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ORIGINAL RESEARCH

Randomized Clinical Trial Examining the Impact of *Lactobacillus rhamnosus* GG Probiotic Supplementation on Cognitive Functioning in Middle-aged and Older Adults

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Purpose: The gut microbiome has been linked to cognitive function and appears to worsen with aging. Probiotic supplementation has been found to improve the health of the gut microbiome. As such, it is possible that probiotic supplementation may protect the aging brain. The current study examined the cognitive benefits of probiotic supplementation (*Lactobacillus rhamnosus* GG) in healthy middle-aged and older adults.

Materials and Methods: The study was a double-blind, placebo-controlled, randomized clinical trial. Two hundred community-dwelling adults aged 52–75 were enrolled (mean age=64.3, SD=5.52). A three-month intervention involved daily consumption of probiotic or placebo. Independent sample *t*-tests, chi-squared tests, and repeated measure ANOVAs compared groups and examined changes over time. Primary outcome was change in NIH Toolbox Total Cognition Score from baseline to follow-up.

Results: A total of 145 participants were examined in primary analyses (probiotic=77, placebo=68) and excluded persons due to discontinuation, low adherence, missing data, or outlier values. Established criteria (ie ≥1 subtest t-scores ≤35; n=19, n=23) were used to operationally define cognitive impairment. Repeated measures ANOVAs revealed that persons with cognitive impairment who consumed probiotics exhibited a greater total cognition score improvement than persons with cognitive impairment in the placebo group and cognitively intact persons in probiotic or placebo groups.

Conclusion: *Lactobacillus rhamnosus* GG probiotic supplementation was associated with improved cognitive performance in middle-aged and older adults with cognitive impairment. Probiotic supplementation may be a novel method for protecting cognitive health in aging.

Keywords: cognitive aging, dementia, microbiota, gastrointestinal microbiome, probiotics

Introduction

Cognitive Aging

Decline in mental abilities is normal with advancing age and coincides with changes in brain structure and function, including reductions in global and regional brain volume.[1–4] These changes are known to be mitigated by education[5] and physical activity,[6] though cognitive aging cannot be avoided.

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Gut Microbiota as Risk Factor for Cognitive Decline

There is increasing reason to believe that gut microbiota may be an important contributor to cognitive aging.[7,8] The term gut microbiota refers to the 10–100 trillion symbiotic microbial cells living in the human gut and the term gut microbiome refers to the catalog of their nucleic acids (DNA and RNA molecules).[9] Four major phyla of gut bacteria are present in mammals, namely: *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, identified through microbial DNA sequencing.[10] The primary microbial phyla in humans are *Firmicutes* and *Bacteroidetes* and comprise up to 90% of our gut microbiome.[11] Dysbiosis refers to a state of the gut microbiota in which the proportions of bacteria are atypical, resulting in a disease-promoting state.[12] Gut dysbiosis can be readily detected in both human and animal models, including the comparison of stool samples of lean with obese individuals or individuals who eat a healthy diet with those who eat a high fat/Western diet.[13,14] More importantly, gut dysbiosis has been associated with a number of medical conditions including inflammatory bowel disease (IBD),[15,16] cardiovascular disease,[17] metabolic disorders,[18,19] and autoimmune disorders,[20,21] each of which has been associated with poorer cognitive function.[22–25] Further, the aging process is associated with progressive decline in gut microbiota diversity and proportions of core microbiota.[26]

Gut-brain-microbiome Axis

The gut, the gut microbiota, and the brain form an interconnected system of processes and communication referred to as the gut-brain-microbiome axis. This complex system involves bidirectional signaling through several pathways, including immune responses,[27] the vagus nerve,[28] enteroendocrine cells,[29] and metabolites that influence the production of neurotransmitters.[30] These pathways provide signaling information among microbiota, the gut, and the brain regarding a wide range of processes, including inflammation and satiety, as well as complex behaviors like social isolation or repetitive movement.[31] The gut microbiota appears to influence the brain by altering axis signaling through bile acids,[7,32] inflammatory markers,[31,33] and metabolites.[31,34] These altered signals lead to changes in key neurochemical processes. Certain bile acids have been associated with brain volume, amyloid beta deposition,[7] and later development of Alzheimer's disease.[35] Short-chain fatty acid (SCFA) metabolites modulate neurotransmission impacting synthesis of noradrenaline and dopamine,[30] and also induce neuronal nerve activation.[34]

Can Probiotics Improve Cognitive Function?

Given the influence of the gut microbiome on the brain, modification of the gut microbiome through probiotic supplementation may protect against cognitive impairment. Probiotics are living microorganisms which, when administered, provide health benefit to the host.[36] Fermented foods can contain beneficial bacteria, which could be considered probiotics. Those include sauerkraut, pickles, yogurt, and miso,[37] though the ease and convenience of taking probiotic supplements containing larger proportions of bacteria appears to be more appealing for some individuals.[10]

A particularly promising strain of probiotic is *Lactobacillus rhamnosus* GG. This bacterium is known for its rapid growth, adhesive properties, and bile resistance[38] allowing it to remain in the gut longer and exert a greater influence than other strains, including protecting the gut lining.[39] Along the gut-brain-microbiome axis, strains of *Lactobacillus* probiotics have been associated with reduced inflammatory cytokines,[40] enhanced levels of cAMP response element binding protein (CREB) and brain derived neurotrophic factor (BDNF) in the hippocampus of rats,[41] and reduced kynurenine metabolites.[42] *Lactobacillus rhamnosus* GG itself has been found to protect intestinal epithelial cells[43] and reduce inflammatory markers such as interleukin-8 (IL-8).[44] It has also been found to improve metabolic factors including glucose tolerance, insulin-sensitivity, adiposity,[45] and inflammation.[46,47] Through these direct and indirect mechanisms, *Lactobacillus rhamnosus* GG may impact brain health and cognitive function. In fact, *Lactobacillus rhamnosus* GG supplementation has been associated with reduced anxiety-like,[48] obsessive compulsive disorder-like,[49] and depressive behaviors[50] in mouse models and has been associated with reduced risk of developing neuropsychiatric disorders in children.[51]

Although previous research has examined the role of *Lactobacillus rhamnosus* GG on physical and psychological outcomes, little is known about its potential impact on cognitive function. One RCT examined the possible cognitive benefits of eight weeks of *Lactobacillus rhamnosus* GG supplementation on cognitive function in young adult

males. No significant changes for inflammatory markers, stress-related anxiety behaviors, or performance on cognitive function were shown in that sample.[52] However, cognitive improvement in a healthy young sample may be unlikely due to range restriction and further investigation on the effects of *Lactobacillus rhamnosus* GG in samples of persons at risk for cognitive impairment is needed.

Current Study

The current study investigated whether *Lactobacillus rhamnosus* GG probiotic supplementation could be associated with improved cognitive function in community-dwelling middle-aged and older adults. Two hundred individuals were recruited into a double-blind RCT. Cognitive function was assessed at baseline and following three months of supplementation of either probiotic or placebo. It was hypothesized that *Lactobacillus rhamnosus* GG supplementation would be associated with improvements in cognitive performance both in persons with and without evidence of cognitive impairment.

Materials and Methods

All data was obtained in compliance with the regulations set forth by the Kent State University Institutional Review Board (approval no. #16-321) and was conducted in accordance with the Declaration of Helsinki. All participants were informed of the purpose of the study. Recruitment and data collection were partly financially supported by i-Health, Inc., a division of Royal DSM. All participants provided written consent acknowledging that any published work would not include identifying information. Participant data has been fully anonymized. All safety precautions (ie university physician oversight, adverse event reporting, eligibility screening for individuals at risk for physical discomfort/symptom exacerbation by probiotic use, participant written agreement to discuss with treating physician before participation) were determined prior to study onset, approved by the Kent State University Institutional Review Board, and adhered to throughout the project. Study methods have been described in detail previously.[53] Briefly, we conducted a parallel, double-blind, placebo controlled, RCT with a 1:1 allocation ratio. See Supplementary Table 1. The study was listed in advance of participant recruitment through clinicaltrials.gov (study no. NCT03080818). Two hundred healthy, middle-aged and older adults (aged 52–75) were recruited from the local community through fliers and advertisements. Sample size was predetermined through power analyses using G*Power 3.0.10 software.

Persons were excluded if they reported history of developmental, neurological, or severe psychiatric disorder, recent consumption of antibiotics, acid-blocking medication, prebiotic, or probiotic supplements, past alcohol or illicit drug dependence, history of severe heart, liver, or kidney problems, immunosuppression, or severe gastrointestinal conditions. Participants were randomized to a study group using a computerized number generator by the principal investigator (JG). The principal investigator was the only staff member aware of participant group assignment and prepared capsules for distribution in advance using unmarked containers. Other research staff, responsible for recruitment and study testing, and participants were blind to group assignment by using unmarked containers and restricted access to randomization documentation. To increase similarity between control and intervention procedures, all participants completed the same study protocol and placebo and probiotic capsules were identical in appearance and packaging.

Lactobacillus rhamnosus GG was selected for two reasons. Although previous research has encouraged the use of multi-strain probiotic supplementation,[54] it is unclear whether *Lactobacillus rhamnosus* GG as a single-strain or multi-strain confers greater benefit for physical and psychological outcomes.[55] Examining *Lactobacillus rhamnosus* GG in isolation also allowed examination of its independent effects on cognitive function, which would be difficult to accomplish in the presence of other probiotic strains. Intervention included Culturelle Vegetarian Capsules containing a 10 billion CFU blend of *Lactobacillus rhamnosus* GG (manufactured by iHealth, Inc., Cromwell, CT, USA) for the experimental group and Culturelle Placebo Veggie capsules containing microcrystalline cellulose for the control group. Participants were instructed to take two capsules daily.

For the study, participants completed telephone eligibility screening, baseline testing, two adherence visits, and follow-up testing. Study visits were conducted at a local retirement community in Northeastern Ohio to promote convenience . Participants were enrolled for approximately 90 days including baseline visit, adherence visits once a month for the following two months and follow-up visit. Baseline and follow-up visits involved brief medical interview, physical measures, computerized neuropsychological assessment using the NIH Toolbox Assessment of Neurological and Behavioral Function—Cognition battery, provision of capsules, and compensation. The NIH Toolbox Total Cognition

Score (ie composite index score based on age, sex, education, race, and mother's education reflecting performance on all cognitive subtests) was used as the primary outcome. NIH Toolbox was selected for its strong psychometric properties,[56,57] close association with traditional pencil-and-paper neuropsychological tests,[58] and ability to assess healthy samples like those found in the current study.

Adherence visits involved brief medical interview, count of nonconsumed capsules, provision of new capsules, and compensation. Study adherence was calculated by dividing self-reported number of capsules consumed by total number of capsules provided and multiplying that value by 100 to obtain a percentage. The primary outcome was the possible change in cognitive function at follow-up across persons randomized to probiotic vs placebo.

Data Analysis

Preliminary Analyses

All participants with missing data, low adherence (ie <80%), and/or incomplete study visits were excluded from analyses. Normality was assumed for variables with skewness <2.0 and kurtosis <6.0. Potential outliers were identified by examining boxplot graphs and clarified using the approach proposed by Iglewicz and Hoalgin[59] for each variable (ie creating $z_{modified}$ transformation scores and removing any values >3.5). Per protocol (PP) and intention-to-treat (ITT) analyses were conducted to determine any significant difference in dropout rates between probiotic and placebo groups.[60,61] To identify possible between-group differences between those who were retained or excluded, independent samples *t*-tests and chi-square d tests compared groups on age, education, sex, BMI, ethnicity, medical history (ie diabetes, hypertension, sleep apnea, anxiety/depression) and baseline NIH Toolbox scores. Finally, Petersen/Winblad criteria (ie one or more NIH Toolbox t scores at or below 35) was used to operationally define cognitive impairment using baseline test results including subtest scores and total composite score.

Hypothesis Testing

A 2 (probiotic vs placebo)×2 (cognitive impairment vs intact)×2 (baseline to follow-up) repeated measures ANOVA was used to identify possible changes in total cognition score over time by the cognitive group. This analysis was then repeated for each individual NIH Toolbox subtest score to clarify any significant omnibus test.

The effect of time, intervention group, and cognitive status were examined independently, as well as multiple interactions (ie time×group, time×cognitive status, time×group×cognitive status).

To minimize potential impact of practice effects, reliable change indices (RCI; ie standardized z-scores) were calculated for individual subtest scores and total cognition score for each of the four final subgroups (ie probiotic vs placebo and intact vs impaired).[62] Steps for these calculations included: (1) calculation of standard error values for each cognition score using the square root of 1 minus the published reliability of each test,[61] (2) calculation of standard error of the difference values for each cognition score using the square root of two times the squared value of the standard error, and (3) calculation of the absolute value of change in each score divided by the standard error of the difference. RCIs (ie, z-scores reflecting standardized amount of change) calculated for subtest and total cognition scores with an absolute value greater than 1.96, reflecting less than 5% chance that scores were due to standard error,[62] were considered to reflect significant change.

Results

Data Cleaning

Recruitment began in May 2017 and ended in September 2019. From the original sample of 200 participants, data from 52 individuals were excluded from primary analyses due to discontinuation (n=15), missing cognitive data (n=28), missing medical history data (n=2), or low compliance (ie <80%; n=7). Reasons for study discontinuation included reported gastrointestinal symptoms (n=6), extenuating personal circumstances (n=1), starting antibiotics (n=1), abnormal liver enzyme panel (n=1), or lost to follow-up (n=6). See Figure 1. At the entire group level (n=148), no outliers for cognitive scores were identified (all $z_{modified}$ <3.5). When examining intervention groups separately ($n_{probiotic}$=78, $n_{placebo}$=70), one outlier was identified and removed from the probiotic group. When examining those with evidence of cognitive impairment (n=44), two outliers were identified and removed. See Table 1 for demographic and medical characteristics of retained study participants (n=145; $n_{probiotic}$=77, $n_{placebo}$=68).

Of the 55 participants excluded from primary analyses, all had complete data for age, education, ethnicity and gender while only 45 had complete data for BMI, 34 had complete data for cognitive subtests, and 32 had complete data for total cognition score. Incomplete cognitive data was due to

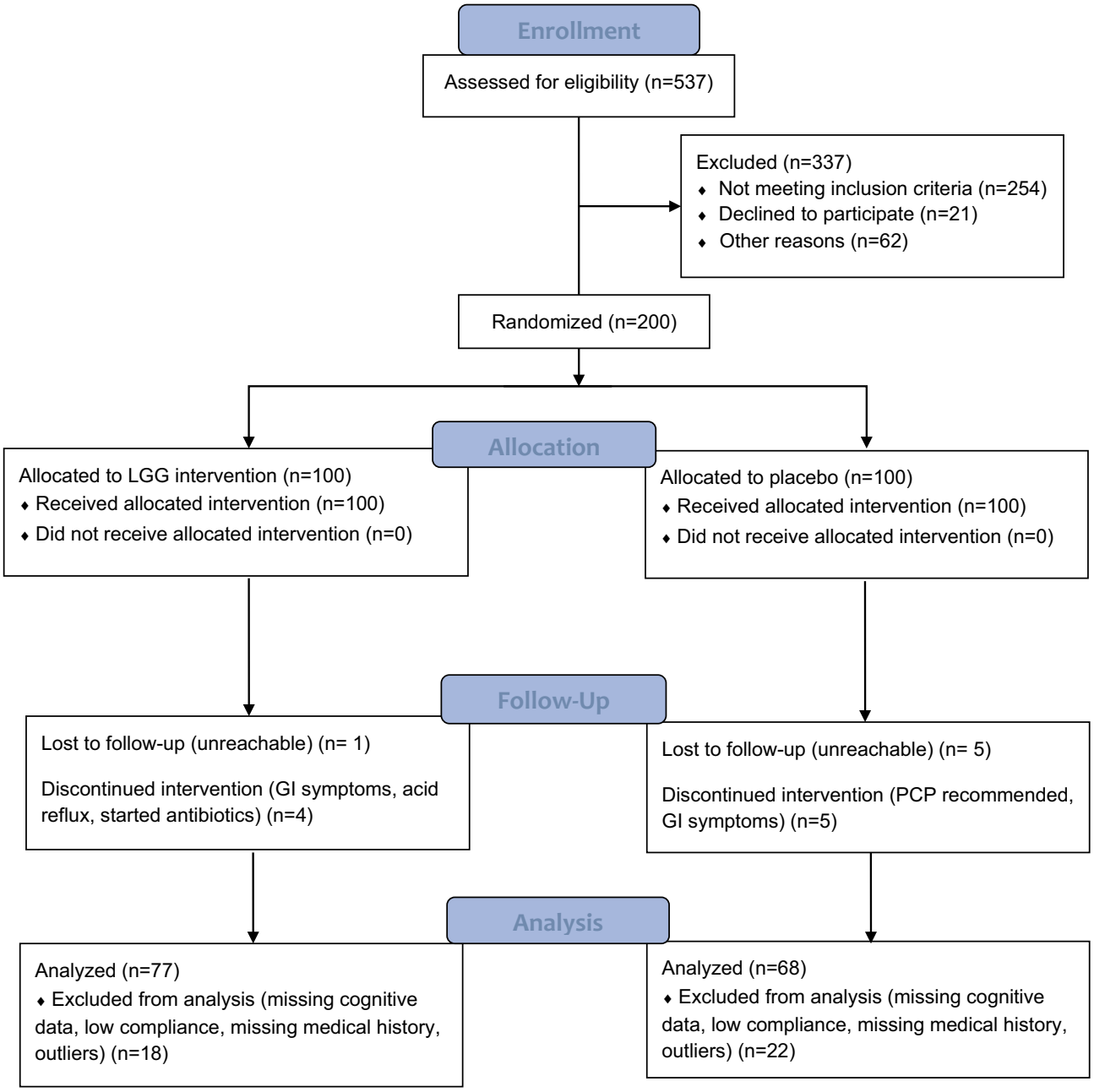

Figure 1 CONSORT (Consolidated Standards of Reporting Trials) flowchart. 2010 CONSORT Flow Diagram, Adapted from Schulz, KF, Altman DG, Moher D, for the CONSORT Group. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. *Obstet Gynecol.* 2010;115(5):1063–70.[63]
Abbreviations: LGG, *Lactobacillus rhamnosus* GG probiotic supplementation; GI, gastrointestinal; PCP, primary care physician.

a malfunction with a tablet used to administer testing. Excluded participants were not significantly different from included participants in demographic, medical, or cognitive function characteristics (all $p>0.05$; See Table 2).

Sample Characteristics

PP analysis showed that no differences in dropout rates emerged between probiotic (6%) and placebo groups (13%). This was confirmed through ITT analysis, which showed comparable dropout rates between probiotic (5%) and placebo groups (10%). These results suggest *Lactobacillus rhamnosus* GG was well-tolerated ($p>0.05$).

Within the probiotic supplementation group, participants that dropped out (M=13.0, SD=0.71) reported fewer years of education than those who completed the study (M=15.1, SD=2.59; t=4.95, df=13.2, $p<0.01$). No other differences emerged in demographic, medical, or cognitive variables (all $p>0.05$). Similarly, no differences

Table 1 Demographic, Medical, and Cognitive Characteristics by Study Group (n=145)

	Entire Sample (n=145)		Probiotic (n=76)		Placebo (n=69)	
	M/% (range)	SD	M/% (range)	SD	M/% (range)	SD
Demographics						
Age	64.4	5.44	64.6	5.58	64.1	5.32
BMI	27.9	6.56	27.5	6.88	28.3	6.21
Education	15.2	2.46	15.1	2.52	15.3	2.40
Gender (female)	59.3%	–	53.9%	–	65.2%	–
Ethnicity						
Caucasian	97.0%	–	100%	–	94.0%	–
Black	2.00%	–	0.00%	–	5.00%	–
Other	1.00%	–	0.00%	–	1.00%	–
Medical history						
Depression/anxiety	22.0%	–	19.7%	–	24.6%	–
Sleep apnea	9.66%	–	5.26%	–	14.5%	–
Diabetes	8.28%	–	6.58%	–	10.1%	–
Hypertension	27.6%	–	19.7%	–	36.2%	–
Cognition						
Picture	48.0 (28.0–84.0)	10.1	47.4 (29.0–78.0)	9.70	48.7 (28.0–84.0)	10.6
Flanker	43.1 (24.0–65.0)	6.94	43.7 (31.0–65.0)	6.60	42.4 (24.0–61.0)	7.28
Card sort	53.2 (29.0–81.0)	9.89	54.3 (35.0–75.0)	8.83	51.9 (29.0–81.0)	10.9
List sort	51.8 (28.0–73.0)	9.19	52.7 (28.0–70.0)	8.28	50.8 (33.0–73.0)	10.1
Pattern	47.9 (10.0–83.0)	13.1	48.6 (20.0–74.0)	11.8	47.2 (10.0–83.0)	14.5
Total	48.0 (27.0–78.0)	9.97	48.8 (27.0–76.0)	9.50	47.1 (27.0–78.0)	10.5

Abbreviations: Picture, picture sequence memory test; Flanker, flanker inhibitory control and attention test; Card sort, dimension change card sort test; List sort, list sorting working memory test; Pattern, pattern comparison processing speed test; Total, total cognition score.

emerged within the placebo group between those that dropped or remained (all $p>0.05$).

