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Mid-Life Microbiota Crises: Middle Age is Associated with Pervasive Neuroimmune Alterations that are Reversed by Targeting the Gut Microbiome

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Running Title: Prebiotics reverse neuroimmune alterations in middle-aged mice

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Abstract

Male middle age is a transitional period where many physiological and psychological changes occur leading to cognitive and behavioural alterations, and a deterioration of brain function. However, the mechanisms underpinning such changes are unclear. The gut microbiome has been implicated as a key mediator in the communication between the gut and the brain, and in the regulation of brain homeostasis including brain immune cell function. Thus, we tested whether targeting the gut microbiome by prebiotic supplementation may alter microglia activation and brain function in ageing. Male young adult (eight weeks) and middle-aged (ten months) C57BL/6J mice received diet enriched with a prebiotic (10% oligofructose-enriched inulin (FOS-Inulin)) or control chow for 14 weeks. Prebiotic supplementation differentially altered the gut microbiota profile in young and middle-aged mice with changes correlating with faecal metabolites. Functionally, this translated into a reversal of stress-induced immune priming in middle-aged mice. In addition, a reduction in ageing-induced infiltration of Ly-6C^{hi}-monocytes into the brain coupled with a reversal in ageing-related increases in a subset of activated microglia (Ly-6C^{+}) was observed. Taken together, these data highlight a potential pathway by which targeting the gut microbiome with prebiotics can modulate the peripheral immune response and alter neuroinflammation in middle age. Our data highlight a novel strategy for the amelioration of age-related neuroinflammatory pathologies and brain function.

1. Introduction

We have trillions of microbes in our gastrointestinal tract, and a growing body of evidence supports a role for them in maintaining health across the lifespan (1-3). Indeed, microbiota has been implicated as a key mediator in the communication between the gut and the brain and regulating brain homeostasis. Diet has been shown to be one of the most important factors in modifying the gut microbiota composition (4, 5). However, the ability of nutritional interventions that target the microbiome to alter brain function has not received much attention (3, 4, 6).

Ageing is defined as a process involving slow deterioration of various homeostatic functions throughout the lifespan. Middle age in particular is a life period where many physiological and psychological changes occur, leading to first cognitive impairments and behavioural alterations, and a deterioration of brain function (7-11). In rodents, increased anxiety-like behaviour occurs in middle-age (7, 11). A few studies reported cognitive decline in middle-aged rodents (8, 11), with variable definitions of “middle-age“ highlighting the need for greater specification (12). Moreover, the levels of neurotransmitters (9) and neurotrophins (10) were shown to decline with age, which may possibly contribute to altered behaviour and brain homeostasis.

Increased age is associated with a shift towards a pro-inflammatory state and inflammageing (13, 14). This, in turn, can make the age brain more vulnerable to various intrinsic and extrinsic disruptive effects including stress, disease and infection (12, 15). Moreover, this vulnerability may result in cognitive alterations (3). However, it remains unclear to what extent an altered brain immune system can contribute to alterations in cognitive functions in middle-aged subjects.

Microglia are the major immune cells in the brain and have been shown to be a key player in neuropsychological and neurodegenerative conditions (16, 17). Increased activation of microglia in the aged brain has been suggested to be indicative of enhanced inflammation and heightened reactivity in the rodent and the human brain (13, 18, 19). Following an immune stimulus, which is exaggerated in ageing, microglia are referred to as “primed“ due to their rapid induction and increased cytokine release upon activation (13, 18). Microglia are specialised cells continuously monitoring their environment (20) and can sense changes in the brain’s milieu (21). In addition, microglia play a crucial role in synaptic plasticity, brain function and cognition across the lifespan (17).

Numerous studies have shown shifts in the composition of the intestinal microbiota with age in rodent models (22, 23) and in humans, including extreme ageing (24, 25). Previous research utilizing

pre-clinical models implicated a role of microbiota from aged mice in driving systemic immunity (26, 27). However, the effect on neuroimmunity and subsequent brain function and behaviour remains unaddressed. Interestingly, the transfer of gut microbiota from young-to-aged subjects might influence healthy ageing as shown in the short-lived killifish model, which exhibited an increase in lifespan and motor behaviour (28). It has been shown that the administration of prebiotics (a substrate that is selectively utilized by host microorganisms conferring a health benefit (29)) results in an increase in the number of beneficial intestinal bacterial species with a reduction in systemic inflammation in humans (30, 31), and both, peripheral and neuroinflammation in rodents (32, 33) which would have important implications for the healthcare system. It however remains unclear what is driving these changes and what is the impact on brain function and behaviour. Therapeutic interventions are thus sought in order to delay ageing, decrease pro-ageing factors, reduce microglia activation and ultimately improve cognition during ageing.

We hypothesise that there is a dysregulation in the communication between the gut microbiota and the brain during middle age, which is critical in mediating age-related functional decline. Thus, targeting the gut microbiota with prebiotics may alter microglia activation state and brain function in ageing. To this end, we hypothesised that targeting the gut microbiome by dietary intervention with a complex short- and long-chain prebiotic, oligofructose-enriched inulin (FOS-Inulin), would have selective effects on (neuro-) immune profile and behaviour in middle-aged male compared to young adult C57BL/6J mice.

2. Methods

2.1 Animals

Male young adult C57BL/6J mice (n = 50; Harlan, Cambridgeshire, UK; 2 months) and middle-aged C57BL/6J mice (n = 38; 10 months) were used in this study. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork (B100/3774). Animals were habituated to the animal facility for two weeks before experiments started and kept under a 12-hour light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10%. Food and water were given *ad libitum*.

Power analysis was performed beforehand using the Software G*Power 3.1 to ensure adequate sample size number to detect changes in behaviour and neuroimmunity (34). Mice were equally assigned to experimental groups based on bodyweight to ensure equally distribution among the groups.

2.2 Prebiotic administration

Mice received chow (ssniff-Spezialdiäten GmbH, Soest, Germany) enriched with 10% Oligofructose-enriched inulin (FOS-Inulin: mixture of 92±2% Inulin and 8±2% Fructooligosaccharide, Orafti®Synergy1; BENEO-Orafti N.V., Tienen, Belgium) or control chow for 3.5 weeks (microglia cohort) and 14 weeks (behavioural cohort). The dosage of FOS-Inulin supplementation was chosen based on previous studies in rodents (35-37). Duration of prebiotic intervention was chosen according to previous studies in rodents showing effects on brain and behaviour (32, 38).

2.3 Study design and experimental timeline

Two separate cohorts of animals were used (see Supplementary Figure S1).

Cohort one investigated the effects of FOS-Inulin on behaviour including cognitive (spontaneous alternation behaviour, Morris water maze, fear conditioning), anxiety-like (open field, elevated-plus maze, marble burying), social (three-chamber social interaction test) and depressive-like behaviour (forced swim test). Following a three-week lead-in of diet, mice (n=9-10 per group) underwent behavioural assessment while continuing dietary supplementation for a total of 14 weeks. In addition, peripheral immune cell activation (pre-/post stress) was assessed in blood using flow cytometry. To correlate the changes in behaviour with specific neuroimmune targets, we subsequently analysed targets in the brain at the end of the study.

To characterize the neuroimmune status in the brain at a time point before animals were tested behaviourally, cohort two (young adult: n=14-16, middle-aged: n=8-10) investigated if a dietary lead-

in phase of 3.5 weeks with FOS-Inulin can alter monocyte infiltration and subsequent microglia activation in the brain, key mediators influencing cognition and anxiety-like behaviour.

See Supplemental Methods for detailed information on procedures (2.4 to 2.9).

2.4 Behaviour

2.4.1 Spontaneous alternation in the Y-Maze

Spontaneous alternation behaviour in the Y-maze tests hippocampal-dependent spatial memory and exploration exploratory activity and was carried out as previously described (22). Behaviour was assessed for five minutes.

2.4.2 Morris water maze

The Morris water maze represents a robust and reliable test for spatial learning that strongly correlates with hippocampal synaptic plasticity (39). Briefly, mice were trained over five days (four trials per day, two minutes each) to spatially locate the submerged platform. On day six, the platform was removed and a probe trial lasting 30s was conducted.

2.4.3 Fear conditioning

Fear conditioning was conducted as previously described (40), over three consecutive days (day 1: conditioning by three pairings with variable inter-pairing interval; day 2: conditioned stimulus recall and extinction in a novel context; day 3: context recall).

2.4.4 Open field

The open field is a widely used test to assess approach-avoidance behaviour, locomotor activity, and the behavioural response to a novel context; and was conducted as previously described (32). Briefly, a test mouse was placed into an open arena with 60 lux lighting and allowed to explore the context for ten minutes.

