**STUDY PROTOCOL**

**Prebiotic supplement use, the gut microbiome and behaviour change in children with autism (ASD)**

Version 7

**Project Team**

Ms. Jacqui Palmer, B App Sci, Grad Dip Nutrition & Dietetics. Accredited Practising Dietitian (APD) PhD Candidate. Principal Researcher. Responsible for background research, study design, ethics approval, recruitment, sample collection, nutrition intervention (dietetics advice) and interpretation of questionnaires, data analysis and reporting.

Dr Rebecca Byrne, PhD. Lecturer, School of Exercise and Nutrition Sciences, QUT. Researcher with expertise in child health and nutrition. Shared role of primary supervisor, replacing A/Prof. Jolieke van der Pols who will be on maternity leave.

A/Prof. Jolieke van der Pols, PhD. Associate Professor in Nutrition Science and Epidemiology. School of Exercise and Nutrition Sciences, QUT. Principal Coordinating Researcher and Primary Supervisor of PhD candidate (JP). In combination with Dr Rebecca Byrne, will oversee this research project and carry overall responsibility.

Prof. Karen Sullivan, PhD. Professor of Psychology. Chief Investigator and secondary supervisor of the PhD candidate (J. Palmer). Support and advice concerning interpretation of behaviour data and stress biomarker data.

Prof. Flavia Huygens, PhD. Head of Laboratory and Associate Director QUT at QIMR. Chief Investigator. Responsible for microbiome analysis and interpretation and storage of stool samples and microbiome data.

Professor Stewart Trost, PhD. Associate Director IHBI, Professor at the School of Exercise and Nutrition Sciences, QUT. Chief Investigator. Internationally recognised expert in the assessment of physical activity in children. Will advise on interpretation of sleep data.

Dr. Heidi Staudacher, PhD. Research Fellow and Accredited practising Dietitian (APD), Deakin University, Melbourne. Advisory role in this project, and Associate Supervisor of PhD candidate. Expert advice for trial design, interpretation of microbiome findings.

**Resources**

The following research funds and in-kind contributions have been received to cover the costs for this pilot project:

Project funding: $10,000 (Mid-Career Research Award from QUT Institute of Health and Biomedical innovation (IHBI), A/Prof. Jolieke Van der Pols). This grant covers analysis of stool samples for laboratory analysis of the gut microbiota and metabolites. It also pays for the stool sample collection equipment.

Project funding: $10,000. Project funding received from the QUT School of Exercise and Nutrition Sciences. This funding is for the analysis of waking and diurnal cortisol levels in saliva samples. We will measure fluctuations in cortisol as a measure of stress.

PhD allocation of Ms. Jacqui Palmer will be used to purchase the SRS-2 survey and manual from Pearson Clinical (cost $320), the prebiotic supplement from Quantum Hi-Tech (China) Biological Co. Ltd (cost $440) and to purchase small equipment needed for transport of samples, dose packaging of the prebiotic and placebo and postage costs ($2,500).

In kind contributions: The placebo product, maltodextrin (Dextrin DE28), was provided by the Manildra Group of Companies (free of any associated conditions). The wrist accelerometers for this project will be made available by Prof. Stewart Trost (QUT) as an in-kind contribution.

**Background**

**Literature review**

Autistic spectrum disorder (ASD) is a neurodevelopmental disorder characterized by difficulties in social interactions, deficits in communication, and restrictive, repetitive thoughts and behaviours (APA, 2013). It is a heterogeneous condition with a number of commonly associated problems. These include high levels of anxiety (Kent and Simonoff 2017), disturbed sleep (Delahaye, Kovacs et al. 2014, Mazurek and Sohl 2016, Mazzone, Postorino et al. 2018), mealtime behavioural problems (Curtin, Hubbard et al. 2015, Reinoso, Carsone et al. 2018), and gastrointestinal (GI) symptoms (Adams, Johansen et al. 2011, Frye, Rose et al. 2015). In addition, quality of life (QoL) of children with ASD and their families has consistently been shown to be lower than in both typically developing children, and children with other developmental disabilities (de Vries and Geurts 2015, Giovagnoli, Postorino et al. 2015, Vasilopoulou and Nisbet 2016).

QoL is defined as an “individuals’ perception of their position in life in the context of the customs and value systems in which they live, in relation to their goals and expectations, standards and concerns”(WHO 1998). It encompasses the idea of wellbeing, of happiness and life satisfaction. Behaviour problems, deficits in social skills and comorbid psychiatric conditions (Chiang and Wineman 2014), as well as poor sleep quality (Delahaye, Kovacs et al. 2014) have been cited as major factors influencing QoL in children with ASD and their families.