In the entire sample (n=145), there was a higher proportion of participants in the placebo group (36%) reporting hypertension than participants in the probiotic group (19%; $p=0.02$, Fisher's exact test). No differences emerged between probiotic and placebo groups for other variables of interest (all $p>0.05$; See Table 1). A significantly greater portion of participants with objective evidence of cognitive impairment (n=42) reported history of sleep apnea compared with cognitively intact participants (n=103). No other differences emerged between cognitively impaired and intact participants on medical or demographic characteristics (all $p>0.05$; See Table 3).

ITT analyses largely replicated PP analyses regarding baseline comparisons between intervention groups. For those with complete data, a significantly greater portion of participants in the placebo group had hypertension (40%) and sleep apnea (14%) than those in the probiotic group (23% and 6%). No significant differences in other demographic or medical characteristics emerged (all $p> 0.05$).

It was noted that though no participants reported taking medications that met exclusion criteria for the current trial (eg antibiotics, prebiotics), number of self-reported medications and supplements ranged from 0 to 13 in the total sample; for participants with cognitive impairment, 13 participants in the probiotic group and 16 in the probiotic group reported taking medications beyond vitamins/supplements. The most common medications included statins, beta-blockers, metformin, antidepressants, anxiolytics, nonsteroidal anti-inflammatories, and pain medications. Groups did not differ on the prevalence of medications and it was not utilized in primary analyses.

Lactobacillus rhamnosus GG Improves Cognition in Persons with Cognitive Impairment

PP analyses using repeated measures ANOVA found a significant group by cognitive status by time interaction for total cognition score ($F[1,141]=4.60$, $p=0.03$, $\eta_p^2=0.03$); see Table 4. Though all groups improved from baseline to

Table 2 Comparing Participants Who Were Excluded or Included in the Study

	Excluded (n=55)		Compared with Entire Sample (n=145)				
	M/% (Range)	SD	t	df	*p*	χ^2	Fisher's
Demographics (n=55)							
Age	64.0	5.77	−0.40	198	0.69	–	–
BMI (n=50)	29.3	6.57	1.39	196	0.17	–	–
Education	15.2	2.40	0.12	198	0.91	–	–
Gender (female)	63.6%	–	–	–	–	–	0.63
Ethnicity							
Caucasian	96.0%	–	–	–	–	0.68	–
Black	4.00%	–	–	–	–	0.68	–
Other	0.00%	–	–	–	–	0.68	–
Medical history (n=45)							
Depression/anxiety	33.0%	–	–	–	–	–	0.12
Sleep apnea	8.89%	–	–	–	–	–	1.00
Diabetes	17.8%	–	–	–	–	–	0.10
Hypertension	42.2%	–	–	–	–	–	0.10
Cognition (n=34)							
Picture	47.6 (27.0–83.0)	12.4	−0.19	177	0.85	–	–
Flanker	42.1 (29.0–55.0)	6.72	−0.75	177	0.46	–	–
Card sort	53.4 (34.0–77.0)	10.5	0.25	177	0.80	–	–
List sort	51.1 (30.0–76.0)	9.56	−0.42	177	0.68	–	–
Pattern	45.4 (17.0–77.0)	16.2	−0.99	177	0.32	–	–
Total (n=32)	46.6 (23.0–76.0)	10.9	−0.72	171	0.47	–	–

Abbreviations: Picture, picture sequence memory test; Flanker, flanker inhibitory control and attention test; Card sort, dimension change card sort test; List sort, list sorting working memory test; Pattern, pattern comparison processing speed test; Total, total cognition score.

follow-up, participants with cognitive impairment in the probiotic group ($M_{baseline}$=38.7, $M_{follow\text{-}up}$=47.6) showed significantly greater improvement in total cognition score than participants with cognitive impairment in the placebo group ($M_{baseline}$=37.7, $M_{follow\text{-}up}$=42.4) and participants without cognitive impairment in the probiotic ($M_{baseline}$=52.1, $M_{follow\text{-}up}$=54.5) and placebo ($M_{baseline}$=51.8, $M_{follow\text{-}up}$=54.6) groups. When comparing change in total cognition score from baseline to follow-up to RCI estimates, it was discovered that change in total cognition score was reliable for participants with cognitive impairment in the probiotic group (RCI=2.07) but not for any other group (cognitive impairment-placebo, RCI=1.34; intact-probiotic RCI=0.54; intact-placebo, RCI=0.63). See Tables 5 and 6.

To clarify this improvement on the total cognition score, repeated measures ANOVA were performed for specific subtests from the NIH Toolbox. No group by cognitive status by time interaction emerged (p>0.05).

ITT analyses using the carry-forward method (ie, inserting baseline values for missing outcome values[63]) largely corroborated these findings. Repeated measures ANOVA (n=173) showed a borderline significant group by time by cognitive status interaction (F[1,169]=3.90, p=0.05, η_p^2=0.02), such that impaired persons in the probiotic group ($M_{baseline}$=39, $M_{follow\text{-}up}$=46) showed greater improvement in cognitive performance than impaired persons in the placebo group ($M_{baseline}$=37, $M_{follow\text{-}up}$=42), intact persons in the probiotic group ($M_{baseline}$=53, $M_{follow\text{-}up}$=54), and intact persons in the placebo group ($M_{baseline}$=51, $M_{follow\text{-}up}$=54). No group by cognitive status by time interaction was identified (>0.05).

Discussion

Summary of Findings

The current study examined the possible cognitive benefits of *Lactobacillus rhamnosus* GG in a sample of healthy middle-aged and older adults. Results showed that *Lactobacillus rhamnosus* GG supplementation was associated with improvement in total cognition score in persons with objective evidence of cognitive impairment, though no such effect emerged in persons with intact cognitive function or those randomized to placebo. Several aspects of these findings warrant brief discussion.

Table 3 Demographic, Medical, and Cognitive Characteristics by Cognitive Status (n=145)

	Entire Sample (n=145)		Intact (n=103)		Impaired (n=42)	
	M/% (range)	SD	M/% (range)	SD	M/% (range)	SD
Demographics						
Age	64.4	5.44	64.5	5.48	64.0	5.41
BMI	27.9	6.56	28.1	6.85	27.4	5.83
Education	15.2	2.46	15.1	2.56	15.3	2.21
Gender (female)	59.3%	–	60.2%	–	57.1%	–
Ethnicity						
Caucasian	97.0%	–	98.0%	–	95.2%	–
Black	2.00%	–	1.00%	–	4.80%	–
Other	1.00%	–	1.00%	–	0.00%	–
Medical history						
Depression/anxiety	22.0%	–	18.6%	–	28.6%	–
Sleep apnea	9.66%	–	5.88%	–	19.0%	–
Diabetes	8.28%	–	8.82%	–	7.10%	–
Hypertension	27.6%	–	25.4%	–	33.3%	–
Cognition						
Picture	48.0 (28.0–84.0)	10.1	50.5 (36.0–84.0)	9.62	42.0 (28.0–67.0)	10.0
Flanker	43.1 (24.0–65.0)	6.94	45.1 (36.0–65.0)	6.49	38.2 (24.0–49.0)	5.54
Card sort	53.2 (29.0–81.0)	9.89	56.0 (38.0–81.0)	8.80	46.1 (29.0–67.0)	9.15
List sort	51.8 (28.0–73.0)	9.19	53.4 (37.0–73.0)	8.98	48.0 (28.0–69.0)	8.53
Pattern	47.9 (10.0–83.0)	13.1	52.4 (36.0–83.0)	10.4	38.2 (10.0–71.0)	13.0
Total	48.0 (27.0–78.0)	9.97	52.0 (37.0–78.0)	8.24	38.1 (27.0–55.0)	7.29

Abbreviations: Picture, picture sequence memory test; Flanker, flanker inhibitory control and attention test; Card sort, dimension change card sort test; List sort, list sorting working memory test; Pattern, pattern comparison processing speed test; Total, total cognition score.

Table 4 RM ANOVAs Examining Effect of Study Group and Cognitive Status on Total Cognition Score and Subtest Scores

Predictor	df_{NUM}	df_{DEN}	Epsilon	SS	*F*	*p*	η2
Total Cog.							
Time	1	141	1	1297.70	77.50	0.00**	0.36
Group	1	141	1	154.75	1.41	0.24	0.01
Time×group	1	141	1	51.81	3.10	0.08	0.21
Time×cognition	1	141	1	268.21	16.03	0.00**	0.10
Time×group×cognition	1	141	1	76.98	4.60	0.03*	0.03
Subtests							
Time	5	137	1	–	19.83	0.00**	0.42
Group	5	137	1	–	0.71	0.62	0.03
Time×group	5	137	1	–	1.07	0.38	0.04
Time×cognition	5	137	1	–	5.07	0.00**	0.16
Time×group×cognition	5	137	1	–	1.11	0.36	0.04

Notes: *p<0.05; **p<0.01.
Abbreviations: Total Cog., total cognition score RM ANOVA; Subtests, subtest scores RM ANOVA.

Test Improvement in Persons with Cognitive Dysfunction

The exact reason for finding cognitive benefits of *Lactobacillus rhamnosus* GG in persons with cognitive impairment, but not those with normal cognitive function, is unclear. One possible explanation involves a limited capacity for improvement. At baseline, the total cognition score for the normal cognition subsample fell in the average range (M=48.0, SD=9.97). As noted above, past studies show that probiotic supplementation is associated with improved

Table 5 Demographic, Medical, and Cognitive Characteristics of Intervention by Cognitive Status Groups

	Intact				Impaired			
	Probiotic (n=57)		Placebo (n=46)		Probiotic (n=19)		Placebo (n=23)	
	M/% (range)	SD	M/% (range)	SD	M/% (range)	SD	M/% (range)	SD
Demographics								
Age	64.2	5.68	64.8	5.25	65.6	5.27	62.7	5.27
BMI	28.3	7.46	27.8	6.08	25.2*	4.09	29.2*	6.48
Education	15.2	2.65	15.1	2.48	14.8	2.14	15.7	2.25
Gender (female)	56.1%	–	65.2%	–	47.4%	–	65.2%	–
Ethnicity								
Caucasian	100%	–	96.0%	–	100%	–	91.3%	–
Black	0.00%	–	2.00%	–	0.00%	–	8.70%	–
Other	0.00%	–	2.00%	–	0.00%	–	0.00%	–
Medical history								
Depression/anxiety	15.8%	–	21.7%	–	31.6%	–	26.1%	–
Sleep apnea	3.50%	–	8.7%	–	10.5%	–	26.1%	–
Diabetes	7.00%	–	10.9%	–	5.30%	–	8.70%	–
Hypertension	19.3%	–	32.6%	–	21.1%	–	43.5%	–
Cognition								
Picture	49.6 (36.0–78.0)	8.61	51.5 (36.0–84.0)	9.72	40.7 (29.0–67.0)	9.96	43.0 (28.0–65.0)	10.1
Flanker	45.3 (36.0–65.0)	6.56	44.9 (36.0–61.0)	6.37	39.2 (31.0–47.0)	4.18	37.4 (24.0–49.0)	6.44
Card sort	56.4 (38.0–75.0)	8.03	55.6 (38.0–81.0)	9.54	48.1 (35.0–67.0)	8.38	44.5 (29.0–67.0)	9.62
List sort	54.5 (40.0–70.0)	7.59	52.0 (37.0–73.0)	10.5	47.5 (28.0–58.0)	8.24	48.4 (33.0–69.0)	8.92
Pattern	52.1 (36.0–74.0)	9.01	52.8 (36.0–83.0)	11.7	38.0 (20.0–68.0)	12.9	36.2 (10.0–71.0)	13.3
Total	52.1 (39.0–76.0)	7.37	51.8 (37.0–78.0)	8.66	38.7 (27.0–55.0)	8.10	37.7 (27.0–53.0)	6.70

Notes: *Significant difference ($p<0.05$) between intervention groups within cognitive status subgroup.

Abbreviations: Picture, picture sequence memory test; Flanker, flanker inhibitory control and attention test; Card sort, dimension change card sort test; List sort, list sorting working memory test; Pattern, pattern comparison processing speed test; Total, total cognition score.

Table 6 Baseline and Follow-up Cognitive Test Scores of Intervention by Cognitive Status Groups

	Intact						Impaired					
	Probiotic (n=57)			Placebo (n=46)			Probiotic (n=19)			Placebo (n=23)		
	Base M (SD)	Post M (SD)	F	Base M (SD)	Post M (SD)	F	Base M (SD)	Post M (SD)	F	Base M (SD)	Post M (SD)	F
Cognition												
Picture	49.6 (8.61)	52.7 (7.33)	8.9	51.5 (9.72)	54.3 (9.31)	3.3	40.7 (9.96)	52.0 (9.79)	34.9	43.0 (10.1)	47.9 (9.49)	14.9
Flanker	45.3 (6.56)	46.3 (6.67)	2.8	44.9 (6.37)	46.3 (6.84)	4.5	39.2 (4.18)	41.7 (6.18)	3.0	37.4 (6.44)	39.7 (7.57)	4.4
Card sort	56.4 (8.03)	56.6 (9.87)	0.0	55.6 (9.54)	55.9 (9.86)	0.1	48.1 (8.38)	54.4 (9.15)	10.5	44.5 (9.62)	48.8 (8.18)	7.7
List sort	54.5 (7.59)	54.4 (7.91)	0.0	52.0 (10.5)	52.5 (9.32)	0.0	47.5 (8.24)	49.1 (8.00)	1.1	48.4 (8.92)	48.9 (7.54)	0.1
Pattern	52.1 (9.01)	55.0 (10.1)	4.8	52.8 (11.7)	56.7 (11.0)	9.7	38.0 (12.9)	45.0 (14.4)	11.8	36.2 (13.3)	40.8 (15.1)	4.5
Total	52.1 (7.37)	54.5 (7.72)	8.4	51.8 (8.66)	54.6 (8.22)	11.5	38.7 (8.10)	47.6 (9.21)	46.9	37.7 (6.70)	42.4 (7.92)	17.0

Notes: Base, baseline; Post, follow-up; Picture, picture sequence memory test; Flanker, flanker inhibitory control and attention test; Card sort, dimension change card sort test; List sort, list sorting working memory test; Pattern, pattern comparison processing speed test; Total, total cognition score.

cognitive function in persons with baseline cognitive impairment[64,65] but, thus far, it has been found to have limited impact in persons with intact abilities.[66] Such findings suggest that probiotics may help to alleviate deficits in cognitive function (ie return to premorbid range) but may not be sufficient to improve test performance beyond that expected by an individual's preexisting genetic and biological capacity. Further examination of this possibility—especially clarification of benefits across the adult lifespan—is much needed.

Mechanisms for Cognitive Improvement from *Lactobacillus rhamnosus* GG

There are also several mechanistic pathways through which *Lactobacillus rhamnosus* GG probiotic supplementation may improve cognitive function in at-risk individuals. Along the gut-brain-microbiome axis, the gut microbiota and the brain engage in bidirectional signaling through bile acids,[7,32] metabolites,[34] and immune responses.[31] These means of signaling have been associated with a wide range of neurodegenerative disorders,[7,35] neuronal protein expression,[33] neurotransmission,[29] synaptic pruning,[67,68] and behavioral change.[69,70] As described previously, *Lactobacillus* strains in particular have been found to reduce inflammatory cytokines[40] and metabolites in humans,[42] and enhance levels of CREB and BDNF in rats.[41] As probiotics can modulate the composition and functionality of the gut microbiota, *Lactobacillus rhamnosus* GG could have improved these signaling markers as well—ultimately leading to better cognitive function.

It is also possible that *Lactobacillus rhamnosus* GG may improve cognitive function through indirect pathways. *Lactobacillus rhamnosus* GG specifically has been shown to improve glucose tolerance and insulin sensitivity[45] and reduce inflammatory responses.[46,71] As metabolic dysfunction[24,72,73] and inflammatory diseases[74–76] are associated with poor cognitive functioning, *Lactobacillus rhamnosus* GG may have indirectly improved cognitive health by reducing pathological responses associated with these conditions. Additional work is much needed to begin to clarify possible neuroprotective effects of *Lactobacillus rhamnosus* GG and other probiotics in aging adults.

Limitations

The current study is limited in several ways. Though concerted efforts were made, the exact number of days of supplementation differed slightly due to participant scheduling conflicts (M_{days}=92, SD=5.17). As previous research has shown that the gut microbiome can change quickly,[77] this variability may have subtle impact on study findings. Similarly, no gold standard for duration of probiotic supplementation has been established. Though the current study followed participants for a sufficient time to identify cognitive changes,[78] it is possible that supplementation could have differential cognitive effects at other timepoints, as past work has examined effects of supplementation from as little as three weeks to as long as six months.[64,79,80] As suggested above, much longer trials (eg 12–24 months) are also needed to clarify the possible protective effects of probiotic supplementation on cognitive decline in at-risk individuals. Relatedly, the absence of a no-treatment follow-up period in enrolled study participants limits the opportunity to clarify specific, acute benefits of *Lactobacillus rhamnosus* GG.

Another limitation is found in the use of self-report to identify psychiatric and neurodegenerative disorders. It is possible that participants denied such disorders to ensure eligibility for participation or may have been unaware that they met criteria for such diagnoses.

The observed practice effects also introduce a possible concern for the current study. Though previous research suggests that NIH Toolbox has limited practice effects,[56] probiotic and placebo groups both showed improvement on testing. Though the current study methodology (including use of RCI) helps to mitigate possible confound, the possibility of test-related issues or familiarity with testing itself,[81] cannot be fully ruled out. Similarly, minor differences were found between analyses that included all persons that fully adhered to study protocol vs those that did not or dropped out. This finding requires clarification in future studies, as explanations may include dose–response relationships for the cognitive benefits of probiotic supplementation or artifact due to the difficulties in adhering to study protocol in persons with cognitive dysfunction.[82]

A final limitation of the current study is the lack of data regarding changes in the gut microbiome during the study period. Past research concerning the effects of probiotics on cognitive and/or health outcomes have directly examined the microbiome using DNA sequencing.[10,83,84] As increases/decreases of certain microbial groups have been associated with changes in a variety of health and psychological outcomes (eg increased *Bacteroidetes* associated with increased inflammatory responses, increased *Lactobacillus* associated with reduced glucoregulatory markers);[69,85,86] DNA sequencing may help clarify the underlying mechanisms responsible for the observed cognitive gains. Stool samples have been collected in a subsample of participants, though are not available for analysis at the present time.

Conclusion

Persons with cognitive impairment who received *Lactobacillus rhamnosus* GG probiotic supplementation showed improvement on neuropsychological testing over a three-month period. These benefits emerged despite no difference in dropout rates between study arms, suggesting that

Lactobacillus rhamnosus GG was well-tolerated and had no deleterious impact on health, consistent with past work.[87–89] Should these findings be replicated in larger and clinical samples including persons with diagnosed neurodegenerative and neuropsychiatric conditions, probiotic supplementation may ultimately prove to be a low-risk and cost-effective approach to promote cognitive health in older adults.

Data Sharing Statement

Data from the current research study is not available for public dissemination. If others are interested in working in collaboration regarding the referenced data, requests can be made directly to the PI (Dr John Gunstad) via email (jgunstad@kent.edu). All data available for sharing will be de-identified.

