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Lactobacillus plantarum DR7 alleviates stress and anxiety in adults: a randomised, double-blind, placebo-controlled study

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RESEARCH ARTICLE

Abstract

Probiotics have been reported to exert beneficial effects along the gut-brain axis. This randomised, double-blind and placebo-controlled human study aimed to evaluate such properties of *Lactobacillus plantarum* DR7 and its accompanying mechanisms in stressed adults. One hundred and eleven (n=111; DR7 n=56, placebo n=55) stressed adults were recruited based on moderate stress levels using the PSS-10 questionnaire. The consumption of DR7 (1×10^9 cfu/day) for 12 weeks reduced symptoms of stress (P=0.024), anxiety (P=0.001), and total psychological scores (P=0.022) as early as 8 weeks among stressed adults compared to the placebo group as assessed by the DASS-42 questionnaire. Plasma cortisol level was reduced among DR7 subjects as compared to the placebo, accompanied by reduced plasma pro-inflammatory cytokines, such as interferon-γ and transforming growth factor-α and increased plasma anti-inflammatory cytokines, such as interleukin 10 (P<0.05). DR7 better improved cognitive and memory functions in normal adults (>30 years old), such as basic attention, emotional cognition, and associate learning (P<0.05), as compared to the placebo and young adults (<30 years old). The administration of DR7 enhanced the serotonin pathway, as observed by lowered expressions of plasma dopamine β-hydroxylase (DBH), tyrosine hydroxylase (TH), indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase accompanied by increased expressions of tryptophan hydroxylase-2 and 5-hydroxytryptamine receptor-6, while stabilising the dopamine pathway as observed via stabilised expressions of TH and DBH over 12 weeks as compared to the placebo (P<0.05). Our results indicated that DR7 fulfil the requirement of a probiotic strain as per recommendation of FAO/WHO and could be applicable as a natural strategy to improve psychological functions, cognitive health and memory in stressed adults.

Keywords: *Lactobacillus plantarum* DR7, stress, anxiety, memory, cognition, serotonin

1. Introduction

Stress is a nonspecific response of the body against threatening demands, resulting in anxiety, discomfort, emotional tension and difficulty in adjustment (George, 2010). It is often triggered by an event and the impacts of stress-induced psychological consequences are gaining increasing interests. Anxiety is the first psychological reaction to stress, where prolonged anxiety often leads to mental illnesses including depression. Globally, over 300 million people are affected by depression, accompanied by nearly 800,000 suicidal deaths annually (WHO, 2018). The physiological of stress responses include activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal axis, leading to increased blood and tissue levels of catecholamines and glucocorticoids. These hormones alter immune functions, such as antigen presentation, leukocyte trafficking and proliferation, antibody secretion

and cytokine release (Dhabhar *et al.,* 1995). Long-term exposure to glucocorticoid leads to increased resistance of the glucocorticoid receptor which ultimately decreases sensitivity of immune cells and impairs downregulation of inflammatory responses (Miller *et al.,* 2002), exemplifying the impacts of stress on the immune system.

A probiotic is defined as 'live microorganisms that confer the health effects to the host when consumed in adequate amounts' (FAO/WHO, 2006). While *Lactobacillus* remains as one of the common residents of gut microbiota, they are also the most common bacterial genera reported with probiotic properties, exerting health benefits ranging from regulation of the gut environment, to alleviation of metabolic disorders and modulation of immune responses (Galdeano and Perdigón, 2004). Increasing evidence has shown the association of gut microbiota with brain health along the gut-brain axis, a bidirectional flow of signalling responses between the gut and brain (Mayer, 2011). Microbial neuroactive substances and their precursors such as tryptophan have been reported to reach the brain through endocrine and afferent autonomic pathways leading to altered behavioural responses (Desbonnet *et al.,* 2008), and brain development, mood and cognition (Romijn *et al.,* 2008). Germ-free mice showed higher levels of plasma corticosterone accompanied by increased anxiety behaviours as compared to specific pathogen-free mice (Bravo *et al.,* 2011), while the administration of *Lactobacillus rhamnosus* JB-1 reduced anxious and depressive behaviours in mice (Neufeld *et al.,* 2011), illustrating the influence of gut microbiota on brain health and behaviours of the host. In humans, a milk drink containing *Lactobacillus casei* Shirota improved mood scores compared to the placebo group after 3-weeks (Benton *et al.,* 2006), while the administration of *L. casei* Shirota at 2.4×10 log cfu/day for 2 months improved anxiety symptoms in patients with chronic fatigue syndrome, a common comorbid of anxiety disorders (Rao *et al.,* 2009). Amid these positive reports, the mechanisms involved remain largely unknown, while issues related to host- and strain-dependencies remain a primary concern prior to further development of probiotics as an adjuvant or preventive therapy for psychological disorders.

We have previously reported the ability of *Lactobacillus plantarum* DR7 (DR7), a bovine milk isolate, to activate the 5'-AMP-activated protein kinase (AMPK) pathway via phosphorylation (Lew *et al.,* 2018), while chronic mild stress has been reported to induce anxiety and depression-like behaviours in mice via the inactivation of AMPK (Zhu *et al.,* 2014). We postulated that DR7 may have the potential to modulate brain health along the gut brain axis. This study aimed to investigate the effects of DR7 on stress, anxiety and depression in stressed adults, in addition to memory capacity and cognitive functions. Considering that little information is available on the mode of actions, we also hope to better understand and elucidate the possible mechanisms involved.

2. Materials and methods

Probiotic properties

DR7 was isolated from fresh cow's milk in Penang, Malaysia. Stock cultures of DR7 were preserved in 20% glycerol (-20 °C), activated in sterile De Mann, 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 (Lew *et al.*, 2018). The cell-free supernatant (CFS) was prepared by centrifuging cell cultures at 12,000×*g* for 5 min at 4 °C. The supernatant was filter-sterilised (0.22 µm) and stored at -20 °C. The adherence of DR7 onto mucin-coated 96-wells was assayed according to the protocol by Tham *et al.* (2012). Resistance of DR7 against simulated gastric and intestinal fluid was determined as described by Lew *et al.* (2011). Antioxidant potentials of CFS from DR7 were determined using the ferric reducing antioxidant power (FRAP) assay according to Benzie and Strain (1996). The presence or absence of antibiotic resistance by DR7 as per requirement of the European Food Safety Authority (EFSA) was determined using the minimum inhibitory concentration microdilution broth method against individual antibiotics (Klare *et al.*, 2007). Ability of DR7 to utilise prebiotics was determined via evaluating growth of DR7 (optical density at 600 nm, 37 °C, 24 h) in media with a similar composition of MRS except glucose, which was replaced with fructooligosaccharide (FOS), galactooligosaccharide (GOS) and inulin (Fuji Neihon Seito, Tokyo, Japan). Carbon metabolism profile of DR7 was determined using the API-50 CHL kit (BioMérieux, Marcy-l'Ètoile, France) as per manufacturer's instructions. Antimicrobial activity of CFS from DR7 against pathogens *Escherichia coli* ATCC 25922, *Salmonella* sp. and *Streptococcus epidermidis* ATCC 12228 was performed in 96-well plate with pathogens growing without any treatment as negative controls, while amoxicillin (1.5 µg/ml), chloramphenicol (256 µg/ml) and vancomycin (2 µg/ml) were used as positive controls, respectively (Hor and Liong, 2014). Pathogens were obtained from School of Industrial Technology, Universiti Sains Malaysia (Penang, Malaysia). *E. coli* and *S. epidermidis* were grown and maintained in trypticase soy broth (Hi-media) while *Salmonella* sp. in nutrient broth (Hi-media). Human liver hepatocellular cell line (HepG2) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) as previously described (Lew *et al.*, 2013). Viability of HepG2 cells were determined via the MTT assay (Tuo *et al.*, 2010) upon treatment with different concentrations of cell free supernatant from DR7 for 24 h at 37 °C, 5% CO_2.

Human study

Lactobacillus plantarum *DR7 (DR7) and placebo products*

Both DR7 and placebo products were manufactured by GN Pharmaceuticals Sdn. Bhd., Selangor, Malaysia under GMP certified manufacturing plant. Each aluminium sachet consisted of 2 g and all were identical in taste and appeared as light-yellow powder. The probiotic product contained 9 log cfu/sachet of DR7 and maltodextrin as excipient while placebo contained only maltodextrin. Each dose was supplied in an aluminium sachet. Sachets were stored away from direct sunlight and below 30 °C.

Selection of subjects

Subjects were recruited from Penang and Kubang Kerian, Malaysia, and screened based on inclusion and exclusion criteria. Inclusion criteria included men or women, aged 18-60 years old, willing to commit throughout the experiment, and a score of moderate stress level on Cohen's Perceived Stress Scale (PSS-10) (Cohen *et al.,* 1983). Exclusion criteria include type-I diabetes, long term medication due to certain severe illness, HIV/AIDS, and glucose-6-phosphate dehydrogenase deficient, and subjects who, in opinion of the investigator, were not likely to complete the trial for whatever reasons. Written informed consent was obtained from all subjects prior to the start of the study.

Study protocol

This was a double-blind, randomised and placebo-controlled design study. Randomisation was conducted upon considering the inclusion and exclusion criteria. Qualified subjects were randomised according to 1:1 ratio to the two arms of the study according to a computer-generated list, assigned to the probiotic group (DR7) and placebo group with treatment codes. Randomisation was performed by the study statistician, who had no contact with the participants. The allocation sequence was not available to any member of the research team until the completion 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) and was registered at ClinicalTrials.gov (identifier number NCT 03370458).

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. Previous data have shown that for an intervention using natural supplement to alleviate stress, anxiety and depression, a standard deviation of 3.32 within treatment group and a mean reduction of 2.33 between treatment and placebo groups were observed for anxiety scores as measured by the DASS-21 questionnaire after 4-weeks of intervention (Daryani *et al.,* 2015). Based on these, a total of 124 subjects were needed for this study; comprising of 62 subjects in each group (treatment and placebo; inclusive of 15% dropout). This calculation was established on the need for a continuous response variable from independent control and experimental subjects, with a ratio of control to subject fixed at 1:1, probability (power) of 0.95 and Type-I error probability associated with this test of null hypothesis of 0.05.

