Gastrointestinal Microbiota in Autistic Spectrum Disorder

by

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# Table of Contents

List of Peer-reviewed Publications Included in this Thesis............................ vi
Conference attendance ................................................................................ vii
List of Appendices .................................................................................... viii
Acknowledgements.................................................................................... ix
Abstract..................................................................................................... 1
Chapter 1: Phenomenology of Autism Spectrum Disorder ......................... 2
  Introduction............................................................................................. 2
  Common signs of ASD ......................................................................... 3
  Types of ASD ..................................................................................... 4
  Autism Diagnosis................................................................................ 5
  Prevalence of Autism.......................................................................... 6
Chapter 2: The pathophysiology of autism and gastrointestinal factors........ 10
Chapter 3: Gastrointestinal microbiology in autistic spectrum disorder: A review............................................................................................................. 19
  Reprint of the original article............................................................... 22
Chapter 4: Faecal microbiota of individuals with autism spectrum disorder ... 29
  Reprint of original article................................................................. 32
Chapter 5: Molecular characterization of gastrointestinal microbiota of children with autism (with and without gastrointestinal dysfunction) and their neurotypical siblings ......................................................................................... 38
  Reprint of original article................................................................. 41
Chapter 6: Evaluation of biogenic amines in the faecal samples of children with autism by LC-MS/MS........................................................................................................... 50
  Reprint of original article................................................................. 54
Chapter 7: General Discussion..................................................................... 65
  Limitations........................................................................................... 70
  Suggestions for future research......................................................... 71
Summary.................................................................................................................. 75
References................................................................................................................ 76
Appendices.............................................................................................................. 88
List of Peer-reviewed Publications Included in this Thesis

Refereed journal articles


Reprints made with the permission of the publishers
Conference attendance

Oral presentation


Poster Presentation

List of Appendices

Appendix A. Ethics approval from SUHREC for pilot study (Chapter 4) ..................89
Appendix B. Ethics approval from SUHREC for molecular study (Chapter 5) ...........92
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28th May, 2013
Abstract

While there is evidence of genetic susceptibility to autism, the degree of autistic individuals with specific genetic backgrounds is unknown. It is likely that other factors, such as environmental conditions, may play a role in this disease. Children with autism have been found to experience gastrointestinal (GI) problems (such as diarrhoea, constipation, abdominal pain and discomfort) more frequently and more severely than children from the general population.

In the present thesis, GI microbiota in children with autism were examined and compared with a matched control group. The results provide new information and a compelling picture of the GI microbiota of children with autism with GI symptomatology. Furthermore, a group of bacterial metabolites - biogenic amines - were examined in the faeces of children with autism and their matched sibling control group.

The thesis results did not indicate any significant differences in the GI microbiota profile when compared with-in the group, regardless of the severity or type of GI dysfunction. Furthermore, no significant difference was found with neurotypical sibling control group. Similarly, there was no significant difference found in the concentration of the bacterial metabolite-biogenic amines when compared with a neurotypical sibling control group. Therefore, these observations suggest that GI dysfunction associated with autism might not be associated with the presence or absence of specific GI microbiota. The mechanisms that underlie GI dysfunction might involve factors other than GI microbiota like stress-induced changes in GI physiology. Animal studies suggest that perturbations of behaviour, such as stress, are associated with increased vulnerability to inflammatory stimuli in the GI tract which lead to GI dysfunction. Therefore, it might be possible that GI dysfunction associated with autism may have related to factor like early life stress.
Chapter 1
Phenomenology of Autism Spectrum Disorder

Introduction

Autism was first identified and proposed as a new psychiatric disorder by psychiatrist, Leo Kanner, in 1943 (Kanner, 1943) and was first included in the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III, APA 1980). Autism spectrum disorders (ASD) are a group of complex neurodevelopmental disorders with similar core features characterized by impairments in social interaction and communication, and restricted, repetitive and stereotyped patterns of behaviour. ASD include Autistic Disorder (typically referred to as autism) which is the most severe form, while other conditions along the spectrum include childhood disintegrative disorder, pervasive developmental disorder not otherwise specified (usually referred to as PDD-NOS) and Asperger’s Disorder. ASD occur throughout the world in people of all ethnic and social backgrounds though it varies significantly in character and severity, with males being 4-10 times more likely to have an ASD than females (Baron-Cohen, 2002; Baron-Cohen et al., 2001; Lord et al., 1982; Wing, 1981). The reason for the higher prevalence of autism in males is yet to be determined (Yeargin-Allsopp et al., 2004).

Experts generally agree that there are at least two types of ASD in terms of development: ‘classical autism’ is defined by abnormal cognitive development which is evident very early in infancy, and ‘regressive autism’ which usually presents between 18 and 24 months of age following apparently normal development (Baron-Cohen, 2002). In cases of regressive autism, it is often reliably detected by the age of 42 months and, in some cases, as early as 20 months (Wing, 1981).
**Common signs of ASD**

The hallmark feature of ASD is impaired social interaction. A child with ASD may appear to develop normally, then withdraw and become indifferent to social engagement after a certain age. Children with ASD may not respond to their names and often avoid eye contact with other people (Baron-Cohen et al., 2001). They have trouble interpreting what others are thinking or feeling because they may not understand social cues (or consider them salient), such as voice tone or facial expressions. These children also tend to avoid eye contact and, therefore, do not watch other people’s faces for clues about appropriate behaviour. Many children with ASD engage in repetitive movements such as rocking, twirling/spinning, staring and finger flapping or self-abusive behaviours such as biting and head-banging. They are likely to start speaking later than other children (if at all) and may refer to themselves by name instead of “I” or “me”. Children with ASD have difficulty playing and interacting with other children. Individuals with ASD may also show unusual responses to sensations such as sound, touch, vision and smell (Lord et al., 1982). Auditory and visual processing problems are common (Dawson et al., 2000). Sensory problems vary from mild to severe with both over and under sensitivities.

Children with characteristics of ASD may also have other associated conditions such as Fragile X syndrome (which causes mental retardation), tuberous sclerosis, epileptic seizures, Tourette’s syndrome, learning disabilities and attention deficit hyperactivity disorder. About 20 to 30 % of children with ASD develop epilepsy by the time they reach adulthood (Canitano, 2007; Kanner, 1943). Apart from these conditions, some individuals with ASD possess unique abilities (savant behaviour) such as an accurate and detailed memory for information and facts, high visual recall and a superb ability to manipulate data for useful purposes. They may be able to focus for long periods of time on particular tasks or
subjects and may be far more attentive to detail compared to people without ASD (Howlin et al., 2009). There is no medical test for diagnosis of ASD; rather, they may only be described as a cluster of specific behaviours. These behaviours are marked before the age of three years and continue, in the majority of cases, throughout the individual’s life.

Types of ASD

Currently, the term ASD is an umbrella description which refers to three different diagnoses according to DSM-IV-TR.

1. Autistic disorder (classic autism):
   The diagnosis of autistic disorder is given to individuals with impairments in social interaction and communication. The condition is often associated with restricted and repetitive interests, activities and behaviours. It is generally diagnosed prior to three years of age.

2. Asperger’s disorder:
   Individuals with Asperger’s disorder have difficulties with social interaction and communication, as well as restricted and repetitive interests, activities and behaviours. Individuals with Asperger’s disorder do not have a significant delay in early language acquisition or other cognitive abilities or self-help skills. Asperger’s disorder is often detected later than autistic disorder.

3. Pervasive developmental disorder – not otherwise specified (PDD-NOS) (Atypical autism):
   The diagnosis of PDD-NOS is made when an individual has a marked social impairment but fails to meet full criteria for either autistic disorder or Asperger’s disorder. These individuals may also have communication impairments and/or restricted and repetitive interests, activities and behaviours.
Autism Diagnosis

There is no specific diagnostic test for ASD. Best-practice assessment utilises a multi-disciplinary approach. ASD varies widely in severity and symptoms and may go unrecognised for many years, especially in children who have milder forms. Autism is currently diagnosed by the presence or absence of certain behaviours and developmental history. The criteria most widely used for diagnosis of autism in Australia and North America come from the Diagnostic and Statistical Manual of Mental Disorders - fourth edition - text revision (DSM-IV-TR, APADSM-IV-TR, 2000). The DSM identifies three areas of functional impairment associated with a diagnosis of autism:

1. Impaired socialisation
2. Impaired communication
3. Restricted and repetitive patterns of behaviour, interests and activities

According to DSM-IV-TR, each of the three areas above contains four specific criteria, making a total of 12 criteria. For a diagnosis of autism, an individual must meet at least six of the 12 criteria (with at least two from the first domain and one each from the second and third domains). Therefore, individuals can meet a variety of different combinations of criteria and still receive a diagnosis of autism. Thus, individuals who have a diagnosis of autism may vary a great deal in their personality, abilities, behaviours and presentation.

The American Psychiatric Association (APA) is currently preparing the fifth edition of the DSM (DSM-V), which is due for publication in May 2013. In this edition, autism diagnostic criteria have been revised along with other mental disorders. It will merge various subtypes of autism into the singular diagnostic category of autism spectrum disorder with severity variants from mild to severe. Further it
will eliminate the DSM-IV-TR categories of Asperger’s disorder and PDD-NOS and this will make a single category of autism spectrum disorder. For the ASD diagnosis, instead of three domains (impaired socialisation, impaired communication and restricted and repetitive behaviour) of diagnostic criteria stated in DSM-IV-TR, only two domains (social communication impairment and restricted interests/repetitive behaviours) will be used in the DSM (DSM-V). According to new proposed diagnosis criteria, individuals would be required to exhibit (1) three deficits in social interaction and communication and, (2) at least two repetitive behaviours.

Prevalence of Autism

Autism was initially thought to be rare (and may indeed have been in the first few decades following Kanner’s seminal paper in 1943) but, during the 1990s, the number of persons reported to be receiving services for ASD increased substantially (Kadesjo et al., 1999; Sponheim et al., 1998; Wing, 1993). According to population-based studies conducted worldwide before 1985, the prevalence of autism and related conditions was 0.4–0.5 per 1,000 children aged less than 18 years (Gillberg et al., 1991; Lotter, 1966). Studies of ASD prevalence during the 1990s in the United States identified rates of 2.0–7.0 per 1,000 children (Fombonne, 1996; Shattuck, 2006; Sturmey et al., 2001; Yeargin-Allsopp et al., 2003). The US Centres for Disease Control (CDC) conducted surveys based on parent reports of diagnosed autism in their children. These studies indicated that 5.5–5.7 per 1,000 children aged 4–17 years received a diagnosis of autism during 2003–2004, approximately 1 child in every 150 (CDC, 2012; Charman, 2002; Rutter, 2005). In 2007, according to the National Survey of Children's Health in the US, ASD prevalence was 110 per 10,000, an average of 1 child in every 100
(Kogan et al., 2009). According to the recent CDC Autism and Developmental Disabilities Monitoring (ADDM) network, about 1 in 88 children has been identified with an ASD and is almost 5 times more common among boys (1 in 54) than among girls (1 in 252).

In Australia, there has been no national survey conducted to estimate the prevalence of ASD. However, surveys that have been carried out at the regional level (see Table 1) support the overseas estimates of the ASD prevalence.

Table 1. Autism prevalence studies in Australia.

<table>
<thead>
<tr>
<th>Region</th>
<th>Period of Study</th>
<th>Prevalence (per 10,000)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1997</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Western Australia</td>
<td>1999</td>
<td>159</td>
<td>Newschaffer et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2001</td>
<td>204</td>
<td></td>
</tr>
<tr>
<td>New South Wales</td>
<td>2000</td>
<td>758</td>
<td>Treffert (1970)</td>
</tr>
</tbody>
</table>

Epidemiological studies of ASD have provided evidence that the prevalence is increasing (Fombonne et al., 2001; Gillberg et al., 1991; Lotter, 1966; McCarthy et al., 1984; Treffert, 1970). Population-based prevalence surveys of autism have been conducted since the 1960s and summarized in recent reviews; these surveys have been used for evaluating changes in reported estimates over time (Fombonne, 2005; Kadesjo et al., 1999; Wing et al., 2002). Prevalence time tendencies for autistic disorder are available for longer time periods than for ASD as a group as PDD-NOS and Asperger’s disorder diagnoses were introduced.
in DSM-III-R and DSM-IV in 1987 and 1994, respectively. Based on the nature of the diagnostic criteria for different subgroups of ASD, it is possible that there were variations in the ways in which these were interpreted by different researchers (Newschaffer et al., 2007; Rutter, 2005). However, differences in survey methodology, particularly changes in case definition and case identification over time, have made comparisons between surveys difficult to perform and interpret (Fombonne, 2005).

Furthermore, the assessment process can also be affected by various other factors such as sample size, proportion of immigrants and methods of case-findings (Newschaffer et al., 2007). Increasing awareness of the existence of ASD among parents, professionals and the general public may also be one of the factors contributing to the reported increase in ASD prevalence (Newschaffer et al., 2007; Wing et al., 2002). ASD can be associated with other conditions including developmental disorders and/or physical disabilities. Therefore, it can cause diagnostic confusion if underlying symptoms are not recognised. Research is now being undertaken on the association of autism with a range of identifiable abnormalities including Fragile X (Turk et al., 1997), Turner’s syndrome (Creswell et al., 1999), epilepsy and Down’s syndrome (Howlin et al., 1995).

It has been observed that because of the stereotyped view of children with the associated disorder, the presence of autism has often not been diagnosed (Howlin et al., 1995). Genetic factors alone are less likely to account for a real increase in incidence that appears to have occurred so rapidly and continuously year by year (Hertz-Picciotto et al., 2006; Persico et al., 2006). If there is a real increase that is continuous, environmental factors are likely implicated.
Many hypotheses and theories have been made concerning possible cause, including environmental pollutants, use of antibiotics in early stages of life, gastrointestinal dysbiosis, constituents of diets, vaccines and exposure to heavy metals and allergies, but none has yet been scientifically validated (Hertz-Picciotto et al., 2006). The assessment of gastrointestinal (GI) microbiota (one of the environmental factors which may contribute to ASD symptomatology) was the main focus of this thesis. Hypotheses relating to the possible involvement of GI microbiota in ASD are discussed in the next chapter.