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Lacticaseibacillus paracasei Lpc-37® improves psychological and physiological markers of stress and anxiety in healthy adults: a randomized, double-blind, placebo-controlled and parallel clinical trial (the Sisu study)

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ABSTRACT

Chronic stress is a risk-factor for the development of mood and stress-related disorders. Clinical evidence indicates that probiotics can influence the stress response and mood. The Sisu study investigated whether *Lacticaseibacillus paracasei* Lpc-37® (Lpc-37®) could modulate stress, mood and well-being. Prior to a two-week run-in period, 120 healthy adults (18-45 y) were stratified for sex and chronic stress and randomized to either 1.75×10^{10} colony forming units (CFU) of Lpc-37 or placebo (1:1) per day for 5 weeks. The primary objective was the effect of Lpc-37 on heart rate (HR) in response to the Trier Social Stress Test (TSST). Secondary objectives were assessed by biomarkers and self-report scales over the study. The primary hypothesis was not met in either the Intention-to-Treat (ITT) or Per Protocol (PP) population, but Lpc-37 reduced the increase in HR in participants with low chronic stress (LCS) and increased HR in participants with high chronic stress (HCS) during the TSST. Supporting significant efficacy in the PP population (n = 113), Lpc-37 reduced perceived stress following intervention. More significant effects were identified within the subgroups where Lpc-37 reduced exhaustion during the TSST and normalized cortisol levels at 8pm in participants with LCS, reduced perceived stress also in females, and increased perceived health and sleep-related recovery in participants with HCS. Adverse events (AEs) were similar between groups, there were no severe AEs, and vital signs remained unchanged. Overall, Lpc-37 reduced perceived stress compared to placebo. Other beneficial effects within biomarkers related to stress indicate that the effects of Lpc-37 may be differentially dependent on sex and chronic stress. (ClinicalTrials.gov: NCT03494725).

1. Introduction

Everyday life can be demanding with many sources of stress. While short-term stress is a beneficial adaption process to stressors (McEwen, 2007), chronic stress is a major risk-factor for the development of a wide range of physical and mental disorders (Chrousos, 2009). According to the American Psychological Association (APA) Stress in America report of 2018, nearly 75% of adults reported experiencing at least one physical or emotional symptom of stress in the past month and almost 50% reported higher average stress levels than their perceived healthy levels of stress within the past month (American Psychological Association, 2018). Understanding the risks to our health and ways to reduce daily stress are therefore paramount.

Overwhelming evidence now indicates that the health benefits of the gut microbiome extend far beyond the gut. Here, host-microbe interactions influence the release of several immunological and neurological signaling molecules, and microbial by-products which communicate along the bi-directional pathway of the microbiota-gut-brain axis through central, enteric and autonomic nervous systems as well as the hypothalamic pituitary adrenal (HPA) axis (Rea et al., 2020). The gut microbiome therefore exerts a regulatory function upon neuroinflammation, neurodevelopment and the neuroendocrine stress response (El Aidy et al., 2014; Foster et al., 2016; Kelly et al., 2015), influencing brain physiology, psychological responses and ultimately, behavior. Infiltrating the realms of psychology and psychiatry, the APA have recognized the gut microbiome as a novel paradigm for studying the psychobiological underpinnings of mental illness (Liu, 2017). Recent clinical data supports the hypothesis that the stress response can be

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influenced through targeted modification of the gut microbiota (Cryan et al., 2019; Foster et al., 2017). Probiotics are one such means of targeting the gut microbiota to deliver health benefits. The physiological and psychological benefits of probiotics on stress and mood outcomes have been described in pre-clinical trials using different models, with some translation to clinical trials in different populations ranging from healthy participants (Allen et al., 2016; Marotta et al., 2019; Messaoudi et al., 2011a, 2011b), to subjects under various stress levels (Benton et al., 2007; Chahwan et al., 2019; Chong et al., 2019; Pinto-Sanchez et al., 2017; Sawada et al., 2017; Slykerman et al., 2017). Of note, one recent study demonstrated that the neurocognitive benefits of a multi-species probiotic became evident only when the participants were stressed, highlighting the need to carefully characterize study populations (Papalini et al., 2019).

Lacticaseibacillus paracasei Lpc-37® (Lpc-37®), formerly known as *Lactobacillus paracasei* Lpc-37®, has proven effective in preventing chronic stress-associated behaviors from developing in two recent pre-clinical experiments of the same model (Stenman et al., 2020). The Sisu study investigated the a priori hypotheses that Lpc-37 could reduce the expected increase in physiological markers of stress such as heart rate (HR) and blood pressure (BP) in response to an acute stress; the Trier Social Stress Test (TSST), and furthermore to normalize the cortisol awakening response (CAR) and evening cortisol levels, improve psychological test scores both in response to the TSST and over the study, and improve sleep, productivity and overall well-being following a five-week intervention compared to placebo. The primary objective was the effect of Lpc-37 on HR in response to the TSST and was chosen mainly due to the suspected mode of action: the vagus nerve activity responsible for the gut-brain interaction. A biomarker for the autonomic nervous system (ANS), HR has also repeatedly been shown to be affected by the TSST. Since the clinical effects of Lpc-37 on outcomes of stress and anxiety were unknown, one single primary objective and endpoint was selected. Although HR was expected to increase in response to the TSST, chronic psychosocial factors have affected HR reactivity to acute psychological stress with mixed results (Chida and Hamer, 2008). To control for chronic stress while investigating the effect of Lpc-37 on the ANS response to acute stress, the population was stratified into low and high chronic stress using cut off values previously defined in an age-related population using the Trier Inventory for Chronic Stress (TICS) (Schulz and Schlotz, 1999). Secondary objectives were measured throughout the study and were assessed by biomarkers and self-report scales. The results serve as an indication that the study design is suitable to investigate clinical stress-related effects of probiotics and confirm that Lpc-37 is a safe and effective probiotic to beneficially impact several outcomes related to physiological and psychological stress in healthy adults.

2. Materials and methods

2.1. Study design

The Sisu study was a randomized, double-blind, placebo-controlled, two-arm (allocation ratio 1:1) and parallel groups clinical trial. The study design included a two-week run-in period between Visit 1 (V1) and Visit 2 (V2) when randomized participants were not permitted to consume products containing concentrated sources of probiotics and/or prebiotics. This was followed by a five-week intervention with the investigational products (IP)s between V2 and Visit 3 (V3). Randomized participants were provided with saliva collection kits and instructed to collect saliva at home during two consecutive working days before V2 and V3 and provided with training and access to an online daily diary from V1 to V3. A detailed outline of the investigation steps at each visit are shown in Fig. 1a and b. The primary objective evaluated the efficacy of Lpc-37 on HR before, during and after the TSST (V3). Secondary

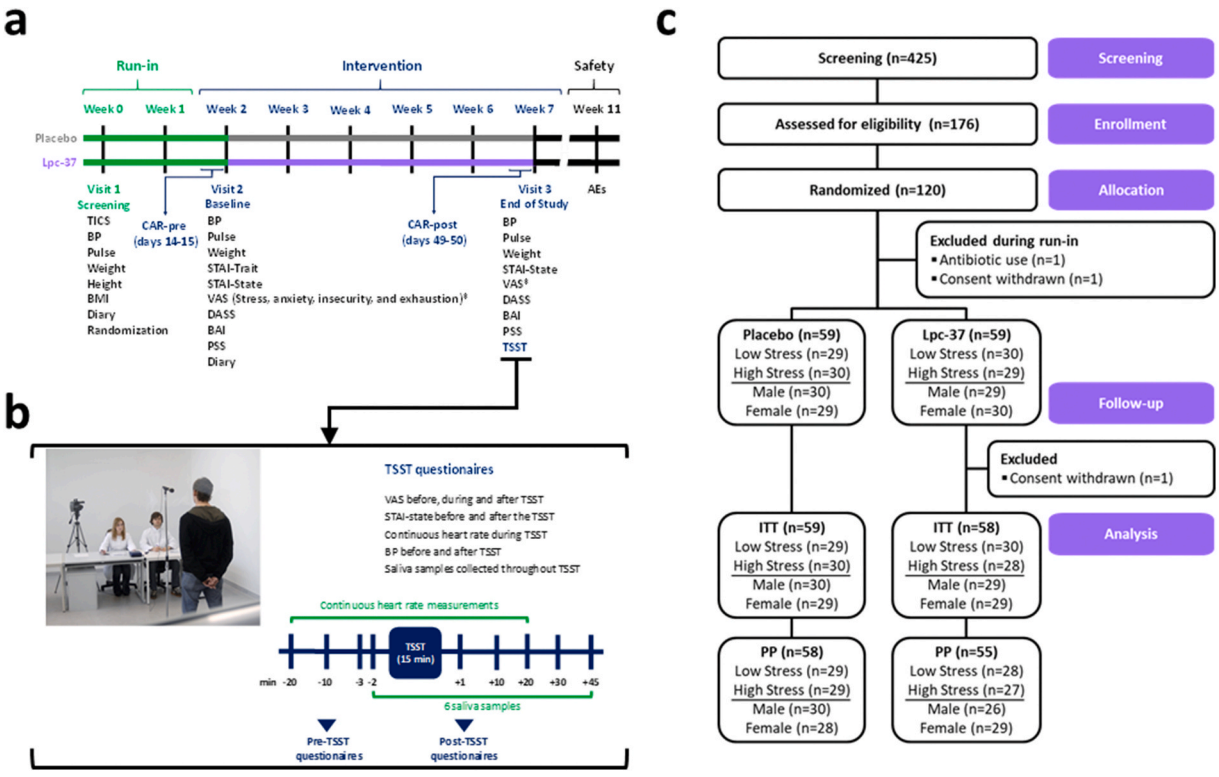

Fig. 1. a. Sisu study design. **b.** Study-specific Trier Social Stress Test (TSST) procedures. **c.** CONSORT flow diagram. Abbreviations: AEs, Adverse Events; BAI, Beck Anxiety Inventory; BMI, Body Mass Index; BP, Blood Pressure; CAR, Cortisol Awakening Response; CONSORT, Consolidated Standards of Reporting Trials; DASS, Depression Anxiety Stress Scale; ITT, Intention to Treat; PP, Per Protocol; PSS, Perceived Stress Scale; STAI, State Trait Anxiety Inventory; TICS, Trier Inventory for Chronic Stress; VAS, Visual Analog Scale.

objectives evaluated the efficacy of Lpc-37 before, during and after the TSST, before and after intervention, and throughout the study period by salivary cortisol analyses, HR, BP, self-report scales, validated inventories and diary entries. Prior to recruitment, the protocol, participant information and the informed consent form (ICF) were reviewed and approved by the Independent Ethics Committee (IEC) of the Chamber of Physicians of the State of Rhineland-Palatinate on March 13, 2018 and the study was registered at ClinicalTrials.gov (NCT 03494725). The study was conducted at a single site at daacro GmbH and Co. KG (Trier, Germany) in accordance with the Declaration of Helsinki (World Medical Association, 2013), and the guidelines for good clinical practice (GCP) (ICH Expert Working Group, 1996), following all applicable laws and regulations for clinical research in Germany. During the study, a minor amendment was added to the ICF to include follow-up calls for ongoing adverse events (AEs) at V3 and was approved by the IEC. Clinical monitoring was performed by an external Clinical Research Associate.

2.2. Study participants

Participants were recruited from daacro's in-house database (www.werdeproband.de). A total of 120 eligible participants signed the ICF and were randomized into the study at V1. The full description of eligibility criteria is included in Supplementary Methods. Randomization was managed by Oy 4Pharma Ltd. (Turku, Finland) and performed using block randomization according to a computer-generated randomization list, with concealed allocation. All randomized participants were assigned to one of two study groups (verum or placebo). Within each study group, the randomization was stratified for sex and prolonged perceived stress levels using the TICS (Schulz and Schlotz, 1999; Schulz et al., 2004). Chronic stress was determined using the Screening Subscale for Chronic Stress, a subscale of the TICS. The classification of low chronic stress (LCS) and high chronic stress (HCS) depended on whether the participant's score was above or below the age-related median score for the frequency of stressful events perceived within the last three months. Participants with a score $\leq$13 were stratified into the LCS subgroup and participants with a score $\geq$14 were stratified into the HCS subgroup (Schulz et al., 2004). A detailed description of the TICS is included in Supplementary Methods.

2.3. IPs

The verum (batch 1103180371) consisted of Lpc-37 at a dose of 1.75 $\times$ 10^{10} colony forming units (CFU), microcrystalline cellulose, magnesium stearate and silicon dioxide in one capsule per day. The matching placebo (batch 1103180369) was the same formulation without Lpc-37, in one capsule per day. Both IPs were identical in appearance and taste and a five-week supply plus some extra capsules was provided to participants at V2. Participants were instructed to consume one capsule of their assigned IP each morning, at least 30 min before breakfast or their first meal of the day, with a glass of plain water.

DuPont Nutrition & Biosciences, Danisco USA Inc. (Madison, WI, USA) produced, packaged and labelled the IPs with individual randomization numbers per capsule bottle as per the unblinded randomization list provided by 4Pharma (Turku, Finland). The identity of the IPs was blinded to participants, site staff, the principal investigator (PI) and all sponsor personnel involved in the trial. The PI, site staff, data manager, biostatistician and all sponsor personnel involved in the trial remained blinded to the group assignments until after the database was locked and the blind data review (BDR) was completed. The integrity of the sealed individual blinded envelopes was inspected during routine interim monitoring visits.

IP compliance was documented by participants each day in the online diary and percentage compliance was calculated by counting the number of remaining capsules in the bottles returned at V3: 35/(40 – number of capsules returned)*100, where 35 was the number of expected capsules to have been taken over the five-week intervention and 40 was the number of capsules provided. All participants had completed the intervention before the expiration date of the IPs.

2.4. Study outcomes

2.4.1. TSST

The TSST is a protocol for inducing an acute and physiological stress, including an endocrine reaction to experimental psychosocial stress in humans (Allen et al., 2017; Dickerson and Kemeny, 2004). All participants completed the TSST (Kirschbaum et al., 1993) at V3 which consisted of the following four components: introduction, preparation, interview and mental arithmetic task. The TSST is described in detail by Kudielka et al. (Kudielka et al., 2007a, 2007b; Kudielka and Wust, 2010) and a study specific description of the TSST is included in Supplementary Methods. Specific procedures measured before, during and after the TSST are described below and outlined in Fig. 1b.

2.4.2. Primary outcome: HR in response to the TSST

The primary outcome was change in HR in response to the TSST. Efficacy was defined as a lower increase in HR in response to the TSST following intervention with Lpc-37, compared to placebo. A Polar watch device (M400, Polar Electro GmbH, Büttelborn, Germany) worn by participants collected HR measurements every second throughout a 55 min test period. Mean values were calculated per group before, during and after the TSST: 10 min sitting pre-TSST; 10 min standing pre-TSST; 5 min during the TSST introduction and preparation; 5 min during the interview; 5 min during the mental arithmetic task; 10 min standing post-TSST; 10 min sitting post-TSST.

2.4.3. Secondary outcomes: TSST-related outcomes

2.4.3.1. Salivary cortisol and alpha amylase (AA). Individual saliva samples were collected from each participant 2 min before and 1-, 10-, 20-, 30- and 45-min after the TSST. Saliva was collected using Salivette® Cortisol, code blue collection tubes (Sarstedt, Nuembrecht, Germany). Briefly, participants gently moved the swab from the Salivette® in the mouth for approximately 1 min to stimulate salivation and to ensure the swab was soaked thoroughly in saliva. The swab containing the absorbed saliva was then returned to the Salivette® and the cap was replaced. All saliva samples collected during the TSST were stored frozen at −20 °C until analysis. Salivary cortisol levels were determined using a high sensitivity salivary cortisol enzyme immunoassay kit (Salimetrics, PA, USA). Salivary AA levels were determined using a kinetic enzyme assay kit (Salimetrics). All samples were analyzed at daacro.

2.4.3.2. BP. BP measurements were taken from each participant 3 min before and 1 min after the TSST. Systolic and diastolic BP were obtained using an automated device (OMRON M10-IT, OMRON Medizintechnik Handelsgesellschaft mbH, Mannheim, Germany).

2.4.3.3. State-trait anxiety inventory (STAI; X1 Form). Participants rated their state anxiety levels using the STAI-X1 Form at 10 min before and 1 min after the TSST. The STAI-X1 Form is a subscale of the STAI self-report questionnaire that measures the presence and severity of current symptoms of anxiety and the propensity to be anxious (Spielberger and Gorsuch, 1983). It comprises 20 items which assess momentary anxiety characterized by *tension, solitude, nervousness, uneasiness* and *fear of future situations*. Participants rated how they felt on a scale ranging from 1 = "not at all" to 4 = "very much so". The total score for the STAI-X1 Form was obtained by summing the scores of all 20 items. The range of the total score for anxiety is 20–80, wherein the higher the score, the higher the anxiety.

2.4.3.4. Perceived -stress, -anxiety, -emotional insecurity and -exhaustion.

Participants rated their individual perception of stress, anxiety, emotional insecurity and exhaustion, using separate visual analog scales (VAS) (Bond and Lader, 1974; Aitken, 1969). These psychological measures were taken 10 min before the TSST, between the interview and mental arithmetic TSST tasks, and 1 min after the TSST. Participants marked a spot on the line representing their perceived stress, anxiety, emotional insecurity and exhaustion; where 0 = "feeling not at all" and 100 = "feeling highly stressed/anxious/insecure/exhausted". Scores were determined with millimeter precision and reported as percentage ranging from 0 to 100. The VAS is a useful and suitable tool to measure perceived psychological reactions to the TSST (Hellhammer and Schubert, 2012).

2.4.4. Secondary outcomes: baseline and end of study-related outcomes

2.4.4.1. CAR and 8pm cortisol. Individual saliva samples were collected from each participant on two consecutive working days before V2 and V3. Participants were provided with saliva collection kits containing Salivette® Cortisol, code blue collection tubes (Sarstedt, Germany) and instructions on how to collect saliva samples at home. The method for saliva sample collection using the swabs from the Salivette® was the same as briefly described in section 2.4.3.1. Saliva samples for the CAR were collected at 0-, 30-, 45- and 60-min post-awakening and one sample was collected at 8pm that evening. Participants stored the saliva samples in either their refrigerator or freezer at home and were instructed to bring the samples with them to the study site at their next scheduled visit. Saliva samples were stored at −20 °C until analysis. Salivary cortisol levels were determined using a high sensitivity salivary cortisol enzyme immunoassay kit (Salimetrics). Mean values were calculated for each time point for the two measuring days. The CAR was summarized using the following variables: area under the curve with respect to increase (AUC_i), area under the curve with respect to ground (AUC_g), peak value (maximum value of the two-day mean of the four CAR samples) and mean increase (two-day mean of cortisol at awakening subtracted from two-day mean peak value). The two AUC measurements aggregated the change in cortisol levels over the time course of the CAR and were calculated as previously described (Pruessner et al., 2003). Efficacy for the CAR variables AUC_g, AUC_i, cortisol at awakening and 8pm cortisol levels were defined in terms of a normalization, *i.e.* number of participants with normal test values (between first and third quantile of reference measures relative to a gender specific control data base) and numbers of participants with low or high values were compared before and after the intervention. The normative database was generated using assay kits manufactured by Salimetrics, including n = 1746 participants (n = 1296 women and n = 450 men), established in 2017.

2.4.4.2. BP. BP measurements were taken for each participant upon arrival at the site at V2 and V3, as described in 2.4.3.2.

2.4.4.3. Self-report questionnaires and VAS. Participants completed a battery of four questionnaires (Perceived Stress Scale (PSS), Beck Anxiety Inventory (BAI), 42-Item Depression, Anxiety and Stress Scale (DASS-42), the STAI-X1 Form (as described in 2.4.3.3) and four VAS' (as described in 2.4.3.4), to investigate the effects of Lpc-37 on self-reported symptoms and perception of anxiety, stress, depression, emotional insecurity and exhaustion following five-weeks of intervention. In all cases the German language versions were used.

2.4.4.3.1. PSS. The PSS is a widely used psychological instrument for measuring the degree to which people perceived their lives as stressful within the last month (Cohen et al., 1983). The PSS comprises of 14 items that are answered on a 5-point scale from 0 = "never" to 4 = "very often". The total score was calculated by summing the scores of the 14 items.

