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Research paper

Gut feelings: A randomised, triple-blind, placebo-controlled trial of probiotics for depressive symptoms

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ABSTRACT

Background: Depression is the leading cause of disability worldwide; with evidence suggesting that decreased gut barrier function and inflammation are correlated with depressive symptoms. We conducted a clinical trial to determine the effect of consumption of probiotic supplements (Winclove's Ecologic® Barrier) on depressive symptoms in a sample of participants with mild to severe depression.
Method: 71 participants were randomly allocated to either probiotic or placebo, which was, consumed daily over eight weeks. Pre- and post-intervention measures of symptoms and vulnerability markers of depression as well as gut microbiota composition were compared. Clinical trial participants were also compared on psychological variables and gut microbiota composition to a non-depressed group ($n = 20$).
Results: All clinical trial participants demonstrated improvement in symptoms, suggesting non-specific therapeutic effects associated with weekly monitoring visits. Participants in the probiotic group demonstrated a significantly greater reduction in cognitive reactivity compared with the placebo group, particularly in the mild/moderate subgroup. Probiotics did not significantly alter the microbiota of depressed individuals, however, a significant correlation was found between *Ruminococcus gnavus* and one depression metric.
Limitations: There was a high attrition rate, which may be attributed to weekly monitoring visits. Additionally, modulation of the gut microbiota may need more specific testing to distinguish subtle changes.
Conclusions: While microbiota composition was similar between all groups, probiotics did affect a psychological variable associated with susceptibility to depression. Further research is needed to investigate how probiotics can be utilised to modify mental wellbeing, and whether they can act as an adjunct to existing treatments.

1. Introduction

Depression is a debilitating psychiatric disorder that is the leading cause of disability world-wide (Kessler and Bromet, 2013; World Health Organization, 2017). Multiple causes of depression have been identified, including genetic, neurological, inflammatory, personality, cognitive, and environmental factors (Beck and Bredemeier, 2016; Disner et al., 2011; Miller and Raison, 2016; Sullivan et al., 2000; Wohleb et al., 2016). A number of different therapy modes exist, including pharmacological treatments (e.g. antidepressant medications), and psychological therapies (e.g. cognitive-behaviour therapy), which aim to alleviate symptoms by targeting the neurological functioning or maladaptive cognitive patterns affected in depression (Beck, 2002; Wallace and Milev, 2017). Research indicates that these treatments are effective in reducing depressive symptoms, with approximately 60–70% of patients responding to treatment (Al-Harbi, 2012). However, an estimated third of patients do not respond to existing treatments, and a significant number of people do not seek treatment due to the associated stigma (Collins et al., 2011; Rieder et al., 2017; Souery et al., 1999). As such, there is need for additional or adjunctive treatment strategies for depression.

The gut microbiota is recognised to play a significant role in human health and disease (Gareau et al., 2010; McCusker and Kelley, 2013; Round and Mazmanian, 2009; Sekirov et al., 2010). Its influence extends beyond the gut and has been associated with diseases including, but not limited to, obesity (Turnbaugh et al., 2009), Type-2 diabetes (Larsen et al., 2010), celiac disease (De Palma et al., 2010), Crohn's disease (Scanlan et al., 2006), and depression (Jiang et al., 2015;

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Kelly et al., 2016; Lin et al., 2017; Naseribafrouei et al., 2014; Zheng et al., 2016). A range of rodent and human studies have indicated that disruption of the gut microbiota modulates stress reactivity (Bravo et al., 2011; Goehler et al., 2007; Lyte et al., 2006), and is linked to poorer mental health outcomes (Logan and Katzman, 2005; Rao et al., 2009). Further evidence linking the gut microbiota to mental health comes from faecal microbiota transplants (FMTs), where BALB/c mice with a natural tendency toward anxious behaviour can be shifted towards the more outgoing behaviour of NIH Swiss mice via FMT (Bercik et al., 2011). Similarly, FMTs from depressed humans results in the development of depressive behaviours in rodents (Kelly et al., 2016; Zheng et al., 2016). These studies suggest that the gut microbiota exerts a strong influence on mental health, and that manipulation of the microbiota could be a viable treatment option.

The gut microbiota may mediate effects on mental health via the gut-brain axis, a bidirectional communication system between the gastrointestinal tract (GI) and the central nervous system (CNS). This axis involves the integration of neural, hormonal and immunological signals (Mayer et al., 2014), and has been recognised to play a role in modulating physical and psychological health(Foster and Neufeld, 2013; Mayer, 2011; Steenbergen et al., 2015). The vagus nerve has been shown to mediate communication between the gut and brain, as antidepressant effects of probiotics in mice are no longer observed when the vagus nerve is severed (Bravo et al., 2011). Another possible mechanism involved in gut-brain interaction are metabolites of tryptophan. Tryptophan can be converted into serotonin, but most of it is converted to kynurenine, especially under inflammatory conditions. Kynurenine can be converted further to anthranilic acid, kynurenic acid, and quinolinic acid of which the latter two have neuromodulatory properties (Kennedy et al., 2017; Waclawiková and El Aidy, 2018). Further, gut microbiota may affect mental health via stimulation of systemic inflammation. Numerous studies have shown that pro-inflammatory cytokines, such as IL-6 and TNF-alpha are increased in people with depression (Cizza et al., 2008; Dowlati et al., 2010; Lanquillon et al., 2000; Maes et al., 1995; Mesquita et al., 2008). The cause of the increased inflammation is not yet understood but one hypothesis is that it results from a 'leaky gut' (Maes et al., 2012). A leaky gut is characterised by an increase in gut permeability through decreased barrier function, which includes the epithelial mucus layer and complex tight junctions. This barrier prevents microbes and other inflammatory stimulants from moving across the epithelium (Arrieta et al., 2006; Turner, 2009), and different bacterial species have the ability to either promote or weaken this barrier (Pedicord et al., 2016). When the barrier is impaired it can lead to gut leakiness allowing microbial products such as lipopolysaccharides (LPS) to activate an inflammatory immune response (Maes et al., 2012; Qin et al., 2007). It is thought that systemic inflammation as a result of leaky gut can influence brain functioning via pro-inflammatory cytokines crossing the blood-brain barrier and affecting central nervous system functioning such as serotonin signaling, contributing to symptoms of depression (Sampson and Mazmanian, 2015).

