Title	Mid-life microbiota crises: middle age is associated with pervasive neuroimmune alterations that are reversed by targeting the gut microbiome
Authors	Boehme, Marcus;van de Wouw, Marcel;Bastiaanssen, Thomaz F. S.;Olavarría-Ramírez, Loreto;Lyons, Katriona;Fouhy, Fiona;Golubeva, Anna V.;Moloney, Gerard M.;Minuto, Chiara;Sandhu, Kiran V.;Scott, Karen A.;Clarke, Gerard;Stanton, Catherine;Dinan, Timothy G.;Schellekens, Harriët;Cryan, John F.
Publication date	2019-05-16
Original Citation	Boehme, M., van de Wouw, M., Bastiaanssen, T. F. S., Olavarría-Ramírez, L., Lyons, K., Fouhy, F., Golubeva, A. V., Moloney, G. M., Minuto, C., Sandhu, K. V., Scott, K. A., Clarke, G., Stanton, C., Dinan, T. G., Schellekens, H. and Cryan, J. F. (2019) 'Mid-life microbiota crises: middle age is associated with pervasive neuroimmune alterations that are reversed by targeting the gut microbiome', Molecular Psychiatry, doi: 10.1038/s41380-019-0425-1
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.nature.com/articles/s41380-019-0425-1 - 10.1038/s41380-019-0425-1
Rights	© Springer Nature Limited 2019. This is a post-peer-review, pre-copyedit version of an article published in Molecular Psychiatry. The final authenticated version is available online at: https://doi.org/10.1038/s41380-019-0425-1
Download date	2024-05-02 18:24:49
Item downloaded from	https://hdl.handle.net/10468/8077

University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

University College Cork, Ireland
Coláiste na hOllscoile Corcaigh

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

3 Marcus Boehme[1,2], Marcel van de Wouw[1,2], Thomaz F. S. Bastiaanssen[1,2], Loreto Olavarria-
4 Ramirez[1,2], Katriona Lyons[4], Fiona Fouhy[1,4], Anna V. Golubeva[1], Gerard Moloney[1,2], Chiara
5 Minuto[1,3], Kiran V. Sandhu[1], Karen A. Scott[1], Gerard Clarke[1,3], Catherine Stanton[1,4], Timothy
6 G. Dinan[1,3], Harriët Schellekens[1,2], John F. Cryan[1,2]

7 [1]*APC Microbiome Ireland, University College Cork, Ireland*
8 [2]*Department of Anatomy and Neuroscience, University College Cork, Ireland*
9 [3]*Department of Psychiatry and Neurobehavioural Science, University College Cork, Ireland*
10 [4]*Teagasc Food Research Centre, Food Biosciences Department, Moorepark, Fermoy, Cork, Ireland*

11

12

13

15

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

17

18 **Correspondence:**   Prof. John F. Cryan
19   j.cryan@ucc.ie
20   Phone: +353-21-420-5426
21   Dept. Anatomy & Neuroscience
22   APC Microbiome Ireland
23   University College Cork, Cork
24   Ireland

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.

25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41

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

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

90   We hypothesise that there is a dysregulation in the communication between the gut microbiota and

91   the brain during middle age, which is critical in mediating age-related functional decline. Thus,

92   targeting the gut microbiota with prebiotics may alter microglia activation state and brain function in

93   ageing. To this end, we hypothesised that targeting the gut microbiome by dietary intervention with

94   a complex short- and long-chain prebiotic, oligofructose-enriched inulin (FOS-Inulin), would have

95   selective effects on (neuro-) immune profile and behaviour in middle-aged male compared to young

96   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-

131   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*

138   Spontaneous alternation behaviour in the Y-maze tests hippocampal-dependent spatial memory and

139   exploration exploratory activity and was carried out as previously described (22). Behaviour was

140   assessed for five minutes.

141

142   *2.4.2 Morris water maze*

143   The Morris water maze represents a robust and reliable test for spatial learning that strongly

144   correlates with hippocampal synaptic plasticity (39). Briefly, mice were trained over five days (four

145   trials per day, two minutes each) to spatially locate the submerged platform. On day six, the platform

146   was removed and a probe trial lasting 30s was conducted.

147

148   *2.4.3 Fear conditioning*

149   Fear conditioning was conducted as previously described (40), over three consecutive days (day 1:

150   conditioning by three pairings with variable inter-pairing interval; day 2: conditioned stimulus recall

151   and extinction in a novel context; day 3: context recall).

152

153   *2.4.4 Open field*

154   The open field is a widely used test to assess approach-avoidance behaviour, locomotor activity, and

155   the behavioural response to a novel context; and was conducted as previously described (32). Briefly,

156   a test mouse was placed into an open arena with 60 lux lighting and allowed to explore the context

157   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*

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

174

175 *2.4.8 Forced swim test*

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

182

183 **2.5 Plasma collection and corticosterone analysis**

184 To investigate the endocrine and immune response to stress, we collected blood samples prior to
185 and following the forced swim test session. Approximately 60 µl of blood per mouse were collected
186 by tail tipping using Lithium-Heparin-coated capillaries (Sigma-Aldrich, St. Louis, Missouri, United
187 States). Blood was centrifuged at 3500 g at 4 °C for 15 min. Plasma was aspirated and stored at
188 −80°C. Blood was taken immediately before the forced swim test (baseline), as well as 15 min, 45 min
189 and 120 min after the baseline. Baseline samples and samples at 120 min post-stress time point were
190 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
193 derived from duplicates with < 15% coefficient of variation (CV) were included in the analysis.

194

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).

228 RNA was reverse transcribed to cDNA using the Applied Biosystems High Capacity cDNA kit (Applied

229 Biosystems, Warrington, UK). *Ccl2* and *Tnf* genes were amplified with probes designed by Integrated

230 DNA Technologies (Coralville, IA, US) (Table S1). PCR was run in triplicates on a LightCycler®480

231 (Roche). Data were analysed with the comparative cycle threshold (Ct) method. Data were

232 normalized using $Actb$ as endogenous control and transformed using the $2^{-\Delta\Delta CT}$ method (45). We

233 confirmed beforehand that the housekeeper $Actb$ is neither changed by age nor by prebiotic

234 treatment.