2.4.5 Marble burying test

The marble burying test assesses compulsive, repetitive and anxiety-like behaviour, and was conducted as previously described (32). Briefly, mice were tested for 30 min and the number of buried marbles was recorded.

2.4.6 Elevated-plus maze

The Elevated-plus Maze test was used to assess anxiety-like behaviour and was conducted as previously described (32). Mice were allowed to explore the maze for five minutes; the time spent in the open arms, as well as number of entries into the open arms was analysed.

2.4.7 Three-chamber social interaction test

Sociability and social novelty were assessed in a three-chamber apparatus as previously described (41). The test consists of three sequential ten-minute trials: (1) habituation; (2) sociability (the analysis of time a test mouse spends in the chamber with the conspecific mouse or with the object); and (3) social novelty preference (the analysis of time a test mouse spends in the chamber with the novel or in the chamber with the familiar mouse).

2.4.8 Forced swim test

The forced swim test (FST) was used to assess depressive-like or despair-like behaviour (42, 43). Mice were individually placed in a transparent glass cylinder for six minutes. Time spent immobile was defined as no movements apart from breathing and considered as depressive-like behaviour. Behaviour was analysed during the last 4 minutes of the test which represents the most common protocol to use in analysing FST in the mouse and accounts for the fact that most mice struggle heavily during the first two minutes as they habituate to the water situation (42, 43).

2.5 Plasma collection and corticosterone analysis

To investigate the endocrine and immune response to stress, we collected blood samples prior to and following the forced swim test session. Approximately 60 µl of blood per mouse were collected by tail tipping using Lithium-Heparin-coated capillaries (Sigma-Aldrich, St. Louis, Missouri, United States). Blood was centrifuged at 3500 g at 4 °C for 15 min. Plasma was aspirated and stored at −80°C. Blood was taken immediately before the forced swim test (baseline), as well as 15 min, 45 min and 120 min after the baseline. Baseline samples and samples at 120 min post-stress time point were used for flow cytometry (see 2.7).

Plasma corticosterone levels were measured in duplicates by ELISA (ENZO Corticosterone ELISA, Enzo Life Sciences, Exeter, UK) as previously described (22). Data were expressed in ng/ml. Only data derived from duplicates with < 15% coefficient of variation (CV) were included in the analysis.

2.6 Blood stimulation cytokine assay

To assess if a prebiotic-enriched diet alters systemic immunity, 100 µl of trunk blood was obtained at the end of the study using Lithium-Heparin-coated tubes (Greiner Bio-One, Kremsmünster, Austria). Blood cells from each mouse were stimulated with lipopolysaccharide (LPS-2 µg/ml) or Concanavalin A (ConA-2.5 µg/ml) for 24 h or left unstimulated as control. Following 24 h-incubation, samples were taken and stored at -80°C. The levels of secreted IL-1β, IL-4, IL-6, IL-10, TNFα and CXCL1 were analysed with the Proinflammatory Panel 1 (mouse) V-PLEX Kit and the MESO QuickPlex SQ 120, SECTOR Imager 2400 (Meso Scale Discovery, Maryland, USA). Only data derived from duplicates with < 15% CV were included in the analysis. Concentrations of cytokines were expressed in pg/ml.

2.7 Flow cytometry

To assess stress-induced immune priming, blood was collected from young adult and middle-aged mice by tail tipping (60 µl) at baseline and 120 min after acute stress (cohort one). Staining was performed using CD11b-VioBright FITC, Ly-6C-PE, LY-6G-PerCP-Vio700 and MHC-II-PE (all Miltenyi Biotec, Bergisch Gladbach, Germany) to assess inflammatory monocytes (CD11b+, SSC^{low}, LY-$6C^{hi}$) and MHC-II+-neutrophils (CD11b+, LY-6G+, MHC-II+). Inflammatory monocyte and MHC-II+-neutrophil counts were normalized to the amount of peripheral blood mononuclear cell (PBMC). Gating strategy is depicted in Supplementary Figure S2a.

Cohort two investigated if the diet lead-in phase with FOS-Inulin modulates monocyte infiltration and subsequent microglia activation in the brain. Following perfusion with ice-cold PBS for five min, brains were carefully dissected, enzymatically digested using the neural dissociation kit (P), followed by incubation in myelin-removal beads and magnetic separation using LS columns (Miltenyi Biotec). Cells were stained using CD11b-Viobright FITC, CD45-APC and Ly-6C-PE (all Miltenyi Biotec). Gating strategy is depicted in Supplementary Figure S2b. Monocyte counts were normalized to CD11b+ cells, microglia to CD11b+, $CD45^{low}$.

2.8 Analysis of gene expression levels in the brain tissues (RT-qPCR)

To assess gene expression brain areas associated with cognition, the right hemisphere of both, the hippocampus and the prefrontal cortex were used (44). Total RNA was extracted using the mirVana™ miRNA Isolation Kit (Ambion, Life technologies, Waltham, MA, US), followed by DNase treatment using the TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer's instructions. RNA was quantified using the NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA kit (Applied Biosystems, Warrington, UK). *Ccl2* and *Tnf* genes were amplified with probes designed by Integrated DNA Technologies (Coralville, IA, US) (Table S1). PCR was run in triplicates on a LightCycler®480 (Roche). Data were analysed with the comparative cycle threshold (Ct) method. Data were normalized using *Actb* as endogenous control and transformed using the $2^{-\Delta\Delta CT}$ method (45). We confirmed beforehand that the housekeeper *Actb* is neither changed by age nor by prebiotic treatment.

2.9 Caecal microbiota composition (16S rRNA gene sequencing) and short-chain fatty acid analysis

Caecum was harvested, snap frozen and stored at -80°C prior to the analysis. DNA from caecal content was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-beating step, as previously described (46). The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol. Samples were sequenced at Teagasc Sequencing Facility on the Illumina MiSeq platform using a 2 × 250 bp kit.

FLASH was used to assemble paired-end reads. Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME (version 1.9.0). Denoising, chimera detection and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7 (64-bit) (47). OTUs were aligned using PyNAST (and taxonomy was assigned using BLAST against the SILVA SSURef database release 123. Alpha and beta diversities were generated in QIIME (48).

Short chain fatty acids (SCFAs) were measured by gas chromatography, using a Varian 3500 GC flame-ionization system fitted with a ZB-FFAP column as previously described (46).

2.10 Metabolomics from faecal water

Faecal pellets were collected at the end of cohort one. Faecal material was freshly collected using sterilized tools to ensure no cross contamination within a time-window of 10 minutes' maximum to ensure least oxygen exposure of the faeces as possible. Subsequently, pellets were directly snap freeze to ensure optimal DNA integrity. Faecal water was prepared by homogenising faecal samples (20-50 mg) with 4x wt/volume sterile PBS followed by vortexing for 20 minutes. Samples were centrifuged at 16000 g for 30 minutes; the supernatant was transferred in a new 2 mL micro centrifuge tube and centrifuged for further 30 minutes. This step was repeated one more time

before filtering the supernatant through Costar Spin-X centrifuge filters 0.2 µM at 10000 g. Faecal water samples were stored at -20°C.

Subsequently, samples were derivatized with methyl chloroformate as previously described (49) and processed by MS-Omics (Copenhagen, Denmark) using Gas Chromatography – Mass Spectometry (GC-MS). Raw data was converted to netCDF format using ChemStation (Agilent technologies) and processed in Matlab R2014b (Mathworks, Inc., Natick, MA, USA) using the PARADISe software described by (50).

2.11 Statistical analysis

Statistical analyses were conducted using SPSS 24 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analysed for normality using the Shapiro-Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed with Kruskal-Wallis test followed by post hoc Dunn's, and are depicted as median with inter-quartile ranges (IQR) and min/max values as error bars. Parametric data were analysed using two-way analysis of variance (ANOVA) post hoc Holm-Sidak, and are shown as mean ± SEM. Changes in corticosterone response, Morris Water Maze learning and fear conditioning were analysed using two-way-repeated measurement (RM)-ANOVA post hoc Sidak. Correlation analyses were performed using Spearman correlations for non-parametric data. Outliers were excluded using the Grubbs test (51). Statistical significance was set at $p \leq 0.05$.

Statistical analysis of microbiota data was performed in an R software environment. For Principal Component Analysis (PCA), Permutational multivariate analysis of variance (PERMANOVA) was used to identify relationships of significance between variables the Adonis function from the vegan package on Aitchison distance matrices calculated with the ALDEx2 package. ALDEx2 was also used to calculate pairwise differential abundance. Hierarchical All-against-All significance (HAllA) was used to investigate between-dataset covariance. For all tests, a Benjamini-Hochberg post hoc test was performed to correct for multiple comparisons with a conservative *q*-value of 0.2 as critical value.