The concept of 'psychobiotics', defined as probiotics which can confer mental health benefits, has emerged in recent years (Dinan et al., 2013; Sarkar et al., 2016; Wall et al., 2014; Zhou and Foster, 2015). Probiotics are defined as live microorganisms that provide a beneficial health effect (Hill et al., 2014). Probiotic bacterial species have been shown to improve gut barrier function (Krishna Rao and Samak, 2013), and preclinical studies in animals and humans have demonstrated improvements in behaviour and mood with probiotic treatment (Benton et al., 2007; Desbonnet et al., 2010; Steenbergen et al., 2015). Clinical trials of probiotics for the treatment of depression have reported conflicting results. Akkasheh and colleagues identified that consumption of probiotics containing *Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacterium bifidum* over an eight-week period led to a significant reduction in depressive symptoms (Akkasheh et al., 2016). Romijn et al. conducted a similar 8-week clinical trial with the probiotic strains *Lactobacillus helveticus* and *Bifidobacterium longum*, but found no evidence of an effect on depression (Romijn et al., 2017). Evidently, further research is required to determine the effectiveness of probiotic consumption including specific strains, dosage and duration of treatment, and whether probiotics can function as an adjunct or standalone therapy.

In this study we report the results from a randomised clinical trial investigating the effect of regular probiotic consumption on depressive symptoms. The primary aim of this study was to determine whether eight-week consumption of a probiotic supplement (Winclove's Ecologic® Barrier) led to a reduction in depressive symptoms in a sample of participants with a range of symptom severity. As depression is typically thought of as existing on a continuum (Ayuso-Mateos et al., 2010), with intensity of treatment varying based on depression severity (Davidson, 2010). The primary outcome was also determined based on the Mini International Neuropsychiatric Interview (M.I.N.I) (Hergueta et al., 1998) clinician ratings. A secondary aim was to determine whether the treatment effect varied with baseline levels of depression; do individuals with milder levels of depression severity respond differently to those with more severe depression to probiotic treatment? Another secondary aim was to investigate any potential effects of the probiotics on cognitive reactivity towards sad mood. This cognitive reactivity, thinking patterns or so-called dysfunction attitudes, are strengthened during depressive episode (Figueroa et al., 2018) and it has been shown earlier in healthy students that probiotic intake could influence the patterns (Steenbergen et al., 2015). Finally, faecal samples were taken before and after treatment to explore potential changes in the gut microbiota and were also compared to a non-depressed control group.

We hypothesised that probiotic treatment would reduce depressive symptoms when compared to the placebo group, particularly for those with milder levels of depression. For the microbiota, we expected to see baseline differences between the depressed cohort and non-depressed group. Additionally, we anticipated that changes in psychological scores during the course of the clinical trial might be associated with changes in the composition of the microbiota.

2. Method

2.1. Design

The study was a triple-blinded parallel, placebo-controlled randomised clinical trial. Participant numbers were randomly allocated into two groups (probiotic and placebo) via a computerised randomiser in blocks of four by a researcher not directly involved with administration of the product (S. van Hemert). Recruited participants were allocated a participant number, with both researchers administering the trial (L. Roberts and S. Kwan) as well as participants being unaware of group allocation throughout the trial and analysis of the results. Sample size calculations were based on clinical trials investigating psychological and antidepressant therapies for depression since when the present study was designed there were no other published clinical trials looking at probiotics for depression to use as a comparison. Power calculations indicated that a sample size of 26–36 participants in each group was sufficient to detect a moderate to large between-group effect size (ES) of 0.67–0.80 with power of 80%, which was the minimum expected based on similar studies using BDI scores as a primary outcome for treatment of depression (Dimidjian et al., 2006; Titov et al., 2011). This study was approved by the University of Technology Sydney Human Research Ethics committee, UTS HREC Reference number: 2015000438. The trial was registered in the ANZCTR, Trial ID: ACTRN12615001081505.

2.2. Participants

71 participants with depressive symptoms were recruited and allocated sequentially over 12 months at the University of Technology Sydney (UTS), Ultimo campus, New South Wales, Australia. Potential participants were screened through the Beck Depression Index - Second Edition (BDI-II) (Beck et al., 1996) by the research team to verify eligibility, with a cut-off score of 12 as a minimum score for entry into the trial. Participants were eligible for the study if they were aged 18 years or above, could provide informed consent, were willing and able to travel to UTS Ultimo campus on a weekly basis to complete questionnaires on mental wellbeing, could provide a stool sample at the start and end of the treatment period, and not consume probiotic-rich foods and drinks such as fermented cheeses during the trial, which may act as a confound to investigating the effects of probiotic supplementation. Participants were required to be relatively healthy apart from a diagnosis of depression and not be taking any medications (i.e. antibiotics, antidepressants). Full inclusion/exclusion criteria can be found in detail in the supplementary material (1.1.1). Participants were then randomly allocated either probiotic ($n = 34$) or placebo ($n = 37$) treatment. A summary of the demographic information for these participants can be seen in Table 1. Given the significant group difference in the number of prior diagnoses of depression results were analysed both with and without this variable as a covariate and the pattern of results did not change hence the data without this covariate is reported.

Table 1
Participant demographic information for three groups: depressed clinical trial participants assigned probiotics or placebo and the non-depressed cohort.

	Depressed participants		
	Probiotic	Placebo	Non-depressed
Age (years) mean (SD)	36.65 (11.75)	35.49 (12.34)	35.95 (11.74)
Gender% (*n*)			
Male	38.2 (13)	24.3 (9)	25.0 (5)
Female	61.8 (21)	75.7 (28)	75.0 (15)
Ethnicity% (*n*)			
Caucasian	68.8 (23)	67.6 (25)	65.0 (13)
Non-Caucasian[a]	31.2 (11)	32.4 (12)	35.0 (7)
Medical History[b] % (*n*)			
Yes	44.1 (15)	25.0 (9)	20.0 (4)
No	55.9 (19)	75.0 (28)	80.0 (16)
Past Antidepressant Use % (*n*)			
Yes	50.0 (17)	56.8 (21)	0 (0)*
No	50.0 (17)	43.2 (16)	100 (20)
Previously Accessed Psychological Treatment% (*n*)	64.7 (22)	64.9 (24)	68.4 (13)*,[e]
Yes			
No	35.3 (12)	35.1 (13)	31.5 (6)
Number of Previous Depression Diagnoses; Mean (SD)	1.24 (1.42)*	2.08 (1.59)*	_[f]
Abdominal Conditions[c]% (*n*)			
Yes	23.5 (8)	18.9 (7)	15.0 (3)
No	76.5 (26)	81.1 (30)	85.0 (17)
Smoking% (*n*)			
Yes	20.6 (7)	29.7 (11)	10.5 (2)
No	79.4 (27)	70.3 (26)	89.5 (18)
Alcohol[d]% (*n*)			
Yes	5.9 (2)	13.5 (5)	0 (0)
No	94.1 (32)	86.5 (32)	100 (20)

* $p < .05$.
[a] *Asian, Hispanic/Latino, African/Middle Eastern, Indian/Sri Lankan, Indian, Polynesian, Other*

Additionally, there was no statistically significant difference between the probiotic and placebo groups in their probiotic supplements or probiotic rich food consumption prior to the intervention (*ps* > .05).

