Microbiota-Gut-Brain Interactions in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Focus on Neuropsychological Symptoms and Sex Comparisons

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ABSTRACT

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic, disabling condition with debilitating fatigue and neuroimmune symptoms. Consensus about diagnosis, pathogenesis and efficacious treatments for ME/CFS are yet to be elucidated. Advances in the understanding of microbiota-gut-brain interactions in healthy and disease states, combined with evidence of gastrointestinal symptoms and gut dysbiosis in individuals with ME/CFS has directed investigation towards the role of enteric microbiota in this condition. The body of work presented in this thesis includes five publications based on reviews and empirical research conducted over the past 3.5 years.

The first review paper (Paper 1) found preliminary evidence to support the proposal that microbiota-gut-brain interactions may contribute to sleep, mood and cognitive symptoms but revealed gaps in knowledge with few empirical studies that have investigated commensal microbiota in patients with ME/CFS. Papers 2 and 3 describe the results of a correlational analyses between microbiota and ME/CFS symptoms in a cross-sectional, retrospective study of 274 ME/CFS patients. A notable finding from this study included sex-specific interactions between gut microbiota and symptom expression in ME/CFS, signaling possible sex differences in microbial function.

The systematic review examining symptom and etiological overlap between D-lactic acidosis and ME/CFS in Paper 4, revealed preliminary support for the hypothesis that subclinical concentrations of D-lactate from bacterial dysbiosis may be a mechanism contributing to several ME/CFS symptoms (including fatigue, neurocognitive impairments, pain, sleep disturbances, motor disturbances, gastrointestinal abnormalities, cardiovascular, respiratory, thermostatic, and comorbid mood and behavioural disturbances). The review highlighted the gaps in knowledge without measurement of D-lactate concentrations in ME/CFS samples.

Paper 5 presents the results of an open-label, repeated-measures trial examining the efficacy of a 4-week treatment (alternate weeks of Erythromycin and D-lactate free probiotic) for an overgrowth of commensal *Streptococcus* species in 44 adult patients with ME/CFS. Large time effects were shown including a reduction in *Streptococcus* count and improvement on several clinical outcomes (sleep, cognition and total symptoms) for the total sample at post intervention. Ancillary results highlighted individual variability in microbial changes and the importance of other genera with changes in *Bacteroides*, *Bifidobacteria* and *Clostridium* and associated with clinical changes in males.

In combination, the analysis of literature and results from both cross-sectional and experimental studies substantiate the theoretical premise that microbiota and gut dysbiosis contribute to specific neuropsychological symptoms in some ME/CFS patients. Our mechanistic understanding of gut dysbiosis will be advanced by multidisciplinary investigations that include assessment of clinical symptoms, the microbiome (combined sequencing and culture techniques), metabolites, oxidative and inflammatory markers, and immune profiles that help identify possible factors contributing to, precipitating or perpetuating imbalances in microbial composition. These advances may help clarify diagnostic discrepancies and inform efficacious treatment alternatives that are responsive to individual variability.

STUDENT DECLARATION

Doctor of Philosophy by Publication Declaration

"I, Amy Wallis, declare that the PhD thesis by Publication entitled **Microbiota-Gut-Brain Interactions in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: Focus on Neuropsychological Symptoms and Sex Comparisons** is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

I declare that I have received a scholarship to complete this PhD with financial support from an industry partner, Bioscreen and Victoria University. This was an untied contribution from Bioscreen administered through Victoria University, with no restrictions on publication. Other Bioscreen funds supporting specific studies were either in kind support or untied grants administered through Victoria University. This has also been declared on each paper for publication.

Signature:  Date: 1/10/17

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Paper 5

Note. Figures in the main text of the thesis and each subsequent publication are presented separately. Both thesis and publication page numbers are provided where applicable.

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ANS	autonomic nervous system
ATP	adeonsine triphosphate
B. breve	*Bifidobacterium breve*
B. lactis	*Bifidobacterium lactis*
B. longum	*Bifidobacterium longum*
BDNF	brain derived neuotrophin factor
BPQ	Bioscreen Patient Questionnaire
CANTAB	Cambridge Neuropsychological Test Automated Battery
CAR	cortisol awakening response
CBT	cognitive behaviour therapy
CCC	Canadian Consensus Criteria (Carruthers et al., 2003)
CDC1	Centre for Disease Control/Holmes Criteria (Holmes, 1988)
CDC2	Centre for Disease Control/Fukuda Criteria (Fukuda, 1994)
cfu/g	colony forming units per gram
CNS	central nervous system
COMT	catechol-O-methyltransferase
CONSORT	consolidated standards of reporting trials
CoQ10	Coenzyme Q10
D-2-HDH	D-2-hydroxy acid dehydrogenase
D-la	D-lactic acidosis
DASS	Depression Anxiety Stress Scale
DNA	deoxyribonucleic acid
EEG	electroencephalogram
EES	erythromycin ethyl succinate
ENS	enteric nervous system
ES	effect sizes
F. prausnitzii	*Faecalibacterium prausnitzii*
FMA	faecal microbial assessment
GABA	gamma-amino butyric acid
GAD	generalized anxiety disorder

GET	graded exercise therapy
HHV	human herpesvirus
HPA	hypothalamic-pituitary-adrenal
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
ICC	International Consensus Criteria (Carruthers et al., 2011)
Ig	Immunoglobulin
IL	interleukin
IO&NS	inflammation, oxidative and nitrosative stress
ITT	intention to treat
LBP	LPS-binding protein
LPS	lipopolysaccharide
MALDI-TOF-MS	Matrix Assisted Laser Absorption & Ionisation Time of Flight Mass Spectrometry
MANOVA	multiple analysis of variance
MDD	major depressive disorder
ME/CFS	myalgic encephalomyelitis/chronic fatigue syndrome
MFI	Multidimensional Fatigue Inventory
MRI	magnetic resonance imaging
NADH	nicotinamide adenine dinucleotide
NATA	National Association of Testing Authorities
NIOF	neuro-inflammatory and oxidative fatigue
NK	Natural Killer
NREM	non-rapid eye movement
OUT	operational taxonomic unit
PNS	parasympathetic nervous system
POMS	Profile of Mood States-Short Form
PRISMA	preferred reporting items for systematic reviews and meta-analyses
PSQI	Pittsburgh Sleep Quality Index
RA	relative abundance
RAS	reticular activating system
RAVLT	Rey Auditory Verbal Learning Test
RCT	randomized clinical trial

RVP	Rapid Visual Attention
SBS	short bowel syndrome
SCFAs	short chain fatty acids
SE	sleep efficiency
SEID	systemic exertion intolerance disease
SFI	sleep fragmentation index
SIBO	small intestinal bacterial overgrowth
SNS	sympathetic nervous system
SOL	sleep onset latency
SPF	specific pathogen free
SPSS	Statistical Package for the Social Sciences
SSH	Symptom Severity and Symptom Hierarchy Profile
SWA	slow wave activity
SWM	Short-term Working Memory
TCA	tricarboxylic acid
TNF	tumor necrosis factor
TRYCAT	tryptophan catabolite pathway
VO2 max	maximum volume oxygen
VUHREC	Victoria University Human Research Ethics Committee
WASO	wake after sleep onset
XMRV	xenotropic murine leukemia virus-related virus

PART A: INTRODUCTION

CHAPTER 1

Contextualising the Research

The Challenge: The Complexities of Chronic Fatigue Syndrome

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a debilitating, multisystemic condition that is frequently misunderstood. The complexities of this condition with heterogeneous symptoms and inconsistent diagnostic criteria have complicated both clinical and research fields (Jason et al., 2012; Jason, Corradi, Torres-Harding, Taylor, & King, 2005). Over the past three decades, ME/CFS conceptualisations have endured several iterations in an attempt to defuse scepticism and accurately identify appropriate assessment and treatment pathways for this clinical population. Often trivialised, the familiar notion of 'fatigue' can minimise the devastating and debilitating experience of patients. Unlike 'normal' levels of fatigue experienced by most, the level of fatigue in ME/CFS is excessive, disproportionate to the patient's level of activity, and associated with significant reductions in mental and physical capacity (Carruthers et al., 2011). Misdiagnosis, inappropriate treatment recommendations, symptom minimisation and delegitimisation are frequent experiences for ME/CFS patients (V. R. Anderson, Jason, Hlavaty, Porter, & Cudia, 2012; Clayton, 2015; Dickson, Knussen, & Flowers, 2007; Nacul et al., 2011). Patients report experiencing stigma from both professionals and within personal relationships where symptoms of fatigue are misunderstood and attributed to personality or maladaptive coping mechanisms (Jason et al., 1997; Jason, Holbert, Torres-harding, & Taylor, 2004).

Perpetual blaming and stigmatisation of ME/CFS patients combined with advances in pathophysiological understandings have prompted refinements in terminology and diagnostic criteria. CFS, ME, ME/CFS and, the most recent term, systemic exertion intolerance disease (SEID) have been used interchangeably in research and clinical settings. Diagnostic discrepancies not only add complexity for patients attempting to navigate an effective treatment pathway but also partially contribute to the inconsistent empirical evidence that plagues ME/CFS research. For these reasons, a brief overview of the history of diagnostic criteria for CFS, ME, ME/CFS and SEID is provided.

Diagnostic criteria: Clarifying terminology

Over the past three decades, increasing evidence of physiological abnormalities in ME/CFS and improvement in statistical analyses have contributed to the development and improvement of diagnostic criteria. Nevertheless, the literature and clinical practice continues to be compromised by the use of different criteria. Whilst a complete appraisal of diagnostic

criteria is beyond the scope of this thesis (see Jason et al., 2012; Jason, Evans, Brown, Sunnquist, & Newton, 2015a), a brief overview of key differences between the most common and recent criteria is provided.

Chronic fatigue syndrome

The first operational definition of CFS was proposed by the Centre for Disease Control (CDC_1/Holmes 1988 criteria; Holmes et al., 1988) to aid research efforts, with the aim of distinguishing between patients who experience excessive fatigue from those who present with a dysfunctional level of fatigue and an array of other symptoms conceptualised as CFS. Originally referred to as chronic Epstein-Barr virus syndrome when recognised in 1985 in response to cluster outbreaks, the change in terminology to CFS was suggested to reflect the unknown etiology (Holmes et al., 1988). This working case definition of CFS requires the presence of symptoms over a 6 month period and the patient to fulfil the two major criteria (1. Debilitating fatigue that does not resolve with bedrest with a 50% reduction in patient activity since onset, and 2. Exclusion of other clinical conditions with similar symptoms); the onset of 6 or more minor criteria (including: 1. mild fever, 2. sore throat, 3. painful lymph nodes, 4. muscle weakness, 5. muscle discomfort or myalgia, 6. prolonged fatigue after exercise, 7. headaches, 8. joint pain, 9. neuropsychological symptoms, 10. sleep disturbance, and 11. main symptoms developing rapidly) and two or more physical criteria (1. Low-grade fever, 2. nonexudative pharyngitis, and 3. tender lymph nodes); or eight of the eleven minor criteria (Holmes et al., 1988). Developed for research purposes, criticisms of this definition related to inconsistent application of the criteria and the complexity associated with heterogeneous symptom presentations (Sharpe et al., 1991).

The Oxford criteria was developed in response to these concerns. Whilst less stringent, it provides detailed definitions of primary symptoms to aid diagnosis (Sharpe et al., 1991, p. 120). Another key development of the Oxford criteria was the proposal of subgroup classifications by suggesting the distinction between two broad symptoms: CFS and post-infectious fatigue syndrome (Sharpe et al., 1991). However, reduced restriction on the number of symptoms required for a diagnosis under this criteria means that the patient group fulfilling the Oxford criteria for CFS is broader than the CDC_1/ Holmes 1988 criteria.

The CDC_1/Holmes 1988 criteria was revised and presented as the Fukuda/Centre for Disease Control case definition (CDC_2/Fukuda 1994 criteria: Fukuda et al., 1994) in an attempt to clarify diagnostic discrepancies and have utility in both clinical and research settings. The CDC_2/Fukuda 1994 criteria stipulates the primary requirement of chronic

fatigue ($\geq$ 6 months) that is disproportionate to the level of activity, has a clear onset, is unresponsive to rest and interferes with daily functioning. The CDC_2/Fukuda 1994 criteria requires the primary criterion of chronic fatigue and four or more additional core symptoms: (a) impaired memory or concentration, b) sore throat, c) tender lymph nodes, d) muscle pain, e) multi-joint pain, f) new headaches, g) unrefreshing sleep, and/or h) post-exertional malaise (Fukuda et al., 1994, p. 955).

The advances of the CDC_2/Fukuda 1994 definition resulted in this criteria being widely used in research (Jason, Sunnquist, Brown, Evans, & Newton, 2016; Maes, 2015) as it selects a smaller clinical population than the Oxford criteria (Flo & Chalder, 2014) but is more inclusive than the CDC_1/Holmes 1988 definition. In a large sample of patients with chronic fatigue (n = 2073), 100% of CFS patients (n = 1578) met the CDC_2/Fukuda 1994 criteria, compared with the more stringent CDC_1/Holmes 1988 definition (n = 951, 60.3% of CFS patients; De Becker, McGregor, & De Meirleir, 2001). Jason and colleagues' (2001) results supported this finding with patients meeting CDC_1/Holmes 1988 criteria reporting more severe symptoms and impairment in functioning. De Becker et al. (2001) suggested the different patient groups were related to symptom severity and that the CDC_2/Fukuda 1994 definition may constrain research efforts as it includes a more heterogeneous patient group. An additional criticism of the CDC_2/Fukuda 1994 definition is that the post-exertional malaise that is the core symptom outlined for ME/CFS (Carruthers et al., 2003, 2011) is not required under a CDC_2/Fukuda 1994 diagnosis (Jason et al., 2016).

Myalgic encephalomyelitis/chronic fatigue syndrome

Both epidemic and endemic occurrences of myalgic and post-viral encephalomyelitis have been described since 1934 (Acheson, 1959; Dowsett, Ramsay, McCartney, & Bell, 1990). ‘Benign’ ME was used to describe the often acute ‘outbreaks’ more commonly observed in females (symptoms included muscle pain and/or weakness, cognitive difficulties, headaches, and symptoms indicating damage to the central or peripheral nervous system, no or low fever, no mortality; Acheson, 1959). Overlap between CFS and ME led to the development of clinical case definitions of ME/CFS proposed by the Canadian Case Criteria (CCC; Carruthers et al., 2003) and International Consensus Criteria (ICC; Carruthers et al., 2011).

ME/CFS, as defined by both CCC and ICC documents, highlight the acquired dysfunction in neural, immune and endocrine systems with the term ‘encephalomyelitis’ reflecting underlying neurological pathology, i.e., inflammation of the central nervous system

(CNS: brain and spinal cord; Carruthers et al., 2003, 2011). There is considerable overlap between CCC and ICC diagnostic requirements. Advances of the more recent ICC include removing the need for symptoms to be present for 6 months duration before diagnosis and clustering symptoms according to systemic dysfunction (Carruthers et al., 2011). This change in length of symptoms encourages earlier diagnoses and opportunities to improve treatment response and reduce relapse. Unlike the CDC definitions of CFS, both CCC and ICC documents indicate that onset can be gradual or distinct (Carruthers et al., 2003, 2011). According to the ICC, a diagnosis of ME/CFS requires patients to have experienced a) at least a 50% reduction in functional capacity, b) post-exertional neuro-immune exhaustion, c) at least three categories of symptoms reflective of neurological impairments, d) at least three categories of symptoms of immune, gastrointestinal and genitourinary dysfunction, e) at least one energy production or ion transportation impairment (Carruthers et al., 2011, pp. 329-331).

Comparison between CDC_2/Fukuda 1994 and ICC definitions in two large samples indicates that ICC diagnostic criteria is more rigorous and identifies approximately 60% of CFS patients (Jason et al., 2016). Jason and colleagues (2016) showed that patients meeting ICC criteria reported more severe symptoms, reduced functional capacity, and worse physical health but comparable mental health problems (Jason et al., 2016). The diagnostic modifications of the ICC attempt to create a more homogenous group and promote early, targeted intervention after biomedical assessment. However, the CCC and ICC criteria for ME/CFS have been criticized for their restrictive criteria that can select a more severe patient group with more somatic complaints that may reflect psychiatric conditions (Jason, Zinn, & Zinn, 2015c; Maes, Anderson, Morris, & Berk, 2013). Whilst still eagerly debated, some clinicians prefer the CCC and ICC to formulate a diagnosis of ME/CFS compared with the less stringent polythetic conceptualisation (i.e. CDC_2/Fukuda 1994). Further diagnostic complications have been raised since the newest proposal by the Institute of Medicine (IOM) to change terminology and refer to CFS and ME/CFS as SEID.

Systemic exertion intolerance disease

The term SEID was proposed to reflect the excessive and disproportionate level of fatigue after physical or mental exertion (i.e. post-exertional malaise, or ‘payback’) that is not explicit in the ME label (Clayton, 2015). A diagnosis of SEID requires four primary criteria a) reduced functional capacity, b) post-exertional malaise, c) unrefreshing sleep, and d) either cognitive impairments and/or orthostatic intolerance (Clayton, 2015). Whilst intentions of the

IOM were in line with the increasing evidence of pathophysiological abnormalities and support validation of the patient symptoms as organic in nature rather than psychosomatic, this new iteration of the condition has been criticised by patients and experts alike (Jason, Sunnquist, Brown, McManimen, & Furst, 2015b). A primary concern is related to decreased specificity, with patients meeting SEID having less functional impairment and less symptoms than patients meeting other ME/CFS diagnostic criteria (Jason et al., 2015b). Without exclusionary conditions, a SEID diagnosis is more likely to include patients from other populations (e.g., depression, cancer, autoimmune conditions) as well as a higher proportion of healthy controls (Jason et al., 2015b).

The more inclusive SEID diagnostic criteria may complicate research efforts by increasing the heterogeneity of the clinical sample. Possible overlap with other primary mood disorders and autoimmune conditions (Jason et al., 2015b) may muddy the already murky diagnostic waters. At the time of writing, SEID was not commonly accepted (see Jason et al., 2015b). Evidence of differential diagnoses (Hawk, Jason, & Torres-Harding, 2006), pathophysiological differences between ME and major depressive disorder (e.g., Maes, 2011) and treatment response (i.e., exercise tolerance/intolerance) justifies the need for careful diagnostic procedures that distinguish subtle differences in clinical presentations. Efforts to formulate diagnostic clarity continue to be examined with proposals of subtype classifications (e.g., Jason et al., 2015c) or differential diagnoses (e.g., neuro-inflammatory and oxidative fatigue: NIOF, Maes, 2015) and pursuit of phenotypic biomarkers (e.g., Hornig et al., 2015; Petty, McCarthy, Le Dieu, & Kerr, 2016; Zhang et al., 2010). The studies presented in this thesis include participants clinically diagnosed with ME/CFS according to CCC criteria (Carruthers et al., 2003). For simplicity, the term ME/CFS will also be used when referring to past research based on either CFS or ME/CFS criteria unless explicitly stated.

Prevalence

As an endemic disorder with acute or gradual onset, ME/CFS appears to afflict all ethnicities and sociodemographic groups (Carruthers et al., 2011). Prevalence rates vary between approximately 0.08% to 2.6% dependent on diagnostic and sample selection methods. Point prevalence of approximately 0.2% has been repeatedly reported (Buchwald et al., 1995; Nacul et al., 2011; Reyes et al., 2003; Steele et al., 1998) but may be conservative based on other findings across population-based (0.2% to 2.54%; Jason et al., 1999; Reeves et al., 2007; Reyes et al., 2003; Steele et al., 1998), community health (0.08% to 0.27%; Buchwald et al. 1995) and primary health settings (0.2% to 2.6%; Fuhrer & Wesseley, 1995; Lawrie, Manders, Geddes, & Pelosi, 1997; Nacul et al., 2011). Difference in prevalence rates

are confounded by bias in selection method and diagnostic variability without clear biomarkers. Additionally, the stigma and etiological confusion may contribute to an underreporting of symptoms and misdiagnoses. Hence, the prevalence rates are estimates at best.

Females are consistently overrepresented in ME/CFS clinical samples, with approximately 2/3 women (Buchwald et al., 1995; Carruthers et al., 2011; Jason et al., 1999; Nacul et al., 2011; Steele et al., 1998). In some non-clinical samples, the proportion of male ME/CFS patients was even smaller (~9%, Reeves et al., 2007; ~22%, Reyes et al., 2003). Results from Bakken et al.'s, (2014) epidemiological study showed similar female predominance and a peak in incidence rates during two age groups for females (10-19 years and 30-39 years) that may indicate a role for hormonal changes in ME/CFS etiology. It is likely that the overrepresentation of females reflects physiological differences (i.e., neuroendocrine and immune; Bakken et al., 2014; Weaver, Janal, Aktan, Ottenweller, & Natelson, 2010; Whitacre, 2001) rather than merely inflated by higher rates of help-seeking (Addis & Mahalik, 2003) and research participation amongst women (e.g., Singer, Van Hoewyk, & Maher, 2000).

The influence of other demographic variables may be more relevant than prevalence data suggests, considering prevalence research has frequently used ambiguous or restricted classification of ethnicity and has been conducted in selected geographic locations. Based on cross-sectional analysis of a large cohort of adults aged 18-64 years accessing primary care services in England (N = 143000), prevalence variability was dependent on region (Nacul et al., 2011). It is difficult to tease apart the interaction and confounding nature of ethnic, occupational, marital or socio-economic circumstances using epidemiological methods (Jason et al., 1999). Some differences in the prevalence rates between geographic location may accurately reflect the endemic nature of the condition or support infectious theory (discussed below).

Prognosis

There is no known cure for ME/CFS (Carruthers et al., 2011). Estimated recovery rates are rare and complicated by diagnostic challenges, selected treatment pursued and unclear methods of differentiating between clinical improvement, remission and recovery. It is difficult to accurately determine prognosis with many articles indicating improvement in selected symptoms but not stating remission or relapse rates. An improvement in health may not equate to the patient's premorbid activity level or health status. This can also be complicated by the inability to accurately determine a patient's premorbid activity level due

to childhood onset of ME/CFS or experiencing the illness for several decades. It has been challenging to develop an agreed operational definition of recovery in ME/CFS and there is an ongoing need to distinguish between 'recovery', 'successful adaptation' and 'clinically significant improvement' (Adamowicz, Caikauskaite, & Friedberg, 2014).

Notwithstanding the issues of an agreed operational definition of recovery, for psychosocial and behavioural interventions, randomized clinical trials (RCTs) that have measured recovery rates at follow-up have shown that between 0 and 31% of patients recovered after intervention with cognitive behaviour therapy (CBT; Deale, Husain, Chalder, & Wessely, 2001; Flo & Chalder, 2014; Knoop, Prins, Stulemeijer, van der Meer, & Bleijenberg, 2007). It is notable that these studies used different CFS (i.e., CDC_1/Fukuda 1994 and Oxford criteria) not ME/CFS diagnostic criteria and also suggested a drop in recovery rates at longer follow-up intervals (Deale et al., 2001). Similarly, the research design of the largest trial (Pacing, graded Activity, and Cognitive behavioural therapy: a randomized Evaluation: PACE) examining the efficacy of adaptive pacing therapy, CBT and graded exercise therapy (GET; White et al., 2011) has been criticized for drawing inaccurate conclusions from inappropriate outcome measures (Twisk, 2016). More specifically, two of the four criteria used to determine 'recovery' were relaxed so that a deterioration in scores after treatment did not preclude a patient from being considered as 'recovered' (Kindlon, 2017). Additionally, variable compliance in the GET protocol alongside variable reporting and classification of adverse events impacts the generalizability of these results and the ability to evaluate the risks and efficacy of exercise treatments (Kindlon, 2017). Therefore, the rates of recovery after adaptive pacing therapy, CBT or GET are unclear and require further evaluation to determine their effectiveness and safety.

Treatment targeting specific pathophysiological dysfunction suggest some symptomatic improvement (e.g., Pall, 2001; Rao et al., 2009; Williams, Waterhouse, Mugarza, Minors, & Hayden, 2002). A recent systematic review of pharmacotherapies showed conflicting evidence for some medications (e.g., hydrocortisone) and possible effectiveness for others targeting immune dysregulation (e.g., rituximab: Fluge et al., 2011; intravenous immunoglobulins: Rowe, 1997) or cellular metabolism (e.g., acetyl-l-carnitine: Malaguarnera et al., 2008) with no universal treatment identified (Collatz, Johnston, Staines, & Marshall-Gradisnik, 2016). Additional research is required to establish treatment efficacy and evaluate individual risks. For a subgroup of ME/CFS with comorbid irritable bowel syndrome (IBS) symptoms, treatment aimed at restoring gut dysbiosis (i.e., an imbalance of enteric microbiota) using a bacteriotherapy approach has also shown high rates of

improvement (70%) and maintenance (58%; Borody, Nowak, & Finlayson, 2012) but results require replication. Most ME/CFS treatment studies have been restricted by sample size, with individual variability influencing remission and relapse rates.

Impact on the individual and society

As a condition with poor prognosis, that is chronically disabling and sometimes life-threatening, ME/CFS has a devastating impact on the individual (Jason et al., 2011). Symptom severity can fluctuate with patients experiencing periods of being incapacitated, unable to perform basic tasks, being bedridden or housebound (Marshall, Paul, & Wood, 2011). Qualitative descriptions from ME/CFS patients report that the reduced energy and debilitating symptoms can often shift social roles, restrict social networks and disrupt intimate relationships (V. R. Anderson et al., 2012). The shift in roles directly and/or indirectly impacts both the sufferer and relatives/friends who may assume caring roles to aid the chronically ill person (V. R. Anderson et al., 2012; Donalek, 2009). Like other chronic illnesses, patients with ME/CFS can experience reduced confidence, self-esteem, and multiple losses associated with reduced physical, social and occupational functioning (Jason et al., 2011). The cost to the individual's life, occupational, social and relational functioning, combined with chronic pain and suffering is further compounded by financial burden. When estimating direct medical costs for ME/CFS patients, this varied between an average annual cost of US$2342 and US$9436 per patient dependent on community or tertiary samples (Jason, Benton, Valentine, Johnson, & Torres-Harding, 2008). Individuals with ME/CFS have higher rates of unemployment, part-time employment and/or are receiving disability pensions compared to controls (Jason et al., 1999). Unemployment has been reported as a direct consequence of cognitive symptoms (Ware, 1998).

Whilst these direct costs are considerable, the societal impact from indirect costs (i.e., loss of income, reduced capacity for self-care and disability reimbursement) were astounding at US$2 billion (community sample) and $US7 billion (clinical sample; Jason et al., 2008). In combination using ME/CFS prevalence of 0.42, the sum of direct and indirect costs of ME/CFS to society was estimated at $18,677,912,000 for the community sample and US$23,972,300,000 for the tertiary sample (Jason et al., 2008). These estimates signify the extent of the problem and highlight the global impact of this disease, indicating relevance for both individuals, health professionals, government and policy. Notably this study used a higher prevalence rate (0.42%; (Jason et al., 2012). However, even if these costs were halved to reflect a more conservative prevalence rate of approximately 0.2%, the total societal costs of $US9-12 billion remain considerable. Debilitating economic and psychosocial costs for the

individual and society highlight the need for continued research efforts to identify causes and treatment options for ME/CFS.

Searching for Pathophysiological Causes in ME/CFS and Options for Treatment

Conflicting psychosocial and bio(psychosocial) frameworks have also added to the complexity and confusion surrounding ME/CFS etiology. Traditionally, ME/CFS has been viewed within psychosocial frameworks (see Harvey & Wessely, 2009; Vercoulen et al., 1998). In both these models, there is minimal focus on biological mechanisms, with physiological symptoms explained as resultant from and maintained by maladaptive coping and personality factors. Consequently, treatment attempts have centred on psychosocial and behavioural therapies, i.e., CBT (see review by Price, Tidy & Hunot, 2009), pacing (see review by Goudsmit, Nijs, Jason, & Wallman, 2012) and GET (see review by Edmonds, McGuire, & Price, 2004). Whilst some benefits have been observed, at present there is inconsistent support for the use of these therapies as a primary treatment for ME/CFS. These reviews support their role as an adjunctive therapy when combined with biomedical treatments. However, in light of the aforementioned criticisms (Kindlon, 2017; Twisk, 2016) of the PACE trial (White et al., 2011), the safety and efficacy of pacing, CBT and GET is unknown.

Mounting biological evidence points to dysfunction of the CNS, immune systems and inflammatory pathways as the underlying pathology for ME/CFS symptom presentation (Anderson, Berk, & Maes, 2014; Carruthers et al., 2011; Maes & Twisk, 2010; Morris, Berk, Galecki, Walder, & Maes, 2015; Twisk, 2014). Maes and Twisk (2010) proposed the bio(psychosocial) medical model for ME/CFS. This model focuses on inflammation, immune and gastrointestinal abnormalities and related impairments in mitochondrial function and oxidative stress. Physiological and psychological stress are included as co-factors rather than primary etiological factors. The model acknowledges a genetic predisposition and onset can be triggered by infection and/or immune dysfunction.

Genetic predisposition

Results from preliminary studies with small ME/CFS samples examining genetic susceptibility, highlight a predisposition for immune dysregulation (Gow et al., 2009; Kerr et al., 2008a; Kerr et al., 2008b; Schlauch et al., 2016). Zhang et al. (2010) showed genetic expression variability amongst post-infectious ME/CFS subtypes, compared to controls, and compared to patients with endogenous depression. Other studies have investigated the role of catechol-O-methyltransferase (COMT) in ME/CFS. COMT regulates adrenergic activity,

primarily the production and clearance of circulating concentrations of dopamine, adrenaline and noradrenaline (Jiang, Qiu, Peng, & Wang, 2006). Reduced clearance of these catecholamines can be associated with more pain sensitivity and migraines and may play a role in neuroimmune dysregulation in ME/CFS. Higher frequency of COMT polymorphisms have been observed in some ME/CFS samples (Goertzel et al., 2006; Lachman et al., 1996; Sommerfeldt, Portilla, Jacobsen, Gjerstad, & Wyller, 2011). More recently, Löbel et al. (2015) showed no difference in the prevalence of genetic polymorphisms in COMT and glucocorticoid receptor genes in healthy control compared with ME/CFS patients. However, their results indicated an association between immune dysfunction and a variant of COMT rs4680 that may increase their vulnerability to infection during periods of stress. Although some results are contradictory, preliminary results suggest promise in pursuing investigation of genetic factors that may underlie ME/CFS development. Genes responsible for immunity may result in an increased susceptibility to viral or bacterial infections.

Infections in ME/CFS

ME/CFS onset after an acute viral or bacterial infection is frequently reported (Carruthers et al., 2011; Royal Australasian College of Physicians, 2002) but no single pathogen has been identified as a consistent causative agent. Claims of xenotropic murine leukemia-related virus (XMRV) as the causal agent of ME/CFS (Lombardi et al., 2009) have since been disputed due to methodological errors (Alter et al., 2012; Paprotka et al., 2011). Some infectious agents appear to be more common than others, including Epstein-Barr virus (EBV; Hickie et al., 2006; Zhang et al., 2010), human herpesvirus 6 and 7 (HHV-6/7; Ablashi et al., 2000; Chapenko et al., 2006; Nicolson, Gan, Haier, & Nicolson, 2003), mycoplasma (Nicolson et al., 2003) chlamydia (Chia & Chia, 1999; Nicolson et al., 2003), and human retroviruses and enteroviruses (Chia et al., 2010; Chia & Chia, 2007; Chia, 2005). Patients with raised markers for EBV and HHV-6 have shown improvement on fatigue and immunological markers after antiviral treatments (Lerner et al., 2010; Montoya et al., 2013).

Several theories have been proposed, with some researchers predicting that ME/CFS symptoms are the result of an ongoing primary infection, a reactivation from a latent infection, and dependent on the site of infection (e.g., heart, brain, vagus nerve; see Jason et al., 2015c; Lerner, Zervos, Dworkin, Chang, & O'Neill, 1997; VanElzakker, 2013). Initial viral activation may result in long-term consequences in bodily regions infected by the virus and/or exacerbation of symptoms during reactivation. For example, even in otherwise healthy individuals, after acute infection, human herpesviruses can remain latent in ganglia and

lymphoid tissues and be reactivated during times of stress producing various neurological symptoms (Gilden, Mahalingam, Cohrs, & Tyler, 2007).

The location of the latent virus (or other infection) may contribute to symptomatic differences and an altered immune response. In non-ME/CFS samples, HHV-6 DNA has been found in the temporal lobe and epileptiform activity has been observed on electroencephalogram (EEG) recordings during the active form of the virus (Epstein & Millichap, 2014). In ME/CFS patients, spikes in the temporal region may have viral origins and precipitate hippocampal dysfunction (i.e., impaired memory and attention; see Jason et al., 2015c). Simultaneously, various viral or bacterial infections affect vagal signaling and the innate immune response. VanElzakker (2013) has proposed the Vagus Nerve Infection Hypothesis suggesting that the excessive fatigue is a consequence of an exaggerated immune response when glial cells surrounding the vagus nerve are infected. It appears that regardless of the type of infection, both central and peripheral infections may stimulate chronic proinflammatory and neuroexcitatory responses. Different infectious agents but results of similar fatigue, neurological and pain symptoms from 3-months post-infection in ME/CFS patients (Hickie et al., 2006) suggest that the initial infection may precipitate immunological and/or CNS disturbances for the subgroup of genetically and/or environmentally susceptible individuals who develop ME/CFS.

Microbiota-gut-brain interaction

Susceptibility to infection, immune and adrenergic system dysregulation, circadian rhythm disruptions and frequent gastrointestinal symptoms (i.e., IBS and diffuse abdominal pain) implies that the gut-brain axis may play a role in ME/CFS. The bidirectional communication between the brain and the gut occurs through CNS, enteric nervous system (ENS), autonomic nervous system (vagus nerve), neuroendocrine, immune and microbial pathways (see Cryan & Dinan, 2012). The microbiota-gut-brain axis recognises the interaction between the host and the trillions of commensal bacteria that resides in the gastrointestinal tract continues to gain attention as a pivotal pathway involved in healthy and disease states (Moloney, Desbonnet, Clarke, Dinan, & Cryan, 2014).

Gut dysbiosis (i.e., an imbalance of commensal microbiota/bacteria; e.g., Frémont, Coomans, Massart, & De Meirleir, 2013; Sheedy et al., 2009) and intestinal permeability in the mucosal lining of the gastrointestinal tract (Maes et al., 2012a; Maes et al., 2012b; Maes & Leunis, 2008) have been shown in some ME/CFS patients. This bacterial dysbiosis may precede the occurrence of intestinal permeability and both states can directly or indirectly precede gastrointestinal, neurocognitive and or immune disturbances (Bested, Logan, &

Selhub, 2013; Morris et al., 2016). As a bidirectional relationship, stress and neurobiological mechanisms can also exert effects on gastrointestinal functioning and microbial composition as evidenced in animal models (e.g., Bailey & Coe, 1999; Desbonnet et al., 2010; O'Mahony et al., 2009). This bidirectional relationship is also supported by evidence that circadian rhythm disruptions can increase intestinal permeability in male mice (Voigt et al., 2016) and increasing evidence that microbial composition and metabolic activity can be altered by the circadian rhythms of the host (for example diet and sleep-wake cycles; Voigt et al., 2014; Voigt, Forsyth, Green, Engen, & Keshavarzian, 2016). Within ME/CFS, preliminary studies have investigated treatment aimed at restoring microbial balance using probiotics (Groeger et al., 2013; Rao et al., 2009; Sullivan, Nord, & Evengård, 2009), antibiotics (Jackson, Butt, Ball, Lewis, & Bruck, 2015) and faecal transplants (Borody et al., 2012). The few treatment studies that have been conducted suggest that microbial-host interactions may be involved in ME/CFS presentations with gut dysbiosis a potential target for treatment.

The D-lactate hypothesis has been proposed as one mechanism for microbiota-gut-brain interactions in ME/CFS. Preliminary evidence of an abundance of D-lactate producing bacteria in ME/CFS patients compared with healthy controls (Sheedy et al., 2009) raised the question of similarities between D-lactic acidosis (D-la) and ME/CFS symptoms and mechanisms. D-la is a condition with acute neurological symptoms that appear to arise from gastrointestinal dysfunction. D-la most commonly presents in patients with a medical history of short bowel surgery (Tappenden, 2014). Carbohydrate malabsorption that is a consequence of the shortened bowel is believed to precipitate an overgrowth of D-lactate producing bacteria, increased D-lactate absorption combined with insufficient excretion that manifests as encephalopathy (Petersen, 2005). Mental confusion, memory loss, cognitive and motor-coordination difficulties are primary symptoms of D-la (Kowlgi & Chhabra, 2015) and are also experienced by patients with ME/CFS (Carruthers et al., 2011). Therefore, the D-lactate hypothesis postulates that higher concentrations of circulating D-lactate through bacterial metabolism and enteric absorption precipitates or exacerbates neurological symptoms in ME/CFS. Some *Streptococcus* species produce excess D-lactate (Petersen, 2005). The increased abundance of *Streptococcus* in ME/CFS patients (Sheedy et al., 2009) raises the possibility of antimicrobial treatments to help restore bacterial balance in ME/CFS patients.

Rationale for Interdisciplinary Investigation

The microbiota-gut-brain axis has relevance for a broad range of health disciplines including biomedical, neuroscience and psychology (Bested et al., 2013; Sekirov, Russell,

Antunes, & Finlay, 2010). Psychologists have commonly supported the psychosocial model of investigation and treatment related to neurological symptoms and mood disturbance in ME/CFS. Psychologists play active roles in the treatment and management of this complex clinical condition by employing CBT techniques to enhance patients' coping strategies to manage stress, comorbid mood symptoms and consequential loss related to the illness. However, the suggestion of a bidirectional microbiota-gut-brain interaction as a possible explanation for cognitive, emotional and behavioural symptoms is stimulating interest in the physiological mechanisms that may precipitate neuropsychological symptoms in this condition.

Psychologists' explanation of brain-gut connections commonly assumes a top-down approach, with practitioners providing psychoeducation about the impact of the stress response on gastrointestinal symptoms. The research investigated within this thesis focuses on examining the opposite direction of interaction i.e., what role gut bacteria plays in neuropsychological symptoms, particularly cognition, sleep and mood symptoms. Through the pursuit of knowledge about this reverse relationship (i.e., from the perspective of how microbial composition is related to ME/CFS symptoms), psychologists can endeavor to enhance understanding of the bidirectional relationship between the gut and the brain. Within research, psychologists provide valuable insights through the assessment of cognitive, sleep and mood symptoms. In order for psychologists to work within their realm of expertise, investigating this topic requires a multidisciplinary team approach. Thus, the research presented in this thesis involved experts from medical, microbiology, biochemistry and psychology disciplines.

The papers in this thesis are a result of collaborative projects between psychologists and biochemists from Victoria University and industry partners, Bioscreen and CFS Discovery Clinic. All participants included in the presented studies involved adult patients from CFS Discovery Clinic. CFS Discovery Clinic adopts clear procedures for assessing ME/CFS (CCC; Carruthers et al., 2003) and investigating possible underlying mechanisms. A primary area includes assessment of gut dysbiosis through specialised faecal microbial assessments conducted by Bioscreen, an independent pathology laboratory. Bioscreen uses a culture-based methodology to profile commensal bacteria from the stool sample provided by patients. Microbiologists and biochemists from Bioscreen also collaborated with a biotechnologist and biomedical scientist from Victoria University. Within this interdisciplinary framework, shared expertise provides the opportunity to accumulate knowledge that has relevance for diverse fields.

Research Focus: Guiding Questions

The overarching question that underpins the research perspective of the interdisciplinary team is *what role does enteric microbiota play in ME/CFS?* The primary focus for the psychologists involved in the research is on neuropsychological symptoms and attempting to ascertain whether microbiota-gut-brain interactions contribute to sleep, mood and cognitive symptoms in ME/CFS.

Five papers are presented in this thesis responding to different objectives and research questions. The aim of Paper 1 (Wallis, Jackson, Ball, Lewis, & Bruck, 2017d) was to evaluate evidence of possible microbiota-gut-brain mechanisms that may be involved in sleep, mood and cognitive symptoms observed in ME/CFS. The purpose of the paper was to present a conceptual framework for the studies that follow by both outlining the type of neuropsychological symptoms experienced by ME/CFS patients and evaluating the possible influence of enteric microbiota in symptom expression. Papers 2 (Wallis, Butt, Ball, Lewis, & Bruck, 2016) and 3 (Wallis, Butt, Ball, Lewis, & Bruck, 2017c) describe the results of a cross-sectional study with the primary objective of exploring associations between gut microbiota and ME/CFS symptoms in a large retrospective sample. Paper 4 (Wallis et al., 2017b) presents a systematic review of D-la case studies that evaluates *what evidence supports/contradicts the relevance of the D-lactate hypothesis for ME/CFS pathogenesis?* This involved determining the extent of symptomatic and mechanistic overlap between D-la and ME/CFS to increase understanding of a possible theoretical explanation for neurological symptoms arising from gut dysbiosis in some ME/CFS patients.

Finally, Paper 5 (Wallis et al., 2017a) presents the results of an open-label trial to evaluate an antibiotic/probiotic treatment for bacterial dysbiosis that was being used within a clinical setting for a subgroup of ME/CFS patients with *Streptococcus* overgrowth. The guiding research question was to determine: *What is the effect of a 4-week antibiotic and probiotic treatment aimed at reducing commensal Streptococcus on sleep, mood and cognitive symptoms in ME/CFS patients?* The primary aims were to a) evaluate if there was a sex-specific treatment response to the intervention and b) examine the results through the lens of the D-lactate theory.

Thesis Structure

This thesis is organized in three parts (A, B and C, see Figure 1). This introductory chapter (Part A) contextualises the body of published work or work submitted for publication that is being presented (Part B). Part C provides the critical review that synthesizes the major

research findings, critically evaluates these findings in the context of new evidence and provides directions for future research. The flowchart in Figure 1 shows the sequential process and cumulative knowledge that informed each study/publication and chapter structure.

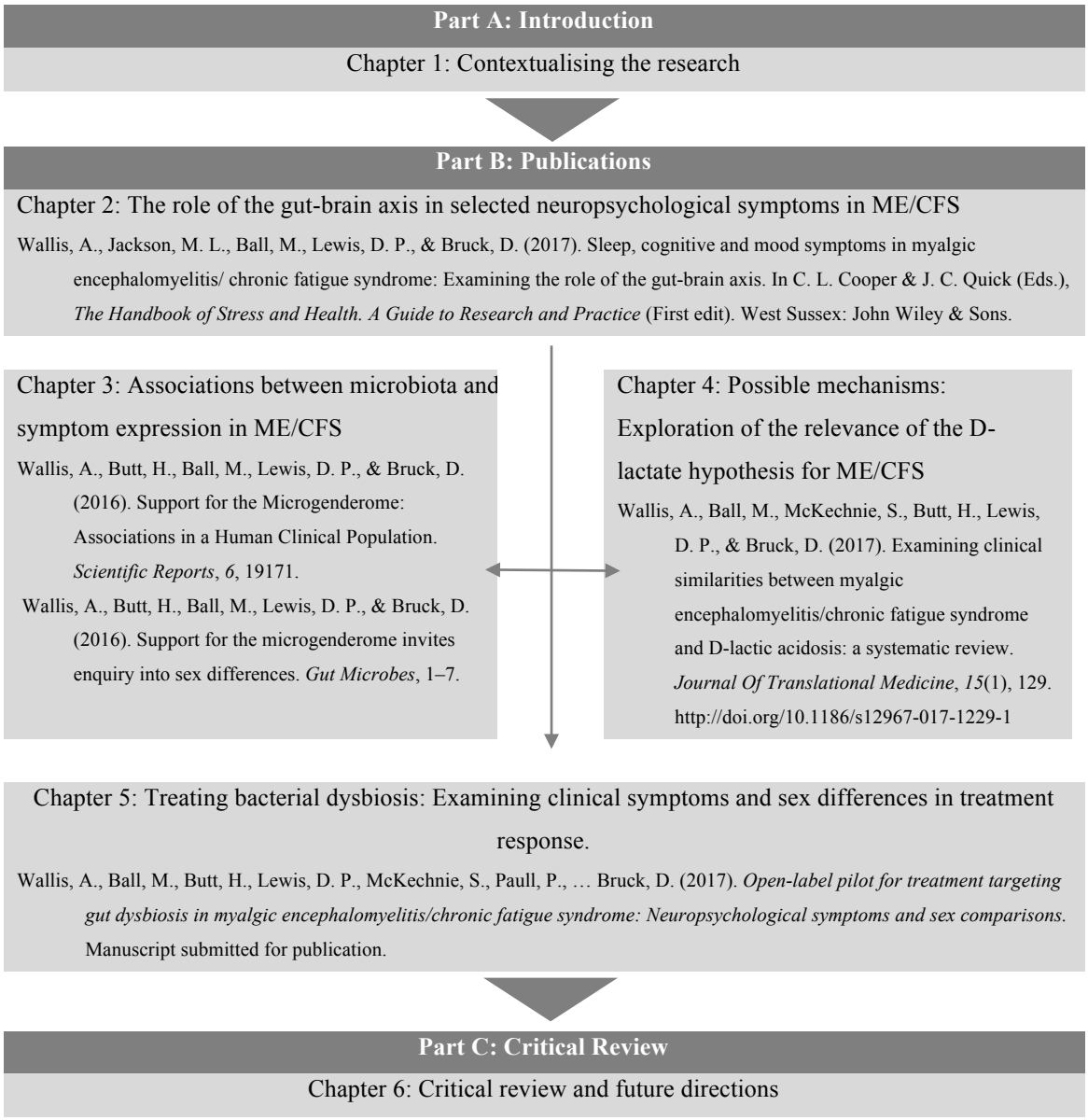

Figure 1. Thesis structure flowchart

Each chapter in Part B presents publications that respond to objectives described above. A brief introduction precedes the manuscripts to explain the rationale for each paper in relation to the broader research questions with any overlapping sections brought to the attention of the reader. Table 1 summarises the articles, publication status and author contributions of papers included in Part B of this thesis. Signed declarations of contribution by co-authors for each publication are presented before commencement of Part B.

