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A gut reaction? The role of the microbiome in aggression

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ABSTRACT

Recent research has unveiled conflicting evidence regarding the link between aggression and the gut microbiome. Here, we compared behavior profiles of control, germ-free (GF), and antibiotic-treated mice, as well as re-colonized GF mice to understand the impact of the gut microbiome on aggression using the resident-intruder paradigm. Our findings revealed a link between gut microbiome depletion and higher aggression, accompanied by notable changes in urine metabolite profiles and brain gene expression. This study extends beyond classical murine models to humanized mice to reveal the clinical relevance of early-life antibiotic use on aggression. Fecal microbiome transplant from infants exposed to antibiotics in early life (and sampled one month later) into mice led to increased aggression compared to mice receiving transplants from unexposed infants. This study sheds light on the role of the gut microbiome in modulating aggression and highlights its potential avenues of action, offering insights for development of therapeutic strategies for aggression-related disorders.

1. Introduction

Aggression is an intentional, complex, multifaceted social behavior, one prevalent in almost all species and intimately associated with host survival. Animals display aggression when defending territory, securing and defending food and mates, and establishing dominance hierarchies. The behavior is modulated by numerous factors including specific genes, neurotransmitters, environmental factors, pheromones, and hormones (Bartholow, 2018; Edwards et al., 2009a; Edwards et al., 2009b; Nelson and Trainor, 2007), which are generally thought to be conserved across species ranging from fruit flies, to mice, to humans (Thomas et al., 2015; Zhang-James et al., 2019). One such hormone is serotonin which is a monoamine neurotransmitter, produced mainly in the gastrointestinal tract (Fidalgo et al., 2013; Glinert et al., 2022). High central serotonin levels have been negatively correlated to aggression in both mice and humans (Holmes et al., 2002). One of the factors affecting serotonin levels is the dietary consumption of tryptophan (Bartholow, 2018). Additionally, recent studies have shown that the gut microbiota play an important role in the regulation of serotonin levels (Frankiensztajn et al., 2020; Weiner et al., 2023; Yano et al., 2015). Mice reared in the absence of microbial colonization (germ-free, GF) have lower levels of serotonin in their serum compared to conventionally-colonized (specific pathogen-free, SPF) controls (Sjogren et al., 2012).

In addition to the direct role of serotonin in aggression, various studies have revealed a correlation between different genes and aggression with a specific focus on understanding how the serotonergic system influences this relationship, as reviewed by Popova in 2006. These studies highlight the crucial role of brain serotonin that underlies genetically determined individual variation in aggressiveness. Additionally, they show genes encoding key enzymes in serotonin metabolism, serotonin transporters, and certain serotonin receptors that play a significant role in modulating aggression (Popova, 2006).

Numerous studies have provided evidence supporting the notion of a gut-brain axis, with crosstalk mediated by the microbiome (Kayyal et al., 2020; Leclercq et al., 2017; Morel et al., 2023; Ritz et al., 2024; Zhang-James et al., 2019). While research on the role of the microbiome in aggression is in its infancy, connections have been identified in fruit flies (Grinberg et al., 2022), dogs (Craddock et al., 2022), mice (Leclercq

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et al., 2017; Watanabe et al., 2021), and humans (Deng et al., 2022). The impact of changes in microbial composition on aggression is still unclear though, as some studies have found that an absence of bacteria or antibiotic treatment led to decreased aggression in fruit flies (Jia et al., 2021) and hamsters, respectively (Sylvia et al., 2017), but several other studies revealed increased aggression following antibiotic treatment or in germ-free settings (Grinberg et al., 2022; Leclercq et al., 2017; Watanabe et al., 2021). Many of the published studies are preliminary or descriptive, and to date, none have uncovered mechanisms or even explained the network of interactions between aggression, hormone levels, gene expression, metabolite profiles, and the gut microbiome.

There is also a growing interest in understanding the relationship between antibiotic use during infancy and child behavior, particularly in the context of the gut-brain connection. We have recently reported that exposure to antibiotic treatment during infancy is linked to significant differences in the gut microbiome, persisting until the age of two (Uzan-Yulzari et al., 2021). In addition, multiple studies have shown the association between antibiotic treatment during early life and neurodevelopmental disorders in mice and humans (Diamanti et al., 2022; Lynch et al., 2023; Neuman et al., 2018). The potential link between early-life antibiotic exposure and aggression may be of clinical importance given that antibiotics are administered to 2–42 % of all newborns in the United States (Schulman et al., 2019) and after the neonatal period, children have on average received nearly three courses of antibiotics by the age of two years (Cox and Blaser, 2015). On the other hand, conduct disorders, which are characterized by aggression and antisocial behavior, have been estimated to affect 3 % of school-aged children (Fairchild et al., 2019).

Here we examine this complex network in a model organism using germ-free, antibiotic-manipulated, conventionalized, and humanized animals to reveal the importance of an intact microbiome in mediating host aggression.

2. Results

2.1. The role of the microbiome in mediating aggression

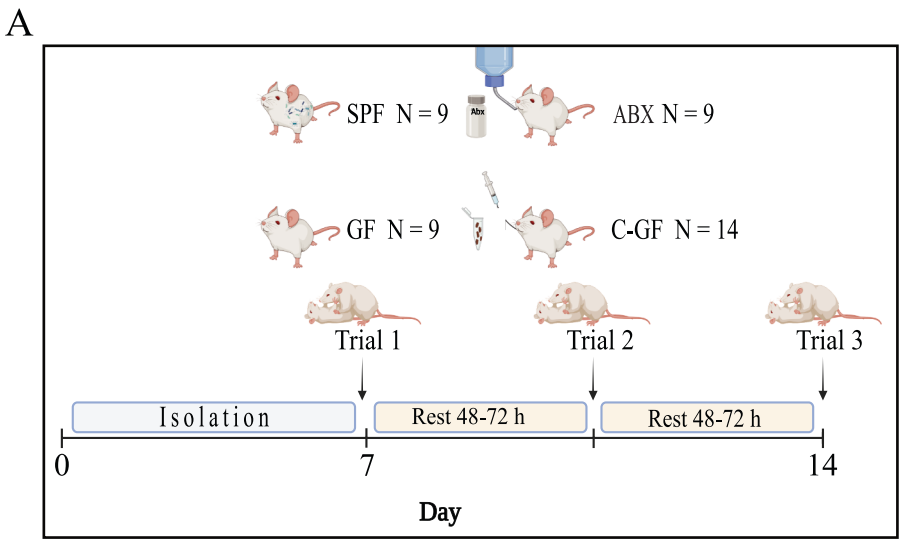
As recent research is inconclusive, we first aimed to examine the impact of the gut bacterial community on aggression in a murine model. To this end, we compared behavior profiles (resident intruder test (Kaliste-Korhonen and Eskola, 2000)) of germ-free (GF) mice, specific pathogen-free (SPF, wild type baseline) mice, antibiotic-treated SPF mice (ABX), and GF mice that were re-conventionalized with SPF fecal samples at the age of 5 weeks (C-GF). Both GF and ABX mice were included as in ABX mice, there could be direct chemical effects on host behavior in addition to microbiota mediated ones which can be controlled for in using GF models. It is important to note that fecal samples from both ABX and GF mice were bacteria-free as confirmed by PCR of the 16S rRNA gene, as described in (Binyamin et al., 2020). Three resident-intruder behavior assays (Kaliste-Korhonen and Eskola, 2000) were performed, with each trial lasting 10 min (Fig. 1A, see Methods for details).

