Probiotic treatment reduces depressive-like behaviour in rats independently of diet

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The gut microbiota has recently emerged as an important regulator of brain physiology and behaviour in animals, and ingestion of certain bacteria (probiotics) therefore appear to be a potential treatment for major depressive disorder (MDD). However, some conceptual and mechanistical aspects need further elucidation.

We therefore aimed at investigating whether the habitual diet may interact with the effect of probiotics on depression-related behaviour and further examined some potentially involved mechanisms underlying the microbe-mediated behavioural effects.

Forty male Sprague-Dawley rats were fed a control (CON) or high-fat diet (HFD) for ten weeks and treated with either a multi-species probiotic formulation or vehicle for the last five weeks. Independently of diet, probiotic treatment markedly reduced depressive-like behaviour in the forced swim test by 34% (95% CI: 22–44%). Furthermore, probiotic treatment skewed the cytokine production by stimulated blood mononuclear cells towards IFNγ, IL2 and IL4 at the expense of TNFα and IL6. In addition, probiotics lowered hippocampal transcript levels of factors involved in HPA axis regulation (Cnr1, Cnr2 and Mtr), whereas HFD increased these levels. A non-targeted plasma metabolomics analysis revealed that probiotics raised the level of indole-3-propionic acid, a potential neuroprotective agent.

Our findings clearly support probiotics as a potential treatment strategy in MDD. Importantly, the efficacy was not attenuated by intake of a “Western pattern” diet associated with MDD. Mechanistically, the HPA axis, immune system and microbial tryptophan metabolism could be important in this context. Importantly, our study lend inspiration to clinical trials on probiotics in depressed patients.

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1. Introduction

The emerging literature on the gut microbiota (GM) has outlined a considerable crosstalk between the residing microbes and physiological systems of the host, including brain and behaviour (Sampson and Mazmanian, 2015). This realization has led to research into the possible importance of the GM in several psychiatric disorders, including major depressive disorder (MDD) (Sampson and Mazmanian, 2015). Indeed, recent studies imply that the GM may play a role in the aetiology of MDD. Firstly, studies have identified a number of changes in GM composition in depressed patients (Jiang et al., 2015; Naseribafrouei et al., 2014). Secondly, two intriguing studies have shown that the depressive phenotype may be transferred from depressed patients to rats and mice through the GM (Kelly et al., 2016; Zheng et al., 2016).

A number of pre-clinical studies have now evaluated whether manipulations of the GM through ingestion of probiotics (defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Hill et al., 2014)) can affect behaviour in animals. Indeed, it appears that probiotics may be used to modulate a wide range of behaviours related to psychiatric disorders, including depressive-like behaviour (Bravo et al., 2011; Desbonnet et al., 2010; Liang et al., 2015; Liu et al., 2016; Savignac et al., 2014). Consequently, the term “psychobiotics” was
conceptualised by Dinan and colleagues and denotes a live organism that produces a health benefit in patients suffering from a psychiatric illness (Dinan et al., 2013).

However, several questions regarding the mechanisms underlying the ability of certain bacteria to modulate depressive-like behaviour remain unanswered. Firstly, several studies have evaluated the activity of the hypothalamic-pituitary-adrenal (HPA) axis through plasma corticosterone measurements with conflicting results (Bravo et al., 2011; Desbonnet et al., 2008; Liang et al., 2015; Liu et al., 2016; Savignac et al., 2014). Since the release of stress hormones occurs in rapid ultradian oscillations, follows a circadian rhythm and is easily affected by stress resulting from the blood sampling itself, crude plasma levels may be considered relatively insensitive in the evaluation of HPA axis regulation (Spiga et al., 2014). So far, the effect of probiotics on central HPA axis regulation by hippocampus and hypothalamus has not been studied in detail. Secondly, several studies have evaluated immunomodulatory properties of probiotics through changes in plasma cytokines (Arseneault-Breard et al., 2012; Desbonnet et al., 2008; Liang et al., 2015; Liu et al., 2016). However, the local and paracrine activity of cytokines holds a strong limitation to the functional interpretation of their plasma concentration. Thirdly, it has been shown that GM exerts a considerable influence on blood metabolite levels (Wikoff et al., 2009). Unfortunately, no comprehensive metabolomic profiling has been carried out in relation to probiotic ingestion and its effect on depressive-like behaviour.

Another issue regarding probiotic treatment may be the concurrent diet. Importantly, MDD is associated with increased rates of dysmetabolic conditions, including obesity and diabetes mellitus type II (Luppino et al., 2010; Rotella and Mannucci, 2013), and this association may be partly caused by an unhealthy diet associated with MDD (Jacka et al., 2011; Le Port et al., 2012). Interestingly, it has been shown that diet has a major impact on the GM (David et al., 2014), and particularly, a “Western pattern” high-fat low-fibre diet is associated with marked changes in GM composition and poor microbial diversity (Altenberg and Wu, 2014). However, it remains unknown whether a such diet interacts with the effects of probiotics.