Analyses

Questionnaires

Eligible subjects who met all the inclusion and exclusion criteria were first screened for levels of psychological distress using the Perceived Stress Scale-10 (PSS-10) questionnaire. Subjects with confirmed moderate levels of stress were recruited to join the study. PSS-10 consisted of ten items, where six items were negatively stated based on a 5-point Likert scale (0=never, 1=almost never, 2=sometimes, 3=fairly often, 4=very often), while the remaining four items were positively stated (items 4, 5, 7, and 8) and were in reversely scored (0=very often, 1=fairy often, 2=sometimes, 3=almost never, 4=never). The total of the 10 items represented the total score, with scores of 0-13 representing low stress, 14-26 representing moderate stress and 27-40 confirming high perceived stress. Different language versions of the PSS-10 questionnaire were used to assess stress perception; the original English language version, the Malay and Chinese languages translated and validated versions (Lee and Crockett, 1994; Sadhu *et al.,* 2015).

Subjects were also assessed for stress, anxiety and depression using the Depression, Anxiety and Stress Scale (DASS-42) questionnaire (Lovibond and Lovibond, 1995) at baseline (week 0) and at intervals of 4-weeks (week 4, 8, 12). DASS-42 comprises of 42 item self-report validated inventory including three scales designed to measure the negative emotional states of depression, anxiety and stress, where each of the three scales contained 14 questions. The depression scale were assessed via summation of items number 3, 5, 10, 13, 16, 17, 21, 24, 26, 31, 34, 37, 38, and 42, while the anxiety scale utilised items number 2, 4, 7, 9, 15, 19, 20, 23, 25, 28, 30, 36, 40 and 41, and the stress scale utilised items number 1, 6, 8, 11, 12, 14, 18, 22, 27, 29, 32, 33, 35 and 39. Subjects were evaluated based on a 4-point Likert scale (0=did not apply to me at all, 1=applied to me to some degree or some of the time, 2=applied to me to a considerable degree or a good part of time, 3=applied to me very much or most of the time). Scores for each subscale were then divided into five severity ranges, including normal, mild, moderate, severe and extremely severe.

Cortisol, cytokines and full blood count

Blood samples (6 ml) were collected from an antecubital vein directly into a K^2EDTA tube at baseline (week 0) and end of the study (week 12). Plasma samples were analysed for concentrations of stress hormone cortisol, interleukin-1β, -4, and -10, tumour necrosis factor (TNF)-α and interferon (IFN)-γ using enzyme-linked immunoabsorbent assay (ELISA) kits (Immunodiagnostik, Bensheim, Germany) according to the manufacturer's instructions. Whole blood was used for full blood count tests (Gribbles Pathology, Penang, Malaysia).

CogState brief battery

At the end of study (week 12), the computerised CogState Brief Battery (CBB; Mielke *et al.,* 2015) was used to assess memory and cognitive functions of all subjects. The test was conducted in a computer laboratory and/or personal laptops, installed with the CogState ClinicalTrials software. All subjects had an initial practice prior to the actual test while a study coordinator was assigned to assist subjects in understanding the tasks during the practice session. During the test session, the coordinator provided minimal supervision or assistance. Each task of the test battery was randomly chosen from the large number of equivalent alternative forms at any one time, resulting in a different set of trials for each individual. The outcomes were reported as number of correct or incorrect responses, speed and accuracy of performance. Each task is described below:

- Detection task. Playing cards were presented individually and faced-down in the centre of the computer screen on a green background. After random intervals of 2.5 and 3.5 s, the card instantly turned face-up, and subjects were required to respond 'Yes' as quickly as possible (constituting 1 trial). The 'Yes' response was indicated by a keyboard 'K' key or the right mouse button. Correct responses following an anticipatory response were ignored. While subjects could visually differentiate correct and incorrect responses, an additional error audible signal also occurred upon incorrect responses. Subjects that presented an anticipatory response before the card turned face-up were also triggered by the error sound. The faced-up card displayed was always the same joker card. The task ended upon recording of 35 correct trials.
- Identification task. This task was presented similarly to the detection task, except that subjects were required to respond 'Yes' if a faced-up card was red, or 'No' if it was not red. The 'Yes' response was indicated by a keyboard 'K' key while 'No' by the 'D' key, or the right and left mouse buttons, respectively. The cards were displayed as red or black joker cards. The task ended upon recording of 30 correct trials.
- One card learning task. This task was presented similarly to the identification task, except that subjects were required to respond 'Yes' if the faced-up card has appeared in the task before, or 'No' if it has not appeared previously. The cards were displayed as normal playing cards without the joker cards. The task ended upon recording of 42 trials.
- One back task. This task was presented similarly to the one card learning task, except that subjects were required to respond 'Yes' if the faced-up card was exactly the same as the immediately previous card, or 'No' if it was not the same as the previous card. The cards were displayed as normal playing cards without the joker cards. The task ended upon recording of 30 trials.
- Groton maze learning task. A 10×10 grid of tiles was presented to the subjects on the computer screen, with a hidden 28-step pathway among these tiles. A 'chase' task required subjects to learn the same hidden pathway starting from a blue-coloured start-tile to trace the path of a target which moved until it reached the red-coloured finish-tile. Subjects must move one step at a time by clicking a tile next to their current location. A green checkmark indicated a correct move while a red cross indicated an incorrect move. Upon completion, subjects returned to the start location to repeat the test using the same hidden pathway they have just completed. The chase task ended upon recording of 5 trials. A 'final recall' task was presented similarly to the 'chase' task, but presented to subjects after the completion of all tasks in the battery. The final recall task ended upon recording of 1 trial.
- Social emotional task. This task presented four pictures on the computer screen, where one of these pictures will be different to the others. Subjects were required to select the odd picture by clicking on it as quickly and accurately as they can. The task ended upon recording of 30 trials.
- Continuous paired associated learning task. Subjects were presented with a set of randomly located simple shapes on the screen. During the task, the locations of all shapes were hidden, while one of the patterns were presented in the centre location. Subjects were required to click on the location of that individual pattern as it previously appeared. Upon incorrect responses, a red cross appeared over the correct location accompanied by a buzzer sound. Subjects were then required to choose a second location, and this process continued until the correct location that has been paired with the pattern was found. The task ended upon recording of 6 trials.
- International shopping list task. Subjects were read a list of 16 shopping items commonly found in the local store at a rate of 1 word per 2 s. Once all 16 items had been read, subjects were required to recall as many items on the shopping list as possible in no particular order. Marks were given for each correct response and deducted for incorrect responses such as repeated entry or naming items that were not on the shopping list. The task was stopped once a subject confirmed that no more items could be recalled. The task ended upon recording of 3 trials.

Gene expression

Gene expression studies were performed as previously described (Hor *et al.,* 2018). In brief, whole blood collected in K^2EDTA tubes was lysed in TRIsure reagent (Bioline, London, UK), according to manufacturer's instructions prior to extraction of RNA. Precipitation of RNA was performed by adding isopropyl alcohol, and the precipitated RNA was washed with 75% ethanol. Purity and concentration of the extracted RNA was determined by measuring the absorbance at 260, 280 and 230 nm using the Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA was synthesised using RevertAid RT Kit (Thermo Scientific) with Random Hexamer primer, according to manufacturer's instructions. Total RNA (1 µg) was reverse transcribed and amplified by incubating the reaction mixture at 25 °C for 5 min, followed 42 °C for 60 min. The reaction was terminated by incubating the mixture at 70 °C for 5 min. The cDNA was used directly as template in qPCR or stored at -80 °C until use. The expression levels of genes of interest were determined by qPCR using the Agilent AriaMx Realtime PCR System (Agilent Technologies, Santa Clara, CA, USA). Twenty microliter of PCR reactions consisted of 10 µl of 2×SensiFAST SYBR mix (Bioline), 0.8 µl each of 10 µM forward and reverse primers and 50 ng of cDNA. The primer sequences are shown in Table 1, using amplification conditions suggested by the manufacturer. Melting curve analysis was performed after each PCR amplification via measuring the dissociation of PCR products between 65 and 95 °C, with reaction mixture without the cDNA template used as the negative control. Threshold cycle (Ct) of each sample was recorded by measuring the cycle number at which the emission of SYBR green was above the threshold level. The data was calculated using comparative Ct method for relative quantification, as previously described (Lowe *et al.,* 2014). The 18S rRNA gene was used as the housekeeping gene for normalisation of data.

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. The score differences between different time points were examined using one sample t-test, where mean of the differences had a hypothesised value of zero. Differences between DR7 and placebo groups were compared using the independent T-test. Comparisons between treatment groups as a measure of time were assessed using between-group repeated measures analysis of variance (ANOVA; general linear model) with group and time as main effects, with additional group-by-time interaction term. All tests were two-sided with $P<0.05$ as considered statistically significant.

3. Results

Probiotic properties

DR7 showed the ability to adhere to mucin (Figure 1A), and tolerated simulated conditions of gastric acidity and bile (Figure 1B,C). DR7 showed better resistance towards acid while maintaining viability (reduction of less than one log cfu) as compared to bile conditions, where 50% of viability was maintained. Cell free supernatant of DR7 showed antioxidant potential and surpassing that of the standard antioxidant Trolox (Figure 1D). DR7 adhered to non-antibiotic resistance as per requirement of EFSA (Table 2). API assessment revealed that DR7 was able to utilise sugars, such as L-arabinose, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, D-sorbitol, methyl-α-D-mannopytanoside, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, gentiobiose, D-turanose and potassium gluconate (Table 3). DR7 could utilise shorter chained galactose-based oligosaccharide prebiotics, such as GOS, better than fructose-based oligosaccharide prebiotics, such as FOS, and could not thrive well in longer-chained oligosaccharide prebiotics,

Table 1. List of primer sequences used for quantitative real time polymerase chain reaction.

Genes	Primer sequences (5'-3')[1]	References
GABA5	F: CTTCTCGGCGCTGATAGAGT R: CGCTTTTTCTTGATCTTGGC	Mendu *et al.*, 2012
TDO	F: CATGGCTGGAAAGAACTC R: CTGAAGTGCTCTGTATGAC	Pilotte *et al.*, 2012
IDO	F: AGAAGTGGGCTTTGCTCTGC R: TGGCAAGACCTTACGGACATCTC	Chen *et al.*, 2008
CREB	F: CACCAGGAGTGCCAAGGATT R: CTGCTGCATTGGTCATGGTT	Chen *et al.*, 2013
TH	F: GCAGGCAGAGGCCATCATGT R: GGCGATCTCAGCAATCAGCT	Ishikawa *et al.*, 2010
GAD65	F: CTGGAAGACAATGAAGAGAGAATG R: TGCGGAAGAAGTTGACCTTATC	Naseri *et al.,* 2017
5-HT6	F: AGGCCTCTTCGATGTCCTCA R: CGCATGAAGAGTGGGTAGATGAT	Hirst *et al.*, 2003
TPH1	F: TAAGGAGAACAAAGACCATTCC R: TTTCTTCTTTTTGATTTTCGGG	Zill *et al.*, 2007
TPH2	F: GCC TTT CCT CTG TGT TCA TTA R: TCA TTC CAA CTG CTG TGT TAC C	Zill *et al.*, 2007
BDNF	F: TAACGGCGGCAGACAAAAAGA R: GAAGTATTGCTTCAGTTGGCCT	Zuccato *et al.*, 2011
DBH	F: CACCACATAATCATGTATGAGCCA R: GTGTGGAGCTGGGAGGCGAAGATG	Hong *et al.*, 2008

[1] F = forward primer; R = reverse primer.