Formatting and references

Each paper has been formatted in accordance with the book/journal requirements. Published papers are presented as the final edited version for print or online publication with references pertaining to each publication. All additional text presented throughout this thesis are presented as the one body of work with consistent layout, heading structure, and continuous page, figure and table numbering. Accordingly, the reference list for in-text citations from introductory, linking and concluding chapters are presented at the end of the thesis document in American Psychological Association 6th edition citation style.

Contribution to the field

Clarification of the pathogenesis and evaluation of treatment options for ME/CFS is essential to minimize the burden of disease. The heterogeneity of ME/CFS presentations complicates assessment and treatment pathways. Efforts to identify subgroups with similar mechanisms or symptomatic profiles (i.e., gut dysbiosis) using objective assessment methods aim to provide a targeted treatment approach. To date, few studies have examined enteric microbiota in ME/CFS and whilst the evidence suggests that there are differences between patient and control groups, interactions between microbial composition and clinical presentations are not clear and the precise mechanisms are yet to be determined. The D-lactate hypothesis has not been tested within ME/CFS populations. Examining overlap between symptoms and mechanisms in D-la and ME/CFS, measurement of D-lactate, and comparison between microbial profiles and D-lactate concentrations in ME/CFS patients will help ascertain the relevance of D-lactate theory for ME/CFS pathogenesis. Examining clinical outcomes after antibiotic and probiotic treatment for gut dysbiosis will help inform current clinical practices and direct future research. The results from the applied research conducted as part of this thesis aim to have direct clinical relevance and extend the current knowledge of ME/CFS etiology and treatment efficacy.

Table 1. Publication status and author contributions for papers presented in Part B

Part B: Publications				
Thesis Chapter	***Publication Title***	***Status***	***Nature and % of student contribution***	***Co-author name(s) Nature and % of Co-author's contribution***
Chapter 2 The role of the gut-brain axis in selected neuropsychological symptoms in ME/CFS	Sleep, cognitive and mood symptoms in myalgic encephalomyelitis/ chronic fatigue syndrome: Examining the role of the gut-brain axis.	Published 18/02/17	Concept, literature review, wrote all manuscript drafts and revisions (80%)	1. M. L. Jackson, concept, input into manuscript (5%) 2. M. Ball, concept, input into manuscript (5%) 3. D. P. Lewis, concept, input into manuscript (5%) 4. D. Bruck, concept, input into manuscript (5%)
Chapter 3 Associations between microbiota and symptom expression in ME/CFS	Support for the Microgenderome: Associations in a Human Clinical Population.	Published 13/01/16	Concept, study design, data analysis, interpretation, wrote all manuscript drafts and revisions (74%)	1. H. Butt: study design, data collection, interpretation, input into manuscript (7%) 2. M. Ball: study design, data analysis, interpretation, input into manuscript (7%) 3. D. P. Lewis: study design, data collection, interpretation, input into manuscript (5%) 4. D. Bruck, study design, data analysis, interpretation, input into manuscript (7%)
	Support for the microgenderome invites enquiry into sex differences.	Published 28/10/16	Concept, data interpretation, wrote all manuscript drafts and revisions (85%)	1. H. Butt, concept, data interpretation, input into manuscript (4%) 2. M. Ball, concept, data interpretation, input into manuscript (4%) 3. D. P. Lewis, concept, data interpretation, input into manuscript (3%) 4. D. Bruck, concept, data interpretation, input into manuscript (4%)
Chapter 4 Possible mechanisms: Exploration of the relevance of the D-lactate hypothesis for ME/CFS	Examining clinical similarities between myalgic encephalomyelitis/chronic fatigue syndrome and D-lactic acidosis: A systematic review	Published 07/06/17	Concept, design, critical appraisal of case reports, data acquisition, data interpretation, wrote all manuscript drafts and revisions. (84%)	1. M. Ball, concept, critical appraisal of case reports, data interpretation, input into manuscript (4%) 2. S. McKechnie, concept, data interpretation, input into manuscript (3%) 3. H. Butt, concept, data interpretation, input into manuscript (3%) 4. D. P. Lewis, concept, data interpretation, input into manuscript (2%) 5. D. Bruck, concept, critical appraisal of case reports, data interpretation, input into manuscript (4%)
Chapter 5 Treating bacterial dysbiosis: Examining clinical symptoms and sex differences in treatment response	Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: Neuropsychological symptoms and sex comparisons	Submitted 30/9/17	Concept, study design, recruitment, data collection, intervention and trial monitoring, data analysis, data interpretation, wrote all manuscript drafts and revisions. (74%)	1. M. Ball, concept, study design, trial monitoring, data analysis, data interpretation, input into manuscript (6%) 2. H. Butt, concept, study design, microbial data analysis and interpretation, input into manuscript (4%) 3. D. P. Lewis, concept, study design, co-ordinated recruitment, data collection and intervention, data interpretation, input into manuscript (4%) 4. S. McKechnie, design, lactate data analysis and interpretation, input into manuscript (2%) 5. P. Paull, design, lactate data analysis and interpretation, input into manuscript (2%) 6. A. Jaa-Kwee design, lactate data analysis and interpretation, input into manuscript (2%) 7. D. Bruck, concept, study design, trial monitoring, data analysis, data interpretation, input into manuscript (6%)

AUTHORSHIP DECLARATIONS

[documentation required by Victoria University]

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GRADUATE RESEARCH CENTRE

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

Title of Paper/Journal/Book:	Wallis, A., Jackson, M. L., Ball, M., Lewis, D. P., & Bruck, D. (2017). Sleep, cognitive and mood symptoms in myalgic encephalomyelitis/chronic fatigue syndrome: Examining the role of the gut-brain axis. In C. L. Cooper & J. C. Quick (Eds.), The Handbook of Stress and Health. A Guide to Research and Practice (First edit). West Sussex: John Wiley & Sons.

Surname:	Wallis	First name:	Amy
College:	Health and Biomedicine	Candidate's Contribution (%):	80

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

[redacted]	18/9/17
Signature	**Date**

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In the case of the above publication, the following authors contributed to the work as follows:

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N/A

Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
Melinda J. Jackson	5	concept, input into manuscript	[redacted]	27/9/17
Michelle Ball	5	concept, input into manuscript	[redacted]	24/9/17
Donald P. Lewis	5	concept, input into manuscript	[redacted]	21/9/17
Dorothy Bruck	5	concept, input into manuscript	[redacted]	24/9/17

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Surname:	Wallis	First name:	Amy
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Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
Henry Butt	7	study design, data collection, interpretation, input into manuscript	[redacted]	28/9/17
Michelle Ball	7	study design, data collection, interpretation, input into manuscript		24/9/17
Donald P. Lewis	5	study design, data collection, interpretation, input into manuscript		21/9/17
Dorothy Bruck	7	study design, data collection, interpretation, input into manuscript		24/9/17

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Donald P. Lewis	3	study design, data collection, interpretation, input into manuscript		21/9/17
Dorothy Bruck	4	study design, data collection, interpretation, input into manuscript		24/9/17

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Surname:	Wallis
First name:	Amy
College:	Health and Biomedicine
Candidate's Contribution (%):	84

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Michelle Ball	4	concept, critical appraisal of case reports, data interpretation, input into manuscript		24/9/17
Sandra McKechnie	3	concept, data interpretation, input into manuscript		26/9/17
Henry Butt	3	concept, data interpretation, input into manuscript		28/9/17
Donald P. Lewis	2	concept, data interpretation, input into manuscript		21/9/17
Dorothy Bruck	4	concept, critical appraisal of case reports, data interpretation, input into manuscript		24/9/17

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

	18/9/17
Signature	**Date**

3. CO-AUTHOR(S) DECLARATION

In the case of the above publication, the following authors contributed to the work as follows:

The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to a) granting bodies, b) the editor or publisher of journals or other publications, and c) the head of the responsible academic unit; and

5. The original data will be held for at least five years from the date indicated below and is stored at the following **location**(**s**):

The Victoria University R:Drive "Amy Wallis PhD" file will continue to store all digital data files for the 15 year period after completion of the study (i.e. until 2032).
Hard copy raw data will be stored in a locked filing cabinet in Michelle Ball's office at the Footscray Park Campus of Victoria University.

Name(s) of Co-Author(s)	**Contribution (%)**	**Nature of Contribution**	**Signature**	**Date**
Michelle Ball	6	concept, study design, trial monitoring, data analysis, data interpretation, input into manuscript		24/9/17
Henry Butt	4	concept, study design, microbial data analysis and interpretation, input into manuscript		28/9/17
Donald P. Lewis	4	study design, co-ordinated recruitment, data collection and intervention, data interpretation, input into manuscript		21/9/17
Sandra McKechnie	2	design, lactate data analysis and interpretation, input into manuscript		26/9/17
Phillip Paull	2	design, lactate data analysis and interpretation, input into manuscript	[redacted]	27/9/17
Amber Jaa-Kwee	2	design, lactate data analysis and interpretation, input into manuscript	[redacted]	25/9/17
Dorothy Bruck	6	concept, study design, trial monitoring, data analysis, data interpretation, input into manuscript		24/9/17

Updated: June 2015

PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
1/2	Sleep, cognitive and mood symptoms in myalgic encephalomyelitis/ chronic fatigue syndrome: Examining the role of the gut-brain axis.	Published	**Text book: Handbook of Stress and Health, published by John Wiley & Sons, 18/2/17**
2/3	Support for the Microgenderome: Associations in a Human Clinical Population.	Published	**Scientific Reports, published 13/1/16, Impact factor 4.259, H index 104, Q1**
3/3	Support for the microgenderome invites enquiry into sex differences.	Published	**Gut Microbes, published 28/10/16, Impact factor 1.959, H index 37, Q1**
4/4	Examining clinical similarities between myalgic encephalomyelitis/chronic fatigue syndrome and D-lactic acidosis: A systematic review	Published	**Journal of Translational Medicine, published 7/6/17, Impact factor 1.482, H index 77, Q1**
5/5	Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: Neuropsychological symptoms and sex comparisons	Currently under review	Submitted to Journal of Translational Medicine

Declaration by [candidate name]: Signature [redacted] Date:

Amy Wallis 1/10/17

PART B: PUBLICATIONS

CHAPTER 2

The Role of the Gut-Brain Axis in Selected Neuropsychological Symptoms in ME/CFS

The paper presented in this chapter (Wallis et al., 2017d) was accepted without any required revisions in June 2015 and subsequently published as a book chapter in *The Handbook of Stress and Health: A Guide to Research and Practice* edited by Professor Cary Cooper and James Quick. The primary aim of the chapter was to provide a review of selected neurological symptoms in ME/CFS and evaluate evidence of microbiota-gut-brain interactions. The overview of the stress response, sleep disturbances, neurocognitive dysfunction, comorbid depression and treatment modalities highlights the complexity of ME/CFS. It is likely that the heterogeneous samples and diagnostic discrepancies exacerbate the inconsistent findings within the literature, making it difficult to determine both clear pathophysiological mechanisms and distinctions between ME/CFS patients and healthy controls using some objective measures of sleep and neurocognitive functioning. At the time of writing, few studies had examined the role of commensal microbiota in ME/CFS presentations with most theories informed by results from animal or preclinical studies, or other clinical populations. This review provides a strong rationale for increasing our understanding of the gut-brain interaction in ME/CFS.

Overlap

Pages 501-504 present conceptually similar information that was discussed in Chapter 1. The text is not identical and adds detail and figures to provide a visual representation of ME/CFS etiology and microbiota-gut-brain interactions.

Paper 1

Wallis, A., Jackson, M. L., Ball, M., Lewis, D. P., & Bruck, D. (2017). Sleep, cognitive and mood symptoms in myalgic encephalomyelitis/chronic fatigue syndrome: Examining the role of the gut-brain axis. In C. L. Cooper & J. C. Quick (Eds.), *The Handbook of Stress and Health. A Guide to Research and Practice* (First edit). West Sussex: John Wiley & Sons.

[One citation as of 18th September 2017]

Wallis, A., Jackson, M. L., Ball, M., Lewis, D. P., & Bruck, D. (2017). Sleep, cognitive and mood symptoms in myalgic encephalomyelitis/chronic fatigue syndrome: Examining the role of the gut-brain axis. In C. L. Cooper & J. C. Quick (Eds.), The Handbook of Stress and Health. A Guide to Research and Practice (First edit). West Sussex: John Wiley & Sons.

CHAPTER 3

Associations between Microbiota and Symptom Expression in ME/CFS

The cross-sectional exploratory study presented in these two papers complements the few preliminary studies suggesting that enteric microbiota may play a role in ME/CFS. The original article (Paper 2, Wallis et al., 2016) presents findings from a large retrospective clinical sample exploring interactions between commensal microbiota and ME/CFS symptoms. After publishing these results in *Scientific Reports* (Paper 2, Wallis et al., 2016), the authors were invited to contribute an article (formatted as an addendum) for publication in *Gut Microbes*. The aim of this addendum (Paper 3, Wallis et al., 2017c) was to provide an expanded summary and commentary of the original findings. The discussion focuses on an appraisal of genera vs species-level analysis and explores the relevance of the results for D-lactate theory.

Overlap

The nature of these two articles, reporting results from the same analyses, means that there is considerable overlap in content presented on pp.46-47 of the addendum (Paper 3, Wallis et al., 2017c) whilst summarising the original study (Paper 2, Wallis et al., 2016).

Paper 2

Wallis, A., Butt, H., Ball, M., Lewis, D. P., & Bruck, D. (2016). Support for the Microgenderome: Associations in a Human Clinical Population. *Scientific Reports*, *6*. http://doi.org/10.1038/srep19171

[Five citations as of 18th September 2017]

The dataset associated with this research has been made publicly available: https://figshare.com/articles/Support_for_the_microgenderome_Associations_in_a_human_clinical_population_Dataset/1377862

Erratum. An inaccurate statement was found when editing this thesis. The sentence on page 5 of the article stating "*Increased D-lactic acid levels have been found in the serum of CFS patients with intestinal bacterial overgrowth*[7]*, associated with cognitive and neurological impairments*[26]*, and reduced in response to treatment in a sample of CFS patients*[27]." should commence with "*Increased D-lactate producing bacteria have been found in the stool of CFS patients*" followed by the remainder of the sentence.

Paper 3

Wallis, A., Butt, H., Ball, M., Lewis, D. P., & Bruck, D. (2017). Support for the microgenderome invites enquiry into sex differences. *Gut Microbes*, *8*(1), 46–52.

[Two citations as of 18th September 2017]

SCIENTIFIC REPORTS

OPEN

Support for the Microgenderome: Associations in a Human Clinical Population

Received: 09 April 2015
Accepted: 02 December 2015
Published: 13 January 2016

Amy Wallis[1], Henry Butt[2], Michelle Ball[1], Donald P. Lewis[3] & Dorothy Bruck[1]

The 'microgenderome' provides a paradigm shift that highlights the role of sex differences in the host-microbiota interaction relevant for autoimmune and neuro-immune conditions. Analysis of cross-sectional self-report and faecal microbial data from 274 patients with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) suggests that commensal gut microorganisms may play both protective and deleterious roles in symptom expression. Results revealed significant sex-specific interactions between *Firmicutes* (*Clostridium*, *Streptococcus*, *Lactobacillus and Enterococcus*) and ME/CFS symptoms (including neurological, immune and mood symptoms), regardless of compositional similarity in microbial levels across the sexes. Extending animal studies, we provide support for the microgenderome in a human clinical population. Applied and mechanistic research needs to consider sex-interactions when examining the composition and function of human microbiota.

Our growing knowledge of the host-microbiota interaction is rapidly informing translational research and therapeutic approaches to an array of chronic health conditions. Flagged as 'the microgenderome', gender differences and the critical role of sex hormones has been emphasized within the brain-gut-enteric-microbial axis[1]. Using an animal model, Markle *et al.* confirmed the bidirectional relationship between commensal gut microbiota, sex hormones and the immune system and provided an explanation of sexual dimorphism in Type 1 diabetes[2]. Their results revealed evidence of sex-specific microbial communities, sex-specific responses to the same microbial communities, the role of sexual maturation impacting changes to microbial communities, and evidence that microbial communities can play a protective and therapeutic role by influencing hormonal, metabolic and immune pathways. Highlighting the need to examine sex-specificity in microbial composition and function, these findings and similar[3,4] suggest that intestinal dysbiosis (marked alterations in gut microbiota[5,6]) may play causative and consequential roles in autoimmune diseases and other health conditions[2].

Intestinal dysbiosis and increased intestinal permeability (aberrations in the mucosal lining and musculature of the gastrointestinal tract) have been observed in the neuro-immune condition, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS[7–9]). The core feature of post-exertional fatigue and multi-systemic symptomatology reflect dysfunction of the central nervous system (CNS), immune systems and inflammatory pathways[10–11]. Overlapping symptom presentation and the 2:1 female-dominant incidence rates are comparative to those found in autoimmune diseases[12]. Researchers have tended to shy away from investigating this vulnerable population since the xenotropic murine leukaemia virus-related virus (XMRV) controversies[13]. However, future research is required to clarify aetiology for this complex and debilitating condition[10]. Applying the microgenderome lens to ME/CFS may provide future opportunities to elucidate unconfirmed pathophysiology and differentiate treatment pathways for this heterogeneous clinical population.

Using a cross-sectional design with a retrospective clinical data sample ($N = 274$, 68.6% female, aged 6–81 years), we were able to provide sex comparisons for a) symptom presentation; b) microbial composition and; c) interactions between microbial communities and ME/CFS symptoms (see Method for detailed explanation).

Results

Sex Differences in Symptom Presentation. To assess sex differences in symptom presentation, self-reported symptoms were categorised into thirteen factors; with twelve factors categorized according to the International Consensus Criteria (ICC[10]), plus a mood symptoms factor (Table S1). Patients rated symptom severity (past 7 days) and frequency (past 12 months) using a 5-point Likert scale (0–4). Impact scores

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	ME/CFS Symptom Factors (*Possible range*)	Females			Males			*Sex Comparison*		
		n	*Mdn* (Range)	*M* (*SD*)	*n*	*Mdn* (Range)	*M* (*SD*)	*U*	*p*	*r*
F1.	Exertion and Fatigue (0–48)	169	31(0–48)	30.01 (*15.70*)	74	31.5 (0–48)	27.77 (*16.19*)	6806.0	0.269	0.07
F2.	Neurocognitive Symptoms (0–144)	161	47 (0–120)	50.07 (*33.52*)	72	43.5 (0–120)	44.85 (*30.13*)	6241.5	0.349	0.06
F3.	Pain Symptoms (0–208)	156	45.5 (0–179)	54.02 (*43.70*)	70	21 (0–160)	31.74 (*32.29*)	7219.0	0.000***	0.26
F4.	Sleep Symptoms (0–64)	167	29 (0–64)	30.89 (*18.66*)	74	24 (0–64)	25.51 (*18.41*)	7244.5	0.033*	0.14
F5.	Neurosensory Symptoms (0–112)	167	24 (0–103)	28.31 (*21.98*)	74	17 (0–82)	21.34 (*18.88*)	7391.5	0.015*	0.16
F6.	Immunity Impairment (0–112)	165	8 (0–72)	13.5 (*15.58*)	74	4 (0–70)	9.76 (*14.15*)	7002.0	0.068	0.12
F7.	Gastrointestinal (GI) Symptoms (0–128)	163	24 (0–113)	27.93 (*22.86*)	73	11 (0–112)	19.71 (*21.85*)	7344.0	0.004**	0.19
F8.	Genitourinary (GU) Symptoms (0–48)	170	2 (0–44)	6.54 (*9.71*)	77	4 (0–48)	8.00 (*10.91*)	5959.5	0.249	-0.07
F9.	Sensitivities (0–32)	168	12 (0–32)	12.94 (*9.71*)	72	4.5 (0–32)	7.58 (*8.24*)	8098.5	0.000***	0.27
F10.	Energy Production/ Transportation Impairments (0–112)	167	22 (0–128)	30.93 (*28.67*)	72	12 (0–86)	17.78 (*19.12*)	7628.5	0.001***	0.21
F11.	Mood (0–128)	159	19 (0–113)	27.16 (*26.44*)	69	12 (0–116)	20.25c (*22.93*)	6424.5	0.040*	0.14
F12	*ICC Symptom Score* [F1-F10] (0–1008)	126	245.5 (2–826)	268.37 (*172.91*)	58	185.5 (11–607)	207.66 (*147.87*)	4480.0	0.014*	0.18
F13	*Total Symptom Score* [F1-F11] (0–1136)	120	291.5 (2–908)	264.81 (*193.41*)	57	196 (11–664)	223.72 (*161.07*)	4301.0	0.006**	0.21

Table 1. Sex differences in self-reported ME/CFS symptoms. Descriptive statistics, Mann-Whitney U test statistics and effect sizes (*r*) comparing symptom scores across the sexes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(frequency × severity of symptoms) were calculated as a measure of each factor with higher scores reflecting greater impairment. Mann-Whitney tests showed sex differences for nine of the thirteen factors with measures of central tendency indicating that females were more likely to report greater impairment (Table 1). Notwithstanding possible gender differences in self-reporting or coping styles, the upregulated serotonergic response observed in female patients with CFS[14] and evidence in parallel clinical populations, e.g., pain (osteoarthritic[15], migraine[16], and deep tissue[17], irritable bowel syndrome (IBS[18]); and depression[19] indicate that an interaction between sex steroids, neuroendocrine and immune systems is a plausible explanation for increased symptom severity and associated functional impairment in women. These results prompted investigation of pathophysiological differences.

Sex Similarity in Microbial Composition. Comparison between sexes for each genus relied on culture-based methods of assessing faecal microbial content. Metagenomic advances provide superior detection of microbial diversity, however, culture-based methods continue to have utility to examine viable count within clinical and applied research settings[6]. Genera were quantified by viable count (frequency as per cfu/g exponent) and relative abundance (RA; ratio of genera count divided by total detectable bacteria count expressed as a percentage). Anaerobic (*Bacteroides, Bifidobacterium, Clostridium, Eubacterium,* and *Lactobacillus*) and aerobic (*Escherichia*, *Streptococcus*, *Enterococcus*) genera were investigated.

Mann-Whitney tests revealed no significant sex differences in the frequency (count) or proportion (RA) of each genus (Table S2). Additionally, sex comparisons of the total detectable bacteria count (Total Bacteria: $Mdn_{\text{males}} = 10^{10}$ cfu/g, $Mdn_{\text{females}} = 10^{10}$ cfu/g, $U = 7097.5$, $P = 0.093$, $r = -0.10$) and the ratio between all detectable aerobic and anaerobic bacteria (Aerobic:Anaerobic Ratio: $Mdn_{\text{males}} = 1.21$, $Mdn_{\text{females}} = 1.10$, $U = 6844.5$, $P = 0.088$, $r = 0.10$) did not differ significantly between the sexes. These results suggest sex-consistency in microbial composition within this clinical sample.

Interactions between Microbial Community and Symptom Expression. Spearman's rank order correlations (r_s) were used to investigate sex-interactions between microbial RA and ME/CFS symptom factors (Table S3). Multiple significant associations between genera and ME/CFS symptoms indicated a pattern of results diverging between the sexes (Fig. 1). The sex-specific interactions observed for *Clostridium*, *Lactobacillus* and *Streptococcus* are discussed.

Clostridium. In females, the *Clostridium* genus was positively associated with eight of the thirteen ME/CFS symptoms. Significant small-medium positive correlations were shown for fatigue (F1: $r_s = 0.18$, $n = 166$, $p = 0.019$), neurocognitive symptoms (F2: $r_s = 0.22$, $n = 158$, $p = 0.005$), sleep (F4: $r_s = 0.24$, $n = 164$, $p = 0.002$), immunity impairments (F6: $r_s = 0.16$, $n = 162$, $p = 0.049$), total ICC symptoms (F12: $r_s = 0.25$, $n = 123$, $p = 0.006$),

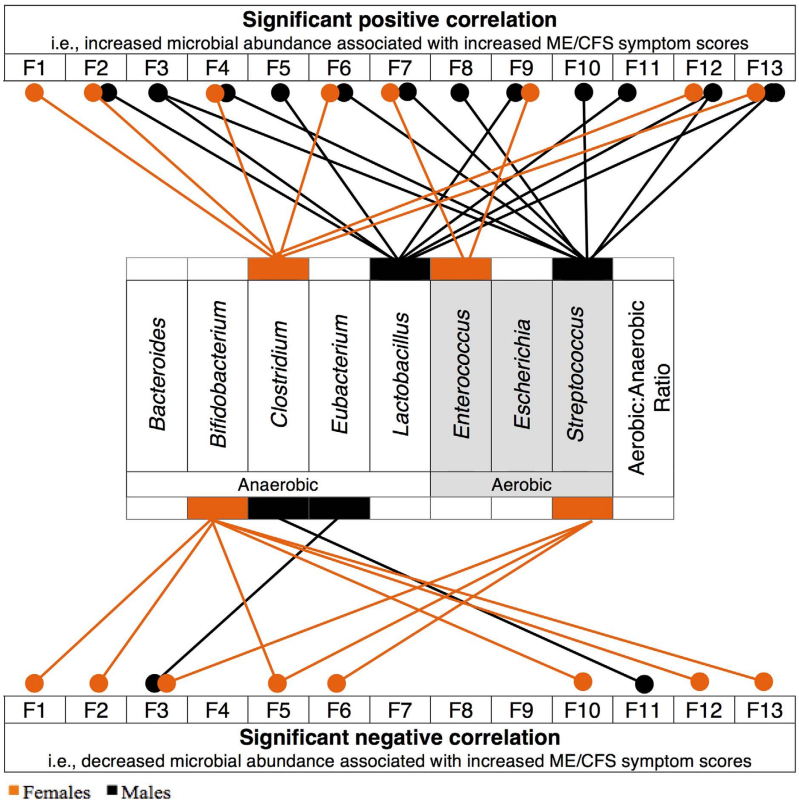

Figure 1. Associations between microbiota relative abundance and ME/CFS symptoms (F1–F13) for females (n_{range} = 120–170, orange), and males (n_{range} = 57–77, black). Only significant results from Spearman's rank correlations are presented ($P < 0.05$). Anaerobic (white) and aerobic (grey) bacteria genera are distinguished. The *Aerobic:Anaerobic Ratio*: total detectable aerobic bacteria divided by total detectable anaerobic bacteria multiplied by 1000 (including but not limited to the selected genera presented above).

and total symptoms score (F13: $r_s = 0.29$, $n = 117$, $p = 0.002$). For males, an opposite association was found, with a significant negative correlation between *Clostridium* RA and mood symptoms (F11: $r_s = -0.25$, $n = 68$, $p = 0.039$). Whilst not reaching significance, a similar pattern of results was observed for pain, gastro-intestinal, and energy production/transportation impairment factors for males (Fig. 2A and Table S3).

Lactobacillus. Figure 2B highlights the positive associations between the distribution of *Lactobacillus* and total ME/CFS symptom factors for males (F12: $r_s = 0.28$, $n = 58$, $p = 0.036$; F13: $r_s = 0.29$, $n = 57$, $p = 0.028$) in this sample (Table S3). However for females, no significant relationships were revealed between these variables. Notably, in males only, analyses recorded moderate effect sizes for neurocognitive (F2: $r_s = 0.34$, $n = 72$, $p = 0.003$) and neurosensory factors (F5: $r_s = 0.35$, $n = 74$, $p = 0.002$). Other symptoms associated with neurological impairment, including pain (F3: $r_s = 0.26$, $n = 70$, $p = 0.031$) and mood factors (F11: $r_s = 0.28$, $n = 69$, $p = 0.019$) also showed consistently significant associations and similar effect sizes for males. When considering the compositional similarity in the frequency and distribution of *Lactobacillus* across the sexes in this sample, the symptom expression differences in males may be best explained by a sex-specific response to the same microbial community.

Streptococcus. The sex-divergent pattern of associations between *Streptococcus* levels and ME/CFS symptoms was consistent across twelve of the thirteen symptom factors (Fig. 2C and Table S3). Correlations for *Streptococcus* RA suggested opposing protective or pathogenic qualities between the sexes. For males, analyses revealed small to moderate significant positive associations between *Streptococcus* RA and pain (F3: $r_s = 0.39$, $n = 70$, $p = 0.001$), sleep (F4: $r_s = 0.26$, $n = 74$, $p = 0.028$), immunity (F6: $r_s = 0.24$, $n = 74$, $p = 0.038$), gastrointestinal (F7: $r_s = 0.24$, $n = 73$, $p = 0.44$, genitourinary (F8: $r_s = 0.27$, $n = 77$, $p = 0.018$), energy production/transportation impairments (F10: $r_s = 0.24$, $n = 72$, $p = 0.045$), ICC symptom (F12: $r_s = 0.33$, $n = 58$, $p = 0.013$), and Total symptom (F13: $r_s = 0.31$, $n = 57$, $p = 0.017$) factors. Conversely for females, there were significant negative correlations between *Streptococcus* RA and pain (F3: $r_s = -0.17$, $n = 154$, $p = 0.034$), neurosensory (F5: $r_s = -0.16$, $n = 165$, $p = 0.040$), and immunity impairments (F6: $r_s = -0.21$, $n = 163$, $p = 0.007$).

Bifidobacterium: Possible sex consistency. Although only reaching significance in the female subgroup, analyses of *Bifidobacterium* RA provided an example of sex consistency in this sample (Fig. 2D and Table S3) and provided support for possible protective properties of these species. Significant, small negative correlations were

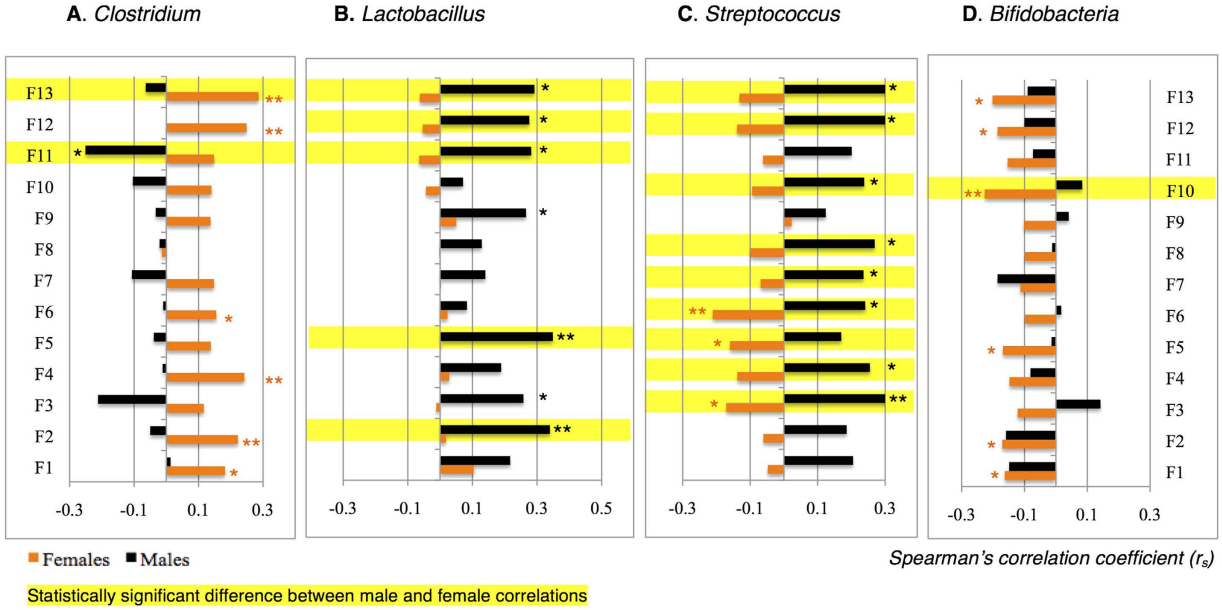

Figure 2. Microbial-dependent sex differences in ME/CFS symptoms (F1–F13) for females (n_{range} = 120–170, orange), and males (n_{range} = 57–77, black). Spearman's correlation coefficient are presented showing the size of the relationship between symptom factors and the relative abundance (RA) of A. *Clostridium*, B. *Lactobacillus*, C. *Streptococcus*, and D. *Bifidobacterium*. Positive correlations indicate that an increase in microbial relative abundance was monotonically associated with an increase in symptom scores. The direction of a positive association could also be explained in reverse. Negative correlations indicate an inverse monotonic relationship between the two variables. Correlations were classified as small (0.01), moderate (0.03) and large (0.05) effect sizes[51]. $^{*}P < 0.05$, $^{**}P < 0.01$. z_{obs} values were calculated[52] to examine whether there was a significant difference between male and female correlation coefficients. Statistically significant differences are highlighted when $z_{obs} < -1.96$ or $z_{obs} > 1.96$.

shown between *Bifidobacterium* RA fatigue (F1: $r_s = -0.16$, $n = 166$, $p = 0.036$), neurocognitive (F2: $r_s = -0.17$, $n = 158$, $p = 0.032$), neurosensory (F5: $r_s = -0.17$, $n = 164$, $p = 0.030$), energy/production and transportation impairments (F10: $r_s = -0.23$, $n = 164$, $p = 0.003$), ICC symptoms (F12: $r_s = -0.19$, $n = 123$, $p = 0.044$), and Total symptoms (F13: $r_s = -0.20$, $n = 117$, $p = 0.029$) factors.

Discussion

Observations in this ME/CFS sample showed a) sex differences in symptom presentation; b) sex consistency in microbial communities and; c) sex-specific interactions with gut microbiota and symptom expression. Associations between symptom level and bacterial level, in the context of sex consistency in microbial communities, imply sex-specific interactions with gut microbiota. Precise mechanisms of sex interactions can only be hypothesized because the hormonal status of patients was not available for this sample. It has been suggested that changes in microbial composition and the associated imbalance in production of estrogen receptor agonists/antagonists may contribute to immune disturbances and other symptoms observed in ME/CFS[20]. Specific bacterial taxa (*Firmicutes*, *Actinobacteria* and *Proteobacteria*) metabolise and consequently modulate homeostasis of sex steroid hormones through genes that encode hydroxysteroid dehydrogenase (HSD) enzymes[21]. Particular species within the genera *Clostridium, Bacteroides* and *Eubacterium* are known to produce the enzymes 7α– and 7β–hydroxysteroid dehydrogenase[22,23], deconjugating primary bile acids enabling humans and animals to absorb cholesterol, the precursor of steroid hormones. The results from this study however question our current understanding of these processes and suggest the need to examine the host relationship with intestinal organisms at the species level of each of the three genera.

The relationship between microbiota and hormones appears bidirectional. In populations with intestinal dysbiosis, the consequence of changes to hormonal metabolism and dysregulation may help explain symptom expression and variability. In reverse, hormonal imbalances may also perpetuate intestinal dysbiosis. The Firmicutes phylum of bacteria include *Clostridium*, *Lactobacillus*, and *Streptococcus* species, all of which showed interesting sex-interactions in our sample. Prospective studies should consider obtaining hormonal status and biomarkers to examine possible interactions with microbial composition in an attempt to delineate the physiology underlying these sex-differences.

Perhaps the associations between *Clostridium* composition and some ME/CFS symptoms in females may reflect the influence of diet and variation at the species level. An increase in Firmicutes has also been associated with a more typically 'Western diet' with opportunistic species *Clostridium difficile* and *Clostridium perfringens* flourishing with increased refined sugar intake[24]. The sex-specific associations in the current sample raise further questions about intestinal dysbiosis in ME/CFS, particularly investigating whether higher levels of Clostridium

species exacerbate neurological symptoms in females and the potential benefits of targeting treatment to restore intestinal balance.

Observations across *Lactobacillus* and *Streptococcus* genera suggest support for D-lactate as a contributing factor to symptom expression, particularly in males. This hypothesis explains the neurological symptoms of ME/CFS as a consequence of neurotoxic effects of bacterial metabolites (i.e., D-lactic acid produced by most species of *Lactobacillus* and *Streptococcus*) on the brain and nervous system[25]. Increased D-lactic acid levels have been found in the serum of CFS patients with intestinal bacterial overgrowth[7], associated with cognitive and neurological impairments[26], and reduced in response to treatment in a sample of CFS patients[27]. The mechanisms of a sex-specific response to D-lactic acid have not been considered.

Potential sex differences in symptom expression as a consequential or contributing factor in microbial composition have clinical and research implications. Treatment aimed at restoring intestinal homeostasis, including faecal transplants, antibiotic and probiotic therapy require consideration of individual variation and potential sex difference affecting treatment responsiveness. Clinical trials need to be designed with appropriately sized samples to enable sex comparisons. Compositional similarity within a clinical population may be falsely interpreted without considering sex interactions. Notably, the findings for *Lactobacillus spp.* in males caution against premature probiotic supplementation with D-lactate producing bacteria. However, results support the health-promoting effects of *Bifidobacteria* as observed across diverse disease states including IBS[28,29], cancer[30], anxiety and depression[31,32].

In combination, our results suggest support for the microgenderome in a human clinical population. The sex-interactions observed using genera analyses do not provide specificity and prompt the need for further examination at the species level. These results call for mechanistic research to examine the role of the sex steroid interaction with microbiota in the modulation of fatigue, pain, neural and immune responses seen within ME/CFS. This is a clinically complex but potentially advantageous research population with overlapping symptomatology relevant for diverse clinical groups. Research efforts that generate phenotypes and mechanistic understanding of the human microbiome require examination of potential sex and functional differences within compositionally similar communities.

Methods

Setting and Participants. The methods for this study were conducted in accordance with the approved guidelines for human experimental research. Ethics approval was obtained from Victoria University Human Research Ethics Committee in May 2013 (HRE13-109). As a retrospective sample, there was no direct contact with participants. Patients obtaining faecal assessment through Bioscreen (Aust.) signed informed consent to allow their microbial results and accompanying self-reported symptoms to be used for research purposes.

The dataset included 274 patients who had signed consent to participate in research during faecal microbial assessment (FMA) through the NATA (National Association of Testing Authorities) accredited laboratory, Bioscreen. All patients received a diagnosis of CFS in accordance with the Canadian Definition Criteria[33] or Fukuda criteria[34] during treatment from one of the co-authors (DPL) between January 2011 and April 2013. Only the earliest available data were included when multiple FMA results were available for the same patient.

Sex representation within this study was equivalent to prevalence ratios amongst clinical ME/CFS populations[10] with 86 male (31.4%) and 188 female (68.6%) participants. The age range of 6 to 81 years ($M = 39.25$, $SD = 14.81$) is consistently representative of the occurrence of ME/CFS across developmental stages[10]. Age was not provided for two participants. Additional demographic information or information about comorbid diagnoses were not available. Therefore, no additional exclusion criteria were applied.

Data sources/measurement. *Faecal Microbial Analysis.* Sample collection: Prior to faecal sample collection, patients were instructed to cease antibiotic and/or probiotic treatment for four and two weeks, respectively. Patients collected a sample of their first morning bowel motion in a faecal container (anaerobic pouch system) with a perforated lid to aid anaerobiasis (achieved by activating Anaero Gen Compact (Oxoid, Thermo Fisher Scientific, Australia)). Samples were immediately transported to the laboratory in cold conditions (<12 °C) for analysis within 48 hours after collection. Laboratory protocol rejects samples subjected to inaccurate collection, transportation, anaerobiasis or refrigeration procedures. Internal quality assurance investigations validated the anaerobic transport and culture methods (see[35]).

Microbial identification and quantification: After removal from the anaerobic pouch system, all faecal samples were processed within 10–15 minutes. Between 0.5–1.0 g was transferred to 10 mL of 1% glucose-saline buffer[36]. Dilution factor was determined by the difference in the weight of the glucose-saline buffer with and without the sample. One hundred and one thousand fold dilutions (beginning from 10^{-1} to 10^{-7}) of homogenised faecal samples were prepared[37]. Dilutions (10 and/or 1 μL amounts) were transferred onto dried Columbia horse blood agar (Oxoid), chromogenic medium (Oxoid), colistin and nalidixic acid blood selective agar (Oxoid), and chloramphenicol-gentamicin selective Sabouraud agar for aerobic incubation. Anaerobic incubation (4 day duration) in anaerobic jars (Oxoid) utilised pre-reduced Columbia horse blood haemin agar and Raka Ray medium. Aerobic media were incubated at 35 °C for 48 hours. A stereomicroscope was used to examine both aerobic and anaerobic culture plates for a minimum of 20 min/plate before bacterial identification. Each colony from each medium was microscopically examined and the colony/viable count were quantified for each plate. To assess purity prior to identification, similar morphotypes were sub-cultured onto horse blood agar.

Identification using MALDI-TOF MS analysis: Following overnight purity checks, index bacterial colonies were transferred to a target polished steel plate (MSP 96, Bruker Daltonics Inc.) for drying under exhaust ventilation in a Class II Biohazard Hood (Gelman Sciences Australia) at room temperature. Air-dried samples were subjected to protein extraction with 1 μL 70% formic acid (Sigma). After repeat air-drying under exhaust ventilation,

samples were overlaid with 1 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) in a mixture of 47.5% ultra-pure water, 2.5% trifluoroacetic acid, and 50% acetonitrile). Dried samples were analysed using Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear mode for the mass range of 2,000–20,000 Da at maximum laser frequency. The MALDI Biotyper 3.0 software package (default settings; Daltonik GmbH, Bremen, Germany) was used to automatically analyse and measure raw spectra without user intervention. This technology can detect approximately 5000 species. The most prevalent microorganisms are quantified (viable count detection limits include anaerobes $>10^8$ CFU/g, facultative anaerobes $>10^5$).

Data Used for Statistical Analysis. Genera investigated: Anaerobic (*Bacteroides, Bifidobacterium, Clostridium, Eubacterium,* and *Lactobacillus*) and aerobic genera (*Escherichia*, *Streptococcus*, *Enterococcus*) were investigated. Species identified during FMA were classified according to genera (data provided is the combined total of species identified within each genus). Species-level analyses were not included due to the heterogeneity of species identified during MALDI-TOF MS assessment and insufficient power to correlate less common species. Whilst genera-level investigations lack specificity, some evidence suggests similar metabolic and functional capacity within taxa and genera[38].

Justifications for selected genera: A priori selection of genera was grounded in the literature. Some of the most abundant strains of enteric microbiota within healthy human samples fall within *Bacteroides*, *Clostridium*, *Eubacterium*, and *Prevotella* as the anaerobic genera and *Escherichia* and *Streptococcus* as the aerobic genera[39]. Within infants, some of the dominant enteric microbiota include *Lactobacillus*, *Bifidobacterium* and *Streptococcus* species[40]. Whilst the abundance of specific microbiota does not necessarily equate to their purpose, function or importance[39], they provide an initial direction for examining specific genera.

The D-lactate hypothesis and relationship between increased D-lactate levels and neurocognitive impairment[26] further guided selection of genera investigated in this research. An association between D-lactic acidosis and an overgrowth of enteric lactic acid bacteria (including some species of *Lactobacillus, Bifidobacterium, Enterococcus* and *Streptococcus*) has been shown[7]. An Australian sample of patients with ME/CFS showed significantly higher levels of *Enterococcus* and *Streptococcus* genera viable count compared with healthy controls[7]. This study also showed variable levels of *Escherichia coli* amongst ME/CFS samples compared with controls, hence the *Escherichia* genus was also investigated.

A possible cause of D-lactic acidosis is from abnormal metabolism of carbohydrate by enteric microbiota[41]. Although not a primary byproduct, *Eubacterium* species can also produce lactic acid[42]. Evidence of dietary influences on microbial composition supported the rationale for including examination of *Eubacterium* (associated with dietary fibre and starch[43]); and *Clostridium* (associated with increased refined sugar inake[26]). Additionally, some strains of *Clostridium* have been associated with health[44] and others with pathology[45].

Some strains of *Lactobacillus* and *Bifidobacteria* are frequently associated with optimal health and used for probiotic supplementation[28–32,46]. Health-promoting functions of these microbiota contrast the D-lactic hypothesis and provided further justification for examining these genera.

The abundance of *Prevotella* as well as evidence of an association between colonic overgrowth and neurological symptoms[47] suggests the need to further investigate this genus. Unfortunately, *Prevotella* species were excluded from the analysis due to variable microbial identification and quantification methods during the data collection period.

Selection of the eight genera was supported by post-hoc analyses of the current dataset showing that they accounted for large proportions of detectable microbiota in the majority of stool samples. To assess the level of representation of selected genera within this ME/CFS sample, the Total RA was calculated as the combined proportion of the eight genera investigated within the total detectable bacteria (i.e., including all genera and not specifically limited to those analyzed in this study). From the 270 samples that were assessed for both aerobic and anaerobic bacteria, the eight genera represented between 5–100% of detectable microbiota ($M = 92.60\%$, $SD = 16.80\%$). The most common Total RA score was 100% with 90% of the sample showing a Total RA of equal to or above 72%. Sex comparisons of the Total RA indicated similarity in representation of the eight genera investigated ($Mdn_{\text{males}} = 99.67\%$, $Mdn_{\text{females}} = 99.77\%$, $U = 8529.0$, $P = 0.263$, $r = 0.068$).

Count: Microbial frequency of each genus was measured in colony-forming units per gram (CFU/g). Genera exponent values were used as a measure of each microbial count per patient.

Total Bacteria: Exponent values for the microbial frequency of all detectable bacteria as measured in CFU/g.

Aerobic:Anaerobic Ratio: Total detectable aerobic bacteria divided by total detectable anaerobic bacteria multiplied by 1000. This includes all genera and not specifically limited to those selected for data analysis.

Relative abundance (RA): Percentages were calculated by dividing the viable count of each genus by the total detectable bacteria count (methods akin to[39,48]). The expanded whole numbers for both counts were used in this calculation.

Total RA: The sum of *RA* percentages for the eight selected genera.

Patient Questionnaire: Concurrently to faecal sample collection, patients completed an 88-item Bioscreen Patient Questionnaire (BPQ): The BPQ is used for all referring patients regardless of clinical presentation.