3. Results

3.1 Prebiotic supplementation reversed stress-induced immune priming in ageing

To assess if ageing triggers stress-induced immune priming in middle-aged mice, and whether age-associated changes are alleviated following prebiotic supplementation, mice were exposed to an acute stress (forced swim test), and blood samples were taken at baseline and 2 hours after stress exposure. We focused on neutrophils, which act as the first responders to any immune challenge and can trigger adaptive immunity, including T-cell priming via expression of major histocompatibility complexes (MHC), a classical activation marker.

In middle-aged mice, acute stress caused a long-lasting increase of the MHC-II+ neutrophil population ($p=0.0280$, Kruskal-Wallis post hoc Dunn's; Figure 1a); the response being absent in young adult animals. Strikingly, prebiotic supplementation prevented the development of the age-associated phenotype and restored the levels of MHC-II+ neutrophils in stressed aged animals to young levels ($p=0.011$).

Since acute stress is known to affect peripheral innate immunity through corticosterone (52), we investigated whether these changes in neutrophil activation status were associated with altered stress axis activity. For this, we measured plasma release of corticosterone (as an indicator of endocrine reactivity to stress) in the same animals, prior to and at different time points following the forced swim stress exposure. Two-way RM-ANOVA revealed an overall effect of age on the corticosterone response ($F_{(1, 28)} = 10.825$, $p=0.003$; Figure 1b). In particular, middle-aged mice exhibited lower plasma corticosterone levels at baseline ($F_{(1, 29)} = 16.68$, $p<0.001$, Figure 1c) and at T15 ($F_{(1, 34)} = 24.65$, $p<0.001$). Two samples in the middle-aged control group did not reached the detection limit and were therefore not included into the analysis. Calculation of area-under-the-curve (AUC) for corticosterone response confirmed that middle-aged mice exhibited a blunted stress axis reactivity ($F_{(1, 28)} = 5.207$, $p=0.03$, Figure 1d). However, we did not observe any modulation on corticosterone response either at baseline or following stress in either young adult or middle-aged mice by prebiotic supplementation suggesting that the changes in peripheral innate immunity are not mediated by corticosterone.

3.2 Effect of prebiotic supplementation on systemic inflammation and immune cell priming

To investigate if systemic inflammation and immune cell priming is altered in middle-aged mice and counteracted by prebiotic supplementation, whole blood was taken after 14 weeks of prebiotic intervention and stimulated with LPS or ConA. Following ConA-stimulation, middle-aged control mice exhibited a trend towards increased IL-1β and IL-10 cytokine release ($p=0.089$ and $p=0.069$, respectively, Supplementary Figure S3b+e), while prebiotic-treated middle-aged mice showed similar

levels as in young controls. Moreover, prebiotic supplementation decreased TNFα in middle-aged mice following ConA-stimulation (p=0.014, Kruskal-Wallis post hoc Dunn's; S3a). No changes were observed at baseline or in response to LPS stimulation.

3.3 Pervasive neuroimmune alterations in middle-aged mice were alleviated by prebiotic supplementation

Given the decline of cognitive function in middle-aged mice (11, 12), we investigated whether the middle-aged brain is more vulnerable to peripheral immune cell trafficking and subsequent microglial activation, and whether this status can be targeted by prebiotic supplementation, utilizing flow cytometry to investigate brain immunity. Two-way ANOVA revealed an effect of age ($F_{(1, 41)} = 11.94$, p=0.001; Figure 2a) and prebiotic treatment ($F_{(1, 41)} = 7.88$, p=0.008) as well as an interaction of both ($F_{(1, 41)} = 6.01$, p=0.019) on trafficking of inflammatory monocytes (Ly-6C^{hi}) into the brain. Specifically, middle-aged control mice showed an increase in Ly-6C^{hi} monocytes compared to young controls (p<0.001), which was alleviated by prebiotic supplementation (p=0.007). We in addition investigated if these changes in infiltrating monocytes are also systemically reflected in the blood. No differences were observed (see Supplementary Figure S7), suggesting that the brain becomes particularly vulnerable in middle-aged mice as inflammatory monocytes traffic to inflamed tissue. Furthermore, we investigated whether the observed increase in monocyte trafficking was associated with microglia activation in the brain. Two-way ANOVA revealed an effect not only of age ($F_{(1, 43)} = 10.75$, p=0.002; Figure 2b), but also prebiotic treatment ($F_{(1, 43)} = 10.95$, p=0.002) and an interaction of both ($F_{(1, 43)} = 13.81$, p<0.001) on Ly-6C+ microglia. Middle-aged controls showed a higher percentage of Ly-6C+ microglia compared to young controls (p<0.001), which was reversed to young control levels following prebiotic supplementation (p<0.001).

In agreement with these findings, the gene expression of *Ccl2* and *Tnf* were up-regulated in the hippocampus of middle-aged mice ($F_{(1, 35)} = 13.60$, $F_{(1, 35)} = 15.79$, p<0.001; Figure 2c-d). *Ccl2* and *Tnf* encode for pro-inflammatory cytokines which are secreted from activated microglia and associated with monocyte infiltration. This supports the observation of microglia activation in the middle-aged brain, including the hippocampus, a key region controlling learning and memory. In contrast, both, *Ccl2* and *Tnf*, were not found to be upregulated in middle-aged mice following prebiotic supplementation. Furthermore, we investigated this phenomenon in another cognition-related brain region, the prefrontal cortex. In contrast to the hippocampus, no effect of age or prebiotic supplementation on *Ccl2* and *Tnf* gene expression was found (Supplementary Figure S4), suggesting a non-universal effect of prebiotic supplementation on cytokine expression across brain regions.

3.4 Prebiotic intervention improved learning and reduced anxiety-like behaviour in young adult mice

To assess whether prebiotic intervention improves spatial learning and memory, mice were trained over five consecutive days to find a hidden platform in the Morris water maze (MWM). Middle-aged mice displayed an impairment in learning ($F_{(1, 35)} = 8.653$, p=0.006; Figure 3a). However, prebiotic supplementation modulated learning ($F_{(1, 35)} = 10.252$, p=0.003), albeit, the improvement was only evident in young adult mice ($F_{(1, 18)} = 10.897$, p=0.004). We did not identify an interaction between age and prebiotic supplementation ($F_{(1, 35)} = 2.073$, p=0.159) suggesting that the prebiotic effects were specific to young adult mice. Although, the average between day four to five is visually different, both days are not statistically different from each other (p=0.19) and mostly explained by a much greater variation compared to day four. Similarly, area-under-the-curve (AUC) analysis confirmed the improved learning in prebiotic-treated young mice (p=0.005). Both, age ($F_{(1, 34)} = 13.10$, p=0.001) and prebiotic supplementation ($F_{(1, 34)} = 12.89$, p=0.001) had a modulatory impact on spatial learning. To assess spatial long-term memory, a probe trial was performed on day six. A trend towards decreased time spent in the target quadrant with age ($F_{(1, 35)} = 3.442$, p=0.072) was observed, however, no improvement by prebiotic supplementation was found (Figure 3a). Neither age or prebiotic exposure affected swim speed, or total distance respectively (data not shown).

We further tested the effect on short-term memory by assessing spontaneous alternation behaviour in the Y-maze. Middle-aged mice showed a decrease in spontaneous alternations ($F_{(1, 35)} = 10.66$, p=0.003) and total number of alternations ($F_{(1, 35)} = 7.986$, p=0.008; Figure 3b) suggesting impairments in short-term memory.

Next, we tested if prebiotic supplementation can modulate fear-dependent learning and memory. For this, mice were tested in a fear conditioning task (Figure 3c). On day one, mice were conditioned to three cued-shock pairings with a variable inter-pairing interval. Middle-aged mice displayed an impaired acquisition ($F_{(1, 36)} = 4.842$, p=0.034, Figure 3c). 24h later, CS recall and extinction learning were assessed. Middle-aged mice showed increased freezing during habituation to the new context ($F_{(1, 35)} = 6.702$, p=0.014) suggesting increased anxiety-like behaviour. Although statistically not significant, the changes in extinction in the prebiotic-treated young adult mice compared to the other groups are explained by the reduced freezing across the cue-shock pairings during acquisition. Similarly, to deficiencies in acquisition, middle-aged mice showed impairments in extinction learning ($F_{(1, 36)} = 4.898$, p=0.034). In contrast, no impact of age nor of prebiotic supplementation on context recall was found (Figure 3c).