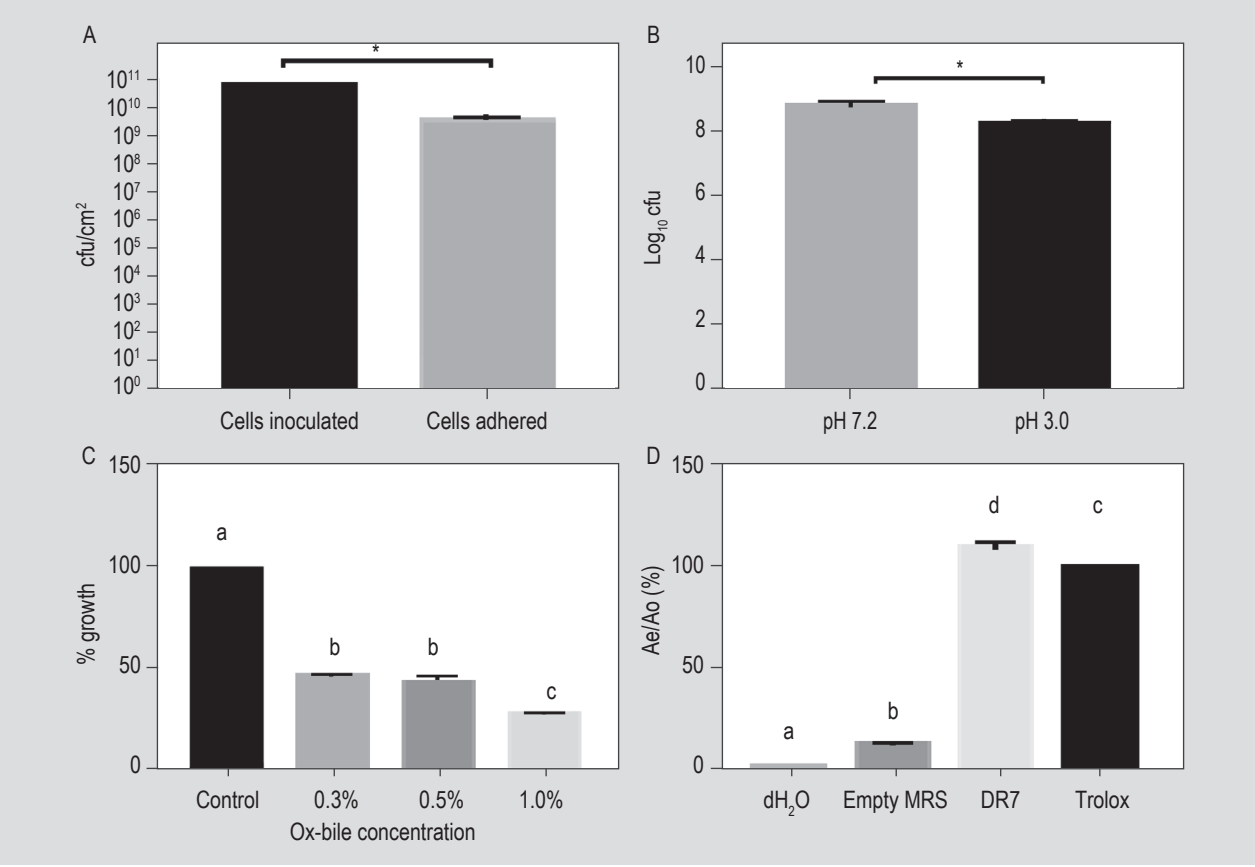

Figure 1. Probiotic properties of *Lactobacillus plantarum* DR7. (A) Adhesion to mucin (cfu/cm^2) of loaded and adhered cells onto mucin in 96-well microplate. (B) Resistance to acid (viability $\log_{10}$ cfu) at pH 7.0 and pH 3.0 phosphate buffered saline after incubation at 37 °C for 3 h. (C) Resistance to bile (percent growth as compared to control) at different concentrations of ox-bile after incubation at 37 °C for 24 h. MRS broth was used as the base medium. (D) Ferric reducing antioxidant power of cell-free supernatant. Trolox was used as positive control, while distilled water (dH_2O) and empty MRS was used as negative control. All data are expressed as mean and error bars represent standard errors of means (n=6). * Significantly different as compared to the control via independent T-test (*P*<0.01). Different letters indicate statistical difference (*P*<0.05) as determined by one-way ANOVA.

Table 2. Minimum inhibitory concentration (mg/l) for *Lactobacillus plantarum* DR7 tested against commercial antibiotics according to the guidelines by European Food Safety Authority (EFSA) using broth microdilution method. MIC value was recorded as the lowest concentration of antibiotic that prevented visible bacterial growth.

Antibiotics	Microbiological cut-off value (mg/l) for *L. plantarum*	
	EFSA	DR7
Gentamicin	16	0.5
Kanamycin	64	8
Tetracycline	32	16
Erythromycin	1	0.25
Clindamycin	2	1
Chloramphenicol	8	8
Ampicillin	2	1

such as inulin (Figure 2). Antimicrobial assays showed that cell free supernatant of DR7 exhibited inhibitory activities against common pathogens such as *Salmonella* sp., *S. epidermidis* and *E. coli,* where DR7 outperformed the antibiotics used against *S. epidermidis* and *E. coli* (Figures 3B and 3C). Cell free supernatant (CFS) of DR7 did not exert toxicity effects on HepG2 cells with viability exceeding 80% for all concentrations of CFS studied, except at concentration of undiluted CFS where a viability of 79.6% was observed (Figure 4).

Human study

Baseline data

Of the recruited 124 subjects, 5 subjects dropped-out during the 12-week period, while 7 subjects did not fully comply with answering questionnaires, computerised CogState test and/or providing blood samples, yielding 111 subjects after the 12-week study (n=56 for DR7, n=55 for placebo).

Table 3. Carbohydrate utilisation of *Lactobacillus plantarum* DR7 as measured using API-50 CHL with 24 h incubation at 37 °C.

Active ingredients	Reaction[1]	Active ingredients	Reaction[1]
control	–	Arbutin	+
Glycerol	–	Esculin ferric citrate	+
Erythritol	–	Salicin	+
D-arabinose	–	D-cellobiose	+
L-arabinose	+	D-maltose	+
D-ribose	+	D-lactose (bovine origin)	+
D-xylose	–	D-melibiose	+
L-xylose	–	D-saccharose	+
D-adonitol	–	D-trehalose	+
Methyl-β-D-xylopyranoside	–	Inulin	–
D-galactose	+	D-melezitose	+
D-glucose	+	D-raffinose	+
D-fructose	+	Amidon (starch)	–
D-mannose	+	Glycogen	–
L-sorbose	–	Xylitol	–
L-rhamnose	–	Gentiobiose	+
Dulcitol	–	D-turanose	+
Inositol	–	D-lyxose	–
D-mannitol	+	D-tagatose	–
D-sorbitol	+	D-fucose	–
Methyl-α-D-mannopytanoside	+	L-fucose	–
Methyl-α-D-glucopyranoside	–	D-arabitol	–
N-acetylglucosamine	+	L-arabitol	–
Amygdalin	+	Potassium gluconate	+
		Potassium 2-ketogluconate	–
		Potassium 5-ketogluconate	–

[1] + indicates positive reaction, – indicates negative reaction.

Subjects from both groups fulfilled the inclusion criteria of moderately stressed. The PSS questionnaire was used as a tool for the diagnostic of stress, where a subject was confirmed to have moderate stress levels based on total scores of 14-26. Insignificant differences were observed in most of the general and demographic characteristics except for height between placebo and DR7 for normal adults (Table 4).

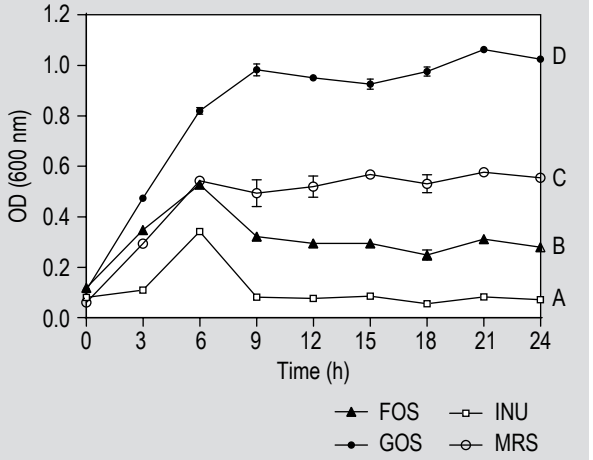

Figure 2. Growth of *Lactobacillus plantarum* DR7 as measured using optical density (600 nm) in the presence of different prebiotics (galactooligosaccharide (GOS), fructooligosaccharide (FOS), inulin (INU), de Man, Rogosa and Sharpe broth (MRS)) at 37 °C and every 3 h intervals for 24 h. All data are expressed as mean and error bars represent standard errors of means (n=6). Statistical difference of between groups (*P*<0.05) as determined using repeated measure ANOVA are indicated by capital letters.