All or some of the factors discussed above may have contributed to the observed increase in the prevalence rate of ASD. However, there is no certainty as to how much of the increase they may explain. It remains an open debate whether there is a genuine increase in the number of children with ASD or if this is due to the changes in the diagnostic criteria and public awareness of the condition (Newschaffer et al., 2007; Wing et al., 2002).
Chapter 2

The pathophysiology of autism and gastrointestinal factors

For more than three decades after autism was first described, it was believed to be a psychogenic disorder. However, according to new research, the high incidence of mental deficiency and epileptic seizures point to biological determinants. Many studies have reported structural abnormalities in various regions of the brain (Brambilla et al., 2003; Sparks et al., 2002). Genetic influences have also been noted; there are high concordance rates in siblings of children with autism, as well as in monozygotic twins (Hallmayer et al., 2011; Rosenberg et al., 2009). Hence, it has now become generally accepted that autism has a biological basis. Nevertheless, the etiology of autism is complex and the underlying pathophysiological mechanisms are unknown in most cases. A number of causes have been proposed, including various medical conditions (Fragile X syndrome, phenylketonuria and Down’s Syndrome), perinatal insult, autoimmune mechanism and genetic susceptibility to environmental factors, gastrointestinal dysfunctions and dysbiosis/infectious agents.

Family and twin studies have provided evidence that autism is a genetic neuropsychiatric disorder, with concordance rates of 60–91% in monozygotic twins depending on whether a narrow or broad phenotype is considered for the diagnosis. This is very much in contrast with dizygotic twins, with 0–10% concordance depending on a narrow or broader phenotypic definition (Folstein et al., 2001; Veenstra-Vander Weele et al., 2004).

However, in recent years, different levels of complexity have emerged in relation to autism and genetics, namely a high degree of genetic heterogeneity (different contributing genes in different patients), a polygenic or oligogenic inheritance mode in most cases (i.e. many
susceptibility gene variants at different loci are required for an individual to develop the disease) and the presence of significant gene–gene and gene–environment interactions (Abrahams et al., 2008). By identifying genetic markers inherited with autism in family studies, numerous candidate genes have been located, most of which encode proteins involved in neural development and function (Persico et al., 2006). However, for most of the candidate genes, the actual mutations that increase the risk for autism have not been identified. Typically, autism cannot be traced to a Mendelian inheritance or to single chromosome abnormality such as Fragile X syndrome (Cohen et al., 2005; Muller, 2007).

According to a recent study, around 103 genes and 44 genomic loci have been reported in ASD (Betancur, 2011) with significant overlap with other conditions such as intellectual disability, epilepsy, schizophrenia and attention deficit hyperactivity disorder (ADHD). These findings therefore suggest that ASD is not a single-gene disorder but rather a complex disorder resulting from coinciding genetic variations in multiple genes (El-Fishawy et al., 2010). Moreover, it supposed to have complex interactions between genetic, epigenetic and environmental factors. Genetic contributors to autism may be associated with a broader range of mechanisms beyond those directly associating with the central nervous system. In particular, these mechanisms may confer increased vulnerability to environmental factors and they involve other organ systems such as the gastrointestinal or immune systems, two systems which are on the frontlines of the individual’s interaction with the environment.

Very few research studies on gastrointestinal symptoms in ASD were reported prior to the 1990s (Goodwin et al., 1971; Walker-Smith et al., 1972) and little attention was given to these findings until last decade when routine gastrointestinal evaluation of children with ASD revealed a
high prevalence of gastrointestinal symptoms, histologic changes in digestive tract, gastrointestinal dysfunction and abnormal gut microbiota compared to control children. A study by Wakefield and colleagues (2000) reported the first systematic evaluation of children with autism and GI symptoms. On endoscopy of the lower GI tract, 93% of the children evaluated had ileal lymphonodular hyperplasia (LNH) and 30% had colonic LNH. Chronic colitis (inflammation) was also present in most of the patients. These children had one or more of the following symptoms at the time of their evaluation: abdominal pain, constipation, diarrhoea (or alternating constipation and diarrhoea) and bloating. A similar study (Horvath et al., 1999b) performed endoscopic evaluations of the upper GI tract in symptomatic children with autism and reported high prevalence of gastrointestinal abnormalities such as inflammation, decreased intestinal carbohydrate digestive enzyme activity, and most individuals experienced symptoms of abdominal pain, chronic diarrhoea, gaseousness/bloating and unexplained irritability. The high prevalence of GI symptoms provide evidence for an association between autism and GI symptoms and have increased clinician’s and researcher’s awareness of the chronic GI problems that can occur in children with an ASD.

In the late 1990s, researchers began studying GI microbiota as a potential factor in the GI dysfunction associated with autism and studies were designed to examine faeces of children with autism for the presence of various microorganisms. Researchers have used a variety of techniques to study the GI microbial profile ranging from conventional culture methods to extensive molecular biology techniques (Bolte, 1998; Finegold et al., 2002; Song et al., 2004). Many hypotheses have been proposed that involve GI microbiota in autism including: the presence of Clostridium spp. (Bolte, 1998; Finegold, 2008); the use of high doses of antibiotics in the early stage of life (Adams et al., 2007; Fallon, 2005); food intolerance (Gorrindo et al., 2012); colonisation by pathogenic
bacteria (Finegold et al., 2002); and, *Candida* infection (Shaw et al., 1995).

However, to date, researchers have not been able to definitively demonstrate a link between GI microbiota and autism. The possible role of GI microbiota in autism is fundamental to this thesis. Therefore, the associated research program was designed to explore GI microbiota in children with autism and determine whether it differs from that of a non-clinical healthy control group and if there is any evidence of an association between GI microbiota and the clinical severity of ASD.

Food intolerance in the autistic population, particularly for wheat and cow’s milk, is very common (Goodwin et al., 1971; Gurney et al., 2006). Based on this observation, other GI conditions like ‘leaky gut syndrome’/intestinal epithelial barrier dysfunction has been hypothesised to co-occur with autism symptoms (Rimland et al., 1996; White, 2003). Two different studies have reported increased intestinal permeability at a high frequency (43% and 75%) in ASD children with GI symptoms (D'Eufemia et al., 1996; Horvath et al., 2000). However, a study with 14 children with autism having current or previous GI complaints found no evidence of altered intestinal permeability (Robertson et al., 2008).

Excessive levels of incompletely metabolised peptides from certain substances found in common foods, particularly the casein in cow's milk and the gluten and gliadin in wheat and other cereal grains, can pass through the intestine and leak into the bloodstream. This can induce immunogenic responses. It is thought that due to deficient enzyme activity in the lumen of the small intestine, wheat (gluten) and milk (casein) proteins are incompletely broken down (Kidd, 2002) into short neuroactive peptides such as β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) generated from casein and gliadinomorphin (Tyr-Pro-Gln-Pro-Gln-Pro-Phe) generated from gluten (Reichelt et al., 1994). These products
are potent psychosis-inducing factors (Lindstrom et al., 1984) which enter into the bloodstream and cross the blood-brain barrier to exert an opioid-like effect in the brain (Reichelt et al., 1994).

In a study by Reichelt and Knivsberg (2003), abnormal opioid peptide concentrations were reported in urine samples from autistic children. Based on analysis of urine samples, dietary intervention involving gluten-and/or casein-free food has been proposed to be effective in some of the behavioural symptoms of autism. However, this diet has not been scientifically established to produce improvements in ASD symptomatology. Following dietary restriction of gluten and/or casein, a number of studies reported that the urine peptide profile normalized in autistic children and significant reductions of autistic behaviour, epileptic fits and aggression were achieved, with an increased level of attention, and acquisition of language and social skills (Knivsberg et al., 1998; Reichelt et al., 1990; Reichelt et al., 1991). Conversely, a smaller number of studies found the reversal of urine peptide profile and behaviours that were not significantly different to control groups (Elder et al., 2006; Sponheim, 1991; Whiteley et al., 1999). However, these studies were not randomised trials and the behavioural assessments were not carried out by evaluators blind to the intervention. In a placebo-controlled trial (Johnson et al., 2011), children with ASD aged between 3 and 5 years were randomly allocated to a gluten-casein free diet \((n = 8)\) or a low-sugar diet \((n = 14)\) for a 3 month period using an open-label design. There were no statistically significant differences observed between groups when compared across a range of behavioural and developmental outcomes. Currently, there is little empirical evidence from randomised control trials to support the use of gluten and/or casein free diets as an effective intervention for children with ASD (Whitehouse, 2013).
Several studies have taken a different approach by focusing on immunological responses to dietary proteins. A study by Jyonouchi et al. (2002), found that peripheral blood mononuclear cells from children with ASD responded to gliadin, cow’s milk protein and soy by producing higher levels of inflammatory cytokines in comparison to typically developing children. Studies looking at mucosal immune responses in children with ASD who have GI symptoms have shown increased infiltration of eosinophil, T-cells and monocytes in the gut mucosa and increased numbers of paneth cells, compared with non-inflamed control subjects or compared with children with celiac disease and inflammatory bowel disease (Kolevzon et al., 2007; Rellini et al., 2004; Sullivan, 2008).

Numerous research studies point to the presence of immune dysfunction in some children with ASD, which is consistent with autoimmunity (Croen et al., 2005; Goines et al., 2010). Most autoimmune diseases result from a complex interaction between genetic and environmental factors. Various genes are thought to be associated with an increased risk of developing certain autoimmune diseases. The human leukocyte antigen (HLA) genes are among the strongest predictors of risk for autoimmune conditions (Wing et al., 2002). Different HLA haplotypes are also associated with schizophrenia and ASD (DeSoto, 2009; Karr et al., 2007; Krakowiak et al., 2012).

Though it is not known whether immune activation plays an initiating or maintaining role in the pathology of ASD, immune activation leading to inflammation can have serious damaging effects and could lead to tissue damage. Increased immune activation is associated with a number of neurodegenerative disorders and is speculated to play a role in psychiatric disorders such as depression, schizophrenia, obsessive compulsive disorder, bipolar disorder as well as ASD (Ashwood et al., 2011; Fombonne et al., 2001; Howlin et al., 1995). Elevated levels of
inflammatory cytokines in the CNS could reflect an inflammatory process that might contribute to abnormal neurodevelopment as seen in ASD.

The presence of antibodies against brain and CNS tissue has been repeatedly reported in children with ASD (Knivsberg et al., 2002; Roberts et al., 2007). These antibodies have various targets, including neuronal proteins, neurotransmitter receptors and nuclear material (Kidd, 2002; Reichelt et al., 1994). However, it is not clear whether these autoantibodies have direct pathogenic relevance or are instead secondary to previous cellular damage or inflammatory reactions. Although some investigators have analysed whether antibodies are directed at specific targets, such as myelin basic protein or glial fibrillary acidic protein, no clear associations between ASD and specific antibodies have been replicated across studies to date. This lack of specificity may suggest that the antibodies are generated as a consequence of some previous undiscriminating damage that involves CNS targets, leading to the generation of a diverse array of antibody specificities, as seen in ASD. Alternatively, the presence of these autoantibodies could reveal a group of ASD individuals who are more prone to immune dysfunction. Notably, antibodies directed at gut epithelium have also been described in ASD children (Careaga et al., 2010). However, no link between gut and brain antibodies has been established.

A theory linking vaccines and autism has also been proposed with many controversial findings across the research literature. In two separate reports by Fudenburg (1996) and Gupta (1996), a close sequential association between the administration of the MMR vaccine and the onset of autistic symptoms in patients was noted. Kawashima et al. (2000) reported genomic RNA of the measles virus vaccine strain in peripheral mononuclear cells in three out of nine autistic patients and one of three patients with ulcerative colitis, while all controls were negative.
The most controversial autism research article of the last decade by Wakefield et al. (1998) (retracted in 2010 because of scientific fraud and ethical violations) noted intestinal pathology, namely the hyperplastic lymph nodes in the ileum and colon, suggesting that the immune system of the gut has been seriously challenged in the autistic children. Although this group did not draw a conclusion as to the cause of the pathology, they noted based on parental reports that the children had received the trivalent measles-mumps-rubella (MMR) vaccine before undergoing behavioural regression. The research by Wakefield et al. has drawn scientist’s and clinician’s attention toward the potential association between vaccines and autism and started a series of investigations in this field.

MMR vaccine has an enormous beneficial effect upon public health and is highly efficacious in limiting childhood measles, mumps, and rubella infections. Therefore, the studies suggesting that the MMR vaccine may be an etiological factor in autism created very high public health concern. Hence, several epidemiological studies of different populations have been carried out to examine the relation between vaccinations and the prevalence of autism. After carefully reviewing the case history of 473 autistic children in London (UK) over a 20-year period starting from 1979, Taylor et al. (1999) found no significant variation in the proportion of children with developmental regression or bowel problems before and after introduction of the MMR vaccine. Similarly, independent studies by Dales et al. (2001), Miller et al. (1997) and Fombonne et al. (2001) have failed to find any significant correlation between the distinct syndrome of MMR-induced autism or of autistic enterocolitis.

There are considerable controversies surrounding the published findings as well as many of the related issues discussed within this thesis. Therefore, it is essential to examine the pathophysiology of autism and
extend research to evaluate the supposed theories and hypotheses as a tool in the clinical diagnosis of autism. All potential links between GI pathophysiology and autistic symptoms must be given cautious and rigorous examination. Even if the GI pathology is unrelated to the etiology of autism or its unique symptomatology, the findings appear to verify what parents and physicians have long suspected in that many autistic children have abnormal GI function. As one of the suspected GI dysfunction theories relates to abnormal GI microbiota in children with autism, the focus of this research was to empirically test this hypothesis.
In 1971, researchers for the first time reported a co-incidence of autism with celiac disease and reported elevated rates of GI dysfunction in autistic individuals (Goodwin et al., 1971). This promoted a series of related investigations of biological abnormalities in autism. The high rates of GI dysfunction in individuals with ASD have been reported in several studies with symptoms including one or more of: abdominal pain, constipation, diarrhoea and alternating constipation. (Ashwood et al., 2011; Gorrindo et al., 2012; Horvath et al., 1999b).

GI dysfunction associated with autism has been hypothesised to be related to the colonization of harmful bacteria in the intestinal tract (Bolte, 1998). This was supported by other theories including: late-onset autism preceded by the use of broad-spectrum antibiotics, and the use of anti- and/or pre/pro-biotics to improve GI symptoms as well as autistic symptoms (Sandler et al., 2000; Tuohy et al., 2003; Weber et al., 2007). Some fungal species, in particular *Candida*, have the ability to establish on gastrointestinal mucosa and induce intestinal permeability which allows the passage of undigested molecules through the intestinal wall and creates a series of reactions often observed in individuals with autism and allergy symptoms.

It also has been hypothesised that viral infection early in life and during pregnancy may trigger ASD (Rosen et al., 2007) but the mechanism is not known. These observations have stimulated a series of investigations of the involvement of microorganisms in the pathophysiology of GI dysfunction associated with autism. However, there is inconsistency in the findings of different studies regarding the
specific group(s) of microorganisms potentially involved with ASD. Given the mixed findings of previous studies, it appeared that mere replication of this research would be unlikely to add substantial new information of the role of GI microbiology in autism.

