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Evaluation of Biogenic Amines in the Faeces of Children with and without Autism by LC-MS/MS

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Abstract

Previous researchers have postulated that gastrointestinal bacteria may contribute to the development and maintenance of Autism Spectrum Disorders (ASD). There is evidence based on quantitative evaluation of the gastrointestinal bacterial population in ASD that this is unlikely and an alternate mechanism will be examined where the bacteria may contribute to the development of ASD via their metabolic products and the role of biogenic amines (BAs) will be investigated. In humans, BAs influence a number of physiological processes via their actions as neurotransmitters, local hormones and gastric acid secretion. Various amines have been implicated in several medical conditions such as schizophrenia and colon cancer. To date, the relationship between BAs and autism has not been explored. This study has been designed to identify differences (and/or similarities) in the level of BAs in faecal samples of autistic children (without gastrointestinal dysfunction: \(n = 14\); with gastrointestinal dysfunction; \(n = 21\)) and their neurotypical siblings (\(n = 35\)) by LC-MS/MS. Regardless of the diagnosis, severity of ASD and
gastrointestinal dysfunction there were no significant differences found between the groups. The findings suggest that BAs in the gastrointestinal tract do not play a role in the pathophysiology of gastrointestinal dysfunction associated with ASD.

**Keywords:** Autism, ASD, biogenic amines, faecal, chromatography.

**Introduction**

Biogenic amines (BAs) are low molecular weight nitrogenous compounds mainly formed and degraded as part of the normal metabolism of animals, plants and microorganisms by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones [1]. The basic structure of BAs can be aromatic (tyramine, phenylethylamine), aliphatic (putrescine, cadaverine, spermine, spermidine) or heterocyclic (histamine, tryptamine). In humans, BAs play a number of physiological roles by acting as neurotransmitters and local hormones [2]. BAs are involved in gastric acid secretion; cell growth and differentiation; they are required for the synthesis of DNA, RNA, and proteins; are involved in the regulation of circadian rhythm; maintenance of body temperature; food intake; learning and memory; immune response and allergic reactions [3; 4; 5]. An essential factor in the formation of BAs in food products and the gastrointestinal (GI) tract is the presence of bacterial strains with the capacity to decarboxylate amino acids. This ability has been described in different genera, species and strains of bacteria, both Gram positive and Gram negative. Amino acid decarboxylases are found in certain Enterobacteriaceae, Clostridium, Lactobacillus, Streptococcus, Micrococcus and Pseudomonas species, among others [6].

The concentrations of BAs present in cells and tissues are strongly controlled at the stages of catabolism, biosynthesis, uptake and efflux, however, the ingestion of foods rich in BAs or production in the intestine by intestinal microbiota can alter the equilibrium [7; 8]. Various BAs have been associated with migraine, schizophrenia, colon cancer and the onset of hypertensive symptoms [4; 9; 10; 11; 12], however, a link between BAs and Autism Spectrum Disorder (ASD) has not been explored despite many researchers suggesting that gastrointestinal bacteria contribute to the development of ASD [13; 14].

ASDs are defined by cognitive defects, impairments of spoken and/or receptive language and social interactions as well as repetitive and stereotyped behaviours. In addition to these core deficits, previous studies have indicated that the prevalence of gastrointestinal distress is high in individuals with ASD [15; 16; 17; 18; 19]. The percentage of individuals suffering from GI problems varies from study to study, but high rates of GI dysfunction in individuals with ASD have been reported in several studies with symptoms including abdominal pain, constipation, diarrhoea, alternating diarrhoea and constipation as well as GI inflammation [20; 21; 22].

