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Dietary tryptophan depletion alters the faecal bacterial community structure of compulsive drinker rats in schedule-induced polydipsia

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Abstract

Rationale: Compulsive behaviour, present in different psychiatric disorders such as obsessive-compulsive disorder, schizophrenia and drug abuse, is associated with altered levels of serotonin (5-hydroxytryptamine, 5-HT). The gut microbiota regulates tryptophan (TRP) metabolism and may affect global 5-H synthesis in the enteric and central nervous systems, suggesting a possible involvement of gut microbiota in compulsive spectrum disorders.

Objectives: The present study investigated whether chronic TRP depletion by diet alters the faecal bacterial community profiles of compulsive *versus* non-compulsive rats in schedule-induced polydipsia (SIP). Peripheral plasma 5-HT and brain-derived neurotrophic factor (BDNF) levels were evaluated.

Methods: Wistar rats were selected as High Drinkers (HD) or Low Drinkers (LD) according to their SIP behaviour and were fed for 14 days with either a TRP-free diet (T-) or a TRP-supplemented diet (T+). The faecal bacterial community structure was investigated with 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis.

Results: Compulsive HD rats showed a lower bacterial diversity than LD rats, irrespectively of the diet. The TRP-depleted HD rats, the only group increasing compulsive licking in SIP, showed a reduction of bacterial evenness and a highly functionally organized community compared with the other groups, indicating that this bacterial community is more fragile to external changes due to the dominance of a low number of species. The chronic TRP depletion by diet effectively reduced peripheral plasma 5-HT levels in both HD and LD rats, while plasma BDNF levels were not altered.

Conclusions: These results highlight the possible implication of reduced microbial diversity in compulsive behaviour and the involvement of the serotonergic system in modulating the gut brain-axis in compulsive spectrum disorders.

Keywords

Compulsivity; Schedule-induced Polydipsia; Gut microbiota; Chronic Tryptophan depletion; PCR-DGGE; Brain-derived neurotrophic factor;

1. Introduction

Compulsivity can be defined as actions inappropriate to the situation which persist, have no obvious relationship to the overall goal, and which often result in undesirable consequences (Dalley et al. 2011). The presence of this symptom is characteristic of various psychiatric disorders, such as obsessive-compulsive disorder (OCD), body dysmorphic disorder, hoarding disorder, hair-pulling disorder, and skin-picking disorder, which comprise the Obsessive Compulsive and Related Disorders cluster in the *Diagnostic and Statistical Manual of Mental Disorders*, 5th Edition (American Psychiatric Association, 2013). Among the potential mechanisms underlying compulsive behaviours, pharmacological treatments in clinical and pre-clinical observations in OCD suggest the involvement of serotonergic dysfunctions (Goddard et al. 2008; Derksen et al. 2020).

Emerging data implicates the gut microbiota (GM) in the regulation of brain function, behaviour and mental health by immune, endocrine and neural pathways of the *brain-gut axis*, which is the bi-directional system of communication between the central nervous system and the gastrointestinal tract (Cryan et al. 2019; Foster et al. 2016). In particular, the GM regulates tryptophan (TRP) metabolism and may affect global serotonin (5-hydroxytryptamine, 5-HT) synthesis in the enteric and central nervous systems, pointing toward the GM as a therapeutic target for serotonin-related brain-gut axis disorders (O'Mahony et al. 2015; Stasi et al. 2019). In fact, central serotonin production represents just 5% of total serotonin synthesis, with the vast majority of serotonin made in the periphery (Gheorghe et al. 2019). Specific bacterial strains can produce serotonin from tryptophan (Ozogul 2004; Ozogul et al. 2012; Shishov et al. 2009) and are susceptible to the effects of serotonergic drugs administered to the host such as selective serotonin reuptake inhibitors (SSRIs) (Munoz-Bellido et al. 2000). Recent evidence from germ-free (GF) animals found that these animals had increased plasma TRP (Clarke et al. 2013) and hippocampal and striatal 5-HT (Clarke et al. 2013; Diaz-Heijtz et al. 2011), and decreased plasma 5-HT levels (Wikoff et al. 2009). When these animals were colonised, circulating levels of TRP decreased to control levels (Clarke et al. 2013). Moreover, these GF animals also displayed a reduction in central brain-derived neurotrophic factor (BDNF), a neurotrophin that is involved in the synaptic plasticity. Reduced peripheral BDNF levels have been linked to the pathogenesis of several neuropsychiatric disorders such as major depressive disorder (Sen et al. 2008),

schizophrenia (Ikeda et al. 2008), bipolar disorder (Machado-Vieira et al. 2007), eating disorders (Nakazato et al. 2003) or autism (Hashimoto et al. 2006). Moreover, a meta-analysis found that the decreased BDNF levels observed in patients with anxiety disorders were mostly due to the effects found in those suffering from OCD (Suliman et al. 2013). Therefore, peripheral BDNF levels have been proposed as a biomarker for OCD (Hall et al. 2003; Hemmings et al. 2008; Katerberg et al. 2009; Suliman et al. 2013) and could be modulated by altered GM in this population.

Several researchers have hypothesized possible GM alterations in OCD patients based on the following observations (Bastiaanssen et al. 2018; Rees 2014; Turna et al. 2016). First, it has been noted that many of the risk factors for the onset of OCD are also known to disrupt the GM including stress, pregnancy, and antibiotic use (Rees 2014). Second, there is preclinical evidence that compulsive behaviour in rodents (frequently based on performance on the marble burying test) may be reduced by probiotic treatments (Kantak et al. 2014; Savignac et al. 2014), and exposure to non-sterile environments in GF animals (Nishino et al. 2013). To date, only one pilot study has shown that medication-free OCD patients present lower species diversity and evenness (Turna et al. 2020). Still, little research exits on examining the implication of GM in OCD (Cryan et al. 2019).

Schedule-induced polydipsia (SIP), a phenomenon characterized by the development of excessive drinking in food-restricted animals exposed to intermittent food reinforcement schedules (Falk 1961, 1971), has been proposed as a useful model to study neuropsychiatric disorders distinguished by the presence of compulsive behaviour (Flores et al. 2014; Ford 2014; Gilpin et al. 2008; Hawken et al. 2011; Hawken and Beninger 2014; Merchán et al. 2019; Moreno and Flores 2012). Important differences among individual subjects in the amount of fluid intake and licks support the differentiation of two phenotypes of rats, one with high or excessive drinking (High Drinkers-HD), and a second group with low or no SIP acquisition (Low Drinkers- LD) (López-Grancha et al. 2008). Previous SIP studies have found alterations of the serotonergic system in HD rats, such as increased 5-HT levels in the medial prefrontal cortex and amygdala (Mora et al. 2018; Moreno et al. 2012) and reduced 5-HT_{2A} receptor binding in the frontal cortex, compared with LD rats (Mora et al. 2018). Therefore, the HD rats in SIP represent a suitable model of compulsivity for studying serotonergic vulnerabilities.