2.4.4.3.2. BAI. The BAI is a self-rating scale designed to measure anxiety in adults and youths within the last week (Beck et al., 1988). It comprises of 21 items that are answered on a 4-point scale from 0 = "not at all" to 3 = "severely – it bothered me a lot". The total score was calculated by summing the scores of the 21 items.

2.4.4.3.3. DASS-42. The DASS-42 is a 42 item questionnaire that collects information about negative emotional states of depression, anxiety and stress during the past week (Lovibond, 1998; Lovibond and Lovibond, 1996). These three subscales include 14 items ranging from 0 = "did not apply to me at all" to 3 = "applied to me very much or most of the time". Scores for depression, anxiety and stress were calculated by summing the scores for the relevant items within each subscale.

2.4.5. Secondary outcomes: online diary-related outcomes measured throughout the study

Following randomization at V1, participants received an individual access to the online diary and were instructed to complete the diary everyday between 3am and 12pm, during the run-in period and throughout the intervention period. If entries were not performed in a timely manner, the study team received an e-mail notification. The respective participants were then contacted the following day by a study team member and asked to provide the information. The online diary collected information on perceived productivity, perceived health, sleep quality (sleep disruptions, both binary and reported number of sleep disruptions (count), sleep duration and sleep related-recovery) and perceived mood.

2.5. Vital signs and assessment of safety

The safety objectives of this study were to evaluate if vital signs (BP and HR), body mass index (BMI) and the incidence and intensity of AEs were comparable between the groups. Systolic and diastolic BP and HR were obtained at V1 using an automated device (OMRON M10-IT) to determine eligibility. At V2 and V3, BMI, BP and HR were obtained following participant arrival at site. AEs were assessed at each visit with open, standardized questions such as *"Have you had any health problems since you were last questioned?"*. Additionally, participants were asked to record any occurring AE as follows: description of the event, onset (date and time), resolution (date and time), whether the AE was ongoing at the end of the study, intensity (mild, moderate, severe), therapy of event, action taken, and outcome. The PI classified causality (definitely, probably, possibly, unlikely, not related, not assessable) and whether it constituted a serious adverse event (SAE) or not. Any AEs still ongoing at study completion on V3 were followed up to 30 days after V3.

2.6. Sample size calculation and statistical analyses

The sample size was computed for a repeated measurement ANOVA with two groups and seven repeated measurements (power = 0.85, α = 0.05, f = 0.1). The calculation resulted in a group size of 56 participants each, which was rounded up to 60 participants per study group to account for attrition. Subgroup analyses were performed for the different strata, i.e. female, male, HCS and LCS. For the subgroup analyses, which relied on 50% of the total sample size, this resulted in a power = 0.55 for the parameters assumed for the sample size calculation (α = 0.05, f = 0.1).

For all endpoints, analyses were performed for the Intention-to-Treat (ITT) and Per Protocol (PP) populations, separately. For the PP analyses, individual decisions on exclusion of participants or data points were made during the BDR, resulting in different Ns for different endpoints. A detailed description of the methodology to define the PP population is included in Supplementary Methods. Table S1a lists the number of participants in the ITT and PP population per endpoint, statistical model, and transformation criteria and Table S1b lists the number of participants in the PP population per endpoint along with reasons for exclusion.

Endpoints with more than two measurements were analyzed using

linear mixed models. Mixed models were built up gradually, first testing how many time polynomials should be included, then testing possible covariates (gender, chronic stress, STAI trait, BMI, weight, age), and lastly adding the effect of study group and time × group interaction terms (e.g. time 1 × group, time 2 × group). Models were built including time and intercept as random factors. In case of convergence difficulties, time was dropped from the random effects. Type II F-tests were conducted using Satterthwaite's degrees of freedom method. Endpoints with two measurements (before and after TSST or intervention) were analyzed using repeated measures ANOVAs including relevant covariates (see above).

If the assumptions of a statistical analysis were violated despite efforts of transformation, alternative parametric or non-parametric tests were used. *P*-values in section 3. Results describe efficacy for a study group based on the interaction between study group and time for all parametric tests and based on group difference between change scores for non-parametric tests. All *P*-values <0.05 were considered as statistically significant and in some cases *P*-values ≥ 0.05 and < 0.10 are reported as trends where interesting. The results described in the main text focus on the PP population because they more accurately represent those participants who strictly followed the protocol, however significant *P*-values found only within the ITT (and not the PP) population are also reported. Fisher's exact test on frequency of compliance in percent between groups was used to compare compliance of IP between the groups. Statistical analyses were conducted using R Version 3.5.2 (R Core Team, 2018).

3. Results

3.1. Participants and baseline characteristics

A total of 425 volunteers were telephone screened, of which 176 were eligible and invited to a screening visit (V1). Of those, 120 participants met the inclusion/exclusion criteria and were enrolled in the study between April and October 2018. Two participants were excluded during the run-in period (use of antibiotics and withdrawn consent) and one participant withdrew consent during the intervention period. A total of 117 participants completed the study. Fig. 1c displays the CONSORT flow diagram with detailed disposition of participants. There were no marked differences in baseline and demographic characteristics between the groups in the general population (Table 1) or in the subgroups (Table S2).

The PP population was identified before database lock, after the BDR and included all randomized participants that satisfied the inclusion/exclusion criteria and had no major protocol deviations (n = 113; Lpc-37, n = 55; placebo, n = 58). For individual endpoints, participants were excluded if they showed deviations that might have affected that endpoint (Tables S1a and S1b). The ITT population included all randomized participants that satisfied the inclusion/exclusion criteria with data available for all endpoints for 117 participants (Lpc-37; n = 58 and placebo; n = 59). The safety population included all participants that received at least one dose of IP and contained 118 participants (Lpc-37; n = 59 and placebo; n = 59).

Table 1
Demographics and other baseline characteristics for randomized participants (n = 120).

	Placebo	Lpc-37
	Mean (SD)	Mean (SD)
TICS (score)	15.32 (8.65)	15.08 (9.28)
Age (years)	23.25 (4.20)	23.73 (4.27)
Height (cm)	173.58 (9.33)	175.58 (8.86)
Weight (kg)	69.79 (12.15)	71.13 (11.05)
BMI (kg/m^2)	23.02 (2.67)	22.97 (2.30)
Systolic BP (mmHg)	120.72 (13.47)	120.75 (12.09)
Diastolic BP (mmHg)	74.88 (8.53)	74.22 (7.25)
Heart rate (bpm)	71.03 (12.43)	72.27 (13.71)

Abbreviations: BMI, Body Mass Index; BP, Blood Pressure; n, number of participants; SD, Standard Deviation; TICS, Trier Inventory for Chronic Stress.

3.2. IP compliance, IP stability and blinding

All participants satisfied the criterion of >80% compliance. Mean compliance in the ITT population was 100.4% for Lpc-37 and 99.9% for placebo ($P = 0.987$). While the target dose of Lpc-37 was 1×10^{10} CFU/capsule, the certificate of analysis recorded the initial dose as 1.75×10^{10} CFU/capsule. Both the presence of Lpc-37 and absence of contaminants and genetic variants was confirmed by genomic sequencing of the IP (DuPont Nutrition & Biosciences, Danisco USA Inc.). From IP bottles stored at the study site until all participants had completed the study, the final dose of Lpc-37 was determined to be 1.68×10^{10} CFU/capsule. The randomization code was not broken for any participant during the study.

3.3. Stress reactivity - physiological response to the TSST

3.3.1. Primary outcome: the effects of Lpc-37 on HR are dependent on chronic stress

As expected, there was a significant change in HR in both groups in response to TSST-induced acute stress ($P < 0.001$). HR increased by 43.7% in the Lpc-37 group from sitting pre-TSST to interview TSST and by 42.1% in the placebo group (Table 2). There was no significant effect of Lpc-37 on HR in the general population (Table 2). The HR-increase in response to acute stress was significantly lower in participants with LCS (Fig. 2a; $P = 0.014$), but significantly higher in participants with HCS (Fig. 2b; $P = 0.034$) in the Lpc-37 group compared to the placebo group. There were no effects of Lpc-37 on HR in either male or female participants (Table S3).

3.3.2. Lpc-37 had no effect on salivary cortisol or AA, but reduced the acute stress induced increase in systolic BP in females

Both salivary cortisol and AA levels significantly changed in both groups in response to the TSST ($P < 0.001$). There was no significant effect of Lpc-37 on either salivary cortisol or AA levels in the general population (Table 2), or in any of the subgroups (Table S3).

The TSST resulted in a significant increase in both systolic ($P < 0.001$) and diastolic ($P < 0.001$) BP in both groups. There were no significant effects of Lpc-37 on either systolic or diastolic BP in response to the TSST (Table 2). In female participants, systolic BP increased significantly less in the Lpc-37 group, compared to the placebo group (Fig. 2c; $P = 0.031$), with no significant difference in diastolic BP between groups (Table S3). There were no significant effects of Lpc-37 on systolic or diastolic BP in the other subgroups (Table S3). Results for the effects of Lpc-37 on the physiological response to the TSST in the ITT population are included in Tables S4 and S5.

3.4. Stress reactivity - psychological response to the TSST

3.4.1. Lpc-37 reduced perceived exhaustion in participants with LCS, but had no effect on state anxiety or perceived -stress, -anxiety or -insecurity

The TSST resulted in a significant increase in state anxiety in both groups ($P < 0.001$). There were no significant effects of Lpc-37 on state anxiety in the general population (Table 2), or in any of the subgroups (Table S3).

Perceived -stress, -insecurity and -anxiety significantly changed in both groups, in response to the TSST ($P < 0.001$), while perceived exhaustion did not. There were no significant effects of Lpc-37 on any of the four outcome measures in the general population (Table 2). In participants with LCS, the increase in perceived exhaustion was significantly lower in the Lpc-37 group compared to the placebo group (Fig. 2d; $P = 0.037$). There were no significant effects of Lpc-37 on perceived exhaustion in the other subgroups (Table S3). Furthermore,

Table 2
Summary measures in response to the Trier Social Stress Test (TSST) for participants in the Per Protocol population.

	Sitting pre-TSST −20 min	Standing pre-TSST −10 min	Pre-TSST −3 min	Pre-TSST −2 min	Interview TSST	Arithmetic TSST	Post-TSST +1 min	Standing post-TSST +10 min	Sitting post-TSST+20 min	Post-TSST +30 min	Post-TSST +45 min	*P*
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	
Heart Rate (bpm)												
Placebo (n = 57)	74.34 (9.04)	86.69 (10.74)	97.62 (16.23)	–	105.66 (18.86)	100.81 (17.20)	–	90.81 (12.11)	74.97 (9.86)	–	–	0.757[T]
Lpc-37 (n = 55)	74.84 (10.20)	88.15 (11.13)	97.34 (17.15)	–	107.56 (21.56)	102.77 (19.57)	–	93.32 (14.08)	75.88 (11.11)	–	–	
Salivary Cortisol (nmol/L)												
Placebo (n = 57)	–	–	–	4.82 (2.60)	–	–	6.85 (3.50)	8.97 (5.84)	9.21 (6.59)	7.71 (5.06)	6.16 (3.79)	0.566[T]
Lpc-37 (n = 55)	–	–	–	4.79 (2.62)	–	–	6.96 (3.73)	9.48 (5.75)	9.89 (6.51)	8.04 (5.36)	6.21 (3.17)	
Salivary Alpha Amylase (U/ml)												
Placebo (n = 57)	–	–	–	161.67 (110.89)	–	–	270.55 (174.85)	158.85 (91.21)	141.49 (93.00)	138.48 (90.31)	148.15 (105.60)	0.815[T]
Lpc-37 (n = 55)	–	–	–	154.04 (98.17)	–	–	246.29 (153.62)	146.53 (86.80)	130.11 (82.45)	125.19 (79.67)	141.13 (92.94)	
Systolic BP (mmHg)												
Placebo (n = 58)	–	–	114.33 (14.07)	–	–	–	129.19 (14.33)	–	–	–	–	0.274
Lpc-37 (n = 55)	–	–	115.11 (12.53)	–	–	–	127.47 (13.67)	–	–	–	–	
Diastolic BP (mmHg)												
Placebo (n = 58)	–	–	78.41 (8.32)	–	–	–	88.36 (9.72)	–	–	–	–	0.345
Lpc-37 (n = 55)	–	–	79.13 (7.83)	–	–	–	90.38 (7.17)	–	–	–	–	
STAI-State (score)												
Placebo (n = 58)	–	36.83 (9.48)	–	–	–	–	43.60 (10.00)	–	–	–	–	0.755
Lpc-37 (n = 55)	–	36.09 (8.45)	–	–	–	–	42.38 (10.91)	–	–	–		
VAS Stress (score)												
Placebo (n = 58)	–	18.52 (21.73)	–	–	51.51 (28.10)	–	32.85 (23.66)	–	–	–	–	0.327[T]
Lpc-37 (n = 55)	–	19.89 (20.61)	–	–	47.71 (27.08)	–	31.72 (24.25)	–	–	–	–	
VAS Insecurity (score)												
Placebo (n = 58)	–	17.19 (21.37)	–	–	52.19 (27.16)	–	23.69 (23.58)	–	–	–	–	0.364[T]
Lpc-37 (n = 55)	–	14.47 (16.96)	–	–	45.08 (28.92)	–	23.92 (23.87)	–	–	–	–	
VAS Anxiety (score)												
Placebo (n = 58)	–	8.50 (14.94)	–	–	22.47 (23.51)	–	11.74 (18.46)	–	–	–	–	0.251[T]
Lpc-37 (n = 55)	–	6.80 (10.95)	–	–	20.85 (23.61)	–	10.68 (15.19)	–	–	–	–	
VAS Exhaustion (score)												
Placebo (n = 58)	-	19.79 (21.88)	–	–	21.30 (22.47)	–	25.68 (26.07)	-	-	-	-	0.101[T]
Lpc-37 (n = 55)	–	21.18 (21.49)	–	–	19.20 (21.11)	–	22.12 (22.46)	–	–	–	–	

Abbreviations: BP, Blood Pressure; n, number of participants; SD, Standard Deviation; STAI; State-Trait Anxiety Inventory; TSST, Trier Social Stress Test; VAS, Visual Analog Scale.
[T] Outcome was subjected to transformation to meet model assumptions.

there were no significant effects of Lpc-37 on perceived -stress, -insecurity or -anxiety in any of the subgroups (Table S3). Results for the effects of Lpc-37 on the psychological response to the TSST in the ITT population are included in Tables S4 and S5.

3.5. Physiological biomarkers of stress – changes over intervention

3.5.1. Lpc-37 normalized 8pm cortisol levels in participants with LCS, and reduced diastolic BP in participants with HCS

For all four variables; AUC_g, AUC_i, cortisol at awakening and cortisol

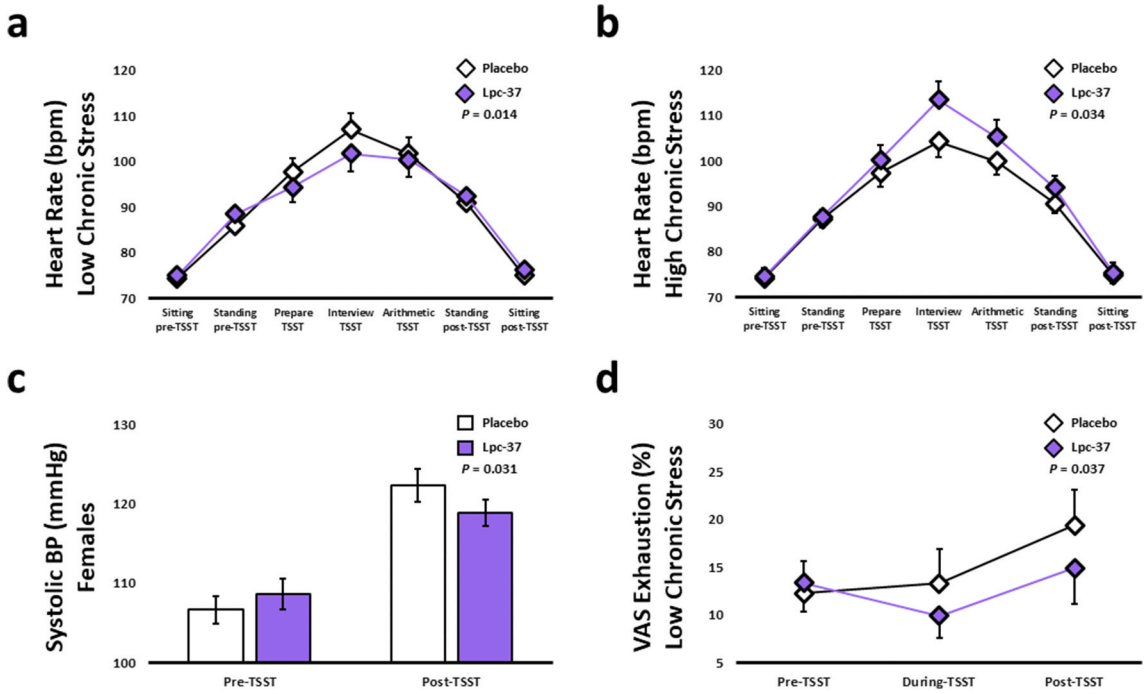

Fig. 2. Trier Social Stress Test (TSST) related outcomes: a. Heart rate in the low chronic stress subgroup (Mean ± SE). b. Heart rate in the high chronic stress subgroup (Mean ± SE). c. Systolic blood pressure in the female subgroup (Mean ± SE). d. Visual analog scale (VAS) exhaustion in the low chronic stress subgroup (Mean ± SE). Abbreviations: BP, Blood Pressure; TSST, Trier Social Stress Test; VAS, Visual Analog Scale.

at 8pm, there were no significant differences between the groups at baseline in the distribution of participants in different cortisol test value categories (low, normal, high) within the general population (Table 3) or in any of the subgroups (Table S6). For the variables AUC_g, AUC_i and cortisol at awakening, Lpc-37 had no significant impact on the distribution of participants in different cortisol test value categories at the end of study (Table 3). There was however an increase of 40.0%, and a decrease of 21.7% of participants in the normal test value category for cortisol at 8pm following intervention with Lpc-37 and placebo, respectively, at the end of study (Table 3; $P = 0.082$), highlighting a marginally favorable effect of Lpc-37 on 8pm cortisol levels. In addition, in participants with LCS, there was an increase of 75.0%, and a decrease of 54.5% of participants in the normal test value category for cortisol at 8pm following intervention with Lpc-37 and placebo, respectively, at the end of the study (Fig. 3a; $P = 0.036$) indicating a significant effect favoring the Lpc-37 group on 8pm cortisol levels. For the variable AUC_g, in participants with HCS, there was an increase of 23.5% in the placebo group and a decrease of 26.3% in the Lpc-37 group of participants in the normal test value category at the end of the study (Table S6; $P = 0.058$). There were no differences between the groups at the end of study in the distribution of participants in different cortisol test value categories for the other subgroups for the variables AUC_g and cortisol at 8pm; for any of the subgroups for the variables AUC_i and cortisol at awakening (Table S6).

Results for the effects of Lpc-37 on the distribution of participants in different cortisol test value categories following intervention in the ITT population are included in Tables S7 and S8.

There were no significant effects of Lpc-37 on either systolic or diastolic BP following intervention (Table 4). In participants with HCS, diastolic BP increased significantly less in the Lpc-37 group from baseline to end of study compared to the placebo group (Fig. 3b; $P = 0.047$). There were no significant effects of Lpc-37 on systolic BP in any of the subgroups or on diastolic BP in participants with LCS, or male and female participants (Table S9). Results for the effects of Lpc-37 on systolic and diastolic BP following intervention in the ITT population are included in Tables S10 and S11.

3.6. Psychological markers of stress – changes over intervention

3.6.1. Lpc-37 reduced perceived stress in the general population and females

PSS scores increased in the placebo group (+0.84 points; +4.1%) and decreased in the Lpc-37 group (−1.40 points; −6.4%) from baseline to end of study in the general population indicating a significant effect of Lpc-37 toward reducing perceived stress compared to placebo (Fig. 3c; $P = 0.048$). In female participants, Lpc-37 significantly reduced perceived stress (−1.00 point; −4.6%) following intervention compared to placebo (+2.36 points; +11.2%; Fig. 3d; $P = 0.049$). There were no significant effects of Lpc-37 on perceived stress in the other subgroups (Table S9).

