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Naturally-diverse airborne environmental microbial exposures modulate the gut microbiome and may provide anxiolytic benefits in mice

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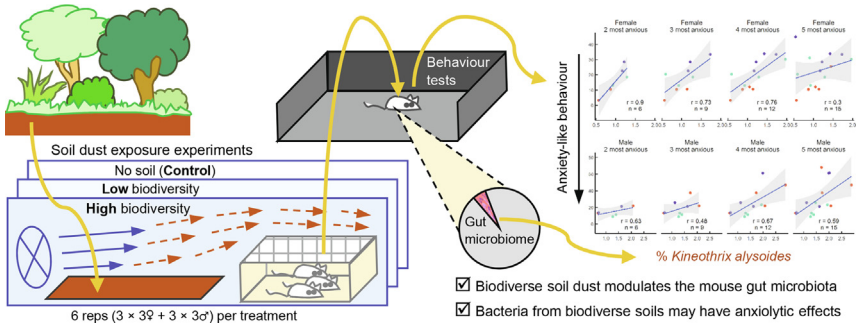
HIGHLIGHTS

- The effect of biodiverse aerobiomes on gut microbiota was previously untested.
- We demonstrate that exposure to biodiverse soil dust modulates mouse gut microbiota.
- Biodiverse soil exposure may help supplement butyrate-producing bacteria in the gut.
- These bacteria appear to moderate anxiety-like behaviour in the most anxious mice.
- We suggest a new hypothesis linking biodiverse soils, gut health and mental health.

GRAPHICAL ABSTRACT



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ABSTRACT

Growing epidemiological evidence links natural green space exposure with a range of health benefits, including for mental health. Conversely, greater urbanisation associates with increased risk of mental health disorders. Microbiomes are proposed as an important but understudied link that may help explain many green space-human health associations. However, there remains a lack of controlled experimental evidence testing possible beneficial effects from passive exposure to natural biodiversity via airborne microbiota. Previous mouse model studies have used unrealistic environmental microbial exposures—including excessive soil and organic matter contact, feed supplements and injections—to demonstrate host microbiota, immune biomarker, and behavioural changes. Here, in a randomised controlled experiment,

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we demonstrate that realistic exposures to trace-level dust from a high biodiversity soil can change mouse gut microbiota, in comparison to dust from low biodiversity soil or no soil (control) (n = 54 total mice, comprising 3 treatments × 18 mice, with 9 females + 9 males per group). Furthermore, we found a nominal soil-derived anaerobic spore-forming butyrate-producer, *Kineothrix alysoides*, was supplemented to a greater extent in the gut microbiomes of high biodiversity treatment mice. Also, increasing relative abundance of this rare organism correlated with reduced anxiety-like behaviour in the most anxious mice. Our results point to an intriguing new hypothesis: that biodiverse soils may represent an important supplementary source of butyrate-producing bacteria capable of resupplying the mammalian gut microbiome, with potential for gut health and mental health benefits. Our findings have potential to inform cost-effective population health interventions through microbiome-conscious green space design and, ultimately, the mainstreaming of biodiversity into health care.

1. Introduction

The influence of environmental microbial communities (microbiotas) and associated genetic material (microbiomes) on human health represents an important knowledge gap with potentially far-reaching implications for cost-effective public health interventions, and the management, design and use of our natural and built environments (Rook, 2013; von Hertzen et al., 2011). Environments are a key factor in shaping our human (e.g., skin, airway, gut) microbiota (Gilbert et al., 2018) and immune system (Carr et al., 2016), particularly from an early age (Wopereis et al., 2014). Gut microbiota are connected to mammalian health and disease at distant body locations (e.g., via immune signalling) (Molloy et al., 2012), and also influence brain development and behaviour (Foster and McVey Neufeld, 2013). Because ambient airborne environmental microbiota may interact with and supplement our gut microbiota (Flandroy et al., 2018; Rook, 2013), differing environments and their characteristic microbiota (e.g., Mhuireach et al., 2016) have potential to influence neurodevelopment and mental health via the bidirectional brain-gut-microbiome axis (Martin et al., 2018).

It is important to build knowledge of possible mechanisms that may underpin associations found between green space exposure and mental health (e.g., Beyer et al., 2014; Engemann et al., 2019; Newbury et al., 2016; Sarkar et al., 2018; Sundquist et al., 2018; Tillmann et al., 2018), given the rapid rates of global urbanisation (Rydin et al., 2012). Beyond mental health, the diversity of health benefits associated with nature contact suggests that a broad, nonspecific physiological pathway of action, a multiplicity of pathways, or a combination of these, may be present (Frumkin et al., 2017). Due to their ubiquitous and immunomodulatory nature, environmental microbiota are suggested to be part of the causal connection (Rook, 2013; von Hertzen et al., 2011), and offer promise for cost-effective solutions (Flies et al., 2017; Mills et al., 2017), to the rapidly increasing prevalence of many modern diseases. Indeed, health benefits from nature contact, biodiversity and microbial diversity exposures have been found in humans (Ege et al., 2011; Hanski et al., 2012; Stein et al., 2016) and animal models (Matthews and Jenks, 2013; Ottman et al., 2018; Schuijs et al., 2015). However, support for a tangible biological link between nature contact and mental health is limited (Gascon et al., 2015; Reber et al., 2016; Rook et al., 2013).

In particular, there is little controlled experimental evidence testing for changes in host gut microbiota and mental health outcomes from normal passive exposure to ambient natural biodiversity. Previous studies have used unrealistic environmental microbiota exposures—including excessive soil and organic matter contact (e.g., ~30–50 g.mouse^{-1}.week^{-1} (Ottman et al., 2018; Zhou et al., 2016); Supplementary Methods, Appendix A), forced feeding (Matthews and Jenks, 2013) and injections (Schuijs et al., 2015)—to demonstrate changes in host microbiota, immune biomarkers, and anxiety-related behaviour.

Here we sought evidence of potential mechanistic links between passive exposure to ambient natural biodiversity, environmental and gut microbiota, and the intriguing outcome of mental health. Specifically, we tested the hypothesis that varying airborne microbial exposures, spanning a gradient from low to high natural biodiversity, may have differential impact on the gut microbiome and anxiety-like behaviour in mice. To achieve this, we ran a randomised controlled study in mice subjected to trace-level soil dust exposures from high biodiversity soil, low biodiversity soil, or no soil (hereafter referred to as *high*, *low* and *control*). Mice were housed in open wire-top cages, with each cage residing in a single environmental enclosure. After a 7 week exposure, changes to gut microbiota were characterised by bacterial 16S rRNA gene sequences and aspects of anxiety-like behaviour were assessed using Open Field and Elevated Plus Maze apparatus (Carola et al., 2002).