Stress, anxiety, depression

In order to better evaluate psychological outcomes, we further divided the subjects into two groups (age below 30 years old, age above 30 years old) for both DR7 and placebo groups. Stress was assessed via the 10-items questionnaire of PSS-10 and the 42-items questionnaire of DASS-42 (Figure 5). Based on PSS-10 (Figure 5A), both DR7 and placebo showed reduction in total scores over 12 weeks. However, the effects between treatment groups across 12 weeks for all age ranges remained insignificantly different from each other. The administration of DR7 benefited young adults (age <30 years old) better than normal adults (age >30 years old), where a higher reduction of total DASS-42 stress scores were observed after week 8 as compared to the placebo for young adults (P=0.016), while insignificant differences were observed in normal adults (Figure 5B). DR7 also showed higher reduction of DASS-42 stress scores compared to the placebo for all subjects after week 8 (P=0.024; Figure 5B). Time (P=0.002) exerted a significant effect on total DASS-42 stress scores over 12 weeks, while treatment groups and the interactions between time and treatment groups had insignificant effects. The administration of DR7 reduced scores for anxiety as assessed by the DASS-42 questionnaire in all populations studied (young adults, normal adults, overall total subjects) after week 8, where a higher reduction of total DASS-42 anxiety scores were observed as compared to the placebo (P=0.017; Figure 5C). Time (P=0.010) and treatment groups (P=0.003) exerted significant effects on DASS-42 anxiety scores over 12 weeks, although the interactions

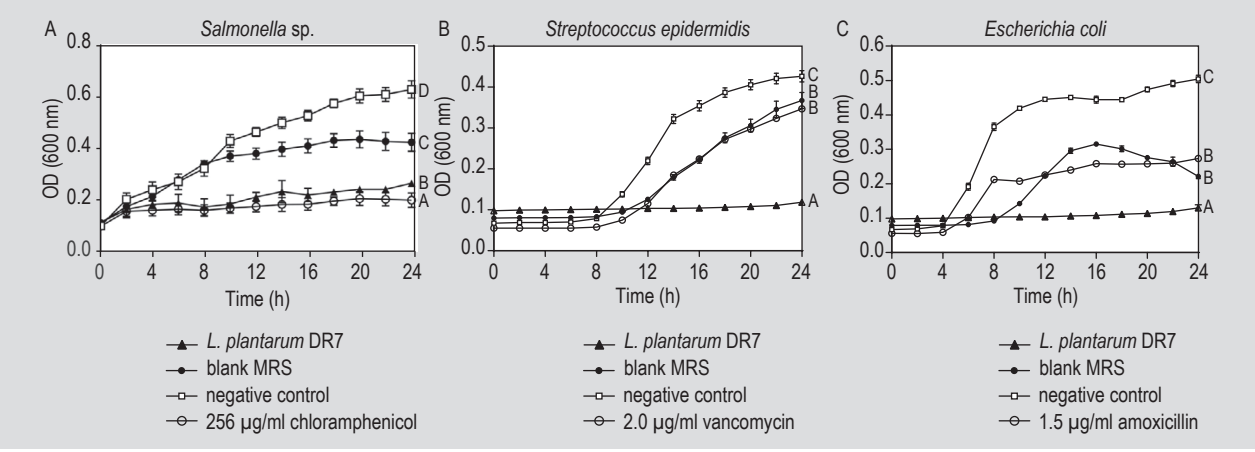

Figure 3. Antimicrobial activity of cell-free supernatant of *Lactobacillus plantarum* DR7 against (A) *Salmonella* sp. (T: *P*<0.001, H: *P*<0.001, TxH: *P*<0.001), (B) *Staphylococcus epidermidis* ATCC 12228 6538P (T: *P*<0.001, H: *P*<0.001, TxH: *P*<0.001), (C) *Escherichia coli* ATCC 25922 (T: *P*<0.001, H: *P*<0.001, TxH: *P*<0.001). Negative control was designed as the growth of pathogen in TSB in the absence of treatment compound. Pathogens treated with antibiotics amoxicillin (1.5 µg/ml), vancomycin (2 µg/ml) or chloramphenicol (256 µl/ml) were used as positive controls. All data are expressed as mean and error bars represent standard errors of means (n=6). Statistical difference of between groups (*P*<0.05) as determined using repeated measure ANOVA are indicated by capital letters.

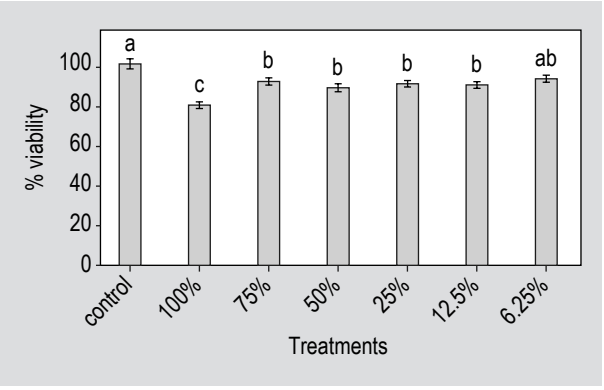

Figure 4. Safety and toxicity of *Lactobacillus plantarum* DR7 using human liver hepatocellular cell line (HepG2) as a model, at varying concentrations of cell free supernatant from DR7 as measured using the MTT assay. All data are expressed as mean and error bars represent standard errors of means (n=6). Different letters indicate statistical difference (*P*<0.05) as determined by one-way ANOVA.

between time and treatment groups had insignificant effects. DR7 did not exert any effects against depression as compared to the placebo over 12 weeks as assessed via the DASS-42 questionnaire (Figure 5D). Although time affected DASS-42 depression scores (P=0.013) over 12 weeks, both treatment groups and interactions between time and treatment groups showed insignificant effects. A higher reduction in total DASS-42 score was observed for all subjects after week 8 for subjects administered with DR7 as compared to the placebo (P=0.022; Figure 5E). The administration of DR7 benefited young adults better than normal adults, where a higher reduction of DASS-42 total scores were observed after week 8 as compared to the placebo for young adults (P=0.044), while insignificant differences were observed in normal adults (Figure 5E). Time (P=0.001) and treatment groups (P=0.036) exerted significant effects on total DASS-42 total scores over 12 weeks although their interactions had insignificant effects.

To better understand individual component effects, we evaluated individual aspects of each item that were significantly improved as assessed by DASS-42 at week 8 compared to week 0 (Figure 6). Of the 14 items designated for stress, DR7 significantly reduced scores for stress in two items (P<0.05) while marginally reduced one item (P<0.10) as compared to the placebo. The efficacy of DR7 in reducing stress as compared to the placebo was predominately attributed to improvement of relaxation (item no. 8, P=0.011) and alleviated use of nervous energy (item no. 12, P=0.012). At a lesser degree, DR7 also suggestively reduced touchiness (item no. 18, P=0.075) as compared to the placebo. The efficacy of DR7 in reducing anxiety as assessed by DASS-42 compared to the placebo was predominately attributed to improvement in swallowing (item no. 23, P=0.001) and reduction in trembling (item no. 41, P=0.002).

Plasma levels of cortisol and cytokines

The administration of DR7 significantly reduced plasma cortisol levels in total subjects compared to the placebo group after 12 weeks (P=0.008) (Figure 7), accompanied by reduced plasma pro-inflammatory cytokines in the DR7 group as compared to the placebo group, such as IFN-γ (P<0.001) and TNF-α (P=0.006), and increased plasma anti-inflammatory cytokines IL-10 (P=0.035) (Figure 7C). The administration of DR7 benefited both the young and normal adult groups differently. DR7 exerted more prevalent effects

Table 4. Baseline characteristics of one hundred and eleven (n=111) adult subjects randomly assigned to 12 weeks of double-blind treatment with either *Lactobacillus plantarum* DR7 or placebo.

Baseline characteristics	Age <30 years old			Age >30 years old			Total		
	Placebo	DR7	*P*-value	Placebo	DR7	*P*-value	Placebo	DR7	*P*-value
Sample size (n)	32	27		23	29		55	56	
Age	24.9±2.9	24.8±2.8	0.704	41.7±9.5	37.0 ±6.0	0.059	32.1±11.0	31.1±7.8	0.595
Body weight (kg)	60.6±15.4	57.5±11.0	0.398	64.1±15.4	63.8±12.0	0.928	62.1±14.8	60.9±11.9	0.642
Height (cm)	161.4±7.5	159.1±8.0	0.257	158.0±9.3	164.2±7.3	0.010*	160.0±8.4	161.8±8.0	0.254
BMI	23.2±5.2	22.8±4.3	0.774	25.7±5.2	23.6±4.0	0.114	24.2±5.3	23.2±4.1	0.277
	% (n)	% (n)		% (n)	% (n)		% (n)	% (n)	
Occupation									
Professional	56.3 (18)	51.9 (14)		43.5 (10)	41.4 (12)		50.9 (28)	46.4 (26)	
Manager	3.1 (1)	7.4 (2)		4.3 (1)	13.8 (4)		3.6 (2)	10.7 (6)	
Clerical support workers	18.8 (6)	22.2 (6)		13.0 (3)	6.9(2)		16.4 (9)	14.3 (8)	
Services & sales workers	9.4 (3)	18.5 (5)		4.3 (1)	17.2 (5)		7.3 (4)	17.9 (10)	
Technician	12.5 (4)	0		21.7 (5)	6.9(2)		16.4 (9)	3.6 (2)	
Self-employed	0	0		4.3 (1)	6.9(2)		1.8 (1)	3.6 (2)	
Retired	0	0		0.0	3.4(1)		0.0	1.8 (1)	
Unemployed	0	0		8.7 (2)	3.4(1)		3.6 (2)	1.8 (1)	
Educational level									
Primary school	0	0		4.3 (1)	0		1.8 (1)	0	
Secondary school	0	14.8 (4)		13.0 (3)	24.1 (7)		5.5 (3)	19.6 (11)	
Diploma	6.3 (2)	0		8.7 (2)	0		7.3 (4)	0.0	
Colleges	28.1 (9)	25.9 (7)		21.7 (5)	17.2 (5)		25.5 (14)	21.4 (12)	
Bachelor's degree	62.5 (20)	55.6 (15)		39.1 (9)	58.6 (17)		52.7 (29)	57.1 (32)	
Master's degree	0	3.7 (1)		4.3 (1)	0		1.8 (1)	1.8 (1)	
Ph.D	3.1 (1)	0		8.7 (2)	0		5.5 (3)	0	
Family income status									
Low (<RM 2,300)	50.0 (16)	37.0 (10)		17.4 (4)	17.2 (5)		36.4 (20)	26.8 (15)	
Middle (RM 2,301-5,999)	31.3 (10)	51.9 (14)		43.5 (10)	13.8 (4)		36.4 (20)	32.1 (18)	
High (>RM 6,000)	18.8 (6)	11.1 (3)		39.1 (9)	69.0 (20)		27.3 (15)	41.1 (23)	
Marital status									
Married	68.8 (22)	66.7 (18)		100.0 (23)	75.9 (22)		81.8 (45)	71.4 (40)	
Divorced	12.5 (4)	0		0	13.8 (4)		7.3 (4)	7.1 (4)	
Single	18.8 (6)	33.3 (9)		0	10.3 (3)		10.9 (6)	21.4 (12)	

[1] Significantly different between groups; * $P<0.05$.

in increasing plasma anti-inflammatory cytokines IL-10 ($P=0.033$) and decreasing pro-inflammatory cytokines IFN-γ ($P=0.015$) in young adults compared to the placebo over 12 weeks (Figure 7A), while only decreasing plasma pro-inflammatory cytokines IFN-γ ($P<0.001$), TNF-α ($P=0.003$) and IL-1β ($P=0.002$) in normal adults compared to the placebo over 12 weeks without any effect on pro-inflammatory cytokines (Figure 7B).

