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Mid-Life Microbiota Crises: Middle Age is Associated with Pervasive Neuroimmune 1 Alterations that are Reversed by Targeting the Gut Microbiome 2 Marcus Boehme^{1,2}, Marcel van de Wouw^{1,2}, Thomaz F. S. Bastiaanssen^{1,2}, Loreto Olavarria-3 Ramirez^{1,2}, Katriona Lyons⁴, Fiona Fouhy^{1,4}, Anna V. Golubeva¹, Gerard Moloney^{1,2}, Chiara 4 Minuto^{1,3}, Kiran V. Sandhu¹, Karen A. Scott¹, Gerard Clarke^{1,3}, Catherine Stanton^{1,4}, Timothy 5 G. Dinan^{1,3}, Harriët Schellekens^{1,2}, John F. Cryan^{1,2} 6 ¹APC Microbiome Ireland, University College Cork, Ireland 7 ²Department of Anatomy and Neuroscience, University College Cork, Ireland 8 ³Department of Psychiatry and Neurobehavioural Science, University College Cork, Ireland 9 ⁴Teagasc Food Research Centre, Food Biosciences Department, Moorepark, Fermoy, Cork, Ireland 10 11 12 13 14 **Molecular Psychiatry** To be submitted to: 15 16 **Running Title:** Prebiotics reverse neuroimmune alterations in middle-aged mice 17 **Correspondence:** 18 Prof. John F. Cryan 19 j.cryan@ucc.ie 20 Phone: +353-21-420-5426 21 Dept. Anatomy & Neuroscience **APC Microbiome Ireland** 22 23 University College Cork, Cork 24 Ireland

25 Abstract

26 Male middle age is a transitional period where many physiological and psychological changes occur 27 leading to cognitive and behavioural alterations, and a deterioration of brain function. However, the 28 mechanisms underpinning such changes are unclear. The gut microbiome has been implicated as a 29 key mediator in the communication between the gut and the brain, and in the regulation of brain 30 homeostasis including brain immune cell function. Thus, we tested whether targeting the gut 31 microbiome by prebiotic supplementation may alter microglia activation and brain function in 32 ageing. Male young adult (eight weeks) and middle-aged (ten months) C57BL/6J mice received diet 33 enriched with a prebiotic (10% oligofructose-enriched inulin (FOS-Inulin)) or control chow for 14 34 weeks. Prebiotic supplementation differentially altered the gut microbiota profile in young and 35 middle-aged mice with changes correlating with faecal metabolites. Functionally, this translated into 36 a reversal of stress-induced immune priming in middle-aged mice. In addition, a reduction in ageing-37 induced infiltration of Ly-6C^{hi}-monocytes into the brain coupled with a reversal in ageing-related 38 increases in a subset of activated microglia (Ly-6C⁺) was observed. Taken together, these data 39 highlight a potential pathway by which targeting the gut microbiome with prebiotics can modulate 40 the peripheral immune response and alter neuroinflammation in middle age. Our data highlight a 41 novel strategy for the amelioration of age-related neuroinflammatory pathologies and brain function.

42 **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).

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50 Ageing is defined as a process involving slow deterioration of various homeostatic functions 51 throughout the lifespan. Middle age in particular is a life period where many physiological and 52 psychological changes occur, leading to first cognitive impairments and behavioural alterations, and a 53 deterioration of brain function (7-11). In rodents, increased anxiety-like behaviour occurs in middle-54 age (7, 11). A few studies reported cognitive decline in middle-aged rodents (8, 11), with variable 55 definitions of "middle-age" highlighting the need for greater specification (12). Moreover, the levels 56 of neurotransmitters (9) and neurotrophins (10) were shown to decline with age, which may possibly 57 contribute to altered behaviour and brain homeostasis.

58

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.