For the purposes of microbiota comparison between people with and without depression, twenty participants were recruited for the non-depressed control group over two months. Participants were eligible for the non-depressed group if they endorsed a BDI-II score of 10 or below and met the exclusion criteria described in the supplementary material. Comparisons of baseline variables between the depressed and non-depressed group showed that there were no significant differences in participant age, gender, ethnicity, medical history, current abdominal conditions, smoking habit, alcohol intake or weekly physical activity. Across both the depressed and non-depressed groups, participants were predominantly female (70%), Caucasian (67%), had no significant medical history (69%) or abdominal conditions (80%), did not smoke (78%) or consume above the recommended daily alcohol intake (92%).

2.3. Procedure

Following enrolment into the study, provision of informed consent and allocation to a participant number, participants were provided with a stool sample kit one week prior to the start of the study. At pre-intervention assessment, participants returned their stool sample, and completed psychological tests including the clinician administered M.I.N.I to determine a clinical or subclinical diagnosis of depression, the Depression Anxiety Stress Scale - 21 Items (DASS-21) (Lovibond and Lovibond, 1995), BDI-II, and Beck Anxiety Inventory (BAI) (Beck and Steer, 1990) to assess their levels of depression and anxiety, and the Leiden Index of Depression Sensitivity-Revised (LEIDS-R) (Van der Does and Williams, 2003) to evaluate participant's cognitive reactivity, as well as demographic, and dietary questionnaires (further details in supplementary material, 1.2). Participants were provided with an eight-week supply of their randomly allocated probiotic/placebo supplement, and information on how to prepare and consume the product twice daily over the eight-week trial period. Participants then attended weekly check-up appointments at UTS over the next seven weeks, and completed the BDI-II, DASS-21 and Weekly Check-up questionnaire to assess mood and monitor for side effects. Participants returned empty probiotic/placebo sachets for compliance assessment. Participants returned a second stool sample at week nine of the trial corresponding to the day of or day after their last probiotic treatment. They also completed the M.I.N.I, BDI-II, DASS-21, BAI and LEIDS-R, as well as the post-assessment questionnaire, dietary questionnaire, and client satisfaction questionnaire. Participants returned to the UTS campus one month later to complete a follow-up assessment, where they were re-administered the M.I.N.I, BDI-II, DASS-21, BAI and LEIDS-R, as well as the dietary, and follow-up questionnaires.

2.4. Materials - Intervention

Participants randomly assigned to the probiotic group were provided two sachets for each day of the trial, containing 2 g of freeze-dried probiotic powder mixture (Ecologic®Barrier; Winclove probiotics, The Netherlands). This product has been shown before to influence depressive like behaviour in rats (Abildgaard et al., 2017b, 2017a), to influence cognitive reactivity towards sad mood (Steenbergen et al., 2015) and to improve working memory under stress (Papalini et al., 2019). Ecologic®Barrier (2.5×10^9 CFU/g) is constituted of the following nine bacterial strains: *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *L. acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58 (total cell count 1×10^{10} CFU/day). With the application of new molecular identification techniques (including whole genome

sequencing), the declaration of bacterial strains has been updated compared to previous publications. It has been confirmed that the probiotic formulation has always contained these nine strains, and has not been changed in ratio or CFU count since it has been (commercially) available. Participants in the placebo group were provided with two 2 g sachets daily of the freeze-dried maize-starch and maltodextrins, which is the medium used to carry the probiotics in the product Ecologic®Barrier. The taste, smell and colour of the placebo was identical to that of the probiotic, but did not contain any probiotic bacteria.

2.5. Psychological data analysis

Analysis of psychological data from the clinical trial was intention to treat, with last value brought forward (LVBF) used for post-treatment outcomes. Analysis of covariance (ANCOVA) were conducted with group (probiotics, placebo) as the between-subjects variable, post-treatment (LVBF) scores as the dependent variable, and pre-treatment scores included as a covariate. Any skewed variables were $\log_{10}$ transformed. Statistical analyses were conducted using IBM SPSS statistics, version 21 (Armonk, NY) (IBM Corp, 2012).

2.6. Microbiome analysis procedures

Stool samples from all participants were kept on ice or in refrigeration storage before being delivered to research staff. Participants were instructed to keep the sample in the fridge from the time of collection until they bought the sample back to UTS. They were asked to use a cold pack to transport the samples, but this was not provided. Upon receipt, samples were placed at 4 °C, and were then aliquoted and stored at −80 within several days. Individual aliquots were defrosted for processing and any remaining sample within the aliquot was then disposed of. DNA extraction was carried out using the PowerFecal DNA Isolation Kit, manufactured by MoBio. The methodology of extraction was completed following the manufacturer's protocol with samples defrosted and weighed to 0.25 g prior to DNA extraction.

The 16S rRNA gene V3-V4 region was amplified from DNA samples and prepared for Illumina sequencing using a 2 stage PCR dual-indexing protocol (supplementary material, 1.4.2). Negative controls were processed at each stage of each procedure including DNA extraction and PCR and were sequenced along with the samples, and a mock community sequencing control (ZymoBIOMICS microbial community DNA standard by Zymo Research). The pooled library was sequenced on an Illumina MiSeq using a V3 600 cycle kit and paired 300 bp sequences from either end of the amplicon.