Items address diverse symptomatology similar to the Symptom Checklist-90-Revised[49] and Beck Depression Inventory-II[50]. Patients rated symptom severity (past 7 days) and frequency (past 12 months) using a 5-point Likert scale (0–4). Frequency scores ranked from *none at all* (0) to *extreme* (4, severity) or *constant* (4, frequency). The BPQ showed high internal consistency within this ME/CFS population (Cronbach's $\alpha = 0.974$).

ME/CFS Symptom Factors: Seventy-six BPQ items were clinically classified into 13 factors reflecting ME/CFS symptoms in accordance with the ICC (F1-F10[10]) and mood symptoms (F11; Table S1). Seventeen items were omitted that were inconsistent with the ICC, could be misinterpreted as representative of two or more factors, or did not pertain to mood symptoms. Whilst psychological or mood symptoms are not specified under the ICC, high comorbidity with depression and anxiety symptoms in the ME/CFS population provided the rationale for further investigation of mood symptoms (predominantly depressive and anxiety symptoms). An impact score (severity × frequency) was calculated for each item (possible range 0–16) and relevant items were added to form corresponding factors. As measures of combined symptomatology, an ICC Symptoms Score (summation of F1-10) and Total Symptoms Score (summation of F1-F11) were calculated.

Bias: To reduce item selection bias, the factor classification was performed according to face validity as assessed by A.W., D.B. and M.B. and confirmed by consultation with clinician, D.P.L. No changes to the factor structure were made after commencing data analysis.

As a retrospective data sample, FMA results were initially performed for clinical purposes. Hence, no *a priori* hypotheses influenced data collection methods reducing the potential for investigator bias or falsification of data.

Statistical Methods. Descriptive statistics were performed for all ME/CFS symptom (Table 1 and Table S1) and microbial variables (Table S2) for the total sample, males and females. No outliers were found for microbial variables. The heterogeneity of symptom scores influenced the decision to include any clusters of outliers identified by SPSS on the ME/CFS Symptom Factors. Pairwise exclusion was used for missing data. All variables defied normality, therefore, nonparametric analyses were employed.

Examining Subgroups and Interactions. Sex comparisons on ME/CFS symptom factors: Descriptive statistics confirmed that each symptom factor (total, females and males separately) defied normality. A series of Mann-Whitney tests were used to compare the distribution of female and male symptom scores for each factor.

Sex comparison for microbial levels: Descriptive statistics confirmed that each microbial genus (count and RA) defied normality. A series of Mann-Whitney tests were used to compare the distribution of female and male microbial levels for count and percentage distribution independently. Effect sizes were calculated using equation:

$$r = \frac{z}{\sqrt{N}}, \tag{1}$$

where N was the total sample used in the analysis. Effect sizes were classified as small (0.01), moderate (0.03) and large (0.05) according to Cohen's (1988) guidelines[51].

Associations between microbial level and ME/CFS symptoms: Spearman's rank order correlations (r_s) were used to investigate sex-interactions between microbial RA and ME/CFS symptom factors (Table S3). Missing data were excluded pairwise from the analyses. Correlations were deemed statistically significant at $P < 0.05$. Positive correlations indicated an increase in microbial relative abundance was monotonically associated with an increase in symptom scores. The direction of a positive association could also be explained in reverse. Negative correlations indicate an inverse monotonic relationship between the two variables. Correlations were classified as small (0.01), moderate (0.03) and large (0.05) effect sizes[51].

Observed z scores were calculated using equation (2)[52] to examine whether there was a statistically significant difference between the sexes for the strength of the correlation between symptom factor and microbial RA. Differences were deemed statistically significant when $z_{obs} < -1.96$ or $z_{obs} > 1.96$[52].

$$z_{obs} = \frac{z_1 - z_2}{\sqrt{\frac{1}{N_1 - 3} + \frac{1}{N_1 - 3}}} \tag{2}$$

Design Limitations and Advantages. We caution against over-interpretation of these findings considering the limitations of cross-sectional, observational research design (unable to establish causation or consequence, difficulty excluding confounding variables[53]) and categorical analysis of genera rather than species. Other genera that were not selected during this investigation may also have relevance for ME/CFS symptomatology. Technological advancement enabling 16S amplicon sequencing of viable count will be able to identify and compare a broader range of genera and species. This will then allow comparison with other ME/CFS samples (*e.g.* [20]) and the ability to examine the representative nature of these results whilst considering the impact of ethnic and geographic diversity on microbial composition. Applied human research has clinical relevance[54] and can appropriately direct the pursuits in animal investigations where mechanistic studies are needed[21]. A symbiotic, interdisciplinary approach that integrates sex differences in clinical observational data and mechanistic data will inform therapeutic directions and treatment utility.

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A.W. wrote the manuscript; A.W., D.B. and M.B. conducted data analysis; H.B. and D.P.L. co-ordinated data collection; and all authors contributed to study design, data interpretation and manuscript editing.

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Supplementary Information for

Support for the Microgenderome: Associations in a Human Clinical Population

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This PDF file includes:

Tables S1 to S3

	Factor Structure from BPQ Items	International Consensus Criteria *(10)*
F1	**Exertion and Fatigue** *(n = 3)*	A. Postexertional neuroimmune exhaustion: Compulsory
14.	Feeling low in energy or fatigued	
50.	Avoiding certain activities due to physical problems	
58.	Unusual post exertion/exercise fatigue	
		B. Neurological Impairments
F2	**Neurocognitive Symptoms** *(n = 9)*	1. Neurocognitive impairments
9.	Trouble remembering things	a. Difficulty processing information
38.	Having to do things slowly to ensure they are correct	b. Short-term memory loss
46.	Difficulty in making decisions	
51.	Mind going blank	
55.	Trouble concentrating	
62.	Forgetfulness	
69.	Feelings of mental tiredness or fatigue	
70.	Difficulty using words or language	
78.	Mental confusion or losing your train of thought	
F3	**Pain Symptoms** *(n = 13)*	2. Pain
1.	Headaches	a. Headaches
7.	Migraine headaches	b. Significant pain
10.	Frequent muscle cramps	
13.	Face pain or tenderness	
15.	Neck pain or tenderness	
16.	Shoulder pain or tenderness	
21.	Arm pain or tenderness	
22.	Leg pain or tenderness	
24.	Stiff or painful joints first thing in the morning	
25.	Joints that hurt when you move	
26.	Locking or clicking of jaw	
27.	Pain or tenderness in your lower back	
42.	Muscle soreness or stiffness	
F4	**Sleep Symptoms** *(n = 4)*	3. Sleep disturbances
34.	Unrefreshed or prolonged sleep	a. Disturbed sleep patterns
44.	Trouble falling asleep	b. Unrefreshed sleep
64.	Trouble waking up in the morning	
66.	Restless or disturbed sleep	
F5	**Neurosensory Symptoms** *(n = 7)*	4. Neurosensory, perceptual and motor disturbances
8.	Unusual muscle twitches	
29.	Tinnitus or noise in the ear	a. Neurosensory and perceptual
33.	Photophobia or dislike of strong light	b. Motor
52.	Loss of feeling, tingling or numbness of the skin	
56.	Muscle weakness or feeling of weakness in the body	
68.	Hypersensitive skin	
71.	Trouble focusing your eyes	
		C. Immune, Gastro-intestinal and Genitourinary Impairments

F6	**Immunity Impairment** *(n = 7)*	1. Flu-like symptoms may be recurrent or chronic and typically activate or worsen with exertion.
2.	Sinusitis or nasal congestion	
31.	Sore throat	
45.	Persistent cough	2. Susceptibility to viral infections with prolonged recovery periods.
53.	Sore or swollen lymph glands in the neck	
59.	Sore or swollen lymph glands	
65.	Sore or swollen lymph glands in the groin	
76.	Recurrent mouth ulcers	
F7	**Gastro-intestinal Symptoms** *(n = 8)*	3. Gastro-intestinal tract
19.	Poor appetite	
23.	Abdominal pain or tenderness	
37.	Unexplained diarrhoea	
40.	Nausea or upset stomach	
41.	Constipation	
77.	Symptoms of irritable bowel	
82.	Gastric reflux or heartburn	
83.	Cravings for certain foods	
F8	**Genitourinary symptoms** (n = 3)	4. Genitourinary
43.	Frequent urination	
57.	Burning or uncomfortable urination	
63.	Urgent urination	
F9	**Sensitivities** (n = 2)	5. Sensitivities to food, medications, odours or chemicals
17.	Allergies, intolerance or reactivity to food	
61.	Reactivity to smells or chemicals	
F10	**Energy Production/Transportation Impairments** *(n = 7)*	D. Energy production/transportation impairments
4.	Faintness or dizziness	1. Cardiovascular
39.	Heart pounding	
85.	Low blood pressure	
48.	Breathlessness or chest pain upon exertion	2. Respiratory
6.	Night sweats, unusual sweating while asleep	3. Loss of thermostatic stability
49.	Hot and cold spells or recurrent feverishness	4. Intolerance of extremes of temperature
75.	Cold hands or feet	
F11	**Mood** *(n = 8)*	*No reference made to psychological or mood symptoms on the ICC.*
3.	Repeated unpleasant thoughts	
5.	Loss of libido or sexual interest	
20.	Crying easily over your problems	
30.	Feeling blue as a results of your problem	
32.	Feeling no interest in things	
54.	Feelings of hopelessness about the future	
72.	Spells of panic related to your problems	

87.	Feeling anxious	
F12	**ICC Symptom Score** *(n = 63)*	
	All items from F1-F10	
F13	**Total Symptom Score** *(n = 71)*	
	All factor items, ICC and mood items combined.	
	Items Omitted *(n = 17)*	**N/A**
11.	Grinding or clenching your teeth	*Unsure about placement*
12.	Chest or heart pain	*Unsure about placement*
18.	Arthritis	*Non-ME/CFS Symptom*
28.	Feeling that your problems are disrupting your life	*Secondary*
35.	Stress from financial problems	*Secondary*
36.	Feeling that others are unsympathetic to your problems	*Secondary*
47.	Ovulation or menstruation pain	*Extensive missing data*
60.	Orchialgia or testicular pain	*Extensive missing data*
67.	Vaginal irritation or discomfort	*Extensive missing data*
73.	Sciatica or numbness/tingling down the back of the leg	*Unsure about placement*
74.	Frequently getting into arguments	*Secondary*
79.	Stressful events in your life related to your problems	*Secondary*
80.	Dermatitis	*Unsure about placement*
81.	Stress over family problems	*Secondary*
84.	High blood pressure	*Unsure about placement*
86.	Stress from work problems	*Secondary*
88.	Feelings of guilt	*Unsure about placement*

Table S1. ME/CFS symptom factor structure. Classification of BPQ items according to International Consensus Criteria *(10).* BPQ: Bioscreen Patient Questionnaire.

			Total *N*			Males			Females			Sex Comparison		
			N	*M* (*SD*)	*Mdn* (*Range*)	*N*	*M* (*SD*)	*Mdn* (*Range*)	*N*	*M* (*SD*)	*Mdn* (*Range*)	*U*	*p*	*r*
Selected Anaerobic Genera	*Bacteroides*	Count (CFU/g)	270	9.02 (2.22)	10 (0-11)	85	8.76 (2.71)	10 (0-11)	185	9.14 (1.95)	10 (0-11)	8033.0	0.752	0.02
		RA (%)	270	58.35 (31.59)	61.45 (0-100)	85	58.21 (31.47)	58.97 (0-100)	185	58.41 (31.73)	62.30 (0-100)	7965.0	0.863	0.01
	Bifidobacterium	Count (CFU/g)	271	5.54 (3.97)	8 (0-10)	86	5.48 (4.10)	8.00 (0-10)	185	5.61 (3.91)	7 (0-10)	7910.0	0.939	-0.01
		RA(%)	271	11.44 (19.66)	0.48 (0-89.26)	86	11.81 (20.17)	0.76 (0-89.26)	185	11.27 (19.46)	0.38 (0-81.35)	7861.0	0.874	-0.001
	Clostridium	Count (CFU/g)	270	3.49 (4.15)	0 (0-10)	85	3.11 (4.07)	0 (0-10)	185	3.66 (4.18)	0 (0-10)	8416.5	0.297	0.06
		RA (%)	270	3.47 (8.26)	0 (0-53.17)	85	2.83 (6.46)	0 (0-35.45)	185	3.76 (8.97)	0.00 (0-53.17)	8380.5	0.333	0.06
	Eubacterium	Count (CFU/g)	270	5.11 (4.57)	8 (0-11)	85	5.00 (4.66)	8 (0-10)	185	5.16 (4.55)	8 (0-11)	7791.5	0.899	0.01
		RA (%)	270	17.10 (24.25)	5.30 (0-100)	85	15.51 (20.43)	4.16 (0-81.55)	185	17.84 (25.84)	5.98 (0-100)	8048.5	0.744	0.02
	Lactobacillus	Count (CFU/g)	271	3.68 (3.45)	5 (0-10)	86	3.83 (3.45)	5 (0-9)	185	3.62 (3.45)	5 (0-10)	7734.5	0.700	-0.02
		RA (%)	271	1.62 (6.76)	0.001 (0-69.75)	86	1.89 (9.25)	0.001 (0-69.75)	185	1.49 (5.24)	0.001 (0-39.20)	7766.0	0.742	-0.02
Selected Aerobic Genera	*Enterococcus*	Count (CFU/g)	274	1.81 (2.83)	0 (0-8)	86	1.78 (2.80)	0 (0-8)	188	1.83 (2.84)	0 (0-8)	8182.0	0.843	0.01
		RA (%)	274	0.44 (6.05)	0 (0-100)	86	0.13 (0.75)	0.00 (0-5.45)	188	0.58 (7.29)	0.00. (0-100)	8155.0	0.886	0.01
	Escherichia	Count (CFU/g)	247	5.84 (2.08)	6 (0-9)	86	6.10 (2.01)	6 (0-9)	188	5.72 (2.11)	6 (0-8)	7097.5	0.093	-0.10
		RA (%)	272	1.15 (7.67)	0.04 (0-87.59)	86	2.76 (13.30)	0.05 (0-87.59)	186	0.40 (1.75)	0.04 (0-22.27)	7108.0	0.140	-0.089
	Streptococcus	Count (CFU/g)	274	4.51 (2.67)	6.00 (0-9)	86	4.38 (2.80)	5.5 (0-8)	188	4.56 (2.61)	6 (0-9)	8291.5	0.726	0.02
		RA (%)	272	0.18 (0.99)	0.01 (0-11.17)	86	2.48 (1.25)	0.004 (0-11.17)	186	0.15 (0.85)	0.01 (0-10.49)	8333.0	0.576	0.03
Total Bacteria		Count (CFU/g)	271	9.73 (0.66)	10 (6-11)	86	9.76 (0.65)	10 (7-11)	185	9.72 (0.67)	10 (6-11)	7097.5	0.093	-0.10
Aerobic:Anaerobic Ratio			270	9.75 (45.94)	1.21 (0-666.73)	85	15.95 (73.40)	1.32 (0-666.73)	185	6.90 (24.50)	1.10 (0-286.85)	6844.5	0.088	0.10

Table S2. Microbial genera descriptive statistics and sex comparison results. Descriptive statistics for each microbial genus (count and relative abundance separately) across total participants, males and females. Count (CFU/g): Exponent value presented (i.e., 9.02 = $10^{9.02}$). Relative abundance (RA): ratio of genera viable count divided by total bacteria count expressed as a percentage. Total Bacteria count: exponent value of total bacteria detectable on MALDI-TOF MS assessment. Aerobic:Anaerobic Ratio: total detectable aerobic bacteria divided by total detectable anaerobic bacteria multiplied by 1000. *U*: Mann Whitney test value calculated by comparing the rank order of scores between two groups *(52)*. Effect sizes (*r*) calculated from Mann-Whitney tests comparing differences in the microbial distribution between males and females. Effect sizes were classified as small (.01), moderate (.03) and large (.05) *(51)*. No significant sex differences were shown ($P > 0.05$).

		Selected Anaerobic Genera										Selected Aerobic Genera									
		Bacteroides RA		*Bifidobacterium* RA		*Clostridium* RA		*Eubacterium* RA		*Lactobacillus* RA		*Enterococcus* RA		*Escherichia* RA		*Streptococcus* RA		Total Bacteria Count		A:AN Ratio	
		M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
F1	$r_{(s)}$	-.02	.07	-.15	-.16*	.01	.18*	.01	.05	.22	.10	-.10	.06	.05	-.02	.21	-.05	-.07	.01	.08	.03
	P	.839	.389	.206	.036	.912	.019	.967	.511	.063	.182	.378	.434	.654	.780	.080	.534	.550	.898	.523	.700
	n	73	166	74	166	73	166	73	166	74	166	74	169	74	167	74	167	74	166	74	166
F2	$r_{(s)}$	.11	.04	-.16	-.17*	-.05	.22**	.05	.05	.34**	.02	-.08	.01	.003	-.06	.19	-.06	-.05	.07	.01	.03
	P	.343	.615	.182	.032	.676	.005	.673	.578	.003	.822	.492	.926	.981	.483	.119	.444	.682	.414	.950	.716
	n	72	158	72	158	72	158	72	158	72	158	72	161	72	159	72	159	72	158	72	158
F3	$r_{(s)}$	.000	-.06	.14	-.12	-.21	.12	-.24*	.06	.26*	-.01	-.12	.15	-.01	-.03	.39***	-.17*	-.20	.01	.14	.04
	P	1.000	.441	.241	.133	.078	.155	.044	.443	.031	.885	.326	.056	.909	.743	.001	.034	.098	.873	.255	.627
	n	70	153	70	153	70	153	70	153	70	153	70	156	70	154	70	154	70	153	70	153
F4	$r_{(s)}$	.03	.05	-.08	-.15	-.12	.24**	-.01	.01	.19	.03	-.21	-.05	.04	.04	.26*	-.14	-.08	-.03	.07	.02
	P	.809	.513	.493	.059	.329	.002	.953	.898	.107	.724	.072	.500	.707	.66	.028	.078	.515	.718	.552	.792
	n	73	164	74	164	73	164	73	164	74	164	74	167	74	165	74	165	74	164	74	164
F5	$r_{(s)}$	-.04	.001	-.01	-.17*	-.04	.14	-.08	-.004	.35**	.003	.07	.06	.05	-.02	.17	-.16*	-.08	-.02	.10	.03
	P	.752	.993	.906	.030	.746	.079	.496	.964	.002	.972	.535	.479	.664	.822	.147	.040	.498	.843	.377	.71
	n	73	164	74	164	73	164	73	164	74	164	74	167	74	165	74	165	74	164	74	164
F6	$r_{(s)}$	-.08	.000	.02	-.10	-.01	.16*	.02	.01	.08	.02	-.06	.14	.10	.004	.24*	-.21**	-.13	-.02	.07	.06
	P	.512	.999	.887	.214	.935	.049	.868	.948	.484	.772	.604	.079	.390	.964	.038	.007	.276	.827	.542	.423
	n	73	162	74	162	73	162	73	162	74	162	74	165	74	163	74	163	74	162	74	162
F7	$r_{(s)}$	.01	-.07	-.19	-.11	-.11	.15	.10	.09	.14	.003	-.14	.15*	-.08	.02	.24*	-.07	-.05	.06	-.001	.07
	P	.950	.414	.115	.156	.369	.063	.391	.280	.237	.974	.232	.049	.503	.841	.044	.383	.71	.478	.995	.359
	n	73	160	73	160	73	160	73	160	73	160	73	163	73	161	73	161	73	160	73	160
F8	$r_{(s)}$	-.14	-.02	-.01	-.10	-.02	-.01	-.02	.09	.13	-.003	-.12	.01	.02	-.03	.27*	-.10	-.22	-.03	.14	-.10
	P	.235	.848	.918	.204	.854	.861	.850	.248	.262	.971	.283	.895	.833	.670	.018	.202	.054	.709	.231	.211
	N	76	167	77	167	76	167	76	167	77	167	77	170	77	168	77	168	77	167	77	167
F9	$r_{(s)}$	-.03	.03	.04	-.10	-.03	.14	.03	.05	.27*	.050	-.18	.21**	-.06	.01	.12	.02	-.001	-.01	-.06	.10
	P	.830	.713	.734	.193	.783	.080	.791	.560	.024	.526	.138	.006	.614	.918	.301	.772	.991	.931	.614	.200
	n	71	165	72	165	71	165	71	165	72	165	72	168	72	166	72	166	72	165	72	165
F10	$r_{(s)}$	-.05	.13	.08	-.23**	-.10	.14	-.05	-.05	.07	-.04	.03	.05	.07	-.01	.24*	-.09	-.33**	-.01	.13	.04
	P	.657	.11	.482	.003	.390	.073	.706	.533	.555	.572	.776	.567	.535	.936	.045	.228	.005	.924	.264	.629
	n	71	164	72	164	71	164	71	164	72	164	72	167	72	165	72	165	72	164	72	164
F11	$r_{(s)}$	.20	.04	-.07	-.15	-.25*	.15	-.10	-.002	.28*	-.07	.03	-.03	.01	.02	.20	-.06	-.001	-.03	.01	.04
	P	.106	.66	.554	.06	.039	.065	.417	.981	.019	.421	.826	.732	.970	.828	.097	.441	.991	.761	.922	.590
	n	68	156	69	156	68	156	68	156	69	156	69	159	69	157	69	157	69	156	69	156
F12	$r_{(s)}$	-.11	.03	-.10	-.18*	-.001	.25**	.04	.02	.28*	-.05	-.05	.11	.04	-.04	.33*	-.14	-.24	.07	.08	.03
	P	.422	.742	.455	.044	.996	.006	.780	.860	.036	.552	.725	.214	.787	.651	.013	.128	.068	.424	.543	.787
	n	58	123	58	123	58	123	58	123	58	123	58	126	58	124	58	124	58	123	58	123
F13	$r_{(s)}$	-.08	.03	-.09	-.20*	-.06	.29**	.03	-.002	.29*	-.06	-.03	.11	.03	-.01	.31*	-.13	-.25	.04	.06	.06
	P	.535	.73	.507	.029	.638	.002	.839	.985	.028	.501	.815	.221	.829	.948	.017	.155	.064	.699	.644	.495
	n	57	117	57	117	57	117	57	117	57	117	57	120	57	118	57	118	57	117	57	117

Table S3. Associations between microbial composition and ME/CFS symptom factors. Spearman's rank order correlations (r_s) are shown for respective male (M) and female (F) subgroups with variable sample sizes (*n*). Relative abundance (RA): calculated from ratio of each genus viable count divided by total bacteria count expressed as a percentage. Total Bacteria count: calculated from exponent value of total bacteria detectable on MALDI-TOF MS assessment. Aerobic:Anaerobic Ratio: total detectable aerobic

bacteria divided by total detectable anaerobic bacteria multiplied by 1000. Correlations (r_s) were classified as small (.01), moderate (.03) and large (.05) *(51)*. *$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

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ADDENDUM

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Support for the microgenderome invites enquiry into sex differences

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ABSTRACT

The microgenderome defines the interaction between microbiota, sex hormones and the immune system. Our recent research inferred support for the microgenderome by showing sex differences in microbiota-symptom associations in a clinical sample of patients with myalgic encephalomyelitis / chronic fatigue syndrome (ME/CFS). This addendum expands upon the sex-specific pattern of associations that were observed. Interpretations are hypothesized in relation to genera versus species-level analyses and D-lactate theory. Evidence of sex-differences invites future research to consider sex comparisons in microbial function even when microbial abundance is statistically similar. Pairing assessment of clinical symptoms with microbial culture, DNA sequencing and metabolomics methods will help advance our current understandings of the role of the microbiome in health and disease.

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Introduction

Evidence of the bidirectional role of the microbiome in human health continues to emerge. Patients with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) present with excessive post-exertional exhaustion and a complex array of symptoms suggestive of multi-systemic abnormalities.[1] Gastrointestinal, immune and neurological symptoms in ME/CFS makes this an appropriate clinical population to examine brain-gut-microbiota interactions. The recent proposition of the 'microgenderome' emphasizes the potential mediating and modulatory role of sex hormones in these interactions.[2] Flak et al.'s explanation and other animal studies have shown that microbiota manipulation can alter hormonal, metabolic, inflammatory and/or immune processes.[3,4] Twin studies have revealed that the once similar microbial composition of opposite-sex twins becomes distinctly different after puberty when compared to same-sex twins that remain compositionally similar.[5] Application of the microgenderome lens has only recently been applied to a human clinical population.[6] The focus of this addendum is to provide a comprehensive summary of the original results and additional commentary on our earlier findings. We discuss further interpretations and implications related to genera compared with species-level analyses and D-lactate theory.

Our research[6] indicated support for the microgenderome by showing sex-specific associations between gut microbiota and symptom presentation in ME/CFS (detailed below). Results from faecal microbial assessments and self-reported symptoms were analyzed from 274 ME/CFS patients. Sex comparisons for self-reported ME/CFS symptoms showed that females tended to report greater impairment than males. The cross-sectional design impeded clear interpretation of the reason for these observed differences. A longstanding belief is that females tend to over-report symptoms compared with under-reporting in males.[7] However, accumulating evidence suggests that increased perception of symptoms in females correlates with higher circulating cytokine levels[8] and more chronic health problems[9] compared to males. Hence, our results may reflect gender differences in self-reporting or pathophysiological differences in ME/CFS presentation.

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kgmi.
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Culture-based methods for bacterial identification (MALDI-TOF MS) were used to measure microbial composition (see methods from original report[6]). The frequency and relative abundance (RA) of selected anaerobic (*Bacteroides, Bifidobacterium, Clostridium, Eubacterium*, and *Lactobacillus*) and aerobic (*Escherichia, Streptococcus*, and *Enterococcus*) genera were similar across the sexes (data available in Table S2 in the original article[6]). Sex-differences between self-reported symptoms in the presence of compositional similarity led to investigation of possible sex-interactions between microbiota and symptoms.

Sex differences in symptom-microbiota associations

Non-parametric correlations between symptoms and the microbial abundance of specific genera showed a sex-divergent pattern of associations. Effect sizes were small to medium suggesting that microbiota-symptom interactions may reflect one piece of the complex ME/CFS puzzle. As highlighted in our original article,[6] sex differences were notable for *Streptococcus, Lactobacillus* and *Clostridium* genera. Significant associations between RA of other genera and symptom factors were also shown for males and females independently. In this addendum we present all significant associations from our original analyses including genera (*Enterococcus, Eubacterium*) and *Total Bacteria Count* (defined in Fig. 1 legend) that were not previously discussed. Figure 1 clearly demonstrates a divergent pattern of associations between the sexes.

Streptococcus and *Clostridium* were the only 2 genera that showed significant associations with symptom factors for both males and females. Surprisingly, an opposite direction of associations was observed. Increased streptococcal colonization was associated with more impairment in males (Fig. 1, eight significant positive correlations noted) but less impairment in females (Fig. 1, three significant negative correlations found). For females, higher levels of clostridial colonization correlated with higher symptom scores (Fig. 1, six positive significant correlations). For clostridial colonisation in males, only one significant negative correlation was identified, whereby mood symptoms and *Clostridium* levels were inversely related (Fig. 1). Possible reasons for these differences are merely speculative at this stage and are explored below.

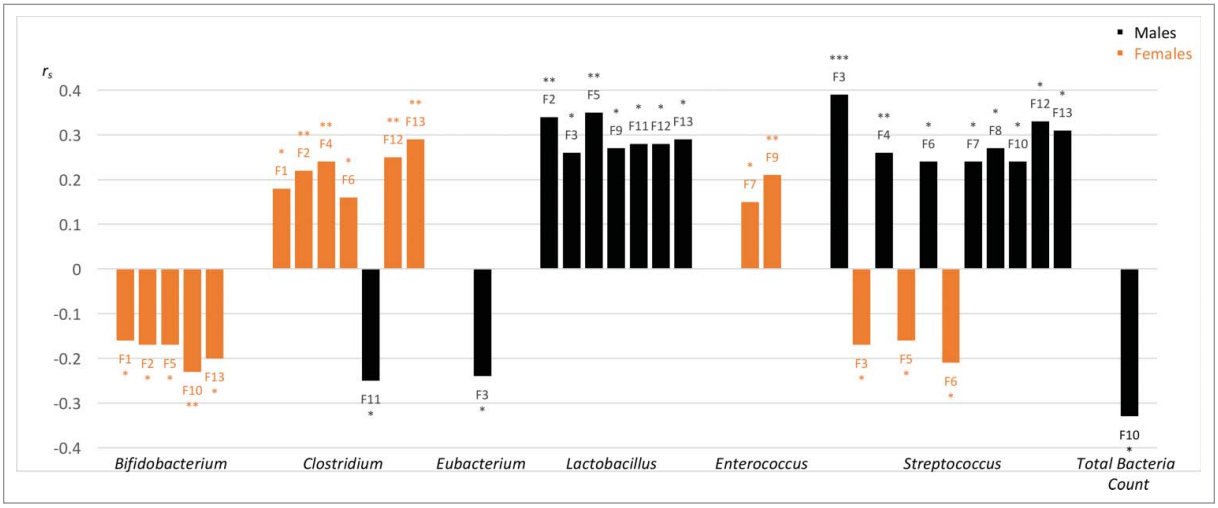

Figure 1. Summary of significant associations between genera relative abundance (RA) and ME/CFS symptom factors (F1-F13). All significant ($P \leq 0.05$) Spearman's rank order correlations (r_s) are shown highlighting differences between males (black) and females (orange). RA: calculated from ratio of each genus viable count divided by t *Total Bacteria Count* expressed as a percentage. Total Bacteria Count: calculated from exponent value of total bacteria detectable on MALDI-TOF MS assessment. Symptom factors included: F1. Fatigue, F2. Neurocognitive, F3. Pain, F4. Sleep, F5. Neurosensory, F6. Immune, F7. Gastro-intestinal, F8. Genitourinary, F9. Sensitivities, F10. Energy Production/Transportation Impairments, F11. Mood, F12 ICC Symptom Score (sum of F1-F10), F13. Total Symptom Score (sum of F1-F11). F12 reflects diagnostic symptoms from the International Consensus Criteria (ICC) for ME/CFS. F13 also includes the mood factor (F11) as these frequently comorbid symptoms are not a diagnostic requirement. Positive correlations show that symptom factor and RA covary in the same direction i.e. either both increasing or both decreasing. An inverse monotonic association is indicated by negative correlations. Correlations can be interpreted as small (0.01), moderate (0.03) and large (0.05) effect sizes.[35] $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$.

For males, *Eubacterium* and *Total Bacteria Count* also showed significant negative correlations (Fig. 1). Pain symptoms (F3) correlated negatively with *Eubacterium* RA. Negative correlations were observed between *Total Bacteria Count* and all symptom factors in males. The one significant negative correlation between *Total Bacteria Count* and self-reported energy production/transportation impairments (F10) in males (see Fig. 1) may reflect reduced capacity for energy production with less bacterial numbers. Colonic bacterial metabolism of volatile fatty acids has been shown to account for approximately 10% of energy production in humans.[10] Using metagenomics sequencing methods, decreased bacterial abundance and diversity has been shown within inflammatory bowel disease (IBD) and obesity populations.[11,12] Sex differences were either not considered[11] or statistically controlled[12] in these studies. Inter-individual differences of bacterial abundance and diversity within healthy populations[13] support the need to investigate functional difference in energy metabolism to more accurately interpret our results.

Genera analyses – A starting point

Analysis at the genus level provides a broad picture of interactions. An example of the complexity of possible interpretations is provided by examining the results for *Clostridium*. *Clostridium* RA and symptom associations highlight the potential importance of this genus in ME/CFS females. However, correlational analyses impede our ability to determine whether the increase in symptoms associated with increased *Clostridium* RA is causative or consequential. Further evidence of functional diversity within the *Clostridium* genus makes interpretation difficult and invites species-level analyses.

Varied protective roles of *Clostridium* species have been described within the literature. The commensal properties of the genus *Clostridium* have been recognized within experimental and clinical research. Atarashi and colleagues[14] found that specific components of the immune system may be modulated by *Clostridium*, with clusters IV and XIVa promoting regulatory T cell (T_{reg}) production and associated anti-inflammatory effects. *Clostridium*-abundant mice evidenced less colitis, improved bowel markers and reduced allergic response. No reference was made to the sex of the mice used in these studies, nor was reference made to the potential effect of bacterial diversity, i.e., multi-strained colonisation (46 strains of *Clostridium*) compared with colonisation with fewer strains (segmented filamentous bacteria, 3 strains of lactobacillus, and 16 strains of Bacteroides). Consistent with animal models, human investigations have shown lower ratios of *Clostridium leptum* and *Clostridium coccoides* in patients with IBD compared with healthy controls.[15] Results to date are not causative, however, the potential beneficial role of *Clostridium* species in gut health warrants further investigation.

Other *Clostridium* species have been associated with disease. Key examples of the potentially deleterious role of *Clostridium* species include the well-documented neurotoxic and enterotoxic effects of many species including *Clostridium botulinum* and *Clostridium difficile*.[16] Higher incidence of *Clostridium* species have been identified in patients with irritable bowel syndrome compared with healthy controls.[17] Additionally, opportunistic species *Clostridium difficile* and *Clostridium perfringens* proliferate with increased refined sugar intake.[18] Conflicting findings call for consideration of species- and host-specific effects. Genetic diversity and dietary interactions with microbiota may promote differing commensal or deleterious effects dependent on the individual.[19]

Results from the current sample raise more questions than answers. The associations between neurological symptoms and *Clostridium* in females may reflect the neurotoxic effects of specific species that may be mediated or modulated by sex hormones in a subset of ME/CFS patients. However, our interpretations are limited because hormonal and metabolic profiles were not collected for this retrospective sample and could not be correlated with bacterial composition. Species level investigations and functional microbial assessment are required to ascertain the role of *Clostridium* in ME/CFS presentations and why this may differ between the sexes. Increased specificity has value for all of the genera examined. Genus-level analyses provide initial insights and demand further investigation at the species-level to aid interpretation.

D-lactate theory

For males, *Streptococcus* was highlighted as a genus positively associated with ME/CFS symptom factors, suggesting a potential deleterious role. This result may support the application of D-lactate theory for ME/

CFS. D-lactic acidosis (D-la) is a condition originally observed in ruminants.[20] In humans, it is primarily reported in patients with short bowel syndrome where an increased level of D-lactate is associated with neurological symptoms reflecting encephalopathy.[21] Certain species of *Streptococcus, Lactobacillus, Bifidobacterium and Enterococcus* produce more D-lactate (the isomer of L-lactate).[22,23] Humans have the capacity to metabolise both D- and L-lactate.[24,25] However, D-lactic acid can accumulate in the presence of bacterial overgrowth, triggered by carbohydrate metabolism and in individuals with impaired or reduced D-lactate metabolism.[26] Increased abundance of D-lactate producing bacteria[22] and symptom overlap between ME/CFS and D-la lead to the suggestion that a similar mechanism may occur for both conditions. While D-la is an acute condition, subclinical levels of D-lactate may play a role in the neurological symptoms of ME/CFS. Our team are currently investigating this possibility.

Streptococcus sanguinis has been shown to produce more D-lactic acid from glucose metabolism and is involved in maintaining a more acidic environment.[22] In ruminants, greater carbohydrate intake increased *Streptococcus bovis* growth, reduced the pH level and encouraged the growth of *Lactobacillus* species.[20] These mechanisms appear consistent in humans. The pH level influences bacterial composition. A more acidic environment (lower pH) encourages the growth of acid-resistant bacteria (including *Lactobacilli*) and increases lactic acid production.[27] Several D-la case studies have shown an overgrowth of *Lactobacillus* species in stool samples.[27-29] With no comparative control group, we could not determine whether ME/CFS patients in our sample had an overgrowth of *Lactobacilli*. Nonetheless, results for *Lactobacillus* support the application of D-lactate theory in male patients. Significant positive correlations were shown between *Lactobacillus* RA and neurocognitive, pain, neurosensory, gastrointestinal and mood symptoms for males (Fig. 1). These ME/CFS symptoms overlap with symptoms of D-la.[30]

Application of the D-lactate theory in females is less clear. No significant associations were yielded for *Lactobacillus* and reverse significant negative associations were found between *Streptococcus* and pain, neurosensory and immune symptoms (Fig. 1). While *Enterococcus* RA was significantly and positively correlated with gastrointestinal symptoms and sensitivities (food and chemical) in females (Fig. 1), neurological symptoms did not reach significance. These results raise the possibility of sex-differences in D-lactate metabolism. The opposing microbial-symptom associations for males and females suggests that the functional role of microbiota, and perhaps D-lactic acid, may differ between the sexes. The role of D-lactate in ME/CFS is only a theoretical proposition at this stage. Sex comparison of species-level analysis of gut microbiota, bacterial metabolites and D-lactic acid levels in ME/CFS patients will help evaluate the validity of this theory.

As discussed in our original paper,[6] results for *Bifidobacteria* add further complexity to the argument. Sex consistency and positive microbial-symptom correlations for this genus do not support the relevance of D-lactate theory for either sex. Similar to other genera discussed, only selected species of the genus *Bifidobacterium* produce excess D-lactate. Investigation at the species level will clarify these unanswered questions.

Future considerations

Clinical and research settings should not underestimate the value of sex comparisons. As indicated by our results, comparison between the proportion of genera in male and female patients revealed sex-similarities. However, further analyses examining symptom-bacterial interactions suggest that merely using a surface-level comparison of bacterial composition is too simplistic. More detailed analyses of the functional differences between similar organisms are likely to provide a more comprehensive picture. Optimally, future studies will also measure sex hormone levels to advance our current understanding of the bidirectional interaction between hormones and microbial composition.

Male mice are preferentially used in animal studies.[31] Historically, this has been due to the suggested variability that occurs throughout the estrous cycle.[32] Recent evidence negates this proposition and encourages the inclusion of female mice in biomedical and neuroscience research.[32] Our results echo the proposed policy changes by the US National Institutes of Health[31] and recommend that animal research and clinical trials are designed to enable sex comparisons to accurately interpret results and establish efficacious treatments across the population.

A limitation of our results is the use of culture-based methods compared to metagenomic sequencing. Advanced sequencing technology has superior capacity to detect bacterial diversity.[33] This raises the possibility that some species and genus unable to be cultured may also be relevant for ME/CFS. However, distinguishing viable genetic material can be limited using sequencing technology. Using culture methods within the context of functional and applied pathology, we have focused on a small selection of viable genera compared to the hundreds of bacterial species with unclear viability that can be revealed through sequencing methods.[11] Hence our results do not exclude the relevance of other organisms not identified in this research. Nevertheless, culture methods remain valuable for gaining information about how bacteria react to other bacteria and respond to their environment.[34] In fact, combining culture and sequencing methods may ensure that we continue to advance our knowledge of microbial function at the same rate as the rapidly growing identification of new bacterial species. Regardless of the selected method, examination of sex differences in bacterial function remains pertinent.

Extension of our results requires the use of metabolomics technology to accurately examine functionality of bacterial species across individuals. Concurrently with metagenomic advances, metabolomics technology allows the genetic potential of bacteria to be compared with the biological metabolites of species.[34] Metabolic profiling of the gut microbiome appears to not only have localized effects. Animal studies showed that both microbiome manipulation and infection can lead to metabolic changes in multiple anatomical sites including the liver and brain.[34] While the technology is still in its infancy, this information is likely to dramatically improve our understanding of mind-gut interactions and the microgenderome. The bacterial environment, related energy production and metabolism can vary according to intrinsic and extrinsic characteristics including sex, age, diet, climate, ethnicity, disease status and hormonal status. It is predicted that measurement of bacterial metabolites, including but not restricted to metabolic hormones, neurotransmitters and lactate production will advance understanding of mechanisms involved in ME/CFS. Inter-individual comparisons will enable exploration of potential sex differences and clarification of the relevance of D-lactate theory for this population.

As authors from psychology, medical and microbiology fields, we encourage inter-disciplinary collaboration and education. The brain-gut-microbial axis and our results in this ME/CFS sample suggest that some symptoms previously considered in isolation (e.g., neurological, gastrointestinal and immune symptoms) may have shared mechanisms. In conjunction with technological advances, collective insights from multiple disciplines will enhance our understanding of the complexities of the microgenderome's role in human health. If we can understand the function of the microbiota/microbiome for each individual, we can more accurately assess gut dysbiosis, metabolic abnormalities, deficiencies or accumulated toxic metabolites that may be related to disease processes. A future with more individualised assessments and targeted interventions appears within closer reach.

Abbreviations

D-la	D-lactic acidosis
F. prausnitzii	*Faecalibacterium prausnitzii*
IBD	inflammatory bowel disease
ME/CFS	myalgic encephalomyelitis/chronic fatigue syndrome
RA	relative abundance

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Contributions

A.W. wrote the manuscript and all authors provided conceptual guidance and contributed to data interpretation, manuscript design and editing.

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CHAPTER 4

Possible Mechanisms: Exploration of the Relevance of the D-Lactate Hypothesis for ME/CFS

The accumulating body of evidence presented thus far in this thesis, suggests that commensal microbiota and specific genera may be related to ME/CFS symptoms. It is unclear the direction of this relationship, but if symptoms are a consequence of underlying dysbiosis, there are several possible mechanisms that could be involved including intestinal permeability, systemic inflammation, and altered neurotransmitter or metabolic production. D-lactate is one metabolite produced by some bacterial species that has been suggested as contributing to ME/CFS symptoms. The systematic and narrative review presented in Paper 4 (Wallis et al., 2017b) aimed to ascertain the relevance of the D-lactate hypothesis in ME/CFS by determining symptomatic and mechanistic overlap between D-la and ME/CFS.

Overlap

The '*Background'* presented on pp.1-2 contextualises this study with a summary of ME/CFS and D-la that is consistent with descriptions in the introduction and earlier papers. Also, the subsections '*Gastrointestinal abnormalities*' (p. 16), '*Bacterial dysbiosis, antibiotic and probiotic treatment*' (pp. 16-18), and '*Implications for gut-brain interactions*' (p. 18) have similar but expanded and more specific content to that discussed in prior chapters.

Paper 4

Wallis, A., Ball, M., McKechnie, S., Butt, H., Lewis, D. P., & Bruck, D. (2017). Examining clinical similarities between myalgic encephalomyelitis/chronic fatigue syndrome and D-lactic acidosis: A systematic review. *Journal Of Translational Medicine*, *15*(1), 129. http://doi.org/10.1186/s12967-017-1229

[No citations as of 18th September, 2017]

The excel spreadsheet for Supplementary Table 1 can be accessed online at https://figshare.com/collections/Examining_clinical_similarities_between_myalgic_encephalomyelitis_chronic_fatigue_syndrome_and_d-lactic_acidosis_a_systematic_review/3798199

Wallis et al. J Transl Med (2017) 15:129
DOI 10.1186/s12967-017-1229-1

Journal of Translational Medicine

REVIEW Open Access

Examining clinical similarities between myalgic encephalomyelitis/chronic fatigue syndrome and D-lactic acidosis: a systematic review

Amy Wallis[1*], Michelle Ball[1], Sandra McKechnie[2], Henry Butt[3], Donald P. Lewis[4] and Dorothy Bruck[1]

Abstract

Background: The pursuit for clarity in diagnostic and treatment pathways for the complex, chronic condition of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) continues. This systematic review raises a novel question to explore possible overlapping aetiology in two distinct conditions. Similar neurocognitive symptoms and evidence of D-lactate producing bacteria in ME/CFS raise questions about shared mechanisms with the acute condition of D-lactic acidosis (D-la).

Methods: D-la case reports published between 1965 and March 2016 were reviewed for episodes describing both neurological symptoms and high D-lactate levels. Fifty-nine D-la episodes were included in the qualitative synthesis comparing D-la symptoms with ME/CFS diagnostic criteria. A narrative review of D-la mechanisms and relevance for ME/CFS was provided.

Results: The majority of neurological disturbances reported in D-la episodes overlapped with ME/CFS symptoms. Of these, the most frequently reported D-la symptoms were motor disturbances that appear more prominent during severe presentations of ME/CFS. Both patient groups shared a history of gastrointestinal abnormalities and evidence of bacterial dysbiosis, although only preliminary evidence supported the role of lactate-producing bacteria in ME/CFS.

Limitations: Interpretation of results are constrained by both the breadth of symptoms included in ME/CFS diagnostic criteria and the conservative methodology used for D-la symptom classification. Several pathophysiological mechanisms in ME/CFS were not examined.

Conclusions: Shared symptomatology and underlying microbiota–gut–brain interactions raise the possibility of a continuum of acute (D-la) versus chronic (ME/CFS) presentations related to D-lactate absorption. Measurement of D-lactate in ME/CFS is needed to effectively evaluate whether subclinical D-lactate levels affect neurological symptoms in this clinical population.

Keywords: Acidosis, lactic, Dysbiosis, Fatigue syndrome, chronic, Encephalomyelitis, myalgic, Microbiota-gut–brain, Neurological symptoms

Background

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a complex condition with evidence of multi-systemic dysfunction. The primary symptom of post-exertional fatigue is accompanied by heterogeneous neurological, immune, cardiovascular, respiratory and/or gastrointestinal manifestations [1]. Research efforts continue to search for biomarkers to aid etiological understandings and treatment options for this debilitating condition. It has been proposed that some neurological symptoms may be related to an imbalance of commensal

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gut bacteria (i.e., gut dysbiosis [2]). Within ME/CFS, evidence of gut dysbiosis [3, 4] and associations between microbial genus and symptom expression [5] raise questions about whether gut dysbiosis plays a causative or mechanistic role in onset, maintenance and/or symptomatic variability. The mechanisms are not clear because microbe–gut–brain interactions can occur through several pathways (i.e., central, autonomic, and enteric nervous systems; neuroendocrine and neuroimmune; enteric microbiota) [6–8]. Investigations of other conditions with similar presentations may aid the current etiological understanding of ME/CFS. D-Lactic acidosis (D-la) is an acute condition that shares some similar features with ME/CFS and provides a clear example of the microbe–gut–brain interaction.

