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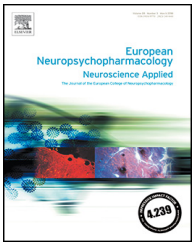

The antidepressant-like effect of probiotics and their faecal abundance may be modulated by the cohabiting gut microbiota in rats

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Abstract
Numerous studies have been published describing the effect of various probiotics (PRO) on behaviours related to psychiatric disease. We have previously shown a robust antidepressant-like effect of PRO in rats, but over time, the treatment effect seems to vary significantly between different sets of rats from the same commercial vendor. Therefore, we hypothesised that the antidepressant-like response may be modulated by the cohabiting gut microbiota.
The aims of the present study were (1) to investigate any differences in the gut microbiota composition between responders (Resp) and non-responders (Non-resp) to PRO with regards to depressive-like behaviour, and (2) to evaluate the effects of PRO on the microbiota composition.
Two sets of 20 male Sprague-Dawley rats each were treated with multi-species PRO (nine *Bifidobacterium*, *Lactococcus* and *Lactobacillus* species) for eight weeks and subjected to a behavioural assessment. Faecal samples were collected for 16 s rRNA (VR4) gene amplicon sequencing (Illumina MiSeq).

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As previously reported, PRO-treated Resp animals showed a marked decrease in depressive-like behaviour, whereas no such response was seen in Non-resp. We observed profound differences in the gut microbiota composition between the two sets of rats, and the relative faecal abundance of the genera that comprised PRO was higher in Resp than in Non-resp although treated with the same dose of PRO. Particularly, the relative abundance of the *Lactobacillus* genus was not increased in PRO-treated Non-resp animals.
In conclusion, the cohabiting microbiota and the faecal abundance of PRO may modulate the antidepressant-like effect of PRO in rats.
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1. Introduction

The gut microbiota has emerged as an important contributor to the regulation of a range of physiological systems throughout the body, including the brain (Sampson and Mazmanian, 2015). Intriguingly, several studies have presented interactions between the residing microbes, brain development and function and behaviour related to psychiatric disease (Sampson and Mazmanian, 2015). On this background, Cryan & colleagues defined the term *psychobiotics* as neuroactive probiotics (PRO) that, when ingested in adequate amounts, produce a mental health benefit in patients (Dinan et al., 2013). Numerous studies have now been published describing the effect of PRO in animals and fewer in healthy humans (Wang et al., 2016), and the results from clinical trials offering different PRO formulations to depressed patients have recently been reported (Akkasheh et al., 2016; Pinto-Sanchez et al., 2017; Romijn et al., 2017). Unfortunately, the results from all those studies appear diverse with mixed positive and negative findings, and even two recent meta-analyses on the effect of PRO in healthy volunteers did not reach the same conclusion (McKean et al., 2017; Ng et al., 2018).

Undoubtedly, not only the results of the published psychiatric studies on PRO suffer from great heterogeneity. Most studies utilise unique formulations comprising privileged or patented strains belonging to the *Bifidobacerium* or *Lactobacillus* genera (Wang et al., 2016), which impede a direct comparison of the outcomes. Also, the most optimal dose of PRO has not been established, and no dose-response studies have been published. Notwithstanding, much attention has been paid to the properties of candidate microbial strains that could, potentially, be effective neuroactive PRO (Bambury et al., 2017). Yet, a specific PRO formulation that showed antidepressant-like effect in one rat model, failed to do so in another although the same dose was used (Arseneault-Breard et al., 2012; Tillmann et al., 2018). Also, not all strains that generate promising results in animal models do so in clinical trials (Kelly et al., 2016a). Consequently, certain factors intrinsic to the recipient of the treatment may be hypothesised to modulate the response. It is well-described that the interindividual differences in gut microbiota composition are enormous: studies have revealed that any two individuals only share 32.8% of their microbial genes (Li et al., 2014), and this circumstance may be of great importance for several reasons. Firstly, it is believed that PRO partly exert their effect by modulating the function of the residing microbes, rather than by affecting the gut microbiota structure (Eloe-Fadrosh et al., 2015; McNulty et al., 2011). Accordingly, the cohabiting microbes may turn out to be pivotal to any beneficial effect of the treatment. Secondly, it is not known whether the beneficial effects of PRO depend on their intestinal colonisation and multiplication, which may be hindered by numerous factors, including the competitive intestinal microbial ecosystem. Unfortunately, not much is known about the interaction between the resident microbes and exogenously supplied PRO, although such knowledge would be of benefit to researchers especially when designing costly clinical trials. Definitely, a large interindividual variation in treatment response would inevitably blur any possible effect and urge researchers to draw misleading conclusions.

We have previously shown that eight weeks of treatment with multi-species PRO (*Bifidobacterium (B.) bifidum, B. lactis, Lactobacillus (Lb.) acidophilus, Lb. brevis, Lb. casei, Lb. salivarius, Lactococcus lactis*) robustly reduced the level of depressive-like behaviour in Sprague-Dawley (SD) rats independently of diet (Abildgaard et al., 2017b). Over time, however, we have experienced major fluctuations in the magnitude of the behavioural response to the treatment, although strict adherence to the same experimental protocol has been undertaken. This immediate challenge has generated the unique opportunity to identify individual factors that may interact with the treatment, and in the present study, we therefore compared a set of animals showing a significant behavioural response with a set of rats characterised by complete absence of treatment response. Accordingly, the specific aims of the present trial *post hoc* analyses were 1) to investigate any differences in the gut microbiota composition between responders and non-responders to PRO, and 2) to evaluate the effects of PRO on the gut microbiota composition in responders and non-responders.

2. Experimental procedures

2.1. Animals

Male Sprague-Dawley (SD) rats (NTac: SD, SPF; Taconic, Denmark) arrived at the age of four weeks and were pair-housed throughout the studies in Eurostandard Type III H cages ($37.5 \times 21.5 \times 18$ cm) with raised lids, a shelter and nesting material. The vivarium had a constant temperature of 20 °C and a 12/12 h light/dark cycle (lights on at 6 AM). All procedures complied with the EU Directive 2010/63/EU and with

the Danish law regulating experiments on animals (permission ID 2012-15-2934-00,254).

2.2. Study design

The present study *post hoc* analysis included 20 rats from each of two similar experiments that were carried out with an interval of 20 months. In the first experiment, PRO significantly reduced the average level of depressive-like behaviour, and the respective animals were, therefore, collectively defined as responders (Resp). Behavioural and molecular data (exclusive of 16 s rRNA gene amplicon sequencing) from this experiment have already been published (Abildgaard et al., 2017b). The animals from the second experiment did not show any changes in the average level of depressive-like behaviour with PRO and were, therefore, collectively defined as non-responders (Non-resp).

From the day of arrival (study week 0), all rats were fed a purified control diet (cat no. E15000, Ssniff, Germany) ad libitum together with tap water throughout the experiment. After a 5 weeks habituation period on the control diet, each cage of two rats was randomised to receive vehicle (VEH) or probiotic (PRO) treatment (study week 5) throughout the rest of the study. After eight weeks of treatment with PRO, the forced swim test (FST) was conducted (study week 13). Faecal samples were collected 14–16 days later to minimise the acute effect of swim stress on the gut microbiota (study week 15–16).