Our first objective in this study was to test whether exposures to ambient aerobiology (airborne microbes) were capable of modulating the gut microbiome. A second objective was to explore potential impacts to anxiety-like behaviour and any links to changes in gut microbiota composition. In particular, we were interested in the possibility of uncovering candidate microbial 'Old Friends' (Rook, 2013)—i.e. species with immunoregulatory or other beneficial properties—that may be supplemented from natural environments. On the first objective, to our knowledge, we provide the first evidence that realistic exposures to trace-level biodiverse soil dust can modulate the gut microbiome; and we report on the nature of changes observed. On the second objective, we found preliminary evidence for sex differences in anxiety-like behaviour and gut microbiota-behavioural responses; and a reduction in base-level anxiety-like behaviour (i.e. anxiolytic effects) linked to a rare, nominally soil-derived organism that increased most in the guts of the high biodiversity treatment mice. In short, we not only show that aerobiology from biodiverse, natural environments can modulate the gut microbiome—our results also point to a new hypothesis, suggesting the existence of beneficial anxiolytic bacteria that may be both part of a normal healthy gut microbiota and supplemented from soils in biodiverse environments.

2. Methods

2.1. Study design

We subjected a total of 54 weaned, inbred 3–5 week old specific-pathogen-free BALB/C mice to a 7-week exposure of trace-level airborne dust from soils that reflected both a macro- and micro-biodiversity gradient. This exposure corresponded to a critical early period in the development of the murine microbiota and immune system (e.g. 4–8 weeks of age; Laukens et al., 2016). Same-sex groups of three mice were housed in open wire-top

cages, with each cage residing in a larger individually-isolated environmental treatment enclosure (Figs. S1 and S2, Appendix A). Each soil biodiversity treatment (i.e. *high*, *low*, *control*) was replicated in 6 enclosures. We did not know if sex effects might occur, so we included an equal number of female and male mice across all treatments. This meant each treatment was represented by 3 all-female and 3 all-male enclosures (i.e. n = 9 females + 9 males per treatment). *Low* soils came from a low plant macro-diversity setting (Figs. S3 and S4, Appendix A) and had low microbial diversity (Fig. 1). Likewise, *high* soils came from a high plant macro-diversity setting and had high microbial diversity. Source soils were homogenised and 1.75 kg was spread over a shallow tray in each soil treatment enclosure. We chose a no-soil *control* to mimic regularly sanitised built environments. Ten cm diameter USB fans adjacent to the trays were used on a 2-hr on/2-hr off cycle to generate the light dust exposures. To focus on the potential influence of the aerobiology, we controlled for known microbiota effects including genetics, birth mode, breeding facility, diet, age, light and sleep cycles, and non-treatment environmental parameters. Littermates were randomised across treatments to help normalise the starting microbiotas (Fig. S5, Appendix A).

2.2. Data collection

Soil, air (dust), faecal and caecal samples were taken to assess microbiota changes over the 7-week study (Fig. S6, Appendix A). Microbiota samples were assessed using bacterial 16S rRNA (V3-V4) marker gene survey data, clustered into operational taxonomic units (OTUs) with ≥97% sequence similarity. We targeted the bacterial 16S rRNA V3-V4 gene region using forward primer 341F (CCTAYGGGRBGCASCAG) and reverse primer 806R (GGACTACNNGGGTATCTAAT). Genomic DNA samples were PCR-amplified and sequenced by the Australian Genome Research Facility (AGRF) using Illumina paired end chemistry on the MiSeq platform with a V3, 600 cycle kit (2 × 300 base pairs paired-end reads). Anxiety-like behaviour was assessed by analysing time spent in the anxiety-provoking zones of the Open Field and Elevated Plus Maze apparatus. We considered the centre zone in the Open Field, and the centre and open arms in the Elevated Plus Maze, as the anxiety-provoking zones. Further details of the enclosure design, mice strain and husbandry, sampling procedures, behaviour testing and analysis, DNA extraction, PCR and sequencing are provided in the Supplementary Methods, Appendix A.

2.3. Bioinformatic and statistical analyses

The processing of 16S rRNA gene sequences, formation of OTUs, taxonomic assignments, data exclusions and decontamination are described in the Supplementary Methods, Appendix A. Microbiota compositions (beta diversity) were visualised using non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis distances based on rarefied OTU abundances. To test for compositional differences between microbiota sample groups we used permutational multivariate analysis of variance (PERMANOVA), followed by testing for homogeneity of group dispersions. We estimated OTU alpha diversity based on rarefied abundances and used the exponential transform of Shannon Index values to derive the effective number of OTUs (Jost, 2006). Merged-sample bootstrap resampling (Liddicoat et al., 2019) was also used to visualise alpha diversity across related samples. We used the Kruskal-Wallis rank sum test (no. groups ≥ 3) or the Wilcoxon rank sum test (no. groups = 2) for testing of statistical differences between groups for microbiome and behaviour data. All such tests excluded outliers, identified as any data points beyond 1.5 times the interquartile range above the upper quartile or below the lower quartile, as defined in the default R *boxplot()* function in base R (R Core Team, 2018). We assessed differentially abundant OTUs between week 0 and week 7 faecal samples within each treatment using the *DESeq2* algorithm (Love et al., 2014), which applies the Benjamini-Hochberg method to control false discoveries. OTUs that were identified as increasing within each treatment were assigned to a putative species based on the closest match in the NCBI 16S database. We used a heatmap to visualise the relative enrichment of increasing OTUs within treatments. Also, we calculated OTU relative abundances for taxa of interest to examine associations with anxiety-like behavioural outcomes; and compared microbiota compositions between the 1/3 most anxious and 1/3 least anxious mice, in each sex, based on Open Field centre times. Further details are provided in the Supplementary Methods, Appendix A.

3. Results

3.1. Biodiverse soil dust exposure modulates gut microbiota

The environmental treatments influenced the composition of mice gut microbiota (Fig. 2), despite very low soil dust exposures (~0.0034 g soil.mouse^{-1}.week^{-1}; Fig. S7, Appendix A); several thousand times less than previous studies. There was no difference in the accumulated weights of dust deposited in the *low* and *high* biodiversity treatment enclosures (Fig. S7, Appendix A). Treatment soil and air samples displayed distinct bacterial signatures (Fig. 1; Fig. S8, Appendix A). The treatments explained 5.5% and 4.2% of the variation in week 7 faecal and caecal microbiota respectively (Fig. 2). Faecal microbiota at week 0 were not associated with sex (PERMANOVA df = 1, F = 1.23, R^2 = 0.014, P = 0.26, n = 27 per group) in a model that included litter as the dominant factor (df = 14, F = 3.33, R^2 = 0.543, P = 0.001). However, sex did help explain 6.4% and 9% of the variation in week 7 faecal and caecal microbiota. As expected, litter and cage/enclosure had prominent effects on gut microbiota. Litter continued to influence the gut microbiota, explaining ~30% and 40% of the variation in week 7 faecal and caecal samples respectively. Cage/enclosure explained ~35% and 29% of the variation in week 7 faecal and caecal samples. Here, cage and enclosure represent the same level of co-housing.