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65 Microglia are the major immune cells in the brain and have been shown to be a key player in 66 neuropsychological and neurodegenerative conditions (16, 17). Increased activation of microglia in 67 the aged brain has been suggested to be indicative of enhanced inflammation and heightened 68 reactivity in the rodent and the human brain (13, 18, 19). Following an immune stimulus, which is 69 exaggerated in ageing, microglia are referred to as "primed" due to their rapid induction and 70 increased cytokine release upon activation (13, 18). Microglia are specialised cells continuously 71 monitoring their environment (20) and can sense changes in the brain's milieu (21). In addition, 72 microglia play a crucial role in synaptic plasticity, brain function and cognition across the lifespan 73 (17).

74

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

77 pre-clinical models implicated a role of microbiota from aged mice in driving systemic immunity (26, 78 27). However, the effect on neuroimmunity and subsequent brain function and behaviour remains 79 unaddressed. Interestingly, the transfer of gut microbiota from young-to-aged subjects might 80 influence healthy ageing as shown in the short-lived killifish model, which exhibited an increase in 81 lifespan and motor behaviour (28). It has been shown that the administration of prebiotics (a 82 substrate that is selectively utilized by host microorganisms conferring a health benefit (29)) results 83 in an increase in the number of beneficial intestinal bacterial species with a reduction in systemic 84 inflammation in humans (30, 31), and both, peripheral and neuroinflammation in rodents (32, 33) 85 which would have important implications for the healthcare system. It however remains unclear 86 what is driving these changes and what is the impact on brain function and behaviour. Therapeutic 87 interventions are thus sought in order to delay ageing, decrease pro-ageing factors, reduce microglia 88 activation and ultimately improve cognition during ageing.

89

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.

97 2. Methods

98 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.

110

111 **2.2 Prebiotic administration**

Mice received chow (ssniff-Spezialdiäten GmbH, Soest, Germany) enriched with 10% Oligofructoseenriched 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).

118

119 **2.3 Study design and experimental timeline**

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

121 Cohort one investigated the effects of FOS-Inulin on behaviour including cognitive (spontaneous 122 alternation behaviour, Morris water maze, fear conditioning), anxiety-like (open field, elevated-plus 123 maze, marble burying), social (three-chamber social interaction test) and depressive-like behaviour 124 (forced swim test). Following a three-week lead-in of diet, mice (n=9-10 per group) underwent 125 behavioural assessment while continuing dietary supplementation for a total of 14 weeks. In 126 addition, peripheral immune cell activation (pre-/post stress) was assessed in blood using flow 127 cytometry. To correlate the changes in behaviour with specific neuroimmune targets, we 128 subsequently analysed targets in the brain at the end of the study.

129 To characterize the neuroimmune status in the brain at a time point before animals were tested

130 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
- 132 activation in the brain, key mediators influencing cognition and anxiety-like behaviour.
- 133
- 134 See Supplemental Methods for detailed information on procedures (2.4 to 2.9).
- 135

136 **2.4 Behaviour**

137 **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.

141

142 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.

147

148 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).

152

153 **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.

158

159 2.4.5 Marble burying test

160 The marble burying test assesses compulsive, repetitive and anxiety-like behaviour, and was 161 conducted as previously described (32). Briefly, mice were tested for 30 min and the number of 162 buried marbles was recorded.

163 **2.4.6 Elevated-plus maze**

164 The Elevated-plus Maze test was used to assess anxiety-like behaviour and was conducted as 165 previously described (32). Mice were allowed to explore the maze for five minutes; the time spent in 166 the open arms, as well as number of entries into the open arms was analysed.

167

168 **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).

174

175 **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).

182

183 **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).

- 191 Plasma corticosterone levels were measured in duplicates by ELISA (ENZO Corticosterone ELISA, Enzo
- 192 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.

195 **2.6 Blood stimulation cytokine assay**

196 To assess if a prebiotic-enriched diet alters systemic immunity, 100 μ l of trunk blood was obtained at 197 the end of the study using Lithium-Heparin-coated tubes (Greiner Bio-One, Kremsmünster, Austria). 198 Blood cells from each mouse were stimulated with lipopolysaccharide (LPS-2 µg/ml) or Concanavalin 199 A (ConA-2.5 µg/ml) for 24 h or left unstimulated as control. Following 24 h-incubation, samples were 200 taken and stored at -80°C. The levels of secreted IL-1 β , IL-4, IL-6, IL-10, TNF α and CXCL1 were 201 analysed with the Proinflammatory Panel 1 (mouse) V-PLEX Kit and the MESO QuickPlex SQ 120, 202 SECTOR Imager 2400 (Meso Scale Discovery, Maryland, USA). Only data derived from duplicates with 203 < 15% CV were included in the analysis. Concentrations of cytokines were expressed in pg/ml.

