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Coláiste na hOllscoile Corcaigh

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

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

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Abstract

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

1. Introduction

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

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

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

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

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

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

195 **2.6 Blood stimulation cytokine assay**

196 To assess if a prebiotic-enriched diet alters systemic immunity, 100 µl 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-1β, IL-4, IL-6, IL-10, TNFα 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**

206 To assess stress-induced immune priming, blood was collected from young adult and middle-aged
207 mice by tail tipping (60 µl) at baseline and 120 min after acute stress (cohort one). Staining was
208 performed using CD11b-VioBright FITC, Ly-6C-PE, LY-6G-PerCP-Vio700 and MHC-II-PE (all Miltenyi
209 Biotec, Bergisch Gladbach, Germany) to assess inflammatory monocytes (CD11b+, SSC^{low}, LY-$6C^{hi}$) and
210 MHC-II+-neutrophils (CD11b+, LY-6G+, MHC-II+). Inflammatory monocyte and MHC-II+-neutrophil
211 counts were normalized to the amount of peripheral blood mononuclear cell (PBMC). Gating strategy
212 is depicted in Supplementary Figure S2a.
213 Cohort two investigated if the diet lead-in phase with FOS-Inulin modulates monocyte infiltration and
214 subsequent microglia activation in the brain. Following perfusion with ice-cold PBS for five min,
215 brains were carefully dissected, enzymatically digested using the neural dissociation kit (P), followed
216 by incubation in myelin-removal beads and magnetic separation using LS columns (Miltenyi Biotec).
217 Cells were stained using CD11b-Viobright FITC, CD45-APC and Ly-6C-PE (all Miltenyi Biotec). Gating
218 strategy is depicted in Supplementary Figure S2b. Monocyte counts were normalized to CD11b+ cells,
219 microglia to CD11b+, $CD45^{low}$.
220

221 **2.8 Analysis of gene expression levels in the brain tissues (RT-qPCR)**

222 To assess gene expression brain areas associated with cognition, the right hemisphere of both, the
223 hippocampus and the prefrontal cortex were used (44). Total RNA was extracted using the mirVana™
224 miRNA Isolation Kit (Ambion, Life technologies, Waltham, MA, US), followed by DNase treatment
225 using the TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA)
226 according to the manufacturer's instructions. RNA was quantified using the NanoDrop™
227 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

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

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

2.11 Statistical analysis

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

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

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

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

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

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

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

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).

371 We further tested the effect on short-term memory by assessing spontaneous alternation behaviour
372 in the Y-maze. Middle-aged mice showed a decrease in spontaneous alternations ($F_{(1, 35)} = 10.66$,
373 p=0.003) and total number of alternations ($F_{(1, 35)} = 7.986$, p=0.008; Figure 3b) suggesting
374 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
379 were assessed. Middle-aged mice showed increased freezing during habituation to the new context
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
385 recall was found (Figure 3c).

386 Next, we analysed anxiety-like behaviour in the elevated plus maze and the open field, as changes in
387 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 ($F_{(1,}$
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
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

409 **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 ($\rho=-$
439 0.63, $p<0.001$, FDR corrected, Figure 4d), valine and isoleucine ($\rho=-0.60$, $p<0.001$, respectively).
440 Similarly, between *Bifidobacterium*, which was significantly over-represented in prebiotic-treated
441 young and middle-aged mice, and the respective amino acids ($\rho=-0.55$, $p=0.001$). 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**

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

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

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.

