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Heat-killed lactobacilli alter both microbiota composition and behaviour

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ABSTRACT

Recently it has been proposed to expand the definition of psychobiotics (beneficial bacteria (probiotics) or support for such bacteria (prebiotics) that positively impact mental health) to any exogenous influence whose effect on the brain is bacterially-mediated. This definition would include inactivated microorganisms with anxiolytic and antidepressant effects. The use of inactivated microorganisms has several advantages over living organisms, including no risk of infection in vulnerable individuals and ease of use in terms of storage and delivery options. It has been reported that consumption of inactivated microorganisms can affect behaviour, particularly in chronic or prolonged stress situations, but effects on healthy populations have not been investigated to the same extent. Also, only limited data is available on the effects of inactivated microorganisms on the microbiota of healthy individuals (animal or human). Therefore, we investigated the effect of feeding a standard mouse chow which incorporates ADR-159, a heat-killed fermentate generated by two *Lactobacillus* strains, on the behaviour and microbiota of healthy mice.

Prolonged consumption of ADR-159 diet had no adverse effect on anthropometrics or general health, but the ADR-159 fed animals demonstrated increased sociability and lower baseline corticosterone levels (stress hormone). The diet also led to subtle but significant changes in the microbiota, with less abundant taxa being most affected. The behavioural, biochemical and microbiological results provide a new light on the impact of inactivated microorganisms and their metabolites on the social behaviour and microbiota of healthy mice.

1. Introduction

Anxiety and depression are common, albeit heterogeneous, disorders in all societies [1,2]. The prevalence of depression and other common mental disorders is increasing, and it is estimated that 4.4% (322 million) of the global population suffer from depression, and 3.6% (264 million) suffer from anxiety disorders [3]. Given that currently available pharmacological and psychological treatments are suboptimal in terms of moderate short-term benefits, severe side effects and age limitations [4,5], there is an urgent need for the development of new approaches.

Many clinical and preclinical studies have linked the gut microbiota to both behaviour and mood [6–9]. Changes in the gut microbiota composition in patients with psychiatric disorders have been widely reported [10,11], yet it is unclear if the microbiota changes are causal or as an appropriate response to the disorder. Faecal microbiota transfer (FMT) from depressed patients to germ-free (GF) mice [12] and antibiotic treated rats [2] suggest that the microbiota have a causal role in certain facets of these disorders including depressive-like and anxiety-like behaviour. Further research has demonstrated that the influence of gut microbiota on central events is bidirectional, with behavioural events such as stress profoundly influencing the gut microbiota complexity and diversity. Interestingly, the behavioural and neurobiological disturbances in stressed mice can be reversed by microbial colonization [13], probiotic bacteria [6,11,14,15] and prebiotic-rich diets [16], supporting the hypothesis that the gut microbiome plays a fundamental role in the pathology of stress-related disorders [8]. Not surprisingly, the brain-gut-microbiota axis has been put forward as a novel paradigm for understanding and treating mental illness [10,17–19].

Psychobiotics (beneficial bacteria (probiotics) or support for such

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bacteria (prebiotics) that influence bacteria–brain relationships) exert anxiolytic and antidepressant effects characterised by changes in emotional, cognitive, systemic, and neural indices [19]. Under normal circumstances the gut microbiota help to maintain general health by maintaining intestinal peristalsis, mucosal integrity, pH balance, immune priming and protection against invading pathogens. However, these microbes can influence events in the brain and influence behaviour and mood through mechanisms including microbially derived bioactive molecules (amino acid metabolites, short-chain fatty acids, neuropeptides and neurotransmitters), mucosal immune and enteroendocrine cell activation, as well as vagal nerve stimulation [1,19–21]. Recently it has been suggested to expand the definition of psychobiotics to any exogenous influence whose effect on the brain is bacterially-mediated [19]. A number of studies have investigated the potential of inactivated microorganisms to confer a benefit on humans or animals; these preparations have also been referred to as 'paraprobiotics' or 'ghost probiotics' [22,23]. The application of inactivated microorganisms could be considered as superior to living organisms on several grounds; (i.) longer shelf life, (ii.) no cold chain requirements, (iii) ease of storage and transport and therefore also potential applications in less developed regions, (iv.) no risk of infection in vulnerable individuals, (v.) no translocation of bacterial-virulence or antibiotic-resistance cassettes if the genetic material has been destroyed by the inactivation step, and (vi.) no loss of activity when used in conjunction with antibiotics or anti-fungal agents [22,23].

Health benefits reported for human and/or animal subjects for preparations containing inactivated microorganisms include treatment of diarrhea, colitis, liver diseases induced by alcohol, respiratory diseases, intestinal lesions, visceral pain, inflammation, modulation of the immune system, impacts on intestinal microbiota, and bacterial translocation [23]. This list is constantly expanding and includes psychobiotic effects. Heat-inactivated *Lactobacillus gasseri* CP2305 had a favourable effect on physical symptoms and sleep quality in a clinical study with a limited number of healthy medical students during a cadaver dissection course [24]. In another clinical study consumption of washed heat-treated CP2305 cells re-suspended in a dairy drink (1×10^{10} cell equivalents per day) by volunteers with low (≤ 4 times per week) or high (≥ 10 times per week) frequencies of bowel movement resulted in improved scores for number of evacuations and faecal odours [25].

Attempts were made in some of these clinical studies to investigate the effect of CP2305 on the gut microbiota but only limited changes were reported [25,26]. Moreover, applied T-RFLP analysis (including qPCR validation) led to contradictory results [25,26].

Although health benefits have been associated with inactivated microorganisms, most data shows these benefits are linked to their ongoing consumption, rather than having a prolonged effect. In this study, we investigated the impact of including inactivated lactobacilli into the murine diet on microbiota and behaviour. ADR-159 was selected as it contains a heat-killed fermentate generated by two *Lactobacillus* strains, *Lactobacillus fermentum* and *Lactobacillus delbrueckii*. Development of new preparations containing inactivated microorganisms (or broadening the application range of existing products) with psychobiotic characteristics will facilitate the use of more natural approaches with potentially fewer side effects compared to traditional treatments for stress related illness.

2. Materials & methods

2.1. Diet preparation

ADR-159 contains a co-fermentate of *Lactobacillus fermentum* and *Lactobacillus delbrueckii*, which also includes culture medium (lactose monohydrate, casein peptone, yeast extract, sodium acetate, dipotassium phosphate). The fermentate is subjected to an extended high-temperature treatment post production. 1 g of ADR-159 contains a minimum of 60 billion intact bacterial cells. ADR-159 is a proprietary product produced and supplied by Adare Pharmaceuticals SAS, Route de Bu, 78550, Houdan, France. ADR-159 was incorporated into standard mice chow (2018S Teklad Global 18% Protein Rodent Diet (Envigo)) to a final concentration of 5%, equivalent to approximately 3×10^9 cell bodies per gram of chow.