The biology of the serotonergic system is often studied by using protocols that deplete the precursor TRP. The dietary manipulation of TRP is a non-invasive and naturalistic method that is able to reduce the 5-HT synthesis, content (Gessa et al. 1974) and release (Stancampiano et al. 1997a, b). In albino rats, acute TRP depletion (ATD) has been shown to produce a moderate serotonergic reduction (Brown et al. 1998; Lieben et al. 2004), while chronic TRP depletions had stronger effects, reducing brain 5-HT levels to 35-40% at 14 days (Fadda et al. 2000) and to 75% at 5-week exposures (Vergnes and Kempf 1981). A recent study found that HD rats increased compulsive drinking after chronic dietary TRP depletion (Merchán et al. 2017), and this increment was accompanied by a reduction in striatal 5-HT_{2A} receptors. However, little is known about the consequences of TRP depletion for serotonergic functions outside the central nervous system, particularly in the gut microbiota.

We propose that compulsive HD rats will be more vulnerable to GM alterations following chronic dietary TRP depletion, and we will expect to find differences in GM between non-depleted HD and LD rats in SIP. To test this hypothesis, we studied the faecal bacterial community profiles of HD and LD rats in SIP, fed with either a TRP-free diet (T−) or a TRP supplemented diet (T+), by 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis, and expressed by Shannon-Weaver diversity index, evenness index, functional diversity (Fo) and clustering analyses. Additionally, plasma 5-HT and BDNF levels were measured in depleted and non-depleted HD and LD rats.

2. Methods

2.1. Subjects

Twenty-eight adult male Wistar rats from Harlan Iberica (Barcelona, Spain), weighing approximately 300-400 grams at the beginning of the experiment, were housed three/cage or two/cage (57×35×20 cm) at 22ºC with a 12 hours light-dark cycle (lights on from 8pm to 8am), with food and water available ad libitum. The animals were gradually reduced to 80%-85% of their free-feeding body weight by controlled feeding and then maintained at this level of food-restriction throughout the experiment. Approximately 30 minutes after each experimental session, a fixed-amount of food was made available by daily feeding of lab chow. Water was always available in the home cages.

These animals were the same as those used in a previous study (Merchán et al, 2017). Rats were classified as High Drinkers and Low Drinkers according to their total number of licks in the previous experimental SIP. The rats were divided as follows: High Drinkers receiving a TRP-free diet (HD T-, *n*=7), High Drinkers receiving a control diet (HD T+, *n*=7), Low Drinkers with a TRP-free diet (LD T-, *n*=7) and Low Drinkers with a control diet (LD T+, *n*=7). Once the animals had begun the specific diets, they were housed in cages individually (50x25x18 cm) to prevent the ingestion of faecal samples from other animals.

All procedures were conducted in accordance with the Spanish Royal Decree 53/2013 on the protection of experimental animals, the European Community Directives for animal experiments (2010/63/EU) and approval was obtained from the University of Almería Animal Research Committee.

2.2. Schedule-induced polydipsia

2.2.1. Apparatus. We conducted the tests in ten standard operant-conditioning chambers (MED Associates, Inc., Cibertec, Madrid, Spain) that were 32-cm long ×25-cm wide ×34-cm high, with stainless-steel grid floors. A detailed description of the apparatus has been provided previously for the SIP (López-Grancha et al. 2008; Moreno et al. 2012). The scheduling and recording of experimental events were controlled by a Med PC computer and commercial software (Cibertec SA, Madrid, Spain).

2.2.2. Behavioural Procedure. First, rats were habituated to the test chambers for 60 min and were given 30 food pellets placed in the food magazine in one day session. Following the habituation session, the animals were daily exposed to a fixed-time 60s (FT-60s) schedule of food pellet presentation throughout 60 min sessions (from 9am to 12pm). Water bottles with fresh water were available. After 20 sessions, the average total licks for each animal were calculated based on the last three SIP sessions. Rats were classified as high drinkers (HD) and low drinkers (LD) if their average total licks were above or below the group median (which were 1017 licks), respectively. After 14 days of exposure to a TRP-free diet, the animals were again subjected to a FT-60s schedule of food pellet presentations for 6 consecutive 60-min sessions. Rats consumed conventional sucrose-flavoured pellets in all SIP sessions (60 pellets per session; TSE systems, Germany). The following measures were shown in the present study: (a) total number of

licks and (b) total number of magazine entries. A full description of the SIP results was reported in Merchán et al. (2017).

2.3. Tryptophan depletion diet

The TRP-free diet (TD08126, Harlan Laboratories S.A., Barcelona, Spain) has a standard nutritional value, but with a complete lack of TRP (T- groups). The control groups (T+ groups) were fed a similar diet containing a standard amount of TRP (1.8 g/Kg diet) (TD99366, Harlan Laboratories S.A., Barcelona Spain). The rats received chronic exposure to a TRP-free diet (T- groups) or a control diet (T+ groups) for 14 days before the behavioural task, in accordance with previous studies (Bortolato et al. 2008; Franklin et al. 2012; Stancampiano et al. 2013), and the diets were maintained until euthanasia. Therefore, rats received their diets for a total of 40 days.

2.4. Faeces–DNA extraction and PCR–DGGE analysis

Rats were rapidly euthanized by decapitation 20 days after the last SIP post-treatment session to prevent the effects of high fluid intake in the faecal bacterial community structure. Faecal samples were collected from the large intestine and stored at -80ºC. The total DNA was extracted from 250 mg faeces by the bead-beating method, following the manufacturer's instructions (MoBio UltraClean Soil DNA Isolation kit, MoBio Laboratories Inc., Solana Beach, CA, USA). For the analysis of the bacterial community, the amplification of the variable region V3–V5 of 16S rRNA was carried out using the primers 341F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTGAGTTT). The primer 341F had at its 5´ end an additional 40-nucleotide GC-rich tail (5´-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3´) (Muyzer et al. 1993). The total reaction mixture of the PCR consisted of 25 µL with the following ingredients: 3 µl of extracted DNA, (0.75µL) 1 mM primer 341F-GC, (0.75µL) 1 mM primer 907R, 4.5 µl AptaTaq Fast PCR (Roche, Mannheim, Germany) and sterile Milli-Q water to a final volume. The PCR was conducted as follows: 7-min initial denaturation of DNA at 94 °C, followed by 35 cycles of 0.45-min denaturation at 94 °C, 0.45-min annealing at 49 °C, and 1.50-min extension at 72 °C. Amplification was completed by a final extension step at 72 °C for 30 min. PCR products were first visualized in a 1.5% (w/v) agarose gel in TBE 1× buffer by ethidium bromide staining and then purified using filters Diffinity Rapid Tip (Sigma-Aldrich, St. Louis, MO, USA). The DNA samples were