Therefore, the following paper (Gondalia et al., 2010b) sought to comprehensively review the possible involvement of GI microbiota, including bacteria, viruses and fungi, in autism. Furthermore, the paper sought to contribute to the literature by reviewing the current hypotheses, outlining previous research and providing discussion of fresh directions or perspectives which researchers in this field could consider to advance the understanding of the possible role of GI microbiota and associated factors in ASD.
**Paper 1**


Reprint of the original article
Gastrointestinal microbiology in autistic spectrum disorder: a review

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Cases of autism have frequently been reported in association with gastrointestinal problems. These observations have stimulated investigations into possible abnormalities of intestinal microbiota in autistic patients. The objectives of this paper were to review the possible involvement and mechanisms of gastrointestinal microbiota in autistic spectrum disorder and explain the possible role of gastrointestinal microbiota in the condition. This review addresses the possible involvement of bacteria, viruses and fungi, and their products in autism. Direct viral damage of neurons or disruption of normal neurodevelopment by immune elements such as cytokines, nitric oxide and bacterial products, including lipopolysaccharides, toxins and metabolites, have been suggested to contribute to autistic pathology. Numerous intestinal microbial abnormalities have been reported in individuals with autism. Research to date exploring possible gastrointestinal problems and infection in autism has been limited by small and heterogeneous samples, study design flaws and conflicting results. Furthermore, interventions designed to modify the intestinal microbial population of autistic patients are few and limited in their generalisation. In order to bring clarity to this field, high-quality and targeted investigations are needed to explore the role of gastrointestinal microbiology in autism. To this end, several promising avenues for future research are suggested.

Reviews in Medical Microbiology 2010, 21:44–50

Introduction

A psychiatrist, Leo Kanner, [1] first described a syndrome in 11 children displaying severe impairments in social interaction and communication, reduced behavioural flexibility as well as obsessive interests and stereotyped behaviours. This landmark study was the first to illustrate the clinical entity that has become known as autistic spectrum disorder (ASD). Despite over 60 decades of research, the cause of autism remains unknown [2].

Nevertheless, recent studies have suggested gastrointestinal disorders and associated symptoms are commonly reported in individuals with ASD, but key issues such as the prevalence of this condition and best treatment of these conditions are incompletely understood [3,4]. Despite the consequences, the clinical picture of ASD is frequently consistent with gastrointestinal disorders including constipation, diarrhoea, foul-smelling stools, gaseousness, abdominal bloating, signs of abdominal discomfort, changes in normal intestinal microbiota and...
decreased intestinal carbohydrate digestive enzyme activity [5]. Several important questions will remain salient regardless: ‘Why do individuals with autism more frequently have co-occurring gastrointestinal conditions?’; ‘What is different about the gut of individuals with autism?’; ‘Are these differences due to diet or intrinsic dysfunctions of the gut?’; ‘Is there a connection between brain and gut dysfunction?’

The microbiological ecosystem of the gut is complex and poorly understood but appears to be of key importance in health and disease [6] and is often conceptualised as an essential ‘organ’ in providing nourishment, regulating epithelial development and instructing innate immunity and a broad range of other activities. Despite this, basic features remain poorly described.

Intestinal bacteria secrete both detrimental as well as beneficial compounds, and overgrowth of certain species of bacteria or other changes to the normal intestinal microbiota have frequently been reported in association with autism [7]. The treatment of gastrointestinal dysfunction in ASD with antibiotics or probiotics has been proposed as a way to regulate intestinal microbiota and subsequently improve symptom severity in ASD [8,9]. Such intervention is not routine practice given the lack of consistent evidence as to the specific microbial adjustment that is desirable. The alterations of intestinal microbiota and the presence of harmful microbiota are descriptive observations and it is uncertain how, or if, it is related to the pathology of autism to the pathology of autism. This review is intended to develop a new direction which may explain the possible role of intestinal microbiota in the symptomatology of autism. In order for any significant advances to be made with respect to gastrointestinal microbial intervention for autism, a more consistent and extensive evidence base is required. We will review the existing evidence and provide recommendations as to promising areas for future research work.

Bacteria

Generally, bacteria are classified as Gram-negative or Gram-positive on the basis of their cell wall composition. Few Gram-positive genera are pathogenic to humans (exceptions include Streptococcus, Staphylococcus, Clostridium, Listeria and Bacillus); however, many Gram-negative bacteria are pathogenic to humans, including Escherichia coli, Salmonella and Helicobacter. Gram-negative bacterial infections are associated with inflammatory and immunological manifestations including fever, injury and neurological dysfunction. Lipopolysaccharide (LPS) is localised to the exterior leaflet of the outer membrane of Gram-negative bacteria and serves as the major surface component of the bacterial cell envelope. This glycolipid is essential to virtually all Gram-negative organisms and represents one of the conserved microbial structures responsible for activation of the innate immune system [10]. Furthermore, LPS is responsible for tissue damage and other pathophysiological changes associated with Gram-negative infection [11]. Singh and Jiang [12] hypothesised that either LPS crossed the blood-brain barrier (BBB) and directly induced the inducible nitric oxide (iNOS) gene or it did not cross the BBB but was bound to the endothelial cell to release nitric oxide (a gaseous neurotransmitter that diffuses into the brain) [13]. According to Gaillard et al. [14] and Xia et al. [15], LPS damages the BBB making it relatively porous, thus being congruent with the hypothesis of Singh and Jiang that LPS may cross the barrier and enter the brain.

The issue of LPS and Gram-negative bacteria relates to one of the dominant causal theories of autism, namely the mercury poisoning hypothesis [16]. A study by Minami et al. [17] showed no differences in the cerebrum of control mice, LPS-injected mice and ethyl mercury (thimerosal)–injected mice, 1 and 3 days after injection. However, when LPS was injected 3 h before the mercury, mercury content increased in the cerebrum at both days 1 and 3. This study provides evidence that LPS increases penetration and storage of mercury in the cerebrum. The same phenomenon was observed for cerebellar levels in the cerebrum after LPS injection [18]. LPS toxicity, therefore, works synergistically with mercury (and possibly other heavy metals) to expand damage [19]. Furthermore, LPS decreases glutathione levels, making it even more difficult for the body to passivate and excrete heavy metals [20]. Therefore, the mechanisms by which LPS may play a role in autism are varied, and may differ across subsets of autistic patients.

Enteric microbiota can modulate intestinal motility. For example, Bifidobacterium bifidum and Lactobacillus acidophilus are able to promote motility; whereas Escherichia species can inhibit it [21]. Metabolic products of intestinal bacteria, such as short-chain fatty acids or chezymic peptides (e.g. N-formyl-methionyl-leucine-phenylalanine), are able to stimulate the enteric nervous system and influence the rate of gut transit [21]. Disruption of the balance that exists between enteric microbial populations might predispose the host to altered gut motility and secretion, which results in diarrhoea or constipation, commonly found amongst ASD populations [22–24].

Clostridial species are part of the normal human intestinal microbiota and are considered a principal group of bacteria that produce both an enterotoxin and a neurotoxin. They are generally very active metabolically and may produce potentially toxic metabolites such as phenol, p-cresol or certain indole derivatives [25]. Boile [26] suggested a causal role for clostridia in ASD, and cultivation and polymerase chain reaction–based studies have shown distinctive clostridial populations in autistic
children compared with healthy controls [27,28]. Such alterations in the indigenous gut microbiota may reflect colonisation of neurotoxin-producing bacteria (including several members of the genus Clostridium).

Finegold et al. [7] demonstrated that the number of clostridial species found in the stools of children with autism was greater than that in the stools of control children. Nine Clostridium species were exclusively isolated from faecal samples of autistic children (i.e. not found in the predominant faecal microbiota of healthy controls). In addition, three species were uniquely found in healthy samples. In a subsequent study, Song et al. [27] identified significantly higher levels of Clostridium bolteae and Clostridium clusters I and XI in 15 autistic children than in eight healthy controls.

It has been hypothesised that the use of antimicrobial (antibiotic) agents might disrupt the indigenous intestinal microbiota and allow colonisation of opportunistic pathogens such as the spore-forming clostridia [28]. There is now clear evidence for a causal relationship between antibiotic use and pseudomembranous colitis caused by Clostridium difficile [29,30]. C. difficile produces two exotoxins: toxin A, which is classified as an enterotoxin, and toxin B, classified as a cytotoxin. These toxins damage intestinal mucosa and cells and result in watery diarrhoea [29–31]. Similarly, Clostridium tetani produces two toxins: cytotoxin and tetanus neurotoxin (TeNT) [30]. C. tetani also produces tetanolyxin, an oxygen-labile haemolysin. The role of tetanolyxin in tetanus infection remains unclear; however, it is thought that it promotes the growth of C. tetani by damaging healthy tissue in the vicinity of the infection [32]. The possibility exists that tetanolyxin’s action in the intestinal tract could increase intestinal permeability. When intestinal permeability is increased, food molecules larger than those which would normally be absorbed from the intestinal tract (e.g. uncleaved proteins such as casein and gluten) enter the bloodstream and can become antigenic [33]. Children with autism frequently develop intolerance to foods containing gluten or casein [34] and are sometimes described as having a ‘fixed smile’, hypertonia or rigidity, and have difficulty in chewing and swallowing food [32]. Hypotonia/flaccid muscle tone is a classical symptom of TeNT [35]. Elevated catecholamine (in plasma and urine), tachycardia, profuse sweating and irregular birthing are all symptoms of autonomic nervous system dysfunction and are documented to occur in both autism and tetanus [36,37].

A further reason Clostridium is suspected to play a role in autism is that vancomycin has proven effective in reducing autistic symptomatology and is known to be highly effective against Gram-positive organisms. When given orally, vancomycin is poorly absorbed by the gut, so any effect seen with its use is likely due to its action on intestinal bacteria. Therefore, reported relapses in autistic behaviours after discontinuation of oral vancomycin may be because of germination of clostralid spores that persist through antibiotic therapy [9,38].

Many species of Streptococcus are used in the fermentation of foods and as probiotics. For example, Streptococcus thermophilus is a food source and is associated with a variety of health benefits [39,40]. Nevertheless, a childhood neurodevelopmental condition is believed to be associated with streptococcal infection. In the 1990s, Swedo et al. [41] at the National Institute of Mental Health coined the term PANDAS (pediatric autoimmune neuropsychiatric disorders associated with streptococcal infection). The accepted cause of PANDAS is that group-A β-haemolytic Streptococcus (GABHS; Streptococcus pyogenes) produces the production of antibodies that cross-react with neural tissues and thereby provoke the neurobehavioural symptoms associated with the condition [41], which include symptoms often shared with autism, such as cognitive inflexibility, obsessive and compulsive behaviours and vocal and/or postural tics [42]. Anti-GABHS antibodies have also been found in a subset of persons with ASD. Vojdani et al. [43] examined antibodies against nine different neuron-specific antigens, including Streptococcus M protein (STM6P), in sera of 40 autistic and 40 healthy children. Analyses of sera of autistic children showed approximately 54, 72 and 40% elevation of IgG, IgM and IgA antibodies, respectively, against STM6P (neurologic antigen). However, in healthy children, approximately 10, 18 and 9% elevation of IgG, IgM and IgA antibodies, respectively, against STM6P were found.

As neurobehavioural symptoms associated with PANDAS are also found in autistic children, a case study by Cosford [42] raised the possibility that, for perhaps some autistic patients, gastrointestinal streptococcal overgrowth triggers autoimmune responses that cross-react with central nervous system (CNS) antigens. Furthermore, a study by Vojdani [43] confirmed the presence of Streptococcus in some autistic children. Nevertheless, further detailed investigation is needed to clarify the role of Streptococcus in autism.

Viruses

Viral infections can trigger autoimmune responses and eventually lead to organ-specific autoimmune diseases [44]. With regard to the cause of autism, the trigger mechanism is not known but viral infections such as rubella, measles, human herpes virus-6 (HHV-6), influenza and cytomegalovirus (CMV) have been suspected [45–47]. The research to date, although far from conclusive, suggests an underlying inability of some ASD children to fully eradicate viral insult and/or mount a functional immune response.
The rubella viral epidemic in 1964 provided investigators with an opportunity to study the possible relationship between an infection and a clinical syndrome. The epidemic resulted in the birth of 20,000-30,000 infants with congenital malformation. Studies of 243 youths from this cohort found a prevalence rate for autism substantially higher than expected [48,49]. To date, over 42 cases connecting congenital rubella to autism have been reported [50,51].

Numerous other cases have been described in which a viral infection was associated with the development of autism. Stubb [52] first reported on the case of a child who developed autism secondary to intrauterine CMV infection. DeLong et al. [53] described an 11-year-old girl who developed symptoms of autism after the onset of herpes simplex virus (HSV) encephalitis. An extensive lesion to the left temporal lobe was found on computed tomography scan. This development was reversible. Gillberg [54] reported a 14-year-old female who developed autism secondary to HSV encephalitis, which persisted at 10-year follow-up.

Nevertheless, Case–control studies have demonstrated mixed findings. Early work by Peterson and Torrey [55] did not find evidence of increased infection by viruses, such as hepatitis B, CMV, rubella and measles, in sera of autistic children versus controls. But at follow-up, there was an increased incidence of antibodies to HSV in the blood of the autistic children. Further examination of serum antibody titres to HSV [56], CMV [57], parvovirus B19 [58], as well as HHV-6 and measles virus [44] revealed no difference between autistic children and controls.

A serological study of measles-mumps-rubella (MMR) antibodies was conducted with serum samples of 125 autistic children and 92 control children [59]. Analyses revealed an atypical MMR antibody in 75 (60%) of 125 autistic sera versus none in control sera. Singh and Jensen [60], in their study of 88 autistic children and 15 siblings and 32 normal controls, found increased measles virus antibody levels in sera of autistic children compared with controls. Serum antibodies to mumps or rubella virus did not differ between the groups. Another investigation found that blood titres of rubella virus antibodies were 10 times normal in 11 (50%) of 22 autistic patients and five (28%) of 18 patients with another pervasive developmental disorders (PDDs) [61]. Recently, Rosen et al. [62] conducted a case–control study (cases, n = 403; controls, n = 2100) to investigate the relationship between infections in the first 2 years of life and subsequent diagnosis of a PDD. The authors found no such relationship to the development of autism, but did note modestly elevated rates of infection in the first 30 days. The onset of autism following early infection with congenital syphilis [63] and varicella encephalitis [64] has also been described.

