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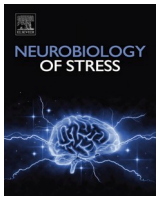

Sex specific gut-microbiota signatures of resilient and comorbid gut-brain phenotypes induced by early life stress

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ABSTRACT

Background: Alterations in gut-brain axis communication pathways and the gut microbiota ecosystem caused by early life stress have been extensively described as critical players in the pathophysiology of stress-induced disorders. However, the extent to which stress-induced gut microbiota alterations manifest in early life and contribute to the sex-specific susceptibility to distinct gut-brain phenotypes in adulthood has yet to be defined.
Methods: Male and female Sprague-Dawley rat offspring underwent maternal separation (3h/day from postnatal day 2–12). Faecal samples were collected before weaning for gut microbiota 16S rRNA sequencing and metabolomic analysis. Visceral pain sensitivity and negative valence behaviours were assessed in adulthood using colorectal distension and the forced swim test respectively. Behavioural data were processed in a two-step cluster analysis to identify groupings within the dataset. Multi-omics analysis was carried out to investigate if the microbial signatures following early life stress were already defined according to the membership of the adult behavioural phenotypes.
Results: Maternal separation resulted in increased visceral hypersensitivity while showing a trend for a sex-dependent increase in negative valence behaviour in adulthood. The cluster analysis revealed four clusters within the dataset representing distinct pathophysiological domains reminiscent of the behavioural consequences of early-life stress: 1. resilient, 2. pain, 3. immobile and 4. comorbid. The early life gut microbiota of each of these clusters show distinct alterations in terms of diversity, genus level differential abundance, and functional modules. Multi-omic integrations points towards a role for different metabolic pathways underlying each cluster-specific phenotype.
Conclusion: Our study is the first to identify distinct phenotypes defined by susceptibility or resilience to gut-brain dysfunction induced by early life stress. The gut microbiota in early life shows sex-dependent alterations in each cluster that precede specific behavioural phenotypes in adulthood. Future research is warranted to determine the causal relationship between early-life stress-induced changes in the gut microbiota and to understand the trajectory leading to the manifestation of different behavioural phenotypes in adulthood.

1. Introduction

Adverse early life events represent stress exposures linked to the manifestation of pathophysiological outcomes later in life (Bradford et al., 2012; Danese and McEwen, 2012; Daníelsdóttir et al., 2024). This early-life period in children is very plastic during which the experience of stress can have long-lasting physiological and psychological consequences. Stress during this period is an important risk factor for later diagnosis with psychiatric and gastrointestinal (GI) disorders including irritable bowel syndrome (IBS), a disorder of gut-brain interaction characterised by comorbid expression of both GI and psychological

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Abbreviations:

CRD	Colorectal distension
ELS	Early-life stress
FDR	False discovery rate
GBM	Gut-brain module
GI	Gastrointestinal
GMM	Gut-metabolic module
MS	Maternal separation
NS	Non-separated
SCFA	Short chain fatty acid

dysfunction (Lee et al., 2024).

Importantly, the female population displays a higher prevalence of stress-related GI and psychiatric disorders including IBS, depression, and anxiety (Bangasser and Valentino, 2014; Kim and Kim, 2018). While the basis of the sex differences in the more prevalent development of pathophysiological consequences is not clear, evidence points towards differential central regulation of the stress response, possibly via sex hormones (Bangasser et al., 2019; Bangasser and Valentino, 2014; Goldfarb et al., 2019). Similarly, it remains to be determined why some individuals are susceptible and others are resilient to these stress exposures. While significant advances have been made in understanding contributing factors to stress susceptibility, the mechanisms behind the individual and sex-specific differences in response to early-life stress (ELS) remains to be elucidated. The gut microbiota is known to be affected by ELS and has been proposed as one of the risk factors promoting resilience (Kalisch et al., 2024).

ELS can compromise the development of both the stress system and the assembly trajectory of the GI microbiota (Bolton et al., 2022; O'Mahony et al., 2009; Otaru et al., 2024). The maternal separation (MS) model is an animal model of brain-gut axis dysfunction that recapitulates many of the key features of IBS, including the comorbid behavioural phenotypes relevant to pain and psychiatric disorders (O'Mahony et al., 2011). The changes reported thus far are on multiple levels of the gut-brain axis, both molecularly and behaviourally in both sexes during adulthood, with male animals most frequently being studied (Aisa et al., 2007; Barreau, 2004; Donoso et al., 2020; Moloney et al., 2016; O'Mahony et al., 2009, 2009, 2009; Tao et al., 2023). Moreover the gut microbiota is critical for inducing key aspects of the behavioural phenotype (De Palma et al., 2015) and gut microbiota-targeted interventions alleviate some of the behavioural phenotypes (Collins et al., 2022; McVey Neufeld et al., 2019). However, the importance of the microbiota during developmental phases has been neglected as a mediator of the subsequent adult behavioural phenotypes, particularly at a functional microbial level (Barouei et al., 2012; Moya-Pérez et al., 2017; Zhou et al., 2016).

Recent studies highlight that not all rodents that undergo ELS develop modified behavioural phenotypes in adulthood, warranting application of the model to study the pathways in the microbiota-gut-brain axis leading to susceptibility and resilience to ELS (Creutzberg et al., 2023; Huang et al., 2023; Kang et al., 2023; Shi et al., 2021). The extent to which stress-induced microbiota-gut-brain axis alterations manifest in early life and contribute to the sex-specific susceptibility to stress has to be defined. Further the distinct development of gastrointestinal or emotional disturbances in adulthood in response to ELS remains to be elucidated. Therefore, our study aims to understand the role of the gut microbiota and associated microbial metabolic pathways in the development of distinct but converging gut-brain dysfunctions induced by ELS.

2. Methods

2.1. Animal and housing

All procedures were conducted with approval from the Animal Experimentation Ethics Committee (AEEC) at University College Cork and the Health Products Regulatory Authority (HPRA), under project authorisation number AE19130/P127, in accordance with the recommendations of the European Directive 2010/63/EU. Male and female Sprague Dawley rats (approximately 6 weeks of age) were purchased from Envigo, UK and were mated in the Biological Services Unit, Western Gateway Building, University College Cork. Two females were mated with one male per cage. The male was removed after one week, and the females were separated into individual cages 1–3 days prior to giving birth. The day of birth was designated as postnatal day 0 (PND0). Dams and littermates were housed in large plastic breeding cages (45 × 28 × 20 cm), after weaning animals were housed in RC2F type cages (56 × 38 × 22 cm) in a humidity 55 ± 10% and temperature-controlled room 21 °C ± 1 °C. The light/dark cycle was set to 12 h (light phase 7am-7pm).

2.2. Experimental design

After birth, rat pups were randomly assigned to MS or control (non-separated, NS) groups. Rat pups underwent the MS paradigm between PND 2–12. Before weaning (from PND22 to PND25), fresh faecal pellets were collected from each MS or NS rat, flash frozen and stored at −80 °C for 16S rRNA sequencing and faecal metabolome analysis. At weaning (PND25), littermates were randomly housed in groups of 3–4 rats/cage/sex. In adulthood NS (n = 30 rats [4 rats/litter]) and MS (n = 90 rats [4–5 rats/litter] offspring were assessed for visceral sensitivity and immobility behaviour as a measure of negative valence using colorectal distension (CRD; PND 59–61) and forced swim test (FST; PND 63–65), respectively. The behavioural data were incorporated into a two-step cluster analysis to identify natural groupings within the dataset. 16S rRNA sequencing and metabolomics were performed to identify if the microbial profile in early life can be used to identify adult behavioural phenotypic differences in response to stress. Experimental design and timeline are shown in Fig. 1A.

2.3. Maternal separation paradigm

ELS was induced by MS as described previously (Collins et al., 2022). Briefly, at PND0 litters were randomly assigned to MS and NS groups. At PND2, the litters assigned to MS were moved from the main colony room to an adjacent room maintained at the same temperature (21 ± 2 °C) and lighting conditions. The dam was first removed from the home cage and placed into a smaller holding cage, following which, the pups (entire litters) were gently transferred together into a small cage and kept there for 3 h. Cages containing the pups were placed on heating pads set to 30–33 °C and were filled with 3 cm of bedding for thermoregulation. The dam was returned to the home cage and transferred back to the main colony room without her pups for this period to avoid communication between the dam and her pups. After 3-h, dams were again brought into the adjacent room and pups were returned to their original home cages. NS litters were also transported to the same room as the MS groups to avoid the confound of transportation stress. NS groups were left undisturbed in their home cages with their dams except for weekly cage cleaning. This procedure was repeated daily from PND2 to PND12 inclusive. The period of separation was carried out at the same time each day (9am–12pm). At weaning (PND25), rat offspring were sexed, weaned and both male and female offspring were used for the remainder of the study.

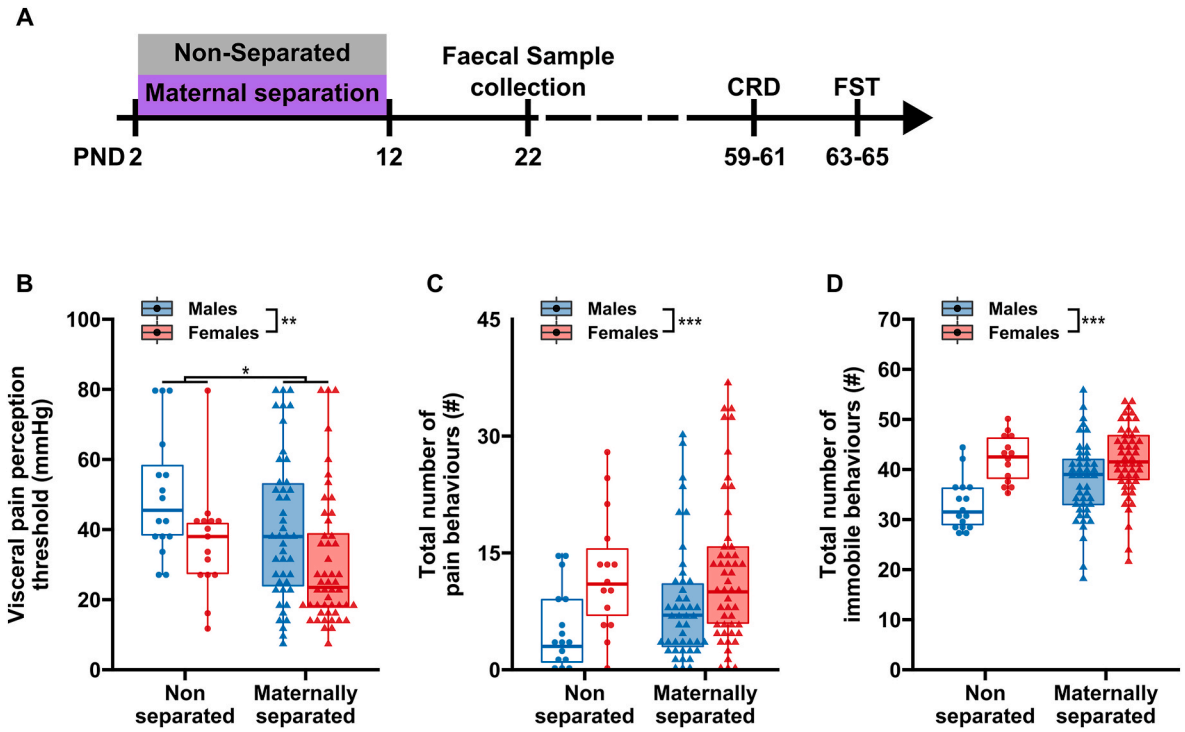

Fig. 1. Maternal separation modulates behaviour in adulthood. (A) Experimental design schematic (PND – Postnatal day). (B) Pressure eliciting the first pain response in adult rats during colorectal distension (CRD). (C) Total amount of pain behaviours during CRD. (D) Total amount of immobility behaviours during the forced swim test (FST) using time-sampling technique (5-sec intervals). n = 45–50 (Maternal separation, MS), n = 15–16 (non-separated, NS). Data expressed as Boxplots (min-max). Data analysed using Two-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001.