checked for concentration and quality using the NanoDrop1 ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). DGGE of the amplified 16S rRNA gene sequences was carried out using the Dcode System (Universal Mutation Detection System, Bio-Rad Laboratories Inc., Hercules, CA, USA). DGGE analyses were conducted using 30 µl (300 ng) of PCR product loaded into a 40–70% urea-formamide–polyacrylamide gel. The run was performed in 1 x Tris-acetate-EDTA buffer at 60 °C and a constant voltage of 70 V for 16 h to separate the fragments. The gels were ethidium bromide stained and photographed under UV light (l = 254 nm) using a Gel DocTM XR (Bio-Rad Laboratories Inc., Hercules, CA, USA). Samples were run to obtain at least three profiles.

The DGGE band patterns in different lanes were compared with the Gelanalyzer2010a (http://www.gelanalyzer.com/). The DGGE data were the means of samples from three rats selected randomly from each group. The lanes were normalized to contain the same amount of total signal after background subtraction and the gel images were straightened and aligned to give a densitometric curve. Band positions were converted to Rf values between 0 and 1. The DGGE profiles obtained were analysed considering each band as a species or individual operational taxonomic unit (OTU) having 16S rRNA sequences with similar melting behaviour, while the band intensity indicated the relative abundance of the species. Processing of the DGGE data resulted in a square matrix containing the presence and abundance of DGGE band types per sample. The Shannon–Weaver (H', diversity), and evenness (E´) indices (Shannon & Weaver 1963; Magurran, 2013) were calculated for each DGGE lane. Shannon-Weaver diversity index (H´) was calculated with the formula, $H' = \sum (p_i * \ln p_i)$ where p_i is the band intensity of the *i*th band divided by the sum of all band intensities in a DGGE lane, i.e. the proportion of each species in the community, and ln p_i is the natural logarithm of this proportion (Magurran, 2013). Evenness index (E) was calculated as E = H′/ln S, where S is the number of bands detected in a DGGE lane. Evenness index can range from near 0, indicating pronounced dominance of a few species in the community, to near 1, indicating complete evenness, i.e. equal abundance of all species. The functional organization (*Fo*) of the community was analysed by using the Pareto-Lorenz (PL) distribution curves that represent the evenness of the bacterial community. The *Fo* is the ability of the community to organize into an adequate distribution of dominant and resilient microorganisms, a condition that should ensure the potentiality of counteracting the effect of a sudden

exposure to stress. For this measurement, the bands in each DGGE lane were ordered from high to low, based on their intensity levels. The cumulative normalized numbers of bands were represented along the x-axis, and their respective cumulative normalized intensity along the y-axis (Marzorati et al. 2008). Mathematically, this yields a convex curve. The 45º diagonal represents the theoretically perfect evenness line, indicating that all species are equally abundant. Thus, higher deviations of the PL curve from the 45° diagonal indicates lower evenness in the structure of the studied community. To numerically interpret the PL curves, the *y*-axis projection of their respective intercepts with the vertical 20% *x*-axis line is scored (Wittebolle et al. 2008).

2.5. Plasma 5-HT and BDNF analyses

Trunk blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was separated by centrifuging (Sigma 3-18KS, Germany) the blood samples at 3,000 rpm (800 g) for 20 min at 4ºC and stored at -80ºC until assay. 5-HT levels were determined using a commercial ELISA kit (RE59121, IBL, Hamburg, Germany), previously described in Sánchez-Mateos et al. (2008). BDNF levels were determined using an Ultrasensitive ELISA kit (SK00752-02, Aviscera-Bioscience, Santa Clara, CA, USA). All samples were assayed in duplicate on each plate. Protocols were performed according to the manufacturer's instructions. The optical density of each well was measured using an automated microplate reader (DTX-880, Beckman Coulter, Inc., USA). Plasma 5-HT levels are expressed in nanogram/millilitre (ng/mL), and plasma BDNF levels in picogram/millilitre (pg/mL).

2.6. Statistical analysis

Analyses of variance (ANOVAs) were conducted with two between-subject factors, "group" (HD and LD) and "treatment" (T+ and T−). Repeated measures ANOVAs were conducted with "SIP sessions" as the within-subject factor. Data was tested for normality (Shapiro-Wilks test) and equality of variances (Levene's Test). The *Fo* of the community was analysed by using the PL distribution curves. The similarity between the communities was calculated using the Bray–Curtis cluster analysis. A Pearson's correlation analysis was used to assess the possible relationship between plasma 5-HT and BDNF levels. When appropriate, *post hoc* comparisons were made using the Newman-Keuls test. Partial eta-squared values (η^2_p) are reported as a measure of the effect size, for which values of .01, .06, and .14 are considered to reflect small, medium,

and large effects, respectively (Cohen, 1973). All analyses were computed using the Statistical Package for the Social Sciences (SPSS) 22.0 software package. Statistical significance was set at $p<0.05$. All statistics were two-tailed.

3. Results

3.1. Schedule-induced Polydipsia pre-treatment and post-treatment

Figure 1 shows the mean total licks and total magazine entries in high-drinker (HD) and low drinker rats (LD) on the SIP pre-treatment and post-treatment FT-60s schedule of food presentation. A repeated measures ANOVA revealed significant differences in total licks from Session 1 to session 20 between HD and LD rats (Fig. 1a; group x session effect $F_{1, 26}=43.595$; $p<0.001$; $\eta^2_p=0.63$). Further, significant main effects of group ($F_{1, 26}=48.016$; $p<0.001$; $\eta^2_p=0.65$) and sessions ($F_{1, 26}=69.264$; $p<0.001$; $\eta^2_p=0.73$) were observed. *Post-hoc* analyses indicated that HD increased total licks from Session 1 to Session 20 ($p<0.001$), showing an increased number of total licks compared with LD rats on Session 20 ($p<0.001$). LD rats did not show increments in total licks from Session 1 to 20 ($p=0.455$). No interaction effect was found for magazine entries (Fig. 1c*;* $F_{1, 26}=0.478$; $p<0.495$).