235

236 **2.9 Caecal microbiota composition (16S rRNA gene sequencing) and short-chain fatty acid analysis**

237 Caecum was harvested, snap frozen and stored at -80°C prior to the analysis. DNA from caecal

238 content was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-

239 beating step, as previously described (46). The V3-V4 hypervariable region of the 16S rRNA gene was

240 amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing

241 Library Protocol. Samples were sequenced at Teagasc Sequencing Facility on the Illumina MiSeq

242 platform using a 2 × 250 bp kit.

243 FLASH was used to assemble paired-end reads. Further processing of paired-end reads including

244 quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences

245 below length thresholds was completed using QIIME (version 1.9.0). Denoising, chimera detection

246 and clustering into operational taxonomic unit (OTU) grouping were performed using USEARCH v7

247 (64-bit) (47). OTUs were aligned using PyNAST (and taxonomy was assigned using BLAST against the

248 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**

253 Faecal pellets were collected at the end of cohort one. Faecal material was freshly collected using

254 sterilized tools to ensure no cross contamination within a time-window of 10 minutes' maximum to

255 ensure least oxygen exposure of the faeces as possible. Subsequently, pellets were directly snap

256 freeze to ensure optimal DNA integrity. Faecal water was prepared by homogenising faecal samples

257 (20-50 mg) with 4x wt/volume sterile PBS followed by vortexing for 20 minutes. Samples were

258 centrifuged at 16000 g for 30 minutes; the supernatant was transferred in a new 2 mL micro

259 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 µM at 10000 g. Faecal

261   water samples were stored at -20°C.

262   Subsequently, samples were derivatized with methyl chloroformate as previously described (49) and

263   processed by MS-Omics (Copenhagen, Denmark) using Gas Chromatography – Mass Spectometry

264   (GC-MS). Raw data was converted to netCDF format using ChemStation (Agilent technologies) and

265   processed in Matlab R2014b (Mathworks, Inc., Natick, MA, USA) using the PARADISe software

266   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

276   two-way-repeated measurement (RM)-ANOVA post hoc Sidak. Correlation analyses were performed

277   using Spearman correlations for non-parametric data. Outliers were excluded using the Grubbs test

278   (51). Statistical significance was set at $p \leq 0.05$.

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**

288 To assess if ageing triggers stress-induced immune priming in middle-aged mice, and whether age-

289 associated changes are alleviated following prebiotic supplementation, mice were exposed to an

290 acute stress (forced swim test), and blood samples were taken at baseline and 2 hours after stress

291 exposure. We focused on neutrophils, which act as the first responders to any immune challenge and

292 can trigger adaptive immunity, including T-cell priming via expression of major histocompatibility

293 complexes (MHC), a classical activation marker.

294 In middle-aged mice, acute stress caused a long-lasting increase of the MHC-II+ neutrophil

295 population (p=0.0280, Kruskal-Wallis post hoc Dunn's; Figure 1a); the response being absent in young

296 adult animals. Strikingly, prebiotic supplementation prevented the development of the age-

297 associated phenotype and restored the levels of MHC-II+ neutrophils in stressed aged animals to

298 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**

315 To investigate if systemic inflammation and immune cell priming is altered in middle-aged mice and

316 counteracted by prebiotic supplementation, whole blood was taken after 14 weeks of prebiotic

317 intervention and stimulated with LPS or ConA. Following ConA-stimulation, middle-aged control mice

318 exhibited a trend towards increased IL-1β and IL-10 cytokine release (p=0.089 and p=0.069,

319 respectively, Supplementary Figure S3b+e), while prebiotic-treated middle-aged mice showed similar

11

320 levels as in young controls. Moreover, prebiotic supplementation decreased TNFα in middle-aged

321 mice following ConA-stimulation (p=0.014, Kruskal-Wallis post hoc Dunn's; S3a). No changes were

322 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

331 ($F_{(1, 41)}$ = 6.01, p=0.019) on trafficking of inflammatory monocytes (Ly-6C^{hi}) into the brain. Specifically,

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.

353

12

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

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 ($F_{(1,34)}$ = 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

402 mainly driven by *Akkermansia,* the predominant genus within the *Verrucomicrobiaceae* ($r_{(38)}$ = -

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

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

410 **fatty acid profile in the gut**

411 Principal Component Analysis (PCA) analysis identified structural differences in microbiota across all

412 four groups (PERMANOVA, p<0.001; Figure 4a). The composition of caecal microbiota was

413 significantly affected by age and by prebiotic supplementation (all p<0.05, pairwise PERMANOVA).

414 Interestingly, no interaction between age and prebiotic was observed, i.e. marked differences

415 between middle-aged and young mice were evident in both control and prebiotic-treated groups,

416 and prebiotic supplementation effectively shifted microbiota composition in both young adult and

417 middle-aged animals.

418 When we looked at structural properties of microbial communities at the genus level, we observed

419 multiple changes in the relative abundance of individual bacterial taxa (Figure 4b). In particular,

420 middle-aged mice displayed an increase in *Clostridum sensu stricto 1, Delftia, Salmonella,*

421 *Enterococcus, Turibacter* (q < 0.1). In contrast, *Parabacteroides* (q < 0.01) was decreased in middle-

422   aged control mice. Interestingly, prebiotic supplementation not only increased the abundance of

423   *Bifidobacterium* in young adult but also middle-aged mice (q < 0.1 and q < 0.01, respectively). In

424   contrast, *Akkermansia* was only increased in middle-aged prebiotic-treated mice (q < 0.1). Moreover,

425   prebiotic supplementation increased the abundance of *Prevotellaceae UCG-001* and *Bacteroides* not

426   only in young adult mice but even more pronounced in middle-aged mice (q < 0.01, respectively),

427   while *Lactobacillus* and *Roseburia* were decreased in prebiotic-treated middle-aged mice (q < 0.1).