GI symptoms and anxiety often occur together which has driven research into the relationship between the GI tract and the central nervous system (CNS). The GI tract is populated by a large number of microorganisms known as the gut (GI) microbiota. These play an important role in homeostasis and health and are now known to play an important role in mental health (Dinan and Cryan 2017). Research has seen the emergence of a bi-directional communication system known as the gut brain axis (Grenham, Clarke et al. 2011, Carabotti, Scirocco et al. 2015). Microbial metabolites such neurotransmitters, neuropeptides and hormones act as signalling molecules along enteroendocrine, immune and neural (vagus nerve) pathways (Carabotti, Scirocco et al. 2015).

Preclinical studies have contributed strong evidence to the role of the microbiota in these pathways and to their anxiolytic tendencies (Sudo, Chida et al. 2004, Bercik, Park et al. 2011, Bravo, Forsythe et al. 2011). Sudo et al. (2004) demonstrated this concept by supplementing mice with the probiotic B. infantis before subjecting them to restraint stress. Reduced HPA axis activity under stress was observed in the group supplemented with Bifidobacteria compared with controls (Sudo, Chida et al. 2004). Similarly, in a landmark study, Bravo et al. (2011) highlighted the role of the vagus nerve in gut-brain communication and the impact that gut microbiota can have on behaviour. They supplemented mice with the probiotic L. rhamnosus and subjected them to controlled stressors (forced swim, open field, stress induced hyperthermia). Treatment fed mice showed reduced stress induced corticosterone and reduced anxiety behaviours compared to control fed mice. A reduction in anxiety behaviours and corticosterone was not seen in vagotomised mice (Bravo, Forsythe et al. 2011).

These preclinical studies were the basis for microbiota-targeted interventions using either probiotics or prebiotics in humans. A probiotic is “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill, Guarner et al. 2014) while a prebiotic is “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson, Hutkins et al. 2017). Prebiotics have the advantage of being stable in most environmental conditions including in the acidic environment of the upper GI tract.

Well known prebiotics include galacto-oligosaccharides (GOS), chains of galactose with a terminal glucose molecule found naturally in pulses, spelt flour and lentils, and inulin type fructans such as fructo-oligosaccharides (FOS), found naturally in chicory root, artichoke, onions, and green bananas.

FOS is also produced commercially from chicory root while GOS is produced commercially from the cleavage of lactose via the enzyme βgalactosidase. Commercially produced β GOS has been shown to be highly bifidogenic (Wilson and Whelan 2017). Prebiotics are indigestible to the host and pass through the small intestine untouched where they become available to colonic bacteria. They are then fermented produce short chain fatty acids which act as substrates and signalling molecules (Derrien and Veiga 2017).

In a double blinded RCT comparing 45 healthy adults (mean age 24 years), Schmidt et al. (2015) randomised participants to take either 5.5g daily of βGOS, FOS, or placebo for a period of 3 weeks. They reported a significant increase in response to positive versus negative stimuli and a lowered cortisol awakening response (an indicator of stress) in the group taking GOS when allowing for baseline data (Schmidt, Cowen et al. 2015).

GOS has also been shown to improve bowel habits and anxiety scores in some patients with Irritable Bowel Syndrome (IBS). In a 12-week parallel cross-over control trial, Silk et al (2009) supplemented the diet of 44 patients with IBS with either 3.5g or 7g of prebiotic GOS or 7g placebo. 3.5g GOS daily resulted in an improvement in stool consistency, flatulence and bloating while 7g GOS daily resulted in improved subjective global assessment (SGA) and anxiety scores, the placebo producing no effect (Silk, Davis et al. 2009). Other studies have shown small effect of prebiotics including a recent systematic review including 11 eligible papers, which concluded that although many symptoms of IBS were not helped by prebiotics, bloating and flatulence were reduced by non-inulin type prebiotics (Wilson, Dimidi et al. 2019).

Very few studies have assessed the effectiveness of using prebiotics to influence social behaviour and emotion in children with ASD, despite a number of probiotic interventions which have provided preliminary evidence indicating improvements in behaviour following supplementation with either bifidobacterial or Lactobacillus (Parracho, Gibson et al. 2010, Tomova, Husarova et al. 2015, Grossi, Melli et al. 2016). The only study to date using prebiotic GOS in children with ASD, Grimaldi et al (2018) assessed the effect of supplementing the diet of 30 children with ASD with 1.8g daily of GOS or placebo for 6 weeks following a period of restricted or unrestricted diets. Improvements in social behaviour skills as assessed by the AQ questionnaire were observed in the group supplemented with GOS (Grimaldi, Gibson et al. 2018) providing preliminary evidence for the use of GOS to improve some of the behaviours associated with ASD.