2.4. Oestrous cycle determination

Oestrous stage in females was evaluated as described previously (Goldman et al., 2007). Cotton swabs moistened with normal saline were inserted 1 cm into the vaginal orifice. Afterwards secretions from the swabs were spread on a glass slide and evaluated under a light microscope. Fresh smears were evaluated, and cycle stage was identified based on the presence of leukocytes, nucleated epithelial cells and cornified cells. To avoid the variance induced by the fluctuating hormones during the oestrus cycle on visceral pain and negative valence behavioural responses, female rat offsprings were subjected to CRD and FST during the metestrus/dioestrus phase of the oestrus cycle as previously conducted (Moloney et al., 2016).

2.5. Visceral sensitivity assessment through colorectal distension

CRD protocol was carried out as previously described (O'Mahony et al., 2009). Rat offspring were fasted for 16 h prior to the start of the procedure. Animals were lightly anaesthetised with isoflurane and a 6-cm long polyethylene balloon with a connecting catheter was inserted into the colon, 1 cm proximal to the anus. The catheter was secured to the tail of the animal with surgical tape to prevent displacement. Animals were allowed to recover from the anaesthesia for 10 min prior to the start of the procedure. The CRD paradigm used was an ascending phasic distension from 0 to 80 mmHg over an 8-min period. Air inflation and pressure were monitored during the procedure using a customised barostat (Distender Series II, G and J Electronics, Toronto, ON, Canada). Pain behaviours were identified as abdominal retraction, withdrawal, and stretching (O'Mahony et al., 2012). A trained observer, blinded to the experimental groups, scored each animal for the threshold pressure, when the first pain behaviour was observed, as well as the total number of pain behaviours displayed across all pressure ranges by each animal.

2.6. Forced swim test

FST was used to assess immobility behaviours associated with negative valence (Molendijk and de Kloet, 2022). The test was carried out in two sessions as described previously (Slattery and Cryan, 2012). Briefly, on the first day rats were carefully placed in a transparent glass cylinder (H: 45 cm; D: 20 cm) filled with 24 ± 0.2 °C water at a depth of 30 cm for a period of 15 min 24 h later, the rat was placed again in a plexiglass cylinder for a 5-min test swim. This test was recorded by a video camera placed above the cylinder. Following each swim, rats were thoroughly hand-dried with a towel and then moved to a recovery cage before being replaced in their home cage. The water was changed between each animal. Behavioural scoring was performed by trained personnel blinded to experimental condition. The 5-min sessions were scored using a time-sampling technique, whereby the predominant behaviour in each 5 s of the 300-s trial was recorded. Climbing behaviour was defined as the upward movements of the animal to escape the cylinder. Swimming behaviour is defined as the vertical movement in the cylinder. Immobility behaviour was noted as the absence of any movements with the exception of those that would ensure the rat was just maintaining its head above the water level.

2.7. Two-step cluster analysis of behavioural data

The clusters were identified using IBM SPSS Statistics 27. To detect subgroups within the dataset, a two-step cluster analysis was performed for both sexes separately. The key behavioural readouts were used as input (continuous) variables from each animal. To ensure that all variables were independent, only one metric was used per behavioural task. For the FST, the input was the number of immobile behaviours. For the CRD, the two readouts (threshold, number of pain behaviours) were summarised using the z-score method to account for both allodynia and hyperalgesia according to the following formula (Becker et al., 2023):

$$Z = \frac{x_i - \overline{x}}{\overline{\sigma}}$$

In which x_i represents the test score of each individual animal, while $\overline{x}$ and $\overline{\sigma}$ represent the mean and standard deviation of the control population respectively. The directionality of scores was adjusted by multiplying with −1 so that an increased z-score indicated increased pain sensitivity. Lastly, a single CRD z-score was calculated using following equation (Becker et al., 2023):

$$Z_{CRD} = \frac{Z_{Threshold} + Z_{Total\ pain}}{2}$$

For the identification of clusters, Log-likelihood was used as a distance measure for the pre-clustering step, while the Akaike information criterion (AIC) was used as a cluster criterion to estimate the most appropriate number of clusters. The main behavioural readouts from each rat were fed into the cluster analysis without predetermining the number of clusters.

2.8. 16S rRNA sequencing

DNA extraction and 16S rRNA sequencing were performed as described previously (Lynch et al., 2023). Briefly, DNA was extracted from stool samples using the QIAamp Power Fecal Pro extraction kit (Qiagen) according to manufacturer's instructions. DNA was quantified using the qubit broad range DNA quantification kit (Invitrogen) and normalised to 5ng/ul. DNA was used as to amplify the V3-V4 region of the 16S rRNA gene using the Illumina 16S metagenomics guide (Illumina) with some minor modifications (McCormack et al., 2017). Pooled libraries were sequenced on an Illumina MiSeq using 2 × 300 chemistry according to manufacturer's guidelines.

2.9. Metabolomics

Metabolomic analysis in stool samples was carried out by MS-Omics using a Thermo Scientific Vanquish LC coupled to Thermo Q Exactive HF Mass spectrometry. An electrospray ionisation interface was used as ionisation source. Analysis was performed in negative and positive ionisation mode. The UPLC was performed using a slightly modified version of the protocol described (Doneanu, 2011). Peak areas were extracted using Compound Discoverer 3.1 (Thermo Scientific). Identification of compounds were performed at four levels; Level 1: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and Mass spectrometry spectra, Level 2a: identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm). Level 2b: identification by accurate mass (with an accepted deviation of 3 ppm), and Mass spectrometry spectra, Level 3: identification by accurate mass alone (with an accepted deviation of 3 ppm). For the metabolomic analysis only metabolites found in annotation level 1 and 2a were considered.

2.9.1. Short chain fatty acid determination

Short-chain fatty acid (SCFA) analysis was carried out by MS-Omics as follows. Stool samples were acidified using hydrochloride acid, and deuterium-labelled internal standards were added. All samples were analysed in a randomised order. Analysis was performed using a high polarity column (Zebron™ ZB-FFAP, GC Cap. Column 30 m × 0.25 mm x 0.25 μm) installed in a GC (7890B, Agilent) coupled with a quadrupole 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. (2017).

2.10. Statistical analysis and bioinformatics

2.10.1. General statistical analysis

Statistical analysis of behavioural data was performed using RStudio. Results are presented as boxplots. Normality was assessed using the Shapiro-Wilk test. Sex*Stress interactions were assessed using two-way ANOVA followed by Holm-Sidak post-hoc test. In cases where data was not normally distributed, log-transformation was performed. Behavioural cluster effects were analysed using one-way ANOVA followed by Dunnet's test. Kruskal-Wallis H test with subsequent Dunnett's test was used for data where normality of data couldn't be assumed. Statistical significance was set at $p < 0.05$. No statistical outliers were removed from the data.

2.10.2. Microbiota taxonomic and functional analysis

Microbiota analysis was performed as described previously (Lynch et al., 2023) using R (version 4.2.1.). 300 base pair sequences were trimmed, checked for quality (phred >30), and chimeras using DADA2 pipeline using default parameters. Samples with less than 10000 reads after quality control were removed from analysis. Taxonomy was inferred with DADA2 against SILVA database version 138 and assigned on the genus level. Features that were detected in less than 5% of the samples or that were unknown on the genus level were removed from the analysis. Functional capacity was predicted using PICRUST2 (Douglas et al., 2020) in form of KEGG orthologs. Gut-brain modules (GBMs) and gut-metabolic-modules (GMMs) abundance were estimated using the Gomixer tool in R (Valles-Colomer et al., 2019).

2.10.3. Single-omics analysis

Statistical analysis was performed in R (R Core Team, n.d.) using the Rstudio GUI (version 2023.12.1.402). Features (metabolites, genera, GBMs, GMMs) were transformed using the centred log-ratio (clr) (Aitchison et al., 2000). Alpha diversity indexes in the form of Chao1, Shannon entropy and Simpson index were calculated using the iNEXT library (Hsieh et al., 2016). Beta diversity was computed in terms of Aitchison distance (i.e., Euclidian distance over the clr transformed values), while compositional differences were assessed using a PERMANOVA with 1000 permutations from the vegan library (Dixon, 2003). Differential abundance of taxa, GBMS and GMMs were assessed by fitting linear models for each value (Bastiaanssen et al., 2023b, 2023c). False discovery rate (FDR) was controlled for using the Benjamini-Hochberg procedure using FDR of $q < 0.2$ as cut-off (Butler et al., 2023; Ritz et al., 2024b).

2.10.4. Multi-omics analysis

The annotation-based analysis of specific interactions, ANANSI (Bastiaanssen et al., 2023a, 2023c), was used to assess associations between 16S-rRNA sequencing data and metabolomics. Faecal metabolites and predicted microbial genes were converted to KEGG orthologues. Pairs of KEGG orthologues that shared membership in KEGG pathways were fitted using linear models (Bastiaanssen et al., 2023a, 2023c). Faecal metabolites showing significant associations with predicted gene orthologues were used for quantitative enrichment analysis using the MetaboAnalyst (Chong et al., 2019) online pipeline using default parameters and the KEGG library as a reference.