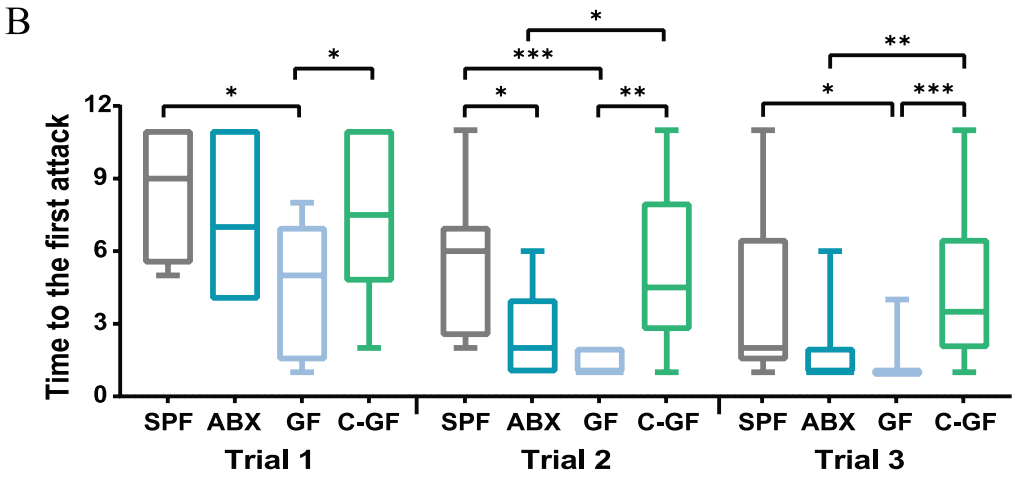
The GF group demonstrated significantly higher aggression levels according to two parameters: they were faster to attack and attacked more frequently compared to SPF mice throughout the three trials (Fig. 1B and 1C respectively). Moreover, the mean number of attacks among GF mice during the 10-minute trials was higher in all three trials compared to SPF mice, with statistically significant differences in the first and second trials (Fig. 1C). Consistent with the GF results, antibiotic administration led to an elevation in aggression; latency to attack in ABX-treated mice was significantly lower compared to the SPF group in the second trial and to the C-GF group in the second and third trials, matching behavior profiles of GF mice (Fig. 1B). In addition, the number of attacks increased in the case of ABX treatment, with significant differences seen in trial 2 (Fig. 1C). Interestingly, colonization of GF mice showed a significant reduction in aggression levels; C-GF mice were slower to attack (Fig. 1B), and the number of attacks was significantly lower compared to GF groups (Fig. 1C). In the resident-intruder test, repeated exposures not only foster aggression as the intruder seeks dominance or territory defense but may also heighten the intruder's responsiveness and aggression in subsequent encounters. This phenomenon is observed across all groups, with aggression escalating over trials.

2.2. Mechanisms underlying increased aggression in ABX-treated mice

After revealing that absence of a microbiome (GF) or significant perturbations to the microbiome (ABX) increased aggression levels, we explored the web of interactions underlying this relationship. As the primary mode of communication among mice is through scents, and urine serves as a main source of volatile and nonvolatile compounds, we first examined the metabolite profile of mouse urine using untargeted metabolomics in search of potential compounds that might be linked to aggression. Principal component analysis (PCA) was performed to evaluate the overall differences in the metabolic profiles between all groups prior to the co-housing with a female mouse and the subsequent aggression assay (T0) and between samples taken from SPF mice prior to and following the resident-intruder test (T14, Fig. 2). A clear separation can be seen between the SPF-T0 and the GF groups, based on the PCA plot. Moreover, antibiotic treatment of SPF mice led to a dramatic shift in the metabolic profile compared to the SPF-T0 group. Similarly, the metabolite profile of the C-GF mice was more similar to the SPF group rather than the GF group. When we examined all the groups together, there was no clear separation between the two time points for the SPF group: T0 and T14 (Fig. 2A). However, when we performed PCA for only these two groups, we saw separation which was likely caused by the aggression trials (Fig. 2B). Altogether, 99 metabolites from the urine samples (identified or putatively annotated) were differentially abundant ($q < 0.05$) in at least one of the statistical comparisons performed between the four experimental groups or nominally different ($p < 0.05$) between T0 and T14 in the SPF group (Supp. Figs. S1-S8, Supp. Table S1). Interestingly, we identified several metabolites that changed with antibiotic treatment and found they also changed after aggression trials in the SPF mice in the same manner – following trials, profiles of unmanipulated SPF mice (exposed to aggression assays) became more similar to the (unexposed) GF and ABX mice (Fig. 2C-H). Tryptophan (Trp) and creatinine are examples of metabolites that changed due to the presence or absence of a bacterial population; the GF group showed significantly higher levels of Trp and creatinine compared to the SPF group. Likewise, antibiotic administration to SPF mice, for a period of 3 weeks, led to elevated Trp and creatinine levels compared to the SPF group, whereas bacterial colonization of GF mice (C-GF) led to a reduction in Trp and creatinine levels (Fig. 2C,E). Trp and creatinine metabolite levels also changed following multiple aggression trials compared to baseline in SPF mice, displaying higher levels similar to those seen after antibiotic administration (Fig. 2D,F). DL-indole-3-lactic acid (I3LA), a metabolite of Trp, showed the same pattern but in the opposite direction. The absence of a bacterial population among the GF and ABX groups led to lower levels of I3LA, and bacterial presence led to higher levels of I3LA (Fig. 2G). The effect of aggression was also observed here; significantly lower levels of I3LA were observed following aggression trials compared to the baseline T0 (Fig. 2H).

To further our understanding of the gut-brain-microbiome-metabolism interplay, we next quantified levels of Trp, serotonin (5-HT) and a serotonin metabolite (5-HIAA) in the whole brain using HPLC (Fig. 3A). The tissue was collected at T0 to serve as a baseline reference before housing with the female and before the aggression trials. This allowed us to assess the initial state of these compounds prior to experimental manipulation. Since GF mice have distinct brain development, which could introduce confounding variables, we compared only SPF mice to antibiotic-treated SPF mice in all experiments involving the brain.





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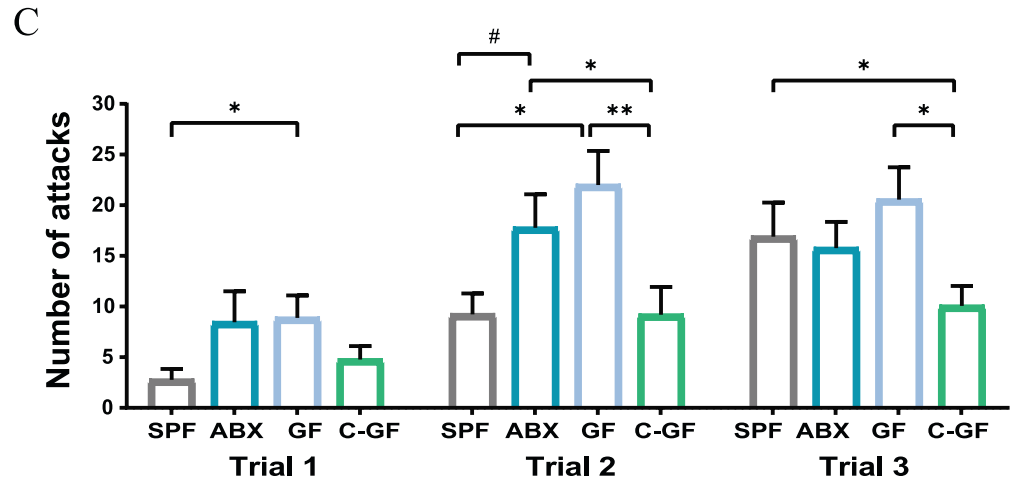

Fig.1. Gut microbiome modulates aggression in mice. (A) Experimental design – aggression was examined using the resident- intruder test. A resident male mouse was isolated with a female for 7 days and tested against an intruder male mouse of the same group (i.e. SPF-SPF, GF-GF) three times at 2–4 day intervals. The female was removed 1 h before the experiment, and each experiment lasted for 10 min. At the end of the experiment, the intruder was removed, and the female was returned to the cage. Aggression was measured using two parameters: (B) attack latency, the time between the introduction of the intruder and the first attack - mice that did not attack within the 10-min trial were scored 11, and (C) number of attacks, the overall number of attacks in each trial (SPF N = 9, GF N = 9, ABX N = 9, C-GF N = 14, n refers to number of pairs, resident and intruder, $^{\#}p=0.06$, $^{*}p<0.05$, $^{**}p<0.01$, number of attacks represents the mean +/- SEM).