204

205 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}.

220

221 **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.

235

236 **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).
- 249 Short chain fatty acids (SCFAs) were measured by gas chromatography, using a Varian 3500 GC 250 flame-ionization system fitted with a ZB-FFAP column as previously described (46).
- 251

252 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

260 before filtering the supernatant through Costar Spin-X centrifuge filters 0.2 μ M at 10000 g. Faecal 261 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).

267

268 2.11 Statistical analysis

269 Statistical analyses were conducted using SPSS 24 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 270 7 (GraphPad Software, Inc., La Jolla, CA, USA). Data were analysed for normality using the Shapiro-271 Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed 272 with Kruskal-Wallis test followed by post hoc Dunn's, and are depicted as median with inter-quartile 273 ranges (IQR) and min/max values as error bars. Parametric data were analysed using two-way 274 analysis of variance (ANOVA) post hoc Holm-Sidak, and are shown as mean ± SEM. Changes in 275 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 276 277 using Spearman correlations for non-parametric data. Outliers were excluded using the Grubbs test 278 (51). Statistical significance was set at $p \le 0.05$.

279 Statistical analysis of microbiota data was performed in an R software environment. For Principal 280 Component Analysis (PCA), Permutational multivariate analysis of variance (PERMANOVA) was used 281 to identify relationships of significance between variables the Adonis function from the vegan 282 package on Aitchison distance matrices calculated with the ALDEx2 package. ALDEx2 was also used to 283 calculate pairwise differential abundance. Hierarchical All-against-All significance (HAllA) was used to 284 investigate between-dataset covariance. For all tests, a Benjamini-Hochberg post hoc test was 285 performed to correct for multiple comparisons with a conservative *q*-value of 0.2 as critical value.

286 **3. Results**

287 **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 ageassociated 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 ageassociated phenotype and restored the levels of MHC-II+ neutrophils in stressed aged animals to young levels (p=0.011).

299 Since acute stress is known to affect peripheral innate immunity through corticosterone (52), we 300 investigated whether these changes in neutrophil activation status were associated with altered 301 stress axis activity. For this, we measured plasma release of corticosterone (as an indicator of 302 endocrine reactivity to stress) in the same animals, prior to and at different time points following the 303 forced swim stress exposure. Two-way RM-ANOVA revealed an overall effect of age on the 304 corticosterone response (F (1, 28) = 10.825, p=0.003; Figure 1b). In particular, middle-aged mice 305 exhibited lower plasma corticosterone levels at baseline (F $_{(1, 29)}$ = 16.68, p<0.001, Figure 1c) and at 306 T15 (F $_{(1, 34)}$ = 24.65, p<0.001). Two samples in the middle-aged control group did not reached the 307 detection limit and were therefore not included into the analysis. Calculation of area-under-the-308 curve (AUC) for corticosterone response confirmed that middle-aged mice exhibited a blunted stress 309 axis reactivity (F $_{(1, 28)}$ = 5.207, p=0.03, Figure 1d). However, we did not observe any modulation on 310 corticosterone response either at baseline or following stress in either young adult or middle-aged 311 mice by prebiotic supplementation suggesting that the changes in peripheral innate immunity are 312 not mediated by corticosterone.

313

314 **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-1 β 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.