In a previous study, we noted that not all ASD individuals suffered from GI dysfunction and that only a sub-population were affected. We also noted that only a subset of this GI dysfunction group had unusual bacterial populations, which included
very high and very low numbers of beneficial bacteria and similarly harmful bacteria, regardless of severity and GI dysfunction [18]. In a subsequent study, we failed to find any significant differences in the microbiome of ASD children (with and without GI dysfunction) and their neurotypical siblings (with and without GI dysfunction) [23], however, given that GI distress is a commonly reported symptom in ASD children, we have designed this follow up study to consider other possible factors that may contribute to GI dysfunction. Therefore, we evaluated BAs which can be produced by microbiota [24] or ingested from food rich in BAs and directly absorbed through the intestinal epithelium. High quantities of tyramine and histamine have been associated with GI symptoms including nausea, abdominal cramping and diarrhoea. Tyramine has been proposed as an initiator of hypertensive crisis [3]. The toxicity of histamine appears to be enhanced by the presence of other amines such as cadaverine, putrescine and tyramine. These biologically active amines have important physiological effects in humans, generally either psychoactive or vasoactive [25]. All have been shown to be involved in the central nervous system responses to stress [26]. BAs also act as releasing agents for the neurotransmitters and abnormally low physiological concentrations often result in clinical depression, whereas abnormally high concentrations have a strong correlation with schizophrenia [27; 28]. To date, no research has evaluated the role of BAs in the GI symptoms of autistic children even though there is good evidence those BAs can influence neurological processes.

Analytical methods for BAs in faecal samples involve high performance liquid chromatography (HPLC). Most biogenic amines require derivatisation to be detected by a UV-Visible or fluorescence detector in HPLC. Therefore, liquid chromatography mass spectrometry (LC-MS) is extensively used for their direct analysis. With LC-MS, the derivatisation step can be avoided and sample processing time can be reduced which is important when a large number of samples are to be analysed. However, LC-MS is often affected by matrix effects where both ion enhancement and suppression have been observed with suggested strategies available if they are apparent [2]. Therefore, the method development process needs to investigate this possibility with faecal samples being a relatively unique matrix compared to other biological matrices such as urine, blood and saliva.

In this study, we have developed a method for the detection of the seven biogenic amines in faecal samples. In this method, amines were extracted with aqueous trichloroacetic acid (TCA), separated by liquid chromatography and detection was achieved by positive ionisation with a triple quadrupole mass spectrometer operating in multiple-reaction monitoring (MRM) mode. This method was applied to a range of faecal samples obtained to identify differences (and/or similarities) in the biogenic amines in children with autism.

2. Experimental
2.1 Sample collection
The study was approved by the Human Research Ethics Committee of Swinburne University of Technology, Australia. Participants were recruited through notices posted on websites and newsletters of various local state and national bodies related to

2.2 Protocol
1. The study was explained to parents and informed consent received.
2. Parents filled out a questionnaire relating to socio-demographic variables and their children’s diagnosis and symptoms (including GI).
3. Stool samples were collected by parents in sterilized faecal sample collection containers during March–June 2011 and transported to Swinburne University in a 1-day express air post satchel containing a frozen gel-iced pack.

Seventy children (n = 35 with ASD and n = 35 neurotypical siblings) with an age range of 2 to 12 years participated in this study (see Table 1 for participant characteristics). The autism severity category was based on CARS (Childhood Autism Rating Scale) assessment conducted by registered psychiatrists or psychologists as reported by the parents of participants. Existing assessments were used; with no confirmatory assessment being conducted. In Australia, formal assessment and reassessment of Autism and Asperger’s disorder occurs annually as part of the requirements of the educational and social services systems. Therefore, we can assume that the assessments were current to within 12 months. The GI dysfunction criteria included one or more of the following listed symptoms: constipation; diarrhoea; abdominal discomfort or pain; gaseousness; foul-smelling stools.

2.3 Reagents and standards
Ultra-pure water from a Millipore Milli-Q system (Milford, MA, USA) was used for the preparation of all the solutions. Trichloroacetic acid (TCA) (≥99%), cadaverine dihydrochloride (>99%), histamine dihydrochloride (>99%), spermidine trihydrochloride (>99.5%), tryptamine hydrochloride (99%), tyramine hydrochloride (99%), agmatine sulphate (≥97%), ammonium formate and formic acid (≥95%) were acquired from Sigma–Aldrich, whereas acetonitrile was obtained from Merck.

A 30mM ammonium formate buffer (pH 4) was prepared and the pH was adjusted with formic acid. Standard solutions of biogenic amines were prepared in Milli-Q water and kept in 2 mL aliquots in plastic vials at −20°C. When required, these were brought to 25°C before preparation of more diluted solutions in 2% TCA each day. A fresh standard mixture of all seven amines (1 mg/mL) was prepared and further diluted for calibration.

