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Complete List of Authors:	Simpson, Carra; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne School of Psychological Sciences Schwartz, Orli; Orygen The National Centre of Excellence in Youth Mental Health, Centre for Youth Mental Health Eliby, Djamila; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne School of Psychological Sciences; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne Neuropsychiatry Centre Butler, Catherine; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Centre for Oral Health Research, Melbourne Dental School, Bio21 Institute Huang, Katherine; The University of Melbourne Bio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology Simpson-O'Brien, Neil; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Centre for Oral Health Research, Melbourne Dental School, Bio21 Institute Callaghan, Bridget; University of California Los Angeles, The Department of Psychology; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne Neuropsychiatry Centre Dashper, Stuart; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Centre for Oral Health Research, Melbourne Dental School, Bio21 Institute Gooley, Paul; The University of Melbourne Bio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology Whittle, Sarah; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne Neuropsychiatry Centre; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne School of Psychological Sciences Haslam, Nick; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne School of Psychological Sciences Simmons, Julian; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne School of Psychological Sciences; The University of Melbourne Faculty of Medicine Dentistry and Health Sciences, Melbourne Neuropsychiatry Centre

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Protocol: Bugs and Brains, the Gut and Mental Health Study - A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome

Carra A. Simpson[1, 2], Orli S. Schwartz[3], Djamila Eliby[1, 2], Catherine A. Butler[4], Katherine Huang[5], Neil O'Brien-Simpson[4], Bridget L. Callaghan[2, 6], Stuart G. Dashper[4], Paul R. Gooley[5], Sarah Whittle[1, 2], Nick Haslam[1], & Julian G. Simmons[1, 2]

[1] Melbourne School of Psychological Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, VIC, 3010, Australia.

[2] Melbourne Neuropsychiatry Centre, Department of Medicine, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne and Melbourne Health, VIC, 3010, Australia.

[3] Orygen, The National Centre of Excellence in Youth Mental Health; Centre for Youth Mental Health, The University of Melbourne, VIC, 3010, Australia.

[4] Centre for Oral Health Research, Melbourne Dental School, Bio21 Institute, The University of Melbourne, VIC, 3010, Australia

[5] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, VIC, 3010, Australia

[6] The Department of Psychology, The University of California, Los Angeles, 90503

Correspondence: Carra A. Simpson, https://orcid.org/0000-0002-8281-5881

Melbourne School of Psychological Sciences, 12th floor Redmond Barry Building, The University of Melbourne, Parkville, VIC 3010, Australia.

Email: carra.simpson@unimelb.edu.au, Phone: +61 03 8344 1845

Declarations of interest: None

Abstract

Introduction: Research has highlighted the relationship between the microorganisms that inhabit our gastrointestinal tract (oral and gut microbiota) with host mood and gastrointestinal functioning. Mental health disorders and functional gastrointestinal disorders co-occur at high rates, although the mechanisms underlying these associations remain unclear. The Bugs and Brains study aims to investigate the complex relationships between anxiety/depression and irritable bowel syndrome (IBS) in two ways. Firstly, its primary component will compare the gut and oral microbiota in females with anxiety/depression and/or IBS relative to controls, and investigate underlying physiological, endocrine, and immune factors, as well as interactions with diet and psychosocial factors. In an ancillary component, the study will also investigate gastrointestinal and mental health symptoms in a larger sample, and explore relationships with diet, medical history, and psychosocial factors. **Methods and analysis:** The Bugs and Brains study aims to recruit 160 females to the primary component: i) 40 controls; ii) 40 participants with a depressive/anxiety disorder, but no IBS; iii) 40 participants with IBS, but no depressive/anxiety disorder; and iv) 40 participants with both depressive/anxiety disorder and IBS. Participation is completed within one month, and involves comprehensive questionnaires, anthropometrics, a diagnostic clinical interview, collection of two saliva samples, and stool, urine, and hair samples. This study aims to use a systems biology approach to characterise oral and gut microbial composition and function using 16S rRNA gene sequencing and nuclear magnetic resonance spectroscopy. As part of the ancillary component, it will collect questionnaire data from 1000 females aged 18-40 years, capturing mental health, gastrointestinal health, oral health, diet, and psychosocial factors. **Ethics and dissemination:** Approval was granted by the University of Melbourne Human Research Ethics Committee (#1749221). All participants voluntarily provided informed consent. Results will be published in peer-reviewed journals and presented at scientific conferences.

Keywords: Gut microbiota, microbiome, depression, anxiety, irritable bowel syndrome

Article summary

Strengths and limitations

- The Bugs and Brains study will compare oral and gut microbiota composition and function using next generation sequencing and nuclear magnetic resonance spectroscopy in individuals with anxiety/depression, IBS, both anxiety/depression and IBS, and controls, as defined by clinical diagnosis and strict inclusion criteria.
- It will assess medical history, gastrointestinal health, early life adversity, mental and physical health symptoms using detailed questionnaires and clinical interview, and investigate associations between symptoms, microbiota, metabolome, and the immune and endocrine systems.
- It will also investigate gastrointestinal and mental health symptoms in a large sample, and examine relationships between diet, medical history, and early life adversity.
- Future research should investigate these associations using shotgun metagenomics, examine relationships longitudinally to disentangle causes and effects, and extend investigations to male participants.

Introduction

Microorganisms have co-existed with humans throughout our evolution, with between 20 and 30 trillion microbial cells occupying ecological niches within and upon the human body. It is therefore unsurprising that bacteria residing throughout the gastrointestinal (GI) tract are implicated in host endocrine, immune, and nervous system functioning [1]. Microbes produce neuroactive compounds, including catecholamines (e.g., noradrenaline, dopamine), GABA, histamine, serotonin, and acetylcholine, each of which plays a critical role in human physiological functioning [1,2]. Multiple GI bacterial genera and their metabolic products also facilitate peristaltic waves to aid with digestion [3], contribute to fermentation of dietary carbohydrates, maintain host membrane function, and produce essential amino acids and vitamins [2,4]. Resident bacterial species also prime the immune system in the early stages of life, teaching our bodies what is 'foreign' rather than commensal [2]. The gut microbiota (i.e., the collection of microorganisms in the GI tract) is therefore essential to normal development.

As we come to appreciate the essential role microorganisms play in host physiology, so too have we opened a novel direction for investigation in conditions with no current clear aetiology. Among these are irritable bowel syndrome (IBS), depression and anxiety disorders. IBS is the most common functional GI disorder, affecting 7 to 21% of individuals worldwide [5]. Similarly, depression remains the highest contributor to non-fatal health burden, and approximately 264 million individuals live with an anxiety disorder globally [6]. These three conditions frequently co-occur [7]: approximately 84% of patients with IBS also have a depressive disorder and 44% an anxiety disorder [8,9]. Moreover, 45% of participants with anxiety and 30% of participants with a depressive disorder develop IBS [7,10,11]. The mechanisms underlying this comorbidity remain unclear.

The complex interplay between host physiology and microbiota promises novel insight into these conditions. Research has revealed that anxiety, depression and IBS share several gut microbial alterations, including decreased overall microbial diversity and a higher relative abundance of pro-inflammatory and toxigenic species [12–15]. Moreover, research indicates alterations may exist along a continuum, in that participants with comorbid depression and IBS have gut microbiota alterations simply more pronounced than those presenting with one disorder alone, including higher Proteobacteria, *Prevotella/Prevotellaceae, Bacteroides* and lower *Lachnospiraceae* [10,16–18]. These implicated taxa further build on the evidence of altered

microbial and host immune system interactions, as taxa reported to have a higher relative abundance in anxiety, depression and IBS are associated with inflammation, whereas a loss of species that secrete anti-inflammatory metabolic products has also been observed [14]. No studies have compared the gut microbiota of participants with anxiety relative to IBS, although studies have identified microbiota-related correlates between anxiety symptoms among IBS patients [18–21].

Although research in this area has primarily investigated the composition of the gut microbiota, the entire length of the GI tract harbours resident microorganisms. The oral microbiota is the second most diverse community in the body following the gut [22], and research indicates that microbial representation in the oral cavity is predictive of the composition in the lower GI tract [23]. Consistently, oral bacteria can translocate throughout the body, with significant effects on GI and systemic health [24]. Although no known studies have examined the oral microbiota in clinical anxiety, depression or IBS, its known association with host physiology (i.e., immune and endocrine functioning [22]) warrants investigation.

Several confounding factors have been neglected in the small literature investigating the microbiota in IBS, anxiety and depression. Severity and type of IBS symptoms interact with gut microbial composition [15,18,25], as may use of anti-depressants/anxiolytic medication [26]. Diet and its interaction with psychosocial factors also act as sources of gut microbiota composition variation [27,28], which has also been inadequately considered. The extant literature has not often employed gold-standard diagnostic measures, including clinical psychological interviews, and has examined microbial composition but neglected microbial function [25].

Aims and objectives

The Bugs and Brains study aims to characterise the microbial and physiological profiles in anxiety, depression, and IBS; to understand interactions between symptoms and confounding variables; and, to investigate varied contributors to disorder presentation (both across and within disorders). In this primary case-control component, key questions relate to the similarities and differences across bacterial, metabolic, endocrine, and immune indices, in a well-controlled cohort. In a large ancillary questionnaire-only component, this research will also investigate how GI and mental health symptoms are associated with diet, exercise, oral health, substance use, medical history, and psychosocial factors. Possible sources of inter-study variation in previous investigations will be considered (i.e., symptom severity, medication use, diet). Understanding

shared and distinct microbiota relationships in IBS, anxiety and depression has implications in resolving the directionality of co-morbidity, and in informing future targeted clinical interventions.

Methods and analysis

Participants

Primary case-control component

Females aged 18 to 40 will be assessed for participation in all phases of the study. The primary case-control component will include 160 community-based female volunteers aged 18 to 40 meeting eligibility criteria for one of four groups:

i) 40 controls
ii) 40 participants with a depressive/anxiety disorder, but no IBS
iii) 40 participants with IBS, but no depressive/anxiety disorder; and
iv) 40 participants with depressive/anxiety disorder and IBS

The study chose to recruit only participants of the female sex due to significantly elevated rates of all three conditions relative to biological males [6,29]. Sex is also an identified source of inter-study variation in microbiota research [30]. All participants are being recruited through online and printed advertisements at the University of Melbourne and using paid Facebook ads. All participants must demonstrate the capacity to provide informed consent and adequate comprehension of English.

Ancillary questionnaire-only component

To investigate associations in a larger sample, the ancillary component aims to collect questionnaire data from 1000 females aged 18 to 40, capturing mental health, diet, exercise, gastrointestinal health, oral health, substance use, medical history, emotion regulation, stress, early life adversity, and medical history.

Inclusion criteria for the case-control component

Participants in the primary component are required to live in Greater Melbourne or Geelong (unless willing to travel to the site of sample collection: Melbourne, Australia). Inclusion and exclusion criteria are presented in Table 1. All participants initially complete the Phase 1 screening questionnaire to assess their likely eligibility for one of the four groups. If participants meet screening criteria, they are invited to take part in a psychiatric diagnostic

interview to confirm eligibility (Phase 2). All ineligible participants, including those who prefer not to complete sample collection, are invited to complete the ancillary questionnaire-only component.

Depression and anxiety group

Participants in this group must meet current diagnostic criteria for at least one of six depressive and/or anxiety disorders, as assessed by the Structured Clinical Interview for DSM-5 Research Version (SCID-5-RV; [31]) at Phase 2. Specific disorders for inclusion are: Major Depressive Disorder, Persistent Depressive Disorder, Social Anxiety Disorder, Panic Disorder, Agoraphobia, and Generalized Anxiety Disorder. Participants with a co-morbid Specific Phobia, Obsessive-Compulsive or Related Disorder, Trauma- or Stressor-Related Disorder, Sleep Disorder, and/or Externalizing Disorder, as assessed in the SCID-5-RV, are included if they also meet criteria for one of the six target disorders. Participants in this group must have no lifetime history of IBS and must not meet current diagnostic criteria for IBS or another FGID.

Irritable bowel syndrome group

Participants must meet current criteria for IBS, as assessed by the Rome IV during Phase 1 screening questionnaires [32,33]. The Rome IV assesses gastrointestinal symptom duration and delineates diagnosis by the prominent altered stool type: constipation (IBS-C), diarrhoea (IBS-D), mixed (IBS-M), or an unspecified dominance (IBS-U). Participants in this group must not meet current or past (i.e., lifetime) diagnostic criteria for any of the DSM-5 disorders mentioned above (including Specific Phobia, etc).

Comorbid irritable bowel syndrome and anxiety/depression group

Consistent with measures outlined above, participants must currently meet criteria for IBS and diagnosis of at least one of the six primary anxiety and/or depressive disorders.

Control group

Participants in this group must have no lifetime history of IBS, must not meet current diagnostic criteria for IBS or another FGID, and must not meet lifetime diagnostic criteria for any of the DSM-5 disorders mentioned above (i.e., current or past).

Exclusion criteria for the primary case-control component (all groups)

Physical health

Participants are excluded from all groups if they report or present with current obesity,

hypertension, cardiovascular disease, respiratory disease, diabetes mellitus, liver cirrhosis, fatty liver disease, inflammatory bowel disease, coeliac disease, other autoimmune disease, short bowel syndrome, or a history of abdominal surgery that involved cutting into the GI tract. Participants who report daily cigarette smoking, a recent colonoscopy (past 12 months), who are pregnant/lactating are also excluded.

Mental health

Participants are excluded if they report a lifetime history or present with a clinical psychotic symptom or disorder, Bipolar Disorder, Somatic Symptom or Related Disorder, or over the past 12 months meet criteria for a Feeding and Eating Disorder or an Alcohol or Substance Use Disorder (at Phase 1 questionnaires or Phase 2 SCID-5 interview).

Medication

Participants are excluded if they report use of anti-depressant or anti-anxiety medications, other psychotropics, steroids, probiotics, prebiotics or synbiotics in the past four weeks. They are also excluded if they report a current bacterial, fungal, or viral infection, or use of non-steroidal anti-inflammatory drugs or antifungal medications in the past two weeks. Specific diets may be used to alleviate GI symptoms (e.g., FODMAP exclusion, casein-free), and therefore participants without IBS are excluded if they are following a restrictive diet (i.e., do not meet criteria, potentially due to dietary reduction of symptoms).

Inclusion criteria for the ancillary questionnaire-only component

Females aged 18-40 who are ineligible for the primary case-control component or elect not to collect biological samples are invited to complete a set of questionnaires that comprise the ancillary questionnaire-only component. No strict eligibility criteria apply, although all participants are required to demonstrate the capacity to provide informed consent and adequate comprehension of written and spoken English. All participants in the case-control component complete the same questionnaires. The phases of participation in the Bugs and Brains study are presented in Figure 1.

