The efficacy of the multi-strain probiotic, Vivomixx, on behaviour and gastrointestinal symptoms in children with autism spectrum disorder (ASD)

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ASD-Probiotic R&D ID:17/0148

An investigation of the parent-observed link between behaviour symptoms and the dietary management of gastrointestinal symptoms in children with autism spectrum disorder

Can gastrointestinal symptoms be managed by dietary intervention and does this bring benefits to children with autism spectrum disorder (ASD) and their families, over and above improvement in gastrointestinal (GI) symptoms?

Short Title: ASD-Probiotic

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Gastropharm

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Protocol Version History

Version Number	Date	Protocol update finalised by:	Reasons for Update
1.0	26/7/17	Sue Simmons	
2.0	30/9/17	Sue Simmons	Provisional opinion from Ethics Committee and questions from Chris Kitchen at HRA
3.0	14/10/17	Sue Simmons	Addition of letter to Educator and requesting referral from the GP
4.0	15/01/18	Sue Simmons	Adding collection of stool sample at three time points
5.0	08/05/18	Sue Simmons	Referral from GP no longer required by the site. GP will be notified once participant is enrolled.
6.0	30/01/2020	Sue Simmons	Added voice recording of interviews with selected participants in phase 3 of the study

Signatures

The Chief Investigator and the JRO have discussed this protocol. The investigator agrees to perform the investigations and to abide by this protocol.

The investigator agrees to conduct the trial in compliance with the approved protocol, the UK Data Protection Act (1998), the Trust Information Governance Policy (or other local equivalent), the current Research Governance Framework, the Sponsor's SOPs, and other regulatory requirements as amended.

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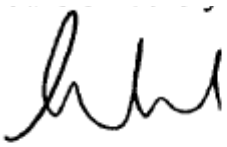

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Glossary of abbreviations

3di	Developmental, Dimensional and Diagnostic Interview
ADHD	Attention Deficit Hyperactive Disorder
ADOS	Autism Diagnostic Observation Schedule
ADR-R	Autism Diagnostic Interview-Revised
APSI	Autism Parenting Stress Index
ASD	Autistic Spectrum Disorder
ATEC	Autism Treatment Evaluation Checklist
CARS	Childhood Autism Rating Scale
CBT	Cognitive Behaviour Therapy
CHARGE	Childhood Autism Risks from Genetics and the Environment
CI	Chief Investigator
CNS	Central nervous system
DISCO	Diagnostic Interview for Social and Communication disorders (DISCO) or (3di).
DMC	Data Monitoring Committee
FMPP	Fermented milk product with probiotic
GI	Gastrointestinal
GIH	Gastrointestinal History questionnaire
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
LPS	Lipopolysaccharides
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs

OCD	Obsessive Compulsive Disorder
OR	Odds Ratio
PD	Parkinson's Disease
PDD/NOS	Pervasive Development Disorder/ Not otherwise specified
PMG	Project Management Group
SCFAs	Short chain fatty acids
TMG	Trial Management Group
TSC	Trial Steering Committee
UCL	University College London
UCLH	University College London Hospital

Trial Personnel

See protocol cover page for Chief Investigator and Sponsor contact details.

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Trial Steering Committee (TSC)

This committee is comprised of independent members along with the Chief Investigator. Representatives from the trial researchers may be invited to attend meetings to provide information as appropriate.

Independent members of the Trial Steering Committee
Mrs Jane Wills, Patient Participation representative
Frauke Elichaoff, member of London Autism Research Advisory Group
Dr Michael Absoud, Consultant in Paediatric Neurodisability, Evelina London Children's Hospital

Data Monitoring Committee (DMC)

Data Monitoring Committee members:
Independent statistician, James Neil, Research Director CNELM
Dr Natalia Zarate-Lopez UCLH
Dr Amalia Tsiami, Associate Professor in Food Science, University of West London

Protocol Summary

Questions addressed:	Is Vivomixx an effective agent in ASD children who also have GI symptoms in terms of improving overall functional level and is this associated with a reduced frequency of gastrointestinal symptoms? Does this intervention improve the quality of life of the patient and their parents?
Type of trial:	A single-site randomised double blind placebo controlled crossover trial in children with autistic spectrum disorder and gastrointestinal symptoms.
Trial duration per participant:	30 weeks
Estimated total trial duration:	1 year
Interventions:	1. Daily administration of Vivomixx probiotic powder mixed into a drink or selected cold foods 2. Daily administration of placebo powder mixed into a drink or selected cold foods
Outcome assessment:	• Parent completed questionnaires after 12 weeks of treatment and after 28 weeks of treatment • Educator completed questionnaires after 12 weeks of treatment and after 28 weeks of treatment • Clinician completed assessments after 12 weeks of treatment and after 28 weeks of treatment
Planned trial sites:	Single-site
Total number of participants planned:	82
Main inclusion/exclusion criteria:	Children aged between 3 – 16 years of age, with a diagnosis of ASD and gastrointestinal symptoms excluding those, • with Retts Syndrome and Fragile X • on NSAIDs • taken antibiotics in the past month • taken a probiotic in the past month • history of intolerance or allergy to probiotics
Statistical methodology and analysis	The intention-to–treat (ITT) analysis will be performed on all patients who are randomised. An additional analysis will be done on only those patients who complete the study per protocol analysis. Changes in ASD scores during follow-up will be examined using the mixed model for analysis of variance to account for missing data (dropouts). The mixed model will use group (treatment versus control) and time as factors. If residuals prove not to be normally distributed the Bonferroni multiple comparisons with Bootstrap multiple comparisons to accommodate for the non-normality of residuals will be done. A *P*-value < 0.05 is considered significant and multiple comparisons

will be undertaken with Fisher's least significant difference test and 95% confidence intervals.

Trial Design and Methods Summary

A plausible relationship exists between the composition of the GI microbiota and the incidence of certain aberrant behaviours seen in ASD children. Gastrointestinal (GI) symptoms (constipation, diarrhoea, abnormal stools, abdominal pain) are common in children with autism (ASD) and evidence suggests that the severity of GI symptoms is related to the severity of aberrant behaviour. Despite this, current treatment options are limited and poorly evaluated.

This research aims to find out whether Vivomixx probiotic can help improve the overall function and GI symptoms of children with ASDs. In our survey data, parents of children with ASD reported improvements in GI symptoms and overall function when their children were taking a probiotic. A controlled study is now required to investigate this reported effect in children with ASD and GI symptoms and use statistical analysis to evaluate whether the change in GI symptoms or behaviour is significant.

Potential trial participants will be recruited via one of two routes,

1. Caudwell Children charity will email parents of children with ASD on their database with a copy of our recruitment poster and asked to contact us if they wish their child to be considered for the study.
2. Information on the trial was given to attendees of a bi-annual charity conference in June 2016 run by Treating Autism charity, which offers support to parents of children with ASD. Contact details were taken from parents who were potentially interested in their child taking part. They will be emailed a copy of our recruitment poster and asked to contact us if they wish their child to be considered for the study.

Parents who respond to the recruitment poster will be emailed the Patient Information Sheet and the Child Information Leaflet. A few days later, they will then be emailed to ask if they are interested in answering some questions to see if their child is eligible for the study. If they are, an appointment will be made to phone them and complete the screening questionnaire.

For those children that fit the requirements for the trial, the parents will be invited for their child to take part. We will notify their General Practitioner (GP) once they have enrolled in the study. The participants recruited to the study will be allocated at random to either group A or B and will be given a unique Study Number. All data collected throughout the study will be anonymised and identified only by the participant's study number.

The first week of study will be a taste evaluation of the placebo powder for all participants (both groups). The participants and their parents will be blinded to the fact that this is the placebo powder. The taste evaluation is to ensure that the participant finds the taste acceptable and is willing to take the treatment product. For those that find the taste acceptable they will then either receive a 12-week course of Vivomixx probiotic or a 12-week course of the placebo powder, according to their random group allocation. The placebo powder has the same look and taste as Vivomixx.

Once the participant has passed the taste evaluation, the child's educator will be sent a letter inviting them to participate in the study. Included with this letter will be a consent slip, 3 copies of the Educator Questionnaire (with instructions on when these should be completed) and 3 stamped addressed envelopes for returning the questionnaires and consent slip to the researcher.

All participants' parents will be phoned by the researcher in Weeks 5 and 9 to adjust the treatment dose, offer support and encourage compliance.

Those parents whose child is eligible but who choose not to take part will be asked for the main reason and this will be logged in the screening log of patients.

After 12 weeks there will be 4 weeks when neither group is taking any treatment (a washout period). After this, participants who complete all 12 weeks of the double-blind treatment period will be eligible to enter the double-blind 12-week withdrawal period. The participants that have been taking the placebo will then receive a 12-week course of Vivomixx probiotic. The participants that have received Vivomixx, will take a 12-week course of the placebo. All participants' parents will be phoned by the researcher in Weeks 21 and 25 to adjust the treatment dose, offer support and encourage compliance. We will also ask about their experience of the study.

We will measure the results after 12 weeks and 28 weeks of treatment. We are primarily interested in whether participants in the intervention group (Vivomixx) have had more of an improvement in their overall function after their 12-week course of Vivomixx compared to that of the control group (receiving the placebo). We will also measure the effect of the active treatment on parent stress compared to the control group and the effect of the active treatment on gastrointestinal symptoms and clinician's global assessment compared to the control group. We will analyse stool samples taken before treatment, after 12 weeks of treatment and at the end of the trial to see if the composition of bacteria in the stool has changed with the active treatment compared to the placebo. This will help us to understand how Vivomixx works in the body.

During the trial we will assess how well the participants kept to the treatment plan. We will also record changes that happen during the trial that may affect the efficacy of treatment e.g. taking antibiotics or a significant change in diet. We will talk to the participant's parents to find how they perceived the treatment and how their child tolerated the treatment.

Individual participation will be entirely voluntary and we do not believe there are any risks associated with taking part.

Background and Rationale

Autistic Spectrum Disorders

Autism is a lifelong, neurodevelopmental disorder that affects how a person communicates with and relates to other people, and how they experience the world around them. Autism and related conditions such as Asperger Syndrome are collectively known as Autism Spectrum Disorders (ASD).

Incidence rates increased significantly in the 1990's but have stabilised in recent years in the UK [1]. A review of the incidence rate of ASD using the General Practice Research Database, concluded that the annual prevalence of autism recorded by UK GPs remained steady for the 7-year period 2004– 2010. During this period the incidence rate for 8-year-old boys varied from 1.02 to 1.3 per 1000. For girls aged 8 years the incidence rate for the same period varied from 0.2 to 0.31 per 1000 [1]. There are around 700,000 adults with ASD in the UK, which equates to prevalence rate of 1.1 per cent [2]

Autism is a neurodevelopmental disorder associated with impaired social ability, especially communication, and restricted, repetitive patterns of behaviour [3] Abnormal sensory responses are also common [4] and are considered part of the diagnostic criteria and core symptoms [5] . Individuals with autism can suffer significant functional impairments that have a profound effect on the affected individual and their family [American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. 5th ed. Arlington,VA: American Psychiatric Association; 2013]. ASDs are associated with long-term health, social and financial costs for the individuals with ASD, their families and society as a whole. The consequences of ASD can potentially be seen in many domains, including family and other relationships, employment, leisure activities, standards of living, social and personal functioning [6] . These problems can substantially affect the person's quality of life and that of their families and carers, and lead to social vulnerability.
High-functioning adults with ASD may achieve independent living but rely heavily on the support of their families in finding jobs and accommodation. The pressure of trying to fit into society may lead to stress and anxiety and even psychiatric breakdown [7].
Higher levels of parenting stress have been found in parents of young children with autism than in other disabilities [8]. This is due to the challenges imposed by co-morbid behavioural and physical symptoms as well as core symptoms that can affect almost every aspect of the individual's functioning. This can challenge the coping skills and affect the mental health of parents.

The aetiology of autism spectrum disorders

The aetiology of autism spectrum disorders (ASD) is still unknown. The MRC Review of Autism research – Epidemiology and Causes in December 2001 concluded that;

- Most researchers believe that ASDs have a variety of causes
- There is a genetic component to ASDs that may be operating to confer susceptibility
- The genetic findings do not preclude the possibility that some form of gene-environment interaction may be involved in the pathogenesis
- In a minority of individuals with ASDs, there is an identifiable probable causal medical condition, usually comprising various single gene disorders or chromosomal abnormalities

More recent research has identified a number of risk factors associated with the onset and course of ASD that appear to involve gene-environment interaction and gene-environment correlation [11]

- Advanced paternal and maternal age at conception
- Maternal exposure to traffic related air pollutants during pregnancy
- Maternal exposure to some metals during pregnancy

- Maternal exposure to several pesticides during pregnancy
- Maternal obesity and gestational diabetes mellitus
- Maternal enhanced steroidogenic activities
- Maternal immune activation
- Prenatal or neonatal exposure to thalidomide, misoprostol and valproic acid and possibly SSRIs

Folate intake is a prenatal protective factor, with a particular window of action around 4 weeks preconception and during the first trimester.

Research has also found a number of chromosomal abnormalities associated with autism including chromosomal abnormalities in regions such as 15q and 7q in some ASD individuals. However, all the currently known genetic variations contributing to autism only account for approximately 5-15% of cases.

