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Bacteria - derived short chain fatty acids restore sympathoadrenal responsiveness to hypoglycemia after antibiotic-induced gut microbiota depletion

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ABSTRACT

The microbiome co-evolved with their mammalian host over thousands of years. This commensal relationship serves a pivotal role in various metabolic, physiological, and immunological processes. Recently we discovered impaired adrenal catecholamine stress responses in germ-free mice suggesting developmental modification of the reflex arc or absence of an ongoing microbiome signal. To determine whether maturational arrest or an absent bacteria-derived metabolite was the cause, we tested whether depleting gut microbiome in young adult animals could also alter the peripheral stress responses to insulin-induced hypoglycemia. Groups of C57Bl6 male mice were given regular water (control) or a cocktail of non-absorbable broad-spectrum antibiotics (Abx) in the drinking water for two weeks before injection with insulin or saline. Abx mice displayed a profound decrease in microbial diversity and abundance of Bacteroidetes and Firmicutes, plus a markedly enlarged caecum and no detectable by-products of bacterial fermentation (sp. short chain fatty acids, SCFA). Tonic and stress-induced epinephrine levels were attenuated. Recolonization (Abx + R) restored bacterial diversity, but not the sympathoadrenal system responsiveness or caecal acetate, propionate and butyrate levels. In contrast, corticosterone (HPA) and glucagon (parasympathetic) resting values and responses to hypoglycemia remained similar across all conditions. Oral supplementation with SCFA improved epinephrine responses to hypoglycaemia. Whole genome shotgun sequence profiling of fecal samples from control, Abx and Abx + R cohorts identified nine microbes (SCFA producers) absent from both Abx and Abx + R groups. These results implicate gut microbiome depletion plus its attendant reduction in SCFA signalling in adversely affecting the release of epinephrine in response to hypoglycemia. We speculate that regardless of postnatal age, a mutable microbiome messaging system exists throughout life. Unravelling these mechanisms could lead to new therapeutic possibilities through controlled manipulation of the gut microbiota and its ability to alter systemic neurotransmitter responsiveness.

1. Introduction

Trillions of bacteria, archaea, fungi, protists, helmints and viruses form our commensal microbiota family. These organisms co-evolved with their mammalian hosts in a mutually beneficial, continuously modifiable relationship of inter-dependent adaptation to survive. An abundance of human microbiota reside in the lumen of the gut and is important in maintaining our health by assimilating host-indigestible dietary nutrients, strengthening the hosts immune system to prevent pathogens from invading tissues and for the commonly recognized production of vitamin K to prevent hemorrhage (Tang et al., 2017; Blacher et al., 2017; Boulange et al., 2016; Camara-Lemarroy et al., 2018; Dominguez-Bello et al., 2019). A bidirectional neurohumoral communication system, known as the gut-brain axis, integrates the host gut and brain activities (Collins et al., 2012; Cryan et al., 2019). Disruption of commensal microbiota homeostasis (gut microbiome

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alteration) causes profound long lasting disturbances and leads to overgrowth of detrimental bacteria linked to a wide range of diseases including neurological disorders (Walker, 2017; Tamburini et al., 2016; Tochitani et al., 2016; Sudo et al., 2004; Vuong and Hsiao, 2017).

Stress is a normal aspect of life experience in all creatures (Gunnar and Quevedo, 2007). Adaptive mechanisms preserve viability in threatening conditions. Principal components of the reflex stress-response involve the sympatho-adrenomedullary (SAM) system and the hypothalamus-pituitary-adrenocortical (HPA) axis (McEwen, 2007); both mature in the early postnatal period in parallel with initial gut colonization and microbiota-gut-brain axis development (rev. in (Foster et al., 2017; Wiley et al., 2017; Dominguez-Bello et al., 2019; Warner, 2019). The ability of gut microbiome to modulate the development and function of the nervous system, host neurophysiology and several complex behaviors has been increasingly recognized (Vuong et al., 2017). Germ free (GF) mice display several abnormal stress-related behaviors – anxiety like behavior associated with altered synapse-related proteins and neurotransmitter turnover, abnormal motor activities, memory dysfunction, etc. many of which could be reversed by conventionalization of gut microbiota but only during a critical window in early postnatal life (rev. in (Jena et al., 2020)). Neurobehavioral effects are not limited to unidirectional gut-to-brain signaling as exposure to chronic stress in turn, influences composition of the gut microbiome providing further feedback on brain homeostasis, behavior and host metabolism (van de Wouw et al., 2018). Well-characterized reflex circuits in intact animals would aid in evaluating testable hypotheses of the role of gut microbiome in the more complex cellular and molecular processes of the central nervous system.