Diet is a strong driver of microbiota composition. A western style diet characterised by highly refined carbohydrates, low fibre, and a high fat and protein intake is associated with a distinctly different and less desirable gut microbiota composition compared with diets high in seeds, wholegrains, fruit and vegetables (De Filippo, Di Paola et al. 2017). A diet high in fruit, vegetables, pulses, seeds and wholegrains provides a good supply of different dietary fibres some of which are prebiotic. This type of diet is linked to health effects (Gibson, Hutkins et al. 2017).

Foods rich in prebiotics are typically low in the diet of a child with ASD as many have a very limited range of foods which often excludes fruit and vegetables (Evans, Must et al. 2012, Hubbard, Anderson et al. 2014). The core trait in ASD of a need for sameness, dictates repeated and rigid food choices, largely based on refined carbohydrates (Hubbard, Anderson et al. 2014). Sensory processing impairments and negative feeding experiences may also contribute to a restricted diet comprised of similar textures, flavours, odours and appearance (Hubbard, Anderson et al. 2014, Curtin, Hubbard et al. 2015). Generally, children with ASD eat fewer serves of fruit, vegetables and wholegrains than typically developing children and consume more energy dense foods (Evans, Must et al. 2012).

The microbiota of children with autism has been shown to be altered compared to typically developing children (De Angelis, Francavilla et al. 2015). Lower abundance of Bifidobacteria, lower diversity of microorganisms, and higher abundance of pathogenic Clostridia and inflammatory Proteobacteria (Parracho, Bingham et al. 2005, Adams, Johansen et al. 2011, Kang, Park et al. 2013, Tomova, Husarova et al. 2015). Parracho et al. (2005) compared the bacterial populations of 58 children with ASD to 12 typically developing siblings and 10 typically developing non-related children using FISH analysis of DNA from stool samples finding a significantly higher abundance of pathogenic Clostridium compared to typically developing siblings and control and that severity of ASD as determined by the ATEC was strongly associated with severity of GI symptoms. In a later study, Parracho et al. (2010) conducted a cross-over double-blind placebo-controlled trial of children 3 to 16 years with ASD, commencing with either 3 weeks or placebo or 3 weeks of the probiotic L. planetarium, followed by a 3-week washout before cross-over. Findings indicated a decrease in Clostridium cluster XIV and an increase in Lactobacillus compared to placebo, as well as a significant difference in stool consistency and improvement in behaviour scores during the probiotic phase (Parracho, Gibson et al. 2010). This study suggests that microbiome targeted interventions may be effective in this population group however it was limited by high attrition rates and inter-individual variation.

Similarly, Adams et al. (2011) compared the GI microbiota of 58 children with ASD to 39 typically developing controls and found a significantly reduced abundance of Bifidobacteria in the ASD group as well as a strong correlation between GI symptoms measured by the 6-GSI, and behavioural problems measured by the ATEC(Adams, Johansen et al. 2011).

**Rationale: Type and dosage of the prebiotic supplement**

βGOS is highly bifidogenic and has been shown to reduce anxiety in both murine and human studies (Schmidt, Cowen et al. 2015, Collins and Reid 2016, Gronier, Savignac et al. 2018). It has also been shown to have a greater effect on basal cortisol levels (stress) and response to positive stimuli in healthy adults compared to FOS (Schmidt, Cowen et al. 2015).

GOS will be given as a supplement βGOS due to the time constraints of the study and food selectivity characteristics of the majority of children with ASD. βGOS has also shown greater selective stimulation of the GI bacteria than dietary sources of GOS.

The supplement will be provided as 4 capsules of 0.6g βGOS or placebo per day which are to be opened and mixed into 100mls of the child’s drink of choice. Doses should be supervised by the parent or carer and the amount consumed noted in the supplement diary. The prebiotic has been matched for colour, taste and texture to the placebo (Dextrin D28, Manildra).

The Prebiotic supplement used in this study

Prebiotics are not classed as drugs, they are classed as functional foods, and the prebiotic supplement used in this study is freely available for purchase in Australia. The following gives details of the prebiotic that will be used:

* + Name: Gosyan GOS®
	+ Manufacturer: Quantum Hi Tech Biological, China
	+ Supplier of supplement Invita
	+ Class: Functional food
	+ Action: Bifidogenic
	+ Excretion: Not digested by host, fermented by gut bacteria, excreted in stools.
	+ Known side effects: may cause increased gas production and abdominal discomfort in high doses.
	+ Contraindications: supervised intake in IBS and gastrointestinal conditions.