2.2. Animals and housing conditions

24 eight-week old male C57BL/6 mice were used. Male mice were selected to avoid the effects of sex-hormone mediated fluctuations on corticosterone during the oestrous cycle in female mice. Animals were randomly divided into six enriched (cardboard tubes and shredded paper) cages, each holding 4 mice, and allowed to acclimatise for one week in the biological services unit, University College Cork, prior to dietary administration. Animals were fed *ad libitum* throughout the whole study with 2018S Teklad Global 18% Protein Rodent Diet (Envigo) 3 cages (12 animals) or diet supplemented with 5% ADR-159 3 cages (12 animals). The holding room was temperature (21 ± 1 °C) and humidity (55 ± 10%) controlled and under a 12-h light/dark cycle. All experiments were conducted in accordance with the European Directive 86/609/EEC, the Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork. Animals were weighed once weekly, and faecal samples were collected for 16S rRNA gene metagenomics. Additionally, feed consumption was monitored by weighing contents of the food hoppers.

After 3 weeks of designated diet all animals were subjected to the same battery of behavioural tests as indicated in Fig. 1. Specifically, the open field/novel object recognition commenced on day 24 of dietary intervention, followed by marble burying test, 3-chamber test, elevated plus maze, carmine red test, tail suspension test and forced swim test. The order of tests were run in incremental order of increasing stress level as determined by European guidelines. Each behaviour is mutually exclusive, has adequate controls, and animals were allowed to recover for at least 2 days after each test to reduce the risk of an accumulation effect of behaviour-related stress. We cannot exclude an accumulative effect of baseline stress as a consequence of handling and experimentation. However, this residual stress is controlled for by subjecting both ADR-159 and control fed animals to identical testing regime. Finally, after 8 weeks of diet and concomitant behaviour testing mice were sacrificed.

2.3. 16S rRNA gene metagenomics – microbiota analysis

2.3.1. DNA isolation, amplification, indexing, normalisation and sequencing

Faecal samples were stored at −80 °C until processing. DNA

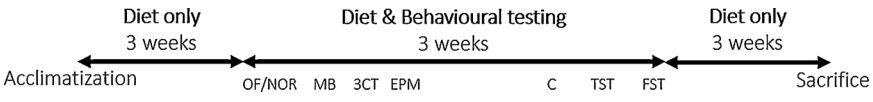


Fig. 1. Schematic representation of behaviour animal experiment. OF/NOR, open field/novel object recognition (starts on day 24 of the diet); MB, marble burying (day 29 of the diet); 3CT, 3 chamber test (day 31 of the diet); EPM, Elevated plus maze (day 33 of the diet); C, Carmine red (day 38 of the diet); TST, tail suspension test (day 39 of the diet); and FST, forced swim test (days 43 and 44 of the diet).

isolation was performed using QIAamp Fast DNA Stool mini kit (Qiagen) according to the manufacturers recommendations, followed by measurement of DNA concentration using Qubit dsDNA HS Assay Kit and running 5 µl sample on a gel for quality assessment. V3 and V4 region of 16S rRNA genes were amplified using Phusion Polymerase Master Mix and V3-V4 (Forward 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′; Revers 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) primers (98 °C 30 s; 25 cycles of 98 °C 10 s, 55 °C 15 s, 72 °C 20 s; 72 °C 5 min). Amplicons were checked for quality and quantity by Qubit dsDNA HS Assay Kit and running on gel, and cleaned using Ampure XP magnetic beads. 5 µl of cleaned amplicon was used as template for Index PCR using Phusion Polymerase Master Mix and Nextera XT Index Kit (95 °C 30 s; 8 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 30 s; 72 °C 5 min). Indexed amplicons were cleaned using Ampure XP magnetic beads and checked for quality and quantity by Qubit dsDNA HS Assay Kit and running on gel. All samples were normalised to 4 nM, followed by pooling together 5 µl of each sample and sending for MiSeq sequencing to GTAC (Germany).

2.3.2. 16S rRNA gene Data analysis

2.3.2.1. Bioinformatic analysis. First, Nextera adapters were removed using Cutadapt 1.9.1 [27] as well as the first 5 bp and low-quality bases (PHRED quality lower or equal than 28) were trimmed using Trimmomatic [28]. Merging all paired FASTQ files, quality filtering by expected error rate, removal of singletons, and clustering sequences into operational taxonomic units (OTUs) was performed using UPARSE [29]. After that, a second chimera removal, based on the Ribosomal Database Project database (RDP v. 14 [30]), was done using USEARCH v8.1 according to the UCHIME algorithm [31]. Taxonomical classification of the 16S rRNA gene sequences was performed using the RDP classifier v. 2.12 [32] against the RDP v 14, and SPINGO [33] for species level.

Sequencing data discussed in this publication have been deposited in Sequence Read Archive (SRA) and are accessible under accession number PRJNA490647.

2.3.2.2. Statistical tests. Prior to any experimention, the R package rnaseqpower [34] was used to estimate (coefficient of variation) of the microbial community and MicroPower R package [35] to assess the power of the PERMANOVA test. It was determined that 12 mice were sufficient to adequately power the microbiota analysis to a power of 90% with an alpha of 0.05.

Several statistical tests were performed to analyse the microbial composition and diversity between control and treatment per week. First, to evaluate potential differences in the microbial composition between control and treatment per week, principal coordinates analyses (PCOA) plots using Bray-Curtis dissimilarity were constructed considering the number of sequences. Then, permutational multivariate analyses of variance (PERMANOVA; [36]) were performed to assess differences in the microbial composition between control and treatment and per weeks. The maximum number of iterations was set to 1000 in all analyses. As PERMANOVA do not discriminate between location and dispersion null hypotheses [37], generalised linear models for multivariate abundance data [38] was performed as suggested by Warton et al. [37]. Second, to identify all species that are differentially expressed in the treatment respect to the control, two tests were performed: Analysis of Communities (ANCOM [39]) was performed using the less stringent correction mode (equivalent to False Discovery Rate), and, differential expression analysis based on the Negative Binomial distribution (DESeq2 [40]) using Wald test. Third, to evaluate potential differences in the microbial diversity between control and treatment per week, nested ANOVA were performed when considering the Shannon and the inverse Simpson indexes. Shannon index was calculated using the decimal logarithm. Fourth, to describe the temporal trend of the microbial diversity over time, a Mann–Kendall trend test [41,42] was performed. All these statistical approaches were carried out at an alpha level of 0.05, except DESeq2 analyses, which were carried out at an alpha level of 0.01, and were performed in R v. 3.4.1 [43] using the *ancom.R* [39], DESeq2 [40], *Kendall* [44], *labdsv* [45], *mvabund* [38], and *vegan* [46] packages.

