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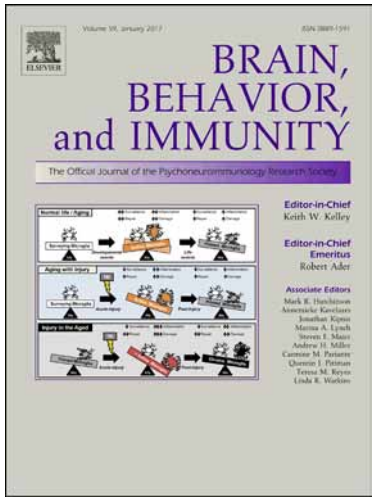

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Revisiting Metchnikoff: Age-related Alterations in Microbiota-Gut-Brain Axis in the Mouse

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Abstract

Over the last decade, there has been increased interest in the role of the gut microbiome in health including brain health. This is by no means a new theory; Elie Metchnikoff proposed over a century ago that targeting the gut by consuming lactic acid bacteria such as those in yogurt, could improve or delay the onset of cognitive decline associated with ageing. However, there is limited information characterising the relationship between the behavioural and physiological sequelae of aging and alterations in the gut microbiome. To this end, we assessed the behavioural, physiological and caecal microbiota profile of aged male mice. Older mice (20-21 months old) exhibited deficits in spatial memory and increases in anxiety-like behaviours compared to younger mice (2-3 months old). They also exhibited increased gut permeability, which was directly correlated with elevations in peripheral pro-inflammatory cytokines. Furthermore, stress exacerbated the gut permeability of aged mice. Examination of the caecal microbiota revealed significant increases in phylum TM7, family Porphyromonadaceae and genus *Odoribacter* of aged mice. This represents a shift of aged microbiota towards a profile previously associated with inflammatory disease, particularly gastrointestinal and liver disorders. Furthermore, Porphyromonadaceae, which has also been associated with cognitive decline and affective disorders, was directly correlated with anxiety-like behaviour in aged mice. These changes suggest that changes in the gut microbiota and associated increases in gut permeability and peripheral inflammation may be important mediators of the impairments in behavioural, affective and cognitive functions seen in ageing.

1. Introduction

Over 100 years ago, Nobel Prize winner Elie Metchnikoff hypothesised that beneficial bacteria contained in fermented foods could influence health and delay cognitive decline by manipulating the intestinal environment (Mackowiak 2013, Cryan and Dinan 2015). Although popular at the time, the theory was largely ignored by the medical community until a recent resurgence in interest over the past 20 years (Mackowiak 2013). The gut microbiome is now recognised to play a critical role in health and disease, and this is especially true at the extremes of life (Brussow 2013, Borre et al. 2014, Candela et al. 2014, Diaz Heijtz 2016, Dinan and Cryan 2016). Indeed, changes in microbial composition have been shown to have a distinct impact on health outcomes in infants and in the elderly and also is thought to play a key role in modulating gut-brain axis function, influencing brain and behaviour (Jeffery and O'Toole 2013, Borre et al. 2014). However, there is limited information on how ageing regulates this axis. With advances in healthcare, longevity has markedly increased; currently it is estimated that within 50 years, approximately 20% of the world population will be classified as elderly (Ellison et al. 2015). Ageing is associated with a number of behavioural and physiological changes, including physical decline, altered mood and cognitive impairments (Ellison et al. 2015, Eshkoor et al. 2015, Joyce and Reich 2015). Although we cannot stop the march of time, the manner in which one ages can vary greatly; thus there is significant research interest in understanding the biological basis of healthy ageing (Li and Schmiedek 2002).

Gastrointestinal (GI) disorders are frequently reported in elderly individuals, with 35-40% of patients reporting at least one GI complaint (Tran and Greenwood-Van Meerveld 2013). These include malnutrition as a result of poor nutrient absorption, reduced gut motility, associated with constipation, diverticulosis from structural weakening of the GI tract, and increased susceptibility to colon cancer (Langille et al. 2014). Ageing in humans and laboratory animals is also related to increased intestinal permeability and increased colonic cytokine expression resulting in chronic systemic inflammation which has been termed “inflammaging,” (Tran and Greenwood-Van Meerveld 2013, Deleidi et al. 2015, Peterson et al. 2015).

The mechanisms underlying the increased intestinal permeability and peripheral inflammation are still unclear, but the gut microbiota is thought to play a critical role. It is now widely recognised that the gut microbiota is a key modulator of homeostasis, and perturbation of its composition can result in gut dysfunction. Furthermore, the composition of the gut microbiota in both humans and laboratory animals varies across the lifespan (Rehman 2012, Biagi et al. 2013, Jeffery and O'Toole 2013, Langille et al. 2014, Lynch et al. 2015). In general, as an individual ages, the microbiota shifts in numerous ways that may predispose one to inflammation.

In addition to the physical deterioration of the body, such as increased risk of disease and frailty, there is great interest in the effects of ageing on the central nervous system (Lee et al. 2000, Prenderville et al. 2015). Psychiatric conditions, most notably anxiety, depression, and social withdrawal are frequently reported in the elderly (Kastenschmidt and Kennedy 2011, Prenderville et al. 2015). It is also well established that peripheral inflammation can directly affect neuroimmune processes in the central nervous system (CNS) resulting in impaired cognitive function (Block et al. 2007, Perry 2010, Zheng et al. 2015). Moreover, circulating inflammatory markers are often elevated in individuals with mood disorders (Block et al. 2007, Perry 2010, Young et al. 2014, Zheng et al. 2015, Kelly et al. 2016). It is therefore likely that age-related alterations in intestinal microbiota and function contribute to chronic systemic inflammation. This may, in turn, lead to central inflammation, manifesting in cognitive impairment.

Taken together, it is clear that the microbiota-gut-brain axis plays a critical role in health, and perturbations in this axis have been implicated in numerous pathological conditions. However, there are a limited number of studies focused on its impact on ageing-induced behavioural and neurobiological outcomes, notwithstanding Metchnikoff's theory that microbes may be the underpinnings of longevity (Cryan and Dinan 2015). To this end, we hypothesised that alterations in the gut microbiota of young and aged mice will correlate with changes in intestinal permeability, inflammation and neurobehavioural outcomes.

2. Methods

2.1. Animals

All animal protocols were approved by the Animal Experimentation Ethics Committee at University College Cork (UCC) and by the Health Products Regulatory Authority (HPRA) of Ireland, in accordance with EU Directive 2010/63/EU. Twelve young (2 month old) and 10 aged (18 month old) male C57BL/6J mice were obtained from Charles River (France) and housed 2-4 per cage. All mice were housed in the same temperature and humidity-controlled animal room with a 12 h light:dark cycle and were maintained on *ad libitum* standard chow and water unless otherwise noted. Mice were given 4 weeks to recover from transportation and acclimate to the UCC animal facility.

2.2 Behavioural testing

All mice were exposed to a battery of well-validated behavioural tests designed to assess cognitive function as well as depressive- and anxiety-like behaviours over a period of 5 weeks. Young mice were approximately 2-3 months old and aged mice were approximately 20-21 months old at the time of behavioural testing. Behavioural tests were performed in what we considered to be increasing severity to lessen the likelihood of the prior testing influencing future tests. Tests were performed in the following order: 1) Object location, 2) Y-maze, 3) 3 chamber, 4) elevated plus maze, 5) open field, 6) forced swim and 7) gut permeability tests. A washout period of 2 days was observed between behavioural tests 1-6. A washout period of 2 weeks followed the FST, and 4 weeks followed gut permeability testing and prior to cull.