The TRP depletion by diet increased the total number of licks in HD T− rats over the course of the sessions (Fig. 1b; group x treatment x session effect $F_{5,120}=2.529$; $p<0.05$; $\eta^2_p=0.095$), whereas magazine entries were unaffected (Fig. 1d; group x treatment x session effect $F_{5,120}=1.018$; $p=0.410$). *Post hoc* analysis indicated that the differences in total licks between HD T+ and HD T− occur from Session 3 ($p<0.01$). HD T− animals significantly increased their lick rate from Session 3 onwards ($p<0.05$). Although an increase in total licks was observed in HD T+ ($p<0.05$) and LD T− ($p<0.01$) rats on Session 6 compared with Session 1, these groups remain statistically different from each other ($p<0.05$). In fact, a significant main effect of group was found (group effect $F_{1,24} = 32.36$; $p<0.001$), indicating that the differences between HD and LD rats remained stable in terms of total licks.

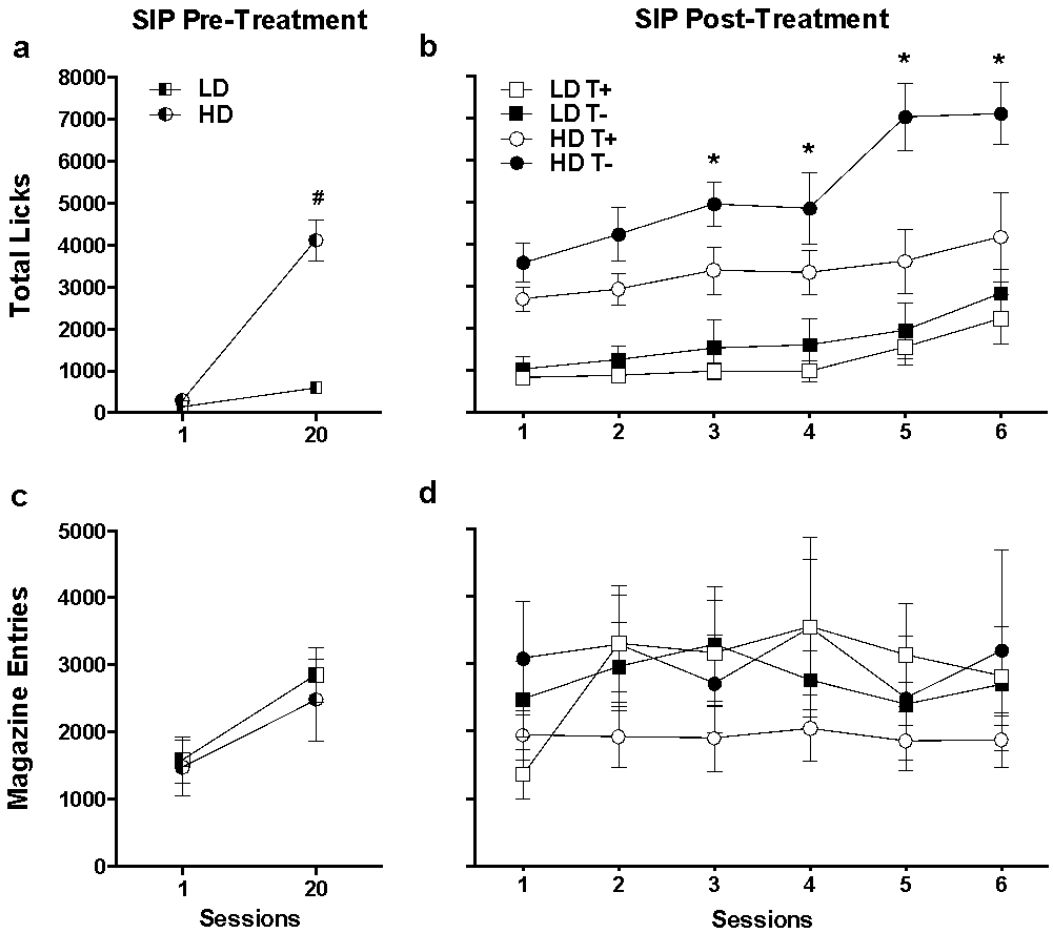

Fig. 1. SIP pre- and post-treatment. The mean (±SEM) total licks (**A**, **B**) and magazine entries (**C**, **D**) on the SIP pre-treatment and SIP post-treatment FT-60s (60 min) sessions. Rats are grouped in the SIP pre-treatment as High Drinkers (HD, *n*=14) and Low Drinkers (LD, *n*=14). Rats are grouped in the SIP post-treatment as TRP non-depleted High Drinkers (HD T+, *n*=7), TRP depleted High Drinkers (HD T-, *n*=7), TRP non-depleted Low Drinkers (LD T+, *n*=7) and TRP depleted Low Drinkers (LD T-, *n*=7). The number sign indicates significant differences between HD and LD (#p<0.001). Asterisks indicate significant differences between HD T+ and HD T− (*p<0.05).

3.2. Shannon-Weaver diversity and Evenness indices

The Shannon-Weaver diversity and evenness indices of the faecal bacterial community from HD T+, HD T-, LD T+ and LD T- rats, calculated from DGGE profiles of PCR-rRNA fragments (Fig. S1), are reported in Figure 2. Chronic dietary TRP depletion significantly altered the bacterial diversity of HD and LD rats (Fig. 2a; treatment x group $F_{1, 8}$=8.148; p<0.05; η^2_p=0.51). *Post-hoc* analysis revealed that chronic TRP depletion caused a reduction in bacterial diversity of LD T- compared with LD T+ rats (p<0.05). Nonetheless, the bacterial diversity of LD T- rats was greater than that for HD T- (p<0.05) and HD T+ rats (p<0.01). HD T+ and HD T- rats had similar diversity values (p=0.202).

In non-depleted groups, HD rats showed a significant decrease in bacterial diversity compared with LD rats ($p<0.001$). Moreover, a main effect of group was found in the diversity index (group effect $F_{1,\ 8}=46.587$; $p<0.001$; $\eta^2_p=0.85$), and *post-hoc* analysis revealed that HD rats had lower bacterial diversity than LD rats, irrespective of diet ($p<0.001$).

Chronic TRP depletion also altered the evenness index in HD rats (Fig. 2b; treatment x group $F_{1,8}=10.596$; $p<0.05$; $\eta^2_p=0.57$). *Post-hoc* analysis indicated that HD T- rats showed significantly lower evenness than the LD T- group ($p<0.05$). Additionally, a trend towards significance was observed between HD T- and HD T+ rats ($p=0.08$). No significant differences were found between HD T+ and LD T+ rats ($p=0.339$).