In the present study, we therefore aimed at evaluating the effect of probiotic treatment on depression-related behaviour in rats on a control or high-fat diet (HFD). Furthermore, we examined three mechanisms that may be involved in MDD and in the gut microbiota-mediated effects on depression-related behaviour as well; namely, hippocampal and hypothalamic HPA axis regulation, cytokine profile of stimulated immune cells and the complete plasma metabolic profile.

2. Materials and methods

2.1. Animals and diets

Forty male Sprague-Dawley (SD) rats (NTac:SD, SPF; Taconic, Denmark) arrived at the age of four weeks and were pair-housed in Eurostandard Type III H cages (37.5 × 21.5 × 18 cm) with raised lids, a shelter and nesting material. The environment was temperature (20 °C) and light-controlled (12/12 h light/dark cycle, lights on at six AM). After arrival, the animals were randomly assigned to a control (CON) or high-fat diet (HFD) (CON cat no. E15000/HFD cat no. E15741, Ssniff, Germany). The CON diet had a fat content of 11 kJ (%soybean oil), a protein content of 23 kJ % and a carbohydrate content of 66 kJ% (mainly corn starch). HFD had a fat content 60 kJ% (mainly beef tallow), a protein content of 20 kJ % and a carbohydrate content of 20 kJ% (maltodextrin and sucrose). The diets were provided ad libitum together with tap water. All rats and diet remnants were weighed once a week. All procedures complied with the EU Directive 2010/63/EU and with the Danish law regulating experiments on animals (permission ID 2012-15-2934-00254). Study design is depicted in Fig. 1.

2.2. Probiotics

After five weeks on the diets, the rats were randomised to vehicle (VEH) or probiotic (PRO) treatment. The probiotics consisted of eight bacterial strains (B. bifidum W23, B. lactis W52, L. acidophilus W37, L. brevis W63, L. casei W56, L. salivarius W24, Lc. Lactis W19, Lc. Lactis W58; “Ecologic Barrier”, Wincolve Probiotics BV, The Netherlands) in a carrier matrix of maize starch, maltodextrins and vegetable protein. Each cage received a bottle containing 4.5 g (2.5 × 10^9 CFU/g) of freeze-dried powder dissolved in 30 mL of tap water. VEH groups were given the carrier matrix only. The bottles were administered daily between four and six pm and completely emptied by the animals during the night. Bottles were chosen over oral gavage to minimise the amount of stress inflicted on the animals. The experimenter was blinded to treatment groups.

2.3. Barnes maze

The barnes maze (BM) is a test of hippocampus-dependent spatial memory which is often impaired in patients suffering from MDD (Campbell and Macqueen, 2004). The maze consisted of a black, circular table (Ø 120 cm) with 18 perimeter holes, and one of the holes was connected to a hidden escape box. Black curtains equipped with spatial cues (simple geometric symbols) surrounded the maze. Each rat was tested twice daily with a 3-h interval for four consecutive days (eight trials in total). In each trial, the rat was allowed to spend a maximum of four minutes to locate the escape hole, otherwise it was gently moved to it by the experimenter. An aversive environment was established by bright light (800 Lux) and loud background noise (White Noise Generator Software: www.sobolsoft.com). Just as the rat entered the escape hole, the noise was turned off, and the rat was collected after a 30-s delay. To exclude any local cues, the maze was turned 120° daily, and the escape box was moved proportionally to maintain the same physical location in the room. Seven days after the last trial, a recall trial was performed. The maze was wiped with an ethanol solution between each trial. The sessions were video recorded and analysed by EthoVision XT9 software (Noldus IT, The Netherlands). Time (s) and distance moved (cm) before locating the escape hole were calculated. In the first trial, velocity (cm/s) was calculated as a measure of locomotor activity.

2.4. Forced swim test

The forced swim test (FST) is a commonly used screening tool for depressive-like behaviour in rodents. Immobility (depressive-like behaviour) is defined as the rat making no movements beyond those needed to keep its head above the water. We utilised the modified version of the test (Detke et al., 1995). Briefly, each rat was immersed in a water-filled cylinder (H: 54 cm; Ø: 24 cm; water depth: 40 cm; 24–25 °C) and allowed to swim for 15 and sevenmin, respectively, on two consecutive days. The water was changed between each trial. All swim sessions were video-recorded. The second day test trial was scored afterwards by an experimenter blinded to group assignment using the time-sampling technique, i.e. the most predominant behaviour during each 5-s time bin was registered. Time spent on active behaviours (struggling and swimming) as well as immobility was calculated.
Fig. 1. Study design and outline. The animals were four weeks old at study initiation. CON: control diet; HFD: high-fat diet; VEH: vehicle; PRO: probiotics; FST: forced swim test; OF: open field test; OGTT: oral glucose tolerance test.

