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Authors	Cruz-Pereira, Joana S.;Moloney, Gerard M.;Bastiaanssen, Thomaz F. S.;Boscaini, Serena;Fitzgerald, Patrick;Clarke, Gerard;Cryan, John F.
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Short Communication

Age-associated deficits in social behaviour are microbiota-dependent

Joana S. Cruz-Pereira [a,b], Gerard M. Moloney [a,b], Thomaz F.S. Bastiaanssen [b], Serena Boscaini [b], Patrick Fitzgerald [a,b], Gerard Clarke [b,c], John F. Cryan [a,b,*]

[a] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland
[b] APC Microbiome Ireland, University College Cork, Cork, Ireland
[c] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

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ABSTRACT

Aging is associated with remodelling of immune and central nervous system responses resulting in behavioural impairments including social deficits. Growing evidence suggests that the gut microbiome is also impacted by aging, and we propose that strategies to reshape the aged gut microbiome may ameliorate some age-related effects on host physiology. Thus, we assessed the impact of gut microbiota depletion, using an antibiotic cocktail, on aging and its impact on social behavior and the immune system.

Indeed, microbiota depletion in aged mice eliminated the age-dependent deficits in social recognition. We further demonstrate that although age and gut microbiota depletion differently shape the peripheral immune response, aging induces an accumulation of T cells in the choroid plexus, that is partially blunted following microbiota depletion. Moreover, an untargeted metabolomic analysis revealed age-dependent alterations of cecal metabolites that are reshaped by gut microbiota depletion. Together, our results suggest that the aged gut microbiota can be specifically targeted to affect social deficits. These studies propel the need for future investigations of other non-antibiotic microbiota targeted interventions on age-related social deficits both in animal models and humans.

1. Introduction

Aging is an intricate process that entails complex and dynamic remodeling of different physiological systems and distinct behavioural changes. Alterations in the gut microbiome and the immune system have been associated with aging (Cruz-Pereira et al., 2022, Ghosh et al., 2022, Scott et al., 2017, Wyss-Coray, 2016). Moreover, aging is intrinsically linked to increased susceptibility to neurological and cognitive impairments (Wyss-Coray, 2016) as well as deficits in social cognition (Moran et al., 2012, Roheger et al., 2022). Indeed, cognitive decline has profound impacts on social functioning (Arioli et al., 2018). There has been renewed interest in linking microbiota and immune alterations with social cognition across the lifespan (Filiano et al., 2016, Ratsika et al., 2022, Sherwin et al., 2019).

The choroid plexus - an epithelial monolayer located in each ventricle in the brain that acts as a key neuroimmunological hub (Baruch et al., 2013) - represents one of the very few sites where T cells are found in the central nervous system (CNS) (Engelhardt & Ransohoff, 2005). In aged mice the choroid plexus is populated with CNS-antigen specific CD4 + T cells that promote *T*-helper type 2 (Th2) responses, which promote cognitive dysfunction (Baruch et al., 2013). Additionally, with age the choroid plexus presents a type I interferon (IFN-I)-dependent gene profile, and in turn blockade of this signalling pathway results in amelioration of cognitive function (Baruch et al., 2014).

Whether there is a relationship among gut microbiota, social behaviour and the immune system remains relatively unknown in the context of aging. Thus, we sought to understand the impact of gut microbiota depletion via antibiotic administration on social behavior and immune readouts in aged mice, while identifying age-related metabolites that could be underlying the changes observed.

2. Materials and Methods

2.1. Animals

Male young C57/BL6 mice (n = 20; 9–10 weeks old; Charles River, Kent, UK) and aged C57/BL6 (n = 20; 18 months old; Charles River, Kent, UK) were used in this study. All experiments were conducted in

* Corresponding author at: Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland.
E-mail address: j.cryan@ucc.ie (J.F. Cryan).