2.4. Behaviour tests

Individual animals (or cages of animals) were tested in random order to prevent bias. All animals (12 per group) were subjected to the same series of behavioural tests. Power calculations were performed using G-power software based on in-house data and published data from our group, and based on the assumption that each behaviour was mutually exclusive, and approved by biostatisticians on the Animal Experimentation Ethics Committee of University College Cork. Animals were allowed to acclimatise to the test room for 30 to 60 min prior the test.

2.4.1. Open field / Novel object recognition (OF/NOR)

The NOR is a well-established paradigm used to assess cognitive function and memory in laboratory rodents [47]. Mice were placed in the middle of a grey plastic rectangular box (40 × 32 × 23 cm, L × W × H) under a dimly light, 60 lx at the level of the arena, for 10 min to assess the response to a novel stressful environment and locomotor activity (habitation phase; OF part of the test). After 24 h mice were placed again in the box, this time with the two identical objects for a total time of 10 min (familiar phase). Mice were placed in the middle of the box at the mid-point of the wall opposite the sample objects. After another 24 h, mice were placed for a total time of 10 min in a box in which one of the two identical objects were substituted with a novel object (novel phase). After each of the 10 min phases mice was returned to its home cage. Box and objects were cleaned with 70% alcohol to avoid any cue smell between each trial. Experiments were videotaped using a ceiling camera for further parameter analysis. Interaction with the objects, including any contact with mouth, nose or paw were scored using a digital stop watch. Climbing on top of the object was not considered an interaction. % Preference for novel object over familiar object was calculated. Additionally, Ethovision XT software v 8.5 (Noldus, TrackSys, Nottingham, UK) was used to measure distance travelled and time spend in central zone (50% of the surface) during habitation phase.

2.4.2. Marble burying (MB)

The MB test is an established test to quantify behaviours relating to neophobia – the fear of having new things in their environment [48]. Mice were individually placed in a novel large mouse cages (RM2; 38 × 25 x 18 cm, L x W x H), filled up with sawdust (5 cm) and 20 marbles on top of it (five rows of marbles regularly spaced 2 cm away from the walls and 2 cm apart). Thirty minutes later, the number of marbles buried for more than 2/3 of their surface was scored by two experimenters blind to condition. A higher number of marbles buried represents higher levels of anxiety. Box and marbles were cleaned with alcohol 70% to avoid any cue smell between each trial.

2.4.3. Three-chambered social test (3CT)

The 3CT is a well-validated ethologically relevant model that assesses social interaction between sex-matched conspecifics and allows for readouts of social novelty and social preference in mice [6]. The social testing apparatus was a rectangle, three-chambered box (36 by 19 cm). Dividing walls (partitions) were made with semi-circular openings (3.5 cm high, 4.5 cm wide) allowing access into each chamber. Two identical wire cup-like cages, with a bottom diameter of 9 cm, 17 cm in height and bars spaced to allow contact but prevented fighting, were placed inside each side chamber in bilaterally symmetric positions. The test has three phases of 10 min each: 1) habitation 2) mouse versus object 3) novel mouse versus familiar mouse.

Experiments were videotaped using a ceiling-mounted camera. Analysis of videos was performed using two stop watches for direct interaction and Ethovision XT software v 8.5 for time spend in each of the chambers as well as speed and distance travelled during each phase. For the first phase the test mouse was placed into the middle chamber and allowed to explore the entire box (with empty small wire cages inside) for a 10-min habituation session. After the habituation period, the test mouse was contained in the middle section by turning the partitions over for short interval while an object (rubber duck) is placed in mesh cage in one side chamber and an unfamiliar conspecific male mouse (no prior contact with the test subject) in the mesh cage in other side chamber. During phase two, the partitions are turned around allowing mice to explore the entire box for 10 min. During the third phase the object was replaced with an unfamiliar mouse serving as a novel mouse and in the other chamber the mouse used in phase two was kept the same, now serving as familiar mouse. After every trial, all chambers and cup-like wire cages were cleaned with 70% ethanol and dried to prevent olfactory cue bias and to ensure proper disinfection. The amount of time spent exploring the object or mouse in each chamber was evaluated. The location of the unfamiliar mouse in the left vs right side chamber was systematically alternated between trials. Lack of innate side preference was confirmed during the initial 10 min of habituation to the entire arena. Time (in seconds) of interaction with each of the wire cages was measured and analysed individually for each phase.

2.4.4. Elevated plus maze (EPM)

The EPM is used to measure anxiety-like behaviour, or to determine the efficacy of anxiolytic drugs [20,49]. The set up was made of a grey plastic cross-shaped maze 1 m elevated from the floor, comprising two open (fearful) and two closed (safe) arms (arm length 30 cm; arm width 5 cm; wall high 20 cm or no wall). Experiments were performed under red light (˜5 lx). Mice were individually placed into the center of the maze (hub) facing an open arm (to avoid direct entrance into a closed one) and were allowed 5-min free exploration. Maze was cleaned with alcohol 70% to avoid any cue smell between each trial. Experiments were videotaped using a ceiling camera for further parameters analysis using Ethovision XT software v 8.5. The time spent, the number of entries (entrance in an arm was defined as all four paws inside the arm) and latency (delay to enter) in each arm were measured along with speed and distance moved.

2.4.5. Carmine red (C)

The animals were housed individually without access to food or water for 3 h, after which time animals were gavaged with 100–200 µl of non-digestible Carmin red (6% solution in 0.5% methylcellulose). After gavage, cages with individual animals were monitored at 20 min intervals until the first occurrence of red faecal pellet for each of the animals (maximum up to 7 h), after which animal was returned to the home cage. Transition time (min) was calculated as follows:

$$Transition\ \ time = (time\ \ of\ \ detection\ \ of\ \ 1st\ \ red\ \ pellet) - (time\ \ of\ \ gavage)$$

2.4.6. Tail suspension test (TST)

The TST is a model of learned helplessness and is a widely used assay for screening potential antidepressant therapeutics [50]. Mice were individually hung by the tail with adhesive tape (2 cm from tail tip) to a grid bar 50-cm elevated from the floor. Two animals, separated by visual divider, were subjected to the test for 6 min in parallel. Experiments were videotaped using a numeric tripod-fixed camera and data were further independently scored (Video Media Player software) by two experimenters blind to conditions. The time spent immobile (s) was scored during last four minutes of the test; immobility is defined as the absence of voluntary or escape-orientated movement (grooming was considered mobility).