2.5. Open field test

To assess locomotor activity, the rats were subjected to a 10-min open field test (OFT) immediately before the second day test trial of FST. Each animal was placed in a black 100 × 100 cm video-recorded square box. The videos were analysed by Noldus Ethovision XT9 software (Noldus IT, The Netherlands), and the distance moved by each animal (cm) was retrieved.

2.6. Oral glucose tolerance test

Following a 6-h fast, a blood sample was taken from a tail snip. Next, the rat was given a glucose solution (2.5 g glucose/kg body weight; 500 g/L) by oral gavage, and blood samples were drawn again 30, 60 and 120 min later. For each time point, blood glucose concentration was measured in duplicate by use of a OneTouch Vita glucose monitor (Lifescan, Cilag GmbH, Switzerland). For insulin measurements, an additional amount of blood was collected in heparinized capillary tubes, and the plasma was immediately frozen on dry ice. Insulin concentrations were measured with an ultra-sensitive rat insulin ELISA kit (DRG Diagnostics GmbH, Marburg, Germany).

2.7. Blood and tissue collection

Animals were given a lethal dose of pentobarbital intraperitoneally. At the absence of all reflexes (after 3–5 min), blood was collected by cardiac puncture. After decapitation, hypothalamus and hippocampus were dissected by an experienced technician and immediately frozen on powdered dry ice.

2.8. Anti-CD3/28 stimulation of PBMC

Peripheral blood (EDTA-anticoagulated) mononuclear cells (PBMC) were isolated by Ficoll-Paque 1.084 (GE Healthcare, Illinois, USA) density gradient centrifugation, washed twice in sterile PBS and finally resuspended in RPMI-1640 (BS758; Sigma-Aldrich, Missouri, USA) supplemented with 10% FBS, 100 U/mL penicillin and 10 µg/mL streptomycin (P4333; Sigma-Aldrich, Missouri, USA). A cell count was obtained from a haemocytometer, and the concentration was adjusted to 10⁶ cells/mL. Cell viability was determined by trypan blue exclusion. Two hundred thousand cells were added to each well of a U-bottom microplate that was pre-coated with anti-rat CD3 (5 µg/mL in 100 µL PBS; clone G4.18; eBioscience, California, USA). Finally, soluble anti-rat CD28 (clone 319; eBioscience, California, USA) was added to the wells at 1.5 µg/mL. After 72 h of stimulated culture, the supernatants were collected and stored at −80°C.

2.9. Measurement of cytokines

Six cytokines were measured in the PBMC culture supernatants. Interleukin 2 (IL2), IL4, IL6 and interferon gamma (IFNγ) were measured with a magnetic-bead based multiplex kit (Bio-Rad, California, USA) according to the manufacturer’s instructions and assayed on a LumineX Bio-Plex 200 system (Bio-Rad, California, USA). IL10 and tumor necrosis factor alpha (TNFα) were measured with Quantikine Elisa kits (R&D Systems, Minneapolis, USA). Absolute levels as well as relative cytokine composition were analysed.

2.10. Plasma endotoxin

Lipopolysaccharide (LPS) was measured in sterile plasma (lithium heparin-anticoagulated) with a limulus amoebocyte lysate assay (Lonza AG, Switzerland). Spiked samples were made to determine the dilution needed in order to avoid any inhibitory matrix components. Consequently, plasma samples were diluted 25 times and heated at 70°C for 15 min. The assay was then done according to manufacturer’s instructions.

2.11. RNA extraction & cDNA synthesis

PARIS kits (Thermo Fisher Sci., Massachusetts, USA) were used to extract RNA from left hippocampus and hypothalamus according to the manufacturer’s instructions. RNA concentration and purity were determined by a Nanodrop spectrophotometer (Thermo Fisher Sci., Massachusetts, USA), and the RNA concentration of each sample was adjusted to the lowest one observed (31 ng/µL (hippocampus) and 64 ng/µL (hypothalamus)). Reverse transcription PCR was performed to generate cDNA using random primers and Superscript IV Reverse Transcriptase (Thermo Fisher Sci., Massachusetts, USA) according to the manufacturer’s instructions. The resulting cDNA was stored undiluted at −80°C until quantitative real-time polymerase chain reaction (real-time qPCR) analysis.

2.12. Real-time qPCR

The expression of seven genes related to structural plasticity (Bdnf, Creb1, Ltf-1, Vegfa, Traak, Trek2, TrrkB) and six genes related to HPA axis regulation (Gr, Mr, 11β-hsd1, Crh-bp, Crh-r1, Crh-r2) was determined in hippocampus by use of real-time qPCR. In hypothalamus, only genes involved in HPA axis regulation were assessed (Gr, Mr, 11β-hsd1, Crh-r1, Crh-r2, Pomc). In addition, eight reference genes were included (ActB, Ywhaz, Hmbs, Hprt, Rpl13, CycA, 18s rRNA, Gapdh). Primers are listed in supplementary Table S1. The real-time qPCR reactions were carried out on a Mx300P system (Stratagene, California, USA) as described previously (Elving et al., 2008). Briefly, each well of a 96-well microplate was loaded with SYBR green master–mix (Bio-Rad, California, USA), 0.5 µM of primer pairs and 3 µl of diluted cDNA (hippocampus 1:11; hypothalamus 1:20) (10 µl in total). A standard curve in duplicate was made on each plate, and all samples were also run in duplicate. Individual sample genes were normalised to the geometric mean of the two most stable reference genes which were selected by Normfinder software (hippocampus: CycA/Rpl13; hypothalamus Actb/18s rRNA) (Bonefeld et al., 2008).
2.13. Metabolomics