BAI scores increased in the placebo group (+0.48 points; +8.2%) and decreased in the Lpc-37 group (−0.76 points; −13.8%) from baseline to end of study, indicating a marginally favorable effect of Lpc-37 toward reducing anxiety compared to placebo (Table 4; $P = 0.099$). There were no significant effects of Lpc-37 on anxiety in any of the subgroups (Table S9).

There was no significant effect of Lpc-37 on DASS-depression, -anxiety and -stress scores, following intervention in either the general population (Table 4) or in any of the subgroups (Table S9).

There was no significant effect of Lpc-37 on VAS-stress -anxiety, -insecurity and -exhaustion in the general population (Table 4). Further, there were no significant effects of Lpc-37 on VAS-stress -anxiety and -exhaustion in any of the subgroups (Table S9) and no significant effects of Lpc-37 on VAS-insecurity in participants with LCS, HCS and female participants. In male participants, the difference for the change score was marginally significant with VAS-insecurity scores decreasing in the placebo group and increasing in the Lpc-37 group from baseline to end of study (Table S9; $P = 0.063$). This result became significant in the ITT population (Table S11; $P = 0.031$).

Table 3
Number of participants by cortisol test value category at baseline and end of study for participants in the Per Protocol population.

	Baseline				End of Study			
	Low	**Normal**	**High**	***P***	**Low**	**Normal**	**High**	***P***
AUC_g (n)								
Placebo (n = 55)	12	30	13	0.270	7	35	13	0.442
Lpc-37 (n = 53)	6	36	11		11	28	14	
AUC_i (n)								
Placebo (n = 55)	22	28	5	0.413	15	36	4	1.000
Lpc-37 (n = 53)	16	34	3		15	34	4	
Cortisol at awakening (n)								
Placebo (n = 55)	16	26	13	0.425	12	34	9	0.265
Lpc-37 (n = 53)	14	31	8		19	26	8	
Cortisol at 8pm (n)								
Placebo (n = 55)	6	23	26	0.718	7	18	30	0.082
Lpc-37 (n = 53)	4	20	29		3	28	22	

Abbreviations: AUC_g, Area Under the Curve with respect to ground; AUC_i, Area Under the Curve with respect to increase; High, above 75% quantile; Low, under 25% quantile; n, number of participants; Normal, between 25% and 75% quantile.

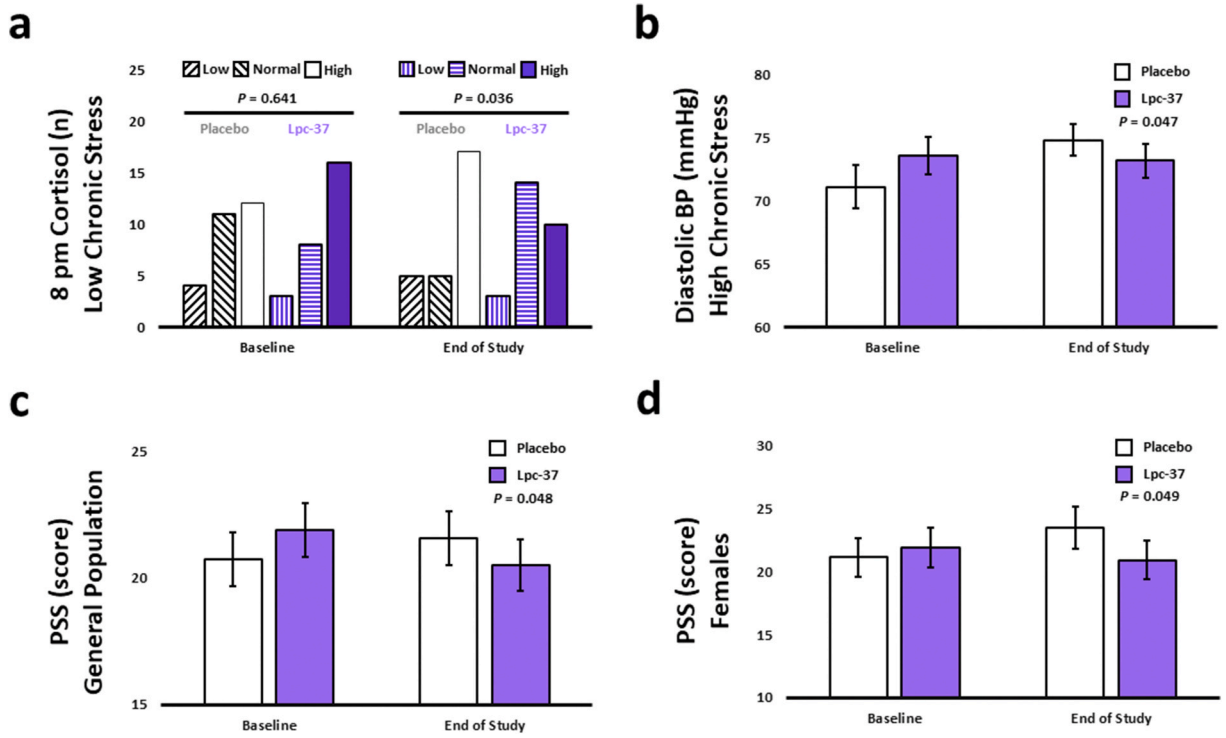

Fig. 3. Baseline and end of study related outcomes: a. 8pm cortisol in the low chronic stress subgroup (Low, under 25% quantile; Normal, between 25 and 75% quantiles; High, above 75% quantile). b. Diastolic BP in the high chronic stress subgroup (Mean ± SE). c. PSS in the general population (Mean ± SE). d. PSS in the female subgroup (Mean ± SE). Abbreviations: BP, Blood Pressure; PSS, Perceived Stress Scale; TSST, Trier Social Stress Test; VAS, Visual Analog Scale.

There was no significant effect of Lpc-37 on STAI-state anxiety in either the general population (Table 4), or in any of the subgroups (Table S9). Results for the effects of Lpc-37 on the psychological markers of stress following intervention in the ITT population are included in Tables S10 and S11.

3.7. Online diary measures of health and well-being. Lpc-37 increased perceived health and sleep-related recovery in participants with HCS

In the general population, Lpc-37 tended to increase perceived productivity scores compared to the placebo group throughout the study (Table 5; $P = 0.054$). Furthermore, Lpc-37 tended to increase perceived productivity in male participants (Table S12; $P = 0.092$). There were no significant effects of Lpc-37 on perceived productivity in the other subgroups (Table S12). In the ITT population, Lpc-37 significantly increased perceived productivity in participants with HCS compared to placebo (Table S14; $P = 0.037$).

Perceived health scores tended to increase in the Lpc-37 group compared to the placebo group throughout the study (Table 5; $P = 0.093$). In participants with HCS, Lpc-37 significantly increased perceived health scores, compared to placebo throughout the study (Fig. 4a; $P = 0.012$). There were no significant effects of Lpc-37 on perceived health in the other subgroups (Table S12).

Lpc-37 tended to reduce sleep disruptions (binary) throughout the study period, compared to placebo (Table 5; $P = 0.061$), but had no significant effect on sleep disruptions (count) (Table 5). In participants

Table 4
Summary measures at baseline and end of study for participants in the Per Protocol population.

	Baseline	End of Study	*P*
	Mean (SD)	**Mean (SD)**	
Systolic BP (mmHg)			
Placebo (n = 58)	119.66 (13.82)	122.86 (14.14)	0.871[missT]
Lpc-37 (n = 55)	119.60 (14.21)	121.87 (14.28)	
Diastolic BP (mmHg)			
Placebo (n = 58)	71.68 (9.16)	74.62 (6.39)	0.327[miss]
Lpc-37 (n = 55)	71.89 (7.74)	73.18 (7.45)	
STAI-State (score)			
Placebo (n = 58)	34.33 (7.73)	35.33 (8.37)	0.715[T]
Lpc-37 (n = 55)	33.65 (6.80)	35.18 (8.38)	
PSS (score)			
Placebo (n = 57)	20.72 (7.97)	21.56 (8.16)	0.048
Lpc-37 (n = 55)	21.89 (7.90)	20.49 (7.51)	
DASS Depression (score)			
Placebo (n = 58)	5.21 (6.38)	5.10 (5.61)	0.221[T]
Lpc-37 (n = 55)	4.60 (4.94)	4.15 (5.52)	
DASS Anxiety (score)			
Placebo (n = 58)	3.07 (4.58)	3.45 (5.08)	0.224[V]
Lpc-37 (n = 55)	2.60 (3.35)	2.44 (3.59)	0.933[B] 0.117[EOS]
DASS Stress (score)			
Placebo (n = 58)	9.41 (7.87)	10.09 (8.17)	0.248[T]
Lpc-37 (n = 55)	9.76 (7.92)	8.91 (7.14)	
BAI (score)			
Placebo (n = 58)	5.85 (5.73)	6.33 (7.26)	0.099[T]
Lpc-37 (n = 55)	5.51 (4.46)	4.75 (4.39)	
VAS Stress (score)			
Placebo (n = 58)	19.34 (21.44)	20.67 (21.63)	0.436[T]
Lpc-37 (n = 55)	19.11 (22.97)	23.32 (23.18)	
VAS Insecurity (score)			
Placebo (n = 58)	15.91 (19.60)	17.30 (20.15)	0.355[V]
Lpc-37 (n = 55)	13.58 (21.41)	16.44 (19.67)	0.234[B] 0.344[EOS]
VAS Anxiety (score)			
Placebo (n = 58)	7.58 (14.05)	7.85 (13.40)	0.204[V]
Lpc-37 (n = 55)	7.29 (15.13)	9.26 (16.48)	0.362[B] 0.584[EOS]
VAS Exhaustion (score)			
Placebo (n = 58)	23.19 (21.08)	18.45 (21.31)	0.609[T]
Lpc-37 (n = 55)	29.56 (27.63)	24.66 (22.78)	

Abbreviations: BAI, Beck Anxiety Inventory; BP, Blood Pressure; DASS, Depression Anxiety Stress Scale; n, number of participants; PSS, Perceived Stress Scale; SD, Standard Deviation; STAI; State-Trait Anxiety Inventory; VAS, Visual Analog Scale.
[V] Model assumptions for ANOVA were violated. Change score = baseline vs end of study.
[miss] Inferential statistics is not based on the same data set as descriptive statistics as records with missing data had to be excluded.
[T] Outcome was subjected to transformation to meet model assumptions.
[B] Model assumptions for ANOVA were violated. *P* value at baseline.
[EOS] Model assumptions for ANOVA were violated. *P* value at end of study.

with LCS, there was a larger decrease observed in the placebo group compared with the Lpc-37 group for sleep disruptions (count), although both groups displayed the same sleep disruptions at the end of study (Fig. 4b; $P = 0.005$). There were no significant effects of Lpc-37 on sleep disruptions (binary) in any of the subgroups and on sleep disruptions (count) in participants with HCS, or male and female participants (Table S12). For sleep duration and sleep-related recovery, the interaction between treatment group and time was not significant throughout the study in the general population (Table 5). In participants with HCS, Lpc-37 significantly increased sleep-related recovery scores compared to placebo (Fig. 4c; $P = 0.006$). There were no significant effects of Lpc-37 on sleep-related recovery scores in the other subgroups and on sleep duration in any of the subgroups (Table S12).

There was no significant effect of Lpc-37 on mood ratings throughout the study in either the general population (Table 5), or in any of the subgroups (Table S12).

Results for the effects of Lpc-37 on the online diary measures of health and well-being in the ITT population are included in Tables S13 and S14.

3.8. Safety parameters

Concerning the safety objectives of this study, no significant differences were observed in either systolic or diastolic BP, HR, weight, and BMI between randomized participants in the study groups at V3. Causality of all AEs reported by the participants were rated as "unlikely" or "not related" by the PI and the study physicians for both groups. Moreover, the maximum severity of these events was "moderate". Thus, no SAEs were recorded in this study for either group. Only two AEs were lost to follow-up, but all other AEs were resolved, and no action was necessary (*i.e.* study interruption or withdrawal). In total, 111 AEs were reported in the placebo group from 74 participants (Table S15) and 100 AEs were reported in the Lpc-37 group from 71 participants (Table S16) over the duration of the study. There were no significant differences in the frequencies of the most frequently occurring AEs; common cold, sore throat, headache or stomach ache between the groups. The number of participants was too small for all other AEs to estimate statistical differences between the groups. The distribution of AEs was similar between the groups.

4. Discussion

Exposure to stress can impact the gut microbial profile and in turn, experimental alteration of the gut microbiota can influence the stress response (Foster et al., 2017). Manipulation of the gut microbiota through probiotic intervention is therefore a novel approach to influence stress, mood and well-being. Previously, Lpc-37 prevented stress-associated behaviors and an anxious phenotype from developing in mice from two experiments using the same chronic stress model (Stenman et al., 2020). The results of this clinical trial point to different directions with respect to efficacy of Lpc-37 on physiological and psychological outcomes when analyzed over the study and in response to an acute stressor (summarized in Table 6 for the PP and Table S17 for the ITT).

The primary objective of this study was selected based on previous studies which demonstrated that the TSST elicits a significant increase in HR (Kirschbaum et al., 1993; Hellhammer and Schubert, 2012; Hellhammer et al., 2014). While HR was expected to increase in response to the TSST, chronic psychosocial factors have been shown to affect cardiovascular reactivity to acute stress, with some studies demonstrating an association between HCS and blunted cardiovascular reactivity (Fries et al., 2005; Teixeira et al., 2015). Such conditions have also been associated with a host of negative behavioral outcomes (Carroll et al., 2017). For this reason, the study population was stratified to investigate the impact of chronic stress on HR as a biomarker of the ANS response to acute stress. In the general population, Lpc-37 had no effect on HR in response to the TSST, however significant effects were observed within the subgroups. While Lpc-37 reduced the increase in HR in response to acute stress in participants with LCS, the opposite was seen in participants with HCS. The exact mechanisms for these effects are unknown but could suggest that the effect of Lpc-37 on HR may be differentially dependent on chronic stress. Although cardiovascular reactivity was not blunted per se in the HCS population, the effect of Lpc-37 could be more

Table 5
Summary online diary measures for participants in the Per Protocol population.

		Week 1 run-in	Week 2 run-in	Week 3 treatment	Week 4 treatment	Week 5 treatment	Week 6 treatment	Week 7 treatment	*P*
Perceived Productivity (score)									
Placebo (n = 47)	Mean (SD)	7.15 (1.07)	7.29 (1.03)	7.30 (1.01)	7.34 (1.18)	7.43 (1.17)	7.31 (1.22)	7.32 (1.25)	0.054
Lpc-37 (n = 44)	Mean (SD)	6.98 (1.02)	7.34 (1.06)	7.53 (0.97)	7.48 (1.19)	7.59 (1.04)	7.57 (1.13)	7.50 (1.17)	
Perceived Health Status (score)									
Placebo (n = 47)	Mean (SD)	7.86 (1.08)	7.92 (1.12)	7.92 (1.06)	8.01 (1.05)	7.92 (1.16)	7.73 (1.26)	7.75 (1.52)	0.093[V]
Lpc-37 (n = 44)	Mean (SD)	7.80 (1.31)	7.89 (1.15)	7.88 (1.20)	7.91 (1.18)	8.05 (1.22)	8.11 (1.20)	7.91 (1.15)	
Sleep Duration (min)									
Placebo (n = 47)	Mean (SD)	447.45 (38.76)	448.13 (41.62)	456.90 (37.08)	459.81 (39.44)	457.26 (42.04)	450.16 (42.04)	459.66 (39.71)	0.737
Lpc-37 (n = 44)	Mean (SD)	447.27 (47.50)	444.01 (44.60)	449.45 (41.47)	450.62 (36.07)	454.50 (39.82)	450.88 (38.95)	445.60 (40.02)	
Sleep Disruptions (binary)									
Placebo (n = 47)	Proportion (yes/total)	0.465	0.426	0.418	0.310	0.292	0.331	0.389	0.061
Lpc-37 (n = 44)	Proportion (yes/total)	0.477	0.435	0.354	0.367	0.306	0.279	0.290	
Sleep Disruptions (count)									
Placebo (n = 47)	Mean of week sum (SD)	6.09 (4.96)	5.49 (4.82)	5.11 (4.89)	4.30 (6.05)	3.53 (3.80)	4.02 (4.68)	5.83 (6.23)	0.084
Lpc-37 (n = 44)	Mean of week sum (SD)	7.30 (6.87)	5.50 (4.62)	4.89 (5.11)	5.43 (9.20)	3.52 (3.48)	3.80 (7.40)	4.66 (6.37)	
Sleep Related Recovery (score)									
Placebo (n = 47)	Mean (SD)	6.91 (1.00)	7.15 (1.07)	7.27 (1.12)	7.29 (1.18)	7.36 (1.19)	7.10 (1.28)	7.28 (1.18)	0.232[T]
Lpc-37 (n = 44)	Mean (SD)	6.71 (1.34)	7.07 (1.28)	7.32 (1.11)	7.30 (1.30)	7.36 (1.22)	7.42 (1.19)	7.31 (1.25)	
Mood Ratings (score)									
Placebo (n = 47)	Mean (SD)	7.27 (1.04)	7.49 (1.10)	7.46 (1.13)	7.53 (1.15)	7.50 (1.24)	7.40 (1.21)	7.55 (1.22)	0.179[T]
Lpc-37 (n = 44)	Mean (SD)	7.31 (1.25)	7.53 (1.21)	7.66 (1.05)	7.77 (1.25)	7.73 (1.17)	7.90 (1.10)	7.77 (1.30)	

Abbreviations: n, number of participants; SD, Standard Deviation.
[V] Model assumptions for linear mixed models were violated. ANOVA on aggregated data.
[T] Outcome was subjected to transformation to meet model assumptions.

pronounced in a clinically stressed population. The ANS is just one component of the microbiota-gut-brain axis and perhaps there is some mechanism mediated through the gut and influenced through probiotic intervention which beneficially influences the response to acute stress differently, dependent on underlying stress. This hypothesis based on the results described herein is purely exploratory and should be investigated in future studies. To our knowledge this is the first time a probiotic has demonstrated different effects on HR under different conditions of chronic stress.

Lpc-37 also significantly reduced perceived exhaustion/fatigue in response to the TSST in participants with LCS. This psychological response could indeed be associated with the reduced HR in response to the acute stress also seen within this subgroup. Furthermore, Lpc-37 significantly decreased both diastolic and systolic BP in participants with HCS and females, respectively. It has previously been shown in mildly hypertensive patients that consumption of a fermented milk drink containing *Lacticaseibacillus casei* strain Shirota (LcS) and gamma-aminobutyric acid (GABA) decreased both systolic and diastolic BP after four weeks and up to twelve weeks of intervention (Inoue et al., 2003). Furthermore, the incidence of hypertension among community-living normotensive elderly participants consuming the same fermented milk drink containing LcS (without GABA), three times or more per week over a five-year interval was lower than those consuming the drink less than three times per week (Aoyagi et al., 2017). The effects of Lpc-37 on biomarkers of the ANS response to stress indicate one pathway through which microbiota-gut-brain signaling could be influenced by Lpc-37.

The secondary objectives of this study included a range of outcomes to measure the stress response following intervention with Lpc-37. The PSS is a global measure of subjective stress, not restricted to any one specific life event or clinical condition and is suitable for use across diverse populations and settings. Other probiotic interventions have proven relatively unsuccessful in reducing self-reported perceived stress using this scale (Messaoudi et al., 2011a; Chung et al., 2014; Ostlund-Lagerstrom et al., 2016; Siegel and Conklin, 2020). While post-hoc analyses from Messaoudi and colleagues demonstrated a within-group effect of the probiotic formulation, the result was expressed as a percentage of change in PSS score from baseline to follow-up and was only found in a subset of participants with low levels of 24-h urinary-free cortisol at baseline (Messaoudi et al., 2011b). In a repeated measures design, Allen and colleagues demonstrated that *Bifidobacterium longum* 1714 reduced PSS score compared between groups using AUC measurements (Allen et al., 2016). Participants in the Sisu study reported significantly lower PSS scores following intervention with Lpc-37 and this result was reflected in absolute scores from baseline to end of study. Perceived stress was also reduced in females taking Lpc-37. Interestingly, covariate analyses revealed that females had higher stress (DASS-stress), sleep disruptions (binary), and lower sleep-related recovery scores compared to males, indicating that sex is an underlying factor influencing the stress response. Thus, female participants in this study could be considered more stressed than males.