Next, we analysed anxiety-like behaviour in the elevated plus maze and the open field, as changes in anxiety levels are known to affect cognitive performance. Overall, middle-aged mice displayed

increased anxiety-like behaviour, as shown by less time spent in the aversive open arms of the elevated plus maze ($F_{(1,33)} = 18.31$ $p<0.001$; Figure 3d), the central zone of the open field arena ($F_{(1, 34)} = 7.337$, $p=0.011$; Figure 3e) as well as decreased number of centre visits ($F_{(1, 34)} = 14.69$, $p<0.001$). The locomotor activity was also marginally reduced in middle-aged mice ($F_{(1,33)} = 4.538$, $p=0.041$; Figure 3c). Prebiotic supplementation did not affect anxiety levels in middle-aged mice. However, a significant increase in the time spent in the open arms of the elevated plus maze was observed in young adult prebiotic-treated mice ($p=0.027$). This suggests that prebiotic supplementation did have an anxiolytic-like effect, but in young animals only. The observed changes in anxiety-like behaviour, i.e. increased anxiety levels in aged mice and selective anxiolytic effect in prebiotic-treated young mice, had a similar pattern seen in the spatial recognition in the MWM. This suggests that impaired cognitive performance in middle-aged mice, as well as improved learning of prebiotic-treated young adults could may be partially mediated by changes in anxiety levels.

Interestingly, learning performance in the Morris water maze correlated with the relative abundance of the *Verrucomicrobiaceae* family ($r_{(38)} = -0.369$, $p=0.023$; Figure 3f); wherein the association is mainly driven by *Akkermansia,* the predominant genus within the *Verrucomicrobiaceae* ($r_{(38)} = -0.323$, $p=0.048$; Figure 3f). Moreover, we identified a significant correlation between hippocampal *Ccl2* expression (as a readout of microglia activation linked to monocyte trafficking) and learning performance (AUC) in the MWM task ($r_{(39)} = 0.349$, $p=0.03$; Figure 3g). To emphasize these correlations further, we displayed which data points relates to which group indicating that prebiotics drive these associations.

3.5 Effect of age and prebiotic supplementation on gut microbiota composition and short-chain fatty acid profile in the gut

Principal Component Analysis (PCA) analysis identified structural differences in microbiota across all four groups (PERMANOVA, $p<0.001$; Figure 4a). The composition of caecal microbiota was significantly affected by age and by prebiotic supplementation (all $p<0.05$, pairwise PERMANOVA). Interestingly, no interaction between age and prebiotic was observed, i.e. marked differences between middle-aged and young mice were evident in both control and prebiotic-treated groups, and prebiotic supplementation effectively shifted microbiota composition in both young adult and middle-aged animals.

When we looked at structural properties of microbial communities at the genus level, we observed multiple changes in the relative abundance of individual bacterial taxa (Figure 4b). In particular, middle-aged mice displayed an increase in *Clostridum sensu stricto 1, Delftia, Salmonella, Enterococcus, Turibacter* ($q < 0.1$). In contrast, *Parabacteroides* ($q < 0.01$) was decreased in middle-

aged control mice. Interestingly, prebiotic supplementation not only increased the abundance of *Bifidobacterium* in young adult but also middle-aged mice ($q < 0.1$ and $q < 0.01$, respectively). In contrast, *Akkermansia* was only increased in middle-aged prebiotic-treated mice ($q < 0.1$). Moreover, prebiotic supplementation increased the abundance of *Prevotellaceae UCG-001* and *Bacteroides* not only in young adult mice but even more pronounced in middle-aged mice ($q < 0.01$, respectively), while *Lactobacillus* and *Roseburia* were decreased in prebiotic-treated middle-aged mice ($q < 0.1$).

The Chao1 index was increased in middle-aged compared to young adult control mice, indicating an increase in overall richness of bacterial species associated with age ($p=0.028$; Kruskal-Wallis post hoc Dunn's; Figure 4c). However, the Shannon and the Simpson indices, which take into account the evenness of species abundance, were not affected by age but were reduced following prebiotic supplementation in young adult mice ($p=0.010$ and $p=0.016$, respectively). This suggest that prebiotic supplementation favoured the selective expansion of certain bacterial taxa in young adult animals only.

To identify if changes in gut microbiota composition correlated with faecal metabolomics, we utilized Hierarchical All-against-All significance testing (HAllA). Among others, HAllA identified a negative association between the relative abundance of *Akkermansia*, which was significantly over-represented in prebiotic-treated middle-aged mice, and several amino acids including leucine ($\rho=-0.63$, $p<0.001$, FDR corrected, Figure 4d), valine and isoleucine ($\rho=-0.60$, $p<0.001$, respectively). Similarly, between *Bifidobacterium*, which was significantly over-represented in prebiotic-treated young and middle-aged mice, and the respective amino acids ($\rho=-0.55$, $p=0.001$). Prebiotic supplementation increased caecum weight ($F_{(1, 35)} = 88.95$, $p<0.001$; Supplementary Figure S6b) in both young adult and middle-aged mice. Among short-chain fatty acids (SCFAs), caecal butyrate, propionate and valerate levels were affected by either age or prebiotic supplementation. No effect was found on acetate and total-SCFA levels (data not shown). Middle-aged mice exhibited higher butyrate levels than young mice ($F_{(1, 35)} = 16.74$, $p<0.001$; Figure S6c). Prebiotic supplementation increased propionate independent of age ($F_{(1, 35)} = 8.75$, $p<0.001$), with a more pronounced increase seen in middle-aged mice ($p=0.035$). While valerate was increased in middle-aged compared to young controls ($p<0.001$), prebiotic supplementation reduced valerate in both, young adult ($p=0.021$) and middle-aged mice ($p<0.001$).

4. Discussion

There is a growing appreciation of the role of the gut microbiota in regulating neuroinflammatory responses. The middle-aged brain remains completely understudied regarding this interrelationship. Our data show that middle age is already associated with pervasive alterations in systemic and brain immunity. Targeting the gut microbiome by prebiotic intervention (FOS-Inulin) reversed many of these age-associated neuroinflammatory impairments.

To our knowledge, this is the first study demonstrating the presence of a strong basal and stress-induced (neuro-) inflammatory profile in middle-aged mice (11 months old), although an exaggerated inflammatory response has been previously reported in middle-aged rodents following immune stimulation (53-55). Moreover, our study implicates the gut microbiome in such processes as dietary targeting with prebiotic supplementation counteracted stress-induced peripheral immune cell activation. Following acute stress, we investigated a subtype of neutrophils that express MHC-II, which plays a role in priming of T-cells and therefore provides a link between the innate and the adaptive immune system (56, 57). Further research is warranted on the functional characterization of these neutrophils and their impact on the brain in ageing particularly following acute stress.

The gut microbiome has emerged as being essential for brain health in ageing and as a key player in the bidirectional communication across the gut–brain axis (58, 59). Previous research points out a role of aged microbiota in driving systemic immunity (26, 27). In addition, key metabolites which are produced by the gut microbiota following i.e. a prebiotic-enriched diet such as short-chain fatty acids (SCFAs) has been implicated in alleviating stress-induced alteration (46). We show that prebiotic supplementation is capable of dampening age-associated systemic inflammation, particularly TNFα, following stimulation with Concanavalin A. As ConA stimulates both, T- and NK-cells, it seems that both cell types are in particular sensitive to prebiotic treatment in middle-aged mice compared to LPS stimulation which stimulates a broad range of immune cells. We previously showed that prebiotic treatment rescues immune alteration induced by chronic psychosocial stress following ConA stimulation exclusively (32) suggesting that prebiotics might have specific effects on immune priming on T- and NK-cells systemically, and may influence brain function and behaviour which warrants further research. Recent research showed a role of T-cell activation in regulating behaviour, anxiety-like and fear-related behaviour (60), cognition (61) and sociability (62), which may possibly be influenced by the gut microbiota. A critical factor for T-cell activation is the availability of specific amino acids such as leucine (63). By using HAIIA, we identified strong correlations between prebiotic-driven changes in gut microbiota, *Bifidobacterium* and *Akkermansia* with several amino acids in faecal water, including valine, leucine and isoleucine amongst others. In fact the gut microbiome has

been implicated in regulating amino acid availability (64). Interestingly, a recent study in a Chinese cohort of middle-aged to elderly individuals found a correlation between *Akkermansia* and CD8+ as well as CD4+ T cells (65).