Abnormalities of the cerebellum have been proposed as a unifying framework for understanding the diversity of autism research findings. The cerebellum has been found to be structurally and functionally abnormal in patients diagnosed with autism. Mutations of several genes that contribute to normal cerebellar development are consistently associated with increased susceptibility to autism [12]. An active neuro-inflammatory process has been shown in the cerebral cortex, white matter and notably the cerebellum of autistic patients.

A number of neuro-immune axis alterations have been found in ASDs and it has been proposed that prenatal immune alterations and early inflammatory processes could be the autism aetiological events. Monocytes or peripheral blood mononuclear cells (PBMCs) show strong dysfunctions in ASD children and are committed to a pro-inflammatory state, which in turn results in long-term immune alterations. In ASD, altered PBMCs are responsible for elevated pro-inflammatory cytokine production leading to blood-brain barrier disruption. Changes in blood-brain barrier permeability directly influence neural plasticity, connectivity and function, potentially triggering impairments in social interaction, communication and behaviour [13].

ASD and Co-morbid Conditions

The most common co-morbid symptoms in young children with autism are sleep disruption (86%), hyperactivity, and gastrointestinal disorders (70%), followed by self-injurious behaviour (34%), aggression/irritability (22%), motor co-ordination and eating problems [14]. Research suggests that children with ASD are more likely to have health problems and illnesses in general compared to typically developing children.

There is an increased risk for epilepsy in autistic individuals and for some subgroups this is significantly higher. In autistic individuals with intellectual disability the prevalence is 21.5% and for those without intellectual disability it is 8% [15].

A longitudinal study in Sweden found the death rate in autistic individuals was a rate 5.6 times higher than expected. Associated medical disorders (including epilepsy with cognitive impairment) and accidents accounted for most of the deaths and it was not possible to determine whether autism per se actually carries an increased mortality risk [16].

In a 2014 survey of 264 parents of children with ASDs in the UK, they were asked about they main health challenges facing their children and their efforts to access services [10]. This report highlighted the scale of the challenges faced by children with ASD and their families including the following:

% reporting	Challenge suffered by child
85	Anxiety/fearfulness/avoidance behaviours
85	Irritability (low mood/tantrums/lack of flexibility/oppositional behaviours)
85	Sensory sensitivity
78	Lethargy or hyperactivity
78	Diarrhoea/constipation/highly abnormal bowel movements
72	Gastrointestinal symptoms (flatulence/bloating/posturing/seeking pressure on stomach)
57	Incontinence (urinary/bowel)

Their report [10] concluded that their survey highlighted the need for more scientific research into the causes and possible treatments of ASD as well as the range of co-morbid conditions associated with ASDs. It also reported how parental experiences of diet and nutritional supplements (such as probiotics) suggested that these can make a significant difference to the lives of children with ASD and warrant further research.

Gastrointestinal Symptoms and ASD

There is a greater prevalence of GI symptoms among children with ASD compared with control children [17]. Functional GI disorders (FGIDs) are a group of chronic GI symptoms found in the absence of structural disease. Phillip Gorrindo et al [18] found that functional constipation is the most common type of GI dysfunction in children with ASD (85%), indicating that the majority of ASD children with GI symptoms would fall into the category of having a FGID.

There is also some evidence that certain behaviours found in ASD correlate to the presence of GI problems in children with Pervasive Development Disorder (PDD). Specifically Nikolov et al [19] found that those with PDD and GI problems (primarily constipation and diarrhoea) showed greater symptom severity on measures of irritability, anxiety and social withdrawal but were no different from subjects without GI problems in demographic characteristics, in measures of adaptive functioning or autism symptom severity. However, an earlier study looking at children with an ASD diagnosis and incorporating those with autism, Pervasive Development Disorder/Not Otherwise Specified (PDD/NOS) and Asperger's, reported a strong correlation between gastrointestinal symptoms and the severity of autism (measured by ATEC Total Score) indicating that children with more severe autism are likely to have more severe gastrointestinal symptoms and vice versa [20]. J.B. Adams et al, mentioned in their conclusion that it is possible that autism symptoms are exacerbated or even partially due to the underlying gastrointestinal problems.

In her 2015 review of current concepts in paediatric functional GI disorders [21], Katja Kovacic states that many patients with FGIDs display numerous comorbidities: She lists common complaints including chronic fatigue, poor sleep, anxiety, migraines, chronic nausea, dizziness and high levels of stress. Some of these co-morbid symptoms are know to be common in ASD children but others, such as migraines and dizziness would be very hard to ascertain in a non-verbal child with learning disability. 40-60% of people with ASDs also have intellectual disability. It is estimated that as many as 25% of individuals living with autism spectrum disorders are non-verbal meaning that they cannot functionally communicate with others using their voice. Those that are verbal may have varying degrees of impairment in expressive speech and language comprehension.

Most FGIDs share common associated symptoms of anxiety, hypervigilance and somatization [21]. Comparing these behavioural symptoms to the core symptoms of ASD, which are social deficits, language

impairment, repetitive behaviours and abnormal sensory responses. There is little overlap in these behaviour symptoms and a treatment that brings an improvement in overall function in a child with ASD and GI dysfunction, is unlikely to be simply a result of addressing the pain and discomfort of the GI symptoms. However this remains to be proven.

Despite the fact that some children with ASD follow a selective diet due to sensory issues, S.E. Levy et al found that their results suggested that GI symptoms are not significantly related to abnormal patterns of dietary intake of macronutrients [22]. Similarly P. Gorrindo et al found that the presence of GI dysfunction in children with ASD was not associated with distinct dietary habits or medication status [18].

Research has indicated that individuals with ASD have an altered gut microbiome and that this may be affecting behaviour [23] [24] [25]. Kang et al [26] found that the presence of autistic symptoms rather than the severity of gastrointestinal symptoms was associated with a less diverse gut microbiome in ASD children.

Many individuals with autism need set routines, familiar people and environments to help them cope, and they find unexpected changes to their routine stressful: This can result in challenging behaviour. Individuals with autism may also have sensory sensitivity and be over- or under-sensitive to stimuli such as sound, touch, smell and taste. This can make busy, brightly lit or noisy environments difficult to cope with and can lead to self-restricted diets.

A change in the pattern of gut microbiota has been noted in a number of diseases, including neurological conditions such as depression [27] [28] [29]. There is a growing body of evidence on the 2-way nature of the gut-brain axis and a realisation of the potential for the gut microbiota to influence behaviour and mental health. It has been suggested that the gut microbiome may play a role in the presentation and severity of ASD symptoms. Research has shown a high rate of gastrointestinal problems in children with ASD, a correlation between symptom severity and GI symptoms, and distinctive profiles of gut microbes and their metabolites in ASD children [24] [25] [20]. Autistic individuals with comorbid GI abnormalities exhibit altered carbohydrate digestion [23]. Increased intestinal permeability is linked to autism and hypothesized to have detrimental effects on intestinal barrier function and with the potential for translocation of intestinal metabolites or bacteria with consequent immune activation. The influence of the gut microbiome may start early in the infant's life when bacterial colonisation of the infant gut, likely modulates host neural development through signalling pathways that include the vagus nerve, a direct conduit between the gut and the central nervous system. Overall, changes in the gastrointestinal tract can influence higher-order behaviour and brain function via the gut-brain axis, driven by direct connections of the intestinal epithelium to the central nervous system via the vagus nerve, and by indirect connections of the gut to the brain via alterations in immunity and metabolism [30].

The cost of Autistic Spectrum Disorders

The cost of autism to the UK government is significant: In 2014 the estimated annual cost of autism in the UK was £32 billion, making it the most costly medical condition in the UK. This included the cost of housing, healthcare, special education and the opportunity cost of lost earnings by the affected individuals and their parents. At financial levels based on a 2014 paper, the cost of supporting an individual with an ASD and intellectual disability during their lifetime is £1.5 million. The cost of supporting an individual with an ASD without intellectual disability during their lifetime is £0.92 million [6]

Only 15% of autistic adults without a learning disability in the UK are estimated to be in full-time paid

employment [9].

NICE guidelines for the support and management of Autism in under-19's recommends a detailed assessment, management and co-ordination of care for children and young people with ASD involving local specialist community-based multidisciplinary teams working together. These teams may include the following professionals: paediatrician, psychologist/psychiatrist, learning disability specialist, speech and language therapist, occupational therapist, education and social care services. Every child or young person diagnosed with ASD should have a care manager or key worker to manage and co-ordinate their treatment, care and support, as well as their transition into adult care. Where a child's behaviour is causing problems, they should be assessed for possible triggers such as a physical health condition, mental health problem or environmental factors. Psychological treatments such as CBT or medication may be given for anxiety. Assessment, psychosocial and pharmacological interventions, and regular reviews may also be needed for the management of sleeping problems, constipation, OCD, depression, epilepsy, ADHD, aggression or self-harming [NICE guidelines (CG170), Aug 2013, Autism in under 19s: support and management].

Despite the NICE guidelines, the 2016 Queen Marys University report [10] found that many parents of children with ASD had difficulty accessing NHS services for their children. 24% (n=48) of those trying to use the NHS had been unsuccessful in getting support for diet and nutritional support, even though in the survey this was identified as the area most parents reported having the greatest success in helping their children. 22% (n=45) were unsuccessful in accessing services for the challenges associated with gastrointestinal and bowel complaints and 20% (n=40) were unsuccessful in accessing services for help with problem behaviour.

The UK 2014 estimated mean annual costs per capita for medical services for children with ASDs varies from £267 for 0-1 years of age, to £1818 for 12-17 years of age with intellectual disability. The UK 2014 estimated mean annual costs per capita for medical services for adults with ASDs are £5142 for those with intellectual disability and £16044 for those without intellectual disability [6].

Probiotics

This trial proposes using a combination probiotic, Vivomixx, as an intervention for children with ASD. Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' [FAO/WHO Working Group (2001), Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties in food including powder milk with live lactic acid bacteria. Cordoba, Argentina: FAO/WHO; 2001]

The interest in the potential of probiotics for treatment of disease symptoms and as a preventative intervention has increased as research has shown the extensive role of the gut microbiota in health and disease. There are a number of functions that the microbiota perform that are beneficial to the host;

- Development of metabolic and immune systems
- Postnatal development and maturation of the endocrine system
- Production of neurotransmitters
- Growth inhibition of pathogens
- Participation in digestion
- Influencing fat absorption and distribution
- Vitamin K synthesis
- Production of short chain fatty acids
- Maintenance of gut barrier function
- Participation in xenobiotic clearance
- Intestinal motility

Thus, the gut microbiome plays a key role in establishment and regulation of the immune system, digestion and assimilation of nutrients, maintenance of intestinal barrier function and also affects communication between the gut and brain. Preclinical research indicates that the gut microbiota have a major impact on cognitive function and fundamental behaviour patterns, such as social interaction and stress management [31]. Over 25 diseases have been linked with an altered intestinal microbiome including obesity, type II diabetes, IBS, Crohn's disease, ulcerative colitis, multiple sclerosis, schizophrenia and autism [27] [28] [29]. Until recently, babies were believed to be born sterile and only populated by microbes during and after the birth. However, there is now a belief that this may begin before birth, with transfer between mother and baby taking place via the placenta. The microbiome matures in the first 3 years of life and factors such as method of delivery, breastfeeding, diet and host immune status appear to colonize the gut, skin, mouth and airways and confer a 'permanent resident status' for some microbes, known as symbionts. Beyond this age, an individual's microbiome can be maintained with only minor changes over many months or even years. Factors that have been shown to alter the adult microbiome are the use of antibiotics, prebiotics, probiotics and a change in diet [29] [32].

Evidence is building which indicates that many chronic diseases are associated with less diverse gut microbiomes. Currently, it is unproven whether reduced diversity occurs as a result of disease or vice versa. However, in some cases e.g. Clostridium difficile infection, disease certainly results from a loss of gut microbiota diversity and robustness. Some have hypothesised that modern lifestyles do not sustain a diverse microbiome and that the extinction of certain 'keystone' microbes could lead to the loss of fundamental functional abilities, which would ultimately contribute to dysbiosis and disease. Dysbiosis is simply an imbalance within the gut microbial ecosystem. Ground-breaking research comparing the microbiome of an isolated Amazonian tribe who have had no documented previous contact with Western people, with the microbiome of the population of the United States, adds some support to this hypothesis. It showed that this isolated tribe have a microbiome with the highest diversity of bacteria and genetic functions ever reported in a human group [33].

In the gut, some probiotics can strengthen the tight junctions between epithelial cells thereby reducing the damaging effect on intestinal permeability of some pathogens and their metabolites [34]. The literature regarding a possible link between autism, intestinal permeability and allergic response is promising. Laura de Magistris et al [35] studied the prevalence of antibodies to gliadin and milk proteins and assessed intestinal permeability in three groups; patients with ASD on a regular diet (ASD-RD), patients with ASD on a gluten and dairy-free diet (ASD-GF/CF) and healthy controls on a regular diet (HC-RD). The ASD-RD group had increased intestinal permeability compared to the HC-RD group and the ASD-GF/CF group. Anti-alpha-Gliadin IgG (AGA-IgG) titres and Anti-Deamidated alpha-Gliadin IgG titres were increased in ASD-RD and ASD-GF/CF groups compared to HC-RD, irrespective of their intestinal permeability result. They also noted that the AGA-IgG mean titre decreased in the ASD-GF/CF group compared to ASD-RD. The ASD-RD group more frequently showed casein IgG titres > 12 mgA/L compared to the HC-RD group and the ASD-GF/CF group had significantly decreased casein IgG titres compared to ASD-RD and HC-RD. Their results support the hypothesis that the immune system in a subgroup of individuals with ASD, is triggered by gluten and casein and confirmed that a large percentage (25.6%) of the individuals with ASDs have impaired intestinal barrier function.