3. Results

3.1. Early-life stress induces behavioural changes in adult rats

To assess the effect of MS on visceral sensitivity rats were exposed to CRD. Both male and female MS rats showed a lower threshold to the distension compared to the NS rats (Stress: $F_{(1,122)} = 5.9661$, $p < 0.05$, Fig. 1B). Overall, female animals showed a lower threshold and a higher total number of pain behaviours compared to males (Threshold: Sex: $F_{(1,122)} = 9.7505$, $p < 0.01$; Total pain behaviours: Sex: $F_{(1,122)} = 12.211$,

$p < 0.001$, Fig. 1B and C). Female rats displayed higher immobility time during the FST (Sex: $F_{(1,\ 121)} = 17.7241$, $p < 0.001$) and a statistical trend for an interaction between stress and sex was observed (Interaction: $F_{(1,121)} = 3.2156$, $p = 0.07544$, Fig. 1C). A lower amount of swimming behaviour was observed in male MS rats (Stress: $F_{(1,121)} = 11.1988$, $p < 0.01$, Supplementary Fig. 1A) and a lower number of swimming and climbing behaviours was observed in female rats compared to males (Sex: $F_{(1,121)} = 13.8134$, $p < 0.001$, Supplementary Fig. 1B).

3.2. Two-step cluster analysis reveals four distinct behavioural phenotypes

The algorithm identified four clusters in both sexes with a silhouette measure of cohesion and separation ≥0.5. For both sexes, the outcome of the FST had the higher predictor importance (1.0) while the outcome of the CRD was slightly lower (0.92 Female, 0.96 Male, Supplementary Fig. 2). In the male rats, the four clusters showed an even distribution in size (22%, 31%, 24%, and 22% of the total population; Fig. 2A and C), whereas the females were predominated by two larger clusters of 36% and 34% respectively (Fig. 2B and D). Animals of cluster 1, showing higher pain behaviour indicated by the CRD z-score and immobility time in the FST, were referred to as "comorbid". While animals only showing a higher immobility time (cluster 2) in the FST or higher z-score during CRD (cluster 3) were referred to as "immobile" or "pain" respectively. Animals showing similar or lower outcomes than the NS group in both variables were categorised as "resilient" (Cluster 4). Multiple cluster affiliation in both male and female litters was noted indicating no litter effects within the clustering approach (Fig. 2E and F).

Statistical comparison of the clusters revealed more negative valence behaviour in both the immobile cluster and the comorbid cluster of both sexes (Males: $\chi2_{(4)} = 37.78$, $p < 0.001$, NS vs Immobile: $p < 0.001$, NS vs Comorbid $p < 0.001$, Females: $\chi2_{(4)} = 41.008$, $p < 0.001$, NS vs Immobile: $p < 0.01$, NS vs Comorbid $p < 0.05$; Fig. 2G and H, respectively). Male and female rats belonging to the pain and comorbid clusters showed an increased CRD score compared to the NS control (Males: $\chi2_{(4)} = 40.208$, $p < 0.001$, NS vs Pain $p < 0.05$, NS vs Comorbid $p < 0.001$; Females: $\chi2_{(4)} = 35.302$, $p < 0.001$, NS vs Pain $p < 0.001$, NS vs Comorbid $p < 0.05$; Fig. 2I and J).

3.3. Maternal separation altered the composition and function of the gut microbiota in early-life

MS resulted in changes in gut microbiota configurations in both sexes collectively. An altered alpha diversity was observed, as measured with Simpson index (Stress: $F_{(1,120)} = 11.676$, $p < 0.001$) and Shannon entropy (Stress: $F_{(1,120)} = 5.737$, $p < 0.05$), in the microbiota of young male and female MS rats was higher compared to age-matched NS rats (Fig. 3A). However, MS did not affect the alpha diversity in terms of richness (Chao1 index, Stress: $F_{(1,120)} = 2.566$, $p = 0.112$; Fig. 3A). Overall, MS resulted in an altered microbiota composition as reflected by differences in beta diversity with respect to Aitchison distance (Stress: $F_{(1,120)} = 2.03$, $p < 0.01$, $R^2 = 0.017$, Fig. 3B). MS resulted in 7 and 6 genera with higher and lower abundance respectively (Table 1). Looking into metabolic pathways associated with the microbiota-gut-brain axis which are predicted based on genus-level abundance, 13 GBMs (4 increased and 9 decreased) and 8 GMMs (5 increased and 3 decreased) were significantly changed in both sexes collectively exposed to MS (Table 1).

3.4. Microbiota analysis of behavioural clusters reveal phenotype-dependent changes in both sexes

3.4.1. Male clusters

To investigate if compositional or predicted functional microbiota changes can be linked to specific behavioural phenotypes, analysis of the microbiota was carried out according to the different behavioural clusters identified in Fig. 2. The comorbid cluster showed reduced richness measured with the chao1 index (Chao1: $F_{(1,60)} = 5.402$, $p < 0.001$) compared to the NS control ($p < 0.01$), the resilient cluster ($p < 0.01$), and the pain cluster ($p < 0.01$) while the immobile cluster showed a reduced complexity indicated by the Simpson index (Simpson index: $F_{(1,60)} = 2.8$, $p < 0.05$) compared to the NS control (Fig. 4A). No differences in beta diversity were observed between clusters in male rats (Fig. 4B). Changes in abundance were found both within genus level, GBM and on the GMM levels and are presented in Table 2 and as heatmaps in Fig. 4C, D and E respectively.

3.4.2. Female clusters

In female rats the Chao1 index revealed an increase in the total amount of different genera in the comorbid cluster compared to the pain cluster (Chao1: $F_{(1,59)} = 2.633$, $p < 0.05$), however no changes in Shannon entropy or the Simpson index were identified (Fig. 5A) nor were changes in beta diversity were observed. Minor changes were seen at genus level, GBMs and GMMs compared to the male clusters and are highlighted in Table 3 and Fig. 5C and D and E respectively.

3.5. Metabolite analysis reveals distinct alterations of microbial-derived metabolic pathways across the treatment groups

To identify if the predicted functional changes across the clusters translate to metabolic changes, faecal SCFAs and semi-polar metabolites were analysed. No significant changes were observed in any of the SCFAs detected (Supplementary Tables 1–3). The beta diversity analysis (Aitchison distance) of untargeted metabolites revealed no effect of stress in metabolite concentrations (Supplementary Fig. 3). Next, we sought to determine which metabolites were associated with the changes in gut microbiota composition. All metabolites were converted to KEGG compounds and predicted genes to KEGG orthologues. We used a multi-omics approach based on known relationships in KEGG pathways between these two outputs. Data were fitted in linear models, revealing metabolites associated with the bacterial genomic content (Fig. 6A). A total of 24 metabolites were correlated with one or more predicted microbiota-related enzymes (Fig. 6B). A list of these metabolites was generated and used for quantitative enrichment analysis against the NS group using default parameters. Overall, MS results in an enrichment of several pathways including the pentose phosphate pathway ($p < 0.05$) and galactose metabolism ($p < 0.05$). While the pentose phosphate pathway ($p < 0.05$) is modulated by males, female rats drive enrichment of galactose metabolism ($p < 0.05$) (Fig. 6C).

Focusing on the clusters, distinct metabolic pathways were highlighted in both sexes. In males (Fig. 6D), resilience was linked to thiamine ($p < 0.01$) and pyruvate metabolism ($p < 0.05$). Rats belonging to the clusters associated with increased pain behaviour were associated with pentose phosphate pathway ($p < 0.01$) and glycerophospholipid metabolism ($p < 0.05$). Within the female clusters, only the immobile and comorbid groups highlight pathways (Fig. 6E). While both groups showed an association with pyrimidine ($p < 0.001$, $p < 0.05$, respectively) and nitrogen metabolism ($p < 0.001$, $p < 0.05$, respectively), only the comorbid cluster showed a link to pentose phosphate pathway ($p < 0.05$) and galactose metabolism ($p < 0.01$).

4. Discussion

In recent decades there has been a large body of research into the various long-term consequences of ELS and the implications for the development of psychiatric and GI disorders, in an attempt to better understand the pathophysiology of complex disorders of gut-brain interaction. Recently, the gut microbiota has been identified as a key player in potentially modulating some of the manifold effects of ELS through the gut-brain axis. In this study we characterised distinct behavioural subgroups of adult rats exposed to MS in early life. Specifically, we identified animals resilient and susceptible to ELS. Within the