2.3. Probiotic treatment

Daily, each PRO cage of two rats was administered a bottle of 4.5 g (2.5×10^9 CFU/g) freeze-dried PRO in a carrier matrix (maize starch, maltodextrins and vegetable protein) dissolved in 30 mL of tap water, whereas the VEH cages were treated with a solution of 4.5 g carrier matrix only. The bottles were administered daily between 4 and 6 pm and completely emptied by the animals during the night, and the rats had concurrently access to their usual bottle of tap water. The multi-species PRO consisted of nine strains (*Bifidobacterium (B.) bifidum* W23, *B. lactis* W51, *B. lactis* W52, *Lactobacillus (Lb.) acidophilus* W37, *Lb. brevis* W63, *Lb. casei* W56, *Lb. salivarius* W24, *Lactococcus (Lc.) lactis* W19 and *Lc. lactis* W58; "Ecologic Barrier", Winclove Probiotics BV, The Netherlands). The declaration of bacterial strains has recently been updated owing to the application of new molecular identification techniques (sequencing). It has been confirmed that the PRO formulation has always contained these nine strains, and the proportion between strains and CFU counts are unaltered.

Enumeration of colonies by anaerobic incubation on MRS agar (cat no AEB621757VAF, BioMérieux, France) was performed to verify the bacterial count and to ensure that the viability in solution remained stable over 24 hours. In addition, random colonies were selected, and their carbohydrate fermentation patterns were assessed to confirm the presence of individual strains by use of API 50 CH kits (BioMérieux, France). The kit insert provided a table of fermentation patterns for *Lactobacillus* and *Lactococcus* species. The fermentation pattern of *Bifidobacteria* was adopted from (Briczinski et al., 2009).

2.4. Forced swim test

Immobility in FST is believed to reflect depressive-like behaviour and is defined as the rat making no further movements than those needed to keep its head above the water. We used the modified version of the test (Detke et al., 1995). Briefly, the animals were individually immersed in a water-filled plastic cylinder (H: 54 cm; diameter: 24 cm; water depth: 40 cm; 24–25 °C) and allowed to swim for 15 and 7 min, respectively, on two consecutive days. After each swimming session, the animals were dried in a towel and placed under a heat lamp for 30 min. The water was changed between each trial. All sessions were video-recorded, and the second day test trial was subsequently scored by an experimenter blinded to treatment groups by use of the prevailing time-sampling technique (Slattery and Cryan, 2012), i.e. the most predominant behaviour during each 5 sec bin was registered. Trials of both experiments (Resp and Non-resp) were carried out according to our standard operating protocol with a minimum of variability in test parameters (same experimenter, room, apparatus, time of day, light intensity; randomised test sequence of rats).

2.5. Open field test

Immediately prior to the second trial of FST, locomotor activity of the animals was assessed in the open field test (OFT) to rule out false positive findings in the FST. Each animal was individually placed in a square arena (100×100 cm) for 10 min (black arena; wall height 20 cm; light intensity 25 lx). The sessions were video-recorded, and distance travelled was analysed by use of Noldus Ethovision XT 11 (Noldus IT, The Netherlands). The arenas were cleaned between each trial.

2.6. Faecal DNA extraction

Fresh faecal samples were collected directly from each animal 18–24 hrs after last PRO dose and immediately frozen on dry ice. Samples were stored at −80 °C. Genomic DNA was isolated from rat faeces by use of the NucleoSpinSoil kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instruction. For cell lysis, buffer SL2 & Enhancer buffer SX were used, and the subsequent vortex step was replaced with repeated bead beating. DNA yield, purity and integrity were assessed with a Qubit 2.0 flourometer, a NanoDrop 2000 spectrometer (Thermo Fisher Scientific Inc., MA USA) and agarose gel electrophoresis, respectively. Samples from both experiments were processed at the same time to minimise variations in DNA extraction.

2.7. Library preparation and 16 s sequencing

Library preparation with PCR amplification was performed with 20 ng bacterial DNA, 0.2 μM of each

barcoded forward and reverse primer, and HotMaster-Mix (5Prime) solution (total volume 25 μL). To target the variable region 4 of the 16S rRNA gene, a forward primer 515F (5′ AATGATACGGCGACCACCGAGATC-TACAC < i5 > TATGGTAATTGTGTGCCAGCMGCCGCGGTAA 3′) and a reverse primer 806R (5′ AAGCAGAAGACGGCATAC-GAGAT < i7 > AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT 3′) were used; each primer consisted of the appropriate Illumina adapter, an 8-nt index sequence i5 and i7, a 10-nt pad sequence, a 2-nt linker, and the gene-specific primer. The PCR reaction conditions were 3 min at 94 °C, followed by 28 cycles of 20 s at 94 °C, 30 s at 55 °C and 54 s at 72 °C on an Eppendorf thermocycler (Eppendorf AG, Germany). The samples were purified with a magnetic-bead based clean-up and size selection kit (Macherey-Nagel GmbH & Co. KG, Germany). Amplicons were visualized by gel electrophoresis and quantified by a Qubit 2.0 fluorometer. A master DNA pool was generated from the purified products in equimolar ratios.

The DNA was sequenced with an Illumina MiSeq platform (MiSeq Reagent Kits v2, 500 cycles). Quality control of sequencing data was done by use of the DADA2 pipeline, the raw sequences were trimmed, de-replicated, de-noised, and merged: chimera were identified and removed. Expected error filtering (Emax = 1.0) was used to exclude low quality contigs. The remaining high-quality sequences were mapped against RDP reference database using closed reference OTU picking (RDP training set 16/release 11.5).

2.8. 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 ($\beta = 0.20$). Normality was assessed by QQ plots, and Bartlett's test was used to test for equal variances when relevant. Body weight, behavioural data, diversity measures and total PRO genus abundance were analysed by two-way ANOVA (responder status x treatment), and significant interactions were followed up by individual Bonferroni-corrected comparisons. Abundance of the three individual PRO genera was analysed by pair-wise Wilcoxon rank-sum tests with Bonferroni correction due to non-normality of data. In addition, relative abundance on the phylum and family levels were analysed by zero-inflated negative binomial regression. Independent factors included responder status, treatment and their interaction. Contrasts of marginal linear predictions were used to test for differences between groups after the models were built, and Bonferroni correction was applied. Families/phylae not detected in >25% of animals were excluded. Also, *a priori* defined pairwise comparisons were made for all ribosomal sequence variants (RSV) identified in at least 20% of animals (RESP VEH vs RESP PRO; NON-RESP VEH vs NON-RESP PRO; RESP VEH vs NON-RESP VEH) by Wilcoxon rank-sum test followed by correction for multiple comparisons with false discovery rate (FDR) at a level of 0.10 (Benjamini and Hochberg, 1995). The *a priori* comparisons were defined according to the study aims. All statistical analyses were performed with Stata 14 (StataCorp LT, Texas, USA). Finally, the overall microbiota composition was analysed by a principal component analysis (PCA) with pareto-scaling of the RSVs and a missing value tolerance of 50% with Simca 14 (MKS Umetrics AB, Sweden), and a two-way PERMANOVA test was performed based on Jaccard (qualitative) and Bray-Curtis (quantitative) similarity indices with PAST 3.20 (Hammer et al., 2001).

