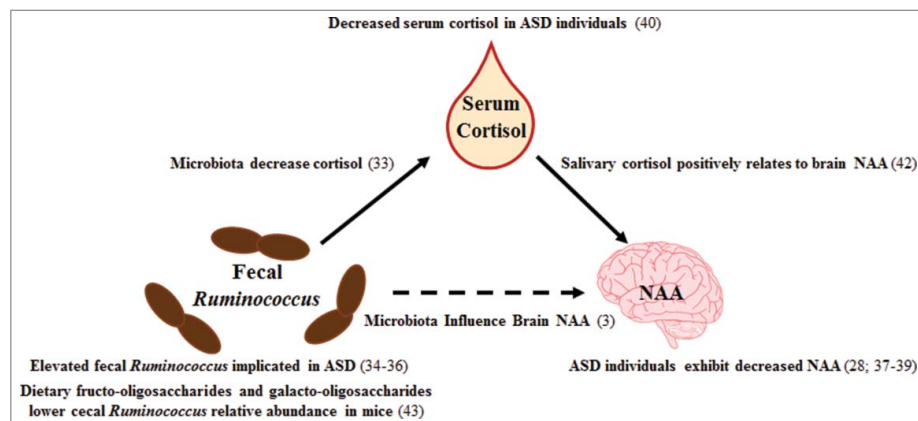
Given the relationships reported in our predictive models, we used a mediation model to characterize the mediatory effects of serum biomarkers on the relationship between fecal microbiota and neurometabolites. Our results provided clear evidence that serum cortisol mediated the relationship between fecal *Ruminococcus* and brain NAA. Interestingly, *Ruminococcus*, cortisol, and NAA have all independently been

implicated in ASD; however, this is the first time a relationship between the 3 has been reported.

First, higher *Ruminococcus* predicted lower NAA. Previous research indicates elevated fecal *Ruminococcus* in subjects with ASD,<sup>34,35</sup> and fecal *Ruminococcus* positively correlated with autistic-like behaviors in a mouse model of ASD.<sup>36</sup> In addition, MRS indicates NAA was decreased in the cerebellum,<sup>37</sup> hippocampus and amygdala,<sup>38</sup> temporal lobe,<sup>39</sup> gray matter and white matter,<sup>28</sup> and whole brains<sup>27</sup> of ASD patients. To our knowledge, no other study has reported a link between *Ruminococcus* and NAA.

Second, higher *Ruminococcus* predicted lower cortisol concentrations. This finding is supported by previous research in which decreased serum cortisol was observed in individuals with ASD.<sup>40</sup> It should be noted that the implications of cortisol in ASD vary widely, with some research suggesting elevated cortisol in ASD patients.<sup>41</sup> Although these inconsistent results could be attributed to differences in methodological approach (i.e., salivary vs. serum cortisol) as well as the heterogeneity of symptoms among individuals with ASD, it is important to note that diverse microbial populations may differentially influence serum cortisol concentrations. As stated previously, supplementation of specific bacterial strains resulted in reduced cortisol in healthy human volunteers.<sup>33</sup> These findings further suggest that specific bacterial strains may differentially impact serum cortisol concentrations, rather than attributing the cortisol changes to a particular disease state.

Third, the indirect pathway of the mediation indicated cortisol has a mediatory effect on the relationship



**Figure 2.** Cited literature for mediation results. Previous studies which support the findings of our mediation analysis are presented in the figure with appropriate literature citations. Abbreviations: autism spectrum disorder (ASD), N-acetylaspartate (NAA).



between *Ruminococcus* and NAA. Previous research shows salivary cortisol positively relates to hippocampal NAA, although it should be noted this observation was made in patients exhibiting posttraumatic stress disorder.<sup>42</sup> Thus, it follows that lower levels of cortisol would relate to lower levels of NAA, thereby supporting our finding. Given the independent reports on *Ruminococcus*, cortisol, and NAA in individuals with ASD (Fig. 2), this mediation analysis could provide a possible mechanism underlying the complex relationship between the microbiota and neurodevelopment in the context of ASD. Interestingly, provision of fructo-oligosaccharides and galacto-oligosaccharides in mice resulted in a reduction in cecal *Ruminococcus* among other bacterial genera, indicating a possible dietary approach to reducing *Ruminococcus* concentrations.<sup>43</sup>

## Conclusion

This study used an interdisciplinary approach through the use of fecal microbiota measures, serum biomarkers, and MRS. To our knowledge, this approach has not been undertaken in previously published studies. The relationships identified herein provide several novel perspectives on the possible mechanisms of microbiome-gut-brain-axis. First, due to the high diversity of the gut microbiota, unravelling specific relationships between bacterial genera and particular brain metabolites is difficult; however, these predictive models identify the most influential genera on individual brain metabolites. Second, identification of these relationships permits a focused approach for selection of mediatory biomarkers. Third, identification of predictive microbes on brain metabolites enables sensitive targeting of the gut microbiota to alter neurometabolites, thereby influencing neurodevelopment. This study reported a predictive relationship between levels of fecal *Ruminococcus*, serum cortisol, and brain NAA, 3 observations that have independently been reported in patients with ASD. While the initial aim of this study was not to focus on ASD etiology, these results provide preliminary data for future research aiming to investigate the microbiota-brain communication in ASD and to develop targeted treatment options for a subgroup of affected individuals.

Our study was limited to assessing fecal microbiota at the genera taxonomic level; thus, future work assessing the impact of specific microbial species may reveal a more detailed microbial signature

as it relates to specific aspects of neurodevelopment. Provided the continued maturation of the brain and the gastrointestinal tract into childhood, longitudinal assessment in the pig model may provide key evidence of critical developmental windows during which microbiota are most influential. Lastly, this novel approach can be implemented in the assessment of other neurodevelopmental disorders that may be influenced by microbiota. While these findings do not specifically identify mechanisms of action between the observed relationships, this exploratory study used a statistical approach that aids in identifying potential mechanisms, which can set the framework for future interventional studies.

From our results we: i) provide a novel perspective into the interaction between fecal microbiota and brain metabolites, ii) provide novel links between fecal microbiota and serum biomarkers implicated in ASD, and iii) bring together 3 independently reported observations in ASD patients to provide a potential mechanism for the communication between one bacterial genera and a specific brain metabolite. The relationships identified in this study may serve as hypothesis-generating findings from which future empirical studies can aim to further investigate the underlying mechanisms proposed in this paper. Additionally, these novel findings may serve as preclinical evidence through which future dietary intervention can be implemented to alter microbial populations and influence neurodevelopment.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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## Author contribution

SMD and RND were involved in initial project conceptualization. ATM was involved in daily project activities. ATM, KB, MW, SMD, and RND were involved in data analysis. All authors were involved in data interpretation and manuscript preparation.

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