According to Gillberg and Gillberg [65], during pregnancy, 43% of the mothers of autistic children compared with 26% of control mothers reported the occurrence of an influenza-like illness, upper respiratory tract infection, urinary tract or vaginal infection. One study found a trend towards increased infections during pregnancy in 25 mothers bearing autistic children versus 25 matched healthy controls. Another study of 183 mothers of children with autism versus 209 healthy controls identified a significant increase in viral infections, including rubella, during pregnancy [66]. Yet other similar studies, including a study of 74 children with autism compared with US population indices [67] and a study of 233 children with autism versus 62 of their nonautistic siblings [68], have found no such correlation.

Lancaster et al. [69] used rats as an animal model of ASD based on neonatal Borna disease virus (BDV) infection. The neonatal BDV infection profoundly affected social behaviour in adult rats. Compared to control rats, both 90-day-old and 180-day-old infected rats spent less time in social interaction and more time in following their partners. The BDV-infected behaviour may be an example of ‘stereotypic’ activity due to BDV-induced abnormal social communication between rats.

The mechanisms by which viral infection may lead to autism, be it through direct infection of the CNS, through infection elsewhere in the body acting as a trigger for disease in the CNS, through alteration of the immune response of the mother or offspring, or through a combination of these, is not yet known. Animal models in which early viral infection results in behavioural changes later in life include the influenza virus model in pregnant mice and the BDV model in newborn Lewis rats. Many studies over the years have presented evidence both for and against the association of autism with various viral infections [60,70] and there exists substantial controversy as to the involvement of measles virus and/or the MMR vaccine in the development of autism [71,72]. Biological assays lend support to the association between measles virus or MMR vaccine and autism, whereas epidemiologic studies show no such association [73]. Further research is needed to clarify the possible mechanisms whereby viral infection early in development may lead to autism [74].

**Fungi**

Crook [75] suggested a causal hypothesis for autism whereby overgrowth of *Candida* in the intestine might occur secondary to antibiotic use or secondary to ingestion of processed sugar and others foods which enhance yeast growth. *Candida* can affect the CNS and the immune system [76]. The carbohydrate alcohol, arabitol, is produced by *Candida albicans* [77] and
measurement of arabinose in human and animal blood has been used as an indicator of the extent of Candida infection. Shaw et al. [78] reported a marked increase in analogies of Krebs cycle metabolites in the urine of two brothers with autistic features. These metabolites included citramalic acid, carboxylic acid, 3-oxoglutaric acid, tartaric acid and arabinose. The mean concentration of arabinose in urine of normal children was 60.4 nmol/mol creatinine whereas in the autistic brothers it was 305 nmol/mol creatinine.

Treatments to minimise the overgrowth of Candida include antifungal agents and probiotic agents such as Lactobacillus [79] as well as dietary modification to decrease the substrates for yeast overgrowth. No clinical trials have been published to date in the peer-reviewed literature examining these treatments for ASD, although they remain popular [80]. The presence of Krebs cycle intermediates of potentially fungal origin in the urine of some children with autism warrants further investigation.

Conclusion

Studies into the gastrointestinal microbiology of autism to date have been diverse and often contradictory. As reviewed, case studies describing an association between autism and infections suggest that, in some cases, infectious agents may be associated with the onset of autism. Are they secondary to disease processes that cause autism or resultant of neurobiological dysfunction? The question as to how microbes may play a role in autism needs to be considered. Possible mechanisms might include direct viral damage to neurons, immune elements (cytokines and interferon), nitric oxide, toxic metabolites (lipopolysaccharide, phenol, p-cresol, indole derivatives and arbutol), toxins (enterotoxin and neurotoxins) and induction of autoimmune responses. Studies that examine risk factors prospectively and studies examining more reliable biomarkers of infection are needed.

The possible aetiological role of these infectious agents (or at least a mechanistic role) is biologically plausible and consistent with the present understanding of the brain-gut axis (BGA). The BGA is a bidirectional nervous connection between the brain (CNS) and gut (enteric and autonomic nervous systems) that serves various physiological functions. The role of the CNS in the modulation of various gut functions, including motility, secretion, blood flow and gut-associated immune function in response to psychological and physical stressors, is well established by preclinical and clinical evidence [81]. Conversely, the possible effect of microbial signalling molecules beyond the gastrointestinal tract and on the nervous system has received little attention. Luminal microorganisms produce a range of signalling molecules (bipeptides and tripeptides, e.g. N-formyl-methionyl-leucine-phenylalanine) [82] that can interact with receptors of other microbes, as well as those on host cells. Through these various mechanisms, enteric microbiota are likely to affect the nervous system via endocrine, immune and neural signalling pathways [83,84]. A possible role for gut infection in triggering brain responses, such as anxious behaviour and mood changes, in the absence of overt gut inflammation or elevation of plasma cytokines has been described in mice [85]. It is thought that an extensive and cordial ‘conversation’ is occurring between host and microbiota, but it is yet to be confirmed that bacteria may use their signals to modulate complex host behaviours and function.

In order to advance our understanding of the role of gastrointestinal microbes in autism, we propose the following as priority areas for future research:

1. Identification of elevated levels of unfavourable or pathogenic bacteria in ASD individuals
2. The effect of LPS on cognitive function and neurodevelopmental processes
3. Improved identification of viral precursors and concomitants in ASD
4. The possible interaction between microbial findings in ASD with other aetiological hypotheses such as mercury poisoning and genetic foundations
5. Microbiological factors as a causal or accessory factor in aetiological models
6. Autoimmune responses to microbiological anomalies in ASD individuals
7. Microbiological factors as targets for novel approaches to autism treatment.

In order to overcome the existing inconsistencies in this field of research, it is important that future studies are conducted using high-quality designs. Obviously, patients and controls must be well matched and, because autism may have more than one cause, attention must be given to subsets of patients, such as those with regressive-onset autism or clinical and behavioural traits that might be strongly associated with gastrointestinal abnormalities (such as behaviours that serve to exert pressure on the abdomen).

Some association of ASD with viruses, fungi and bacterial populations seems apparent, although we are unable to consistently identify or even quantify their precise nature or mode of action as it relates to the development or maintenance of autism. If abnormalities in gastrointestinal microbiota are found to play an integral role in the pathophysiology of autism, then therapies based on correcting these abnormalities may indeed prove helpful. At present, the findings are so inconsistent that routine treatment of autism that focuses on gastrointestinal microbiology is not easily justified. Despite this, the available data indicate a likely relationship between ASD
Gastrointestinal microbiota in ASD

and gastrointestinal microbial aberrations, and therefore further and urgent research on the role of intestinal microbiota in autism is warranted.

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50 Review in Medical Microbiology 2010, Vol 21 No 3


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Chapter 4

Faecal microbiota of individuals with autism spectrum disorder

The paper presented in the previous chapter (Gondalia et al., 2010b) suggested that research into GI microbiology and autism to date has provided mixed findings and often contradictory conclusions. Specifically, when trying to find an association between autism and GI microbiota, it is difficult to make a hypothesis about whether GI microbiota are related with the onset of autism or whether they are secondary to the disease processes that cause autism or subsequent to neurobiological dysfunctions. Previous research has suggested that certain bacteria such as *Clostridium* spp. are associated with the onset of autism (Finegold, 2008; Finegold et al., 2002). It has also been observed that the GI tracts of children with autism have lower counts of ‘good’ bacteria (such as *Lactobacillus, Bifidobacterium*) and are colonised with more harmful bacteria (such as clostridia) compared to neurotypically developing children (Finegold, 2008; Finegold et al., 2010; Finegold et al., 2002; Song et al., 2004).

GI microbial ecosystem is very diverse and complex to study in the laboratory. The GI microbiota is highly influenced by the surrounding environment, personal hygiene conditions and dietary factors. It can be altered by treatment with antibiotics, probiotics and prebiotics. The GI microbial ecosystem plays an important role in an individual’s health. Any disruption of this system may lead to disease conditions. Recent research in this field has indicated the involvement of bacterial infection and disruption of the normal microbial profile in the GI tract of patients with Crohn’s disease (Sartor, 2006) and irritable bowel syndrome (Posserud et al., 2007; Pyleris et al., 2012). Research has suggested that the GI microbiota is greatly affected by anxiety and stress-like conditions.
(Ait-Belghaoui et al., 2012; Lutgendorff et al., 2008). Therefore, it is possible that a condition like autism may affect the resident GI microbiota rather than vice versa. Of course, the relationship could be bidirectional (at least in a population of ASD patients). The association of autism and GI microbiota seems apparent, although we are unable to consistently identify, even quantify, their detailed nature or mode of action as it relates to the increase or maintenance of autism. Furthermore, determining the role of GI microbiota in the pathophysiology of autism and identifying all microbial entities, including viruses and fungi, are challenging and require rigorous research. Thus, this thesis has focused on the role of GI bacteria in relation to ASD.

The following study was designed as a pilot study on the basis of the aforementioned research literature that suggests gastrointestinal microbiota may play a role in the pathophysiology of ASD. This was the first study of its kind conducted in Australia. The aim of this study was to determine if a relationship exists between ASD severity and GI microbial populations. This was done by examining whether any particular bacterial group was consistently present or absent (or in higher or lower numbers) in autistic children of various clinical severities. This study also attempted to determine whether the microbial profile of children with autism differed from a laboratory reference range.
Paper 2


Reprint of original article
Faecal microbiota of individuals with autism spectrum disorder

Abstract
Many children with autism spectrum disorders (ASDs) suffer from gastrointestinal problems such as diarrhoea, constipation and abdominal pain. Such symptoms may be due to a disruption of the indigenous gut microbiota promoting the overgrowth of potentially pathogenic microorganisms. These observations have stimulated investigations into possible abnormalities of intestinal microbiota in autistic patients. The purpose of the present study was to determine if a relationship exists between ASD severity (mild – severe) and gastrointestinal (GI) microbial populations. The faecal microbiota of 22 male and 6 female participants with ASDs (aged 2-14 years) were analyzed by standard microbial culture methods and compared within-group (based on ASD severity) and with a standard laboratory reference range. Comparisons between children with mild ASD and those with moderate to severe ASD, as well as comparisons to a neurotypical control group previously reported, revealed that no significant differences appear to exist in the composition of the gut microbiota. Nevertheless, examination of each individual’s gut microbial composition showed 10 cases of unusual findings which means 1 out of 3 cases have unusual microbiota. Our data do not support consistent GI microbial abnormalities in ASD children, but the findings do suggest that aberrations may be found in a minority of ASD children. Further studies are required to determine the possible association between the microbiota and gastrointestinal dysfunctions in a subset of children with both ASD and gastrointestinal problems.

Keywords: Microbiota; ASD; gastrointestinal tract; bacteria

Introduction
Autism spectrum disorders (ASDs) are behaviorally defined developmental disorders (including autistic and Asperger’s disorder) with a wide range of behaviours. Although the etiology of ASD is unknown, data suggest that autism may have multiple etiologies with both genetic and environmental contributions (Herbert, 2010). This diverse etiology may explain the spectrum of observed behaviours seen in affected individuals. Nevertheless, recent studies have suggested gastrointestinal (GI) disorders and associated symptoms are commonly reported in individuals with ASD, but key issues such as the prevalence and best treatment of these conditions are incompletely understood (Buie, Campbell et al., 2010; Buie, Fuchs et al., 2010; Galliatsatos, Gologan, & Lamoureux, 2009; Gondalia, Palombo, Knowles, & Austin, 2010). The clinical picture of GI disturbance includes constipation, diarrhoea, foul-smelling stools, gaseousness, abdominal bloating, signs of abdominal discomfort, and decreased intestinal carbohydrate digestive enzyme activity (Horvath, Papadimitriou, Rabsztyn, Drachenberg, & Tildon, 1999). Nevertheless, the incidence and nature of GI symptoms in those with ASD remains somewhat uncertain and, even, controversial (Ibrahim, Voigt, Katusic, Weaver, & Barbaresi, 2009; Kuddo & Nelson, 2003; Molloy & Manning-Courteny, 2003).

Reasons to consider that microorganisms may be involved in autism include the following: gastrointestinal symptoms are common at ASD onset and often persist, antimicrobials (e.g., oral vancomycin) may lead to a clear-cut response and relapse may occur when the vancomycin is discontinued, and some patients have responded to several courses of vancomycin and relapsed each time it was discontinued. Vancomycin has proven effective in reducing autistic symptomatology and is known to be highly effective against Gram-positive organisms. When given orally, vancomycin is poorly absorbed by the gut, so any effect seen with its use is likely due to its action on intestinal bacteria (Finegold, 2008; Sandler et al., 2000).

One hypothesis is that the toxins produced by microorganisms may play an important role in the cause of ASD, in particular, lipopolysaccharides (LPS), the bacterial toxins from Gram-negative bacteria (Gondalia...
The present study was, therefore, to examine microbiology and ASD severity (Finegold et al., 2010). One has examined the relationship between faecal microbiota of individuals with ASD to controls (Finegold et al., 2002; Parracho, Bingham, Gibson, & Probert, Smejkal, & Gibson, 2003). The variable results reported are not surprising given the lack of consistent evidence as to the specific microbial adjustment that is desirable. While many studies have compared the faecal microbiota of individuals with ASD to controls (Finegold et al., 2002; Parracho, Bingham, Gibson, & Probert, Smejkal, & Gibson, 2003), the treatment of GI dysfunction in ASD with anti- or probiotics has been proposed as a way to regulate intestinal microbiota and subsequently improve symptom severity in ASD (Sandler et al., 2000; Tuohy, Probert, Smejkal, & Gibson, 2003). The variable results reported are not surprising given the lack of consistent evidence as to the specific microbial adjustment that is desirable. While many studies have compared the faecal microbiota of individuals with ASD to controls (Finegold et al., 2002; Parracho, Bingham, Gibson, & Probert, Smejkal, & Gibson, 2003), only one has examined the relationship between faecal microbiology and ASD severity (Finegold et al., 2010). The purpose of the present study was, therefore, to determine if a relationship exists between ASD severity (mild – severe) and GI microbial populations. Such an association would be consistent with a dose-response relationship whereby GI microflora aberrations are considered to play a role in the aetiology of the disorder or, alternatively at least, a mechanistic role related to symptom severity.

Method

Participants

Twenty-eight individuals aged 2-14 years (M = 4.8, SD = 3.3; 22 male and 6 female) with ASD participated in this study. None of the participants were taking antibiotics within 14 days prior to the sampling. Dietary intake was not controlled and so this likely varied across individuals. The participants were recruited from consecutive patients presenting to the private psychology practice of the fourth author. The study was approved by the Human Research Ethics Committee of Swinburne University of Technology, and informed consent was given by parents on behalf of their children who participated in the study.