2.4.7. Forced swim test (FST)

The FST is a rodent behavioural test used for evaluation of antidepressant drugs, antidepressant efficacy of new compounds, and experimental manipulations that aimed at rendering or preventing depressive-like states [51,52]. Mice were individually placed in a clear glass cylinder (22 cm diameter, 45 cm high), containing 15-cm-depth water (23–25 °C). Water was changed between each animal tested to remove odors. The test lasted 6 min and experiments were videotaped using a ceiling camera; data were further scored using the videos (Video Media Player software) by two experimenters blind to conditions. The time of immobility (s) as well as time of passive swim (s) were scored over last 4 min of the test. The immobility is defined as a total absence of movement, while passive swim is defined as floating and movement without direction (motions to maintain the head above the water). The cessation of escape-oriented behaviour is often termed 'behavioural despair'. Blood samples for measurement of Corticosterone were collected directly before the FST (swims from 9.30 to 11.30) and 30, 60, 90 and 120 min after the test from each animal.

2.4.7.1. Blood & plasma collection. End of the tail of free moving mice was held against the workbench, using a single edge razor blade a diagonal incision of 1–2 mm long was made from the tip of the tail (tail-tip procedure takes approximately 20 s). Approximately 75 µl blood was collected in a heparinised glass capillary by gently milking the blood from the tail to avoid blood coagulation. Blood was expelled into collection tube and centrifuged at 3500 × g at 4 °C temperature for 15 min. Plasma was carefully aspirated and stored at −80 °C.

2.4.7.2. Corticosterone assay. Samples were analyzed in duplicate in a single assay using Corticosterone ELISA Kit (Enzo Life Scientific) according to manufacturer's recommendations. Steroid displacement reagent was used to inhibit steroid binding to proteins. Plasma from base line samples (T0) was diluted 1:20 while for remaining samples 1:40; the threshold detection was less than 32 pg/ml (standard curve 32 - 20,000 pg/ml). Light absorbance was read with a multi-mode plate reader (Synergy 2, BioTek Instruments, Inc.) at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of corticosterone in either standards or samples. The concentrations are expressed in ng/ml.

2.4.8. Statistical analysis for behavioural and physiological responses

Statistical analyses were conducted using SPSS software (IBM Corporation). Behavioural data above or below two standard deviations from the mean were classified as outliers and were removed prior to statistical analysis. In case of interaction time during 3CT, a two standard deviation criteria was applied to discrimination ratio rather than to individual interaction times. Data that were normally distributed according to Shapiro-Wilk test were analysed using parametric tests, an independent *t*-test (when necessary with correcting for data violating the condition of homogeneity of variances). Behavioural non-parametric data were analysed using the non-parametric Mann-Whitney *U* test or Wilcoxon Signed Rank test. Body weight, change in body weight and corticosterone data were analysed using a repeated measures analysis of variances (ANOVA). Statistical significance was set at $p < 0.05$. Initial weight of animals (week 0) was considered as possible covariate for each of the behavioural measurements.

3. Results

3.1. Behaviour

Control and ADR-159 fed animals were subjected to a battery of behavioural tests as illustrated in Fig. 1.

3.1.1. Open field (OF)

ADR-159 fed animals spent less time in the central zone during the

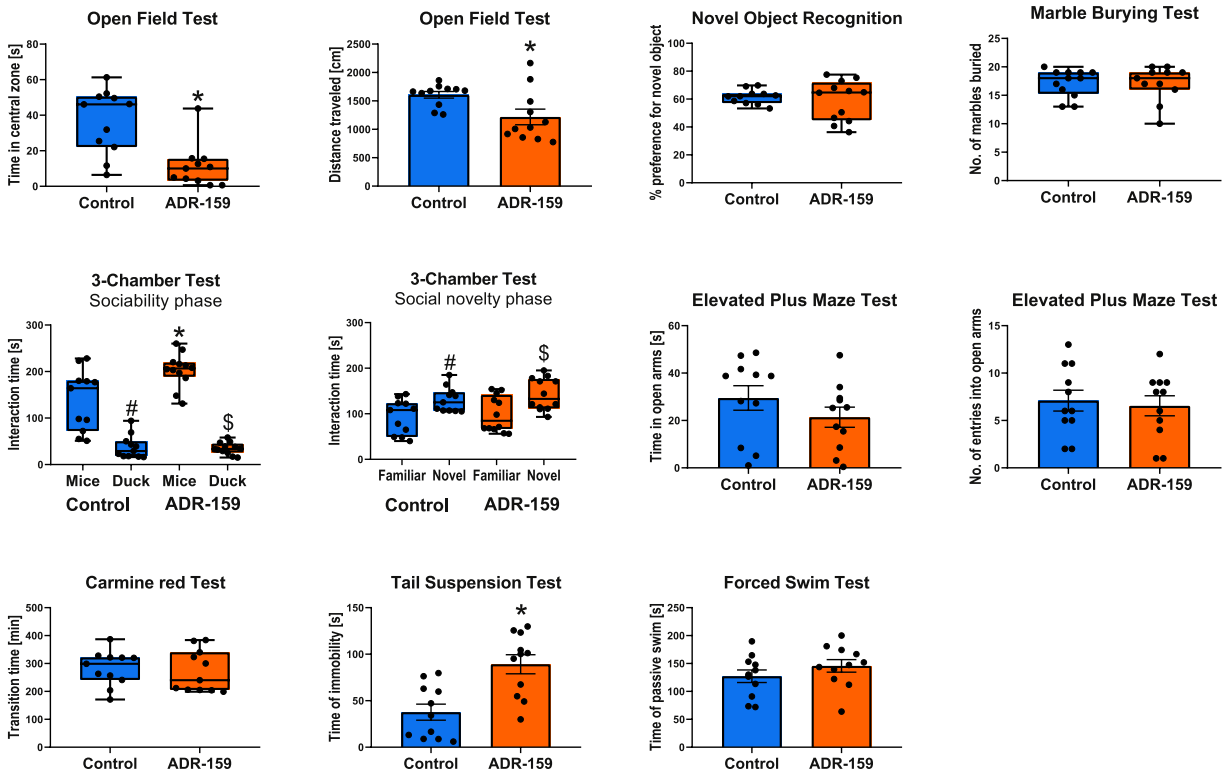


Fig. 2. Average measures for control (blue) or ADR-159 (orange) fed animals regarding: time spent in central zone during OF, distance traveled during OF, preference for novel object during NOR, number of buried marbles, interaction time with (un)familiar mouse and a rubber duck during sociability and social novelty phase of 3CT, time spend in open arms during EPM, number of entries into open arms during EPM, gut transition time during carmin red test, and immobility time during TST and FST. Box and wiskers graphs represent comparisons of median and min-max range for non-parametric data, while bar graphs represent mean and SEM for comparisons of parametric data. * indicates significant difference compared to control fed animals. Differences in interaction time mice vs. duck and familiar vs. novel were significant for both control (#) and ADR-159 ($) fed animals.