The custom-designed online diary proved a successful tool for gathering exploratory data throughout the study. The diary results have alluded to some mechanistic insights into the significant effect of Lpc-37 on perceived stress. Participants in the general population and males consuming Lpc-37 had a marginally significant increase in productivity. In participants with HCS within the ITT population, those consuming Lpc-37 had a significant increase in productivity. These results indicate Lpc-37 could increase *feelings* of productivity. Probiotic interventions have proven to support various aspects of work-place healthiness (Tubelius et al., 2005), a healthy immune system (Turner et al., 2017; Weizman et al., 2005), and prevent the onset of symptoms in participants exposed to stress (Sawada et al., 2017; Culpepper et al., 2016; Kato-Kataoka et al., 2016). Interestingly, while these studies suggest a link between probiotics and productivity, none have measured the individual perception of such. The association between productivity and chronic stress is of major relevance as workplace stress and burn-out are increasingly prevalent (Street and Lacey, 2019). Perhaps while reducing perceived stress, Lpc-37 might be beneficial in targeting

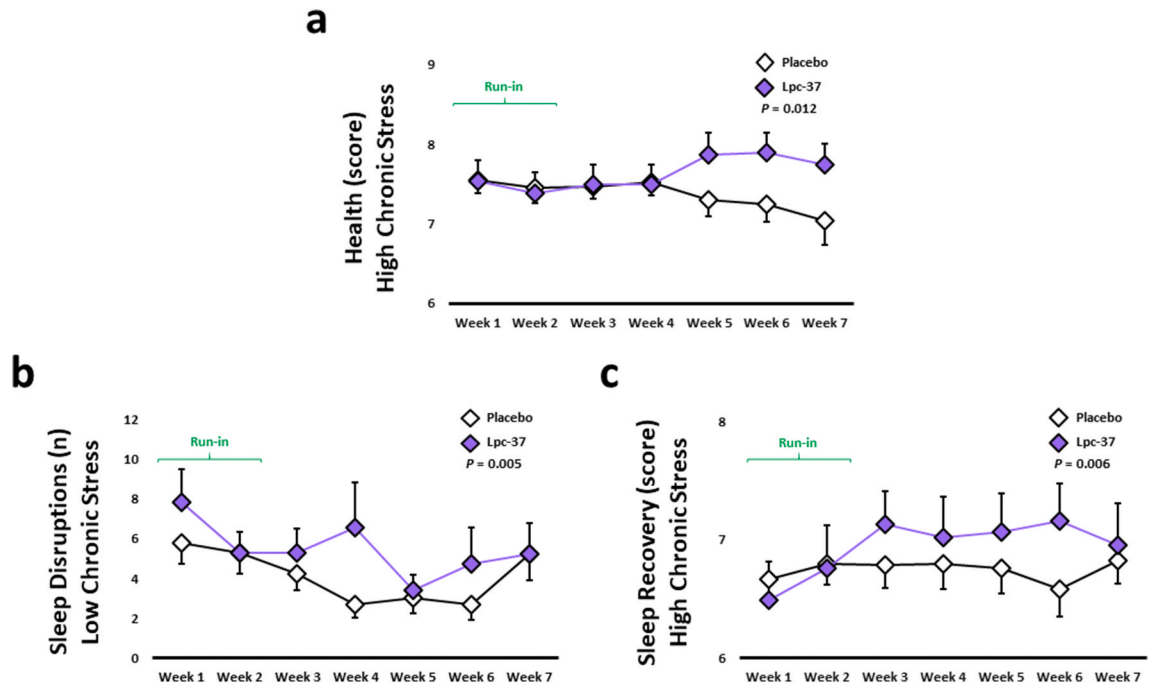

Fig. 4. Online diary related outcomes: a. Perceived health in the high chronic stress subgroup (Mean ± SE). b. Number of sleep disruptions in the low chronic stress subgroup (Mean ± SE). c. Sleep related recovery in the high chronic stress subgroup (Mean ± SE).

stress-associated dips in productivity. In addition, Lpc-37 marginally increased perceived health throughout the study, becoming significant in participants with HCS. These results suggest some potential pathways through which Lpc-37 may influence symptoms of stress, be it through increasing perceived productivity or health, or vice versa.

The TSST successfully induced an endocrine stress response in both the HPA axis (cortisol) and sympatho-adreno-medullary system (AA), however there was no effect of Lpc-37 on either system's acute stress response. Lpc-37 marginally normalized the 8pm cortisol levels, *i.e.* more participants in the normal-test value category in the Lpc-37 group at the end of study. This trend became significant in participants with LCS and is worth exploring in future studies. Vreeberg and colleagues previously reported that depressed participants in a large community-based study had higher evening cortisol levels when compared to non-depressed participants (Vreeburg et al., 2009). Therefore, there is some indication that evening cortisol directly correlates with stress-associated disorders. Some studies have found an impact of probiotics on the cortisol response in stressed participants (Chong et al., 2019), in particular in the response to exam stress (Sawada et al., 2017; Kato-Kataoka et al., 2016; Andersson et al., 2016; Takada et al., 2016). Manipulation of the gut microbiota can therefore alter the neuroendocrine stress response through the HPA axis.

The gut microbiome has been implicated in sleep disturbances (Benedict et al., 2016), and some studies support the role of probiotics in improving sleep patterns in humans (Marotta et al., 2019; Takada et al., 2016; Yamamura et al., 2009). While participants taking Lpc-37 tended to have less reported sleep disruptions (binary), those with LCS had significantly higher self-reported sleep disruptions (count) throughout the intervention. Lpc-37 increased sleep-related recovery – *or* – how rested participants with HCS felt after a night sleep. In a recent meta-analysis, probiotics had a significant effect on the Pittsburgh Sleep Quality Index-total score but had no significant effect on other subjective sleep scales or objective parameters of sleep (Irwin et al., 2020). Therefore, the effects of Lpc-37 on sleep observed in this study should be considered exploratory, and future study designs with Lpc-37 to explore the effect of this strain on sleep should include more comprehensive measures of sleep quality and efficiency. Indeed, stress is closely linked with sleep disruption which plays a central role in mediating psychiatric disorders (Simon et al., 2020).

4.1. Limitations

Although considered the gold standard in clinical experimental stress research, perhaps the most obvious limitation of the TSST (Allen et al., 2017), was its single administration and lack of comparative baseline data. The TICS was used to stratify the population according to chronic stress over the past three months and while this inventory has delivered helpful results in previous TSST studies (Hellhammer et al., 2010, 2012, 2014; Schult et al., 2010), it may not differentiate enough to fully depict the large variety of chronic stress as a predecessor for physical and mental health problems. Finally, while Lpc-37 did have a beneficial impact on many endpoints in this study, the mechanisms are largely unknown and will be explored in future studies.

5. Conclusion and future perspectives

The intake of Lpc-37 for five weeks significantly reduced perceived stress. In addition, Lpc-37 tended to improve many other biomarkers related to stress in the general population and other significant beneficial effects were identified within the subgroups. Concerning safety, there were no SAEs and only mild to moderate AEs were recorded throughout the study, with no significant differences between the groups. The occurrence of AEs was therefore not connected to any study group. Vital signs remained unaffected at the end of the study. Thus, the findings from this study do not raise any concerns over the safety of Lpc-37. In the sample studied, the mean scores for the screening scale of the TICS were still in a relatively normal range, even for the HCS subgroups. Therefore, one could speculate that the reported effects of Lpc-37 in participants with HCS would be enhanced in participants under more pronounced chronic stress. Considering the unexpected findings that Lpc-37 decreased HR in response to the TSST in participants with LCS, but increased the same biomarker for the ANS response to stress in

Table 6
Summary of effects of Lpc-37 for participants in the Per Protocol population and subgroups.

	All data	Low chronic stress	High chronic stress	Male	Female
TSST-related endpoints†					
Heart Rate (bpm)		↓	↑		
Systolic BP (mmHg)					↓
VAS Exhaustion (score)		↓			
Baseline and End of Study‡					
Cortisol Normalization AUC_g			$(\downarrow)^{EOS}$		
Cortisol Normalization at 8pm	$(\uparrow)^{EOS}$	$\uparrow^{EOS}$			
Diastolic BP (mmHg)			↓		
PSS (score)	↓				↓
BAI (score)	(↓)				
DASS-anxiety (score)				$(\downarrow)^{EOS}$*	
VAS Insecurity (score)				$(\uparrow)^{V}$	
VAS Anxiety (score)				$\uparrow^{V,B}$	
Online diary measures∅					
Perceived Productivity (score)	(↑)			(↑)	
Perceived Health Status (score)	(↑)		↑		
Sleep Disruptions (binary)	(↓)	(↑)*			
Sleep Disruptions (count)	(↑)*	↑		(↑)*	
Sleep Related Recovery (score)			↑		

The data in Table 6 report p-values according to the following criteria: a) significant p-values ($P < 0.05$) and b) marginal/trend p-values ($P < 0.1$). All p-values in Table 6 describe effects for the Lpc-37 group based on the interaction between treatment group and time. Abbreviations: AUCg, Area Under the Curve with respect to ground; BAI, Beck Anxiety Inventory; BP, Blood Pressure; DASS, Depression, Anxiety, Stress Scale; PSS, Perceived Stress Scale; TSST, Trier Social Stress Test; VAS, Visual Analog Scale.
^{B} Baseline.
^{EOS} End of study.
^{V} Change score = baseline vs end of study.
* Due to the patterns presented by the groups, the effect cannot be confirmed.
↑ Increase for the Lpc-37 group as compared to the placebo group.
↓ Decrease for the Lpc-37 group as compared to the placebo group.
† Based on the mean difference over the duration of the TSST.
‡ Based on the mean difference from baseline to end of study.
∅ Based on the mean difference over the entire study period.
↑,↓ $P < 0.05$.
(↑,↓) $P < 0.1$.

participants with HCS, future probiotic intervention studies should include elaborated psychobiological diagnostics for chronic stress and could combine the TSST innovative methods assessing the psychological and physiological response to an acute stressor. Such an approach would decipher whether the effects of probiotics are somewhat dependent on daily/chronic stress. Finally, Lpc-37 maintained stability and did not fall below the target dose throughout the study, thereby there are no stability concerns for Lpc-37.

CRediT authorship contribution statement

Elaine Patterson: Conceptualization, Methodology, Writing - original draft, Visualization, Project administration. **Síle M. Griffin:** Writing - original draft, Visualization, Project administration. **Alvin Ibarra:** Conceptualization, Methodology, Writing - original draft, Visualization. **Emilia Ellsiepen:** Methodology, Software, Formal analysis, Data curation, Writing - original draft. **Juliane Hellhammer:** Conceptualization, Methodology, Resources, Writing - original draft, Supervision.

Declaration of competing interest

Lacticaseibacillus paracasei Lpc-37 is a commercial product of DuPont Nutrition & Biosciences. Lpc-37® is a registered trademark or trademark of DuPont de Nemours, Inc. or its affiliated companies. Elaine Patterson, Alvin Ibarra and Síle M. Griffin are employees of DuPont Nutrition & Biosciences, the manufacturer of *Lacticaseibacillus paracasei* Lpc-37 and were employed by DuPont Nutrition & Biosciences during the study. Juliane Hellhammer and Emilia Ellsiepen are employees of daacro GmbH and Co. KG, a company that was hired by DuPont Nutrition & Biosciences to conduct the study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2020.100277.

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Lactobacillus plantarum DR7 Modulated Bowel Movement and Gut Microbiota Associated with Dopamine and Serotonin Pathways in Stressed Adults

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Abstract: We have previously reported that the administration of *Lactobacillus plantarum* DR7 for 12 weeks reduced stress and anxiety in stressed adults as compared to the placebo group, in association with changes along the brain neurotransmitters pathways of serotonin and dopamine-norepinephrine. We now aim to evaluate the effects of DR7 on gut functions, gut microbiota compositional changes, and determine the correlations between microbiota changes and the pathways of brain neurotransmitters. The administration of DR7 prevented an increase of defecation frequency over 12 weeks as compared to the placebo ($p = 0.044$), modulating the increase of stress-induced bowel movement. Over 12 weeks, alpha diversity of gut microbiota was higher in DR7 than the placebo group across class ($p = 0.005$) and order ($p = 0.018$) levels, while beta diversity differed between groups at class and order levels ($p < 0.001$). Differences in specific bacterial groups were identified, showing consistency at different taxonomic levels that survived multiplicity correction, along the phyla of Bacteroides and Firmicutes and along the classes of Deltaproteobacteria and Actinobacteria. Bacteroidetes, Bacteroidia, and Bacteroidales which were reduced in abundance in the placebo group showed opposing correlation with gene expression of dopamine beta hydrolase (DBH, dopamine pathway; $p < 0.001$), while Bacteroidia and Bacteroidales showed correlation with tryptophan hydroxylase-II (TPH2, serotonin pathway; $p = 0.001$). A correlation was observed between DBH and Firmicutes ($p = 0.002$), Clostridia ($p < 0.001$), Clostridiales ($p = 0.001$), *Blautia* ($p < 0.001$), and *Romboutsia* ($p < 0.001$), which were increased in abundance in the placebo group. *Blautia* was also associated with TDO ($p = 0.001$), whereas *Romboutsia* had an opposing correlation with TPH2 ($p < 0.001$). Deltaproteobacteria and Desulfovibrionales which were decreased in abundance in the placebo group showed opposing correlation with DBH ($p = 0.001$), whereas *Bilophila* was associated with TPH2 ($p = 0.001$). Our present data showed that physiological changes induced by *L. plantarum* DR7 could be associated with changes in specific taxa of the gut microbiota along the serotonin and dopamine pathways.

Keywords: *Lactobacillus plantarum*; probiotic; microbiota; stress; serotonin; dopamine; clinical trial

1. Introduction

The symptoms of stress affect human anatomy beyond that of psychological perceptions. Increasing consumer awareness on health have led to better characterization and understanding of stress, on both aetiology and consequences. One of the more vastly reported bodily changes as influenced by stress includes gastrointestinal functions, where symptoms of heartburn, indigestion, nausea and vomiting,

bowel movement, and abdominal pain are reportedly increased amid stress. Recent advances in gut microbiota profiling have garnered much evidence that these gut inhabitants play major roles in host physiological signaling and responses, including psychiatric conditions along the gut–brain axis. Mounting evidence suggests that microbial cellular components and metabolites of the complex gut microbiota may influence brain functions via neuroimmune and neuroendocrine pathways as well as the nervous system, while differences in microbial diversity and taxonomic compositions were observed between stressed and control individuals [1]. Although gut microbiota changes with growth, primarily attributed to external factors such as environment, mode of delivery, diet, and lifestyles, dysbiosis also occurs upon changes in health conditions such as bowel disorders and inflammatory and metabolic diseases [2]. It is thus suggested that the modulation of gut microbiota is crucial towards a healthier general well-being, including that of mental health.

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [3]. *Lactobacillus* remains one of the most commonly administered probiotic genera with a long history of safe use and comprehensive documentation on gut health and antimicrobial protective properties [4]. Increasing evidence has shown the potentials of probiotics as a natural agent to influence brain health and psychological well-being. Anxiolytic and antidepressant-like behaviors were observed in mice administered with *Lactobacillus rhamnosus* while exaggerated hypothalamic–pituitary–adrenal stress response in germ-free mice was partially reversed upon oral consumption of *Bifidobacterium infantis* [5]. Although depression-like behaviors in a rat model were reversed upon administration of *Bifidobacterium infantis* [6], studies on the association of major depressive disorders (MDD) with gut microbiota revealed that MDD patients showed a lower abundance of gut *Bifidobacterium* and *Lactobacillus* than healthy controls [7].

We have previously reported on the use of *Lactobacillus plantarum* DR7 (now *Lactoplantibacillus plantarum* DR7 [8]) in the alleviation of stress and anxiety in stressed adults, accompanied by improved traits of memory and cognition such as basic attention, emotional cognition, and associate learning as compared to the placebo group [9]. DR7 was administered in a randomized, double-blind, and placebo-controlled study, where plasma cortisol levels were reduced accompanied by reduced plasma pro-inflammatory cytokines, such as interferon-γ (IFN- γ) and tumor necrosis factor-α (TNF-α), and increased plasma anti-inflammatory cytokines, such as interleukin-10 (IL-10), compared to the placebo. Plasma gene expression analysis revealed that DR7 exerted these psychological effects along the brain neurotransmitters pathway of serotonin synthesis from tryptophan and the dopamine-norepinephrine pathway. The study started during the year-end period of 2017 in East Peninsular Malaysia, which also corresponded to the period of annual monsoon season, for 12 weeks, which coincided with the aftermath of a flood. During the monsoon of 2017, a massive flood occurred in East Peninsular Malaysia, affecting over 14,000 individuals and displacing over 2000 homes where families were housed in shelters [10], attributed to increased rainfall in November and December 2017. We have also previously reported that these monsoon and flood seasons imposed great stress, gut dysbiosis, and abdominal disorders in flood victims and the general communities of East Peninsular Malaysia [11].

Considering the gut–brain axis is bidirectional while probiotics are orally administered to reach the gut, we aimed to evaluate and better understand the effects of DR7 on gut disorders and gut microbiota compositional changes. More importantly, we aimed to determine the potential relationships between microbiota changes and the pathways of brain neurotransmitters that had led to improved psychological effects in stressed subjects as previously reported [9].

2. Results and Discussion

2.1. Baseline

As a continuation of a previous study, the general characteristics for all subjects were as previously reported, where the subjects from both groups fulfilled the inclusion criteria of moderately stressed, and insignificant differences were observed in most of the general and demographic characteristics

between placebo and DR7 groups [9]. A total of 124 subjects were assessed for eligibility, recruited, and randomized into either the placebo or DR7 group (Figure 1). A total of 13 subjects dropped-out during the 12-weeks period, either due to failure to track or did not comply in answering the gut health questionnaire, yielding a total of 111 subjects (n = 55 for placebo and n = 56 for DR7). No adverse effects were reported. A total of 44 subjects from the placebo group and 55 from the DR7 group provided complete sets of faecal samples, yielding a total of 99 completed faecal sample sets.