Faecal sampling and analysis

Faecal microbial analysis by standard microbial culture methods was carried out by a commercial laboratory, Bioscreen Medical (www.bioscreenmedical.com). Participants were provided with a sample collection kit containing a specimen container (lid with two holes), an air-locked plastic bag, two gel freezer packs and an AnaeroGen Compact foil sachet (Oxoid, UK) to create anaerobic conditions inside the plastic bag.

Faecal samples were collected in specimen containers with a scoop attached to the lid. Participants were instructed to collect enough faeces to fill at least 1/3 of the scoop with faeces from used toilet paper. Participants were instructed to screw the lid on firmly, place the container in the plastic bag along with an opened AnaeroGen Compact foil sachet and seal the bag to make it airtight. For transport to the laboratory, samples were sealed in a polystyrene box together with two freezer packs.

Statistical methods

Faecal microbial analysis was carried out to identify the presence and quantities of bacteria (aerobes and anaerobes), and the yeast, Candida. Analysis of variance was used to examine difference between mild and severe ASD groups. Data analysis was also carried out between the ASD groups as a whole and the commercial laboratory reference ranges. Various groups of organisms were also analysed for possible significant associations and data from this study were compared with that from published control group data (Finegold, Flora, Attebery, & Sutter, 1975) as well as to the laboratory reference ranges. Data were analyzed using the Statistical Package for Social Sciences (SPSS).

Results & Discussion

Participants were divided into two groups: those with moderate to severe ASD (n = 16) and those with mild ASD (n = 12) (Table 1). The severity category was based on CARS (Childhood Autism Rating Scale) assessments conducted by registered psychiatrists or psychologists and the child’s score reported by parents’ of participants. Scores of less than 37 are considered mild and those at or above 37 are considered moderate – severe (Rellini, Tortolani, Trillo, Carbone, & Montecchi, 2004). Details on the specific bacteria recovered with ranges and converted mean counts are given in Table 2. These counts represent a conversion from the means obtained after log transformation of the
Individuals from different groups (mild vs. moderate-severe) of ASD showed significant differences in faecal microbiota. Analyses of variance revealed no significant differences between the two ASD severity groups on mean levels for any of the analytes (see Table 1). Furthermore, no relationships were evident between the levels of any bacterial populations examined across age and gender. Inspection of each individual case revealed some aberrant results, but not in a consistent or common enough pattern to significantly affect the mean group levels. Ten ASD children appeared to show more variation in microbial profile than the other 18 ASD children (see Table 3). Given the lack of associations between GI microbiota population levels and autism severity we decided to compare our total sample means with the normal laboratory reference ranges as well as means reported for neurotypical individuals in a previously published study (Finegold et al., 1975).

### Table 1: Mean values of faecal microbiota examined for different groups (mild vs. moderate-severe) of ASD individuals

<table>
<thead>
<tr>
<th></th>
<th>Mild ASD group (n=12)</th>
<th>Moderate to severe ASD group (n=16)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total bacterial count</td>
<td>10.26</td>
<td>10.36</td>
<td>.62</td>
</tr>
<tr>
<td>Total aerobe count</td>
<td>7.31</td>
<td>7.37</td>
<td>.83</td>
</tr>
<tr>
<td>Total anaerobe count</td>
<td>10.26</td>
<td>10.35</td>
<td>.64</td>
</tr>
<tr>
<td><em>Candida</em></td>
<td>2.48</td>
<td>3.98</td>
<td>.07</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>6.44</td>
<td>6.69</td>
<td>.55</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>6.79</td>
<td>6.51</td>
<td>.58</td>
</tr>
<tr>
<td><em>Staphylococcus</em> spp.</td>
<td>5.62</td>
<td>5.63</td>
<td>.98</td>
</tr>
<tr>
<td><em>Streptococcus</em> spp.</td>
<td>6.71</td>
<td>6.73</td>
<td>.96</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> spp.</td>
<td>9.14</td>
<td>9.38</td>
<td>.52</td>
</tr>
<tr>
<td><em>Clostridium</em> spp.</td>
<td>7.24</td>
<td>7.47</td>
<td>.79</td>
</tr>
</tbody>
</table>

The kind of individual aberrations found in our sample are consistent with the current consensus that GI dysfunction does occur more frequently in ASD than non-ASD children (Buie, Campbell et al., 2010), but possibly only in a minority of individuals. It is critical, therefore, for future research to attempt to identify this minority a priori in order to conduct more advanced testing beyond group mean comparisons which our study show may dilute (at a group level) any aberrations apparent in the minority of children. Furthermore, future researchers should cast a wider net in their search for microbial correlates in ASD. All studies conducted to date have examined only a fraction of the microbial species known to inhabit the gut, both pathogenic and commensal. Additionally, microbial species beyond bacteria should be examined, including viruses, fungi and protozoa. If microbial correlates to the GI disturbance through appropriate intervention may be developed, such as pro- or antibiotic therapy or dietary intervention.
## Table 2

*Comparison of bacterial counts between individuals with an ASD, control group and laboratory reference range*

<table>
<thead>
<tr>
<th>Lab ref Range (n = 177)</th>
<th>Total ASD Range (n = 28)</th>
<th>Control Range (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Range</td>
</tr>
<tr>
<td>Total bacterial count</td>
<td>11-12</td>
<td>28</td>
</tr>
<tr>
<td>Total aerobe count</td>
<td>7-8</td>
<td>28</td>
</tr>
<tr>
<td>Total anaerobe count</td>
<td>8-12</td>
<td>28</td>
</tr>
<tr>
<td><em>Candida</em></td>
<td>&lt;4</td>
<td>18</td>
</tr>
</tbody>
</table>

**Facultative Gram negative bacteria**

- *Escherichia coli*: 7-8 | 27 | 4-8 | 6.59 | 23 | 4-11 | 8.10
- *Klebsiella spp.*: <5 | 3 | 4-7 | 5.48 | 12 | 4-10 | 4.81
- *Citrobacter spp.*: <6 | 0 | - | - | 1 | 8 | 2.76
- *Enterobacter spp.*: <6 | 4 | 5-7 | 6.24 | 1 | 4 | 2.17

**Facultative Gram positive bacteria**

- *Enterococcus spp.*: <6 | 9 | 6-7 | 6.41 | - | - | -
- *Staphylococcus spp.*: <5 | 8 | 5-7 | 5.63 | 9 | 4-10 | 4.44
- *Streptococcus spp.*: <6 | 25 | 5-9 | 6.73 | - | - | -
- *Alpha-haemolytic Streptococcus*: <6 | 14 | 4-8 | 6.66 | 9 | 5-11 | 3.91
- *Beta-haemolytic Streptococcus*: <6 | 5 | 4-6 | 5.17 | - | - | -
- *Non-haemolytic Streptococcus*: <6 | 22 | 4-9 | 6.43 | - | - | -

**Anaerobic Gram negative bacteria**

- *Prevotella spp.*: <10 | 19 | 9-11 | 9.73 | - | - | -
- *Porphyromonas spp.*: <9 | 2 | 9-10 | 9.80 | - | - | -
- *Eubacterium*: <9 | 7 | 9-11 | 9.67 | - | - | -

**Anaerobic Gram positive bacteria**

- *Eubacterium*: <9 | 0 | - | - | - | - | -
- *Bifidobacterium spp.*: 7-9 | 22 | 7-10 | 9.29 | 10 | - | 8.31
- *Lactobacillus spp.*: 6-8 | 8 | 7-10 | 8.24 | 10 | - | 6.90
- *Clostridium spp.*: <9 | 28 | 0-10 | 9.71 | - | - | -
- *Peptostreptococcus spp.*: <5 | 1 | 0-10 | 9.71 | - | - | 5.91

*Note:* *Bioscreen (2009), Finegold et al (1975), Log10 no. of organisms per g of faeces (rounded off to nearest log in the case of range)
Table 3

Microbiological data from examination of faecal samples obtained from 10 selected children with abnormal microbiota compared with a control group

<table>
<thead>
<tr>
<th>Organism isolated</th>
<th>Microbiological data, by patient number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group converted mean&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total bacterial count</td>
<td>10.55</td>
</tr>
<tr>
<td>Total aerobe count</td>
<td>9.92</td>
</tr>
<tr>
<td>Total anaerobe count</td>
<td>11.58</td>
</tr>
<tr>
<td>Candida</td>
<td>3.39</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>8.10</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>4.81</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>-</td>
</tr>
<tr>
<td>Sphingobacterium spp.</td>
<td>4.44</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>-</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>8.31</td>
</tr>
<tr>
<td>Lactobacillus spp.</td>
<td>6.90</td>
</tr>
<tr>
<td>Clostridium spp.</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Note: Finegold et al (1975);<sup>b</sup>Log<sub>10</sub> no. of organisms per g of faeces (rounded off to nearest log in the case of range)

References


The paper presented in the previous chapter (pilot study) (Gondalia et al., 2010a) suggested that the kind of individual aberrations found in the participants were consistent with the current consensus that GI dysfunction does occur more frequently in ASD than non-ASD children. However, the findings failed to support the hypothesis that the GI microbiota of individuals with autism was significantly different from neurotypically developing children. Nevertheless, these results did indicate that not all the autistic individuals had disrupted GI microbiota and that possibly only a minority of children with ASD showed evidence of GI microbial aberrations.

The previous paper (pilot study) also suggested that limitations inherent in the study may, in part, explain the mixed findings. Specifically, the limitations of such research may be reduced with larger numbers of participants, related control (sibling) groups and by using high throughput bacterial analytical techniques rather than culture methods. Furthermore, the consistency of the results is potentially increased by comparisons based on the degree of severity and the GI dysfunctions.

The aim of the following paper, therefore, was to conduct experiments that overcame the limitations of previous studies. This study utilised advanced technology, namely bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP), to explore the GI microbial community in faeces collected from autistic individuals and their neurotypical siblings. The technique enables testing of the bacterial
community in a more comprehensive way and with a greater validity, in comparison to culture-based method.

Participants were recruited across 4 groups; (1) children with autism with GI dysfunction, (2) children with autism without GI dysfunction, (3) neurotypical siblings with GI dysfunction and (4) neurotypical siblings without GI dysfunction. Children with autism and their neurotypical siblings were recruited for the detailed comparison of GI microbiota whilst keeping other variables, such as the surrounding environment, similar. Participants were excluded from the study if they were using any antibiotic/probiotic treatment 15 days prior to sample collection to remove any effect of antibiotics on resident GI microbiota.
Paper 3


Reprint of original article
Molecular Characterisation of Gastrointestinal Microbiota of Children With Autism (With and Without Gastrointestinal Dysfunction) and Their Neurotypical Siblings

Shakuntla V. Gondalia, Enzo A. Palombo, Simon R. Knowles, Stephen B. Cox, Denny Meyer, and David W. Austin

Many children with autism spectrum disorders (ASDs) suffer from gastrointestinal problems such as diarrhea, constipation and abdominal pain. This has stimulated investigations into possible abnormalities of intestinal microbiota in autistic patients. Therefore, we designed this study to identify differences (and/or similarities) in the microbiota of children with autism (without gastrointestinal dysfunction: \( n = 23 \)) and children with gastrointestinal dysfunction (\( n = 28 \)) and their neurotypical siblings (\( n = 53 \)) who share a similar environment using bacterial tag-encoded FLX amplicon pyrosequencing. Regardless of the diagnosis and sociodemographic characteristics, overall, Firmicutes (70%), Bacteroidetes (20%) and Proteobacteria (4%) were the most dominant phyla in samples. Results did not indicate clinically meaningful differences between groups. The data do not support the hypothesis that the gastrointestinal microbiota of children with ASD plays a role in the symptomatology of ASD. Other explanations for the gastrointestinal dysfunction in this population should be considered including elevated anxiety and self-restricted diets. *Autism Res* 2012, 5: 419–427. © 2012 International Society for Autism Research, Wiley Periodicals, Inc.

**Keywords:** faecal microbiota; pyrosequencing; autism spectrum disorder; gastrointestinal dysfunction

**Introduction**

Autism spectrum disorders (ASDs) are defined by cognitive defects, impairments of spoken and/or receptive language, social interactions, and repetitive and stereotyped behaviours. In addition to these core deficits, previous studies indicate that the prevalence of gastrointestinal (GI) symptoms is high in individuals with ASD [Buie et al., 2010; Finegold et al., 2002; Gondalia, Palombo, Knowles, & Austin, 2010a,b]. The percentage of individuals suffering from GI problems varies from study to study [Buie et al., 2010], but high rates of GI dysfunction in individuals with ASD have been reported in several studies with symptoms including abdominal pain, constipation, diarrhoea, diarrhea and alternating constipation, and GI inflammation [Horvath, Papadimitriou, Rabsztyn, Drachenberg, & Tildon, 1999; Levy et al., 2007, Molloy & Manning-Courtney, 2003]. An elevated prevalence of GI dysfunction in individuals with autism was first reported by Goodwin, Goodwin, and Cowen [1971]. In a study of 137 children with ASD, 24% had a history of at least one GI symptom. Another study found that 70% of children with ASD presented with GI symptoms compared with 28% of neurotypically developing children and 42% of developmentally disabled children [Valicenti-Mcdermott et al., 2006]. Additionally, in a metal toxicity study of 51 children with ASD compared with 40 typical controls aged 3–15, 63% of children with autism were reported to have diarrhoea and/or constipation compared with 2% of the control children [Adams, Holloway, George, & Quig, 2006]. In summary, these studies consistently demonstrate that GI symptoms are common in ASD paediatric populations, although the precise nature of these symptoms varies considerably both between and within study populations.

GI dysfunctions, like diarrhea, constipation and abdominal pain, are unpleasant and may lead to frustration, decreased ability to concentrate, behavioural problems, and possibly aggression and self-abuse, especially in children unable to communicate [Hollander & Anagnostou, 2008], although there is little direct evidence for this. The cause of GI dysfunction in the ASD population is unclear, but it may partly relate to the use of oral antibiotics that can alter gut microbiota. Many studies have
reported significantly higher oral antibiotic use in children with autism than typical children [Adams et al., 2006; Adams, Romdalvik, Levine, & Hu, 2008; Konstantareas & Homatidis, 1987; Niehus & Lord, 2006]. Furthermore, occurrence of ear infections and the use of antibiotics were significantly higher in children with ASD than typically developing children [Niehus & Lord, 2006]. These antibiotics can disrupt the protective microbiota that plays an important role in normal GI function. The disruption of this normal gut microbiota can create favourable conditions for colonisation by pathogenic and/or harmful bacteria, and it is hypothesised that this can cause or contribute to GI dysfunction commonly reported in ASD populations.