Cognition and memory

The administration of DR7 enhanced the speed needed for social emotional cognition ($P=0.001$) and verbal learning and memory ($P=0.021$) while marginally reducing errors for associate learning in all subjects over 12 weeks as compared to the placebo (Table 5). Upon separation into age groups, the administration of DR7 benefited normal adults better than younger adults. Speed needed for basic attention ($P=0.034$), social emotional cognition ($P<0.001$) and associate learning ($P=0.010$) were enhanced in the normal adults population upon administration of DR7 compared

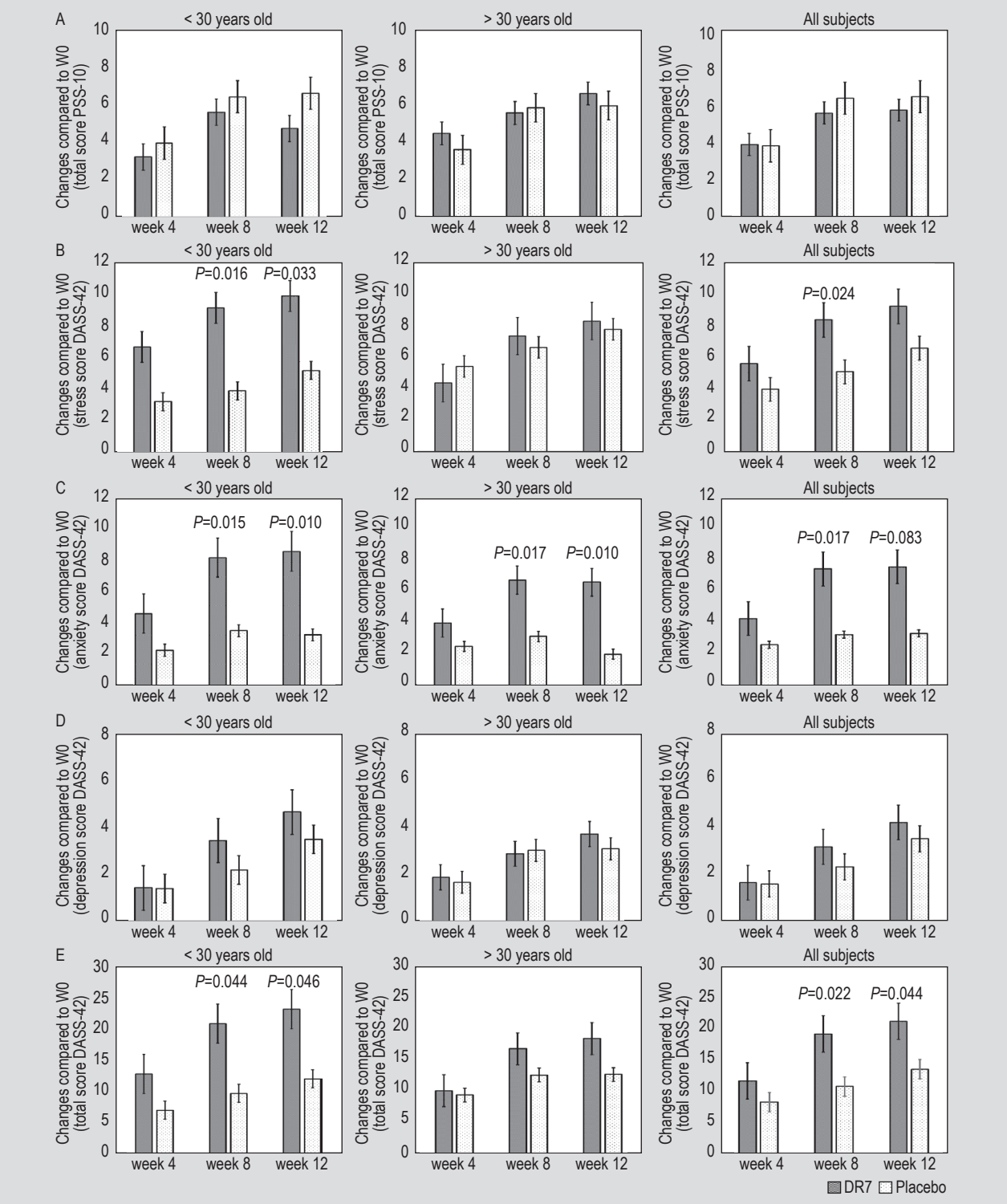

Figure 5. Effects of a 12 week administration of *Lactobacillus plantarum* DR7 or placebo, on changes in scores as compared to week 0 for (A) PSS-10, (B) Stress, (C) Anxiety, (D) Depression, (E) Total scores DASS-42 in young adults (aged <30 years old), normal adults (aged >30 years old) and all subjects. Repeated measure ANOVA for DASS-42; stress for <30 years old: W: *P*=0.130; T: *P*=0.016, T×W: *P*=0.628; stress for all subjects: W: *P*=0.003; T: *P*=0.036, T×W: *P*=0.593; anxiety for <30 years old: W: *P*=0.040; T: *P*=0.016, T×W: *P*=0.291; anxiety for >30 years old: W: *P*=0.127; T: *P*=0.045, T×W: *P*=0.433; anxiety for all subjects: W: *P*=0.007; T: *P*=0.002, T×W: *P*=0.170; total score for <30 years old: W: *P*=0.061; T: *P*=0.044, T×W: *P*=0.484; total score for all subjects: W: *P*=0.005; T: *P*=0.028, T×W: *P*=0.448. *P*-values indicated difference between treatment groups at individual time points. Repeated measures ANOVA provided statistical significance on W: effect of weeks; T: effect of treatment groups DR7 and placebo; T×W: interaction between weeks and treatment. Results are expressed as mean; error bars (SEM); n=111 (DR7 n=56, placebo n=55).

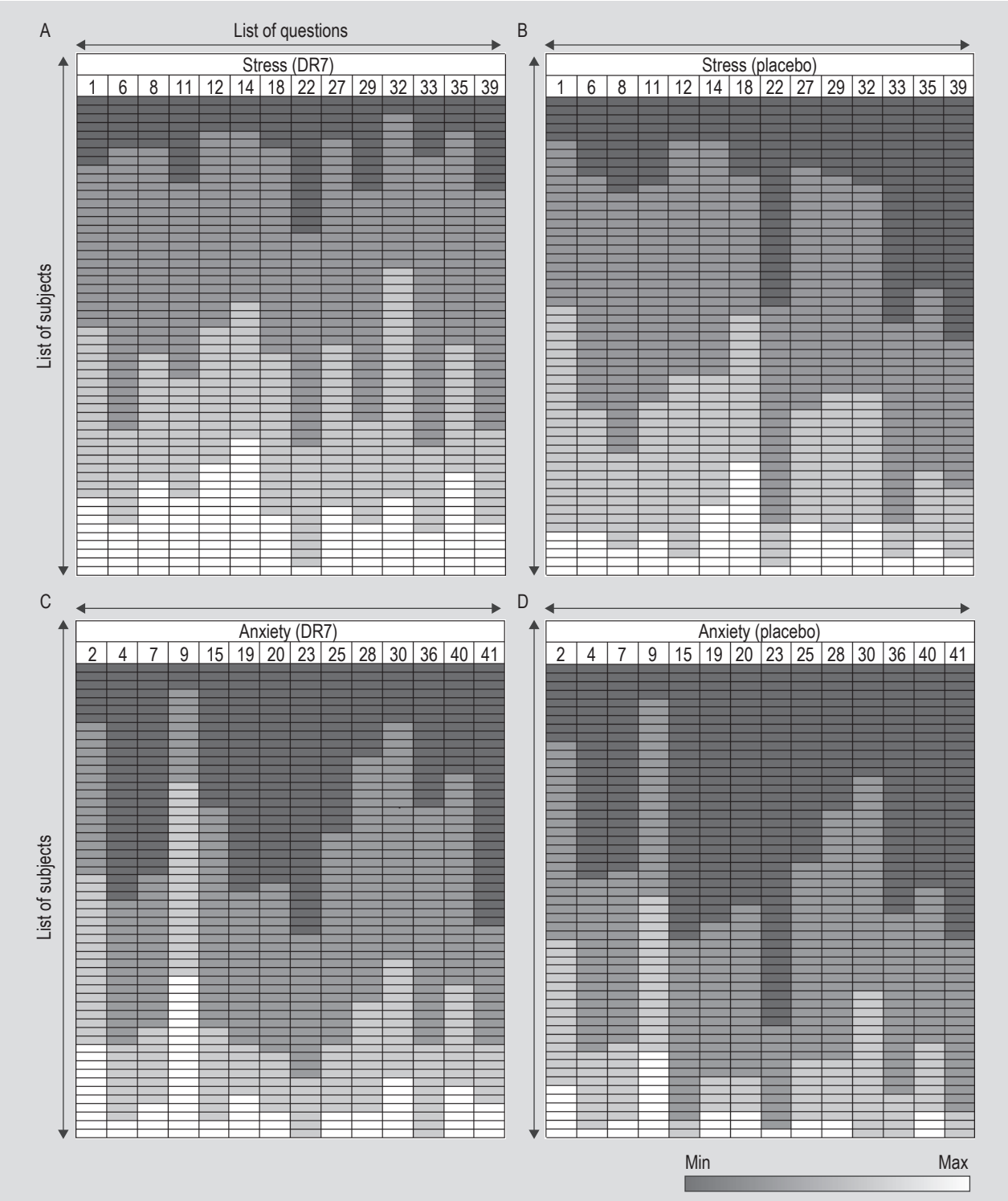

Figure 6. Heat map illustrating the effects of placebo or *Lactobacillus plantarum* DR7 on the different scores of individual subjects across individual items for the parameters of stress (A = DR7, B = placebo) and anxiety (C = DR7, D = placebo) as assessed by the DASS-42 questionnaire. Data are presented as differences in scores between week 8 and 0; n=111 (DR7 n=56, placebo n=55). A darker colour indicated lower reduction of scores. Individual items are arranged independently based on a descending order, with lower reduction of scores at the top and higher reduction of scores at the bottom. Significant differences were observed between DR7 and placebo for stress items no. 8 (*P*=0.011), no. 12 (*P*=0.012), and marginal difference for no. 18 (*P*=0.075). There were significant differences between DR7 and placebo for anxiety items no. 23 (*P*=0.001) and no. 41 (*P*=0.002).