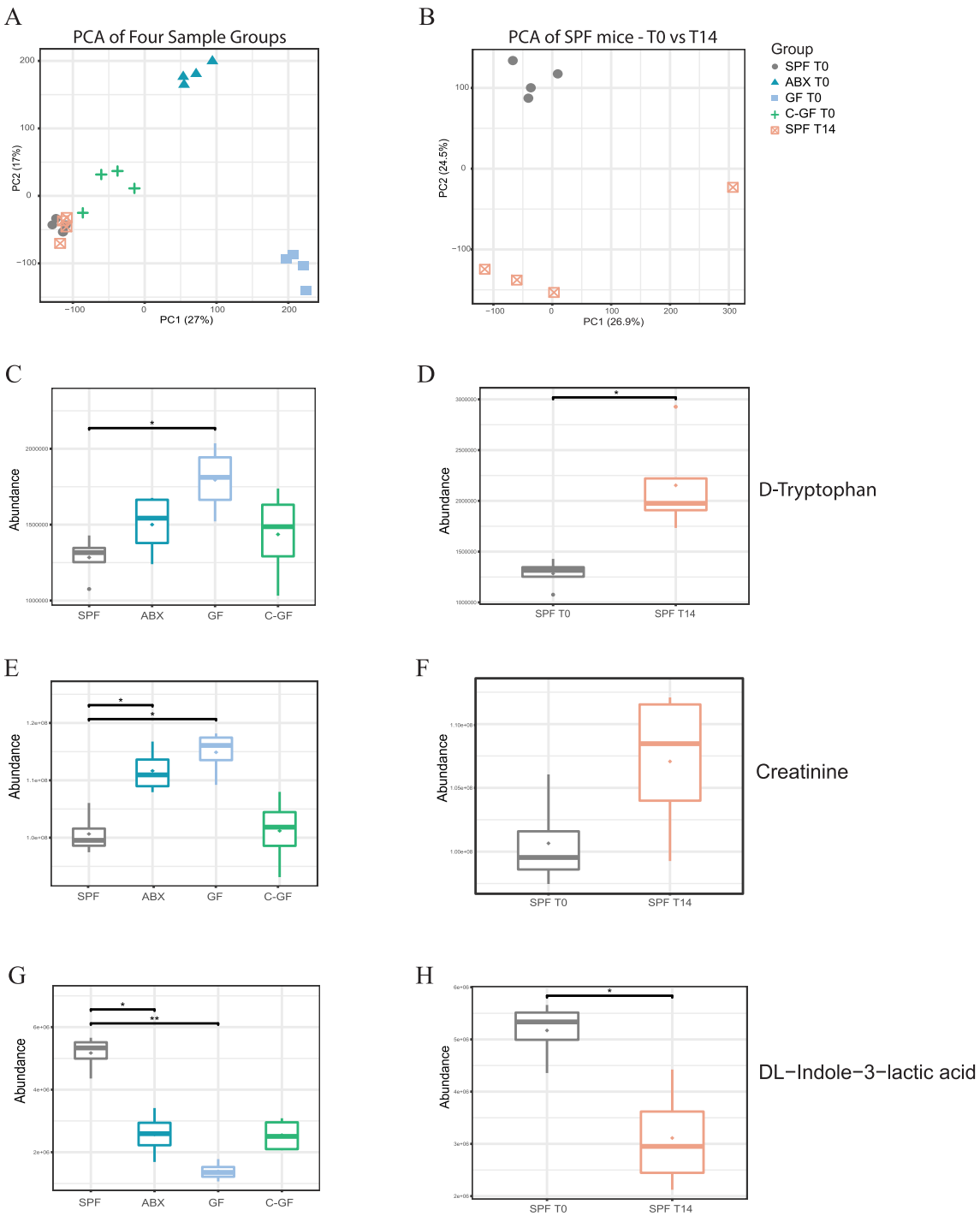

Fig.2. Gut microbiome and aggression induce changes in urine metabolite profiles in male mice. PCA of the overall metabolite profile in urine samples from male mice using untargeted urine metabolomics. The analysis compares two sets of samples: (A) Samples from four groups, including SPF (N = 4), ABX (N = 4), GF (N = 4), and C-GF (N = 4), taken at time point T0, before aggression tests, to assess the effect of gut microbiome perturbation on urine metabolite profiles. (B) Samples from SPF mice taken before (T0, N = 4) and after (T14, N = 4) aggression trials, to investigate the impact of aggression on urine metabolite profiles. Box plots illustrating the differences in (C+D) D-Tryptophan, (E+F) creatinine and (G+H) DL-Indole-3-lactic acid abundance between (C+E+G) SPF (gray), ABX (Dark blue), GF (light blue), and C-GF (green) mice and (D+F+H) SPF-T0 (gray) and SPF-T14 (orange) aggression trials. The levels are shown as normalized abundances (peak areas) (N = 4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Bacterial depletion using antibiotic treatment to the SPF group (Fig. 3A) induced significant elevation in Trp levels (Fig. 3B) in addition to significant reduction in 5-HT (Fig. 3C). Furthermore, 5-HIAA and 5-HT turnover (5-HIAA/5-HT ratio), were significantly higher in the ABX group compared to the SPF group (Fig. 3D,E respectively).

Next, we performed transcriptomics profiling on five brain regions (prefrontal cortex, amygdala, hippocampus, hypothalamus and septum), which revealed differential expression of thousands of genes and pathways between the SPF and ABX groups (Fig. 4A-E and Supp. Table S2). We analyzed our data focusing on the 40 aggression-related genes published by Zhang-James et al. (Zhang-James et al., 2019) and key 5-HT related genes (Soga et al., 2020) (Fig. 4F-J). Within this set of genes, we identified aggression-related genes that changed significantly following antibiotic treatment. Notably, the expression levels of several