We found that all *high* females showed an increase ($\Delta > 0$) in faecal alpha diversity within individual animals between week 0 to week 7, in contrast to *control* and *low* females (Fig. S9, Appendix A). Also, there was a rising pattern in week 7 faecal alpha diversity towards the *high* females that bordered on significance (Kruskal-Wallis χ^2 = 5.58, df = 2, P = 0.06, n = 8 to 9 per group; Fig. S10). No trend was found in caecal alpha diversity (Fig. S11, Appendix A). Setting aside the borderline indications in females, we conclude that exposure to the *high* biodiversity environmental microbiota did not translate to increased alpha diversity of gut microbiota in this study. There was a considerable drop in alpha diversity of the *low* and *high* environmental microbiota exposures towards the end of the experiment (Fig. 1D), likely due to the progressive windblown loss of the finest soil particles. Therefore, we speculate that the treatment influence on gut microbiota composition would have largely relied on exposures that occurred earlier, rather than later, in the experiment.

3.2. Putative butyrate-producing environmental bacteria were supplemented most in high biodiversity mice

We identified the differentially abundant taxa between week 0 and week 7 faecal microbiota separately within each treatment (Figs. S12 and S13, Tables S1–S3; Appendix A). Among many recognised intestinal bacteria, we found an environmentally-derived, putative butyrate-producer, OTU 37, in *high* biodiversity soils and in corresponding air, faecal and caecal samples, which showed

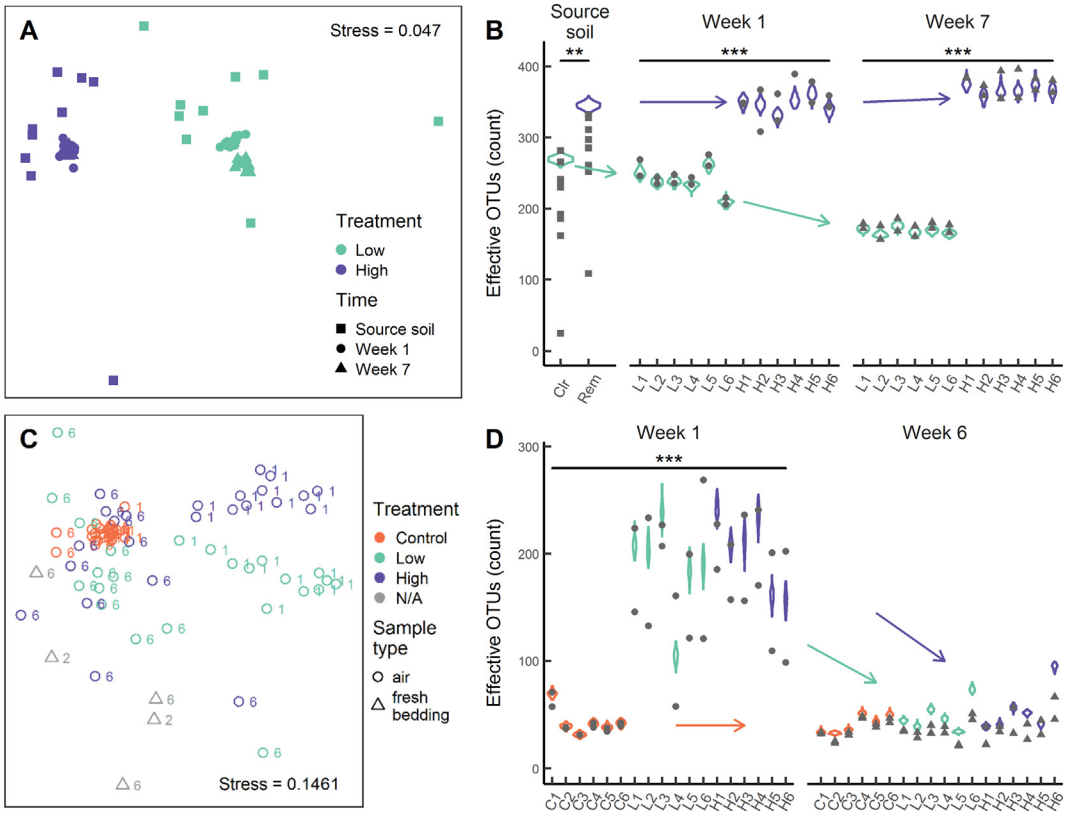

Fig. 1. Characteristics of the soil and air bacteria exposures. (A) NMDS visualisation of microbiota in treatment soils from week 1, week 7, and source soils (cleared vs. remnant). Rarefied OTU abundance data (sequence depth 12524) show different compositional centroids by treatment (PERMANOVA df = 1, F = 75.4, R^2 = 0.545, P = 0.001, n = 32 and 33 per group). Beta dispersions of treatment groups are comparable (df = 1, F = 0.072, P = 0.78). (B) Alpha diversity of soil bacteria, showing individual subsamples (points) and overall diversity density distributions. *High* soils have higher alpha diversity than *low* soils at source (Wilcoxon W = 6, P = 0.0047, n = 8 per group), week 1 (Wilcoxon W = 120, P < 0.001, n = 10 and 12 per group) and week 7 (Wilcoxon W = 144, P < 0.001, n = 12 per group). (C) NMDS visualisation of low biomass air (dust) and fresh bedding microbiota, labelled by week of collection. Rarefied OTU abundance data (sequence depth 839) showed different compositional centroids by treatment (PERMANOVA df = 2, F = 7.21, R^2 = 0.162, P = 0.001, n = 24 per group) and time (df = 1, F = 6.84, R^2 = 0.077, P = 0.001, n = 36 per group), although beta dispersions of treatment groups were different (df = 2, F = 47.5, P = 0.001). Final air samples came from week 6. Fresh bedding was excluded from testing of compositional differences (N/A = not applicable). (D) Alpha diversity of air microbiota samples, showing subsamples (points) and overall diversity density distributions. Week 1 *low* and *high* air samples are more diverse than *controls* (Kruskal-Wallis χ^2 = 20.6, df = 2, P < 0.001, n = 10 to 12 per group), however diversity is comparable by week 6 (Kruskal-Wallis χ^2 = 0.5, df = 2, P = 0.78, n = 11 to 12 per group). Alpha diversity density distributions are estimated from merged-sample bootstrap resampling (B = 100; Supplementary Methods, Appendix A).