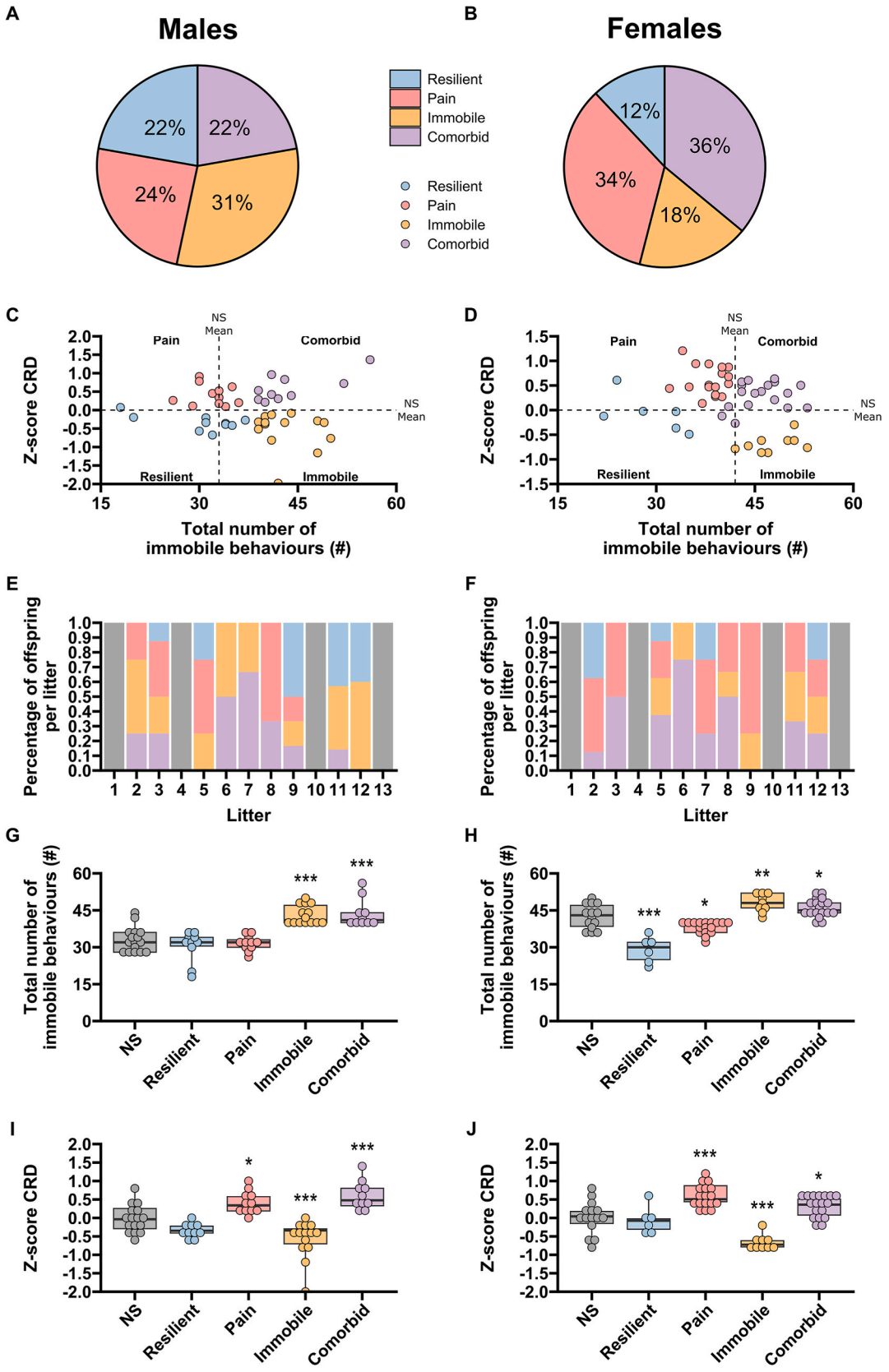

Fig. 2. Stratification of maternally separated animals into behavioural subgroups. (A, B) Pie charts illustrating the distribution of clusters across the offspring in percentage in both sexes. (C, D) Scatterplots illustrating the behavioural characteristics of each animal in both sexes. Y-axis showing the outcome of colorectal distension (CRD) and the x-axis showing the outcome of the forced swim test (FST). Dotted lines represent the mean outcome of the non-separated control group. (E, F) Distribution of all clusters across litters (N = 2–8, per litter/sex). Boxplots showing the outcome of the CRD (G, H) and the FST (I, J) for subgroups identified by the two-step cluster analysis. Males n = 10 (Resilient), 11 (Pain), 14 (Immobile), 10 (Comorbid). Females n = 6 (Resilient), 17 (Pain), 9 (Immobile), 18 (Comorbid). Data analysed using One-Way ANOVA followed by Dunnet's test, *p < 0.05, **p < 0.01, ***p < 0.001.

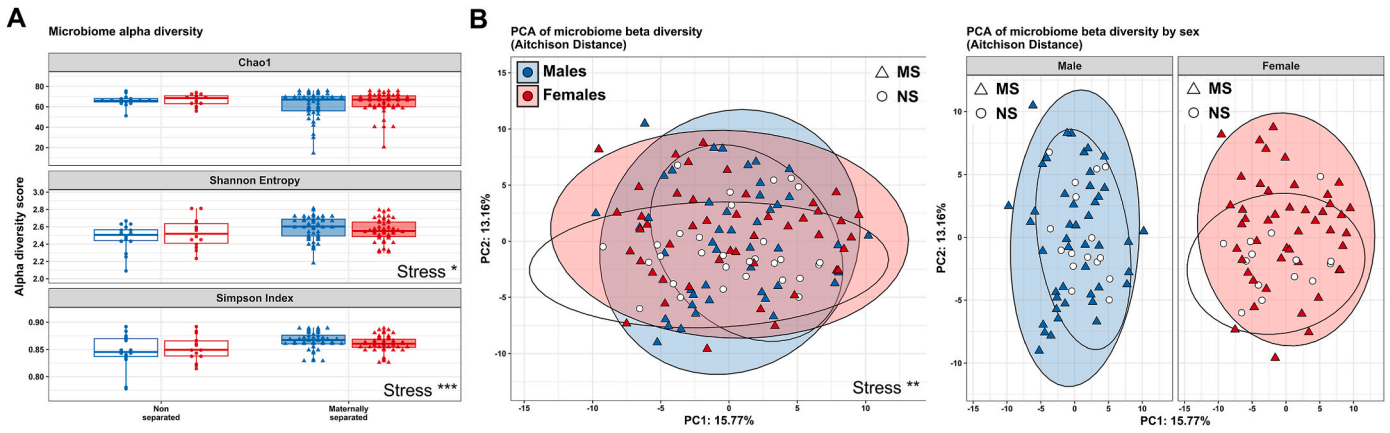

Fig. 3. Maternal separation results in changes in gut microbiota configurations in early life. (A) Alpha diversity indices of faecal microbiota in early life,n = 45–50 (Maternal separation, MS), n = 15–16 (non-separated, NS), analysed with Two-way ANOVA. (B) Principal component analysis (PCA) showing the first 2 principal components (PC1, PC2) in terms of Atchison distance performed to estimate the β-diversity between experimental groups. *p < 0.05, **p < 0.01, ***p < 0.001.

susceptible animals, we characterised animals by either increased visceral pain sensitivity, increased negative valence, or both phenotypes. The behavioural clusters are characterised by distinct and converging alterations in gut microbiota composition and function detectable early in life.

MS is a widely used model to study ELS-induced gut-brain axis dysfunction, yielding phenotypes relevant for both GI and psychiatric disorders (O'Mahony et al., 2011). Most studies focus on male animals and the composition of the adult microbiota and do not scrutinise a broader scope of microbial regulation of resilience and susceptibility at functional levels. Our study is the first exploring the link between MS-induced alterations in the gut microbiota at multi-omic levels in early life and the subsequent development of stress-resilient or susceptible gut-brain axis behavioural phenotypes in adulthood in both male and female animals.

While not directly comparable, in the human population, not every individual exposed to adverse events or environmental challenges during early life will develop negative health outcomes later in adulthood. Some individuals are disproportionally affected by negative environmental stimuli, increasing the risk of pathological disorders. This is partly explained in multiple models including the diathesis-stress and differential susceptibility frameworks. In short, certain "susceptibility factors" result in an increased plasticity in some individuals, which make them more susceptible to both negative and positive stimuli (Homberg and Jagiellowicz, 2022). The study of stress in animal models experienced a "paradigm shift" as stress is not considered as the cause of a predetermined outcome, but rather as a trigger for a spectrum of changes resulting in varied behavioural outcomes. This has been demonstrated in the model of social defeat (Gururajan et al., 2019), but also outside models of stress such as maternal immune activation (Mueller et al., 2021). Interestingly, while variations in stress exposure such as duration or intensity could explain differences in pathophysiological outcomes in humans, preclinical models are often treated as identical organisms and yet they result in differential outcomes. We propose the early-life microbiota as a one of the factors exhibiting variable stress signatures that can potentially explain the spectrum of individual behavioural differences in adulthood from resilience to susceptibility.

In this study, we aimed to identify subpopulations within our data that exhibit predominant stress-related changes either in the brain or the gastrointestinal system. Given the two-dimensional nature of these distinct organ systems affected by stress, we choose against applying arbitrary thresholds or calculating standard deviations from control groups. These methods, commonly employed in models like social defeat stress and social fear extinction to identify subpopulations were deemed unsuitable in our study (Golden et al., 2011; Laudani et al., 2023; Ritz et al., 2024a). Instead, we pursued an alternative approach to better capture the complexity of these physiological responses. To define subgroups in our dataset, we employed a two-step cluster analysis, an algorithm designed to reveal natural groups within a dataset. The benefit of this approach is the unbiased method in which the amount and characteristics of the clusters are not predetermined. Due to the sex differences in the behavioural parameters, we performed the cluster analysis separated by sex to avoid this as a confounding factor. The algorithm identified four clusters in both sexes. The identification of clusters prompted us to assess if changes in the gut microbiota early in life can precede the behavioural outcomes adulthood. Importantly, it has been shown that the microbiota is necessary to induce the negative behavioural consequences of ELS in mice (De Palma et al., 2015). In line with previous studies in our lab focused on adulthood, we show that MS resulted in substantial changes in the gut microbiota in both composition and functional potential (Donoso et al., 2020; O'Mahony et al., 2009). Additionally, MS led to a reduction in alpha diversity metrics, commonly associated with negative health outcomes (Ma et al., 2019).

We hypothesised that the presence or absence of distinct adult behavioural phenotypes would be already visible with specific microbiota configurations early in life. While we found no change in beta diversity in both males and females, alpha diversity revealed cluster specific changes in a sex-dependent manner. Male animals of the comorbid and immobile clusters show a reduced alpha diversity in the Chao1 and Simpson index respectively, while female animals of the comorbid cluster show the reverse pattern. Chao1 describes the total amount of different genera within a sample. This indicates that the animals with the most severe phenotype display the most pronounced differences early in life in the microbiota diversity in a sex-specific manner. While pathophysiological states are usually connected with a reduction in diversity (Spragge et al., 2023) including depression and IBS (Kelly et al., 2016; Yang et al., 2023), some studies, especially in early life, show an increased Chao1 index associated with negative outcomes (Carlson et al., 2018). However, global metrics like alpha diversity should not be taken as the principal measure of healthy or disease-associated microbiota at all stages across the lifespan (Clarke et al., 2014; Ghosh et al., 2022). Indeed, taxonomic differences and the associated functional potential are likely more critical for determining the biological importance of these observations for the subsequent behavioural phenotypes.

Unique differentially abundant bacteria were identified across all clusters, suggesting both a common footprint of stress in addition to a bespoke cluster-dependent impact with as yet unclear implications for behavioural phenotypes. However, several bacteria were found to be

Table 1
Differential abundance at the level of genus, gut-brain module and gut-metabolic modules between maternally separated rats and non-separated control rats.