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accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC, and approved by the Animal Experimentation Ethics Committee of University College Cork AE19130/P087. Animals were kept under a 12-h light/dark cycle, with a temperature of 21 ± 1 °C and humidity of 55 ± 10 %. Food and water were given *ad libitum*.

2.2. Antibiotic treatment

To deplete gut microbiota, mice were treated for 10 consecutive days with an antibiotic cocktail (ampicilin (1 g/L), neomycin (0.5 g/L) and vancomycin (0.35 g/L)) or water (as previously described (Boscaini et al., 2021). Antibiotics were dissolved in water and changed every second day. Control animals received just water, which was also replaced every second day.

2.3. Behavior

On day 9 post initiation of antibiotic treatment, social cognition was evaluated using the 3-chamber social interaction test, in which time spent interacting with a novel conspecific is examined against time spent with a novel object or familiar conspecific. It is based on the premise that mice are attracted to novelty, hence would prefer a novel conspecific to a familiar one (Moy et al., 2004).

The test arena consisted of 3 chambers; the left and right chambers measured 13.5 × 20 × 20 cm and the center chamber was 9 × 20 × 20 cm. A solid partition divided the chambers, with a small hole allowing access to the other chambers. During the habituation phase, the mouse was placed into the center chamber and then allowed access to the empty left and right chambers for 10 min. For the social novelty trial, an aged-matched novel conspecific mouse was placed in a mesh cage, on the opposite chamber to a familiar conspecific mouse. Placement of the novel mouse was randomised between animals to eliminate side preferences. The 3-chamber apparatus was cleaned with 70 % ethanol and allowed to evaporate between animals. The time spent in each chamber was then measured. The animals were habituated to the room for 45 min before the test, and the test was conducted under dim light (60 lx). The time spent in each chamber was then scored using the Behavioral Observation Research Interactive Software (BORIS) (Friard & Gamba, 2016).

2.4. Tissue collection

Animals were killed by anesthetic overdose with sodium pentobarbital and transcardially perfused in a random fashion regarding testing groups between 09.00 h and 15.00 h. The caecum and ileum were quickly removed before the transcardial perfusion, weighed, snap-frozen on dry ice and stored at − 80 °C. The brain was excised and kept in paraformaldehyde (PFA) for 24 h, and then dehydrated by a sucrose gradient before being snap frozen.

2.5. Cytokine quantification

The levels of secreted interferon gamma (IFN-γ) was analysed with the Pro-inflammatory Panel 1 (mouse) V-PLEX Kit, and IL-17a was analysed with the U-PLEX Mouse IL-17a assay, using the *MESO* QuickPlex SQ 120, SECTOR Imager 2400 (*Meso* Scale Discovery, Maryland, USA). Only data derived from duplicates with CV < 15 % were included in the analysis. Concentrations of cytokines were expressed in pg/mg of tissue.

2.6. Immunofluorescence

Serial coronal sections (40 um) were cut in a cryostat (Leica) and stored at −20ºC. For staining, the sections were washed three times in phosphate buffered saline (PBS) for 5 min each. For antigen retrieval, slides were immersed in citrate buffer, and placed in a water bath for 15 min at 80ºC. Next, a blocking solution (10 % normal donkey serum (NDS) in 0.3 % TritonX-100 PBS) was applied once the slides achieved room temperature, for 1 h30 in a humid chamber. The blocking solution was replaced with primary antibody probing solution (2 % NDS, 0.3 % TritonX-100 PBS and primary antibody rabbit anti-$CD4^+$ (1:500, Abcam)), and was incubated overnight at 4ºC. After primary incubation, the sections were washed four times with PBS, for 5 min per wash. The Alexafluor secondary antibody donkey anti-rabbit 488 (1:500, Invitrogen) was applied in a carrier solution (2 % NDS, 0.1 % PBS Tween) and incubated for 2 h at room temperature. Following secondary incubation and 10 min of incubation with DAPI, the sections were washed three times with PBS, mounted with Immu-Mount (Fisher Scientific) and coverslipped. The number of $CD4^+$ cells was quantified in the wall of the lateral ventricles and the 3rd ventricle, using Olympus BX43 optical microscope at 20x.