2.2.1 Spontaneous alternation in the Y-maze

The Y-maze was performed as a test of spontaneous alternation, used to assess exploration, cognition and hippocampal-dependent memory (Hughes 2004, Senechal et al. 2007). It is based on the premise that mice will alternate between arms visited when exploring a new environment. The test was performed as previously described with minor adaptation (Senechal et al. 2007).

Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The Y-maze was constructed from black plastic and each arm measured 16 x 6.5. Lighting within the Y-maze was 25 lux. The mouse was placed at the end of the first arm facing the wall. Behaviour was videorecorded from above for 5 min after placement within the maze. The Y-maze was wiped down with 70% ethanol between tests. An entry into an arm was noted when all 4 paws crossed into the area. Alternation was noted as the number of consecutive entries into the three maze arms. This was calculated as the number of alternations divided by the total number of arm entries during the 5 min test period.

2.2.2 Novel Object/Object Displacement test

The combined Novel Object/Object Displacement test (NOR) was used to assess cognitive function, including and spatial working memory and is based on the premise that mice will spend more time interacting with a novel or displaced object in comparison with a familiar object in a familiar location (Antunes and Biala 2012). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The test arena was a grey plastic box, measuring 32 x 40 cm. Lighting within the test arena was approximately 60 lux. The arena was wiped with ethanol after each test. Day one consisted of habituation to the empty test arena; the mouse was placed in the centre and allowed to explore for 10 min, while behaviour was videorecorded from above. After this period, the mouse was returned to its home cage. The acquisition phase of the study was performed on day 2. Again, mice were given at least 30 min to habituate to the testing room. During the acquisition phase, the mouse was placed in the centre of the test arena where 2 identical objects were placed. The mouse was allowed to explore for 10 min, after which it was returned to its home cage. After a 3 h period, the mouse was returned to the test arena with the same objects, however one of the objects was now in a different location. The mouse was allowed 5 min to explore and time spent interacting with the 2 objects was videorecorded, after which it was returned to its home cage. This comprised the Object Displacement phase of the study. Following a 5 min break, the mouse was returned to the test arena where the displaced “familiar” object was replaced with a novel one. Behaviour was videorecorded from above for 5 min, and

afterwards the mouse was returned to its home cage. However, due to technical issues no useable data was obtained for this Novel Object phase of the study. Because the novel object phase followed the object displacement phase, object displacement results were unaffected. Exploration of an object was defined as orientation of the mouse with its nose 2 cm or closer to the object.

2.2.3 Open field test

The open field (OF) test was used to assess locomotor activity and anxiety-like behaviour and is based on the premise that mice will avoid open areas (the centre of the test arena), which are anxiety-provoking and will spend more time near the walls (thigmotaxis) (Hall and Ballachey 1932, Gould 2009). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The OF arena was a white plastic box measuring 32 x 40 cm and the test was performed under full light conditions (1000 lux). Mice were placed in the centre of the arena and behaviour was recorded from above for 10 min. Mice were returned to their home cage following the test and the arena was wiped down with ethanol. Distance travelled and entries into centre of the arena was analysed using Ethovision (Noldus, Waeginingen, Netherlands).

2.2.4 Elevated Plus Maze

The elevated plus maze (EPM) was used to assess anxiety-like behaviour and is based on the premise that mice prefer enclosed areas when exposed to a novel environment and will spend less time in open areas, which are anxiety-inducing (Pellow et al. 1985). Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The room was lit by one red light and the lighting in the centre of the EPM measured 9 lux. The elevated plus maze was constructed of black plastic and had 4 arms measuring 50 x 5 cm. Walls on the 2 closed arms were 15 cm high whereas the 2 open arms had no walls. The plus maze rested on a platform 1 m high. For testing, the mouse was placed in the centre of the EPM facing an open arm. Behaviour was videorecorded from above for 5 min, after which the mouse was returned to its home cage. The EPM was wiped down with ethanol solution between each test. Entry into each

arm was noted when all 4 paws crossed into the arm. The number of entries and percentage of time spent in open and closed arms were calculated.

2.2.5 Forced swim test

The forced swim test (FST) was used to assess antidepressant-sensitive behaviours and is based on the premise that immobility, performing the minimum amount of movement to keep one's head above water, is a marker of depressive-like or despair-like behaviour (Porsolt et al. 1978, Porsolt 1979, Cryan and Mombereau 2004). FST was performed under full light conditions (1000 lux). The tank was a glass cylinder measuring 21 cm in diameter and filled to a depth of 15 cm with 24℃ tap water. The test was videorecorded from above for 6 min, and afterwards the mouse was removed from the tank, dried with a towel and placed into an individual cage for recovery. Ninety minutes post-test, mice were returned to their home cages. Water was changed between each test. Immobility was assessed for the last 4 min of the FST and was defined as the animal not actively swimming or moving and performing the minimal amount of activity necessary to keep the head above water.

2.2.6 3-chamber test of social behaviour

Sociability was assessed using the 3-chamber social interaction test (SIT), in which time spent interacting with a novel conspecific is compared to time spent with a novel object or familiar conspecific. It is based on the premise that mice will spend more time interacting with a novel conspecific than novel object, and that they will prefer a novel conspecific to a familiar one. Mice were brought into the testing room and allowed to habituate for at least half an hour before testing. The 3-chamber social interaction test was performed as previously described (O'Tuathaigh et al. 2007, Desbonnet et al. 2014). The test arena consisted of 3 chambers; the left and right chambers measured 13.5 x 20 x 20 cm and the centre chamber was 9 x 20 x 20 cm. A solid partition separated the chambers, which could be replaced with partitions with a small hole enabling access to the other chambers. There were 3 phases of the test: habituation, sociability, and social novelty preference. All phases of the test were 10 min in duration, performed sequentially and

videorecorded from above for later analysis. During phase 1, the habituation phase, the mouse was placed into the centre chamber and then allowed access to the empty left and right chambers for 10 min. The mouse was then returned to the centre chamber and a novel mouse was placed in a mesh cage in one of the side chambers, whereas a novel object (a small rubber duck) was placed in a mesh cage in the other side chamber for phase 2. Location of the novel mouse and novel objects were randomised between animals to eliminate side preferences. The mouse was then allowed to explore these chambers for 10 min, after which it was returned to the centre chamber. For the 3rd phase, a new, novel mouse was placed in the mesh cage that had previously housed the novel object. The mouse was then allowed to explore the chambers, which held the familiar mouse (from phase 2) and the novel mouse, for 10 min. The 3-chamber apparatus was cleaned with ethanol between animals. The number of entries and time spent in each chamber were then measured.

2.3 Intestinal permeability (FITC-D)

Intestinal barrier function was assessed using fluorescein isothiocyanate-labelled dextran (FITC-D) (FD4, Sigma Aldrich, Ireland). FITC-D (MW= 4 kDa) was dissolved in phosphate buffered saline (pH 7.4) to make a solution of 80 mg/ml. Mice were fasted overnight, prior to the study, and in the morning (9.00), they were gavaged with FITC-D (600 mg/kg). Two hours following gavage, mice were placed in ventilated plastic restrainers and a basal blood sample was taken. Briefly, a scalpel blade was used to remove the very tip (<1mm) of the tail. Blood was then collected using a heparinised capillary tube and transferred to a microcentrifuge tube. To assess the impact of acute stress on gut permeability, a small piece of gauze was used to gently remove the clot at the tip of the tail and another blood sample was collected 1 h post-restraint. Mice were then returned to their home cages. Blood samples were kept on ice and then centrifuged at 2500 x g. Plasma was collected and stored at -20°C for later analysis. To assess FITC-D, samples were analysed using a spectrometer (Victor Spectrometer, excitation max= 490 nm, emission max= 520 nm). Serial dilutions of FITC-D in PBS were used to generate a standard curve. A separate aliquot of plasma

was collected at each time point to assess basal and stressed corticosterone levels (as described in section 2.4).