428   The Chao1 index was increased in middle-aged compared to young adult control mice, indicating an

429   increase in overall richness of bacterial species associated with age (p=0.028; Kruskal-Wallis post hoc

430   Dunn's; Figure 4c). However, the Shannon and the Simpson indices, which take into account the

431   evenness of species abundance, were not affected by age but were reduced following prebiotic

432   supplementation in young adult mice (p=0.010 and p=0.016, respectively). This suggest that prebiotic

433   supplementation favoured the selective expansion of certain bacterial taxa in young adult animals

434   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 (ρ=-

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).

15

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

16

486   been implicated in regulating amino acid availability (64). Interestingly, a recent study in a Chinese

487   cohort of middle-aged to elderly individuals found a correlation between *Akkermansia* and CD8+ as

488   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

492   studies suggest a constant influx of immune cells, inflammatory monocytes (Ly-6C^{hi}-monocytes), into

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

499   behaviour (67). To characterize if these Ly-6C^{hi}-monocytes also affect the brain in middle-aged mice

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

505   analysed targets in the brain at the end of the study. Ly-6C^{hi}-monocytes are recruited to the brain in a

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

518   microglia expressed Ly-6C (73, 75, 76) and have been suggested to arise from Ly-6C^{hi}-monocytes (70).

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

17

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

548 We hypothesized that the dysregulated gut-microbiome-brain axis in middle-aged mice can be

549 ameliorated by targeting the gut microbiome with prebiotics known to promote beneficial bacteria,

550 like *Bifidobacteria*. It was previously shown that the prebiotic, inulin, can alter the microbiome

551 composition under pathophysiological conditions such as following high-fat diet (87) or in extreme

552 ageing (33); however, its effects remained unexplored in healthy ageing/middle age. In fact, by

553 utilizing FOS-Inulin, we show a profound yet differential alteration of the gut microbiota composition

554 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

567 We have previously reported a shift in microbial composition by prebiotics in adult mice (32) but the
568 impact on middle-aged remained unexplored. Interestingly, we found an increase in species richness
569 in middle-aged mice, which is in line with previous findings in rodents (22) and humans (90). In fact, it
570 has been shown in humans that the gut microbiota remarkably changes with ageing not only in
571 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.

589

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.

613 **Author contributions**

614 MB, GC, CS, TGD, HS and JFC have contributed to the conception and design of the work. Acquisition,

615 analysis and interpretation of data were performed by MB, MVDW, TFS, LOR, KL, FF, AVG, GM, CM,

616 KVS, KAS. MB and JFC wrote the manuscript. MB, MVDW, TFS, FF, AVG, GM, KVS, KAS, GC, CS, TGD,

617 HS and JFC critically revised the manuscript. All authors approve the final version of the manuscript

618 and agree to be accountable for all aspects of the work.

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. c*ontrol 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.

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,
666 Shannon). **(d)** Hierarchical All-against-All significance testing (HAllA) representing the 100 strongest
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.

5. References

1.   Lynch SV, Pedersen O. The Human Intestinal Microbiome in Health and Disease. N Engl J Med. 2016;375(24):2369-79.

2.   Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous systems in health and disease. Nature neuroscience. 2017;20(2):145-55.

3.   Miquel S, Champ C, Day J, Aarts E, Bahr BA, Bakker M, et al. Poor cognitive ageing: Vulnerabilities, mechanisms and the impact of nutritional interventions. Ageing Research Reviews. 2018;42:40-55.

4.   Sandhu KV, Sherwin E, Schellekens H, Stanton C, Dinan TG, Cryan JF. Feeding the microbiota-gut-brain axis: diet, microbiome, and neuropsychiatry. Transl Res. 2017;179:223-44.

5.   David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559-63.

6.   Donovan SM. Introduction to the special focus issue on the impact of diet on gut microbiota composition and function and future opportunities for nutritional modulation of the gut microbiome to improve human health. Gut microbes. 2017;8(2):75-81.

7.   Ennaceur A, Michalikova S, van Rensburg R, Chazot PL. Detailed analysis of the behavior and memory performance of middle-aged male and female CD-1 mice in a 3D maze. Behavioural brain research. 2008;187(2):312-26.

8.   Bensalem J, Servant L, Alfos S, Gaudout D, Laye S, Pallet V, et al. Dietary Polyphenol Supplementation Prevents Alterations of Spatial Navigation in Middle-Aged Mice. Frontiers in behavioral neuroscience. 2016;10:9.

9.   Duarte JM, Do KQ, Gruetter R. Longitudinal neurochemical modifications in the aging mouse brain measured in vivo by 1H magnetic resonance spectroscopy. Neurobiol Aging. 2014;35(7):1660-8.

10.   Francia N, Cirulli F, Chiarotti F, Antonelli A, Aloe L, Alleva E. Spatial memory deficits in middle-aged mice correlate with lower exploratory activity and a subordinate status: role of hippocampal neurotrophins. The European journal of neuroscience. 2006;23(3):711-28.

11.   Shoji H, Takao K, Hattori S, Miyakawa T. Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. Molecular brain. 2016;9:11.

12.   Prenderville JA, Kennedy PJ, Dinan TG, Cryan JF. Adding fuel to the fire: the impact of stress on the ageing brain. Trends in neurosciences. 2015;38(1):13-25.

13.   Sparkman NL, Johnson RW. Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. Neuroimmunomodulation. 2008;15(4-6):323-30.

14.   Franceschi C, Salvioli S, Garagnani P, de Eguileor M, Monti D, Capri M. Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity. Frontiers in immunology. 2017;8:982.

15.   Norden DM, Godbout JP. Review: microglia of the aged brain: primed to be activated and resistant to regulation. Neuropathology and applied neurobiology. 2013;39(1):19-34.

16.   Hickman S, Izzy S, Sen P, Morsett L, El Khoury J. Microglia in neurodegeneration. Nature neuroscience. 2018;21(10):1359-69.

17.   Tay TL, Savage JC, Hui CW, Bisht K, Tremblay ME. Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. J Physiol. 2017;595(6):1929-45.

18.   Perry VH, Newman TA, Cunningham C. The impact of systemic infection on the progression of neurodegenerative disease. Nature reviews Neuroscience. 2003;4(2):103-12.