OF test (Mann-Whitney *U* test; p = 0.001) (Fig. 2), but also displayed a decrease in exploratory behavioure as determined by locomotor activity (t_{20} = 2.615, p = 0.021). This suggests that ADR-159 may have either an anxiogenic or mild sedative / calming effect under certain conditions.

3.1.2. Novel object recognition (NOR)

Cognitive function and memory was evaluated using the well-established NOR paradigm [47,53]. There was no effect of diet on preference for a novel object (t_{21} = 0.586, p = 0.564) (Fig. 2).

3.1.3. Marble burying (MB)

In this study, ADR-159 fed animals showed no significant difference in number of buried marbles (Mann-Whitney *U* test; p = 0.833) (Fig. 2), suggesting that ADR-159 neither enhanced nor suppressed neophobia in this behavioural model.

3.1.4. Three chamber test (3CT)

During the sociability phase [6], both ADR-159 and control fed animals showed a preference for a conspecific animal over an inanimate object (Fig. 2). However, the ADR-159 fed animals showed an increase in direct interaction time (t_{21} = −2.877, p = 0.011) (Fig. 2) as well as in time spent in the chamber (t_{22} = −4.149, p < 0.0005) (data not shown) with the sex-matched conspecific mice.

Interestingly, when an inanimate object was replaced with a novel sex-matched conspecific mouse during the social novelty phase, providing an opportunity to interact with either this novel mouse or a familiar mouse, we observed no difference in behaviour (Fig. 2, data not shown). Both groups of animals showed preference for a novel mouse over a familiar one, as would have been expected based on the social novelty preference paradigm.

There was no significant difference during the habitation (data not shown) phase in time spent in either of the side chambers (each containing an empty basket). However, animals on the control diet spent significantly longer time in the central zone.

3.1.5. Elevated plus maze (EPM)

Both ADR-159 and control fed animals spend a majority of the time in the closed arms of the EPM (Fig. 2). This is a typical behaviour as mice display a strong approach-avoidance behaviour towards the open arms of the EPM and prefer the closed arms. The difference in time spent in either open arms (t_{20} = 1.210, p = 0.240), frequency of entering open arms (t_{20} = 0.358, p = 0.724) and latency to enter open arms (Mann-Whitney *U* test; p = 0.797) were not statistically significantly different between ADR-195 and control fed animals (Fig. 2).

3.1.6. Carmine red (C)

The impact of diet on the gut transition time was tested. There was no difference in gut mobility between ADR-159 and control fed animals (Mann-Whitney *U* test; p = 0.748) (Fig. 2).

3.1.7. Tail suspension test (TST)

The TST model uses immobility as an index of learned helplessness. In our study, ADR-159 fed animals showed a significant increase in time of immobility during the TST (t_{20} = −3.859, p = 0.001) (Fig. 2), suggesting either an increase in anxiety-like profile or a sedative effect of ADR-159 on behaviour.

3.1.8. Forced swim test (FST)

The FST was employed to determine the physiological response to acute stress, as well as being used as an animal model of behavioural despair. There was no significant effect on immobility (t_{22} = −0.927,

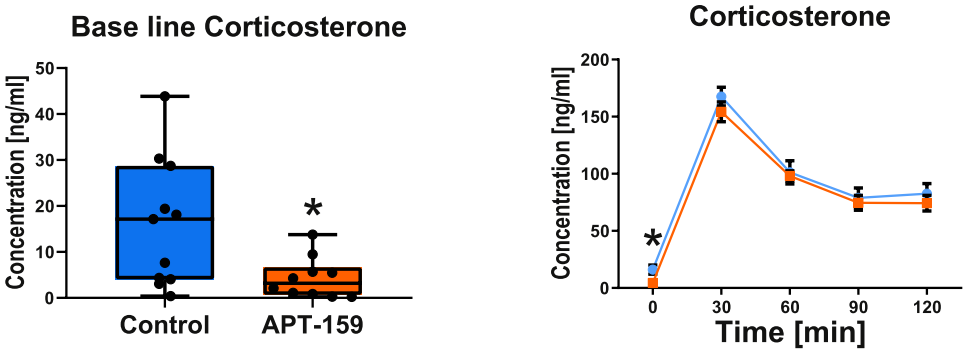


Fig. 3. Endocrinology changes in control (blue) and ADR-159 (orange) fed animals. (Left) base line corticosterone levels and (Right) change in corticosterone levels before (T0) and 30, 60, 90 and 120 min after FST. Wiskers represent min-max range, error bars represent SEM. * indicates significant difference compared to control fed animals.

p = 0.364) or swimming behaviour between control and ADR-159 fed animals (Fig. 2).

3.2. Endocrinology

After 6 weeks of dietary intervention, ADR-159 fed animals had significantly lower base line corticosterone levels (Mann-Whitney *U* test; p = 0.019) (Fig. 3, left). Following the acute stress induced by forced swim test (FST), corticosterone levels increased in both control and ADR-159 fed animals. Corticosterone is a stress related hormone (equivalent to human cortisol) whose levels increase in response to stress. ADR-159 had no effect on corticosterone levels, an index of the physiological response to acute stress, neither in terms of the initial increase due to the stress of the test, nor the rate of the corticosterone decrease during recovery. Repeated ANOVA for 11 animals on control diet and 10 ADR-159 fed animals revealed no statistically significant differences between treatments ($F(4,76) = 0.383$, p = 0.820) (Fig. 3, right).

3.3. General effects of ADR-159 diet

The body weight of animals was different between control and ADR-159 fed animals during the course of experiment (repeated measures ANOVA; $F_{1,20} = 4.580$ p = 0.045) (Figure S1 left). Post-hoc analysis using an independent *t*-test showed significantly lower weight of ADR-159 fed animals at 4, 5 and 7 weeks of diet. However, the change in body weight (repeated measures ANOVA; $F_{1,20} = 0.667$ p = 0.424) (Fig. S1 right) between control and ADR-159 fed animals was not statistically significantly different. Therefore, it was concluded that *ad libitum* ADR-159 diet provided sufficient caloric intake.

In our statistical analysis of behaviour we added initial body weight as a covariate and there was no effect.