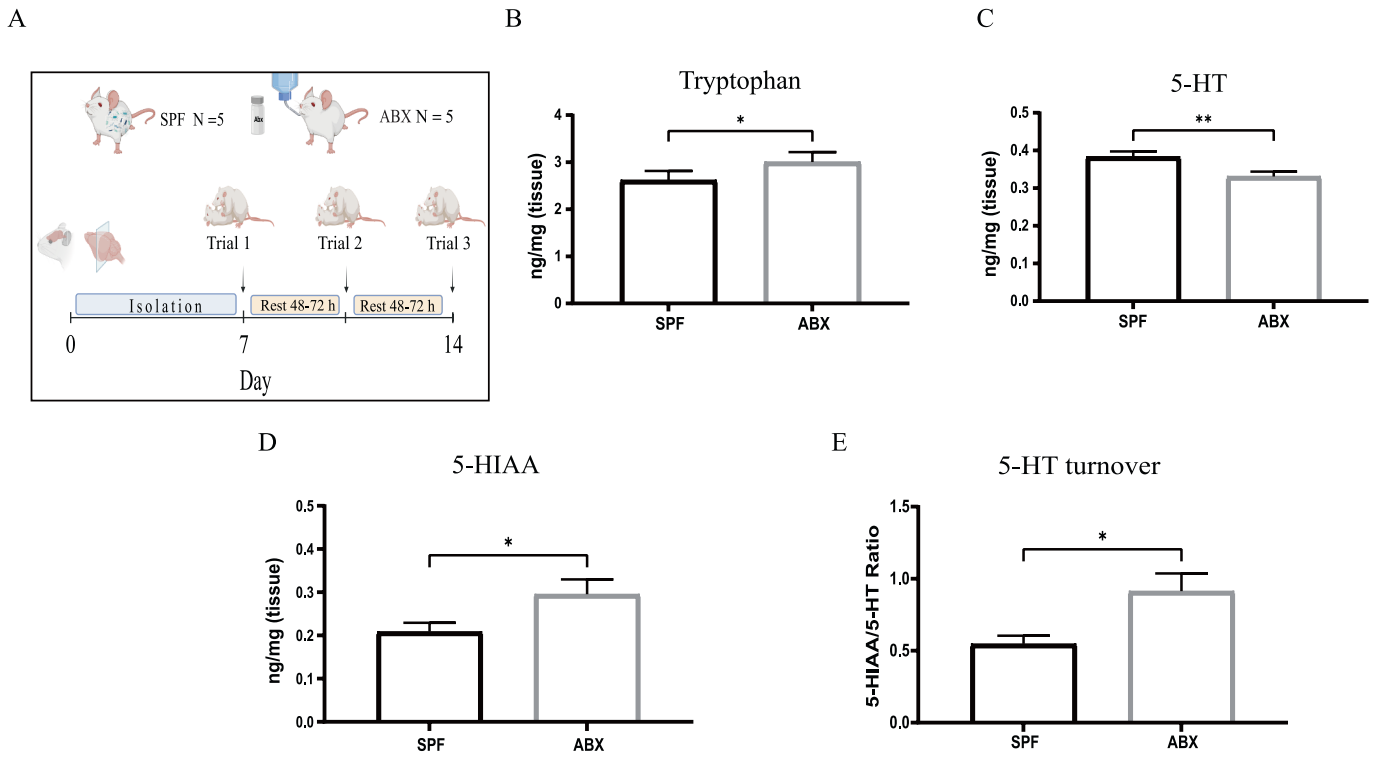

Fig.3. Alterations in gut bacterial composition, using antibiotic treatment, lead to changes in Tryptophan 5-HT, 5-HIAA and 5-HT turnover. (A) Experimental design- High pressure liquid chromatography (HPLC) performed on the whole brain of SPF (N = 5) and antibiotic-treated (N = 5) male mice at 8 weeks of age before isolation and aggression trials. After three weeks of antibiotic treatment, we observed significant differences in the levels of (B) Tryptophan, (C) 5-HT, (D) 5-HIAA, and (E) 5-HT turnover. (n = 5, *p<0.05, **p<0.01, values represent the mean +/- SEM).

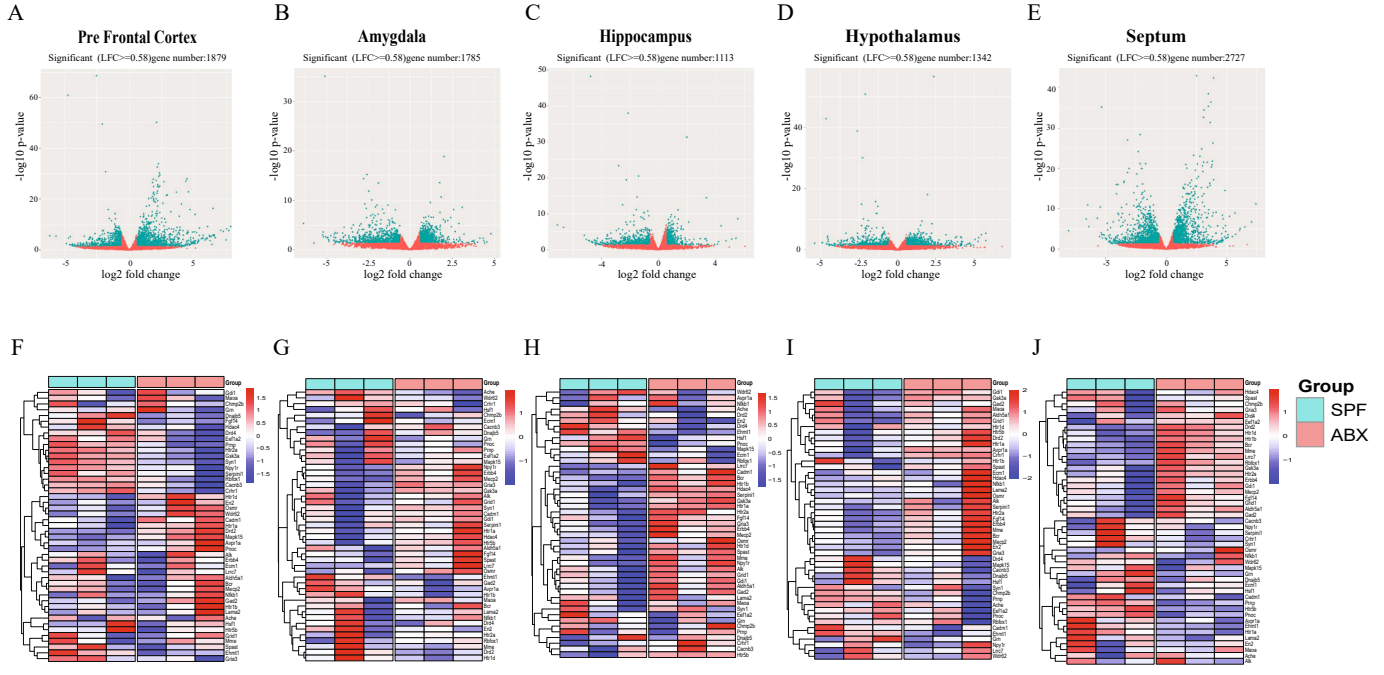

Fig.4. Antibiotic-induced alterations in gut bacterial composition impact gene expression in five brain regions. RNA sequencing analysis was conducted on two groups of mice: SPF (blue N = 3) and ABX (pink, N = 3). The study focused on five distinct brain regions: Prefrontal cortex (A+F), Amygdala (B+G), Hippocampus (C+H), Hypothalamus (D+I), and Septum (E+J). (A-E) Volcano plots displaying the differential gene expression patterns between the SPF and ABX groups in each brain region. The blue dots represent thousands of differentially expressed genes that were identified as being significantly upregulated (on the right) or downregulated (on the left) in the ABX group compared to the SPF group. The red dots represent insignificant differentially expressed genes. (F-J) Heatmaps illustrate the relative expression levels (z-score), of 47 genes linked to aggression across the five brain regions for both SPF and ABX groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

members of the serotonin receptor superfamily, including serotonin receptor genes 1A (htr1A), 1B (htr1B), and 2A (htr2A), were significantly altered in at least three brain regions after antibiotic treatment (Fig. 4F-J). RBFOX1, which encodes a splicing factor involved in the alternative splicing of extensive neuronal gene networks essential for brain development (Fernandez-Castillo et al., 2020), was expressed at significantly higher levels in the septum following antibiotic treatment compared with the SPF group, and therefore emerges as another potential candidate for regulating aggression.