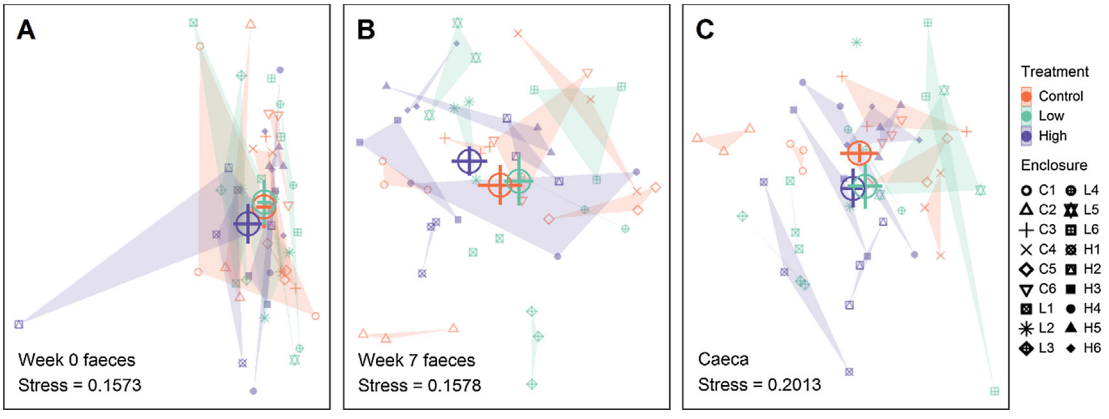

Fig. 2. Changes in mouse gut microbiota. Plots show individual mouse gut microbiota and linked cagemates, superimposed with treatment-based centroids (large circles) and two-dimensional NMDS-dissimilarity standard errors. (A) The treatment centroids were not significantly different in week 0 faecal samples (PERMANOVA[†] df = 2, F = 1.50, R^2 = 0.034, P = 0.072, n = 18 per group; [†]model included litter as dominant factor), but showed significant separation in (B) week 7 faecal and (C) caecal samples. Mice faecal bacteria in week 0 associated with litters (Fig. S5, Appendix A). (B) Week 7 faecal bacteria associated with treatment (PERMANOVA df = 2, F = 2.50, R^2 = 0.055, P = 0.020, n = 17 to 18 per group), sex (df = 1, F = 5.83, R^2 = 0.064, P = 0.002, n = 26 to 27 per group), litter (df = 14, F = 1.95, R^2 = 0.30, P = 0.001, n = median 4, interquartile range 2 to 4.5 per group), and enclosure/cage (df = 14, F = 2.31, R^2 = 0.353, P = 0.001, n = 2 to 3 per group). Beta distributions were similar across treatments (df = 2, F = 0.42, P = 0.66). (C) Similarly, caecal bacteria associated with treatment (PERMANOVA df = 2, F = 2.47, R^2 = 0.042, P = 0.007, n = 17 to 18 per group), sex (df = 1, F = 10.5, R^2 = 0.090, P = 0.001, n = 26 to 27 per group), litter (df = 14, F = 3.32, R^2 = 0.395, P = 0.001, n = median 4, interquartile range 2 to 4.5 per group), and enclosure/cage (df = 14, F = 2.47, R^2 = 0.294, P = 0.001, n = 2 to 3 per group); with similar beta distributions across treatments (df = 2, F = 1.97, P = 0.14). NMDS visualisations are based on rarefied OTU abundances with respective sequence depths: faecal week 0 = 11450, faecal week 7 = 14195, caecal = 14632.

increasing relative abundance and prevalence among *high* faecal samples between week 0 and week 7 (Table S3, Appendix A). This OTU shared 97% sequence similarity to the closest match in the NCBI 16S database, identified as the spore-forming, anaerobic, butyrate-producing environmental bacteria *Kineothrix alysoides* (Haas and Blanchard, 2017). Three additional taxa (OTU 60, OTU 5520, OTU 5790), with 95–96% identity to the closest NCBI match of *K. alysoides*, also exhibited similar increasing relative abundance and prevalence within *high* faecal samples. As discussed later, the beneficial properties of butyrate production, nominal environmental origins, and the potential viability of this organism in both natural soils and the gut warranted our attention.

Additional OTUs with the closest NCBI match to the soil-inhabiting environmental microbe *K. alysoides* were also present, and increased to a lesser degree in *control* and *low* faecal samples (Tables S1 and S2, Appendix A). That is, *K. alysoides*–like OTUs were found in week 0 faecal samples from a number of animals spanning all treatments. However, the greatest supplementation of OTUs corresponding to this putative species was found in the *high* treatment, where they were largely detected at higher mean % OTU relative abundance (compared to *low* or *control* samples) and more often than not in 100% of post-exposure animals (Fig. 3).

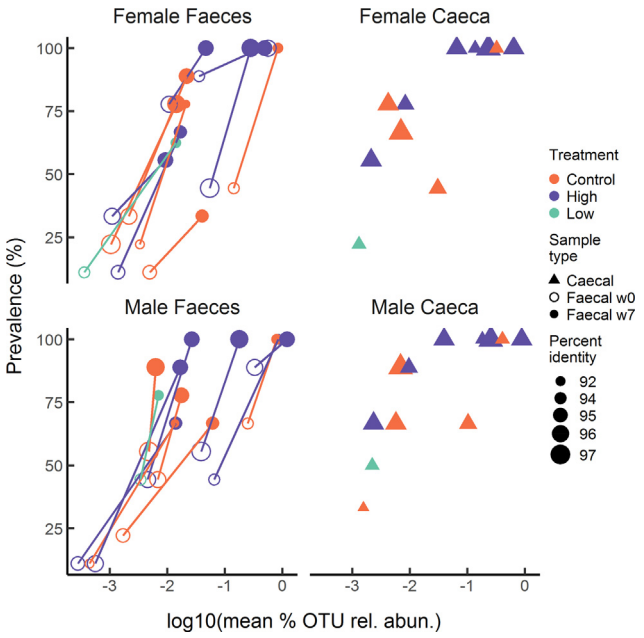

Fig. 3. High biodiversity treatments provided greater supplementation to the gut microbiota of putative butyrate-producing *K. alysoides*-like OTUs. The plot considers only *K. alysoides*-like OTUs that significantly increased within treatments during the experiment (i.e. identified from differential abundance testing between week 0 and week 7 faecal samples). Faecal week 7 and caecal samples from *high* biodiversity treatments generally dominate the top-right portion of each plot, indicating high mean % OTU relative abundance and high percent prevalence in post-exposure animals.

3.3. Putative butyrate-producing environmental bacteria may moderate the most anxious behaviour in both sexes

We examined associations between base-level anxiety-like behaviour (based on Open Field centre times) in both sexes and the total % OTU relative abundance (in week 7 faecal and caecal samples) of all *K. alysoides*-like OTUs that increased in faecal samples during the experiment. From the female caecal samples we found strong positive correlations (e.g., r = 0.90, for n = 2 mice per treatment; r = 0.76 for n = 4 mice per treatment; Fig. 4A) between the total % OTU relative abundance of *K. alysoides*-like OTUs and Open Field centre times for the most anxious female mice across the treatments. Also, a moderate correlation (r = 0.58, n = 9; Fig. 4B) was observed for the '1/3 most anxious' females, but not the '1/3 least anxious' females, as described in Fig. 5. Similarly, from the male caecal samples we found moderate positive correlations (e.g., r = 0.63, for n = 2 mice per treatment; r = 0.67 for n = 4 mice per treatment; Fig. 4C) between the total % OTU relative abundance of *K. alysoides*-like OTUs and Open Field centre times for the most anxious male mice across the treatments. No such relationships were observed for this taxon of interest in the week 7 faecal samples in either females or males.