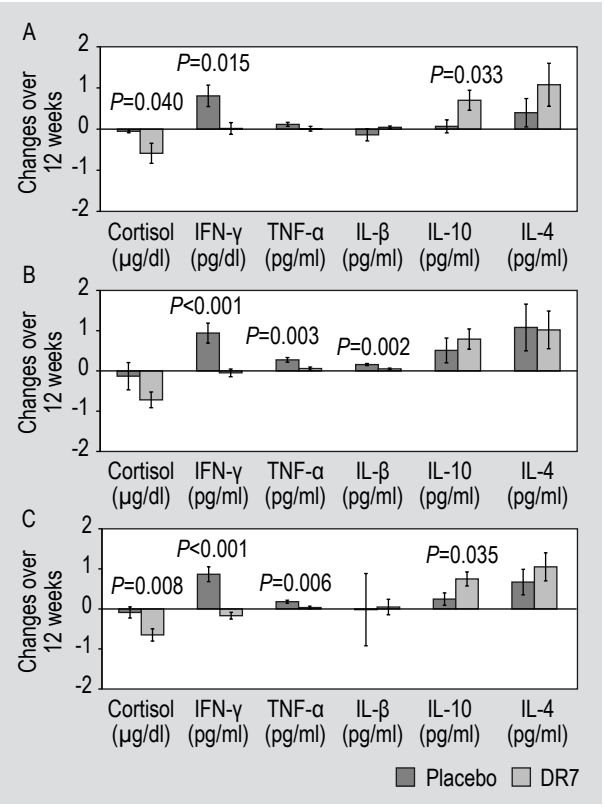

Figure 7. Changes of plasma levels of cortisol and cytokines [interferon gamma (IFN-γ); tumor necrosis factor alpha (TNF-α); interleukin (IL)-1β; IL-10; IL-4] after 12-week administration of *Lactobacillus plantarum* DR7 compared to placebo group in (A) young adults (aged <30 years old), (B) normal adults (aged >30 years old) and (C) all subjects. Concentrations of cytokines were standardised using total white blood cell count obtained from full blood count test. *P*-values indicated difference between treatment groups at individual time points. Results are expressed as mean, error bars (SEM); n=111 (DR7 n=56, placebo n=55).

to placebo over 12 weeks, with marginal improvement in speed for visual learning and memory, working memory and long term memory, total scores for basic attention, and accuracy for associate learning ($P<0.10$). Meanwhile, the administration of DR7 only marginally benefited the younger adult's population via enhancing verbal learning and memory upon administration of DR7 compared to placebo over 12 weeks ($P=0.066$).

Plasma neurotransmitters parameters

The administration of DR7 lowered the expression of dopamine β-hydroxylase (DBH) and tryptophan 2,3-dioxygenase (TDO) while increasing the expressions of tryptophan hydroxylase-2 (TPH2) and 5-hydroxytryptamine receptor-6 (5-HT6) as compared to the placebo over 12 weeks in young adults (Figure 8A). An almost similar trend was also observed in the normal adult population where DR7 lowered the expressions of DBH, tyrosine hydroxylase (TH), indoleamine 2,3-dioxygenase (IDO) and TDO while increasing the expressions of tryptophan hydroxylase-2 (TPH2) as compared to the placebo over 12 weeks (Figure 8B). In all subjects, the administration of DR7 lowered the expressions of DBH, TH, IDO and TDO while increasing the expressions of TPH2 and 5-HT6 over 12 weeks (Figure 8C). The administration of DR7 did not affect the expressions of glutamic acid decarboxylase (GAD65), gamma aminobutyric acid A-receptor α-5 (GABRA5), brain derived neurotrophic factor (BDNF) and cAMP response element binding (CREB) over 12 weeks (Figure 8).

Table 5. Cognition and memory parameters as assessed via the computerised CogState Brief Battery at week 12 for stressed adults (n=111; DR7 n=56, placebo n=55) upon administration of placebo or *Lactobacillus plantarum* DR7.

Parameter[1]	Age <30 years old			Age >30 years old			Total subjects		
	DR7	Placebo	*P*-value[2]	DR7	Placebo	*P*-value[2]	DR7	Placebo	*P*-value[2]
Detection (measures psychomotor)									
Main	92.83±9.79	91.19±11.60	0.581	97.70±12.68	97.39±6.46	0.915	95.26±11.63	93.83±10.15	0.506
LMN	2.59±0.12	2.61±0.13	0.532	2.55±0.16	2.58±0.09	0.538	2.57±0.14	2.60±0.12	0.353
COR	33.09±9.13	33.61±6.73	0.808	34.8±4.92	33.83±5.11	0.505	33.98±7.29	33.70±6.04	0.849
ERR	7.74±16.59	13.71±28.36	0.372	3.60±7.56	5.96±13.38	0.452	5.70±12.85	10.41±23.34	0.222
ACC	1.33±0.40	1.27±0.39	0.553	1.37±0.24	1.37±0.31	0.926	1.35±0.33	1.31±0.36	0.565
Identification (measures basic attention)									
Main	96.04±7.36	97.42±6.61	0.469	101.85±6.23	98.26±6.57	0.053#	97.78±7.39	97.78±16.71	0.350
LMN	2.77±0.09	2.75±0.08	0.488	2.70±0.08	2.75±0.07	0.034*	2.73±0.09	2.75±0.12	0.344
COR	27.61±6.34	29.16±8.62	0.469	29.48±2.14	29.74±1.94	0.663	28.55±4.74	29.41±6.04	0.463
ERR	10.00±15.82	8.68±19.91	0.794	5.16±7.75	5.17±8.98	0.995	7.64±12.48	7.19±23.34	0.876

Table 5. Continued

Parameter[1]	Age <30 years old			Age >30 years old			Total subjects		
	DR7	Placebo	*P*-value[2]	DR7	Placebo	*P*-value[2]	DR7	Placebo	*P*-value[2]
Identification (measures basic attention)									
ACC	1.22±0.38	1.22±0.35	0.948	1.27±0.23	1.29±0.24	0.716	1.24±0.31	1.25±0.36	0.794
One card learning (measures visual learning & memory)									
Main	94.38±9.81	95.00±8.91	0.806	97.37±10.34	94.48±8.06	0.282	95.80±10.14	94.78±8.48	0.577
LMN	3.00±0.15	2.99±0.11	0.655	2.97±0.10	3.04±0.10	0.058#	2.98±0.13	3.01±0.13	0.332
COR	55.68±10.80	54.65±9.45	0.704	57.44±12.06	55.26±3.48	0.490	56.43±11.45	54.91±9.49	0.458
ERR	34.52±12.97	35.55±11.09	0.750	32.78±16.75	33.83±9.04	0.795	33.82±15.00	34.81±10.69	0.696
ACC	0.91±0.14	0.90±0.12	0.701	0.93±0.16	0.91±0.23	0.571	0.92±0.15	0.90±0.12	0.458
One card back (measures working memory)									
Main	95.46±15.13	94.23±12.91	0.746	93.96±10.17	91.65±12.54	0.475	94.44±12.66	93.13±12.70	0.600
LMN	2.95±0.10	2.92±0.10	0.219	2.91±0.10	2.96±0.10	0.094#	2.93±0.10	2.93±0.10	0.987
COR	29.56±5.16	29.52±5.05	0.975	29.96±3.42	29.48±3.48	0.623	29.75±4.41	29.50±4.41	0.972
ERR	7.60±10.80	7.87±10.43	0.925	7.19±7.36	9.57±9.04	0.310	7.51±9.81	8.59±9.81	0.958
ACC	1.20±0.28	1.17±0.26	0.742	1.17±0.19	1.12±0.23	0.461	1.18±0.24	1.15±0.24	0.946
Maze chase (measures executive function)									
Main	100.88±8.28	101.23±5.87	0.855	99.41±8.33	100.39±6.75	0.652	99.98±8.30	100.87±6.22	0.588
DUR	232,293.00± 95,611.90	217,986.00± 65,543.97	0.517	277,087.00± 218,020.47	270,854.00± 100,464.51	0.901	258,091.36± 171,451.56	240,503.78± 85,543.17	0.508
TER	55.56±21.43	53.29±15.89	0.651	60.44±21.36	60.00±16.63	0.936	58.41±21.42	56.15±16.40	0.543
PER	0.20±0.65	0.32±0.83	0.548	0.22±0.42	0.61±1.20	0.123	0.22±0.54	0.44±1.00	0.152
Maze final recall (measures long term memory)									
DUR	31,846.00± 16,416.62	31,896.00± 13,124.52	0.990	34,576.00± 17,108.13	44,421.00± 21,846.71	0.080#	33,583.27± 16,675.53	37,230.70± 18,294.73	0.289
TER	8.16±5.33	7.77±4.89	0.779	10.00±6.63	9.57±4.95	0.797	9.25±6.03	8.54±4.95	0.505
Social emotional task (measures social emotional cognition)									
RES	48.68±1.14	48.55±0.85	0.624	48.59±1.22	48.52±0.73	0.808	48.65±1.18	48.54±0.79	0.574
LMN	3.49±0.10	3.51±0.13	0.573	3.43±0.12	3.59±0.11	<0.001*	3.46±0.11	3.54±0.13	0.001*
COR	38.12±6.21	38.00±4.84	0.936	35.26±9.91	38.74±4.11	0.123	36.55±8.44	38.31±4.52	0.181
ERR	10.56±6.36	10.55±5.06	0.994	13.33±10.41	9.78±4.08	0.131	12.10±8.79	10.22±4.64	0.171
ACC	1.10±0.14	1.10±0.12	0.997	1.04±0.23	1.10±0.12	0.281	1.06±0.19	1.10±0.12	0.309
Continuous paired (measures associate learning)									
RES	127.64±54.00	114.84±42.30	0.324	131.41±80.01	158.35±55.29	0.180	130.80±68.32	133.37±52.47	0.749
LMN	3.32±0.15	3.27±0.28	0.462	3.34±0.22	3.49±0.18	0.010*	3.33±0.19	3.37±0.27	0.413
COR	54.24±8.80	56.00±0.00	0.269	52.37±13.08	56.00±0.00	0.190	53.22±11.26	56.00±0.00	0.075#
ERR	71.76±53.94	58.84±42.3	0.319	78.30±76.61	102.35±55.29	0.216	76.39±66.17	77.37±52.47	0.848
ACC	0.78±0.16	0.85±0.21	0.216	0.84±0.32	0.70±0.22	0.084#	0.80±0.26	0.78±0.22	0.549
Shopping list (measures verbal learning & memory)									
Total scores from 3×	27.08±4.05	24.70±5.13	0.066#	26.19±4.12	24.17±4.66	0.134	26.69±4.08	24.50±4.92	0.021
Score from recall	10.44±1.78	10.80±1.27	0.387	9.81±1.96	10.00±2.66	0.795	10.08±1.89	10.52±1.88	0.289

[1] LMN = speed; COR = correct; ERR = errors; ACC = accuracy; TER = total errors; RES = responses; DUR = duration; PER = perseverance; Main = main score (with colour).