A commercially available plasma metabolomic analysis was performed by Metabolon Inc. (North Carolina, USA) to identify metabolites associated with probiotic treatment. An untargeted analysis was chosen in order to take advantage of the hypothesis-generating potential of the method. Ultrahigh performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) was used. Briefly, proteins were precipitated with methanol, and each sample was analysed on four platforms: two separate reverse phase (RP)/UPLC–MS/MS with positive ion mode electrospray ionization (ESI), an RP/UPLC–MS/MS with negative ion mode ESI and a HILIC/UPLC–MS/MS with negative ion mode ESI. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. Metabolites that were only detected in less than 75% of the animals were excluded.

2.14. Statistical analyses

A significance level of $\alpha = 0.05$ was used in all analyses. Group sizes were chosen on the basis of a power calculation for FST ($\beta = 0.2$). Normality was assessed by QQ plots, and Bartlett’s test was used to test for equal variances. Struggling time in FST and real-time qPCR data were power-transformed, whereas LPS and cytokine levels as well as data from BM and OGTT were log-transformed before analysis. Trek2 was analysed using Wilcoxon rank-sum test. Two-way ANOVA was used in analysis of body weight, fasting glucose/insulin, FST, OF, LPS, BM recall trial and metabolomics with diet and probiotic treatment as independent factors. Significant interactions were followed up by use of pair-wise contrasts with Bonferroni-corrected $p$-values. Metabolomics data were also analysed by use of a principal component analysis (PCA) to detect major changes in analyte composition. In analysis of BM, OGTT and cytokines, a linear mixed model analysis was conducted with rat identity as the random effect (intercept). Fixed effects included day and session (BM), time (OGTT) or analyte (cytokines) and their interactions with diet, treatment and diet × treatment. REML estimation was used, and residuals were considered independent by trial (BM), time (glucose in OGTT) or analyte (cytokines). Contrasts of marginal linear predictions were used to test for differences between groups after the models were built. False discovery rate (FDR) correction (Benjamini and Hochberg (Benjamini and Hochberg, 1995)) was applied to real-time qPCR data at a FDR level of 0.10. For metabolomics, a less conservative FDR level of 0.20 was applied because of the hypothesis-generating purpose of the analysis. All analyses were performed with Stata 14 (StataCorp LP, Texas, USA).

3. Results

3.1. Body weight & caloric intake

Body weights at study initiation were similar between groups (supplementary Table S2). As expected, HFD led to a marked increase in total caloric intake ($F_{1,36} = 31.7; p < 0.001$) and in body weight at study end ($F_{1,36} = 18.8; p < 0.001$). Probiotic treatment did not affect these measures.

3.2. Forced swim test

Probiotic treatment had a clear antidepressant-like effect (Fig. 2). Namely, the treatment reduced immobility time by 34% (95% CI: 22–44%; $F_{1,36} = 24.1; p = 0.001$), and the effect was completely independent of diet ($F_{1,36} = 0.02; p = 0.9$; diet × treatment: $F_{1,36} = 0.15; p = 0.7$). An antidepressant-like effect under both dietary conditions was further confirmed by Bonferroni-adjusted post-hoc comparisons (CON-VEH vs CON-PRO, $p = 0.006$; HFD-VEH vs HFD-PRO, $p = 0.001$). Correspondingly, probiotics increased the duration of swimming ($F_{1,36} = 10.8; p = 0.002$), whereas struggling remained largely unaffected ($F_{1,36} = 2.64; p = 0.1$).

3.3. Open field test

No differences between groups were observed in distance travelled during the 10-min open field session (diet: $F_{1,36} = 0.81; p = 0.4$; probiotics: $F_{1,36} = 0.18; p = 0.7$; diet × treatment: $F_{1,36} = 0.15; p = 0.7$).

3.4. Barnes maze

Data are presented in supplementary Fig. S3. Across the four days, a clear improvement was seen in distance travelled before locating the escape hole (day: $\chi^2(3) = 307; p < 0.001$) and also in time spent to complete the trial (day: $\chi^2(3) = 557; p < 0.001$). In addition, an advance was evident between the two sessions within the first and the second day in analysis of distance travelled (day × session: $\chi^2(3) = 9.38; p = 0.02$) as well as in time to complete (day × session: $\chi^2(3) = 21.4; p < 0.001$).