Genus level differential abundance: Maternal separation vs non separated control				
Genus	non-separated - CLR genus abundance	MS - CLR genus abundance	p-value	q-value
Caldicoprobacteraceae Caldicoprobacter	−1.98 ± 0.16	−2.51 ± 0.09	<0.01	0.053
Eggerthellaceae Enterorhabdus	−0.34 ± 0.21	−0.91 ± 0.1	<0.01	0.09
Erysipelotrichaceae Turicibacter	0.83 ± 0.18	0.19 ± 0.12	<0.01	0.07
Lachnospiraceae A2	−0.03 ± 0.37	1.32 ± 0.15	<0.001	<0.01
Lachnospiraceae Eisenbergiella	−1.58 ± 0.26	−2.15 ± 0.12	<0.05	0.18
Lachnospiraceae AC2044 group	−2.76 ± 0.09	−2.14 ± 0.15	<0.05	0.16
Lachnospiraceae NK4B4 group	−2.76 ± 0.09	−1.49 ± 0.18	<0.001	<0.01
Lachnospiraceae UCG-008	−2.69 ± 0.12	−1.79 ± 0.16	<0.01	<0.05
Lachnospiraceae Marvinbryantia	0.25 ± 0.27	0.95 ± 0.16	<0.05	0.18
Monoglobaceae Monoglobus	−0.21 ± 0.16	−0.86 ± 0.1	<0.01	<0.05
Peptostreptococcaceae Romboutsia	5.10 ± 0.11	4.80 ± 0.6	<0.05	0.16
Prevotellaceae Paraprevotella	−2.28 ± 0.16	−1.77 ± 0.11	<0.05	0.18
Rikenellaceae Alistipes	2.71 ± 0.12	3.29 ± 0.07	<0.01	<0.01
GBM level differential abundance: Maternal separation vs non separated control				
Gut-brain module	non-separated - CLR GBM abundance	MS - CLR GBM abundance	p-value	q-value
Acetate synthesis I	2.60 ± 0.02	2.52 ± 0.02	<0.05	0.06
Acetate synthesis III	2.25 ± 0.03	2.13 ± 0.02	<0.01	<0.05
ClpB (ATP-dependent chaperone protein)	2.47 ± 0.02	2.41 ± 0.02	<0.05	0.13
Glutamate synthesis II	2.56 ± 0.03	2.48 ± 0.02	<0.05	0.07
Inositol degradation	−1.01 ± 0.06	−0.57 ± 0.04	<0.001	<0.001
Isovaleric acid synthesis II (KADC pathway)	2.22 ± 0.02	2.13 ± 0.02	<0.01	<0.05
Menaquinone synthesis (vitamin K2) II (alternative pathway: futalosine pathway)	−1.55 ± 0.11	−0.99 ± 0.06	<0.001	<0.01
Quinolinic acid synthesis	2.29 ± 0.02	2.21 ± 0.01	<0.05	0.058
S-Adenosylmethionine (SAM) synthesis	2.61 ± 0.02	2.53 ± 0.02	<0.05	0.07
Tryptophan degradation	−0.54 ± 0.05	−0.23 ± 0.03	<0.001	<0.01
Tryptophan synthesis	2.24 ± 0.03	2.16 ± 0.02	<0.05	0.09
g-Hydroxybutyric acid (GHB) degradation	−1.90 ± 0.16	−1.39 ± 0.07	<0.01	<0.05
p-Cresol synthesis	2.43 ± 0.03	2.32 ± 0.02	<0.01	<0.05
GMM level differential abundance: Maternal separation vs non separated control				
Gut-metabolic module	non-separated - CLR GMM abundance	MS - CLR GMM abundance	p-value	q-value
ethanol production I	0.76 ± 0.04	0.92 ± 0.02	<0.001	<0.05
formate conversion	−1.57 ± 0.09	−1.78 ± 0.03	<0.01	0.12
hydrogen metabolism	−1.24 ± 0.06	−1.51 ± 0.04	<0.001	<0.05
NADH:ferredoxin oxidoreductase	−5.91 ± 0.18	−5.27 ± 0.13	<0.05	0.14
succinate consumption	−2.28 ± 0.09	−1.98 ± 0.04	<0.01	<0.05
threonine degradation II	0.92 ± 0.02	−0.84 ± 0.01	<0.01	0.058
tryptophan degradation	−1.49 ± 0.05	−1.13 ± 0.03	<0.001	<0.01
tyrosine degradation II	−6.05 ± 0.24	−5.19 ± 0.15	<0.01	0.07

unchanged in the male resilient cluster compared to the others. For example, the genera *Enterorhabdus*, *Lachnoclostridium*, *Monoglobus* were not altered in the male resilient cluster compared to control group but were changed in at least one of the other clusters. Conversely, animals showing a comorbid phenotype were associated with the most distinct gut microbiota profile compared to the other clusters. The abundance of *Monoglobus* and *Enterohabdus* was decreased only in the male immobile cluster. Both genera have been previously associated with changes in emotional behaviours (Liang et al., 2022; Vicentini et al., 2022). On the other hand, changes in abundance of *Tuciribacter* in the male pain cluster can be linked to previous studies investigating MS induced visceral hypersensitivity (Enqi et al., 2021).

We were not able to pinpoint specific genus-level relative abundance alterations coinciding with distinct behavioural characteristics. Multiple bacteria are capable of performing the same function within a community. Therefore, understanding which biological functions are present or absent within the microbiota is essential to understand which metabolic pathways are driving a pathological phenotype. Therefore, we investigated the functional potential of bacterial pathways in our metagenomic dataset. In particular we inferred the abundance of gut-brain and gut-metabolic modules, functional modules that can influence the gut-brain axis and gut metabolic processes, respectively (Valles-Colomer et al., 2019; Vieira-Silva et al., 2016).

Similarly to the compositional alterations at genus level, the male resilient cluster showed a more limited set of changes in functional potential compared to the control group, while the comorbid cluster showed the most differentially abundant modules in both sexes. Notably, in male animals, both the pain and comorbid clusters showed increased isovaleric acid synthesis compared to the resilient cluster. This SCFA has been strongly associated with visceral pain in recent years as it stimulates enterochromaffin cells to release serotonin that binds 5HT-3 expressed in enteric nerve endings transmitting nociceptive signals (Bayrer et al., 2023; Bellono et al., 2017). The clusters characterised by higher negative valence behaviours had a decreased abundance of modules related to the production of acetate. Acetate is produced in the gut and has been linked to gut-brain signalling via various routes such as G-protein coupled receptors, histone deacetylase, neuroinflammation and mitochondrial function (Dalile et al., 2019; Lynch et al., 2021). Particularly the immune pathway modulated by acetate has been linked to the pathogenesis of psychiatric and other brain disorders (Erny et al., 2021; Miller and Raison, 2016). However, these results could not be confirmed in the SCFA analysis. In contrast to previous reports (Donoso et al., 2020), no changes in the production in any SCFAs, including acetate, were evident in this study. The samples used for this analysis may be a limitation that requires cautious interpretation of the results, as most studies perform SCFA acid analysis from caecal content as the main site of bacterial fermentation. Faecal SCFA measurements are more accessible but less informative about exclusive microbial production as these levels are also determined by absorption and use by colonocytes for energy production or release into the blood stream where they can reach other tissues (Dalile et al., 2019). Therefore, while faecal measurements of SFCAs are a proxy measurement that give some indications about the metabolic output of the microbiota, they are insufficient in

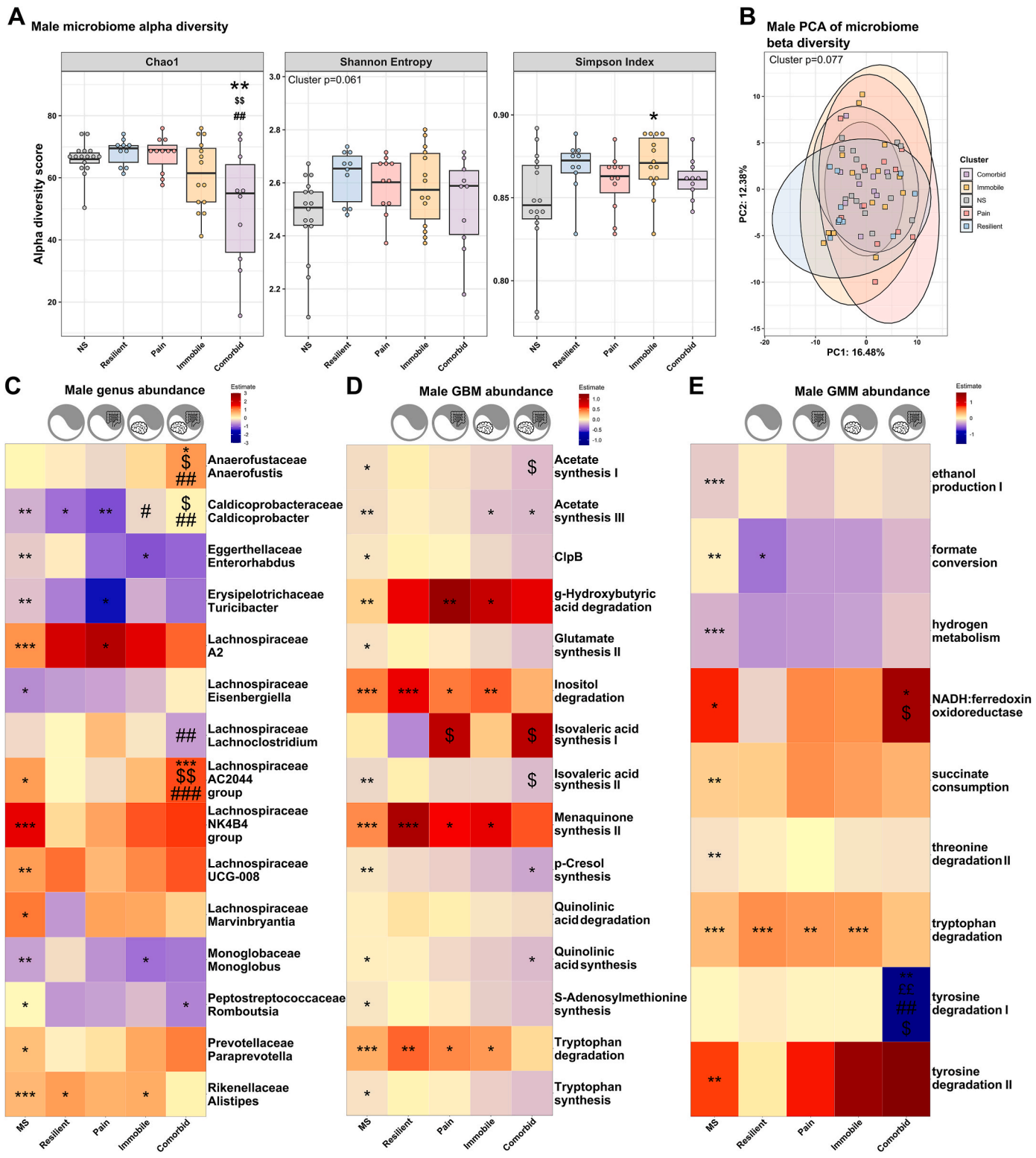

Fig. 4. Male clusters show individual differences in microbiota configurations. (A) Alpha diversity indices of faecal microbiota. (B) Principal component analysis (PCA) showing the first 2 principal components (PC1, PC2) in terms of Atchison distance performed to estimate the β-diversity between male clusters. (C–E) Heat maps, showing significant changes of all maternally separated (MS) animals and male cluster compared to the non-separated control: (C) differential abundant genera, (D) gut-brain modules (GBM) and (E) gut-metabolic modules (GMM). Colour indicates estimate with blue indicating higher abundance in control group and red indicating higher abundance in stressed groups. Resilient: n = 10, Pain: n = 11, Immobile: n = 14, Comorbid: n = 10. Data is shown if FDR<0.2 in maternally separated animals overall or in Clusters. *p < 0.05, **p < 0.01, ***p < 0.001 vs non-separated (NS) control. $p < 0.05 vs Resilient. #p < 0.05, ##p < 0.01 vs Pain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Differential abundance at the level of genus, gut-brain module and gut-metabolic modules between the male clusters.