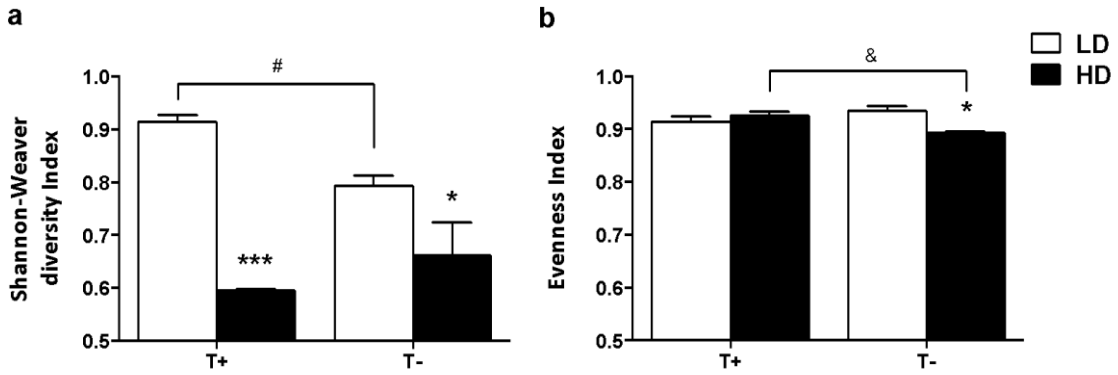

Fig. 2. Shannon-Weaver Diversity and Evenness Indices. The mean (±SEM) Shannon-Weaver diversity (**A**) and Evenness (**B**) indices calculated from DGGE profiles of the faecal bacterial community of TRP non-depleted High Drinkers (HD T+, $n=3$), TRP depleted High Drinkers (HD T-, $n=3$), TRP non-depleted Low Drinkers (LD T+, $n=3$) and TRP depleted Low Drinkers (LD T-, $n=3$). Asterisks indicate statistical differences between HD and LD groups (*$p<0.05$; ***$p<0.001$). The number sign indicates a significant difference between LD T+ and LD T- (#$p<0.05$). Ampersand indicates a trend toward a significant difference between HD T+ and HD T- (&$p=0.08$).

3.3. Functional organization

In order to graphically represent the evenness of the bacterial community (species distribution), PL curves were constructed based on the DGGE profiles (Fig. 3). The samples analysed in this study showed *Fo* values ranging from 53% to 66%. The curves of HD T+ and LD T+ rats were positioned similarly (*Fo*=62%), indicating similar species evenness. However, chronic dietary TRP depletion caused significant differences in functional organization depending on the group. The most specialized community (in

which a small number of the species is dominant and all the others are present in low numbers) was found in HDT- rats, which indicates a more functionally organized community (high *Fo*, 66%), that implies that the original microbiome of HD rats was more vulnerable to TRP depletion. In contrast, LD T- rats had a more balanced community (lower Fo values, 56%), even when compared with non-depleted groups.

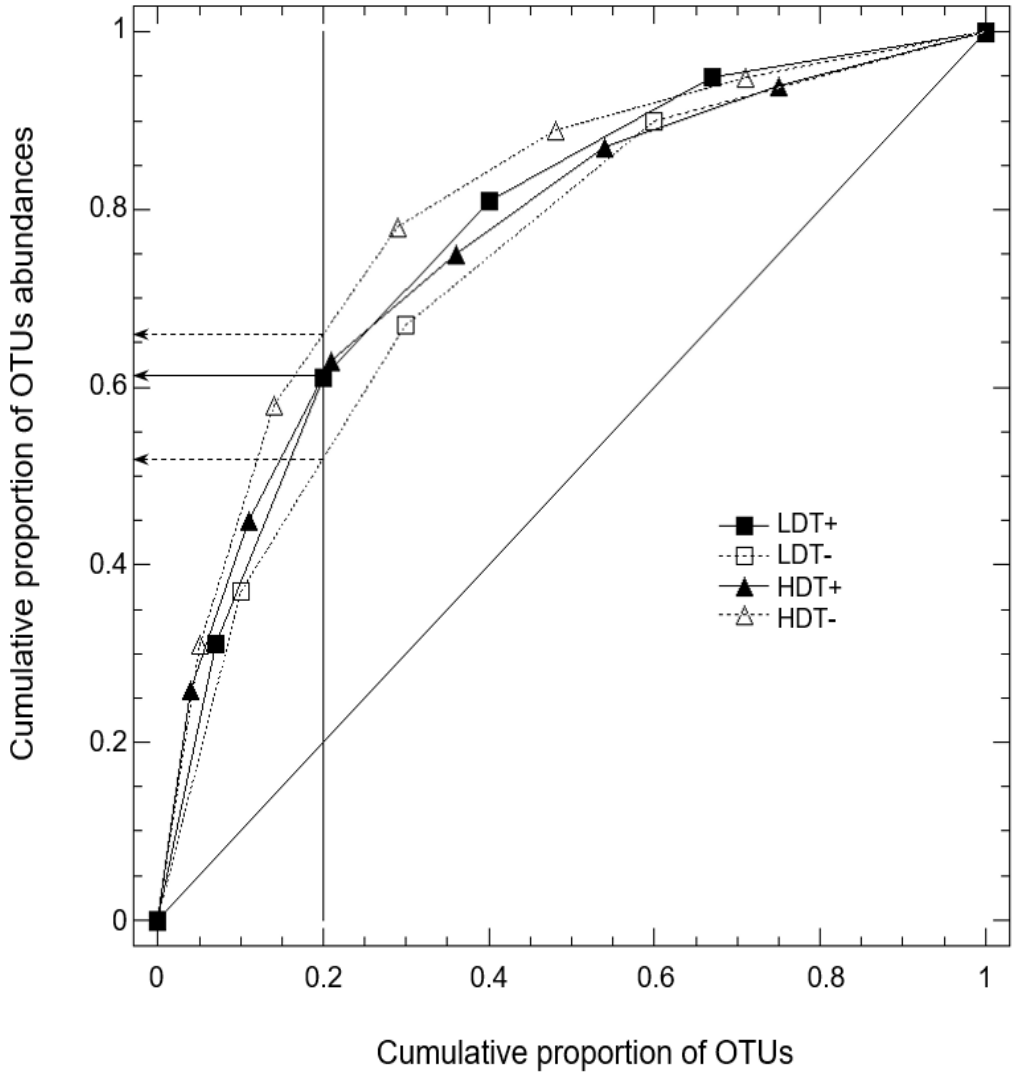

Fig. 3. Functional Organization. Pareto-Lorenz distribution curves based on the DGGE profiles of the faecal bacterial community of TRP non-depleted High Drinkers (HD T+, *n*=3), TRP depleted High Drinkers (HD T-, *n*=3), TRP non-depleted Low Drinkers (LD T+, *n*=3) and TRP depleted Low Drinkers (LD T-, *n*=3). The dashed vertical line at the 20% x-axis level is plotted to evaluate the range of the Pareto values. The 45° diagonal represents the perfect evenness of a community. The y-axis projection of their respective intercepts with the vertical 20% x-axis line is scored.