323

324 3.3 Pervasive neuroimmune alterations in middle-aged mice were alleviated by prebiotic 325 supplementation

326 Given the decline of cognitive function in middle-aged mice (11, 12), we investigated whether the 327 middle-aged brain is more vulnerable to peripheral immune cell trafficking and subsequent microglial 328 activation, and whether this status can be targeted by prebiotic supplementation, utilizing flow 329 cytometry to investigate brain immunity. Two-way ANOVA revealed an effect of age (F (1, 41) = 11.94, 330 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, 331 332 middle-aged control mice showed an increase in Ly-6C^{hi} monocytes compared to young controls 333 (p<0.001), which was alleviated by prebiotic supplementation (p=0.007). We in addition investigated 334 if these changes in infiltrating monocytes are also systemically reflected in the blood. No differences 335 were observed (see Supplementary Figure S7), suggesting that the brain becomes particularly 336 vulnerable in middle-aged mice as inflammatory monocytes traffic to inflamed tissue. Furthermore, 337 we investigated whether the observed increase in monocyte trafficking was associated with microglia 338 activation in the brain. Two-way ANOVA revealed an effect not only of age (F $_{(1, 43)}$ = 10.75, p=0.002; 339 Figure 2b), but also prebiotic treatment (F $_{(1, 43)}$ = 10.95, p=0.002) and an interaction of both (F $_{(1, 43)}$ = 340 13.81, p<0.001) on Ly-6C+ microglia. Middle-aged controls showed a higher percentage of Ly-6C+ 341 microglia compared to young controls (p<0.001), which was reversed to young control levels 342 following prebiotic supplementation (p<0.001).

343 In agreement with these findings, the gene expression of Ccl2 and Tnf were up-regulated in the 344 hippocampus of middle-aged mice (F $_{(1,35)}$ = 13.60, F $_{(1,35)}$ = 15.79, p<0.001; Figure 2c-d). Ccl2 and Tnf 345 encode for pro-inflammatory cytokines which are secreted from activated microglia and associated 346 with monocyte infiltration. This supports the observation of microglia activation in the middle-aged 347 brain, including the hippocampus, a key region controlling learning and memory. In contrast, both, 348 Ccl2 and Tnf, were not found to be upregulated in middle-aged mice following prebiotic 349 supplementation. Furthermore, we investigated this phenomenon in another cognition-related brain 350 region, the prefrontal cortex. In contrast to the hippocampus, no effect of age or prebiotic 351 supplementation on Ccl2 and Tnf gene expression was found (Supplementary Figure S4), suggesting a 352 non-universal effect of prebiotic supplementation on cytokine expression across brain regions.

354 3.4 Prebiotic intervention improved learning and reduced anxiety-like behaviour in young adult 355 mice

356 To assess whether prebiotic intervention improves spatial learning and memory, mice were trained 357 over five consecutive days to find a hidden platform in the Morris water maze (MWM). Middle-aged 358 mice displayed an impairment in learning (F $_{(1, 35)}$ = 8.653, p=0.006; Figure 3a). However, prebiotic 359 supplementation modulated learning (F $_{(1, 35)}$ = 10.252, p=0.003), albeit, the improvement was only 360 evident in young adult mice (F (1, 18) = 10.897, p=0.004). We did not identify an interaction between 361 age and prebiotic supplementation (F $_{(1, 35)}$ = 2.073, p=0.159) suggesting that the prebiotic effects 362 were specific to young adult mice. Although, the average between day four to five is visually 363 different, both days are not statistically different from each other (p=0.19) and mostly explained by a 364 much greater variation compared to day four. Similarly, area-under-the-curve (AUC) analysis 365 confirmed the improved learning in prebiotic-treated young mice (p=0.005). Both, age (F (1, 34) = 366 13.10, p=0.001) and prebiotic supplementation (F $_{(1, 34)}$ = 12.89, p=0.001) had a modulatory impact on 367 spatial learning. To assess spatial long-term memory, a probe trial was performed on day six. A trend 368 towards decreased time spent in the target quadrant with age (F $_{(1, 35)}$ = 3.442, p=0.072) was 369 observed, however, no improvement by prebiotic supplementation was found (Figure 3a). Neither 370 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.