D-la is a type of metabolic acidosis with the primary presentation of encephalopathy (i.e., impaired mental state including confusion, loss of memory or cognitive capacity) [9]. D-la has also been referred to as "D-lactate neuropathy" or "D-lactate encephalomyelitis" in humans and "floppy kid syndrome", or "drunken lamb syndrome" in animals. Originally described in ruminants [10], the condition has now been observed in multiple human case reports since 1979 [11].

The neurological symptoms and associated biochemical imbalances of D-la appear to result from gastrointestinal dysfunction. D-la is most commonly observed in patients with short bowel syndrome (SBS), often after surgery or removal of a section of the small bowel [12]. This reduced length diminishes the small bowel's functional capacity to effectively metabolise carbohydrates leading to excessive bacterial fermentation in the colon [13]. Small intestinal carbohydrate malabsorption precipitates an increase in colonic acidity and the consequential overgrowth of certain species of colonic microbiota that produce an abundance of D-lactate. Healthy humans have the capacity to effectively metabolise D-lactate [14, 15]. However, the combination of high levels and insufficient D-lactate metabolic capacity can result in excess accumulation of D-lactate in the blood and absorption within the brain, resulting in the neurological symptoms characteristic of D-la [13].

Higher levels of D-lactate producing bacteria (such as *Streptococcus* and *Enterococcus*) have been identified in stool samples from patients with ME/CFS [4]. This evidence, combined with some similar neurological symptoms in both conditions, has led to comparison with D-la and proposal of the D-lactate hypothesis for ME/CFS. Accordingly, this hypothesis suggests that an increased abundance of D-lactate producing bacteria and suspected higher circulating levels of D-lactate may contribute to the neurological manifestations of ME/CFS. However, this hypothesis has not been systematically evaluated. Neither plasma nor urine D-lactate levels have been documented in ME/CFS to date. This lack of clinical D-lactate data coupled with confusion surrounding the degree of symptom overlap between D-la and ME/CFS provide the rationale for this qualitative review. To help ascertain the relevance of the D-lactate hypothesis for ME/CFS, Part A of this review aims to (a) systematically summarise published D-la episodes that report neurological symptoms and D-lactate levels; and (b) examine the overlap between D-la and ME/CFS symptom. Part B provides a narrative review of proposed neurological mechanisms in D-la to examine its relevance for ME/CFS aetiology.

Main text

Part A. Systematic qualitative review

Method

MEDLINE (via Ebscohost) and PubMed databases were searched for publications from 1965 to April 1 2016. To obtain papers referring to D-la, the following search terms were used: D-lact* AND (acidosis OR encephalopathy OR neuropathy). These databases were also searched for ME/CFS articles referring to acidosis (search terms: acidosis AND ("chronic fatigue syndrome" OR "myalgic encephalomyelitis"). Reference lists from articles obtained were manually screened to find other relevant references. Figure 1 shows the PRISMA flowchart for identification, screening and article exclusion.

Qualitative synthesis

Sixty-eight case reports were screened for inclusion in the qualitative synthesis (see Table 1). Case reports were screened in a two-step process. The first stage of this process involved identifying case reports that reported both D-lactate levels and neurological symptoms during an episode of D-la. Fifteen case reports were excluded at this stage due to an inability to obtain full-text or inadequate reporting of neurological symptoms and/or D-lactate levels. A serum D-lactate level of greater than 3.0 mmol/L has been proposed as a marker for D-la diagnosis [16]. However, using this criterion for exclusion was considered to be inappropriate when there were varying measurement methods used throughout the case reports. To reduce bias in case report selection, all cases that measured D-lactate and indicated that the patient's D-lactate level was 'high' or above the 'normal' range, as stipulated by the authors and relevant measurement method, were included. Only one episode was excluded [17] when plasma D-lactate fell within the normal range according to the chosen method of measuring D-lactate defined within this case report. Across the episodes reviewed, there were considerable discrepancies between sampling and measurement methods (see Additional file 1:

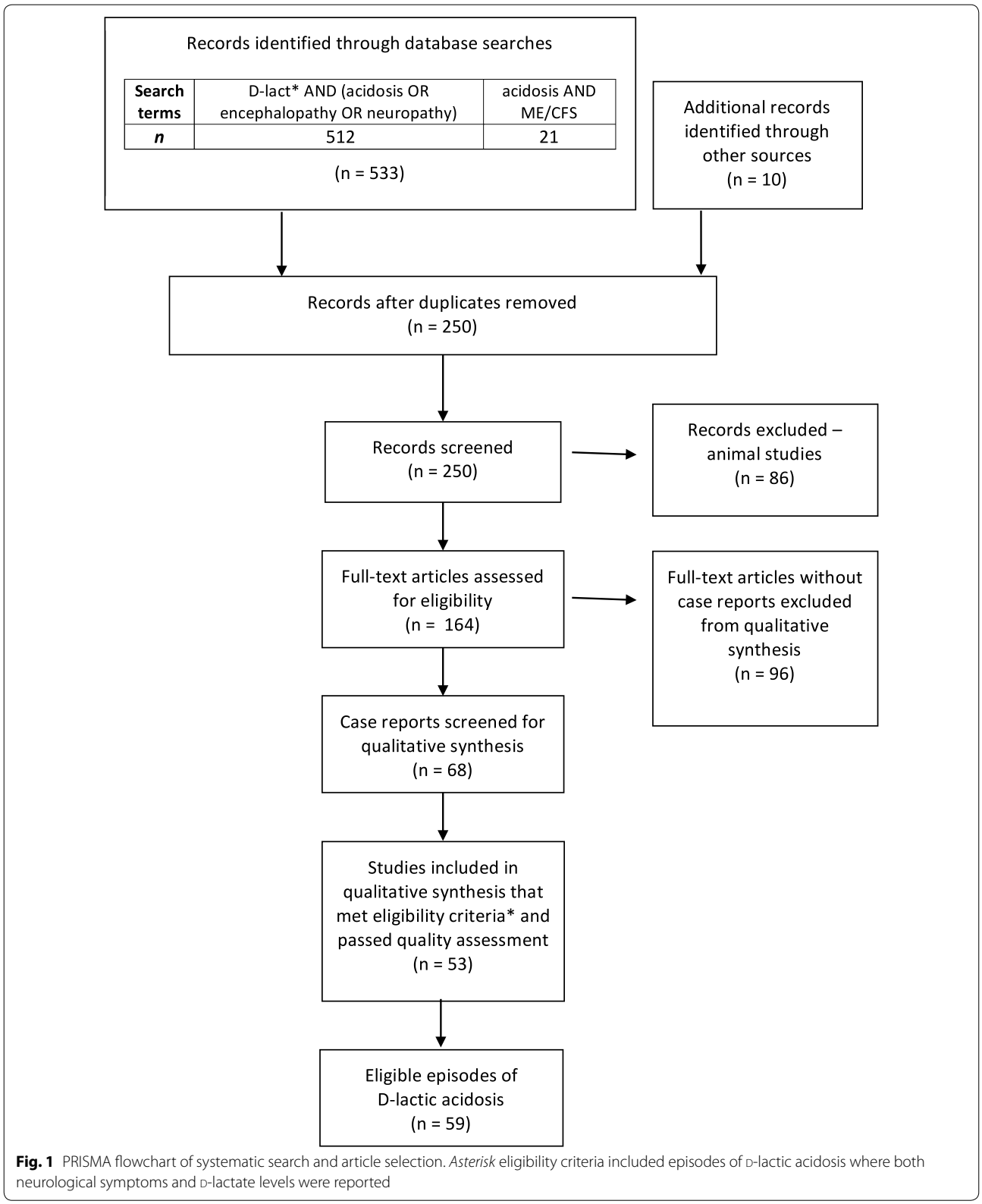

Fig. 1 PRISMA flowchart of systematic search and article selection. *Asterisk* eligibility criteria included episodes of D-lactic acidosis where both neurological symptoms and D-lactate levels were reported

Table 1 D-Lactic acidosis case reports screened for qualitative synthesis

Episode code #	References	Included	Reasons for exclusion
1	[20]	N	D-Lactate measurement not specified
2	[17]	N	Plasma D-lactate within normal range
3	[21]	N	D-Lactate measurement not specified
4	[22]	Y	
5	[23]	N	D-Lactate measurement not specified
6	[24]	Y	
7	[25]	N	D-Lactate not D-la; No comparison between D-lactate and neurological symptoms
8	[26]	Y	
9	[27]	Y	
10	[28]	Y	
11	[29]	Y	
12a and 12b	[30]	Y	
13	[31]	Y	
14	[32]	Y	
15	[33]	Y	
16_1 and 16_2	[34]	Y	
17	[35]	Y	
18	[36]	Y	
19	[37]	Y	
20	[38]	Y	
21	[39]	Y	
22	[15]	N	D-Lactate levels not presented in relation to neurological symptoms
23	[40]	Y	
24	[41]	Y	
25	[42]	Y	
26*	[43]	Y	
27	[44]	Y	
28	[45]	Y	
29	[46]	Y	
30	[47]	Y	
31	[48]	Y	
32	[49]	N	Unable to obtain full-text
33	[50]	Y	
34_1 and 34_2	[51]	Y	
35	[52]	Y	
36*	[53]	Y	
37	[54]	Y	
38	[55]	N	Neurological symptoms not specified
39*	[56]	Y	
40	[57]	Y	
41	[58]	Y	
42*	[59]	Y	
43	[60]	Y	
44	[11]	Y	
45	[61, 62]	N	Same case for both references; D-lactate levels not specified
46	[63]	N	D-Lactate not measured
47a and 47b	[64]	Y	
48	[65]	Y	
49	[66]	Y	
50	[67]	Y	
51a and 51b	[68]	Y	
52	[69]	Y	
53	[70]	Y	
54	[71]	N	Unable to obtain English full-text
55	[72]	Y	
56	[73]	Y	
57	[74]	Y	
58a and 58b	[75]	Y	
59	[76]	Y	
60	[77]	Y	
61	[78]	N	D-Lactate measurement not specified
62	[79]	Y	
63	[80]	N	Neurological symptoms not specified
64	[16]	Y	
65	[81]	Y	
66	[82]	Y	
67	[83]	N	D-Lactate only measured during intervention

Subscript numbers ($_1$ and $_2$) indicate separate episodes for the same patient. The letters *a* and *b* identify different patient cases reported in the same reference. Episodes from non-SBS patients are marked with an asterisk. Episodes included in qualitative synthesis simultaneously reported at least one high D-lactate level (from blood or urine analysis) and documented neurological symptoms

Table S1). A discussion paper on measurement issues and analyses is being compiled by our team and beyond the scope of the current review.

During the second stage of screening, the remaining 53 case reports were independently assessed for quality by a team of three critical appraisers. Each article was assessed by two appraisers using the checklist developed by The Joanna Briggs Institute [18] based on the CARE Guidelines [19] established to improve the quality of reporting clinical cases. Appraisers rated (Yes, Unclear, No or N/A) on the eight items pertaining to (1) Demographic characteristics; (2) Patient history; (3) Current clinical condition; (4) Diagnostic tests; (5) Treatment/intervention; (6) Post-intervention clinical condition; (7)

Adverse events; and (8) Take away lessons. Items 1–4 were prioritised as they were most relevant for the focus of this review. When comparing ratings across these four items, most articles (47/53, 90.1%) were rated as 'Yes' by both critical appraisers. For the remaining 6 articles, at least one reviewer provided a rating of 'Unclear' on an item. Discrepancies in ratings were discussed and the appraisers agreed that all articles adequately covered these priority domains and were deemed appropriate for inclusion in the qualitative synthesis.

Case reports that described multiple episodes (either for the same patient or different patients) were included as separate episodes if they met the eligibility criteria. From the 53 case reports, a total of 59 episodes were identified and included for qualitative synthesis. Each episode was reviewed with information about patient demographics, medical history, comorbid conditions, proposed triggers, neurological symptoms, non-neurological symptoms, D-lactate levels, L-lactate levels, anion gap, pH levels, microbial composition and treatment tabulated (Additional file 1: Table S1).

Determining ME/CFS and D-la symptom overlap

Reported D-la symptoms (neurological and non-neurological) were compared with ME/CFS International Consensus Criteria (ICC; [1]. Comorbid mood symptoms (including anxiety and depression) not required for ME/CFS diagnosis but frequently experienced by patients were also included for comparison with D-la presentations. D-la symptoms were classified as *'matching'* ME/CFS symptoms or *'ambiguous/other'*. D-la symptoms were only classified as *matching* if terminology was directly comparable to the symptoms described in the ICC (see Table 2). All other symptoms were categorised as *ambiguous/other.*

Neurological symptoms

As neurological symptoms were the primary focus of this review, further categorisation was used to obtain more information about the types of neurological symptoms that accompany a D-la presentation. The *ambiguous/other* neurological symptoms were delineated into ME/CFS categories B1–B4, *speech* and *consciousness* subgroups (see Table 2). Reports of altered consciousness formed a distinct subcategory (*consciousness*) to identify the proportion of patients that presented with this more severe neurological symptom.

Speech and language impairment may have shared pathophysiology with other motor or neurocognitive disturbances. The ME/CFS ICC describes 'slow speech' without mentioning any other specific speech or language impairments [1]. Impaired information processing and word retrieval have been described as cognitive manifestations of ME/CFS, with speech therapy being a suggested treatment option [84]. The transient speech and language symptoms (e.g. dysarthria and/or slurred and incoherent speech) in D-la are likely to be overt behavioural manifestations of underlying muscle weakness and/or neurocognitive disturbances. However, without further information from each report, speech symptoms (excluding 'slow speech') were grouped as a subcategory for further investigation.

Conservative methodology

As highlighted by the aforementioned distinct classification of speech and language symptoms, we chose to pursue a conservative method of symptom categorisation. Several other *ambiguous* D-la symptoms were highly suggestive of ME/CFS and more likely to reflect discrepancies in terminology rather than different symptomatology per se. Inconsistent assessment and reporting of symptoms can reflect differences between patient demographics (i.e., age or sex), disciplines, and professional settings. This is particularly pertinent when considering comparisons between terminology used to describe chronic (i.e., ME/CFS) versus acute (i.e., D-la) presentations. For example, a patient presenting with 'fluctuating consciousness' or 'comatose' may preclude further neurological assessment and thus limit reporting of other covert symptoms. Similarly, the observable nature of motor and speech/language symptoms may be more frequently reported during an acute hospital presentation unlike some neurocognitive symptoms that require more specific testing and comparative measures to notice, for example a deterioration in memory, attention and clarity of thought. In another example, 'slowed cerebellar function' was used to describe D-la symptoms. This term is likely to reflect similar ME/CFS motor disturbances. However, the ICC does not specifically refer to 'slowed' movement, hence this symptom was classified as *ambiguous/other*. Consequently, our method of clinically comparing ME/CFS and D-la symptoms was conservative and chosen to ensure that symptom overlap was not inflated.

The presence of each reported D-la symptom was identified by episode number (see Table 1). This enabled frequencies and percentages to be calculated for both broad (A–D) and specific (B1–B4, C1–5, D1–4) ME/CFS ICC categories. Many episodes reported multiple neurological symptoms both within and between different subcategories i.e. *neurocognitive impairments* (B1) and *motor disturbances* (B4b). In these circumstances, each episode was only counted once for each specific subcategory. Likewise, an episode was only included once when calculating the presence of symptoms in each broad category, i.e. *neurological impairments* (B). Frequencies and percentages were calculated for each symptom category and

Table 2 Mapping overlap between myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and D-lactic acidosis (D-la) symptoms

ME/CFS International Consensus Criteria [1]	D-la symptoms mapped to ME/CFS criteria	
	Matching	**Ambiguous/other**
A. Postexertional neuroimmune exhaustion (compulsory)	A. Lethargy/fatigue	
B. Neurological impairments (at least one symptom from 3 of the 4 categories) B1. Neurocognitive impairments B2. Pain B3. Sleep disturbances B4a. Neurosensory and perceptual and B4b. Motor disturbances	B1. Encephalopathy/Mental confusion/disorientation/dazed/Concentration difficulties/Slow processing and responding to questions/slow speech B2. Headaches/Muscle pain B3. Drowsiness/sleepiness/somnolence B4a. Blurred vision B4b. Weakness/hypotonic (lowered muscle tone)/flaccidity/impaired gait (staggering/wide/ataxic/unsteady/instability)/ataxia (movement and co-ordination difficulties)/impaired balance	B1. Altered mental state/cortical dysfunction (e.g., disoriented to date, time, place and space)/delirium/blunted judgment/abnormal EEG B4a. Hallucinations (visual and auditory)/delusions/paranoid ideation B4b. Slowed cerebellar function/movement/dysiadochokinesia (difficulty performing rapid movement)/impaired reflexes/Neuropathy (fine motor coordination difficulties)/unable to grasp objects/Ptosis (eye drooping)/Asterixis (hand 'flapping'/tremor)/Spasms: nystagmus (eye spasms)/opisthotonos (muscle spasms leading to hyperextended posture)/Bruxism *Speech symptoms:* Slurred and incoherent speech/dysarthria (speech pronunciation difficulties, weak muscles effecting speech)/thickened speech/ataxic speech (explosive—pauses between syllables) *Consciousness:* Altered/fluctuating/comatose/intermittent coma/stupor/induced sleep/depressed level of consciousness/obtunded/fluctuating from unrousable to alert
C. Immune, gastro-intestinal and genitourinary impairments (at least one symptom from 3 of the 5 categories) C1. Flu-like symptoms C2. Prone to viral infections C3. Gastro-intestinal abnormalities: nausea, abdominal pain, irritable bowel syndrome, bloating C4. Genitourinary symptoms C5. Sensitivities to food, medication, odours or chemicals	C3. Gastrointestinal symptoms*: Increased diarrhea/bowel movements Nausea/vomiting Diffuse abdominal pain	
D. Energy production/transportation impairments (at least 1 symptom) D1. Cardiovascular: orthostatic intolerance (inability to tolerate an upright position), postural orthostatic tachycardia syndrome, palpitations, arrhythmias, hypotension, dizziness, pallor D2. Respiratory: labored breathing, air hunger, fatigue of chest wall muscles D3. Thermostatic instability: lowered body temperature, cold extremities, marked diurnal fluctuations, sweating, episodic feverishness D4. Intolerance to temperature extremes	D1. Inability to stand/sit upright/Tachycardia (rapid heart rate)/Respiratory arrhythmia/Hypotension/low blood pressure/Dizziness/Pallor D2. Breathing difficulties: hyperpnoea (deep breathing)/dyspnoea (shortness of breath)/tachypnea (rapid breathing)/Kussmaul (deep and laboured)/breathlessness/hyperventilation D3. Body temperature changes (high or low)	D1. Bradycardia (slowed heart rate) D2. Respiratory acidosis and hypercarbic respiratory failure
Comorbid Mood and Behavioural Disturbances 1. Depressive symptoms 2. Anxiety symptoms	1. Unhappy/agitation/irritability 2. Anxiety	Irrational/unusual/disturbed behavior/aggressive/hostile/abusive/combative/uncooperative behavior/euphoria/aloofness
Uncategorized D-la symptoms		*Metabolic acidosis* *Other abnormalities:* dehydration/cravings (water, cigarettes)/excessive thirst Acute renal failure/hyperchloremic acidosis/liver dysfunction

ME/CFS broad category B. Neurological impairments are highlighted as the primary focus of this review and to show three subcategories of delineation under *ambiguous/other* symptoms (i.e., in accordance with specific ICC criteria (B1 – B4), speech/language symptoms, and level of consciousness)

* Gastro-intestinal symptoms associated with short bowel syndrome or the patient's medical history were not included as symptoms of D-la. Only reports of a *change* in gastrointestinal symptoms were included

ambiguous/other: symptoms that were not clearly identified as consistent with ME/CFS presentation (see Table 2 for detailed symptom delineation), *D-la* D-lactic acidosis, *matching*: mapped overlap between ME/CFS and D-la symptoms, *ME/CFS* myalgic encephalomyelitis/chronic fatigue syndrome

delineated by available demographic details (age and sex). Episodes were classified as paediatric (≤17 years) or adult (≥18 years).

Results

Systematic summary of D-la episodes

A total of 59 episodes of D-la reported both neurological symptoms and D-lactate levels. The average patient age during D-la presentation was 29.9 years ($SD = 21.0$). Twenty-two paediatric (age range 10 months to 16 years, $M = 7.1$ years, $SD = 4.5$ years) and 37 adult (age range 18–60 years, $M = 43.4$ years, $SD = 13.9$ years) episodes were examined. There were 35 male and 23 female episodes with similar sex ratios documented for adult males ($n = 20$) and females ($n = 17$). Patient sex was not identified in one paediatric case. A predominance of male paediatric episodes ($n = 15$) were found compared with female paediatric episodes ($n = 6$). D-la episodes were primarily from patients with a history of SBS (55/59, 93.2%). The four patient episodes without SBS presented with propylene glycol intoxication [43], chronic pancreatitis [53], acute lymphoblastic leukaemia [56], and surgery error [59]. Table 3 summarises the frequency and percentage of reported D-la symptoms by age (paediatric and adult), sex (male and female) and total episodes.

Table 3 shows some evidence of shared symptomatology across each broad ME/CFS ICC category. The highest percentage of overlap was found for neurological symptoms. Other symptoms specific to D-la were also frequently reported (e.g., metabolic acidosis). ME/CFS symptom categories are discussed sequentially to examine similarities with D-la symptoms.

Overlap between D-la and ME/CFS symptoms

Post-exertional neuroimmune exhaustion

This ME/CFS symptom describes a chronic pattern of excessive and disproportionate fatigue upon exertion. This is the core compulsory symptom of ME/CFS [1]. In the context of the chronicity of ME/CFS symptoms, it is difficult to directly compare this pattern of post-exertional exhaustion with an acute presentation of D-la. Nevertheless, one quarter of patients reported symptoms of lethargy and fatigue during a D-la episode (15/59, 25.4%). In contrast, all ME/CFS patients experience fatigue and lethargy as it is a required diagnostic criterion. The lower frequency of fatigue reported in D-la, may accurately reflect characteristic distinctions between the two conditions. Alternatively, an acute presentation of D-la can include fluctuating levels of consciousness and hence symptoms of fatigue may be less relevant and/or underreported within this emergency hospital setting.

Neurological impairments

Episodes reviewed in this qualitative synthesis required neurological symptoms to be reported (as an inclusion criterion), accordingly, all episodes of D-la reported at least one neurological impairment. The majority of neurological symptoms that were reported overlapped with ME/CFS symptomatology (52/59, 88.1%). The frequencies of *matching* ME/CFS neurological symptoms were similar when comparing paediatric (19/22, 86.4%) and adult (33/37, 89.2%) episodes as well as male (30/35, 85.7%) and female (21/23, 91.3%) episodes. *Ambiguous/other* neurological impairments (e.g., altered mental state or cortical dysfunction) were also frequently reported (45/59, 76.3%). The more severe neurological symptom of an altered level of consciousness was reported in 13 episodes (22.0%). Five case reports documented the patient's altered consciousness as the only neurological symptom during the D-la episode. The remaining reports described additional neurological symptoms and a deterioration in symptoms affecting consciousness.

When considering more specific types of neurological impairments, motor disturbance (B4b) was the most frequently reported *matching* ME/CFS neurological symptom (42/59, 71.2%, see Fig. 2). This was notably higher than the other neurological symptoms (B1. Neurocognitive = 25/59, 42.4%, B2. Pain = 3/59, 5.1%, B3. Sleep = 10/59, 16.9%, B4a. Neurosensory and Perceptual = 2/59, 3.4%). Common motor disturbances in ME/CFS include muscle weakness, clumsiness, balance and coordination difficulties [84]. The ICC noted that the presence of balance and gait instabilities are more frequently observed in severe cases [1]. *Ambiguous/other* neurocognitive, neurosensory, perceptual and motor disturbances were reported in 37.3% of total D-la episodes (22/59). Within these 22 episodes, 90.9% (20/22) of episodes simultaneously reported at least one *matching* neurological symptom akin with ME/CFS diagnostic criteria. Therefore, there was considerable overlap between *matching* symptoms and *ambiguous/other* neurological symptoms.

Approximately half of D-la episodes reported impairments in speech (30/59, 50.9%). Notably, all episodes that reported speech and language impairments also reported at least one other ME/CFS-*matching* neurological impairment, which may reflect the shared pathophysiology that underlies the behavioural manifestation of overt speech symptoms.

Immune, gastrointestinal and genitourinary impairments

The majority of D-la episodes were from patients with SBS (55/59, 93.9%). As such, these patients had a history of extensive gastrointestinal abnormalities. The case report of the patient with leukaemia [56] was the only

Table 3 Frequency of episodes that reported *matching* and/or *ambiguous/other* D-lactic acidosis (D-la) symptoms as a function of age and sex

ME/CFS ICC	D-La symptom overlap with ME/CFS	Episode frequencies															
		Paediatric (≤17 years)							Adult (≥18 years)						Total	
		Male (15 episodes)		Female (6 episodes)		NI (1 episode)	Total (22 episodes)		Male (20 episodes)		Female (17 episodes)		Total (37 episodes)		(59 episodes)	
		n	%	*n*	%	*n*	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
A. Postexertional neuroimmune exhaustion	Matching	8	53.3	1	16.7	–	9	40.9	2	20.0	4	23.5	6	16.2	15	25.4
	Ambiguous/other	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. Neurological impairments	Matching	12	80.0	6	100	1	19	86.4	18	90.0	15	88.3	33	89.2	52	88.1
	Ambiguous/other	11	73.3	2	33.3	1	14	63.6	17	85.0	14	82.4	31	83.8	45	76.3
	Ambiguous/other B1–B4	3	20.0	1	16.7	1	5	22.7	11	55.0	6	35.3	17	45.9	22	37.3
	Speech/language	7	46.7	2	33.3	1	10	45.5	11	55.0	9	52.9	20	54.1	30	50.8
	Consciousness	5	33.3	–	–	–	5	22.7	5	25.0	3	17.6	8	21.6	13	22.0
C. Immune, gastrointestinal, genitourinary impairments	Matching	3	20.0	–	–	–	3	13.6	6	30.0	4	23.5	9	24.3	12	20.3
	Ambiguous/other	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D. Energy production/transportation impairments	Matching	8	53.3	3	50.0	–	11	50.0	7	35.0	2	11.8	9	24.3	20	33.9
	Ambiguous/other	–	–	–	–	–	–	–	2	10.0	–	–	2	5.4	2	3.4
Mood/behavior	Matching	2	13.3	1	16.7	–	3	13.6	2	10.0	3	17.6	5	13.5	8	13.6
	Ambiguous/other	6	40.0	–	–	–	6	27.3	4	20.0	3	17.6	7	18.9	13	22.0
Uncategorized D-la symptoms																
	Metabolic acidosis	15	100	6	100	1	22	100	20	100	16	94.1	36	97.3	58	98.3
	Other abnormalities	4	26.7	–	–	–	4	18.2	6	30.0	2	11.8	7	18.9	11	18.6

Percentages were calculated from fractions of the number of episodes that reported relevant symptoms (*n*) against the number of possible episodes (noted in column subheadings) within each sex and age category. ME/CFS broad category B Neurological impairments are highlighted as the primary focus of this review. The *ambiguous/other* symptoms are further delineated into three subcategories (ICC criteria B1–B4, speech/language symptoms, and level of consciousness; see Table 2 for explanations). In each subcategory the same episode code number can be shown several times to represent multiple symptoms during each D-la episode. See Additional file 2: Table S2 for an expansion of these results showing episode code numbers that were included for each symptom category

Ambiguous/other symptoms that were not clearly identified as consistent with ME/CFS presentation (see Table 2 for detailed symptom delineation), *B1–B4* neurocognitive impairments, pain, sleep disturbances, neurosensory and perceptual, motor disturbances, *D-la* D-lactic acidosis, *ICC* International Consensus Criteria, *Matching* mapped overlap between ME/CFS and D-la symptoms, *ME/CFS* myalgic encephalomyelitis/chronic fatigue syndrome, *NI* sex not identified

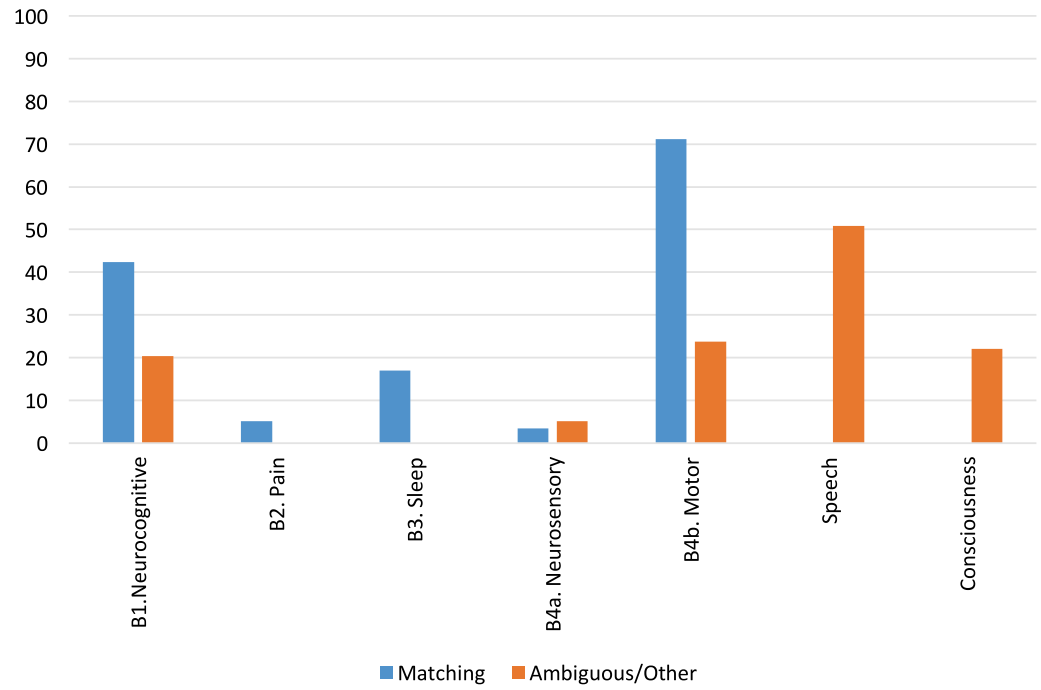

Fig. 2 Percentages of D-lactic acidosis (D-la) episodes that reported ME/CFS *matching* and *ambiguous/other* neurological impairments. Total percentages are reported for neurocognitive symptoms (B1), pain (B2), sleep disturbance (B3), neurosensory and perceptual disturbances (B4a), motor disturbances (B4b), speech symptoms, and altered consciousness. N.B. The same episode may be represented multiple times for both *matching* and *ambiguous/other* symptom groups across all neurological impairment subcategories

episode of D-la that did not report any gastrointestinal symptoms during the acute stage or prior history. This episode was also an exception as it was the only episode of D-la without metabolic acidosis (discussed below). A *change* in ME/CFS-*matching* gastrointestinal symptoms associated with the D-la presentation was only reported in 22.0% of the total episodes (13/59). These changes included an increase in diarrhoea, nausea, vomiting and/or abdominal pain/distension.

Immune or genitourinary impairments (*matching* or *ambiguous/other*) were not specifically reported during D-la episodes. Conversely, immune symptoms are a primary component of ME/CFS as a neuro-immune condition with evidence of immune abnormalities [85] and autoimmune mechanisms [86].

Energy production and transportation impairments

ME/CFS-*matching* energy production and transportation impairments were reported in 33.9% (20/59) of total D-la episodes. These symptoms were more frequently reported in male (15/35, 42.9%) compared with female episodes (5/23, 21.7%; see Table 3). *Ambiguous/other* cardiovascular (bradycardia) and respiratory symptoms (respiratory acidosis and hypercarbic respiratory failure) were documented during two adult male episodes (2/59, 3.4%).

Comorbid mood and behavioural disturbances

Mood disturbances are not included in ME/CFS diagnostic criteria. However, patients with ME/CFS frequently experience comorbid anxiety and depressive symptoms [1, 87]. *Matching* mood (depressive and anxiety) symptoms were reported in 13.6% of D-la episodes (8/59). *Ambiguous/other* ME/CFS mood and behavioural disturbances were described in 22.0% of D-la episodes (13/59). The higher frequency of *ambiguous/other* mood and behavioural disturbances seen in paediatric male (6/15, 40.0%) compared to paediatric female episodes (0/6) may reflect the tendency for boys to externalise behaviours more than girls [88].

Other symptoms (non-ME/CFS)

Metabolic acidosis as defined by blood pH values below 7.35 [89] and/or as stipulated in each case report based on patients' anion gap, was confirmed in all except one episode of D-la (58/59, 98.3%). Metabolic acidosis occurs when there is a decrease in serum bicarbonate, excess hydrogen ions and, commonly, a lower pH value suggestive of acidosis [90]. However, in some situations an underlying metabolic acidosis can be reflected in higher pH values that are indicative of alkalosis but are secondary to a metabolic acidosis, sometimes referred to as

a compensatory process [90]. In Mendu et al. [56] the authors described the normal serum pH values (7.35–7.45) as a "compensated metabolic acidosis" due to simultaneous higher L-lactate levels observed in this patient (p. 90). Metabolic acidosis is a primary marker of D-la but is not described in ME/CFS diagnostic criteria. Blood pH values are not routinely measured in ME/CFS, therefore, the symptomatic overlap cannot be determined.

Other abnormalities such as dehydration, cravings and excessive thirst were infrequently reported in the D-la episodes (9/59, 15.3%). Acute renal failure, hyperchloremic acidosis and liver dysfunction were reported in three separate episodes (3/59, 5.1%).

Discussion

Examples of *matching* ME/CFS and D-la symptoms were found throughout the D-la case reports. More than 96.6 per cent (57/59) of D-la episodes reported at least one *matching* ME/CFS symptom. Whilst there was considerable overlap, some symptoms of both ME/CFS and D-la were distinct. Figure 3 provides an overview of shared and distinct symptoms in these acute and chronic clinical conditions.

This qualitative synthesis has confirmed that the type of neurological impairments reported during D-la episodes are similar to those experienced by ME/CFS patients. However, the most commonly reported motor disturbances in D-la are considered a more severe presentation within ME/CFS [1]. This may reflect differing pathophysiology or alternatively may support a proposal for both conditions to lie on a continuum. ME/CFS may fall at one end as a chronic condition with fluctuating severity and D-la at the other extreme as an exacerbation of an acute presentation (see Fig. 3). The fluctuating neurological symptoms that present in both D-la and ME/CFS may vary in severity and the corresponding treatment response [66]. Htyte et al. [40] described these transient symptoms as "usually mild and self-limiting in patients with normal renal function" (p. 1435), highlighting the individual variation in presentation and reporting of symptoms with less severe symptoms unlikely to prompt acute emergency care.

Some key areas of disparity between D-la and ME/CFS symptoms related to immune impairments and metabolic acidosis. These results may accurately reflect pathophysiological differences between the two conditions. Alternatively, some other plausible explanations warrant consideration. The lack of reports relating to specific immune symptoms in D-la may be related to symptom prioritisation during an acute presentation. Reports of bacterial infection preceding D-la onset, bacterial overgrowth during the D-la episode and response to antibiotic treatment (see Additional file 1: Table S1), suggest that immune dysfunction may still be relevant for D-la patients.

Without measurement of blood pH levels the prevalence of metabolic acidosis in ME/CFS is unknown. Other research raises questions about the possibility of similar mechanisms of metabolic acidosis (or the compensatory acidosis described above) in ME/CFS. Alkalosis in skeletal muscles may result in a compensated acidosis in the blood, precipitating hyperventilation [91]. This theory

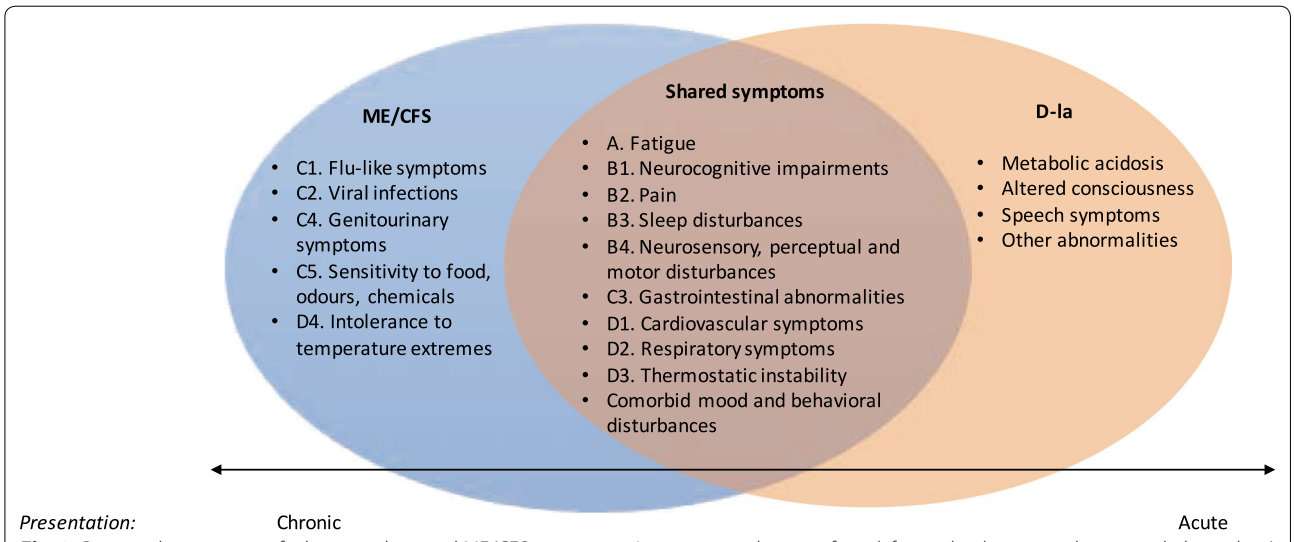

Fig. 3 Proposed continuum of D-lactic acidosis and ME/CFS symptoms. A continuum showing, from *left* to *right*; *distinct* myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) symptoms, *shared* and *distinct* D-lactic acidosis (D-la) symptoms. Continuum also shows *chronic* compared to *acute* presentations

has been proposed from evidence of hyperventilation in patients with ME/CFS [92] and an inverse association between skeletal muscle pH and cerebral blood flow [91]. Compared to sedentary controls, ME/CFS patients have higher skeletal muscle pH at rest [93] and at recovery after exercise [93]. Alkalosis in skeletal muscle has been proposed as a mechanism effecting orthostatic and neurocognitive ME/CFS symptoms [91]. Blood acidosis can also directly alter the function of cellular membranes [91], therefore, our current understanding of the mechanisms involved remain rudimentary. Routine assessment of blood pH levels in ME/CFS will ascertain the prevalence of metabolic acidosis/alkalosis for this clinical population.

Limitations

These results need to be considered with an awareness of potential methodological limitations. Firstly, the inclusion criteria for selected case reports in this review may have been problematic. Although unavoidable, the requirement of neurological symptoms during D-la episodes may have increased reporting bias during this review process leading to an exaggerated focus on neurological symptoms. However, the effect of this limitation may be moderated when considering the high percentage of case reports meeting both the eligibility criteria of describing neurological symptoms and D-lactate measurement during the episode (80.0%).

Findings from this qualitative review are also limited by the lack of standardised procedures used when reporting symptoms in case reports. Differences in assessment procedures and terminology used for reporting neurological symptoms may impede accurate interpretation. Some *ambiguous/other* symptoms described as distinct may share similar pathophysiology. This may be particularly pertinent for speech symptoms. On the one hand, the results may underestimate the level of overlap based on the conservative classification of symptoms. Alternatively, the breadth of ME/CFS symptoms included in the ICC diagnostic criteria may inflate the findings. Reliance on qualitative symptom report comparisons only provide a preliminary guide to shared symptomatology. Whilst useful for theoretical purposes it is insufficient to draw confirmatory conclusions.

Implications

Mindful of these limitations, the proposal of a continuum of acute and chronic encephalopathy related to D-lactate warrants further investigation. Within D-la, several authors have proposed that the level of acidosis and associated encephalopathy may result in differing severity and either an acute or chronic presentation [27, 28, 32]. A subclinical elevation of D-lactate has been reported in SBS patients and diverse populations [94]. Higher D-lactate levels were recorded in 2.8% of randomly selected hospital patients [40]. Minimal details were provided about this sample other than noting that 40% of these patients did not have a history of gastrointestinal surgery [40]. Higher levels of D-lactate have also been recorded in response to trauma or infection [95]; and in patients with diabetes compared with healthy controls [96]. Thornalley et al. [97] showed positive correlations between the level of D-lactate and duration of diabetes. They found that the duration of disease was positively associated with retinopathy, neuropathy and nephropathy complications of diabetes. The relevance of D-lactate for diverse presentations is currently unknown.

Even within SBS populations, D-la has been under-recognised and frequently misdiagnosed [9]. Misdiagnosis is complicated by issues related to accurate and efficient measurement of D-lactate. A further diagnostic complication related to the clinical presentation of D-la is that the neurological manifestations can present without gastrointestinal complications or a change in gastrointestinal symptoms. Less than one-quarter of D-la episodes analysed in this review described a worsening of gastrointestinal symptoms. Therefore, it is plausible that clinicians may focus on the neurological presentation and overlook the underlying gastrointestinal mechanism. The case report from Scully et al. [70] highlighted this when the 16-year old male patient was first treated by a psychiatrist with lithium carbonate for suspected bipolar disorder and tested for illicit drug use before being diagnosed with D-la. The patient presented with aggression, somnolence and weight loss without current gastrointestinal symptoms, although had an abdominal trauma one year prior that required short-bowel surgery [70]. The presence of neurological symptoms in the absence of current gastrointestinal symptoms may lead to frequent misdiagnoses. The proposed mechanisms of D-la (i.e., carbohydrate malabsorption and related bacterial overgrowth [32, 36]) may have relevance for patients presenting with neurological symptoms but without an observable change in gastrointestinal symptoms.

Carbohydrate malabsorption is not exclusive to SBS populations and can vary in severity. Altschule et al. [98] found that D-lactate was more slowly metabolised in patients with schizophrenia, manic-depression and psychosis compared with healthy controls. Even earlier studies have shown increased lactate after fructose or glucose ingestion and disturbed lactate metabolism after exercise within these populations [98], suggesting difficulties with carbohydrate metabolism. Within ME/CFS, carbohydrate restriction (e.g., avoidance of sugars and grains) may be advantageous [99, 100]. Whilst there is minimal empirical support, clinical reports suggest that dietary triggers can exacerbate symptoms and that some patients

benefit from dietary exclusions [99]. The response to treatment for small intestine bacterial overgrowth (SIBO) in ME/CFS patients [101], suggests that carbohydrate malabsorption may be relevant for a subgroup of this population. An analysis of mechanisms involved in D-la is provided to help identify shared pathophysiology between D-la and ME/CFS.

Part B. Narrative review

Proposed mechanisms in D-lactic acidosis

Understanding D-la involves firstly establishing the reason for increased D-lactate levels before examining proposed neurological mechanisms. Figure 4 summarises the contextual factors, triggers and proposed mechanisms leading to D-la. The presentation of D-la requires both an increase in D-lactate absorption that exceeds the metabolic and/or excretion capacity of the patient.

Bacterial dysbiosis

Bacterial dysbiosis (i.e., an imbalance in commensal bacteria [2]) has been suggested as the primary mechanism influencing D-la presentation in SBS populations. The dysbiosis is distinguished by an increased colonisation of lactic acid-producing bacteria, particularly bacteria that produce D-lactate (e.g., *Lactobacillus fermenti, L acidophilus,* and *Streptococcus*; see review by Petersen [13]). An overgrowth of *Lactobacilli* has been identified in SBS patients with increased D-lactate levels [26, 28, 37, 47, 64, 68, 72, 74, 75, 103]. This dysbiosis has been proposed as a result of an impaired small intestine, either due to congenital causes, surgery for midgut volvulus, gangrene or inflammatory bowel disease [104]; functional SBS and carbohydrate malabsorption [32, 36]; or colonic stagnation [62]. With reduced absorptive capacity of the small intestine, malabsorbed carbohydrates are more likely to enter the colon and provide fuel for colonic bacteria leading to increased bacterial fermentation [54, 81]. Increased bacterial fermentation can further reduce bowel motility [31], alter colonic pH and change the level of bacterial metabolites. This can include a reduction in volatile fatty acids [26] and increased D-lactate production [shown in 26, 54, 64, 66, 75, 105].