3.4. High biodiversity treatments appeared to reduce anxiety-like behaviour in females only

We observed reduced anxiety-like behaviour in females only with the higher biodiversity treatments, as assessed by time spent in the centre of the Open Field (Fig. 5). In a different assessment of anxiety-related behaviour in the Elevated Plus Maze, we found no overall trend by sex or treatment (Fig. S14, Appendix A). Pre-treatment baseline (week 0) behavioural tests indicated no differences between treatment groups or sexes in either the Open Field or Elevated Plus Maze (Figs. S15 and S16, Appendix A). Total distances travelled and counts of entries into anxiety-provoking zones showed no trend by sex or treatment (Figs. S17 and S18, Appendix A), indicating that the Open Field reduced anxiety-like behavioural response in females to our *high* biodiversity treatment was not due to underlying differences in activity levels. These behavioural results are considered preliminary due to low samples sizes as discussed in the Limitations. We considered the noise from the small USB fans in each enclosure to be negligible, and fans were consistently placed and operated across all treatments and sexes. The fans did not impact on the behaviour test area.

3.5. High and low anxiety-like behaviour groups associated with gut microbiota in females only

We found a connection in females between high and low anxiety-like behaviour groups and week 7 faecal microbiota at the microbial community level. In each sex, we compared the top third of Open Field centre times ('1/3 least anxious') to the bottom third ('1/3 most anxious'). In females, but not males, the microbiota of these groups were different (Fig. 6). No such differentiation in microbial community compositions by the anxiety groups was observed in the caecal samples.

4. Discussion

4.1. Biodiverse aerobiology modulates the gut microbiome

We have demonstrated that realistic exposure to aerobiology associated with natural, high microbial diversity soils exerts an influence on the composition of the gut microbiota, compared to low biodiversity soil or no soil. The evidence that contact with the natural environment is important for human health has increased greatly in the last few years (Frumkin et al., 2017), and the suspicion that the microbiota of the natural environment is an important part of that contact has been growing (Flandroy et al., 2018; Rook, 2013; von Hertzen et al., 2011), but this link has not previously been subjected to carefully controlled experiments using realistic exposures. Given the importance of the gut microbiota in underpinning health (Gilbert et al., 2018; Molloy et al., 2012), this result lends support to notion that microbiome-conscious landscapes and urban green spaces in cities might be developed to provide supplementary, cost-effective, microbiome-

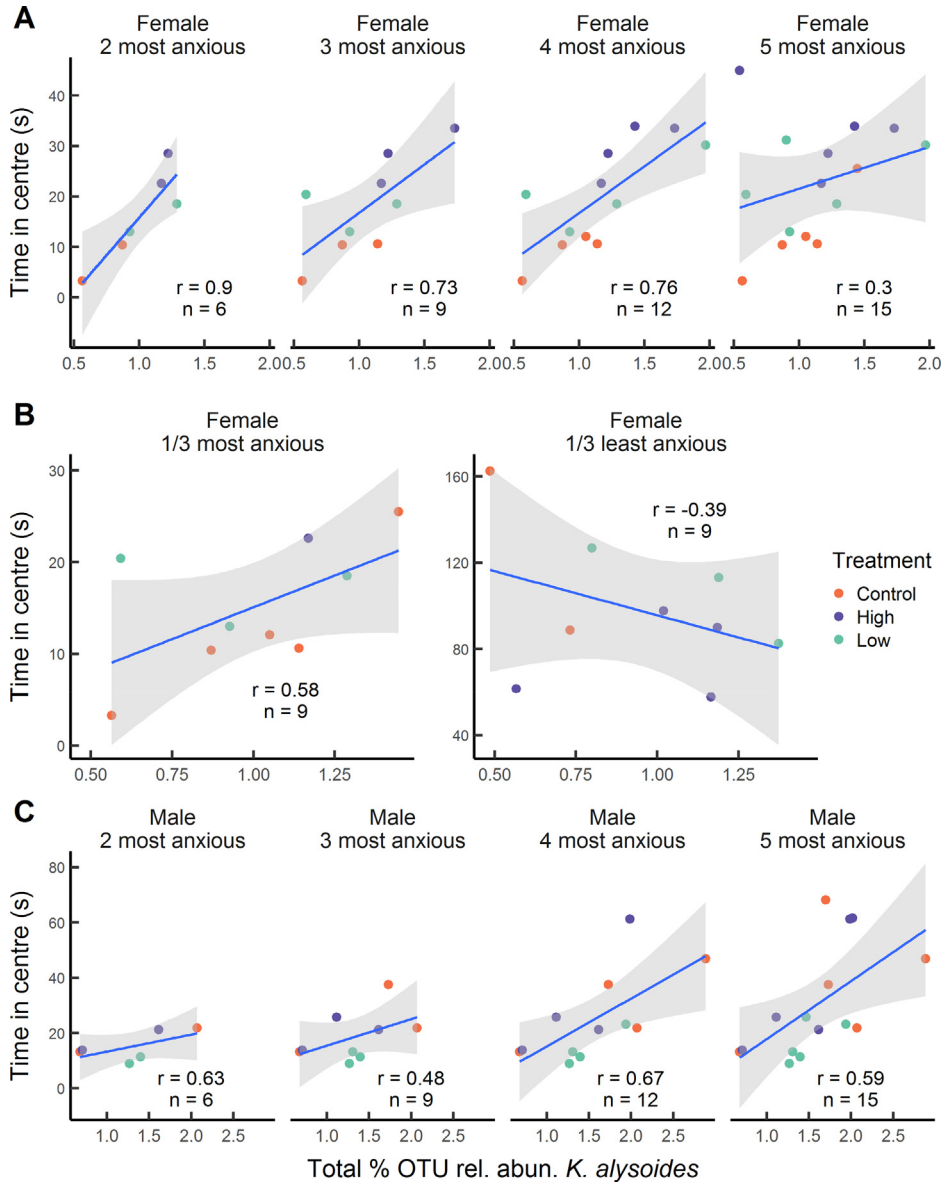

Fig. 4. Putative butyrate-producing *K. alysoides* appears to moderate the most anxious behaviour. Plots show total % OTU relative abundance within caecal samples of all *K. alysoides*-like OTUs that increase during the experiment (x-axes) versus Open Field centre times (y-axes), considering: (A) the 'x' most anxious female mice from each treatment, (B) the third most anxious and third least anxious groups based on Open Field centre times (as per Fig. 6), and (C) the 'x' most anxious male mice from each treatment. Pearson correlation coefficients (r) are noted. Shading indicates the 95% confidence intervals for each linear regression.

mediated population health benefits (Flies et al., 2017; Mills et al., 2017).

4.2. A new hypothesis: can biodiverse soils boost mammalian health via butyrate-producing bacteria?

Our results also point to an intriguing hypothesis—and provide preliminary evidence—for a plausible biological link between exposure to trace-levels of natural biodiversity, gut health and mental health, via soil-derived butyrate-producing bacteria. Butyrate is a short-chain fatty acid that is essential to gut health (Nicholson et al., 2012) and linked to immunological homeostasis (Furusawa et al., 2013), protection from metabolic diseases (Sanna et al., 2019), and improved quality of life and reduced depression (Valles-Colomer et al., 2019). Butyrate in the gut environment has been shown to suppress damage and enhance repair of key central nervous system tissues in a mouse model for multiple sclerosis (Chen et al., 2019). Increased diversity of butyrate-producing bacteria may also support functional stability through life disturbances, such as periods of antibiotic treatment and disease (Vital et al., 2017).