Following the identification of aggression-related genes that changed significantly following antibiotic treatment, the next step was to perform Gene Set Enrichment Analysis (GSEA) to identify gene sets associated with aggression (Fig. 5). The analysis identified numerous enriched pathways, in all brain regions, that were significantly up or down-regulated following antibiotic treatment (Supp. Table S3). We found that the Rho GTPase pathway was one of the top 20 most enriched pathways in the hippocampus (Fig. 5A,B, q = 0.01), while the Reelin pathway showed significant enrichment in the amygdala (Fig. 5C,D, q = 0.003). In addition to these pathways, it is worth noting that serotonin-related pathways were also identified, although they did not rank among the top 50 enriched pathways. The serotonin neurotransmitter release cycle in the amygdala (q = 0.03) and serotonin receptor activity in the hippocampus (q = 0.04, Supp. Table S3) were among the other significantly enriched pathways.

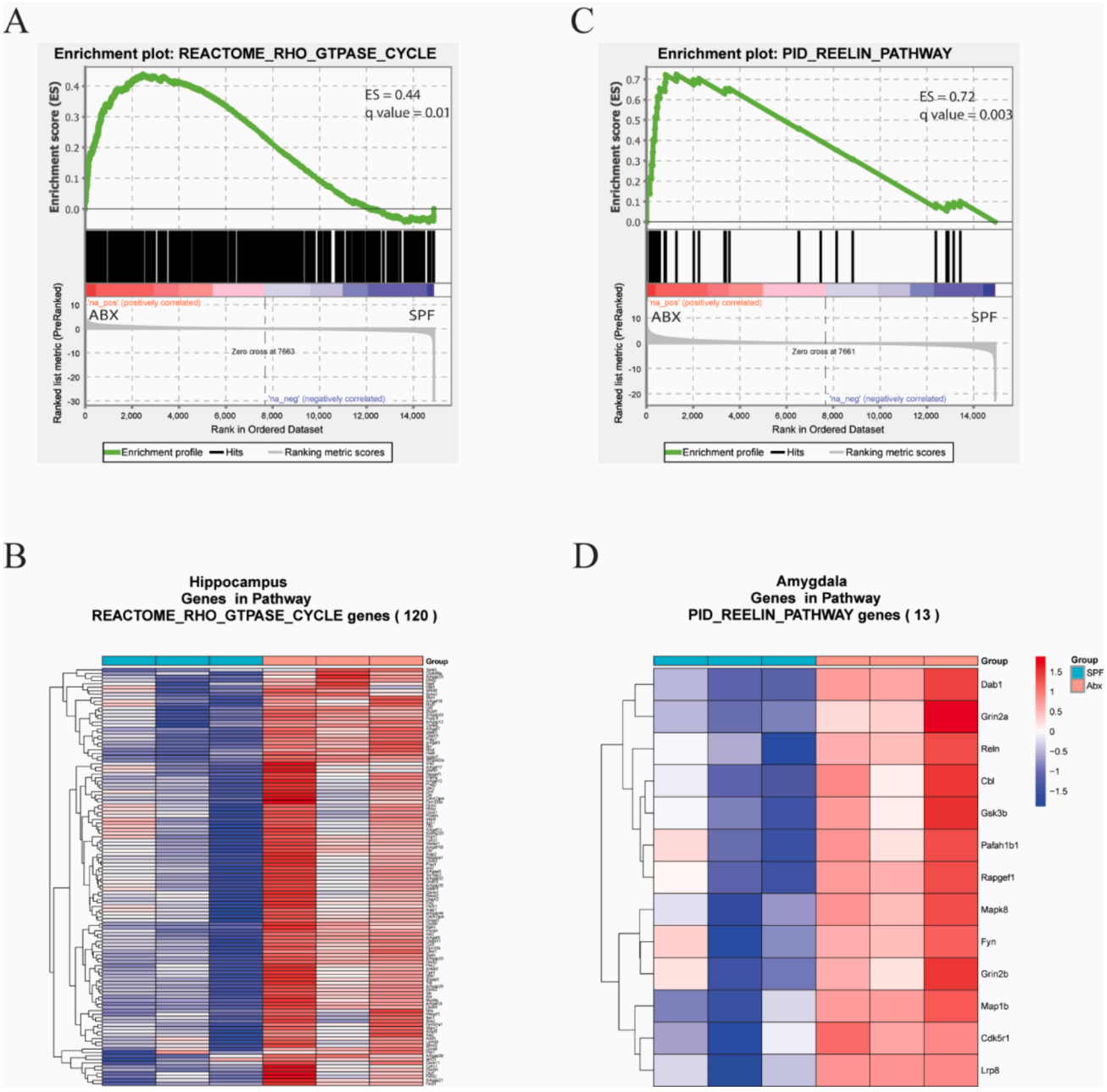

Fig.5. Antibiotic-induced alterations in gut bacterial composition led to changes of hundreds of pathways in five brain regions. Gene Set Enrichment Analysis, based on RNA sequencing, was employed to identify enriched pathways. (A+C) enrichment plot of Rho GTPase cycle in the hippocampus and Reelin pathway in the amygdala, respectively, illustrate the differentially expressed genes (DEGs) identified between the SPF and ABX groups. The top portion of plots show the enrichment scores for each gene, and the bottom portion shows the ranked genes. (B+D) Heatmap displays the core enrichment genes' relative expression (z-scores) identified for each pathway, Rho GTPase cycle and Reelin pathway.

2.3. Early-life antibiotic use increased aggression in humanized mice

Next, we aimed to translate our findings into a clinical context, exploring how antibiotic use in early life affects aggression. Towards this end, we performed fecal microbiome transplant (FMT) from feces of one-month-old infants who were or were not exposed to antibiotics in the first 48 h of life, into five-week-old GF mice (see Supp. Table S4 for infant demographics). In previously published work, we validated this approach determining that the microbiota in the stools of donors are still altered one month post-treatment and that these altered stools can lead to phenotypic differences in mice (namely growth) (Uzan-Yulzari et al., 2021). This FMT approach also allowed us to control for direct chemical effects of antibiotics on behavior as nearly one month elapsed between antibiotic course and sample collection. Pools of ABX and control samples differed significantly in microbiota profile (Supp. Fig. S9). Impressively, behavioral assays in mature mice 4 weeks post-transplant confirmed our above findings: antibiotic-altered microbial communities, here of infants, led to increased aggression, even when antibiotic use has ended, and the microbiome has begun to recover (samples were collected 1 month after antibiotic exposure) (Fig. 6).

3. Discussion

The present study provides insights into the role of the gut microbiome in modulating aggression in a murine model and in humanized mice, supporting the involvement of the microbiota-gut-brain axis in regulating social behaviors – namely aggression – consistent with previous research (Jossin, 2020). Our findings not only demonstrate the causative impact of gut microbiome on aggression, through use of FMT, but also reveal its influence on multiple factors and pathways that regulate this behavior. To gain a deeper understanding of the gut-brain-microbiota crosstalk, we first explored urine metabolite profiles using untargeted metabolomics. We demonstrate distinct shifts in metabolite profiles following bacterial perturbation and aggression trials, associated with increased tryptophan, and creatinine levels, as well as a decrease in DL-indole-3-lactic acid. Tryptophan (Trp), an essential amino acid and precursor to the neurotransmitter serotonin, has been extensively studied in relation to psychological disorders, including aggression. A recent study on twins revealed lower Trp levels in aggressive twins compared to their less aggressive counterparts (Hagenbeek et al., 2020). Contrasting findings from previous studies have shown a positive association between plasma Trp levels and aggression though (Suarez and Krishnan, 2006). Our results on creatinine levels align with Hagenbeek *et al.*, who also identified a positive link between childhood aggression and creatinine levels (Hagenbeek et al., 2020). Indole and its derivatives play a crucial role as essential mediators in the microbiota-gut-brain axis, influencing the brain in various ways and contributing significantly to the onset and progression of neurological and neuropsychiatric diseases such as depression (Zhou et al., 2023). However, there is a lack of data concerning aggression. Our insights not only highlight the impact of the gut microbiome on aggression but also demonstrate how it affects the complex network of metabolites involved in the regulation of aggression. These findings contribute to the broader understanding regarding the mechanism of the gut-brain axis in modulating aggression. Continued research is needed to gain a deeper understanding of these complex interactions.