Genus level differential abundance: Male clusters					
Genus	non-separated	Resilient	Pain	Immobile	Comorbid
Anaerofustaceae Anaerofustis	−2.61 ± 0.14	−2.72 ± 0.18	−2.86 ± 0.16	−2.30 ± 0.19	−1.76 ± 0.27[bc]
Caldicoprobacteraceae Caldicoprobacter	−1.86 ± 0.23	−2.78 ± 0.16[a]	−2.98 ± 0.14[a]	−2.11 ± 0.2[c]	−1.80 ± 0.29[bc]
Eggerthellaceae Enterorhabdus	−0.12 ± 0.28	−0.22 ± 0.21	−1.04 ± 0.28	−1.21 ± 0.26[a]	−1.09 ± 0.26
Erysipelotrichaceae Turicibacter	0.93 ± 0.23	0.12 ± 0.49	−0.54 ± 0.38[a]	0.43 ± 0.22	0.04 ± 0.31
Lachnospiraceae A2	−0.52 ± 0.58	1.30 ± 0.28	1.73 ± 0.53[a]	1.13 ± 0.48	0.65 ± 0.49
Lachnospiraceae Eisenbergiella	−1.64 ± 0.35	−2.27 ± 0.24	−2.21 ± 0.39	−2.04 ± 0.3	−1.72 ± 0.35
Lachnospiraceae Lachnoclostridium	2.33 ± 0.15	2.3 ± 0.15	2.89 ± 0.13	2.18 ± 0.22	1.71 ± 0.32[c]
Lachnospiraceae AC2044 group	−2.77 ± 0.12	−2.78 ± 0.16	−2.98 ± 0.14	−2.19 ± 0.26	−1.47 ± 0.37[abc]
Lachnospiraceae NK4B4 group	−2.76 ± 0.12	−2.46 ± 0.34	−2.01 ± 0.57	−1.51 ± 0.46	−1.39 ± 0.41
Lachnospiraceae UCG-008	−2.76 ± 0.12	−1.66 ± 0.5	−2.17 ± 0.6	−1.81 ± 0.36	−1.52 ± 0.37
Lachnospiraceae Marvinbryantia	0.37 ± 0.39	−0.27 ± 0.6	1.13 ± 0.33	1.10 ± 0.45	0.75 ± 0.43
Monoglobaceae Monoglobus	0.01 ± 0.21	−0.21 ± 0.23	−0.68 ± 0.37	−0.91 ± 0.19[a]	−0.84 ± 0.25
Peptostreptococcaceae Romboutsia	5.27 ± 0.13	4.61 ± 0.12	4.68 ± 0.2	4.86 ± 0.15	4.51 ± 0.27[a]
Prevotellaceae Paraprevotella	−2.18 ± 0.25	−2.25 ± 0.31	−1.97 ± 0.36	−1.44 ± 0.36	−1.17 ± 0.25
Rikenellaceae Alistipes	2.75 ± 0.16	3.55 ± 0.17[a]	3.40 ± 0.12	3.43 ± 0.13[a]	2.82 ± 0.34
GBM level differential abundance: Male clusters					
Gut-brain module	non-separated	Resilient	Pain	Immobile	Comorbid
Acetate synthesis I	2.6 ± 0.03	2.64 ± 0.04	2.53 ± 0.03	2.49 ± 0.07	2.43 ± 0.05[b]
Acetate synthesis III	2.23 ± 0.03	2.2 ± 0.04	2.17 ± 0.03	2.07 ± 0.06[a]	2.06 ± 0.05[a]
ClpB (ATP-dependent chaperone protein)	2.47 ± 0.03	2.49 ± 0.04	2.44 ± 0.03	2.36 ± 0.06	2.31 ± 0.05
g-Hydroxybutyric acid (GHB) degradation	−2.11 ± 2.24	−1.47 ± 0.26	−1.01 ± 0.18[a]	−1.27 ± 0.15[a]	−1.43 ± 0.23
Glutamate synthesis II	2.69 ± 0.03	2.71 ± 0.04	2.68 ± 0.03	2.45 ± 0.06	2.55 ± 0.05
Inositol degradation	−1.02 ± 0.07	−0.38 ± 0.13[a]	−0.58 ± 0.10[a]	−0.53 ± 0.11[a]	−0.75 ± 0.15
Isovaleric acid synthesis I (KADH pathway)	0.29 ± 0.23	−0.03 ± 0.32	1.17 ± 0.27	0.47 ± 0.27	1.21 ± 0.24[b]
Isovaleric acid synthesis II (KADC pathway)	2.21 ± 0.03	2.26 ± 0.04	2.11 ± 0.02[b]	2.11 ± 0.07	2.04 ± 0.05[b]
Menaquinone synthesis (vitamin K2) II	−1.62 ± 0.14	−0.62 ± 0.17[a]	−1.02 ± 0.12[a]	−0.95 ± 0.15[a]	−1.11 ± 0.19
p-Cresol synthesis	2.45 ± 0.03	2.35 ± 0.06	2.33 ± 0.04	2.27 ± 0.08	2.23 ± 0.06[a]
Quinolinic acid degradation	2.39 ± 0.03	2.48 ± 0.04	2.42 ± 0.03	2.33 ± 0.06	2.29 ± 0.05
Quinolinic acid synthesis	2.31 ± 0.03	2.28 ± 0.04	2.22 ± 0.03	2.18 ± 0.06	2.13 ± 0.04[a]
S-Adenosylmethionine (SAM) synthesis	2.61 ± 0.03	2.61 ± 0.03	2.57 ± 0.03	2.48 ± 0.06	2.44 ± 0.05
Tryptophan degradation	−0.58 ± 0.05	−0.1 ± 0.11[a]	−0.19 ± 0.07[a]	−0.24 ± 0.09[a]	−0.46 ± 0.15
Tryptophan synthesis	2.24 ± 0.03	2.26 ± 0.04	2.21 ± 0.03	2.12 ± 0.06	2.08 ± 0.05
GMM level differential abundance: Male clusters					
Gut-metabolic module	non-separated	Resilient	Pain	Immobile	Comorbid
ethanol production I	0.78 ± 0.05	0.73 ± 0.07	0.59 ± 0.04	0.69 ± 0.07	0.65 ± 0.05
Formate conversion	−1.44 ± 0.14	−1.92 ± 0.06[a]	−1.79 ± 0.10	−1.78 ± 0.06	−1.68 ± 0.09
hydrogen metabolism	−1.23 ± 0.10	−1.95 ± 0.11	−1.57 ± 0.09	−1.54 ± 0.10	−1.41 ± 0.12
NADH:ferredoxin oxidoreductase	−5.79 ± 0.25	−5.93 ± 0.42	−5.28 ± 0.34	−5.32 ± 0.38	−4.42 ± 0.35[ab]
succinate consumption	−2.32 ± 0.14	−2.10 ± 0.15	−1.83 ± 0.11	−1.89 ± 0.12	−1.98 ± 0.19
threonine degradation II	0.91 ± 0.04	0.86 ± 0.04	0.91 ± 0.03	0.83 ± 0.04	0.82 ± 0.03
tryptophan degradation	−1.53 ± 0.05	−1.05 ± 0.11[a]	−1.09 ± 0.05[a]	−1.10 ± 0.08[a]	−1.27 ± 0.12
tyrosine degradation I	0.95 ± 0.05	0.89 ± 0.02	0.92 ± 0.03	0.93 ± 0.04	−0.46± 0.71[abcd]
tyrosine degradation II	−6.10 ± 0.34	−6.02 ± 0.56	−5.34 ± 0.50	−4.66 ± 0.33	−4.54 ± 0.38

fully assessing the impact on complex processes as pain sensitisation or behaviour (Verbeke et al., 2015).

The gut microbiota is a metabolically active ecosystem breaking down complex carbohydrates, proteins, and other compounds while also producing micronutrients and other molecules used by the host (Vieira-Silva et al., 2016). Overall gut microbiota metabolism was assessed using gut-metabolic modules and metabolomics. Changes in processes modulating amino acid metabolism including tryptophan,