2.7. Caecal metabolomics

The caecal metabolome was analysed by MS-Omics (Copenhagen) using Mass Spectrometry. Briefly, samples were acidified using hydrochloride acid, and deuterium labelled internal standards where added. All samples were analyzed in a randomized order. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column 30 m × 0.25 mm × 0.25 μm) installed in a GC (7890B, Agilent) coupled with a quadropole detector (5977B, Agilent). The system was controlled by ChemStation (Agilent). Raw data was converted to netCDF format using Chemstation (Agilent), before the data was imported and processed in Matlab R2014b (Mathworks, Inc.) using the PARADISe software described by Johnsen et. Al (Johnsen et al., 2017).

Peaks were quantified using area under the curve (AUC). Biostatistics were run in R (version 4.1.2) with the Rstudio GUI (version 1.4.1717). Principal-component analysis was performed on CLR-transformed values (Aitchison et al., 2000). The PERMANOVA implementation from the vegan library was used to find structural differences between treatments on a compositional level. To find metabolites that were differentially abundant based on either age or antibiotic treatment, we fitted linear models using the CLR-transformed metabolite levels with both factors as explanatory variables. To correct for multiple testing (FDR) in tests involving metabolomics features, Storey's q-value posthoc procedure was performed with a q-value of 0.2 as a cut-off (Storey, 2002). Metabolomics figures were generated using ggplot2. Metabolites were filtered where the absolute β estimate of Young-Control vs Aged-Control > absolute β of Young-Control vs Aged-ABX (*i.e.*, when the difference between young and aged controls is greater than the difference between young controls and aged mice exposed to antibiotics).

2.8. Statistical analysis

Statistical analyses were conducted using SPSS 27 (IBM, USA). Normality was assessed employing the Shapiro–Wilk test and for equality of variances using the Levene's test. Non-parametric data were analysed with independent-samples Kruskal–Wallis test followed by pairwise comparisons adjusted by the Bonferroni correction for multiple tests, with a 95 % confidence interval. Parametric data were analysed using two-way analysis of variance (ANOVA) *post hoc* Tukey HSD. All data is represented as mean ± SEM. Social novelty was analysed using a general linear model integrating age, treatment and stimulus. Further, we utilized simple main effects to explore the individual group differences in these complex models, adjusted by the Bonferroni correction for multiple tests, as we hypothesized a priori that the Aged-control group would be significantly different from the other groups. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Microbiota depletion Restores the Age-Dependent social cognitive Defects

Young (9–10 weeks old) and aged (18 months old) C57BL/6 mice were assessed in the 3-chamber test after 9 days of antibiotic exposure (Fig. 1a). A distinct preference between either chamber was observed (Novel mouse vs Familiar mouse, ($F_{(1,35)} = 14.796$, $p = 0.0005$), as young animals show a preference for the novel mouse chamber (Young-CTRL, $p = 0.039$; Young-ABX, $p = 0.088$), which is not seen in Aged-Control animals ($p = 0.822$) (Fig. 1b).

3.2. Age did not significantly impact Pro-Inflammatory cytokine levels in the ileum

To understand how microbiota depletion influences pro-inflammatory cytokine secretion in the gut of aged animals, cytokine levels were quantified in the ileum. The proinflammatory cytokines IFN-γ and IL-17a showed no significant differences in response to aging (Fig. 1c-d).