[2] Significance * $P<0.05$; # $P<0.10$.

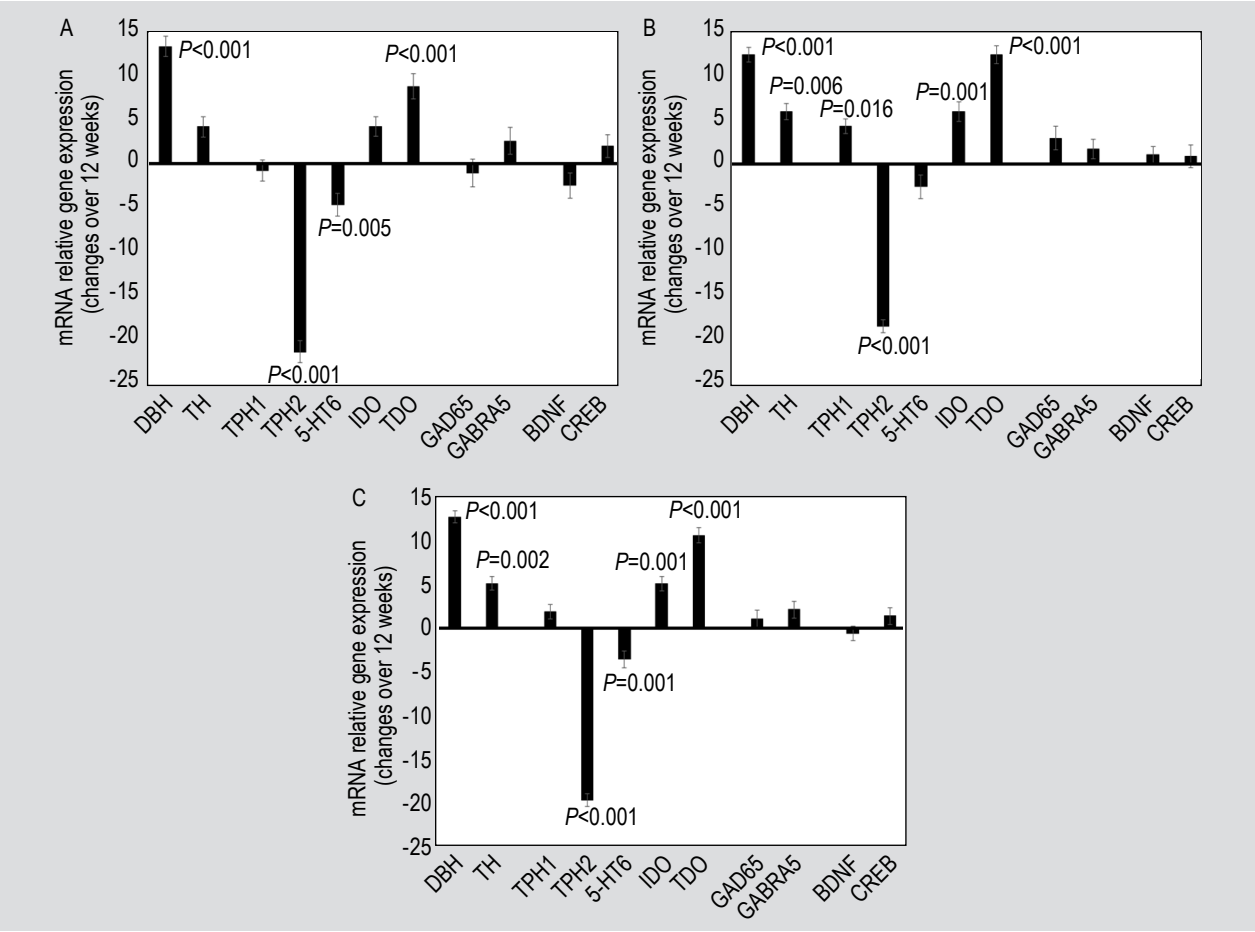

Figure 8. Effects of a 12-week administration of *Lactobacillus plantarum* DR7 on blood mRNA relative gene expressions (normalised using placebo as a control) for dopamine β-hydroxylase (DBH), tyrosine hydroxylase (TH), tryptophan hydroxylase-1 (TPH1), tryptophan hydroxylase-2 (TPH2), 5-hydroxytryptamine receptor-6 (5-HT6), indoleamine 2,3-dioxygenase (IDO), tryptophan 2,3-dioxygenase (TDO), glutamic acid decarboxylase (GAD65), gamma aminobutyric acid A-receptor α-5 (GABRA5), brain derived neurotrophic factor (BDNF) and cAMP response element binding (CREB) in (A) young adults (aged <30 years old), (B) normal adults (aged >30 years old) and (C) all subjects. *P*-values indicated difference between treatment groups at individual time points. Results are expressed as mean, error bars (SEM); n=111 (DR7 n=56, placebo n=55).

4. Discussion

Probiotics have been long reported to exert an array of gut health benefits, with recent evidences suggesting new roles on mental wellbeing along the gut-brain-axis. This study was designed to evaluate the effects of *L. plantarum* DR7 in modulating brain health and physiological properties. Two types of questionnaires were used as assessment tools to evaluate stress, anxiety and depressive parameters. Both the PSS-10 and DASS-42 questionnaires are validated psychological instruments that correlated with psychological, clinical emotional and behavioural measures (Cohen *et al.,* 1983; Lovibond and Lovibond, 1995). While all subjects were recruited based on moderate stress levels as assessed by PSS-10, the administration of DR7 showed reduced stress and anxiety levels as compared to the placebo after 8 weeks upon assessment by DASS-42, while assessment by PSS-10 did not yield significant changes. This difference may be attributed to the different levels of appraised stress by both tools. DASS-42 comprises broader natured items which are sensitive to varying levels of chronic non-specific arousal, such as difficulty in calming, nervous stimulation, and being easily upset/disturbed, irritable/over-reactive and intolerance. Due to its extensive ranges of evaluations, DASS-42 has been widely used in both clinical and non-clinical settings, while PSS-10 is primarily used in research samples. Predictive validity of PSS-10 reportedly fell off rapidly after four weeks, while DASS-42 has been shown to have a high internal consistency to yield meaningful discriminations in diverse settings (Cohen *et al.,* 1983; Lovibond and Lovibond, 1995).

Despite improving stress and anxiety levels, DR7 showed no impact on depression scores as compared to the placebo. Depression is a deeper psychological condition evolving from prolonged exposure to anxiety and stress, to

include deteriorative mental conditions, such as dysphoria, hopelessness, devaluation of life and self-deprecation. This may need medical interventions that are beyond the curative scopes of natural alternatives. Meanwhile, anxiety has a lesser degree of psychological severity as compared to depression, to include health circumstances such as autonomic arousal, skeletal muscle effects and anxiousness, while stress has a lesser degree of acuteness as compared to anxiety to cover psychological impairment such as difficulty in relaxing, nervous arousal, easily upset/agitated, irritable/over-reactive and impatience. Our present data suggested that natural interventions, such as probiotics may be beneficial for less severe psychological conditions, such as anxiety and stress as compared to depression. Our further evaluations of individual anxiety and stress items in DASS-42 confirmed that the effects of DR7 were primarily associated with a calming outcome, such as increased relaxation, improved in swallowing, reduced nervousness, touchiness and trembling attributes.

As the global population ages and human life span increases, mortality has also been expanded beyond 85 years old in developed countries, resulting in lengthening the period of adulthood. Considering that most of the working years occur during adulthood, we aimed to target a healthier adult population that would subsequently form healthier aging populations. In the context of nutrition and diet, little information is available on the effects of probiotic interventions on health of the young adults (age <30 years old) as most studies emphasised on normal/older adults (age 31-60 years old). Our data showed that although DR7 benefited both the young and normal adult populations for anxiety, DR7 only benefited the younger adult population for stress as compared to the placebo, while such an effect was not observed in the normal adult population. This may be attributed to higher perceived stress among young adults as compared to normal/older adults, resulting in much room for improvements via natural interventions. Stress scores as assessed by the DASS-42 questionnaire were reduced from moderate to normal in the young adult population while only reduced from mild to normal in the normal adult population for both DR7 and placebo groups (data not shown). Young adults and millennials have been labelled as the most stressed-out generation in the US with 64% young adults surveyed reported middle to extreme stress levels (Jayson, 2013) while young adults in the UK spend over 6 h daily feeling stressed-out (Gemma, 2018). The administration of DR7 also increased anti-inflammatory cytokines and decreased pro-inflammatory cytokines in young adults, while only decreased pro-inflammatory cytokines in normal adults over 12 weeks. As stress often triggers inflammatory responses, DR7 may have decreased stress levels in association with decreased inflammatory parameters in young adults, while a lesser reduction of both stress and inflammatory parameters were observed in normal adults.

While DR7 benefited the young adult populations better for stress and plasma inflammatory cytokine parameters, memory and cognitive traits were better enhanced in the normal adult populations upon administration of DR7 over 12 weeks. The administration of DR7 only enhanced verbal learning and memory in young adults but basic attention, social emotional cognition and associate learning were boosted in normal adults over 12 weeks. Normal cognitive aging has been described as non-pathologic declines that occur gradually and inevitably with aging, that affects an array of cognitive and memory parameters such as crystalised and fluid intelligence, processing speed, attention, language, visual construction abilities and executive functioning (Harada *et al.,* 2013). It was conventionally argued that adults at age 30 years old remain young with an active neuropathology state. However, recent evidences have shown that synaptic density decreased by approximately 14% comparing age 25 versus 38 years old, while crystalised abilities declined by 8 and 28% at ages 30 and 40 years old, respectively, with fluid abilities decreased by 36% at age 30-40 years old, compared to 20 years old (Murman, 2015). We thus postulate that normal adults experienced declines in cognitive and memory functions, which were less observed in young adults, thus had larger rooms for enhancement upon administration of DR7.