The QIIME 1.9.1 pipeline (Caporaso et al., 2010) was utilised for demultiplexing, assigning sequences to samples and quality filtering. Additionally, operational taxonomic units (OTUs) (used as a proxy for bacterial species) were clustered at 97% similarity using the pick_open_reference.py script against the GreenGenes database (DeSantis et al., 2006) with parameters specifying the RDP classifier algorithm (Wang et al., 2007) trained on the GreenGenes database for taxonomy assignment. Chimeric sequences were identified and removed prior to further sequence processing. Samples were rarefied to an equal sequence depth of 28,170 sequences for statistical comparisons of alpha and beta diversity. The OTU table was filtered to the top 100 most abundant OTUs to increase statistical power for test of differential relative abundance and correlation to the BDI, DASS-21 and LEIDS-R measures, as has been applied in similar studies (Naseribafrouei et al., 2014). The Kruskal Wallis test was used to determine significant differences in relative abundance of individual taxa between groups, with the False Discovery Rate (FDR) correction for multiple testing. Spearman's Correlations (with FDR correction for multiple testing) were calculated to determine significant correlation of the relative abundance of individual OTUs or taxa to the BDI, cognitive reactivity and DASS scales for depression, anxiety and stress. Further details on DNA sequence processing are provided in the supplementary materials (1.4.4). Quality filtered DNA sequence data has been deposited in the European Nucletide Archive under study accession number PRJEB30099.

The R environment (R Core Team, 2016) and the Phyloseq package (McMurdie and Holmes, 2013) were used to calculate alpha and beta diversity as well as Principal Coordinates Analysis (PCoA) Three alpha diversity metrics (Richness, Chao1, Shannon) were calculated and differences between groups tested with the Wilcoxon Rank Sum test (R base stats package). Beta diversity was calculated using weighted Unifrac distances (Lozupone et al., 2011), which were used for PCoA. Differences between groups were tested with Permutational Analysis of Variance (PERMANOVA) (Anderson, 2001) as implemented in the adonis function in the Vegan package (Oksanen et al., 2017). The Dplyr package (Wickham and Francois, 2015) was used for data manipulation and graphs produced with the ggplot2 package (Wickham, 2009).

3. Results

3.1. Clinical trial–psychological data

The attrition rate was 34% (24/71 participants), further the details of this and the study design can be seen in Fig. 1. The attrition rate was calculated based on the number of participants who attended the week 9 post-intervention assessment. Further details on attrition distribution can be found in supplementary material (2.4) Psychological data was analyzed for 71 participants, as data analysis was intention-to-treat as described in the previous section. The most commonly reported side effect in the probiotic group was nausea (11/34) and the most common in the placebo group was dehydration (9/37). The probiotics group reported significantly more drowsiness (20.6%) compared to the placebo group ($p = .02$). There was a trend for participants in the placebo group to report dry mouth (13.5%) more than those in the probiotics group ($p = .06$). For further details on the side effects refer to Supplementary Table 1 in the supplementary material (1.1.3). Participants reported these side effects were temporary and occurred early on in the study, subsided quickly, and did not interfere with their ability to complete the clinical trial.

3.2. Primary outcomes

Analysis of 71 participants using LVBF revealed no significant main effect of group ($ps > 0.05$) for BDI, DASS, and BAI. . See Table 2 for means and standard deviations for the BDI. See Supplementary Table 4 for test statistics and exact significance values for between-group analyses.

The sample was split into two groups based on initial depression severity on the BDI-II; mild/moderate (scores from 12 to 28) and severe (scores >28). An ANCOVA revealed no significant main effect of group (probiotics, placebo) in either the mild/moderate or severe subgroups ($ps > 0.05$). See Table 3 for means and standard deviations. See Supplementary Table 5 for test statistics and exact significance values for sub-group analyses.

Scores on the clinician administered psychiatric interview, the M.I.N.I were next analyzed to determine whether there were any changes in the number of participants with no depression diagnosis, a subclinical depression diagnosis or a clinical depression diagnosis, before and after intervention, and one month follow-up, by group allocation. Friedman Tests (non-parametric alternatives to one-way ANOVA with repeated measures) indicated that there was a statistically significant difference in the level of clinical diagnoses (none, subclinical, clinical) across time points in the probiotics, but not placebo group, $\chi2(2) = 19.14$, $p = .00$, and $\chi2(2) = 0.89$, $p = 0.64$, respectively.