3.3. T-Helper cells accumulate in the aged choroid plexus and are partially restored by microbiota depletion

As the choroid plexus relays signals interchangeably between the CNS and the circulating immune system, and also has a prominent role in T-cell mediated neuroinflammation in aging (Baruch et al., 2013), we quantified CD4 + T cells in the choroid plexus, focusing our analysis on the number of cells along the walls of the lateral and third ventricles. Considering the cells accumulated along the ventricle wall, aged animals show a significant increase in CD4 + T cells in the lateral ventricle walls, which is partially reduced towards the levels present in young animals by antibiotic treatment (Two-Way ANOVA; $F_{(1,16)} = 5.308$, $p = 0.035$; Young-CTRL vs Aged-CTRL, $p < 0.001$; Aged-CTRL vs Aged-ABX, $p = 0.052$) (Fig. 1f). Likewise, in the third ventricle aged animals show an increase in T cells, which is reversed by antibiotic treatment ($F_{(1,15)} = 6.354$, $p = 0.024$; Young-CTRL vs Aged-CTRL, $p = 0.001$; Aged-CTRL vs Aged-ABX, $p = 0.023$) (Fig. 1g).

3.4. Age-Dependent Shifts in caecal metabolites can be Successfully restored by antibiotic treatment

We next set out to assess the extent of which gut microbiota depletion impacts cecal metabolite concentrations in young and aged animals with or without antibiotic exposure (Fig. 2a-b). Seventy metabolites were differentially altered by both age and gut microbiota depletion - after the filtering criteria (see Supplementary Methods), we focused on which metabolites if any are restored with antibiotic treatment in aged mice. After this filtering criteria, four metabolites are highlighted: 2-Methylbutyrylglicine, Argininosuccinic acid, Gentisic acid and *N*-formylmethionine (Fig. 2c). The levels of 2-Methylbutyrylglycine, an acyl glicine, a minor metabolite of fatty acids, were significantly reduced in aged animals ($\beta = 0.506$, $p = 0.003$, BH = 0.028; Young-CTRL vs Aged-CTRL, $p = 0.001$), and restored by antibiotic treatment in aged mice (Aged-CTRL vs Aged-ABX, $p < 0.001$) as was argininosuccinic acid, an amino acid generated during the urea cycle ($\beta = 1.023$, $p = 0.002$, BH = 0.019; Young-CTRL vs Aged-CTRL, $p < 0.001$; Aged-CTRL vs Aged-ABX, $p = 0.03$).