19.   Streit WJ, Sammons NW, Kuhns AJ, Sparks DL. Dystrophic microglia in the aging human brain. Glia. 2004;45(2):208-12.

20.   Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science. 2005;308(5726):1314-8.

21.   Hickman SE, Kingery ND, Ohsumi TK, Borowsky ML, Wang LC, Means TK, et al. The microglial sensome revealed by direct RNA sequencing. Nature neuroscience. 2013;16(12):1896-905.

22.   Scott KA, Ida M, Peterson VL, Prenderville JA, Moloney GM, Izumo T, et al. Revisiting Metchnikoff: Age-related alterations in microbiota-gut-brain axis in the mouse. Brain, behavior, and immunity. 2017;65:20-32.

724 23. van der Lugt B, Rusli F, Lute C, Lamprakis A, Salazar E, Boekschoten MV, et al. Integrative
725 analysis of gut microbiota composition, host colonic gene expression and intraluminal metabolites in
726 aging C57BL/6J mice. Aging (Albany NY). 2018;10(5):930-50.
727 24. Biagi E, Franceschi C, Rampelli S, Severgnini M, Ostan R, Turroni S, et al. Gut Microbiota and
728 Extreme Longevity. Curr Biol. 2016;26(11):1480-5.
729 25. Biagi E, Nylund L, Candela M, Ostan R, Bucci L, Pini E, et al. Through ageing, and beyond: gut
730 microbiota and inflammatory status in seniors and centenarians. PloS one. 2010;5(5):e10667.
731 26. Fransen F, van Beek AA, Borghuis T, Aidy SE, Hugenholtz F, van der Gaast – de Jongh C, et al.
732 Aged Gut Microbiota Contributes to Systemical Inflammaging after Transfer to Germ-Free Mice.
733 Frontiers in immunology. 2017;8(1385).
734 27. Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, et al. Age-Associated
735 Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage
736 Dysfunction. Cell Host Microbe. 2017;21(4):455-66 e4.
737 28. Smith P, Willemsen D, Popkes M, Metge F, Gandiwa E, Reichard M, et al. Regulation of life
738 span by the gut microbiota in the short-lived African turquoise killifish. Elife. 2017;6.
739 29. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert
740 consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP)
741 consensus statement on the definition and scope of prebiotics. Nature Reviews Gastroenterology
742 &Amp; Hepatology. 2017;14:491.
743 30. Vulevic J, Drakoularakou A, Yaqoob P, Tzortzis G, Gibson GR. Modulation of the fecal
744 microflora profile and immune function by a novel trans-galactooligosaccharide mixture (B-GOS) in
745 healthy elderly volunteers. The American journal of clinical nutrition. 2008;88(5):1438-46.
746 31. Schiffrin EJ, Thomas DR, Kumar VB, Brown C, Hager C, Van't Hof MA, et al. Systemic
747 inflammatory markers in older persons: the effect of oral nutritional supplementation with
748 prebiotics. J Nutr Health Aging. 2007;11(6):475-9.
749 32. Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, et al. Targeting the
750 Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the
751 Impact of Chronic Stress in Mice. Biological psychiatry. 2017.
752 33. Matt SM, Allen JM, Lawson MA, Mailing LJ, Woods JA, Johnson RW. Butyrate and Dietary
753 Soluble Fiber Improve Neuroinflammation Associated With Aging in Mice. Frontiers in immunology.
754 2018;9:1832.
755 34. Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis
756 program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;39(2):175-91.
757 35. Messaoudi M, Rozan P, Nejdi A, Hidalgo S, Desor D. Behavioural and cognitive effects of
758 oligofructose-enriched inulin in rats. The British journal of nutrition. 2005;93 Suppl 1:S27-30.
759 36. Rozan P, Nejdi A, Hidalgo S, Bisson JF, Desor D, Messaoudi M. Effects of lifelong intervention
760 with an oligofructose-enriched inulin in rats on general health and lifespan. The British journal of
761 nutrition. 2008;100(6):1192-9.
762 37. Rault-Nania MH, Gueux E, Demougeot C, Demigne C, Rock E, Mazur A. Inulin attenuates
763 atherosclerosis in apolipoprotein E-deficient mice. The British journal of nutrition. 2006;96(5):840-4.
764 38. Savignac HM, Tramullas M, Kiely B, Dinan TG, Cryan JF. Bifidobacteria modulate cognitive
765 processes in an anxious mouse strain. Behavioural brain research. 2015;287:59-72.
766 39. Vorhees CV, Williams MT. Morris water maze: procedures for assessing spatial and related
767 forms of learning and memory. Nature protocols. 2006;1(2):848-58.
768 40. Izquierdo A, Wellman CL, Holmes A. Brief uncontrollable stress causes dendritic retraction in
769 infralimbic cortex and resistance to fear extinction in mice. The Journal of neuroscience : the official
770 journal of the Society for Neuroscience. 2006;26(21):5733-8.
771 41. Desbonnet L, O'Tuathaigh C, Clarke G, O'Leary C, Petit E, Clarke N, et al. Phenotypic effects of
772 repeated psychosocial stress during adolescence in mice mutant for the schizophrenia risk gene
773 neuregulin-1: a putative model of gene x environment interaction. Brain, behavior, and immunity.
774 2012;26(4):660-71.