The intestinal barrier can be disturbed by various causes including certain medicines, exercise, mast cell activation, a high fat diet and stress, leading to increased permeability. This then has the potential for translocation of intestinal metabolites e.g. lipopolysaccharides (LPS) or bacteria with consequent immune activation and the release of pro-inflammatory cytokines. Increased intestinal permeability has been associated with a number of gastrointestinal diseases but can also lead to systemic inflammatory diseases

(allergy, metabolic syndrome, diabetes, atherosclerosis, rheumatoid arthritis). There is also evidence of systemic inflammation in autism [34].

Gut microbiota produce a wide range of lipid molecules from short chain fatty acids like butyrate and propionate to polyunsaturated fatty acids including conjugated linoleic acid (CLA), which has a potent anti-inflammatory effect. It is becoming evident that these lipids can have an immune modulating effect. In a mouse model of colitis, both oral CLA treatment and oral VSL#3 probiotic treatment were compared to controls to explore the mechanism of the protective effect of VSL#3 and CLA on colitis [36]. VSL#3 treated mice showed increased levels of colonic CLA but systemic levels were not raised suggesting a local effect whereas oral CLA raised serum and colonic levels of CLA. Both showed histological improvements on the colonic specimens indicating that both treatments ameliorated colitis. Both treatments showed a decrease in colonic bacterial diversity and both reduced the immune response shown by a decrease in macrophage accumulation in the mesenteric lymph nodes and a lower percentage of inflammatory macrophages . The researchers showed that the protective effect of VSL#3 against colitis was not present in conditional PPAR y knockout mice suggesting the mechanism of protection is via activation of PPAR *y* in macrophages, with subsequent anti-inflammatory effect.

There are many potential direct and indirect pathways through which the gut microbiota can modulate the gut-brain axis including epigenetic, neural, immune and endocrine pathways. The gut microbiota can alter the levels of circulating cytokines and this can have a marked effect on brain function. Some probiotics are able to modulate the effect of the gut microbiota on the local immune and inflammatory systems, thereby down-regulating an over-stimulated inflammatory response. A lot of animal research is being done in this area and the exact mechanism of how the gut microbiota modulate the gut-brain axis is not yet agreed but is emerging as a complex multifactorial process.

In animal models, treatment with a probiotic has been shown to modulate the expression of genes in the brain tissue and also to reverse changes in genetic expression caused by an early life stress. Treating rats with the oral probiotic VSL#3 was shown to modulate the expression of a large group of genes in brain tissue, with evidence of a change in genes that impact on processes mediating inflammation and neuronal plasticity[37]. This same research found that an age-related deficit in long-term potentiation (LTP) was attenuated by treatment with VSL#3 probiotic and this was accompanied by a modest decrease in markers for microglial activation and an increase in expression of brain derived neurotrophic factor (BDNF) and synapsin. LTP is impaired in a number of neurodegenerative disease models that are associated with inflammatory changes and BDNF is essential for maintaining LTP. They concluded that their data supports the notion that intestinal microbiota can be manipulated to positively impact on neuronal function.

In earlier research [38], Distrutti el al used a rat model to investigate the pathophysiology of visceral hypersensitivity in IBS [38]. They found that neonatal maternal separation (NMS) of the rat pups caused allodynia and hyperalgesia, which are also found in IBS sufferers, while influencing the expression of a wide range of genes, including those known to mediate inflammation and pain. Oral administration of VSL#3 probiotic was effective in reversing NMS-induced visceral hypersensitivity and resetting the genes involved in inflammation and pain. They comment that their data supports a hypothesis that VSL#3 probiotic inhibits visceral hypersensitivity by reversing the alterations in genetic expression caused by an early life stress.

Probiotics have long been known to have an anti-inflammatory effect and animal research shows that this effect reaches the brain. Using a rat model of systemic inflammatory disease, D'Mello et al [39] found that administering oral VSL#3 probiotic reduced microglial activation and cerebral monocyte infiltration thereby reducing development of inflammation-associated sickness behaviours. They also found parallel changes in markers for systemic inflammation including a decrease in tumour necrosis factor alpha (TNF-*a)* levels. They comment that peripheral TNF-*a* signalling plays a key role in mediating microglial activation and cerebral

monocyte recruitment in this context. They conclude that where there is inflammation in the body, the administration of probiotics may beneficially affect brain function.

Research in animals indicates that the gut microbiota tightly control the maturation and function of microglia [40]. As the tissue macrophages of the CNS, microglia are critically involved in diseases of the CNS. They respond rapidly to even minor pathological changes in the brain and contribute directly to neuro-inflammation by producing various pro-inflammatory cytokines and free radicals. Human studies have revealed microglial activation in schizophrenia, Alzheimer's disease [41] and ASD [40].

Microglia have been found to be critical for synaptic pruning and remodelling during brain development and in adulthood [40]. Proper synaptic pruning is essential for the development of functional neural circuits. Impairments in synaptic pruning disrupt the excitatory versus inhibitory balance of synapses, which may cause neurodevelopmental disorders such as ASD [40]. Microglia are particularly affected when a complex microbiome is absent and microglia from germ-free mice display global defects in microglia. Even during adulthood, a continuous input from complex microbiota is necessary for maintenance of normal microglia. If the input signals are lost e.g. after antibiotics, microglia return to an immature status. Recolonization with complex gut microbiota partially restored microglia features. Limited microbiota complexity also resulted in defective microglia. This animal research determined that short-chain fatty acids (SCFAs) regulate microglia homeostasis [41].

There is growing interest in the role of short chain fatty acids as neuroactive molecules. SCFAs are produced in the large intestine by bacteria fermentation of dietary fibre. These include propionic, butyric and acetic acids. SCFAs have been reported to have anti-inflammatory properties. Butyric acid is a major source of fuel for the enterocytes. In preclinical research, butyrate has been shown to improve insulin sensitivity and increase energy expenditure. Circulating SCFAs can be carried by monocarboxylate transporters, which are abundantly expressed at the blood-brain barrier. It has been hypothesized as a plausible mechanism through which they can cross the blood-brain barrier and enter the CNS. In rat research[42], intracerebroventricular administration of propionic acid induced reversible behavioural, electrographic, neuroinflammatory, metabolic and epigenetic changes that closely resembles those found in autism. Altered fecal concentrations of SCFAs have been found in autistic individuals [34]. Together, the evidence suggests that enteric SCFAs play a major role in host physiology and provide further evidence that gut microbiota can modulate brain function and behaviour in health and disease conditions, including ASD.

Another mechanism by which some probiotics can influence health is the modification of expressed functions of the gut microbiota. Some probiotics are able to change the enzymatic activities of the gut microbiota, e.g. the nitrogen metabolism as reflected by urinary concentration of p-cresol [29].

The evidence for the use of probiotics

There have been some very promising results in clinical trials using probiotics as a treatment for certain gastrointestinal disorders.

A meta-analysis of the use of Lactobacillus rhamnosus GG for abdominal pain-related functional GI disorders in childhood concluded that its use moderately increases treatment success in children with this type of GI disorders, particularly among children with IBS [43]. A multicentre, randomised, placebo-controlled, double-blind, crossover study giving VSL#3 to children with IBS, found the probiotic to be significantly superior to placebo in the subjective assessment of relief of symptoms, severity and frequency of abdominal pain/discomfort and bloating/gassiness as well as caregivers' assessment of life disruption [18].

There is some evidence that giving an oral probiotic can affect changes in the gut microbiota in some patients. A non-randomised controlled study looking at the VSL#3 probiotic for adults with functional constipation (FC) and healthy controls [44] measured 5 bacteria species (Lactobacillus, Bacteroides, Clostridium, Escherichia coli, Bifidobacterium) using qRT-PCR analysis, before and after 2 weeks of treatment. At baseline, patients with FC had a significantly low levels of Bifidobacterium and Bacteroides species compared to controls. After treatment, fold differences of Lactobacillus, Bifidobacterium and Bacteroides species increased significantly in controls, but not in patients with FC: This was despite an improvement in measures of stool consistency and complete spontaneous bowel movements (CSBM) in the patients with FC.

There is some evidence that probiotics may affect brain activity in adults. In a study of the effect of 4 weeks consumption of a fermented milk product with probiotic (FMPP) by healthy women, the participants underwent functional magnetic resonance imaging before and after the intervention to measure brain response to an emotional faces attention task and resting brain activity. The researchers concluded that the 4-week intake of an FMPP by healthy women affected activity of brain regions that control central processing of emotion and sensation [45].

A Finnish study concluded that probiotic supplementation early in life may reduce the risk of neuropsychiatric disorder development later in childhood. In this study, 75 infants received Lactobacillus rhamnosus GG or placebo during the first 6 months of life and were followed up for 13 years. At the age of 13 years, ADHD or Asperger Syndrome was diagnosed in 17.1% of the placebo group and none in the treatment group. The mean numbers of Bifidobacterium species bacteria in the faeces during the first 6 months of life was lower in affected children than in healthy children [46].

There have been five studies published on probiotics for children with ASD, the results of which have all indicated that probiotics may be beneficial for more than just GI symptoms. However, there are methodological issues with each study (small sample size, non-standard measures, incomplete reporting) which still leaves this unproven.

Parracho et al [Parracho, H. M. R. T., Gibson, G. R., Knott, F. , Bosscher, D., Kleerebezem, M. and McCartney, A. L. (2010) *A double-blind, placebo-controlled, crossover-designed probiotic feeding study in children diagnosed with autistic spectrum disorders.* International Journal of Probiotics and Prebiotics, 5 (2). pp. 69-74. ISSN 1555-1431] conducted a double-blind placebo-controlled crossover trial of a 3-week treatment of a daily dose of 4.5×10^{10} colony forming units (CFU) of the probiotic Lactobacillus plantarum WCFS1, (a single strain probiotic) for ASD children aged 3-16 years. They measured the faecal microbiota, gut function and behaviour scores throughout the trial. Giving the probiotic showed the following results compared to placebo;

- significantly increased the Lab158 counts (lactobacilli and enterococci group)
- significantly reduced Erec482 counts (Clostridium cluster XIVa) compared to placebo
- significant differences in stool consistency compared to placebo
- significant difference in behaviour score (total score and some subscales)

This study did not target a defined subgroup of ASD subjects and suffered a very high dropout rate.

Another case control study [47] of 22 ASD children aged 4-10 years treated with Lactobacillus acidophilus strain Rosell-11 (single strain probiotic), 5×10^9 twice daily, for 2 months, showed an improvement in mental concentration and the ability to follow instructions but failed to show a significant impact on behaviour responses to other people's emotions or eye contact. However, this study does not describe how the behaviours were assessed.

Research Questions which the trial will address

A plausible relationship exists between the composition of the GI microbiota and the incidence of certain aberrant behaviours seen in ASD children. Our hypothesis is that Vivomixx probiotic is an effective agent in ASD children with GI symptoms in terms of improving overall functional level.

Treatment for Autistic Spectrum Disorders

Treatments are divided into those for the core features of autism and those that address co-morbid symptoms. Core features of autism are qualitative differences and impairments in reciprocal social interaction and social communication, combined with restricted and stereotyped interests and activities, and rigid and repetitive behaviours.
Social-communication interventions may be used to address the core features of autism in children and young people, including play-based strategies to increase joint attention, engagement and reciprocal communication. Drug treatments have been shown to be ineffective in addressing the core features of autism and carry significant potential risks.
For co-morbid symptoms in individuals with autism, psychosocial interventions are recommended for behaviour that challenges [NICE quality standard (QS51), Jan 2014, Autism]. Any other treatments relate to symptoms that are also suffered by the general population such as insomnia, depression, anxiety, allergy and constipation.

Why this research is needed

There is existing weak evidence of the benefit of probiotic preparations in some individuals with ASD. This needs to be formally confirmed with a well-conducted independent study.

As described above, the only treatments for ASD core symptoms including repetitive behaviours are social-communication interventions such as play therapies that aim to increase social interaction and reciprocal language. These therapies are long-term programmes with a significant cost associated with them and require significant commitment from parents in terms of attending appointments with their child and continuing the therapy at home. Parental stress is known to be increased amongst parents of children with disabilities, particularly those with ASD [48], so any treatment plan should take this into account. Giving a probiotic daily would require relatively little commitment from the parents and is unlikely to add to their stress levels although this is something we will assess in the trial.

There are many probiotic products available over-the-counter and the consumer is often unsure as to what to use. The NICE guidelines regarding probiotics relate to IBS and state that people with IBS who choose to try probiotics should be advised to take the product at the dose recommended by the manufacturer for at least 4 weeks while monitoring the effect. Patients' use of probiotics will vary according to carer knowledge, finance and personal preference for mode of delivery (capsules, powder or liquid). Wills and Evans in their 2014 UK survey of parents of ASD individuals found that 90% of their survey respondents had used nutritional supplements for an ASD person in their care. Probiotics were mentioned as one of the nutritional supplements commonly given. With such a prevalence in use, there is a need for robust research evidence to guide parents and health professionals.