Interestingly, HFD was found to improve time to complete the trials (diet × day: $\chi^2(3) = 8.2; p = 0.04$). Specifically, HFD led to a faster completion on day 4 only ($p = 0.02$). Diet did, however, not affect distance travelled (diet × day: $\chi^2(3) = 5.24; p = 0.2$). No effect of probiotic treatment was observed (treatment × day: $\chi^2(3) = 0.84; p = 0.8$ (time)/$\chi^2(3) = 0.53; p = 0.9$ (distance)).

In the recall-session 7 days later, HFD lowered the distance travelled before locating the escape hole ($F_{1,36} = 4.64; p = 0.04$), whereas a significant diet × treatment interaction was seen for time to com-
complete ($F_{1,36} = 0.20; p < 0.05$). Here, post-hoc comparisons revealed that HFD led to faster completion of the trial in VEH treated animals only ($p = 0.03$).

As regards locomotor activity, no differences in velocity or distance travelled were observed in the first trial (data not presented).

### 3.5. Oral glucose tolerance test

Baseline fasting levels of blood glucose and plasma insulin were not affected by diet or probiotic treatment, although HFD strongly tended to increase insulin level ($F_{1,33} = 3.9; p = 0.06$). During the OGTT, glucose levels rose to a higher level in HFD rats than in CON rats ($time \times diet: \chi^2(1) = 7.48; p = 0.006$) (Fig. 3a). Over-all, HFD-fed rats also had higher insulin levels that CON rats ($\chi^2(1) = 5.83; p = 0.02$), although diet did not interact with time course ($time \times diet: \chi^2(1) = 0.02; p = 0.9$) (Fig. 3b). Probiotic-treated animals had lower glucose and insulin levels during the test, but the improvements were not statistically significant (glucose: treatment: $\chi^2(1) = 0.03; p = 0.9$; time \times treatment: $\chi^2(1) = 1.33; p = 0.2$) (insulin: treatment: $\chi^2(1) = 0.93; p = 0.3$; time \times treatment: $\chi^2(1) = 0.14; p = 0.7$).

### 3.6. PBMC cytokine production

The absolute cytokine levels in the supernatant of anti-CD3/28 stimulated PBMCs are shown in Fig. 4a. Over-all, probiotic treatment affected the cytokine levels (treatment \times analyte: $\chi^2(5) = 25.38; p < 0.001$), whereas diet had no such effect (diet \times analyte: $\chi^2(5) = 6.67; p = 0.24$). Specifically, probiotics increased the level of IL2, IL4 and IFN; yet, the treatment did not affect the total amount of cytokines in general ($\chi^2(1) = 2.07; p = 0.15$). In analysis of each cytokine in percent of the total amount produced, an overall effect of probiotics was observed again (treatment \times analyte: $\chi^2(5) = 25.68; p < 0.001$). Namely, probiotics skewed the production towards IL2, IL4 and IFN at the expense of the highly pro-inflammatory cytokines IL6 and TNF$\alpha$ (Fig. 4b). Again, no effect of diet was observed (diet \times analyte: $\chi^2(5) = 8.37; p = 0.14$).
3.7. Real-time qPCR

The real-time qPCR data are presented in Table 1. In hippocampus, HFD increased the transcript levels of the HPA axis regulating genes Mr (F1,36 = 7.29; p = 0.01), Crh-r1 (F1,36 = 8.18; p = 0.007), Crh-r2 (F1,36 = 4.36; p = 0.04), 11β-hsd1 (F1,36 = 5.79; p = 0.02), whereas probiotics lowered the expression of these genes (Mr: F1,36 = 5.93; p = 0.02; Crh-r1: F1,36 = 10.6; p = 0.003; Crh-r2: F1,36 = 11.6; p = 0.002), with the exception of 11β-hsd1 (F1,36 = 2.05; p = 0.16). The hippocampal expression of Bdnf (F1,36 = 17.0; p < 0.001), Trek2 (z = 3.30; p = 0.001) and Traak (F1,36 = 5.02; p = 0.03), which are related to structural plasticity and neuroprotection, was lowered by HFD, but probiotics oppositely increased the level of Trek2 (z = 2.33; p = 0.02) and Traak (F1,36 = 5.65; p = 0.02). In hypothalamus, only one change was observed. Namely, HFD increased the level of Crh-r2 (F1,36 = 10.3; p = 0.003). Two significant diet × treatment interactions did not pass the FDR correction (Traak in hippocampus (F1,36 = 5.62; p = 0.02) and Pomp in hypothalamus (F1,36 = 5.05; p = 0.03)).

3.8. Plasma endotoxin

HFD led to an increased plasma LPS level (F1,36 = 8.83; p = 0.005), whereas probiotics did not affect the concentration (F1,36 = 0.4; p = 0.5) (Fig. 3c).