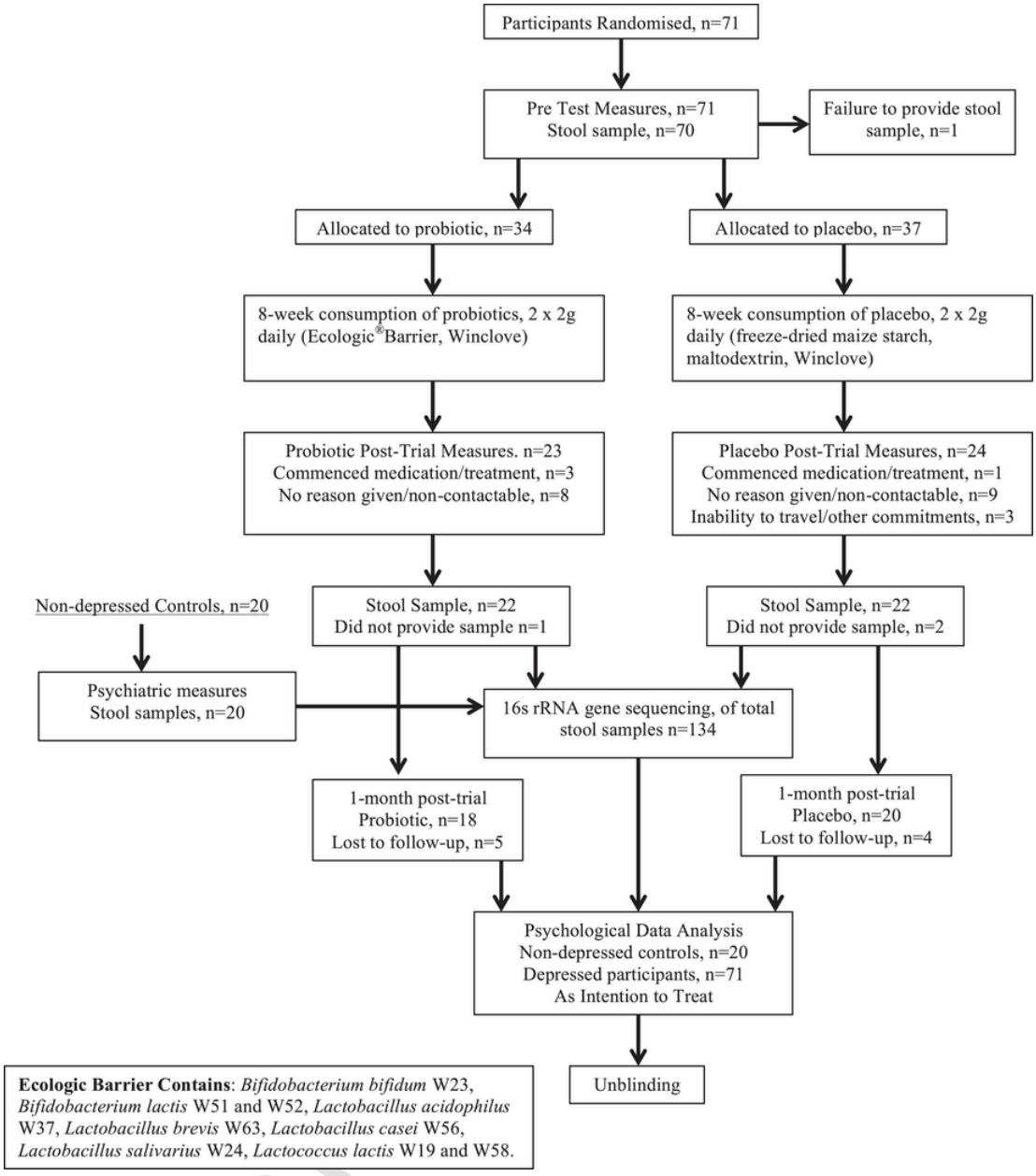

Fig. 1. Flow chart of the study design. 71 participants were recruited for the clinical trial, and 20 non-depressed controls were recruited separately for the purposes of microbiota comparison. For depressed participants the attrition rate was 34%.

Post hoc analysis with Wilcoxon signed-rank tests was conducted with a Bonferroni correction applied, resulting in a significance level set at $p < .017$. There was no significant difference in clinical diagnoses between pre and post in the probiotics group ($Z = -2.31$. $p = .02$), however there were significant differences between pre and follow-up ($Z = -3.29$. $p = .00$), and post and follow-up, ($Z = -2.64$. $p = .00$) within the probiotics group. Over these time points participants in the probiotics group moved from a median rank of a subclinical diagnosis to a median rank closer to no diagnosis.

Subgroup analyses indicated that there was a statistically significant difference in the level of clinical diagnoses (none, subclinical, clinical) across time points in the severe probiotics group ($\chi 2(2) = 15.55$, $p = .00$), but not in the mild/moderate probiotics group ($\chi 2(2) = 3.85$, $p = .15$), the mild/moderate placebo group ($\chi 2(2) = 0.15$, $p = .93$), or the severe placebo group ($\chi 2(2) = 0.77$, $p = .68$). Post-hoc analyses were conducted as described above and indicated a significant difference for the severe probiotics group in the level of clinical diagnosis between pre and follow-up tests ($Z = -2.89$. $p = .00$), but not between pre and post ($Z = -2.12$. $p = .03$), or post and follow-up ($Z = -2.27$. $p = .02$). From pre to follow-up participants in the severe probiotics group moved from a median rank of a clinical diagnosis to a median rank of subclinical diagnosis. See Table 4 for frequency data.

3.3. Secondary outcomes

Means and standard deviations for all 71 participants using LVBF secondary outcomes (DASS scales, Cognitive Reactivity, BAI) are reported in Tables 2 and 3. We examined whether there were group differences on cognitive reactivity scores, which are a vulnerability marker for depression. One pair of values was missing, as the participant did not complete the measure in its entirety at baseline. Participants in the probiotics group reported lower cognitive reactive

Table 2
Psychological measures for pre and post 8-week intervention and one month follow up for depressed participants.

	Probiotic	Placebo
BDI pre[a]	28.91 (10.10)	27.97 (9.79)
BDI post	19.88 (13.44)	19.25 (11.96)
BDI follow-up	18.50 (12.40)	19.42 (12.40)
DASS depression pre	22.88 (9.96)	20.43 (10.76)
DASS depression post	15.18 (11.03)	12.97 (9.34)
DASS depression follow-up	14.53 (11.22)	13.08 (10.57)
DASS anxiety pre	12.29 (8.62)	13.51 (10.15)
DASS anxiety post	6.94 (9.25)	8.81 (7.45)
DASS Anxiety follow-up	7.35 (9.36)	9.08 (8.80)
DASS stress pre	22.82 (10.61)	21.49 (8.60)
DASS stress post	15.29 (12.42)	14.16 (11.12)
DASS stress follow-up	14.65 (11.73)	15.51 (11.85)
Cognitive Reactivity pre[a]	63.94 (19.90)	66.68 (16.98)
Cognitive Reactivity post	55.24 (21.64)*	61.22 (13.86)
Cognitive Reactivity follow-up	52.91 (22.51)	60.49 (16.40)
BAI pre	17.94 (11.56)	18.03 (9.80)
BAI post	12.59 (11.27)	13.84 (9.11)
BAI follow-up	11.82 (11.19)	14.16 (11.40)

* $p < .05$.
[a] *One pair of values missing. BDI – Beck Depression Index, DASS – Depression Anxiety Stress Scale, BAI – Beck Anxiety Index.* Results reported as Mean (SD).

scores following intervention than those in the placebo group ($F(1,67) = 4.21$, $p = .04$, seen in Table 2. Subgroup analyses revealed that participants in the mild to moderate severity range at baseline reported lower cognitive reactivity scores following intervention, while participants in the severe range did not ($F(1,30) = 7.89$, $p = .01$, and $F(1,33) = 0.02$, $p = .89$ respectively). This result can be seen in Table 3, denoted by an asterisk.

There was no significant main effect of group on anxiety (BAI), or on the depression, anxiety, stress scale scores on the DASS-21 ($ps > 0.05$). There was also no effect of group (probiotics, placebo) within the subgroup analyses (mild/moderate, severe) on either the BAI or DASS-21, ($ps > 0.05$).

3.4. One-month follow-up analyses

Analyses of one-month follow-up data revealed no significant between group differences on follow-up versus post data on BDI, BAI, cognitive reactivity and DASS scores ($ps > 0.05$). There were also no differences between groups on the BDI, BAI, and DASS when conducting subgroup analyses. The follow-up M.I.N.I analysis has previously been discussed in the aforementioned primary outcomes section. Interestingly, when conducting subgroup analyses for cognitive reactivity scores, there was a trend, where participants in the severe (but not mild/moderate group) group who received probiotics reported lower cognitive reactivity scores at one month follow-up compared to post-test scores, relative to those consuming the placebo ($F(1,34) = 0.3.69$, $p = .06$). However, this trend disappears when controlling for the number of previous diagnoses of depression ($F(1,32) = 2.15$, $p = .15$).