We propose that butyrate-producing bacteria such as *K. alysoides* may be favoured in biodiverse environments; in particular within biodiverse soils. Butyrate is a key intermediate in the breakdown of organic matter in anaerobic soils (Liu et al., 2011). However, anaerobic conditions can impact well-drained soils to some extent, particularly under conditions of high moisture and organic matter (i.e. oxygen-consuming) content (Tiedje et al., 1984). High aboveground vegetation diversity is associated with increased soil bacterial diversity (Coleman et al., 2004; Delgado-Baquerizo et al., 2018) and increased soil organic matter (Chen et al., 2018). There are multiple pathways and contributing taxa involved in butyrate production (Vital et al., 2017), so an array of supporting microbes (i.e. microbial diversity) is likely to be important. In short, we

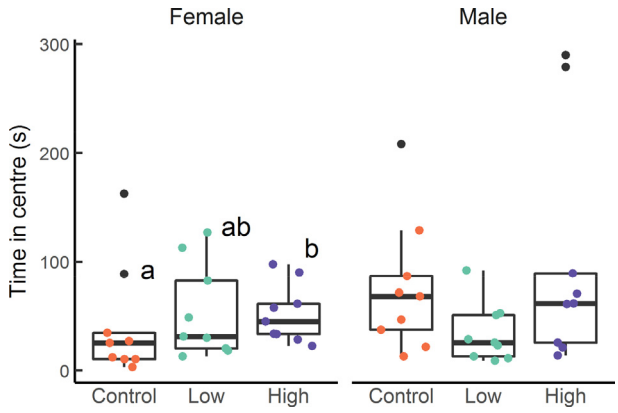

Fig. 5. Post-treatment Open Field behaviour test results. Boxplots (with outliers in black) indicate that treatments involving higher soil biodiversity exposure associated with reduced anxiety-like behaviour in females only, in the form of increased time spent in the centre of the Open Field apparatus (Kruskal-Wallis $\chi^2 = 8.08$, df = 2, $P = 0.018$, n = 7 to 9 per group). Groups not sharing a letter were significantly different. No trend was observed in males.

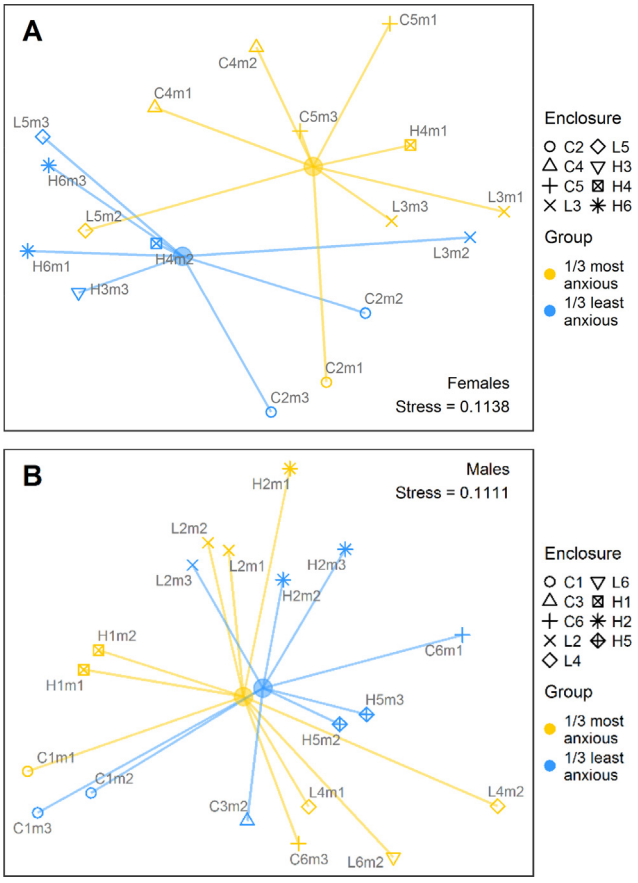

Fig. 6. Faecal microbiota and anxiety-like behaviour. NMDS visualisation of week 7 faecal microbiota for both sexes, showing individuals in the top third (*1/3 least anxious*) and lower third (*1/3 most anxious*) of results for time spent in the centre of the Open Field. (A) Rarefied OTU abundance data for females (sequence depth 17606) showed different compositional centroids associated with anxiety group (PERMANOVA df = 1, F = 3.38, $R^2 = 0.137$, $P = 0.017$, n = 8 and 9 per group) and enclosure/cage (df = 7, F = 1.89, $R^2 = 0.538$, $P = 0.018$, n = 1 to 3 per group). Beta dispersions of the anxiety groups were comparable (df = 1, F = 0.516, $P = 0.49$). (B) Rarefied OTU abundance data for males (sequence depth 19385) were associated with enclosure/cage (PERMANOVA df = 8, F = 2.61, $R^2 = 0.686$, $P = 0.002$, n = 1 to 3 per group) but not anxiety group (df = 1, F = 0.542, $R^2 = 0.018$, $P = 0.77$, n = 9 and 10 per group).

hypothesise that many biodiverse soils may contain the necessary biological resources to support butyrate-producing bacteria. Although, they may only register as rare members of the soil microbiota (e.g. ~0.001% sequence relative abundance for the *K. alysoides* type strain in the initial soil sample reported by Haas and Blanchard (2017); and ~0.01% mean OTU relative abundance for putative *K. alysoides* OTU_37 in Table S3, Appendix A). It should be noted that rare taxa can play important ecological roles (Hol et al., 2010). As we discuss later, it is possible the apparent rare nature of this taxon may be exaggerated due to the formation of resistant spores.

Although derived from soil, Haas and Blanchard (2017) suggest *K. alysoides* may be a normal resident in the mammalian gut or rumen, given its optimal temperature range which matches the internal temperature of humans and large animals. Indeed, we detected putative *K. alysoides* in a number of mice at week 0 with increasing presence to varying degrees in faecal samples across all treatments (Tables S1–S3, Appendix A). These results support the notion that gut microbiota may have an affinity for these nominally soil-associated organisms. The environmental range for growth of *K. alysoides* is pH 5.5–8.0 (optimal pH 7) and temperature 15–40 °C (optimal 35–40 °C), with increasingly slower growth observed towards 15 °C (Haas and Blanchard, 2017). *K. alysoides* resides in the family *Lachnospiraceae*, which is one of the most abundant in the human gut microbiome, and contains many known plant degraders and most of the butyrate-producers in the gut (Haas and Blanchard, 2017). After the stomach (pH < 3), median pH values fluctuate along the human intestinal tract from the duodenum to the rectum, and in faeces, but fall within the range pH 5.5 to 8.0 (Fallingborg et al., 1989) suited to *K. alysoides*.