3. Results

3.1. Assessment of the probiotic formulation confirmed the product declaration

The CFU count provided by the manufacturer was verified by anaerobic cultures on MRS agar, and it remained stable for at least 24 h (Supplementary Table S1). Assessment of carbohydrate fermentation patterns revealed the presence of strains compatible with *Lb. paracasei*, *Lb. brevis*, *Lb. salivarius*, *Lb. acidophilus* and two different *Lc. lactis* species according to the kit insert (Supplementary Table S2). Also, a number of colonies showing a fermentation pattern compatible with *Bifidobacteria* were observed.

3.2. All groups had similar weight gain and locomotor activity

Body weight at arrival was not affected by treatment ($F_{1,36=}0.74$; $p = 0.4$; Table 1), but Resp animals had a lower body weight than Non-resp animals ($F_{1,36=}16.6$; $p < 0.001$). The weight gain over the course of the study was not affected by responder status ($F_{1,36=}0.51$; $p = 0.5$) or treatment ($F_{1,36=}0.37$; $p = 0.6$). Similarly, the locomotor activity in OFT was also not affected by responder status ($F_{1,36=}1.91$; $p = 0.2$) or treatment ($F_{1,36=}3.13$; $p = 0.1$; Table 1 & Supplementary Fig. S3).

3.3. PRO had antidepressant-like effect in resp only

Ten animals from each separate experiment (Resp and Non-resp) were treated with PRO. Resp animals were characterised by a 30% (95% CI: 10–49%; Fig. 1) reduction in immobility duration in FST (depressive-like behaviour) as previously reported (Abildgaard et al., 2017b). The Non-resp set of rats, however, were characterised by an unaltered mean immobility level, since PRO non-significantly increased the immobility duration by 2% (95% CI: −22–26%). Consequently, a responder x treatment interaction was observed for immobility ($F_{1,36=}4.82$; $p = 0.03$). PRO reduced depressive-like behaviour in Resp ($p = 0.02$; Bonferroni-corrected), but not in Non-resp ($p = 1.00$; Bonferroni-corrected).

3.4. PCA analysis revealed a clear separation between resp and Non-resp

All faecal samples were sequenced successfully (median reads: 16,579; range: 9,345–25,855). A PCA plot of the 16 s rRNA gene sequences (Fig. 2) showed that Resp and Non-resp had fundamentally distinct gut microbiota compositions. A clear separation between Resp and Non-resp

Table 1 Body weight, weight gain and locomotor activity. All values presented as mean ± SD.

	Non-resp VEH	Non-resp PRO	Resp VEH	Resp PRO	Resp vs. Non-resp	VEH vs PRO	Resp x PRO interaction
Group size (n)	10	10	10	10	-	-	-
Body weight, at arrival (g)	91.9 ± 11.0	91.2 ± 12.4	**78.1 ± 14.5**[#]	**71.8 ± 13.4**[#]	$F_{1,36=}$ **16.6; p < 0.001**[#]	$F_{1,36=}$ 0.74; p = 0.4	$F_{1,36=}$ 0.48; p = 0.5
Weight gain during study (g)	405 ± 21.1	400 ± 43.0	415 ± 36.6	406 ± 43.8	$F_{1,36=}$ 0.51; p = 0.5	$F_{1,36=}$ 0.37; p = 0.5	$F_{1,36=}$ 0.03; p = 0.9
Open Field activity (cm)	5,589 ± 1,371	4,369 ± 1,202	5,466 ± 714	5,455 ± 1007	$F_{1,36=}$ 1.91; p = 0.2	$F_{1,36=}$ 3.13; p = 0.1	$F_{1,36=}$ 3.02; p = 0.1

Non-resp: non-responders; Resp: responders; VEH: vehicle treatment; PRO: probiotic treatment;
[#] Resp groups had significantly lower body weight at arrival than Non-resp ($p < 0.001$). However, Resp animals were weighed at the day of arrival, whereas Non-resp animals were weighed on the 2nd day after arrival, and this circumstance may account for the observed difference.

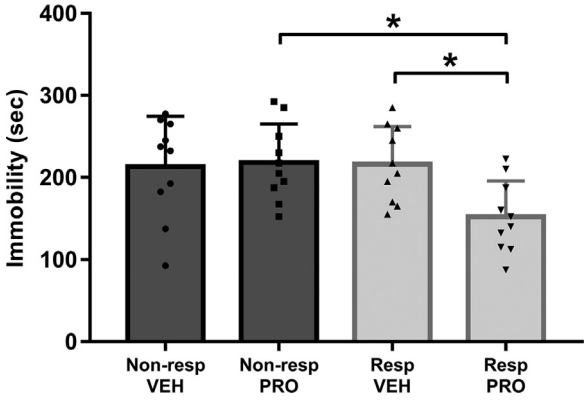

Fig. 1 Depressive-like behaviour (immobility) in the Forced Swim Test (mean ± SD). Probiotic treatment (PRO) had antidepressant-like effect in responders (Resp), but not in non-responders (Non-resp).
*: $p < 0.05$ (Bonferroni-corrected).

animals was seen along the first principal component ($R^2 = 23.2\%$), but the PRO groups did not separate from the VEH groups. A PERMANOVA test based on the Bray-Curtis dissimilarity index confirmed a highly significant effect of responder status ($F_{1,36} = 17.6$; $p = 0.0001$), but showed neither an effect of PRO ($F_{1,36} = 1.36$; $p = 0.17$) nor an interaction ($F_{1,36} = 1.20$; $p = 0.2$). PERMANOVA based on the Jaccard dissimilarity index, however, showed an effect of responder status ($F_{1,36} = 13.1$; $p = 0.0001$) and treatment as well ($F_{1,36} = 1.89$; $p = 0.03$), but still no interaction ($F_{1,36} = 1.35$; $p = 0.12$). Since the Jaccard index is only based on presence/absence (qualitative), whereas the Bray-Curtis index incorporates abundances (quantitative), such results may likely indicate that a number of infrequent PRO species were introduced with the treatment. No correlations were observed between immobility in FST and the first ($r = -0.20$; $p = 0.4$) or second ($r = 0.16$; $p = 0.5$) principal component.

3.5. PRO increased shannon's diversity index in resp only

No differences in observed richness were seen between groups (Fig. 3A; Resp: $F_{1,36} = 0.87$; $p = 0.4$; PRO: $F_{1,36} = 0.00$; $p = 1.0$; Resp x PRO: $F_{1,36} = 0.79$; $p = 0.4$). However, a treatment x responder interaction was seen for the Shannon's diversity index (Fig. 3B; $F_{1,36=}11.2$; $p = 0.002$). Indeed, the Shannon index was increased by PRO in Resp ($p = 0.02$; Bonferroni-corrected), but remained unaltered in Non-resp ($p = 0.3$; Bonferroni-corrected).