A bidirectional relationship between the brain and the peripheral immune system exists (66), which can promote neuroinflammation and exacerbate neuronal damage in the hippocampus. Recent studies suggest a constant influx of immune cells, inflammatory monocytes (Ly-6C^{hi}-monocytes), into the brain even under steady-state conditions (67-69). Previously these cells were thought to only play a role in inflammatory conditions such as following viral infection and associated encephalitis (70) or after social defeat stress (71, 72). However, recent research suggests that trafficking of Ly-6C^{hi}-monocytes into the brain is crucial for brain plasticity and influence cognitive behaviour (67). This was mediated by the gut microbiome as antibiotic depletion and subsequent reconstitution of the gut microbiome restored the antibiotic-induced deficits in brain plasticity and cognitive behaviour (67). To characterize if these Ly-6C^{hi}-monocytes also affect the brain in middle-aged mice before animals were tested behaviourally, we assessed their neuroimmune status in cohort two. Here we show that middle-aged mice exhibited an increased influx of inflammatory monocytes into the brain. Following the determination of their neuroimmunity baseline response, we then subjected the mice to the behavioural assessment. To correlate the changes in behaviour with specific neuroimmune markers which link monocyte trafficking to microglia activation, we subsequently analysed targets in the brain at the end of the study. Ly-6C^{hi}-monocytes are recruited to the brain in a CCL2-dependent manner (70, 72, 73). We show that *Ccl2* is specifically upregulated in the hippocampus of middle-aged mice, but not present following prebiotic supplementation suggesting that this is may be a potential pathway in which gut-microbiota-immune-brain communication can affect brain function and behavioural traits in this key region for learning and memory. However, despite these changes in neuro-immunity, we have not identified any overt cognitive impairments in middle-aged control mice. Although it is worth noting that the dynamics of hippocampal *Ccl2* expression correlated with cognitive behaviour assessed in the Morris water maze paradigm. Interestingly, prebiotic-driven changes in the neuroinflammatory profile are not universal across brain regions as there were no changes in the prefrontal cortex. This is in line with previous findings that there are marked regional differences in microglia activation across brain regions (74). Interestingly, we found that middle-aged mice exhibited increased microglia activation under basal conditions before animals were behaviourally assessed. This subset of inflammatory activated microglia expressed Ly-6C (73, 75, 76) and have been suggested to arise from Ly-6C^{hi}-monocytes (70). Recent work has demonstrated a modulatory effect of the gut microbiota on microglia function (77-79). Of note, germ-free mice exhibited deficits in microglia maturation and function while addition of

SCFAs rescued these deficits. However, the short-chain fatty acid receptor FFAR2 is actually not present on microglia (77), but on monocytes (80). Future studies are needed to investigate the mechanistic relationship between these receptors and prebiotic-induced effects on microglia activation across the lifespan.

Microglia activation has been shown to alter cognitive and anxiety-like behaviour (17, 81). Here, we show that prebiotic supplementation improves anxiety-like behaviour and cognition in young adult mice. This is in accordance with previous studies which targeted the gut microbiome by dietary interventions in rodents (32, 38, 82-84). Interestingly, studies using a probiotic mix (VSL#3) failed to show improvements in anxiety-related behaviour (85) suggesting that strain selection is very important and that prebiotics might be a better approach to improve behaviour. Moreover, we show that middle-aged control mice showed a decreased number of centre visits in the open field suggesting increased anxiety-like behaviour (7), which may have influenced cognitive performance (11). Middle-aged mice displayed mild cognitive impairments, which were not present following prebiotic supplementation. It is worth noting that neuroinflammation at this stage was not significant enough to manifest in major cognitive impairments. However, our data imply that prebiotic intervention may have some potential to counteract cognitive decline. As the impact of prebiotic supplementation on behaviour, particularly the cognitive tests, is clearly stronger in adult subjects, the data suggests that prebiotics may be less effective as we age. On the other side, a much longer exposure to prebiotics might be needed to achieve significant effects suggesting that supplementation may have to start earlier to be effectively preventative before alterations in the brain occur. This is particularly evident for the behaviour. On the other side, particularly in light of the stress-induced peripheral immune data, the system may need to be challenged to potentially exert negative behavioural effects (86) before prebiotic supplementation can act beneficially (32). Future studies focused on long-term effects of this mid-life microbiota manipulation are now warranted.

We hypothesized that the dysregulated gut-microbiome-brain axis in middle-aged mice can be ameliorated by targeting the gut microbiome with prebiotics known to promote beneficial bacteria, like *Bifidobacteria*. It was previously shown that the prebiotic, inulin, can alter the microbiome composition under pathophysiological conditions such as following high-fat diet (87) or in extreme ageing (33); however, its effects remained unexplored in healthy ageing/middle age. In fact, by utilizing FOS-Inulin, we show a profound yet differential alteration of the gut microbiota composition in both young adult but also in middle-aged mice. This was concomitant with a change in short-chain

fatty acids with propionate increased in prebiotic-treated middle-aged mice while prebiotic supplementation decreased valerate in both, young adult and middle-aged mice.

Previous research has shown that diet-driven modulation of the gut microbiota by administration of prebiotics can modulate peripheral immune response in the serum of naïve mice (32) and we recently showed that SCFAs attenuate the effect of chronic stress (46). It was shown previously that propionate can inhibit the production of pro-inflammatory cytokines (88). Moreover, *in-vitro* experiments suggests pro-inflammatory capabilities of valerate while it enhanced LPS-induced inflammatory response in a murine N9 microglial cell line (89). Although the effects on SCFA levels is relatively modest it is possible that some of the anti-inflammatory effects of prebiotic supplementation might have been mediated by the changes observed in SCFA concentrations.

We have previously reported a shift in microbial composition by prebiotics in adult mice (32) but the impact on middle-aged remained unexplored. Interestingly, we found an increase in species richness in middle-aged mice, which is in line with previous findings in rodents (22) and humans (90). In fact, it has been shown in humans that the gut microbiota remarkably changes with ageing not only in diversity but also representation of specific taxa (91-93).

Prebiotic supplementation increased the relative abundance of *Bifidobacterium*, which is in accordance with previous studies in humans (94). Interestingly, *Bifidobacteria* has been reported to be reduced in the elderly (95). In addition, supplementation increased the relative abundance of *Akkermansia* in middle-aged mice suggesting that prebiotics might promote a young microbiota phenotype, compared to a previous study where *Akkermansia* abundance strongly declined in 12- vs. 4-months-old control mice (23). When transferring faecal matter from old mice to young germ-free (GF) mice, *Akkermansia* was lower abundant in those recipients than in GF mice that received young microbiota (26). Interestingly, *Akkermansia* has been associated with immune modulation (26), has shown to protect against inflammation and promote gut health in diet-induced obesity (96), and restored intestinal permeability and subsequent immunomodulation in aged mice (97). Moreover, *Akkermansia* has been found to be enriched in super-centenarians (24). Together with *Bifidobacterium*, *Akkermansia* are claimed as longevity-adapted and possibly health-promoting taxa and therefore might be involved in healthy ageing (24). It is worth noting that learning performance strongly correlated with the abundance of *Akkermansia* suggesting a link between microbiota and cognitive performance. Future studies are warranted to investigate the potential beneficial impact of *Akkermansia* on cognitive performance and healthy ageing.

590 It is now clear that the microbiota-gut-brain axis communicates through multiple channels (98). Thus
591 targeting the gut microbiota as we have done with a prebiotic, can affect the brain and subsequent
592 behaviour through a variety of potential pathways including SCFAs, amino acids and immune
593 pathways. All of these are interconnected and future studies are needed to better deconvolve the
594 primacy of such pathways in eliciting the beneficial effects of inulin.
595
596 In conclusion, the present study identified a strong neuroimmune phenotype in middle-aged mice.
597 Moreover, prebiotic-driven changes in gut microbiota composition are beneficial for host health and
598 associated well-being in middle-aged mice. Prebiotic supplementation is capable of altering age-
599 induced changes in brain homeostasis, particularly alleviation of microglia activation, suggesting a
600 preventative strategy towards preservation of cognitive health in ageing. Taken together, the
601 modulatory effects of prebiotic supplementation on monocyte infiltration into the brain and
602 accompanied regulation of age-related microglia activation highlight a potential pathway by which
603 prebiotics can modulate peripheral immune response and alter neuroinflammation in ageing. Our
604 data thus suggest a novel strategy for the amelioration of age-related neuroinflammatory
605 pathologies and brain function.