2.4 Extraction of biogenic amines
Two millilitres of 10% TCA was added to 1 g of faecal matter that was contained in a 15 mL Falcon tube. The mixture was homogenized by vortex mixing for 1 min, 8 mL of Milli-Q water was added and the mixture was incubated for 30 mins at 4°C. The
mixture was filtered through a Whatman no. 1 filter paper using a vacuum system. The extraction process was carried out on ice to minimise the loss of the more volatile amines. The filter was washed three times by adding 5 mL of 2% TCA each time and the final volume was adjusted to 25 mL with 2% TCA. This solution was filtered through a 0.45 μm nylon syringe filter and stored at −20°C until required for analysis.

2.5 Analysis conditions
Analyses were performed with an Agilent 6410 Triple Quadrupole LC/MS system (Agilent Technologies Inc.). Chromatographic separation was developed on a Luna® 5 μm NH2 100 Å (250×3.0 mm) (Phenomenex, NSW, Australia). Isocratic elution with 30mM ammonium formate adjusted to pH 4 with formic acid and acetonitrile in a ratio of 3:1 was used with a flow rate of 0.5 mL/min and the injection volume was 50 μL. Quantitation of the amines was based on the fragmentation transitions MRM mode as listed in Table 2. The ESI probe tip was 3.5 kV, nitrogen was used as the nebulizer gas and heated to 300°C at 15 psi, and the insource fragmenting voltage was 35 V for putrescine and 70 V for the other amines.

2.6 Statistical analysis
A one-way ANOVA and independent sample t-test were conducted to compare amines between the groups based on ASD severity and GI dysfunction. Statistical significance was defined as a p value of less than 0.05 with BA concentration data presented as means with standard deviations. In addition, Principal Component Analysis (PCA) was performed to further explore if there were any influential factors. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows Statistical Package Version 19.0.

3. Method validations
3.1 Linearity
The linearity of the method for each amine was determined from data collected for six different concentrations of biogenic amines in mixtures ranging from 0.05 - 10 mg/L, using triplicate injections for each mixture of different concentrations. Calibration graphs for each amine were constructed by plotting the peak area against the amine concentration and the correlation coefficient is listed in Table 2. The correlation coefficients indicate that good linearity exists for the range of concentrations tested, which is also given in Table 2.

3.2 Limits of detection and quantification
The limits of detection (LOD) were calculated from the concentration of amines required to give a signal-to-noise ratio of 3. The limits of quantification (LOQ) were determined with a signal-to-noise ratio of 10. Both LOD and LOQ are presented in Table 2.

3.3 Matrix effect
To study the effect of the matrix, 10 mL of faecal extract from the extraction
procedure described in section 2.4 was spiked with a known amount of standard mixture of amines. The faecal extract contained some amines and the areas for the spiked samples were corrected by subtraction (the results of this analysis were compared with a set of external standard mixtures using a four-point calibration curve - 0.5, 1, 2.5, 5 μg/mL prepared in the 2% TCA). Matrix effect (ME) values were calculated according to the following formula [29].

\[ ME = \left( \frac{B}{A} - 1 \right) \times 100\% \]  

Where, \( A \) is the peak area for the external standards and \( B \) is the peak area from the spiked faecal samples. The values in Table 2 indicate that significant and varied ion suppression prevents the use the external standards as prepared in section 2.3 and that standardisation requires the use of standards prepared using spiked faecal samples.

3.4 Recovery
The overall recovery of the sample preparation process as described in section 2.4 was determined by spiking 1 g of faecal sample with a known amount of standard mixture of seven amines in three replicates. Once again the amount of amines initially present was subtracted from the spiked samples. It can be seen from Table 2 that the recovery of amines from the extraction process is quantitative but considering the matrix effects previously described, it has not totally eliminated components from the faeces that influence the ionisation process for the amines. However, the quantitative recovery indicates that linearity is maintained in the sample matrix and standardisation is possible using standards prepared in the faecal matrix. Consequently single point standardisation (2 μg/mL) was used for the samples.

3.5 Final Protocol
The final optimised method was as follows: Sample prepared as specified in section 2.4 and were analysed under condition described in section 2.5. The concentration of the each compound was calculated based on single point standard (2 μg/mL) prepared in the faecal matrix.