In the current project, there will be a one-week period of incremental dosing, increasing from 2 capsules (1.2g: half dose) to 4 capsules (2.4g: full dose) of prebiotic or placebo per day, to encourage gut adaptation in the study participants. Based on reports from other studies, 2.4g/d is judged to be safe and is expected to be tolerated very well—for comparison, infant formulas use the dose of 0.8g/100ml with a 1:9 mix of GOS: FOS (fructo-oligosaccharides) (Bakker-Zierikzee, Alles et al. 2005). Further, a recently published study, the first of its kind by Grimaldi et al. (2018) used a dose of 1.8g per day in children with ASD with minimal adverse GI effects. This dose was calculated from a previous study using a 3 chamber in vitro model, using faecal samples from both typically developing children and children with ASD and the substrate βGOS (Grimaldi, Cela et al. 2017).

**Overall Aim:**

The hypothesis is that prebiotic βGOS will improve the quality of life of children with ASD and their families through a reduction in stress and improvement in mealtime behaviour, social behaviour, sleep and GI symptoms.

**Significance of the proposed study**

This study will be one of the first studies to investigate effects of the use of a prebiotic supplement on quality of life, stress and behaviour in children with ASD. Preclinical studies as well as preliminary studies in healthy humans strongly suggest that prebiotics may have anxiolytic properties as they increase beneficial gut bacteria such as Bifidobacteria and Lactobacillus. Supplementation with prebiotics represents a low risk strategy which may lead to improved social and mealtime behaviours, improved sleep patterns and subsequent improved quality of life for the child with ASD and their family.

**Research aims**

This project will address the following research aims. In children with ASD, it will:

1. Assess whether 6-week supplementation with prebiotic βGOS results higher parental and familial quality of life compared with placebo as assessed by the QoLA questionnaire.
2. Assess whether 6-week supplementation with prebiotic βGOS results in a lower basal cortisol levels and enhanced mealtime behaviour, social behaviour, GI symptoms and sleep patterns compared with placebo.
3. Assess whether changes in the outcome variables in the prebiotic group are associated with changes in the GI microbiota.

**Project Design**

**Project Overview**

This project is a small RCT involving 50 children with ASD (aged 4-10yrs), who will be randomised to receive either a prebiotic supplement βgalacto-oligosaccharide (βGOS) or placebo (maltodextrin) daily for 6 weeks. All participants (parent or carer of child in both intervention and control group) will receive usual dietetic advice from one paediatric dietitian (JP), focusing on setting up positive mealtime environments. Questionnaire data assessing parental and family quality of life, mealtime behaviour, social behaviour, and GI symptoms will be collected at baseline and completion, and clinical measurements of sleep via an accelerometer, cortisol (as a biomarker of stress) via saliva samples and microbiota profile via stool sample analysis will also be collected at baseline and completion.

This study will serve as a pilot for collection of proof-of-principle data, to support grant applications and larger scale data collections in the future.

**Setting**

Participants for this project will be recruited from Brisbane and surrounding areas (see recruitment details below). All clinical samples will be collected by the parent or carer in the participants home, the questionnaire booklets are to be completed by the parent at home, and the supply of dose of the supplement is to be supervised by the parent. Consultations between the researcher and parents will take place at IHBI, QUT, 41 Blamey Street, Kelvin Grove, 4059.

**Participants**

We aim to enrol 50 children aged 4 to 10 years with a clinical diagnosis of ASD (refer to the section below on Recruitment, rolling recruitment, participant withdrawal, further enrolments).

Inclusion criteria:

a formal diagnosis of ASD (presentation of medical letter of diagnosis),

child participants aged between 4 and 10 years,

parents / carer English speaking

naïve to dietary intervention involving prebiotic supplementation

Parents of participants must agree to all aspects of the protocol.

Exclusion criteria:

no recent use (within last 3 months) of antibiotics,

no recently commenced prebiotics (within the past month),

no recently commenced probiotics (within the past month)

no new medications (or dose change) for anxiety, sleep or behaviour (within the past 2 months).

The role of Ms Palmer will be well defined. She will act as dietitian only for the scope of this project, that is, for the 2 dietetic consultations focusing on behavioural strategies for selective eating. Any other concerns that arise during this study will be referred to the appropriate health practitioner.

**Recruitment**

Flyers will be distributed to targeted health clinics in the Brisbane area including private speech and language therapists, occupational therapists, paediatricians and psychologists. Online advertisements will be posted via forums including Autism Queensland, Asperger’s Queensland, Autism Awareness Australia and Raising Children Network.