Furthermore, our analysis of serotonin in the brain demonstrated lower serotonin levels and higher levels of Trp, 5-HIAA, and serotonin turnover following antibiotic treatment. These results are supported by a previous study in rats showing lower serotonin levels together with higher levels of serotonin metabolites and serotonin turnover in the brain following antibiotic treatment. In addition, Trp measured in plasma was similarly elevated following antibiotic treatment (Hoban et al., 2016). Such changes in serotonin and related metabolites following antibiotic treatment highlight the interconnection between gut health and brain function, emphasizing the crucial role of the gut-brain axis in modulating neurochemistry and behavior. In addition to our whole brain examination, gene expression analysis showed alterations in serotonin receptor genes across specific brain regions following antibiotic treatment. The majority of differences regarding the 47 aggression-related genes (40) and genes associated with 5-HT (7) were found in the septum followed by the prefrontal cortex and the amygdala. The first two regions have been identified as potential critical sites for the control of aggression (Lischinsky and Lin, 2020). The altered genes are thought to play a role in aggression regulation as they encode factors that can modulate serotonergic neurotransmission. A study on silver foxes found that domesticated foxes have a significantly lower density of 5-HT1A in hypothalamic membranes compared to their wild counterparts (Popova et al., 1991). Additionally, knockout mice lacking 5-HT1B receptors demonstrate higher aggression levels than wild-type mice (Saudou et al., 1994), and additional studies have reported an association between the HTR2A gene, a gene encoding one of the receptors of serotonin, and aggression in both mice and humans (Rosell et al., 2010; Sakaue et al., 2002). Interestingly, the protein encoded by RBFOX1, another potentially relevant gene that changed following antibiotic treatment, regulates the expression of 15 out of the top 40 genes mentioned earlier (Lee et al., 2016). The GSEA aligns with finding from Zhang-James *et al.* (Zhang-James et al., 2019), who associated the function of Rho GTPase and Reelin pathways with aggression. While these pathways may not have a direct relationship with aggression, they are involved in various cellular processes in the brain. Rho GTPase signaling plays a crucial role in regulating various aspects of neuronal development, growth, and survival (Stankiewicz and Linseman, 2014), and the Reelin signaling pathway is crucial for nervous system development and has been linked to various neuropsychiatric disorders (Jossin, 2020). These findings provide valuable insights into the complex gut-brain interplay between the gut microbiome, gene expression, and aggression and offer further support for the involvement of serotonin signaling pathways in mediating the gut microbiome's effects on aggression.

After investigating the role of the microbiome in aggression-related neurobiological mechanisms, we were interested in considering a clinical scenario – how early life antibiotic exposure affects behavior. To that end, we established a humanized mouse model. Our results demonstrate that FMT from infants exposed to neonatal antibiotics led to increased aggression in humanized mice. Aggression in children has complex etiology with contributions of both genetic and environmental risk factors but, interestingly, early-life antibiotic use was recently reported to be associated with behavioral problems and conduct disorder. According to an epidemiological study of 5,589 children from New Zealand, antibiotic exposure during the first 12 months of life was associated with increased behavioral difficulty scores at 4.5 years (Slykerman et al., 2023). Furthermore, early exposure to antibiotics in the first year of life was associated with lower executive function scores and lower receptive language ability. In a population-based register study of more than one million children and young adults from Denmark, antibiotic exposure was also associated with an increased risk of subsequent psychiatric diagnoses including conduct disorder (Kohler-Forsberg et al., 2019). It is often impossible to distinguish between the impact of antibiotic exposure and the underlying infection in epidemiological studies. However, our experimental data using FMT from antibiotic-exposed infants suggests that the antibiotic-induced gut microbiome perturbations may play a causal role in the development of behavioral problems, and the reduced aggression in conventionalized GF mice gives hope of ameliorating the detrimental impact of early antibiotics by gut microbiota targeted intervention.

These findings highlight the complex interplay between the gut microbiome and aggression, providing valuable insights into the underlying biological processes involved. However, further research is needed to fully understand the multi-faceted interactions between gut microbiota, gene expression, and aggression, and how these findings can be translated into clinical applications.

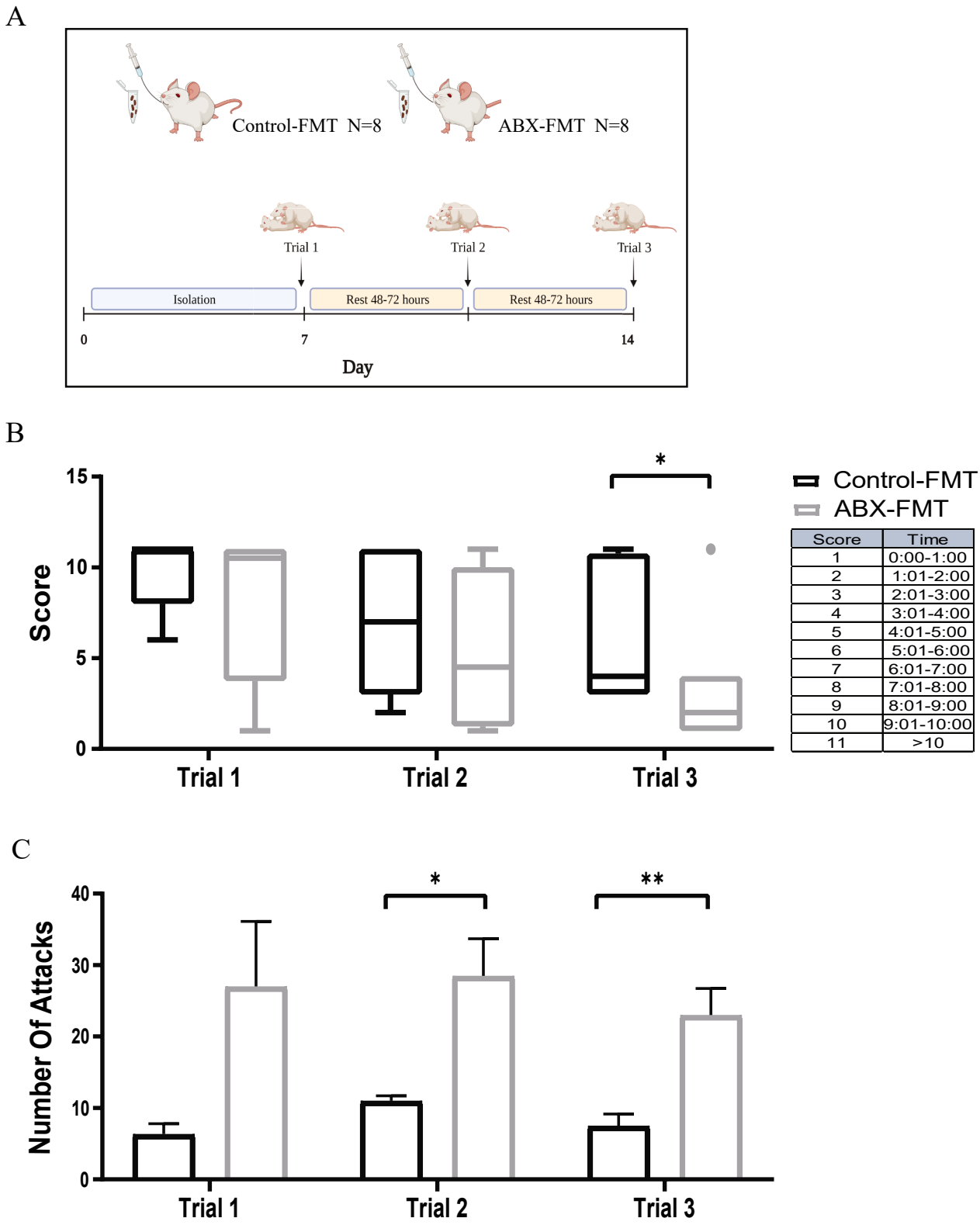

Fig.6. Fecal microbiome transplantation (FMT) from antibiotic-treated babies, collected 1 month after exposure, to GF mice induces increased aggression in mice. (A) Experimental design – FMT from antibiotic-treated and control infants to five-week-old GF mice. At the age of 8 weeks, aggression was examined in both groups; control-FMT (N = 8) and ABX-FMT (N = 8), using the resident-intruder test as described above. (B) Attack latency, the time between the introduction of the intruder and the first attack - mice that did not attack within the 10-min trial were scored 11, and (C) number of attacks, the overall number of attacks in each trial (*p<0.05, **p<0.01, number of attacks represents the mean +/- SEM).