3.6. Several phylae, families and RSVs differed between resp and Non-resp

At the phylum level, Resp showed a higher relative abundance of Bacteroidetes ($p < 0.0001$), Actinobacteria ($p = 0.02$) and Cyanobacteria ($p = 0.0002$) than Non-resp, but a lower relative abundance of Deferribacteres ($p = 0.008$; Table 2 and Fig. 4A). At the family

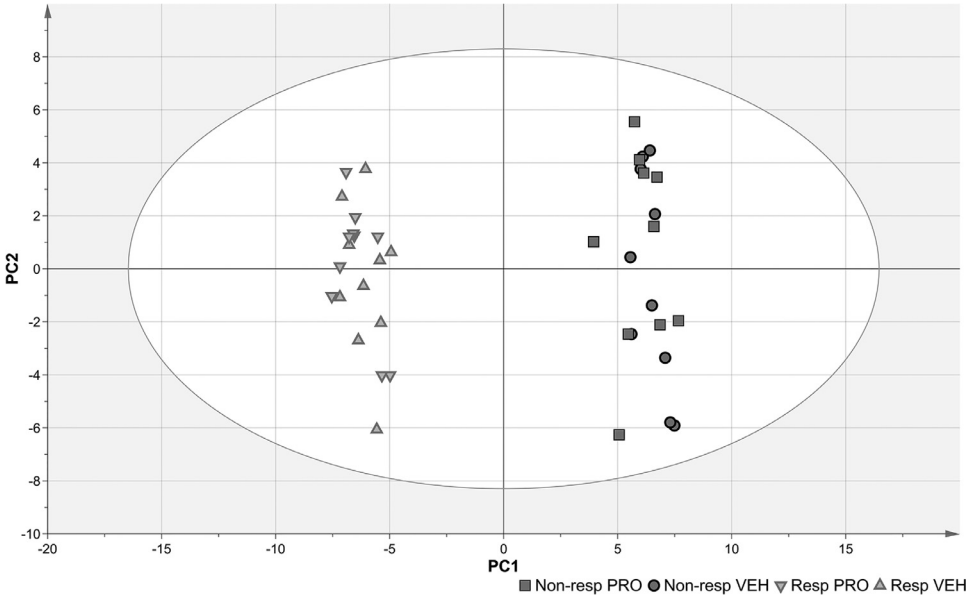

Fig. 2 Principal component analysis of faecal microbiota composition. A clear separation between responders (Resp) and non-responders (Non-resp) was seen along principal component 1 (PC1; $R^2 = 23.2\%$), whereas vehicle (VEH) and probiotic (PRO) treatments could not be distinguished.

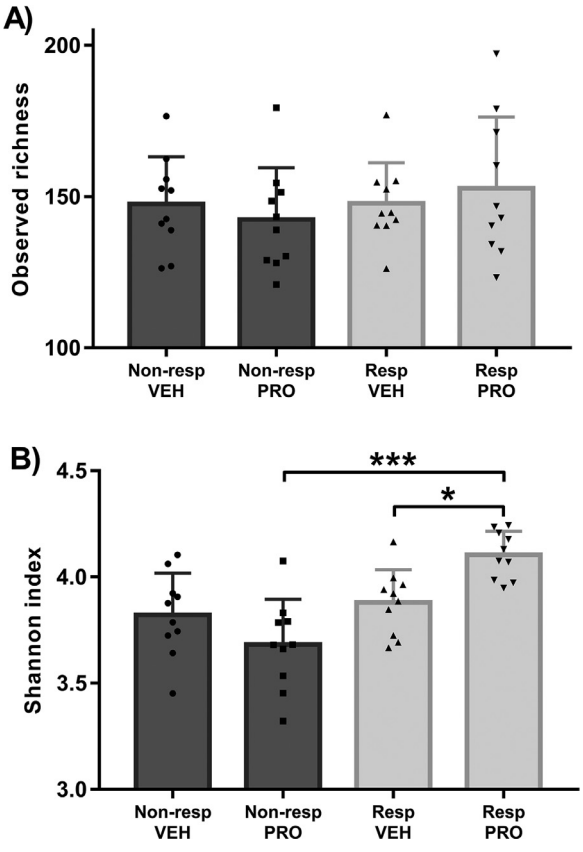

Fig. 3 Diversity of faecal microbiota (mean ± SD). Panel A: No changes in observed richness were seen. Panel B: Probiotic treatment (PRO) increased the Shannon's diversity index in responders (Resp), but not in non-responders (Non-resp). Data were normalised to a sequencing depth of 9,345.
*: $p < 0.05$; ***: $p < 0.001$ (Bonferroni-corrected).

level, *Bacteroidales_S24-7_group* ($p = 0.008$), *Peptococcaceae* ($p < 0.0001$), *Bacteroidaceae* ($p < 0.0001$), *Porphyromonadaceae* ($p < 0.0001$), *Rikenellaceae* ($p < 0.0001$), *Family_XIII* ($p = 0.0003$), *Coriobacteriaceae* ($p = 0.0002$) and *Clostridiales_vadinBB60_group* ($p = 0.01$) were more common in Resp animals, whereas the relative abundance of *Ruminococcaceae* ($p = 0.0008$) and *Deferribacteraceae* ($p = 0.02$) were higher in Non-resp than Resp animals (Table 3 and Fig. 4b).

In total, 906 different ribosomal sequence variants (RSVs) were identified. Of these, 307 were unique to Resp, 345 were unique to Non-resp, and 254 were found in both sets of rats. Group-wise comparisons revealed that 61% of all sequence variants found in at least 20% of the animals differed between VEH-treated Resp and Non-resp (Supplementary Table S4) after FDR correction. Interestingly, approximately two thirds of the differing RSVs belonged to the *Lachnospiraceae* and *Ruminococcaceae* families.

3.7. PRO had limited effects on the phylum, family and RSV level

At the phylum level, Actinobacteria was increased by PRO ($p < 0.0001$; Table 2 and Fig. 4A), and this may likely be caused by the *Bifidobacterium* content of the PRO. Interestingly, PRO were associated with a decrease in Tenericutes in Non-resp ($p < 0.05$), but not in Resp ($p = 0.4$). At the family level, an interaction between responder status and treatment was observed for the *Lactobacillacea* family (Table 3 and Fig. 4B). Specifically, a clear increase was observed in Resp ($p < 0.001$), whereas the relative abundance was unaltered by PRO in Non-resp ($p = 0.9$). The *Streptococcacea* family, that comprises the *Lactococcus* genus, was increased by PRO in both groups ($p < 0.0001$), and this was also the case for the *Bifidobacteriacea* family ($p = 0.001$).

Table 2 Relative bacterial abundance at the phylum level. Data were analysed by zero-inflated negative binomial regression, and predicted number of events are presented as percentage of 9,345 reads with 95% CI. Phylae that were observed in < 25% of animals were excluded. All p-values are Bonferroni-adjusted. n = 10 animals / group.