The viable pH and temperature range for *K. alysoides* may also correspond to many soils, particularly in temperate climates. Anaerobes generally require redox potential (Eh) values ranging from +100 to less than −250 mV (Ray, 2004). Soil redox potential can fluctuate between −300 and +900 mV, and vary over short distances, depending on water, nutrient and organic matter content, proximity to growing roots and soil microbiota (Hinsinger et al., 2009; Husson, 2013). For example, the centre of a soil aggregate of 6–7 mm in diameter can have a redox potential 100 to 200 mV lower than its surface (Husson, 2013). In short, with appropriate plant material to degrade, along with sufficient moisture and other necessary inputs, it is feasible that *K. alysoides* may be viable in fertile, organic-rich (i.e. typically biodiverse) soils—even if conditions for growth are sub-optimal and intermittent, e.g. due to fluctuating soil temperature and redox potential.

Spore-forming bacteria (such as *K. alysoides*) can persist and be transported in aerobic environments and then activate under anaerobic conditions. Spore-formers also dominate the human gut, comprising 50–60% of bacterial genera, and they show greater changes in terms of abundance and species over time (compared to non-spore-formers) (Browne et al., 2016), implying that spore-forming organisms can be lost and then regained. Also, their capacity to survive in aerobic external environments allows them to be shared widely (Kearney et al., 2018). We know that poor diets can induce individual and intergenerational loss of key gut microbiota (Sonnenburg et al., 2016), with flow-on impacts to health. Our results suggest there may be an opportunity to supply key bacteria, such as butyrate-producers, from exposure to biodiverse soils.

Soil-associated microbes represent a large component of the aerobiology derived from environments (Polymenakou, 2012). Therefore, it is plausible that normal passive exposures to biodiverse, natural environments may often involve contact with diverse airborne immunomodulatory soil microbiota including beneficial butyrate-producers. This hypothesis is consistent with the large number of human health benefits, particularly to mental health, associated with nature contact (e.g., Beyer et al., 2014;

Engemann et al., 2019; Newbury et al., 2016; Sarkar et al., 2018; Sundquist et al., 2018; Tillmann et al., 2018). We speculate that contact with potentially beneficial airborne soil microbiota might be enhanced through active interactions, such as environmental volunteering (e.g., planting, weeding) or working in biodiverse, organic-rich gardens or farms. With potential for inheritable microbiomes that influence offspring health (Myles et al., 2013), there may also be potential for such exposures to have intergenerational effects.

4.3. Are there sex differences in responses to environmental microbiota exposure and microbiota-behaviour relationships?

In females and males we found strong to moderate correlations suggesting that the putative butyrate-producer *K. alysoides* may help moderate base-level anxiety-like behaviours in both sexes. However, males and females appeared to differ in their overall behavioural response to the environmental microbiota treatments, and regarding associations of gut microbiota composition in animals expressing low and high-level anxiety-like behaviour.

The microbiota-behaviour relationships we observed suggest that contact with biodiverse environmental microbiota including butyrate-producers has potential for providing a protective role in mental health, particularly in the most anxious female mice. This is a particularly interesting idea, given the consistent large-scale epidemiological evidence for higher prevalence rates of anxiety disorders in women compared to men (McLean et al., 2011), and points to opportunities for nature-based public health interventions.

Some sex differences in behavioural responses to stress may be attributed to sex hormones, which play a role in regulating the hypothalamo-pituitary-adrenal response (Handa et al., 1994). Also, sex hormones can influence the microbiota, as shown in mouse models of immune-related disease (Yurkovetskiy et al., 2013). Microbiota can influence anxiety-like behaviour through a variety of mechanisms, for example, particular members of the gut microbiota can activate neural pathways and central nervous system signalling systems involved in stress responses (Foster and McVey Neufeld, 2013). Ultimately, the expression of social and affective (including anxiety-like) behaviours involves the interplay of many different physiological systems including the neuroendocrine and immune systems—and the gut microbiome is expected to play an important mediating role across these systems (Sylvia and Demas, 2018). Sex differences in mouse gut microbiota composition and anxiety-like behaviour have been previously observed in studies investigating responses to dietary change and stress (Bridgewater et al., 2017).

Also, there may be sex differences in the sensitivity to stressors, and robustness of host microbiota, with flow-on implications for behaviour (Sylvia and Demas, 2018). Sylvia et al. (2017) found a strong sex difference in the behavioural response, and microbiota resistance to change, following antibiotic treatment in Siberian hamsters. They found that two broad-spectrum antibiotic treatments (causing changes to gut microbiota) were required to cause marked decreases in aggressive behaviour in males; with aggression returning to normal levels following a recovery period. However, only a single antibiotic treatment was required for females to show decreased aggression, and, unlike males, female aggression did not return to normal during the recovery period.

Extending this information, we might speculate on possible explanations for the apparent sexually dimorphic behavioural results in our study. It might be possible that the gut microbiota, physiological systems and associated behaviours of the male mice were more resistant to the influence of the biodiversity treatments, in comparison to females. However, for males to benefit from the mechanism we have proposed, we might speculate that higher exposure rates or longer durations of exposure may be required to see treatment effects. Also, environmentally supplemented butyrate-producers might be passed on to both sexes through maternal and intergenerational transmission, particularly during the critical early microbiome colonization phases at birth and during early feeding and social contact. To speculate further, it may be possible that among the male mice at the start of our study there was a sufficient initial presence of butyrate-producers, including *K. alysoides*, that additional supplementation from the high biodiversity treatment did not overtly influence their microbiota composition or behaviour. However we did not have week 0 caecal samples to test this idea, and week 7 faecal samples were deemed unsuitable (see later discussion).

4.4. Adding to knowledge of gut microbiota responses

The biodiversity treatment explained a relatively low percentage of variation in the gut microbiota. However, we note that rare taxa can play important ecological roles (Hol et al., 2010), and our results will help to build knowledge towards the construction of environmental microbiota dose-health outcome response relationships. We observed considerable influences on gut microbiota associated with litter, sex, and cages/enclosures (which reflected cohousing). These variables, particularly litter and cage effects are known major influences on gut microbiota in mice studies (Laukens et al., 2016).

Our results add to known links between gut microbiota, brain function and behavioural outcomes (Foster and McVey Neufeld, 2013). For example, early life exposure to normal gut microbiota has been shown to affect brain development and behaviour (Heijtz et al., 2011). Particular gut microbiome profiles have been linked to autism spectrum disorder-like behaviours in mice (Sharon et al., 2019). Also, certain probiotic bacteria have been shown to reduce anxiety in mice (Bravo et al., 2011). Our study contrasts with previous work (e.g. Heijtz et al., 2011) where sterile germ-free mice displayed reduced anxiety compared to specific-pathogen-free mice with normal gut microbiota. Here, we started with specific-pathogen-free mice and found indications of reduced anxiety (particularly in females) following exposure to naturally-diverse environmental microbiota. We used a specific-pathogen-free mouse model to avoid the artificially stunted host microbiome of sterile (e.g., germ-free) animals, while providing greater control over known key microbiome-influencing factors than is possible in human studies.