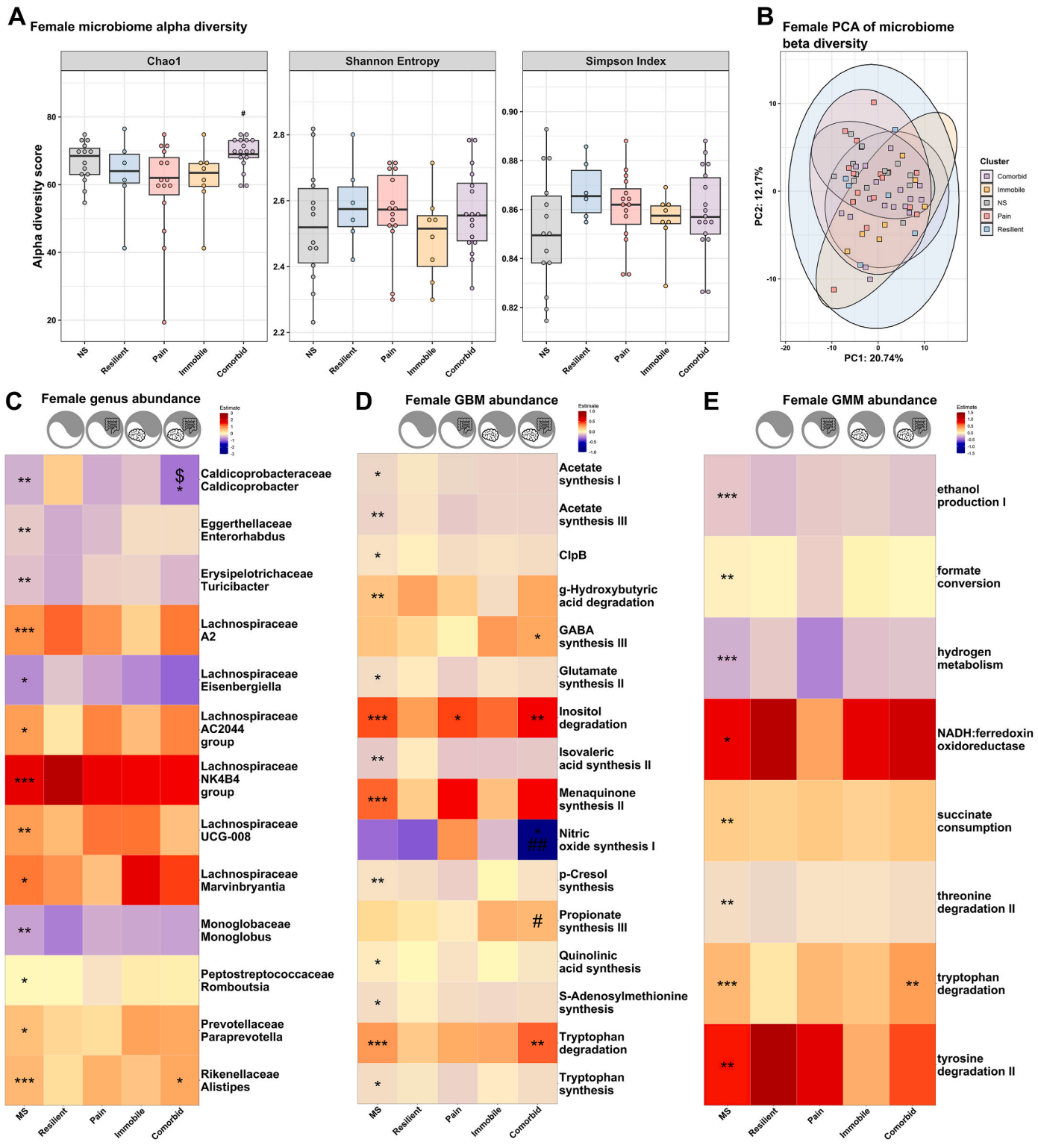

Fig. 5. Female clusters show individual differences in microbiota configurations. (A) Alpha diversity indices of faecal microbiota. (B) Principal component analysis (PCA) showing the first 2 principal components (PC1, PC2) in terms of Atchison distance performed to estimate the β-diversity between female clusters. (C–E) Heat maps, showing significant changes of all maternally separated (MS) animals and female cluster compared to the non-separated control. (C) differential abundant genera, (D) gut-brain modules (GBM) (E) gut-metabolic modules (GMM). Colour indicates estimate with blue indicating higher abundance in control group and red indicating higher abundance in stressed groups. Resilient: n = 6, Pain: n = 17, Immobile: n = 9, Comorbid: n = 18. Data is shown if FDR<0.2 in maternally separated animals overall or in Clusters. *p < 0.05, **p < 0.01, ***p < 0.001 vs non-separated (NS) control. $p < 0.05, $$p < 0.01 vs Resilient. #p < 0.05, ##p < 0.01, ###p < 0.001 vs Pain. ££p < 0.01 vs Immobile. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Differential abundance at the level of genus, gut-brain module and gut-metabolic modules between the female clusters.

Genus level differential abundance: Female clusters					
Genus	non-separated	Resilient	Pain	Immobile	Comorbid
Caldicoprobacteraceae Caldicoprobacter	−2.12 ± 0.21	−1.73 ± 0.51	−2.63 ± 0.26	−2.51 ± 0.19	−3.00 ± 0.10[ab]
Eggerthellaceae Enterorhabdus	−0.6 ± 0.32	−1.12 ± 0.48	−1.01 ± 0.23	−0.80 ± 0.28	−0.80 ± 0.28
Erysipelotrichaceae Turicibacter	0.71 ± 0.29	0.18 ± 0.38	0.44 ± 0.31	0.44 ± 0.34	0.26 ± 0.24
Lachnospiraceae A2	0.54 ± 0.38	1.71 ± 0.43	1.41 ± 0.38	0.90 ± 0.44	1.60 ± 0.28
Lachnospiraceae Eisenbergiella	−1.52 ± 0.41	−1.88 ± 0.46	−2.09 ± 0.37	−2.26 ± 0.24	−2.50 ± 0.25
Lachnospiraceae Lachnoclostridium	2.54 ± 0.23	2.17 ± 0.74	2.31 ± 0.29	2.25 ± 0.30	2.37 ± 0.14
Lachnospiraceae AC2044 group	−2.75 ± 0.13	−2.55 ± 0.25	−1.75 ± 0.53	−2.19 ± 0.38	−1.75 ± 0.45
Lachnospiraceae NK4B4 group	−2.75 ± 0.13	−0.53 ± 0.74	−1.24 ± 0.43	−1.24 ± 0.64	−1.28 ± 0.49
Lachnospiraceae UCG-008	−2.59 ± 0.20	−2.01 ± 0.40	−1.52 ± 0.39	−1.49 ± 0.64	−2.08 ± 0.42
Lachnospiraceae Marvinbryantia	0.12 ± 0.38	1.01 ± 0.87	0.65 ± 0.31	1.73 ± 0.47	1.47 ± 0.25
Monoglobaceae Monoglobus	−0.45 ± 0.23	−1.26 ± 0.28	−0.96 ± 0.28	−1.00 ± 0.36	−1.05 ± 0.27
Peptostreptococcaceae Romboutsia	4.90 ± 0.16	4.93 ± 0.25	4.73 ± 0.14	5.06 ± 0.27	4.99 ± 0.14
Prevotellaceae Paraprevotella	−2.40 ± 0.21	−2.06 ± 0.28	−2.12 ± 0.28	−1.63 ± 0.38	−1.66 ± 0.26
Rikenellaceae Alistipes	2.66 ± 0.18	2.91 ± 0.28	3.32 ± 0.15	3.15 ± 0.21	3.39 ± 0.15[a]
GBM level differential abundance: Female clusters					
Gut-brain modules	non-separated	Resilient	Pain	Immobile	Comorbid
Acetate synthesis I	2.60 ± 0.04	2.56 ± 0.08	2.52 ± 0.03	2.25 ± 0.07	2.51 ± 0.03
Acetate synthesis III	2.22 ± 0.05	2.16 ± 0.08	2.11 ± 0.04	2.13 ± 0.07	2.13 ± 0.03
ClpB (ATP-dependent chaperone protein)	2.47 ± 0.04	2.44 ± 0.08	2.41 ± 0.03	2.41 ± 0.06	2.41 ± 0.03
g-Hydroxybutyric acid (GHB) degradation	−1.66 ± 0.2	−1.41 ± 0.24	−1.52 ± 0.21	−1.73 ± 0.30	−1.42 ± 0.013
GABA synthesis III	0.89 ± 0.07	1.00 ± 0.09	0.91 ± 0.05	1.16 ± 0.10	1.13 ± 0.05[a]
Glutamate synthesis II	2.56 ± 0.04	2.52 ± 0.08	2.46 ± 0.03	2.50 ± 0.05	2.49 ± 0.02
Inositol degradation	−1.00 ± 0.11	1.38 ± 0.05	1.26 ± 0.06[a]	−0.63 ± 0.09	−0.51 ± 0.09[a]
Isovaleric acid synthesis II (KADC pathway)	2.23 ± 0.04	2.20 ± 0.09	2.12 ± 0.03	2.12 ± 0.07	2.12 ± 0.03
Menaquinone synthesis (vitamin K2) II	−1.46 ± 0.18	−1.32 ± 0.20	−0.97 ± 0.15	−1.28 ± 0.20	−0.97 ± 0.12
Nitric oxide synthesis I (NO synthase)	−5.56 ± 0.26	−5.91 ± 0.47	−5.26 ± 0.24	−5.70 ± 0.36	−6.47± 0.18[ac]
p-Cresol synthesis	2.4 ± 0.04	2.33 ± 0.08	2.31 ± 0.04	2.41 ± 0.06	2.34 ± 0.04[c]
Propionate synthesis III	1.50 ± 0.06	1.56 ± 0.10	1.47 ± 0.05	1.72 ± 0.08	1.70 ± 0.05
Quinolinic acid synthesis	2.26 ± 0.02	2.27 ± 0.06	2.21 ± 0.04	2.27 ± 0.06	2.22 ± 0.02
S-Adenosylmethionine (SAM) synthesis	2.61 ± 0.04	2.58 ± 0.07	2.54 ± 0.03	2.53 ± 0.07	2.54 ± 0.03
Tryptophan degradation	−0.50 ± 0.09	−0.36 ± 0.11	−0.27 ± 0.07	−0.26 ± 0.12	−0.11 ± 0.07[a]
Tryptophan synthesis	2.24 ± 0.04	2.19 ± 0.08	2.15 ± 0.04	2.20 ± 0.06	2.17 ± 0.03
GMM level differential abundance: Female clusters					
Gut-metabolic modules	non-separated	Resilient	Pain	Immobile	Comorbid
ethanol production I	0.75 ± 0.05	0.54 ± 0.07	0.59 ± 0.04	0.60 ± 0.10	0.56 ± 0.04
formate conversion	−1.72 ± 0.12	−1.74 ± 0.20	−1.86 ± 0.09	−1.69 ± 0.14	−1.74 ± 0.08
hydrogen metabolism	−0.25 ± 0.08	−1.41 ± 0.18	−1.63 ± 0.10	−1.45 ± 0.18	−1.43 ± 0.08
NADH:ferredoxin oxidoreductase	−6.04 ± 0.27	−4.94 ± 0.40	−5.66 ± 0.30	−5.23 ± 0.46	−5.10 ± 0.32
succinate consumption	−2.23 ± 0.11	−2.05 ± 0.12	−2.01 ± 0.08	−2.06 ± 0.17	−2.00 ± 0.08
threonine degradation II	0.93 ± 0.03	0.81 ± 0.02	0.84 ± 0.03	0.85 ± 0.05	0.83 ± 0.03
tryptophan degradation	−1.43 ± 0.09	−1.35 ± 0.14	−1.14 ± 0.08	−1.18 ± 0.12	−1.03 ± 0.07[a]
Tyrosine degradation II	−5.99 ± 0.36	−4.82 ± 0.36	−5.17 ± 0.32	−5.66 ± 0.12	−5.34 ± 0.33

tyrosine, and threonine were identified in both sexes in response to ELS. Alterations in these processes could affect downstream processes such as conversion into neurotransmitters or antibodies (O'Mahony et al., 2015). Similarly, energy metabolic processes were affected by MS including increased abundance of succinate consumption, NADH:ferredoxin oxidoreductase an important enzyme in anaerobic respiration or reduced formate conversion, an end-product of fermentation.

Due to the inferred functional changes in the gut microbiota, we decided to analyse faecal metabolites. Contrary to previous studies reporting alterations in early-life faecal metabolomics following MS in mice (Chen et al., 2023), untargeted analysis revealed no global changes in our study in rats. Therefore, we used a multi-omics approach, by identifying the metabolites in our samples that correlated with bacterial enzymes within the same KEGG pathway (Bastiaanssen et al., 2023c).