775   42.   Porsolt RD, Bertin A, Jalfre M. Behavioral despair in mice: a primary screening test for
776   antidepressants. Arch Int Pharmacodyn Ther. 1977;229(2):327-36.
777   43.   Cryan JF, Mombereau C. In search of a depressed mouse: utility of models for studying
778   depression-related behavior in genetically modified mice. Molecular psychiatry. 2004;9(4):326-57.
779   44.   Schellekens H, Clarke G, Jeffery IB, Dinan TG, Cryan JF. Dynamic 5-HT2C receptor editing in a
780   mouse model of obesity. PloS one. 2012;7(3):e32266.
781   45.   Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative
782   PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif). 2001;25(4):402-8.
783   46.   van de Wouw M, Boehme M, Lyte JM, Wiley N, Strain C, O'Sullivan O, et al. Short-chain fatty
784   acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations. J Physiol.
785   2018;596(20):4923-44.
786   47.   Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics.
787   2010;26(19):2460-1.
788   48.   Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME
789   allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7(5):335-6.
790   49.   Smart KF, Aggio RB, Van Houtte JR, Villas-Boas SG. Analytical platform for metabolome
791   analysis of microbial cells using methyl chloroformate derivatization followed by gas
792   chromatography-mass spectrometry. Nat Protoc. 2010;5(10):1709-29.
793   50.   Johnsen LG, Skou PB, Khakimov B, Bro R. Gas chromatography - mass spectrometry data
794   processing made easy. J Chromatogr A. 2017;1503:57-64.
795   51.   Grubbs FE. Procedures for Detecting Outlying Observations in Samples. Technometrics.
796   1969;11(1):1-21.
797   52.   Dhabhar FS, Malarkey WB, Neri E, McEwen BS. Stress-induced redistribution of immune cells-
798   -from barracks to boulevards to battlefields: a tale of three hormones--Curt Richter Award winner.
799   Psychoneuroendocrinology. 2012;37(9):1345-68.
800   53.   Nikodemova M, Small AL, Kimyon RS, Watters JJ. Age-dependent differences in microglial
801   responses to systemic inflammation are evident as early as middle age. Physiol Genomics.
802   2016;48(5):336-44.
803   54.   Bardou I, Brothers HM, Kaercher RM, Hopp SC, Wenk GL. Differential effects of duration and
804   age on the consequences of neuroinflammation in the hippocampus. Neurobiol Aging.
805   2013;34(10):2293-301.
806   55.   Lee DC, Ruiz CR, Lebson L, Selenica ML, Rizer J, Hunt JB, Jr., et al. Aging enhances classical
807   activation but mitigates alternative activation in the central nervous system. Neurobiol Aging.
808   2013;34(6):1610-20.
809   56.   Culshaw S, Millington OR, Brewer JM, McInnes IB. Murine neutrophils present Class II
810   restricted antigen. Immunology letters. 2008;118(1):49-54.
811   57.   Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Loré K. Neutrophils acquire the capacity
812   for antigen presentation to memory CD4(+) T cells in vitro and ex vivo. Blood. 2017;129(14):1991-
813   2001.
814   58.   Dinan TG, Cryan JF. Gut instincts: microbiota as a key regulator of brain development, ageing
815   and neurodegeneration. J Physiol. 2017;595(2):489-503.
816   59.   Leung K, Thuret S. Gut Microbiota: A Modulator of Brain Plasticity and Cognitive Function in
817   Ageing. Healthcare (Basel). 2015;3(4):898-916.
818   60.   Miyajima M, Zhang B, Sugiura Y, Sonomura K, Guerrini MM, Tsutsui Y, et al. Metabolic shift
819   induced by systemic activation of T cells in PD-1-deficient mice perturbs brain monoamines and
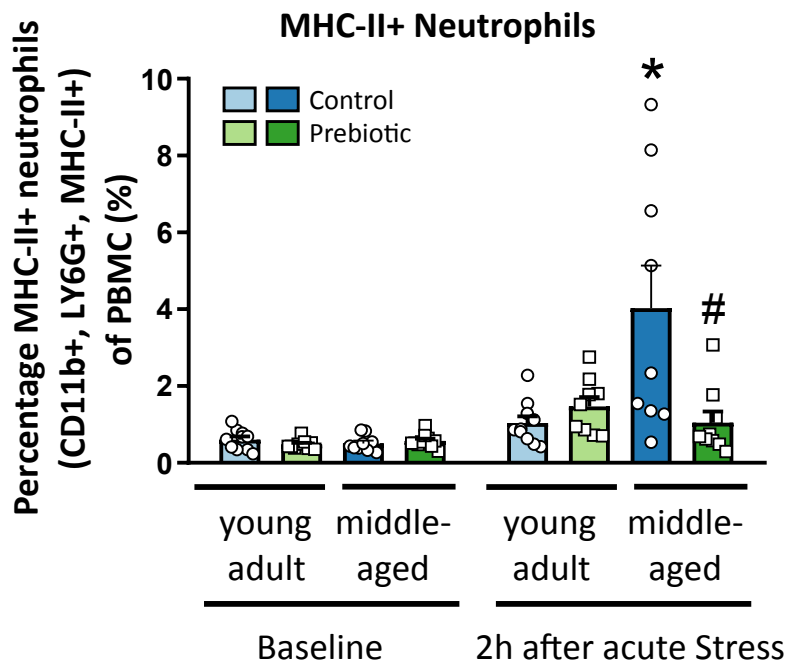820   emotional behavior. Nature immunology. 2017.
821   61.   Derecki NC, Cardani AN, Yang CH, Quinnies KM, Crihfield A, Lynch KR, et al. Regulation of
822   learning and memory by meningeal immunity: a key role for IL-4. J Exp Med. 2010;207(5):1067-80.
823   62.   Filiano AJ, Xu Y, Tustison NJ, Marsh RL, Baker W, Smirnov I, et al. Unexpected role of
824   interferon-γ in regulating neuronal connectivity and social behaviour. Nature. 2016;advance online
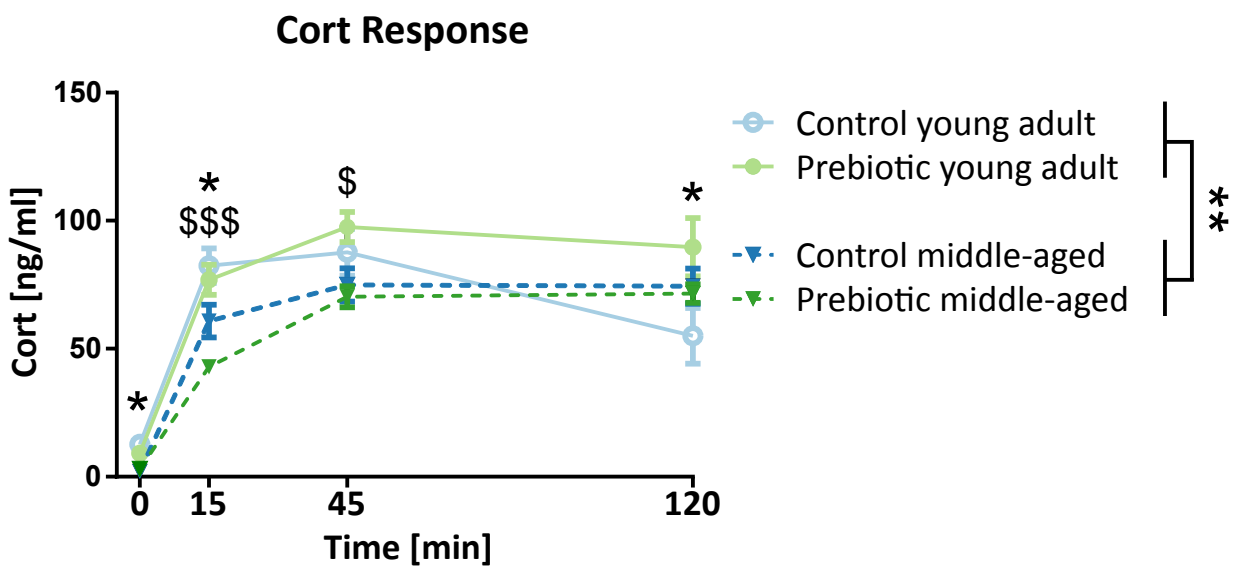825   publication.