Dietary, probiotic and antibiotic intake have preceded bacterial dysbiosis and D-la presentations. Some episodes of D-la have been triggered by increased sugars/carbohydrate (e.g., [31, 42, 66]) or a change from parenteral to oral intake (e.g., [48, 70]). In patients with bacterial dysbiosis, diet and probiotic supplementation can increase bacterial fermentation and further alter bacterial composition. It appears that the type of diet or probiotics can influence D-lactate production in either a beneficial or detrimental manner. Whilst antibiotics are commonly used as a treatment for D-la, indiscriminate and inappropriate use of antibiotics has also been shown to precede and potentially trigger D-la [36]. The way antibiotics alter bacterial composition may lead to

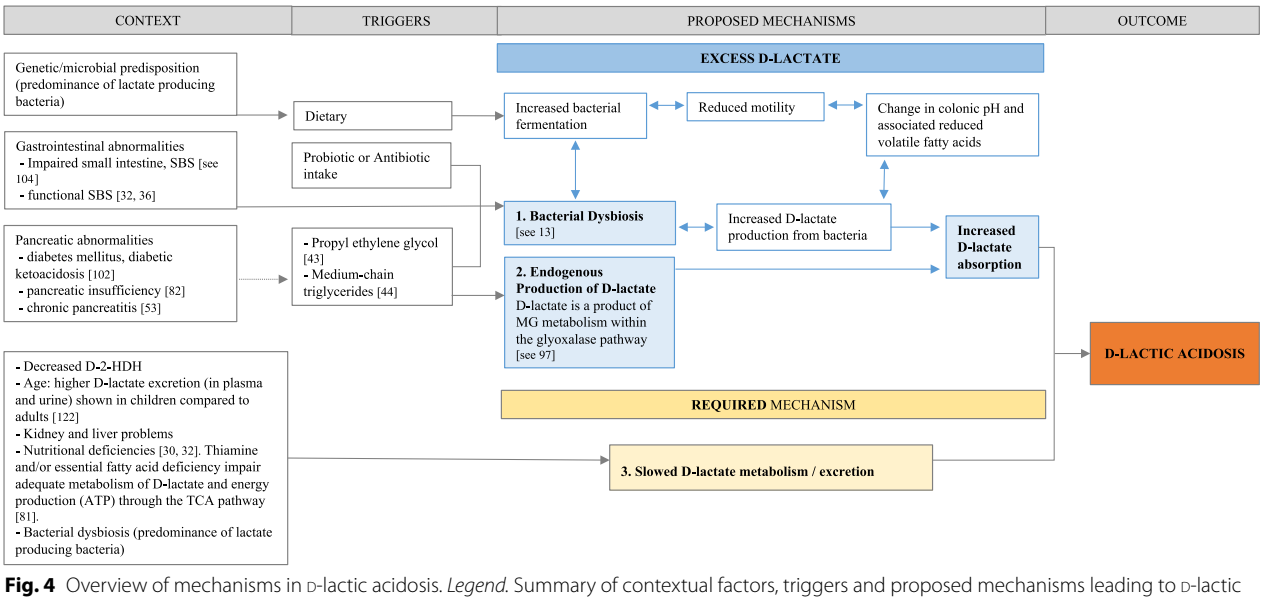

Fig. 4 Overview of mechanisms in D-lactic acidosis. *Legend.* Summary of contextual factors, triggers and proposed mechanisms leading to D-lactic acidosis (D-la). A presentation of D-la requires an increase in D-lactate absorption. The proposed mechanisms of increased D-lactate production can be through (1) bacterial dysbiosis and/or (2) endogenous production of D-lactate. Simultaneous to the increased absorption, patients who present with D-la also require (3) slowed metabolism of D-lactate. Hence, the D-lactate production exceeds the body's metabolic and/or excretion capacity. Short bowel syndrome (SBS); D-2-hydroxy acid dehydrogenase (D-2-HDH); adenosine triphosphate (ATP); tricarboxylic acid (TCA)

further dysbiosis and an increased D-lactate production in vulnerable patients.

Although bacterial dysbiosis is the primary mechanism used to explain the occurrence of D-la, enteric microbial composition was only measured prior to treatment for 21 of the 59 episodes screened for the qualitative review (35.6%). More consistent measurement of the gut microbiome may add clarity to D-la etiology and individual treatment.

Slowed D-lactate metabolism/excretion

Whilst it is beyond the scope of this review to explain lactate metabolism (see [13, 106]) a brief overview of D-lactate metabolism in relation to D-la is provided. Humans can effectively metabolise large amounts of D-lactate. Hove and Mortensen [15] confirmed that humans have the enzyme D-2-hydroxy acid dehydrogenase (D-2-HDH) to enable conversion of D-lactate to pyruvate. Certain conditions such as increased oxalate and low pH can inhibit the activity of D-2-HDH enzymes, as shown in animal tissue [107]. The kidney and liver have the highest concentrations of D-2-HDH. Therefore, kidney and liver impairments can reduce effective metabolism of D-lactate indicated by D-lactate accumulation in patients with renal dysfunction [40] and liver cirrhosis [108]. The presence of adequate D-2-HDH is required for D-lactate metabolism.

Colonic bacteria can be involved in both lactate production and excretion during pyruvate metabolism. Human and some bacterial mitochondria have the enzyme DL-lactate racemase which enables conversion between D-and L-lactate [15]. For example, *Lactobacillus* species are common producers of lactate but the ratio of D- and L-lactate production and the direction of conversion is dependent on the species (see [109]). Therefore, impaired colonic metabolism of D-lactate may also be a consequence of bacterial dysbiosis. Colonic flora that is predominated by lactate-producing bacteria and a reduced capacity to convert lactate to short chain fatty acids (SCFA) will result in less SCFA and reduced metabolism of D-lactate [13].

Impaired metabolism of consequential D-lactate accumulation is required for the presentation of D-la [25]. It may be beneficial to categorise patients into lactate accumulators vs non-lactate accumulators. When examining bacterial composition in a sample of SBS patients, Mayeur et al. [110] showed that some patients preferentially accumulated D-lactate compared with L-lactate, suggesting the influence of bacterial composition on D-lactate profiles. The D-lactate accumulators were more likely to experience encephalopathy symptoms. Therefore, multiple factors including increasing bacterial D-lactate production, other endogenous production of D-lactate, nutritional status and altered D-lactate metabolism will effect D-lactate accumulation and the clinical presentation of an episode of D-la.

Proposed neurological mechanisms in D-lactic acidosis

Metabolic acidosis and increased D-lactate levels are synonymous with the presentation of D-la. However, neither condition can predict the development of neurological symptoms. Acidosis can occur without associated neurological symptoms and in reverse, encephalopathy can be present without the accompanying acidosis [13]. Similarly, whilst increased D-lactate levels are required in D-la, the presence of high D-lactate is not the sole cause or determinant of neurological symptoms. Some studies have shown a temporal association between D-lactate level and symptom onset [30] as well as severity [103]. However, this has not always been replicated (e.g., [48]). Other factors must also be required because higher D-lactate levels have been shown in patients with SBS and other gastrointestinal disorders but without concurrent encephalopathy [111]. These inconsistencies suggest that there are several possible direct and indirect mechanisms responsible for the neurological manifestations in D-la (see Fig. 5).

Possible pathways of gut–brain interaction

Three pathways have been proposed to explain how excess D-lactate production in the colon can impact neurological symptoms [112]. Firstly, a colonic environment with lowered pH and high lactate levels can increase intestinal permeability (i.e., aberrations in the mucosal lining of the gastrointestinal tract [113, 114]) and result in systemic inflammation. For example, in ruminants, preliminary findings showed that lactic acidosis (lowered pH and increased faecal lactate) was significantly associated with increased tumor necrosis factor-alpha (TNF-α [115]). Translocation of luminal content, including endotoxins, to bodily fluid or tissue may result in an increased immune response and associated neurological sequelae [116].

A second pathway of gut–brain interaction is through neural mechanisms. The bidirectional communication between the enteric nervous system (ENS) and central nervous system (CNS) via the vagus nerve can regulate or dysregulate neurotransmitter production [117]. Bacterial dysbiosis can have both direct and indirect effects on neurotransmitter production and associated neurological symptoms (see [2]). Dahlquist et al. [30] suggest that the effect of D-lactate on neurotransmitter production is one possible explanation for the temporal association between neurological symptoms and D-lactate levels observed during some D-la episodes. Alternatively, D-lactate may act by reducing neuronal energy metabolism as explored below.

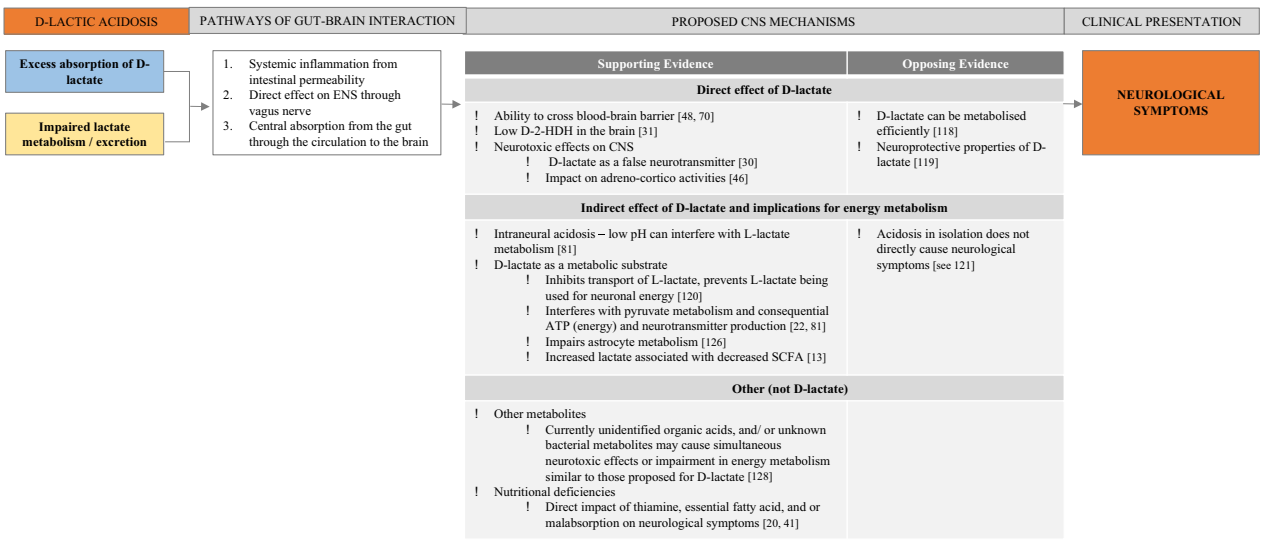

Fig. 5 Proposed mechanisms of gut–brain interaction leading to neurological symptoms observed in D-lactic acidosis. Supporting and opposing evidence for proposed central nervous system (CNS) mechanisms are categorised according to direct effects of D-lactate, indirect effects of D-lactate and other possible mechanisms unrelated to D-lactate. Abbreviations: enteric nervous system (ENS); D-2-hydroxy acid dehydrogenase (D-2-HDH); adenosine triphosphate (ATP); short chain fatty acids (SCFA)

Thirdly, excess D-lactate can act centrally in the colon and be absorbed and transported from the gut to the brain through the circulation. Hanstock et al. [112] provided support for this being a primary mode of action finding significant associations between plasma and colon/caecum D-lactate levels in rats. D-Lactate can cross the blood–brain barrier with evidence of D-lactate in both circulating plasma and cerebrospinal fluid in human case reports of D-la [48, 70]. Whilst murine models show reduced uptake of D-lactate compared with L-lactate within the brain [112], slowed metabolism due to low D-2-HDH in the brain may explain the subsequent neurological symptoms [31]. An increased D-lactate level within the brain may exert direct and indirect effects on the CNS.

Proposed central nervous system mechanisms

Direct effect of D-lactate As a substrate on its own, the direct neurotoxic effect of D-lactate is questionable and unlikely. Intravenous infusion of D-lactate in healthy males did not result in evidence of neurological symptoms [118]. However, at substantially higher levels, as shown in propylene glycol intoxication [43], or in combination with nutritional deficiencies [30], direct neurotoxic effect may be possible. D-Lactate may act as a "false neurotransmitter" [30, p. 145]. Similar fluctuations in biochemistry concurrent with non-specific EEG abnormalities during an adult episode of D-la may support this proposal [74]. However, this remains propositional without further evidence of the precise mechanisms involved.

The neuroprotective properties of D-lactate have also been described raising doubt about the neurotoxicity of D-lactate alone. Castillo et al. [119] showed that both L- and D-lactate can exert neuroprotective properties in a male mouse model of cerebral ischemia (stroke). Unexpectedly, they found that D-lactate showed near equivalent neuroprotective properties (i.e., reduced cell death, less damage observed on behavioural measures) to that shown with L-lactate. Unlike earlier findings, their results indicated that D-lactate can be metabolised by the rodent brain. This raises the possibility that D-lactate may also be able to be metabolised within human cerebral tissue. Notably this evidence is from a stroke animal model and requires investigation before generalising to D-la. It further highlights that D-lactate alone may not be neurotoxic but can play deleterious roles in certain environments when combined with other necessary conditions (e.g., nutritional deficiencies, excess glutamate, or mitochondrial toxicity) to produce the encephalopathy observed in D-la.

It has also been proposed that D-lactate can inhibit L-lactate transportation [120]. Considering that L-lactate can have an inhibitory effect on the adrenal cortex [46], it could be surmised that excess D-lactate may reduce available L-lactate and consequentially increase adreno-cortico activity. Further investigation of this mechanism is warranted. Growing evidence provides support for the role of D-lactate in energy metabolism.

Indirect effects of D-lactate and implications for energy metabolism Previous research has proposed that D-lac-

tate levels can reduce the pH balance within the brain and impede neurological processes [81]. Low pH can interfere with L-lactate metabolism [42]. However, in animal models it appears that the intraneural acidosis itself is not the primary mechanism at play (see [121]). Similarly in clinical D-la cases, in isolation the acidosis does not directly cause neurological symptoms [32]. Reduced D-lactate is more closely related to clinical improvement than neutralizing pH [39]. Bongaerts et al. [122] also showed that there was not a direct correlation between D-la and acidosis. Rather than intraneural acidosis, the competing role of L- and D-lactate for cerebral metabolism is a preferred explanation [121].

Pairing lactate and glutamate in the first in vivo studies in male rats demonstrated the neuroprotective properties of L-lactate and neurotoxic properties of D-lactate [120]. The mechanism appears to be related to D-lactate's influence on energy metabolism. When D-lactate was combined with glutamate, larger cortical lesions were produced [120]. This result suggests that D-lactate inhibits its transport of L-lactate and prevents L-lactate being used for neuronal energy. Ros et al. [120] findings indicate the compounding neurotoxic effects of D-lactate when combined with excess glutamate. In a comparable murine study, Cassady et al. [121] showed that compared to D-lactate, L-lactate is the preferred substrate for cerebral energy. D-Lactate increased the excretion of amino acids and therefore was unable to act as an efficient metabolic substrate [121]. Variable levels of glutamate and other amino acids may explain why some people experience neurological symptoms and others do not.

Overlap between symptoms of pyruvate metabolism disorders and D-la presentation suggest that D-lactate can interfere with pyruvate metabolism and consequently reduce energy (adenosine triphosphate: ATP) and neurotransmitter production [81, 123]. Lower levels of a primary enzyme required for pyruvate metabolism, pyruvate dehydrogenase, have been found in the healthy cerebellum [124]. An increased D-lactate level that further impedes pyruvate metabolism may explain the predominance of motor/cerebellar symptoms observed in D-la [81]. Pyruvate metabolism abnormalities can interfere with optimal mitochondrial energy production [123]. This has potentially more revealing implications for organs that require greater energy, such as the brain and heart [123]. Ling et al. [123] found that D-lactate was an inadequate metabolic substrate and produced lower respiration in murine brain and heart mitochondria, however equivalent respiration rates were shown in liver tissue. D-Lactate was shown to inhibit L-lactate and pyruvate metabolism in brain and heart tissue.

The inhibition of L-lactate by D-lactate effects memory formation in day old chickens [125, 126]. The impaired metabolism may not only occur in neuronal cells as suggested by Baker and Edwards [125]. Gibbs and Hertz [125] results revealed that the inhibitory action of D-lactate occurs in astrocytes either through an extracellular effect or an intracellular effect impairing pyruvate metabolism in astrocytic mitochondria. Astrocytes play a primary role in maintaining homeostasis in the brain, including regulating glutamate use and removal, neuronal energy, and neuronal pH [127]. Gibbs and Hertz's [125] results demonstrated that the presence of D-lactate prior to a learning task prevented memory formation, but memory loss was delayed by 20 min when D-lactate was injected 10 min after the learning task. The authors suggest that impaired memory formation in day-old chicks is comparable with the encephalopathy observed in D-la. Therefore, similar mechanisms may be responsible for neurological symptoms in the mammalian brain.

Other possible mechanisms, not D-lactate Most research has focused on D-lactate's role in precipitating the neurological symptoms observed in D-la. However, other metabolites and nutritional deficiencies may play causative and/or contributory roles in the encephalopathy observed in this condition. The suggestion to investigate other causative factors has been supported by evidence of increased D-lactate levels in healthy populations [14, 118] and poor direct association between D-lactate level and clinical symptoms [13]. Colonic bacteria can produce several other metabolites (including alcohol, organic acids, amines, mercaptans, and aldehydes) that may exert neurotoxic or neuromodulating effects by influencing neurotransmitter production [128]. Indirectly, higher D-lactate produced by an increased abundance of lactic-acid producing bacteria may reduce the presence of other bacteria that metabolise SCFAs. The reduced availability of SCFA can impact energy metabolism and neurotransmitter production [13]. Currently unidentified organic acids or unknown bacterial metabolites may cause simultaneous neurotoxic effects or impairment in energy metabolism similar to those proposed for D-lactate [20].

As alluded to earlier, the nutritional deficiencies commonly present in SBS populations may exacerbate the clinical presentation [20]. Adequate nutrition is required for brain development with nutrient deficiency or insufficiency having both broad and specific effects on regions of the brain and neural functioning (see Georgieff [129]). Within D-la, nutritional deficiencies may directly impact neurological symptoms or the reduced availability of nutritional substrates may alter D-lactate metabolism, clearance or utilization within the brain. Hudson et al. [40] presented a case report of a patient with D-la and thiamine deficiency where thiamine supplementation effectively resolved neurological symptoms. Interestingly

in Wernicke encephalopathy, another condition that presents with acute confusion, delirium and ataxia, thiamine deficiency is responsible for these neurocognitive symptoms that resolve once adequate thiamine levels are restored (see Latt and Dore [130]). Thiamine is required for effective pyruvate metabolism in the brain, particularly within the cerebellum, hence thiamine deficiency may contribute to the encephalopathy seen in some patients with D-la.

Summary

There is more support for the indirect effect of D-lactate interfering with energy metabolism in the CNS compared with the direct neurotoxic effects of D-lactate. Multiple mechanisms may be at play. Evidence of the inhibitory action of D-lactate on utilisation of L-lactate in neural cells and astroglia appears a particularly pertinent mechanism that may explain the neurological symptoms observed in D-la. The relevance of other bacterial metabolites remains in question. The vulnerability of certain individuals related to predisposing genetic, microbial factors or nutritional status that influence D-lactate production and/or adequate excretion/metabolism is likely to contribute to the presentation of D-la.

What is the relevance for ME/CFS?

Whilst D-lactate levels have not been specifically measured in ME/CFS patients, elevated lactate levels within ventricular cerebrospinal fluid have been observed. Significantly higher levels of ventricular lactate were recorded in the ME/CFS patient group compared to both generalized anxiety disorder (GAD) and control groups. From this small sample of 16 CFS patients, 10 patients had high ventricular lactate levels, indicated by lactate levels above 2 standard deviations above control mean whereas the remaining 6 participants had equivalent lactate levels to both the GAD and healthy control groups. This distinction between clinical and control groups gives promise for ventricular lactate being a potential biomarker useful for establishing ME/CFS subgroups. Interestingly lactate level was not associated with any other demographic or clinical variables, including severity of illness. Notably, clinical measures of anxiety, depression, fatigue, sleep quality and fibromyalgia were used as outcome variables but cognitive symptoms were not measured. More detailed analysis of associations between objective neurocognitive symptoms and ventricular lactate level would be valuable. The authors explained the potential mechanisms related to mitochondrial dysfunction and/or oxidative stress that precede reduced cerebral blood flow which in turn upregulates anaerobic glycolysis and consequential lactate accumulation [131]. Mitochondrial dysfunction or increased oxidative stress may have bacterial and/or viral origins, or be related to underlying gastrointestinal abnormalities.

Gastrointestinal abnormalities

Examination of gastrointestinal abnormalities in ME/CFS indicate some similarities between D-la mechanisms and ME/CFS pathophysiology. Gastrointestinal dysfunction is included as one of the multiple symptoms in ME/CFS. Although not required for a diagnosis, gastrointestinal abnormalities and comorbid irritable bowel syndrome are frequently reported by patients with ME/CFS [132]. ME/CFS patients more frequently experience gastrointestinal symptoms and use corresponding treatments (i.e., antacids, H2 blockers, proton pump inhibitors) compared with healthy controls [133]. Estimates based on a clinical patient group of 1400 patients show recurring gastrointestinal symptoms are experienced by between 80 and 90% of patients [134]. In a sample of 165 CFS patients, Chia and Chia [134] identified evidence of chronic inflammation and enterovirus of the stomach in 95 and 82% of patient biopsies respectively. As the authors suggest, the presence of viral markers in the stomach years after initial infection suggest that chronic viral infections of the stomach may contribute to continued pathophysiology. Viral infections have been proposed to precipitate and perpetuate the bacterial dysbiosis observed in ME/CFS (see review by Navaneetharaja et al. [135]).

Bacterial dysbiosis, antibiotic and probiotic treatment

Evidence of gut dysbiosis has been observed through measurement of fecal microbial composition in ME/CFS populations. Differences between microbial composition of healthy compared with ME/CFS populations have been reported using both culture-based [136, 137] and genetic sequencing methods [3, 138]. Treatment using antibiotic [139], probiotic [140–142] or bacteriotherapy [143] have also been used to help modulate the gut microbiota in ME/CFS with somewhat unpredictable and varied success.

Using culture-based methods, we have previously observed a predominance of D-lactate producing bacteria (*Enterococcus* and *Streptococcus* species) in ME/CFS patients [4]. These bacteria produce high levels of lactate in vitro, compared with fecal isolates [4] which would support the maintenance of a more acidic colonic environment as one of the mechanisms in D-la that was previously described. This inference about the acidity of the colon in ME/CFS patients has been deduced from in vitro methods only, as we are not aware of any research that has measured colonic pH in this population. Within our prior clinical investigations, responders to a short-term antibiotic treatment for *Streptococcus* overgrowth was associated with increased vigor on self-reports and

selected improvement on objective sleep markers [139]. Extending from these preliminary findings, we are currently examining interactions between microbiota, broader neuropsychological symptoms and D-lactate levels in a clinical pilot evaluating treatment aimed at reducing an overgrowth of *Streptococcus* in a subgroup of ME/CFS patients. Our group have also compared culture-based fecal assessment and symptom expression within a larger sample ($N = 274$) of ME/CFS patients [5]. This observational study showed partial support for D-lactate theory in ME/CFS whilst raising questions about sex differences. Significant positive associations between some lactate producing bacteria (*Lactobacillus* and *Streptococcus* genera) and ME/CFS symptoms were shown for males but not females [5, 144]. Notably, the relative abundance of genera measured was consistent across the sexes raising questions about the functional differences of microbiota or a differing response to D-lactate for males compared to females. The heterogeneity of presentation and differing response to treatments could have varied explanations. Through the D-lactate lens, a preferential uptake of D-lactate (i.e., D-lactate accumulation as proposed by Mayeur et al. [110]) may contribute to variable symptoms and/or treatment response.

Using sequencing methods, Frémont et al. [3] examined ME/CFS patients and healthy controls from Norway and Belgium. Comparison between patient and control groups revealed no significant difference in bacterial diversity across the groups but differences in composition were observed. When comparing Norwegian patient and control samples there was a significant difference in bacterial composition, with ME/CFS patients showing a lower proportion of genus within the *Firmicutes* phylum. Interestingly, microbial differences between culturally diverse control samples (i.e., Norwegian compared with Belgian; [3] highlight the importance of considering inter-individual characteristics that may contribute to microbial variation.

Unlike Frémont et al. [3] findings of similar bacterial diversity, Giloteaux et al. [138] reported evidence of decreased diversity of microbial composition and instability in the microbial community in ME/CFS patients compared with controls. Significant differences were not shown when comparing the composition of ME/CFS and control samples at the phylum or genus level. However, at the operational taxonomic unit (OTU) level, proportions significantly differed for 40 OTU's. For example, the proportion of *Faecalibacterium* and *Bifidobacterium* was significantly lower in ME/CFS patients compared with controls. The few studies that have examined fecal microbial composition in ME/CFS have shown some inconsistent results making current interpretation incomplete suggesting that evaluation of subgroups, species-level comparison and measurement of metabolites is required. Replication using a combination of culturing and genetic sequencing methods with larger samples and varied demographics will help ascertain the relevance of D-lactate neurotoxicity in ME/CFS.

Bifidobacterium are high lactate-producing bacteria. Whilst the ratio of D/L lactate vary between species, a lower proportion of *Bifidobacterium* species raises some doubt about the relevance of D-lactate theory for ME/CFS. Selected *Bifidobacterium* (*Bifidobacterium adolescentis, Bifidobacterium breve*) and *Lactobacillus* (*L. plantarum, L. salivarius, L. casei subspecies rhamnosus, L. delbrueckii* subsp. *Lactis, L. acidophilus, L. fermentum, L. buchneri*) species have been identified as predominant in patients with D-la [26, 28, 29, 37, 47, 54, 64, 68, 74, 75, 145]. Similarity between species identified as overgrown in D-la patients and those used in probiotic studies could also generate skepticism about the relevance of D-lactate theory for ME/CFS. Both a small open-label [140] and two randomized, double-blind placebo-controlled studies [141, 142] examining the efficacy of probiotic supplementation in ME/CFS have indicated modest improvements for selected symptoms.

ME/CFS patients supplemented with a lactic-acid producing bacterial strain probiotic showed clinical improvement in self-reported neurological symptoms but no significant changes in fatigue or activity levels [140]. Rao et al.'s (2009) small double-blind RCT used an eight-week probiotic supplementation of *Lactobacillus casei* to examine changes in emotional symptoms. ME/CFS patients in the treatment group reported a significant reduction in anxiety symptoms compared with controls. No change was recorded for subjective reports of depression. More recently, treatment using *Bifidobacterium infantis* 35,624 resulted in reduced inflammation in ME/CFS patients, however neurological symptoms were not measured [142]. Preliminary results indicate the need for further investigation of the efficacy of probiotic treatment in ME/CFS. Of relevance to the current hypothesis in question, the D-lactate potential of selected strains used in the aforementioned studies is unknown. Therefore, results from these treatment studies suggest support for gut–brain interaction in ME/CFS but fail to provide additional information about the relevance of D-lactate for this population.

Bacterial overgrowth in the small intestine may also have implications for D-lactate production. Logan et al. [146] hypothesized that SIBO is involved in ME/CFS and related to the immune alterations observed in this condition. SIBO can be a cause of functional short bowel and result in carbohydrate malabsorption. Patients with comorbid SIBO and CFS have shown clinical improvement (on subjective reports of depression, memory/

concentration and pain) following antibiotic treatment [101]. D-Lactate levels were not measured in this study but dependent on the type of bacterial overgrowth, excess production of bacterial metabolites (including but not limited to D-lactate) may act centrally, through ENS activation or systemically due to intestinal permeability.

Implications for gut–brain interaction

Systemic inflammation as a consequence of gut mucosal damage and intestinal permeability as the first proposed pathway of gut–brain interaction in D-la has also been suggested as a pathophysiological mechanism in ME/CFS [116]. Initial support for this hypothesis in ME/CFS is reflected by findings of an increased immune response to lipopolysaccharide (LPS) (as measured by serum IgA and IgM to selected bacteria [116] and clinical improvement after treatment to restore intestinal permeability [113]. Measurement of plasma levels of LPS have been used as an indicator of microbial translocation as they are produced in response to Gram-negative bacteria [138]. Chronic LPS stimulation can be measured by plasma sCD14 and plasma LPS-binding protein (LBP) levels [138]. Recently, additional evidence of intestinal permeability in ME/CFS patients has been shown through significantly higher proportions of plasma LPS, LBP and sCD14 compared with controls [138]. These results support the hypothesis of an inflammatory and/or immune response to microbial translocation that occurs when there is chronic gut mucosal damage and intestinal permeability in ME/CFS patients.

Nutritional deficiencies in ME/CFS

Nutritional status can be impaired for individuals with chronic health conditions and comorbid gastrointestinal abnormalities. Nutritional deficiencies require careful monitoring and treatment for ME/CFS patients [1]. Coenzyme Q10 (CoQ10) was shown to be significantly lower in the plasma of ME/CFS patients compared with healthy controls [147]. Treatment that includes nutritional supplementation is frequently employed with CoQ10, magnesium, l-carnitine and S-adenosylmethionine indicated as potentially beneficial for this population [148, 149]. Improvements in cognitive symptoms (mental fatigue, attention, concentration) have been described after supplementation with acetyl-L-carnitine and propionyl-L-carnitine for patients with ME/CFS [150]. Colabamin (B12) injections are proposed to exert effects by reducing oxidative stress [151] but the implications of B12 deficiency may also be relevant when considering the role of B12 in the TCA cycle and lactate metabolism (see [152]). Considering the impact of nutritional deficiencies in D-la, this may interact with the symptom presentation in ME/CFS and the potential for excess D-lactate accumulation or issues with metabolism. Nutritional deficiencies in ME/CFS may have varied origins, including but not limited to, genetic vulnerabilities, stress, infection, inadequate dietary sources and/or impaired metabolism that are factors involved in the etiology of ME/CFS [153]. Dietary modifications appear helpful for some ME/CFS patients (self-report in [154]) and in clinical case reports [148]. Similarly, dietary treatments and reduced carbohydrate intake were common recommendations for D-la patients (see Additional file 1: Table S1). It would be useful to understand the role of diet as a potential moderating factor (precedent, perpetuating or consequential) in bacterial dysbiosis and D-lactate production in ME/CFS patients and whether this varies for moderately impaired compared to severely impaired (i.e., bedbound) patients.

Conclusions

D-la is an acute condition that provides a clear example of the microbe–gut–brain interaction with encephalopathy similar to ME/CFS. Growing evidence supports the proposal of the microbiota–gut–brain interaction in ME/CFS. Specific mechanisms are yet to be confirmed. Our qualitative review of D-la case studies shows considerable overlap between D-la and ME/CFS neurological symptoms. Subclinical levels of D-lactate may be related to fluctuating neurological symptoms in ME/CFS. Our review of the D-la literature has led us to propose the hypothesis that D-la and ME/CFS may lie on a continuum, with notable distinctions related to differences in acute versus chronic presentations (see Fig. 3). Increased prevalence of D-lactate producing bacteria in an ME/CFS sample compared with controls [4] provides the only clear evidence supporting D-lactate theory in ME/CFS. Gut dysbiosis in fecal microbiota, SIBO, and preliminary responses to antibiotics warrant measurement of D-lactate levels in this clinical population.

We acknowledge the complexity and heterogeneity of ME/CFS. Explanation of other pathophysiological mechanisms in ME/CFS (including but not limited to neuro-immune, oxidative stress and inflammatory pathways, [116, 147, 153, 155, 156] was beyond the scope of the current review. We stress that D-lactate theory may be relevant for a select subgroup and if not causative, may be a factor that perpetuates or exacerbates neurological symptoms. To date, there is no research that has measured D-lactate levels in ME/CFS. Improved efficiency and availability of D-lactate measurement in urine and blood samples is needed. Measurement of D-lactate will clarify its role of D-lactate in this population and may generate an avenue for alternative treatments. Subclinical levels of D-lactate in diverse populations suggest that this may be

extended to other conditions. The proposed continuum is relevant for general physicians, gastroenterologists, psychiatrists and psychologists alike. Awareness of gastrointestinal origins for neurological presentations may hasten diagnostic accuracy, prevent misdiagnosis and improve treatment outcomes.

Additional files

Additional file 1: Table S1. Demographic and clinical data summary of D-lactic acidosis episodes ($n = 59$) included in the qualitative synthesis. All episodes simultaneously reported at least one high D-lactate level (from blood or urine analysis) and documented neurological symptoms. Episodes were screened for information about patient demographics, neurological symptoms, non-neurological symptoms, D-lactate levels, L-lactate levels, anion gap, pH levels, microbial composition, proposed triggers, medical history/comorbid conditions and treatment. Numbers in brackets (1) and (2) indicate separate episodes for the same patient. The letters *a* and *b* identify different patient cases reported in the same reference. Episodes from non-SBS patients are marked with an asterisk.

Additional file 2: Table S2. Episodes that reported *matching* or *ambiguous/other* D-lactic acidosis (D-la) symptoms as a function of age and sex.

Abbreviations

D-la: D-lactic acidosis; SBS: short bowel syndrome; ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome; D-2-HDH: D-2-hydroxy acid dehydrogenase; ENS: enteric nervous system; CNS: central nervous system; ATP: adenosine triphosphate; TCA: tricarboxylic acid.

Authors' contributions

AW conducted data acquisition and drafted the manuscript. AW, MB and DB were involved in the critical appraisal of case reports and data analysis. All authors contributed to study conception and design, interpretation of data and critical revision. All authors read and approved the final manuscript.

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Availability of data and materials

All data is provided as Additional material supporting this manuscript.

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Supplementary Information for

Examining Clinical Similarities between Myalgic Encephalomyelitis/Chronic Fatigue Syndrome and D-Lactic Acidosis: A Systematic Review

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This PDF file includes:

Supplementary Table 2. Episodes that reported *matching* or *ambiguous/other* D-lactic acidosis (D-la) symptoms as a function of age and sex.

Other supplementary material for this manuscript includes the following:

Additional file 1: Supplementary Table 1 (excel file) Demographic and clinical data summary of D-lactic acidosis episodes (n = 59) included in the qualitative synthesis.

Legend: All episodes simultaneously reported at least one high D-lactate level (from blood or urine analysis) and documented neurological symptoms. Episodes were screened for information about patient demographics, neurological symptoms, non-neurological symptoms, D-lactate levels, L-lactate levels, anion gap, pH levels, microbial composition, proposed triggers, medical history/comorbid conditions and treatment. Numbers in brackets (1) and (2) indicate separate episodes for the same patient. The letters *a* and *b* identify different patient cases reported in the same reference. Episodes from non-SBS patients are marked with an asterix (*).

Supplementary Table 2. Episodes that reported *matching* or *ambiguous/other* D-lactic acidosis (D-la) symptoms as a function of age and sex.

ME/CFS ICC	$D\text{-}la_b$ symptom overlap with ME/CFS	Episode Case Numbers				
		Paediatric (≤ 17 years)			Adult (≥ 18 years)	
		Male	*Female*	*NI*	*Male*	*Female*
A. Postexertional neuroimmune exhaustion	Matching	14, 48, 50, 66, 53, 8, 47a, 47b	40	-	36, 42	12b, 13, 58b, 39
	Ambiguous/other	-	-	-	-	-
B. Neurological impairments	Matching	48, 47a, 30, 19, 52, 56, 66, 43, 53, 14, 50, 8	37, 17, 51b, 4, 40, 35	11	12a, 10, 29, 44, 58a, 25, 24, 49, 64, 59, 25, 42, 62, 9, 15, 23, 27, 33	6, 16_1, 16_2, 65, 20, 34_2, 57, 28, 41, 58b, 60, 21, 12b, 13, 34_1
	Ambiguous/other	43, 56, 66, 14, 18, 51a, 8, 19, 48, 53, 30	35, 37	11	9, 29, 44, 58a, 42, 64, 15, 24, 25, 49, 55, 23, 26, 10, 62, 27, 33	57, 21, 13, 31, 39, 58b, 43_2, 6, 16_1, 16_2, 65, 20, 34_1, 12b
	Ambiguous/other B1-B4	43, 56, 66	35	11	9, 29, 44, 58a, 42, 64, 15, 24, 25, 49, 33	57, 21, 13, 31, 39, 58b
	Speech/Language	14, 48, 66, 53, 8, 30, 19	37, 35	11	10, 15, 44, 62, 64, 9, 23, 25, 58a, 27, 33	6, 16_1, 16_2, 65, 20, 34_1, 57, 21, 12b
	Consciousness	14, 18, 51a, 8, 19	-	-	55, 9, 23, 26, 49	34_2, 57, 21
C. Immune, gastrointestinal and genitourinary	Matching	50, 47b, 66	-	-	10, 62, 42, 44, 36, 15	16_2, 60, 16_1, 65
	Ambiguous/other	-	-	-	-	-

D. Energy production/ transportation impairments	Matching	36, 66, 48, 51a, 52, 8, 43, 19	51b, 17, 35	-	55, 42, 25, 49, 9, 29, 62	60, 39
	Ambiguous/other	-	-	-	42, 23	-
Mood / Behavior	Matching	52, 53	40	-	9, 10	60, 31, 16_2
	Ambiguous/other	50, 56, 30, 66, 53, 19	-	-	44, 58a, 62, 33	31, 58b, 20
Uncategorized D-la Symptoms						
	Metabolic acidosis	14, 18, 48, 50, 51a, 52, 66, 53, 56, 8, 43, 47a, 47b, 30, 19	35, 51b, 37, 40, 4, 17	11	10, 15, 29, 44, 55, 62, 64, 9, 12a, 23, 24, 25, 26, 49, 58a, 59, 27, 36, 42, 33	6, 12b, 16_1, 16_2, 60, 65, 13, 20, 31, 34_1, 34_2, 57, 58b, 21, 28, 41
	Other abnormalities	66, 43, 47a, 47b	-	-	9, 2, 15, 26, 59, 33	16_1, 58b

ambiguous/other: symptoms that were not clearly identified as consistent with ME/CFS presentation (see Table 2 for detailed symptom delineation); D-la: D-lactic acidosis; ICC: International Consensus Criteria; *matching*: mapped overlap between ME/CFS and D-la symptoms; ME/CFS: myalgic encephalomyelitis/chronic fatigue syndrome; NI: sex not identified.

Legend. Subscript numbers ($_1$ and $_2$) indicate separate episodes for the same patient. The letters *a* and *b* identify different patient cases reported in the same reference. ME/CFS broad category B. Neurological impairments are highlighted as the primary focus of this review and to show three subcategories of delineation under *ambiguous/other* symptoms (i.e., in accordance with specific ICC criteria (B1 – B4), speech/language symptoms, and level of consciousness; see Table 2). Therefore, the same episode code number can be shown several times to represent multiple symptoms during each D-la episode (see Table 1 for references). Descriptions of drunkenness were referred to in several studies. Adult males self-reported *feeling* "drunk" (12a, 27) whereas females were described as *appearing* "drunk" (16_1, 16_2, 20,

58b). Rather than using this ambiguous term, the specific symptoms that were also referred to in each of these studies were categorized in the table. See Table 3 for a summary of symptom frequencies.

CHAPTER 5

Treating Bacterial Dysbiosis: Examining Clinical Symptoms and Sex Differences in Treatment Response

The results of the review in Paper 4 (Wallis et al., 2017b) suggest overlap between symptoms and mechanisms in D-la and ME/CFS. The lack of D-lactate measurement in ME/CFS samples indicates a critical gap in knowledge that is required to determine the relevance of this mechanism for the fluctuating neurological symptoms in ME/CFS. *Streptococcus* overgrowth was highlighted in the culture-based measurement of microbial composition and proposed to be a primary producer of D-lactate (Sheedy et al., 2009). Clinicians at CFS Discovery Clinic routinely measure bacterial dysbiosis through patient stool samples (analysed professionally by Bioscreen) and pursue interventions to restore microbial balance. ME/CFS patients from CFS Discovery Clinic who are identified as having an overgrowth of *Streptococcus* species are treated with antibiotic and probiotic intervention. Recently, a small pilot study by our research team examined sleep and mood symptoms in these ME/CFS patients after 6 days of low-dose Erythromycin treatment (Jackson et al., 2015). Small improvements were observed on some sleep and mood outcomes for a subgroup of patients who responded to the treatment based on reduction in *Streptococcus* at post intervention.

As an extension from this pilot (Jackson et al., 2015), the longer treatment protocol (i.e., alternate weeks of Erythromycin and D-lactate free probiotic across 4 weeks duration) and measurement of cognitive symptoms was added to the treatment design. Initially, the preferred research design was using a randomized placebo-controlled trial. However, in light of results from the cross-sectional study (Papers 2 and 3: Wallis et al., 2016, 2017c) that suggested potential sex differences in microbial function, sex comparisons were prioritised to evaluate whether males and females respond differently to the treatment. An open-label design with adequate sample size to enable male and female comparisons was employed (Paper 5: Wallis et al., 2017a).

Overlap

The concepts presented in the '*Background*' (pp. 4-7) section of this report will be familiar to the reader as it summarises the theoretical premise of the research that has been described in all prior chapters.

Paper 5

Wallis, A., Ball, M., Butt, H., Lewis, D. P., McKechnie, S., Paull, P., … Bruck, D. (2017). *Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: Neuropsychological symptoms and sex comparisons.* Manuscript submitted for publication.

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Supplementary tables S2-S5 are available at https://figshare.com/s/abb8d26889a798db5a4b

Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: Neuropsychological symptoms and sex comparisons

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ABSTRACT

Background

Preliminary evidence suggests that the enteric microbiota may play a role in the expression of neurological symptoms in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Overlapping symptoms with the acute presentation of D-lactic acidosis has prompted the use of antibiotic treatment to target the overgrowth of species within the *Streptococcus* genus found in commensal enteric microbiota as a possible treatment for neurological symptoms in ME/CFS.

Methods

An open-label, repeated measures design was used to examine treatment efficacy and enable sex comparisons. Participants included 44 adult ME/CFS patients (27 females) from one specialist medical clinic with *Streptococcus* viable counts above 3.00 x 10^5 cfu/g (wet weight of faeces) and with a count greater than 5% of the total count of aerobic microorganisms. The 4-week treatment protocol included alternate weeks of Erythromycin (400mg of erythromycin as ethyl succinate salt) twice daily and probiotic (D-lactate free multistrain probiotic, 5x10^{10} cfu twice daily). 2 x 2 repeated measures ANOVAs were used to assess sex-time interactions and effects across pre- and post-intervention for microbial, lactate and clinical outcomes. Ancillary non-parametric correlations were conducted to examine interactions between change in microbiota and clinical outcomes.

Results

Large treatment effects were observed for the intention-to-treat sample with a reduction in *Streptococcus* viable count and improvement on several clinical outcomes including total symptoms, some sleep (less awakenings, greater efficiency and quality) and cognitive symptoms (attention, processing speed, cognitive flexibility, story memory and verbal fluency). Mood, fatigue and urine D:L lactate ratio remained similar across time. Ancillary

results infer that shifts in microbiota were associated with more of the variance in clinical changes for males compared with females.

Conclusions

Results support the notion that specific microorganisms interact with some ME/CFS symptoms and offer promise for the therapeutic potential of targeting gut dysbiosis in this population. *Streptococcus spp*. are not the primary or sole producers of D-lactate. Further investigation of lactate concentrations are needed to elucidate any role of D-lactate in this population. Concurrent microbial shifts that may be associated with clinical improvement (i.e., increased *Bacteroides* and *Bifidobacterium* or decreased *Clostridium* in males) invite enquiry into alternative strategies for individualised treatment.

Trial Registry

Australian and New Zealand Clinical Trial Registry (ACTRN12614001077651)

9th October 2014

https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=366933&isReview=true

Keywords

Antibiotic; chronic fatigue syndrome; clinical outcomes; gut dysbiosis; microbiota-gut-brain; myalgic encephalomyelitis; neuropsychological symptoms; open-label pilot; probiotic; sex comparisons; *Streptococcus*; treatment

Background

Background and Objectives

ME/CFS is a complex, neuroimmune condition characterised by post-exertional mental and physical fatigue that is disproportionate to the level of exertion[1]. The multisystemic dysregulation results in pathophysiological abnormalities affecting a combination of central nervous, immune, gastrointestinal, energy metabolism, cardiovascular and respiratory systems manifesting in heterogeneous symptomatic presentations[1]. The history of diagnostic discrepancies (see [1–4]) is reflected in varied prevalence rates between 0.08% and 2.6% [5–11] but the burden on both the patient, their family and society is unequivocal [12]. This burden is not only a result of the devastating impact that the condition has on the patient's daily, occupational and social functioning [13–15] but can also be attributed to the direct cost of medical care that is often exacerbated by misdiagnosis and unclear treatment pathways [16, 17]. This awareness provides the rationale to examine the efficacy of treatments targeting pathophysiological abnormalities in ME/CFS patients with the hope of minimizing clinical exploration and identifying subgroups that may be more responsive to specific treatments.

Gastrointestinal disturbance and comorbid irritable bowel syndrome (IBS) are common in ME/CFS [18]. Estimates from a clinical sample of 1400 patients found that 80-90% experienced recurring gastrointestinal symptoms [19]. Intestinal permeability of the mucosal lining of the gastrointestinal tract [20, 21] and an imbalance in commensal enteric bacteria (i.e., gut dysbiosis) using culture-based methods (i.e., microbiota [22, 23]) and DNA sequencing (i.e., microbiome [24–26]) have also been shown in this population. These imbalances in both the microbiota and microbiome appear distinct from healthy controls [24, 26], and associated with inflammation [25] and symptom expression [23, 26–29]. Accumulating evidence suggests that microbial imbalances (whether consequential or

causative) should not be viewed in isolation as they may be relevant for multiple ME/CFS symptoms, including but not limited to neurological manifestations.

Gut-brain interaction occurs through multiple bidirectional pathways including through central, autonomic, and enteric nervous systems; neuroendocrine and neuroimmune pathways; and enteric microbiota [30–32]. Our understanding of the importance of the symbiotic relationship between enteric microbiota and health is becoming well accepted, with research efforts directed towards understanding mechanisms of microbial/host communication (see [33]). Gut dysbiosis may directly or indirectly precede gastrointestinal, neurocognitive and immune disturbances [34] or may be a consequence of stress and neurobiological mechanisms (e.g., in animal models [35–37]). Results of antibiotic [27], probiotic [38–40] and faecal transplant [41] interventions provide preliminary support for microbiota-gut-brain interactions in ME/CFS.

The D-lactate theory has been proposed as a possible mechanism for the neurological disturbances associated with gut dysbiosis in this population [23, 34, 42]. D-lactic acidosis is an acute metabolic acidosis with associated encephalopathy that is observed in patients with a history of small bowel resections [43]. The shortened small bowel can lead to impaired absorption of carbohydrates, preferential growth of selected gut bacteria (e.g., increase in some species of *Lactobacillus* and *Streptococcus*) that promotes an acidic colonic environment and excess production of D-lactic acid [44]. This abundance of D-lactic acid combined with decreased metabolic capacity can lead to excess absorption within the blood and brain believed to play a role in the neurological symptoms of D-lactic acidosis [44]. Within ME/CFS, an overgrowth of *Streptococcus* and *Enterococcus* species (D-lactic acid producing bacteria) has been observed in culture-based microbial studies [23]. This bacterial imbalance, combined with overlapping neurological symptoms and possible mechanisms have contributed to the proposal that subclinical concentrations of D-lactate may play a role

in ME/CFS presentations [42]. To date, measurement of D-lactate concentrations in ME/CFS have not been published.