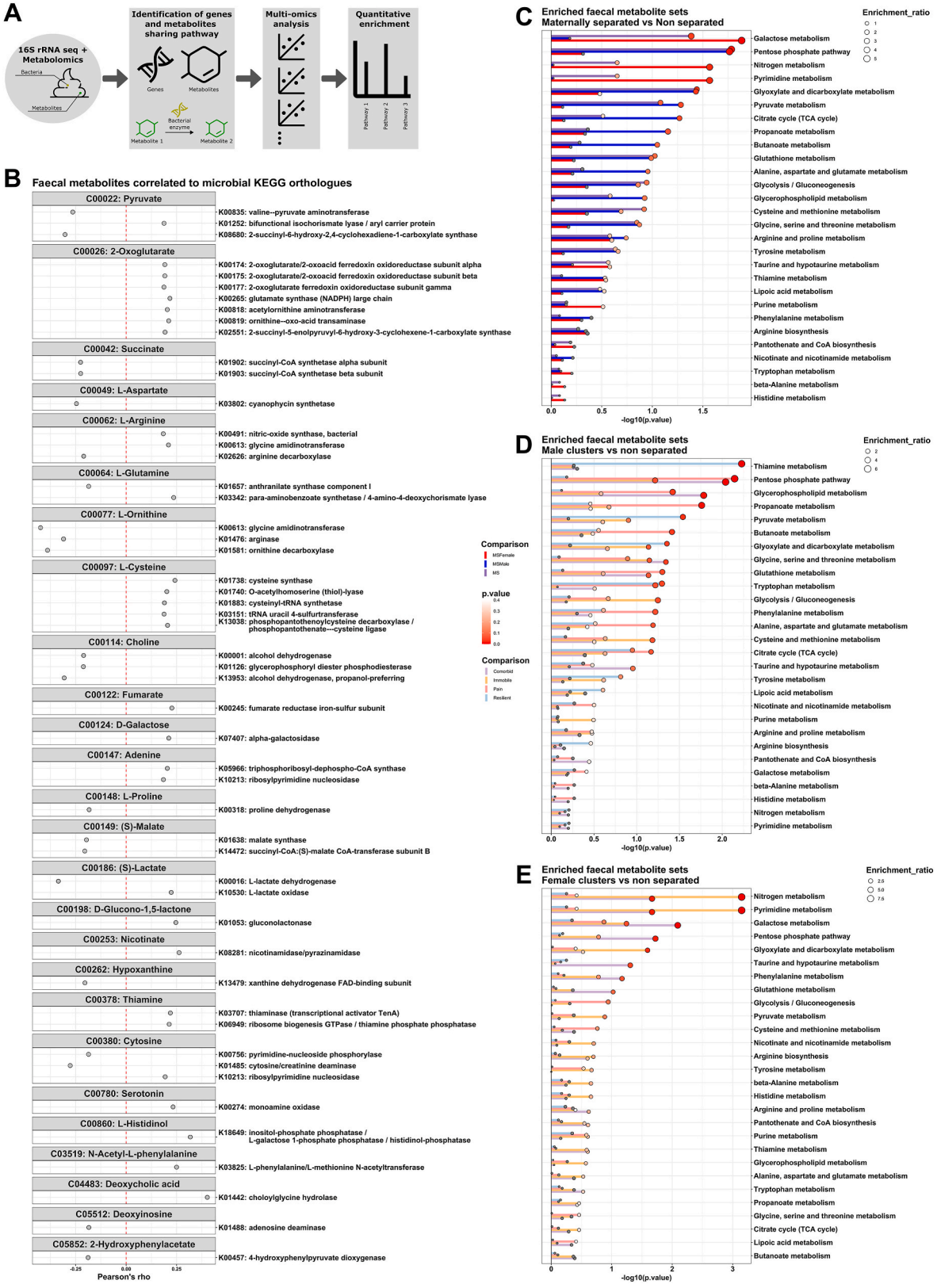

Fig. 6. Faecal metabolomics reveal enriched pathways in a cluster specific manner. (A) Schematic of multi-omics analysis approach. (B) Associations between the 16S and the metabolome in faecal samples collected in early life (N = 119), in which x-axis is depicting pearson's correlations coefficients between the metabolites and predicted functionally related transcripts as kyoto encyclopaedia of genes and genomes (KEGG) functions. Only associations of shared KEGG pathways passing $p < 0.05$ are shown. (C–E) Enrichment of metabolic pathways based on metabolites correlated with functional relevant genes in the 16S dataset. (C) Maternal separated animals compared to controls. (D) Male clusters compared to control (E) Female clusters compared to control.

Twenty-four metabolites were used for quantitative enrichment analysis of any pathways changed in our treatment groups compared to the NS control. The results showed that pathways related to carbohydrate metabolism, the pentose phosphate pathway, and galactose metabolism were enriched in MS animals. Pentose phosphate pathway describes a parallel pathway to glycolysis resulting in the production of NADPH, while galactose metabolism describes the conversion of galactose to glucose-6-phosphate which subsequently can be used for glycolysis or the pentose phosphate pathway respectively. The pentose phosphate pathway was mainly driven by males, while galactose metabolism was driven by the female population in our study. Similar to the findings of the GMMs, these results point towards changes in bacterial energy metabolism, possibly linked to the changed metabolic demand induced by chronic stress induced glucocorticoid dysregulation, a key feature of MS (Moussaoui et al., 2017; O'Mahony et al., 2009; Walker et al., 2017). In both male and female clusters, the changes in pathways related to carbohydrate metabolism are congruent. However, changes in pathways are modulated in a cluster specific manner. In the male clusters, both thiamine and pyruvate metabolism are enriched in the resilient cluster pointing towards an increase conversion of pyruvate into the citric acid cycle. The male clusters with visceral pain showed disproportionally more changes. They are characterised by increased pentose phosphate pathway and glycerophospholipid metabolism. Conversely, in the female animals, changes in metabolic pathways were restricted to the cluster showing increased negative valence. While the male animals were predominated by changes in pathways related to glucose metabolism, female clusters show changes in pyrimidine and nitrogen metabolism. Changes in nitrogen, and by extension of pyrimidine as a source of nitrogen modulates gut ammonia levels. Changes in gut ammonia and nitrogen metabolism have been linked to stress vulnerability by changing the availability of glutamine, influencing the glutamate/GABA levels in the brain and modulating negative valence behaviour in mice (Wang et al., 2023).

Investigation of sex differences is an ongoing area of research, particularly important given that women are disproportionately affected by both gastrointestinal and psychiatric disorders (Bangasser and Valentino, 2014). Interestingly, our study revealed that while males and females exhibited similar behavioural phenotypes, these behaviours were driven by distinct microbiota-related mechanisms, detectable early in life. There is an increasing appreciation for the role of sex differences across the microbiota-gut-brain axis, yet the mechanisms remain unresolved (Jaggar et al., 2020). It has been shown previously that sex hormones modulate microbiota configurations via oestrogen receptors (Menon et al., 2013; Tetel et al., 2018) and that sex hormone-driven behavioural effects are microbiota dependent (Tramullas et al., 2021). The different microbiota-dependent mechanisms underlying the same but proportionally different behavioural phenotypes across sexes might therefore be due to hormone-related reorganisation of the microbiota resulting in similar phenotypes (Moreno-Indias et al., 2016). Notably, most microbiota-related effects have been observed in male animals. Similar trends are found in other stress models, such as chronic variable stress, which produces stronger alterations in males (Kropp et al., 2024). Additionally, microbiota-targeted interventions like faecal microbiota transplantation have shown greater effect sizes in male rodents (Keubler et al., 2023). However, some models show stronger effects in females, such as the single prolonged stress model (Tanelian et al., 2024). This potentially highlights sex-specific microbiota and host determines of behaviour as well as stress-specific responsiveness of the microbiota.

One limitation of our study lies in the minimalistic approach of the behaviour. Neither CRD nor the FST fully captures the complexity of symptoms seen in human disorders. Due to the heterogeneity of IBS, we decided to focus on visceral hypersensitivity as a measure of gastrointestinal impairment following ELS rather than measurements of gastrointestinal transit. Similarly, the FST does not represent depressive-like behaviour (Commons et al., 2017; von Mücke-Heim et al., 2023). It is rather a test measuring changes in emotional behaviour and stress-coping which is sensitive to early-life stress. These behavioural measures act as surrogates for specific gastrointestinal and brain-related changes, allowing us to infer how seemingly distinct phenotypes are driven by different but converging underlying mechanisms. This study serves as a proof of concept rather than offering a comprehensive explanation of the full comorbidities between IBS and its associated psychiatric symptoms.

Another limitation of the study lies within the clustering approach. Clustering using biological data does not always form fully separable clusters, in part due to the sensitivity to outliers. This can result in failure to identify meaningful subgroups. Similarly, individuals of distinct cluster can show seemingly similar behavioural responses and obstruct the identification of biological mechanisms underlying the subgroups. Nonetheless, despite these limitations we identified meaningful associations in our microbiota data and linked known mediators of pain (isovaleric acid) (Bayrer et al., 2023; Bellono et al., 2017) and valence related behaviour (Acetate) (Dalile et al., 2019) to the respective phenotypes. This highlights the benefits of exploratory tools. Nonetheless, a validation cohort of this experiment would be required to identify the reliability of the data.

5. Conclusion

Taken together, our results suggest that not all animals exposed to the same ELS show the full repertoire of pathophysiological consequences in adulthood. Using a clustering approach, we revealed gut-brain phenotypes susceptible or resilient to ELS in both sexes. We demonstrated a potential role for the gut microbiota in early life preceding the trajectory of ELS-induced gut or brain alterations in adulthood. Future studies are warranted to elucidate the pathways of the microbiota-gut-brain axis which temporally connect the distinct early microbiota profiles to adult behavioural changes. Furthermore, faecal microbiota transplantation will be required to determine if there is an ongoing casual role of the adult microbiota in modulating these distinct behavioural phenotypes. This research sets the base for a novel, individual-based temporal approach to treat stress-induced disorders of gut-brain interaction such as IBS.

CRediT authorship contribution statement

Lars Wilmes: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Valentina Caputi:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Thomaz F.S. Bastiaanssen:** Writing – review & editing. **James M. Collins:** Writing – review & editing. **Fiona Crispie:** Writing – review & editing. **Paul D. Cotter:** Writing – review & editing, Methodology, Data curation. **Timothy G. Dinan:** Writing – review & editing. **John F. Cryan:** Writing – review & editing. **Gerard Clarke:** Writing – review & editing. **Siobhain M. O'Mahony:** Writing – review & editing.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used no service in order to write this manuscript.

Declaration of competing interest

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and is a paid consultant for Yakult and Zentiva.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2024.100686.

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