3.4. Impact of ADR-159 on the microbial community

Considering the potential of the microbiome to affect behaviour via the brain-gut-microbiota axis, we investigated the impact of ADR-159 on the murine microbiota using 16S rRNA gene amplicon sequencing. At the phylum level, Bacteroidetes and Firmicutes (followed by Verrucomicrobia) dominated the microbiota of both control and ADR-159 fed animals (data not shown), with no clear difference between groups. At a genus level, the microbiota of all animals were dominated by unclassified *Porphyromonadaceae* and unclassified *Lachnospiraceae* (Fig. 4 and Fig. S2 for individual animals), reflecting the division at the phylum level. At the genus level differences were observed between control and ADR-159 fed animals. In particular, the relative abundance of *Alistipes* and *Odoribacter* was consistently reduced in ADR-159 fed animals, while *Prevotella* was increased. The lack of major differences in microbiota composition between the two groups of animals suggests that prolonged consumption of ADR-159 did not cause a major disruption to the initial microbiota of healthy animals, but rather led to subtle modifications. We can speculate that these subtle modifications could nonetheless have a substantial effect on the animals, as many characteristics (such as probiotic properties) are strain rather than species or genus specific. It is important to notice that microbiota of both control and ADR-159 fed animals underwent changes during the course of the experiment, however our main focus is comparison between control and ADR-159 fed groups.

To further investigate microbiota diversity between individuals (β diversity) we used a principal coordinate analysis (PCoA) approach together with PERMANOVA analysis. It revealed that, despite the high overall similarity in the dominant taxa, a clear shift in microbiota composition occurred during the eight weeks of the trial. At the beginning all animals clustered together (Fig. 5), but after diet differentiation a gradual modification of microbiota composition occurred. This was obvious even after one week, and continued in a time-dependent manner leading to a clear separation based on β diversity by the end of the feeding period.

Changes in relative abundance between control and ADR-159 fed animals were analysed at the OTU level, with 229 OTUs differently abundant in at least one time point. To focus on the long term effect of the dietary intervention we looked particularly at 41 OTUs (representing at least 15 species) showing abundance changes in the period from 5 to 8 weeks of the experiment but only including those OTU's which showed changes in at least two of the final three time points (Fig. 6). The majority of the OTUs belonged ether to Bacteroidetes or Firmicutes, showing both increased and decreased abundance in ADR-159 animals. In particular, some OTUs belonging to unclassified *Porphyromonadaceae*, unclassified *Lachnospiraceae* and *Barnesiella intestinihominis* showed increased abundance in ADR-159 animal while other OTUs from the same taxa were reduced.

The most long-lasting changes (at least five of eight time points) which were detected after one week included a decrease in one of the OTUs of unclassified *Lachnospiraceae* along with increase in abundance of two other OTUs belonging to the same taxa. These were followed by changes detected after two weeks of the diet with one OTU within the unclassified *Porphyromonadaceae* showing a strong increase in abundance while another showed a strong decrease.

Selected OTUs which significantly decreased in abundance in ADR-159 fed animals included *Barnesiella* spp., *Alistipes* spp., unclassified *Rikenellaceae*, unclassified *Bacteroidales*, *Clostridium piliforme*, *Eubacterium siraeum* and unclassified bacteria. An increase was observed for OTUs belonging to *Clostridium fusiformis*, *Flavonifractor plautii*, unclassified *Ruminococcaceae*, unclassified *Clostridiales*, and unclassified *Proteobacteria*.

α diversity was evaluated using both Shannon and Inverse Simpson indexes (Figure S3). Despite variations in the index levels, overall diversity was considered stable, particularly according to the Mann-Kendall trend test.

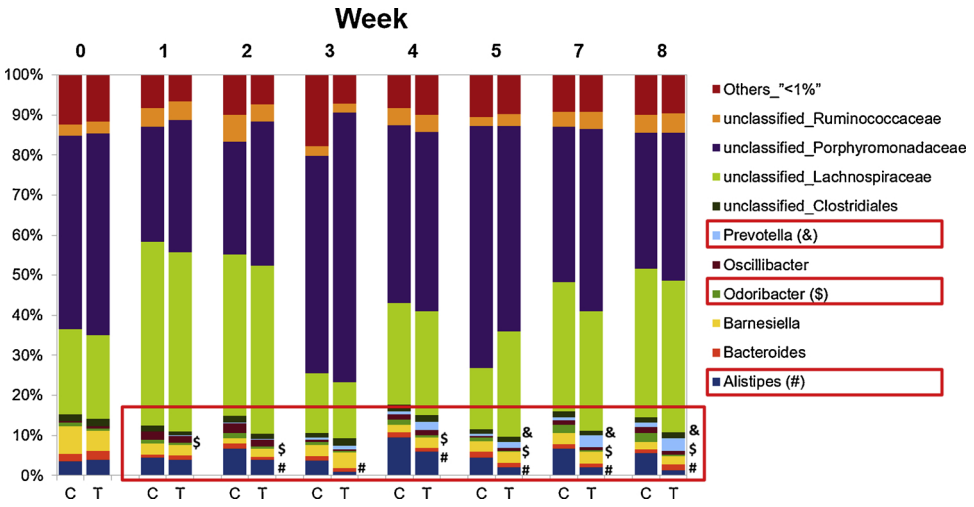


Fig. 4. The median relative abundance of major OTUs (assigned to genera level) based on 16S rRNA gene sequences across time points in control (C) and ADR-159 (T) fed animals. Colour legend presented at the side. For simplicity, all OTU's (assigned to genera level) with abundances below 1% were grouped together. Red squares indicate taxa undergoing significant changes. & indicates significant increase in *Prevotella*; $ indicates significant decrease in *Odoribacter*; # indicates significant decrease in *Alistipes* in ADR-159 fed animals.

4. Discussion

The objective of this study was to evaluate the effect of ADR-159 on a series of behaviours relevant to stress, depression, anxiety, social interaction and cognition, and also to determine any impact on microbiota composition. The intervention comprised feeding male mice chow supplemented with 5% ADR-159, a heat-killed lactobacilli fermentate, while control animals received standard chow.