Table 1. Inclusion criteria for full participation in the Bugs and Brains study (i.e., primary case-control component)

Inclusion criteria	Controls	Depression/Anxiety	IBS	Depression/Anxiety and IBS
Female sex	✓	✓	✓	✓
Fluent in English	✓	✓	✓	✓
Capacity to provide consent	✓	✓	✓	✓
Live in Melbourne or Geelong (Australia)	✓	✓	✓	✓
Meet DSM-5 criteria for current Depression or Anxiety disorder*	✗	✓	✗	✓
OCD, specific phobia, Trauma- or stressor-related disorder, Sleep Disorder, Externalizing disorders	✗	-	✗	-
Irritable bowel syndrome (Rome IV)	✗	✗	✓	✓

Notes. ✓= inclusion criteria; ✗= exclusion criteria (current or lifetime history [as assessed by SCID-5-RV]); - = neither exclusion nor inclusion; * Specific disorders for inclusion are: Major Depressive Disorder, Persistent Depressive Disorder, Social Anxiety Disorder, Panic Disorder, Agoraphobia, and Generalized Anxiety Disorder as assessed by the Structured Clinical Interview for the Diagnostic and Statistical Manual fifth edition, research version (SCID-5-RV)

[INSERT FIGURE 1 HERE]

Figure 1. Phases of participation in the Bugs and Brains study. Participants eligible for the primary case-control component at Phases 1 and 2 complete all phases of the study within a month, including biological sample collection ($N = 160$; $n = 40$ with IBS, $n = 40$ with anxiety and/or depressive disorder, $n = 40$ with IBS and anxiety and/or depressive disorder, $n = 40$ controls with no lifetime history of either IBS or an anxiety/depressive disorder). Participants who decline sample collection or are not eligible for the primary component complete the questionnaire-only ancillary component (Phases 1 and 4; $N = 1000$). GAF = Global Assessment of Functioning; IBS = irritable bowel syndrome; SOFAS = Social and Occupational Functioning Assessment Scale; Structured Clinical Interview for the Diagnostic and Statistical Manual (DSM) 5th edition (Research version [SCID-5-RV]). Created with BioRender.com.

Phases of participation

Phase 1 - screening questionnaire (all participants)

Participants who provide informed consent are sent a Phase 1 questionnaire link online, using the Qualtrics platform. This component takes approximately 20 minutes and comprises a brief demographic overview, the Psychiatric Diagnostic Screening Questionnaire (PDSQ; [34]), and the Rome IV IBS module [32]. It also captures participants' regular diet category (e.g., omnivore, low FODMAP), medication/supplements use, and alcohol/substance use. Eligible participants are invited for further Phase 2 screening. Excluded participants, or those who decline Phases 2 or 3, are invited to complete Phase 4 questionnaires as part of the ancillary component, (bypassing Phases 2 and 3).

Phase 2 - assessment of current and past mental health diagnoses (SCID-5; case-control component only)

Participants invited for the primary case-control component are administered the SCID-5-RV [31] to further assess eligibility. Interviews are conducted in-person at the University of Melbourne or online via a video application. Trained interviewers administer a modified version of the SCID-5-RV to assess the inclusion and exclusion psychiatric disorders displayed in Table

2. The interviewer then assesses functioning using the Global Assessment of Functioning (GAF) and Social and Occupational Functioning Assessment Scale (SOFAS) [35]. Fortnightly clinical meetings are conducted with the research clinical psychologist (OS) to maintain interviewer consistency and discuss any risks presented by participants. All interviewers are trained in psychological interviewing and are required to complete Management of Clinical Aggression training (MOCA) [36] and Applied Suicide Intervention Skills Training (ASIST). Interrater reliability will be conducted for at least 20% of interviews from eligible participants.

Table 2. Disorders assessed by the modified SCID-5-RV utilised by the Bugs and Brains study

Disorder	Duration assessed at interview	Eligibility Status
Bipolar and related disorders		
Bipolar I Disorder	Lifetime and past month (current)	✗ Exclusion (all groups)
Bipolar II Disorder	Lifetime and past month (current)	
Cyclothymic Disorder	Past 2 years (current)	
Other Specified Bipolar Disorder	Lifetime and past month (current)	
Bipolar Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Bipolar Disorder	Lifetime and past month (current)	
Depressive disorders		
Major Depressive Disorder	Lifetime and past month (current)	✓ Current = Inclusion (MH and Comorbid)
Persistent Depressive Disorder	Lifetime and past two years (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Premenstrual Dysphoric Disorder	Past 12 months (current)	- Lifetime = Neither inclusion nor exclusion
Other Specified Depressive Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Depressive Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Depressive Disorder	Lifetime and past month (current)	
Schizophrenia and other psychotic disorders		
Clinical psychotic symptoms	Lifetime and current	✗ Exclusion (all groups)
Substance use disorders		
Alcohol	Past 12 months (current)	✗ Exclusion (all groups)
Sedative-Hypnotic-Anxiolytic	Past 12 months (current)	

Disorder	Duration assessed at interview	Eligibility Status
Cannabis	Past 12 months (current)	
Stimulants/Cocaine	Past 12 months (current)	
Opioids	Past 12 months (current)	
Phencyclidine (PCP)	Past 12 months (current)	
Other Hallucinogens	Past 12 months (current)	
Inhalants	Past 12 months (current)	
Other/Unknown	Past 12 months (current)	
Anxiety disorders		
Panic Disorder	Lifetime and past month (current)	✓ Current = Inclusion (MH and Comorbid)
Agoraphobia	Lifetime and past six months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Social Anxiety Disorder	Lifetime and past six months (current)	
Generalized Anxiety Disorder	Lifetime and past six months (current)	✗ Lifetime = Exclusion (Controls and IBS)
Specific Phobia	Lifetime and past six months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Other Specified Anxiety Disorder	Lifetime and past month (current)	
Anxiety Disorder Due to Another Medical Condition	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)
Substance/Medication-Induced Anxiety Disorder	Lifetime and past month (current)	
Obsessive-compulsive and related disorders		
Obsessive Compulsive Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Other Specified Obsessive Compulsive and Related Disorder	Lifetime and past month (current)	
Obsessive-Compulsive and Related Disorder Due to Another Medical Condition	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)
Substance/Medication-Induced Obsessive-Compulsive and Related Disorder	Lifetime and past month (current)	

Disorder	Duration assessed at interview	Eligibility Status
<u>Sleep-wake disorders</u>		
Insomnia Disorder	Past three months (current)	– Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Hypersomnolence Disorder	Past three months (current)	
Substance-Induced Sleep Disorder	Past three months (current)	
<u>Feeding and Eating Disorders</u>		
Anorexia Nervosa	Past 12 months and past 3 months (current)	✗ Exclusion (all groups)
Bulimia Nervosa	Past 12 months and past 3 months (current)	
Binge Eating Disorder	Past 12 months and past 3 months (current)	
Other Specified Feeding or Eating Disorder	Past 12 months and past 3 months (current)	
<u>Somatic Symptom and Related Disorders</u>		
Somatic Symptom Disorder	Past 6 months (current)	✗ Exclusion (all groups) IBS-related symptoms not included for IBS and comorbid groups
Illness Anxiety Disorder	Past 6 months (current)	
<u>Externalizing Disorders</u>		
Adult Attention-deficit Hyperactivity Disorder	Past 6 months (current)	– Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
<u>Trauma- and Stressor-Related Disorders</u>		
Acute Stress Disorder	Past month (current)	– Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Posttraumatic Stress Disorder	Lifetime and past month (current)	
Adjustment Disorder	Past 6 months (current)	
Other Specified Trauma- and Stressor-Related Disorder	Lifetime and past month (current)	

Notes. MH = mental health anxiety/depression group; Comorbid = comorbid IBS and anxiety/depression group; IBS = irritable bowel syndrome

Phase 3 - sample collection and anthropometrics (case-control component only)

At Phase 3, participants collect biological samples in their own home. Instructions are provided verbally during a telephone call and included in written form with the collection kits. On day one, a stool sample is collected; on day two, a urine sample (first urination) and saliva sample are collected on awakening; on day three, another saliva sample is collected on awakening. Instructions on sterile storage are provided, and samples are immediately placed in participants' freezers following collection. A home visit is scheduled to pick up the biological samples, collect the hair sample, and take anthropometric measurements (height, weight and waist circumference), ideally within one week of collection. Samples are then transported on ice to the university and stored at -80 °C until processing.

Faecal sample. Participants collect a faecal sample at their first bowel movement of their chosen day, but not if they are menstruating or sick. Participants are asked to wait at least three days after they cease menses to begin collection, and to not collect samples within one week of menstruation. They are asked to consume a diet 'normal for them' for at least two weeks prior. Approximately 2.5 g is collected into sterile faecal collection containers. Participants are instructed not to collect the faecal sample if they have very loose stools, and to wait until they return to 'normal' (for them), or to a firmer state (i.e., Bristol stool chart type 5 or 6, if diarrhoea is frequent).

Urine sample. A mid-stream urine sample of approximately 10 to 20 ml is collected into sterile urine specimen containers at first urination on the morning the day after collection of the faecal sample.

Saliva sample. Participants collect (by passive drool) a total of two 3 ml saliva samples, one per day across two days, into Techno Plas 10 ml sterile centrifuge tubes. The first saliva sample is collected on awakening, and before eating or drinking, the morning of day 2 (day following collection of the faecal sample). The second saliva sample is collected on awakening the following morning on day 3. Participants are asked to complete a brief saliva record form.

Hair sample. A hair sample is collected by researchers at participants' homes. Hair sampling is carried out by combing and pinning the surrounding hair with clips, such that a 2 cm long by 0.5 cm wide scalp area is isolated on the posterior vertex (back of the head below the inion; ~1 cm^2). Hair is then bound together (marking the scalp end) and cut with blunt-nose scissors as close to the scalp as possible.

Phase 4 - additional questionnaire data (all participants)

Participants in the primary case-control component of the study receive the Phase 4 questionnaires online, via the Qualtrics platform. Phase 4 questionnaires are completed within 24 hours of faecal collection. Participants in the ancillary questionnaire-only study complete Phase 4 questionnaires following Phase 1 (or whichever phase they are excluded from the case-control component/decline to participate). The Phase 4 questionnaires (see Table 3) cover demographic information (e.g., country of birth), early life history (e.g., mode of birth, breastfeeding), history of gastric infections, and scales assessing psychopathology symptoms, stressful life events, early life adversity, diet, emotion regulation, and oral and physical health. The Phase 4 questionnaires take approximately 60 min.

Table 3. Summary of questionnaire measures included in the Bugs and Brains study

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
Demographic information	Phase 1 Health Questionnaire*	✓		
	Phase 4 Demographics, Health and Exercise Questionnaire*			✓
Emotional functioning	Difficulties in Emotion Regulation Scale (DERS) [37]			✓
Mental health and behaviour	Psychiatric Diagnostic Screening Questionnaire (PDSQ) [34]	✓		
	Global Assessment of Functioning (GAF) † [35]		✓	
	Social and Occupational Functioning Assessment Scale (SOFAS) † [35]		✓	
	Beck Depression Inventory (BDI-II) [38]			✓
	Beck Anxiety Inventory (BAI) [39]			✓
	Adult Self Report (ASR) [40]			✓
Diet	Dietary Questionnaire for Epidemiological Studies (DQES) [41]			✓
	Phase 1 Health Questionnaire* • Specific diet information (past four weeks; e.g. gluten-free, specific carbohydrates, FODMAP, vegetarian)	✓		
Exercise	Phase 4 Demographics, Health and Exercise Questionnaire*			✓
Quality of life/wellbeing	The World Health Organization quality of life assessment (WHOQOL-BREF) [42]			✓
Oral health	The World Health Organization Oral Health Questionnaire for Adults [43]			✓
Physical health /medical history	The Physical Health Questionnaire (PHQ) [44]			✓
	Phase 1 Health Questionnaire* • Current bacterial, fungal, or viral infection • Obesity, hypertension, cardiovascular disease, respiratory disease, diabetes mellitus, liver cirrhosis, fatty liver disease, coeliac disease, other autoimmune	✓		

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
	disease (e.g., rheumatoid arthritis, lupus, etc.), and polycystic ovary syndrome (PCOS). • Current pregnancy/lactation • Mode of birth • Medication (past month): anti-depressants/anti-anxiolytics, antibiotics, psychotropics, oral or injected steroids, probiotics, prebiotics, synbiotics, dietary supplements including laxatives, other Medication (past two weeks): NSAIDs, probiotics, antifungals			
Gastrointestinal health/history	Rome IV Diagnostic Questionnaire (R4DQ) [32]	✓		
	Phase 1 Health Questionnaire* • Non-surgical procedures related to the digestive system (past six months): enema, colonoscopy, gastroscopy Inflammatory bowel disease (e.g., ulcerative colitis, Crohn's disease), short bowel syndrome, history of abdominal surgery (e.g., appendectomy, ileostomy, bowel surgery, caesarean section, etc.),	✓		
	Phase 4 Demographics, Health and Exercise Questionnaire* • Lifetime history of gastric infections: Salmonella, Shigella, Campylobacter, Giardia, Virus, Other			✓
Stressful life events	The Lifetime Incidence of Traumatic Events (LITE) [45]			✓
	Childhood Trauma Questionnaire (CTQ) [46]			✓
Substance use	Phase 1 Health Questionnaire* • Alcohol (past month) • Smoking (past month): tobacco cigarettes, vape, e-cigarettes containing nicotine Illicit drug use (past month)	✓		

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
	• Dietary Questionnaire for Epidemiological Studies (DQES) [41]			✓

Notes. * = designed specifically for the Bugs and Brains study; † = measure completed by researchers, rather than participants; NSAIDS= nonsteroidal anti-inflammatory drugs; SCID-5-RV = Structured Clinical Interview for the Diagnostic and Statistical Manual fifth edition, research version

Gut and oral microbiota sequencing

Amplicon 16S ribosomal ribonucleic acid (rRNA) gene sequencing will be used to estimate participants' microbiota, using a faecal sample (gut microbiota) and a saliva sample (oral microbiota). Genomic DNA will be extracted using PowerSoil® HTP kit and amplified using universal prokaryotic 515F and 806R primers targeting the V4 region of the 16S small subunit rRNA gene [47]. Sequences are generated using paired-end sequencing on the Illumina MiSeq platform. Should additional funding be secured, shotgun metagenomic sequencing will be performed.

Metabolomics

Nuclear magnetic resonance spectroscopy (NMR) will be run on faecal and urine samples to quantify metabolites. If additional funding is obtained, mass spectrometry metabolite assays will also be conducted.

Sample Preparation

Faecal water. A small proportion of the frozen faecal sample (0.5-1 g) will be homogenized by vortex in deuterated saline (0.9% NaCl) at 1:5 w/v before being centrifuged (3,200 × g) at 4 °C for 15 min. Faecal water will be syringe filtered (0.22 µm, 33 mm diameter) and dispensed into 700 µL aliquots.

Urine. Frozen urine samples will be thawed in ice baths, syringe filtered (0.22 µm, 33 mm diameter) and each dispensed into 700 µL aliquots.

Metabolite extraction. Faecal and urine samples will be prepared for NMR analysis using a liquid-liquid extraction technique [48]. A 250 µL aliquot of each faecal water/urine sample will be added to 250 µL ice-cold deuterated chloroform and 250 µL ice-cold deuterated methanol and mixed by vortex before being left on ice for 15 min. Samples are then centrifuged (16,000 × g) at 4 °C for 10 min to produce a biphasic mixture with a hydrophilic phase of water/deuterated methanol containing polar metabolites and lipophilic phase of deuterated chloroform containing lipophilic compounds.

Nuclear magnetic resonance spectroscopy (NMR)

A 297 µL sample of the top hydrophilic layer will be added to equal volume of 200 mM sodium phosphate in $^{2}H_2O$ (pH 7.4) and 66 µL of $^{2}H_2O$ containing 5 mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt and 0.2% (w/v) sodium azide to make a total of 660 µL. The

sample will be mixed by vortex, centrifuged at 16,000 × g for 1 min, and 550 µL of supernatant transferred to a 7-inch 5-mm 507-grade Bruker NMR tube for NMR analysis.

All NMR spectra of faecal water and urine will be collected using Bruker 600 MHz spectrometer set to a constant temperature of 300 K. Standard 1D ^{1}H spectra will be acquired for faecal water using 1D NOESY pulse sequence (recycle delay-90°-t_1-90°-t_m-acquire) with t_1 corresponding to 3 µs, t_m to 10 ms and the 90° pulses optimised. Water suppression will be achieved by pre-saturation of the water signal during t_m and the recycle delay (4 s). Each spectrum will be collected over 64 K data points and 256 scans with spectral width of 20 ppm [49].