A number of studies have looked at the gut microbiome and metabolome in ASD [24][25][26] and found several imbalances. The findings of some studies have been contradictory about specific species abundance, possibly due to difference methods of analysis and different procedures for collection and storage of the

samples. However, there is general agreement that there is a distinct difference in the gut microbiota of ASD children compared to healthy, non-sibling controls. A 2017 [49] study of the bacterial and fungal gut microbiota of a cohort of 40 ASD children, using 16s rRNA showed "an altered microbial community structure" compared to 40 neurotypical controls. They found a significant increase in the Firmicutes/Bacteroidetes ratio due to a reduction in Bacteroidetes relative abundance. They also observed a number of differences in the relative abundance of bacteria at the genus level and a relative abundance of the fungal genus Candida. They took into account whether the study participants suffered constipation and found that constipation was not a factor in determining the differences in the gut microbiota of the ASD children compared to controls.

There is some evidence that probiotics can be used to correct an imbalance in the gut microbiota of ASD children. Tomova et al [50] compared the results of real time polymerase chain reaction (PCR) stool analysis from 10 ASD children to those of 9 non-autistic siblings and 10 non-autistic children as controls. They reported several differences in the fecal microbiota of children with autism compared to controls including that the Bacteriodetes/Firmicutes ratio was significantly lower. They also assessed the autism severity of the participants using the Childhood Autism Rating Scale (CARS). They then gave the ASD children a daily probiotic supplement containing 3 strains of lactobacillus and 2 strains of Bifidumbacteria and 1 strain of streptococcus for 4 months. Real time PCR stool analysis after this intervention showed the amount of Firmicutes had significantly reduced and there was a concomitant increase in the Bacteriodetes/Firmicutes ratio to the level of the control group. The study size was small and they did not repeat the CARS assessment after the probiotic intervention so we have no knowledge of how the probiotic treatment affected sociability and behaviour.

To date there have been no published randomised placebo controlled clinical trials of probiotics for children with ASD that have assessed overall function before and after treatment. However, a case report has been published of a 12-year-old boy with ASD and severe cognitive disability who showed an unexpected improvement in core ASD symptoms after 4-month treatment with a probiotic [51]. The boy's rehabilitation programme, which he had followed for 6 years, and his diet were maintained stable in the treatment period and in the follow-up. Autism Diagnostic Observation Schedule-2 (ADOS-2) was assessed six times; twice before starting treatment, twice during treatment and twice after interruption of treatment. The probiotic reduced the severity of the boy's abdominal symptoms and also reduced the Social Affect domain of ADOS-2 (from 20 to 18) after 2 months of treatment, with a further reduction of 1 point in the following two months. The level of 17 was maintained for the 10-month follow-up period after treatment ended.

In addition, a recent small open-label trial of Microbiota Transfer Therapy (MTT) for children with ASD [52], found a significant improvement in GI symptoms (constipation, diarrhoea, abdominal pain, indigestion) and a significant improvement in behavioural ASD symptoms after treatment, which continued at the follow-up 8 weeks after treatment finished. The MTT involved a 2-week course of antibiotics followed by a bowel cleanse and an extended fecal transplant protocol over 8 weeks. In their analysis of before and after stool samples they found an increase in bacteria diversity and a significant increase in relative abundance of Bifidobacterium, Prevotella and Desulfovibrio. In particular, the relative abundance of Bifidobacterium increased fourfold and aligned with its comparable abundance in neurotypical children who provided stool samples as controls in the study. We should bear in mind that this was an open-label study and there may be a placebo effect.

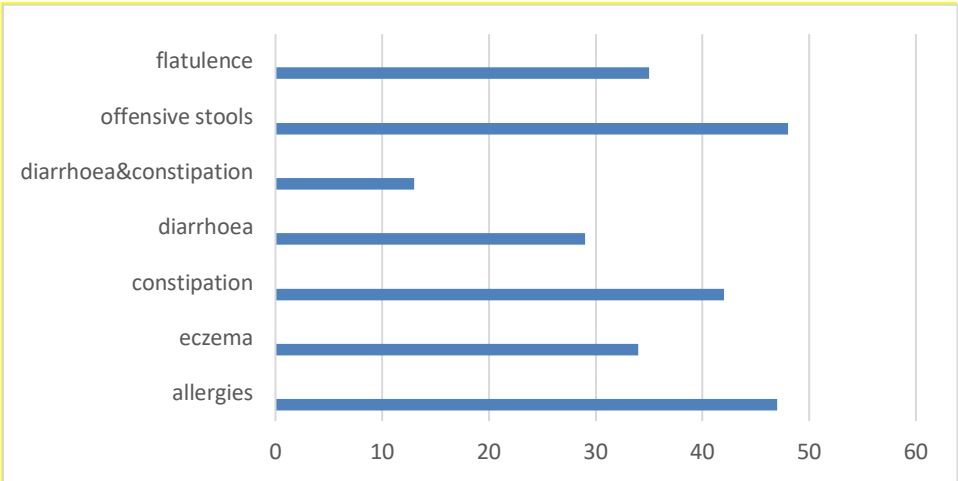
A controlled trial is needed to assess a particular probiotic (Vivomixx) in this complex patient group. All trial participants will receive the active treatment at some stage of the trial. The wider benefit of the trial for society will be the generation of evidence regarding an intervention which may provide significant benefit for children with ASDs, reducing symptoms that are impacting the child's mental and physical health, improving the quality of life for the child and their family and reducing costs to the family and the NHS of products and other treatment. Potential savings to the NHS would be by way of a reduction in the uptake of other treatment if Vivomixx is found to be effective. Additional laboratory data would help to identify if there are any microbial changes which either predict response to therapy, or which can be produced by successful therapy.

Scoping survey data

An online survey was developed to gather

(1) pilot data on gastrointestinal symptoms,
(2) demographic and ASD factors that may help inform parental interest in participation in a clinical trial of a probiotic for their child .

The survey was titled "ASD and GI symptoms" and was not specifically Vivomixx or probiotic related. A link to the online survey was emailed to members of the Treating Autism charity, which is a charity providing support to parents of individuals who have ASDs. A total of 73 parents completed the survey on behalf of their child with the following results with regard to symptom burden;



95% have a diagnosis ASD, autism or Asperger Syndrome
44% suffer chronic constipation
30% suffer regular diarrhoea or loose stools
14% suffer both constipation and regular diarrhoea/loose stools
48% suffer allergies
36% suffer eczema
51% have offensive stools
37% suffer excess flatulence

The survey also asked whether parents would consider allowing their child to take part in a clinical study of a probiotic and 48 out of 73 (66%) respondents expressed an interest in this.

Rationale for Trial Design

A previous trial of VSL#3 probiotic (a formulation now known as Vivomixx) for children with IBS, showed it to be an effective treatment for relieving symptoms, severity and frequency of abdominal pain/discomfort and bloating/gassiness [53]. That trial used a randomised, placebo-controlled, double-blind crossover design and specifically excluded children with significant concomitant psychiatric or neurological illness, which would excludes those with diagnosed ASDs [18]. They adopted this study design because it offered the most stringent criteria for efficacy by minimising the variability between patients. This is particularly important in our study given the significant variability in GI and behavioural symptoms in those diagnosed with ASD and the varying levels of severity of these symptoms and is the reason we have chosen a randomised, placebo-controlled, double-blind crossover design. One potential drawback of the crossover design is the possibility of a "carryover" effect between treatments. To minimise this the IBS study used a 2-week washout period. Following advice from professor Claudio De Simone, the inventor of Vivomixx probiotic and asking the opinion of some parents of ASD children, we have designed the trial with a 4-week washout period. Other probiotic clinical trials have used washout periods varying from 3 weeks to 2 months [54], [55]. There may still be some carryover effect even after the 4-week washout but as we are not re-assessing until 16 weeks after the end of the first treatment phase, we do not consider that this will hamper results interpretation.

In setting the age range for the participants, we considered the fact that children in the UK are rarely formally diagnosed with ASD before age 3. Many of the patient referrals will come via Caudwell Children charity who support children with disabilities up to the age of 17 years so this influenced our upper cut off age. The other factor we considered was the evidence that the gut microbiome matures by the age of 3 years and then remains largely the same until old age. Keeping the age range as wide as possible means that if the treatment proves successful, the recommendations for treatment will apply to a wide group.

We have chosen to include only those children with ASD and GI symptoms as we believe these are the subset that are most likely to have a positive response to probiotic treatment. This is based on clinical experience and the fact that symptoms such as constipation and IBS have been associated with an altered gut microbiome, which probiotics have the potential to moderate. Parracho et al [as above] emphasised the importance of future trials using defined subgroups of ASD because of the relatively high inter-individual variability within the ASD diagnosis.

In their 2016 review of the potential effect of probiotics on neurologic disease, Umbrello and Esposito [56] state that the efficacy of probiotics is influenced by the dose and that probiotic combinations appear to be more effective than single component probiotics. Vivomixx is a combination probiotic with a high dose per sachet. In deciding the dosage schedule for the trial, we considered the dose being used by an ongoing trial using the same probiotic with younger (18-72 months) ASD children [57]. This trial uses a randomised placebo controlled crossover design with 3 months of treatment in each phase of the crossover. The dose of Vivomixx they are using is 2 sachets daily (900 billion lyophilized bacterial cells) for the first month and then 1 sachet daily. We have also consulted with Professor Claudio Simone, the inventor of Vivomixx for guidance on the dose to use. We have to balance the need to reach a therapeutic dose against the possibility of the volume of treatment product becoming a problem for the parents to deliver effectively or causing temporary side effects of loose bowels. For this reason, we have decided to start at a dose of 1 sachet of Vivomixx daily for children aged 3 – 10 years and 2 sachets of Vivomixx daily for those between 11 and 16 years. After 4 weeks the dose will then be increased by 1 sachet of Vivomixx daily in both age groups, provided the participant is tolerating the product.

An introductory taste exposure has been incorporated into the design as palatability can be a particular issue that affects adherence in this patient group. Therefore, the children will be given three doses of placebo

(which is identical in appearance and presentation to the actual product, based on previous studies) to ensure they can tolerate the preparation.

A treatment duration of 12 weeks per arm of the crossover, has been chosen in order to allow a good colonization of the gut, which requires 2-3 weeks on average, and its maintenance for a period of time long enough to determine possible significant clinical changes in gastrointestinal function and behaviour. Outcomes will be assessed at the end of the treatment periods once a stable state has been reached.

Assessment and Management of Risk

The ASD-Probiotic trial involves a treatment for children with autistic spectrum disorders who have gastrointestinal symptoms. It is well established in clinical practice that children with ASDs often present with these symptoms and adverse effects will be those observed in everyday practice associated with management of GI symptoms.

There are no tests being performed during the trial that are above standard care. The table below summarises the risks and mitigations of the intervention that are being performed in a table:

Intervention	Potential Risk	Risk Management
Oral age-related dose of Vivomixx probiotic	Allergic reaction	Those with a previous allergic reaction to any probiotic will be excluded from the study
Oral age-related dose of placebo powder containing maltose and silicon dioxide	Allergic reaction	The placebo contains maltose and silicon dioxide which has a very low allegenic profile
Oral age-related dose of Vivomixx	Temporary loose bowel motions	Participants are made aware of this possible temporary side effect which occurs in 1 in 10 patients and does not last typically beyond 2 days. Participants will be able to contact the research clinician at any time if they have concerns about this.

Objectives

Our primary objective is to assess the effectiveness of Vivomixx as an agent in improving overall function in children with ASD and GI symptoms.
Secondary objectives of the trial are

- To identify changes in GI function
- To identify factors predicting outcome with this therapy so we can identify the subset of ASD children that should respond to this treatment
- To identify whether the intervention reduces parenting stress
- To explore the participant's experience of the trial

In summary, our objective is to explore the relationship between the gut microbiota and ASD core symptoms using tools known to alter the GI microbiota, and measuring outcomes using standard assessments of overall function and aberrant behaviour in children with ASDs. At the same time, we will conduct qualitative research to assess the effect of these interventions on the quality of life of the patient and their parent(s).