The patient information flyer will describe the purpose of the research, what will be expected of the participant, the risks and benefits, and by whom the research is funded. It will also provide contact details and qualifications of the lead researchers. Interested participants will then receive the ‘Participant Information Sheet’ and ‘Consent’ form. Parents will be required to sign an informed consent form and return it to the researchers before a child’s acceptance into the study. After written consent is obtained, a trial dose of 0.6g of product will be sent to the participant to ensure that the child will accept the dose. This will be an unlabelled capsule. Parents will then be contacted and if the dose was accepted, the child will be enrolled in the study.

Recruitment will be on a rolling basis, therefore it will remain open until we meet the study quota of 50 children. If a participant withdraws from the study, it may be possible to replace the withdrawn participant with another recruited participant if funding allows, as enrolment will be managed on a rolling basis.

An initial individual information session will be planned for each participating child’s parent(s) at QUT Kelvin Grove Campus in the IHBI building (41 Blamey Street, Kelvin Grove, 4059) to which parents are asked to bring a copy of the letter of diagnosis, or medical document stating clinical diagnosis of ASD. Participants will receive a study identification number, the study kit and instructions on all aspects of the study (completion of questionnaires, collection storage and transport of clinical samples, and placement of the accelerometers). Participants will be randomised to either prebiotic or placebo in a double blinded manner.

A letter will be sent to their GP stating that they will partake in this study with an explanation of the aims and possible side-effects.

**Randomisation**

Children will be randomised to take either a prebiotic (βGOS) or a placebo (maltodextrin) supplement for 6 weeks. Randomisation will take place using randomised block sizes and researchers and participants will remain blinded to the allocations (i.e. double-blind design). A third party, QUT statistician A/Prof. Dimitrios Vagenas, will randomise the participants to either product A or product B and the identity of product A and B will be known only to the compounding pharmacist and the statistician. The two products βGOS and maltodextrin are similar in colour, taste and texture and have been packaged in identical capsules and containers.

**Risk of side-effects**

Although the dose proposed for this study (incremental dosing, increasing from 1.2g to 2.4g of prebiotic per day) is designed to minimise possible gastrointestinal discomfort (as explained in detail above), it is not possible to guarantee that participants will not experience bloating and increased flatus. It is possible that any change in routine could cause distress for a child with ASD. Inclusion of a supplement albeit colourless, odourless, tasteless when dissolved in water may result in refusal in this population group. Wearing a wristband or dribbling into a container may also cause stress in this group of children.

To mitigate these risks, the participants are free to withdraw at any time and parents will be encouraged to contact the study team if any aspect of the protocol is challenging, and strategies to help adherence may be provided. Children will not be excluded from the study if they do not wish to wear the wristband accelerometer. Parents will be provided with training on how to collect saliva and stool samples with minimum disruption to their child.

Negative Events

Parents are asked to communicate negative events to the researchers when and if they occur. Negative events will be defined as a noticeable worsening of GI symptoms or behaviour over the 6-week intervention period as compared with baseline.

**Timeline**

A timeline will be provided to the study participant and will detail the following:

After written consent has been obtained, parents will attend an information session and the participant will receive a study kit containing the relevant questionnaires in booklet form, a 6-week dose of either βGOS prebiotic or the placebo (maltodextrin), a wrist band accelerometer, 2 stool sample kits, 8 saliva sample kits, an esky and food grade dry ice (for transport of samples). The dry ice is a product made by the company Global National Australia Group, Techniice Division.

The participant will be randomised to the prebiotic group or the control group and allocated study ID which will contain a 5-digit number. This study ID will be used on all questionnaire booklets and sample collection tubes and will not show the participant’s full name or other identifiable details. The study ID will only be re-identifiable by the Chief Investigators of this project