Gentisic acid is a metabolite that can have neuroprotective

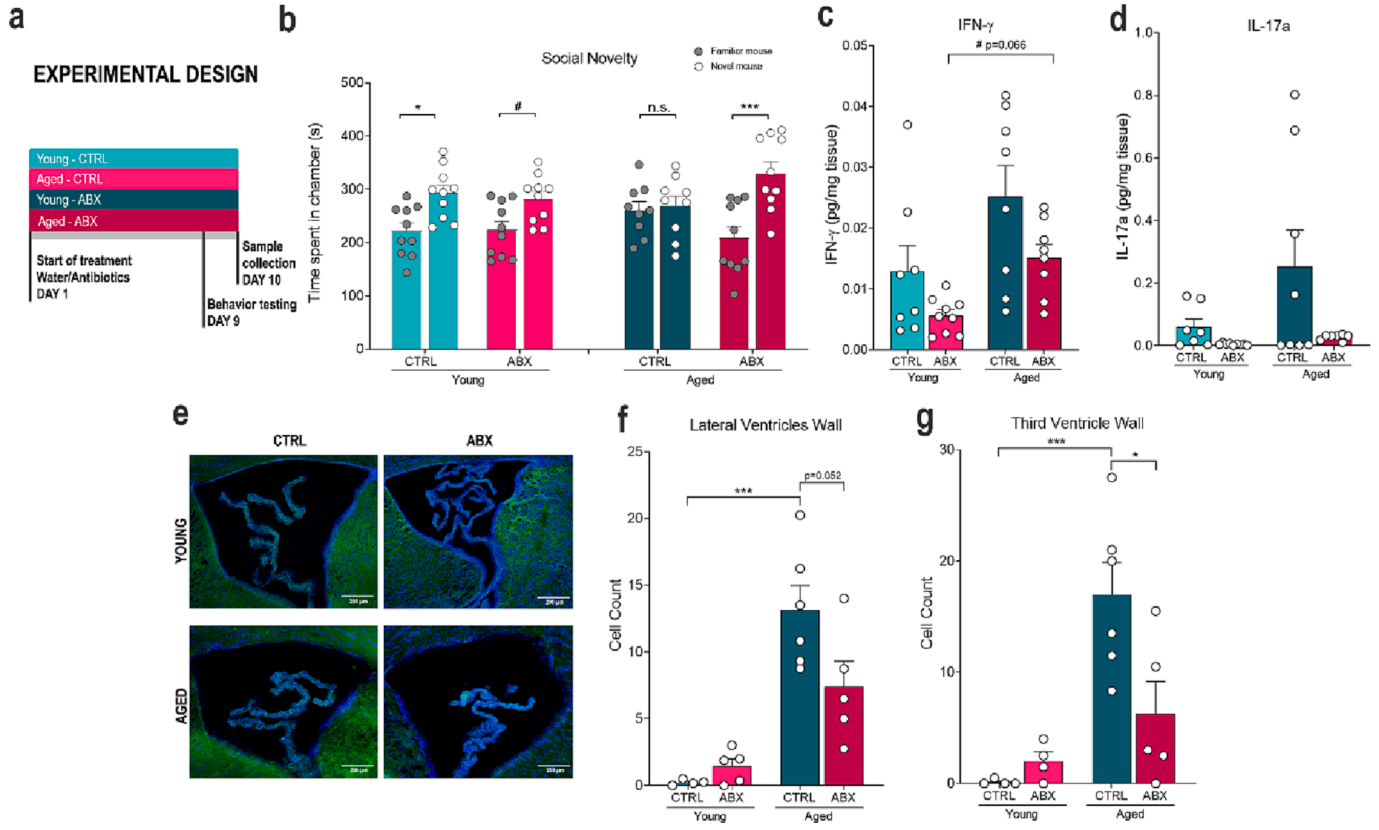


Fig. 1. Microbiota Depletion Restores the Age-Dependent Social Cognitive Defects and Modulates *T*-Helper cell numbers in the Choroid Plexus. a) Experimental Design. b) Social novelty assessed by 3-Chambers (n = 9–10 per group). Ileal levels of c) IFN-γ and d) IL-17a. e) Representative images of the lateral ventricles (10x, scale bar – 200 μM). CD4 + T cell numbers in the wall of the f) lateral ventricles and g) third ventricle (n = 4–6 per group). Results presented as mean + standard error of the mean (SEM). *p < 0.05, **p < 0.01, ***p < 0.001.