3.9. Metabolomics

In total, 455 plasma metabolites were detected in at least 30 animals. Thirteen metabolites were found to be affected by probiotic treatment, and three of these changes remained significantly different after FDR correction (Table 2). Interestingly, two microbial tryptophan metabolites (indole-propionic acid and indole-acrylic acid) were upregulated by probiotics (F1,36 = 16.4; p < 0.001 and F1,36 = 13.5; p < 0.001, respectively) in addition to a metabolite related to amino acid metabolism; namely, acetylornithine (urea cycle metabolite) (F1,36 = 143; p < 0.0001). However, no further changes in tryptophan or tryptophan catabolites (kynurenine pathway metabolites) were seen in animals on probiotics (Table 2), nor was any clear distinction between VEH and PRO groups evident in the PCA (supplementary Fig. S5). On the other hand, HFD was associated with marked changes in the plasma metabolome (supplementary Fig. S5). Indeed, HFD affected the level of 209 metabolites (data not presented), including a lower plasma tryptophan level (F1,36 = 4.18; p < 0.05) and higher levels of the tryptophan catabolite quinolinic acid (F1,36 = 31.5; p < 0.0001) (Table 2). No diet × treatment interactions were found.

4. Discussion

The main finding of the present study is that probiotic treatment markedly reduced the level of depressive-like behaviour independently of diet. In addition, we identified corresponding changes in three physiological systems that could potentially mediate the observed antidepressant-like effect. Firstly, pronounced shifts in the pattern of produced cytokines were observed. Secondly, changes in the hippocampal expression of genes related to HPA axis regulation were evident; and finally, the concentration of a number of plasma metabolites were altered.

To our knowledge, we are the first to show a reduction in depressive-like behaviour with probiotics in healthy rats, and clearly, these findings are in accord with the novel concept of psychobiotics (Dinan et al., 2013). Previous pre-clinical studies have also reported that probiotic ingestion may lead to a reduction in depressive-like behaviour in healthy mice (Bravo et al., 2011; Savignac et al., 2014), and in rats, probiotics have been shown to ameliorate stress-induced depressive-like behaviour (Arseneault-Bréard et al., 2012; Desbonnet et al., 2010; Liang et al., 2015). Whereas each of these previous studies has utilised treatment with only one or two bacterial strains, we used a multi-species composition of eight different strains. Importantly, certain studies suggest that such a combination of several species may hold an additive effect (Chapman et al., 2011), and this notion may partially explain the unequivocally antidepressant-like effect in our non-stressed rats. Although a lot of pre-clinical evidence now supports a role for probiotics in the treatment of depression, there remains a paucity of clinical trials with depressed patients. Our present finding that
HFD did not interact with the antidepressant-like effect of probiotics only further expands the potential group of amenable patients. Encouragingly, a couple of trials on probiotic treatment in healthy volunteers show improvements on depression-related rating scales (Messauodi et al., 2011; Steenbergen et al., 2015).

The cytokine pattern was altered as a consequence of probiotic treatment. Specifically, we found a distributional shift towards production of the T lymphocyte-derived cytokines IL2, IL4 and IFNγ and away from production of cytokines primarily associated with macrophages, namely TNFα and IL6. Interestingly, the observed immunomodulatory properties of probiotics appeared to be independent of the highly pro-inflammatory LPS. In several meta-analyses, the levels of macrophage-derived plasma cytokines, including TNFα and IL6, are consistently found to be modestly increased in depressed patients (Dowlati et al., 2010). On the other hand, a meta-analysis concluded that T cell activity is generally compromised in MDD (Zorrilla et al., 2001). Importantly, certain T cell subsets have recently been found to support the structural plasticity of the CNS and to play a pivotal role in normal brain functioning of mice (Kipnis, 2016). Collectively, MDD may therefore be associated with an overactivity of the innate arm of the immune system to the detriment of the adaptive arm. Interestingly, our present cytokine findings may reflect that probiotics shifted the balance towards the adaptive arm. Furthermore, our data are substantiated by the use of standardised ex vivo measures, i.e. stimulated PBMC cultures. Although most similar studies report crude plasma cytokine levels (Arseneault-Beard et al., 2012; Desbonnet et al., 2008; Liang et al., 2015; Liu et al., 2016), such measurements remain challenging due to the low levels found in peripheral blood; furthermore, plasma cytokines are prone to variations caused by e.g. sample collection issues, diurnal rhythm and diet (Zhou et al., 2010). In stimulated cultures, the concentration of cytokines is higher than in plasma, and no environmental factors affect the production. We therefore believe that our method eases the measurement of cytokines and enhances their interpretative value. Nevertheless, further studies are needed to evaluate a causal link between the cytokine pattern and the behavioural changes.