826 63.   Sinclair LV, Rolf J, Emslie E, Shi YB, Taylor PM, Cantrell DA. Control of amino-acid transport by
827 antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation.
828 Nature immunology. 2013;14(5):500-8.
829 64.   Lin R, Liu W, Piao M, Zhu H. A review of the relationship between the gut microbiota and
830 amino acid metabolism. Amino Acids. 2017;49(12):2083-90.
831 65.   Shen X, Miao J, Wan Q, Wang S, Li M, Pu F, et al. Possible correlation between gut microbiota
832 and immunity among healthy middle-aged and elderly people in southwest China. Gut Pathog.
833 2018;10:4.
834 66.   Varvel NH, Neher JJ, Bosch A, Wang W, Ransohoff RM, Miller RJ, et al. Infiltrating monocytes
835 promote brain inflammation and exacerbate neuronal damage after status epilepticus. Proceedings
836 of the National Academy of Sciences of the United States of America. 2016;113(38):E5665-74.
837 67.   Möhle L, Mattei D, Heimesaat Markus M, Bereswill S, Fischer A, Alutis M, et al. Ly6Chi
838 Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult
839 Hippocampal Neurogenesis. Cell Reports. 2016;15(9):1945-56.
840 68.   Korin B, Ben-Shaanan TL, Schiller M, Dubovik T, Azulay-Debby H, Boshnak NT, et al. High-
841 dimensional, single-cell characterization of the brain's immune compartment. Nature neuroscience.
842 2017;advance online publication.
843 69.   Mrdjen D, Pavlovic A, Hartmann FJ, Schreiner B, Utz SG, Leung BP, et al. High-Dimensional
844 Single-Cell Mapping of Central Nervous System Immune Cells Reveals Distinct Myeloid Subsets in
845 Health, Aging, and Disease. Immunity. 2018;48(3):599.
846 70.   Getts DR, Terry RL, Getts MT, Müller M, Rana S, Shrestha B, et al. Ly6c(+) "inflammatory
847 monocytes" are microglial precursors recruited in a pathogenic manner in West Nile virus
848 encephalitis. The Journal of Experimental Medicine. 2008;205(10):2319-37.
849 71.   Wohleb ES, Powell ND, Godbout JP, Sheridan JF. Stress-induced recruitment of bone marrow-
850 derived monocytes to the brain promotes anxiety-like behavior. The Journal of neuroscience : the
851 official journal of the Society for Neuroscience. 2013;33(34):13820-33.
852 72.   Sawicki CM, McKim DB, Wohleb ES, Jarrett BL, Reader BF, Norden DM, et al. Social defeat
853 promotes a reactive endothelium in a brain region-dependent manner with increased expression of
854 key adhesion molecules, selectins and chemokines associated with the recruitment of myeloid cells
855 to the brain. Neuroscience. 2015;302:151-64.
856 73.   Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, et al. Microglia in the adult
857 brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions. Nature neuroscience.
858 2007;10(12):1544-53.
859 74.   Grabert K, Michoel T, Karavolos MH, Clohisey S, Baillie JK, Stevens MP, et al. Microglial brain
860 region-dependent diversity and selective regional sensitivities to aging. Nature neuroscience.
861 2016;advance online publication.
862 75.   Stirling DP, Cummins K, Mishra M, Teo W, Yong VW, Stys P. Toll-like receptor 2-mediated
863 alternative activation of microglia is protective after spinal cord injury. Brain. 2014;137(3):707-23.
864 76.   Mrdjen D, Pavlovic A, Hartmann FJ, Schreiner B, Utz SG, Leung BP, et al. High-Dimensional
865 Single-Cell Mapping of Central Nervous System Immune Cells Reveals Distinct Myeloid Subsets in
866 Health, Aging, and Disease. Immunity. 2018;48(2):380-95 e6.
867 77.   Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host
868 microbiota constantly control maturation and function of microglia in the CNS. Nature neuroscience.
869 2015;18(7):965-77.
870 78.   Rea K, Dinan TG, Cryan JF. The microbiome: A key regulator of stress and neuroinflammation.
871 Neurobiol Stress. 2016;4:23-33.
872 79.   Vuong HE, Yano JM, Fung TC, Hsiao EY. The Microbiome and Host Behavior. Annu Rev
873 Neurosci. 2017;40:21-49.
874 80.   Ang Z, Er JZ, Tan NS, Lu J, Liou YC, Grosse J, et al. Human and mouse monocytes display
875 distinct signalling and cytokine profiles upon stimulation with FFAR2/FFAR3 short-chain fatty acid
876 receptor agonists. Sci Rep. 2016;6:34145.