Bolte [1998] first hypothesised a possible role for Clostridium tetani in the pathophysiology of autism, which was followed by many studies showing over-growth of Clostridium species and other harmful bacteria in ASD populations. Sandler et al. [2000] studied the effects of a minimally absorbed oral antibiotic (vancomycin) on 11 children with regressive-onset autism. This treatment produced short-term improvement in autistic symptoms using multiple pre- and post-therapy evaluations. The same research team then studied the GI microbiota in 13 children with late-onset autism and eight controls using conventional culture and polymerase chain reaction (PCR) methods. Results indicated higher counts of Clostridium and Ruminococcus species in autistic children and a few species of these genera present only in the faeces of children with autism [Finegold et al., 2002]. In a follow-up study, Song, Liu, and Finegold [2004] used quantitative real-time PCR to evaluate Clostridium species in the faeces of children with autism and controls. They found that mean cell counts of Clostridium cluster groups I and XI were significantly higher in autistic children than those of control children. Finegold et al. [2010] investigated GI microbiota in 33 autistic children, 7 ne-urotypical siblings and 8 non-sibling controls using the bacterial tag-encoded FLX amplicon pyrosequencing (bTTEFAP) procedure. Study data indicated several differences at the phylum level, including higher levels of Bacteroidetes in the severely autistic group and higher levels of Firmicutes in the control group. Additionally, Desulfovibrio species and Bacteroides vulgatus were present in higher numbers in autistic than control children. Nevertheless, this study did not show a significant difference between the autistic and neurotypical sibling control groups. In a follow-up study, Finegold [2011] hypothesised that Desulfovibrio may play a role in autistic symptomology. This species produces important virulence factors like lipopolysaccharides (LPSs) and hydrogen sulphide. Its unique physiology is congruent with some of the pathophysiology seen in autism.

GI microbiota can produce toxic metabolites that can gain access to the central nervous system (CNS) via the vagus nerve. Some studies have reported increased intestinal permeability that increases the absorption and pas-sages of toxic metabolites. Studies of urinary biomarkers for autism have reported elevated levels of p-cresol in severely affected males [Altieri et al., 2011; Yap et al., 2010]. P-cresol is a metabolically active toxic compound produced primarily by Clostridia. Similarly, the bacterial metabolic end-product, propionic acid [Shultz et al., 2008], produces social impairment in experimental mice when injected into the cerebrum.

In short, disruption of normal neurodevelopment by GI microbiota and its metabolites has been theorised to contribute to autistic pathology. We noted previously that not all autistic individuals suffer from GI dysfunction and that only a subpopulation is affected [Gondalia et al., 2010a]. Although numerous intestinal microbial abnormalities have been identified in autism, conflicting results have often been reported. The purpose of this study was to identify whether a difference exists between the resident GI microbiota in children with autism (with and without GI dysfunction) and their neurotypical siblings.

The intestinal microbial ecosystem is very complex, and therefore, it is very difficult to study the entire ecosystem using conventional microbiological methods like culture methods. Nevertheless, researchers examining GI microbial variables in ASD populations have most commonly used this method. Culture methods have many limitations over nonculture (molecular) methods. For example, some microorganisms are not able to grow in culture media in laboratory conditions, and therefore, it is very difficult to conduct a comprehensive ecological study. Newly developed molecular methods overcome this limitation [Finegold, 2008, 2011; Finegold et al., 2002, 2010; Song et al., 2004] and allow for the identification of a comprehensive range of microorganisms in samples compared with culture methods by directly extracting the DNA and then carrying out sequencing for determining the specific bacterial composition. Therefore, we used advance technology, bTTEFAP, to study the GI microbial community in the faeces of children with autism and their neurotypical siblings.

**Method**

This publication reports on a study of children with autism compared with their neurotypical siblings. The study was approved by the Human Research Ethics Committee of Swinburne University of Technology, Australia.

**Participant Recruitment**

Participants were recruited through notices posted on websites and newsletters of various local state and...
national bodies related to autism (Autism Victoria, Autism South Australia, Autism Spectrum Australia and Autism Australian Capital Territory, and autism schools in Victoria (Wantirna Heights School, Southern Autistic School, Western Autism School, Northern School for Autism, and Mansfield Autism Statewide Services) and New South Wales (Aspet Central Coast School, Aspect Hunter School, Aspect South Coast School, Aspect South East Sydney School, Aspect Vern Barnett School and Aspect Western Sydney School)).

**Enrolment Criteria**

1. Family having at least two children: one with autism or Asperger's disorder, and one neurotypical sibling, aged 2–12 years.
2. No use of any type of antibiotic or antifungal at least 15 days prior to faecal sampling.
3. Autism group: formal diagnosis of autism/Asperger's by a psychiatrist or psychologist.

**Protocol**

1. The study was explained to parents, and informed consent was received.
2. Parents filled out a questionnaire on their children’s diagnosis and symptoms (including GI).
3. Stool samples were collected by parents in sterilised faecal sample collection container during March–June 2011 and transported to Swinburne University in a 1-day express air post satchel containing a frozen gel ice pack. In the laboratory, samples were thawed and homogenised in sterile MilliQ water, and DNA was extracted. The extracted DNA was stored at −20°C until further analysis.

One hundred and four children (n = 51 with ASD and n = 53 neurotypical siblings) with an age range of 2–12 years participated in this study (see Table I for participant characteristics). The autism severity category was based on Childhood Autism Rating Scale (CARS) assessments [Rellini, Tortolani, Trillo, Carbone, & Montecchi, 2004] conducted by registered psychiatrists or psychologists, as reported by the parents of participants. Existing assessments were only used; no confirmatory assessment was conducted. In Australia, formal assessment and reassessment of autism and Asperger's disorder occur 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, and foul-smelling stools.

**DNA Extraction**

One-millilitre aliquots of stool, previously diluted 1:3 in sterile MilliQ water, was thoroughly homogenised and centrifuged at 13,000 × g for 3 min to pellet faecal bacterial cells. The supernatant was carefully removed and discarded. Two hundred milligrams of cell pellet were transferred to a fresh tube and subjected to DNA extraction using a commercial extraction system (QIAamp DNA stool mini kit, Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The concentration of extracted DNA was measured by a nanodrop spectrophotometer (Biowave-II, Biochrom, Cambridge, England).

**Massively Parallel bTEFAP**

bTEFAP was performed, as described previously, using Gray28F 5′TTTGATCNTGGTCAG and Gray519r 5′GTNTACNGCGGCAGCGCTG [Dowd, Callaway et al., 2008; Dowd, Sun et al., 2008; Dowd, Wolcott et al., 2008; Ishak et al., 2011; Sen et al., 2009]. Initial generation of the sequencing library utilised a one-step PCR, with a total of 30 cycles, with a mixture of Hot Start and HotStar high-fidelity Taq polymerases. Tag-encoded FLX amplicon pyrosequencing analyses utilised a Roche 454 FLX instrument with titanium reagents (Roche, Nutley, New Jersey). Titanium procedures were performed at the Research and Development and QIAseq facilities at Swinburne University.

---

**Table I. Summary of Participant Characteristics**

<table>
<thead>
<tr>
<th>Participant characteristic</th>
<th>Subcategory</th>
<th>Autism (n = 51)</th>
<th>Neurotypical sibling (n = 53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>42</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>9</td>
<td>34</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>21</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Mild</td>
<td>30</td>
<td>NA</td>
</tr>
<tr>
<td>Twins pair</td>
<td>Identical</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Identical</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Current GI dysfunction, n (%)</td>
<td>GI dysfunction</td>
<td>28 (55)</td>
<td>4 (8)</td>
</tr>
<tr>
<td></td>
<td>Constipation</td>
<td>12 (23.5)</td>
<td>3 (5.6)</td>
</tr>
<tr>
<td></td>
<td>Diarrhoea</td>
<td>5 (10)</td>
<td>1 (2)</td>
</tr>
<tr>
<td></td>
<td>Abdominal pain</td>
<td>7 (14)</td>
<td>2 (6)</td>
</tr>
<tr>
<td></td>
<td>Gaseousness</td>
<td>6 (12)</td>
<td>1 (2)</td>
</tr>
<tr>
<td></td>
<td>Foul-smelling stool</td>
<td>12 (23.5)</td>
<td>4 (7.5)</td>
</tr>
<tr>
<td></td>
<td>No GI dysfunction</td>
<td>23 (45)</td>
<td>49 (92)</td>
</tr>
<tr>
<td>Past history of GI dysfunction</td>
<td>All subjects</td>
<td>51</td>
<td>3</td>
</tr>
<tr>
<td>Medically diagnosed GI disorder</td>
<td>All subjects</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Current oral antibiotic</td>
<td>All subjects</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Current probiotics, n (%)</td>
<td>All subjects</td>
<td>17 (33)</td>
<td>8 (15)</td>
</tr>
<tr>
<td>Feeding till the age of 6 months</td>
<td>Breastfeed</td>
<td>23</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Formula feed</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Bath</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Food allergy, n (%)</td>
<td>All subjects</td>
<td>21 (43)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>Immunisation</td>
<td>All subjects</td>
<td>50</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>No immunisation</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

1Gluten-, casein-, dairy-, lactose- or preservative-free.
2Australian standard immunisation schedule according to age.
3GI, gastrointestinal; NA, Not Applicable.
Bacterial Diversity Data Analysis

Following sequencing, all failed sequence reads, low-quality sequence ends, and tags and primers were removed; sequence collections were depleted of any non-bacterial ribosome sequences and chimeras using B2C2 [Gontcharova, Wolcott, Hollister, Gentry, & Dowd, 2010], as has been previously described [Dowd, Zaragoza, Rodriguez, Oliver, & Payton, 2005]. To curate the data, short reads (<150 bp), sequences with ambiguous base calls and sequences with homopolymers >6 bp were removed. To determine the identity of bacteria in the remaining sequences, sequences were denoised, assembled into operational taxonomic unit (OTU) clusters at 96.5% identity and queried using a distributed-
.NET algorithm that utilises Blastn (KrakenBLAST http://www.krakenblast.com) against a database of high-quality 16S bacterial sequences. Using a .NET and C# analysis pipeline, the resulting BLASTn outputs were compiled, and data reduction analysis was performed, as described previously [Dowd, Callaway et al., 2008; Dowd, Wolcott et al., 2008; Wolcott, Gontcharova, Sun, & Dowd, 2009].

Bacterial Identification

Based upon the earlier BLASTn-derived sequence identity (percent of total length query sequence that aligns with a given database sequence), the bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences identified to known or well-characterised 16S sequences with identity scores greater than 97% identity (<3% divergence) were resolved at the species level between 95% and 97% at the genus level, between 90% and 95% at the family level, between 85% and 90% at the order level, between 80% and 85% at the class level, and between 77% and 80% at phyla level. After resolving based upon these parameters, the percentage of each bacterial identification was individually analysed for each sample, providing relative abundance of information within and among the individual samples based upon relative numbers of reads within each. Evaluations presented at each taxonomic level, including percentage compilations, represent all sequences resolved to their primary identification or their closest relative [Dowd, Callaway et al., 2008; Dowd, Sun et al., 2008; Finegold et al., 2010; Ishak et al., 2011; Sen et al., 2009].

Principal Coordinates Analysis

Differences among microbial communities within groups were characterised using the weighted UniFrac distance [Lozupone & Knight, 2005]. Principal coordinates analysis (PCoA) was then used to illustrate weighted UniFrac distances in two dimensions. Weighted UniFrac is a measure of community composition that takes into account the proportion of bacteria found in each sample [Fierer et al., 2010, Lozupone & Knight, 2007]. Multivariate differences among groups were evaluated using distance-based redundancy analysis (dbRDA). For the dbRDA, distances among samples were first calculated using weighted UniFrac distances, and then an analysis of variance (ANOVA)-like simulation was conducted to test for group differences. UniFrac distances were calculated using Quantitative Insights into Microbial Ecology [Caporaso et al., 2010], and all other analyses were conducted in R [Team, 2010] using the vegan [Jari Oksanen et al., 2011] and labdsv [Roberts, 2010] packages.

Indicator species analysis [Dufrene & Legendre, 1997] was used also to identify individual bacterial genera that were indicative of each of the treatment groups. Indicator species analysis synthesises information about occurrence and abundance of individual taxa, and this information is summarised in an indicator score. The analysis also provided a randomisation test of the degree to which taxa are indicative of a particular state. First, genera that were indicative of each of the levels of severity were identified. Then, genera that were indicative of GI dysfunction were examined for autistic and neurotypical samples separately.

Results

The numbers of species identified in a sample or the numbers of organisms detected at any given phylogenetic level are strongly affected by the number of sequences analysed [Schloss & Handelsman, 2005]. Pyrosequencing reads yielded an average of 4000 sequences >350 bp (average length 450 bp) per sample after passing through all quality controls and covered 19 phyla and 666 species. At any phylogenetic level, it is necessary to model and extrapolate the rarefaction curve or to use non-parametric methods to estimate the true maximum value. The non-parametric methods provide estimates that also vary with sample size, so their approach to a richness maximum must also be modelled and extrapolated. Differences in OTU richness estimated via both raw richness and Chao’s method (Fig. 1A,B) were assessed using ANOVA. Both methods produce similar outcomes. The ANOVA results obtained from these two methods are $F = 2.74, P = 0.071$ for raw richness and $F = 1.72, P = 0.1865$ for Chao’s method. Rarefaction and Chao’s maximum predicted curve modelling indicated that 98% OTUs at the 3% divergence were achieved for each sample, which suggests adequate depth of coverage. To make comparisons of richness, rarefaction was used to standardise the number of sequences per samples at 2824.
The average number of OTUs present in the faeces of all autistic samples at 3% sequence divergence (the species level) indicated no significant difference between the autistic and neurotypical sibling control groups.

Information at the phylum level was also analysed on the basis of severity and GI dysfunction. The microbiome mainly comprised 19 phyla overall. Of the major phyla, Firmicutes, Bacteroidetes, Proteobacteria and Verrucomicrobia were the most abundant in all the samples. These four phyla collectively constituted almost 99% of the microbiome. All four phyla were consistently present in all the groups (Fig. 2A,B). Firmicutes comprised 71%, 69% and 72% of the bacteria in the severe autism, mild autism and control groups, respectively. Figure 2A shows the composition of autistic children and sibling controls with and without GI dysfunction, respectively, emphasising no difference between the autistic and control groups. We also divided the group on the basis of the severity (severe and mild) to check the microbiome composition (Fig. 2B) and again showed no significant difference within and between the severity groups and controls.