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Author contributions

MB, GC, CS, TGD, HS and JFC have contributed to the conception and design of the work. Acquisition, analysis and interpretation of data were performed by MB, MVDW, TFS, LOR, KL, FF, AVG, GM, CM, KVS, KAS. MB and JFC wrote the manuscript. MB, MVDW, TFS, FF, AVG, GM, KVS, KAS, GC, CS, TGD, HS and JFC critically revised the manuscript. All authors approve the final version of the manuscript and agree to be accountable for all aspects of the work.

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Figure Legends

Figure 1. Prebiotic supplementation reversed stress-induced immune priming in middle-aged mice. **(a)** MHC-II+ neutrophils at baseline and 2h after acute stress. **(b)** Plasma Corticosterone (Cort) response curve at baseline, immediately before exposure to acute stress, and 15, 45 and 120 min after exposure to acute stress. **(c)** Plasma corticosterone at baseline. **(d)** Area-under-the-curve (AUC) of corticosterone response. Mean ± SEM. (a) n = 9-10, (b-d) n = 7-10. (a) Kruskal-Wallis post hoc Dunn's, (b) two-way-repeated measurement (RM)-ANOVA post hoc Sidak, (c-d) two-way ANOVA post hoc Holm-Sidak (Cort T0, Cort Area-under-the-curve). vs. *control young adult* * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. *control middle-aged* # $p < 0.05$, ## $p < 0.01$, vs. *prebiotic middle-aged vs prebiotic adult* $ < 0.05.

Figure 2. Middle-aged mice exhibited elevated infiltration of Ly-6C^{hi} monocytes into the brain and increased microglia activation; the phenotype was reversed by prebiotic supplementation. (a) Monocyte infiltration in the brain. **(b)** Microglia expression pattern in the brain. **(c-d)** Pro-inflammatory cytokine expression in the hippocampus. Mean ± SEM. (a-b) n = 14-16 (young adult), n = 8-10 (middle-aged), (c-d) n = 10 (young adult), n = 9-10 (middle-aged). (a-d) two-way ANOVA post hoc Holm-Sidak. *vs. control young adult* * $p < 0.05$, *** $p < 0.001$, *vs. control middle-aged* ## $p < 0.01$, ### $p < 0.001$.

Figure 3. Prebiotic supplementation improved learning and reduced anxiety-like behaviour in young adult mice. (a) Learning and memory in Morris water maze (MWM). Latency-to-find platform over five training days. Summarized as area-under-the-curve (AUC), as well as the probe trial 24h after the last training day is depicted. **(b)** Short-term memory assessed by Spontaneous Alternation Behaviour (Y-Maze). **(c)** Fear Conditioning: Conditioning (Acquisition, day one) including AUC. Extinction (day two) – two consecutive cue presentations were depicted as one trial block. AUC for trial block 1-20 is depicted. Context recall (day three). **(d)** Time spent in open arms in elevated-plus maze. **(e)** Behaviour in open field. **(f)** Spearman correlation analysis of learning efficacy in Morris water maze (AUC) vs. relative abundance of bacteria from the *Verrucomicrobiaceae* family and *Akkermansia* genus **(g)** Spearman correlation learning in Morris water maze vs. hippocampal *Ccl2* expression. Mean ± SEM. n = 9-10. (a – MWM – latency-to-find-platform, c – acquisition day one) two-way RM ANOVA post hoc Sidak, (a-e) two-way ANOVA post hoc Holm-Sidak. vs. *control young adult* * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. *control middle-aged* # $p < 0.05$, *prebiotic middle-aged vs prebiotic adult*: $ < 0.05, $$ < 0.01, $$$ $p < 0.001$.

Figure 4. Middle age and prebiotic treatment have distinct effects on the gut microbiota composition accompanied with changes in faecal metabolomic profile. (a) PCA plot **(b)** Heat map representing differentially abundant taxa (genus with higher hierarchy family name), which reach a Benjamini-Hochberg FDR q value < 0.2 at least once. Asterisks in the heat map represent the following q values: * <0.1, ** < 0.01, *** < 0.001. **(c)** Alpha-diversity Indices (Chao1, Simpson, Shannon). **(d)** Hierarchical All-against-All significance testing (HAllA) representing the 100 strongest significant correlations (q<0.2) between gut microbiota composition and faecal metabolomics. Numbers (1-100) indicate the strongest correlations in a descendant order. n = 9-10. (a) PERMANOVA, followed by pairwise PERMANOVA post hoc Benjamini-Hochberg, (b) Mann-Whitney U test post hoc Benjamini-Hochberg (c) Kruskal-Wallis post hoc Dunn's, (d) Spearman post hoc Benjamini-Hochberg.