Urine spectra are acquired using the same 1D NOESY pulse sequence where recycle delay corresponded to 2 s, t_1 to 3 µs and t_m to 10 ms. Each spectrum will be collected over 32 K data points and 64 scans with spectral width of 20 ppm [50]. Using the Chenomx NMR Suite Professional software package, the free induction decay is multiplied by 0.3 Hz exponential line broadening and zero-filled before Fourier transformation. Spectra are manually phased, baseline corrected and referenced to DSS with a chemical shift of 0 ppm. The compound libraries in the Chenomx software will be used to identify and quantitate metabolites in the NMR 1D ^{1}H spectra based on their characteristic chemical shifts using 5 mM DSS as an internal chemical shift reference.

Immune and endocrine assays

Hormones will be measured from hair and saliva samples to estimate longer and shorter-term functioning, respectively. The hormones cortisol, DHEA, and testosterone will be assayed from the two saliva samples collected at awakening to provide indications of hypothalamic-pituitary-adrenal axis hormonal excretion. To estimate systemic inflammation, the acute phase C-reactive protein (CRP) will also be estimated from the two saliva samples, as previously described [51]. Assays for hormones and CRP will be conducted using enzyme-linked immunosorbent assay kits and performed in duplicate following manufacturer protocols.

Pre-processing of bacterial sequencing data

Sequencing reads will be quality filtered, trimmed, denoised and merged reads using QIIME2 [52]. The DADA2 denoising pipeline will be used for correcting sequence errors and identifying amplicon sequence variants (ASVs, i.e., sequences not containing errors) [53]. All low-quality reads will be discarded. ASVs will be taxonomically assigned using a Naïve-Bayes

classifier against the most recent version of the Human Oral Microbiome Database (saliva) and SILVA databases (faecal samples). Sequences will be aligned using MAFFT [54], and phylogeny built using FastTree [55].

Sample size calculation

A sample of 132 would provide 80% power at $p = 0.01$ to detect an effect size of 0.35 when comparing outcomes across the four groups (i.e., anxiety/depression, IBS, comorbidity, controls). This estimate is conservative given the effect sizes reported in related research, and our sample size of 160 exceeds this recommendation.

Data analysis

Proposed analyses will be discipline-specific (microbial, metabolomics, immune and endocrine), but will also utilise systems biology to integrate the wide range of acquired biological, health, and psychosocial data. General linear models will first investigate the relationships between mental health, dietary patterns, exercise, GI health, oral health, early life adversity, substance use, and medical history in the questionnaire-only ancillary study. These analyses may inform necessary metadata to consider in analyses of the primary case-control component. Sources of inter-study variation hypothesised in previous investigations of the gut microbiota will be examined [25].

A primary aim of the case-control component involves characterising and comparing the microbiota and metabolome in the four participant groups (i.e., anxiety/depression, IBS, comorbidity, controls). Proposed microbial analyses include group comparisons at several levels of the taxonomy (phylum, class, order, family, genus levels), which will be analysed using non-parametric tests suited to compositional data (e.g., Mann-Whitney U test). Between and within-sample diversity will also be conducted using alpha diversity (Shannon and Simpson indices, observed ASVs), and beta diversity (PCoA with PERMANOVA using weighted and unweighted UniFrac). Differential abundance, and associations between taxa abundance and metadata, will be analysed using DESeq2 and MaAsLin2 [56], correcting for multiple comparisons (Benjamini Hochberg False Discovery Rate). Statistical analyses and graphical representations will be conducted using R, with packages that enable linear multivariate methods and visualizations (e.g., vegan [57]; phyloseq [58]; ggplot2 [59]; DESeq2 [60]; MaAsLin2 [56]). It will also integrate the wide range of biological data and adopt a systems biology approach using the package mixOmics [61].

Ethics and dissemination

Ethical approval for this project was granted by the University of Melbourne Human Research Ethics Committee (#1749221). After reading our Plain Language Statement, a comprehensive consent procedure is conducted via telephone with all participants. The voluntary nature of the study is explained, including the limits of confidentiality, secure and de-identified nature of all data, and the right to withdraw at any time without prejudice or explanation. Verbal recorded consent is obtained at the end of the first phone call should the participant wish to begin their participation. Following completion of the sample collection, participants' written consent is obtained from those who were eligible for the case-control component. Participants who complete biological samples are monetarily reimbursed for their time. Participants who complete questionnaire-only participation are reimbursed for their time with a personalized dietary analysis.

Patient and public involvement

There were no funds or time allocated for patient and public involvement, therefore we were unable to involve patients in study design and recruitment. We have invited patients to assist with development of our dissemination strategy.

Progress to date

Of the 775 participants who have completed the Phase 1 questionnaire since the end of 2017, 211 have met screening criteria and were invited to the SCID-5-RV interview (Phase 2). At interview, 53 participants met exclusion criteria relating to psychiatric diagnosis or related reasons. Several participants were unable to complete kit collection, moved locations, or began exclusionary medications. Accordingly, 140 of 160 participants have completed the case-control component. A total of 352 of the planned 1000 participants have completed the ancillary questionnaire-only component (Phases 1 and 4).

Discussion

The high rates of co-occurrence between anxiety, depression and IBS remain poorly understood, and interactions between host physiology and the microbiota provide a promising avenue to investigate their shared underlying mechanisms. The Bugs and Brains study will be the first research project to investigate and compare the oral and gut microbiota and metabolome of participants with anxiety/depression, IBS, comorbid anxiety/depression and IBS, and controls, as assessed by clinical diagnosis and gold standard criteria. Moreover, it will analyse hypothesised

underlying mechanisms, including immune, endocrine, and microbial factors, and maintain strict inclusion and exclusion criteria. The questionnaire-only study will examine correlates of GI and mental health symptoms that have been insufficiently examined to date. Utilising comprehensive symptom-based questionnaire data and detailed physiological, microbial and psychological characterisation, study findings may facilitate the development of interventions for individuals with these highly prevalent and burdensome conditions.

Author contributions

JGS conceived the study, with critical input from NH, OS, SW, CAS, BC, SD, PG, NOS, CB, KH, and DE. CAS, JGS and KH wrote the manuscript. All authors critically revised and approved the final manuscript.

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Competing interests statement: None declared

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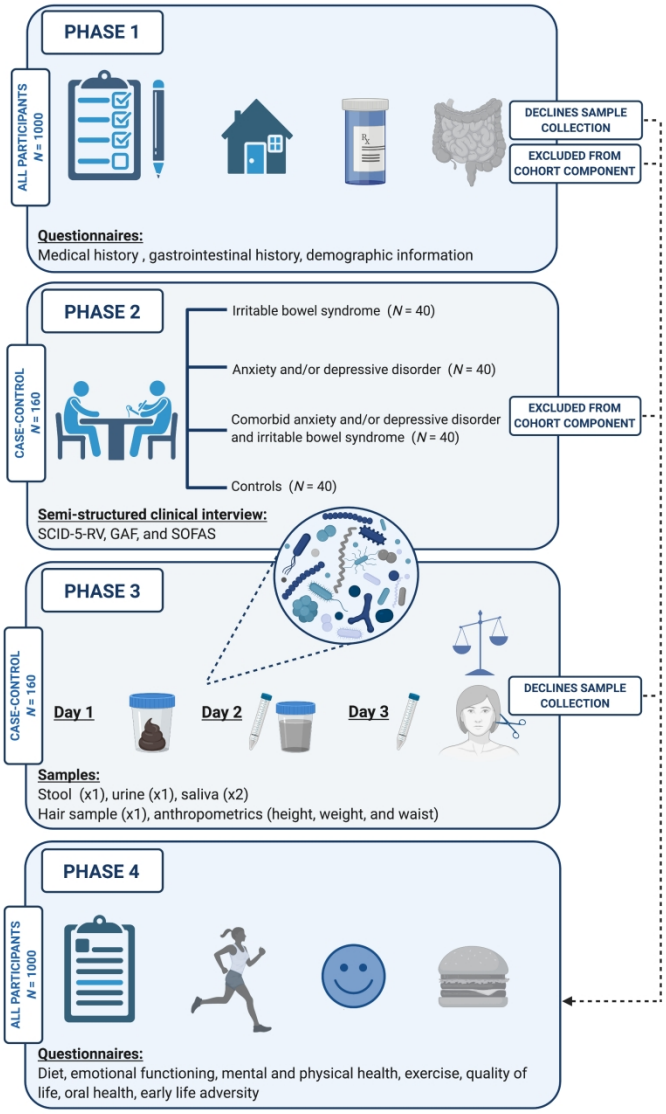

Figure 1. Phases of participation in the Bugs and Brains study. Participants eligible for the primary case-control component at Phases 1 and 2 complete all phases of the study within a month, including biological sample collection (N = 160; n = 40 with IBS, n = 40 with anxiety and/or depressive disorder, n = 40 with IBS and anxiety and/or depressive disorder, n = 40 controls with no lifetime history of either IBS or an anxiety/depressive disorder). Participants who decline sample collection or are not eligible for the primary component complete the questionnaire-only ancillary component (Phases 1 and 4; N = 1000). GAF = Global Assessment of Functioning; IBS = irritable bowel syndrome; SOFAS = Social and Occupational Functioning Assessment Scale; Structured Clinical Interview for the Diagnostic and Statistical Manual (DSM) 5th edition (Research version [SCID-5-RV]). Created with BioRender.com.

BMJ Open

Protocol: Bugs and Brains, the Gut and Mental Health Study - A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome

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Primary Subject Heading:	Mental health
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Keywords:	MICROBIOLOGY, Anxiety disorders < PSYCHIATRY, Depression & mood disorders < PSYCHIATRY, Functional bowel disorders < GASTROENTEROLOGY, PSYCHIATRY

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Protocol: Bugs and Brains, the Gut and Mental Health Study - A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome

Carra A. Simpson[1, 2], Orli S. Schwartz[3], Djamila Eliby[1, 2], Catherine A. Butler[4], Katherine Huang[5], Neil O'Brien-Simpson[4], Bridget L. Callaghan[2, 6], Stuart G. Dashper[4], Paul R. Gooley[5], Sarah Whittle[1, 2], Nick Haslam[1], & Julian G. Simmons[1, 2]

[1] Melbourne School of Psychological Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, VIC, 3010, Australia.

[2] Melbourne Neuropsychiatry Centre, Department of Medicine, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne and Melbourne Health, VIC, 3010, Australia.

[3] Orygen, The National Centre of Excellence in Youth Mental Health; Centre for Youth Mental Health, The University of Melbourne, VIC, 3010, Australia.

[4] Centre for Oral Health Research, Melbourne Dental School, Bio21 Institute, The University of Melbourne, VIC, 3010, Australia

[5] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, VIC, 3010, Australia

[6] The Department of Psychology, The University of California, Los Angeles, 90503

Correspondence: Carra A. Simpson, https://orcid.org/0000-0002-8281-5881

Melbourne School of Psychological Sciences, 12th floor Redmond Barry Building, The University of Melbourne, Parkville, VIC 3010, Australia.

Email: carra.simpson@unimelb.edu.au, Phone: +61 03 8344 1845

Declarations of interest: None

Abstract

Introduction: Research has highlighted relationships between the microorganisms that inhabit our gastrointestinal tract (oral and gut microbiota) with host mood and gastrointestinal functioning. Mental health disorders and functional gastrointestinal disorders co-occur at high rates, although the mechanisms underlying these associations remain unclear. The Bugs and Brains study aims to investigate complex relationships between anxiety/depression and irritable bowel syndrome (IBS) in two ways. Firstly, its primary component will compare the gut and oral microbiota in females with anxiety/depression and/or IBS relative to controls, and investigate underlying physiological, endocrine, and immune factors, as well as associations with diet and psychosocial factors. In an ancillary component, the study will also investigate gastrointestinal and mental health symptoms in a larger sample, and explore relationships with diet, exercise, oral health, substance use, medical history, early life adversity and psychosocial factors.

Methods and analysis: The Bugs and Brains study aims to recruit 160 females to the primary component: i) 40 controls; ii) 40 participants with a depressive/anxiety disorder, but no IBS; iii) 40 participants with IBS, but no depressive/anxiety disorder; and iv) 40 participants with both depressive/anxiety disorder and IBS. Participation is completed within one month, and involves comprehensive questionnaires, anthropometrics, a diagnostic clinical interview, collection of two saliva samples, and stool, urine, and hair samples. This study aims to use a systems biology approach to characterise oral and gut microbial composition and function using 16S rRNA gene sequencing and nuclear magnetic resonance spectroscopy. As part of the ancillary component, it will collect questionnaire data from 1000 females aged 18-40 years, capturing mental health, gastrointestinal health, oral health, diet, and psychosocial factors. **Ethics and dissemination:** Approval was granted by the University of Melbourne Human Research Ethics Committee (#1749221). All participants voluntarily provided informed consent. Results will be published in peer-reviewed journals and presented at scientific conferences.

Keywords: Gut microbiota, microbiome, depression, anxiety, irritable bowel syndrome

Article summary

Strengths and limitations

- The Bugs and Brains Study will compare oral and gut microbiota composition and function using next generation sequencing and nuclear magnetic resonance spectroscopy in individuals with anxiety/depression, IBS, both anxiety/depression and IBS, and controls, as defined by clinical diagnosis and strict inclusion criteria.
- It will assess diet, medical history, gastrointestinal health, early life adversity, mental and physical health symptoms using detailed questionnaires and clinical interview, and investigate associations between symptoms, microbiota, metabolome, and the immune and endocrine systems.
- It will also investigate gastrointestinal and mental health symptoms in a large sample, and examine relationships with diet, exercise, oral health, substance use, medical history, early life adversity and psychosocial factors.
- This study chose to recruit females aged 18 to 40 to control for the confounding effects of biological sex and age, therefore its generalisability is limited to this specific population.
- This study is cross-sectional in nature and employs 16S rRNA gene sequencing; future research should investigate these associations using deeper resolution sequencing (i.e., shotgun metagenomics), examine relationships longitudinally to disentangle causes and effects, and extend investigations to male participants.

Introduction

Microorganisms have co-existed alongside humans throughout our evolution, with between 20 and 30 trillion microbial cells occupying ecological niches within and upon the human body. It is therefore unsurprising that bacteria residing throughout the gastrointestinal (GI) tract are implicated in host endocrine, immune, and nervous system functioning [1]. Microbes produce neuroactive compounds, including catecholamines (e.g., noradrenaline, dopamine), GABA, histamine, serotonin, and acetylcholine, each of which plays a critical role in human physiological functioning [1,2]. Multiple GI bacteria and their metabolic products also facilitate peristaltic waves to aid with digestion [3], contribute to fermentation of dietary carbohydrates, maintain host membrane function, and produce essential amino acids and vitamins [2,4]. Resident bacterial species also prime the immune system in the early stages of life, teaching our bodies what is 'foreign' rather than commensal [2]. The gut microbiota (i.e., the collection of microorganisms in the GI tract) is therefore essential to normal development.

As we come to appreciate the essential role microorganisms play in host physiology, so too have we opened a novel direction for investigation in conditions with no current clear aetiology. Among these are irritable bowel syndrome (IBS), depression and anxiety disorders. IBS is the most common functional gastrointestinal disorder (FGID), affecting between 7 to 21% of individuals worldwide [5]. Similarly, depression remains the highest contributor to non-fatal health burden, and approximately 264 million individuals live with an anxiety disorder globally [6]. These three conditions frequently co-occur [7]: approximately 84% of patients with IBS also have a depressive disorder and 44% an anxiety disorder [8,9]. Moreover, 45% of participants with anxiety and 30% of participants with a depressive disorder develop IBS [7,10,11]. The mechanisms underlying this comorbidity remain unclear.