4. Materials and methods

4.1. Mice

Swiss Webster mice were obtained from Taconic Farms Inc. (Germantown, NY, USA) and maintained at the animal facility of the Azrieli Faculty of Medicine, BIU. Mice were maintained in standard 12 h:12 h light:dark housing conditions; specific pathogen-free (SPF) mice and antibiotic-treated SPF mice (ABX) were housed in the SPF animal house of the Azrieli Faculty of Medicine, Bar-Ilan University (BIU); conventionalized germ-free mice (C-GF) and humanized mice were held in the conventional room of the animal house; and the GF group was housed in cages inside sterile isolators under the same dark/light conditions but with autoclaved water, bedding and nesting material. In addition, GF mice were provided with enriched, autoclaved food. Further details are found in Supplementary Text 1. Both male and female mice were used in this study, which was performed under a protocol approved by the Bar Ilan University Animal Studies Committee (approval number 43–07-2015) – males were the focal mice, the residents and intruders, and females were used in the behavior paradigm (below) to increase territorial behaviors in resident mice.

4.1.1. Antibiotic-treated mice

For the antibiotic-treated SPF group, mice at the age of 5 weeks were treated with a combination of ciprofloxacin (0.04 gl^{-1}) (Sigma-Aldrich Corporation, St. Louis, MO, USA), metronidazole (0.2 gl^{-1}) (Santa Cruz Biotechnology, Santa Cruz, CA) and vancomycin hydrochloride (0.1 gl^{-1}) (Gold Biotechnology, St. Louis, MO, USA) in drinking water, refreshed twice per week for 3 weeks before and during the experiment. Bacterial DNA extraction from mouse fecal pellets, followed by PCR of the 16S rRNA gene and visualization on agarose gel (Neuman et al., 2019) revealed that ABX treatment completely depleted the fecal microbiota.

4.1.2. Re-conventionalized mice

At five weeks of age, GF mice were colonized by oral transplantation with stool samples collected from SPF mice of the same age. Stool samples were suspended in sterile phosphate-buffered saline (PBS) (1 fecal pellet/1 ml of PBS) and dissolved by vortex for 1 min. A total of 150 μl of the fecal suspension was administered by oral gavage to GF mice. The process was performed once, immediately after mice were removed from the sterile isolator, followed by housing in the conventional animal facility under the same conditions as SPF.

4.2. Humanized mice

4.2.1. Fecal sample donor

Fecal samples were obtained from subjects from a clinical study conducted at the Turku University Hospital in Turku, Finland (Uzan-Yulzari et al., 2021). We included 5 infants, administered antibiotic therapy due to symptoms or signs suggesting early-onset sepsis, who received intravenous benzylpenicillin and gentamicin during the first 48 h of life, and 5 non-exposed infants were selected (as controls) based on sample availability (Table S4). The samples (taken when infants were one month old) were maintained at −80 °C and shipped on dry ice to the Azrieli Faculty of Medicine, BIU. Enrollment criteria are presented by Uzan-Yulzari et al. (Uzan-Yulzari et al., 2021), and the study was approved by the Finnish Institute for Health and Welfare, a national expert agency under the jurisdiction of the Finnish Ministry of Social Affairs and Health (Uzan-Yulzari et al., 2021).

4.2.2. Fecal transplantation

Fecal samples from antibiotic-treated (N = 5) and control (N = 5) infants were pooled according to group and then transplanted to GF male mice by oral gavage at 5 weeks of age. Each pool was suspended in 800 μl of sterile phosphate buffered saline (PBS) and dissolved by vortex for 1-minute. A total of 200 μl of the fecal suspension was administered by oral gavage to GF male mice (Control-FMT N = 8, ABX-FMT N = 8). The process took place once, immediately after the mice were taken out of the isolator, followed by housing in the conventional animal facility under the same conditions as SPF mice.

4.2.3. Resident intruder test

Aggression was measured using the resident-intruder paradigm (Kaliste-Korhonen and Eskola, 2000) among all four groups of mice: SPF (N = 9), GF (N = 9), colonized GF (N = 14), and antibiotic-treated SPF (N = 9), between 8–12 weeks of age (n refers to number of pairs, resident and intruder). Mice were tested three times at 2–4 day intervals, and the test was performed within groups only (i.e. GF resident with GF intruder). All trials were carried out during the light phase of the light–dark cycle between 12–3 pm. Each male was housed with a companion female for one week before the start of the experiments; females were removed from the residential cage one hour before the trial. Each resident was tested in his home cage against a group-housed intruder for 10 min. After completion of the trial, the intruder male was removed from the cage and the companion female was reunited with the resident male until the next trial. Aggression was measured using two parameters: attack latency and number of attacks. Attack latency was scored as the time to first aggressive attack; mice that attacked during the first minute were scored 1, during the second minute were scored 2 and so on. Mice that did not attack within the 10-min trial were scored 11. For the germ-free (GF) groups, the resident-intruder test was conducted within isolators to avoid the risk of contamination. Consequently, blinding of the assessment was not feasible. Instead, the behavioral observations were visually monitored and recorded by two individuals, with no differences observed. In addition, to distinguish between the resident and the intruder at the end of the test, we performed an ear punch on the intruder's ear before the isolation phase. In the recorded movies, it was not possible to see the punch and identify each mouse individually. Instead, we calculated the total aggression score for both the resident and the intruder. Initial statistical comparisons for mouse aggression tests were performed by Kruskal-Wallis, with uncorrected Dunn post-hocs (SPF, GF, C-GF, ABX) and subsequent two-group comparisons (SPF vs. ABX, ABX-FMT-recipients vs. Control-FMT-recipients) were made with one-tailed Mann Whitney *U* test, using Prism 9.5.0 (GraphPad Software, San Diego, CA, USA).

4.3. Untargeted metabolomics of mouse urine

4.3.1. Sample preparation

Urine samples were collected from male mice between 8–9 weeks of age directly into 1.5 ml eppendorf tubes using the bladder massage method (Hoban et al., 2016). The groups included GF (N = 4), SPF (N = 4), colonized GF (N = 4) and antibiotic-treated SPF (N = 4). Additionally, for the SPF group, urine samples were compared before (T0, N = 4) and after (T14, N = 4) the aggression trials. Because urine collection is invasive, mice that did not readily provide sample(s) were excluded from this analysis. Samples were maintained at −80 °C and shipped on dry ice to Afekta Technologies Ltd., Finland. Upon arrival, mouse urine samples were thawed in an ice-water bath, vortexed (10 s), and for dilution, aliquots were transferred into filter plate (Captiva ND filter plate 0.2 μm) and Class 1 ultrapure water was added and mixed in a ratio of 300 μl per 100 μl of sample. For metabolite extraction, cold acetonitrile was added in a ratio of 400 μl per 100 μl per of urine sample and mixed. The samples were then centrifuged for 5 min at 700 × g at 4 °C and kept at 4 °C until analysis. The pooled quality control (QC) sample was prepared by collecting 50 μl from each supernatant and combining the material in one tube.