Results and discussion
Participants were divided into two groups: those with moderate to severe ASD (n = 19) and those with mild ASD (n = 16) (Table 1). The severity category was based on CARS assessments conducted by registered psychiatrists or psychologists and the child’s score reported by parents of participants. CARS scores of less than 37 are considered mild and those at or above 37 are considered moderate – severe [30]. Details of the specific amine concentrations are given in Table 3 with all concentrations being based on the weight of the wet faecal sample. Analyses of variance revealed no significant differences between the ASD severity groups and the sibling controls on mean levels of all analytes (Table 3). Furthermore, no relationships for the biogenic amines were evident between the GI dysfunction and GI normal
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group (Table 4). However, PCA reveals correlation between GI dysfunction and severity (correlation coefficient 0.578). In autism group, 39% of participants with severe autism and 61% with mild autism suffered from GI dysfunctions compared to 2.8% in sibling control participants. Similar to ANOVA, no correlation was found between any of the biogenic amines, autism severity and GI dysfunctions. However, interestingly there was a correlation between histamine and cadaverine (correlation coefficient 0.807). It has been observed that the presence of cadaverine potentiates the biological activity of histamine in laboratory animals [31] most likely due to increasing the histamine transport across the gastrointestinal wall [32; 33].

Several researchers have hypothesized that the numbers and the species of several GI bacteria such as the *Clostridium* group, *Ruminococcus* and *Desulfovibrio* may contribute to the development of ASD [13; 14; 34]. However, this hypothesis has little consistency with other studies suggesting no significant difference in the bacterial count in the autistic individuals and their neurotypical siblings [13; 17; 23; 35]. By contrast, this study was designed to evaluate the level of BAs in autistic children and determine whether BAs have any possible role in the GI dysfunction associated with autistic disorder. The comparison of BAs in autistic children, with and without GI dysfunction, and comparison of these children with their neurotypical siblings (again, with and without GI dysfunction) did not reveal any significant differences, which suggest that BAs in the GI tract may not play a significant role in the pathophysiology of GI dysfunction associated with ASD. This follow-up study is consistent with our previous study which did not support the hypothesis of a GI bacterial pathophysiological mechanism triggering or maintaining ASD [23] as there were no significant differences in the bacterial composition of faecal material of our paediatric autistic group and their neurotypical siblings.

<table>
<thead>
<tr>
<th>Participant characteristic</th>
<th>Subcategory</th>
<th>Autism (n=35)</th>
<th>Neurotypical sibling (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>Age (years)</td>
<td>All subjects</td>
<td>2-12</td>
<td>2-12</td>
</tr>
<tr>
<td>Severity</td>
<td>Moderate-Severe</td>
<td>22</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>13</td>
<td>NA</td>
</tr>
<tr>
<td>Current GI dysfunction,</td>
<td>GI dysfunction</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Constipation</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Abdominal Pain</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Gaseousness</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Foul-smelling stool</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No GI dysfunction</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Past history of GI dysfunction</td>
<td>All subjects</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Medically diagnosed GI disorder</td>
<td>All subjects</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2 Method validation values of biogenic amines for LC-MS/MS

<table>
<thead>
<tr>
<th>Biogenic Amines</th>
<th>Fragmentation transitions (m/z)</th>
<th>LOD (µg/L)</th>
<th>LOQ (µg/L)</th>
<th>Linearity (mg/L)</th>
<th>R²</th>
<th>ME (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermidine</td>
<td>146.2 to 72.2</td>
<td>20</td>
<td>50</td>
<td>0.02-5</td>
<td>0.9966</td>
<td>-25±4</td>
<td>97±3</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>144.0 to 117.2</td>
<td>25</td>
<td>85</td>
<td>0.025-5</td>
<td>0.9956</td>
<td>-30±3</td>
<td>94±1</td>
</tr>
<tr>
<td>Tyramine</td>
<td>138.1 to 121.1</td>
<td>20</td>
<td>50</td>
<td>0.02-5</td>
<td>0.9992</td>
<td>-24±4</td>
<td>98±1</td>
</tr>
<tr>
<td>Agmatine</td>
<td>131.1 to 114.1</td>
<td>30</td>
<td>100</td>
<td>0.03-5</td>
<td>0.9965</td>
<td>-17±5</td>
<td>105±14</td>
</tr>
<tr>
<td>Histamine</td>
<td>112.1 to 95.0</td>
<td>50</td>
<td>150</td>
<td>0.05-2.5</td>
<td>0.9997</td>
<td>-11±3</td>
<td>105±10</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>103.2 to 86.2</td>
<td>500</td>
<td>1500</td>
<td>0.5-5</td>
<td>0.9947</td>
<td>-28±1</td>
<td>100±2</td>
</tr>
<tr>
<td>Putrescine</td>
<td>89.2 to 72.2</td>
<td>500</td>
<td>1500</td>
<td>0.5-2.5</td>
<td>0.9972</td>
<td>-30±3</td>
<td>100±8</td>
</tr>
</tbody>
</table>