The complex interplay between host physiology and microbiota promises novel insight into these conditions. Research has revealed that anxiety, depression and IBS share several gut microbial alterations, including decreased overall microbial diversity and a higher relative abundance of pro-inflammatory and toxigenic species [12–15]. Moreover, research indicates alterations may exist along a continuum, in that participants with comorbid depression and IBS have gut microbiota alterations simply more pronounced than those presenting with one disorder, including higher Proteobacteria, *Prevotella/Prevotellaceae, Bacteroides* and lower *Lachnospiraceae* [10,16–18]. These implicated taxa further build on the evidence of altered

microbial and host immune system interactions, as the taxa reported to have a higher relative abundance in anxiety, depression and IBS are associated with inflammation, whereas a loss of species that secrete anti-inflammatory metabolic products has also been observed [14]. No studies have compared the gut microbiota of participants with anxiety relative to IBS, although studies have identified correlations between bacterial taxa and anxiety symptoms among IBS patients [18–20].

Although research in this area has primarily investigated the composition of the gut microbiota, the entire length of the GI tract harbours resident microorganisms. At its entryway, the microbiota of the oral cavity is the second most diverse community in the human body [21], and research indicates that microbial representation in the mouth is predictive of composition in the lower GI tract [22]. Oral bacteria can also translocate throughout the body, with significant effects on GI and systemic health [23]. No known studies have examined the oral microbiota in clinical anxiety, depression or IBS; however, our existing research observed associations between oral microbiota composition with anxiety and depression symptoms [24]. Characterisation of the oral microbiota in clinical mental health and IBS cohorts therefore warrants investigation.

Several confounding factors have been neglected in the small literature investigating the microbiota in IBS, anxiety, and depression. Due to their effects on microbial composition, future research should consider psychotropic medication use [25, 26], smoking [27], alcohol consumption [27], IBS subtype and severity [15,18], as well as biological sex [28]. Diet, and its interaction with psychosocial factors, is another source of gut microbiota variation that has been insufficiently considered in existing research [29,30]. Finally, the extant literature has not often utilised gold-standard diagnostic measures to examine mental health (i.e., clinical interviews), and studies have examined microbiota composition but neglected microbial function [31].

Aims and objectives

Bugs and Brains, the Gut and Mental Health Study (The Bugs and Brains Study) aims to characterise the microbial and physiological profiles in anxiety, depression, and IBS; to examine associations between symptoms and confounding variables; and, to investigate varied contributors to disorder presentation (both across and within disorders). In this primary case-control component, key questions relate to the similarities and differences across bacterial, metabolic, endocrine, and immune indices, in a well-controlled cohort. Given the associations

between biological sex and the microbiota, this study chose to recruit females only and exclude current smokers or participants with a substance abuse disorder. Possible sources of inter-study variation in previous investigations will be considered (i.e., symptom severity, medication use, diet). In a large ancillary questionnaire-only component, this research will also investigate how GI and mental health symptoms are associated with diet, exercise, oral health, substance use, medical history, early life adversity, and psychosocial factors. Understanding shared and distinct microbiota relationships in IBS, anxiety and depression, particularly within a well-characterised sample, has implications in resolving the directionality of co-morbidity, and in informing future targeted clinical interventions.

Methods and analysis

Participants

Primary case-control component

Females aged 18 to 40 will be assessed for participation in all phases of the Bugs and Brains Study. Recruitment for the study began in October 2017 and all phases are expected to be completed by December 2021. The primary case-control component will include 160 community-based female volunteers aged 18 to 40 who meet eligibility criteria for one of four groups:

i) 40 controls
ii) 40 participants with a depressive/anxiety disorder, but no IBS
iii) 40 participants with IBS, but no depressive/anxiety disorder; and
iv) 40 participants with depressive/anxiety disorder and IBS

The study chose to recruit only participants of the female sex due to significantly elevated rates of all three conditions relative to biological males [6,32]. All participants are being recruited through online and printed advertisements at the University of Melbourne and using paid Facebook ads. All participants provide informed consent and demonstrate adequate comprehension of English. Participants who complete biological samples are monetarily reimbursed for their time, and participants who complete the ancillary questionnaire-only component are reimbursed with a personalised dietary analysis.

Ancillary questionnaire-only component

To investigate associations in a larger sample, the ancillary component aims to collect

questionnaire data from 1000 females aged 18 to 40, capturing mental health, diet, exercise, gastrointestinal health, oral health, substance use, medical history, emotion regulation, stress, early life adversity, and medical history.

Inclusion criteria for the case-control component

Participants in the primary component are required to live in Greater Melbourne or Geelong (unless willing to travel to the site of sample collection: Melbourne, Australia). Inclusion and exclusion criteria are presented in Table 1. All participants initially complete the Phase 1 screening questionnaire to assess their likely eligibility for one of the four groups. If participants meet screening criteria, they are invited to take part in a psychiatric diagnostic interview to confirm eligibility (Phase 2). All ineligible participants, including those who prefer not to complete sample collection, are invited to complete the ancillary questionnaire-only component.

Depression and anxiety group

Participants in this group must meet current diagnostic criteria for at least one of six depressive and/or anxiety disorders at Phase 2, as assessed by the Structured Clinical Interview for DSM-5 Research Version (SCID-5-RV; [33]). Specific disorders for inclusion are: Major Depressive Disorder, Persistent Depressive Disorder, Social Anxiety Disorder, Panic Disorder, Agoraphobia, and Generalized Anxiety Disorder. Participants with a comorbid Specific Phobia, Obsessive-Compulsive or Related Disorder, Trauma- or Stressor-Related Disorder, Sleep Disorder, and/or Externalizing Disorder, as assessed in the SCID-5-RV, are included if they also meet criteria for one of the six target disorders. Given the possibility that participants may experience a Major Depressive Episode of relatively brief duration (minimum of two weeks), those reporting Major Depressive Disorder can be included if they meet full criteria at the time of assessment, or if the disorder is in partial remission (i.e., where symptoms of the previous episode are present, but full criteria are not met; or there is a period lasting less than 2 months without any significant symptoms of a major depressive episode following the end of such an episode). Participants in this group must have no lifetime history of IBS and must not meet current diagnostic criteria for IBS or another FGID.

Irritable bowel syndrome group

Participants must meet current criteria for IBS, as assessed by the Rome IV during Phase

1 screening questionnaires [34,35]. The Rome IV assesses gastrointestinal symptom duration and delineates diagnosis by the prominent altered stool type: constipation (IBS-C), diarrhoea (IBS-D), mixed (IBS-M), or an unspecified dominance (IBS-U). Participants in this group must not meet lifetime diagnostic criteria for any of the DSM-5 disorders mentioned above (including Specific Phobia, etc).

Comorbid irritable bowel syndrome and anxiety/depression group

Consistent with measures outlined above, participants must currently meet criteria for IBS and diagnosis of at least one of the six primary anxiety and/or depressive disorders.

Control group

Participants in this group must have no lifetime history of IBS, must not meet current diagnostic criteria for IBS or another FGID, and must not meet lifetime diagnostic criteria for any of the DSM-5 disorders mentioned above (i.e., current or past).

Exclusion criteria for the primary case-control component (all groups)

Physical health

Participants are excluded from all groups if they report or present with current obesity, hypertension, cardiovascular disease, respiratory disease, diabetes mellitus, liver cirrhosis, fatty liver disease, inflammatory bowel disease, coeliac disease, other autoimmune disease, short bowel syndrome, or a history of abdominal surgery that involved cutting into the GI tract. Participants who report daily cigarette smoking, a recent colonoscopy (past 12 months), or who are pregnant/lactating are also excluded.

Mental health

Participants are excluded if they have a lifetime history of a clinical psychotic symptom or disorder, Bipolar Disorder, Somatic Symptom or Related Disorder, or over the past 12 months meet criteria for either a Feeding and Eating Disorder or an Alcohol or Substance Use Disorder (at Phase 1 questionnaires or Phase 2 SCID-5 interview).

Medication

Participants are excluded if they report use of anti-depressants, anxiolytics, other psychotropics, steroids, probiotics, prebiotics or synbiotics in the past four weeks. They are also excluded if they have a current bacterial, fungal, or viral infection, or if they have used non-steroidal anti-inflammatory drugs or antifungal medications in the past two weeks. Specific diets may be used to alleviate GI symptoms (e.g., FODMAP exclusion, casein-free), and therefore

participants without IBS are excluded if they are following a restrictive diet (i.e., do not meet IBS criteria, potentially due to alleviation of symptoms via diet).

Inclusion criteria for the ancillary questionnaire-only component

Females aged 18-40 who are ineligible for the primary case-control component, or who elect not to collect biological samples, are invited to complete the ancillary questionnaire-only component. No strict eligibility criteria apply, although all participants are required to provide informed consent and demonstrate adequate comprehension of English. All participants in the case-control component complete the same questionnaires. The phases of participation in the Bugs and Brains Study are presented in Figure 1.

Table 1. Inclusion criteria for full participation in the Bugs and Brains Study (i.e., primary case-control component)

Inclusion criteria	Controls	Depression/Anxiety	IBS	Depression/Anxiety and IBS
Female sex	✓	✓	✓	✓
Fluent in English	✓	✓	✓	✓
Capacity to provide consent	✓	✓	✓	✓
Live in Melbourne or Geelong (Australia)	✓	✓	✓	✓
Meet DSM-5 criteria for current Depression or Anxiety disorder*	✗	✓	✗	✓
OCD, specific phobia, Trauma- or stressor-related disorder, Sleep Disorder, Externalizing disorders	✗	-	✗	-
Irritable bowel syndrome (Rome IV)	✗	✗	✓	✓

Notes. ✓= inclusion criteria; ✗= exclusion criteria (current or lifetime history [as assessed by SCID-5-RV]); - = neither exclusion nor inclusion; * Specific disorders for inclusion are: Major Depressive Disorder, Persistent Depressive Disorder, Social Anxiety Disorder, Panic Disorder, Agoraphobia, and Generalized Anxiety Disorder as assessed by the Structured Clinical Interview for the Diagnostic and Statistical Manual fifth edition, research version (SCID-5-RV)

[INSERT FIGURE 1 HERE]

Phases of participation

Phase 1 - screening questionnaire (all participants)

Participants who provide informed consent are sent a Phase 1 questionnaire link online, using Qualtrics (Provo, UT). This component takes approximately 20 minutes and comprises a brief demographic overview, the Psychiatric Diagnostic Screening Questionnaire (PDSQ; [36]), and the Rome IV IBS module [34]. It also captures participants' regular diet category (e.g., omnivore, low FODMAP), medication/supplement use, and alcohol/substance use. Eligible participants are invited for Phase 2 screening. Excluded participants are invited to complete Phase 4 questionnaires as part of the ancillary component (bypassing Phases 2 and 3).

Phase 2 - assessment of current and past mental health diagnoses (SCID-5; case-control component only)

Participants invited for the primary case-control component are administered the SCID-5-RV [33] to further assess eligibility. Interviews are conducted in-person at the University of Melbourne or online via video application. Trained interviewers administer a modified version of the SCID-5-RV to assess the inclusion and exclusion psychiatric disorders, described in detail in Supplementary Materials. Researchers also collect a hospitalisation and mental health treatment history (e.g., psychotherapeutic and pharmacological) and assess functioning using the Global Assessment of Functioning (GAF) and Social and Occupational Functioning Assessment Scale (SOFAS) [37]. All interviewers hold a tertiary degree in psychological sciences or related fields, are trained in diagnostic psychological interviewing, and are required to complete Management of Clinical Aggression training (MOCA) [38] and Applied Suicide Intervention Skills Training (ASIST, LivingWorks). Fortnightly clinical meetings are conducted with the research clinical psychologist (OS) to maintain interviewer consistency and discuss any risks presented by participants. Interrater reliability will be conducted for at least 20% of interviews from eligible participants to assess diagnostic-level consistency.

Phase 3 - sample collection and anthropometrics (case-control component only)

In Phase 3 of the study, participants collect biological samples in their own home. Instructions are provided verbally and included in written form with the collection kits. On day one, a stool sample is collected; on day two, a urine sample and saliva sample are collected on awakening; on day three, another saliva sample is collected at awakening. Instructions on sterile storage are provided, and samples are immediately placed in participants' freezers following collection. A home visit is scheduled to pick up the biological samples, collect the hair sample, and take anthropometric measurements (height, weight and waist circumference), ideally within one week of collection. Samples are then transported on ice to the university and stored at -80 °C until processing.

Faecal sample. Participants collect a faecal sample at first bowel movement on day 1 of sample collection, but not if they are menstruating or sick. Participants are asked to wait at least three days after they cease menses to begin collection, and to not collect samples within one week of menstruation, due to the effect of menstrual cycling on endocrine outcomes [39]. A preliminary literature also describes an oestrogen-gut microbiota axis [40], therefore we sought to avoid peaks in oestrogen secretion that may confound microbial analyses. Prior to sample collection, participants are also asked to consume a diet 'normal for them' for at least two weeks. Approximately 2.5 g is collected into sterile faecal collection containers. Participants are instructed not to collect the faecal sample if they have very loose stools, and to wait until they return to a firmer state (i.e., Bristol stool chart type 5 or 6, if diarrhoea is frequent).

Urine sample. A mid-stream urine sample of approximately 10 to 20 mL is collected on day 2 (at first urination) into sterile urine specimen containers (the day after faecal sample collection).

Saliva sample. Participants collect two 3 mL saliva samples (by passive drool), one per day across two days (day 2 and day 3), into 10 mL Techno Plas sterile centrifuge tubes. Saliva samples are collected at awakening before eating or drinking. Participants are asked to complete a brief saliva record form.

Hair sample. A hair sample is collected by researchers at participants' homes. Hair sampling is carried out by combing and pinning the surrounding hair with clips, such that a 2 cm long by 0.5 cm wide scalp area is isolated on the posterior vertex (back of the head below the

inion; ~1 cm^2). Hair is then bound together (marking the scalp end) and cut with blunt-nose scissors as close to the scalp as possible.

Phase 4 - additional questionnaire data (all participants)

Participants receive the Phase 4 questionnaires online, via Qualtrics. Phase 4 questionnaires are completed within 24 hours of faecal sample collection. Participants in the ancillary questionnaire-only study complete Phase 4 questionnaires following Phase 1 (or whichever phase they are excluded from the case-control component). The Phase 4 questionnaires cover demographic information (e.g., country of birth), early life history (e.g., mode of birth, breastfeeding), history of gastric infections, and scales assessing psychopathology symptoms, stressful life events, early life adversity, diet, emotion regulation, and oral and physical health. The Phase 4 questionnaires take approximately 60 min. All questionnaires included in the Bugs and Brains Study are displayed in Table 2.