Probiotic treatment dramatically altered the expression of several hippocampal genes related to HPA axis feedback. Clearly, our results are consistent with earlier studies that demonstrate an important interplay between the gut microbiota and HPA axis (Desbonnet et al., 2008; Sudo et al., 2004). Interestingly, a study in healthy human volunteers reported a reduction in urinary cortisol after 30 days of probiotic treatment (Messauodi et al., 2011). Our data therefore further justify the microbiota as a substantial regulator of the neuroendocrine stress axis at the level of the CNS. Momentously, it is well-established that a large proportion of depressed patients have a dysregulated HPA axis, and hippocampus is believed to play an important role in the negative feedback mechanism (Pariente and Lightman, 2008). Noticeably, the hippocampal transcript levels of Crh-r1 and Crh-r2 were found to be markedly lowered by probiotics in our study. Importantly, increased CRH-R1 signalling has been implicated in stress and depression (Dale et al., 2015), and receptor antagonists were shown to possess antidepressant-like effect in animals. Although clinical studies with such antagonists have been conflicting (Dale et al., 2015), the altered expression may still be of pathophysiological importance. Nevertheless, our findings may serve to corroborate the observed antidepressant-like behavioural effect of the treatment.

HFD also changed the expression of several genes in hippocampus. Intriguingly, HFD and probiotics had oppositely directed effects on all affected genes, and HFD thus increased the mRNA level of several factors involved in HPA axis regulation. Previous studies have shown that chronic HFD consumption may lead to increased basal and stress-induced plasma corticosterone levels (Abildgaard et al., 2014; Tammenga et al., 1997), and it is possible that this observation also involves the gut microbiota. Namely, fatty acids generally possess antimicrobial effects (Kabara et al., 1972), and HFD is known to reduce gut microbiota diversity in mice (Hildebrandt et al., 2009). Similarly, a recent study that utilised antibiotic treatment to induce gut microbial depletion found a consequent increase in depressive-like behaviour associated with altered hippocampal Gr and Crh-r1 transcript levels (Hoban et al., 2016). We also detected lower Bdnf, Trafak and Trek2 mRNA levels in animals on HFD. The latter two have been associated with protection against glutamatergic excitotoxicity (Lauritzen et al., 2000), a phenomenon that has been associated with depression. Although these marked diet-induced changes in gene expressions were not

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**Table 2**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CON-VEH</th>
<th>CON-PRO</th>
<th>HFD-VEH</th>
<th>HFD-PRO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-delta-acetylornithine</td>
<td>0.69 ± 0.05</td>
<td><strong>2.00 ± 0.22 P</strong></td>
<td>0.62 ± 0.05 P</td>
<td><strong>1.45 ± 0.10 P</strong></td>
</tr>
<tr>
<td>Indolepropionate</td>
<td>0.85 ± 0.12</td>
<td><strong>1.34 ± 0.22 P</strong></td>
<td>0.67 ± 0.09</td>
<td><strong>1.37 ± 0.14 P</strong></td>
</tr>
<tr>
<td>Indoleacrylate</td>
<td>0.92 ± 0.12</td>
<td><strong>1.43 ± 0.24 P</strong></td>
<td>0.86 ± 0.08</td>
<td><strong>1.66 ± 0.14 P</strong></td>
</tr>
<tr>
<td>Phenylactate (PLA)</td>
<td>0.80 ± 0.08</td>
<td><strong>1.38 ± 0.19</strong></td>
<td>0.87 ± 0.13</td>
<td>1.18 ± 0.12</td>
</tr>
<tr>
<td>Caprate (10:0)</td>
<td>1.01 ± 0.06</td>
<td>1.16 ± 0.07</td>
<td>0.85 ± 0.03</td>
<td>1.11 ± 0.09</td>
</tr>
<tr>
<td>N-methylproline</td>
<td>1.10 ± 0.11</td>
<td><strong>1.31 ± 0.12</strong></td>
<td>0.82 ± 0.05 P</td>
<td><strong>1.14 ± 0.11 D</strong></td>
</tr>
<tr>
<td>Trigonelline (N-methylnicotinate)</td>
<td>1.21 ± 0.20</td>
<td><strong>2.33 ± 0.72</strong></td>
<td>0.75 ± 0.09 D</td>
<td><strong>1.00 ± 0.17 D</strong></td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>0.81 ± 0.12</td>
<td>0.95 ± 0.09</td>
<td><strong>0.96 ± 0.14 D</strong></td>
<td><strong>1.49 ± 0.21 D</strong></td>
</tr>
<tr>
<td>4-Ethylphenylsulfate</td>
<td>1.32 ± 0.17</td>
<td><strong>0.96 ± 0.10</strong></td>
<td>1.13 ± 0.13</td>
<td>0.85 ± 0.11</td>
</tr>
<tr>
<td>N6-carboxymethyllysine</td>
<td>0.98 ± 0.08</td>
<td>1.32 ± 0.15</td>
<td>0.97 ± 0.09</td>
<td>1.14 ± 0.11</td>
</tr>
<tr>
<td>Carnosine</td>
<td>1.54 ± 0.25</td>
<td>0.97 ± 0.08</td>
<td><strong>1.32 ± 0.30 D</strong></td>
<td>0.91 ± 0.14</td>
</tr>
<tr>
<td>Dihomo-γ-linolenate</td>
<td>1.35 ± 0.15</td>
<td>1.16 ± 0.15</td>
<td><strong>1.01 ± 0.13 D</strong></td>
<td><strong>0.71 ± 0.07 D</strong></td>
</tr>
<tr>
<td>N-acetylproline</td>
<td>0.87 ± 0.09</td>
<td>1.51 ± 0.27</td>
<td>0.88 ± 0.09</td>
<td>1.06 ± 0.11</td>
</tr>
</tbody>
</table>