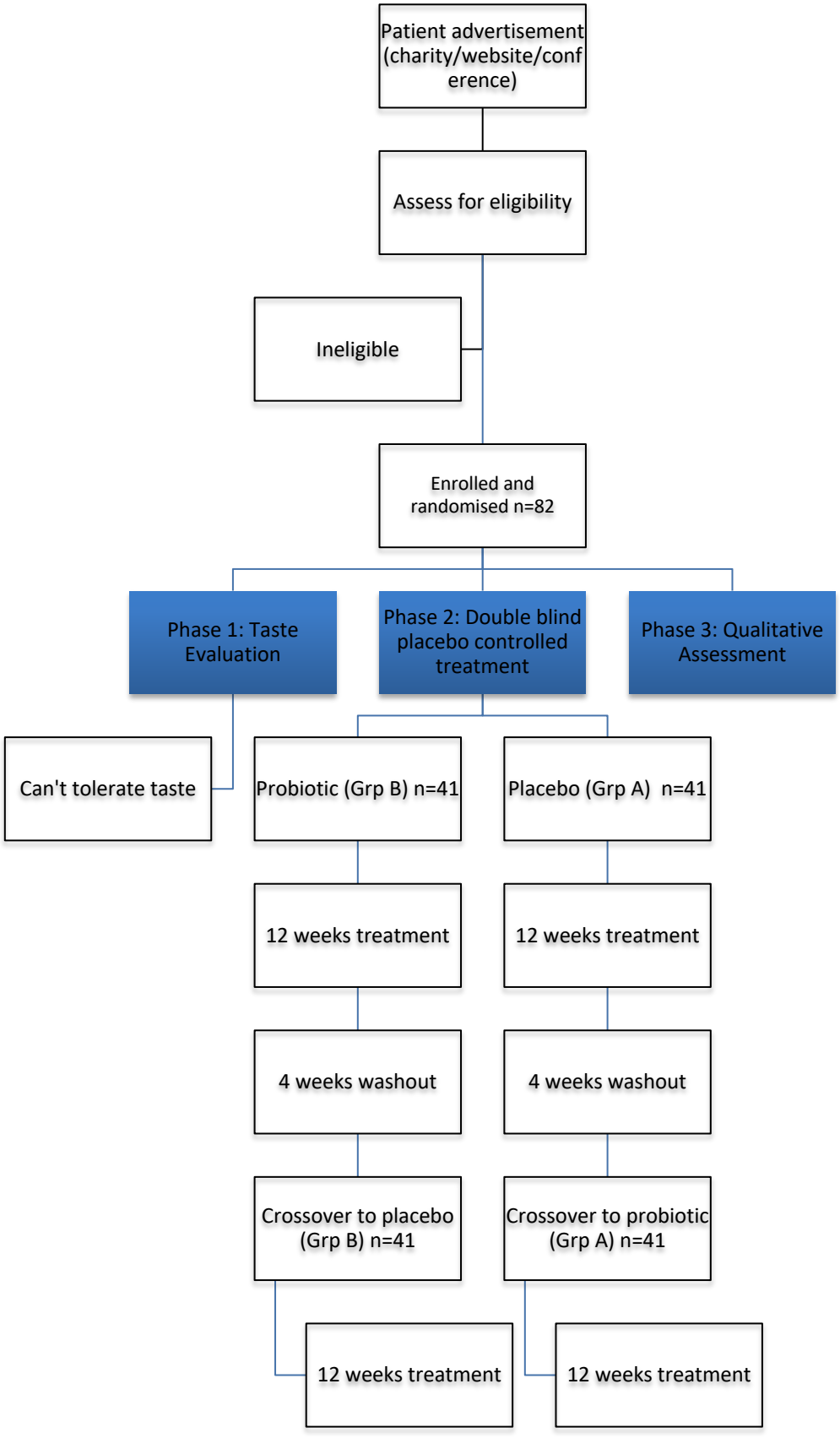
Trial Design

The research comprises four phases:

1. A one week taste evaluation to assess its acceptability within this patient group.
2. A parallel, two-arm randomised, placebo-controlled, double-blind, crossover trial comprising of two periods of 12 weeks with a 4-week washout between, to assess the effectiveness of Vivomixx probiotic at improving overall function of ASD patients as assessed by the Autism Treatment Evaluation Checklist (ATEC).
3. A qualitative assessment looking at the practical issues parents found with the treatment protocol and how it affected their and their child's quality of life.
4. Data analysis including using statistical tools to identify the ASD phenotype that responds to the treatment.

The study participants will participate in the trial for a total of 30 weeks. All assessment data will be anonymised.

Overall Study Flow



Selection of Participants

Inclusion criteria

- Must have a diagnosis of ASD confirmed by a medical professional using one of the following standard assessment tools: Autism Diagnostic Interview – Revised (ADI-R), Diagnostic Interview for Social and Communication disorders (DISCO) or Developmental, Dimensional and Diagnostic Interview (3di).
- Have one or more gastrointestinal symptoms (constipation, diarrhoea, abnormal stools, pain on defecation, abdominal pain, gaseousness/bloating, reflux) for the past 6 months.
- Are either not taking any medication or have been on the same medication for the last 3 months.
- The patient or the patient's parents/guardian are willing and able to provide a written informed consent
- Be willing and able to continue with current medication or nutritional supplements throughout the 30-week trial.
- The patient's primary carer must be willing and able to complete the questionnaires at three time-points in the study. These questionnaires are only available in written English.
- Be willing to refrain from starting any kind of special diet for the duration of the study.
- Be between 3 years and 16 years of age

Exclusion criteria

- Has a diagnosis of Retts Syndrome or Fragile X
- Aged over 16 years or under 3 years
- On NSAIDs
- Taken antibiotics in the past month
- Taken a probiotic in the past month
- History of intolerance or allergy to probiotics
- The patient's primary carer is not willing or are not able to complete the questionnaires at three time-points in the study. These questionnaires are only available in written English.
- Has taken part in a clinical trial in the past 3 months

Specific considerations

- Participants will be asked to refrain from starting any kind of special diet for 30 weeks, but no recommendation will be made about the diet to be consumed. Being on a special diet at the time of assessment for eligibility will not preclude joining the study but a note will be taken of the diet being followed.
- Carers: ability to complete outcome measures on behalf of patient is crucial, so this will be specifically considered during the pre-enrolment assessment.
- Travel and phone contact – since there are 4 clinic visits to UCLH and up to 4 phone calls, it is important that carers and patients are aware of this necessity from the outset. Their attention will be drawn to this in the patient information sheet and during the screening interview. Participants are free to withdraw from the study at any point without an obligation to give a reason. We have approached both of the funders involved in the study to ask for reimbursement of participant's travel expenses but have been unsuccessful.
- In view of length of study, patients/carers will be counselled that it is important to continue treatment throughout.
- There is no evidence that severity of ASD influences outcome from dietary therapy: In the data mining of the Scan-Brit study of gluten and casein free diet for ASD [58] there were responders to the diet even from the severe ASD category. Consequently, we are not stratifying by ASD

severity. However, we will look at whether baseline ASD severity (based on ATEC) influences response to Vivomixx.

Recruitment

A recruitment poster has been produced that will be emailed to all parents of disabled children on the database of Caudwell Children charity. The recruitment poster will also feature on the Caudwell Children website. A notification of the study will feature on the website of Vivomixx UK with the researcher's email address to contact for more information. Anybody contacting us via this route will be emailed a copy of the recruitment poster. The study was presented at a charity conference in London run by Treating Autism charity in June 2016 and we have a list of parents that expressed interest in knowing more about the study. These parents will be contacted by email with a copy of the recruitment poster. The poster asks those interested in knowing more about the trial to contact the researcher by email giving their consent to answer some questions to determine whether their child meets the eligibility criteria for the study. An appointment will be made to do a short pre-enrolment eligibility assessment by phone. A Confidential Screening Questionnaire administered by the researcher, will be used to assess eligibility for the trial. All participants of the pre-enrolment assessment will be told at the time whether their child is eligible for the trial. Those that meet the eligibility criteria will then be emailed a copy of the Parent Information Sheet and the Child Information Sheet. The parent information sheet invites them to participate in the study and asks them to contact the researcher to ask any questions they have and also to provide their GP's contact details, if they wish to participate. If the parent(s) and child participant wish to proceed to enrolment in the study, they will be offered an outpatient appointment at University College London Hospital (UCLH). If they wish to discuss the trial with friends or family before making a decision, an appointment will be made for the researcher to phone again and answer any further questions before asking them for a decision on whether they wish to enrol in the study.

Study timetable and milestones

Before Start	Refine and agree the Intervention protocols and patient documents within the Project Management Group for NRES approval.
9.10.17	Begin patient recruitment
w/b 12.3.18	Patient recruitment complete
w/b 8.1.18	First patient completes 12 week first phase of trial
w/b 5.2.18	First patient begins second phase of trial
w/b 30.4.18	First patient finishes trial treatment
w/b 1.10.18	Last patient finishes trial treatment
October '18	Begin analysis of final results
December '18	Finalise results of the study
December '18	Disseminate results of the study

Informed consent

On arrival at their outpatient appointment, parents will be asked if they and their child understand the relevant patient information sheets and will again be given the opportunity to ask questions of the researcher about the trial. The researcher will explain to the participant and their parent(s) that they are under no obligation to enter the trial and that they can withdraw at any time during the trial, without giving a reason. If they wish to enrol in the trial, the parents will be asked to complete a consent form on behalf of their child. With children over 12 years old, we will discuss with the

parent/guardian whether they feel their child is capable of understanding the Child Information Leaflet and signing an assent form. If so, the child will also be asked to complete an assent form.
The participant will be allocated a unique Study Number and all trial data will be anonymised.

Intervention

Name and description of intervention under investigation

Vivomixx is the probiotic preparation selected for this study. Each sachet contains 450 billion of lyophilized bacterial cells belonging to eight probiotic strains;

- Streptococcus thermophilus DSM 24731
- Bifidobacterium breve DSM 24732
- Bifidobacterium longum DSM 24736
- Bifidobacterium infantis DSM 24737
- Lactobacillus acidophilus DSM 24735
- Lactobacillus plantarum DSM 24730
- Lactobacillus paracasei DSM 24733
- Lactobacillus delbrueckii subsp. bulgaricus DSM 24734

Vivomixx is a patented and marketed product and it has been approved for use in children. It is categorised as a food supplement and is available to purchase in the marketplace without prescription.

Vivomixx and a placebo powder (containing maltose and silicon dioxide) with a matching taste and appearance will be provided by Gastropharm who are the distributors of this product in the UK.

Storage and handling of Vivomixx at UCLH

Vivomixx and the placebo will be stored in the pharmaceutical refrigerator at UCLH.

Packs of Vivomixx and placebo will be packaged in a unit for each participant and labelled only with the participant number and identifier for the arm of the crossover to be used in (1 or 2).

Vivomixx is given orally mixed into a drink or cold food such as yoghurt or fruit puree. The first dose will be given during visit 1 at UCLH. The researcher will mix the product into a drink of juice, fruit puree or yoghurt to demonstrate to parents how this is done.

Accountability of Vivomixx

Vivomixx is a low risk product that is available for purchase without a prescription. Therefore there are no special procedures in place for shipment and receipt at UCLH. Participants will be asked to return any unused product and this will be counted and recorded before being disposed of.

Concomitant medication

The taking of antibiotics or probiotics in the month prior to enrolment will not be permitted. Children that are regularly taking NSAID medication will not be eligible for the trial. Any other regular medication will be recorded but will not affect their ability to take part.

If a child becomes unwell during the trial and is prescribed antibiotics, we will record the medication and the dates given and ask the participant's parent to give the trial product at least 2 hours apart from the antibiotics to minimize the impact on results. They will be allowed to continue on the trial.

If a child is prescribed a new medication during the trial that is not an antibiotic or NSAID, we will note the name of the medication and the date started and the participant will be allowed to continue on the trial.

Dosages and method of administration

The following applies to both arms of the trial and both parts of the crossover;

- Children aged 3 – 10 years take 1 sachet of Vivomixx/placebo daily for the first 4 weeks, then following a phone call with the researcher, they will increase the dose to 1 sachet twice daily for a further 8 weeks, provided there are no contraindications
- Children aged 11-16 years take 1 sachet of Vivomixx/placebo twice daily for the first 4 weeks, then following a phone call with the researcher, they will increase the dose to 1 sachet 3 times daily for a further 8 weeks, provided there are no contraindications

See Appendix 2 for a Schedule of Treatment.
The treatment powder, Vivomixx or placebo, should be mixed into a cold drink or selected cold foods such as yoghurt or fruit puree. The participant's parents will be asked to ensure that the participant consumes all of the food or drink that the treatment is mixed into.
During the 4-week washout period, no treatment will be given.

Trial procedures

Pre-intervention assessments

There are no trial specific procedures that will be carried out to assess the participant's eligibility.

All pre-treatment procedures will be carried out as specified in the schedule of assessments (appendix 1)

Randomisation procedures

Participant randomisation will be managed remotely by Gastropharm, the UK suppliers of Vivomixx, who will employ a contract research organisation. The randomisation protocol will be 2x2 block randomisation, generated electronically. The number of participant treatment packs delivered will be varied from one delivery to another although they will always be multiples of 4, e.g. the first delivery might be 32 packs and the second might be 16 packs.

Consenting participants are assigned to treatment groups through consecutive allocation of subject number and their identity logged in the central record and nowhere else.

The randomisation list is drawn by a specific software and prepared by a contract research organisation (CRO). They will send the list to Gastropharm for labelling of the product and placebo for each participant. The CRO will prepare a set of sealed envelopes containing the randomisation list. This will be sent to the research office of the Chief Investigator responsible for the study. The envelopes are used to break the code of a single patient in case of adverse event and will be kept in the principal investigator's file and at UCLH by Dr Natalia Zarate-Lopez.

Intervention procedures

During visit 1, the first dose for the taste assessment (1 sachet of the placebo powder) will be given by the participant's parent under the guidance of the researcher. The placebo powder will be mixed into a drink or cold liquid food such as yoghurt or fruit puree.

The parents will be sent home with the following;

- 2 day's supply of the placebo product as a taste assessment with instructions on how to administer the product. The parents will be blinded to the fact that this is the placebo product. We need to give all participants the placebo at this stage to reduce waste as we are expecting some participants to drop out of the trial at this stage due to being unable to tolerate the taste.
- A kit and instructions for collection of a stool sample the day before the next clinic visit.

They will be booked to return to UCLH the following week bringing any unused placebo sachets with them.

At visit 2, the participant's parents will be asked how the participant tolerated the taste of the product and whether they feel they can successfully administer this daily for two periods of 12 weeks with a 4-week break in between. Those who can, will continue to phase 2 of the study. The first dose of Vivomixx/placebo will be given during the consultation mixed into a drink or cold liquid food such as yoghurt or fruit puree. The participant's parents will be sent home with;

- 12 week's supply of Vivomixx/placebo with instructions to bring back any unused sachets when they come to UCLH for their next appointment.
- written instructions on the dose to be given;
    - children aged 3 – 10 years take 1 sachet daily for the first 4 weeks, then following a phone call with the research clinician, they will increase the dose to 1 sachet twice daily provided there are no contraindications
    - children aged 11-16 years take 1 sachet twice daily for the first 4 weeks, then following a phone call with the research clinician, they will increase the dose to 3 sachets daily, split over 2 or 3 doses, provided there are no contraindications

- written instructions on how the parent(s) should give the Vivomixx/placebo powder i.e. mixed into a cold drink or selected cold foods such as yoghurt or fruit puree.
- A kit and instructions for collection of a stool sample the day before the next clinic visit.