Table 2. Summary of questionnaire measures included in the Bugs and Brains Study

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
Demographic information	Phase 1 Health Questionnaire*	✓		
	Phase 4 Demographics, Health and Exercise Questionnaire*			✓
Emotional functioning	Difficulties in Emotion Regulation Scale (DERS) [41]			✓
Mental health and behaviour	Psychiatric Diagnostic Screening Questionnaire (PDSQ) [36]	✓		
	Global Assessment of Functioning (GAF) † [37]		✓	
	Social and Occupational Functioning Assessment Scale (SOFAS) † [37]		✓	
	Beck Depression Inventory (BDI-II) [42]			✓
	Beck Anxiety Inventory (BAI) [43]			✓
	Adult Self Report (ASR) [44]			✓
Diet	Dietary Questionnaire for Epidemiological Studies (DQES) [45]			✓
	Phase 1 Health Questionnaire* • Specific diet information (past four weeks; e.g. gluten-free, specific carbohydrates, FODMAP, vegetarian)	✓		
Exercise	Phase 4 Demographics, Health and Exercise Questionnaire*			✓
Quality of life/wellbeing	The World Health Organization quality of life assessment (WHOQOL-BREF) [46]			✓
Oral health	The World Health Organization Oral Health Questionnaire for Adults [47]			✓
Physical health /medical history	The Physical Health Questionnaire (PHQ) [48]			✓
	Phase 1 Health Questionnaire* • Current bacterial, fungal, or viral infection • Obesity, hypertension, cardiovascular disease, respiratory disease, diabetes mellitus, liver cirrhosis, fatty liver disease, coeliac disease, other autoimmune	✓		

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
	disease (e.g., rheumatoid arthritis, lupus, etc.), and polycystic ovary syndrome (PCOS). • Current pregnancy/lactation • Mode of birth • Medication (past month): anti-depressants/anti-anxiolytics, antibiotics, psychotropics, oral or injected steroids, probiotics, prebiotics, synbiotics, dietary supplements including laxatives, other Medication (past two weeks): NSAIDs, probiotics, antifungals			
Gastrointestinal health/history	Rome IV Diagnostic Questionnaire (R4DQ) [34]	✓		
	Phase 1 Health Questionnaire* • Non-surgical procedures related to the digestive system (past six months): enema, colonoscopy, gastroscopy Inflammatory bowel disease (e.g., ulcerative colitis, Crohn's disease), short bowel syndrome, history of abdominal surgery (e.g., appendectomy, ileostomy, bowel surgery, caesarean section, etc.),	✓		
	Phase 4 Demographics, Health and Exercise Questionnaire* • Lifetime history of gastric infections: Salmonella, Shigella, Campylobacter, Giardia, Virus, Other			✓
Stressful life events	The Lifetime Incidence of Traumatic Events (LITE) [49]			✓
	Childhood Trauma Questionnaire (CTQ) [50]			✓
Substance use	Phase 1 Health Questionnaire* • Alcohol (past month) • Smoking (past month): tobacco cigarettes, vape, e-cigarettes containing nicotine Illicit drug use (past month)	✓		

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
	• Dietary Questionnaire for Epidemiological Studies (DQES) [45]			✓

Notes. * = designed specifically for the Bugs and Brains Study; † = measure completed by researchers, rather than participants; ‡ = modified version utilised, see Supplementary Materials; NSAIDS= nonsteroidal anti-inflammatory drugs; SCID-5-RV = Structured Clinical Interview for the Diagnostic and Statistical Manual fifth edition, research version

Gut and oral microbiota sequencing

Amplicon 16S ribosomal ribonucleic acid (rRNA) gene sequencing will be used to estimate participants' microbiota from a faecal sample (gut microbiota) and a saliva sample (oral microbiota). Genomic DNA will be extracted using DNeasy PowerSoil® HTP kit (QIAGEN) and amplified using universal prokaryotic 515F and 806R primers targeting the V4 region of the 16S gene [51]. Sequences will be generated using paired-end sequencing on the Illumina MiSeq platform. Should additional funding be secured, shotgun metagenomic sequencing will be performed.

Metabolomics

Nuclear magnetic resonance spectroscopy (NMR) will be run on faecal and urine samples to quantify metabolites. If additional funding is obtained, mass spectrometry metabolite assays will also be conducted.

Sample Preparation

Faecal water. A small proportion of the frozen faecal sample (0.5-1 g) will be homogenized by vortex in deuterated saline (0.9% NaCl) at 1:5 w/v before being centrifuged (3,200 × g) at 4 °C for 15 min. Faecal water will be syringe filtered (0.22 µm, 33 mm diameter) and dispensed into 700 µL aliquots.

Urine. Frozen urine samples will be thawed in ice baths, syringe filtered (0.22 µm, 33 mm diameter) and each dispensed into 700 µL aliquots.

Metabolite extraction. Faecal and urine samples will be prepared for NMR analysis using a liquid-liquid extraction technique [52]. A 250 µL aliquot of each faecal water/urine sample will be added to 250 µL ice-cold deuterated chloroform and 250 µL ice-cold deuterated methanol and mixed by vortex before being left on ice for 15 min. Samples are then centrifuged (16,000 × g) at 4 °C for 10 min to produce a biphasic mixture with a hydrophilic phase of water/deuterated methanol containing polar metabolites and lipophilic phase of deuterated chloroform containing lipophilic compounds.

Nuclear magnetic resonance spectroscopy (NMR)

A 297 µL sample of the top hydrophilic layer will be added to equal volume of 200 mM sodium phosphate in $^{2}H_2O$ (pH 7.4) and 66 µL of $^{2}H_2O$ containing 5 mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt and 0.2% (w/v) sodium azide to make a total of 660 µL. The

sample will be mixed by vortex, centrifuged at 16,000 × g for 1 min, and 550 µL of supernatant transferred to a 7-inch 5-mm 507-grade Bruker NMR tube for NMR analysis.

All NMR spectra of faecal water and urine will be collected using Bruker 600 MHz spectrometer set to a constant temperature of 300 K. Standard 1D ^{1}H spectra will be acquired for faecal water using 1D NOESY pulse sequence (recycle delay-90°-t_1-90°-t_m-acquire) with t_1 corresponding to 3 µs, t_m to 10 ms and the 90° pulses optimised. Water suppression will be achieved by pre-saturation of the water signal during t_m and the recycle delay (4 s). Each spectrum will be collected over 64 K data points and 256 scans with spectral width of 20 ppm [53].

Urine spectra are acquired using the same 1D NOESY pulse sequence where recycle delay corresponded to 2 s, t_1 to 3 µs and t_m to 10 ms. Each spectrum will be collected over 32 K data points and 64 scans with spectral width of 20 ppm [54]. Using the Chenomx NMR Suite Professional software package, the free induction decay is multiplied by 0.3 Hz exponential line broadening and zero-filled before Fourier transformation. Spectra are manually phased, baseline corrected and referenced to DSS with a chemical shift of 0 ppm. The compound libraries in the Chenomx software will be used to identify and quantitate metabolites in the NMR 1D ^{1}H spectra based on their characteristic chemical shifts using 5 mM DSS as an internal chemical shift reference.

Immune and endocrine assays

Hormones will be assayed from hair and saliva samples to estimate longer and shorter-term endocrine functioning, respectively. The hormones cortisol, DHEA and testosterone will be assayed to provide an indication of hypothalamic-pituitary-adrenal axis secretion. Systemic inflammation will be estimated using the acute phase C-reactive protein (CRP), as previously described [55]. Assays for hormones and CRP will be conducted using enzyme-linked immunosorbent assay kits and performed in duplicate, following manufacturer protocols.

Pre-processing of bacterial sequencing data

Sequencing reads will be quality filtered, trimmed, denoised and merged using QIIME2 [56]. The DADA2 denoising pipeline will be used for correcting sequence errors and identifying amplicon sequence variants (ASVs, i.e., sequences not containing errors) [57]. ASVs will be taxonomically assigned using a Naïve-Bayes classifier against the most recent version of the

Human Oral Microbiome Database (saliva) and SILVA databases (faecal). Sequences will be aligned using MAFFT [58], and phylogeny built using FastTree 2 [59].

Sample size calculation

A sample of 132 would provide 80% power at $p = 0.01$ to detect an effect size of 0.35 when comparing outcomes across the four groups (i.e., anxiety/depression, IBS, comorbidity, controls). This estimate is conservative given the effect sizes reported in related research, and our sample size of 160 exceeds this recommendation.

Data analysis

Proposed analyses will be discipline-specific (microbial, metabolomics, immune and endocrine), but will also utilise systems biology to integrate the wide range of acquired biological, health, and psychosocial data. General linear models will investigate the relationships between mental health, dietary patterns, exercise, GI health, oral health, early life adversity, substance use (including alcohol), and medical history in the questionnaire-only ancillary study. These analyses may inform necessary metadata to consider in analyses of the primary case-control component. Sources of inter-study variation identified in previous investigations of the gut microbiota will be examined [31, 60].

A primary aim of the case-control component involves characterising and comparing the microbiota and metabolome in the four participant groups (i.e., anxiety/depression, IBS, comorbidity, controls). Between and within-sample diversity will be conducted using alpha diversity (Shannon and Simpson indices, Faith's phylogenetic diversity, observed number of ASVs [richness]), and beta diversity (PCoA using weighted and unweighted UniFrac distances, examined statistically using PERMANOVA). Differential abundance of microbial taxa will be examined at several levels of the taxonomy (phylum, order, family, genus levels) using packages suited to compositional data: ANCOM-BC [61] and MaAsLin2 [62], correcting for multiple comparisons (Benjamini Hochberg False Discovery Rate). Associations between taxa abundance and metadata will also be analysed using these packages. All statistical analyses and graphical representations will be conducted using R, with packages that enable multivariate modelling and visualizations (e.g., vegan [63]; phyloseq [64]; ggplot2 [65]; ANCOM-BC [61]; MaAsLin2 [62]). It will also integrate the wide range of biological data and adopt a systems biology approach using the package mixOmics [66].

Ethics and dissemination

Ethical approval for this project was granted by the University of Melbourne Human Research Ethics Committee (#1749221). After reading our Plain Language Statement, a comprehensive consent procedure is conducted via telephone with all participants. The voluntary nature of the study is explained, including the limits of confidentiality, the secure and de-identified nature of all data, and the right to withdraw at any time without prejudice or explanation. Verbal consent is recorded at the end of the first phone call, should the participant wish to begin their participation. Written consent is obtained from participants in the case-control component following sample collection.

Patient and public involvement

There were no funds or time allocated for patient and public involvement, therefore we were unable to engage patients in study design and recruitment. We have invited patients to assist with the development of our dissemination strategy.

Progress to date

Of the 775 participants who have completed the Phase 1 questionnaire since the end of 2017, 211 have met screening criteria and were invited to the SCID-5-RV interview (Phase 2). At interview, 53 participants met exclusion criteria relating to psychiatric diagnosis. Several participants were unable to complete kit collection, moved locations, or began exclusionary medications. Accordingly, 140 of 160 participants have completed the case-control component. A total of 352 of the planned 1000 participants have completed the ancillary questionnaire-only component (Phases 1 and 4).

Discussion

The high rates of co-occurrence between anxiety, depression and IBS remain poorly understood, although interactions between host physiology and the microbiota provide a promising avenue to investigate possible shared underlying mechanisms. The Bugs and Brains Study will be the first research project to investigate and compare the oral and gut microbiota and metabolome of participants with anxiety/depression, IBS, comorbid anxiety/depression and IBS, and controls, as assessed by clinical diagnosis and gold standard criteria. Moreover, it will analyse hypothesised underlying mechanisms, including immune, endocrine, and microbial factors, and maintain strict inclusion and exclusion criteria. The questionnaire-only study will examine correlates of GI and mental health symptoms that have been insufficiently examined to date, including diet, exercise, oral health, substance use, medical history, early life adversity and

psychosocial factors. Via a comprehensive analysis of symptom-based questionnaire data and detailed characterisation of physiological, microbial and psychological profiles, the Bugs and Brains Study may inform the development of targeted interventions for the millions of individuals living with anxiety, depression and/or IBS.

Author contributions

JGS conceived the study, with critical input from NH, OS, SW, CAS, BC, SD, PG, NOS, CB, KH, and DE. CAS, JGS and KH wrote the manuscript, and CAS devised the analytical strategy. All authors critically revised and approved the final manuscript.

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Competing interests statement: None declared

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Figure caption

Figure 1. Phases of participation in the Bugs and Brains Study. Participants eligible for the primary case-control component at Phases 1 and 2 complete all phases of the study within a month, including biological sample collection ($N = 160$; $n = 40$ with IBS, $n = 40$ with anxiety and/or depressive disorder, $n = 40$ with IBS and anxiety and/or depressive disorder, $n = 40$ controls with no lifetime history of either IBS or an anxiety/depressive disorder). Participants who decline sample collection, or are not eligible for the primary component, complete the questionnaire-only ancillary component (Phases 1 and 4; $N = 1000$). GAF = Global Assessment of Functioning; IBS = irritable bowel syndrome; SOFAS = Social and Occupational Functioning Assessment Scale; Structured Clinical Interview for the Diagnostic and Statistical Manual (DSM) 5th edition (Research version [SCID-5-RV]). Created with BioRender.com.

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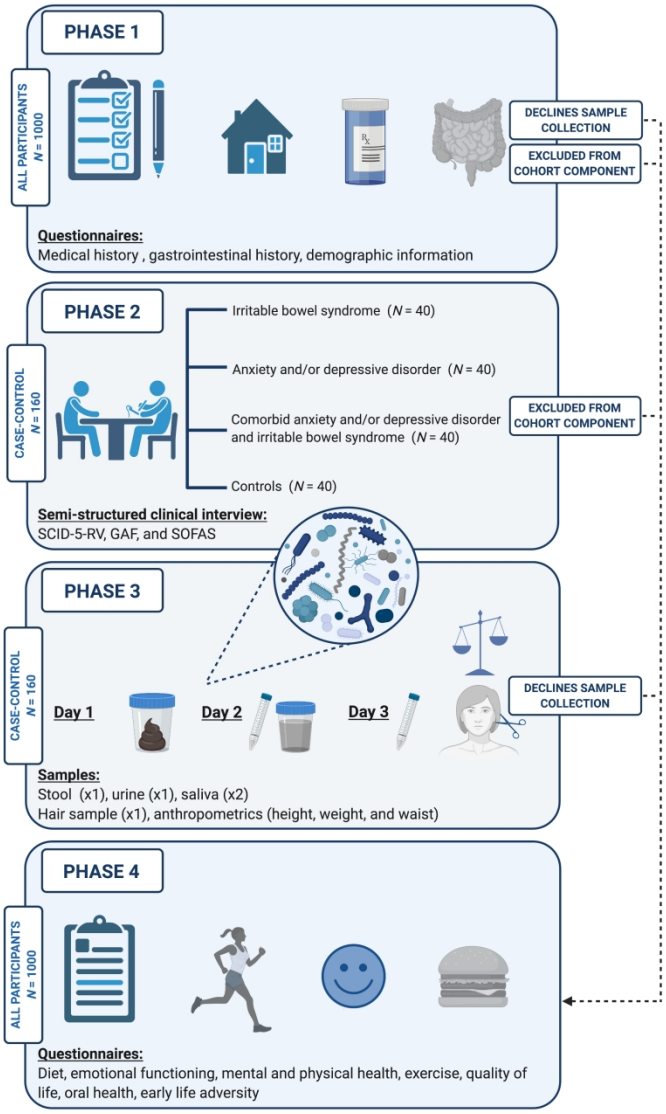

Figure 1. Phases of participation in the Bugs and Brains study. Participants eligible for the primary case-control component at Phases 1 and 2 complete all phases of the study within a month, including biological sample collection (N = 160; n = 40 with IBS, n = 40 with anxiety and/or depressive disorder, n = 40 with IBS and anxiety and/or depressive disorder, n = 40 controls with no lifetime history of either IBS or an anxiety/depressive disorder). Participants who decline sample collection or are not eligible for the primary component complete the questionnaire-only ancillary component (Phases 1 and 4; N = 1000). GAF = Global Assessment of Functioning; IBS = irritable bowel syndrome; SOFAS = Social and Occupational Functioning Assessment Scale; Structured Clinical Interview for the Diagnostic and Statistical Manual (DSM) 5th edition (Research version [SCID-5-RV]). Created with BioRender.com.