	Non-resp VEH	Non-resp PRO	Resp VEH	Resp PRO	Resp vs. Non-resp	VEH vs PRO	Resp x PRO interaction
Bacteroidetes	16.7% (14.1-19.4)	17.2% (14.4-20.0)	26.9% (24.6-29.2)	25.0% (21.0-29.0)	**$\chi^2 = 34.1$; $p < 0.0001$**	$\chi^2 = 0.11$; $p = 1.00$	$\chi^2 = 0.46$; $p = 1.00$
Verrucomicrobioa	8.65% (4.79-12.51)	7.72% (4.27-11.16)	5.69% (3.15-8.24)	4.18% (2.31-6.05)	$\chi^2 = 5.12$; $p = 0.21$	$\chi^2 = 0.86$; $p = 1.00$	$\chi^2 = 0.18$; $p = 1.00$
Firmicutes	71.4% (66.4-76.4)	72.5% (67.4-77.6)	64.8% (60.2-69.3)	65.9% (61.2-70.5)	$\chi^2 = 7.22$; $p = 0.06$	$\chi^2 = 0.19$; $p = 1.00$	$\chi^2 = 0.00$; $p = 1.00$
Proteobacteria	1.12% (0.70-1.54)	1.01% (0.63-1.38)	0.82% (0.51-1.13)	0.90% (0.56-1.23)	$\chi^2 = 1.27$; $p = 1.00$	$\chi^2 = 0.00$; $p = 1.00$	$\chi^2 = 0.28$; $p = 1.00$
Actinobacteria	0.11% (0.05-0.16)	0.70% (0.39-1.01)	0.23% (0.13-0.34)	1.38% (0.78-1.98)	**$\chi^2 = 9.61$; $p = 0.02$**	**$\chi^2 = 61.72$; $p < 0.0001$**	$\chi^2 = 0.03$; $p = 1.00$
Tenericutes	1.64% (0.83-2.45)	**0.64% (0.32-0.96)**[#]	0.95% (0.48-1.42)	1.84% (0.93-2.76)	$\chi^2 = 1.02$; $p = 1.00$	$\chi^2 = 0.30$; $p = 1.00$	**$\chi^2 = 10.1$; $p = 0.01$**[#]
Cyanobacteria	0.06% (0.00-0.11)	0.04% (0.00-0.09)	0.33% (0.10-0.56)	0.33% (0.11-0.54)	**$\chi^2 = 18.0$; $p = 0.0002$**	$\chi^2 = 0.12$; $p = 1.00$	$\chi^2 = 0.11$; $p = 1.00$
Deferribacteres	0.11% (0.03-0.19)	0.11% (0.02-0.19)	0.05% (0.01-0.09)	0.01% (0.00-0.02)	**$\chi^2 = 11.0$; $p = 0.008$**	$\chi^2 = 2.84$; $p = 0.83$	$\chi^2 = 2.80$; $p = 0.85$
Unknown	0.15% (0.09-0.22)	0.12% (0.07-0.17)	0.16% (0.09-0.23)	0.33% (0.19-0.48)	**$\chi^2 = 6.61$; $p = 0.09$**	$\chi^2 = 1.05$; $p = 1.00$	$\chi^2 = 5.04$; $p = 0.22$

Non-resp: non-responders; Resp: responders; VEH: vehicle treatment; PRO: probiotic treatment.
[#] "Non-resp PRO" significantly lower than "Non-resp VEH" and "Resp PRO" ($p < 0.05$).

In the analyses of sequence variants, six RSVs were found to be increased by PRO in the Resp group after FDR correction (Supplementary Table S5). Five of these most likely originated from PRO, since their 16 s rRNA DNA sequences were identical with PRO sequences provided by the manufacturer. In Non-resp animals, PRO only increased the abundance of three RSVs originating from the PRO treatment (Supplementary Table S6). Interestingly, the abundance of a single non-PRO RSV from the *Clostridiales Family XIII* was increased by PRO in Resp animals, whereas a single non-PRO RSV from the *Lachnospiraceae* family (*Lachnospiraceae NK4A136 group*) was almost superseded by PRO in Non-resp rats.

3.8. The relative *lactobacillus* genus abundance only rose with PRO in resp

Overall, PRO increased the relative abundance of all three genera (*Lactobacillus, Streptococcus* and *Bifidibacterium)* that comprise the PRO strains in both Resp and Non-resp ($F_{1,36} = 56.9$; $p < 0.0001$; Fig. 5), although the effect did interact with responder status ($F_{1,36} = 13.8$; $p < 0.001$). Indeed, the increase was larger in Resp compared with Non-resp ($p < 0.001$). In the analysis of each genus separately, we confirmed that the *Lactobacillus* genus abundance only rose in the Resp group ($p < 0.001$), but remained unaltered in Non-resp ($p = 1.0$). Although the *Lactococcus* abundance was increased by PRO in both Non-resp ($p = 0.01$) and Resp ($p < 0.001$), the increase also tended to be higher in Resp animals compared with Non-resp animals ($p = 0.05$). For the *Bifidobacterium* genus, however, an equal ascent was generated by PRO in both Resp ($p < 0.001$) and Non-resp ($p < 0.01$) groups. Consequently, no difference was seen between PRO-treated Resp and Non-resp ($p = 0.1$).

4. Discussion

In the present trial *post hoc* analysis, we found that the previously reported antidepressant-like effect of PRO in SD rats was completely absent in another set of rats from the same commercial vendor. We also observed profound differences in the gut microbiota composition between the two sets of rats. Interestingly, the relative faecal abundance of the genera that comprised the PRO was approximately four times higher in the set of responding animals than in the non-responders treated with the same dose of the same PRO.

A plausible explanation for the dissimilar behavioural treatment responses may be the observed differences in the relative faecal abundance of PRO that likely depends on the cohabiting gut microbiota. Although VEH-treated Resp and Non-resp showed the same microbial richness and diversity, numerous differences were observed at the genus and family level, and as many as 61% of all sequence variants were significantly different between the two sets of animals. Concordantly, a clear separation of the two samples of rats was seen in the PCA analysis. The multispecies PRO were composed of strains from the *Lactobacillus*, *Lactococcus* and *Bifidobacteria* genera. Intriguingly, the *Lactobacillus* genus was expanded by PRO in Resp only, and the *Lactococcus* genus abundance tended to be higher in Resp

Table 3 Relative bacterial abundance at the family level. Data were analysed by zero-inflated negative binomial regression, and predicted number of events are presented as percentage of 9,345 reads with 95% CI. Families that were observed in < 25% of animals were excluded. All p-values are Bonferroni-adjusted. n = 10 animals / group.