877 81.   Wohleb ES, McKim DB, Sheridan JF, Godbout JP. Monocyte trafficking to the brain with stress
878 and inflammation: a novel axis of immune-to-brain communication that influences mood and
879 behavior. Frontiers in neuroscience. 2014;8:447.
880 82.   Vazquez E, Barranco A, Ramirez M, Gruart A, Delgado-Garcia JM, Martinez-Lara E, et al.
881 Effects of a human milk oligosaccharide, 2'-fucosyllactose, on hippocampal long-term potentiation
882 and learning capabilities in rodents. The Journal of nutritional biochemistry. 2015;26(5):455-65.
883 83.   Mika A, Gaffney M, Roller R, Hills A, Bouchet CA, Hulen KA, et al. Feeding the developing
884 brain: Juvenile rats fed diet rich in prebiotics and bioactive milk fractions exhibit reduced anxiety-
885 related behavior and modified gene expression in emotion circuits. Neurosci Lett. 2018;677:103-9.
886 84.   Tarr AJ, Galley JD, Fisher Sydney E, Chichlowski M, Berg BM, Bailey MT. The prebiotics
887 3'Sialyllactose and 6'Sialyllactose diminish stressor-induced anxiety-like behavior and colonic
888 microbiota alterations: Evidence for effects on the gut–brain axis. Brain, behavior, and immunity.
889 2015;50:166-77.
890 85.   Beilharz JE, Kaakoush NO, Maniam J, Morris MJ. Cafeteria diet and probiotic therapy: cross
891 talk among memory, neuroplasticity, serotonin receptors and gut microbiota in the rat. Molecular
892 psychiatry. 2017;23:351.
893 86.   Fonken LK, Frank MG, D'Angelo HM, Heinze JD, Watkins LR, Lowry CA, et al. Mycobacterium
894 vaccae immunization protects aged rats from surgery-elicited neuroinflammation and cognitive
895 dysfunction. Neurobiol Aging. 2018;71:105-14.
896 87.   Zhang S, Yang J, Henning SM, Lee R, Hsu M, Grojean E, et al. Dietary pomegranate extract and
897 inulin affect gut microbiome differentially in mice fed an obesogenic diet. Anaerobe. 2017;48:184-93.
898 88.   Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty
899 acids. Nutrients. 2011;3(10):858-76.
900 89.   Huuskonen J, Suuronen T, Nuutinen T, Kyrylenko S, Salminen A. Regulation of microglial
901 inflammatory response by sodium butyrate and short-chain fatty acids. British journal of
902 pharmacology. 2004;141(5):874-80.
903 90.   Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao JZ, et al. Age-related changes
904 in gut microbiota composition from newborn to centenarian: a cross-sectional study. BMC Microbiol.
905 2016;16:90.
906 91.   Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, et al. Human
907 gut microbiome viewed across age and geography. Nature. 2012;486:222.
908 92.   Claesson MJ, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, et al. Gut microbiota
909 composition correlates with diet and health in the elderly. Nature. 2012;488(7410):178-84.
910 93.   Claesson MJ, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, et al.
911 Composition, variability, and temporal stability of the intestinal microbiota of the elderly.
912 Proceedings of the National Academy of Sciences. 2011;108(Supplement 1):4586-91.
913 94.   Gibson GR, Beatty ER, Wang X, Cummings JH. Selective stimulation of bifidobacteria in the
914 human colon by oligofructose and inulin. Gastroenterology. 1995;108(4):975-82.
915 95.   Hopkins MJ, Sharp R, Macfarlane GT. Age and disease related changes in intestinal bacterial
916 populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles.
917 Gut. 2001;48(2):198-205.
918 96.   Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between
919 Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity.
920 Proceedings of the National Academy of Sciences. 2013;110(22):9066-71.
921 97.   Bodogai M, O'Connell J, Kim K, Kim Y, Moritoh K, Chen C, et al. Commensal bacteria
922 contribute to insulin resistance in aging by activating innate B1a cells. Science translational medicine.
923 2018;10(467).
924 98.   Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain
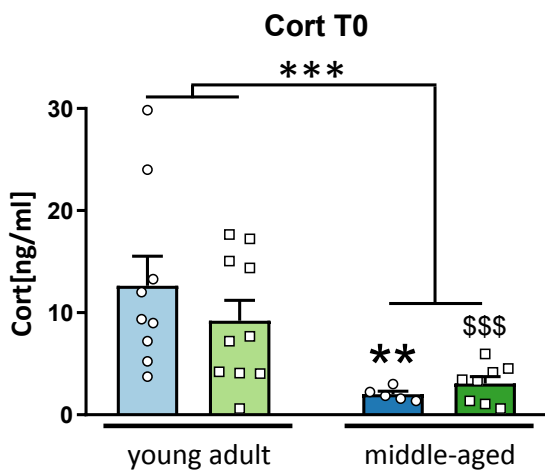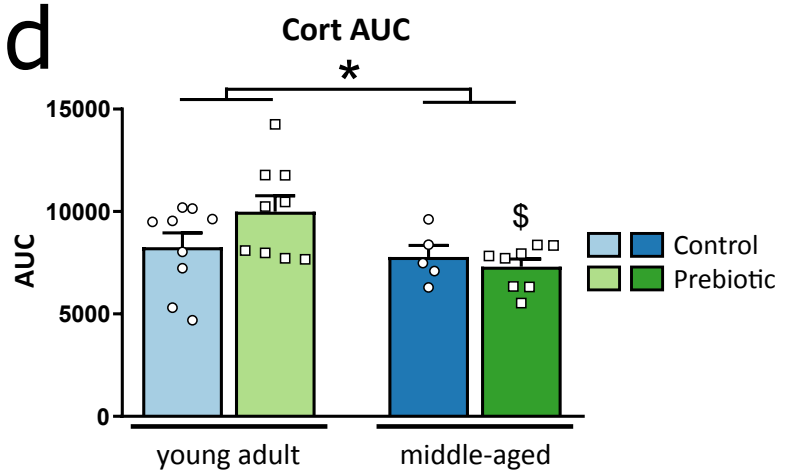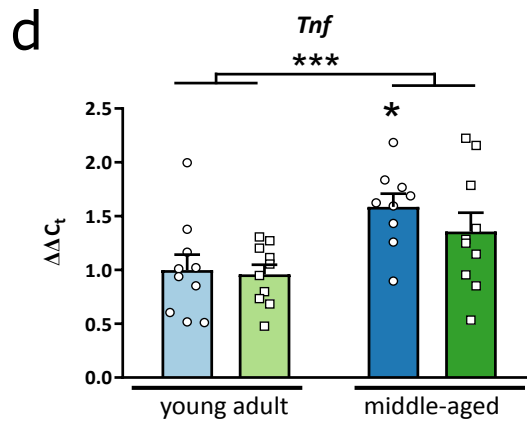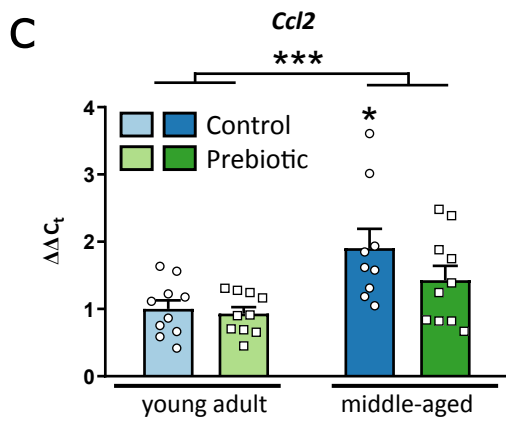925 and behaviour. Nature reviews Neuroscience. 2012;13(10):701-12.
926