375 Next, we tested if prebiotic supplementation can modulate fear-dependent learning and memory. 376 For this, mice were tested in a fear conditioning task (Figure 3c). On day one, mice were conditioned 377 to three cued-shock pairings with a variable inter-pairing interval. Middle-aged mice displayed an 378 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 379 380 (F (1, 35) = 6.702, p=0.014) suggesting increased anxiety-like behaviour. Although statistically not 381 significant, the changes in extinction in the prebiotic-treated young adult mice compared to the 382 other groups are explained by the reduced freezing across the cue-shock pairings during acquisition. 383 Similarly, to deficiencies in acquisition, middle-aged mice showed impairments in extinction learning 384 $(F_{(1, 36)} = 4.898, p=0.034)$. In contrast, no impact of age nor of prebiotic supplementation on context recall was found (Figure 3c). 385

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 388 increased anxiety-like behaviour, as shown by less time spent in the aversive open arms of the 389 elevated plus maze (F $_{(1,33)}$ = 18.31 p<0.001; Figure 3d), the central zone of the open field arena (F $_{(1,33)}$ 390 ₃₄₎ = 7.337, p=0.011; Figure 3e) as well as decreased number of centre visits (F (1, 34) = 14.69, p<0.001). 391 The locomotor activity was also marginally reduced in middle-aged mice (F (1,33) = 4.538, p=0.041; 392 Figure 3c). Prebiotic supplementation did not affect anxiety levels in middle-aged mice. However, a 393 significant increase in the time spent in the open arms of the elevated plus maze was observed in 394 young adult prebiotic-treated mice (p=0.027). This suggests that prebiotic supplementation did have 395 an anxiolytic-like effect, but in young animals only. The observed changes in anxiety-like behaviour, 396 i.e. increased anxiety levels in aged mice and selective anxiolytic effect in prebiotic-treated young 397 mice, had a similar pattern seen in the spatial recognition in the MWM. This suggests that impaired 398 cognitive performance in middle-aged mice, as well as improved learning of prebiotic-treated young 399 adults could may be partially mediated by changes in anxiety levels.

400 Interestingly, learning performance in the Morris water maze correlated with the relative abundance 401 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 $_{(38)}$ = -402 403 0.323, p=0.048; Figure 3f). Moreover, we identified a significant correlation between hippocampal 404 Ccl2 expression (as a readout of microglia activation linked to monocyte trafficking) and learning 405 performance (AUC) in the MWM task (r $_{(39)}$ = 0.349, p=0.03; Figure 3g). To emphasize these 406 correlations further, we displayed which data points relates to which group indicating that prebiotics 407 drive these associations.

408

409 3.5 Effect of age and prebiotic supplementation on gut microbiota composition and short-chain 410 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.

435 To identify if changes in gut microbiota composition correlated with faecal metabolomics, we utilized 436 Hierarchical All-against-All significance testing (HAllA). Among others, HAllA identified a negative 437 association between the relative abundance of Akkermansia, which was significantly over-438 represented in prebiotic-treated middle-aged mice, and several amino acids including leucine (p=-439 0.63, p<0.001, FDR corrected, Figure 4d), valine and isoleucine (ρ =-0.60, p<0.001, respectively). 440 Similarly, between Bifidobacterium, which was significantly over-represented in prebiotic-treated 441 young and middle-aged mice, and the respective amino acids (ρ =-0.55, p=0.001). Prebiotic 442 supplementation increased caecum weight (F $_{(1, 35)}$ = 88.95, p<0.001; Supplementary Figure S6b) in 443 both young adult and middle-aged mice. Among short-chain fatty acids (SCFAs), caecal butyrate, 444 propionate and valerate levels were affected by either age or prebiotic supplementation. No effect 445 was found on acetate and total-SCFA levels (data not shown). Middle-aged mice exhibited higher 446 butyrate levels than young mice (F $_{(1, 35)}$ = 16.74, p<0.001; Figure S6c). Prebiotic supplementation 447 increased propionate independent of age (F $_{(1,35)}$ = 8.75, p<0.001), with a more pronounced increase 448 seen in middle-aged mice (p=0.035). While valerate was increased in middle-aged compared to 449 young controls (p<0.001), prebiotic supplementation reduced valerate in both, young adult (p=0.021) 450 and middle-aged mice (p<0.001).