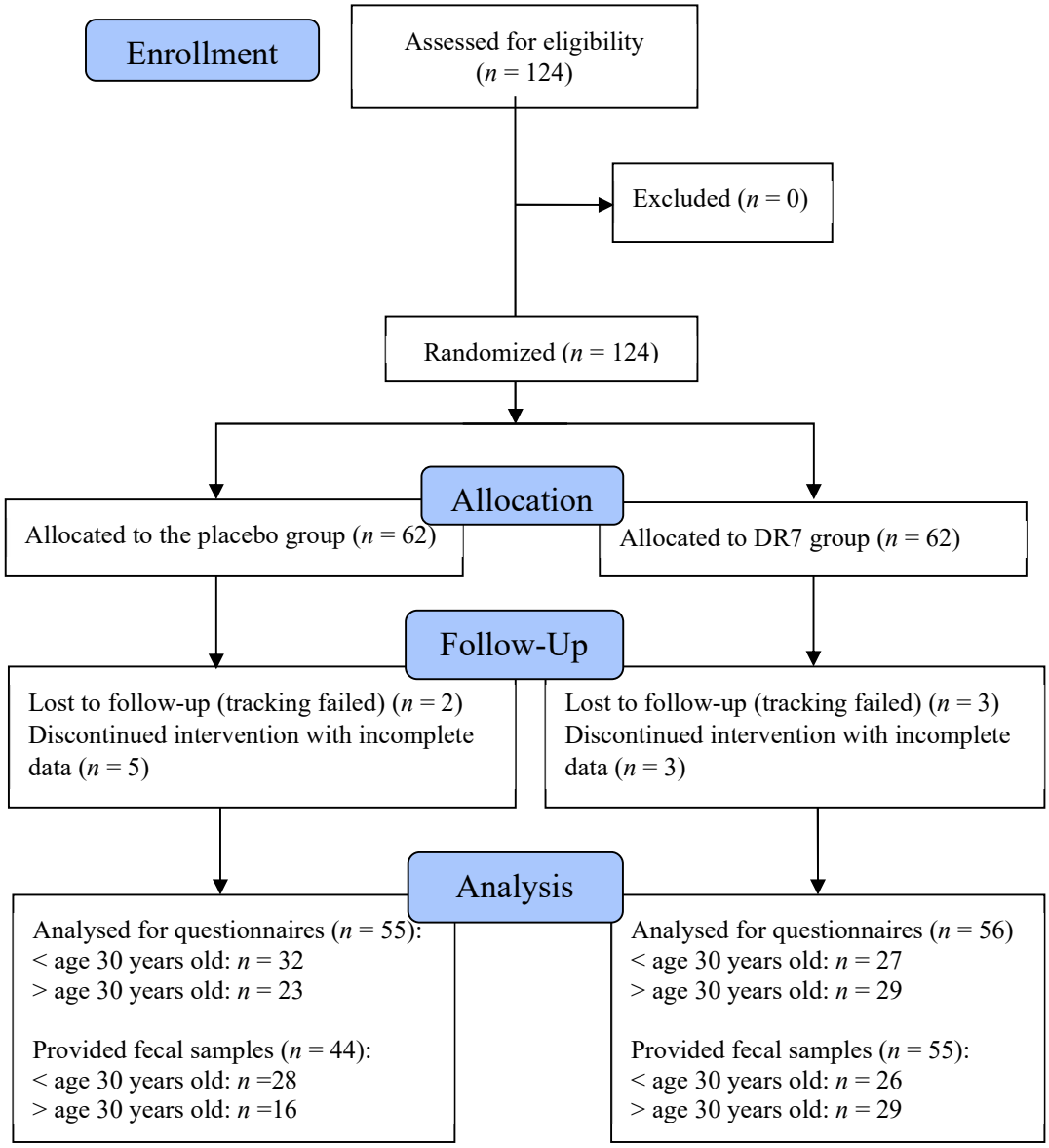

Figure 1. Consort flow chart of recruitment for both intervention groups.

2.2. Gastrointestinal Clinical Outcome

The gastrointestinal questionnaire was developed to evaluate on several parameters involving defecation frequency and direct and indirect gastrointestinal symptoms, which is relevant for the use in this study. It has been reported that natural disasters such as flood and monsoon often affect gastrointestinal health, attributed to changes in diets, lifestyles, availability of clean water supply, and higher spread of infectious diseases in shelters and evacuation centres [11]. Although monsoons and coastal storms often increase the incidences of diarrhoea attributed to poorer sanitation and contaminated water, infectious diarrhoea has also been reported to rise after floods [12]. The incidences

of diarrhoea have been reported by subjects in both placebo and DR7 groups in the present study, although an insignificant difference in frequency was observed between groups over 12 weeks (data not shown). Amid this, the administration of DR7 decreased the frequency of defecation in all subjects as compared to the placebo after week-12 (Figure 2A). This may be explained via the modulatory effects of DR7 against increased bowel movement and defecation as triggered by the central nervous system (CNS) upon stress. Stress affects the regulatory mechanisms and responses of the CNS leading to stimulated colonic motor activity, increased movements of the bowel, and subsequently, increased frequency of defecation [13]. Thus, although DR7 did not exert a significant diarrhoea-reducing effect as compared to the placebo, the administration of DR7 may have alleviated unnecessary bowel movement as induced by stress.

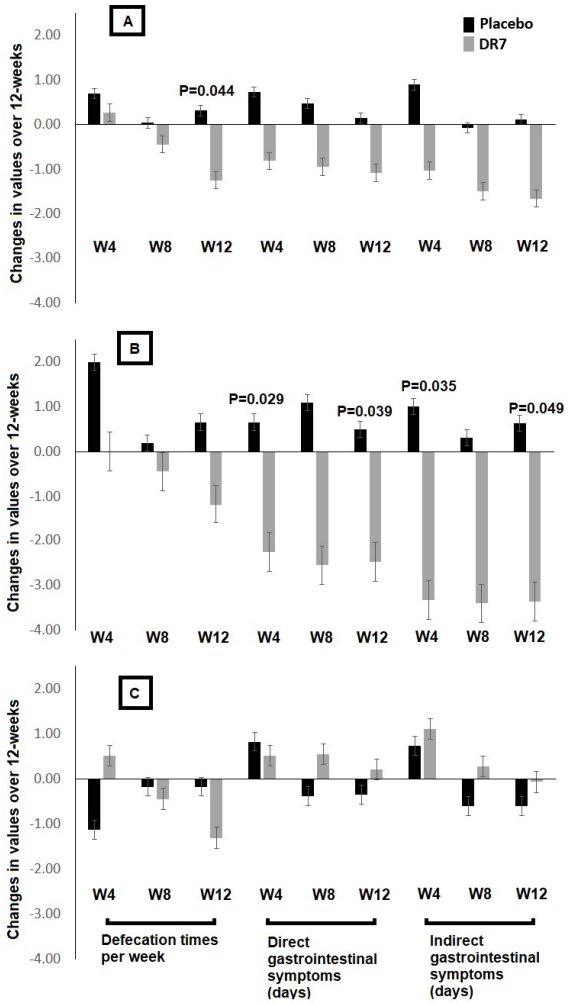

Figure 2. Changes in gastrointestinal clinical outcomes after 12 week administration of *Lactobacillus plantarum* DR7 compared to the placebo group in (**A**) all subjects, (**B**) young adults (aged <30 years old), and (**C**) normal adults (aged >30 years old). Direct gastrointestinal symptoms included vomiting, dysentery (blood in stool), abdominal pain, nausea, and rectal pain (sharp dull, burning, feels like a hard object in the rectum); indirect gastrointestinal symptoms included loss of appetite, fatigue, dizziness, headache, dehydration, and fever; number of defecation times were calculated based on a weekly basis. *p*-values indicated the difference between treatment groups at individual time points. Results are expressed as mean, error bars (SEM); $n = 111$ (DR7 $n = 56$ and placebo $n = 55$).

We have previously reported that the administration of DR7 was more effective in reducing symptoms of stress and plasma levels of cortisol in younger adults as compared to the placebo while such an effect was less observed in normal adults [9]. Thus, in the present assessment, we have also

evaluated gastrointestinal clinical symptoms in both subgroups. Although the administration of DR7 did not show any significant effects against gastrointestinal disorders in normal adults aged above 30 years old, younger adults below 30 years old seemed to benefit from the administration of DR7, where decreased durations for both direct and indirect gastrointestinal symptoms were observed as compared to the placebo group as early as week-4 and continuously till week-12 (Figure 2B,C). The response to either pain or discomfort-related stressors often increases cortisol secretion leading to a sensitized physiologic stress response. To facilitate the consolidation of fear for survival and avoidance of danger, glucose reserves are mobilized for energy and modulating inflammation by cortisol [14]. Reductions in direct and indirect gastrointestinal symptoms have led to reduced levels of discomfort and subsequently reduced levels of stress and cortisol in younger adults.

2.3. Alpha/Beta Diversity

Alpha diversity measures differences within samples. The Chao1 Index provides a measure of alpha diversity in terms of operational taxonomic unit (OUT) "richness," equally taking into account frequent and rare OTUs. Meanwhile, the Shannon Diversity Index also considers OTU's "evenness," as it takes into account the frequency of each OTU [15]. Both study groups were comparable in terms of richness and evenness at baseline at the class, order, and genus level (Figure 3). However, at the end of the intervention period, the placebo group displayed significantly lower richness compared to DR7 at both class ($p = 0.005$) and order ($p = 0.018$) levels, as well as reduced evenness at class level ($p = 0.034$). Our present data showed that the administration of DR7 prevented the reduction of within-group ecological diversity while taking into account the number of different taxa and relative abundances, which was evidently reduced in the placebo group over time. Beta diversity measures differences between samples. The Bray–Curtis Index considers both the co-occurrence and differential abundance of OTUs. Compositional differences were not observed at week-0 (Figure 4). However, at the end of the intervention, significant differences were consistently observed between DR7 and placebo at class, order, and genus levels (all with $p < 0.001$). These indicated that DR7 prevented a shift in microbial community compositional changes over time, which evidently occurred in the placebo group. A recent medium-scaled study involving 671 human subjects showed that a lower gut microbial diversity was associated with increased levels of stress and anxiety, accompanied by an altered overall composition of the gut microbial community [16]. In addition, gut microbiota diversity has been greatly reported to play an important role in maintaining the stability of the intestinal ecosystem as well as normal ecological functions. A reduced microbial diversity has been reported in an array of gastrointestinal diseases such as Crohn's disease [17] and inflammatory bowel disease (IBD) [18], while milder cases such as diarrhea have been associated with decreased phylotype richness [19]. Recent evidence has also shown that a shift in gut microbiota diversity is associated with mental health and psychiatric disorders such as stress, sociability, cognition, anxiety, depression, and autism [20]. Young patients with attention deficit hyperactivity disorder were reported to display reduced alpha diversity and differ in microbial composition as compared to healthy controls [15].

Animal studies have shown that stress caused reduced abundance and diversity in gut microbiota profiles, where stressed pregnant monkeys showed lower abundance of lactobacilli and bifidobacterial [21], mice exposed to social disruption stress led to a shifted colonic clustering compared to the control as observed via beta diversity Principal Coordinates Analysis (PCoA) plots accompanied by a reduced abundance of *Porphyromonadaceae* and *Lactobacillaceae* [22], while depressed mice due to chronic mild stress showed a reduced abundance of *Lactobacillus* and *Turicibacter* [23]. A study involving undergraduate students also showed reduced total faecal microbial load amid exam stress [24]. Amid stress, within-sample microbial richness reduced leading to increased intersample dissimilarities in the present study. The administration of DR7 has aided in the maintenance of gut microbial richness and evenness, to prevent a shift in diversity amid stress.

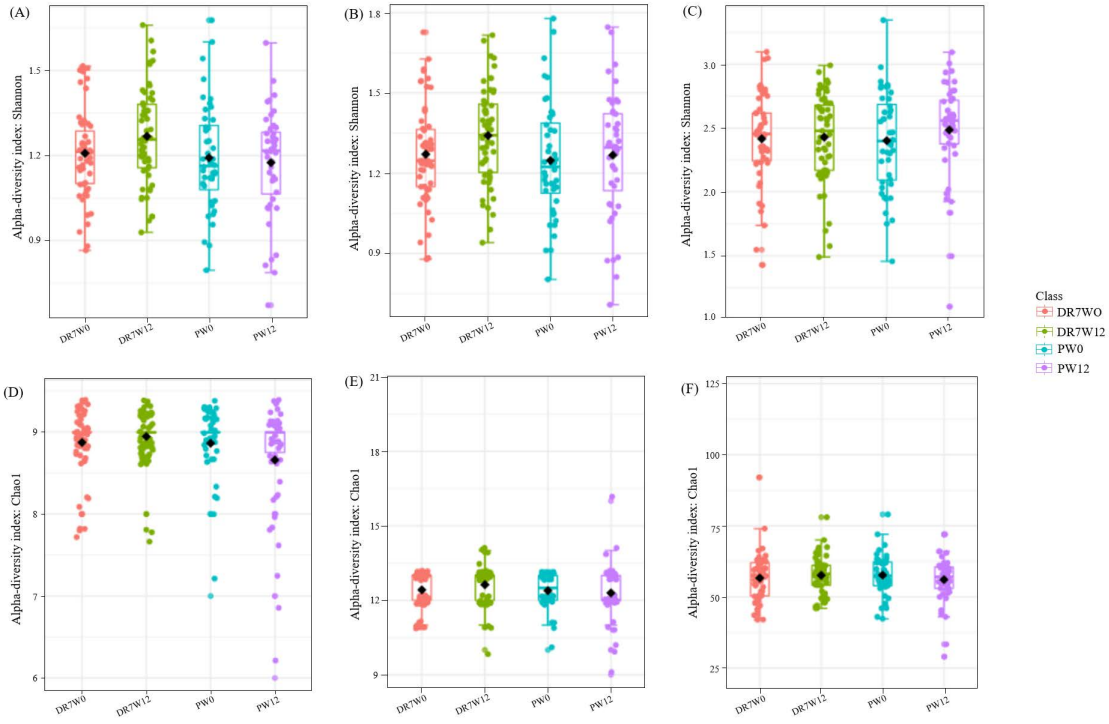

Figure 3. Alpha diversity plots for stressed adults at baseline (W0) and after week-12 (W12), upon administration of *Lactobacillus plantarum* DR7 or placebo. Diversity measured by Shannon Evenness Index for (**A**) class (W0: $p = 0.264$; W12: $p = 0.034$), (**B**) order (W0: $p = 0.316$; W12: $p = 0.116$), and (**C**) genus (W0: $p = 0.919$; W12: $p = 0.351$), and Chao1 Richness Index for (**D**) class (W0: $p = 0.876$; W12: $p = 0.005$), (**E**) order (W0: $p = 0.781$; W12: $p = 0.018$), and (**F**) genus (W0: $p = 0.416$; W12: $p = 0.123$). The line inside the box represents the median, whereas the whiskers represent the lowest and highest values within the interquartile range. Outliers, as well as individual sample values, are shown as dots. Statistical significance was analyzed using the Mann–Whitney *U* test. $n = 99$ (DR7 $n = 55$ and placebo $n = 44$).

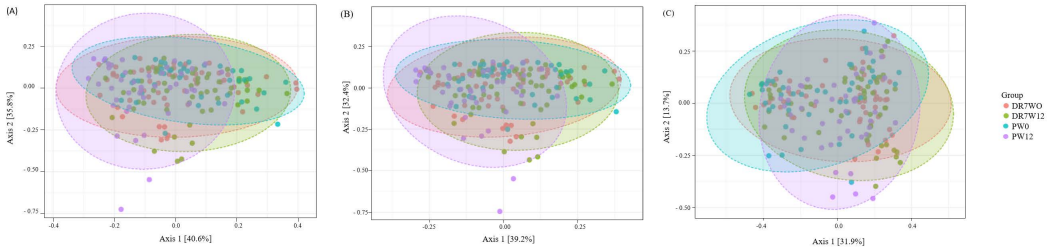

Figure 4. Beta diversity measured by Bray–Curtis dissimilarity and Principal Coordinates Analysis (PCoA) for class (**A**), order (**B**), and genus (**C**) are plotted for stressed adults at baseline (W0) and after week-12 (W12), upon administration of *Lactobacillus plantarum* DR7 or placebo. Diversity in microbial community composition was achieved by permutational analysis of variance (PERMANOVA) for class, order, and genus. PERMANOVA: class (W0: $p = 0.343$, W12: $p < 0.001$), order (W0: $p = 0.338$, W12: $p < 0.001$), and genus (W0: $p = 0.338$, W12: $p < 0.001$). $n = 99$ (DR7 $n = 55$ and placebo $n = 44$).

2.4. Compositional Changes Between DR7 and Placebo

As alpha and beta diversity analyses yielded significant changes between DR7 and placebo groups over 12 weeks, we further analysed faecal microbiota changes along different taxonomic levels. Compared to DR7, subjects administered placebo had a drop in phylum Bacteroidetes over 12 weeks (Supplementary Figure S2; $p < 0.001$), which could be traced to subsequent taxonomic levels of class Bacteroidia (Supplementary Figure S3; $p < 0.001$) and order Bacteroidales (Supplementary Figure S4; $p < 0.001$). Of note, a lower count of Bacteroidetes has been observed in patients with altered

colonic movements such as young children hospitalized for infectious diarrhoea [25] and patients with functional constipation [26].

Conversely, although both study groups displayed a comparable abundance of phylum Firmicutes (and of lower taxonomic levels class Clostridia and order Clostridiales), these bacteria were reduced at the end of the study in subjects receiving DR7, while were increased in the placebo group, resulting in significant differences at phylum (Supplementary Figure S2; $p = 0.002$), class (Supplementary Figure S3; $p = 0.001$), and order Clostridiales (Supplementary Figure S4; $p = 0.001$). These differences could be further traced down to genera *Blautia* and *Romboutsia* (Supplementary Figure S5; $p < 0.001$), which were increased in the placebo group at the end of the study. This effect was compensated by a larger abundance of class Negativicutes (Supplementary Figure S3; $p = 0.003$) and order Selenomonadales (Supplementary Figure S4; $p = 0.003$) in subjects consuming DR7 as compared the placebo group. Of note, although both groups showed a decrease in the abundance of the genus *Acidaminococcus* (belonging to order Selenomonadales), the administration of DR7 contributed to a larger decrease than the placebo over 12 weeks (Supplementary Figure S5; $p = 0.001$). Orders Clostridiales and Selenomonadales are largely gut commensal inhabitants without exerting much detrimental effects on hosts. At the genus level, although *Blautia* has been reported as a butyrate producer that is beneficial for colonocytes, a higher abundance of *Blautia* has also been reported in patients with inflammatory pouchitis [27], nonalcoholic fatty liver disease [28], systemic lupus erythematosus [29], and breast cancer [30]. *Acidaminococcus* has been positively correlated with stunting heights in malnourished children [31], attributed to its fastidious requirement for glutamate as a sole source of carbon and energy, where glutamate in the gut is an essential oxidative fuel for intestinal epithelium [32].

Subjects administered with DR7 had an increased abundance of class Deltaproteobacteria (Figure S3; $p < 0.001$), order Desulfovibrionales (Supplementary Figure S4; $p < 0.001$), and genera *Bilophila* (Supplementary Figure S5; $p < 0.001$) and *Desulfovibrio* ($p = 0.001$) as compared to subjects on placebo, which showed a decreased abundance over 12 weeks. Although many classes of the phylum Proteobacteria are associated with human pathogens such as Alphaproteobacteria (for the genera *Brucella* and *Rickettsia*), Betaproteobacteria (for the genera *Bordetella* and *Neisseria*), Gammaproteobacteria (for the genera *Escherichia*, *Shigella*, *Salmonella*, and *Yersinia*), and Epsilobacteria (for the genus *Helicobacter*), genera *Bilophila* and *Desulfovibrio* of the class Deltaproteobacteria are widely identified as sulphate reducers yielding hydrogen sulphide (H_2S) in the gut. Although past knowledge has associated H_2S with damages of the gut and onset of IBD, recent advances have shown that H_2S is an important mediator for gastrointestinal mucosal defence, repairing of epithelial injury, preventing dysbiosis due to the use of nonsteroidal anti-inflammatory drugs, and promoting resolution of inflammation [33]. The increased abundance of levels along the lineage of Deltaproteobacteria seemed compensated by a decreased abundance in subjects consuming DR7 for the class of Actinobacteria (Supplementary Figure S3; $p = 0.011$), order Actinomycetales (Supplementary Figure S4; $p = 0.002$), and genus *Actinomyces* (Supplementary Figure S5; $p < 0.001$) as compared to subjects on placebo over 12 weeks. Although Actinobacteria is commensal for the human oral mucosa, nasopharyngeal, gastrointestinal, and urogenital tracts, the *Actinomyces* genus has been isolated from colon, cecum, and appendix of patients with actinomycosis, primarily attributed to its ability to form biofilm, induce inflammation, and aggravate injuries caused by inflammation [34].

In addition to physiological and pathophysiological processes, stress has also been reported to affect the gut environment, namely, loss of gut barrier functions [35], relapses of IBD [36], and structural weakening of the colonic mucosal layers [37]. DR7 had modulated gut microbiota as a first targeted site, leading to a healthier gut ecosystem that subsequently alleviated the stress clinical outcomes and inflammatory parameters as observed in our previous study [9].

Limitations have been raised on the accuracy and use of higher taxonomic levels such as phylum to predict and correlate with diseases and biological functions, attributed to the vast diversity along lower taxonomic levels of the human gut microbiota. It is thus crucial to note that in our present study, the changes in gut microbiota upon the administration of DR7 was consistent along different lower

taxonomic levels such as that along the phyla of Bacteroides and Firmicutes (Figure 5A,B), and those along the classes of Deltaproteobacteria and Actinobacteria (Figure 5C,D).