The researcher will phone the participant's parent in Week 5 when they will be asked whether their child is suffering any side effects from treatment (bloating or loose bowels) and how well they are taking the treatment. If there are no bothersome side effects and the child is tolerating the product well, then the parents will be asked to increase the dose by adding 1 extra sachet of Vivomixx/placebo daily until the next appointment at UCLH.

- After 12 weeks, there is a 4-week washout period during which the participants will attend UCLH for a 45-minute outpatient consultation (Visit 3). The participant's parents will be sent home with 12 week's supply of Vivomixx/placebo and written instructions on how to administer this, and also a kit and instructions for collection of a stool sample the day before the next clinic visit.

They will be asked to bring any unused product sachets back to the final outpatient consultation. Those participants that have been taking the placebo product will now take the treatment product. Those participants that have been taking the treatment product will now take the placebo.

The researcher will phone the participant's parent in Week 21 when they will be asked whether their child is suffering any side effects from treatment (bloating or loose bowels) and how well they are taking the treatment. If there are no bothersome side effects and the child is tolerating the product well, then the parents will be asked to increase the dose by adding 1 extra sachet of Vivomixx/placebo daily until the next clinic visit at UCLH.

Subsequent assessments and procedures

At visit 1, each participant and their parent(s), will be seen by the researcher who will make a global assessment of the participant and use the Childhood Autism Risks from Genetics and the Environment (CHARGE) Gastointestinal History Questionnaire (GIH) and a dietary assessment as pro-forma for interview and assessment. The parents will complete a questionnaire pack that consists of:

- A clinical assessment form recording basic demographic details, medical history, birth details, details about the mother's health in pregnancy and information on whether certain medical conditions are present in family members.
- Autism Treatment Evaluation Checklist (ATEC)
- Aberrant Behaviour Checklist (ABC)
- Autism Parenting Stress Index questionnaire.

After visit 2, if the participant tolerated the taste, the child's educator will be sent a letter inviting them to participate in the study with a consent slip to complete and return. Included with this letter will be;

- Parent Information Sheet
- Baseline questionnaire pack for the child's educator to complete in respect of the child
- Midpoint questionnaire pack for the child's educator to complete in respect of the child
- Final questionnaire pack for the child's educator to complete in respect of the child
- 3 stamped addressed envelopes for returning the questionnaires and consent slip
- provided.

The educator questionnaire packs consists of an Autism Treatment Evaluation Checklist and Aberrant Behaviour Checklist

The researcher will phone the participant's parent in Week 5 and Week 9. During the phone calls in Week 5 and Week 9, questions will be asked of the participant's parents to assess and encourage compliance and to check for any change in circumstances that might affect treatment efficacy.

After 12 weeks, there is a 4-week washout period during which the participants will attend UCLH for a 45-minute outpatient consultation (Visit 3). During this consultation, the researcher will make a global assessment and assess the frequency of GI symptoms using the GIH questionnaire. The participant's parents will again complete an ATEC and ABC evaluation for their child, and an Autism Parenting Stress Index questionnaire.

The researcher will phone the participant's parent in Week 21 and Week 25. During the phone calls in Week 21 and Week 25, questions will be asked of the participant's parent to assess and encourage compliance and to check for any change in circumstances that might affect treatment efficacy. Qualitative data on their experience of the trial will also be collected by the researcher.

After 29 weeks, participants will attend UCLH for a 45-minute outpatient consultation (Visit 4). They will be asked to bring any remaining Vivomixx/placebo sachets to the consultation. During this consultation, the researcher will make a global assessment and assess the frequency of GI symptoms

using the GIH questionnaire. The participant's parent will again complete an ATEC and ABC evaluation for their child, and an Autism Parenting Stress Index questionnaire.

Both groups will have the same amount of health professional contact.
The placebo's packaging, taste and appearance of the powder will be indistinguishable to Vivomixx.

Discontinuation/withdrawal of participants

In consenting to participate in the trial, participants are consenting to intervention, assessments, follow-up and data collection.
A participant may be withdrawn from trial whenever continued participation is no longer in the participant's best interests, but the reasons for doing will be recorded. Reasons for discontinuing the trial may include:

- Participant's withdrawing consent
- Intercurrent illness
- Persistent non-compliance to protocol requirements

The decision to withdraw a participant from treatment will be recorded in the case report form (CRF) and medical notes. If a participant explicitly states they do not wish to contribute further data to the trial, their decision will be respected and recorded in the CRF and medical notes.

Definition of End of Trial

The expected duration of the trial is 1 year from recruitment of the first participant.
The end of the trial is the date of the last visit of the last participant.

Recording and reporting of adverse events

Definitions

Term	Definition
Adverse Event (AE)	Any untoward medical occurrence in a patient or trial participant, which does not necessarily have a causal relationship with the intervention involved.
Serious Adverse Event (SAE)	Any adverse event that: • results in death, • is life-threatening*, • requires hospitalisation or prolongation of existing hospitalisation**, • results in persistent or significant disability or incapacity, or • consists of a congenital anomaly or birth defect.

*A life-threatening event refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.

**Hospitalisation is defined as an in-patient admission, regardless of length of stay. Hospitalisation for pre-existing conditions, including elective procedures do not constitute an SAE

Assessments of Adverse Events

Each adverse event will be assessed for severity, causality, seriousness and expectedness as described below.

Severity

Category	Definition

Mild	The adverse event does not interfere with the participant's daily routine, and does not require further intervention; it causes slight discomfort
Moderate	The adverse event interferes with some aspects of the participant's routine, or requires further intervention, but is not damaging to health; it causes moderate discomfort
Severe	The adverse event results in alteration, discomfort or disability which is clearly damaging to health

Causality

The assessment of relationship of adverse events to the intervention is a clinical decision based on all available information at the time of the completion of the case report form.

The following categories will be used to define the causality of the adverse event:

Category	Definition
Definitely	There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out.
Probably	There is evidence to suggest a causal relationship, and the influence of other factors is unlikely
Possibly	There is some evidence to suggest a causal relationship (e.g. the event occurred within a reasonable time after administration of the trial intervention). However, the influence of other factors may have contributed to the event (e.g. the participant's clinical condition, other concomitant events).
Unlikely	There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial intervention). There is another reasonable explanation for the event (e.g. the participant's clinical condition, other concomitant treatments).
Not related	There is no evidence of any causal relationship.
Not assessable	Unable to assess on information available.

Expectedness

Category	Definition
Expected	An adverse event which is consistent with the information about the intervention clearly defined in this protocol.
Unexpected	An adverse event which is not consistent with the information about the intervention clearly defined in this protocol.

Recording adverse events

All adverse events will be recorded in the medical records in the first instance.

Procedures for recording and reporting Serious Adverse Events

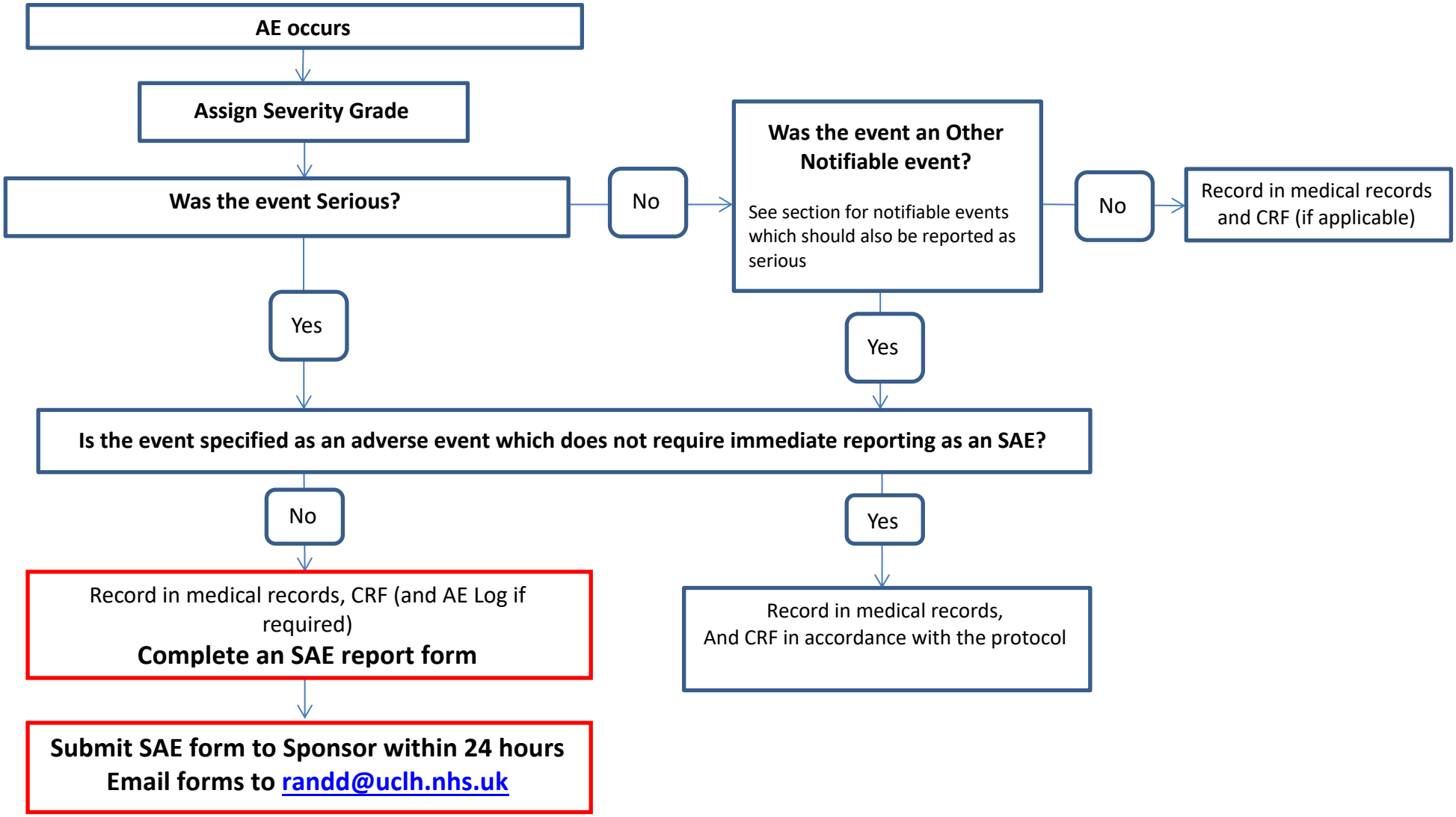
All serious adverse events will be recorded in the medical records and the CRF, and the sponsor's AE log. The AE log of SAEs will be reported to the sponsor at least once per year.

In the ASD-Probiotic trial all SAEs occurring to a research participant will be Data Monitoring Committee if they occur within 24 hours of the participant taking a dose of the treatment and where in the opinion of the Chief Investigator and the Chair of the DMC the event was:

- Related – that is, it resulted from administration of any of the research procedures, and
- Unexpected – that is, the type of event is not listed in the protocol as an expected occurrence.

In addition, SAE forms will record all deaths from any cause during the course of the trial.
SAEs will be reported to the sponsor until the end of the trial.
Participants must be followed up until clinical recovery is complete and laboratory results have returned to normal, or until the event has stabilised. Follow-up should continue after completion of protocol treatment and/or trial follow-up if necessary.
Follow-up SAE forms (clearly marked as follow-up) should be completed and emailed to the JRO as further information becomes available.

Flow Chart for SAE reporting



Unblinding

Emergency unblinding:

The trial code should only be broken for valid medical or safety reasons e.g. in the case of a severe adverse event where it is necessary for the investigator or treating health care professional to know which treatment the participant is receiving before the participant can be treated. Subject always to clinical need, where possible, members of the research should remain blinded.

The code breaks for the trial are held by the GI Physiology Unit at UCLH and are the responsibility of Dr Natalia Zarate-Lopez.

In the event that unblinding is required, a formal request will be made by the investigator/treating health care professional to an individual authorised and delegated to perform code break.

If the person requiring the unblinded information is a member of the Investigating team then a request to the authorised individual to unblind will be made and the treatment allocation information obtained.

If the person requiring the unblinding information is not the CI/PI then that healthcare professional will contact the Investigating team to request the code break. Unblinding will take place if in the opinion of the treating physician a patient's health is compromised. The authorised individual will break the code and immediately inform the treating healthcare professional of the participant's treatment allocation. The treating physician has the ultimate decision and right to unblind the patient.

On receipt of the treatment allocation details, the CI/PI or treating health care professional will treat the participant's medical emergency as appropriate.

The CI/PI will document the breaking of the code and the reasons for doing so on the CRF and unblinding log and will file this, in the site file and medical notes. It will also be documented at the end of the trial in any final trial report and/or statistical report.

The CI/Investigating team will notify the JRO in writing as soon as possible following the code break detailing the necessity of the code break.

The written information will be disseminated to the Data Monitoring Committee for review in accordance with the DMC Charter.