451 **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.

457

458 To our knowledge, this is the first study demonstrating the presence of a strong basal and stress-459 induced (neuro-) inflammatory profile in middle-aged mice (11 months old), although an exaggerated 460 inflammatory response has been previously reported in middle-aged rodents following immune 461 stimulation (53-55). Moreover, our study implicates the gut microbiome in such processes as dietary 462 targeting with prebiotic supplementation counteracted stress-induced peripheral immune cell 463 activation. Following acute stress, we investigated a subtype of neutrophils that express MHC-II, 464 which plays a role in priming of T-cells and therefore provides a link between the innate and the 465 adaptive immune system (56, 57). Further research is warranted on the functional characterization of 466 these neutrophils and their impact on the brain in ageing particularly following acute stress.

467

468 The gut microbiome has emerged as being essential for brain health in ageing and as a key player in 469 the bidirectional communication across the gut-brain axis (58, 59). Previous research points out a 470 role of aged microbiota in driving systemic immunity (26, 27). In addition, key metabolites which are 471 produced by the gut microbiota following i.e. a prebiotic-enriched diet such as short-chain fatty acids 472 (SCFAs) has been implicated in alleviating stress-induced alteration (46). We show that prebiotic 473 supplementation is capable of dampening age-associated systemic inflammation, particularly TNF α , 474 following stimulation with Concanavalin A. As ConA stimulates both, T- and NK-cells, it seems that 475 both cell types are in particular sensitive to prebiotic treatment in middle-aged mice compared to 476 LPS stimulation which stimulates a broad range of immune cells. We previously showed that 477 prebiotic treatment rescues immune alteration induced by chronic psychosocial stress following 478 ConA stimulation exclusively (32) suggesting that prebiotics might have specific effects on immune 479 priming on T- and NK-cells systemically, and may influence brain function and behaviour which 480 warrants further research. Recent research showed a role of T-cell activation in regulating behaviour, 481 anxiety-like and fear-related behaviour (60), cognition (61) and sociability (62), which may possibly 482 be influenced by the gut microbiota. A critical factor for T-cell activation is the availability of specific 483 amino acids such as leucine (63). By using HAIIA, we identified strong correlations between prebiotic-484 driven changes in gut microbiota, Bifidobacterium and Akkermansia with several amino acids in 485 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).

489

490 A bidirectional relationship between the brain and the peripheral immune system exists (66), which 491 can promote neuroinflammation and exacerbate neuronal damage in the hippocampus. Recent studies suggest a constant influx of immune cells, inflammatory monocytes (Lv-6C^{hi}-monocytes), into 492 493 the brain even under steady-state conditions (67-69). Previously these cells were thought to only 494 play a role in inflammatory conditions such as following viral infection and associated encephalitis 495 (70) or after social defeat stress (71, 72). However, recent research suggests that trafficking of Ly-496 6C^{hi}-monocytes into the brain is crucial for brain plasticity and influence cognitive behaviour (67). 497 This was mediated by the gut microbiome as antibiotic depletion and subsequent reconstitution of 498 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 499 500 before animals were tested behaviourally, we assessed their neuroimmune status in cohort two. 501 Here we show that middle-aged mice exhibited an increased influx of inflammatory monocytes into 502 the brain. Following the determination of their neuroimmunity baseline response, we then subjected 503 the mice to the behavioural assessment. To correlate the changes in behaviour with specific 504 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 505 506 CCL2-dependent manner (70, 72, 73). We show that Ccl2 is specifically upregulated in the 507 hippocampus of middle-aged mice, but not present following prebiotic supplementation suggesting 508 that this is may be a potential pathway in which gut-microbiota-immune-brain communication can 509 affect brain function and behavioural traits in this key region for learning and memory. However, 510 despite these changes in neuro-immunity, we have not identified any overt cognitive impairments in 511 middle-aged control mice. Although it is worth noting that the dynamics of hippocampal Ccl2 512 expression correlated with cognitive behaviour assessed in the Morris water maze paradigm. 513 Interestingly, prebiotic-driven changes in the neuroinflammatory profile are not universal across 514 brain regions as there were no changes in the prefrontal cortex. This is in line with previous findings 515 that there are marked regional differences in microglia activation across brain regions (74). 516 Interestingly, we found that middle-aged mice exhibited increased microglia activation under basal 517 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). 518 519 Recent work has demonstrated a modulatory effect of the gut microbiota on microglia function (77-520 79). Of note, germ-free mice exhibited deficits in microglia maturation and function while addition of 521 SCFAs rescued these deficits. However, the short-chain fatty acid receptor FFAR2 is actually not 522 present on microglia (77), but on monocytes (80). Future studies are needed to investigate the 523 mechanistic relationship between these receptors and prebiotic-induced effects on microglia 524 activation across the lifespan.