	Non-resp VEH	Non-resp PRO	Resp VEH	Resp PRO	Resp vs. non-resp (p)	VEH vs PRO (p)	Resp x PRO interaction (p)
Prevotellaceae	0.03% (0.01-0.04)	0.02% (0.00-0.04)	0.04% (0.03-0.06)	0.03% (0.02-0.05)	$\chi^2 = 3.26$; p = 1.00	$\chi^2 = 1.09$; p = 1.00	$\chi^2 = 0.08$; p = 1.00
Verrucomicrobiaceae	8.65% (4.79-12.5)	7.72% (4.27-11.2)	5.69% (3.15-8.24)	4.18% (2.31-6.05)	$\chi^2 = 5.12$; p = 0.47	$\chi^2 = 0.86$; p = 1.00	$\chi^2 = 0.18$; p = 1.00
Bacteroidales_S24-7_group	14.7% (12.3-17.0)	15.3% (12.8-17.7)	20.7% (17.4-24.0)	19.3% (16.2-22.4)	**$\chi^2 = 12.5$; p = 0.008**	$\chi^2 = 0.03$; p = 1.00	$\chi^2 = 0.43$; p = 1.00
Lachnospiraceae	30.0% (24.5-35.5)	34.0% (27.8-40.3)	29.6% (24.2-35.0)	25.9% (21.1-30.6)	$\chi^2 = 2.36$; p = 1.00	$\chi^2 = 0.00$; p = 1.00	$\chi^2 = 1.91$; p = 1.00
Ruminococcaceae	36.5% (31.6-41.5)	31.0% (26.8-35.2)	24.9% (21.5-28.3)	25.7% (22.2-29.2)	**$\chi^2 = 16.9$; p = 0.0008**	$\chi^2 = 0.91$; p = 1.00	$\chi^2 = 2.05$; p = 1.00
Peptococcaceae	0.78% (0.49-1.08)	1.09% (0.68-1.51)	3.37% (2.10-4.64)	3.26% (2.03-4.48)	**$\chi^2 = 43.6$; p < 0.0001**	$\chi^2 = 0.60$; p = 1.00	$\chi^2 = 0.89$; p = 1.00
Bacteroidaceae	1.31% (0.97-1.65)	1.39% (1.03-1.75)	3.67% (2.73-4.60)	3.29% (2.45-4.13)	**$\chi^2 = 52.2$; p < 0.0001**	$\chi^2 = 0.03$; p = 1.00	$\chi^2 = 0.41$; p = 1.00
Alcaligenaceae	0.98% (0.59-1.37)	0.91% (0.55-1.27)	0.69% (0.41-0.96)	0.78% (0.47-1.09)	$\chi^2 = 1.55$; p = 1.00	$\chi^2 = 0.02$; p = 11.0	$\chi^2 = 0.24$; p = 1.00
Porphyromonadaceae	0.59% (0.39-0.80)	0.33% (0.21-0.44)	1.45% (0.96-1.94)	1.09% (0.72-1.46)	**$\chi^2 = 35.7$; p < 0.0001**	$\chi^2 = 6.36$; p = 0.23	$\chi^2 = 0.77$; p = 1.00
Peptostreptococcaceae	0.76% (0.25-1.27)	1.65% (0.49-2.81)	1.24% (0.46-2.02)	2.04% (0.75-3.32)	$\chi^2 = 1.08$; p = 1.00	$\chi^2 = 3.57$; p = 1.00	$\chi^2 = 0.17$; p = 1.00
Clostridiaceae_1	0.82% (−0.05-1.70)	2.10% (0.22-3.98)	1.00% (0.25-1.75)	1.39% (0.22-2.57)	$\chi^2 = 0.05$; p = 1.00	$\chi^2 = 1.93$; p = 1.00	$\chi^2 = 0.44$; p = 1.00
Erysipelotrichaceae	1.29% (0.73-1.84)	1.01% (0.58-1.44)	1.47% (0.84-2.10)	2.12% (1.22-3.03)	$\chi^2 = 4.06$; p = 0.88	$\chi^2 = 0.08$; p = 1.00	$\chi^2 = 1.94$; p = 1.00
Rikenellaceae	0.15% (0.10-0.20)	0.17% (0.11-0.23)	1.04% (0.70-1.37)	1.21% (0.83-1.60)	**$\chi^2 = 129$; p < 0.0001**	$\chi^2 = 0.63$; p = 1.00	$\chi^2 = 0.02$; p = 1.00
Lactobacillaceae	0.22% (0.12-0.32)	0.36% (0.19-0.52)	0.18% (0.08-0.27)	1.27% (0.72-1.81)	$\chi^2 = 4.82$; p = 0.56	**$\chi^2 = 25.6$; p < 0.0001**	**$\chi^2 = 9.26$; p = 0.047** [#]
Christensenellaceae	0.64% (0.28-0.99)	0.50% (0.22-0.77)	1.25% (0.59-1.91)	1.06% (0.50-1.62)	$\chi^2 = 6.72$; p = 0.19	$\chi^2 = 0.56$; p = 1.00	$\chi^2 = 0.02$; p = 1.00
Bifidobacteriaceae	0.02% (0.00-0.07)	0.37% (0.11-0.63)	0.01% (0.00-0.02)	0.59% (0.22-0.97)	$\chi^2 = 0.23$; p = 1.00	**$\chi^2 = 16.0$; p = 0.001**	$\chi^2 = 0.97$; p = 1.00
Streptococcaceae	0.06% (0.02-0.10)	0.38% (0.19-0.57)	0.08% (0.02-0.14)	0.87% (0.47-1.28)	$\chi^2 = 3.15$; p = 1.00	**$\chi^2 = 46.3$; p < 0.0001**	$\chi^2 = 0.75$; p = 1.00
Enterobacteriaceae	0.18% (0.08-0.27)	0.08% (0.04-0.11)	0.08% (0.04-0.11)	0.10% (0.05-0.15)	$\chi^2 = 1.30$; p = 1.00	$\chi^2 = 1.20$; p = 1.00	$\chi^2 = 4.57$; p = 0.65
Family_XIII	0.15% (0.08-0.22)	0.18% (0.10-0.25)	0.37% (0.20-0.55)	0.40% (0.23-0.57)	**$\chi^2 = 14.4$; p = 0.003**	$\chi^2 = 0.26$; p = 1.00	$\chi^2 = 0.05$; p = 1.00
Micrococcaceae	0.07% (0.04-0.09)	0.08% (0.05-0.11)	0.11% (0.07-0.15)	0.13% (0.08-0.17)	$\chi^2 = 6.39$; p = 0.23	$\chi^2 = 0.76$; p = 1.00	$\chi^2 = 0.03$; p = 1.00
Deferribacteraceae	0.11% (0.03-0.19)	0.11% (0.02-0.19)	0.05% (0.01-0.09)	0.01% (0.00-0.02)	**$\chi^2 = 11.0$; p = 0.02**	$\chi^2 = 2.84$; p = 1.00	$\chi^2 = 2.80$; p = 1.00
Coriobacteriaceae	0.03% (0.02-0.05)	0.05% (0.03-0.08)	0.10% (0.07-0.13)	0.10% (0.07-0.14)	**$\chi^2 = 19.4$; p = 0.0002**	$\chi^2 = 1.41$; p = 1.00	$\chi^2 = 1.29$; p = 1.00
Clostridiales_vadinBB60_group	0.05% (0.00-0.13)	0.02% (0.00-0.04)	0.14% (0.01-0.26)	0.28% (0.04-0.52)	**$\chi^2 = 11.5$; p = 0.01**	$\chi^2 = 0.06$; p = 1.00	$\chi^2 = 2.53$; p = 1.00
Unknown	2.00% (1.30-2.69)	1.00% (0.65-1.35)	2.28% (1.48-3.07)	3.14% (2.05-4.23)	**$\chi^2 = 12.8$; p = 0.007**	$\chi^2 = 1.07$; p = 1.00	$\chi^2 = 8.01$; p = 0.09

Non-resp: non-responders; Resp: responders; VEH: vehicle treatment; PRO: probiotic treatment.