To better visualize the relationships among samples, the binary matrix based on the presence/absence of bands and their quantity was analysed using the Bray–Curtis correlation, a distance matrix was calculated, and a cluster analysis was conducted which resulted in a dendrogram (Fig. 4). The generated dendrogram revealed two clear clusters. The first one exclusively included HD T- rats which clustered separately from the remaining samples. The remaining samples were grouped into two sub-clusters, one for LD T+ and LD T-, and another for HD T+ rats.

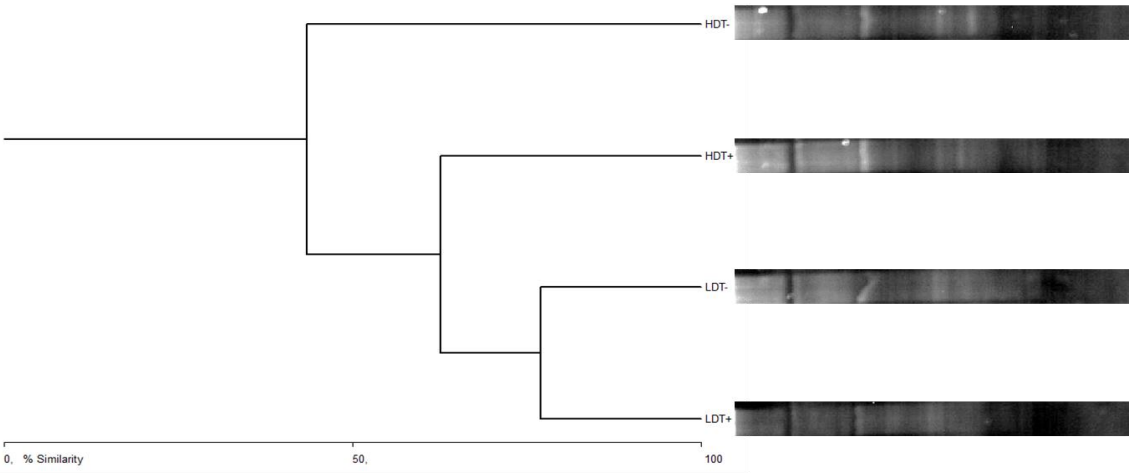

Fig. 4. **Cluster Analysis of DGGE profiles.** Dendrogram obtained from the Bray–Curtis cluster analysis based on the DGGE profiles of the faecal bacterial community of TRP non-depleted High Drinkers (HD T+, *n*=3), TRP depleted High Drinkers (HD T-, *n*=3), TRP non-depleted Low Drinkers (LD T+, *n*=3) and TRP depleted Low Drinkers (LD T-, *n*=3).

3.4. Plasma 5-HT and BDNF levels

The mean plasma 5-HT (ng/mL) and BDNF levels (pg/mL) in HD T+, HD T-, LD T+ and LD T- rats are depicted in Figure 5. The chronic TRP depletion by diet altered plasma 5-HT levels (Fig. 5a; treatment effect $F_{1, 24}$=10.754; p<0.01; η^2_p=0.31), but no significant differences were observed between HD and LD rats (group x treatment effect $F_{1, 24}$=0.041; p<0.842; group effect $F_{1, 24}$=1.522; p<0.229). *Post-hoc* analysis revealed that the depleted groups had lower plasma 5-HT levels than the non-depleted groups (p<0.01). Plasma BDNF levels were not altered by the serotonergic manipulation between HD and LD rats (Fig. 5b; group x treatment effect $F_{1, 23}$=1.062; p<0.313). Moreover, the main effects of treatment ($F_{1, 23}$=0.894; p<0.354) and group ($F_{1, 23}$=2.572; p<0.122) were not

significant. However, a positive Pearson's correlation was found between plasma 5-HT and BDNF levels (Fig. 5c; $r = +0.514$, $p < 0.01$, $n = 27$).

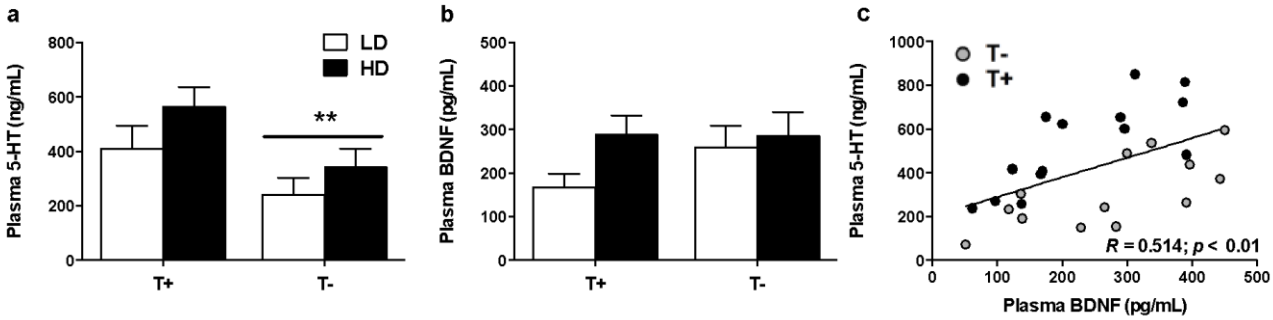

Fig. 5 Plasma 5-HT and BDNT levels. The mean (±SEM) plasma 5-HT (**a**) and BDNF levels (**b**) in TRP non-depleted High Drinkers (HD T+, $n=7$), TRP depleted High Drinkers (HD T-, $n=7$), TRP non-depleted Low Drinkers (LD T+, $n=7$) and TRP depleted Low Drinkers (LD T-, $n=6$-7), and correlation between plasma 5-HT and BDNF levels ($n=27$) (**c**). Asterisks indicate significant differences between TRP non-depleted and depleted rats (**$p<0.01$).