525

526 Microglia activation has been shown to alter cognitive and anxiety-like behaviour (17, 81). Here, we 527 show that prebiotic supplementation improves anxiety-like behaviour and cognition in young adult 528 mice. This is in accordance with previous studies which targeted the gut microbiome by dietary 529 interventions in rodents (32, 38, 82-84). Interestingly, studies using a probiotic mix (VSL#3) failed to 530 show improvements in anxiety-related behaviour (85) suggesting that strain selection is very 531 important and that prebiotics might be a better approach to improve behaviour. Moreover, we show 532 that middle-aged control mice showed a decreased number of centre visits in the open field 533 suggesting increased anxiety-like behaviour (7), which may have influenced cognitive performance 534 (11). Middle-aged mice displayed mild cognitive impairments, which were not present following 535 prebiotic supplementation. It is worth noting that neuroinflammation at this stage was not significant 536 enough to manifest in major cognitive impairments. However, our data imply that prebiotic 537 intervention may have some potential to counteract cognitive decline. As the impact of prebiotic 538 supplementation on behaviour, particularly the cognitive tests, is clearly stronger in adult subjects, 539 the data suggests that prebiotics may be less effective as we age. On the other side, a much longer 540 exposure to prebiotics might be needed to achieve significant effects suggesting that 541 supplementation may have to start earlier to be effectively preventative before alterations in the 542 brain occur. This is particularly evident for the behaviour. On the other side, particularly in light of 543 the stress-induced peripheral immune data, the system may need to be challenged to potentially 544 exert negative behavioural effects (86) before prebiotic supplementation can act beneficially (32). 545 Future studies focused on long-term effects of this mid-life microbiota manipulation are now 546 warranted.

547

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

555 fatty acids with propionate increased in prebiotic-treated middle-aged mice while prebiotic 556 supplementation decreased valerate in both, young adult and middle-aged mice.

557

558 Previous research has shown that diet-driven modulation of the gut microbiota by administration of 559 prebiotics can modulate peripheral immune response in the serum of naïve mice (32) and we 560 recently showed that SCFAs attenuate the effect of chronic stress (46). It was shown previously that 561 propionate can inhibit the production of pro-inflammatory cytokines (88). Moreover, in-vitro 562 experiments suggests pro-inflammatory capabilities of valerate while it enhanced LPS-induced 563 inflammatory response in a murine N9 microglial cell line (89). Although the effects on SCFA levels is 564 relatively modest it is possible that some of the anti-inflammatory effects of prebiotic 565 supplementation might have been mediated by the changes observed in SCFA concentrations.

566

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).