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Author contributions

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

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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 ± 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

638 **Figure 2. Middle-aged mice exhibited elevated infiltration of Ly-6C^{hi} monocytes into the brain and**
639 **increased microglia activation; the phenotype was reversed by prebiotic supplementation. (a)**
640 Monocyte infiltration in the brain. **(b)** Microglia expression pattern in the brain. **(c-d)** Pro-
641 inflammatory cytokine expression in the hippocampus. Mean ± SEM. (a-b) n = 14-16 (young adult), n
642 = 8-10 (middle-aged), (c-d) n = 10 (young adult), n = 9-10 (middle-aged). (a-d) two-way ANOVA post
643 hoc Holm-Sidak. *vs. control young adult* * $p < 0.05$, *** $p < 0.001$, *vs. control middle-aged* ## $p <$
644 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
655 *Akkermansia* genus **(g)** Spearman correlation learning in Morris water maze vs. hippocampal *Ccl2*
656 expression. Mean ± 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*
659 *vs prebiotic adult*: $ < 0.05, $$ < 0.01, $$$ $p < 0.001$.
660

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.

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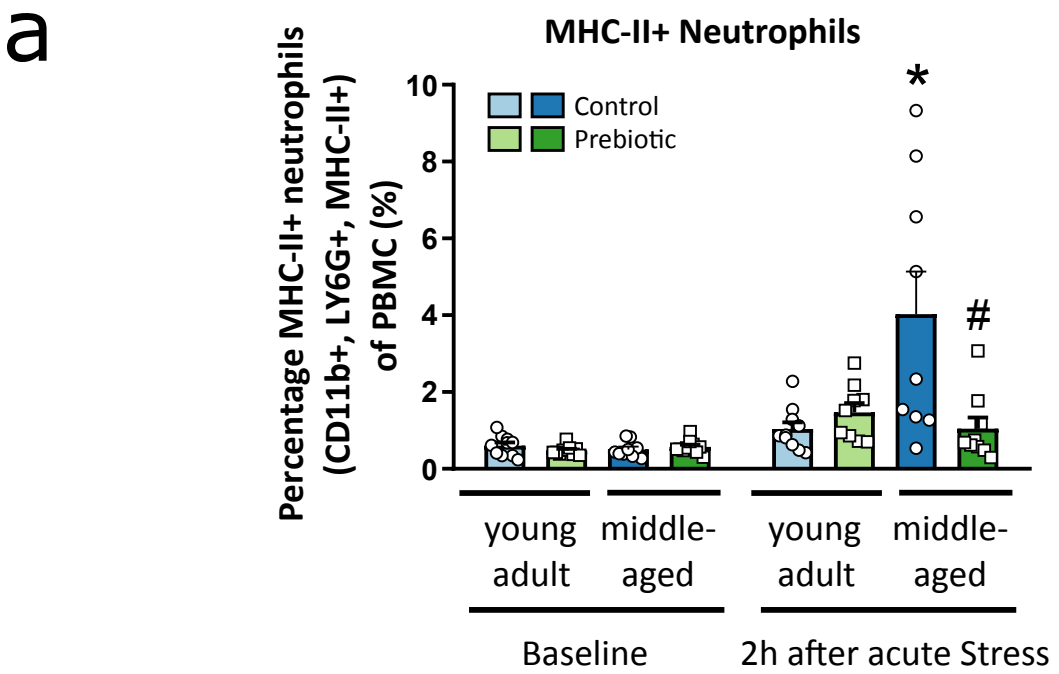
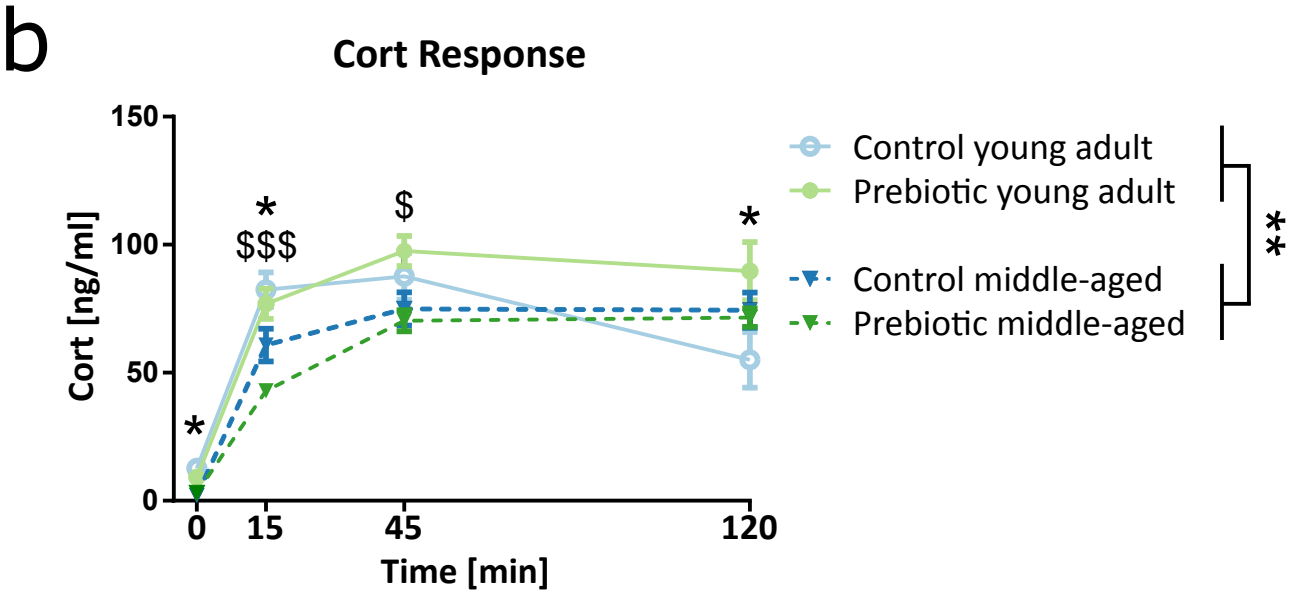
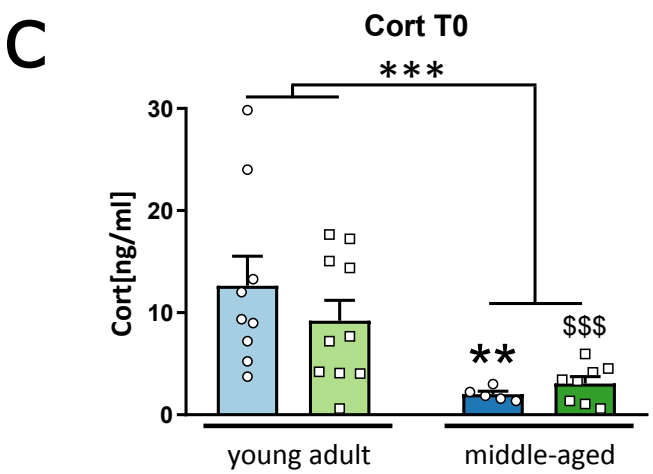
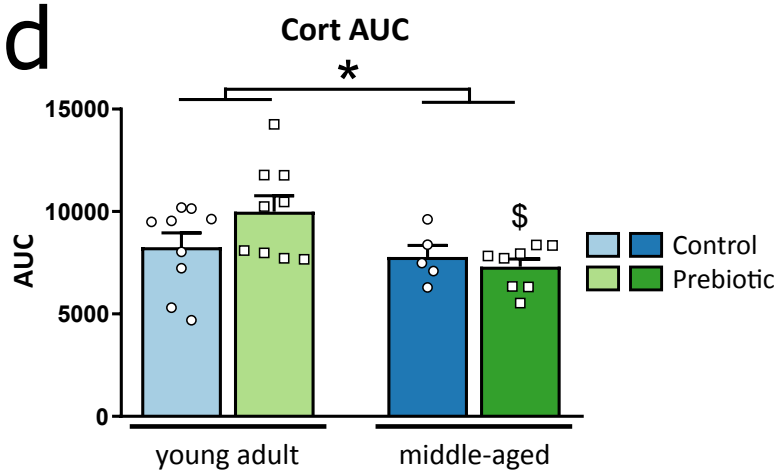
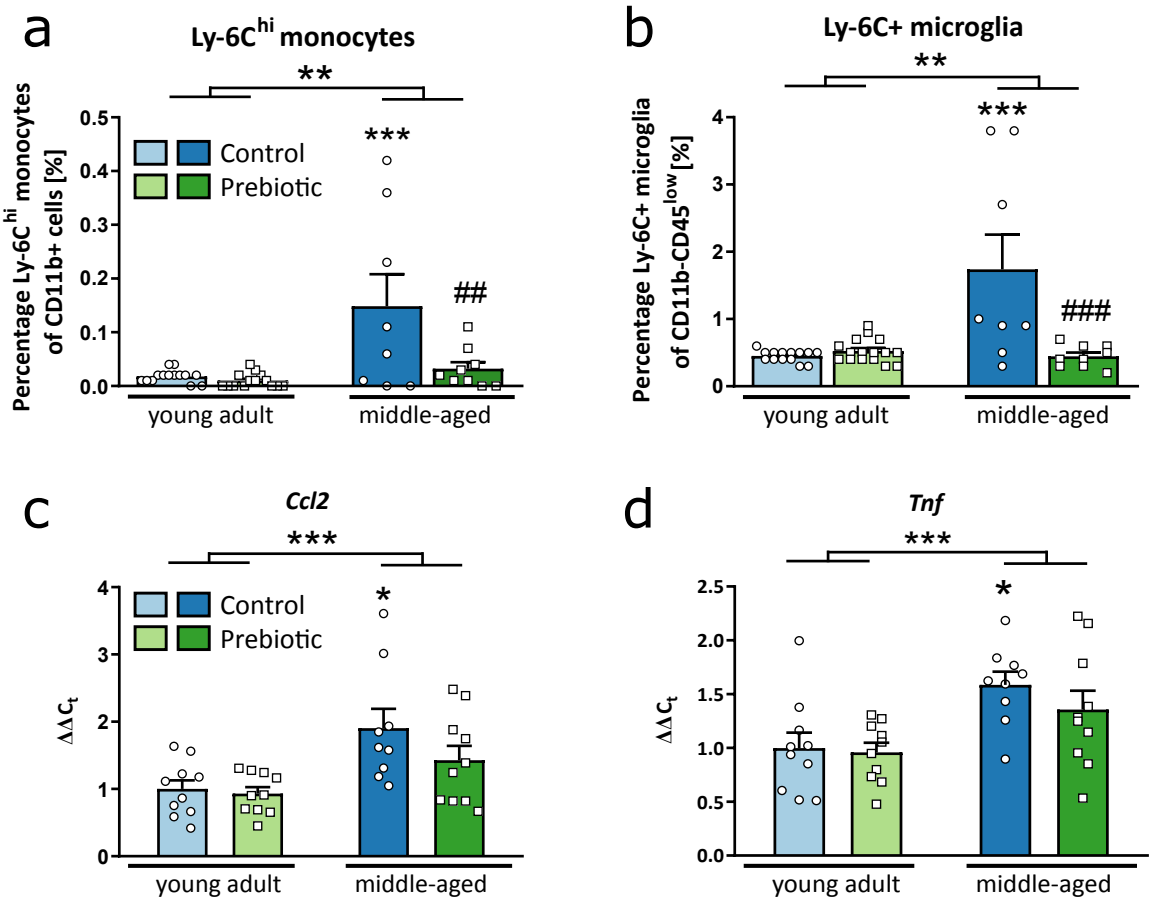
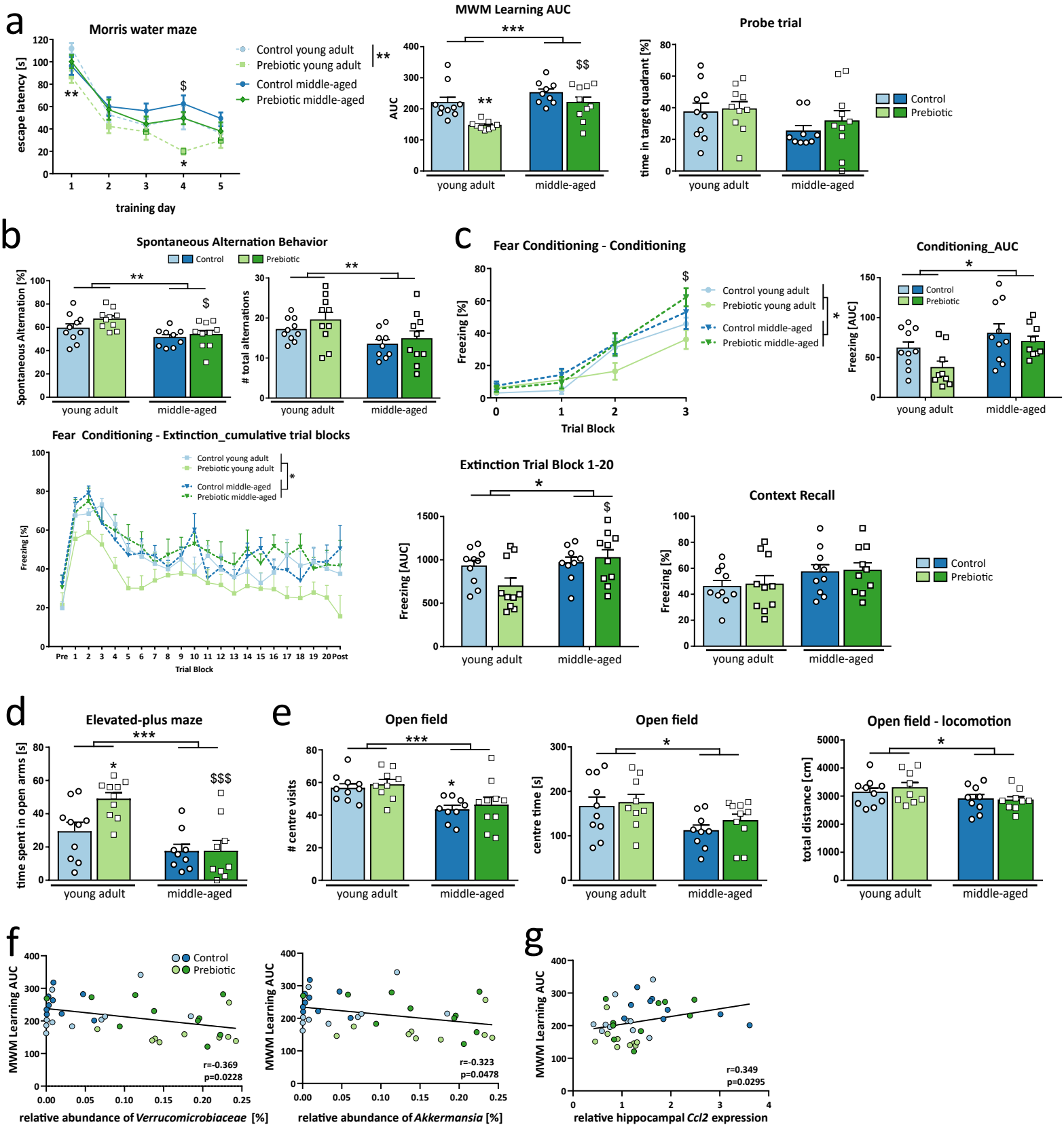