3.5. Clinical trial–microbiota data

43 participants provided both a pre and post stool sample, which resulted in microbiota data that passed quality filtering. Of these, 22 were from the probiotic group and 21 from placebo. Details of general sequencing results are provided in the supplementary material (2.1). Overall, no significant differences were detected between groups for participant demographics or the M.I.N.I, BDI-II, DASS-21, BAI and LEIDS-R psychological test scores before treatment (ANOVA, $ps > 0.05$).

Within the microbiota, no significant differences were found for alpha diversity (number of distinct taxa, Wilcoxon Rank Sum test $ps > 0.05$) or beta diversity (community composition, PERMANOVA test $ps > 0.05$) between the pre and post treatment samples within both placebo and probiotic groups, or between groups.

Tests for differential relative abundance of bacterial taxa also found no significant differences between groups at either pre or post

Table 3
Psychological test scores for pre and post probiotic or placebo treatment for depressed participants and one month follow up, split into groups based on BDI severity.

	Probiotic	Placebo	Probiotic	Placebo
	Mild/Moderate	Mild/Moderate	Severe	Severe
Number	15	18	19	18
BDI pre	19.80 (1.38)	20.00 (1.14)	36.11 (1.46)	35.94 (1.47)
BDI post	13.87 (10.86)	13.50 (8.74)	24.63 (13.62)	25.00 (12.18)
BDI follow-up	14.13 (9.29)	13.56 (8.85)	21.95 (13.38)	25.28 (12.86)
DASS depression pre	16.13 (2.62)	13.44 (1.86)	28.21 (1.34)	26.89 (2.13)
DASS depression post	9.60 (9.17)	8.67 (6.93)	19.58 (10.55)	16.33 (9.39)
DASS depression follow-up	11.33 (11.90)	8.22 (7.06)	17.05 (10.27)	17.00 (11.34)
DASS anxiety pre	7.73 (1.76)	7.00 (1.34)	15.89 (1.91)	20.11 (2.30)
DASS anxiety post	3.33 (4.10)	4.67 (4.06)	9.79 (10.87)	12.78 (8.092)
DASS anxiety follow-up	4.00 (5.86)	5.11 (5.95)	10.00 (10.83)	12.89 (9.80)
DASS stress pre	15.87 (2.09)	16.33 (1.60)	28.32 (2.09)	27.17 (1.55)
DASS stress post	10.00 (8.75)	10.11 (9.71)	19.47 (13.46)	18.33 (11.46)
DASS stress follow-up	10.40 (7.97)	10.00 (9.82)	18.00 (13.27)	21.22 (11.56)
Cognitive Reactivity pre	54.93 (5.04)	54.72 (2.75)	71.44 (4.09)	78.17 (3.15)
Cognitive Reactivity post	45.00 (20.72)*	53.78 (10.47)	63.78 (18.92)	67.89 (13.43)
Cognitive Reactivity follow-up	41.87 (5.40)	51.11 (14.07)	61.63 (20.16)	69.06 (13.72)
BAI pre	13.47 (2.41)	12.17 (1.28)	21.47 (2.78)	24.06 (2.34)
BAI post	9.20 (6.20)	10.11 (7.51)	15.26 (13.63)	17.50 (9.51)
BAI follow-up	9.33 (7.72)	10.72 (11.29)	13.79 (13.62)	17.56 (11.09)

Two groups based on initial depression severity on the BDI-II. The sample was split into mild/moderate severity (scores from 12 to 28) and severe (scores >28).
An ANCOVA revealed no significant main effect of group (probiotics, placebo) in either the mild/moderate or severe subgroups for BDI, DASS or BAI scores, ps > 0.05.
BDI – Beck Depression Index, DASS – Depression Anxiety Stress Scale, BAI – Beck Anxiety Index. Results reported as Mean (SD).
* *p < .05.*

Table 4
Frequency changes in percentage of participants with diagnosis of depression based on M.I.N.I psychiatric interview.

	Probiotic	Placebo
Before Treatment% (*n*)		
No Diagnosis	20.6 (7)	27.0 (10)
Subclinical	38.2 (13)	40.5 (15)
Clinical	41.2 (14)	32.4 (12)
After Treatment% (*n*)		
No Diagnosis	32.4 (11)	29.7 (11)
Subclinical	38.2 (13)	45.9 (17)
Clinical	29.4 (10)	24.3 (9)
One Month Follow Up% (*n*)		
No Diagnosis	50.0 (17)	35.1 (13)
Subclinical	32.4 (11)	40.5 (15)
Clinical	17.6 (6)	24.3 (9)

treatment time-points (Kruskal Wallis, *ps* > 0.05). Similarly no significant differences were detected between time-points within each group (Kruskal Wallis, *ps* > 0.05).

3.6. Relative abundance of genera contained in the probiotic treatment

To determine if the bacterial taxa in the probiotic treatment could be detected in the gut microbiota, the relative abundance of the genera consistent with those in the probiotic mixture used in the trial, *Bifidobacteria, Lactobacillus* and *Lactococcus*, were compared in pre and post treatment samples for the probiotic treatment group. After probiotic treatment no significant difference was detected for the *Lactobacillus* genus and *Bifidobacteria* genus (Wilcoxon Rank Sum test *ps* > 0.05). The *Lactococcus* genus was not detected in the 16S rRNA gene data and therefore significance could not be tested.

3.7. Comparisons between depressed and non-depressed groups

The psychological data for both the depressed and non-depressed groups can be found in the supplementary material (2.2 Table 3). As expected, participants in the depression group showed significantly higher mean scores on measures of depression, anxiety, stress and cognitive reactivity, as measured by BDI-II, BAI-II, DASS-21 Depression, Anxiety and Stress subscales, and LEIDS-R. Additionally, healthy participants were confirmed to be in the normal ranges for these psychological tests.

For the comparison between depressed and non-depressed gut microbiota, faecal samples from the clinical trial participants prior to starting either probiotic or placebo treatment were compared to samples from the 20 non-depressed participants. Three of the 71 enrolled participants were removed from this comparison as they did not provide a stool sample ($n = 1$) or had low sequence coverage ($n = 2$). Therefore, a total of 68 depressed participants were included.