2.4 Corticosterone response to acute stress

Plasma corticosterone (CORT) levels prior to, and following exposure to forced swim and restraint stress were used to assess hypothalamic-pituitary-adrenal (HPA) axis activity. On the day of the forced swim test, each mouse was removed from its home cage and moved to a testing room where a basal blood sample was taken (as described in section 2.3). Blood samples were also taken 15, 45, and 90 min following the onset of the FST to assess peak and recovery CORT levels. Blood was processed as previously described, and stored at -20℃ for later analysis. Blood samples were also collected to assess HPA axis response to restraint stress (in conjunction with the FITC-D test of intestinal permeability, described in 2.3). Plasma CORT was assessed by ELISA, following vendor instructions (ENZO Corticosterone ELISA, ADI-900-097, Enzo Life Sciences, Exeter, UK).

2.5 Plasma cytokines

At the end of the study, trunk blood was collected and processed as described above for collection of plasma. Samples were analysed using the MSD V-Plex Custom Mouse Cytokine kit (MesoScaleDiscovery, Brinny, Ireland) as per vendor instructions. The lower limits of detection for the kit ranged from 0.11 pg/ml (IL-1β) to 0.95 pg/ml (IL-10).

2.6 Caecal microbiota analysis

2.6.1 DNA extraction

Caecal contents were snap frozen at the end of the study and stored at -80℃ until the samples could be processed. DNA was extracted from caecum using the QIAmp Fast DNA Stool Mini Kit (Qiagen, UK) according to manufacturer's instructions with the addition of a 3 min vortex step using 2 ml screw-cap tubes (Sarstedt, Wexford, Ireland) containing 0.25 g of a 1:1 mix of 0.1 mm and 1.5 mm diameter sterile zirconia beads plus a single 2.5mm diameter bead (BioSpec

Products, Bartlesville, USA). Briefly, 200 mg of each caecal sample was added to a screw-cap tube containing beads with 1 ml of Qiagen InhibitEX® Buffer and vortexed for 3 min. Samples were then incubated at 70℃ for 5 min to lyse cells. Samples were centrifuged and the DNA was pelleted and treated with proteinase K. The DNA was then washed with buffers AW1 and AW2 and eluted in 200 µl Buffer ATE. DNA was quantified using the Qubit™ 3.0 Fluorometer (Bio-Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit (Bio-Sciences, Dublin, Ireland).

2.6.2 PCR and 16S compositional sequencing

The V3-V4 regions of the 16S rRNA gene were amplified and prepared for sequencing according to the 16S Metagenomic Sequencing Library Protocol http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf. The protocol involved two PCR reactions on the extracted DNA. The DNA was first amplified using primers specific to the V3-V4 regions of the 16S rRNA gene: (Forward primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Each reaction contained 2.5 µl genomic DNA, 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM) and 12.5 µl 2X Kapa HiFi Hotstart ReadyMix (Kapa Biosystems Ltd., UK). PCR amplification was carried out using the following program: 95℃ x 3 min, 25 cycles of 95℃ x 30s, 55℃ x 30s, 72℃ x 30s, 72℃ x 5 min and held at 4℃. PCR products were visualised using gel electrophoresis and then purified using AMPure XP beads (Labplan, Kildare, Ireland). Following this, a second PCR reaction was carried out on the purified DNA using two indexing primers per sample (Illumina Nextera XT indexing primers, Illumina, Netherlands). Each reaction contained 5 µl purified DNA, 5 µl index 1 primer (N7xx), 5 µl index 2 primer (S5xx), 25 µl 2x Kapa HiFi Hot Start Ready mix and 10 µl PCR grade water. PCR amplification was completed using the previous program but with only 8 amplification cycles instead of 25. PCR products were visualised and purified as described above. Samples were quantified using the Qubit™ 3.0 Fluorometer (Bio-

Sciences, Dublin, Ireland) along with the high sensitivity DNA quantification assay kit and then pooled in an equimolar fashion (20 nM). The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Clinical Microbiomics, Denmark using standard Illumina sequencing protocols.

2.6.3 Bioinformatic Analysis

Paired-end reads were assembled using FLASH. Raw sequence reads were quality trimmed using the QIIME suite of tools (version 1.8.0). This included the filtering of reads which failed to reach a quality score of > 25 and the removal of mismatched barcodes and sequences below length thresholds. Denoising, chimera detection and operational taxonomic unit (OTU) grouping at 97% similarity were performed in QIIME using USEARCH v7. OTU sequences were aligned using PyNAST and the SILVA SSURef database release 111 was used to determine taxonomy. The vegan R package (v. 2.4-1) was used to for statistical calculations of alpha and beta diversity. Alpha diversity was calculated using the Shannon Index, Mann Whitney U test was performed to assess significance between groups. Principal coordinate analysis (PCoA) plots were used to visualise beta diversity between groups based on Bray-Curtis distance matrices; significance of beta diversity between groups was computed by performing an ADONIS PERMANOVA test.

2.7 Statistical Analyses

All data, with the exception of microbiota data, are presented as mean +/- SEM. Data were excluded from analyses if greater than 2 standard deviations from the mean. Two-way ANOVAs were used to assess percentage of time interacting for Object location, social interaction, plasma FITC-D and CORT. When a main effect was detected, a Bonferroni post-hoc test of multiple comparisons was used. Unpaired t-tests were used to analyse behaviour in the OF, EPM, FST and plasma cytokines. Correlations were performed using Spearman correlation coefficient (r). Mann Whitney U analysis was used to assess statistical differences in microbiota compositions and p-values were corrected for multiple comparisons using the Benjamini Hochberg (BH) correction (FDR<0.05). Microbiota were analysed by subject rather than cage.

3. Results

3.1 *Aged mice do not differ in spontaneous alternation behaviour, but exhibit impaired displaced object recognition.*

Ageing is associated with cognitive decline, including impaired memory. Therefore, we performed the Y-maze test of spontaneous alternation and a novel object/object location test to assess memory in aged and young mice. Aged mice performed similarly to young mice in the spontaneous alternation test (Fig. 1A). Whereas young mice spent a significantly greater percentage of time interacting with a displaced familiar object ($p<0.01$), aged males did not (Fig. 1B).

3.2 *Aged mice exhibit behaviours associated with anxiety but not depression.*

Because ageing is often associated with changes in mood, we also investigated anxiety- and depressive- like behaviours using the open field, elevated plus maze, and forced swim tests. Aged mice spent less time in the centre arena and made fewer entries into this area during the open field test ($p<0.01$) (Fig. 1C and 1D). Aged animals also spent a significantly lower percentage of time in the open arms of the plus maze ($p<0.05$) (Fig 1E), and together, these findings suggest increased anxiety. Immobility, used as a marker of depressive- or despair- like behaviour did not differ between young and aged mice in response to the forced swim test (Fig. 1F).

3.3 *Aged mice exhibit behaviours associated with reduced social recognition and/or preference for social novelty.*

As ageing is also associated with social withdrawal, social preference and social recognition were assessed using the 3-chamber social interaction test. Both aged and young mice spent more time investigating a novel mouse as opposed to a novel object, suggesting that there were no differences in preference for a conspecific ($p<0.0001$)(Fig. 2A). However, whereas younger mice spent significantly more time interacting with a novel mouse than a familiar mouse ($p<0.0001$),

aged mice spent similar amount of time investigating the novel and familiar mice, suggesting an impairment in social recognition or a reduced preference for social novelty (Fig. 2B).