1. Questionnaires will then be completed at home in the week prior to commencing the intervention (baseline). Questionnaires are assembled into booklet form, with booklet 1 to be completed at baseline. This booklet includes the following questionnaires: a QoLA, an SRS-2, a BAMBI, a 6-GSI and the following records: a 7-day stool chart, a 7-day sleep diary, and 3-day food record. During this week, the accelerometer will be fitted to the child’s wrist for a period of seven days to measure sleep.
2. Day 0: On the day prior to the first dietetic consultation (see below), one stool sample along with 4 saliva samples will be collected by the participants and stored in the freezer in the container provided (at the participant’s home) and transported to the QUT clinic (where the consultation will take place) within the esky on dry ice the following day. Note that as some children may be constipated, collection on day 0 may not be possible. The stool sample should be collected as close to the research visit as possible (up to 3 days prior to dietetic consultation) and stored in the freezer. Parents will be shown how to use this equipment during the individual information session and a video on stool collection method will be available throughout the study for reference.
3. Day 1: The first dietetic consultation will take place in the QUT Health Clinic or in room 403, IHBI, 44 Musk Ave, Kelvin Grove. Setting up a positive mealtime environment will be discussed based on “Steps to eating” and a “Learning plate”. Advice will be identical for both groups.
4. Day 1: The child will commence the prebiotic or placebo taking 2 x 0.6g capsules per day for the first 7 days.
5. Day 8: The dose will increase to the full dose of 4 x 0.6g capsules of prebiotic or placebo per day for the duration of the study.
6. Day 36 to 42: completion of booklet 2 (questionnaires and records) and fitting of the wristband accelerometer for the final seven days of the study.
7. Day 42: Repeat collection of one stool sample and four saliva samples to be stored in the freezer overnight in the container provided and transported to the second dietetic consultation using the esky and dry ice. Again, the stool sample collection should occur as close to the second dietetic consultation as possible (allowing for constipation).
8. Day 43: Second dietetic consultation. Clinical samples will be transferred to QIMR for storage at -18°C. Progress in eating behaviours will be reviewed and follow-up strategies suggested. The wrist band will also be collected at this point along with the other completed questionnaires and records.
9. Closure of the study. Parents of the Participants will be offered a summary of the results once analysis has been finalised.

All parking related to the research visits will be arranged for participants and covered.

**Data collection tools**

**Self-administered Questionnaires**

*Methods for assessing Quality of Life in the family*

The Quality of Life Autism (QoLA)

The QoLA has been chosen as it is specific to ASD and focuses on both parents and how problematic the child’s behaviour is for them which gives an indication of family quality of life. The QoLA is a 48-item questionnaire divided into 2 subsets, part A uses typical QoL questions related to the parents perceived QoL, while part B addresses how problematic the child’s behaviour is to the parent. Possible scores range from 48 to 240 with higher scores equate to higher perceived quality of life. The QoLA has been validated against a number of QoL scales including the World Health Organisations WHOQOL and WHOQoL Bref (Eapen, Crncec et al. 2014). It has shown good sensitivity and specificity.

*Method for assessing relevant child behaviours:*

The Social Responsiveness Scale-2 (SRS-2)

The Social Responsiveness Scale (SRS) is a 65-item questionnaire focusing on five elements of social interaction including social awareness, cognition, communication, motivation, and mannerisms. It has been validated against the Autism Diagnostic Interview (ADI), and has high reliability (McMahon, Vismara et al. 2013). It is also compatible with the DSM-5, the predominant diagnostic tool for autism. It is both sensitive enough to detect subtle traits, and specific enough to differentiate between clinical groups (Pearson Clinical, 2018). In a heterogeneous cohort, this is an important factor as it enables the tool to be used in children with level 1 (mildest) to level 3 (most severe) forms of autism.

Scores of 59 or less are considered normal social behaviour, scores of 60 to 65 are considered mildly impaired social behaviour, 66 to 75 is considered moderately impaired social behaviour and greater than 75 is considered severely impaired social behaviour.

For the purposes of this project, the SRS-2 questionnaire has been chosen as an appropriate tool to capture behaviours, because it is able to monitor progress and reflect subtle differences in the subsets of behaviours. The SRS-2 questionnaire uses a Likert Scale and is less onerous than some other assessment measures, taking approximately 15 to 20 minutes to complete.

Modified Brief Autism Mealtime Behaviour Inventory (BAMBI)

Mealtime behaviour is of interest to this study as food is often a source of stress for children with ASD. An autism specific questionnaire known as the BAMBI (Brief Autism Mealtime Behaviour Inventory) will be used to assess changes in mealtime behaviour in response to the intervention. This 15-item questionnaire has 4 subscales addressing food selectivity, disruptive mealtime behaviour, food refusal and mealtime rigidity and has been validated for use with children with ASD. The modified 15 item version of the BAMBI has shown excellent sensitivity and good specificity. Possible scores range from 15 to 75 with a score of 34 and above indicating significant mealtime behavioural problems. It addresses feeding behaviours and mealtime attitudes and provides an indication of how problematic the child’s behaviour is around food and mealtimes.