The gut microbiota for all participants was dominated by two phyla, the Bacteroidetes (total mean 47.42% ± 17.63) and Firmicutes (total mean 49.66 ± 14.96). However, there were no significant differences in the relative abundance between groups at any taxonomic level. No significant differences were found in alpha diversity (Wilcoxon Rank Sum, $p > .05$). Similarly, no significant difference in community composition (beta diversity) (PERMANOVA, $p > .05$) was found between the depressed and non-depressed groups.

The depressed cohort was then subdivided based on BDI severity levels into two groups corresponding to severe (BDI > 28, $n = 36$) and mild/moderate (BDI = 12–28, $n = 31$). No significant differences were detected between controls and any of the severity levels of depression for alpha diversity, beta diversity, or the relative abundance of any taxa. Additionally, no significant differences were found between the two depressed sub-groups (Kruskal Wallis, *ps* > 0.05).

The relative abundance of OTUs and bacterial taxa were compared to psychological test scores using Spearman's correlation. An OTU classified as *Ruminococcus gnavus* (OTU ID = 360015) had a significant (Spearman's correlation $p = .04$) and positive correlation (0.37) to the DASS depression score. The relative abundance of this OTU was low with a maximum of 2.79% of sequences per sample. This OTU was present in 72% of the depressed participants compared to only 25% of non-depressed, and was found in higher relative abundance in the severe BDI range of depression (mean relative abundance 0.33 ± 0.72) compared to both the mild/moderate depressed range (mean relative abundance 0.13 ± 0.42) and the non-depressed group (mean relative abundance 0.0041 ± 0.013) (Fig. 2).

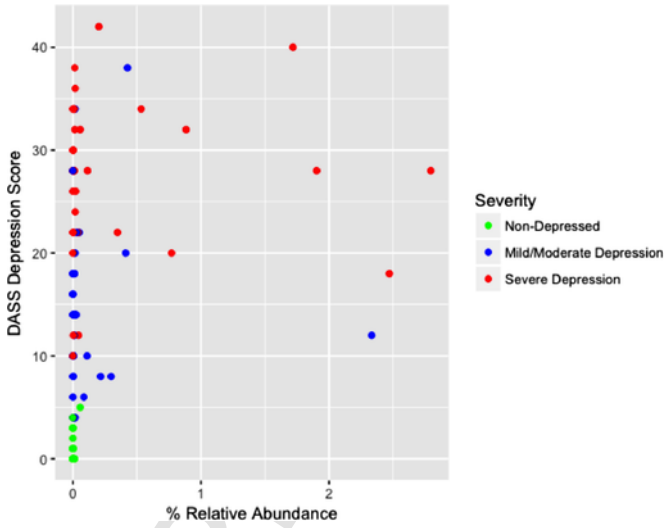

Fig. 2. Scatter plot of the relative abundance of an OTU identified as R. gnavus with corresponding DASS depression scores.

4. Discussion

In this study we investigated the effectiveness of the multispecies probiotic Ecologic® Barrier for reducing symptoms in adults with mild to severe levels of depression. This study was built on emerging literature linking the gut microbiota with mental wellbeing and examined the utility of probiotics as a potential mechanism to improve mental health. Overall, all participants across both probiotic and placebo groups exhibited a reduction in depressive symptoms over the time-period of the trial; levels of depression as measured through BDI-II scores were found to have decreased between pre-intervention and post-intervention assessment. These results suggest that the routine involved with daily preparation and consumption of the probiotic and scheduled appointments, as well as involvement in these behaviours with the aim of seeking improvement in depressive symptoms had positive impacts on mood, irrespective of whether the probiotic or placebo was consumed. This is in line with the evidence suggesting that routines and engagement in planned activities is beneficial for reducing symptoms of depression (Cuijpers et al., 2007), which forms the basis of activity scheduling as a component of CBT for depression (Veale, 2008).

In contrast to our hypotheses, the probiotic group did not demonstrate greater levels of reduction in depressive symptoms compared to the placebo group. Similarly, there was no significant difference in the number of participants with clinical and sub-clinical diagnoses of depression between the two groups at post-intervention assessment. These findings indicate that these probiotics alone are not an effective treatment option for symptom reduction, with any positive effect equal to that produced by the placebo. Similar results were observed for measures of anxiety and stress. Further analysis was conducted after separating participants into mild/moderate and severe levels of depressive symptoms at pre-intervention assessment. Again, no significant differences between groups were observed in measures of depression, anxiety, or stress at any level of severity.

However, a significant effect was observed between the probiotic and placebo groups, particularly in the mild/moderate depression severity subgroup on a measure of cognitive reactivity towards sad mood, which is a vulnerability marker of depression (Kruijt et al., 2013; Steenbergen et al., 2015; Van der Does, 2005). Participants in the probiotic group reported lower cognitive reactivity after the intervention compared to the placebo group. Subgroup analysis revealed that participants in the mild/moderate subgroup who received

probiotics reported lower cognitive reactivity scores following intervention compared to those in the placebo group. This effect was not seen in participants with severe levels of depression. This is consistent with work by Steenbergen and colleagues, where probiotic supplementation was associated with positive effects on cognitive reactivity in a sample of non-depressed individuals. Results from the current study provides further evidence that some probiotic mixtures can influence thinking and cognition (Steenbergen et al., 2015). Although probiotics did not appear to have had a direct effect on depressive symptoms, our results suggest that probiotics potentially act on cognitive processes contributing to depression. Specifically, the cognitive patterns measured in this study are noted to predict and are associated with depression (Antypa et al., 2010; Moulds et al., 2008); implications for future research are that a longer trial may reveal differences between groups in depressive symptoms as changes in cognition translate to changes in mood over time. Interestingly, follow-up analyses indicated that one month after completing the intervention, participants in the probiotics but not placebo group were more likely to move from a subclinical diagnosis to no depression diagnosis. In particular, participants in the severe probiotics group showed a significant change in diagnostic level from pre to follow-up assessment with a tendency to move from a clinical to a subclinical diagnosis over these time points. One account for these follow-up findings is that as suggested earlier, the effect of probiotics may present over a longer time period than the 8-week intervention period. A longer trial may be needed to more fully assess the effect of probiotics on mood. Yet, there is no consensus on how long a probiotic trial should run (Wallace and Milev, 2017). Alternatively, the reported changes may be more reflective of natural or spontaneous recovery with time.