[#] "Resp PRO" significantly higher than all other groups (p < 0.001).

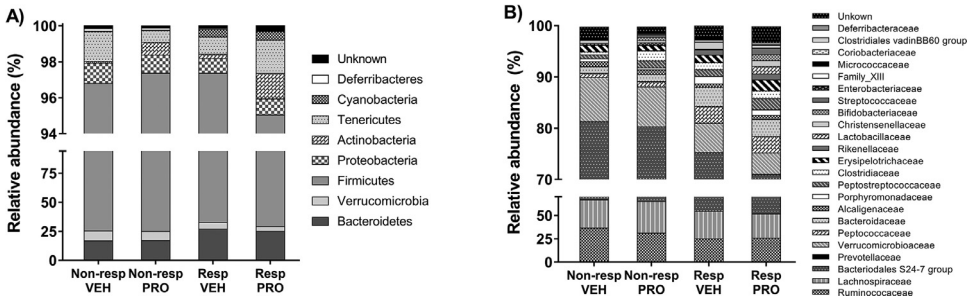

Fig. 4 16 s rRNA gene amplicon sequencing of faecal microbiota. Relative abundance on the phylum (panel A) and family (panel B) level. Cf. Table 2 & 3 for the corresponding statistical analyses.
Non-resp: non-responders; Resp: responders; VEH: vehicle treatment; PRO: probiotic treatment.

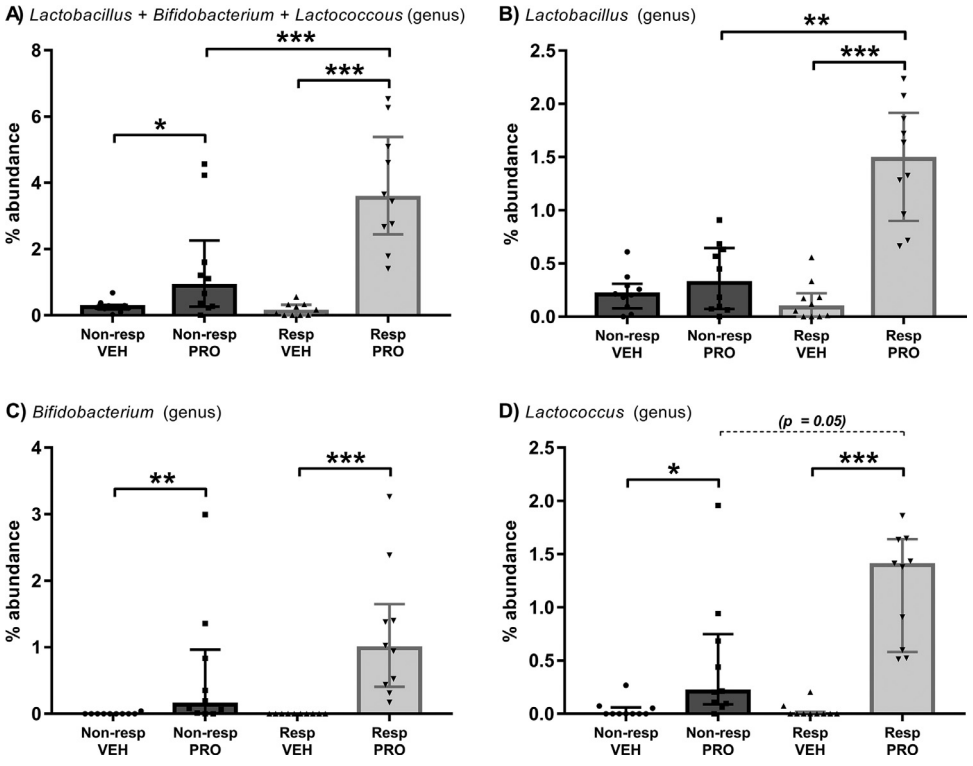

Fig. 5 Relative abundance of the genera that comprised the probiotic strains (medians with interquartile range). Panel A: Responders (Resp) had a larger increase in relative abundance of all three genera compared with non-responders (Non-resp). Panel B: The *Lactobacillus* genus abundance was increased by probiotic treatment (PRO) in Resp, but not in Non-resp. Panel C: The *Bifidobacterium* genus abundance was increased by PRO in both Resp and Non-resp. Panel D: PRO increased the relative abundance of the *Lactococcus* genus, and the rise tended to be larger in Resp compared with Non-resp.
*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (Bonferroni-corrected).

compared with Non-resp. On the contrary, the *Bifidobacteria* genus was found at similar levels in Resp and Non-resp groups. Therefore, it appears likely that the gut microbiota of Non-resp was incompatible with survival, multiplication or colonisation of particularly the *Lactobacillus* and *Lactococcus* strains. At least some *Lactobacillus* species have been shown to transiently colonize the host and multiply on the colonic surface at a rate that nearly counterbalances its shedding (Alander et al., 1999; Firmesse et al., 2008). Our findings are consistent with the notion that such a multiplication only took place in Resp. On the other hand, failure to detect ingested PRO in the faeces has been described in several studies (Firmesse et al., 2008; Fuentes et al., 2008; Hsiao et al., 2013; Smith et al., 2014), and it was speculated that this absence may be due to a too low dose of PRO or a limited amount of bacteria surpassing the hostile environment of the upper gastrointestinal tract (gastric acid, bile).

Since the treatment in our study continued until 18–24 h before faecal sample collection, we can't evaluate whether a permanent colonisation took place. Nonetheless, the gut microbiota is believed to be fundamentally based on competitive interactions between the residing microbes (Coyte et al., 2015), and competitive exclusion of certain strains

of PRO is a plausible mechanism behind our present results. Although we have not shown a causal relation between the relative faecal abundance of PRO and the behavioural response, our findings are novel and definitely warrant further investigations.