In accordance with the D-lactate theory, an antibiotic treatment has been proposed to target the overgrowth of commensal enteric microbiota within the *Streptococcus* genus. Results from our group's earlier pilot showed initial promise on some sleep and mood outcomes for a subgroup of participants who decreased in *Streptococcus* after six days of oral erythromycin treatment [27]. Other probiotic interventions used with ME/CFS patients may contradict the D-lactate hypothesis. Results indicating improved neurocognitive [38] and anxiety [39] symptoms using lactic acid-producing bacteria (predominantly *Lactobacillus* strains) question the mechanisms at play. Notably, colonic bacteria can produce D- and L-lactate with the ratio and rate of metabolism dependent on the species [45]. The proportion of D:L lactate produced by the bacterial strains used in the probiotic studies were not measured. The validity of the D-lactate theory as well as the efficacy of antibiotic and probiotic interventions in ME/CFS requires further examination.

Findings from our cross-sectional study correlating commensal microbiota and clinical symptoms in 274 ME/CFS patients [28] provided an interesting perspective on the role of D-lactate in males and females. Results showed small to moderate positive correlations for both *Streptococcus* and *Lactobacillus* with symptoms in males, suggesting that increased abundance of these genera were related to more impairment across several ME/CFS symptoms [28]. For *Streptococcus,* opposite associations were shown in females with small negative correlations suggesting that higher *Streptococcus* was associated with less pain, neurosensory and immune symptoms. These results highlighted the importance of considering sex differences in microbial function and supported the notion of the 'microgenderome', i.e. the critical role of sex hormones on host-microbiota interactions [46].

Positioned within the context of D-lactate and microgenderome theory, this pilot study aimed to compare the treatment response of male and female ME/CFS patients with high counts of bacteria of the *Streptococcus* genus. To enable sufficient sample sizes for sex comparisons, an open-label design was used with the primary feasibility objective of determining the appropriateness of the intervention for both sexes rather than placebo control. The intervention was an extension of the earlier pilot [27] with alternate weeks of erythromycin and D-lactate-free probiotic supplementation across a 4-week period. Clinical outcomes measuring sleep, mood and cognitive symptoms were prioritised.

Methods

Trial Design and Participant Recruitment

This open-label, non-randomised pilot used a repeated measures design with a baseline, intervention and post-intervention protocol across 6 weeks (see Table 1). The prospective intention was to recruit 40 patients with equal proportions of males and females to enable sex comparisons. Screening and recruitment was continuous, with consecutive commencement dates according to patient presentation at CFS Discovery Clinic, Melbourne, Australia.

New or current patients at the clinic aged above 18 years who met Canadian Consensus diagnostic Criteria for ME/CFS [47] were invited to be screened for participation in this study. Eligible participants were patients with *Streptococcus* viable counts above 3.00×10^5 cfu/gm and more than 5% of the total count of aerobic microorganisms. Participants were asked to refrain from taking other antibiotics (from 4 weeks prior), probiotics (from 2 weeks prior), and substantially altering their diet, prescription medications or over-the-counter supplements across the screening and trial period. Known adverse reactions, contra-indications to the treatment protocol and/or significant comorbid physical or psychiatric illnesses excluded participation.

Trial methods were conducted in accordance with the guidelines for human experimental research and the Australian Clinical Trial Handbook [48]. Ethics approval was obtained from Victoria University Human Research Ethics Committee in June 2015 (HRE15-010). Additional trial details are available on the Australian and New Zealand Clinical Trial Registry (ACTRN12614001077651).

Intervention

The treatment protocol combined antibiotic and probiotic therapy taken on alternate weeks. Tablets of Erythromycin 400mg were given twice daily during weeks 2 and 4 (Erythromycin was given as the Ethyl Succinate salt and supplied by Amdipharm Mercury Pty Ltd or by Alphapharm Pty Ltd). Two capsules of Pro4-50 D-Lactate Free Multistrain Probiotic (Spectrumceuticals Pty Ltd, Belrose, New South Wales, Australia) were taken daily during weeks 3 and 5. Each probiotic capsule contained *Lactobacillus rhamnosus* (2.5×10^{10} cfu), *Bifidobacterium lactis* (1.5×10^{10} cfu), *Bifidobacterium breve* (5×10^{6} cfu), *Bifidobacterium longum* (5×10^{6} cfu). The off-label use of Erythromycin required notification to the Therapeutic Goods Administration under the Clinical Trial Notification scheme (Trial Number: 2015/0492) and approval was obtained on 29 June 2015.

Participants completed the intervention in their own homes. Compliance and adverse events were monitored with weekly phone calls throughout the intervention phase and participant completion of treatment adherence schedules.

[INSERT TABLE 1]

Outcomes

Table 1 provides an overview of the timing of the outcomes assessed. Sleep patterns were measured objectively (actigraphy) using wrist Actiwatch monitors (Respironics Actiwear 2) that estimate movement and light. Participants completed a *Response Booklet* that included the sleep diary and self-report scales. Participants attended two external

appointments for administration of the Cognitive Test Battery. The Cognitive Test Battery included measures of attention, memory, verbal fluency and executive functioning (see Supplementary Method for additional details of all clinical measures and selected outcome variables).

The faecal microbial counts were performed on specimens that were preserved by cooling and then controlling the temperature until the commencement of laboratory analysis (see Supplementary Method). Classical cultural methods, on a variety of media, were used to perform the counts (see [28] for details of microbial identification and microbial quantification procedures). Identification of bacteria was performed by Matrix Assisted Laser Absorption & Ionisation Time of Flight Mass Spectrometry (MALDI-TOF-MS) using a proprietary peptide data base (MALDI Biotyper Bruker Daltonics, Bremen, Germany). Microbial variables included the count and relative abundance (RA) of selected aerobic (*Streptococcus, Enterococcus, Escherichia*) and anaerobic bacteria (*Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Lactobacillus*). These variables were selected based on prior research [28]. RA_{total} was calculated by the ratio of each genus count divided by total detectable bacteria count (aerobic and anaerobic). The proportion of *Streptococcus* within total aerobic bacteria count (RA_{aerobe}) was also used as an outcome measure to be consistent with inclusion criteria and aid clinical interpretation.

The D-lactate and L-lactate concentrations in the urine samples were determined using High Performance Liquid Chromatography and Triple Quadrupole Mass Spectrometry (HPLC-TMS). Briefly, urine samples were acidified with hydrochloric acid and extracted with ethyl acetate. The ethyl acetate extracts were evaporated in a centrifugal vacuum evaporator. The residues were derivitised with an optically active reagent, (+)-O,O-diacetyl-L-tartaric anhydride, as originally described by Scheijen et al. [49]. These data are presented as the ratio of the concentrations of D-lactate to L-lactate. It is common to determine the ratio

of analyte concentration in the urine sample the concentration of creatinine in the sample in order to correct for dilute or concentrated urine samples that arise from variation in the state of hydration of the subject. This was considered inappropriate in the current trial because there is evidence that the excretion of creatinine is increased in subjects suffering from ME/CFS (see [50]).

Primary and secondary outcomes

Primary and secondary endpoints were the change in scores on psychological outcomes at post-intervention for the intention-to-treat (ITT) population (i.e., all participants who commenced at baseline). *A priori* allocation of primary outcome status was based on evidence from research indicating sensitivity measuring treatment effects in this [27] and other clinical populations [51]. Primary outcome variables included a measure of sleep (actigraphic sleep efficiency; SE), mood (Profile of Mood States-Short Form Total Mood Disturbance, POMS [52]) and a measure of sustained visual attention (Rapid Visual Processing-A', RVP-A' from the Cambridge Neuropsychological Test Automated Battery, CANTAB[53]).

Multiple secondary endpoints were selected to evaluate change in microbiota (*Streptococcus*, *Bifidobacteria* and *Lactobacillus* count and RA), urinary D-lactate (D:L lactate ratio) and clinical symptoms including: objective sleep symptoms (Actigraphy sleep onset latency (SOL), wake after sleep onset (WASO), and restlessness/sleep fragmentation index (SFI)); subjective sleep symptoms (Sleep Diary SOL, WASO, SE, and the Pittsburgh Sleep Quality Index, PSQI – Global Score [54]; mood (Depression, Anxiety and Stress Scale, DASS-21 [55]; cognition (word memory, story memory, spatial working memory, visual learning, verbal fluency, processing speed, cognitive flexibility and planning); fatigue (General Fatigue subscale from the Multidimensional Fatigue Inventory, MFI-20 [56]; and

the *Brain Fog* subscale of the Multiple Fatigue Types Questionnaire, MTFQ [57]; and total symptoms (Symptom Severity and Symptom Hierarchy Profile, SSH-Total score [47].

Uncertainty about the suitability of endpoints suggested a less hierarchical approach to outcome classification. Subsequently, the results of both primary and secondary outcome variables are presented together with discussion based on outcomes with large effect sizes (ES).

Sample Size

The study aimed to recruit equal proportions of males and females to conduct sex comparisons. Power analyses conducted by G*Power 3.1 indicated that the minimum sample size of n = 20 per group (alpha = .05, power = .8) would enable moderate to large ES estimates to achieve significance using analysis of variance (2x2 repeated measures ANOVA). A sample size of 40 for the combined group (alpha = .05, power = .8) was required to identify significant, moderate ES estimates using repeated measures ANOVA within factors (f = 0.23).

Statistical Methods

Group comparisons for primary and secondary outcomes

Using SPSS version 22.0 [58], mixed between-within subjects analysis of variance (2x2 repeated measures ANOVA) assessed the sex-time interaction effect and main effects (time and sex) for each outcome. These were performed for the whole sample (according to ITT protocols). Cases with missing data were excluded for pairwise analyses to retain maximum representation for each variable.

Focus on effect estimates

As encouraged by the CONSORT guidelines, it was decided to prioritise estimates of ES values and their confidence intervals (CI) [59]. Partial eta squared (μ^2) values are reported as the ES estimate produced by ANOVA analyses in SPSS software. Cohen's [60]

guidelines for interpreting partial eta squared were employed (small = 0.01, moderate = 0.06, large = 0.14). A conservative approach was used to avoid over-interpretation and the risk of Type 1 errors with multiple outcomes. Therefore, only outcomes with large effect sizes were used to examine treatment efficacy. Wuensch's [61] explanations and Smithson's syntax scripts for use in SPSS software were used to obtain 90% ES confidence intervals. Wuensch [61] explains that 90% confidence intervals are preferred because they are consistent with the ANOVA results and the .05 criterion of statistical significance. Additionally, partial eta squared (μ^2) values can only be positive values and a 95% confidence interval can include negative values. Exact significance values (*P*) are provided without use of Bonferroni corrections or the dichotomous categorisation of significance levels.

Assumptions: Tests used and managing violations

Normality

Each outcome variable was assessed for normality using the Shapiro-Wilk test in SPSS. Mild violations in normality were seen across several clinical variables, microbial RA and D:L lactate ratio variables. These variables were not transformed in accordance with criticisms of using transformations in psychosocial and biomedical research [62].

However, large violations in normality were seen on all microbial count variables. The nature of exponentially large values provided the rationale to transform these variables. Log10 transformations were applied and resulted in improvements in normality. Results were back-transformed after analysis and presented in the original scale as recommended [63].

Parametric tests were performed with minor violations of normality after considering that a) ANOVA is robust to violations of normality for samples larger than 30 [64] and b) nonparametric alternatives (Wilcoxon Signed Rank and Sign Test) exclude ties and, therefore, oppose the theoretical premise of ITT analyses. Means and standard deviations at baseline and post are presented based on cases with pairwise comparisons in each 2x2

ANOVA. In order to address possible concerns about the spread of scores and appropriately describe the data, median and range scores for ITT data at baseline and post are presented in Table S1.

Homogeneity tests

Homogeneity tests were calculated during repeated measures ANOVA procedures. The Levene's Test was used to determine equality of error variances. Given that *p* values are provided, violations of this assumption ($p < .05$) are highlighted in Table 3 to attempt to mitigate inaccurate interpretation. For readers focusing on probability statistics, it is recommended to use a more stringent interpretation of significance values for interaction and main effects when the Levene's test is violated (i.e., $p < .01$; [64]). The Box's *M* test was used to determine if the assumption of homogeneity of intercorrelations was met ($p \geq .001$; [65]).

Ancillary exploratory analyses: Correlations

The results of primary analyses indicated the need for further investigation to understand outcomes and examine interactions between change in bacteria and change in symptoms. Correlations were chosen as the preferred method due to restrictions with sample size and violations of assumptions with other statistical techniques (i.e., MANOVA or regression). Proportional change scores were created for each clinical, microbial count and D:L lactate ratio variable using equation (1).

$$X_{Change} = \frac{PostX}{PreX} \times 100 \quad (1)$$

where *X* represents each variable analysed

Therefore, scores of 100 reflect no change at post and numbers above or below reflect an increase or decrease at post, respectively. Spearman's rho correlations (r_s) between change in clinical variables and change in microbial variables were chosen due to violations in normality. Missing cases were excluded pairwise. To allow for consistent interpretation of

313 correlations, some r_s values were reversed (multiplied by -1) so that a decrease in the clinical
314 outcome score always represented improvement. Correlations were classified as small
315 (0.01), moderate (0.03) and large (0.05) effect sizes [66]. Only large effect sizes (i.e., r_s
316 >.05) were interpreted to reduce the risk of Type 1 errors from multiple correlations.

317 ## Results

318 ### Participant Recruitment and Demographics

319 Figure 1 shows the participant flow diagram with 44 patients deemed eligible and
320 consenting to participate from the 98 screened during recruitment (44.9%). A predominance
321 of females (n = 27) were recruited compared with males (n = 17). The recruitment period was
322 between 29th July 2015 and 8th November 2016. The date of the last data collection was 26th
323 December 2016. All participants completed both baseline and post-intervention stages.

324 **[INSERT FIGURE 1]**

325 Baseline demographics for all participants are presented in Table 2. Participants were
326 aged between 18 and 65 years with mean ages similar between the sexes. On average females
327 spent less time working per week with 15/23 females (65.2%) not working compared with
328 5/14 males (35.7%). The mean years since diagnosis of ME/CFS was approximately 10 years
329 for the total sample, female and male participants. The majority of participants (39/44)
330 adhered to the treatment protocol (self-reported taking >90% of the combined antibiotic and
331 probiotic intervention).

332 **[INSERT TABLE 2]**

333 ### Outcomes and Estimation

334 Descriptive results, ES estimates and exact significance levels obtained from 2x2
335 ANOVAs are presented for the total ITT sample and stratified by sex (Table 3). Some
336 outcomes had missing data due to incomplete responses (questionnaires), collection error
337 (stool and urine samples), and/or technical error (actigraphy). Management procedures for

missing and ambiguous data are presented in the Supplementary Method. Only outcomes with large effect sizes (i.e., μ^2>0.14) are highlighted and discussed with a focus on sex-time interaction and time effects.

[INSERT TABLE 3]

No large ES estimates were observed for sex-time interactions. The cognitive measure of attention (RVP A') was the only primary outcome with a large effect for time (μ^2=0.53, p<.001) suggesting an improvement in sustained attention from baseline (M=0.91, SD=0.42) to post (M=0.94, SD=0.04). Secondary outcomes measuring other cognitive symptoms also revealed large ES estimates indicating improvements in processing speed (μ^2=0.19, p=.004), cognitive flexibility (μ^2=0.43, p<.001), story memory (μ^2=0.21, p=.002), and verbal fluency (μ^2=0.14, p=.014).

Time effects on secondary outcomes measuring sleep symptoms indicated improvements in perceived (Diary) sleep efficiency (μ^2=0.14, p=.035), sleep quality (PSQI: μ^2=0.15, p=.027) and shorter duration of wake periods throughout the night measured both objectively (Actigraphy WASO: μ^2=0.21, p=.004) and subjectively (Diary WASO: μ^2=0.20, p=.007). The final clinical variable that suggested improvement was self-reported total symptoms (SSH), with a large effect indicating improvement (μ^2=0.29, p=.001). Notably, large sex effects were also observed for this variable with females (M=31.14, SD=8.16) reporting worse total symptoms compared to males (M=23.00, SD=11. 27) at baseline (μ^2=0.18, p=.015).

Streptococcus count was the only microbial variable that showed a large effect for time (μ^2=0.21, p=.003) with a reduction at post-intervention. No interaction, time or sex effects were observed on the D-lactate outcome variable. Interestingly split-plot graphs of *Streptococcus* count (Figure 2A), RA_{aerobe} (Figure 2B), and RA_{total} (Figure 2C) showed a spread of individual responses to the treatment with several participants increasing at post

(Count = 12/42, RA_{aerobe} = 17/42, RA_{total} = 13/42). In addition to this individual variability, accurate interpretation of results from ITT analyses were limited by no placebo control and the possibility of practice effects on cognitive outcomes. To better understand associations between bacterial change and symptom expression the ancillary exploratory analyses were performed.

[INSERT FIGURE 2]

Ancillary Exploratory Analyses: Correlations

Results of non-parametric correlations of variable change scores for the total sample, males and females are presented in Tables S2-S4. Detailed examination of the breadth of information provided by these ancillary analyses are beyond the scope of this paper. For the purposes of this article, only correlations with large effect sizes (r_s >.5) are discussed to avoid over-interpretation with smaller samples and the risk of Type 1 error with multiple correlations. There were no large correlations between change in microbiota and clinical symptoms for the total sample (Table S2). For females, results showed negative correlations (i.e., increased bacteria associated with clinical improvement) between change in: *Clostridium* and cognitive flexibility (r_s = -.58, p = .002), *Lactobacillus* and planning (r_s = -.50, p = .010), and *Enterococcus* and story memory (r_s = -.50, p = .015; Table S3). The majority of large correlations were shown for males (see Table S4). Table 4 provides a summary of large correlations between change in clinical symptoms and microbial and lactate change variables in males.

[INSERT TABLE 4]

The correlations presented in Table 4 indicate some consistency across several clinical outcomes for the genera *Bacteroides, Bifidobacterium, Clostridium* and D:L Lactate variables. Negative correlations suggest that an increase in *Bacteroides* (as observed in 11/16 males) was associated with improvements in sleep (Actigraphy WASO, Sleep Quality -

PSQI), mood (Mood Disturbance – POMS Total; Stress - DASS), general fatigue (MFI-GF) and total symptoms (SSH). An association in the opposite direction was found for change on the cognitive measure of planning (SWM-Strategy), which was reduced.

Negative correlations were shown between change in *Bifidobacterium* and sleep quality (PSQI), general fatigue (MFI), anxiety (DASS), and visual learning. Alternatively, positive correlations were revealed between change in *Clostridium* and total symptoms (SSH) and some cognitive outcomes (verbal fluency, story memory, processing speed). Notably, change in *Streptococcus* correlated negatively with perceived sleep onset (Diary SOL) indicating that reduced *Streptococcus* was associated with subjectively longer time taken to fall asleep in males.

D:L Lactate

A small, negative correlation was observed between change in D:L lactate concentration ratios and change in *Streptococcus* count for the total sample (r_s = -.243, p = .142). Correlations with clinical symptoms revealed that the change in D:L lactate concentration ratios was positively associated with change in sleep onset latency (Actigraphy SOL), mood disturbance (POMS Total) general fatigue (MFI) and total symptoms (SSH) in males. This would suggest proportionally higher concentrations of D-lactate were associated with adverse symptoms in males. Changes in the D:L lactate concentration ratio of our sample revealed that 9/15 males and 12/23 females increased had proportionally higher concentrations of D-lactate at post intervention.

Harms

Six unexpected adverse events were reported from 5 participants. One participant (a) experienced severe diarrhoea, vomiting and cramping after taking the first antibiotic. This participant also experienced a respiratory allergic reaction to a non-protocol medication taken to attempt to relieve the gastrointestinal symptoms. Four other participants experienced an

adverse event including (b) blood in stool (bloating but no pain reported), (c) difficulty sleeping, (d) rash on torso, and (e) exacerbation of Seborrheic dermatitis. Of these participants, the first (a) discontinued all treatment after the first antibiotic dose. The other participants (b) completed the treatment protocol, (c) reduced antibiotics (consumed 20/24 capsules), or reduced probiotics (d: consumed 11/28 capsules, e: consumed 14/28 capsules), respectively. All participants participated in post-intervention assessments.

Discussion

ITT analysis of effects across outcome variables showed reduction in *Streptococcus* count and improvement across multiple clinical outcomes with no clear sex difference in treatment effect. The clinical changes observed with this short intervention included large effects likely to reflect modest clinical improvement on some secondary sleep outcomes (wakefulness, efficiency, quality), primary and secondary cognitive outcomes (attention, processing speed, cognitive flexibility, story memory, verbal fluency) and total symptoms. Measures of mood, fatigue and D-lactate showed no (or low) treatment effects.

Improvement on some sleep and cognitive measures appear promising considering this short intervention (4-weeks) and the complexity of this chronic condition (average illness duration ~10 years). Differences between outcome variables makes it difficult to clearly ascertain whether the clinical changes at post were a direct response to the treatment or better explained by placebo, practice effects (particularly cognitive outcomes) or symptom variability of unknown origin. Although it must be noted that if placebo effects are the primary explanation for the results observed, then we would have predicted consistent improvements across subjective variables (i.e., sleep, mood and fatigue variables) that were not shown. With these confounding factors in mind, improvement on objective sleep parameters may provide the most reliable indicator of change. Using these conservative

parameters, reduced wakefulness after sleep onset (actigraphic WASO) may be the best indicator of clinical improvement.

Unexpectedly, individual variability of treatment response was highlighted by the proportion of participants who increased in *Streptococcus* counts at post (Count = 28%, RA_{aerobe} = 41%, RA_{total} = 31%). This prompted exploration of relationships between change in microbial count and clinical symptoms. Ancillary results showed that shifts in microbiota were associated with more of the variance in clinical changes for males compared with females. Smaller correlations for females may (i) suggest non-monotonic relationships, (ii) raise questions about the benefits of the intervention for this group, (iii) suggest that other unmeasured factors may contribute to the variance observed (i.e., changes in the microbiome, hormonal, immune, other stressors) or (iv) indicate an alternate mode of action in females (i.e., not revealed by the methods carried out in this pilot study).

In males, change in *Bacteroides*, *Bifidobacterium* and *Clostridium* were associated with change across several symptoms respectively. Intercorrelations between change in microbial and clinical variables suggest that an increase in *Bacteroides* (count) was associated with improvement on some clinical measures of sleep, mood, fatigue and total symptoms. Similarly, increased *Bifidobacterium* was associated with improvement in sleep quality, general fatigue, anxiety and visual learning. For *Clostridium*, a reduction was associated with more clinical improvements (cognitive and total symptoms).

Using the same culture-based methods for microbial analysis, Armstrong et al. [67] observed distinctions in both *Bacteroides* and *Clostridium*. In an exclusively female sample, results revealed ME/CFS patients had reduced frequency of *Bacteroides* and proportionally greater *Clostridium* compared to controls. Decreased *Bacteroides spp*. in ME/CFS compared with controls and positive associations with serum amino acids [67] may be particularly relevant considering the role of amino acids for cellular energy [68]. Nagy-Szakal et al. [26]

462 also found reduced proportion of *Bacteroides vulgatus* but an increased abundance of
463 'unclassified' *Bacteroides* using sequencing techniques in ME/CFS patients without IBS
464 symptoms. Prior evidence combined with our results raise questions about the abundance,
465 diversity and functional role of *Bacteroides* in ME/CFS.

466 In light of Armstrong et al.'s [67] findings for *Bacteroides* and *Clostridium* in females
467 and positive correlations between *Clostridium* and symptom presentation for females in
468 cross-sectional data [28], it would be premature to conclude that these genera are only
469 relevant for males with ME/CFS. A more reasonable explanation for our ancillary results
470 may be related to observed changes in our sample. For example, a larger proportion of males
471 (11/16, 68.8%) increased in *Bacteroides* count at post compared with females (10/26, 38.5%).
472 Rather than pointing to sex differences as a primary factor relevant for treatment response,
473 our results could merely reflect individual variability or could imply increased complexity in
474 females (i.e., the influence of other confounding factors such as hormonal shifts that may
475 account for a larger percentage of the variance).

476 The growth in *Bacteroides* species at post for 11/16 males may have occurred from
477 cross-feeding through probiotic supplementation. Metabolic by-products from one bacteria
478 can become a food source (i.e., prebiotic) for other commensal bacteria [69]. Several
479 *Bifidobacteria* species produce complex carbohydrates (exopolysaccharides) that can become
480 substrates for other bacteria and subsequently promote their growth [69]. Some strains of
481 *Bifidobacterium* have been shown to increase species of *Bacteroides* using culture methods
482 *ex vivo* [69, 70]. The probiotic used in this trial included three strains of *Bifidobacteria* (*B.*
483 *lactis*, *B. breve*, *B. longum*). Whilst the strains analysed in prior studies are not directly
484 comparable to the strains administered in this study, the possibility of similar metabolic
485 processes should be considered. Our increasing understanding of cross-feeding and microbial

communication (see review [33]) may be useful to identify probiotic or prebiotic treatment alternatives to restore microbial homeostasis.

Relevance for D-lactate Theory

The results of ITT outcome and ancillary analyses showing no change in D:L lactate ratio at post and small negative correlations between change in D:L lactate and *Streptococcus,* raise doubts about D-lactate metabolism from *Streptococcal* species. Considering, 21/38 participants increased in D:L lactate ratio after the intervention, it appears that the reduction of *Streptococcus* did not decrease D-lactate concentrations as expected. Given the enteric microbiota consists of more than 1000 species of bacteria [33], the limitations with culture-based identification methods, and the uncertainty around which species are producing lactate, it is possible that a reduction in *Streptococcus* may have allowed another D-lactate producing organism to proliferate. Some ancillary results provide partial support for D-lactate theory in males with change scores indicating decrease of D:L lactate ratio associated with improvement on some clinical outcomes (sleep onset (actigraphy SOL), mood disturbance (POMS), general fatigue (MFI), and total symptoms (SSH)). Perhaps our results reflect the relative change in reduced L-lactate production that would impact the ratio measured. Further research is needed to compare D-lactate concentrations (optimally in urine, faecal and serum samples) in ME/CFS with healthy controls and investigate other possible D-lactate producing bacteria, to adequately evaluate the relevance of the D-lactate hypothesis for either sex.

Limitations

Our interpretation of D:L lactate is restricted by methodological limitations requiring the use of a lactate ratio. Whilst creatinine is routinely used for normalising urinary metabolites (Barr et al., 2005), significantly higher concentrations of creatinine have been shown in ME/CFS patients compared with controls [50]. These results suggest that it may be

inappropriate to normalise lactate concentrations using creatinine concentrations in this population [50]. Without an appropriate method for normalisation, absolute D-lactate concentrations and absolute L-lactate concentrations could not be statistically analysed because of the known wide variation in the concentration of spot urine samples in contrast to 24 hour timed collections used to calculate daily excretion rates. Similarly, using genera rather than species data for microbial outcomes has reduced specificity and restricts interpretation.

The open-label design without placebo-control and using repeated measures carries inherent limitations restricting interpretation and generalisability of findings. It is possible that changes observed could be attributed to placebo (i.e., unintended therapeutic effects; [71]) rather than the direct action of the treatment for a proportion of the sample. Notably, the placebo response appears to be lower in ME/CFS than other medical conditions (e.g., depression, migraine, gastro-intestinal conditions; see [72]). However, the influence of participant expectation appears to be greater for interventions with physiological targets (i.e., infectious or immunological) compared with psychosocial interventions in ME/CFS [72].

Discrepancies between cognitive measures and other symptoms raise questions about the influence of practice effects inherent in repeated testing over a short interval. Alternate forms and outcomes with reduced practice effects were prioritised (see Supplementary Method). Additional baseline measurement of cognitive symptoms may be deemed ethically inappropriate for this sample due to concerns about post-exertional fatigue and participant burden. Ideally, controlled comparison can be used in future research to ascertain the proportion of change that can be attributed to familiarity with cognitive tests.

Other confounding factors included the influence of diet, concurrent medication and fluctuating symptomatology. Whilst we attempted to control for these factors by asking participants to remain stable on their diet and medication, the possibility of effects from other

treatments or dietary intake cannot be excluded. The nature of the condition is that it has symptomatology that can be exacerbated or diminished without clear attributional cause. These fluctuations and other environmental (change in education or employment status, family stressors) and/or physiological (e.g., stage of menstrual cycle, viral/bacterial exposure) factors could not be controlled.

Statistical limitations include reduced power with smaller male samples, consideration of multiplicity of analyses and restricted interpretation with correlations. Results from correlational data only provide information about monotonic relationships, cannot attribute causation and have limited capacity to infer direct treatment effects. Cautious interpretations have been made focusing on large effects to attempt to reduce bias and improve generalisability. However, this conservative approach excludes small and moderate correlations that may also be relevant.

Other Modes of Action

Some lactate results that contradict D-lactate theory prompt consideration of whether *Streptococcus spp.* or the intervention could have other modes of action. Streptococcal throat infections have been proposed as precipitating encephalitis and neurological symptoms in childhood. Both paediatric autoimmune neuropsychiatric disorders associated with Group A streptococcal infections (PANDAS; [73, 74]) and Sydenham's chorea [75] have been described as conditions with an acute onset of obsessive-compulsive symptoms and vocal or motor tics that can occur in some children after Group A streptococcal infection. Evidence of abnormal basal ganglia imaging and antibasal ganglia antibodies suggests that streptococcal infections may trigger autoimmune responses in some individuals [75]. Within the context of ME/CFS, it seems reasonable to explore whether the overgrowth of commensal enteric *Streptococcus*, as observed in 58/92 (59.2%) patients screened, may exert immunological or autoimmune effects that contribute to neurological symptoms. Future research could also

evaluate a history of Group A streptococci infections and monitor immune and inflammatory markers to establish whether similar mechanisms are at play in ME/CFS.

One possible mechanism of the intervention is through the prokinetic qualities of erythromycin. Erythromycin is a macrolide that inhibits protein synthesis in specific bacteria [76] and can increase gastric motility [77]. Low doses of erythromycin have been used for its prokinetic qualities in patients with delayed gastric emptying [78]. The stimulation of oesophageal, gastric and small intestinal contractions are likely to partially explain commonly reported gastrointestinal side effects (i.e., diarrhoea, nausea, vomiting) of oral erythromycin (see [79]). Therefore, the prokinetic effect of erythromycin may be particularly beneficial for this sample when we consider that constipation is a common symptom for patients with comorbid IBS and/or small intestinal bacterial overgrowth (SIBO; [80]), and the prevalence of intestinal permeability in ME/CFS [20, 21]. Increased monitoring of gastrointestinal changes, SIBO and IBS symptoms would be useful in further studies.

Probiotics may also increase bowel transit [81] or have other modes of action. Possible mechanisms of probiotics include modulating inflammatory and immune responses through enhancing the epithelial barrier, adherence to the mucosal wall, direct (antimicrobial) or indirect (competitive exclusion) effects on pathogenic microbiota, and vagal signalling (see [33, 82–84]). Metabolic by-products from specific bacterial strains may also effect clinical presentations through the production of neurotransmitters (see [85]), short chain fatty acids through fermentation (see [33]), and cross-feeding, as discussed above. Advances in metabolomics methods would be useful to monitor functional changes during probiotic supplementation in ME/CFS patients.

Conclusions

These results add to the accumulating evidence that microbiota-gut-brain interactions play a role in the clinical presentations of a subgroup of ME/CFS patients. This antimicrobial

586 and probiotic treatment showed concurrent reduction in enteric *Streptococcus* counts and
587 improvement in some neurological symptoms for the ITT sample. Precise mechanisms
588 remain to be determined because results for D-lactate challenged the premise that
589 *Streptococcus* species are the primary producers of D-lactic acid.

590 Ancillary results infer that shifts in microbiota were associated with more of the
591 variance in clinical changes for males compared with females. It is unclear whether the
592 reduction in *Streptococcus* is particularly beneficial in some ME/CFS patients or whether
593 other concurrent microbial shifts are equally or more valuable (i.e., reduced *Bacteroides*
594 and/or increased *Clostridium*). These results prompt the use of sequencing methods to
595 elucidate other microbial shifts that may be relevant and not revealed through culture-based
596 methods. Analysis of the microbiome through sequencing techniques should be examined
597 before pursuing a randomised placebo controlled trial. Whilst sex differences were not
598 obvious through primary analyses, ancillary results reinforce the need to recruit sufficient
599 samples to enable sex comparisons in clinical trials.

600 Individual differences in microbial and clinical changes observed across this
601 intervention are unsurprising considering other prominent findings in gut microbiome and
602 ME/CFS research. For example, ground-breaking research with a large healthy cohort has
603 shown the microbiome as a primary predictor of varied glucose response to the same foods,
604 supporting the need for personalised nutrition [86]. Within ME/CFS, duration of illness [87]
605 and genetic variability [88–90] appear to be key factors that contribute to differences in
606 immune markers, pathophysiology and clinical presentation. Considering the bidirectional
607 role of the gut microbiome in immune modulation (e.g., [91]), epigenetic regulation [92], and
608 the influence of genetics on microbial composition [93], continued efforts to understand the
609 function of the microbiome in ME/CFS is warranted.

610

611

LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CANTAB	Cambridge Neuropsychological Test Automated Battery
CONSORT	consolidated standards of reporting trials
DASS	Depression Anxiety Stress Scale
EES	erythromycin ethyl succinate
ES	effect sizes
IBS	irritable bowel syndrome
ITT	intention to treat
MALDI-TOF-MS	Matrix Assisted Laser Absorption & Ionisation Time of Flight Mass Spectrometry
MANOVA	multiple analysis of variance
ME/CFS	myalgic encephalomyelitis/chronic fatigue syndrome
MFI	Multidimensional Fatigue Inventory
POMS	Profile of Mood States-Short Form
PSQI	Pittsburgh Sleep Quality Index
RA	relative abundance
RVP	Rapid Visual Attention
SE	sleep efficiency
SFI	sleep fragmentation index
SIBO	small intestinal bacterial overgrowth
SOL	sleep onset latency
SPSS	Statistical Package for the Social Sciences
SSH	Symptom Severity and Symptom Hierarchy Profile
SWM	Short-term Working Memory
WASO	wake after sleep onset

DECLARATIONS

Ethics Approval and Consent to Participate

Ethics approval was obtained from Victoria University Human Research Ethics Committee in June 2015 (HRE15-010). Participation was voluntary with detailed information about the trial design, intervention and possible risks and benefits provided in oral and written form. Signed informed consent was obtained by all participants.

Consent for Publication

Not applicable

Availability of Data and Materials

The datasets generated and analysed during the current study are not publicly available due to concerns about participant anonymity and concurrent data analyses that are being conducted but are available from the corresponding author on reasonable request.

Competing Interest

Bioscreen (Aust.) Pty Ltd. and Victoria University provided trial funding and post-graduate scholarship funding to A.W. without restriction on publication. D.B., M.B., D.P.L., H.B., P.P., S.M., A.J.K., declare no competing financial interest.

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Bioscreen (Aust.) Pty Ltd. and Victoria University provided trial funding and post-graduate scholarship funding to A.W. without restriction on publication. SpectrumCeuticals provided the probiotic to participants without charge.

Author's Contributions

A.W. wrote the manuscript; A.W. and D.P.L. co-ordinated recruitment, data collection and intervention; raw data analysis was conducted by A.W. (clinical outcomes), H.B. (microbial outcomes), S.M., P.P. and A.J.K. (lactate outcomes); statistical data analysis was conducted by A.W., M.B., and D.B.; all authors contributed to study design, data interpretation and manuscript editing.

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TABLES

Table 1. Trial design

Week		**1**	**2**	**3**	**4**	**5**	**6**
Day		1-7	8-14	15-21	22-28	29-35	36-42
Phase	**Screening/ Recruitment**	**Baseline**	**Intervention**				**Post-Intervention**
Treatment prescribed			**Antibiotic** Erythromycin 400mg/b.d.	**Probiotic** Pro4-50 D-Lactate Free Multistrain 1 tablet b.d.	**Antibiotic** Erythromycin 400mg/b.d.	**Probiotic** Pro4-50 D-Lactate Free Multistrain 1 tablet b.d.	
Measures/ Monitoring	▪ Consent forms ▪ Screening and background questions ▪ Stool and urine collection	*Day 1* ▪ Cognitive test battery *Days 1-7* ▪ Actigraphy ▪ Sleep diary ▪ MAC ▪ MFTQ *Day 7** ▪ SSH ▪ DASS-21 ▪ MFI-20 ▪ POMS ▪ PSQI ▪ ISI	*Day 14* ▪ Phone call, symptom monitoring	*Day 21* ▪ Phone call, symptom monitoring	*Day 28:* ▪ Phone call, symptom monitoring	*Day 35* ▪ Phone call, symptom monitoring	*Day 1* ▪ Cognitive test battery *Days 1-7* ▪ Actigraphy ▪ Sleep diary ▪ MAC ▪ MFTQ ▪ Stool and urine collection *Day 7** ▪ SSH ▪ DASS-21 ▪ MFI-20 ▪ POMS ▪ PSQI ▪ ISI

* Participants had the option of completing these scales at any time during Baseline and Post-intervention phases if they wanted to reduce the risk of post-exertional mental fatigue.

Table 2. Baseline demographics for intention-to-treat sample stratified by sex

Demographics	**Total Sample** (*N* = 44)			**Females** (*n* = 27)			**Males** (*n* =17)		
	n	*M(SD)*[a]	*Range*	*n*	*M(SD)*[a]	*Range*	*n*	*M(SD)*[a]	*Range*
Age (years)	44	44.1(13.5)	18-65	27	43.8(12.8)	19-62	17	44.5(14.9)	18-65
Hours worked per week (not parenting)	37	13.0(16.8)	0-45	23	9.4(15.5)	0-40	14	19.0(17.7)	0-45
Duration of illness (years)	35	9.9(6.9)	0.5-26	23	9.6(7.1)	0.5-26	12	10.4(6.6)	1-20
Frequency short duration (≤3 years)		*7(15.9%)*			*5(18.5)*			*2(11.8%)*	
Frequency long duration (>3 years)		*28(63.6%)*			*18(66.7%)*			*10(58.8%)*	
Unknown		*9(20.45%)*			*4(14.81%)*			*5(29.41%)*	

[a] Data reflects *mean* (*standard deviation*) or *frequency (%)*.
Information about the duration of the illness was also described by classifying into two groups of shorter (≤3 years) and longer (>3 years) duration using the same ranges as in Hornig et al. [87]

Table 3. Results of 2x2 ANOVAs for all outcome variables with descriptive statistics, effect size estimates and exact significance values

Outcomes	Total Sample			Females			Males			Sex-Time Interaction		Time		Sex	
		Baseline	Post		Baseline	Post		Baseline	Post						
	n	*M(SD)*	*M(SD)*	*n*	*M(SD)*	*M(SD)*	*n*	*M(SD)*	*M(SD)*	μ^2 *(90%CI)*	*P*	μ^2 *(90%CI)*	*P*	μ^2 *(90%CI)*	*P*
Sleep															
Actigraphy Sleep Efficiency^	38	82.94 (10.95)	83.80 (9.85)	23	81.94 (13.14)	82.52 (11.68)	15	84.48 (6.42)	85.75 (5.93)	0.00(0.00, 0.09)	0.697	0.03(0.00, 0.16)	0.297	0.02(0.00, 0.14)	0.395
Actigraphy Sleep Fragmentation Index*	38	25.99 (11.28)	24.46 (11.44)	23	26.93 (12.97)	25.93 (13.38)	15	24.55 (8.27)	22.20 (7.47)	0.02(0.00, 0.14)	0.402	0.11(0.02, 0.27)	0.042	0.02(0.00, 0.14)	0.416
Actigraphy Sleep Onset Latency	38	22.39 (19.72)	21.23 (30.54)	23	22.24 (20.57)	23.59 (37.54)	15	22.62 (19.05)	17.61 (15.07)	0.02(0.00, 0.14)	0.381	0.01(0.00, 0.11)	0.612	0.00(0.00, 0.09)	0.723
Actigraphy Wake After Sleep Onset*	38	48.87 (24.75)	43.28 (24.06)	23	53.49 (27.02)	49.85 (27.53)	15	41.78 (19.54)	33.21 (12.66)	0.04(0.00, 0.18)	0.228	0.21(0.04, 0.38)	0.004	0.09(0.00, 0.25)	0.07
Diary Sleep Efficiency^	33	89.01 (5.26)	91.74 (4.92)	21	87.91 (4.72)	91.41 (5.02)	12	90.93 (5.79)	92.31 (4.90)	0.03(0.00, 0.17)	0.345	0.14(0.01, 0.32)	0.035	0.06(0.00, 0.22)	0.188
Diary Sleep Onset Latency	44	30.77 (24.17)	25.47 (23.42)	27	31.56 (22.48)	25.76 (22.63)	17	29.52 (27.30)	25.02 (25.32)	0.00(0.00, 0.05)	0.823	0.07(0.00, 0.21)	0.082	0.02(0.00, 0.15)	0.84
Diary Wake After Sleep Onset	34	27.55 (21.49)	15.62 (14.19)	22	31.55 (21.39)	16.67 (12.88)	12	20.20 (20.50)	13.69 (16.75)	0.04(0.00, 0.18)	0.271	0.20(0.03, 0.38)	0.007	0.05(0.00, 0.21)	0.184
Sleep Quality - PSQI-Global	32	9.78 (4.24)	8.13 (3.72)	21	9.76 (4.61)	8.10 (3.51)	11	9.82 (3.63)	8.18 (4.29)	0.00(0.00, 0.00)	0.983	0.15(0.01, 0.34)	0.027	0.00(0.00, 0.00)	0.958
Mood															
Mood Disturbance - POMS-Total*	32	45.08 (24.11)	43.18 (27.32)	20	43.58 (22.41)	41.79 (32.04)	12	47.58 (27.56)	45.50 (17.91)	0.00(0.00, 0.01)	0.972	0.01(0.00, 0.12)	0.649	0.01(0.00, 0.11)	0.655
Depression - DASS-21	40	5.98 (4.76)	5.00 (4.78)	26	5.85 (4.81)	5.19 (5.34)	14	6.21 (4.84)	4.64 (3.67)	0.01(0.00, 0.12)	0.478	0.07(0.00, 0.23)	0.09	0.00(0.00, 0.00)	0.951
Anxiety - DASS-21	40	4.25 (3.68)	3.63 (3.63)	26	4.50 (3.44)	4.04 (3.74)	14	3.79 (4.17)	2.86 (3.42)	0.01(0.00, 0.09)	0.678	0.04(0.00, 0.17)	0.221	0.02(0.00, 0.14)	0.386
Stress - DASS-21	38	8.24 (5.36)	6.87 (5.16)	25	8.16 (5.23)	6.68 (5.60)	13	8.39 (5.81)	7.23 (4.38)	0.00(0.00, 0.04)	0.857	0.06(0.00, 0.21)	0.151	0.00(0.00, 0.06)	0.808
Cognition															
Executive functioning															
Attention - RVP A'^*	43	0.91 (0.42)	0.94 (0.04)	26	0.91 (0.04)	0.94 (0.05)	17	0.91 (0.04)	0.94 (0.03)	0.00(0.00, 0.07)	0.786	0.53(0.34, 0.65)	0	0.00(0.00, 0.07)	0.753
Procesisng speed - RVP Mean latency*	43	474.82 (116.49)	442.21 (113.51)	26	499.85 (137.73)	454.62 (135.14)	17	436.53 (58.36)	423.23 (68.25)	0.06(0.00, 0.21)	0.104	0.19(0.04, 0.35)	0.004	0.05(0.00, 0.18)	0.173
Cognitive flexibility - AST Median Switching Cost - Block 7	44	284.82 (141.35)	213.05 (149.63)	27	311.15 (147.92)	235.28 (150.92)	17	243.00 (122.94)	177.74 (144.90)	0.00(0.00, 0.08)	0.677	0.43(0.23, 0.56)	0	0.49(0.00, 0.18)	0.149
Planning - SWM - Strategy	44	30.75 (7.74)	28.65 (8.08)	27	29.30 (7.05)	27.11 (7.28)	17	33.06 (8.43)	31.12 (8.87)	0.00(0.00, 0.02)	0.881	0.13(0.02, 0.29)	0.015	0.07(0.00, 0.21)	0.093

Memory														
Word memory - RAVLT–Immediate^	44	49.66 (9.51)	52.45 (8.58)	27	52.07 (7.95)	55.48 (6.60)	17	45.82 (10.73)	47.65 (9.33)	0.01(0.00, 0.11)	0.514	0.10(0.00, 0.25)	0.035	0.18(0.04, 0.34)	0.004
Story memory - LM Immediate^	44	23.23 (7.70)	26.68 (7.04)	27	24.96 (7.07)	28.07 (7.38)	17	20.47 (8.07)	24.47 (7.38)	0.00(0.00, 0.08)	0.681	0.21(0.05, 0.37)	0.002	0.09(0.00, 0.24)	0.044
Spatial working memory - SWM - Between errors*	44	22.80 (19.10)	18.11 (18.02)	27	17.93 (16.01)	12.71 (11.38)	17	30.53 (21.46)	26.71 (23.12)	0.00(0.00, 0.02)	0.739	0.10(0.00, 0.25)	0.036	0.14(0.02, 0.30)	0.011
Visual learning - PAL - Total errors	44	16.07 (23.31)	12.91 (14.16)	27	14.04 (23.30	9.96 (11.88)	17	19.29 (23.65)	17.59 (16.48)	0.01(0.00, 0.09)	0.6	0.04(0.00, 0.16)	0.205	0.03(0.00, 0.15)	0.25
Verbal fluency - COWAT Corrected Score^	44	36.82 (11.99)	39.21 (8.97)	27	38.93 (12.49)	39.85 (8.51)	17	33.47 (10.65)	38.18 (9.84)	0.07(0.00, 0.21)	0.093	0.14(0.02, 0.29)	0.014	0.03(0.00, 0.15)	0.252
Other ME/CFS															
Symptoms															
General fatigue - MFI	40	17.45 (2.74)	16.88 (2.96)	26	18.12 (2.39)	16.88 (3.00)	14	16.21 (2.99)	16.86 (3.01)	0.10(0.01, 0.30)	0.042	0.01(0.00, 0.12)	0.513	0.04(0.00, 0.17)	0.247
Brainfog - MFTQ	43	10.52 (4.31)	8.35 (4.10)	27	10.72 (3.86)	8.35 (4.10)	16	10.19 (5.10)	9.12 (3.11)	0.01(0.00, 0.12)	0.45	0.09(0.00, 0.24)	0.05	0.00(0.00, 0.02)	0.898
Total symptoms - SSH	33	28.42 (9.93)	22.76 (9.81)	22	31.14 (8.16)	25.27 (9.22)	11	23.00 (11.27)	17.73 (9.36)	0.00(0.00, 0.04)	0.852	0.29(0.08, 0.46)	0.001	0.18(0.02, 0.36)	0.015
Microbiota															
Streptococcus Count	42	$8.69x10^6$ (6.39)	$6.88x10^5$ ($1.39x10^2$)	26	$5.44x10^6$ (4.58)	$2.87x10^5$ ($3.58x10^2$)		$1.87x10^6$ (8.39)	$2.86x10^5$ ($1.04x10^1$)	0.01(0.00, 0.12)	0.485	0.21(0.05, 0.37)	0.003	0.09(0.00, 0.24)	0.053
Bifidobacteria Count	42	$1.49x10^4$ ($2.04x10^4$)	$5.08x10^2$ ($8.05x10^3$)	26	$6.38x10^3$ ($1.83x10^4$)	$3.90x10^2$ ($9.44x10^3$)	16	$5.85x10^4$ ($2.90x10^4$)	$7.80x10^2$ ($8.17x10^3$)	0.01(0.00, 0.09)	0.64	0.11(0.01, 0.26)	0.034	0.01(0.00, 0.10)	0.574
Lactobacillus Count*	42	$4.69x10^2$ ($5.33x10^3$)	$1.91x10^2$ ($2.12x10^3$)	26	$8.85x10^1$ ($2.08x10^3$)	$2.45x10^2$ ($2.56x10^3$)	16	$7.03x10^3$ ($1.42x10^4$)	$1.27x10^2$ ($1.96x10^3$)	0.11(0.01, 0.27)	0.032	0.11(0.01, 0.27)	0.032	0.02(0.00, 0.13)	0.425
Streptococcus RA_{aerobe}	42	57.59 (32.17)	40.64 (43.66)	26	56.74 (32.22)	40.64 (43.66)	16	58.96 (33.09)	42.33 (37.59)	0.00(0.00, 0.01)	0.974	0.09(0.00, 0.25)	0.048	0.00(0.00, 0.05)	0.823
Streptococcus RA_{total}*[M]	42	0.86 (4.61)	0.88 (4.29)	26	0.13 (0.22)	1.23 (5.43)	16	2.05 (7.46)	0.31 (0.75)	0.05 (0.00, 0.18)	0.165	0.00(0.00, 0.07)	0.750	0.00(0.00, 0.12)	0.620
Bifidobacteria RA_{total}	42	5.78 (14.98)	1.96 (6.19)	26	6.33 (16.92)	2.46 (7.22)	16	4.88 (11.61)	1.16 (4.12)	0.00(0.00, 0.00)	0.976	0.06(0.00, 0.20)	0.121	0.06(0.00, 0.09)	0.625
Lactobacillus RA_{total}*	42	2.59 (9.05)	1.76 (7.36)	26	0.35 (1.61)	2.61 (9.27)	16	6.24 (14.03)	0.37 (1.29)	0.12(0.01, 0.28)	0.025	0.03(0.00, 0.16)	0.258	0.03(0.00, 0.15)	0.305
Lactate															
D:L lactate ratio*	38	0.30 (0.21)	0.34 (0.20)	23	0.34 (0.24)	0.34 (0.23)	15	0.24 (0.18)	0.33 (0.16)	0.04(0.00, 0.17)	0.247	0.04(0.00, 0.17)	0.254	0.03(0.00, 0.16)	0.322

μ^2 = partial eta squared / effect size estimate; * = Levene's test violated, use p<.01; [M] = Box M test of equality of covariance violated
Lower scores reflect better symptoms for all clinical variables unless indicated by ^
Units of measurement for microbial variables: count = cfu/g back-transformed from Log10; RA_{aerobe} = proportion of genus count within total aerobic bacterial counts as a percentage; RA_{total} = relative abundance of each genus within total bacteria count (aerobic + anaerobic) presented as a percentage.
Large effect size estimates (μ^2>0.14) are highlighted.