4. Discussion

The present study has shown different faecal bacterial community profiles in HD and LD rats in SIP, and the specific effects of chronic dietary TRP depletion on these bacterial communities. We have observed that HD rats had lower bacterial diversity than LD rats, suggesting a possible altered gut microbiota in these vulnerable populations to compulsivity. Moreover, TRP-depleted HD rats, the only group that exhibited an increase in compulsive licking in SIP, showed a reduction of bacterial evenness values and a highly functionally organized community compared with the other groups, indicating that the bacterial community of the HD animals could be more vulnerable to external changes. Despite the fact that the TRP-depleted LD rats showed a reduction in bacterial diversity, the values were greater that those from TRP-depleted HD rats. In fact, the reduction of bacterial diversity observed in TRP-depleted LD rats was not sufficient to alter the functional organization of the bacterial community, with these rats showing the most balanced microbiota according to the PL curves. In addition, chronic dietary TRP depletion effectively reduced peripheral plasma 5-HT levels in depleted HD and LD rats, while plasma BDNF levels were unaffected.

As previously reported by Merchán et al. (2017), chronic TRP depletion produced an increase of compulsive licking in HD, which was not observed in LD rats, and a reduction of striatal 5-HT_{2A} receptor binding, a serotonin receptor subtype that has been proposed as a candidate for mediating compulsive behaviour (Aznar and Hervig 2016; Aznar and Klein 2013; Fineberg et al. 2010, 2011). This receptor subtype was found to mediate the anti-compulsive effect of the serotonin 5-$HT_{2A/C}$ receptor agonist DOI in SIP (Mora et al. 2018; Navarro et al. 2015). Moreover, previous studies have shown that vulnerable HD rats exhibited increased 5-HT levels in the medial prefrontal cortex and amygdala (Mora et al. 2018; Moreno et al. 2012), and reduced 5-HT_{2A} binding in the frontal cortex (Mora et al. 2018). Collectively, these results point to the involvement of the 5-HT system in the development of compulsive drinking in SIP. Additionally, the present study has explored differences in the faecal bacterial community structure of HD and LD rats. Interestingly, compulsive HD rats have shown a reduction in bacterial diversity compared with LD rats. The faecal samples were collected 20 days after the last post-treatment SIP session, thus we consider that the lower bacterial diversity observed in HD rats is unlikely to be a consequence of the high fluid intake (i.e. diarrhea). In support of our results, a recent human study has found reduced species diversity in non-medicated OCD patients compared with controls (Turna et al. 2020). Previous studies with rodents have found that maternal separation (O'Mahony et al. 2009), prolonged restrain stressors (Bangsgaard Bendtsen et al. 2012), and social stressors (Bailey et al. 2011) altered the microbiota composition and reduced the abundance of species. However, few studies have explored the relationship between compulsive behaviour and gut microbiota. For instance, GF mice have been shown to spend an increased amount of time on self-grooming (Desbonnet et al. 2013) and had a higher number of buried marbles compared with controls (Nishino et al. 2013). In innately anxious male BALB/c mice, the number of marbles buried was reduced by probiotics in a manner similar to the SSRI escitalopram (Savignac et al. 2014). In line with this, a study in mice showed that 2 weeks of pre-treatment with probiotics reduced compulsive behaviours such as perseverative open-field locomotion, stereotypic turning and marble burying, induced by the acute 5-HT1A/1B agonist RU24969, in a similar way to mice pre-treated for 4-weeks with fluoxetine, a first-line treatment for OCD (Kantak et al. 2014). Similarly, a study in healthy humans found that probiotics administered daily for 30 days reduced "obsessive-compulsive" sub-scores on the Hopkins symptoms checklist (Messaoudi et al. 2011). Taken together, the findings in the literature suggest that the modulation of the gut

microbiota by probiotics may be beneficial in reducing compulsive behaviour and, therefore, an altered microbiota-gut-brain system may be implicated in the pathogenesis of such behaviour. Further studies could test compulsive behaviour on SIP in germ-free animals, as well as the possible benefits of probiotics in compulsive HD rats.

Chronic dietary TRP depletion had different effects in the faecal bacterial community of LD and HD rats. TRP-depleted LD rats showed a reduction of faecal bacterial diversity, without affecting the evenness of the community. In spite of this, the bacterial diversity values of TRP-depleted LD were greater than those from TRP-depleted HD rats. In fact, this reduction in bacterial diversity was not sufficient for altering the functional organization of the bacterial community of TRP-depleted LD rats, showing the most balanced microbiota according to the PL curves, whilst not increasing their rates of compulsive licking in SIP. In contrast, TRP-depleted HD rats showed an increase in compulsive licking in SIP along with a bacterial community structure that was different from the remaining groups according to the cluster analysis. In this vulnerable group, the chronic TRP depletion reduced the evenness of species, which, together with a lower bacterial diversity observed in the HD groups, resulted in a highly functionally organized community. In support of our findings, a previous study found that mice with dietary TRP insufficiency had an altered gut microbial composition, and these effects were reversed by a TRP supplemented diet (Hashimoto et al. 2012). The alteration of the bacterial community structure after TRP deficiency confirms the modulating role of TRP in the GM. Although most microorganisms can synthesize their own tryptophan, some depend on an exogenous source of amino acids (Zouali 2009). In fact, microorganisms are sensitive to the activity of the Indoleamine 2,3 dioxygenase-1 enzyme (IDO1), the first and rate-limiting step in TRP catabolism along the kynurenine pathway in the gut (Gao et al. 2018; Ciorba 2014). The IDO1 enzyme has been shown to play an essential role in maintaining microbial diversity (Le Floc'h et al. 2011). For instance, IDOI1-Knockout mice exhibit increased production of bacterial TRP metabolites (Zelante et al. 2013). Similarly, IDOI1 activation leading to host TRP depletion can reduce microbial proliferation, possibly by microbial amino acid deprivation and immune tolerance (Gao et al. 2008). Therefore, the reduction of bacterial diversity observed in LD rats may be a consequence of microbial TRP deprivation. However, the mechanisms through which the TRP-deficient diets had a more marked effect on the vulnerable compulsive HD rats in terms of gut microbiota remains unknown. Further studies should investigate the

mechanisms underlying the serotonergic modulation of the microbiota-gut-brain axis in vulnerable populations to compulsivity.