572

573 Prebiotic supplementation increased the relative abundance of Bifidobacterium, which is in 574 accordance with previous studies in humans (94). Interestingly, Bifidobacteria has been reported to 575 be reduced in the elderly (95). In addition, supplementation increased the relative abundance of 576 Akkermansia in middle-aged mice suggesting that prebiotics might promote a young microbiota 577 phenotype, compared to a previous study where Akkermansia abundance strongly declined in 12- vs. 578 4-months-old control mice (23). When transferring faecal matter from old mice to young germ-free 579 (GF) mice, Akkermansia was lower abundant in those recipients than in GF mice that received young 580 microbiota (26). Interestingly, Akkermansia has been associated with immune modulation (26), has 581 shown to protect against inflammation and promote gut health in diet-induced obesity (96), and 582 restored intestinal permeability and subsequent immunomodulation in aged mice (97). Moreover, 583 Akkermansia has been found to be enriched in super-centenarians (24). Together with 584 Bifidobacterium, Akkermansia are claimed as longevity-adapted and possibly health-promoting taxa 585 and therefore might be involved in healthy ageing (24). It is worth noting that learning performance 586 strongly correlated with the abundance of Akkermansia suggesting a link between microbiota and 587 cognitive performance. Future studies are warranted to investigate the potential beneficial impact of 588 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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612

613 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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627 Figure Legends

628 Figure 1. Prebiotic supplementation reversed stress-induced immune priming in middle-aged mice. 629 (a) MHC-II+ neutrophils at baseline and 2h after acute stress. (b) Plasma Corticosterone (Cort) 630 response curve at baseline, immediately before exposure to acute stress, and 15, 45 and 120 min 631 after exposure to acute stress. (c) Plasma corticosterone at baseline. (d) Area-under-the-curve (AUC) 632 of corticosterone response. Mean \pm SEM. (a) n = 9-10, (b-d) n = 7-10. (a) Kruskal-Wallis post hoc 633 Dunn's, (b) two-way-repeated measurement (RM)-ANOVA post hoc Sidak, (c-d) two-way ANOVA post 634 hoc Holm-Sidak (Cort T0, Cort Area-under-the-curve). vs. *control young adult* * p < 0.05, ** p < 0.01, 635 *** p < 0.001, vs. control middle-aged # p < 0.05, ## p < 0.01, vs. prebiotic middle-aged vs prebiotic 636 adult \$ < 0.05.

637

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) Proinflammatory cytokine expression in the hippocampus. Mean \pm 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.

645

646 Figure 3. Prebiotic supplementation improved learning and reduced anxiety-like behaviour in 647 young adult mice. (a) Learning and memory in Morris water maze (MWM). Latency-to-find platform 648 over five training days. Summarized as area-under-the-curve (AUC), as well as the probe trial 24h 649 after the last training day is depicted. (b) Short-term memory assessed by Spontaneous Alternation 650 Behaviour (Y-Maze). (c) Fear Conditioning: Conditioning (Acquisition, day one) including AUC. 651 Extinction (day two) – two consecutive cue presentations were depicted as one trial block. AUC for 652 trial block 1-20 is depicted. Context recall (day three). (d) Time spent in open arms in elevated-plus 653 maze. (e) Behaviour in open field. (f) Spearman correlation analysis of learning efficacy in Morris 654 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 655 656 expression. Mean \pm SEM. n = 9-10. (a – MWM – latency-to-find-platform, c – acquisition day one) 657 two-way RM ANOVA post hoc Sidak, (a-e) two-way ANOVA post hoc Holm-Sidak. vs. control young 658 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. 659

661 Figure 4. Middle age and prebiotic treatment have distinct effects on the gut microbiota 662 composition accompanied with changes in faecal metabolomic profile. (a) PCA plot (b) Heat map 663 representing differentially abundant taxa (genus with higher hierarchy family name), which reach a 664 Benjamini-Hochberg FDR q value < 0.2 at least once. Asterisks in the heat map represent the 665 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 666 667 significant correlations (q<0.2) between gut microbiota composition and faecal metabolomics. 668 Numbers (1-100) indicate the strongest correlations in a descendant order. n = 9-10. (a) 669 PERMANOVA, followed by pairwise PERMANOVA post hoc Benjamini-Hochberg, (b) Mann-Whitney U 670 test post hoc Benjamini-Hochberg (c) Kruskal-Wallis post hoc Dunn's, (d) Spearman post hoc 671 Benjamini-Hochberg.

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Shift in gut microbiota composition by middle-age and prebiotic supplementation

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C Effect of middle-age and prebiotic supplementation on alpha diversity