Using the genus (301 genera) composition data to analyse the microbiome further, differences among diagnosis groups in the overall microbiome were not significant (dbRDA P > 0.05). Furthermore, as can be seen in the PCoA plot (Fig. 3), there was no systematic differences among the three groups—severe, mild and control. Regardless of the autism severity and GI dysfunction, there was no clustering of samples, which again indicates no significant difference in the bacterial composition within and between the autistic and control groups. Indicator species analysis (Figs. 4 and 5) [Dufrene & Legendre, 1997] synthesises information about occurrence and abundance of individual bacterial genera that is indicative of each of the treatment groups, and this information is summarised in an indicator score.

Discussion

The findings indicate that there was no significant difference in the bacterial composition of faecal material of our paediatric autistic group and their neurotypical siblings.
Our data also indicated that there were no significant differences within the ASD group when comparing those with and without GI dysfunction. The results, therefore, are not consistent with several previous reports [Finegold, 2008; Finegold et al., 2002; Song et al., 2004].

Previous studies comparing autistic and control groups have suggested that some microorganisms are more related to autistic symptoms, as more species are found in autistic cases than in neurotypical controls and those are related to disease severity [Finegold et al., 2002]. It has been hypothesised that *Clostridium* species are associated with autistic symptomology and the spore-forming property of *Clostridia* is of major importance for the recurrence of autistic symptoms after obtaining a good response to oral vancomycin [Sandler et al., 2000]. This property could also play a key role in the spread of bacteria to the GI tract of siblings and playmates. However, in the recent study by Finegold et al. [2010] using high-throughput technology (pyrosequencing), *Clostridia* were not found to be particularly prevalent in the stools of autistic individuals, and there was a suggestion by the author that *Desulfovibrio* might be important because it was more common in autistic children than in controls. Furthermore, although not a spore-forming organism, *Desulfovibrio* is very resistant to deleterious influences, and so it is possible that it may play some role in recurrence of autistic symptoms after treatment, although this needs to be tested in future research. In another study, *Bacteroidetes* were found at high levels in the severely affected autistic children, whereas *Firmicutes* were more common in the control children. They also indicated that siblings of autistic children had more similar results to autistic children than to the healthy controls. [Finegold et al., 2010]. Similarly, Parracho, Bingham, Gibson, and McCartney [2005] reported intermediate levels of *C. histolyticum* group in healthy siblings of autistic children, which was not significantly different from that of the autistic and healthy control groups. Thus, these studies suggest little consistency in the microbial profile of autistic children, suggesting that there are no particular bacteria clearly responsible for the pathophysiology of autism.

It is possible, therefore, that the elevated rates of GI dysfunction found in ASD populations might be based on factors other than GI microbiota. For example, it is well established that individuals with ASD have elevated levels of anxiety and distress [Hutman et al., 2010; Mayes, Calhoun, Murray, Ahuja, & Smith, 2011; Mayes, Calhoun, Murray, & Zahid, 2011; Wood et al., 2009]. GI research has highlighted the importance of the “gut-brain axis,” especially in relation to depression and irritable bowel syndrome, which is mainly focused on “top-down” control, or the examination of the impact that the brain can have on general gut function [Aziz & Thompson, 1998]. New work involving GI symptoms in anxiety and distress is indicating that stress and anxiety...
have an impact on GI function and condition. More likely, GI symptoms appear as a significant indicator for the presence of depression and anxiety [Mussell et al., 2008]. Using the top-down approach, research supports the theory that early life stress will lead to altered stress reactivity later in life and an altered gut microbial profile [O’mahony et al., 2009]. Therefore, it is possible that psychological rather than physiological factors may better account for the GI distress in the autistic individual. Nevertheless, GI dysfunction, irrespective of a psychological or physiological basis, is likely to cause stress in the individual.

Overall, the results of the current study do not support the hypothesis that the GI bacterial composition of children with ASD (both with and without GI dysfunction) is significantly different from their neurotypical siblings. This then further supports rejection of the hypothesis that there may be a GI bacterial pathophysiological mechanism triggering or maintaining ASD.

A particular strength of this study was that it compares microbiota in autistic children with and without GI dysfunction and compares these children with their neurotypical siblings—again, with and without GI dysfunction. Another strength was the large sample size and the molecular characterisation of the bacterial population extant in the faecal samples that included 666 different bacterial species. Nonetheless, there are several important limitations to this study that should be considered. First, this study used a prior assessment of autism (via the CARS) as a dependent variable and associated this to currently reported GI dysfunction. Ideally, thorough validated current assessments such as the Autistic Disorder Observational Schedule [Lord et al., 1989] would be conducted; however, this was beyond the resources of the present study. Furthermore, this study only evaluated the bacterial community. Microbial species beyond bacteria should ideally also be examined, including viruses, fungi and protozoa. In addition, we did not control for dietary differences among our participants, and as ASD children typically have a self-limited diet, it would be better to experimentally control dietary intake to remove its impact on GI microbiology. Furthermore, future research should focus on other microbial correlates to ASD rather than just the composition of GI microbiota. Importantly, microbial end-products/metabolites (i.e. neurotoxins, phenolic compounds and LPS) should be investigated, as these metabolites can be transmitted to the CNS and have the ability to modulate its function. Of course, it is also important to explore psychological factors such as anxiety and distress in future research in order to clarify their relationship with both GI function and autistic symptomatology.

Figure 5. Heatmap summary of the indicator species analysis performed on the bacterial genera. Only those genera that contained indicator values with \( P < 0.05 \) are shown. Gastrointestinal (GI) dysfunction: 0, without GI dysfunction; 1, with GI dysfunction.
References


Chapter 6

Evaluation of biogenic amines in the faecal samples of children with autism by LC-MS/MS

The findings of the papers presented in the previous chapters (Gondalia et al., 2010a; Gondalia et al., 2012) contribute significantly to the literature by confirming that GI dysfunction is more common in children with autism than typically developing control. However, the findings do not support the hypothesis that the GI microbiota are different and have a particular role in triggering or maintaining ASD. It is likely, therefore, that the higher rates of GI dysfunction found in ASD populations may be based on other factors.

Intestinal bacteria produce metabolites and release them into the surrounding environment as a result of host-microbial interactions and as part of their metabolic activities (Mishra et al., 2012). These metabolites can have significant effects on the host system. To date, very little research has been carried out to investigate the involvement of these metabolites in the pathophysiology of autism (MacFabe et al., 2011; Yap et al., 2010).

Yap et al. (2010) investigated the urine metabolic profile of children with autism and matched controls and noted that certain bacterial metabolites, namely dimethylamine, hippurate and phenyacetylglutamine (PAG), were in significantly different amounts in the affected and control groups. This observation has been associated with disrupted GI microbiota. Depletion of hippurate and PAG are reported after the ingestion of antibiotics like vancomycin (Yap et al., 2008) and gentamicin (Lenz et al., 2005).
Baker (1995) has reported that a significant percentage of individuals with autism have a history of extensive antibiotic use, supporting the observation that different amounts of bacterial metabolites reported in autism may have links with antibiotic use (Cryan et al., 2012; Parracho et al., 2005; Willing et al., 2011). Other bacterial metabolites that may relate to autism include short chain fatty acids (SCFA), biogenic amines (BA) and lipopolysaccharides (LPS) (Darlington et al., 2009; Kirsten et al., 2012; Wang et al., 2012).

The group of metabolites termed biogenic amines (BAs) includes cadaverine, putrescine, spermidine, histamine, agmatine, tryptamine, tyramine, serotonin, spermine and dopamine. These amines play a number of physiological roles in humans by acting as neurotransmitters (Medina et al., 2003). Research has indicated varied levels of these BAs in the blood and cerebrospinal fluids in individuals with autism (McCauley et al., 2004; Prasad et al., 2009).

The concentrations of biogenic amines present in cells and tissues are strongly controlled. However, the ingestion of foods rich in BAs or their production in the intestine by intestinal microbiota can alter the equilibrium (Mah et al., 2002; Taylor et al., 1986). This may cause host responses which can play an etiologic role in various conditions. BAs have been associated with migraine, schizophrenia, colon cancer and the onset of hypertensive symptoms (Agostinelli et al., 2007; Bardocz, 1995; Issa et al., 1994; Jansen et al., 2003; Onal, 2007). However, an association between BAs, GI dysfunction and autism has not been made.

The following study was therefore designed to quantitatively examine seven BAs, namely tryptamine, tyramine, histamine, spermidine, agmatine, cadaverine and putrescine, in the faeces of children with autism and their neurotypically developing siblings. A
liquid chromatography mass spectrometry-based method was developed for the analysis of BAs in faecal samples.
Paper 4


Reprint of original article
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 re-assessment 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%), 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].

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

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
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 1** Summary of participant’s characteristic

<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></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></td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Medically diagnosed GI disorder</td>
<td></td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
Current oral antibiotic | All subjects | 0 | 0
---|---|---|---
Current probiotics | All subjects | 12 | 4
Other supplements | Fish oil | 9 | 5
| Multivitamins | 10 | 8
| Laxative | 2 | 0
Food allergy | All subjects | 15 | 4
Immunization | Immunization | 35 | 31

1Gluten, casein, dairy, lactose or preservative free. NA, not available.
2Australian standard immunization schedule according to age.

Table 2 Method validation values of biogenic amines for LC-MS/MS

<table>
<thead>
<tr>
<th>Biogenic amine</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±8</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>0.9975</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>246</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>
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<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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Evaluation of Biogenic Amines in the Faeces of Children


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Chapter 7: General Discussion

This thesis used a variety of methodologies to explore the microbial profile of autistic children, including conventional microbiological culture methods, bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) to examine a comprehensive range of bacterial species (666 species), and bacterial metabolite investigation. The findings of this thesis go some way towards answering questions regarding the GI microbial profile of autistic children and neurotypically developing children.

The pilot study in Chapter 4 (Gondalia et al., 2010a) did not find any within-group differences in the GI microbial profile of an ASD group when compared across clinical severity and gender. Furthermore, there were no significant differences found when compared to standardised laboratory reference ranges. Therefore, we hypothesised that not all children with autism have an abnormal GI microbial profile and that perhaps only a subset of individuals have disrupted GI microbiota.

The findings of the pilot study described in Chapter 4 (Gondalia et al., 2010a) was further supported by a subsequent study (Gondalia et al., 2012) (Chapter 5) conducted on a larger sample size (n = 104). This study used a superior experimental design than previous studies, with participants being children with ASD and their non-clinical (typically developing) siblings. It utilised a high-throughput technique for identification of a comprehensive range of GI bacterial populations. The results of the study did not provide any evidence for differences in the GI bacterial profile between the autistic group and the neurotypical sibling group, therefore, the results do not support the hypothesis of such a difference proposed by some other researchers (Finegold et al., 2010;
that disruption of the gut microbiota, overgrowth of some pathogenic bacteria or suppression of beneficial bacteria play major roles in the development and/or maintenance of autism and related conditions.

Some researchers have gone so far as to suggest that atypical microbial populations may actively contribute to autism symptoms. For example, research has shown that the metabolite propionic acid, a short chain fatty acid produced by gut bacteria, can alter the brain and behaviour (Finegold, 2008; MacFabe et al., 2011). Likewise, some other bacterial metabolites, such as LPS and amines, are also believed to interfere with normal physiological and psychological functions (Darlington et al., 2009; Jyonouchi et al., 2005). This thesis examined this latter hypothesis by evaluating the levels of a group of GI bacterial metabolites, biogenic amines (which act as neurotransmitters), in an autistic group compared to a neurotypical sibling group. The findings of this work concord with the previous two microbial study findings of this thesis in that, overall, the autistic group showed no significant differences in concentrations of biogenic amines to the typically developing sibling group. These results, therefore, suggest that these metabolites are unlikely to be playing a role in the GI dysfunction associated with autism. Nevertheless, it is too early to discount completely a connection between biogenic amines and autism.

The findings of the thesis did not show any differences in microbial distribution and selected metabolites between children with autism and their neurotypically developing siblings when compared based on clinical severity and GI dysfunction. This indicates the possibility that the elevated rates of GI dysfunction found in ASD populations might be based on factors other than GI microbiota.
It is well established that individuals with ASD have elevated levels of anxiety and distress (Hutman et al., 2010; Mayes et al., 2011a; Mayes et al., 2011b; Wood et al., 2009). GI research has highlighted the importance of the “gut–brain axis,” especially in relation to depression and irritable bowel syndrome (IBS) based on examination of the impact that the brain can have on general gut function (Aziz et al., 1998). New research involving GI symptoms in anxiety and distress is indicating that stress and anxiety have an impact on GI function and condition. More likely, GI symptoms appear as a significant indicator for the presence of depression and anxiety (Mussell et al., 2008).

There are several mechanisms by which stress can affect the GI system and alter the bacterial composition, including changes in epithelial cell function, mucus secretion and GI motility (Ghia et al., 2008; Varghese et al., 2006). Research supports the theory that early life stress will lead to altered stress reactivity later in life and an altered GI microbial profile (O'Mahony et al., 2009). The changes in the microbiota induced by infection, antibiotics or other events such as stress may have caused physiologic inflammation and perturbed GI physiology at the time of early life development. A change in GI physiology provides an altered habitat that in turn supports a different microbiota and this might have influenced the brain and behaviour.

Animal studies have demonstrated that adverse early life events are associated with a maladaptive stress response system and might increase the vulnerability to disease in adulthood (Ackerman et al., 1978; O'Mahony et al., 2009). In a study by O’Mahony et al., a maternally separated and non-separated rat model was used to identify the response against stress by examining endocrine and immune measurements, whole blood stimulation and GI microbiota composition. The results indicated significantly higher concentration of cytokine – TNF-α (inflammation measure) and corticosterone (regulate physiological processes,
inflammation and behavior). Perturbations in GI microbiota also have been observed. Together, these results indicate that early psychological factors induce physiological changes. Many other studies conducted on animal model have demonstrated that early life stress (i.e. maternal deprivation) can modify adult immune and gastrointestinal tract functions (Barreau et al., 2004; Overmier et al., 1997; Spencer et al., 2006). Stress can trigger a significant increase in colonic permeability, alter mucosal mast cell density and significantly higher inflammatory reaction.

Therefore, it is possible that psychological, rather than physiological, factors may explain much of the GI distress in autistic individuals. Nevertheless, GI dysfunction, irrespective of a psychological or physiological basis, is very likely to be causal of stress in individuals. Additionally, it is very difficult for many children to maintain a proper diet and hygiene. Therefore, such children may be more likely to acquire intestinal infections compared to normally developing children. A restricted diet may also eventually result in other GI dysfunctions. Although the possible mechanisms are unknown, it has long been suggested that some intestinal lesions that increase intestinal permeability to exogenous peptides of dietary origin may lead to the disruption of neuroregulatory mechanisms and normal brain development (enterocolonic encephalopathy) (Wakefield et al., 2002).