*Method for assessing gastrointestinal problems:*

Bristol Stool Form Scale (BSFS)

Parents will be asked to complete a 7-day record of BSFC which is the most widely used clinical measurement tool for assessing stool form and stool frequency. The BSFC divides stools into seven categories ranging from constipation (type 1 and 2), normal (type 3 and 4), tending to diarrhoea (type 5) to diarrhoea (type 6 and 7). It allows calculation of mean stools per day and mean stool consistency and will be completed at baseline and on completion allowing comparison between treatment and control groups. Difficulties arise from distinguishing type 2 from type 3 and type 5 from type 6 although overall the BSFC has good validity and intra-rater reliability (Chumpitazi, Cejka et al. 2015, Raker, Blake et al. 2015).

6-Gastrointestinal severity index (6-GSI)

Parents will be asked to complete a 6-item questionnaire focusing on the 6 most common GI symptoms in children with ASD (Adams, Johansen et al. 2011). These include abdominal pain, constipation, diarrhoea, bloating, smelly stools and frequency of stools. The 6-GSI uses a Likert scale of 0 to 2 with higher scores indicating higher levels of symptoms. The 6-GSI has been used with children with ASD and has been correlated to the Autism Treatment Evaluation Checklist (ATEC) (Adams, Johansen et al. 2011). Scores above 3 (on a possible scale of 0 to 12) have been associated with increased behavioural problems (Adams, Johansen et al. 2011) This questionnaire was adapted from the longer GSI (Gastrointestinal symptoms severity) scale (Schneider, Melmed et al. 2006). It will be completed at baseline and in week 6 to allow for comparison between treatment and control allowing for baseline.

7-day Sleep diary

A 7-day sleep diary will also be used to collect parent reported information on time to bed, time to sleep, night waking, time to rise, from which the following variables can be derived: sleep latency, sleep duration, frequency of night waking and longest duration of un-interrupted sleep.

**Measured**

*Method for collecting sleep data using wrist band accelerometers*

The collection of activity and sleep data will be overseen by JP. Professor Stewart Trost (QUT), a recognised expert in the field of sleep and activity data collection will assist in the analysis of data collected from the wristband accelerometer. The use of wrist band accelerometers has been subject in several previous studies (Trost, Zheng et al. 2014) and this method has been validated and used previously in atypically developing children (Trost, Fragala-Pinkham et al. 2016) (QUT HREC approval numbers 15000001028, 1600000902, 1700000423). In the present study, the wrist band accelerometers are used to obtain an objective measure of sleep and physical activity over two 7-day periods, however, if the child refuses to wear the wristband they will still be included in the study and sleep data will be derived solely from the sleep diary.

*Method for assessing stress: Basal cortisol measures (cortisol awakening response (CAR) + diurnal slope)*

Measuring cortisol via saliva is a non-invasive method of obtaining a biomarker for hypothalamic pituitary adrenal (HPA) axis activity (Keil 2012) and has therefore been chosen for use in this research project. Abnormal patterns in waking cortisol response indicate deregulation of the HPA activity, a pattern which has been associated with autism in both children and adolescents (Muscatello and Corbett 2018).

Saliva collected upon waking and up to 60 minutes after waking represent the cortisol awakening response (CAR). Cortisol peaks 30 to 45 minutes after waking in typically developing subjects (Stalder, Kirschbaum et al. 2016), however, children with ASD have shown altered cortisol patterns with elevated levels early evening and a flattened diurnal decline (Tomarken, Han et al. 2015). For this reason, two further time points will be measured mid-afternoon (3.30 to 4.30pm) and before bed (15 minutes before child’s bedtime). The combination of these time points has been shown to be the optimal method for recognising deregulated cortisol patterns (Jessop, 2008).

Enzyme linked immunosorbent assays (ELISA) will be used to determine the levels of the hormone cortisol in the saliva samples. Saliva samples will be taken over 4 timepoints on day 0 and day 42 of the study and a comparison made between the treatment and control groups allowing for baseline measures. Saliva samples will be collected by passive drool collection kits with salivation into a purpose designed vial (Salimetrics). Cortisol analysis will be carried out by JP under the supervision of Professor Flavia Huygens (IHBI/ QIMR). All samples will be marked with a de-identified study ID of the participant. Samples will be destroyed after analysis.

Principal component analysis indicates two indices which are important in the interpretation of cortisol data; total cortisol production and change in cortisol levels (Khoury, Gonzalez et al. 2015). This project will measure total cortisol and a change in total cortisol over time. The reference range to be used is by Salimetrics with a range of 0.084 to 0.839 for morning values and pm values up to 0.215 (Salimetrics, 2009) with a focus on change in mean cortisol levels between group.

*Method for assessing the gut microbiota*

The well-established method of 16S rRNA gene sequencing will be used to characterise the gut microbiome in stool samples collected from the study participants. 16S rRNA genes are ubiquitous – all bacteria have this gene. This enables 16S rRNA gene sequencing to provide taxonomic classification down to a genus level (and in many cases to a species level).