Table 4. Summary of large spearman's rho (r_s) correlations (>.5) between clinical change and microbial or lactate change variables in males

Microbial Count and Lactate Change	**Clinical Change**	r_s	p	n	*Direction of change in bacteria associated with clinical improvement*
Bacteroides	Sleep Quality - PSQI	-.758	.011	10	Increase
	General Fatigue - MFI	-.738	.004	13	Increase
	Stress - DASS	-.701	.011	12	Increase
	Total Symptoms - SSH	-.600	.067	10	Increase
	Mood Disturbance - POMS Total	-.573	.066	11	Increase
	Actigraphy Wake After Sleep Onset	-.529	.043	15	Increase
	Planning - SWM - Strategy	.588	.017	16	Decrease
Bifidobacterium	Sleep Quality - PSQI	-.681	.030	10	Increase
	General Fatigue - MFI	-.602	.030	13	Increase
	Anxiety - DASS	-.567	.043	13	Increase
	Visual learning - PAL - Total errors	-.529	.035	16	Increase
Clostridium	Total Symptoms - SSH	.582	.078	10	Decrease
	Verbal fluency - COWAT Corrected Score^	.550	.027	16	Decrease
	Story memory - LM Immediate^	.522	.038	16	Decrease
	Processing speed - RVP Mean latency	.507	.045	16	Decrease
Enterococcus	General Fatigue - MFI	-.516	.071	13	Increase
Lactobacillus	Actigraphy Sleep Onset Latency	.610	.016	15	Decrease
	Attention - RVP A'^	-.571	.021	16	Increase
	Actigraphy Sleep Efficiency^	.512	.051	15	Decrease
Streptococcus	Diary Sleep Onset Latency	-.656	.006	16	Increase
D:L lactate ratio	Actigraphy Sleep Onset Latency	.802	.001	14	Decrease
	Total Symptoms - SSH	.567	.112	9	Decrease
	General Fatigue - MFI	.532	.061	13	Decrease
	Mood Disturbance - POMS Total	.509	.110	11	Decrease

N.B. Lower scores on clinical outcomes = improvement, ^ = variables with reversed correlations (i.e., multiplied by -1) to allow for consistent interpretation

FIGURES

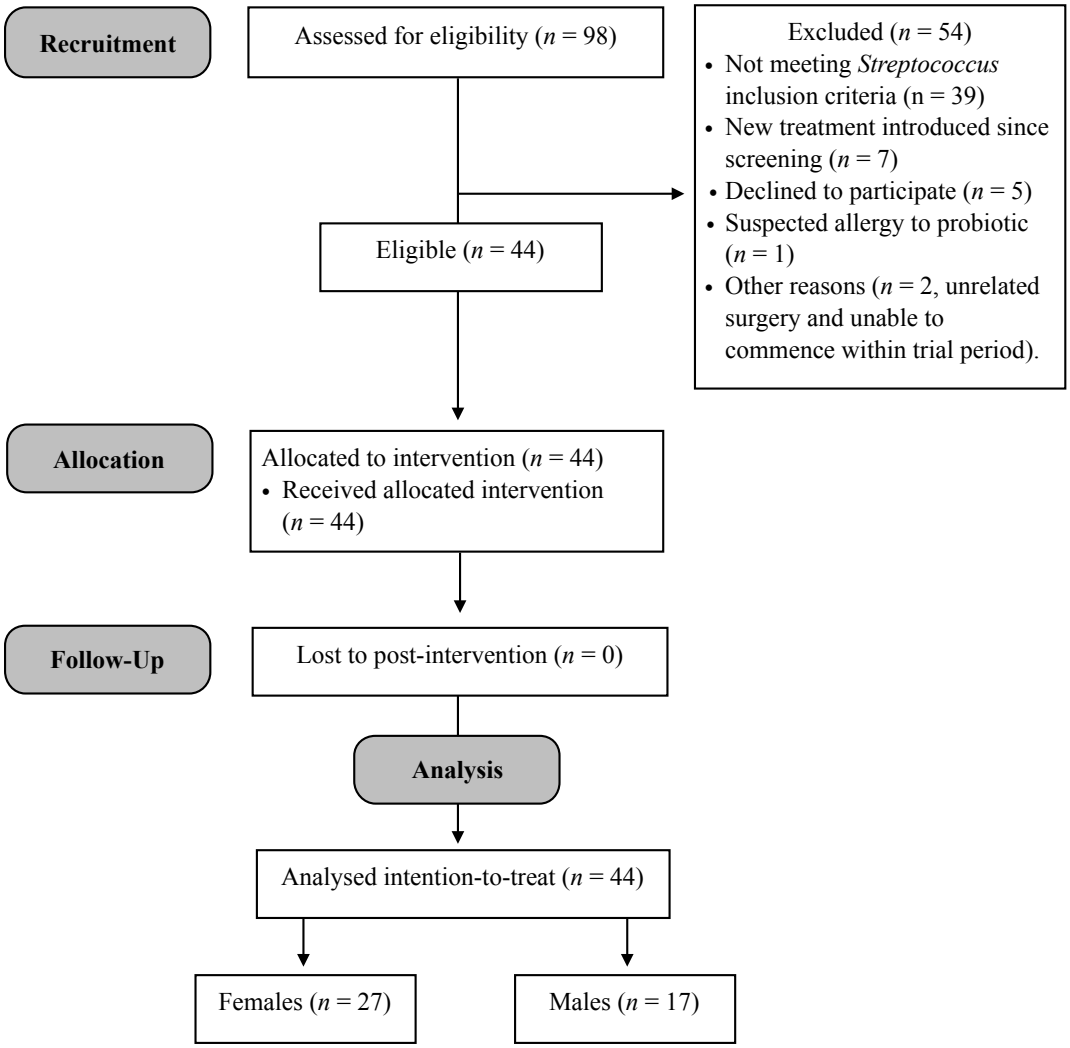

Figure 1. Participant flow diagram

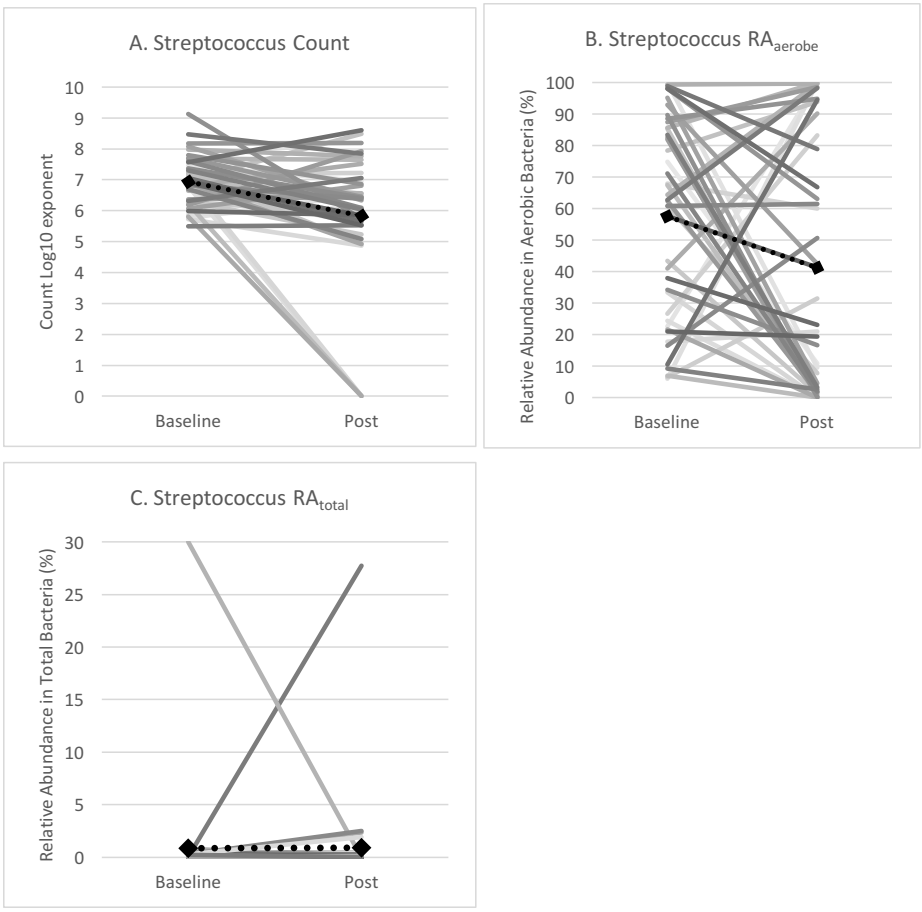

Figure 2. Change in *Streptococcus* (A) count, (B) relative abundance within aerobic bacteria (RA_{aerobe}), and (C) relative abundance within total bacteria (RA_{total}) for individual cases before and after intervention. ◆·····◆ indicates *mean* scores at baseline and post.

SUPPLEMENTARY MATERIAL

Open-label pilot for treatment targeting gut dysbiosis in myalgic encephalomyelitis/chronic fatigue syndrome: Neuropsychological symptoms and sex comparisons

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This PDF File Includes:

Supplementary method

Tables S1, S6-S8

SUPPLEMENTARY METHOD

This supplementary material provides detailed information about clinical, microbial and lactate measures and procedures used during the trial. Operational definitions of sleep terminology, measurement methods, and procedures for managing ambiguous and missing data are presented to aid replication. The rationale for selected outcome variables is provided with reference made to Spearman's rho correlations between baseline clinical outcome variables (see Table S5).

Measuring Sleep Symptoms

Objective Sleep - Actigraphy

Overview and considerations

Actigraphy was used as an objective measure of sleep patterns in this sample. Actigraphy instruments provide a non-invasive measure of sleep/wake behavior through analysis of activity and light intensity. Actigraphy methods have been shown to reliably estimate sleep and wake patterns for several clinical populations across the lifespan [1]. The use of actigraphy within ME/CFS populations has high ecological validity [2] and has been previously employed [3, 4].

Actigraphy has high sensitivity but low specificity. Intervention studies have shown that actigraphy is sensitive to measuring change after both pharmacological and psychological interventions (see [5]). Sensitivity measurement of sleep onset latency (SOL), wake after sleep onset (WASO) and sleep efficiency (SE) variables seem to vary dependent on the clinical population being examined (see Table S6 for operational definitions of sleep terminology). The primary methodological issue relates to the device's low specificity or accuracy when detecting wakefulness during a sleep period [5]. This has been repeatedly indicated for the measurement of SOL and beckons cautious interpretation of this outcome variable [6]. Low specificity is likely to partially explain inconsistencies between some subjective and objective sleep parameters.

Actigraphy data provides information about movement (rather than sleep) that is then used as an indicator of sleep/wake states [5]. Therefore, the data could be influenced by neurobehavioural or motor system disorders [5]. Whilst these factors influence the reliability and validity of actigraphy assessment, with the available technology, actigraphy provides an unobtrusive form of assessing sleep patterns within the home setting [7] and measuring treatment efficacy [5]. Compensatory measures (i.e., recording duration over 5 days; use of established scoring protocols) and adjunctive measures (i.e., concurrent completion of a sleep diary) were used to help overcome some limitations of actigraphy devices [1, 5–7].

Participant procedures

Participants wore the Actiwatch monitors (Respironics Actiware 2) on their non-dominant hand for 7 consecutive days/nights during baseline and post-intervention weeks. Participants were asked to press the silver button on the actiwatch (to signal an 'event marker' on the data) to indicate when they attempted to fall asleep for the night and at final awakening. Participants were encouraged to leave the watch on for the 7 day period and attempt to keep the watch uncovered during the day to prevent disruption to light sensor data. To reduce variability between Actiwatch monitors, the same watch was used for baseline and post-intervention data collection when possible.

Data Scoring Protocol

Procedures outlined by the Society of Behavioral Sleep Medicine were followed to score sleep and wake periods [1]. Sleep/wake patterns during the main sleep interval were used to obtain objective outcome measures. Actiware 6.08 default analysis properties for 30 second epoch lengths were used to calculate sleep parameters (*Sleep* = activity counts $\leq$ 40; *Wake* = activity counts > 40). Sleep onset and final awakening were determined by 10 immobile minutes (see Table S6 for definitions of sleep terminology).

The Actiware program automatically predicted the main sleep interval by inserting a *rest* interval. However, this default *rest* interval did not always accurately reflect the main

sleep interval. The extended bed rest and lowered activity observed in this population frequently resulted in ambiguous start and endpoints and inaccurate placement of the *rest* interval. As a common issue in many sleep disorders and other medical conditions [1], the following decision hierarchy was used to determine the start (*lights out*) and end (*rise* i.e., the time the participant got out of bed for the day) of the main sleep period in accordance with the recommended guidelines (see [1]).

1. All automatic *'rest'* intervals were screened to ensure they met the following criteria:
    1.1. Sleep period start time coincided with
        1.1.1. a marked decrease in light
        1.1.2. a marked decrease in activity
        1.1.3. event marker signaling that the participant pressed the button to indicate attempting to fall asleep (if used)
    1.2. Sleep period end time (rise) coincided with:
        1.2.1. an increase in light
        1.2.2. an increase in activity
        1.2.3. event marker signaling that the participant pressed the button to indicate final awakening (if used)
2. In the event of discrepancies between any of the above conditions, a *manual 'rest'* interval was inserted with the following conditions considered in order of priority:
    2.1. Sleep start time:
        2.1.1. decrease in light
        2.1.2. decrease in activity
        2.1.3. event marker

2.1.4. sleep diary – time the participant turned off the lights and attempted to fall asleep

2.2. Sleep period end time (*rise*):

2.2.1. increase in activity

2.2.2. increase in light

2.2.3. sleep diary *rise* time.

2.2.3.1. In the event that the participant noted that they had remained in bed due to symptoms of the illness, the *final awakening* time was used as the end of the sleep period.

2.2.4. event marker

3. All incomplete main sleep intervals were omitted from analysis. The occurrences of this were when:

3.1.1. The watch was removed and forgotten to be replaced prior to sleep.

3.1.2. Data was not recorded due to technical errors or watch removal that lasted for longer than 1 hour during the main sleep interval.

Possible outcome variables

Default algorithms were used to determine approximate values for *total sleep time* (TST), *SOL, WASO, wake bouts* (WB), *SE* and *sleep fragmentation index* (SFI). See Table S6 for an explanation of terminology and methods of measurement.

Subjective Sleep

Sleep diary

Overview and considerations

Objective measures of sleep complement, rather than replace, subjective sleep assessment. Sleep diaries have been routinely used and proclaimed as the 'gold standard' method of measuring subjective reports of sleep in healthy and clinical populations [8].

Whilst adding to participant burden and confounded by expectation bias, sleep diaries provide an advantage over objective measures when considering the individual experience of sleep and cognitive-affective factors impacting the sleep experience [9]. This is particularly pertinent considering the discrepancies that have been shown between subjective and actigraphic assessment of sleep in both healthy and clinical populations (e.g., [10, 11]) and specifically in ME/CFS samples (see [2]). Within ME/CFS, comparison between sleep diary and objective measures (polysomnography and actigraphy) suggest moderate to high consistency between methods, particularly for TST, SE and WASO [9]. Additionally, the accuracy of sleep diary data as a measurement of SOL appears preferable to actigraphy [9].

Sleep diary material and procedure

Selected items from the standardized protocol procedures for sleep diaries (see [8]) were incorporated in the sleep diary for this study. Relevant items were chosen to gather information about sleep time, quality, wakefulness and sleep-related behavior.

During baseline (7 nights) and post-intervention (7 nights), participants completed the sleep diary each morning in relation to the previous night's sleep. Participants reported the use of any prescription or non-prescription sleep aids (including alcohol). Participants noted the time they a) got into bed, b) turned off the lights to fall asleep (*lights out*), c) of their final awakening, and d) got out of bed for the day (*rise*). They also indicated the number of minutes it took them to fall asleep (SOL), and the number (WB) and duration of awakenings (WASO) during the night. On two separate 5-point Likert scales participants rated sleep quality (1 = very poor, 5 = very good) and how rested or refreshed they felt when they awoke (1 = not at all rested, 5 = very well-rested).

Possible outcome variables

Subjective sleep parameters were operationally defined to avoid construct confusion and for the purpose of replication (see Table S6). Possible outcomes included TST, SOL, WASO, WB, SE, and *duration of sleep episode* (DSE). Subjective measures are referred to as

'Diary' to distinguish between actigraphic measures. SE is frequently calculated in sleep disorder research and clinical practice to reflect difficulties with falling asleep or staying asleep as indicated by the ratio between *total sleep time* (TST) to *time in bed* (TIB; [12]). Prior research has used inconsistent and ill-defined methods to determine TIB resulting in recent recommendations to clarify terminology and the suggestion of using *duration of sleep episode* (DSE = SOL + TST + WASO + *time attempting to sleep after final awakening*: TASAFA) as the denominator in the SE equation (see [12]). Sleep diary data in this study did not obtain a measurement of TASAFA because participants were not asked to describe their intentions between their *final awakening* (FA) and the *time they got out of bed for the day* (Rise). Considering other ME/CFS symptoms could affect the length of time participants spent in bed in the morning, FA was used to indicate the end of the sleep period.

- SE was calculated as TST/ DSE x 100, with higher percentages indicating more efficient sleep.
- When there was missing data for SOL and WASO, DSE was calculated by the time between *lights out* and FA in minutes.

Sleep questionnaires

Two scales were used to measure sleep quality (*Pittsburgh Sleep Quality Index,* PSQI, [13]) and sleep disturbance (*Insomnia Sleep Index*, ISI, [14]). The PSQI has high reliability and validity as a brief measure (10 items) of perceived sleep quality with the rater reflecting on their sleep habits over the previous month [13]. The PSQI has been used to quantify non-restorative sleep in ME/CFS populations [15, 16]. Some alterations to the PSQI were made to suit our study design. Firstly, participants were instructed to rate their sleep habits based on the 'past 2 weeks' to increase specificity of post-intervention ratings. Item 10 was also removed from this scale. This item requires completion by a 'bed partner' and answers are not included when calculating the Global Score. The PSQI Global Score was calculated following instructions by [13] with scores ranging between 0-21 and lower scores indicating

better sleep quality. Normative data on the PSQI from the original validation study using US samples with 'healthy' controls aged 24-83 years (n = 52) indicated that a Global PSQI scores greater than 5 is indicative of "poor" sleep quality [13].

The ISI is a 7-item, 5-point Likert scale that provides subjective information about the nature and severity of insomnia symptoms and impact on the individual's functioning [14, 17]. The Patient version was selected to enable self-administration with total scores ranging between 0 and 28. Comparison with sleep diary data suggests that the ISI has adequate internal consistency, albeit lower correlations with sleep diary variables indicative of insomnia symptoms (i.e., SOL, WASO; [17]). The ISI has validity for use as an outcome measure in treatment research [18].

Selected Outcome Variables for Sleep

The methods used to obtain information about sleep symptoms resulted in numerous variables that needed to be reduced to aid interpretation. Table S6 provides an explanation of sleep parameters and the rationale for retaining or excluding measures of sleep through actigraphic and sleep diary assessment. Baseline correlations with all clinical symptoms (see Table S5) and prior research helped form these decisions. Factor analysis methods were considered but not employed due to small sample size, inadequate case:variable ratio and considering intercorrelations < .3 between several sleep variables [19]. Therefore, selected sleep outcomes included: Actigraphy SOL, WASO, SE, SFI; Diary SOL, WASO, SE; and PSQI Global Score. The PSQI was retained as a measure of sleep quality and the unique contribution suggested by intercorrelations. The ISI was excluded due to intercorrelations with the PSQI, mood and fatigue variables that could be difficult to distinguish the unique contribution of this scale. Diary SOL and WASO were considered sufficient measures of self-reported insomnia symptoms.

Measuring Mood Symptoms

Profile of Mood States

The Profile of Mood States (POMS) Short Form is a list of 37 adjectives asking participants to rate current mood states on a 5-point Likert scale (*Not at all* = 0 to *Extremely* = 4; [20]). POMS clusters adjectives into 6 factors: tension/anxiety, depression/dejection, anger/hostility, fatigue/inertia, vigour/activity, and confusion/bewilderment. The POMS Total Mood Disturbance score is calculated from the sum of all negative clusters and subtraction of the Vigour/Activity cluster. Possible scores range from -24 to 148 with lower scores indicative of less mood disturbance. The POMS provides a measure of psychological distress [20] and appears to be useful as a treatment sensitive measure for this population [4].

Participants were asked to rate their mood on Days 7 and 42 of the study, based on their experiences 'over the past week including today'. The POMS Total Mood Disturbance score was selected as the primary outcome variable a priori.

Depression Anxiety Stress Scale

The Depression Anxiety Stress Scale (DASS-21; [21]) was selected as a psychometrically sound non-diagnostic measure of self-reported symptoms of anxiety, depression and stress. Developed from the original 48-item scale [21], this shorter version is frequently used in clinical and research settings for its ease of administration, brevity and sensitivity to treatment change. The DASS-21 has Australian normative data [22] and moderate-strong psychometric properties observed in clinical [23] and nonclinical populations [24].

This 21-item scale asks raters to indicate their agreement to statements based on their experience over the past week using a 4-point likert scale (*Did not apply to me at all* = 0 to *Applied to me very much, or most of the time* = 3). Seven items pertain to each of the three subscales (DASS-Depression, DASS-Anxiety, DASS-Stress) with maximum scores of 21 indicating more distress on each dimension respectively. The DASS-21 has high reliability and discriminant validity supporting the three-factor structure in this scale [25]. It is frequently employed in clinical and nonclinical populations in both clinical and research

settings [26]. Please note that the DASS-21 manual suggests doubling total scores, however, this study chose to use the raw subscale scores to be consistent with the Australian validation study [22].

Mood Adjectives Checklist

The Mood Adjectives Checklist (MAC; [27]) was chosen as a daily rating of positive and negative mood states to be completed during Baseline and Post-intervention weeks. The MAC has adequate reliability and validity as a daily measure of mood [27] and evidence of being able to separate positive (MAC-Positive) and negative affect as two independent subscales [28]. The extended scale was employed with 13 adjectives aligned with positive affect (happy, joyful, enjoyment/fun, pleased, energetic, relaxed, alert) and negative affect (depressed/blue, unhappy, angry/hostile, frustrated, worried/anxious, fatigued) (Porter et al., 2000). Participants rated their current mood on a 7-point Likert scale (*Not at all* = 0, *Extremely* = 7). Daily scores were calculated for MAC-Positive (higher scores, more positive) and MAC-Negative (higher scores, more negative) subscales. Mean weekly scores on each subscale were calculated from daily scores.

Selected Outcome Variables for Mood

Baseline intercorrelations between POMS subscale and total scores, MAC factors and the DASS subscales suggested overlapping measurement of similar dimensions (see Table S5). Baseline intercorrelations between the POMS Total Mood Disturbance and other POMS subscales were moderate to strong (r = .65 to .86). Therefore, the POMS Total Mood Disturbance score was considered representative of POMS subscale scores. To reduce the number of variables for analysis, the POMS Total Mood Disturbance score (primary outcome) and DASS subscales (DASS-Depression, DASS-Anxiety and DASS-Stress) were selected for further analysis. The POMS measure was prioritized compared with the MAC factors because of a priori selection as a primary outcome variable measuring mood.

Measuring Cognitive Symptoms

Cognitive Test Battery

Seven standardized tests were selected to measure attention, memory, verbal fluency, inhibition and planning. Table S7 provides a summary of the skills assessed by each test, administration information and selected outcome variables. These tests were chosen after evaluating psychometric properties, suitability for use with ME/CFS patients, length of administration, cost and availability.

Alternate forms were used when available to reduce practice effects (see Table S7). The use of alternate forms has been shown to reduce practice effects on the RAVLT and COWAT [29]. Parallel forms (Form A and B) of the test battery were counterbalanced using random allocation to reduce possible differences in the level of difficulty that could interfere with treatment effects [30]. Outcome variables with reduced practice effects were prioritized.

Participant procedures

The total administration time for the test battery was approximately 60 minutes, with 90 minutes allocated to allow for sufficient rest period between testing. Test administrators noted the length and activity type during each rest interval at baseline testing to allow for replication at post-intervention. A touchscreen laptop was used to administer the 4 tests from the Cambridge Neuropsychological Test Automated Battery (CANTAB [31]) and the remaining tests were delivered orally. Standardized test conditions and the order of tests remained consistent for baseline and post-intervention sessions. Before commencing the trial, participants selected their preferred location (either CFS Discovery Clinic or Victoria University campuses) to conduct the sessions during baseline (Day 1) and post-intervention (chosen day during week 6).

Selected Outcome Variables for Cognitive Symptoms

Selected outcome variables and the corresponding rationale for inclusion are summarized in Table S7.

Measuring Other ME/CFS Symptoms

Total ME/CFS Symptoms

The *Symptom Severity and Symptom Hierarchy Profile* (SSH; [32]) was used as an indicator of total ME/CFS symptoms. The scale provides a list of ME/CFS symptoms and asks respondents to rate their severity of symptoms (*Absent* = 0 to *Severe* = 3) and rank their three most severe symptoms according to their experience over the past week. This symptom profile was developed to aid diagnosis and treatment in accordance with the ME/CFS Clinical Working Case Definition [32]. Total scores are weighted based on the severity of ratings (summed responses are multiplied: absent (x0) mild (x1), moderate (x2) and severe (x3)). Higher scores indicate more severe symptoms.

Selected outcome variable for global symptoms

The total score was selected as an outcome measure that is indicative of overall frequency and severity of ME/CFS symptoms (*Total Symptoms-SSH*).

Fatigue

The Multidimensional Fatigue Inventory (MFI-20) was originally validated with ME/CFS patients to assesses fatigue across five dimensions (General Fatigue, Physical Fatigue, Motivation Level, Activity Level and Mental Fatigue [33]). Using a 5-point likert scale (*yes, that is true* = 1 to *no, that is not true* = 5) participants were asked to indicate their level of agreement with each statement considering how they have felt 'over the past 7 days'. The MFI-20 has shown support for a 5-factor model, good internal consistency, test-retest reliability, construct and convergent validity (see [34, 35]). This scale has been used in multiple international studies with evidence for treatment sensitivity and use as a primary outcome measure for ME/CFS (e.g., [36]). Items were scored according to instructions by Smets, Garson and Bonke [37] with higher scores indicating more fatigue. Scores for each subscale range from 4 to 20 with higher scores indicating greater fatigue.

The *Brain Fog* subscale of the Multiple Fatigue Types Questionnaire (MTFQ, [38]) was used as a measure of 'brain fog' that is a common symptom of ME/CFS related to mental

fatigue/exhaustion and associated disruption to thinking, attention, processing and memory [38]. This measure was specifically developed to assess fatigue in individuals with ME/CFS. As a new scale, it has adequate internal reliability and substantiates the notion of cognitive fatigue distinct from other fatigue types [38]. MFTQ items were scored on the same 7-point likert scale as the MAC items to reduce confusion because these items were presented together within the Participant Response Booklet. Scores from the three items of the MFTQ were summed as an indicator of daily cognitive fatigue (*Brain Fog* subscale range of scores: 3-21). A weekly mean score was calculated from daily ratings for the MFTQ-Brainfog outcome variable.

Selected outcome variables for fatigue

With the primary focus on sleep, mood and cognitive symptoms in this current paper, only two outcome measures were chosen for fatigue. The authors of the MFI-20 recommend using the General Fatigue subscale (MFI-GF) as a preferred global estimation of fatigue rather than summation of all subscales [37]. Therefore, MFI-GF was selected as a secondary outcome to represent general/global fatigue in participants in this study. The MFTQ-Brainfog mean weekly score (Brainfog-MFTQ) was selected as another fatigue outcome variable due to low correlations with MFI-GF at baseline (r_s = .061, p = .701) and other MFI subscales (see Table S5).

Microbiota and Lactate Measurement

During screening and post-intervention phases, participants were asked to collect their first morning stool and mid-stream urine samples (after 5am) independently in their own home. Participants were provided with detailed instructions of how to collect a mid-stream urine sample to avoid cross-contamination with bacteria from other sources. Participants were asked to refrain from food and beverages from 10pm on the night prior to collection. After passing the first portion (5-10mL) of urine, a sample of 5-10mL of urine was collected in a sterile specimen container, stored in a zip lock bag and stored in the fridge. Both urine

and stool samples were collected by courier and transported in cold conditions (<12 °C) to Bioscreen laboratory within 48 hours after sample collection.

Methods of faecal collection, transportation and identification of microbiota using MALDI-TOF MS analysis were the same as those described in [39]. Urinary lactate concentrations were determined using High Performance Liquid Chromatography and Triple Quadrupole Mass Spectrometry (HPLC-TMS) as outlined in the main article.

Microbial Outcome Variables

Three genera were prioritised for analysis of microbial outcomes based on treatment target (*Streptococcus*) and probiotic supplementation (*Bifidobacterium* and *Lactobacillus*). Both count and relative abundance (RA) variables were used to examine change in frequency and proportion of each genus. RA_{total} was calculated by the ratio of each genus count divided by total detectable bacteria count. The proportion of *Streptococcus* within total aerobic bacteria (RA_{aerobe}) was also used as an outcome measure to be consistent with inclusion criteria and aid clinical interpretation.

Lactate Outcome Variable

Higher concentrations of creatinine have been shown in ME/CFS compared with controls [40]. Therefore, routine normalisation with creatinine [41] was deemed inappropriate and restricted use of absolute D-lactate concentrations and absolute L-lactate concentrations. Hence, the D:L lactate concentrations ratio was used as a secondary outcome variable.

Procedure for Handling Missing or Ambiguous Data

Table S8 provides a summary of the scoring procedures used for handling missing and ambiguous data on self-report measures and faecal microbial analysis.

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TABLES

Table S1. Median and range for primary and secondary outcomes at baseline and post intervention for total sample

Outcomes	Baseline *Mdn[range]*	Post *Mdn[range]*
Sleep		
Actigraphy Sleep Efficiency^	86.01[31.72, 94.10]	86.64[45.94, 95.56]
Actigraphy Sleep Fragmentation Index	22.17[12.33, 61.57]	20.96[10.81, 55.93]
Actigraphy Sleep Onset Latency	21.60[1.93, 98.71]	11.18[0.71, 177.64]
Actigraphy Wake After Sleep Onset	37.93[16.83, 100.71]	34.04[15.29, 104.93]
Diary Sleep Efficiency^	88.52[79.52, 98.28]	92.57[79.26, 98.96]
Diary Sleep Onset Latency	27.93[0.36, 106.67]	19.02[0.57, 110.00]
Diary Wake After Sleep Onset	20.64[0, 85]	9.48[0, 56]
Sleep Quality - PSQI-Global	9[1, 18]	7.5[1, 14]
Mood		
Mood Disturbance - POMS-Total	39[7, 94]	35[-3, 105]
Depression - DASS-21	5[0, 18]	4[0, 21]
Anxiety - DASS-21	3[0, 14]	2[0, 13]
Stress - DASS-21	7.5[1, 20]	6.5[0, 20]
Cognitive Outcomes		
Executive functioning		
Attention - RVP A'^	0.91[0.82, 0.99]	0.94[0.85, 1.00]
Procesisng speed - RVP Mean latency	469.26[323.00, 1044.33]	417.22[289.00, 917.69]
Cognitive flexibility - AST Median Switching Cost - Block 7	252.25[20.50, 627.00]	178.00[0.00, 575.00]
Planning - SWM - Strategy	31[18, 47]	30[11, 47]
Memory		
Word memory - RAVLT - Immediate^	51.5[28, 71]	52[33, 72]
Story memory - LM Immediate^	24[4, 40]	27[10, 45]
Spatial working memory - SWM - Between errors	17.0[0, 65]	11.5[0, 65]
Visual learning - PAL - Total errors	10.5[0, 114]	7.5[0, 67]
*Verbal fluency - COWAT Corrected Score^*	36[14, 83]	38.5[21, 64]
Other ME/CFS Symptoms		
General fatigue - MFI	18[11, 20]	18[11, 20]
Brainfog - MFTQ	10.83[3.43, 19.00]	8.00[3.00, 17.57]
Total symptoms - SSH	31[6, 52]	21[8, 44]
Microbiota		
Streptococcus - Count	7.76x10^6[3.16x10^5, 1.32x10^9]	1.70x10^6(1, 3.98x10^8)
Bifidobacteria - Count	7.94x10^2[1, 5.50x10^10]	1(1, 1.38x10^9)
Lactobacillus - Count	1[1, 1.38x10^9]	1(1, 3.24x10^8)
Streptococcus - RA_aerobe	63.62[5.93, 100.00]	22.05[0.00, 100.00]
Streptococcus - RA_total	0.00[0.00, 0.30]	0.00[0.00, 0.28]
Bifidobacteria - RA_total	0.00[0.00, 0.81]	0.00[0.00, 031]
Lactobacillus - RA_total	0.00[0.00, 0.46]	0.00[0.00, 0.29]

Lactate		
D:L Lactate Ratio	0.25[0.02, 1.02]	0.29[0.03, 0.92]

Lower scores reflect better symptoms for all clinical variables unless indicated by ^
Units of measurement for microbial variables: count = cfu/g back-transformed from Log10;
RA_{aerobe} = percent distribution within total aerobic organisms determined via culture methods;
RA_{total} = relative abundance of each genus within total bacteria determined via culture methods presented as a percentage.

Table S6. Operational definitions of sleep terminology, measurement method and selected outcome variables

Sleep Terminology	Abbrev.	Unit	Definition	Measurement method		Outcome variables		
				Actigraphy	Sleep Diary	Selected Outcome Variables	Reason for selection	Reason for exclusion
Main sleep interval			Period between *lights out* and *rise*	*Actiware* use sleep/wake algorithms to determine mobility/ immobility and distinguish between *Rest* and *Sleep* intervals. *Rest* interval: Estimate of period between *lights out* and *Rise* *Sleep* interval: Differentiated by Actiware as an estimate of the period between sleep onset and FA	N/A		N/A	
Lights out		Time	Time participant turned off the lights to fall asleep	Start of *Rest* interval	Time turned off lights to fall asleep		N/A	
Sleep onset		Time	Time participant fell asleep	Start of *Sleep* interval (default detection algorithm = 10 immobile minutes)	Actual time not used but could be approximated from participant's estimate of SOL.		N/A	
Final awakening	FA	Time		End of *Sleep* interval (default detection algorithm = 10 immobile minutes)	Final awakening time		N/A	
Rise		Time	Time out of bed to start the day	End of *Rest* interval	Time out of bed to start the day		N/A	

Epoch				Selection of time to determine activity (immobile or mobile) counts Default analysis properties were used for 30 second epoch lengths: *Immobile* = < 2 activity counts *Mobile* = ≥ 2 activity counts *Sleep* = activity counts ≤ 40 *Wake* = activity counts > 40 *Invalid Time SW* = not enough data to determine sleep/wake scoring algorithm			N/A	
Duration of sleep episode	DSE	Minutes	Time between *lights out* and *rise* (FA in diary)	*Rest* interval duration	DSE = total time from *lights out* to *final awakening* calculated as the total of SOL + TST + WASO			Inability to accurately interpret directional change as beneficial or detrimental
Sleep efficiency	SE	%	The percentage of time spent sleeping during main sleep interval.	SE = TST / (DSE – Invalid Time SW)) x 100	Ratio between total sleep time (TST) and duration of sleep episode (DSE), multiplied by 100 SE = (TST/ DSE) x 100	▪ Actigraphy SE ▪ Diary SE	Difference between ME and controls [42]	
Sleep fragmentation index	SFI	%+%	Measure of restlessness, higher scores suggest increase sleep disturbance	SFI = (Percent mobile + percent one minute immobile bouts) / number of immobile bouts during *Rest* interval. Summary data from the Rest interval (rather than sleep) was chosen to reflect movement across the main sleep interval (i.e. *Rest*).	N/A	▪ Actigraphy SFI	Difference between ME and controls [42]	
Sleep onset latency	SOL	Minutes	Time taken to fall asleep after initiating the intent	Time between start of *Rest* interval and start of *Sleep* interval determined by 10 immobile minutes.	Approximate minutes taken to fall asleep	▪ Actigraphy SOL ▪ Diary SOL	Difference between ME and controls [42]	

			to sleep (i.e. *lights out*)					
Time attempting to sleep after final awakening	TASAFA	Minutes	Interval between FA and *Rise* when attempting to fall back to sleep	N/A	N/A Information about intentions during this interval were not obtained.		N/A	
Total sleep time	TST	Minutes	Amount of time asleep within main sleep interval	Number of epochs scored as 'sleep' multiplied by length of epoch (30 seconds) within *Sleep* interval.	Time between *lights out* and FA minus SOL and WASO			Inability to accurately interpret directional change as beneficial or detrimental
Wake after sleep onset	WASO	Minutes	Length of time awake after falling asleep and before FA	Number of epochs scored as 'wake' during *Sleep* interval, multiplied by epoch length (30 seconds)	Total time awake after sleep onset and before FA	▪ Actigraphy WASO ▪ Diary WASO	Difference between ME and controls [42]	
Wake bouts	WB	Number	Number of awakenings after falling asleep and before FA	Number of continuous epoch blocks scored as 'wake' during *Sleep* interval	Total number of awakenings after sleep onset and before FA			Significant, positive correlations between Actigraphy WB and WASO and Diary WB and WASO, respectively.

Table S7. Overview of cognitive test battery and selected outcome variables

	Cognitive Skill	Test	Duration	Form	Alternate form	Outcome Variable
Attention	Sustained Visual Attention	Rapid Visual Information Processing (RVP) [43, 44]	10 minutes	CANTAB (touchscreen tablet)	N/A	▪ *RVP A' ***Attention*** Measure of attention based on accuracy and sensitivity of participant detecting the number target sequence. Higher scores reflect better performance (scores from 0.00 to 1.00). ▪ *RVP Mean Latency ***Processing speed*** Measure of processing speed when accurately detecting the target sequence. Lower scores suggest faster speed.
Executive Functioning	Visual attention, reaction time and inhibition	Attention Switching Task (AST) [44]	8 minutes	CANTAB (touchscreen tablet)	N/A	▪ *AST Median Switching Cost – Block 7 ***Cognitive flexibility*** Measure of cognitive flexibility based on the difference in reaction times across congruent and incongruent trials. Higher scores indicate slower reaction time on switching (incongruent) compared to non-switching (congruent) trials. A score of zero indicates the same reaction speed on both types of trials. Block 7 was selected to reduce practice effects.
	Visual working memory and attention	Spatial Working Memory (SWM) [44–46]	8 minutes	CANTAB (touchscreen tablet)	N/A	▪ *SWM – Between Errors ***Spatial working memory*** Measure of visual memory and attention based on the total number of errors a respondent makes by incorrectly checking a box where a token has previously been found during assessed trials. Lower scores indicate better performance.

						▪ *SWM – Strategy Score* ***Planning*** Measure of planning based on trials with more than 6 boxes. Lower scores indicate better strategy.
Visual Memory	Visual learning and memory	Paired Associate Learning (PAL) [44]	8 minutes	CANTAB (touchscreen tablet)	Available	▪ *PAL – Total Errors (adjusted)* ***Visual learning*** Measure of visual memory and learning based on the number of errors on both completed and incompleted (if test aborted due to failed attempts). Lower scores indicate better memory.
Verbal Memory	Verbal Memory (list learning)	Rey Auditory Verbal Learning Test (RAVLT) Form A [47–49]	8 minutes (with 30 minute delay)	Pen and paper	Form B developed by Jones-Gotman, Sziklas & Majdan (1993, in [49])	▪ *RAVLT Total Score Trials 1-5* (raw) ***Word memory*** Measure of word memory for unrelated auditory information. Higher scores indicate better performance. More reliable and robust outcome measure rather than reliance on individual trial scores [30, 50].
	Verbal Memory (Story)	Form A: Weschler Memory Scales (WMS-IV): Logical Memory Subscale [51]	8 minutes (with 30 minute delay)	Pen and paper	Form B: Morris Revision-Fourth Edition (MR-IV; [52])	▪ *Story Memory Total Immediate* (raw) ***Story memory*** Measure of story/lexical memory for conceptually related information. Total raw scores were used to enable comparison between forms. Higher scores indicate better performance. Selected because lower practice effects than delayed total score.
Verbal fluency	Verbal Fluency	Controlled Oral Word Association Test (COWAT) [53, 54]Form A: C, F, L	3 x 1 minute trials	Pen and paper	Form B: P, R, W	▪ *COWAT Corrected Score* ***Verbal fluency*** Measure of verbal fluency and word retrieval. Higher scores indicate better performance.

** Denotes outcome variable label used within main body of the article.*

Table S8. Scoring procedures for managing missing and ambiguous data.