Taken together, the results of the current study and that of Steenbergen and colleagues indicate that the impacts of probiotics may be more prominent in individuals with lower levels of depression severity (Steenbergen et al., 2015). Further research is needed into qualitative and quantitative differences between individuals with mild/moderate and severe levels of depression, the potential impact of probiotics for varying severity levels, and the utility of probiotics as a preventative measure for depression.

In general, no significant differences in the feacal microbiota were observed between pre- and post-intervention subjects, in either probiotic or placebo groups. However analysis revealed a significant positive correlation between one OTU, *R. gnavus* and the DASS depression score. No other depression microbiota study has reported *R. gnavus* to be associated with depression, conversely; one microbiota study found the *Ruminococcus* genera decreased in depressed populations (Jiang et al., 2015). In contrast, *R. gnavus* was observed to be increased in Crohn's disease (Joossens et al., 2011). In vitro *R. ganvus* produces ß-Glucuronidase, which is involved in toxin generation that could contribute to local inflammation (Beaud et al., 2005). However, the relative abundance of this OTU is very low so the biological significance is unclear, further testing is required to establish if this bacterium plays a role in depression. There is current contention on whether probiotic treatments do successfully alter microbiota composition (e.g. (Cha et al., 2012; Kristensen et al., 2016)). However, probiotics have been shown to modulate gut microbiota gene expression in the absence of compositional changes, with potential anti-inflammatory effects (Eloe-Fadrosh et al., 2015). This is one potential mechanism by which probiotics may affect cognitive function. Alternatively, probiotic bacterial species may exert effect on the host directly, as Ecologic® Barrier probiotics have been shown *in vitro* to improve gut barrier function (Van Hemert and Ormel, 2014).

The dosage size of the probiotic may not have been sufficient to be detected in the stool but still have resulted in the psychological effects seen in the study. For example, it was shown that probiotic supplementation of *Lactobacillus rhamnosus* GG at 10^8 CFU was detectable in only 1 of 10 faecal samples, however this same strain at a higher dose of 10^{12} CFU was detected in all 10 faecal samples (Saxelin et al., 1995). The dose used in this study (10^{10} cfu/day) is above the minimum dose requirement for probiotics without strain specific claims (Hill et al., 2014), and the probiotic supplementation was double that of the dose used with healthy controls in Steenbergen et al. (2015). Further research using a range of concentrations in a dose response study may be warranted to determine the optimal dose. Potentially, a greater dose, or longer consumption of probiotics would have produced a detectable change in gut microbiota, as well as further differences in psychological data between probiotic and placebo groups.

When comparing the gut microbiota of depressed individuals before intervention with a non-depressed cohort there were no significant differences between groups, inconsistent with previous literature (Jiang et al., 2015; Kelly et al., 2016; Lin et al., 2017; Naseribafrouei et al., 2014; Zheng et al., 2016). This may be due to differences in the populations surveyed in terms of geography and diet, which have been shown to have an impact on the gut microbiota (Mueller et al., 2006), or differences in inclusion/exclusion criteria. It is important to note that all previous studies exploring differences in the gut microbiota in depressed humans included many depressed participants taking antidepressant medications. Some antidepressants have been shown to be anti-microbial, and any form of medication is a confounding factor in a gut microbiota study (Ayaz et al., 2015; Coban et al., 2009; Devkota, 2016; Kristiansen, 1990; Kruszewska et al., 2012; Lieb, 2004; Munoz-Bellido et al., 2000). In this study, participants were excluded if they took any type of medication that could influence mood or gut functioning (including antidepressants), and no changes in the gut microbiota were observed. Further investigation into microbiota associations with depression in humans should be undertaken which specifically considers possible confounding factors such as medication.

One of the major limitations of this study is the attrition rate of 34%. Aside from occurrences where patients were removed for no longer meeting inclusion/exclusion criteria (for instance taking medications during the trial), the cause of the high attrition rate was most likely due to the requirement for depressed participants to attend weekly monitoring visits. Similar probiotic studies where weekly visits were not required had lower attrition rates (Rao et al., 2009; Romijn et al., 2017). Further, increasing the frequency of visits in clinical trials of antidepressants has been associated with higher attrition rates in depressed populations (Rutherford et al., 2013). Additionally, non-compliance to medical treatment recommendations is much more likely in depressed cohorts compared to non-depressed patients (DiMatteo et al., 2000), thus, they are more likely to drop-out of clinical trials. In the current study, weekly visits were implemented due to ethics board requirements for weekly participant safety monitoring, with utilisation of intention-to-treat analysis to diminish attrition bias (Jüni et al., 2001; May et al., 1981; Sackett and Gent, 1979). However, future research should consider alternative monitoring methods such as Skype or phone reviews, rather than face-to-face.

Overall, this study offers evidence to indicate that probiotic consumption can exert change on cognitive patterns associated with depression. The study may have benefitted from the inclusion of more sensitive measures of physiological stress, such as cortisol analysis of urine, saliva or blood samples to complement participant's self-report scores. Nevertheless, these preliminary results are promising and offer a number of future research and clinical avenues to build upon. For instance, future research would benefit from additional analysis methods to investigate specific gut microbiota strains, as well as examination of how to maximise the benefits of probiotics, for example dosages and timeframes. In clinical practice, probiotics may be a useful adjunct to potentiate the effects of therapies, such as CBT, which involves changing cognitive patterns. Finally, the use of probiotics promotes the concept of managing physical health as part of mental health

treatment; this holistic view may be a perspective that greatly improves treatment acceptability for individuals with depression.

Declaration of interest

Saskia van Hemert is an employee of Winclove Probiotics.

Conflict of interest

Author Saskia van Hemert Saskia van Hemert is an employee of Winclove Probiotics. All other authors declare that they have no conflicts of interest.

CRediT authorship contribution statement

Bahia Chahwan: Data curation, Formal analysis, Writing - original draft. **Sophia Kwan:** Data curation, Methodology, Writing - original draft. **Ashling Isik:** Data curation, Formal analysis, Writing - original draft. **Saskia van Hemert:** Writing - original draft. **Catherine Burke:** Data curation, Formal analysis, Writing - original draft. **Lynette Roberts:** Data curation, Formal analysis, Writing - original draft, Methodology.

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Supplementary materials

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