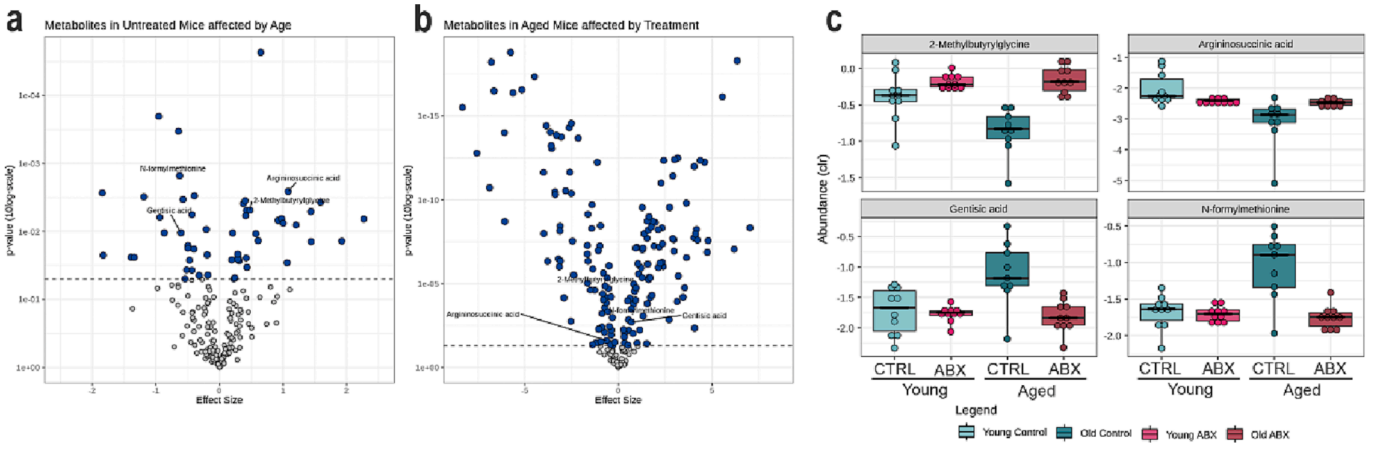


Fig. 2. Age-Dependent Shifts in Caecal Metabolites can be Successfully Restored By Antibiotic Treatment. Volcano plots showing cecal metabolites affected by age a) and by treatment, within aged animals b), respectively. The x-axis represents the estimated difference in means by groups (β), whereas the y-axis depicts p-values. Dark blue points represent metabolites that reach a p-value of < 0.05 and a Benjamini-Hochberg adjusted q-value of < 0.2. dashed horizontal lines represent the $p = 0.05$ mark. c) Boxplots showing the centered log-ratio transformed (clr) abundance of cecal metabolites altered by age, which are restored by antibiotic treatment. For the boxplots, boxes represent the limits of the interquartile range, the horizontal line represents the median point, and the whiskers represent the full data range.

properties (Abedi et al., 2020), but can also be a nephrotoxic metabolite (McMahon et al., 1991). In this study, gentisic acid levels were increased in the cecum of aged animals (β = -0.647, p = 0.007, BH = 0.042; Young-CTRL vs Aged-CTRL, p = 0.003), being restored to control levels by the antibiotic (Aged-CTRL vs Aged-ABX, p = 0.001). *N*-Formylmethionine (fMet) is a metabolite linked to mortality, potentially by promoting metabolic shift in critical illness (Cai et al., 2021, Sigurdsson et al., 2022). Curiously, in this study fMet levels in the cecum were significantly increased in aged mice (β = -0.674, p = 0.0004, BH = 0.007; Young-CTRL vs Aged-CTRL, $p < 0.001$) and completely reduced to young animal concentrations in aged mice receiving antibiotics (Aged-CTRL vs Aged-ABX, $p < 0.001$). Taken together, these results suggest that microbiota depletion can reshape the metabolomic landscape, by reducing levels of metabolites previously implicated in age-dependent physiological alterations.

4. Discussion

As the investigation of the biological processes underlying aging continues to advance, the contributions of the immune system and the gut microbiota have gained attention. In this study, we demonstrate that gut microbiota depletion by a mild antibiotic cocktail not only modulates components of the gut metabolome and immune system features in aging, but also has a beneficial impact on social behavior in aged mice.

In agreement with previous observations from our group (Scott et al., 2017), we show that aged mice display social novelty recognition deficits. Intriguingly, now we show that antibiotic treatment in aged mice restored the preference for the novel over the familiar mouse. These data demonstrate that the gut microbiota is an important player in age-associated social impairments. Interestingly, studies have shown beneficial effects of antibiotics in improving cognitive outcomes, possibly by reducing neuroinflammation in mouse models of Alzheimer's Disease (Angelucci et al., 2019).

Considering the age-dependent synergistic links between the peripheral and neural immune systems (Boehme et al., 2020), we sought to explore the impact of antibiotics on the immune system by *a)* measuring levels of pro-inflammatory cytokines in the ileum and *b)* quantifying the numbers of T cells at a key neuroimmunological interface – the choroid plexus.

Aging showed a tendency for increasing IL-17a and IFN-γ in the ileum, which even though not statistically different seems to be decreased following microbiota depletion. Given that ileal pro-inflammatory cytokines are mostly impacted by microbiota depletion, it is possible that the commensal bacteria are required for driving pro-inflammatory cytokines.