a
MHC-II+ Neutrophils
Percentage MHC-II+ neutrophils (CD11b+, LY6G+, MHC-II+) of PBMC (%)
Control
Prebiotic
young adult
middle-aged
young adult
middle-aged
Baseline
2h after acute Stress

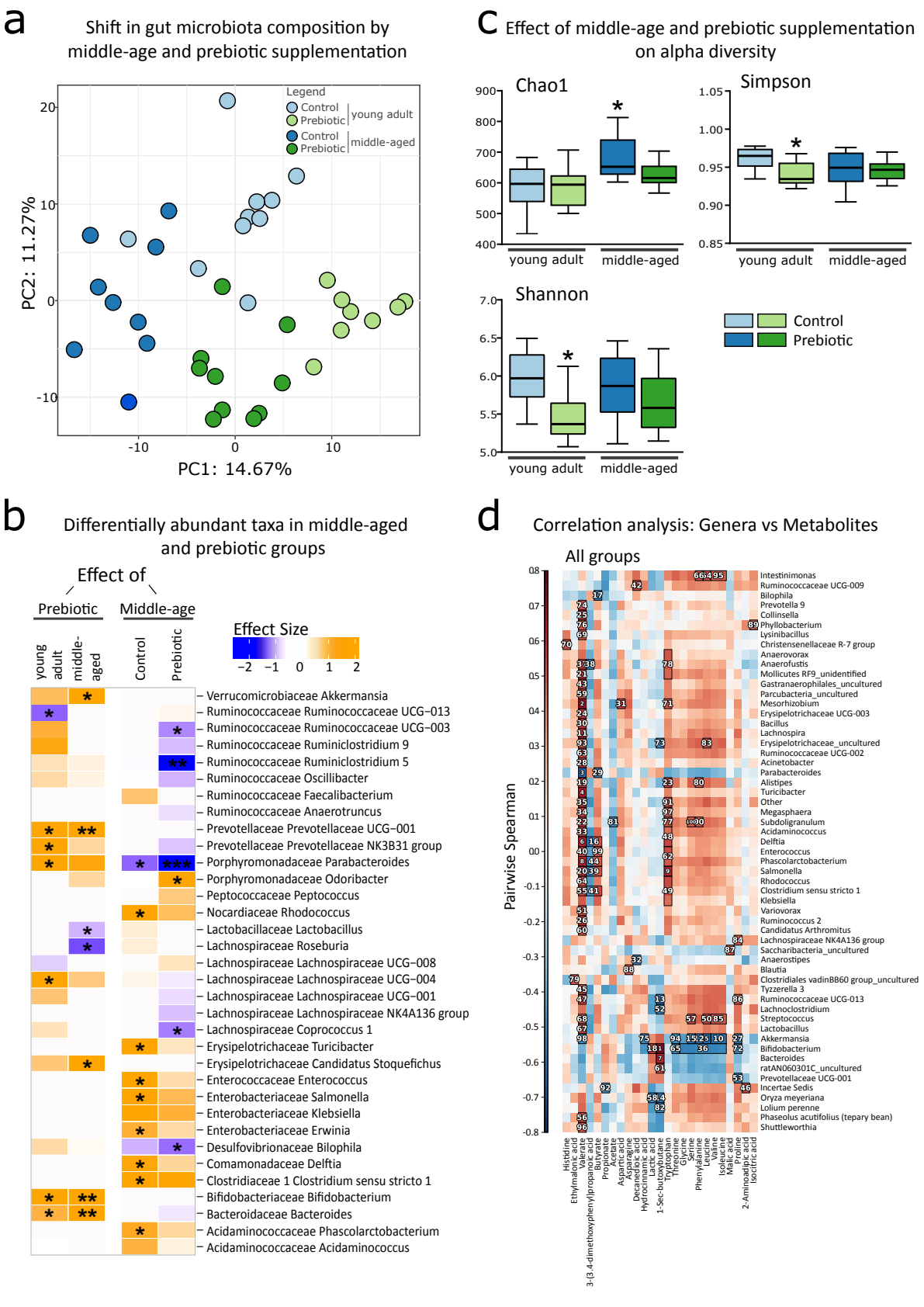

b
Cort Response
Cort [ng/ml]
Time [min]
Control young adult
Prebiotic young adult
Control middle-aged
Prebiotic middle-aged


c
Cort T0
Cort[ng/ml]
young adult
middle-aged


d
Cort AUC
AUC
Control
Prebiotic
young adult
middle-aged


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


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


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