4.3.2. LC–MS analysis

The samples were analyzed by liquid chromatography–mass spectrometry (LC-MS), consisting of a 1290 Infinity II UHPLC (Agilent

Technologies, Santa Clara, USA) coupled with a high-resolution QTOF mass spectrometer (Agilent 6546 with Jet Stream ion source, Agilent Technologies). The analytical method has been described previously (Hanhineva et al., 2015; Klavus et al., 2020). In brief, a Zorbax Eclipse XDB-C18 column (2.1 × 100 mm, 1.8 μm; Agilent Technologies) was used for the reversed-phase (RP) separation and an Aqcuity UPLC BEH amide column (Waters) for the HILIC separation. After each chromatographic run, the ionization was carried out using jet stream electrospray ionization (ESI) in the positive and negative mode, yielding four data files per sample. The collision energies for the MS/MS analysis were selected as 10, 20, and 40 V, for compatibility with spectral databases.

4.4. Data preprocessing

Peak detection and alignment were performed in MS-DIAL ver. 4.90 (Tsugawa et al., 2015). For the peak collection, *m/z* values between 50 and 1500 and all retention times were considered. The amplitude of minimum peak height was set at 5000. The peaks were detected using the linear weighted moving average algorithm. For the alignment of the peaks across samples, the retention time tolerance was 0.2 min and the *m/z* tolerance was 0.015 Da. Solvent background was removed using solvent blank samples under the condition that to be kept for further data analysis, the maximum signal abundance across the samples had to be at least five times that of the average in the solvent blank samples.

After the peak picking, 86,194 detected molecular features were included in the data preprocessing and clean-up step. Low-quality features were flagged and discarded from statistical analyses. Molecular features were only considered high-quality if they met all the following quality criteria: low number of missing values, present in more than 70 % of the QC samples, present in at least 60 % of samples in at least one study group, RSD* below 20 %, D-ratio* below 10 %. In addition, if either RSD* or D-ratio* was above the threshold, the features were still considered high-quality if their classic RSD, RSD* and basic D-ratio were all below 10 %. The signals were normalized for signal drift and batch effect. After the preprocessing and data clean-up, 62,837 molecular features were considered high-quality and included in the FDR correction calculations. The high number of molecular features before data clean-up is due to the high sensitivity of the instrument, collecting several signals from each actual metabolite, but also from the solvent background and detector noise.

4.4.1. Data analysis

For statistical analyses of urine sample metabolite profiles, we used feature-wise Welch's t-tests. Fold changes (relative change or difference between two groups as a ratio of the group means (the first divided by the latter)) and Cohen's D values (a measure of effect size as a difference in standard deviations) were computed to measure the effect size in each analysis. P-values of high-quality features were adjusted for multiple testing with FDR correction. All analyses were conducted with R version 4.1.2.

4.4.2. Compound identification

The chromatographic and mass spectrometric characteristics (retention time, exact mass, and MS/MS spectra) of the significantly differential molecular features were compared with entries in an in-house standard library and publicly available databases, such as METLIN and HMDB, as well as with published literature. The annotation of each metabolite and the level of identification was given based on the recommendations published by the Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI) (Sumner et al., 2007).

4.5. High performance liquid chromatography (HPLC) of whole brains

To quantify levels of serotonin, tryptophan, and associated metabolite 5-HIAA, we performed high performance liquid chromatography (HPLC) analysis between SPF (N = 5) and antibiotic-treated SPF (N = 5) whole brains from male mice 8–9 weeks of age that did not undergo behavioral assays (sacrificed by rapid decapitation) and prepared as described in (Mosienko et al., 2012). Tissue levels of Trp, 5-HT and its metabolite 5-HIAA (Cat Nrs. T0254, 14927, 55697, respectively Merck KGaA, Darmstadt, Germany) were analyzed using uHPLC with fluorometric detection.

4.5.1. Statistical analysis

Amounts of 5-HT, Trp and 5-HIAA were normalized to the wet tissue weight for statistical analysis and calculation of substance levels was based on external standard values. Statistical comparisons of 5-HT, Trp, 5-HIAA and 5-HT turnover were performed by one-tailed Mann Whitney *U* test, using Prism 9.5.0 (GraphPad Software, San Diego, CA, USA). Graphs are presented as mean ± SEM, asterisks indicate significance (*p < 0.05, **p < 0.01, ***p < 0.0001).

4.6. RNA sequencing (transcriptomics)

Brain punches were taken from sacrificed SPF (N = 3) and antibiotic-treated SPF (N = 3) mice between 8–9 weeks of age that did not undergo behavioral testing. Mice were sacrificed by rapid decapitation and brains were removed. Five regions of interest were dissected and frozen on dry ice: the hippocampus, prefrontal cortex, and septum were isolated using a 13-gauge needle, the amygdala using a 16 gauge needle, and the hypothalamus using a 14 gauge needle. Samples were kept in −80 °C until processing. Total RNA was extracted using the RNAeasy Mini Kit according to the manufacturer's instructions (QIAGEN, Manchester, UK). Integrity of the isolated RNA was tested using the Agilent RNA Pico Kit and Bioanalyzer at the Genome Technology Center at the Azrieli Faculty of Medicine, BIU. Total RNA was taken for mRNA isolation using a NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Inc., Ipswich, MA, USA)) and libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Inc., Ipswich, MA, USA)). Quantification of the library was performed using dsDNA HS Assay Kit and Qubit 2.0. Four nM of the library was denatured in 0.2 M NaOH for 5 min at room temperature; 1.45 pM was loaded onto the Flow Cell with 1 % PhiX library control. Libraries were sequenced on an Illumina NextSeq550 instrument, 75 cycles single-read sequencing.

4.6.1. RNA data analysis

For RNA sequencing data analysis, reads were aligned to the *Mus musculus* reference genome GRCm39 using STAR (version 020201) (Dobin et al., 2013), and quantification of reads was performed using htseq-count (version 0.12.4) (Anders et al., 2015) and a list of genes (Ensembl gtf file) (Howe et al., 2021). Differential gene expression analysis was then performed using DESeq2 (version 1.30.1) (Love et al., 2014). Significant differentially expressed genes were selected using threshold values of p-value smaller than 0.05 and $\log_2$fold change greater or equal to 0.58. A volcano plot was rendered using ggplot2 (version 3.3.2) and heatmaps were generated using pheatmap (version 1.0.12) (Wickham, 2016). For pathway enrichment analysis, Gene Set Enrichment Analysis (GSEA version 4.0.3) (Subramanian et al., 2005) was used for all the genes ranked ($-\log_{10}$(pvalue)/sign($\log_2$FoldChange)), converted to human genes, using three datasets: hallmark, Curated Canonical Pathways and GO gene sets. GSEA results (q-value≤0.05) were categorized and plotted (using ggplot2 version 3.3.2).

CRediT authorship contribution statement

Atara Uzan-Yulzari: Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Sondra Turjeman:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology. **Lelyan Moadi:** Methodology. **Dmitriy Getselter:** Methodology. **Efrat Sharon:** Methodology. **Samuli Rautava:** Writing – review & editing, Resources. **Erika Isolauri:**

Writing – original draft, Resources. **Soliman Khatib:** Methodology. **Evan Elliott:** Supervision, Methodology. **Omry Koren:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Metabolomics as a supplementary file

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Appendix A. Supplementary data

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