Overall, our results suggest that the ADR-159 diet has a 'calming' or sedative effect on healthy males. This is perhaps best illustrated by the marked reduction in baseline corticosterone levels in ADR-159 fed animals, and the decrease in locomotor activity in the open field test. Corticosterone, an endocrine biomarker used as an index of physiological response to acute stress, levels are affected by complex network of interactions including a sympathetic nervous system and HPA axis that safeguards the body's homeostasis, preparing it for the 'fight or flight' response, when exposed to internal or external stressors [54]. The HPA axis is regulated by negative feedback loop that prevents from over or under secretion of cortisol / corticosterone [54]. Despite the lower base line corticosterone levels in ADR-159 fed animals the regulation of HPA axis in response to acute stress (FST) does not seem to be affected as both control and ADR-159 fed animals followed the same pattern of induction and reduction of the corticosterone levels. These findings suggest that the resting stress level of these animals is lower in ADR-159 fed animals, but that in response to an acute stress response the ADR-159 fed animals are still capable of mounting an appropriate stress response to the stimulus. A number of interventions have been shown to have an effect on cortisol/corticosterone levels. Administration of the prebiotic (Bimuno-galactooligosacharides) to healthy volunteers over a 3-week period significantly lowered the waking cortisol levels in saliva [55]. Similarly, 'Probio'Stick' (probiotic preparation of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) lowered 24 h urinary cortisol levels in healthy volunteers after 30 days' consumption [56]. The administration of the probiotic mixture of *Lactobacillus rhamnosus* R0011 (95%) and *Lactobacillus helveticus* R0052 (5%) reduced corticosterone levels in mouse pups, after their elevation following maternal separation (3 h per day; days 4–19) [57]. In addition, administration of the probiotic *Bifidobacterium pseudocatenulatum* during maternal separation abolished the effects of maternal separation on the microbiota and animal behaviour (EPM, OF) in adulthood [58]. Besides live bacteria, dead bacteria were also reported to affect cortisol levels. In fact, ingestion of washed and heat-inactivated cells of

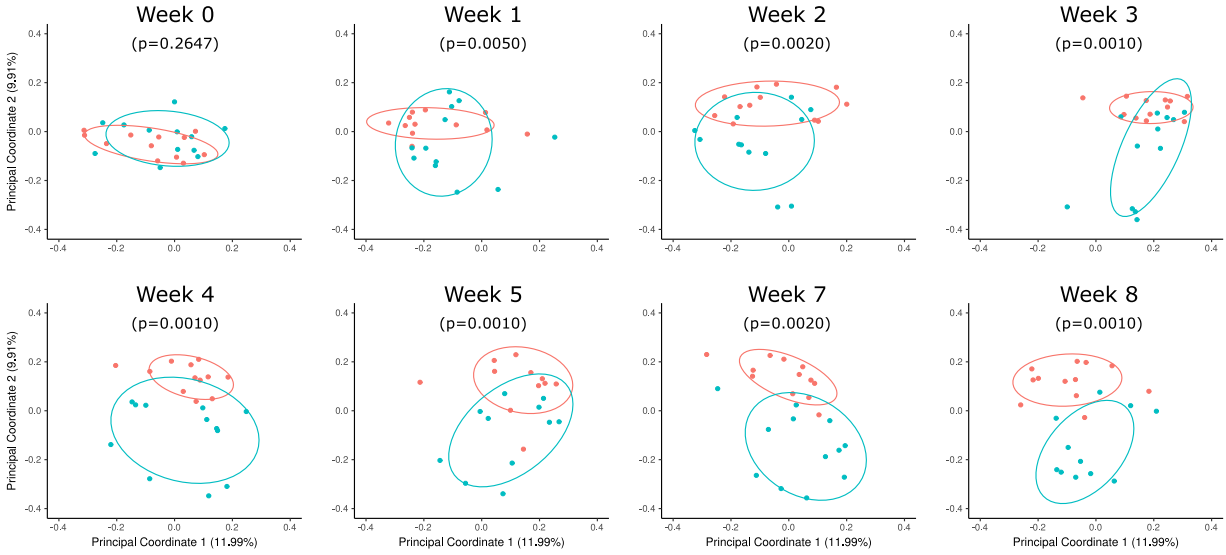


Fig. 5. PCoA plots of microbiota composition before (week 0) and during the diet intervention with control (pink) and ADR-159 (blue) fed animals. Each dot represents individual animal at each given time point. Ellipses represents the confidence interval of every group at 75%.

Species	OUT_Id	Week							
		0	1	2	3	4	5	7	8
Barnesiella intestinihominis	OTU_783							2.02	2.15
	OTU_872				-2.41		-3.22	-3.71	
Barnesiella spp	OTU_886						-3.76	-3.54	
unclassified Porphyromonadaceae	OTU_654				-3.59	-3.78	-3.72	-5.43	-3.28
	OTU_3038				-2.47	-2.67		-2.64	-2.81
	OTU_1689				-2.20			-3.30	-2.52
	OTU_62							-2.48	-2.46
	OTU_23				1.19			1.97	1.33
	OTU_1131			2.61	1.93			2.73	2.35
	OTU_2765				2.54			2.79	2.52
	OTU_1510							2.25	2.83
	OTU_1128							2.83	2.89
	OTU_629					3.01	3.36	2.91	3.26
	OTU_482			4.78	4.43	4.61	4.82	6.11	5.23
	OTU_888						-3.82	-3.34	
Alistipes spp	OTU_1263							-3.82	-5.74
	OTU_912							-3.80	-4.05
	OTU_1159							-3.24	-2.90
unclassified Rikenellaceae	OTU_3035				-4.19	-3.71	-3.73	-5.96	
unclassified Bacteroidales	OTU_66				-4.50	-3.42	-3.39	-4.92	
Clostridium fusiformis	OTU_124						1.67		2.83
Clostridium piliforme	OTU_520							-3.91	-3.77
unclassified Lachnospiraceae	OTU_31							-4.49	-6.00
	OTU_2099		-5.45	-6.57	-4.31			-4.71	-5.86
	OTU_222						-4.34	-4.86	-5.82
	OTU_274						-3.72	-4.28	-4.01
	OTU_437							-3.75	-3.40
	OTU_748							-2.03	-2.18
	OTU_165		1.48	1.93				2.04	1.70
	OTU_75		2.45	2.86		1.94		2.12	1.93
	OTU_65		2.16	2.65	1.81	1.88	1.98	3.62	2.68
	OTU_158						2.76		2.69
	OTU_20					3.06	4.31	2.95	3.62
	OTU_164						-4.19	-3.13	
Flavonifractor plautii	OTU_219						2.40	2.14	2.80
Eubacterium siraeum	OTU_225					-3.20		-3.04	-3.49
unclassified Ruminococcaceae	OTU_174					2.11	2.05	1.82	1.70
unclassified Clostridiales	OTU_297						3.07	3.31	2.64
	OTU_1327							4.55	3.65
unclassified Proteobacteria	OTU_139							6.31	4.81
unclassified Bacteria	OTU_2512				-3.89			-4.86	-3.22

Fig. 6. Selected differentially abundant OTUs within control and ADR-159 fed animals following 8 week of feeding trial. OTUs were selected based on criteria of differential abundance at a minimum two out of the final three time points (weeks 5, 7 and 8).

Lactobacillus gasseri CP2305 supressed the escalation of salivary cortisol levels in medical students (prior to an examination) as compared to the placebo and was also reported to improve stress-related adverse behaviours associated with chronic stress and sleep disturbance [59].