The 16S RNA sequencing will take place in Professor Flavia Huygens’ laboratory (Chief Investigator in this project) at the QIMR Berghofer Medical Research Institute in Brisbane.

This study will assess the differences in abundances of specific taxa including Bifidobacteria, between prebiotic and placebo groups at 6 weeks. In order to determine if changes in outcome variables in the prebiotic group are associated with changes in the GI microbiota, LEfSe will be performed.

**Covariates**

*Method for measuring dietary intake*

3-day food record

A parent-completed 3-day food record using non-consecutive days with 1 weekend day included will be used to measure food intake at baseline and on study completion. Parents will be asked if the food record is representative of usual diet to gain an insight into long-term diet. A weighed food record is considered to be a gold standard method of measuring food intake along with the biomarker doubly labelled water. However, these methods are costly and require extremely motivated participants (Johnson 2002) however, for this study, a weighed food record was considered too burdensome. An unweighed food record will instead be used. Data will be coded and analysed using Foodworks 9 Professional software (XYRUS).

Habitual fibre intake has been shown to have a significant impact on the effect of microbiota-targeted interventions (Healey, Murphy et al. 2018) however this has not been evaluated in children. It is important to estimate fibre intake from the diet to ensure that changes in outcome variables are not due to diet driven changes in the microbiota. Daily mean intakes of fibre and where information is available, daily mean intake of FOS and GOS will be calculated. In addition, mean serves of fruit and vegetables will be reported.

*Method for measuring activity*

Accelerometer

The wristband accelerometer measures movement and by default, lack of movement, allowing the distinction between sleep and activity (Trost, Cliff et al. 2016). The primary use of the accelerometer in this study is to gather data on sleep, however, during two 7-day periods of wearing the wristband, activity data will also be collected as a covariate. Sustained exercise can impact the GI microbiota (Monda, Villano et al. 2017) therefore mean activity on completion will be compared to baseline data.

**Data Collection, Analysis and Statistics:**

**Data Collection**

If a participant withdraws from this study, we will stop collecting research data, however data collected to date will be retained. If possible, the reason for withdrawal will be documented.

**Data Analysis**

The primary data analysis will use an intention to treat (ITT) analysis, the secondary will use a per protocol analysis (PP). Simple descriptive statistics will be used to analyse the outcome variables.

Questionnaires will be checked at each research visit to minimise the risk of missing data. If data is missing unintentionally, this will be completed by the parent during the research visit. Parents will receive a reminder text and email 72 hours before clinical sample collection to minimise the risk of forgotten samples. No statistical power calculation was performed because the intended number of participants for this pilot study is necessarily small (n=25 in each group).

It may be possible to replace the withdrawn participant with another recruited participant if funding allows, because enrolment will be managed on a rolling basis. Numbers (n) will be adjusted for the ITT analysis and include all participants who commenced the study where (n) may be greater than 50.

**Data Management:**

Questionnaires will be stored in locked storage areas in room A409, O Block QUT, Kelvin Grove. Questionnaire data will be entered into databases, with these data stored on QUT computer drives which are fully pass-word protected. Only named investigators will have access to data.

Clinical de-identified samples will be stored at QIMR (stool and saliva) until analysis

Data will be stored in a re-identifiable manner indefinitely as per Queensland Archives directive. De-identified data will be available for reuse in the archives of the QUT repository. All publications will report findings in a de-identified form.

The study will be registered with the ANZCTR clinical trials register. Progress will be reported throughout on the public domain of clinical trials and participants can access this through the link <https://www.australianclinicaltrials.gov.au/>.

No identifiable information will be published. Results will be published in the scientific literature following the completion of the research project.

***Compliance to prebiotic/placebo supplementation:***

Participants will be asked to return all unused capsules at the end of the study. Compliance will be measured by counting unused product at the close of the intervention. Per protocol will be considered to be 75% or greater of the recommended dose and no major protocol violations.

In addition, participants will be provided with a diary of compliance where they are able to record the number of capsules taken daily over the 6-week period. There is provision for estimation of the amount of dose actually consumed.

**Results, Outcomes and Future Plans**

Results of the study will be summarised for interested participants in lay terms. Findings will be communicated to patient interest groups such as Autism Queensland and to the general public through general media commentaries. Research will be presented to peers for review, published in the peer-reviewed scientific literature and presented at conferences.

The data collected in this pilot study will provide proof-of-principle data and will provide valuable information for the design of larger data collections. The pilot study data will be used for grant applications to fund these future studies.

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