Bold values are significantly affected by diet or probiotics (also noted with Ds and Ps). Mean ± SEM (arbitrary units). In total, 455 plasma metabolites were identified in at least 30 animals. In the upper part of the table, the 13 metabolites affected by probiotic treatment before FDR correction are presented. In the lower part, metabolites of the kynurenine pathway are presented. Data were analysed with 2-way ANOVA. No diet × probiotics interaction was identified for any metabolite. P Significant effect of probiotics after FDR correction. D Significant effect of diet after FDR correction. CON: control diet; HFD: high-fat diet; VEH: vehicle; PRO: probiotics.
The plasma metabolomic profile was only modestly affected by probiotic treatment. Tryptophan and its metabolites, including kynurenic pathway metabolites, are believed to play an important role in MDD, and two microbial tryptophan metabolites were found to be upregulated by probiotics. One of these, IPA, has been shown to possess neuroprotective properties and restrain CNS inflammation (Hwang et al., 2009; Rothhammer et al., 2016). In addition, a recent study demonstrated that IPA improved intestinal barrier function via the gut endothelial PXR receptor and, in turn, downregulated Toll-like receptor 4 (TLR4) signalling (Venkatesh et al., 2014). Thus, IPA could potentially mediate some of the immunomodulatory effects of the probiotics. Another important metabolite, 4-EPS, was lowered by probiotics in our study although the change did not pass FDR correction. Interestingly, 4-EPS was recently found to be responsible for some of the aberrant behaviours associated with an autism-like phenotype in mice, and the level of 4-EPS was similarly reduced by probiotic treatment (Hsiao et al., 2013). Our results therefore urge for intensified research into these metabolites and their potential role in MDD.

HFD markedly affected the plasma metabolome, but no interactions between diet and probiotic treatment were seen. Interestingly, HFD increased the level of plasma quinolinic acid, and this finding is in line with a previous study in SD rats and is likely caused by an increased need for NAD for fatty acid metabolism (Shibata and Murata, 1985). Interestingly, it has been hypothesised that microglia-derived quinolinic acid may hold a neurotoxic effect in MDD (Reus et al., 2015). However, since quinolinic acid does not pass the blood–brain-barrier (Fukui et al., 1991), further studies are needed to evaluate any cerebral implications of the altered peripheral tryptophan metabolism. Importantly, HFD did not affect depressive-like behaviour.

High-fat diet led to a clear metabolic stress as indicated by increased body weight, caloric intake, glucose and insulin levels. Although probiotics have been reported to improve metabolic parameters (Razmpoo et al., 2016), these measures were not significantly affected in the present study, and this may be due to differences in probiotic species. Furthermore, no reductions in plasma LPS by probiotics were detected in our animals. Yet, LPS has been suggested to activate especially monocytes and induce depressive-like symptoms (Reichenberg et al., 2001). Unexpectedly, we found that HFD improved memory in the BM, and this is in obvious conflict with previous literature. The diet-induced improvement was, however, only seen on day 4 and in the recall trial. Since an aversive environment was utilised to expedite completion of the trials, an alternative explanation could be reduced fear extinction in rats on HFD. Interestingly, a recent study suggested that increased CRH signalling may lead to impaired extinction (Abiri et al., 2014), but more research is needed to conclude on this topic.

A certain limitation of our study is related to the age of the rats since only young adult rats were included. It is well-described that the gut microbiota changes with age (Claesson et al., 2011), and the effect of probiotics may thus be different in older rats. Furthermore, we examined the effect of probiotics in SD rats and not in a depression-related disease model. However, clinically efficient antidepressants traditionally exert an effect in the FST in non-diseased laboratory rats (Detke et al., 1995), and it may be hypothesised that the effect size would be even larger in a disease model owing to a reduced floor effect.

In conclusion, the multi-species probiotics used in the present study clearly lowered depressive-like behaviour, and HFD did not affect the effectiveness of the treatment. The latter point is of great importance given the high metabolic comorbidity in depression, and it further strengthens the generalisability of our findings. On the molecular level, probiotics were shown to interact with pathophysiological mechanisms believed to play an important role in MDD, including the immune system, hippocampal HPA axis regulation and microbial tryptophan metabolism. Our study therefore confirms probiotics as a promising candidate for the treatment of depression. Now we just need the clinical trials.

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Contributors

All authors contributed to formation of study concept/design and selection of methods. AA and BE contributed to acquisition of data. AA performed data analysis and drafted the manuscript. All authors critically reviewed content and approved the final manuscript.

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Appendix A. Supplementary data

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References