In the forced swim test ADR-159 had no effect on swimming or immobility, suggesting no effect of the ADR-159 on depressive-like behaviour. However, in the tail suspension test, ADR-159 significantly increased immobility time. Classically this model is used to screen for antidepressant-like compounds that decrease immobility, translating to a positive effect of these compounds against learned helplessness. When we combine this finding with the decrease in locomotor or exploratory activity in the novel object arena model, these data suggest a sedative or 'calming' like effect of ADR-159. Furthermore, should the ADR-159 promote depressive-like behaviour, one might expect a significant comorbid increase in anxiety and social behaviours which was not apparent. ADR-159 enhanced social interaction in social cognition (preference) while it had no effect on social novelty nor on cognitive performance in the novel object recognition test. Even though mice are social creatures, they still may experience some degree of 'social anxiety', to unknowns such as territory, size and/or aggression when encountering and possibly interacting with the unknown mouse. Therefore, increase sociability could be interpreted as increased tolerance to such 'social stress / anxiety'.

Corticosterone levels may be affected by factors such as stress, for instance rats stressed during their prenatal life showed increased and extended elevation of corticosterone levels in response to a physical stressor [60]. Those rats also demonstrated a higher abundance of *Oscillibacter* spp., *Anaerotruncus* and *Peptococcus* and a lower abundance of *Streptococcaceae.* In fact, higher corticosterone levels at the end of 1 h long acute restraint stress was correlated with low abundance of cyanobacteria (Cyano4COd-2 uncultured bacteria) [60]. In another study, rats stressed early in life in adult life had blunted corticosterone response to a FST [21], and a follow up study revealed that this response correlated with high *Akkermansia* spp. and low *Rikenella* spp. abundance in adulthood [61]. Still, in our study change in corticosterone levels following the FST both control and ADR-159 fed animals followed the same pattern.

ADR-159 also led to significant changes in the microbiota (Fig. 5). Shift in microbiota of ADR-159 fed animals started already in the first week of the diet and continued during the course of experiment. In time difference in microbiota between standard and ADR-159 fed animal was increasing so that after eight weeks of diet a complete separation was observed (Fig. 5, bottom right).

At the same time microbiota composition at the genus showed only

subtle changes revealing that ADR-159 lead to changes of less abundant taxa while maintaining the overall composition. The main changes included *Odoribacter*, *Alistipes*, *Prevotella*, *Porphyromonadaceae*, and *B. intestinihominis*.

Already after one week of diet abundance of *Odoribacter* was reduced in the ADR-159 fed animals and this reduction was maintained until the end of the experiment. Scott et al. [62] showed an increased in genus *Odoribacter* in aged mice (20–21 months) correlated with increases in anxiety-like behaviours and deficits in spatial memory compared to younger mice (2–3 months) [62]. In other pre-clinical studies, the caecal abundance of both *Odoribacter* and *Alistipes* in female BALBc mice increased in response to prolonged stress (gridded cages) at the same time stressed animals showed a longer immobility time during the TST, and higher number of immobility episodes [63]. In fact, a stable decrease in representatives of *Alistipes* was observed in ADR-159 fed animals starting from week two onward. Interestingly, increase in *Alistipes* has been observed in patients with major depressive disorder [64], chronic fatigue syndrome [65] and IBS [66]. It has also been suggested that *Alistipes* is associated with inflammation in clinical studies [65], and therefore potentially linked to depression through inflammatory pathways [17]. Additional, potentially beneficial changes in perspective of autistic disorders are reduction in *E. siraeumi* [67] and prolonged increase in *Prevotella* [68]. Considering the effect of ADR-159 on reducing both *Alistipes* and *Odoribacter*, as well increasing *Prevotella*, we postulate that these microbiota effects may well drive the positive effects observed in terms of reduced stress hormone (corticosterone) and increased sociability.

In a number of pre-clinical and clinical studies, increases in *Porphyromonadaceae* have been associated with anxiety-like behaviour in older mice [62], and in major depressive disorder in humans [64]. However, our results suggest that the story of *Porphyromonadaceae* might be more complicated, as selected members of the unclassified *Porphyromonadaceae* seem to be involved in the microbiota separation in ADR-159 and control fed animals. Similarly, an increase in the relative proportion of *B. intestinihominis* among metabolically active bacteria was observed in autistic children [67], while a decrease of *B. intestinihominis* was observed in children with Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) [67]. Interestingly we see OTUs representing *B. intestinihominis* showing both increased and decreased abundance in ADR-159 fed animals, suggesting that individual strains or clonal complexes may have a crucial role in observed changes in behaviour.

Limited number of clinical studies administering different heat-killed *Lactobacillus* strains have previously demonstrated effects on microbiota composition. In adult female volunteers consuming a heat-inactivated *Lactobacillus kunkeei* YB38 a decrease in intestinal levels of *Bacteroides fragilis* group members was reported [69]. Meanwhile, a clinical study with a small group of young children fed a cow's milk fermented product containing the heat-killed probiotic strain *Lactobacillus paracasei* CBA L74 showed an increase in butyrate producers (such as *Oscillospira* and *Faecalbacterium*), *Bacteroides* and certain *Roseburia* and *Blautia* oligotypes [70]. However, even when administering the same heat-inactivated *L. gasseri* CP2305a contradictory observation of decreased [25] or increased [26] abundance of *Clostridium* cluster IV (including *E. siraeum* and *F. plautii* [71]) was reported. In fact we observed decreased abundance of *E. siraeum* and increased abundance of *F. plautii* in ADR-159 fed animals. While oral treatment with lysate of *Lactobacillus casei* DN-114 001 cells in a pre-clinical study was associated with changes in the mouse intestinal microbiota composition, the changes prior infection within the placebo group seem to be more pronounced than in the treatment group [72].

Overall, we can conclude that prolonged consumption of ADR-159 diet had no adverse effect on overall health of male mice. ADR-159 diet had a significant effect on behaviour, and subtle but distinct changes in the composition of the microbiota. The overall impact was to reduce the resting corticosterone levels and exhibit behaviours consistent with calming, albeit in a healthy male animal model. In future studies, it would be of interest to study the response of female mice to ADR-159, to determine if sex-depended differences exist and to disentangle the respective roles of sex and treatment and their interaction on microbiota and subsequent behavioural effects [73,74]. Findings from limited number of studied (including ours) indicate need for further study into effects of heat-killed fermentates on human health and microbiota. The behavioural, biochemical and microbiological tests performed with the animals in the experimental series described here provide a new light on the effects of inactivated bacteria and their metabolites on the microbiota and social behaviour of healthy mice.

Declaration of interest

Alicja K. Warda, Timothy G. Dinan and Colin Hill are inventors of a patent application filed covering aspects of the work presented herein.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2018.12.047.

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