However, there are varied theories of the GI dysfunction associated with autism, therefore, clinically, it is necessary to evaluate each individual with autism based on the symptoms described by parents/guardian/doctors for possible intervention and treatment of GI dysfunction and the underlying autistic condition. A central difficulty in recognising and characterising GI dysfunction in ASD is the communication difficulties of many affected individuals. Thus, GI problems in individuals with ASD can be challenging to evaluate. Clinical practice guidelines exist for the evaluation and management of
ASD by primary carers and other physicians but do not include routine consideration of potential GI and other medical problems. Nevertheless, some anecdotal evidence indicates that antibiotics, probiotics, special diet supplements or some restricted diets (e.g. gluten/casein free diet) can be used to successfully treat patients. Because of markedly differing presentations of autism conditions and associated GI dysfunctions, it is challenging to scientifically evaluate these interventions.

The American Academy of Paediatrics reported criteria for the diagnostic evaluation of GI symptoms and disorders in individuals with ASD (Buie et al., 2010). According to this report, GI symptoms are related to sleep disturbance associated with gastrointestinal reflux disease (GERD), self-injurious behaviour, tantrums, aggression, oppositional behaviour with constipation, gastritis, intestinal inflammation, chronic diarrhoea with malabsorption and maldigestion, perceived abdominal discomfort, holding abdomen and crying, problem behaviours related to constipation, lactose intolerance and enteric infections. These symptoms and associated possible GI conditions lead to possible diagnostic tests such as stool analysis for occult blood, enteric pathogens, ova/parasites, Clostridium difficile infection, abdominal radiograph, lactose breath test, colonoscopy and diagnostic trial of proton-pump inhibitors.

GI conditions associated with autism have often been hypothesised as being linked to GI microbiota disruption and infections. Many researchers have suggested the treatment of GI dysfunction in ASD with anti- or pre/probiotics as a way to regulate intestinal microbiota and subsequently improve symptom severity in ASD (Sandler et al., 2000; Tuohy et al., 2003). However, it is challenging to accept these interventions for all autistic individuals because of wide variations in patient presentations and the lack of consistency and reproducibility in the research findings examining the involvement of GI microbiota in autism.
In conclusion, the program of research reported here found no specific bacterial species or associated metabolites (amines) that reliably differentiated between children with autism and their neurotypical siblings. Therefore, it would appear that if gastrointestinal microbiota are involved in the etiology of ASD, it may only be evident at the time of commencement of the original pathogenesis of autism. Therefore, looking at microbiota years after diagnosis of autism may be too late. Furthermore, the results of this thesis do not provide evidence of involvement of microbiota in ASD severity, which may indicate that GI microbiota are not a relevant variable in maintenance mechanisms in autism. Nevertheless, it appears important clinically, that each ASD child be thoroughly assessed and cared for on an individualised basis in terms of both their ASD and any reported or identified GI distress.

Limitations

The studies described in this thesis have some limitations which were difficult to avoid or overcome. These would have had some effect on the assessment and analysis of the indigenous microbial and metabolite profile of each individual.

Each individual has a different diet (non-controlled) which would likely have influenced the composition of the GI microbiota. The diet not only dictates the available nutrients for the microbiota but also helps to establish predictable and competitive relationships between intestinal bacteria. However, dietary differences were not controlled in the participants of the studies reported in this thesis. Given that ASD children typically have a self-limited diet, it would be scientifically better to experimentally control dietary intake to remove its impact on GI microbiology. More generally, the microbial profile of each individual is
very different depending on lifestyle, personal hygiene and eating habits. Therefore, it is difficult to define a “typical” profile of microbiota for healthy individuals and, perhaps even more so, for individuals with ASD.

As with any other culture independent method, pyrosequencing has its limitations. The short length of reads generated by pyrosequencing may represent a limitation when bacterial identification is intended (Siqueira Jr et al., 2012). However, a pyrosequencing approach provides richer descriptive information about microbiota than any other molecular method of bacterial identification in terms of taxonomic levels and relative abundance values.

For the autistic and control groups, GI symptoms were measured by parental report only. No further evaluation was carried out by medical professionals. Furthermore, prior assessment of autism (via the childhood autism rating scale, CARS) was used as a dependent variable and this was associated with currently reported GI dysfunction. Ideally, thorough validated current assessments such as the autistic disorder observational schedule (ADOS) would be conducted; however, this was beyond the resources of the research program. Nevertheless, in Australia, formal assessment and re-assessment of autism and Asperger’s disorder occurs annually as a part of the requirements of the educational and social services systems. Therefore, it can be assumed that the assessments were current to within 12 months.

**Suggestions for future research**

In this thesis, the main focus was on bacteria and selected bacterial metabolites (biogenic amines) and their possible association with autism severity and associated GI dysfunctions. In order to advance
our understanding of the role and cause of GI dysfunction in autism, other factors need to be evaluated in future research.

Microbial species beyond bacteria should also be examined, including fungi, parasites and protozoa. It is hypothesised that overgrowth of Candida in the intestine might occur secondary to antibiotic use or ingestion of processed sugar and other foods which enhance yeast growth (Levy et al., 2005). Also, little research has been conducted evaluating GI parasites and protozoa associated with autism and, yet, research has reported that the abnormalities in behaviour and clinical features associated with autism resemble those of chronic latent toxoplasmosis in humans and rodents (Prandota, 2010) caused by Toxoplasma gondii infections.

It has also been reported that viral infections can trigger autoimmune responses and eventually lead to organ-specific autoimmune disease. With regards to the cause of autism, the trigger mechanism is not known but viral infections such as rubella, measles, human herpes virus-6, influenza and cytomegaloviruses have been suspected (Brambilla et al., 2003; Hallmayer et al., 2011; Rosenberg et al., 2009; Sparks et al., 2002). More through research is warranted to evaluate this possible mechanism where viral infection in early development may lead to autism.

Lipopolysaccharide (LPS) is localised to the exterior leaflet of the outer membrane of Gram-negative bacteria and serves as the major surface component of the bacterial cell envelope. It has been hypothesised that LPS can damage the blood brain barrier and induces the release of nitric oxide (neurotoxic) which is a gaseous neurotransmitter that diffuses in to the brain. Furthermore, LPS toxicity works synergistically with mercury (and possibly other heavy metals) to expand the damage (Minami et al., 1998). In addition, LPS decreases
glutathione levels, making it even more difficult for the body to excrete heavy metals (Zhu et al., 2007). The mechanisms by which LPS may play a role in autism are varied and may differ across subsets of autistic patients. Therefore, careful assessment is needed to identify the role, if any, of LPS in autism symptomatology, cognitive function and neurodevelopmental process.

Identifying an underlying association between infection with a microbial agent and a specific brain disease can be complex. In some cases, for example, herpes simplex encephalitis, the virus destroys infected brain tissue through replication. Immune responses to microbial agents can also lead to breakdown of tolerance to host antigens and result in tissue damage (Lipkin et al., 2008). The original infection may have been peripheral, as it is anticipated for tic disorder and obsessive compulsive disorder following streptococcal infection (Murphy M, 2002). Another mechanism for psychological disorder is persistent non-cytopathic viral infection (Pearce, 2003). Such infections can profoundly impact neurotransmitter function or brain development, yet remain unknown. Therefore it is necessary to study autoimmune responses to microbiological abnormalities in individuals with ASD.

Psychological factors such as anxiety and distress have to be evaluated in future research in order to clarify their relationship with both GI function and autistic symptomatology. Digestive system disorders, such as IBS, are often associated with affective disorders, such as depression, anxiety, panic and post-traumatic stress disorder (PTSD). Some of these associations are observed not only in clinical populations, but also in population-based samples, suggesting a relationship with pathophysiological mechanisms underlying both GI dysfunction and certain affective disorders. Continuous and severe life-threatening stressors can play an important role in the onset and modulation of GI symptoms as well as in the development of affective disorders and
PTSD. A neurobiological research model is needed to better explain such an underlying association of functional GI disorders with anxiety and distress.

The possible interactions between microbial factors in ASD with other proposed aetiological factors such as mercury poisoning, metabolic relationships and genetic foundations are other factors to consider in future research. Higher rates of autism diagnosis have led to hypotheses among researchers that environmental factors operate in concert with genetic risk factors in causing this developmental disorder. Autistic children exhibit evidence of oxidative stress and impairment in some metabolic functions, such as methylation and energy metabolism, which may reflect effects of toxic exposure on metabolism. It has been hypothesised that bacterial products and metabolites like LPS work synergistically with toxins like mercury and other heavy metals to expand the damage. The possible interactions between ‘competing’ hypotheses warrant attention.

The treatment of children with ASD is complex and time-intensive. Because the of the elevated levels of GI distress in this population, treatments targeting the GI tract such as restricted diets (e.g., gluten- or casein-free diets) and use of supplements have been adopted to decrease the frequency of symptoms or to regulate GI functions. Further research is needed to (1) describe the relationship between nutritional intake and GI symptoms, (2) determine nutritional risk factors for children with ASD and selective diets, and (3) determine the etiology of GI dysfunction in children with ASD.
Summary

Overall, the series of studies reported in this thesis found no evidence to support the hypothesis that disruptions of the GI microbiota, overgrowth of some pathogenic bacteria or suppression of beneficial bacteria play a major role in the development of autism. This was supported by the findings of all three studies conducted based on culture methods, genetic analysis and metabolite investigation.

Nevertheless, we know children with ASD have increased GI disturbance than typically developing children. Therefore, it is critical for researchers to continue to explore possible mechanisms of this phenomenon. Given GI problems are associated with behavioural problems in ASD children, finding reasons for these GI problems (and their effective treatment) would be an enormous advance in the clinical management of this condition.
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Appendices
Appendix A

Ethics approval from Swinburne University of Technology research committee for study entitled ‘faecal microbiota of individuals with autism spectrum disorder’ (Chapter 4).

>>> Keith Wilkins 25/05/2009 2:32 PM >>>

To: Assoc Prof David Austin/Ms Shakuntla Gondalia, FLSS

Dear David and Shakuntla

SUHREC Project 2008/114 Autism and the Human Gut Microbiota Dr David Austin, FLSS; Ms Shakuntla Gondalia et al

Approved Duration: 25/05/2009 to 25/05/2010 [Adjusted] [New Student Investigator May 2009]

I refer to the ethical review of the above project protocol by Swinburne's Human Research Ethics Committee (SUHREC). Your responses to the review were as emailed on 22 May 2009 with attachments and details pertaining to a new student investigator. A signed Section F declaration was submitted separately. Your responses were put to the Chair of SUHREC for consideration.

I am pleased to advise that, as submitted to date, the project may proceed in line with standard on-going ethics clearance conditions here outlined.

- All human research activity undertaken under Swinburne auspices must conform to Swinburne and external regulatory standards, including the National Statement on Ethical Conduct in Human Research and with respect to secure data use, retention and disposal.
- The named Swinburne Chief Investigator/Supervisor remains responsible for any personnel appointed to or associated with the project being made aware of ethics clearance conditions, including research and consent procedures or instruments approved. Any change in chief investigator/supervisor requires timely notification and SUHREC endorsement.

- The above project has been approved as submitted for ethical review by or on behalf of SUHREC. Amendments to approved procedures or instruments ordinarily require prior ethical appraisal/clearance. SUHREC must be notified immediately or as soon as possible thereafter of (a) any serious or unexpected adverse effects on participants and any redress measures; (b) proposed changes in protocols; and (c) unforeseen events which might affect continued ethical acceptability of the project.

- At a minimum, an annual report on the progress of the project is required as well as at the conclusion (or abandonment) of the project.

- A duly authorised external or internal audit of the project may be undertaken at any time.

Please contact me if you have any queries about on-going ethics clearance, citing the SUHREC project number. A copy of this communication should be retained as part of project record-keeping.

Best wishes for the project.
Yours sincerely
Keith Wilkins
Secretary, SUHREC
**************************************************************
Keith Wilkins
Research Ethics Officer
Swinburne Research (H68)
Swinburne University of Technology
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Fax +61 3 9214 5267
Appendix B

Ethics approval from Swinburne University of Technology research committee for study entitled ‘Molecular characterization of gastrointestinal microbiota of children with autism (with and without gastrointestinal dysfunction) and their neurotypical siblings’ (Chapter 5).

To: Assoc Prof David Austin, Ms Shakuntla Gondalia, Assoc Prof Enzo Palombo, Dr Simon Knowles

Dear David, Shakuntla, Enzo and Simon,

SUHREC Project 2010/024 Faecal microbiota in Autistic Spectrum Disorder: Characterisation using culture and molecular methods
Proposed Duration: 01/04/2010 to 01/04/2011
Assoc Prof David Austin, FLSS; Ms Shakuntla Gondalia et al

I refer to the ethical review of the above project protocol by Swinburne’s Human Research Ethics Committee (SUHREC). Your responses to the review, as emailed on 31 March 2010 with attachments, were put to a SUHREC delegate for consideration.

I am pleased to advise that, as submitted to date, the project may proceed in line with standard on-going ethics clearance conditions here outlined.

- All human research activity undertaken under Swinburne auspices must conform to Swinburne and external regulatory standards, including the National Statement on Ethical Conduct in Human Research and with respect to secure data use, retention and disposal.
- The named Swinburne Chief Investigator/Supervisor remains responsible for any personnel appointed to or associated with the project being made aware of ethics clearance conditions, including research and consent procedures or instruments approved. Any change in chief investigator/supervisor requires timely notification and SUHREC endorsement.

- The above project has been approved as submitted for ethical review by or on behalf of SUHREC. Amendments to approved procedures or instruments ordinarily require prior ethical appraisal/clearance. SUHREC must be notified immediately or as soon as possible thereafter of (a) any serious or unexpected adverse effects on participants and any redress measures; (b) proposed changes in protocols; and (c) unforeseen events which might affect continued ethical acceptability of the project.

- At a minimum, an annual report on the progress of the project is required as well as at the conclusion (or abandonment) of the project.

- A duly authorised external or internal audit of the project may be undertaken at any time.

Please contact me if you have any queries about on-going ethics clearance, citing the SUHREC project number. Chief Investigators/Supervisors and student researchers should retain a copy of this email as part of project record-keeping.

Best wishes for the project.

Yours sincerely,

Ann Gaeth
For Keith Wilkins
Secretary, SUHREC
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