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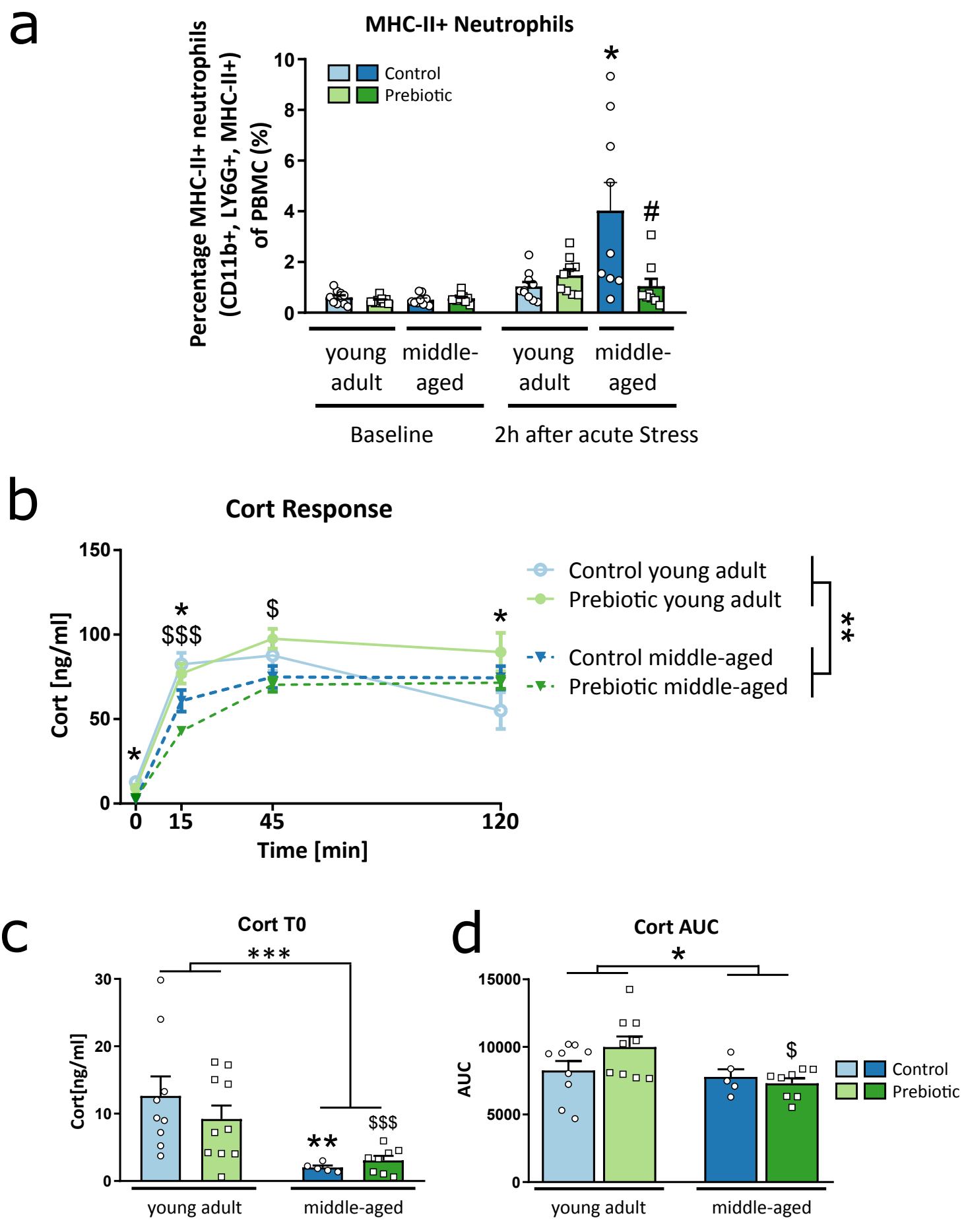
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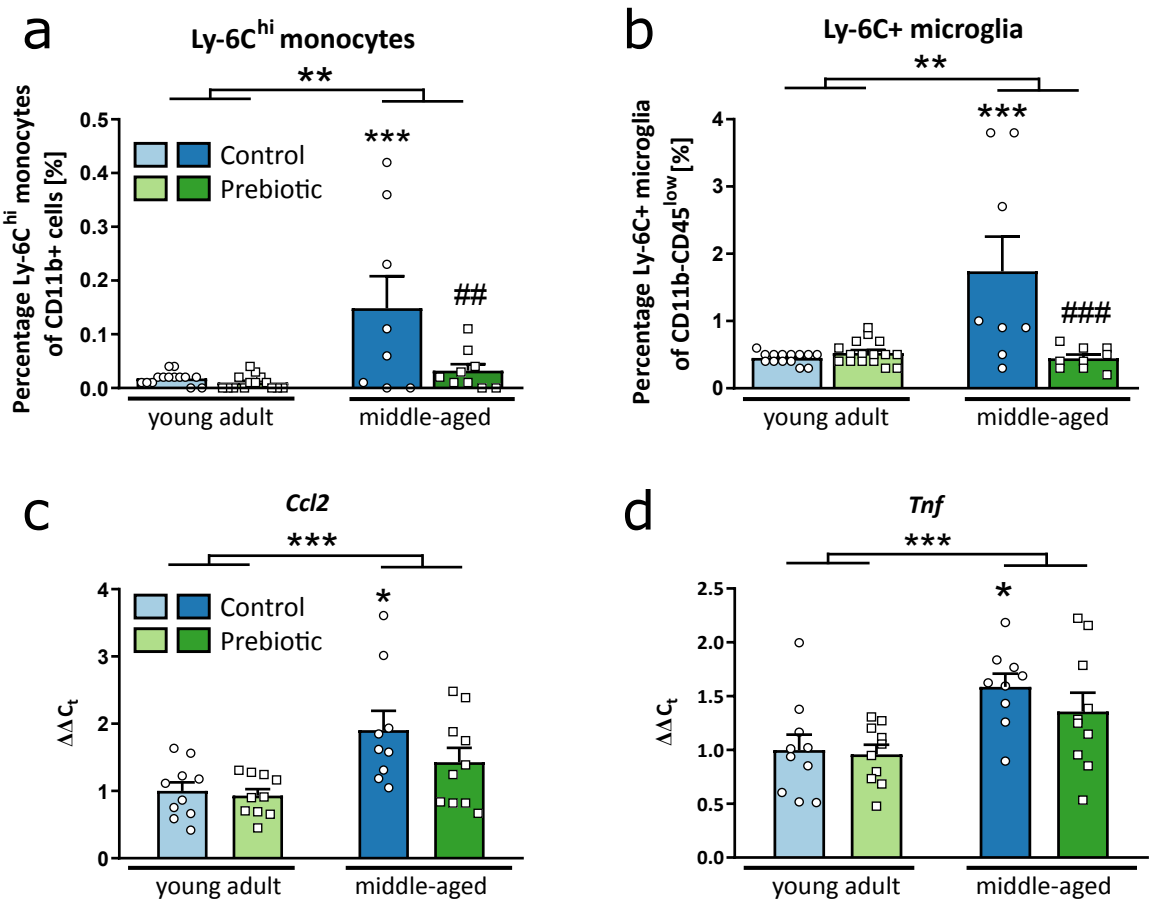
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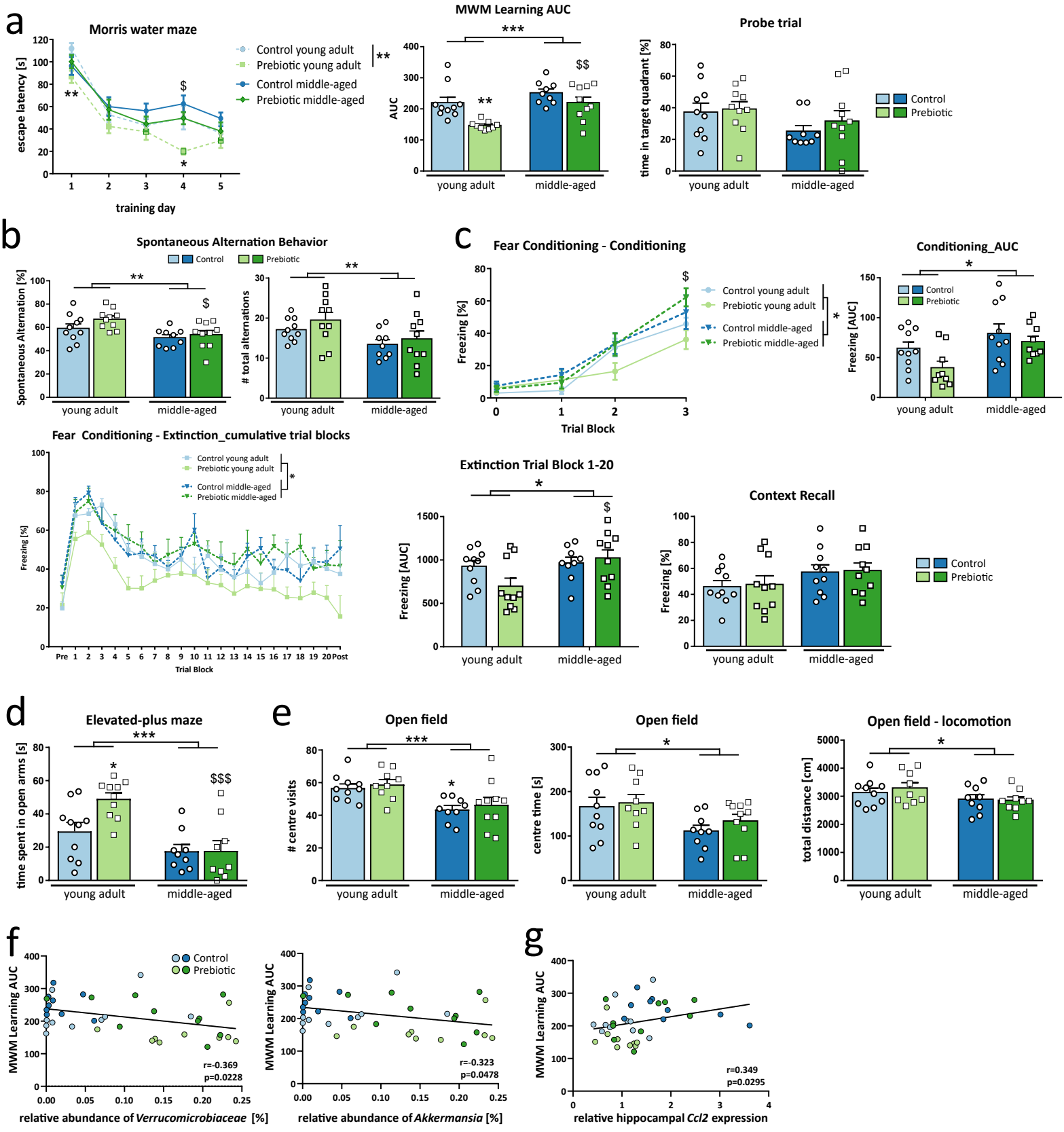
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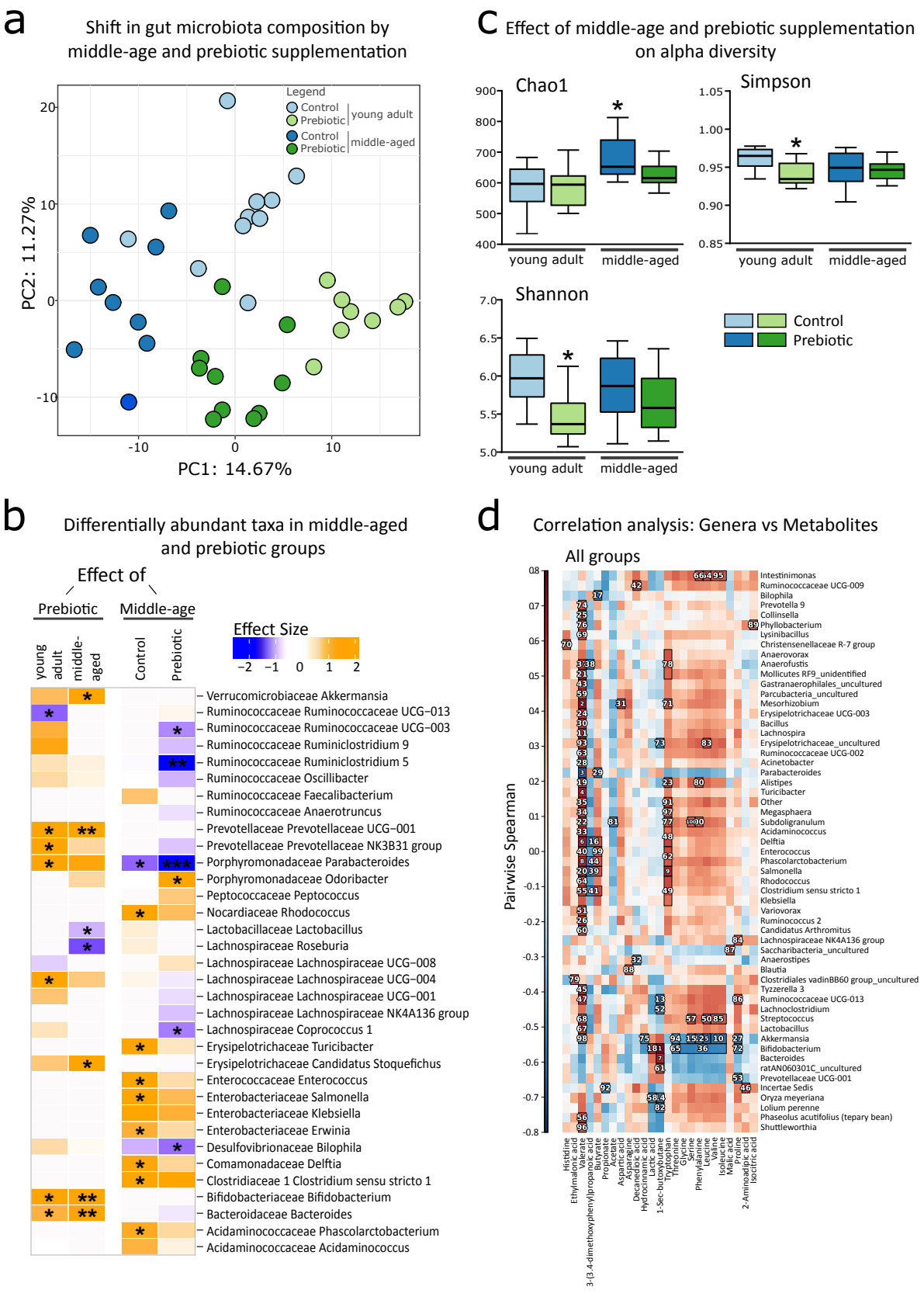
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a
MHC-II+ Neutrophils
Percentage MHC-II+ neutrophils (CD11b+, LY6G+, MHC-II+) of PBMC (%)
Control
Prebiotic
young adult
middle-aged
young adult
middle-aged
Baseline
2h after acute Stress
b
Cort Response
Cort [ng/ml]
Time [min]
Control young adult
Prebiotic young adult
Control middle-aged
Prebiotic middle-aged
c
Cort T0
Cort[ng/ml]
young adult
middle-aged
d
Cort AUC
AUC
young adult
middle-aged
Control
Prebiotic