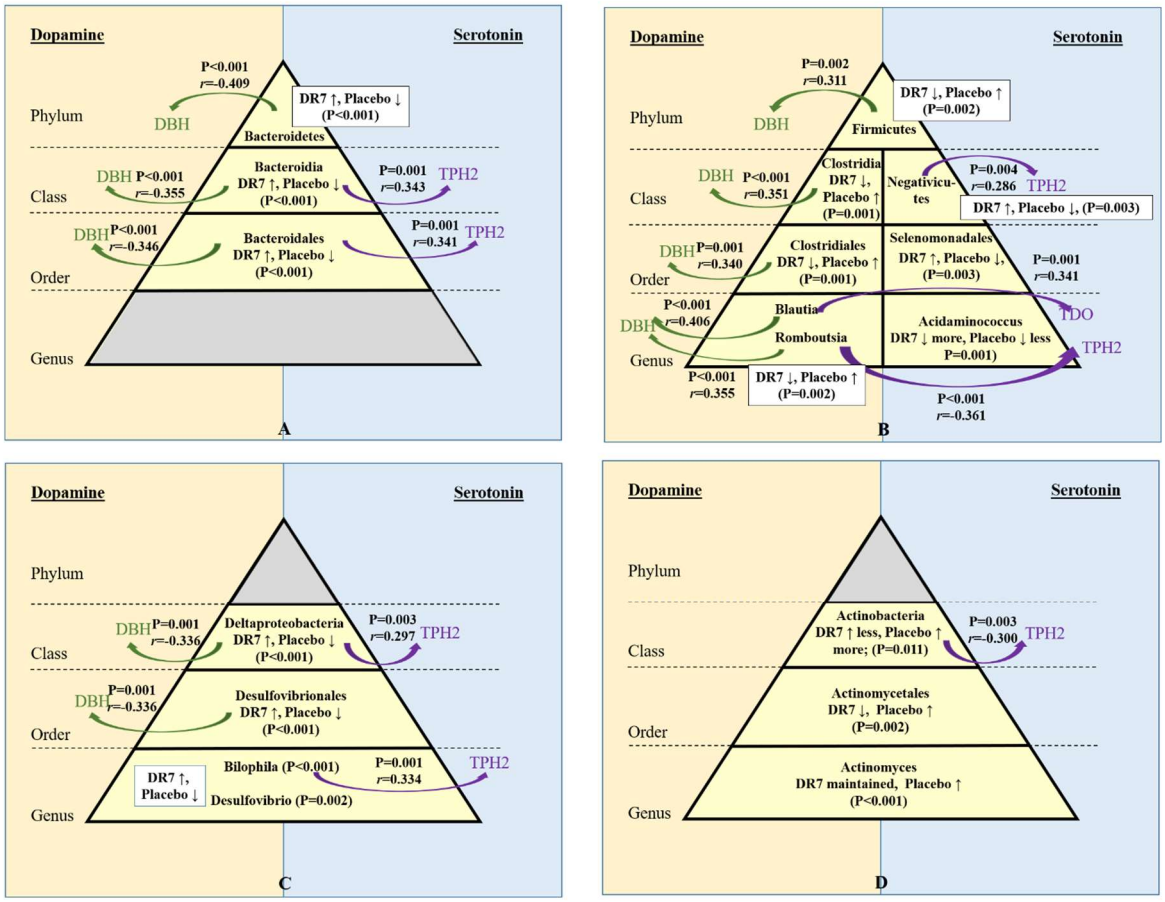

Figure 5. Changes in gut microbiota after 12-week administration of *Lactobacillus plantarum* DR7 compared to placebo group along the lineage of phylum Bacteroidetes (**A**) and Firmicutes (**B**) and classes Deltaproteobacteria (**C**) and Actinobacteria (**D**). Only microbiota groups which showed significant changes over 12 weeks between placebo and DR7 groups are shown (as indicated by *p*-values inside the pyramid; Mann–Whitney *U* test; ↑ indicates an increase over 12 week and ↓ indicates a reduction over 12 weeks). Curved arrows indicate correlations between gut microbiota and blood gene expressions of dopamine *β*-hydroxylase (DBH), tryptophan hydroxylase-2 (TPH2), and tryptophan 2,3-dioxygenase (TDO) (*p*-value and rho (*r*) obtained from Spearman's rank correlations. $n = 99$ for gut microbiota (DR7 $n = 55$ and placebo $n = 44$); $n = 111$ for blood gene expressions (DR7 $n = 56$ and placebo $n = 55$).

2.5. Correlation of Gut Microbiota and Stress Neurotransmitters

We have previously reported that DR7 reduced plasma cortisol levels and exerted changes along the pathways of two neurotransmitters, namely, serotonin, and dopamine. The administration of DR7 for 12-weeks had lowered the expressions of dopamine β-hydroxylase (DBH) and tyrosine hydroxylase (TH) along the dopamine pathway and also lowered the expressions of indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), while increasing the expressions of tryptophan hydroxylase-2 (TPH2) and 5-hydroxytryptamine receptor-6 (5-HT6) along the serotonin pathway as compared to the placebo [9]. Thus, in the present study, correlation analyses were performed to evaluate correlations between microbiota groups that were changed upon the administration of DR7, with those of genes involved along the serotonin and dopamine pathways. Although plasma cortisol levels and defecation frequency did not show any significant correlations with gut microbiota profiles, our present correlation analyses showed significant correlations involving DBH, TPH2, and

TDO (Figure 5). We have previously reported that upon the administration of DR7, the expressions of TPH2 in blood was higher than that of placebo by 3.7 times, while the expressions of DBH and TDO was lower by 1.1 times and 1.3 times than the placebo, respectively [9]. Here, we report these changes correlated to changes in the relative abundance of specific bacterial groups in the gut microbiota. It is noted that baseline TPH2 expression in blood was low, as TPH2 is primarily expressed in the brain, whereas TPH1 is expressed in the brain, gastrointestinal tract, and pituitary glands [38]. However, no significant correlation was observed for TPH1.

Although changes in gut microbiota upon the administration of DR7 was consistent along different taxonomic levels, the correlations of the changes in these microbial groups with changes in neurotransmitters gene expression also showed such consistency. The phylum Bacteroidetes showed a negative correlation with gene expression of DBH ($p < 0.001$, $r = -0.409$; Figure 5A), which was consistent along lower taxonomic levels such as class Bacteroidia ($p < 0.001$, $r = -0.355$) and order Bacteroidales ($p < 0.001$, $r = -0.346$). DBH catalyses the conversion of dopamine to norepinephrine, an indication of increased stress as seen in the placebo group with a lower abundance of Bacteroidetes, Bacteroidia, and Bacteroidales. As the levels of cortisol increase, the brain noradrenergic is activated, where the postsynaptic effects of norepinephrine are triggered to induce alertness, awareness, wakefulness, and also attention amid stressful conditions. Bacteroidia and Bacteroidales, which were maintained upon the administration of DR7 compared to placebo also showed positive correlations with TPH2 ($p = 0.001$; Figure 5A). TPH2 converts tryptophan to serotonin in the brain, where an imbalanced level of serotonin has been reported in patients with psychological disorders including mood and anxiety [39]. Bacteroidetes are reportedly reduced in children with autism [40] and patients with dementia [41], whereas patients with depression have been reported to have lower abundance of Bacteroidia and Bacteroidales [42].

A positive correlation was observed between gene expression of DBH and taxonomic levels along the phylum Firmicutes ($p = 0.002$, $r = 0.311$), class Clostridia ($p < 0.001$, $r = 0.351$), order Clostridiales ($p = 0.001$, $r = 0.340$), and genera *Blautia* ($p < 0.001$, $r = 0.406$) and *Romboutsia* ($p < 0.001$, $r = 0.355$) (Figure 5B). The administration of DR7 had also prevented the increase in abundance of all taxonomic levels along the phylum Firmicutes, whereas the placebo showed an increase amid stress. *Blautia* also has a positive correlation with TDO ($p = 0.001$, $r = 0.341$), which competes with TPH2 for tryptophan in the brain for the conversion into kynurenine. Reduced level of serotonin and increased level of kynurenine has been shown in patients with anxiety and depressive disorders [39]. *Romboutsia* has a negative correlation with TPH2 ($p < 0.001$, $r = -0.361$), whereas class Negativicutes, which was increased in abundance upon the administration of DR7, showed a positive correlation with TPH2 ($p = 0.004$, $r = 0.286$). Although DR7 prevented the increase in abundance of all taxonomic levels along the phylum Firmicutes, several other studies have associated Firmicutes with mental health, where a higher abundance of Firmicutes was seen in demented patients compared to nondemented controls [41], Clostridiales was more abundant in patients with major depression [43], and a higher abundance of *Blautia* was observed in patients with Alzheimer's Disease [37] and major depression disorder [44].

Deltaproteobacteria and its lower taxonomic level of order Desulfovibrionales which were both increased in abundance upon administration of DR7 showed negative correlations with DBH ($p = 0.001$, $r = -0.336$; Figure 5C). Deltaproteobacteria also showed a positive correlation with TPH2 ($p = 0.003$, $r = 0.297$), while its genus of *Bilophila* also showed a similar trait ($p = 0.001$, $r = 0.002$). Meanwhile, Actinobacteria, which showed a lesser increase in abundance upon administration of DR7 as compared to the placebo, exhibited a negative correlation with TPH2 ($p = 0.003$, $r = -0.300$; Figure 5D). Past reports have shown different associations of these microbial groups with different mental disorders. Bilophila was shown to decrease in abundance, in subjects with autism spectrum disorders [45] but increased in abundance in patients with Alzheimer's Disease [37]. Although Actinobacteria has been positively associated with signalling in the thalamus, hypothalamus, and amygdala leading to better cognitive

speed, attention, and flexibility in humans [46], 16s rRNA sequences of Actinobacteria were found more abundant in frozen and fixed autopsied brain samples from patients with multiple sclerosis [47].

While serotonin is one of the main brain neurotransmitters, approximately 90% of total serotonin in humans is located in enterochromaffin cells in the gastrointestinal (GI) tract [48], where serotonin plays important roles in promoting immunity and reducing inflammation in various models of mucosal infections [49]. This is in contrast with the other metabolite of tryptophan and kynurenine, where an increased conversion to kynurenine from tryptophan has been reported to increase incidences of upper respiratory tract infections (URTI) [50]. We have previously reported the effects of DR7 on improving symptoms of URTI via modulating systemic immunity and inflammatory responses in adults [51]. Information on the effects of gut microbiota on the pathways of dopamine remains limited as compared to that of serotonin, where a decrease in serotonin level has been associated with the absence of certain gut bacteria in germ-free animals as early as 1967 [52]. As serotonin is produced in gut by enterochromaffin cells while gut microbiota influences the number and function of enterochromaffin cells thereby promoting the release of serotonin in the gut, serotonin has been widely evaluated for gut–brain axis properties. Meanwhile, dopamine and norepinephrine are reportedly produced by gut microbiota thus associated to play some roles as signalling molecules mediating the function of the microbiota–gut–brain axis less than 10 years ago [53]. Emerging studies in all these years have thus reported on the effects of gut microbiota on serotonin and dopamine levels, and in certain general extents, reported on some psychological effects. To our best knowledge, the present data is the first to report a correlation between gut microbiota and genes related to the serotonin and dopamine pathways, which is also consistent across multiple taxonomic levels.

3. Materials and Methods

3.1. *Lactobacillus plantarum* DR7 and Placebo Products

Lactobacillus plantarum DR7 was isolated from fresh cow's milk in Penang, Malaysia [9]. The preserved stock cultures of DR7, in 20% glycerol at −20 °C, were activated in sterile De Man, Rogosa, Sharpe (MRS) broth (Hi-media, Mumbai, India) for three successive times using 10% (*v*/*v*) inoculums and incubated at 37 °C for 24 h [54]. The identity of all working cultures was reconfirmed using 16S rRNA sequencing and two strain-specific primers of (i) (F) GCAAGGCCACTTGATCGTTG and (R) AATCAGTCGCATCCAGCCAA and (ii) (F) AGCCATTCTCAGTTCGGATTGT and (R) GCTCTTGTTCGACTTCCCCTAA. PCR amplification of 16S rDNA was performed using the universal 16S rDNA primer, with the forward sequence, 27F; 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse primer sequence, 1492R; 5′-GGTTACCTTGTTACGACTT-3′, in a thermal cycler (Bio-Rad, Hercules, CA, USA). The program for amplification consisted of (i) denaturation at 95 °C for 5 min, (ii) 35 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 2 min, and (iii) final extension at 72 °C for 4 min, and finally, maintained at 4 °C until further use. The PCR amplicons were subjected to agarose gel electrophoresis at 100 V for 60 min and visualized using the GeneGenius Imaging System (Syngene, Cambridge, UK). The PCR products purification and sequencing were performed by Apical Scientific Sdn Bhd. The nucleotide sequences of the isolates were analysed using the BLAST program from NCBI (http://www.ncbi.nlm.nih.gov). The accession number for the whole genome of DR7 is CP031318. Both DR7 and placebo products were manufactured by GN Pharmaceuticals Sdn. Bhd., Selangor, Malaysia under GMP-certified manufacturing plant which was also certified HALAL by JAKIM, Malaysia. Live cultures of DR7 is proven stable in a lactose-free medium such as soymilk at 4 °C for 168 h (Supplementary Figure S1). Daily consumption consisted of one aluminium sachet (2 g) containing light-yellow powder of either 10^9 cfu/sachet of DR7 and maltodextrin as excipient or placebo with only maltodextrin. All products were stored away from direct sunlight and at below 30 °C.

3.2. Selection of Subjects

Subjects were recruited from Penang and Kubang Kerian, Malaysia, and screened based on the inclusion and exclusion criteria. The inclusion criteria were men or women, aged 18–60 years old, willing to commit throughout the experiment, and scored moderate stress level on Cohen's Perceived Stress Scale (PSS-10) [55]. The exclusion criteria included subjects with type-I diabetes, taking term medication due to certain severe illness, having HIV/AIDS, deficient in glucose-6-phosphate dehydrogenase, and who, in the opinion of the investigator, were not likely to complete the trial for whatever reasons. Before the start of the study, written informed consent was obtained from all participating subjects.

3.3. Study Protocol

This was a double-blind, randomized, and placebo-controlled design study. Qualified subjects were randomized according to a 1:1 ratio to the two arms of the study according to a computer-generated list with treatment codes. This list was prepared by the study statistician, who had no contact with the participants, and the allocation sequence was not available to any member of the research team until the end of the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects were approved by the JEPeM-USM Review Panel on Clinical Studies (Approval number USM/JEPeM/17040228, 24 August 2017) and was registered at ClinicalTrials.gov (identifier number NCT 03370458, 12 December 2017). The sample size was calculated for a parallel-group study design involving one prevention arm and one placebo arm and was based on power design analysis as previously described [9], where a total of 124 subjects were needed comprising 62 subjects in each group (treatment and placebo; inclusive of 15% dropout).

3.4. Analyses

Questionnaires

Eligible subjects were requested to complete a gut health condition questionnaire at baseline, week-4, week-8, and week-12, which recorded the occurrence for direct gastrointestinal symptoms (such as vomiting, dysentery (blood in stool), abdominal pain, nausea, rectal pain (sharp dull, burning, and feels like a hard object in the rectum)), indirect gastrointestinal symptoms (such as loss of appetite, fatigue, dizziness, headache, dehydration, and fever), and the number of defecation times. The development of all questionnaires as assessment tools also included translational processes to the Malay and Chinese languages, where all versions were validated via stages of development and face validation [56,57] and were also used in our previous study on gastrointestinal health [58].

3.5. Gut Microbiota Analyses

3.5.1. DNA Extraction, PCR Amplification, and Sequencing

Faecal samples were collected at baseline (week-0) and week-12 in faecal collection tubes containing RNAlater™ solution (Qiagen, Hilden, Germany) and glass beads by the subjects and stored at refrigerated temperature (4–8 °C) for not more than 3 days, prior to delivery to the laboratory and stored at −80 °C until further analyses. The faecal samples were homogenized by vortexing the tube containing glass beads prior to DNA extraction and purification as previously described [10,11]. Purified DNAs were determined by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The V3–V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) by thermocycler PCR system (GeneAmp 9700, ABI, San Diego, CA, USA). The PCR reactions were performed in triplicates with 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA, in the following sequence: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s for annealing at

55 °C, and 45 s for elongation at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were then extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Madison, WI, USA) according to the manufacturer's protocol. The purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, USA).

3.5.2. Bioinformatics Analysis on 16s rRNA Gene Profiling

The 16s rRNA gene sequences were processed using QIIME v.1.9.1 (ref QIIME allows analysis of high-throughput community sequencing data) and USEARCH v.10.0 (ref Search and clustering orders of magnitude faster than BLAST). Raw FASTQ files were quality filtered by Trimmomatic and merged by USEARCH with the following criteria: removing of barcodes and primers, filtering of low-quality reads, and finding nonredundancy reads. The merged raw reads were at least 50,000 per sample. Operational taxonomic units (OTUs) were clustered with 97% similarity cut-off using UPARSE. The taxonomy for each 16S rRNA gene sequence was analysed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva 132 16S rRNA database using 60% confidence threshold.

Alpha (within-sample richness) and beta-diversity (between-sample dissimilarity) estimates were computed using MicrobiomeAnalyst phyloseq-R package version 3.6.1 (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/home.xhtml) for class, order and genus.

3.6. Statistical Analyses

Data were analysed using SPSS version 20.0 (SPSS Inc, Chicago, IL, USA). The primary hypothesis of this study involved differential efficacy between the two treatment groups of DR7 and placebo. Considering the skewed distribution and nonparametric nature of our data, differences in OTU relative abundance between DR7 and placebo groups were compared using the Mann–Whitney *U* test, whereas the correlations between OTUs relative abundance and gene expression data were evaluated using Spearman's rank correlations with rho (*r*) as the correlation coefficient. Alpha diversity of gut microbiota was measured by Shannon and Chao1 Indexes and compared using Mann–Whitney *U* test, whereas beta diversity was calculated by principal coordinates analysis (PCoA) on Bray–Curtis dissimilarity and compared using permutational analysis of variance (PERMANOVA).

Data of blood gene expressions of dopamine β-hydroxylase (DBH), tryptophan hydroxylase-2 (TPH2), and tryptophan 2,3-dioxygenase (TDO) used in correlation analyses were obtained from our previous study [9]. All tests were two-sided with $p < 0.05$ as considered statistically significant. Both differences in relative abundance and correlation analyses were adjusted for multiplicity based on the number of OTUs detected at each taxonomical level (class, order, and genus) by using Benjamini and Hochberg procedure at a False Discovery Rate (FDR) threshold of 0.1.

4. Conclusions

Taken altogether, our present data showed that the administration of DR7 modulated stress-induced bowel movement by decreasing the frequency of defecation as compared to the placebo after 12 weeks. Alpha diversity analysis also showed that DR7 prevented the decrease of gut microbiota OTU's richness and evenness, whereas beta diversity analysis showed that DR7 maintained the distribution of gut microbial profiles amid stress. It is noted that these changes in gut microbiota upon the administration of DR7 was consistent along different taxonomic levels along the phyla of Bacteroides and Firmicutes and the classes of Deltaproteobacteria and Actinobacteria. Correlation analyses subsequently revealed that the changes of gut microbiota along different taxonomic levels were consistent with several gene expressions of key enzymes involved along the neurotransmitter pathways of serotonin and dopamine. To our knowledge, this is the first study to correlate the effects of a probiotic towards gut microbiota changes and brain neurotransmitters genes, strengthening the hypothesis of health benefits along the gut–brain axis. Considering that probiotic microorganisms

are frequently utilized in foods, the current finding will benefit the food industries by enabling the development of new functional food products specifically targeting those for mental health promotions.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/13/4608/s1.

Author Contributions: M.-T.L. and Y.L. conceived and designed the experiments. G.L., H.-X.C., F.Y.-L.C., and M.-T.L. performed the study and analyzed the data. G.L., Y.L., and M.-T.L. drafted the work, revised critically for intellectual content, and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

CNS	Central nervous system
DBH	Dopamine β-hydroxylase
GI	Gastrointestinal
MDD	Major depressive disorders
MRS	De Man, Rogosa, Sharpe
OTUs	Operational taxonomic units
PCoA	Principal coordinates analysis
PERMANOVA	Permutational analysis of variance
URTI	Upper respiratory tract infections
TDO	Tryptophan 2,3-dioxygenase
TPH2	Tryptophan hydroxylase-2

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