Reporting Urgent Safety Measures

If any urgent safety measures are taken the CI/ PI shall immediately and in any event no later than 3 days from the date the measures are taken, give written notice to the relevant REC and Sponsor of the measures taken and the circumstances giving rise to those measures.

Notification of reportable protocol violations

A reportable protocol violation is a breach which is likely to effect to a significant degree:

(a) the safety or physical or mental integrity of the participants of the trial; or

(b) the scientific value of the trial.

The sponsor will be notified immediately of any case where the above definition applies during the trial conduct phase.

Trust Incidents and Near Misses

An incident or near miss is any unintended or unexpected event that could have or did lead to harm, loss or damage that contains one or more of the following components:

a. It is an accident or other incident which results in injury or ill health.
b. It is contrary to specified or expected standard of patient care or service.
c. It places patients, staff members, visitors, contractors or members of the public at unnecessary risk.
d. It puts the Trust in an adverse position with potential loss of reputation.
e. It puts Trust property or assets in an adverse position or at risk.

Incidents and near misses must be reported to the Trust through DATIX as soon as the individual becomes aware of them.
A reportable incident is any unintended or unexpected event that could have or did lead to harm, loss or damage that contains one or more of the following components:

a) It is an accident or other incident which results in injury or ill health.
b) It is contrary to specified or expected standard of patient care or service.
c) It places patients, staff members, visitors, contractors or members of the public at unnecessary risk.
d) It puts the Trust in an adverse position with potential loss of reputation.
e) It puts Trust property or assets in an adverse position or at risk of loss or damage.

Data management

Confidentiality

All data will be handled in accordance with the UK Data Protection Act 1998.

The Case Report Forms (CRFs) and questionnaires will not bear the participant's name or other personal identifiable data. The participant's trial identification number, will be used for identification and this will be clearly explained to the patient in the Patient information sheet. Patient consent for this will be sought.

Data collection tools and source document identification

Data will be collected on trial specific case report forms (CTFs) and data collection tools such as standardised questionnaires.
Source data are contained in source documents and must be accurately transcribed on to the CRF. Examples of source documents are medical records which include laboratory and other clinical reports etc.
A source document list will be implemented prior to the start of the trial to identify:

- which data is to be recorded directly onto the CRF;

- which data is recorded firstly into source documents, such as medical notes, and then transcribed into the CRF; and
- which data is not to be recorded in the CRF but only recorded in source documents, e.g. participant questionnaires.

The participant's parents will complete the Parent questionnaires while at the clinic appointments at UCLH.
The child's educator will be provided with stamped addressed envelopes to post back the completed questionnaires to the researcher.

The educator will complete the Educator questionnaires and post to the researcher using the stamped addressed envelopes provided.

We will take active measures to minimise loss to follow-up, such as back-up 'best contact' addresses, sending reminders after 2 weeks and allowing telephone completion of questionnaires at this stage. All patients will receive the treatment product at some stage of the trial, which should encourage continued involvement. All patient's parents will receive phone calls from the researcher in Week 5, 9, 21 and 25 to offer support and answer any questions.

It is the responsibility of the investigator to ensure the accuracy of all data entered in the CRFs. The delegation log will identify all those personnel with responsibilities for data collection and handling, including those who have access to the trial database.

Methods to protect against other sources of bias

Phone interviews and follow-up clinic interviews will explore treatment fidelity. Treatment fidelity data collected will assess compliance with treatment plan (dose, administration, whether given daily, vehicle drink or food used to deliver the treatment powder, number of unused Vivomixx/placebo sachets).

<u>Ensuring standardisation of intervention and outcome measurement (performance bias)</u>
Standardisation of intervention delivery will be the responsibility of the researcher who has clinical expertise in this area. Parents will be given written and verbal instructions to give the age-related dose of Vivomixx/placebo daily, mixed into a cold drink or selected cold foods. The powder can be mixed into a cold drink or mixed into selected cold food such as yoghurt or fruit puree. Time of day of administration is not important and can be selected to suit the child. Compliance with these instructions will be checked at phone and clinic follow-ups throughout the trial.

Contextual data will be gathered on what drink or food is being used as a vehicle for delivery of the treatment and whether there have been any significant changes in the child's diet and any new medications or nutritional supplements will be noted.
The same researcher will conduct the interviews for all participants at all stages of the trial.

<u>Detection bias</u>
Group allocation will be concealed from the participants and the researcher. It is thought very unlikely that the participant's parents will be able to detect whether they are giving the placebo or active treatment, even if they talk to other parents in the study. The placebo product has already been used in previous clinical trials and is indistinguishable in taste and appearance from the treatment product.
A collaborator will ensure completeness and accuracy of data entry of questionnaire responses to the database.

Completing Case Report Forms

All CRFs must be completed and signed by staff that are listed on the site staff delegation log and authorised by the CI/ PI to perform this duty. The CI/PI is responsible for the accuracy of all data reported in the CRF.

Once completed the original CRFs are kept at site. Source data verification of a CRF page should be completed and all data queries answered prior to submission where possible.

Data handling

In the study, the data described in the section Trial Procedures, sub-section Subsequent assessments and procedures, will be collected from patients in accordance with the patient consent form, patient information sheet and Trial Procedures section of this protocol.

The completed questionnaires and assessments will be secured in a filing cabinet in an access-card controlled Unit at UCLH. UCL will act as the data controller of such data for the study.

Sue Simmons, UCL Gower Street, will process, store and dispose of the completed questionnaires and assessments in accordance with all applicable legal and regulatory requirements, including the Data Protection Act 1998 and any amendments thereto.

When participants have been recruited into the study and given informed consent, they will be assigned a non-identifiable code and all data (paper and electronic) will use this code. Identifiable data (e.g. contact details) will be held in a manual file that will be locked between clinics and will only be used to contact the participant about the study. All data will be held on a secure, password protected University computer.

The completed questionnaires and assessments will not be transferred to any party not identified in this protocol and are not to be processed and/or transferred other than in accordance with the patients' consent.

Statistical Considerations

Primary Outcome

Our primary outcome is a change in the baseline Autism Treatment Evaluation Checklist (ATEC) total score to the total score after 12 weeks and 28 weeks of treatment.

The ATEC was developed in 1999 by Bernard Rimland and Stephen M. Edelson of the Autism Research Institute, as a measure to evaluate treatment effectiveness in the ASD population. It was developed to address the need for an easy to administer, sensitive to change and valid instrument specifically developed for children with ASD.

The ATEC is a one-page form, designed to be completed by parents, teachers, or caregivers. It consists of 4 subtests: I. Speech/Language Communication (14 items); II. Sociability (20 items); III. Sensory/

Cognitive Awareness (18 items); and IV. Health/Physical/Behavior (25 items). The scale covers 77 items and gives a total score and scores for each of the 4 sub-sections. The higher the score, the greater the disability overall or in a sub-section. The ATEC total score can range from 0 – 180 and is calculated by summing the scores of each subsection. Geier et al [60] compared the ATEC to the Childhood Autism Rating Scale (CARS) and found significant correlation between total ATEC and CARS scores. Many studies have successfully used the ATEC to measure treatment effects in autism or to measure the severity of autism [61][62][63][64][65][20][66][67][68].

Other instruments such as the Autism Diagnostic Interview-Revised (ADI-R), the Autism Diagnostic Observation Schedule (ADOS) and the Childhood Autism Rating Scale (CARS) have been used to assess progress or response to treatment, but these were primarily developed as diagnostic instruments and as such are designed to show overall *stability* over time and not to be sensitive to change. These measures also do not assess the physical and systemic issues in ASD. The ATEC is unique in that it does address these issues. It has recently been validated against the CARS, which is an established, professional-related measure of autism [60].

Secondary Outcomes

1. A change in the frequency of GI symptoms as assessed by the Gastrointestinal History questionnaire.
   A secondary outcome of this study is the postulated improvement in the participant's GI symptom frequency scores over the 12 and 28-week treatment period. Assessing the severity of symptoms in children with ASD is difficult as many are non-verbal or unable to answer questions regarding symptoms. In 2003 the Childhood Autism Risks from Genetics and the Environment (CHARGE) study [70] developed and began using a frequency assessment of GI symptoms in children with ASD, which they called the Gastrointestinal History Questionnaire. The Gastrointestinal History questionnaire (GIH) includes 10 Likert scale items for the following symptoms: abdominal pain, gaseousness/bloating sensation, diarrhoea, constipation, pain on stooling, vomiting, sensitivity to foods, difficulty swallowing, blood in stools and blood in vomit. In addition, the GIH includes four yes/no questions asking about the presence of food allergies, diet restrictions, food dislikes, and whether any GI diagnosis has ever been given. Finally there are open-ended questions asking parents to list; food allergies, reasons for diet restrictions, and what GI condition has been diagnosed. For this study, the GIH will be used by the clinician as a pro forma for interview with patients and their parents.

2. A change in Aberrant Behaviour Checklist (ABC) score
   A secondary outcome of this study is the postulated improvement in the participant's behavior. Maladaptive behavior will be measured using five subscales of the ABC: irritability (15 items), lethargy/social withdrawal (16 items), stereotypy (7 items), hyperactivity (16 items) and inappropriate speech (4 items) as validated by Kaat et al. [59].
   This one-page, parent completed assessment is the gold standard evaluation for the assessment of autism severity and can also be used for assessing response to treatment interventions in terms of behavior.

3. A change in Autism Parenting Stress Index (APSI)
   A secondary outcome is a change in the baseline APSI score to the score after 12 weeks of treatment and after 28 weeks of treatment.

This one-page, self-assessment questionnaire was developed and validated by Silva and Schalock in 2012 [14]. It is designed to assess parent stress in 13 aspects of autism of concern to parents and to provide a measure of parenting stress specific to core and co-morbid symptoms of autism. It reflects the time, effort and actual difficulty of parenting in the light of the physical, social and communication barriers imposed by the disability. It is intended for use by clinicians to assess the effects of intervention on parenting stress. It is designed to be completed by parents of children with ASD and asks them to rate 13 aspects of their child's health on a 0 – 5 scale according to how much stress it causes them and/or their family, (0 – Not stressful, 1 -Sometimes creates stress, 2 – Often creates stress, 3 – Very stressful on a daily basis, 5 – So stressful sometimes we feel we can't cope). It provides a total score that ranges from 0 – 65 and the higher the score, the more parenting stress. The Autism Parenting Stress Index (APSI) was used to assess the change in parental stress in a study of parent-delivered massage for ASD children [69].

4. A change in clinician's global assessment
   An improvement in the researcher's global assessment of the participant after 12 weeks of treatment and after 28 weeks of treatment.
5. To explore parents' experience of the study and delivering the intervention to guide future research and optimize the treatment experience.

Sample size calculation

Sample size calculation

To determine an effect size of 0.50 with 80% power, a sample size of 72 with a type 1 error of 5% using a two-sided test would be sufficient. Assuming a 15% drop out rate, 82 patients will be enrolled. The sample size calculation is based on the assumed effect size of the primary outcome measure, the Autism Treatment Evaluation Checklist (ATEC), which is a ASD composite function score). The minimally clinically important difference based on the primary outcome measure with this instrument was 15 points. Given previous probiotic efficacy data, the effect size of 15 points is a reasonable assumption. Previous studies have shown that assuming ATEC data to be normally distributed is reasonable. Assuming a dropout rate of 10%, our sample size is reduced from 82 to 74. With a type 1 error rate of 5%, and required power of 80%, a non-parametric matched pairs analysis (Wilcoxon Signed-Rank Test) with an assumed low correlation between the subjects in the two conditions (rho=0.2), G*Power 3.1.7 calculated that the study would achieve and 80% with a Cohen's dz effect size of 0.34. This equates to a small to medium Cohen's standard effect size of 0.37, as adjusted using the correction given in Myers, Well, & Lorch (2010) on page 147 equation 6.28.

[Myers, J.,, Well, A., and Lorch, R., 2010. *Research design and statistical analysis*. [online] Available at: <https://books.google.co.uk/books?hl=en&lr=&id=nbsOIJ_saUAC&oi=fnd&pg=PR2&dq=Research+Design+and+Statistical+Analysis:+Third+Edition&ots=ZwDnkDkrpH&sig=IVDU30D2l5sa5MMmvqAOw5FstZc> [Accessed 10 May 2017].]

Planned Recruitment Rate

The maximum possible recruitment rate will be 15 participants per week. The expected recruitment rate is estimated as 4 participants per week. Given the number of participants seen by the two charities, we would expect approximately 50 patients per week to be self-referred.

Randomisation methods

The participants will be randomised in blocks that are multiples of 4 so that in each block of 4 there will be 2 participants in group A and 2 participants in group B (ie "2x2"). There will be no stratification. There will be equal allocation between the treatment arms.

The randomisation list is drawn by a specific software and prepared by a contract research organisation (CRO). Participant numbers will be allocated sequentially to the recruited participants on the basis of their order of recruitment to the study.

Statistical analysis

Summary of baseline data and flow of participants

As the study is a crossover study, each participant becomes their own control so there is not a need to compare characteristics between the two study arms. Baseline variables as follows will be collected and used in statistical analyses to see whether they are associated with treatment response:

Age
ATEC score (done by parent)
ATEC score (done by educator)
ABC total score and subscale scores
GIH frequency score
Clinician's global assessment

We plan to produce a CONSORT flow diagram.