While the human body houses various types of neurotransmitters, only three neurotransmitters were conventionally reported to exist in the human brain, namely serotonin, dopamine and norepinephrine. Gamma-aminobutyric acid (GABA) has been added as the fourth brain neurotransmitter upon detection of its wide distribution in the neurons of cortex, with inhibitory roles of balancing the excitatory nature of glutamate. Serotonin plays crucial roles in the management of anxiety and learning memory thus affecting perceptions, while dopamine affects cognition via influencing working memory and apathy. Norepinephrine controls attention thus affecting recall memory and perseverance. Tryptophan is an essential amino acid used in building various proteins and an important starting point for the biosynthesis of serotonin and kynurenine (Figure 9). While an imbalanced level of serotonin (5-HT) has been associated with inflammation and psychological functions including mood and anxiety (Field *et al.,* 2005), an increased in kynurenine has been associated with depressive disorders. Upon activation, the kynurenine pathway diverts available tryptophan away from serotonin production (Davis and Liu, 2015). The kynurenine pathway can be activated by IFN-γ, leading to inflammation accompanied by increased amounts of kynurenine pathway metabolites such as kynurenine, kynurenic acid, and quinolinic acid in cerebrospinal fluid (Raison *et al.,* 2010). Along the serotonin and kynurenine pathways, several enzymes play crucial roles in the conversion of tryptophan; TPH converts tryptophan to serotonin while IDO and/or TDO convert tryptophan to kynurenine. The serotonin

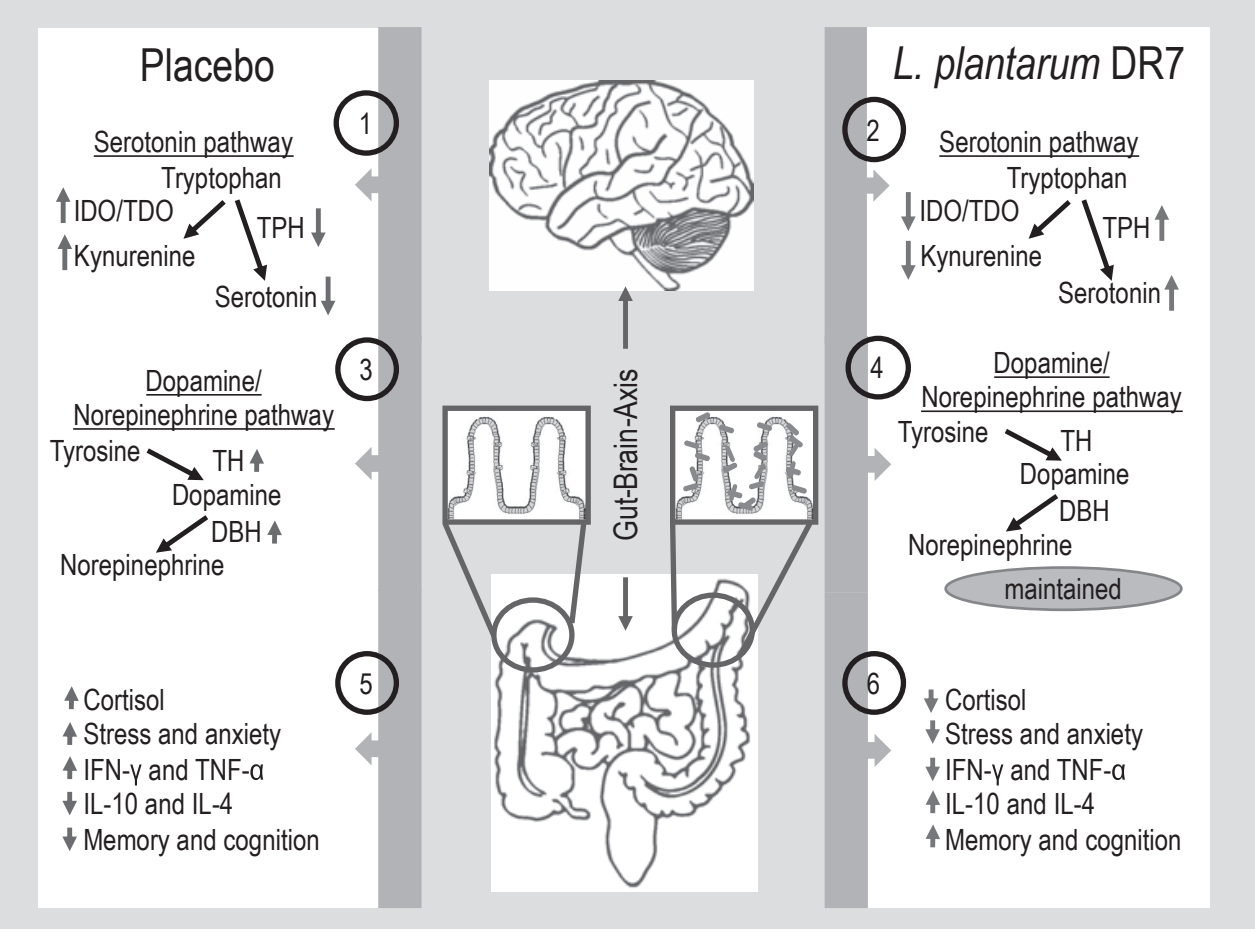

Figure 9. Regulations of brain neurotransmitters pathways in subjects administered with *Lactobacillus plantarum* DR7 or placebo, and associations with blood, questionnaires and cognition battery parameters along the gut-brain axis. Tryptophan is an important starting point for the biosynthesis of serotonin and kynurenine. Tryptophan hydroxylase (TPH) converts tryptophan to serotonin (5-HT) while indoleamine 2,3-dioxygenase (IDO) and/or tryptophan 2,3-dioxygenase (TDO) convert tryptophan to kynurenine. Stressed subjects administered with placebo showed higher expressions of IDO and TDO which potentially channelled tryptophan towards the production of kynurenine (1) as compared to those administered with DR7 which showed higher expressions of TPH and 5-HT6, thus potentially channelled tryptophan towards the production of serotonin (2). Tyrosine hydroxylase (TH) converts tyrosine to dopamine (DA) while dopamine β-hydroxylase (DPH) converts DA to norepinephrine (NE). Stressed subjects administered with placebo showed higher expressions of TH and DPH leading to higher production of DA and NE (3) as compared to those administered with DR7 which showed a maintained level of DA and NE (4). An increased level of NE is often observed amid stress, as supported by higher stress and anxiety parameters in stressed subjects administered with placebo (5), while those administered with DR7 showed lower stress and anxiety parameters (6).

5-HT6 receptor regulates the serotonergic pathway, involves in learning and memory processes and improves cognitive functions (González-Vera *et al.,* 2017). Our present data showed that DR7 affected the serotonin pathway in stressed subjects where higher expressions of TPH and 5-HT6 were observed, thus potentially channelling tryptophan towards the production of serotonin (Figure 9, step 2), as compared to the placebo which showed higher expressions of IDO and TDO, thus potentially channelling tryptophan towards the production of kynurenine (Figure 9, step 1). IDO is increasingly recognised as a link between the immune system and the kynurenine pathway with some anti-inflammatory effects, as it is activated by cytokines, such as IFN-γ (Mandi and Vecsei, 2012). Our data on plasma cytokine also showed a higher level of IFN-γ in placebo subjects compared to those administered with DR7. Notably, serotonin is also produced from enterochromaffin cells in the gut, thus affecting sensory motor functions, immune cell functions and inflammatory properties in gut homeostasis (Banskota *et al.,* in press). As DR7 was orally administered, we postulate that DR7 may also increase the production of gut serotonin that benefited mental health along the gut-brain axis. The brain noradrenergic system is activated by increasing levels of cortisol, where post-synaptic effects of norepinephrine is triggered to induce alertness, awareness, wakefulness and attention

amid stressful conditions. Our present data showed that stressed subjects administered with placebo had higher plasma cortisol levels, accompanied by higher expressions of TH and DBH, two enzymes that convert tyrosine to dopamine and dopamine to norepinephrine, respectively (Figure 9, steps 3,5), justifying the need for an increased level of norepinephrine amid stress. Meanwhile, stressed subjects administered with DR7 showed lower stress and anxiety levels (as per DASS outcomes) and plasma cortisol levels, thus avoided the necessity for a higher level of norepinephrine (Figure 9, steps 4,6). The GABA pathway has been targeted in treatments used for anxiety disorders in human (Swanson *et al.,* 2005), while BDNF and CREB have been shown to reactivate brain plasticity amid strategies to develop antidepressant drugs (Castren and Rantamaki, 2010) and mediation of memory consolidation along neural plasticity pathways (Chen and Wu, 2013). However, our present study indicated that DR7 did not exert a stress and anxiety reducing mechanism via these pathways.

Putative probiotic strains are required to fulfil probiotic criteria and generally regarded as safe (GRAS) prior to applications and commercialisation as food and/or health ingredients. Our data showed that DR7 complied with the selection characteristics for probiotic microorganisms (FAO/WHO, 2006). In addition, DR7 was safe without toxicity effects on HepG2 cells. HepG2 cells have been widely used as a model to evaluate cytotoxicity of a wide variety of chemicals and drugs attributed to its susceptibility against DNA damage and apoptosis, and to evaluate genotoxicity attributed to its ability to express metabolising enzymes required for activation of DNA-reactive carcinogens (Carney and Settivari, 2013). In addition to these criteria, a probiotic microorganism must also exert health benefits to the hosts, and thus the importance of human clinical evidence. Our present human study showed that DR7 exerted certain brain health properties, and thus could be of great potentials for industrialisation and applications.

Taken altogether, the administration of *L. plantarum* DR7 in stressed adults have resulted in reduced symptoms of stress and anxiety, accompanied by improvement of several cognitive and memory functions, and reduced levels of plasma cortisol and pro-inflammatory cytokines. Our present study also showed that DR7 may have exerted these effects via mechanisms involving the upregulation of serotonin pathways and stabilising the pathways of dopamine along the gut-brain axis.

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Conflict of interest

All authors declare no competing financial or conflict of interest.

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