3.4 *Aged mice exhibit increased basal gut permeability, which is further exacerbated by acute stress exposure.*

Gut permeability was assessed in young and aged mice prior to and following 1 h of restraint stress. Aged mice had significantly greater basal intestinal permeability than young mice ($p<0.05$). Whereas gut permeability of young mice was unaffected by restraint stress, permeability was further enhanced in aged mice (Aged basal v. aged stressed, $p<0.01$, Young stressed v. aged stressed, $p<0.001$) (Fig. 3).

3.5 *Aged mice exhibit alterations in corticosterone response to acute stress exposure.*

Despite seeing no behavioural effect, aged mice exhibited altered plasma CORT responses to the FST. Although basal CORT levels did not differ between groups, aged mice had significantly lower plasma CORT levels 15 min following the onset of the test ($p<0.001$) (Fig. 4A). Plasma CORT did not differ 45 and 90 min post FST; however, the trajectory of the CORT response differed. Although levels were dropping at these time points in young mice, suggesting recovery, they were rising in aged mice, suggesting prolonged CORT release. CORT was also measured pre- and post- acute restraint, which was employed during the FITC-D test of intestinal permeability. Similar to our findings in the FST, basal CORT levels did not differ between young and aged mice. However, while plasma CORT was significantly increased in young mice following 1 h of restraint ($p<0.001$), CORT was unchanged in aged mice (young-stressed v. aged-stressed, $p<0.001$) (Fig. 4B).

3.6 *Circulating plasma cytokines are elevated in aged mice and correlate with gut FITC-D permeability*

Plasma cytokines were assessed as markers of peripheral inflammation at the end of the study. A number of pro-inflammatory cytokines were elevated in aged mice, including IL-1β (Fig. 5A,

p<0.01), TNF-α (Fig. 5B, p<0.0001) and IL-2 (Fig. 5C, p<0.01). There was a trend towards increased IL-6 expression in aged mice (Fig. 5E, p=0.07). Anti-inflammatory cytokine IL-10 was unchanged in aged mice (Fig. 5F). Plasma cytokines assessed at the end of the experiment were also plotted against plasma FITC-D levels assessed prior to and following restraint stress. In the basal state, IL-6 and TNF-α were significantly and positively correlated with plasma FITC-D levels (p<0.05), while there was a trend towards positive correlation between IL-1β (p=0.07), (Fig. 6A). In the stressed state, all 3 of these cytokines were significantly and positively correlated with plasma FITC-D levels (p<0.05) (Fig. 6B).

3.7 *The caecal microbiota of mice is significantly altered with ageing*

MiSeq sequencing yielded a total of 8,339,660 raw reads, ranging from 108,541 to 339,456 reads per sample. Following quality filtering, reads were clustered into 1033 OTUs, which were assigned to taxa from phylum to genus level. Due to the variation in read number, the OTU table was rarefied to 108,000 reads, to allow for comparison between samples. The microbiota of aged mice differed significantly from that of young mice. Interestingly, only significant increases in bacterial taxa were seen in aged mice compared to young (Table 1).

At phylum level the most prevalent microbial taxa in both sample groups were Bacteroidetes and Firmicutes accounting for a combined relative abundance of >95% in the young and aged samples (Fig. 7). Other bacterial phyla had relative abundances of <2% in both groups. The large abundance of S24-7 and Rikenellaceae at family level was reflected at genus level as *S24-7 uncultured*, *Alistipes*, and *RC9 gut group*. Together, these highly abundant genera account for approximately 50% relative abundance at the genus level in both aged and young mice.

Several statistically significant changes in bacterial groups were observed in aged mice (Table 1). At the phylum level, TM7 was significantly higher in aged mice (p<0.01). At family level abundance less than 1%, significant increases in Porphyromondaceae (p<0.01), TM7 uncultured (p<0.01) Clostridiaceae (p<0.05), Thermoanaerobacteraceae (p<0.05), Desulfovibrionaceae (p<0.05) and

Oxalobacteraceae ($p<0.05$) were seen in aged mice compared to young. Genera *Odoribacter* was much higher in the aged group (5.14%) than in the young mice (0.16%) ($p<0.01$). In addition, aged mice had significant increases in other bacterial genera, including *Butyricimonas* ($p<0.01$), *TM7 uncultured* ($p<0.01$), *Gelria* ($p<0.05$), *Anaerosporobacter* ($p<0.05$), *Clostridium* ($p<0.05$), *Oxalobacter* ($p<0.05$).

The Chao 1 index (which estimates species richness) was significantly higher in the aged group ($p=0.01$). In addition, the number of observed species and the phylogenetic diversity were also higher in the aged mice ($p=0.01$, $p=0.03$). The Shannon Index (alpha diversity) was significantly higher in aged mice ($p<0.0001$, Fig. 8A). Beta diversity was significantly different between aged and young groups ($p=0.001$, Fig. 8B).

3.8 *The relative abundance of Porphyromonadaceae correlates with anxiety-like behaviour in aged mice*

Because some of the microbiota changes observed have been associated with cognitive dysfunction, we compared relative abundance of 3 taxa that were significantly altered in aged mice, Porphyromonadaceae, Odoribacter and TM7, with behavioural data. Interestingly, in aged mice, the relative abundance of members of the family Porphyromonadaceae was significantly and negatively correlated with the number of entries and time spent in the open arms of the elevated plus maze (Fig. 9A and 9B, $p<0.05$). That is, as Porphyromonadaceae increased in caecal contents, so did anxiety-like behaviour in aged mice. No correlations with anxiety-like behaviours were found in young mice, as presence of taxa from the Porphyromonadaceae family was so low. We found no other correlations between behaviours and the relative abundance of Porphyromonadaceae, Odoribacter or TM7 bacterial groups.

Discussion

Although the microbiome has been implicated in the ageing process, there have been few studies investigating the age-related changes in microbiota concomitant with gut permeability, stress response, peripheral inflammation and behaviour. Here we show that ageing is associated with marked changes in microbiota composition coupled with behavioural and physiological alterations.

Some of the most troubling features associated with ageing are negative affect and cognitive decline (Eshkoor et al. 2015, Prenderville et al. 2015). In these studies, we found that aged mice exhibited several behavioural changes that are thought to reflect these symptoms. The most robust of these were increases in anxiety-like behaviours, as assessed by thigmotaxis during the open field test and less time spent in the open arms of the elevated plus maze. It should be noted that aged mice exhibit reductions in overall locomotor activity; therefore, all data were expressed as percentages to avoid this as a confounding variable. However, we cannot rule out the possibility that reduced activity influenced the results. Anxiety is often reported in the elderly, as are anxiety-like behaviours in aged laboratory animals (Kastenschmidt and Kennedy 2011, Brouwer-Brolsma et al. 2014, Andreescu and Varon 2015, Ellison et al. 2015, Creighton et al. 2016). Importantly, epidemiological studies suggest that anxiety disorders in the elderly predispose or exacerbate the onset of cognitive impairments and other conditions, including cardiovascular disease (Andreescu and Varon 2015).

Impaired performance of aged mice in the object displacement task is in line with other studies suggesting age-associated deficits in hippocampal-dependent spatial memory (Klencklen et al. 2012, Maasberg et al. 2012, Wimmer et al. 2012). Whereas both young and aged mice demonstrated a preference for a novel conspecific over a novel object, aged mice did not exhibit a preference for novel mice when presented with the choice between novel and familiar mice. However, it is unclear whether these findings reflect a true impairment in social recognition or a lack of preference for a novel conspecific.