a MHC-II+ Neutrophils

Percentage MHC-II+ neutrophils (CD11b+, LY6G+, MHC-II+) of PBMC (%)

Control / Prebiotic

young adult, middle-aged — Baseline

young adult, middle-aged — 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

b Ly-6C+ microglia

c *Ccl2*

d *Tnf*

a

Morris water maze

MWM Learning AUC

Probe trial

b

Spontaneous Alternation Behavior

c

Fear Conditioning - Conditioning

Conditioning_AUC

Fear Conditioning - Extinction_cumulative trial blocks

Extinction Trial Block 1-20

Context Recall

d

Elevated-plus maze

e

Open field

Open field

Open field - locomotion

f

r=-0.369
p=0.0228

r=-0.323
p=0.0478
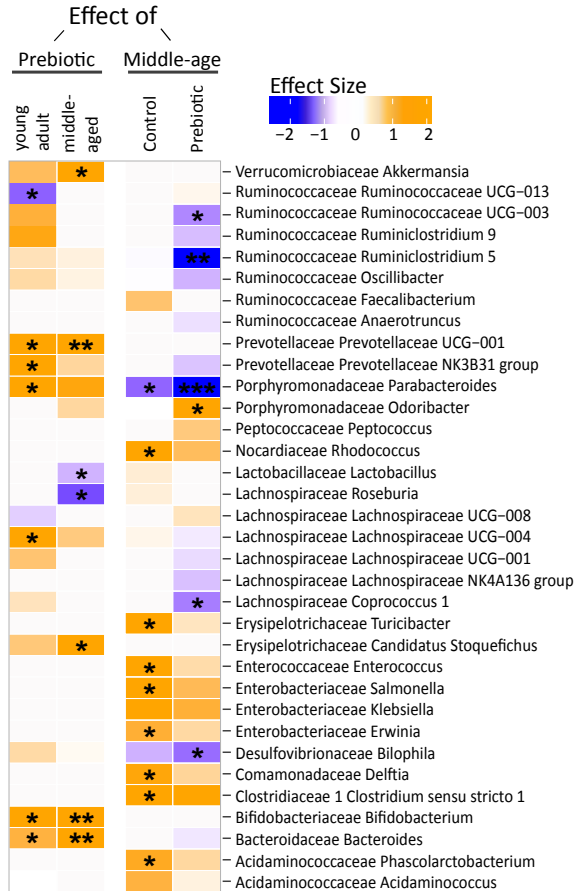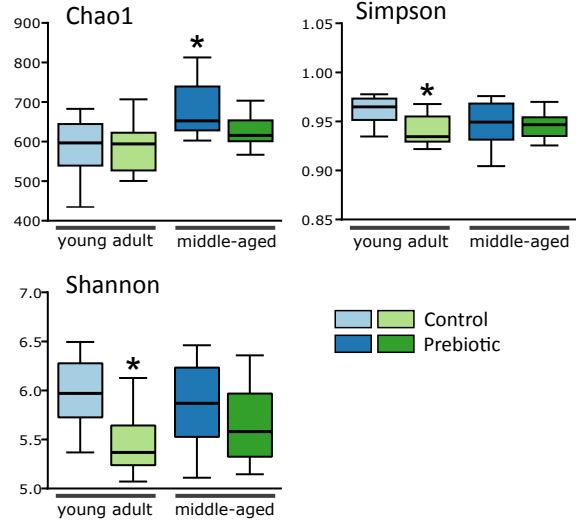
g

r=0.349
p=0.0295

a Shift in gut microbiota composition by middle-age and prebiotic supplementation
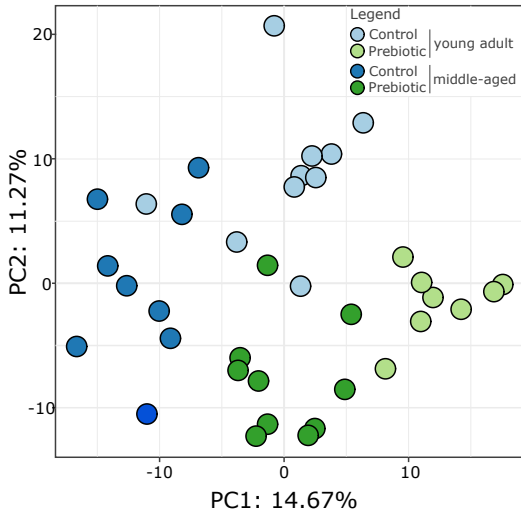
b Differentially abundant taxa in middle-aged and prebiotic groups

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

d Correlation analysis: Genera vs Metabolites