Only minor effects of PRO on the gut microbiota composition were found except for the clear enrichment of the PRO strains themselves. Although no changes in observed richness was seen, PRO caused a higher bacterial diversity index (Shannon) in Resp animals, whereas no changes were observed in Non-resp animals. This finding substantiates the presence of an interaction between PRO and the inherent microbiota. However, whether this finding is causally related to the behavioural outcome or simply indirectly reflects the reduction in *Lactobacillus* multiplication, needs to be resolved. Interestingly, measures of diversity have also been a matter of investigation in clinical trials on depression. One study reported increased diversity in depressed patients (Jiang et al., 2015), two studies found unaltered levels (Naseribafrouei et al., 2014; Zheng et al., 2016), and, lastly, one study detected reduced diversity (Kelly et al., 2016b). Thus, the direct effect of diversity in relation to depression is currently unclear. Another interesting observation was an apparent depletion of the Tenericutes phylum by PRO in Non-resp only. Two studies have linked a lower Tenericutes level to anxiety and depression-related behaviour in animals (Kang et al., 2014; Yang et al., 2017), and the depletion of Tenericutes could potentially counteract any beneficial effect of PRO. Beyond that, other studies confirm that PRO do not usually cause noticeable changes in the microbiota composition (Kristensen et al., 2016). Rather, they have been shown to affect the function of the microbiota as reflected by metatranscriptomic and metabolomic analyses (Eloe-Fadrosh et al., 2015; McNulty et al., 2011), and they may also interact with the immune system (Ashraf and Shah, 2014). Indeed, we have previously shown that the same PRO led to an altered pattern of cytokine production by peripheral leukocytes and a reduced cerebral T lymphocyte CD4/8 ratio (Abildgaard et al., 2017a; Abildgaard et al., 2017b). Our present data, therefore, confirm that the antidepressant-like effect of PRO does not rely on seminal structural changes in the gut microbiota composition.

A strength of the present study is that the two separate experiments were conducted as identical as possible. The only apparent difference was the gut microbiota composition, which may be affected by a number of factors, including age, host genetics and diet (Rodriguez et al., 2015; Turnbaugh et al., 2010; Zhernakova et al., 2016). All animals had the same age at the arrival at our facilities (four weeks), and this is of major importance since age is also known to affect the ability of PRO to adhere to the gastrointestinal mucus (Kirjavainen et al., 1998). According to the commercial breeder, the two sets of rats were of the same SD substrain, and, consequently, major genetic differences are unlikely. Also, all rats were fed the same purified diet. Regardless, we have previously shown that PRO had antidepressant-like effect in animals on a high-fat diet as well (Abildgaard et al., 2017a; Abildgaard et al., 2017b), and this circumstance may suggest that the effect of PRO is relatively robust to major differences in dietary conditions. Owing to the high temporal stability of faecal samples at −80 °C (Kia et al., 2016; Shaw et al., 2016), it also appears unlikely that the differences found could be explained by different storage time of samples. Another notion, that needs to be considered, is experimenter effects that have been suggested to interact with outcomes of animal behavioural tests (Holman et al., 2015). However, in the present study, all behavioural tests were conducted by the same experienced experimenter in accordance with our standardised operating protocols, and the analyses of test videos were performed by an experimenter blinded to treatment groups. The highly similar behavioural read-outs observed in VEH-treated Resp and Non-resp also corroborate our standardised behavioural assessments. Lastly, a final issue that needs to be addressed, relates to the quality of the PRO. It has been described that deviations from the product declaration are commonly seen with commercially available PRO (Kolacek et al., 2017). According to the manufacturer, the PRO utilised in the present study fulfilled all quality controls. In addition, we verified that the stated CFU count of the PRO was correct, and assays of the carbohydrate fermentation patterns of random colonies also confirmed the presence of PRO strains, including four different *Lactobacillus* strains.

A limitation of the present study includes the fact that we were not able to control the early life of the animals due to their originating from a commercial breeding facility. The observed profound differences between the two sets of animals may render early environmental changes probable. Indeed, it is generally believed that the gut microbiota is established at a very early age and remains relatively stable throughout life (Rodriguez et al., 2015). According to the breeder, no changes in husbandry/housing practices or diets had taken place. However, although all rats used in our study were bred in the same animal facility, the dams of the first set were relocated from a geographically different animal facility belonging to the same breeder. This is likely to explain the observed differences in the gut microbiota composition owing to the considerable vertical transmission of the microbiome (Inoue and Ushida, 2003). Interestingly, we observed a minor difference in body weight at arrival between the two sets of rats, although all groups gained the same weight during the study. It has recently been shown that manipulations of the gut microbiota of dams may affect the weight gain of their pups (Tulstrup et al., 2018). The observed difference in body weight is, therefore, compatible with that notion that the microbiota of the dams differed in a physiologically important manner. Unfortunately, we did not collect pre-intervention faecal samples, and, consequently, we are not able to evaluate how or if the microbial composition shifted over time. Although it remains highly speculative whether such early life environmental factors could also entail the observed treatment failure of PRO independently of any gut microbiota alterations, these concerns should always be kept in mind in every pre-clinical research study that includes animals from commercial vendors.

In the present study, we only used the forced swim test to evaluate depressive-like behaviour owing to its high validity in the qualitative screening of anti-depressants (Kara et al., 2018). Consequently, we do not know whether the two cohorts of animals would differ in other behavioural tests, such as measures of anhedonia. Also, since the mechanisms underlying the anti-depressant effect of probiotics are

unclear, it would be difficult to select a suitable positive control.

Our present findings pose two important perspectives. Firstly, they make it emphatically clear that challenges for the reproducibility of research on PRO exist, also within the behavioural field. It may not come as a surprise given earlier reports that the initial state of the gut microbiota affects outcome in metabolic and immunologic pre-clinical research (Denning et al., 2011; Robosky et al., 2005; Ussar et al., 2015). Poor reproducibility of especially animal experiments is a recognised major concern (Collins and Tabak, 2014; Perrin, 2014), and the microbiota probably plays a significant role. Actually, it has already been suggested that the gut microbiota of research animals should be standardised (Bleich and Hansen, 2012). Consequently, at least when it comes to studies on manipulations of the microbiota with anti- or probiotics, a description of the gut microbiota composition ought to be provided. Secondly, our present results imply that PRO may need to be personalised and that the same treatment may not fit everyone. Still, it remains unknown whether this issue may be applied to clinical studies as well. Yet, it has been proposed that the rat gut microbiota is more similar to the human microbiota than the mouse microbiota (Nguyen et al., 2015), and this notion is partly corroborated by our present data indicating that the two dominant families *Lachnospiraceae* and *Ruminococcaceae* constituted 50–70% of the microbes, which is similar to the human situation (Flint et al., 2012). Notwithstanding, specific PRO that have yielded promising results in animal models do not always turn out to be effective in humans (Kelly et al., 2016a), and the cohabiting gut microbiota composition could potentially explain such inconsistencies, but further research on this topic is warranted.

In conclusion, we found that the antidepressant-like effect of PRO as previously described was completely absent in another set of rats in the same experimental setup. We also observed a lower relative faecal abundance of the genera that comprise the PRO in Non-resp than in Resp, especially the *Lactobacillus* species, and this may be caused by competitive exclusion by the inherent gut microbiota that was found to differ noticeably between the two sets of animals. Our results, therefore, lend inspiration to further research into a conceivably causal association between the relative faecal abundance of PRO and behavioural outcomes. The findings have wide implications and may suggest that PRO should be personalised. Importantly, differences in the gut microbiota composition may potentially hold a strong limitation to the reproducibility of research, including studies on PRO.

Conflict of interest

All authors report no conflict of interest.

Contributors

AA, GW and SL designed the study. AA and TK collected and analysed the data. All authors undertook data interpretation. AA wrote the first draft of the manuscript. All authors have approved the final version of the manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.euroneuro.2018.10.011.

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