In addition to alterations in behaviour, aged mice exhibited several physiological changes. It is well established that many aspects of gut function, including intestinal barrier function, are impaired with age, and we observed a similar impairment in aged mice (Tran and Greenwood-Van Meerveld 2013, Man et al. 2014, Saffrey 2014). Interestingly, acute restraint stress exacerbated gut permeability in aged animals, despite a lack of rise in plasma glucocorticoid levels; in our studies, aged mice actually had a blunted and slower HPA axis response to acute stress, although it appears that they may also experience prolonged corticosterone release. The reason for this disconnect between plasma glucocorticoids and increased gut permeability is unclear, as the general consensus is that elevations in glucocorticoids contribute to increased gut permeability (Meddings and Swain 2000, Bhatia and Tandon 2005). However, other studies suggest that glucocorticoids may actually serve to protect the gastrointestinal tract (Filaretova 2007). Furthermore, Crohn's disease, marked by increased inflammation and gut permeability has been associated with hypoactivity of the HPA axis; this may arise because glucocorticoids have anti-inflammatory effects, and insufficient corticosteroid levels may result in excessive inflammation (Stasi and Orlandelli 2008). Although ageing is often associated with basal hyperactivity of the hypothalamic-pituitary-adrenal axis, blunted and delayed activation and recovery of the HPA axis has also been previously reported (Veldhuis et al. 2013, Buechel et al. 2014). Furthermore, it has been previously demonstrated that the intestinal microbiota is sensitive to stress exposure, and this may in turn, modulate immune responses (Bailey 2014, Mackos et al. 2016). In the current study we employed acute stressors, but future studies will incorporate chronic stress, which is particularly relevant to unhealthy ageing.

Numerous factors are thought to contribute to age-associated impairments in gut function. These include histological changes within the structure of the gut, including decreased expression of tight junction proteins and altered morphology of intestinal villi (Tran and Greenwood-Van Meerveld 2013, Ren et al. 2014). Mucous and bicarbonate secretion by the gut and short chain fatty acid (SCFA) production by the gut microbiota, which serve to protect the intestinal epithelia, also decrease with ageing (Woodmansey 2007, Rehman 2012, Saffrey 2014). Moreover, gut motility is

also often reported to decrease in the elderly and in aged laboratory animals, likely due to changes in the smooth muscle structure, gut innervation and disrupted signalling.

Previous studies have demonstrated that the gut microbiota also influences gut permeability and behaviour (Saffrey 2014, Yarandi et al. 2016). The age-related changes in microbiota that we observed have been previously implicated in inflammation and cognitive decline. The most significant increases that we observed are seen in phylum TM7, family Porphyromonadaceae and genus *Odoribacter.* These bacteria have been previously associated with inflammatory diseases such as cirrhosis and inflammatory bowel disease (IBD) (Collins et al. 2012, Giannelli et al. 2014). Interestingly, our studies also revealed changes in bacteria previously associated with cognitive decline. In a study of encephalopathic patients with cirrhosis, increases in Porphyromonadaceae positively correlated with increased cognitive dysfunction (Bajaj et al. 2012, Collins et al. 2012). A more recent study found that poor cognitive performance in the elderly was associated with increases in Porphyromonadaceae irrespective of cirrhosis (Bajaj et al. 2016). Caecal microbiota analysis in our studies revealed bacterial changes that have been previously observed in studies of depression and exposure to psychological stressors (Bangsgaard Bendtsen et al. 2012, Desbonnet et al. 2015, Watanabe et al. 2016). We found that relative abundance of Porphyromonadacea was directly correlated with anxiety-like behaviours in aged mice. Increases in bacterial taxa from this family have also been observed in faecal samples from individuals with Major Depressive Disorder (Jiang et al. 2015). While we did not observe behavioural changes in the forced swim test, a common assay of antidepressant-sensitive behaviours, this may reflect the overall sensitivity of the test. In future studies we may incorporate other methods of assessing depressive-like behaviour across different endophenotypes of the disorder (Slattery and Cryan 2012, Slattery and Cryan 2014, Kelly et al. 2016). The increased anxiety that we observed in aged mice may reflect microbiota changes in stress sensitivity, as noted above (Bangsgaard Bendtsen et al. 2012, Desbonnet et al. 2014, Watanabe et al. 2016). Interestingly, the caecal microbiota of aged mice was significantly more rich and diverse than that of young mice. Reductions in microbial diversity are often reported with ageing, but recent studies have challenged these findings (Biagi et al. 2012). The reasons for increased diversity in aged mice is unclear; however, decreases in

diversity are often reflective of unhealthy ageing and frailty, which we did not subjectively observe in this study (Claesson et al. 2012, Jackson et al. 2016). Another possibility is that with ageing, the stability of the microbiota is reduced, leading to proliferation of opportunistic bacteria (Biagi et al. 2012).

Perturbations in gut structure, function, and microbiota are believed to contribute to an increased risk of infection and inflammation, and markers of inflammation and immune responses have been reported in aged humans and in laboratory animals (Krabbe et al. 2004, Chung et al. 2009, Tran and Greenwood-Van Meerveld 2013, Man et al. 2014, Mabbott 2015). Similarly, we observed that plasma levels of several pro-inflammatory cytokines were elevated in aged mice, and IL-6, IL-1β and TNF-α correlated positively with gut permeability. Indeed, chronic systemic inflammation is linked to numerous neurodegenerative disorders and cognitive deficits associated with ageing, including Alzheimer's disease (Chung et al. 2009, Perry 2010, Deleidi et al. 2015). Moreover, there is a growing literature linking microbiome-based changes with susceptibility to Alzheimer's disease and neurodegenerative disorders (Cattaneo et al. 2016, Frohlich et al. 2016, Xu and Wang 2016).

In conclusion, we show, for the first time to our knowledge, that perturbations of the microbiome-gut-brain axis, resultant of normal ageing, may contribute to peripheral inflammation and the development of altered anxiety behaviours and cognitive impairments. Aged male mice exhibited significant shifts in gut microbiota and marked differences in stress responsivity, gut permeability and peripheral inflammation in comparison with young adult mice. In addition, they exhibited behavioural changes associated with cognitive deficits and increased anxiety. These increases in anxiety-like behaviour were directly correlated with abundance of bacteria from the Porphyromonadaceae family; this is in agreement with recent studies finding an association between this family and cognitive dysfunction and mood disorders (Collins et al. 2012, Jiang et al. 2015, Bajaj et al. 2016). Future studies should focus on the mechanisms that are at play in driving these changes. There is increasing emphasis on understanding pathways of microbiome to brain signalling (including vagus nerve, neuroendocrine pathways, enteric nervous system short chain

fatty acids, tryptophan metabolism and the immune system) but there is still a lack of knowledge in this field (Cryan and Dinan 2012, Galland 2014, O'Mahony et al. 2015, Rogers et al. 2016). Moreover, the microbiome has been shown to regulate adult hippocampal neurogenesis (Ogbonnaya et al. 2015, Mohle et al. 2016), microglia activation (Erny et al. 2015), blood brain barrier function (Braniste et al. 2014) and neuroinflammation (D'Mello et al. 2015), all of which are altered in ageing. Future studies should also address the relative contribution of these to the alterations in behaviour seen in ageing. These studies suggest that the gut microbiota may prove a worthy target for the development of novel therapies to ameliorate or prevent some of the adverse neurobehavioural consequences of unhealthy ageing.