Table S1. Disorders assessed by the modified SCID-5-RV utilised by the Bugs and Brains study

Disorder	Duration assessed at interview	Eligibility Status
Bipolar and related disorders		
Bipolar I Disorder	Lifetime and past month (current)	✗ Exclusion (all groups)
Bipolar II Disorder	Lifetime and past month (current)	
Cyclothymic Disorder	Past 2 years (current)	
Other Specified Bipolar Disorder	Lifetime and past month (current)	
Bipolar Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Bipolar Disorder	Lifetime and past month (current)	
Depressive disorders		
Major Depressive Disorder	Lifetime and past month (current)	✓ Current = Inclusion (MH and Comorbid) - Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Persistent Depressive Disorder	Lifetime and past two years (current)	
Premenstrual Dysphoric Disorder	Past 12 months (current)	- Lifetime = Neither inclusion nor exclusion
Other Specified Depressive Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Depressive Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Depressive Disorder	Lifetime and past month (current)	
Schizophrenia and other psychotic disorders		
Clinical psychotic symptoms	Lifetime and current	✗ Exclusion (all groups)
Substance use disorders		
Alcohol	Past 12 months (current)	✗ Exclusion (all groups)

Disorder	Duration assessed at interview	Eligibility Status
Sedative-Hypnotic-Anxiolytic	Past 12 months (current)	
Cannabis	Past 12 months (current)	
Stimulants/Cocaine	Past 12 months (current)	
Opioids	Past 12 months (current)	
Phencyclidine (PCP)	Past 12 months (current)	
Other Hallucinogens	Past 12 months (current)	
Inhalants	Past 12 months (current)	
Other/Unknown	Past 12 months (current)	
Anxiety disorders		
Panic Disorder	Lifetime and past month (current)	✓ Current = Inclusion (MH and Comorbid)
Agoraphobia	Lifetime and past six months (current)	
Social Anxiety Disorder	Lifetime and past six months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Generalized Anxiety Disorder	Lifetime and past six months (current)	✗ Lifetime = Exclusion (Controls and IBS)
Specific Phobia	Lifetime and past six months (current)	
Other Specified Anxiety Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Anxiety Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Anxiety Disorder	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)
Obsessive-compulsive and related disorders		
Obsessive Compulsive Disorder	Lifetime and past month (current)	
Other Specified Obsessive Compulsive and Related Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Obsessive-Compulsive and Related Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Obsessive-Compulsive and Related Disorder	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)

Disorder	Duration assessed at interview	Eligibility Status
Sleep-wake disorders		
Insomnia Disorder	Past three months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Hypersomnolence Disorder	Past three months (current)	
Substance-Induced Sleep Disorder	Past three months (current)	
Feeding and Eating Disorders		
Anorexia Nervosa	Past 12 months and past 3 months (current)	✗ Exclusion (all groups)
Bulimia Nervosa	Past 12 months and past 3 months (current)	
Binge Eating Disorder	Past 12 months and past 3 months (current)	
Other Specified Feeding or Eating Disorder	Past 12 months and past 3 months (current)	
Somatic Symptom and Related Disorders		
Somatic Symptom Disorder	Past 6 months (current)	✗ Exclusion (all groups) IBS-related symptoms not included for IBS and comorbid groups
Illness Anxiety Disorder	Past 6 months (current)	
Externalizing Disorders		
Adult Attention-deficit Hyperactivity Disorder	Past 6 months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Trauma- and Stressor-Related Disorders		
Acute Stress Disorder	Past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Posttraumatic Stress Disorder	Lifetime and past month (current)	
Adjustment Disorder	Past 6 months (current)	

Disorder	Duration assessed at interview	Eligibility Status
Other Specified Trauma- and Stressor-Related Disorder	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)

Notes. MH = mental health anxiety/depression group; Comorbid = comorbid IBS and anxiety/depression group; IBS = irritable bowel syndrome

Description of the Modified Psychiatric Diagnostic Screening Questionnaire

The Psychiatric Diagnostic Screening Questionnaire (PDSQ) will be modified for the Bugs and Brains Study to screen for the likely presence/absence of mental health diagnoses that relate to the inclusion and exclusion criteria of this study, particularly Major Depressive Disorder, anxiety disorders, substance use disorders, psychotic symptoms, and somatic disorders

The PDSQ has been modified as follows:

- 6 items relating to suicidal ideation and behavior have been removed. Suicidal ideation and behavior will be assessed by a research assistant during the SCID-5-RV diagnostic interview in Phase 2, and in Phase 4 self-report questionnaires.
- Questions relating to Post-Traumatic Stress symptoms and Obsessive Compulsive behaviours will be excluded as information pertaining to these disorders is not required for inclusions/exclusion criteria.
- Additional questions relating to mania symptoms have been added to assess for likely diagnosis of Bipolar disorders.
- Two additional questions assessing Persistent Depressive Disorder were added.
- An additional question assessing Anorexia Nervosa was added.
- For each diagnostic category, a question assessing lifetime history of experience of several symptoms from that diagnostic category has been added.

BMJ Open

Protocol: Bugs and Brains, the Gut and Mental Health Study - A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome

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Primary Subject Heading:	Mental health
Secondary Subject Heading:	Dentistry and oral medicine, Gastroenterology and hepatology, Genetics and genomics, Immunology (including allergy), Nutrition and metabolism
Keywords:	MICROBIOLOGY, Anxiety disorders < PSYCHIATRY, Depression & mood disorders < PSYCHIATRY, Functional bowel disorders < GASTROENTEROLOGY, PSYCHIATRY

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Protocol: Bugs and Brains, the Gut and Mental Health Study - A mixed-methods study investigating microbiota composition and function in anxiety, depression and irritable bowel syndrome

Carra A. Simpson[1, 2], Orli S. Schwartz[3], Djamila Eliby[1, 2], Catherine A. Butler[4], Katherine Huang[5], Neil O'Brien-Simpson[4], Bridget L. Callaghan[2, 6], Stuart G. Dashper[4], Paul R. Gooley[5], Sarah Whittle[1, 2], Nick Haslam[1], & Julian G. Simmons[1, 2]

[1] Melbourne School of Psychological Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, VIC, 3010, Australia.

[2] Melbourne Neuropsychiatry Centre, Department of Medicine, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne and Melbourne Health, VIC, 3010, Australia.

[3] Orygen, The National Centre of Excellence in Youth Mental Health; Centre for Youth Mental Health, The University of Melbourne, VIC, 3010, Australia.

[4] Centre for Oral Health Research, Melbourne Dental School, Bio21 Institute, The University of Melbourne, VIC, 3010, Australia

[5] Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, VIC, 3010, Australia

[6] The Department of Psychology, The University of California, Los Angeles, 90503

Correspondence: Carra A. Simpson, https://orcid.org/0000-0002-8281-5881

Melbourne School of Psychological Sciences, 12th floor Redmond Barry Building, The University of Melbourne, Parkville, VIC 3010, Australia.

Email: carra.simpson@unimelb.edu.au, Phone: +61 03 8344 1845

Declarations of interest: None

Abstract

Introduction: Research has highlighted relationships between the microorganisms that inhabit our gastrointestinal tract (oral and gut microbiota) with host mood and gastrointestinal functioning. Mental health disorders and functional gastrointestinal disorders co-occur at high rates, although the mechanisms underlying these associations remain unclear. The Bugs and Brains study aims to investigate complex relationships between anxiety/depression and irritable bowel syndrome (IBS) in two ways. Firstly, its primary component will compare the gut and oral microbiota in females with anxiety/depression and/or IBS relative to controls, and investigate underlying physiological, endocrine, and immune factors, as well as associations with diet and psychosocial factors. In an ancillary component, the study will also investigate gastrointestinal and mental health symptoms in a larger sample, and explore relationships with diet, exercise, oral health, substance use, medical history, early life adversity, and psychosocial factors.

Methods and analysis: The Bugs and Brains study aims to recruit 160 females to the primary component: i) 40 controls; ii) 40 participants with a depressive/anxiety disorder, but no IBS; iii) 40 participants with IBS, but no depressive/anxiety disorder; and iv) 40 participants with both depressive/anxiety disorder and IBS. Participation is completed within one month, and involves comprehensive questionnaires, anthropometrics, a diagnostic clinical interview, collection of two saliva samples, and stool, urine, and hair samples. This study aims to use a systems biology approach to characterise oral and gut microbial composition and function using 16S rRNA gene sequencing and nuclear magnetic resonance spectroscopy. As part of the ancillary component, it will collect questionnaire data from 1000 females aged 18-40 years, capturing mental health, gastrointestinal health, oral health, diet, and psychosocial factors. **Ethics and dissemination:** Approval was granted by the University of Melbourne Human Research Ethics Committee (#1749221). All participants voluntarily provided informed consent. Results will be published in peer-reviewed journals and presented at scientific conferences.

Keywords: Gut microbiota, microbiome, depression, anxiety, irritable bowel syndrome

Article summary

Strengths and limitations

- The Bugs and Brains Study will compare oral and gut microbiota composition and function using next generation sequencing and nuclear magnetic resonance spectroscopy in individuals with anxiety/depression, IBS, both anxiety/depression and IBS, and controls, as defined by clinical diagnosis and strict inclusion criteria.
- It will assess diet, medical history, gastrointestinal health, early life adversity, mental and physical health symptoms using detailed questionnaires and clinical interview, and investigate associations between symptoms, microbiota, metabolome, and the immune and endocrine systems.
- It will also investigate gastrointestinal and mental health symptoms in a large sample, and examine relationships with diet, exercise, oral health, substance use, medical history, early life adversity, and psychosocial factors.
- Due to the possible effects of host sex, age and substance use on microbial outcomes, this study chose to recruit females aged 18 to 40 who were non-smokers, medication free, and without a substance abuse disorder; the generalisability of this study is therefore limited to this specific population.
- This study is cross-sectional in nature and employs 16S rRNA gene sequencing; future research should investigate these associations using deeper resolution sequencing (i.e., shotgun metagenomics), examine relationships longitudinally to disentangle causes and effects, and extend investigations to male participants.

Introduction

Microorganisms have co-existed alongside humans throughout our evolution, with between 20 and 30 trillion microbial cells occupying ecological niches within and upon the human body. It is therefore unsurprising that bacteria residing throughout the gastrointestinal (GI) tract are implicated in host endocrine, immune, and nervous system functioning [1]. Microbes produce neuroactive compounds, including catecholamines (e.g., noradrenaline, dopamine), GABA, histamine, serotonin, and acetylcholine, each of which plays a critical role in human physiological functioning [1,2]. Multiple GI bacteria and their metabolic products also facilitate peristaltic waves to aid with digestion [3], contribute to fermentation of dietary carbohydrates, maintain host membrane function, and produce essential amino acids and vitamins [2,4]. Resident bacterial species also prime the immune system in the early stages of life, teaching our bodies what is 'foreign' rather than commensal [2]. The gut microbiota (i.e., the collection of microorganisms in the GI tract) is therefore essential to normal development.

As we come to appreciate the essential role microorganisms play in host physiology, so too have we opened a novel direction for investigation in conditions with no current clear aetiology. Among these are irritable bowel syndrome (IBS), depression and anxiety disorders. IBS is the most common functional gastrointestinal disorder, affecting between 7 to 21% of individuals worldwide [5]. Similarly, depression remains the highest contributor to non-fatal health burden, and approximately 264 million individuals live with an anxiety disorder globally [6]. These three conditions frequently co-occur [7]: approximately 84% of patients with IBS also have a depressive disorder and 44% an anxiety disorder [8,9]. Moreover, 45% of patients with anxiety and 30% of patients with a depressive disorder develop IBS [7,10,11]. The mechanisms underlying this comorbidity remain unclear.

The complex interplay between host physiology and microbiota promises novel insight into these conditions. Research has revealed that anxiety, depression and IBS share several gut microbial alterations, including decreased overall microbial diversity and a higher relative abundance of pro-inflammatory and toxigenic species [12–15]. Moreover, research indicates alterations may exist along a continuum, in that participants with comorbid depression and IBS have gut microbiota alterations simply more pronounced than those presenting with one disorder, including higher Proteobacteria, *Prevotella/Prevotellaceae, Bacteroides* and lower *Lachnospiraceae* [10,16–18]. These implicated taxa further build on the evidence of altered

microbial and host immune system interactions, as the taxa reported to have a higher relative abundance in anxiety, depression and IBS are associated with inflammation, whereas a loss of species that secrete anti-inflammatory metabolic products has also been observed [14]. No studies have compared the gut microbiota of participants with anxiety relative to IBS, although studies have identified correlations between bacterial taxa and anxiety symptoms among IBS patients [18–20].

Although research in this area has primarily investigated the composition of the gut microbiota, the entire length of the GI tract harbours resident microorganisms. At its entryway, the microbiota of the oral cavity is the second most diverse community in the human body [21], and research indicates that microbial representation in the mouth is predictive of composition in the lower GI tract [22]. Oral bacteria can also translocate throughout the body, with significant effects on GI and systemic health [23]. No known studies have examined the oral microbiota in clinical anxiety, depression or IBS; however, our existing research observed associations between oral microbiota composition with anxiety and depression symptoms [24]. Characterisation of the oral microbiota in clinical mental health and IBS cohorts therefore warrants investigation.

Several confounding factors have been neglected in the small literature investigating the microbiota in IBS, anxiety, and depression. Due to their effects on microbial composition, it seems reasonable to consider psychotropic medication use [25, 26], smoking [27], alcohol consumption [27], IBS subtype and severity [15,18], as well as biological sex [28]. Diet, and its interaction with psychosocial factors, is another source of gut microbiota variation that has been insufficiently considered in existing research [29,30]. Finally, the extant literature has not often utilised gold-standard diagnostic measures to examine mental health (i.e., clinical interviews), and studies have examined microbiota composition but neglected microbial function [31].

Aims and objectives

Bugs and Brains, the Gut and Mental Health Study (The Bugs and Brains Study) aims to characterise the microbial and physiological profiles in anxiety, depression, and IBS; to examine associations between symptoms and confounding variables; and, to investigate varied contributors to disorder presentation (both across and within disorders). In this primary case-control component, key questions relate to the similarities and differences across bacterial, metabolic, endocrine, and immune indices, in a well-controlled cohort. Given the associations

between biological sex and the microbiota, this study chose to recruit females only and exclude current smokers or participants with a substance abuse disorder. Possible sources of inter-study variation in previous investigations will be considered (i.e., symptom severity, medication use, diet). In a large ancillary questionnaire-only component, this research will also investigate how GI and mental health symptoms are associated with diet, exercise, oral health, substance use, medical history, early life adversity, and psychosocial factors. Understanding shared and distinct microbiota relationships in IBS, anxiety and depression, particularly within a well-characterised sample, has implications in resolving the directionality of co-morbidity, and in informing future targeted clinical interventions.

Methods and analysis

Participants

Primary case-control component

Females aged 18 to 40 will be assessed for participation in all phases of the Bugs and Brains Study. Recruitment for the study began in October 2017 and all phases are expected to be completed by December 2021. The primary case-control component will include 160 community-based female volunteers aged 18 to 40 who meet eligibility criteria for one of four groups:

i) 40 controls
ii) 40 participants with a depressive/anxiety disorder, but no IBS
iii) 40 participants with IBS, but no depressive/anxiety disorder; and
iv) 40 participants with depressive/anxiety disorder and IBS

The study chose to recruit only participants of the female sex due to significantly elevated rates of all three conditions relative to biological males [6,32]. All participants are being recruited through online and printed advertisements at the University of Melbourne and using paid Facebook ads. All participants provide informed consent and demonstrate adequate comprehension of English. Participants who complete biological samples are monetarily reimbursed for their time, and participants who complete the ancillary questionnaire-only component are reimbursed with a personalised dietary analysis.

Ancillary questionnaire-only component

To investigate associations in a larger sample, the ancillary component aims to collect

questionnaire data from 1000 females aged 18 to 40, capturing mental health, diet, exercise, gastrointestinal health, oral health, substance use, medical history, emotion regulation, stress, early life adversity, and medical history.

Inclusion criteria for the case-control component

Participants in the primary component are required to live in Greater Melbourne or Geelong (unless willing to travel to the site of sample collection: Melbourne, Australia). Inclusion and exclusion criteria are presented in Table 1. All participants initially complete the Phase 1 screening questionnaire to assess their likely eligibility for one of the four groups. If participants meet screening criteria, they are invited to take part in a psychiatric diagnostic interview to confirm eligibility (Phase 2). All ineligible participants, including those who prefer not to complete sample collection, are invited to complete the ancillary questionnaire-only component.