Stool Microbiome Analysis

After collection and prior to processing, the samples will be anonymised by means of a bar-coding system. This will ensure that any laboratory staff handling the samples will not be able to identify the donor. The samples will be analysed using a non-culture technique involving 16s rRNA gene amplification and analysis. The prepared samples will be analysed using the Illumina high-throughput sequencing platform. The 16s rRNA gene is ubiquitous to bacteria, but is not found in humans. The use of targeted DNA amplification ensures that only bacterial DNA can be sequenced. The results will then be analysed using bioinformatics and standard statistical software.

Primary outcome analysis

The intention-to-treat (ITT) analysis will be performed on all participants who are randomised. An additional analysis will be done on only those participants who complete the study per protocol analysis.

Change in the ATEC score during follow-up will be examined using the mixed model for analysis of variance to account for missing data (dropouts). This model allows the evaluation of the effect of each factor on the outcome as well as the interactions between factors. The mixed model will use group (treatment

versus control) and time as factors. If residuals prove not to be normally distributed, the Bonferroni multiple comparisons with Bootstrap multiple comparisons to accommodate for the non-normality of residuals will be done. A P-value <0.05 is considered significant and multiple comparisons will be undertaken with Fisher's least significant difference test and 95% confidence intervals.

Secondary outcome analysis

GIH

Analyse the frequency score after treatment with the active product compared to baseline and contrast that to the score after the placebo treatment. To report what proportion and number of participants showed a reduction in the frequency score after active treatment, what the mean reduction was with confidence interval.

ABC

Analyse the ABC scores after treatment with the active product compared to baseline and contrast that to the scores after the placebo treatment. To report what proportion and number of participants that showed a reduction in the total score and the subscale scores for Irritability, Lethargy/social withdrawal, Stereotypic behaviour and Hyperactivity after active treatment, what the mean reduction was with confidence interval.

APSI

Analyse the APSI score after treatment with the active product compared to baseline and contrast that to the score after the placebo treatment. To report what proportion and number of participants that showed a reduction in the score after active treatment, what the mean reduction was with confidence interval.

Clinician's global assessment

Analyse the Clinician's global assessment after treatment with the active product compared to baseline and contrast that to the assessment after the placebo treatment. To report what proportion and number of participants that showed an improvement after active treatment.

Sensitivity and other planned analyses

Sensitivity analysis of data outliers will be presented by showing data as box-plots.

Non-adherence to the protocol will be handled by performing intention to treat and per protocol analyses as necessary.

Missing data will be handled by inputting the missing data and redoing the analysis.

Data will be analysed for normality, and those which are not normally distributed will be non-parametrically assessed.

A more detailed statistical analysis plan will be produced prior to the final analysis. The plan will be reviewed and possibly updated as a result of the blind review of the data and will be finalised before breaking the blind. Formal records should be kept of when the statistical analysis plan was finalised as well as when the blind was subsequently broken.

Qualitative Assessment of the Study experience – Phase 3

The proposed randomised placebo controlled crossover trial, includes a theory-driven process evaluation. This will be informed by realist evaluation methodology [R. Pawson, N. Tilley, Realistic Evaluation. London: Sage; 2009], which goes beyond the evaluation question "What works?" to: "What works, for whom and in what context?" The aim of this approach is to situate and explain outcomes within the contexts in which they are achieved in order to explain potential discrepancies between expected and observed outcome and to assess fidelity to the treatment plan. Furthermore, the results of the process evaluation will provide data to inform the optimisation of the intervention.
The objectives of the process evaluation are as follows:

- To explore the fidelity to the treatment plan (to be embedded within data collection on implementation contexts)
- To explore implementation contexts (including demographics, delivery of the treatment; adherence and non-completion),
- To explore intervention optimisation

Contextual data will be gathered on all participants via the clinical assessment form, GIH and dietary assessment, in order to explore pre-existing background contexts and any changes (e.g. change in diet, treatment with medication) that might have an impact on efficacy of the treatment.
Data on the fidelity to treatment plan will be gathered via the semi-structured phone and clinic interviews with participants and also by recording the number of unused sachets brought back to the clinic by participants.
Qualitative data relevant to the optimisation of the intervention will be gathered during final interviews with the parents, either at completion of the study or on exiting the study

Interview study methods

Interviews will be conducted with the parents of participants at six time points when we will collect Phase 3 qualitative data alongside primary and secondary outcome measures. In-person interviews will be conducted at the beginning of the study, at the crossover point and on completion of the study, or on exiting the study. Support phone calls in Week 5, 9, 21 and 25 or at drop-out, will be made by the researcher who will also include a short semi-structured interview with participants. During the support phone calls the researcher will collect qualitative data about how the parents are coping with administering the treatment and how the experience compares to their expectations. The interviews will not be recorded. The researcher will use a checklist for the interview and notes will be written on this checklist and this will be stored in the CRF.

Phase 3 will explore the acceptability of treatment, expectations of participants versus actual experience; barriers and facilitators to compliance; the quality of information provision; the parent's experience of implementation processes such as questionnaire completion. Particular attention will be paid to including the views of non-completing participants to explore reasons for non-adherence and their views on how the intervention experience might have been improved. The telephone interviews will last approximately 30 minutes. With the parent's consent, the interview will be recorded on a digital voice recorder and stored temporarily in a locked drawer until transcribed. Once it has been transcribed the voice recording will be deleted. The transcript will be identified by the participant number and no identifying details of the participant or parent will be recorded in the transcript.

Record keeping and archiving

At the end of the trial, all essential documentation will be archived securely by the CI for a minimum of 20 years for the declaration of the end of the trial.

Essential documents are those which enable both the conduct of the trial and the quality of the data produced to be evaluated and show whether the site complied with all applicable regulatory requirements.

The sponsor will notify UCLH when trial documentation can be archived. All archived documents must continue to be available for inspection by appropriate authorities upon request.

Oversight Committees

Trial Management Group (TMG)

Data Monitoring Committee (DMC)

Trial Steering Committee (TSC)

Trial Management Group

The TMG will include the Chief Investigator and trial staff. The TMG will be responsible for overseeing the trial. The group will have regular meetings (approximately 3 times per year).

The TMG will review recruitment figures, SAEs and substantial amendments to the protocol prior to submission to the REC.

Other committees

Trial Steering Committee

The role of the TSC is to provide overall supervision of the trial. The TSC will review the recommendations of the Data Monitoring Committee and, on consideration of this information, recommend any appropriate amendments/actions for the trial as necessary. The TSC acts on behalf of the funders and Sponsor.

The Trial Steering Committee will meet/phone the Chief Investigator and Principal Investigator on a fixed monthly basis to confirm the procedures are being followed. They will have access to randomly inspect this process according to their discretion. This Committee will include a patient parent representative.

Data Monitoring Committee

The role of the DMC is to provide advice on data and safety aspects of the trial but where not all members are independent. Meetings of the Committee will be held at 6 monthly intervals to review interim analyses, or as necessary to address any issues.

It is anticipated that the members will meet once to agree the terms of reference and on at least two further occasions to monitor accumulating data and oversee safety issues. During the period of recruitment to the trial, interim analyses will be supplied, in strict confidence, to the DMC, together with

any other analyses that the committee may request. This may include analyses of data from other comparable trials. In the light of these interim analyses, the DMC will advise the Trial Steering Committee if, in its view Vivomixx has been proved, beyond reasonable doubt, to be different from the control (placebo) for all or some types of patients (in respect of either effectiveness or unacceptable safety concerns).

The Trial Steering Committee can then decide whether or not to modify intake to the trial. Unless this happens, however, the Trial Management Group, clinical collaborators and trial office staff (except those who supply the confidential analyses) will remain blinded of the interim results.
The frequency of interim analyses will depend on the judgement of the DMC. However, we anticipate that there will be one interim analysis and one final analysis.

Ethical requirements and participant and public involvement

Ethics

The sponsor will ensure that the trial protocol, participant information sheet, consent form, GP letter and submitted supporting documents have been approved by the appropriate research ethics committee, prior to any participant recruitment. The protocol, all other supporting documents including and agreed amendments, will be documented and submitted for ethical and regulatory approval as required. Amendments will not be implemented prior to receipt of the required approval(s).

Before any NHS site may be opened to recruit participants, the Chief Investigator/Principal Investigator must receive NHS permission in writing from the Trust Research & Development (R&D). It is the responsibility of the CI/ PI to ensure that all subsequent amendments gain the necessary approvals, including NHS Permission (where required) at the site. This does not affect the individual clinician's responsibility to take immediate action if thought necessary to protect the health and interest of individual participants (see earlier section on Reporting Urgent Safety Measures).

An annual progress report (APR) will be submitted to the REC within 30 days of the anniversary date on which the favourable opinion was given, and annually until the trial is declared ended. The chief investigator will prepare the APR.

Within 90 days after the end of the trial, the CI/Sponsor will ensure that the main REC is notified that the trial has finished. If the trial is terminated prematurely, those reports will be made within 15 days after the end of the trial.

The CI will supply the Sponsor with a summary report of the trial, which will then be submitted to the REC within 1 year after the end of the trial.

Patient and public involvement (PPI)

Patients and the public have been involved in the design of the research. We used the results of our survey of parents of children with ASDs to inform our choice of outcome measures. We consulted with parents of children with ASDs about the acceptability of a 4-week washout period and we sought the

views of children with ASDs on our Child Information Sheet. The study protocol has been reviewed by parents' of children with ASDs and also by the London Autism Research Advisory Group (https://m.facebook.com/laragresearch).

Patients and the public will be involved in the management of the research and analysis of results as the Trial Steering Committee will include a Patient Participation representative and a member of London Autism Research Advisory Group.

The dissemination of the findings to parents of children with ASDs will be via parent-led and parent-focused charities.

Monitoring

The sponsor will determine the appropriate level and nature of monitoring required for the trial. Risk will be assessed on an ongoing basis and adjustments made accordingly.

The degree of monitoring will be proportionate to the risks associated with the trial.

A trial specific oversight and monitoring plan will be established for studies. The trial will be monitored in accordance with the agreed plan.

Finance

This trial is supported by,

1. Principal Investigator's salary from Caudwell Children
2. Provision of product and placebo by company grant
3. Administration support from Caudwell Children

The CI, PI and TMG members hold no financial interests in Vivomixx or Gastropharm.

Insurance

University College London holds insurance against claims from participants for injury caused by their participation in the trial. Participants may be able to claim compensation if they can prove that UCL has been negligent. However, as this trial is being carried out in a hospital, the hospital continues to have a duty of care to the participant of the trial. University College London does not accept liability for any breach in the hospital's duty of care, or any negligence on the part of hospital employees. This applies whether the hospital is an NHS Trust or otherwise.

Participants may also be able to claim compensation for injury caused by participation in this trial without the need to prove negligence on the part of University College London or another party. Participants who sustain injury and wish to make a claim for compensation should do so in writing in the first instance to the Chief Investigator, who will pass the claim to the Sponsor's Insurers, via the Sponsor's office.

Hospitals selected to participate in this trial shall provide negligence insurance cover for harm caused by their employees and a copy of the relevant insurance policy or summary shall be provided to University College London, upon request.

Publication policy

The success of the trial depends entirely on the wholehearted collaboration of a large number of participants with ASD and their parents or carers. A trial publication policy will be developed. The results of the trial will be reported first to study collaborators. The main report will be drafted by the Trial Management Group and circulated to all collaborators for comment. The final version will be agreed by the Data Monitoring Committee and Trial Steering Committee before submission for publication, on behalf of all the trial collaborators.

All proposed publications will be discussed with and reviewed by the Sponsor prior to publishing other than those presented at scientific forums/meetings. Please refer to UCL publication policy.

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Appendices

Appendix 1 Schedule of Assessments and Procedures

	Taste evaluation	12 week randomised treatment												Washout				12 week crossover treatment												Exit Interview
Placebo	Day 1-7																													
Placebo or Probiotic		Day 8 - 76																												
No Treatment														Day 77-104																
Placebo or Probiotic																		Day 105 - 189												
Final Assessment																														Day 190 - 196
WEEK	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
Medical History	**P**																													
GI History	**R**													**R**																**R**
Diet assessment	**R**																													
ATEC	**P O**													**P O**																**P O**
ABC	**P O**													**P O**																**P O**
APSI	**P**													**P**																**P**
Global assessment	**R**													**R**																**R**
Phone interview					**P R**				**P R**												**P R**				**P R**					
Clinic visit	**P R**	**P R**												**P R**																**P R**
Collect stool sample	**P**													**P**																**P**

KEY **P =** Parent/guardian **O** = Educator **R =** Researcher

Schedule of Treatment

	Treatment stage	Treatment product	Dose for 3 – 10 year old	Dose for 11 – 16 year old
Week 1	Taste evaluation	Placebo	1 sachet daily for 3 days	1 sachet daily for 3 days
Week 2	Part 1	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 3	Part 1	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 4	Part 1	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 5	Part 1	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 6	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 7	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 8	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 9	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 10	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 11	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 12	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 13	Part 1	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 14	Washout	No treatment		
Week 15	Washout	No treatment		
Week 16	Washout	No treatment		
Week 17	Washout	No treatment		
End of Week 17	Crossover			
Week 18	Part 2	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 19	Part 2	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily

Week 20	Part 2	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 21	Part 2	Randomised Vivomixx or placebo	1 sachet daily	1 sachet twice daily
Week 22	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 23	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 24	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 25	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 26	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 27	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 28	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 29	Part 2	Randomised Vivomixx or placebo	1 sachet twice daily	3 sachets daily split over 2 or 3 doses
Week 30	Exit interview			