Concerning the choroid plexus, with age, there was an accumulation of CD4 + T cells at the lateral and 3rd ventricles, which is in line with previous observations of a marked age-associated increase of these cells, particularly at the 3rd ventricle (Xu et al., 2010). Remarkably gut microbiota depletion improved this effect, by partially reversing CD4 + T cell counts in the ventricles. This suggests that abolishing gut microbial signals in aged animals could partially reduce the CD4 + T cell accumulation at an important neuroimmune interface in the brain.

The gut metabolome is inherently linked to the gut microbiome (Garza et al., 2020, Valles-Colomer et al., 2019), and indeed microbial-derived metabolites have been suggested to be involved in age-related cognitive decline (Connell et al., 2022), caecal metabolomic analysis was performed to examine the metabolic impact of antibiotics in aged animals. 2-Methylbutyrylglicine and Argininosuccinic acid were reduced in the caecum of aged mice, and restored by antibiotic treatment, while inversely, gentisic acid and *N*-formylmethionine were found increased in the aged caecum and recovered by gut microbiota depletion. 2-Methylbutyrylglicine, a metabolite that in high concentrations has been linked to mental retardation and neuronal damage (Kanavin et al., 2007, Knebel et al., 2012), and which can be modulated by administration of *Akkermansia muciniphila,* was in fact reduced in the cecum of aged animals, which was eliminated following microbiota depletion, suggesting a new pattern in an aging context. Argininosuccinic acid is part of the arginine pathway, which has been previously reported to be altered in the aged brain (Rushaidhi et al., 2012) and is also reduced in the aged cecum and recovered by antibiotic treatment. In contrast, gentisic acid and *N*-formylmethionine were increased in the aged cecum and reduced following microbiota depletion. Interestingly, gentisic acid has been suggested to inhibit angiogenesis – which in the context of tumors is beneficial as a therapeutic target (Fernández et al., 2010) – however, given that angiogenesis is impaired with age (Hodges et al., 2018, Reed & Edelberg, 2004), perhaps the increased levels of gentisic acid in the aged cecum may reflect a maladaptive age-response, which is modulated by gut microbiota depletion. Finally, *N*-formylmethionine, a metabolite that has been linked to age-related disease risk and all-cause mortality (Cai et al., 2021), was likewise elevated in the cecum of aged animals and restored with microbiota depletion to young-matched control levels. In light of the relevance of these metabolites in aging physiological processes, and that such levels can be improved by gut microbial depletion, it is feasible to delineate a crucial role of the gut microbiota in the mediation of age-dependent

physiological alterations.

These data are in agreement with a recent study showing that antibiotics can alter the effects of social stimulus on reward behaviour (García-Cabrerizo et al., 2023). Moreover, a growing literature is showing that specific strains of bacteria or microbiota-targeting prebiotics (Cruz-Pereira et al., 2022) can enhance social behavior in the mouse. Therefore, the identification of specific metabolites involved in age-dependent physiological processes, would help advance personalized treatment with probiotics, synbiotics or synthetically engineered microbes (Long-Smith et al., 2020, Skelly et al., 2019) which could potentially provide benefits in alleviating the impact of aging in the host.

In summary, this study demonstrates that the aged gut microbiota is associated with age-dependent immune changes, which is reflected in social behavioral outputs. This suggests that during aging, a targeted gut microbiota depletion can have favorable outcomes, as particular pernicious bacterial populations may expand with age, and their metabolites can therefore have pervasive effects on the host. Future studies aiming to identify other gut microbial communities, their metabolites and their involvement in age-dependent processes would provide invaluable information that could guide new more targeted, non-antibiotic-based microbiome therapeutics.

Author Contributions

JCP and JFC designed research; JCP, GM, SB and PF performed research; JCP and TFSB analysed data; JCP, GM, GC and JFC wrote the paper.

Declaration of Competing Interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.02.008.

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