Depression and anxiety group

Participants in this group must meet current diagnostic criteria for at least one of six depressive and/or anxiety disorders at Phase 2, as assessed by the Structured Clinical Interview for DSM-5 Research Version (SCID-5-RV; [33]). Specific disorders for inclusion are: Major Depressive Disorder, Persistent Depressive Disorder, Social Anxiety Disorder, Panic Disorder, Agoraphobia, and Generalized Anxiety Disorder. Participants with a comorbid Specific Phobia, Obsessive-Compulsive or Related Disorder, Trauma- or Stressor-Related Disorder, Sleep Disorder, and/or Externalizing Disorder, as assessed in the SCID-5-RV, are included if they also meet criteria for one of the six target disorders. Given the possibility that participants may experience a Major Depressive Episode of relatively brief duration, participants with Major Depressive Disorder will also be included if a clinical episode is in partial remission (i.e., although full diagnostic criteria are not met, symptoms of the immediately preceding episode are present and have not fully resolved at the time of assessment). Participants in this group must have no lifetime history of IBS and must not meet current diagnostic criteria for IBS or another functional gastrointestinal disorder.

Irritable bowel syndrome group

Participants must meet current criteria for IBS, as assessed by the Rome IV during Phase 1 screening questionnaires [34,35]. The Rome IV assesses gastrointestinal symptom duration and

delineates diagnosis by the prominent altered stool type: constipation (IBS-C), diarrhoea (IBS-D), mixed (IBS-M), or an unspecified dominance (IBS-U). Participants in this group must not meet lifetime diagnostic criteria for any of the DSM-5 disorders mentioned above (including Specific Phobia, etc).

Comorbid irritable bowel syndrome and anxiety/depression group

Consistent with measures outlined above, participants must currently meet criteria for IBS and diagnosis of at least one of the six primary anxiety and/or depressive disorders.

Control group

Participants in this group must have no lifetime history of IBS, must not meet current diagnostic criteria for IBS or another functional gastrointestinal disorder, and must not meet lifetime diagnostic criteria for any of the DSM-5 disorders mentioned above (i.e., current or past).

Exclusion criteria for the primary case-control component (all groups)

Physical health

Participants are excluded from all groups if they report or present with current obesity, hypertension, cardiovascular disease, respiratory disease, diabetes mellitus, liver cirrhosis, fatty liver disease, inflammatory bowel disease, coeliac disease, other autoimmune disease, short bowel syndrome, or a history of abdominal surgery that involved cutting into the GI tract. Participants who report daily cigarette smoking, a recent colonoscopy (past 12 months), or who are pregnant/lactating are also excluded.

Mental health

Participants are excluded if they have a lifetime history of clinical psychotic symptoms or disorder, Bipolar Disorder, Somatic Symptom or Related Disorder, or over the past 12 months meet criteria for either a Feeding and Eating Disorder or an Alcohol or Substance Use Disorder (at Phase 1 questionnaires or Phase 2 SCID-5 interview).

Medication

Participants are excluded if they report use of anti-depressants, anxiolytics, other psychotropics, steroids, probiotics, prebiotics or synbiotics in the past four weeks or throughout the duration of their participation. They are also excluded if they have a current bacterial, fungal, or viral infection, or if they have used non-steroidal anti-inflammatory drugs or antifungal medications in the past two weeks or throughout their participation. If participants report short-

term medication use or illness, they are invited to participate when they meet eligibility criteria. Specific diets may be used to alleviate GI symptoms (e.g., FODMAP exclusion, casein-free), and therefore participants without IBS are excluded if they are following a restrictive diet (i.e., do not meet IBS criteria, potentially due to alleviation of symptoms via diet).

Inclusion criteria for the ancillary questionnaire-only component

Participants who are ineligible for the primary case-control component, or who elect not to collect biological samples, are invited to complete the ancillary questionnaire-only component. No strict eligibility criteria apply, although all participants are required to provide informed consent and demonstrate adequate comprehension of English. All participants in the case-control component complete the same questionnaires. The phases of participation in the Bugs and Brains Study are presented in Figure 1.

Table 1. Inclusion criteria for full participation in the Bugs and Brains Study (i.e., primary case-control component)

Inclusion criteria	Controls	Depression/Anxiety	IBS	Depression/Anxiety and IBS
Female sex	✓	✓	✓	✓
Fluent in English	✓	✓	✓	✓
Capacity to provide consent	✓	✓	✓	✓
Live in Melbourne or Geelong (Australia)	✓	✓	✓	✓
Meet DSM-5 criteria for current Depression or Anxiety disorder*	✗	✓	✗	✓
OCD, specific phobia, Trauma- or stressor-related disorder, Sleep Disorder, Externalizing disorders	✗	-	✗	-
Irritable bowel syndrome (Rome IV)	✗	✗	✓	✓

Notes. ✓= inclusion criteria; ✗= exclusion criteria (current or lifetime history [as assessed by SCID-5-RV]); - = neither exclusion nor inclusion; * Specific disorders for inclusion are: Major Depressive Disorder, Persistent Depressive Disorder, Social Anxiety Disorder, Panic Disorder, Agoraphobia, and Generalized Anxiety Disorder as assessed by the Structured Clinical Interview for the Diagnostic and Statistical Manual fifth edition, research version (SCID-5-RV)

[INSERT FIGURE 1 HERE]

Phases of participation

Phase 1 - screening questionnaire (all participants)

Participants who provide informed consent are sent a Phase 1 questionnaire link online, using Qualtrics (Provo, UT). This component takes approximately 20 minutes and comprises a brief demographic overview, the Psychiatric Diagnostic Screening Questionnaire (PDSQ; [36]), and the Rome IV IBS module [34]. It also captures participants' regular diet category (e.g., omnivore, low FODMAP), medication/supplement use, and alcohol/substance use. Eligible participants are invited for Phase 2 screening. Excluded participants are invited to complete Phase 4 questionnaires as part of the ancillary component (bypassing Phases 2 and 3).

Phase 2 - assessment of current and past mental health diagnoses (SCID-5; case-control component only)

Participants invited for the primary case-control component are administered the SCID-5-RV [33] to further assess eligibility. Interviews are conducted in-person at the University of Melbourne or online via video application. Trained interviewers administer a modified version of the SCID-5-RV to assess the inclusion and exclusion psychiatric disorders, described in detail in Supplementary Materials. Researchers also collect a hospitalisation and mental health treatment history (e.g., psychotherapeutic and pharmacological) and assess functioning using the Global Assessment of Functioning (GAF) and Social and Occupational Functioning Assessment Scale (SOFAS) [37]. All interviewers hold a tertiary degree in psychological sciences or related fields, are trained in diagnostic psychological interviewing, and are required to complete Management of Clinical Aggression training (MOCA) [38] and Applied Suicide Intervention Skills Training (ASIST, LivingWorks). Fortnightly clinical meetings are conducted with the research clinical psychologist (OS) to maintain interviewer consistency and discuss any risks presented by participants. Interrater reliability will be conducted for at least 20% of interviews from eligible participants to assess diagnostic-level consistency.

Phase 3 - sample collection and anthropometrics (case-control component only)

In Phase 3 of the study, participants collect biological samples in their own home. Instructions are provided verbally and included in written form with the collection kits. On day one, a stool sample is collected; on day two, a urine sample and saliva sample are collected on awakening; on day three, another saliva sample is collected at awakening. Instructions on sterile storage are provided, and samples are immediately placed in participants' freezers following collection. A home visit is scheduled to pick up the biological samples, collect the hair sample, and take anthropometric measurements (height, weight and waist circumference), ideally within one week of collection. Samples are then transported on ice to the university and stored at -80 °C until processing.

Faecal sample. Participants collect a faecal sample at first bowel movement on day 1 of sample collection, but not if they are menstruating or sick. Participants are asked to wait at least three days after they cease menses to begin collection, and to not collect samples within one week of menstruation, due to the effect of menstrual cycling on endocrine outcomes [39]. A preliminary literature also describes an oestrogen-gut microbiota axis [40], therefore we sought to avoid peaks in oestrogen secretion that may confound microbial analyses. Prior to sample collection, participants are also asked to consume a diet 'normal for them' for at least two weeks. Approximately 2.5 g is collected into sterile faecal collection containers. Participants are instructed not to collect the faecal sample if they have very loose stools, and to wait until they return to a firmer state (i.e., Bristol stool chart type 5 or 6, if diarrhoea is frequent).

Urine sample. A mid-stream urine sample of approximately 10 to 20 mL is collected on day 2 (at first urination) into sterile urine specimen containers (the day after faecal sample collection).

Saliva sample. Participants collect two 3 mL saliva samples (by passive drool), one per day across two days (day 2 and day 3), into 10 mL Techno Plas sterile centrifuge tubes. Saliva samples are collected at awakening before eating or drinking. Participants are asked to complete a brief saliva record form.

Hair sample. A hair sample is collected by researchers at participants' homes. Hair sampling is carried out by combing and pinning the surrounding hair with clips, such that a 2 cm long by 0.5 cm wide scalp area is isolated on the posterior vertex (back of the head below the

inion; ~1 cm^2). Hair is then bound together (marking the scalp end) and cut with blunt-nose scissors as close to the scalp as possible.

Phase 4 - additional questionnaire data (all participants)

Participants receive the Phase 4 questionnaires online, via Qualtrics. Phase 4 questionnaires are completed within 24 hours of faecal sample collection. Participants in the ancillary questionnaire-only study complete Phase 4 questionnaires following Phase 1 (or whichever phase they are excluded from the case-control component). The Phase 4 questionnaires cover demographic information (e.g., country of birth), early life history (e.g., mode of birth, breastfeeding), history of gastric infections, and scales assessing psychopathology symptoms, stressful life events, early life adversity, diet, emotion regulation, and oral and physical health. The Phase 4 questionnaires take approximately 60 min. All questionnaires included in the Bugs and Brains Study are displayed in Table 2.

Table 2. Summary of questionnaire measures included in the Bugs and Brains Study

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
Demographic information	Phase 1 Health Questionnaire*	✓		
	Phase 4 Demographics, Health and Exercise Questionnaire*			✓
Emotional functioning	Difficulties in Emotion Regulation Scale (DERS) [41]			✓
Mental health and behaviour	Psychiatric Diagnostic Screening Questionnaire (PDSQ) [36]	✓		
	Global Assessment of Functioning (GAF) † [37]		✓	
	Social and Occupational Functioning Assessment Scale (SOFAS) † [37]		✓	
	Beck Depression Inventory (BDI-II) [42]			✓
	Beck Anxiety Inventory (BAI) [43]			✓
	Adult Self Report (ASR) [44]			✓
Diet	Dietary Questionnaire for Epidemiological Studies (DQES) [45]			✓
	Phase 1 Health Questionnaire* • Specific diet information (past four weeks; e.g. gluten-free, specific carbohydrates, FODMAP, vegetarian)	✓		
Exercise	Phase 4 Demographics, Health and Exercise Questionnaire*			✓
Quality of life/wellbeing	The World Health Organization quality of life assessment (WHOQOL-BREF) [46]			✓
Oral health	The World Health Organization Oral Health Questionnaire for Adults [47]			✓
Physical health /medical history	The Physical Health Questionnaire (PHQ) [48]			✓
	Phase 1 Health Questionnaire* • Current bacterial, fungal, or viral infection • Obesity, hypertension, cardiovascular disease, respiratory disease, diabetes mellitus, liver cirrhosis, fatty liver disease, coeliac disease, other autoimmune	✓		

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
	disease (e.g., rheumatoid arthritis, lupus, etc.), and polycystic ovary syndrome (PCOS) • Current pregnancy/lactation • Mode of birth • Medication (past month): anti-depressants/anxiolytics, antibiotics, psychotropics, oral or injected steroids, probiotics, prebiotics, synbiotics, dietary supplements including laxatives; other Medication (past two weeks): NSAIDs, antifungals			
Gastrointestinal health/history	Rome IV Diagnostic Questionnaire (R4DQ) [34]	✓		
	Phase 1 Health Questionnaire* • Non-surgical procedures related to the digestive system (past six months): enema, colonoscopy, gastroscopy, inflammatory bowel disease (e.g., ulcerative colitis, Crohn's disease), short bowel syndrome, history of abdominal surgery (e.g., appendectomy, ileostomy, bowel surgery, caesarean section, etc.)	✓		
	Phase 4 Demographics, Health and Exercise Questionnaire* • Lifetime history of gastric infections: Salmonella, Shigella, Campylobacter, Giardia, Virus, Other			✓
Stressful life events	The Lifetime Incidence of Traumatic Events (LITE) [49]			✓
	Childhood Trauma Questionnaire (CTQ) [50]			✓
Substance use	Phase 1 Health Questionnaire* • Alcohol (past month) • Smoking (past month): tobacco cigarettes, vape, e-cigarettes containing nicotine	✓		
	Illicit drug use (past month)			

Category	Questionnaire	Phase 1 (Screening questionnaire)	Phase 2 (SCID-5-RV interview)	Phase 4 (During sample collection)
	• Dietary Questionnaire for Epidemiological Studies (DQES) [45]			✓

Notes. * = designed specifically for the Bugs and Brains Study; † = measure completed by researchers, rather than participants; ‡ = modified version utilised, see Supplementary Materials; NSAIDS= nonsteroidal anti-inflammatory drugs; SCID-5-RV = Structured Clinical Interview for the Diagnostic and Statistical Manual fifth edition, research version

Gut and oral microbiota sequencing

Amplicon 16S ribosomal ribonucleic acid (rRNA) gene sequencing will be used to estimate participants' microbiota from a faecal sample (gut microbiota) and a saliva sample (oral microbiota). Genomic DNA will be extracted using DNeasy PowerSoil® HTP kit (QIAGEN) and amplified using universal prokaryotic 515F and 806R primers targeting the V4 region of the 16S gene [51]. Sequences will be generated using paired-end sequencing on the Illumina MiSeq platform. Should additional funding be secured, shotgun metagenomic sequencing will be performed.

Metabolomics

Nuclear magnetic resonance spectroscopy (NMR) will be run on faecal and urine samples to quantify metabolites. If additional funding is obtained, mass spectrometry metabolite assays will also be conducted.

Sample Preparation

Faecal water. A small proportion of the frozen faecal sample (0.5-1 g) will be homogenized by vortex in deuterated saline (0.9% NaCl) at 1:5 w/v before being centrifuged (3,200 × g) at 4 °C for 15 min. Faecal water will be syringe filtered (0.22 µm, 33 mm diameter) and dispensed into 700 µL aliquots.

Urine. Frozen urine samples will be thawed in ice baths, syringe filtered (0.22 µm, 33 mm diameter) and each dispensed into 700 µL aliquots.

Metabolite extraction. Faecal and urine samples will be prepared for NMR analysis using a liquid-liquid extraction technique [52]. A 250 µL aliquot of each faecal water/urine sample will be added to 250 µL ice-cold deuterated chloroform and 250 µL ice-cold deuterated methanol and mixed by vortex before being left on ice for 15 min. Samples are then centrifuged (16,000 × g) at 4 °C for 10 min to produce a biphasic mixture with a hydrophilic phase of water/deuterated methanol containing polar metabolites and lipophilic phase of deuterated chloroform containing lipophilic compounds.

Nuclear magnetic resonance spectroscopy (NMR)

A 297 µL sample of the top hydrophilic layer will be added to equal volume of 200 mM sodium phosphate in $^{2}H_2O$ (pH 7.4) and 66 µL of $^{2}H_2O$ containing 5 mM 3-(Trimethylsilyl)-1-propanesulfonic acid-d6 sodium salt and 0.2% (w/v) sodium azide to make a total of 660 µL. The

sample will be mixed by vortex, centrifuged at 16,000 × g for 1 min, and 550 µL of supernatant transferred to a 7-inch 5-mm 507-grade Bruker NMR tube for NMR analysis.