		Missing Data	Ambiguous Data
Sleep Diary			
Duration of sleep episode (final awakening)	DSE	Not calculated if missing lights out and FA times	
Sleep efficiency	SE	Not calculated if missing TST or DSE	
Sleep onset latency	SOL	Not calculated if missing	▪ Mean calculated when a range was given (e.g. 40-60 minutes = 50 minutes).
Total sleep time	TST	Not calculated if missing lights out, FA, SOL or WASO	
Wake after sleep onset	WASO	Not calculated if the number of estimated wake lengths (in minutes) was not equivalent to the number of wake bouts for that night.	▪ Mean calculated when a range was given. ▪ "a few" = 3 minutes
Wake bouts	WB	Not calculated if missing	▪ Mean calculated when a range was given.
Sleep Quality		Not calculated if missing	
Rested		Not calculated if missing	
Weekly Mean scores for all above sleep diary variables		Only calculated when data was available for **4 of the 7 nights**	
Questionnaires			
MAC_Positive		Not calculated if missing any items	The middle value was calculated if two responses were provided to the one item and all other items were completed. If two responses were provided and the next item left blank (i.e. possible error of placement), both items were calculated as missing data.
MAC_Negative		Not calculated if missing any items	As above for MAC_Positive
MFTQ_Brainfog		Not calculated if missing any items	As above for MAC_Positive
Weekly Mean scores for all above day diary variables		Only calculated when data was available for **4 of the 7 nights**	
SSH_Total Symptoms		Not calculated if missing any items	
DASS-21		Individual subscales were not calculated if missing any items.	

MFI-20	Individual subscales were not calculated if missing any items. Total score not calculated if missing one or more subscale scores.	
POMS	Individual subscales were not calculated if missing any items. Total score not calculated if missing one or more subscale scores.	An average of all other FI items was used for the item 'bushed' when a participant indicated they were unsure of the meaning (i.e., '?') but answered all other items on the POMS.
PSQI	PSQI factors not calculated if any missing data. PSQI_Total not calculated if missing any of the PSQI factors.	Mean calculated when a range was given for items 1-4.
ISI	ISI_Total not calculated if missing any items.	
Faecal microbial analysis	An arbitrary value (1) was used for Count variables to indicate that the analysed genera was not detected through culture methods but was not missing. This was also required for Log10 transformations. Inadequate (due to collection error) or unreturned samples were entered as missing.	
Urine lactate analysis	Only cases with both baseline and post-intervention urine samples were analysed. Inadequate (due to collection error) or unreturned samples were entered as missing.	

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PART C: CRITICAL REVIEW

CHAPTER 6

Critical Review and Future Directions

Synthesis of Findings

The review of literature and empirical reports presented in this thesis provides insights into (i) microbiota-gut-brain interactions in ME/CFS; (ii) D-lactate theory; and (iii) treatment possibilities. Findings in relation to each of these areas are discussed in turn before critiquing the limitations of the work presented to inform future research.

Microbiota-gut-brain interactions in ME/CFS

The review of literature in the book chapter presented in Chapter 2 (Wallis et al., 2017d) highlighted the limited research investigating microbiota-gut-brain interactions in ME/CFS and provided a strong rationale to investigate microbial changes and associations with neurological symptoms. Exploration of possible gut-brain mechanisms contributing to sleep, mood and cognitive symptoms revealed the value in examining microbial-symptom associations and gut dysbiosis to attempt to increase etiological understandings and possible treatment options in this condition. In combination with increasing evidence (Frémont et al., 2013; Giloteaux et al., 2016; Nagy-Szakal et al., 2017; Sheedy et al., 2009; Shukla et al., 2015), our cross-sectional (Papers 2 and 3: Wallis et al., 2016; 2017c) and experimental (Paper 5: Wallis et al., 2017a) findings support the microbiota-gut-brain interaction contributing to some symptomatic presentations in ME/CFS. Since writing the literature review (Paper 2: Wallis et al., 2016), several studies have examined gut dysbiosis in ME/CFS patients that have added to the body of knowledge and require discussion.

There is now sufficient evidence to confirm the presence of intestinal dysbiosis in ME/CFS patients based on controlled comparison studies (Frémont et al., 2013; Giloteaux et al., 2016; Nagy-Szakal et al., 2017; Sheedy et al., 2009; Shukla et al., 2015). Different methodologies (i.e., culture versus DNA sequencing) have been used making direct comparison difficult but substantiating the presence of dysbiosis in ME/CFS samples. Using culture-based methods, Sheedy et al. (2009) found higher counts of *Streptococcus* and *Enterococcus* (both *Firmicutes* phylum) compared with controls and Armstrong, Gooley, McGregor, Lewis, and Butt (2017) observed reduced *Bacteroides* (*Bacteroidetes* phylum) and increased *Clostridium* (*Firmicutes* phylum) species. Both Frémont et al. (2013) and Giloteaux et al. (2016) revealed differences between ME/CFS patients and controls using 16s rRNA sequencing methods. Frémont et al. (2013) showed that Norwegian ME/CFS patients had reduced proportion of genera within the *Firmicutes* phylum. Giloteaux et al. (2016) did

not see differences at the phylum or genus level but at the operational taxonomic unit level (equivalent to species-level) findings indicated lower proportion of *Faecalibacterium* (*Firmicutes* phylym) and *Bifidobacterium* species (*Actinobacteria* phylum), reduced bacterial diversity and increased instability in the microbial community within ME/CFS patients compared to controls.

Most recently, shotgun metagenomic sequencing methods observed greater intragroup variability in ME/CFS patients compared with controls highlighting the benefits of distinguishing between subtypes of ME/CFS patients with and without comorbid IBS (Nagy-Szakal et al., 2017). Results revealed that microbial profiles were predictive of health status (i.e., increased unclassified *Alistipes* and decreased *Faecalibacterium* (same as Giloteaux et al., 2016), predicted ME/CFS patients with IBS, whereas increased unclassified *Bacteroides* but decreased *Bacteroides vulgatus* predicted ME/CFS patients without IBS). It is unsurprising that there is variability between findings, given the heterogeneity of patient presentations, the complexity of the microbiome, and the methodological differences. However, what remains consistent is the observation of microbial differences between ME/CFS patients and controls. Our understanding of the functional relevance of this is being extended by examining interactions with clinical symptoms, metabolic pathways and immune markers.

Results from the cross-sectional, retrospective study of 274 patients with ME/CFS presented in this thesis (Papers 2 and 3: Wallis et al., 2016; 2017c) revealed small-moderate associations between selected microbial genera and self-reported symptoms. Sex comparisons indicated similar microbial composition but some differences in microbial-symptom associations for males and females. A sex-divergent pattern of associations was revealed for *Clostridium*, *Lactobacillus*, and *Streptococcus*. *Clostridium* was positively associated with most symptom factors in females (suggesting possibly detrimental) but negatively associated with pain and energy production/transportation impairments in males (suggesting possibly protective/beneficial). Whereas, *Lactobacillus* and *Streptococcus* were positively associated with most symptoms for males (suggesting possibly detrimental) with no (*Lactobacillus*) or negative associations (*Streptococcus* with pain, neurosensory and immunity impairments) for females (suggesting possibly protective/beneficial). *Bifidobacterium* was shown to correlate negatively (possibly beneficial) with most symptom factors for both sexes. Sex consistency in microbial composition but some notable differences in the direction of associations for males and females raised questions about functional differences in microbiota, potentially influenced by sex characteristics (i.e., how

do sex hormones interact with microbiota and clinical presentations?). Without information about hormonal status or immune status, mechanistic interpretations could not be made but the findings lend support for the microgenderome (see Flak, Neves, & Blumberg, 2013) in a human clinical sample.

The divergent pattern of associations revealed in the cross-sectional study (Wallis et al., 2016) and the notion of sex differences in microbial function provided the rationale to examine whether treatment response (primarily sleep, mood and cognitive symptoms) to an intervention targeting gut dysbiosis varied between the sexes. Primary and secondary outcome results from the pilot study (Paper 5: Wallis et al., 2017a) with 27 female and 17 male participants who received a short antibiotic and probiotic intervention (4 weeks alternating weeks of erythromycin and D-lactate-free probiotic) aimed at reducing an overgrowth of commensal enteric *Streptococcus* revealed sex consistency. Reductions in *Streptococcus* and improvement on several clinical outcomes (wakefulness, sleep efficiency, sleep quality, attention, processing speed, cognitive flexibility, story memory, verbal fluency, and total symptoms) was observed at post-intervention for the total intention-to-treat sample.

Unexpectedly, ancillary results correlating change in microbiota and change in symptoms showed that the change in *Streptococcus* was not related to change on clinical outcomes for the whole sample. Further to this, for males, clinical improvements were associated with increased *Bacteroides* and *Bifidobacterium*, and reduction in *Clostridium*. Ancillary findings provide support for microbiota-gut-brain interactions and suggest that microbial change may account for more of the variance in males compared with females. As discussed in more detail in the manuscript (see Paper 5: Wallis et al., 2017a, pp. 19-20), there appears to be some overlap between genera associated with clinical improvement in males and altered abundance highlighted by recent control comparison results (Armstrong et al., 2017; Giloteaux et al., 2016). Notably, these studies used solely (Armstrong et al., 2017) or predominantly female (38/49, Giloteaux et al., 2016) samples and did not indicate sex differences but these and other studies have suggested possible mechanisms for microbial-host communication that may be particularly relevant for ME/CFS presentation.

Faecal microbial composition has been compared with serum inflammatory markers (Giloteaux et al., 2016), metabolites from microbiota and host biofluids (Armstrong et al., 2017), and clinical symptoms, immune molecules and proposed bacterial metabolic pathways (Nagy-Szakal et al., 2017). Of particular note are findings that implicate altered bacterial metabolism as possible mechanisms for dysregulated cellular and energy production pathways. For example, Nagy-Szakal et al. (2017) observed associations between severity of

symptoms (more fatigue and impaired physical function) and reduced polyamine production. Polyamine metabolism reduces with age and plays an essential role in multiple cellular functions required for cell stability, cellular growth and repair (see Pegg, 2009). Armstrong et al. (2017) have suggested that the gut dysbiosis observed in ME/CFS patients is related to increased production of short chain fatty acids (SCFAs, particularly amino acids) and asserted that this microbial fermentation may exacerbate intestinal permeability and contribute to impaired energy metabolism pathways in the host. Both studies revealed several differences between metabolites in ME/CFS patients compared with controls. Without replication at this stage, these results encourage the utilisation of metabolomics investigations in future studies.

In addition to observations of gut dysbiosis at discrete time points, differences between ME/CFS and controls have been observed with temporal changes in blood and stool microbiome following a maximal exertion exercise task (Shukla et al., 2015). The increase in the relative abundance of some bacterial taxa in the blood stream 15 minutes to 48 hours after exercise paralleled an increase in clinical symptoms (fatigue, pain and confusion; Shukla et al., 2015). The authors suggest that these shifts may be due to increased intestinal permeability and bacterial translocation in ME/CFS patients that has been shown in other samples (Giloteaux et al., 2016; Maes et al., 2012a; Maes & Leunis, 2008; Maes, Mihaylova, & Leunis, 2007b). It appears to be important to acknowledge distinctions between microbial properties because some bacteria appear to have stronger cell walls and increased resilience to survive in different conditions (e.g., *Clostridium* genus within *Firmicutes* phylum and *Bacilli*) and may survive in the bloodstream for longer (Shukla et al., 2015).

Bacterial translocation may have a direct effect on symptomatic expression but also may precede other pathophysiological mechanisms in ME/CFS (e.g., immune dysregulation, neuro-inflammation, oxidative stress) and has also been proposed as one factor that may induce autoimmunity in these patients (see Morris & Maes, 2014; Morris et al., 2016)). Results from Giloteaux et al. (2016) suggest that ME/CFS patients have a pro-inflammatory gastrointestinal tract that may damage the mucosal barrier, increase bacterial translocation and alter the immune response. However, this mechanism may not be relevant for some patients because Giloteaux et al.'s sample revealed similar minimum values for markers of intestinal permeability in ME/CFS and control groups. Considering the varied pathways of host-bacteria communication, other direct or indirect modes of communication may also explain the neurological symptoms, particularly in patients with gut dysbiosis but without gastrointestinal symptoms.

D-lactate theory

The systematic review comparing 59 episodes of D-la with corresponding ME/CFS diagnostic criteria (ICC; Carruthers et al., 2011) indicated the possibility of shared mechanisms based on similar neurological symptoms and underlying microbiota-gut-brain interactions (Paper 4: Wallis et al., 2017b). Key results from the review indicated substantial overlap in neurological symptoms with motor disturbances identified as the most prevalent D-la neurological symptoms. ME/CFS patients commonly present with muscle weakness, clumsiness, balance and co-ordination difficulties (Carruthers et al., 2011). However, the frequent presentation of gait instability in D-la appears to be observed more frequently in severe ME/CFS cases (Carruthers et al., 2011). Overlap between D-la and some ME/CFS symptoms prompted the proposal of a continuum with ME/CFS (the chronic condition with fluctuating severity and clinical presentations) at one end, and the acute exacerbation of symptoms in D-la (often requiring hospitalisation) at the opposite end.

The review highlighted several microbiota-gut-brain mechanisms in D-la that have also been proposed in ME/CFS. Carbohydrate malabsorption due to reduced surface area after short-bowel surgery is assumed to be a primary precipitating factor leading to the bacterial dysbiosis and presentations of D-la. Reduced functional capacity of the small intestinal villi (associated with bacterial overgrowth; see Dukowicz, Lacy, & Levine, 2007) may also precipitate bacterial dysbiosis in ME/CFS patients. Identification of small intestinal bacterial overgrowth (SIBO) and proposed carbohydrate malabsorption in some ME/CFS may precede or exacerbate colonic bacterial dysbiosis (Logan, Rao, & Irani, 2003; Pimentel, Chow, & Lin, 2000) as well as nutritional deficiencies observed in some ME/CFS patients (Carruthers et al., 2011; Maes et al., 2009).

Evidence of bacterial dysbiosis has been shown in both conditions, with increased *Streptococcus* and *Enterococcus* species (identified as dominant producers of D-lactate *in vitro*) in ME/CFS patients compared with controls (Sheedy et al., 2009). In D-la most case studies identified increased *Bifidobacteria* or *Lactobacillus* species (see Wallis et al., 2017b, p. 17). The proposed increase in D-lactate production caused from bacterial dysbiosis can exert several effects that have been discussed in both conditions. Systemic inflammation from intestinal permeability, activation of the ENS through the vagal nerve, and central absorption from the gut, through the circulation to the brain are all possible pathways of gut-brain communication in D-la and ME/CFS. Regardless of the route of communication, bacterial dysbiosis and the central or systemic presence of D-lactate may directly or indirectly exert neurological effects. D-lactate may be an inefficient metabolic substrate for cerebral energy

production (Cassady, Phillis, & O'Regan, 2001) and can interfere with pyruvate metabolism (i.e., reduced ATP and neurotransmitter production; Ling et al., 2012; Vella & Farrugia, 1998), particularly in the context of other nutritional deficiencies (Al Chekakie, Al Kotoub, & Nielsen, 2004; Hudson, Pocknee, & Mowat, 1990).

Evidence from the D-lactate review paper (Paper 4: Wallis, et al., 2017b), the pilot study (Paper 5: Wallis et al., 2017a), and more recent findings highlight (e.g., Armstrong et al., 2017) gaps in knowledge and measurement. A key distinction between literature describing ME/CFS and D-la conditions was the presence/absence of metabolic acidosis. Metabolic acidosis based on blood pH measurement was observed in all but one episode of D-la but is not routinely measured in ME/CFS. The exceptional D-la episode described a 'compensatory acidosis', with the presenting alkalosis as measured by blood pH proposed to result from simultaneous increased L-lactate concentrations (Mendu, Fleisher, McCash, Pessin, & Ramanathan, 2015, p. 90). Routine measurement of blood pH would provide information about acidotic and/or alkalotic mechanisms in ME/CFS.

Findings indicating reduced abundance of *Bifidobacterium* (Giloteaux et al., 2016) and supporting probiotic intervention with lactic-acid producing bacteria (Groeger et al., 2013; Rao et al., 2009; Sullivan et al., 2009) in ME/CFS patients may contradict D-lactate theory. However, the proportion of D- and L-lactate production varies between species. Some species of colonic bacteria are able to both produce and excrete lactate during pyruvate metabolism (see Goffin et al., 2005). Also, Armstrong et al.'s (2017) results revealed reduced absolute lactate concentrations in stool and serum in female patients compared with controls that contradicted other reports of increased lactate in ventricular cerebrospinal fluid (Shungu et al., 2012) and significantly raised venous lactate concentrations in some patients after exercise (Lane et al., 1998; Lane, Burgess, Flint, Riccio, & Archard, 1995). The relevance of these findings for D-lactate theory in ME/CFS can not be ascertained without measurement of D- and L-lactate ratios, from the species identified in stool samples, the probiotic strains used or metabolites (bacterial or host) measured.

Results of the open-label pilot (Paper 5: Wallis et al., 2017a) with no change in D:L lactate ratio contradict the proposal of *Streptococcus* as the sole or primary producer of D-lactate in the ME/CFS sample and prompt consideration of other D-lactic acid producing species. For males there were associations between D:L lactate and some clinical symptoms (sleep onset, mood disturbance, general fatigue, and total symptoms; Paper 5: Wallis et al., 2017a). These results combined with moderate positive correlations between *Lactobacillus* and severity of neurocognitive and neurosensory symptoms in the cross-sectional study

(Paper 2: Wallis et al., 2016) add to the evidence supporting D-lactate theory, particularly for males with ME/CFS.

Both the review paper (Paper 4: Wallis, et al., 2017b) and the pilot study (Paper 5: Wallis et al., 2017a) indicated issues with D-lactate measurement. Findings of raised creatinine concentrations in ME/CFS (Armstrong, McGregor, Lewis, Butt, & Gooley, 2015), suggest that alternate methods for normalisation need to be identified to enable discrete measurement of absolute D-lactate concentrations and absolute L-lactate concentrations rather than reliance on D:L lactate ratio. A control comparison as well as temporal monitoring across shorter intervals with dietary control and/or provocation (i.e., carbohydrate heavy meal) will clarify whether subclinical concentrations of D-lactate are interacting with neurological symptoms in ME/CFS. Examining change in species rather than genera and bacterial metabolites will provide valuable information to help interpret the information obtained from these investigations.

Neurological presentations in the absence of gastrointestinal symptoms may partially explain frequent misdiagnoses of D-la, even within patients with a history of short bowel resection (Kowlgi & Chhabra, 2015). Sometimes the neurological presentation of D-la can be mistaken for a psychiatric condition rather than attributed to gastrointestinal causes (e.g., Scully, Kraft, Carr, & Harig, 1989). Gastrointestinal surgery is not a prerequisite for higher D-lactate concentrations (Htyte, White, Sandhu, Jones, & Meisels, 2011) and higher D-lactate concentrations have been shown in diabetes (Hasegawa et al., 2003; Thornalley, McLellan, Lo, Benn, & Sönksen, 1996) and after trauma/infection (Ewaschuk, Naylor, & Zello, 2005). Considering the divergent presentation, the presence of raised or subclinical D-lactate concentrations in ME/CFS and other neurological or metabolic conditions is unclear without efficient, routine measurement of D-lactate.

The proposed continuum of clinical manifestations associated with D-lactate concentrations presented in the review paper (Paper 4: Wallis et al., 2017b, Fig. 3, p. 10) may have relevance for some patients with ME/CFS. The mixed evidence to date does not confirm or negate this theory. However, it is clear from other metabolomics investigations (Armstrong et al., 2017; Nagy-Szakal et al., 2017) that lactate is only one metabolic by-product of bacterial fermentation. Therefore, an individual's specific dysbiosis is likely to present the most influence on metabolites produced, pathways disrupted and associated symptomatology. Results from both D-lactate and ME/CFS research suggest that it is unlikely that D-lactate is the only bacterial metabolite that could have deleterious effects on the host at large concentrations. If D-lactate is raised as a consequence of dysbiosis,

identification of causative factors will need to be prioritised to help identify appropriate treatment strategies that may vary between individuals.

Treatment possibilities for gut dysbiosis

Results from the pilot study are promising as they showed improvement on several clinical outcomes (cognitive, sleep, total symptoms) following a treatment targeting gut dysbiosis involving excess *Streptococcus* (Paper 5: Wallis et al., 2017a). Improvements indicated across this short intervention (4 weeks) are particularly encouraging considering the average illness duration was approximately 10 years in our sample. Unlike findings from the earlier pilot with the 6-day antibiotic treatment, where changes across time were only observed in the subgroup of participants who reduced in *Streptococcus* (Jackson et al., 2015), our study showed large effect sizes for the total sample. Without placebo control or adequate control of possible practice effects on cognitive outcomes, the clinical changes can not be directly attributed to the antibiotic/probiotic intervention with certainty at this stage. Unexpected ancillary findings with no correlation between change in *Streptococcus* and change in clinical outcomes and no correlation between change in *Streptococcus* and change in D:L lactate may result from insufficiency of our methods in isolating D- and L-lactate in urine. Alternatively, the results raise the possibility of the intervention having other modes of action. Potentially a reduction in *Streptococcus* could support immune regulation (e.g., similar to that observed in other streptococcal infections; Dale et al., 2001; Swedo et al., 2015; Swedo et al., 1998) or the antibiotics and/or probiotics may have broader implications for microbial balance, bowel motility, gut health, inflammation, or on metabolite production (i.e., SCFAs and neurotransmitters; see discussion in '*Other Modes of Action'* section in Paper 5: Wallis et al., 2017a, pp. 23-24).

The results of the open-label pilot (Paper 5: Wallis et al., 2017a) add support to previous preliminary findings indicating some improvement after antibiotic (Jackson et al., 2015) and probiotic interventions (Groeger et al., 2013; Rao et al., 2009; Sullivan et al., 2009) in this population and emphasise the importance of considering individual response to treatment. Individual variability in treatment response was indicated by adverse events and an increase in *Streptococcus* concentrations at post-intervention for 12/42 patients. Ancillary findings support the notion that change in other genera (possibly a result of *Streptococcus* reduction or probiotic supplementation) may be crucial indicators of treatment success. Analysis of the microbiome using DNA sequencing techniques may highlight shifts at the phylum, family and species level that are not possible using the culture-methods employed in our pilot to date. This investigation is currently being conducted.

We again return to the search for underlying causes that should, ideally, direct treatment prescription. Even if we were to assume that the antibiotic/probiotic intervention (rather than placebo or practice effects) was responsible for the clinical changes observed, we are yet to understand the mechanism involved. The D:L lactate results demonstrate the need to examine other reasons for improvement. When considering the complexities of the microbial ecosystem and pathways of communication, it is possible that the dysbiosis in one patient may be contributing to symptomatology but may be an adaptive response in another patient dependent on their pre-disease microbial composition. From the studies to date, what remains unanswered is whether an imbalance of commensal enteric microbiota is a precipitating or maintaining factor, a compensatory mechanism, and/or a consequence of other underlying pathology. Therefore, it is fundamental that we understand the cause of the dysbiosis and the role that this dysbiosis may be playing in the clinical manifestation of the condition (see Figure 2).

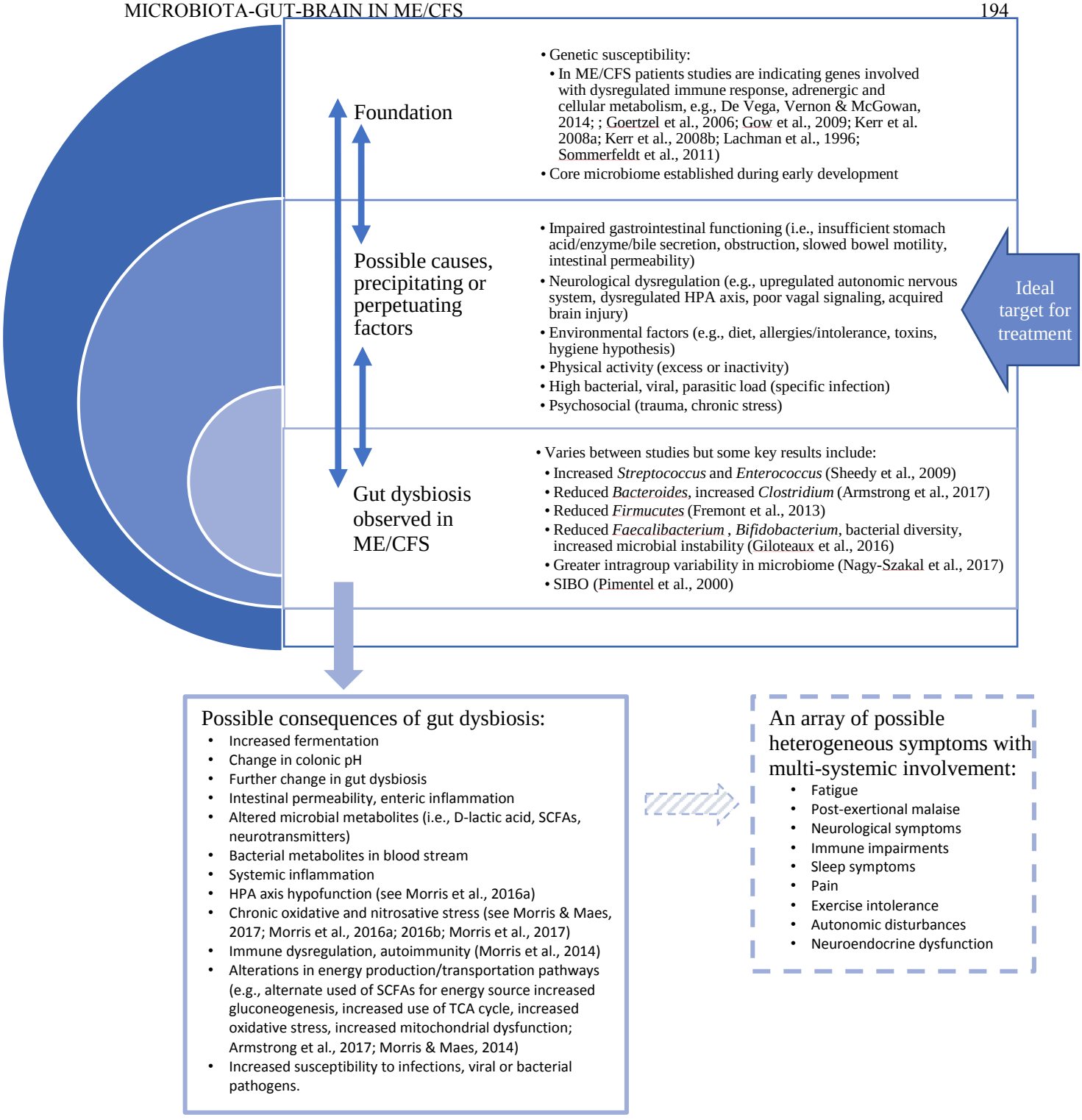

Figure 2. A simplified conceptualisation of complex interactions between genetic, environmental and precipitating factors manifesting in gut dysbiosis, pathophysiological disturbances and symptoms in ME/CFS.
Genetic vulnerability and microbiome composition established from birth provides the foundation of homeostasis (i.e., health or illness progression). Multiple possible causes, maintaining or precipitating factors can contribute to gut dysbiosis in animal, healthy and disease states are highlighted as an optimal treatment target. The gut dysbiosis that has been reported in ME/CFS studies is presented with possible consequences of gut dysbiosis.

N.B. This figure is only from the perspective of gut dysbiosis and does not include other pathophysiological mechanisms that may be involved in ME/CFS presentations.

Abbreviations: hypothalamic-pituitary-adrenal (HPA), myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), short chain fatty acids (SCFAs), tricarboxylic acid (TCA)

Animal studies and results from healthy and other clinical populations offer several possible causes that may precipitate, exacerbate or maintain the gut dysbiosis observed in some ME/CFS patients (Figure 2). The varied origins of gut dysbiosis are likely to be preferable targets of treatment compared with symptomatic management. From this framework, treatment for gut dysbiosis with antibiotic or probiotic intervention may also require dietary modifications (e.g., Craig, 2015), support with detoxification pathways/processes, nutritional/antioxidant supplementation for intestinal permeability (see Maes & Leunis, 2008; Maes, Coucke, & Leunis, 2007a), psychological interventions (for trauma, primary or secondary stressors), and/or support with methylation (dependent on genetic susceptibilities). Individual treatment prescription targeting the hypothesised underlying cause or contributing factors may result in more predictable and sustained clinical improvements that seem to be elusive in the ME/CFS population at large. There are several limitations specific to research in ME/CFS field and the microbiome that need to be addressed to expand our current understanding of etiology and treatment options.

Limitations and Considerations for Future Research

Study-specific limitations have been explored within each paper and will not be repeated here but some consistent methodological limitations between studies will be discussed with reference to considerations for future research. These limitations include issues about measuring symptomatology in ME/CFS (i.e., diagnostic discrepancies, heterogeneity of samples, and selection of outcomes) and the complexity of the microbiome.

Measuring symptomatology in ME/CFS

An issue that was highlighted by both cross-sectional (Paper 2 and 3: Wallis et al., 2016; 2017c) and experimental (Paper 5: Wallis et al., 2017a) results were concerns about accurate measurement of symptoms. This included issues with diagnostic discrepancies and prioritisation of symptoms, selecting primary and secondary outcomes, interpreting variability between symptoms as well as inconsistency across objective and subjective data.

Heterogeneity of samples in ME/CFS

Heterogeneity is a major confounding factor in ME/CFS research. Our attempts to minimise heterogeneity by using consistent diagnostic criteria (CCC; Carruthers et al., 2003) and one site (CFS Discovery Clinic) for participant recruitment appear inadequate methods to obtain homogenous samples. Data from the retrospective study (see Table 1, Paper 2: Wallis et al., 2016) showed that each clinical symptom factor included participants with ratings of zero. Therefore, regardless of a diagnosis of ME/CFS (CCC, Carruthers et al., 2003), several

participants self-reported no or low symptoms on the scale used. Similarly, in the open-label pilot (Paper 5: Wallis et al., 2017a), even within the predefined inclusion criteria that selected a subgroup of ME/CFS patients with high *Streptococcus,* there was large variability in symptoms by both subjective and objective measurement. Standard deviations for clinical measures at baseline reflect the heterogeneity within this sample (see Table 3, Paper 5: Wallis et al., 2017a, pp. 41-42). These results support the continued efforts to pursue diagnostic clarification and classification of subgroups that have been proposed by several authors.

Diagnostic clarity and subtyping

Diagnostic discrepancies plague ME/CFS research. In November 2013, a systematic review of the literature showed 20 case definitions of ME/CFS (Brurberg, Fønhus, Larun, Flottorp, & Malterud, 2014). Since publication of this review, at least three new case definitions or refinements have been proposed, i.e., SEID (Clayton, 2015), Neuro-Inflammatory and Oxidative Fatigue (NIOF; Maes, 2015), and ME subgrouping by Jason and colleagues (Jason et al., 2015c). As discussed in the introduction to this thesis, SEID diagnostic criteria appears to include a more heterogeneous sample of patients than ME/CFS definitions (particularly compared with ICC, Carruthers et al., 2011) and greater overlap with mood and autoimmune conditions (Jason et al., 2015b). NIOF represents a more homogeneous group than ME/CFS (based on CDC_2/Fukuda et al., 1994 definitions). The more restrictive criteria of NIOF requires the presence of chronic fatigue for more than 6 months and at least four of six neuro-inflammatory and oxidative symptoms (muscle tension, memory disturbances, sleep disturbances, irritable bowel, headache, flu-like malaise; Maes, 2015). Maes (2015) also recommends additional categorisation of 1) gastrointestinal symptoms, 2) post-exertional malaise, 3) hyperalgesia/fibromyalgia, 4) depression, or 5) comorbidity with psychiatric, neuroinflammatory or immune disorders. The strength of NIOF diagnostic criteria is the ability for subtype classification of symptoms based on statistically rigorous factor clustering of symptoms and cross-validation with biomarkers (i.e., immune response to lipopolysaccharides; and inflammation, oxidative and nitrosative stress: IO&NS).

Jason and colleagues (2015) also present a compelling case for prioritisation of core symptoms (1. Post-exertional malaise, 2. Neurocognitive symptoms, and 3. Sleep) and subgroup categorisation according to secondary symptoms (1. Immune, 2. Autonomic, 3. Neuroendocrine, 4. Pain). A clear distinction between the diagnostic clarifications presented by Maes (2015) compared with SEID, ME/CFS defined by ICC criteria (Carruthers et al., 2011) and Jason et al.'s (2015) approach is that post-exertional malaise is not a requirement

of NIOF compared with ME/CFS. Therefore, the presence/absence of this symptom may form the basis of differential diagnoses.

Combined use of NIOF diagnostic criteria and gastrointestinal subtyping may be helpful for future research examining microbiota-gut-brain interactions to attempt to identify clear patient subgroups and reduce outcome variability. This is particularly relevant when we consider that intestinal permeability, gastrointestinal symptoms, oxidative stress and systemic inflammation may be a cause and/or consequence of gut dysbiosis (see Figure 2). At a relatively simplistic level, it appears that subgrouping based on IBS comorbidity is valuable in ME/CFS samples. Nagy-Szakal et al.'s (2017) findings of microbial changes in ME/CFS patients with IBS were similar to changes in animal and clinical IBS samples (i.e., reduced *Faecalibacterium* and *Coprococcus* species compared with controls). Associations between microbial composition and proposed bacterial metabolic pathways that were highlighted in the ME/CFS with IBS cohort, encourage the use of subtyping based on IBS symptoms (i.e., diarrhoea, constipation and combined type) to help identify more specific therapeutic targets (Nagy-Szakal et al., 2017).

Sex is another factor that requires consideration with sex differences having implications for clinical and biological markers. For example, our pilot study showed baseline differences between male and female performance on a verbal (females better on RAVLT) and a computer-based visuo-spatial task (males better on Attention Switching Task on CANTAB). These sex differences are similar to those observed in non-clinical populations (Kimura & Hampson, 1993) and highlight the importance of measuring sex-aggregated baseline and outcome data for neurocognitive tests. Sex may be even more critical for immune or neuroendocrine markers in biofluid where differences between the sexes are expected in healthy samples (Markle & Fish, 2014) and have been observed in ME/CFS (e.g., Smylie et al., 2013). The results of the cross-sectional study, supporting the microgenderome, also indicate the need to examine functional sex-differences in microbiota, even with compositional similarity. Sex, like age, ethnicity and duration of illness, are all essential factors to consider when phenotyping in ME/CFS and conducting clinical trials.

Genomic clustering and gene expression studies are attempting to identify subtypes and biomarkers within ME/CFS. Results suggest differentiation between genomic clusters associated with clinical symptoms (Kerr et al., 2008a) and some viral pathogens (particularly Epstein-Barr virus and enterovirus; Zhang et al., 2010). Measurement of microRNA in blood samples of ME/CFS patients suggests that Natural Killer (NK) cells may be useful diagnostic biomarkers (Petty et al., 2016). However, limited concordance between studies (e.g.,

variability in the seven and eight subtypes identified by the same research team with different size samples: Kerr et al., 2008a; Zhang et al., 2010), intra-individual temporal differences for microRNA measurement, and overlap between ME/CFS and controls (Petty et al., 2016) indicate the preliminary nature of these findings. Replication is required to ascertain the generalisability to other samples of ME/CFS patients. Cross validation using IO&NS markers may also extend our understanding of differences between clinical presentations that could support treatment specificity. From the most recent diagnostic advances, discriminant and/or network analysis of ME/CFS subtypes with microbial, metabolomics, genomic, biochemical and symptom expression will inform both our etiological understandings and help prioritise outcomes for treatment.

Selecting outcome measures

The research conducted in this thesis prioritised neurocognitive, sleep and mood symptoms. A challenge faced during data analysis for the pilot study was difficulty interpreting variability across clinical outcomes, e.g., discrepancies between objective and subjective sleep outcomes. Differences between objective (e.g., actigraphy) and subjective (e.g., diary or Pittsburgh Sleep Quality Index: PSQI, Buysse et al., 1989) reports of sleep are common (Kobayashi, Lavela, Mellman, & Huntley, 2012; Wang, Hung, & Tsai, 2011). Issues of validity and reliability of measurement methods that all research fields face are compounded in ME/CFS research by inconsistent findings, overlap with healthy controls, and lack of agreement on biomarkers that are further confounded by temporal fluctuations. One benefit of reaching consensus on both diagnostic issues and pathophysiological and symptom measurement is clarification of appropriate and effective outcome measures.

Many neurological tests and biomarkers hold promise as plausible outcome measures in ME/CFS research (see reviews by Jason et al., 2015c; Fischer et al., 2014; Klimas, Broderick, & Fletcher, 2012). Neurocognitive tests of memory and attention may be particularly useful as unobtrusive, inexpensive means of subtyping (see Fischer et al., 2014). The contradictory findings may be indicative of phenotypic variability within samples tested in ME/CFS or may require alternative assessment that captures post-exertion fatigue. Other more invasive/expensive biomarkers to assess structural/functional brain abnormalities and neurochemical dysregulation (e.g., cortisol awakening response, see Jason et al., 2015c; Powell, Liossi, Moss-Morris, & Schlotz, 2013; Tak et al., 2011) may be useful for subtyping and as outcome measures (if practically feasible). Difference in serum markers of oxidative and antioxidant capacity in ME/CFS compared with controls could implicate

pathophysiological differences underlying neurocognitive disturbances. Fukuda et al. (2016) showed that healthy controls had higher oxidative activity (reactive oxygen metabolites-derived compounds) and lower antioxidant capacity (biological antioxidant potential) after a mentally fatiguing acute (3 hour) task with their post-testing levels similar to the ME/CFS group. ME/CFS patients were not administered the cognitive task but it would be valuable to monitor these markers in ME/CFS patients before and after similar mentally fatiguing tasks (or cognitive testing) to determine whether these markers could be reliable clinical endpoints. Recommended advances to the assessment of neurocognitive symptoms in ME/CFS include a standardised scale assessing cognitive effort, repeat cognitive testing 24 hours after to ascertain cognitive post-exertional malaise, and/or monitoring of neurochemical/endocrine biomarkers before and after assessment.

Sleep disruptions can precipitate neurocognitive symptoms or may be a consequence of neurological disturbances (see Jackson & Bruck, 2012; Landis, 2011) with specific mechanisms yet to be determined. Our findings suggest that Actigraphic WASO was the optimal objective outcome measure without placebo-control in the open-label pilot (Paper 5: Wallis et al., 2017a). However, whilst mindful of potential placebo effects, the patient's subjective experience is imperative, particularly considering vast discrepancies between reports of sleep quality and objective measures of sleep dysfunction. Measurement of delta slow wave activity may be preferable (see Jason et al., 2015c) but less realistic across clinical and research settings due to the expense and reduced ecological validity of polysomnographic assessments compared with actigraphy and self-report methods.

A recent systematic review of pharmacotherapies in ME/CFS indicated a reliance on subjective ratings of symptoms (fatigue, pain, mood, neurocognition, sleep quality and total symptoms) and overall functioning (functional status, well-being, and global health status) as primary outcome measures in RCTs (Collatz et al., 2016). This study demonstrated that 21 out of 26 studies selected only self-report questionnaires or scales as primary outcome measures (see Supplementary Material from Collatz et al., 2016). The studies that also used objective endpoints included measurement of steps (Sulheim et al., 2014), blood pathology results (Cleare, O'Keane, & Miell, 2001) or cognitive tests (Blacker et al., 2004; Montoya et al., 2013; Randall et al., 2005). The inconsistency in endpoints is one confounding factor that requires diligence to select suitable endpoints with high sensitivity and specificity. It is apparent that both subjective (sleep diaries, self-report scales) and objective (actigraphy, neurocognitive testing) measures of symptoms need to be correlated, where possible, with physiological markers that may include measures of immune dysregulation (i.e., cytokine

profiling, NK cell function, viral antibodies, B-cells; see Fischer et al., 2014; Huth, Staines, & Marshall-Gradisnik, 2016; Maes, Bosmans, & Kubera, 2015; Montoya et al., 2017; Petty et al., 2016), IO&NS (see Fukuda et al., 2016; Maes, 2015), host and bacterial metabolites (see Armstrong et al., 2017; Armstrong et al., 2015; Fletcher et al., 2010), enteric microbiota, and genetic profiling.

Suggestions for future research

Solutions to the issues of heterogeneity are dependent on diagnostic clarity and research design. There is an increasing focus on recruiting large samples and extensive biodata in ME/CFS (e.g., Montoya et al., 2017) and microbiome studies (e.g., Human Microbiome Project, see Hollister, Gao, & Versalovic, 2014). Large, multisite studies following the same diagnostic parameters and a longitudinal design using repeat assessment of biomarkers and symptoms are likely to shed light on unanswered questions about ME/CFS pathology (Fischer et al., 2014; Jason et al., 2015c). Control and comorbid condition comparison appears to be particularly important for translational results and because several proposed biomarkers have cyclical fluctuations and age-related changes that have been observed in healthy populations. The substantial costs associated with long-term, co-ordinated research is appropriate considering the continued and growing costs of chronic conditions. This approach will be useful for subtyping but also could enable detailed individual case analysis that may inform treatment opportunities and outcomes. There is merit in looking at different endpoints for respective symptoms and not just a broad measure of global change, particularly when we consider the heterogeneity of symptoms in this condition. Results from large studies with longitudinal designs can also inform methods for determining clinically meaningful improvement (i.e., endpoints for primary and secondary outcomes in clinical trials) that will increase the scientific rigour of treatment studies in ME/CFS. Any methods that enable increased specificity and responsivity to individual variability hold promise for clinical utility and improvement for ME/CFS patients.

Complexities of the microbiome

Research conducted by our team to date has relied solely on culture-based sequencing methods as a representation of microbiome composition. The strength of culture methods is the ability to examine viable counts of microbiota from stool analysis within clinical and research settings. However, advances with metagenomic sequencing allow for a more accurate representation of the diversity of strains, species, genera and phylum within an individual's enteric microbiome (Qin et al., 2010; Sekirov et al., 2010). The next stage of

research that needs to be pursued is examining whether there is concordance between culture-based and sequencing techniques. Both methods have strengths that may complement each other and aid interpretation, whilst considering the constraints of stool analysis as an accessible method of measuring colonic bacteria.

Colonic bacteria continue to be prioritised in research, with our growing knowledge of its role in health and disease (Ohland & Jobin, 2015). Our current understanding presumes that lower bacterial diversity and abundance reside in the upper gastrointestinal tract but advances in measuring difficult to reach areas (i.e., sections of the small intestine) may provide alternate perspectives about bacterial overgrowth or dysbiosis associated with some conditions, e.g., SIBO. Some researchers are currently developing an unobtrusive capsule that can measure gas production (i.e., a proxy measure of bacteria or archaea) throughout all areas of the gastrointestinal tract (Rotbart et al., 2017). Methodological advances like this, coupled with improved measurement of bacterial functioning, i.e., metabolomics, and examination of clinical relevance will be crucial for understanding the complex role of the microbiome in ME/CFS and other chronic conditions.

There is a growing body of evidence (particularly in animal studies) that demonstrates the crucial role of the microbiome in regulating the stress response (see Foster, Rinaman, & Cryan, 2017) and supports treatment directly targeting gut dysbiosis (i.e., nutritional, probiotic supplementation, antibiotic interventions, and bacteriotherapy). However, the multidirectional communication between microbiota, the gastrointestinal system and the CNS must not be forgotten. This has been effectively illustrated within animal studies showing alterations to the microbiome after acute and chronic psychosocial stress (e.g., Bailey & Coe, 1999; Bharwani et al., 2016; O'Mahony et al., 2009). Acknowledging the role of psychosocial stress affecting microbial composition, raises ideas about adjunctive psychological/interventions to modulate the stress response and potentially improve bacterial homeostasis. It would be interesting to consider how psychological interventions (e.g., cognitive, mindfulness, relaxation, hypnosis) focused on stress reduction may be useful as an adjunctive therapy for treatment targeting gut dysbiosis and possibly for ME/CFS prevention (i.e., following acute bacterial/viral infections in genetically/environmentally susceptible individuals).

It needs to be explicitly stated that the term 'dysbiosis' can be conflated to infer causation. The implication can be that an imbalance in microbiota has a negative consequence on the host, even though the evidence remains correlational. Hooks and O'Malley's (2017) recent article summarises the issues with inconsistent application and

interpretation of the term ‘dysbiosis’ within microbiome research. The authors highlight the need for specificity when defining dysbiosis and clarification between dysbiosis as a diagnostic or explanatory term. Identification of differences between the microbiome of healthy compared to disease states is becoming increasingly accepted. These differences (often referred to as dysbiosis) need to be clarified as to whether they are diagnostic or explanatory, causal or consequential, functional or pathogenic, to inform mechanistic understanding and treatment. Hooks and O’Malley (2017) query the focus on taxonomic composition compared with physiological function and suggest that establishing causality requires functional definitions of dysbiosis and nondysbiosis (i.e., healthy or stable microbial composition sometimes referred to as homeostasis, eubiosis, or normobiosis). A functional definition of nondysbiosis in healthy populations and functional analysis of dysbiosis (e.g., metabolomic profiling) within ME/CFS will help clarify the role of microbial imbalances within ME/CFS and guide treatment possibilities.

Concluding Remarks

There is now compelling evidence for microbiota-gut-brain interactions in ME/CFS. Whilst there is no clear microbial phenotype that has been discovered to date, with increased specificity, diagnostic differentiation (i.e., between NIOF and ME/CFS) and subtype classifications within ME/CFS, predictable microbial changes may be observed. The body of work presented in this thesis suggests promise for treatment opportunities targeting gut dysbiosis in ME/CFS. It acknowledges our limited understanding of mechanisms to date and beckons investigation of possible mechanisms involved in neuropsychological presentations. Interdisciplinary collaboration was a key attribute of the research presented in this thesis. If we are to understand the complexities of ME/CFS, the multidisciplinary approach used in this body of research that has involved examining neurocognitive, clinical and microbiological data needs to be expanded to include collection of immune, IO&NS, genome and metabolome data.

Our current understanding of ME/CFS etiology and treatment possibilities will be advanced by research examining possible factors contributing, precipitating or perpetuating gut dysbiosis. Increased awareness of bidirectional microbe-host communication that may underlie neuropsychological and neurological presentations has application for multiple disciplines (e.g., psychology, psychiatry, neurology, medicine) and conditions (e.g., acute and chronic illnesses, autoimmunity, developmental disabilities). A functional understanding of

the role of an individual's microbiome in health and disease offers hope for personalised medicine.

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