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FIGURE LEGENDS

Figure 1. Behavioural tests of cognitive function, anxiety-, and depressive-like behaviours in young (n=9-12) and aged (n=8-10) mice. (A) No differences in spontaneous alternation were observed. (B) Young mice spent a significantly greater percentage of time interacting with a displaced, familiar object compared to a stationary, familiar object. (C) Aged mice spent significantly lower percentage of time in the centre of the open field. (D) Aged mice made fewer entries in to the centre of the open field. E. Aged mice spent a significantly lesser percentage of time in the open arms of the EPM. F No differences in time spent immobile in the FST.
*p<0.05,**p<0.01, ***p<0.001

Figure 2. Behaviours of young (n=9) and aged (n=9) mice in the 3-chamber test. (A) Aged and young mice spent significantly more time investigating a novel mouse than a novel object. (B) Young mice spent significantly more time investigating a novel mouse than a familiar mouse. Aged mice exhibited no preference for the novel mouse.
****p<0.0001

Figure 3. Basal and stress-induced gut permeability in young (n=12) and aged (n=10) mice. Basal gut permeability of aged mice was significantly greater than that of young mice and was significantly increased in response to acute restraint. Gut permeability of young mice was not increased in response to acute restraint stress.
*p<0.05 **p<0.01 ***p<0.001

Figure 4. Corticosterone response to acute stress exposure in young (n= 12) and aged (n=10) mice. (A) Young mice had a significantly greater peak CORT response 15 min after the onset of FST (B) Young mice had significantly higher plasma CORT following 1 h of restraint in comparison with basal levels and significantly higher plasma CORT than aged mice exposed to restraint.
***p<0.001

Figure 5. Plasma cytokines in young (n= 11-12) and aged (n=7-8) mice. (A) Plasma IL-1β was significantly elevated in aged mice. (B) Plasma TNF-α was significantly elevated in aged mice. (C) Plasma IL-2 was significantly elevated in aged mice. (D) There was no difference in plasma IL-4. (E) There was a trend towards increased plasma IL-6 levels in aged mice (p=0.07). (F) Plasma IL-10 did not differ between young and aged mice.

**p<0.01 ***p<0.001

Figure 6. Correlation between plasma cytokines and gut permeability in young (n= 11-12) and aged (n= 8) mice. A positive correlation was found between plasma cytokines and basal (unstressed) gut permeability (A-C) and in response to acute restraint stress (D-F). Open circles represent young and shaded circles represent aged mice.

*p< 0.05

Figure 7. Visual representation of relative abundance of caecal bacterial changes on the order of (A) Phylum, (B) Family and (C) Genus of young (n=10) and aged (n=7) mice. Significant changes are denoted by bold type.

Figure 8. Alpha diversity, quantified by the Shannon index (A) and beta diversity, represented by principle coordinate analysis (B) of caecal bacteria of young (n=10) and aged (n=7) mice.

Figure 9. Correlation between behaviours and relative abundance of Porphyromonadaceae. Porphyromonadaceae and (A) time spent in the EPM and (B) entries into open arms of the EPM were negatively correlated for aged (n=6) mice.

*p<0.05

Table 1. Differences in caecal microbiota between young (n=10) and aged (n=7) mice. Arrows in bold type denote significant changes within aged mice.

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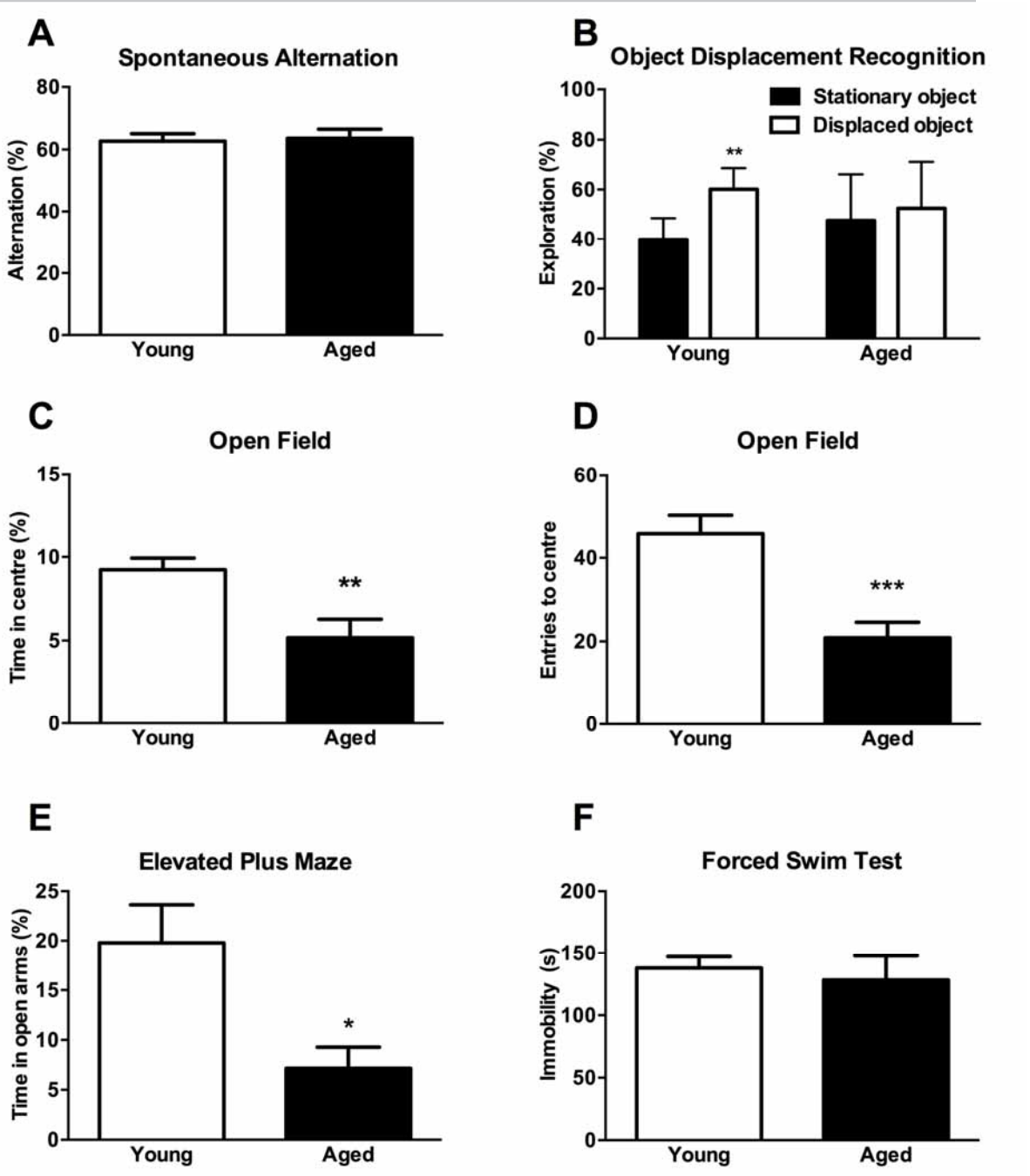