On the other hand, chronic dietary TRP depletion significantly reduced plasma 5-HT levels, independently of the SIP groups. Previous studies in rodents have shown that chronic TRP depletion by diet effectively reduced plasma TRP, brain 5-HT levels and its metabolite (Browne et al. 2012; Cahir et al. 2007; Franklin et al. 1995, 1999, 2012; Koot et al. 2012; Merchán et al. 2017; Vergnes and Kempf 1981) and altered 5-HT_{2A} receptor levels (Franklin et al. 2012; Merchán et al. 2017). As far as we know, this is the first study that measures plasma 5-HT levels following chronic TRP depletion by diet, which may serve as an indirect measure of 5-HT depletion in the peripheral nervous system. Similarly, a previous study reported increments in plasma 5-HT levels after 7 days of oral TRP administration in rats (Sánchez-Mateos et al. 2008). Moreover, acute intra-gastric TRP administration increased 5-HT levels in intestinal tissue (Teff and Young 1988), indicating that intestinal 5-HT could be altered by dietary intake (Biggio et al. 1977). Although we did not find differences in plasma 5-HT levels between HD and LD groups after dietary TRP depletion, HD rats seems to have a vulnerable serotonergic system mediating the specific alteration of the faecal bacterial community structure. In fact, SSRIs, which are effective in reducing OCD symptoms, are also capable of decreasing pain and other symptoms associated with chronic gastro-intestinal disorders (Vanuytsel et al., 2014). Specifically, irritable bowel syndrome has been associated with small intestinal bacterial overgrowth (Stern and Brenner 2018), and the criteria for this syndrome are met in 35,1% of patients with OCD in contrast to 2.5% of controls (Masand et al. 2006).

Regarding the plasma BDNF levels, HD and LD rats showed similar levels of this protein despite of the chronic TRP depletion. In support of our findings, previous ATD studies in rats found no alterations in plasma and central BDNF levels (Cahir et al. 2008; Van Donkelaar et al. 2009). Moreover, serotonin depletion by para-chloroamphetamine administration or 5,7-dihydroxytryptamine injections into the dorsal and median raphe nuclei did not change hippocampal BDNF levels compared with non-depleted rats (Hamani et al. 2012; Zhou et al. 2008). On the other hand, non-depleted HD rats did not exhibit reduced plasma BDNF levels compared with non-depleted LD rats, which does not support the notion that peripheral BDNF is a biomarker for compulsive behaviour in the SIP model. Nonetheless, a positive correlation between plasma 5-HT and BDNF

levels was observed in the present study, which is in accordance with previous studies showing positive correlations between TRP and BDNF levels in the hippocampus and prefrontal cortex in rats (Van Donkelaar et al. 2009) and SERT availability and BDNF levels in humans (Chan et al. 2018; Chou et al. 2013). Therefore, our data confirm the relationship between serotonergic transmission and BDNF expression that has been reported by previous authors (Martinowich and Lou, 2008; Sen et al. 2008).

One limitation of the present study is that the approach used does not allow for identifying the specific bacterial strains that were reduced in diversity and evenness in the TRP-depleted HD group. Nonetheless, from a more general perspective, the data provided from the DGGE profiles showed a clear cluster of the microbiota from depleted HD rats, which is not evident in their non-depleted HD counterparts, suggesting that the serotonin manipulation specifically affected the microbiota of HD rats vulnerable to compulsivity. Further studies should investigate the influence of neurotransmitters on the gut microbiota of subjects vulnerable to compulsive spectrum disorders, and explore possible alterations in the gut-brain axis that could underlie the vulnerability to compulsive behaviour. In fact, the degradation of specific bacteria populations that require TRP to survive could induce a gut dysbiosis that would finally alter the function of 5-HT-producing species, finally affecting the general activity of this neurotransmitter in the CNS and at a systemic level.

In conclusion, the primary findings of the present study highlight the involvement of reduced microbial diversity in the vulnerability to develop compulsive drinking in SIP, and the role of the serotonergic system in the alteration of the gut microbiota of vulnerable populations to compulsivity. In the TRP-depleted HD rats, the chronic dietary TRP depletion increased compulsive licking in SIP and produced a reduction of species evenness that, together with a lower microbial diversity, resulted in a higher specialized bacterial community compared with the other groups, indicating an unbalanced, less adaptive and more vulnerable bacterial community structure of HD rats when facing this kind of challenges. However, the main limitation of the present study is the lack of more in-depth analyses concerning gut bacteria-specific populations at different Taxa levels (e.g., Genus or Species). Although our results provide novel and relevant information regarding the faecal bacterial community structure in an animal model of compulsive behaviour, further studies should complement our findings using other techniques such as Next Gene Sequencing (NGS). Future NGS studies could investigate how TRP

depletion alter specific bacteria that are known to play an important role in producing 5-HT, as well as the mechanisms by which the 5-HT pathways influence the brain-gut axis in compulsive spectrum disorders.

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Conflicts of Interest Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Supplementary Material

Dietary tryptophan depletion alters the faecal bacterial community structure of compulsive drinker rats in schedule-induced polydipsia

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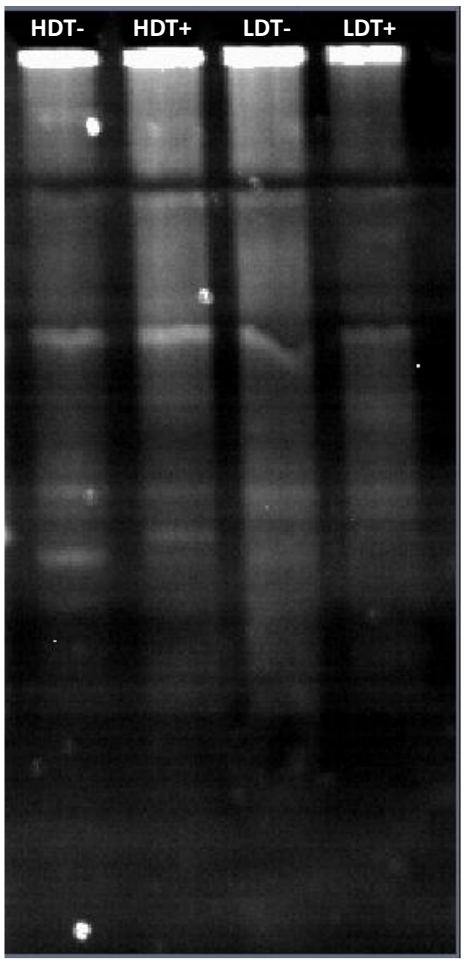

Fig. S1. DGGE fingerprints of 16S rRNA gene sequences amplified from DNA templates extracted from faeces of rats: TRP non-depleted High Drinkers (HD T+), TRP depleted High Drinkers (HD T-), TRP non-depleted Low Drinkers (LD T+) and TRP depleted Low Drinkers (LD T-)