All NMR spectra of faecal water and urine will be collected using Bruker 600 MHz spectrometer set to a constant temperature of 300 K. Standard 1D ^{1}H spectra will be acquired for faecal water using 1D NOESY pulse sequence (recycle delay-90°-t_1-90°-t_m-acquire) with t_1 corresponding to 3 µs, t_m to 10 ms and the 90° pulses optimised. Water suppression will be achieved by pre-saturation of the water signal during t_m and the recycle delay (4 s). Each spectrum will be collected over 64 K data points and 256 scans with spectral width of 20 ppm [53].

Urine spectra are acquired using the same 1D NOESY pulse sequence where recycle delay corresponded to 2 s, t_1 to 3 µs and t_m to 10 ms. Each spectrum will be collected over 32 K data points and 64 scans with spectral width of 20 ppm [54]. Using the Chenomx NMR Suite Professional software package, the free induction decay is multiplied by 0.3 Hz exponential line broadening and zero-filled before Fourier transformation. Spectra are manually phased, baseline corrected and referenced to DSS with a chemical shift of 0 ppm. The compound libraries in the Chenomx software will be used to identify and quantitate metabolites in the NMR 1D ^{1}H spectra based on their characteristic chemical shifts using 5 mM DSS as an internal chemical shift reference.

Immune and endocrine assays

Hormones will be assayed from hair and saliva samples to estimate longer and shorter-term endocrine functioning, respectively. The hormones cortisol, DHEA and testosterone will be assayed to provide an indication of hypothalamic-pituitary-adrenal axis secretion. Systemic inflammation will be estimated using the acute phase C-reactive protein (CRP), as previously described [55]. Assays for hormones and CRP will be conducted using enzyme-linked immunosorbent assay kits and performed in duplicate, following manufacturer protocols.

Pre-processing of bacterial sequencing data

Sequencing reads will be quality filtered, trimmed, denoised and merged using QIIME2 [56]. The DADA2 denoising pipeline will be used for correcting sequence errors and identifying amplicon sequence variants (ASVs, i.e., sequences not containing errors) [57]. ASVs will be taxonomically assigned using a Naïve-Bayes classifier against the most recent version of the

Human Oral Microbiome Database (saliva) and SILVA databases (faecal). Sequences will be aligned using MAFFT [58], and phylogeny built using FastTree 2 [59].

Sample size calculation

A sample of 132 would provide 80% power at $p = 0.01$ to detect an effect size of 0.35 when comparing outcomes across the four groups (i.e., anxiety/depression, IBS, comorbidity, controls). This estimate is conservative given the effect sizes reported in related research, and our sample size of 160 exceeds this recommendation.

Data analysis

Proposed analyses will be discipline-specific (microbial, metabolomics, immune and endocrine), but will also utilise systems biology to integrate the wide range of acquired biological, health, and psychosocial data. General linear models will investigate the relationships between mental health, dietary patterns, exercise, GI health, oral health, early life adversity, substance use (including alcohol), and medical history in the questionnaire-only ancillary study. These analyses may inform necessary metadata to consider in analyses of the primary case-control component. Sources of inter-study variation identified in previous investigations of the gut microbiota will be examined [31, 60].

A primary aim of the case-control component involves characterising and comparing the microbiota and metabolome in the four participant groups (i.e., anxiety/depression, IBS, comorbidity, controls). Between and within-sample diversity will be conducted using alpha diversity (Shannon and Simpson indices, Faith's phylogenetic diversity, observed number of ASVs [richness]), and beta diversity (PCoA using weighted and unweighted UniFrac distances, examined statistically using PERMANOVA). Differential abundance of microbial taxa will be examined at several levels of the taxonomy (phylum, order, family, genus levels) using packages suited to compositional data: ANCOM-BC [61] and MaAsLin2 [62], correcting for multiple comparisons (Benjamini Hochberg False Discovery Rate). Associations between taxa abundance and metadata will also be analysed using these packages. All statistical analyses and graphical representations will be conducted using R, with packages that enable multivariate modelling and visualizations (e.g., vegan [63]; phyloseq [64]; ggplot2 [65]; ANCOM-BC [61]; MaAsLin2 [62]). It will also integrate the wide range of biological data and adopt a systems biology approach using the package mixOmics [66].

Ethics and dissemination

Ethical approval for this project was granted by the University of Melbourne Human Research Ethics Committee (#1749221). After reading our Plain Language Statement, a comprehensive consent procedure is conducted via telephone with all participants. The voluntary nature of the study is explained, including the limits of confidentiality, the secure and de-identified nature of all data, and the right to withdraw at any time without prejudice or explanation. Verbal consent is recorded at the end of the first phone call, should the participant wish to begin their participation. Written consent is obtained from participants in the case-control component following sample collection.

Patient and public involvement

There were no funds or time allocated for patient and public involvement, therefore we were unable to engage patients in study design and recruitment. We have invited participants to assist with the development of our dissemination strategy.

Progress to date

Of the 775 participants who have completed the Phase 1 questionnaire since the end of 2017, 211 have met screening criteria and were invited to the SCID-5-RV interview (Phase 2). At interview, 53 participants met exclusion criteria relating to psychiatric diagnosis. Several participants were unable to complete kit collection, moved locations, or began exclusionary medications. Accordingly, 140 of 160 participants have completed the case-control component. A total of 352 of the planned 1000 participants have completed the ancillary questionnaire-only component (Phases 1 and 4).

Discussion

The high rates of co-occurrence between anxiety, depression and IBS remain poorly understood, although interactions between host physiology and the microbiota provide a promising avenue to investigate possible shared underlying mechanisms. The Bugs and Brains Study will be the first research project to investigate and compare the oral and gut microbiota and metabolome of participants with anxiety/depression, IBS, comorbid anxiety/depression and IBS, and controls, as assessed by clinical diagnosis and gold standard criteria. Moreover, it will analyse hypothesised underlying mechanisms, including immune, endocrine, and microbial factors, and maintain strict inclusion and exclusion criteria. The questionnaire-only study will examine correlates of GI and mental health symptoms that have been insufficiently examined to date, including diet, exercise, oral health, substance use, medical history, early life adversity, and

psychosocial factors. Via a comprehensive analysis of symptom-based questionnaire data and detailed characterisation of physiological, microbial and psychological profiles, the Bugs and Brains Study may inform the development of targeted interventions for the millions of individuals living with anxiety, depression and/or IBS.

Author contributions

JGS conceived the study, with critical input from NH, OS, SW, CAS, BC, SD, PG, NOS, CB, KH, and DE. CAS, JGS and KH wrote the manuscript, and CAS devised the analytical strategy. All authors critically revised and approved the final manuscript.

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Competing interests statement: None declared

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Figure caption

Figure 1. Phases of participation in the Bugs and Brains Study. Participants eligible for the primary case-control component at Phases 1 and 2 complete all phases of the study within a month, including biological sample collection ($N = 160$; $n = 40$ with IBS, $n = 40$ with anxiety and/or depressive disorder, $n = 40$ with IBS and anxiety and/or depressive disorder, $n = 40$ controls with no lifetime history of either IBS or an anxiety/depressive disorder). Participants who decline sample collection, or are not eligible for the primary component, complete the questionnaire-only ancillary component (Phases 1 and 4; $N = 1000$). GAF = Global Assessment of Functioning; IBS = irritable bowel syndrome; SOFAS = Social and Occupational Functioning Assessment Scale; Structured Clinical Interview for the Diagnostic and Statistical Manual (DSM) 5th edition (Research version [SCID-5-RV]). Created with BioRender.com.

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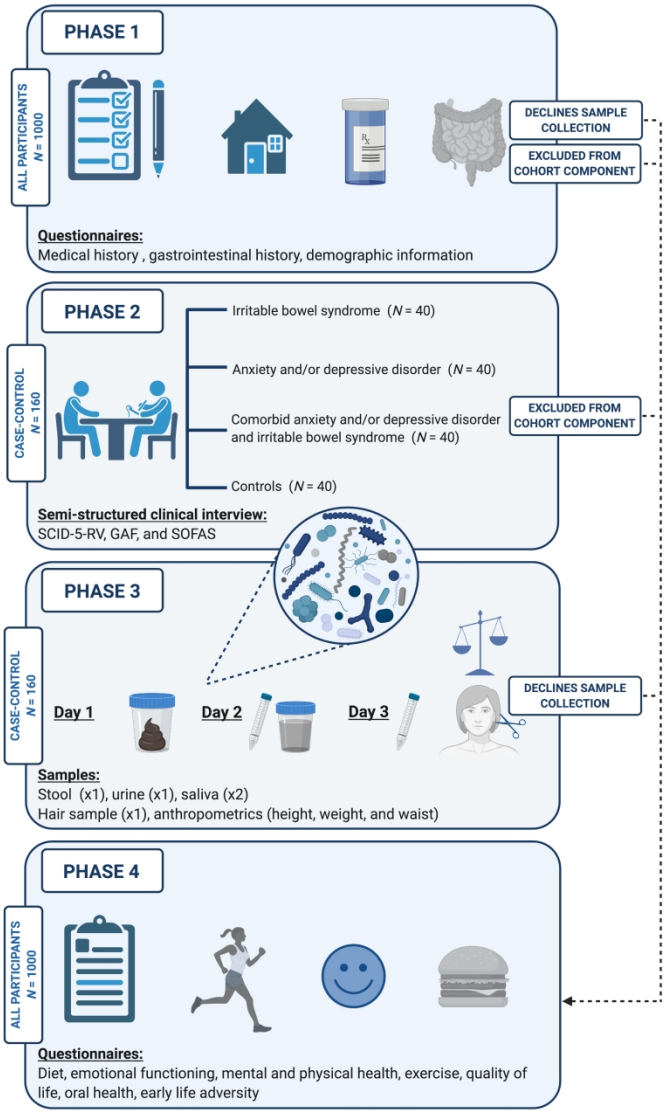

Figure 1. Phases of participation in the Bugs and Brains study. Participants eligible for the primary case-control component at Phases 1 and 2 complete all phases of the study within a month, including biological sample collection (N = 160; n = 40 with IBS, n = 40 with anxiety and/or depressive disorder, n = 40 with IBS and anxiety and/or depressive disorder, n = 40 controls with no lifetime history of either IBS or an anxiety/depressive disorder). Participants who decline sample collection or are not eligible for the primary component complete the questionnaire-only ancillary component (Phases 1 and 4; N = 1000). GAF = Global Assessment of Functioning; IBS = irritable bowel syndrome; SOFAS = Social and Occupational Functioning Assessment Scale; Structured Clinical Interview for the Diagnostic and Statistical Manual (DSM) 5th edition (Research version [SCID-5-RV]). Created with BioRender.com.

Table S1. Disorders assessed by the modified SCID-5-RV utilised by the Bugs and Brains study

Disorder	Duration assessed at interview	Eligibility Status
Bipolar and related disorders		
Bipolar I Disorder	Lifetime and past month (current)	✗ Exclusion (all groups)
Bipolar II Disorder	Lifetime and past month (current)	
Cyclothymic Disorder	Past 2 years (current)	
Other Specified Bipolar Disorder	Lifetime and past month (current)	
Bipolar Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Bipolar Disorder	Lifetime and past month (current)	
Depressive disorders		
Major Depressive Disorder	Lifetime and past month (current)	✓ Current = Inclusion (MH and Comorbid) - Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Persistent Depressive Disorder	Lifetime and past two years (current)	
Premenstrual Dysphoric Disorder	Past 12 months (current)	- Lifetime = Neither inclusion nor exclusion
Other Specified Depressive Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Depressive Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Depressive Disorder	Lifetime and past month (current)	
Schizophrenia and other psychotic disorders		
Clinical psychotic symptoms	Lifetime and current	✗ Exclusion (all groups)
Substance use disorders		
Alcohol	Past 12 months (current)	✗ Exclusion (all groups)

Disorder	Duration assessed at interview	Eligibility Status
Sedative-Hypnotic-Anxiolytic	Past 12 months (current)	
Cannabis	Past 12 months (current)	
Stimulants/Cocaine	Past 12 months (current)	
Opioids	Past 12 months (current)	
Phencyclidine (PCP)	Past 12 months (current)	
Other Hallucinogens	Past 12 months (current)	
Inhalants	Past 12 months (current)	
Other/Unknown	Past 12 months (current)	
Anxiety disorders		
Panic Disorder	Lifetime and past month (current)	✓ Current = Inclusion (MH and Comorbid)
Agoraphobia	Lifetime and past six months (current)	
Social Anxiety Disorder	Lifetime and past six months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Generalized Anxiety Disorder	Lifetime and past six months (current)	✗ Lifetime = Exclusion (Controls and IBS)
Specific Phobia	Lifetime and past six months (current)	
Other Specified Anxiety Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Anxiety Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Anxiety Disorder	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)
Obsessive-compulsive and related disorders		
Obsessive Compulsive Disorder	Lifetime and past month (current)	
Other Specified Obsessive Compulsive and Related Disorder	Lifetime and past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Obsessive-Compulsive and Related Disorder Due to Another Medical Condition	Lifetime and past month (current)	
Substance/Medication-Induced Obsessive-Compulsive and Related Disorder	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)

Disorder	Duration assessed at interview	Eligibility Status
Sleep-wake disorders		
Insomnia Disorder	Past three months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Hypersomnolence Disorder	Past three months (current)	
Substance-Induced Sleep Disorder	Past three months (current)	
Feeding and Eating Disorders		
Anorexia Nervosa	Past 12 months and past 3 months (current)	✗ Exclusion (all groups)
Bulimia Nervosa	Past 12 months and past 3 months (current)	
Binge Eating Disorder	Past 12 months and past 3 months (current)	
Other Specified Feeding or Eating Disorder	Past 12 months and past 3 months (current)	
Somatic Symptom and Related Disorders		
Somatic Symptom Disorder	Past 6 months (current)	✗ Exclusion (all groups) IBS-related symptoms not included for IBS and comorbid groups
Illness Anxiety Disorder	Past 6 months (current)	
Externalizing Disorders		
Adult Attention-deficit Hyperactivity Disorder	Past 6 months (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid) ✗ Lifetime = Exclusion (Controls and IBS)
Trauma- and Stressor-Related Disorders		
Acute Stress Disorder	Past month (current)	- Lifetime = Neither inclusion nor exclusion (MH and Comorbid)
Posttraumatic Stress Disorder	Lifetime and past month (current)	
Adjustment Disorder	Past 6 months (current)	

Disorder	Duration assessed at interview	Eligibility Status
Other Specified Trauma- and Stressor-Related Disorder	Lifetime and past month (current)	✗ Lifetime = Exclusion (Controls and IBS)

Notes. MH = mental health anxiety/depression group; Comorbid = comorbid IBS and anxiety/depression group; IBS = irritable bowel syndrome

Description of the Modified Psychiatric Diagnostic Screening Questionnaire

The Psychiatric Diagnostic Screening Questionnaire (PDSQ) will be modified for the Bugs and Brains Study to screen for the likely presence/absence of mental health diagnoses that relate to the inclusion and exclusion criteria of this study, particularly Major Depressive Disorder, anxiety disorders, substance use disorders, psychotic symptoms, and somatic disorders

The PDSQ has been modified as follows:

- 6 items relating to suicidal ideation and behavior have been removed. Suicidal ideation and behavior will be assessed by a research assistant during the SCID-5-RV diagnostic interview in Phase 2, and in Phase 4 self-report questionnaires.
- Questions relating to Post-Traumatic Stress symptoms and Obsessive Compulsive behaviours will be excluded as information pertaining to these disorders is not required for inclusions/exclusion criteria.
- Additional questions relating to mania symptoms have been added to assess for likely diagnosis of Bipolar disorders.
- Two additional questions assessing Persistent Depressive Disorder were added.
- An additional question assessing Anorexia Nervosa was added.
- For each diagnostic category, a question assessing lifetime history of experience of several symptoms from that diagnostic category has been added.