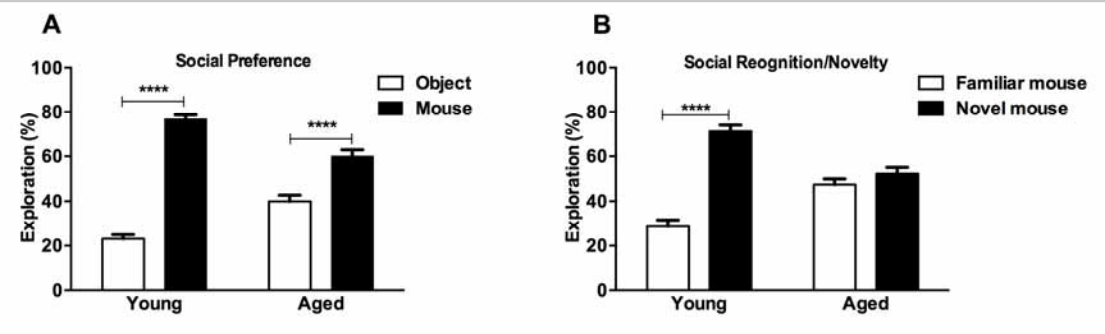

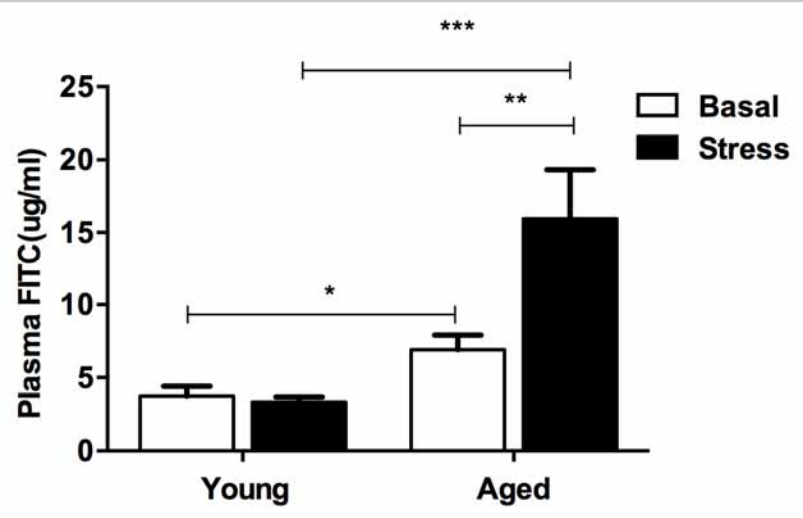

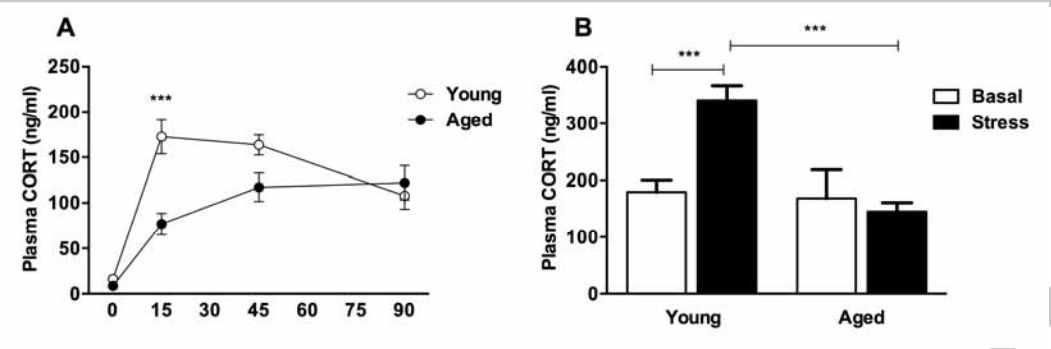

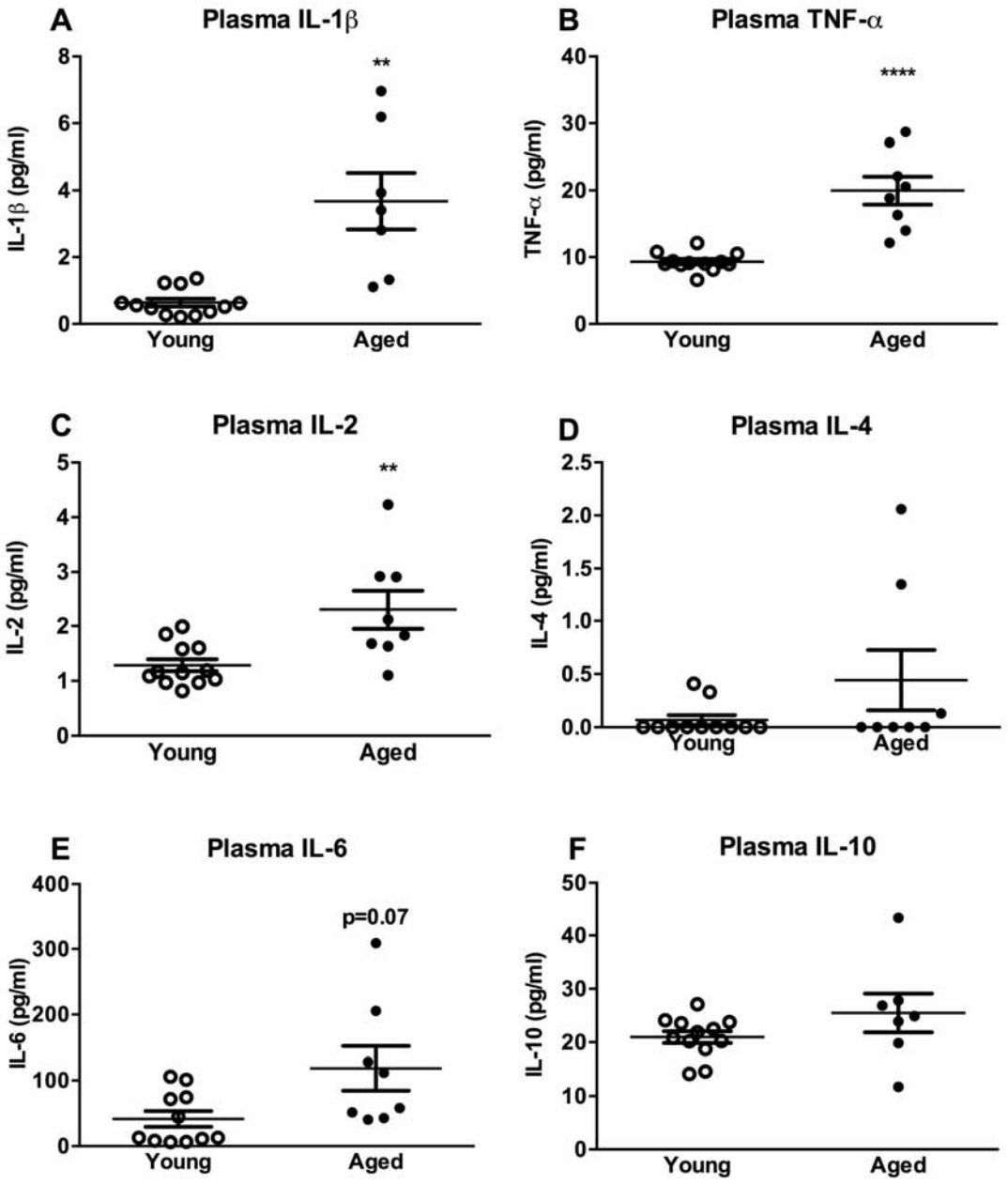

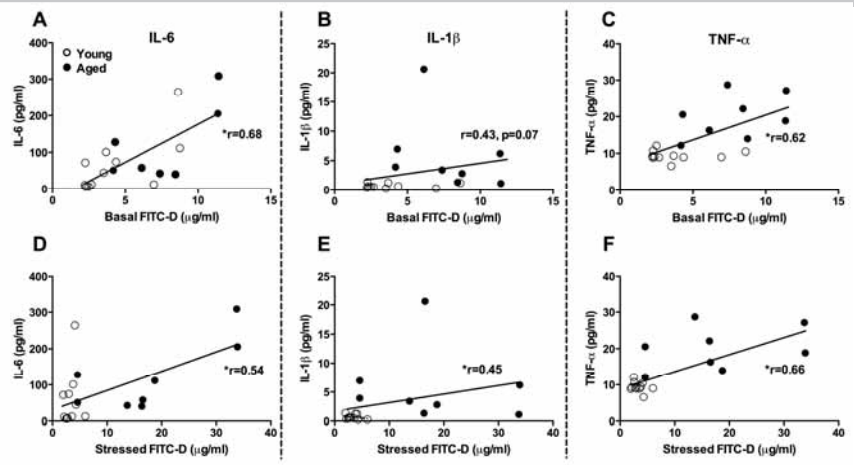

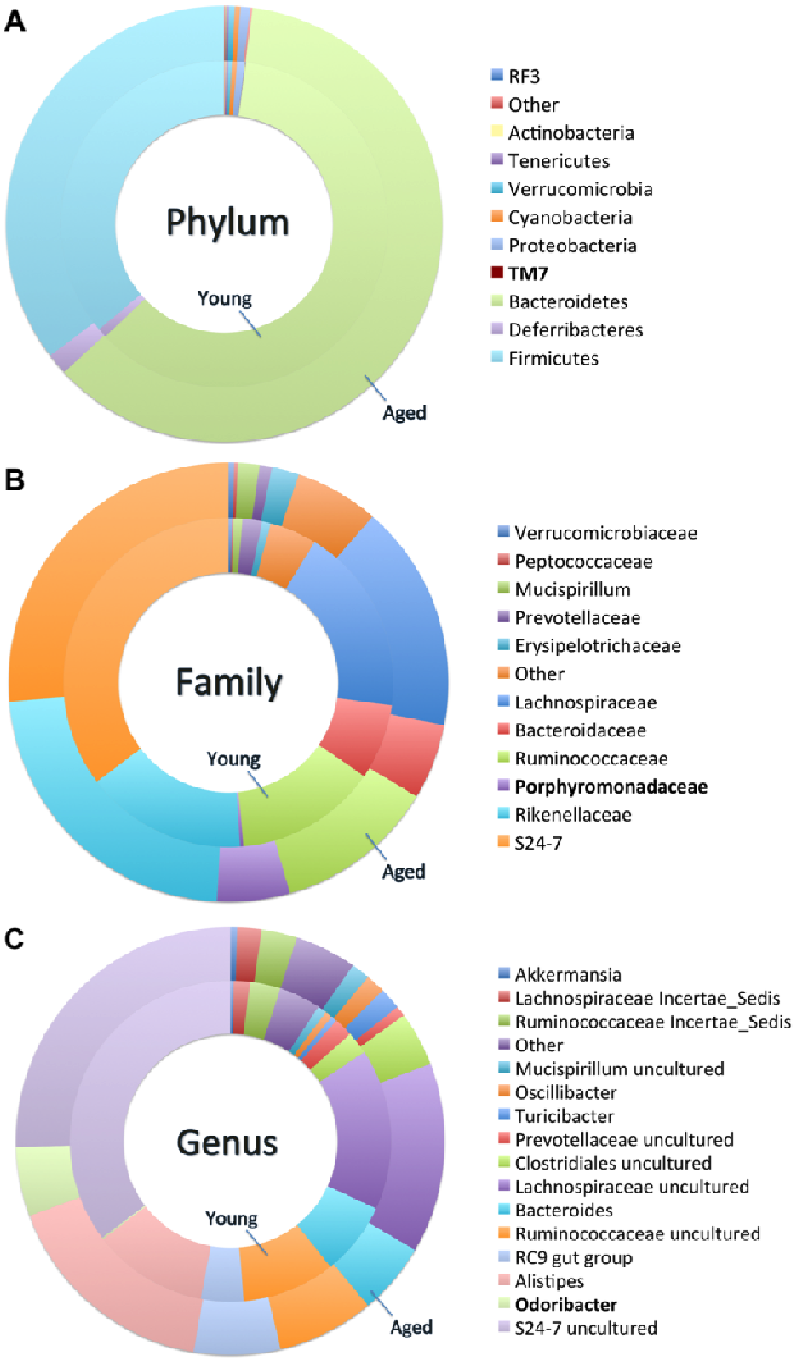

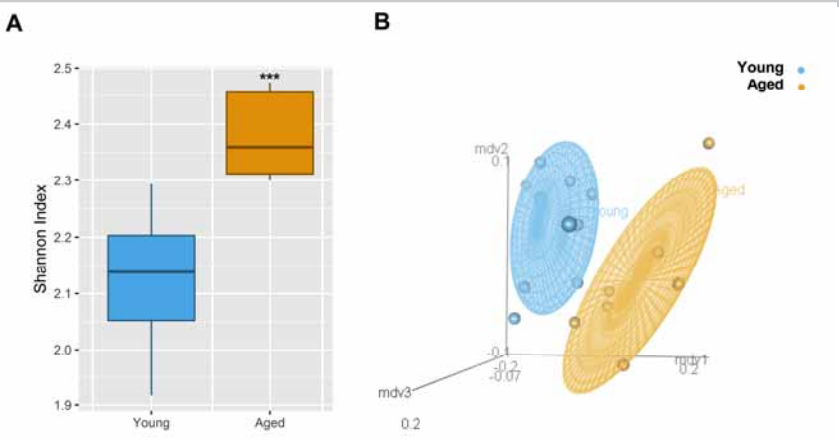

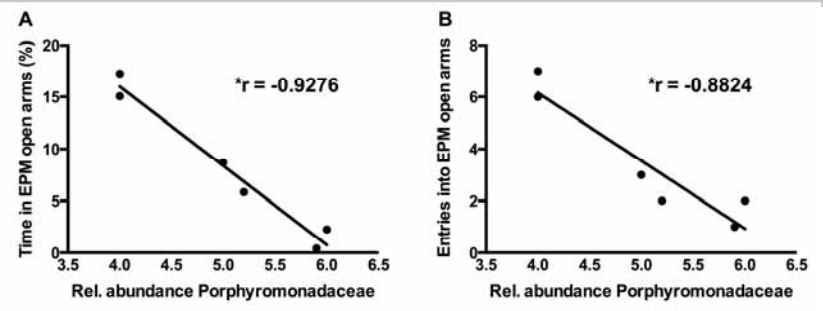

Highlights

- The behaviour and physiology of young and aged mice were compared, with an emphasis on gut microbiota, gut permeability and markers of peripheral inflammation.
- Aged male mice exhibited impaired cognitive function and increased anxiety-like behaviour in comparison with young adult mice.
- Gut permeability was increased in aged mice and was exacerbated by acute stress exposure.
- Gut permeability was positively correlated with increases in plasma proinflammatory cytokines IL-6, IL-1β and TNF-α.
- Gut microbiota was altered in aged mice, with increases in phylum TM7, family Porphyromonadaceae and genus *Odoribacter*, which have been implicated in inflammatory disorders and cognitive impairments.
- Relative abundance of Porphyromonadaceae was significantly correlated with anxiety-like behaviour of aged mice.

Mann-Whitney	Phylum	Class	Order	Family	Genus
p-value < 0.01	Bacteroidetes	Bacteroidia	Bacteroidales	↑ **Porphyromonadaceae**	↑ ***Butyricimonas***
	Bacteroidetes	Bacteroidia	Bacteroidales	↑ **Porphyromonadaceae**	↑ ***Odoribacter***
	↑ **TM7**	Uncultured	Uncultured	↑ **TM7 uncultured**	↑ ***TM7 uncultured***
p-value < 0.05	Firmicutes	Clostridia	Thermoanaerobacterales	↑ **Thermoanaerobacteraceae**	↑ ***Gelria***
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	↑ ***Anaerosporobacter***
	Firmicutes	Clostridia	Clostridiales	↑ **Clostridiaceae**	↑ ***Clostridium***
	Proteobacteria	Betaproteobacteria	Burkholderiales	↑ **Oxalobacteraceae**	↑ ***Oxalobacter***
	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	↑ **Desulfovibrionaceae**	N/A

↑ Increase, ↓ Decrease in Aged relative to Young

significant at taxamonic level