a
Ly-6C^hi monocytes
**
***
##
Control
Prebiotic
Percentage Ly-6C^hi monocytes of CD11b+ cells [%]
0.5
0.4
0.3
0.2
0.1
0.0
young adult
middle-aged
b
Ly-6C+ microglia
**
***
###
Percentage Ly-6C+ microglia of CD11b-CD45^low [%]
4
3
2
1
0
young adult
middle-aged
c
Ccl2
***
*
Control
Prebiotic
ΔΔC_t
4
3
2
1
0
young adult
middle-aged
d
Tnf
***
*
ΔΔC_t
2.5
2.0
1.5
1.0
0.5
0.0
young adult
middle-aged


a
Morris water maze
escape latency [s]
training day
Control young adult
Prebiotic young adult
Control middle-aged
Prebiotic middle-aged
MWM Learning AUC
AUC
young adult
middle-aged
Probe trial
time in target quadrant [%]
Control
Prebiotic
b
Spontaneous Alternation Behavior
Spontaneous Alternation [%]
total alternations
c
Fear Conditioning - Conditioning
Freezing [%]
Trial Block
Conditioning_AUC
Freezing [AUC]
Fear Conditioning - Extinction_cumulative trial blocks
Extinction Trial Block 1-20
Context Recall
d
Elevated-plus maze
time spent in open arms [s]
e
Open field
centre visits
centre time [s]
Open field - locomotion
total distance [cm]
f
MWM Learning AUC
r=-0.369
p=0.0228
relative abundance of Verrucomicrobiaceae [%]
r=-0.323
p=0.0478
relative abundance of Akkermansia [%]
g
r=0.349
p=0.0295
relative hippocampal Ccl2 expression


a
Shift in gut microbiota composition by middle-age and prebiotic supplementation
Legend
Control
Prebiotic
young adult
Control
Prebiotic
middle-aged
PC2: 11.27%
PC1: 14.67%
c
Effect of middle-age and prebiotic supplementation on alpha diversity
Chao1
Simpson
Shannon
young adult
middle-aged
Control
Prebiotic
b
Differentially abundant taxa in middle-aged and prebiotic groups
Effect of
Prebiotic
Middle-age
young adult
middle-aged
Control
Prebiotic
Effect Size
−2 −1 0 1 2
Verrucomicrobiaceae Akkermansia
Ruminococcaceae Ruminococcaceae UCG−013
Ruminococcaceae Ruminococcaceae UCG−003
Ruminococcaceae Ruminiclostridium 9
Ruminococcaceae Ruminiclostridium 5
Ruminococcaceae Oscillibacter
Ruminococcaceae Faecalibacterium
Ruminococcaceae Anaerotruncus
Prevotellaceae Prevotellaceae UCG−001
Prevotellaceae Prevotellaceae NK3B31 group
Porphyromonadaceae Parabacteroides
Porphyromonadaceae Odoribacter
Peptococcaceae Peptococcus
Nocardiaceae Rhodococcus
Lactobacillaceae Lactobacillus
Lachnospiraceae Roseburia
Lachnospiraceae Lachnospiraceae UCG−008
Lachnospiraceae Lachnospiraceae UCG−004
Lachnospiraceae Lachnospiraceae UCG−001
Lachnospiraceae Lachnospiraceae NK4A136 group
Lachnospiraceae Coprococcus 1
Erysipelotrichaceae Turicibacter
Erysipelotrichaceae Candidatus Stoquefichus
Enterococcaceae Enterococcus
Enterobacteriaceae Salmonella
Enterobacteriaceae Klebsiella
Enterobacteriaceae Erwinia
Desulfovibrionaceae Bilophila
Comamonadaceae Delftia
Clostridiaceae 1 Clostridium sensu stricto 1
Bifidobacteriaceae Bifidobacterium
Bacteroidaceae Bacteroides
Acidaminococcaceae Phascolarctobacterium
Acidaminococcaceae Acidaminococcus
d
Correlation analysis: Genera vs Metabolites
All groups
Pairwise Spearman
Intestinimonas
Ruminococcaceae UCG-009
Bilophila
Prevotella 9
Collinsella
Phyllobacterium
Lysinibacillus
Christensenellaceae R-7 group
Anaerovorax
Anaerofustis
Mollicutes RF9_unidentified
Gastranaerophilales_uncultured
Parcubacteria_uncultured
Mesorhizobium
Erysipelotrichaceae UCG-003
Bacillus
Lachnospira
Erysipelotrichaceae_uncultured
Ruminococcaceae UCG-002
Acinetobacter
Parabacteroides
Alistipes
Turicibacter
Other
Megasphaera
Subdoligranulum
Acidaminococcus
Delftia
Enterococcus
Phascolarctobacterium
Salmonella
Rhodococcus
Clostridium sensu stricto 1
Klebsiella
Variovorax
Ruminococcus 2
Candidatus Arthromitus
Lachnospiraceae NK4A136 group
Saccharibacteria_uncultured
Anaerostipes
Blautia
Clostridiales vadinBB60 group_uncultured
Tyzzerella 3
Ruminococcaceae UCG-013
Lachnoclostridium
Streptococcus
Lactobacillus
Akkermansia
Bifidobacterium
Bacteroides
ratAN060301C_uncultured
Prevotellaceae UCG-001
Incertae Sedis
Oryza meyeriana
Lolium perenne
Phaseolus acutifolius (tepary bean)
Shuttleworthia
Histidine
Ethylmalonic acid
Valerate
3-(3,4-dimethoxyphenyl)propanoic acid
Butyrate
Propionate
Acetate
Aspartic acid
Alanine
Decanedioic acid
Hydrocinnamic acid
Leucine
1-Sec-butoxybutane
Tryptophan
Threonine
Glycine
Serine
Phenylalanine
Lysine
Valine
Isoleucine
Malic acid
Proline
2-Aminoadipic acid
Isocitric acid