Table 3 Mean values of biogenic amines (mg) examined per gram of faecal sample for different groups (mild and severe) of ASD individuals and sibling control with the associated ANOVA based p-value.

<table>
<thead>
<tr>
<th>Biogenic Amines</th>
<th>Control (n=35)</th>
<th>Mild (n=16)</th>
<th>Severe (n=19)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. dev</td>
<td>Mean</td>
<td>Std. dev</td>
</tr>
<tr>
<td>Spermidine</td>
<td>4.11</td>
<td>1.94</td>
<td>3.64</td>
<td>2.23</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>0.16</td>
<td>0.44</td>
<td>0.04</td>
<td>0.16</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.09</td>
<td>0.21</td>
<td>0.18</td>
<td>0.61</td>
</tr>
<tr>
<td>Agmatine</td>
<td>2.45</td>
<td>6.65</td>
<td>1.08</td>
<td>2.34</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.04</td>
<td>0.13</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>2.46</td>
<td>3.03</td>
<td>1.87</td>
<td>2.12</td>
</tr>
<tr>
<td>Putrescine</td>
<td>5.41</td>
<td>7.24</td>
<td>4.84</td>
<td>8.29</td>
</tr>
</tbody>
</table>
**Table 4** Mean values of biogenic amines (mg) examined per gram of faecal sample for different groups (GI dysfunction and No GI dysfunction) of ASD individuals with the associated ANOVA based $p$-value.

<table>
<thead>
<tr>
<th>Biogenic Amines</th>
<th>Autism (n=35)</th>
<th>GI dysfunction (n=21)</th>
<th>No GI dysfunction (n=14)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. dev</td>
<td>Mean</td>
<td>Std. dev</td>
</tr>
<tr>
<td>Spermidine</td>
<td>3.88</td>
<td>1.97</td>
<td>4.44</td>
<td>2.11</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>0.10</td>
<td>0.38</td>
<td>0.10</td>
<td>0.26</td>
</tr>
<tr>
<td>Tyramine</td>
<td>0.16</td>
<td>0.38</td>
<td>0.41</td>
<td>0.91</td>
</tr>
<tr>
<td>Agmatine</td>
<td>1.15</td>
<td>1.47</td>
<td>1.39</td>
<td>2.55</td>
</tr>
<tr>
<td>Histamine</td>
<td>0.18</td>
<td>0.58</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Cadavarine</td>
<td>4.37</td>
<td>7.03</td>
<td>1.40</td>
<td>1.43</td>
</tr>
<tr>
<td>Putrescine</td>
<td>3.76</td>
<td>6.08</td>
<td>5.27</td>
<td>7.14</td>
</tr>
</tbody>
</table>

**Conclusions**

We have demonstrated that the LC-MS/MS analysis technique can be used with direct sample extraction and this decreases the sample processing time, which is desirable for BAs analysis as they are very unstable. Furthermore, the reproducibility of the analytical results is greatly improved due to automation. The recommended technique can be easily integrated into future research assessing BAs in clinical samples amongst a larger sample size.

The findings of our present study revealed no significant differences between the ASD severity groups and the sibling controls. Furthermore, no relationships were evident between the GI dysfunction and GI normal group. Therefore, this suggests that BAs may not play a significant role in the pathophysiology of ASD. Nevertheless, further research is warranted to investigate BAs in the other body fluids and tissues before the role of BAs in ASD is ruled out.

**Acknowledgements**

The authors are very grateful to the participants, their parents and guardians for taking part in this study and for providing samples.

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