4.5. Interpreting caecal vs. faecal results

In our results we found relationships involving the taxon of interest, *K. alysoides*, emerged from post-treatment caecal samples, however similar patterns did not occur in faecal samples. Conversely, we found relationships involving microbial community measures such as beta diversity (composition) and alpha diversity (effective no. of OTUs) from faecal samples, but not from caecal samples. We speculate that different constraints on microbiota (e.g. redox potential, pH, chemistry) within the caeca compared to faeces (Tanca et al., 2017) may help explain the differences we observed. We expect that caecal samples will be constrained to a smaller range of redox potential, suited to anaerobic bacteria such as *K. alysoides*. In contrast, faecal samples will be exposed to increased oxygen content and more variable redox potential from the exterior to the interior of each faecal bolus. These variable conditions are likely to trigger sporulation of anaerobic spore-formers, activation of aerobic spore-formers, and switching of facultative species to varying degrees within faeces. We speculate that these variable conditions in faeces may both: diminish any signals from key anaerobic species, and also encourage more diverse expression

of microbial community members with varying environmental niches. This may explain why we obtained signals relating to a potential key species from the caecal samples, yet signals relating to microbiota composition and diversity largely emerged from the faecal samples.

4.6. Limitations

Our study has important limitations. We suggest that butyrate production may underpin the associations we found between anxiety-like behaviour and microbiota changes, however our evidence is observational and correlational. More conclusive evidence is required to support our hypothesis. Recommended future work includes focussed experiments to specifically test whether exposure to *K. alysoides* is linked to increased butyrate levels and a reduction in anxiety-like behaviour in mice. Although, this requires consideration of additional supporting microbiota and inputs as required for butyrate production pathways (Vital et al., 2017). Experimental work may also need to examine the possible production of additional metabolites beyond butyrate, which may contribute to beneficial immunomodulatory, anti-inflammatory, and/or anxiolytic effects (e.g., Sokol et al., 2008). Targeted culture is required to demonstrate the colonization, metabolism, and proliferation of *K. alysoides* within the murine intestine. Also, due to our focus on the environmental treatment, we did not consider the influence and transmission of other butyrate-producing bacteria that are routinely associated with gut microbiota, and may contribute to behavioural outcomes. Furthermore, we only analysed caecal and faecal samples. We recommend that future work consider sampling gut microbiota from other regions of the intestinal tract where key species may interact with specific tissues and environmental niches, for example Peyer's patches (involved in immune signalling) found in the ileum region of the small intestine.

We were unable to use statistical methods to predetermine sample sizes due to a lack of pre-existing data regarding the effect size from airborne microbial exposures on gut microbiota. Although, for our sex-specific analyses we used similar sample sizes to other studies examining the influence of environmental microbial exposures on mouse gut microbiota. For example, both Zhou et al. (2016) and Ottman et al. (2018) use n = 8 per group in comparing microbiota effects between environmental treatment groups. However, our sample sizes may not provide sufficient statistical power for testing behavioural data (i.e. n = 7 to 9 per group; Fig. 5) and examining gut microbiome-behaviour associations in the female mice (i.e. n = 8 to 9 per group; Fig. 6). Elsewhere, Reber et al. (2016) use sample sizes of n = 7 to 16 per group in Elevated Plus Maze testing, and Matthews and Jenks (2013) use sample sizes of 8 and 10 per group in anxiety-related behaviour testing. Although it is possible our behavioural results may be underpowered, we believe the exploration of these data is justified. We contend that the strong to moderate correlations indicating *K. alysoides* may moderate anxiety-like behaviour in the most anxious mice (Fig. 4), and our findings that *K. alysoides* is supplemented to a greater degree in the guts of mice exposed to high biodiversity soil dust (Fig. 3) warrant attention. We recommend further work to provide conclusive support for our behavioural findings.

It was beyond the scope of our study to examine causal mechanisms that may underpin the sex differences discussed earlier. Also, our Elevated Plus Maze and Open Field results did not align, however these apparatus often display discordant results and may encompass multiple dimensions of anxiety-related behaviour (Carola et al., 2002). We only analysed bacterial microbiomes in this study, however, other agents (e.g., fungi, viruses, volatile organic compounds) may have a modulating influence on the gut microbiome and host health. We evaluated effects from a limited exposure period during the development of the murine microbiota and immune system (Laukens et al., 2016). Our findings suggest that further studies would be worthwhile, for example, to explore possible differences between short-term vs. long-term exposure effects. For faecal DNA extractions we aimed to use two typically-sized faecal boli at week 7 (averaging ~50 mg) as the basis for post-treatment microbiota comparisons. However the young mice at week 0 supplied much smaller quantities of faeces over a reasonable timeframe without causing undue stress. Consequently, based on a limited supply, we only used an average of ~30 mg for faecal DNA extractions from week 0. This difference may have introduced a confounding variable which may have affected the microbiome analyses associated with time. Lastly, spore-forming environmental taxa have historically been underrepresented in culture-independent studies due to greater resistance to universal DNA extraction techniques (Wunderlin et al., 2014), and the generally low OTU relative abundances for the putative *K. alysoides* found here may reflect this limitation.

5. Conclusions

We have shown that exposure of specific-pathogen-free mice to dust from a soil known to contain a high microbial biodiversity (compared to dust from a soil of low biodiversity, or no soil) leads to changes in their gut microbiota composition, including an increase in the putative soil-derived butyrate-producing bacterium, *K. alysoides*. Our results indicate that increased relative abundance of *K. alysoides* within the intestinal tract (as sampled in caeca, but not faeces) may moderate anxiety-like behaviour in the most anxious mice of both sexes. Our results also indicate the presence of sexual dimorphism in the sensitivity to environmental microbiota exposures and gut microbiota associations between low and high anxiety-like behaviour groups. *K. alysoides* appeared to be viable in the gut (as we detected it in week 0 faeces) and was supplemented in mouse gut microbiota to a greater extent from high biodiversity soil dust (compared to low biodiversity soil dust or no soil dust). *K. alysoides* is involved in the anaerobic breakdown of plant-derived carbohydrates and may be favoured in high biodiversity soils because microbial processes of organic matter breakdown and turnover are routine in many biodiverse, organic-rich soils. This butyrate-producing bacteria warrants attention because it may be viable in both soils and the mammalian gut, and butyrate is a critical metabolite that supports gut health and mental health. We provide evidence that this bacterium can be transmitted from environmental samples through airborne exposure pathways, and supplements the gut microbiota to a greater extent from biodiverse sources. Such environmental spore-forming, butyrate-producing bacteria provide a plausible explanation for the reduced anxiety-like behaviours indicated here, and may help explain beneficial biodiverse green space-human health and mental health associations seen elsewhere. Our key finding—that aerobiology from biodiverse, natural environments can modulate the gut microbiome—has potential to inform new cost-effective microbiome-conscious nature-based health interventions, with potential for an array of disease prevention outcomes given the fundamental role of gut microbiomes in underpinning health.

6. Ethics Statement

All animal procedures performed in this study were approved by the University of Adelaide Animal Ethics Committee (Approval no. S-2017-112).

Data availability

All data and code used in this study are available on *figshare* at http://doi.org/10.25909/5caaf18c3450d. All 16S rRNA gene sequences have been deposited in the European Nucleotide Archive (accession no. PRJEB31983).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134684.

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