When activated, stress pathways relay signals received by the brain feedback to the periphery via the HPA axis and the autonomic nervous system (sympathetic & parasympathetic SNS, (Ulrich-Lai and Herman, 2009). While HPA activation culminates in secretion of glucocorticoid hormones into the circulation from the adrenal cortex, activation of the SNS releases neurohormones like epinephrine (via a single synapse to the adrenal medullary chromaffin cells) and the neurotransmitter norepinephrine (from nerve terminals directly to various effector organs: heart, blood vessels, liver, etc.). Exposure to microbes in the perinatal period was found to be essential for normal HPA axis programming and stress reactivity over the remaining life span (Sudo et al., 2004; Moloney et al., 2014; Gareau et al., 2011; Diaz Heijtz et al., 2011). The impact of gut microbiome on the SAM axis has not been explored.

We recently showed that only the adrenal catecholamine responses to hypoglycemic stress (and not HPA or parasympathetic responses) were selectively impaired in GF mice; an effect associated with markedly altered components of the synaptic machinery, synaptic vesicle exocytosis and neuropeptide signaling networks (Giri et al., 2019). Given that the development of various central and peripheral nervous systems pathways is affected when mice are raised GF, in the current study we sought to verify the involvement of gut microbiome in reflex responses in young adult animals born and raised with normal microbiota. We again used a peripheral stress model of insulin-induced hypoglycemia but after antibiotic-induced gut microbiome alteration (Farzi et al., 2018).

We hypothesize that the gut microbiome and diet derived metabolites serve an important role in regulating stress responsivity and catecholaminergic pathways. We now provide the first evidence that even in mature animals, Abx-induced gut microbiome depletion results in prolonged sympathoadrenal hyporesponsiveness with intact hypothalamic/adrenal cortical and parasympathetic responses to hypoglycemia. The impaired epinephrine release in response to stress persisted even after re-colonization along with reduced levels of caecal SCFA. Of note, adrenal medullary epinephrine responsiveness was partially recovered after oral SCFA supplementation. After Abx, and even after recolonization, we observed a decreased relative abundance of several Firmicutes (Lachnospiraceae bacterium A4, 28–4 and 10–1; Christensenella, Anaerotruncus, Clostridium clostridioforme and Firmicutes bacterium ASF300) and Bacteroidetes species (B. xylanisolvens and B. fragilis), all of which are producers of SCFA making them candidate bacterial modulators of peripheral catecholaminergic pathways. The implications of these findings portend pragmatic consequences for patient care regarding use of antibiotics, repopulation of gut flora and maintenance of successful stress-adaptations.

2. Materials and methods

2.1. Animals

Male C57BL/6N mice were purchased from Taconic Farms Inc., Germantown, NY, USA. Mice were housed 3–4 per cage in sterile conditions (autoclaved cages and bedding, chow diet and water) with temperature and humidity – controlled room at NYMC animal facility. Cage changes were performed in a laminar flow hood. After one week of acclimation, animals were randomly assigned to three main experimental groups: control (normally colonized mice, C); mice given mixture of broad spectrum non-absorbable antibiotics for microbiome depletion in the drinking water for two weeks (Abx) and recolonized mice (Abx + R, mice with Abx treatment followed by two weeks recolonization with co-housed age matched controls). The antibiotic cocktail comprised of ampicillin (1 g/L; Sigma Aldrich, St. Louis, MO), vancomycin (0.5 g/L; Sagent Pharmaceuticals, Schaumburg, IL), neomycin (0.5 g/L; Fisher Scientific), gentamycin (100 mg/L; Sigma Aldrich), and erythromycin (10 mg/L; Sigma Aldrich) and it was made fresh every 48 h as described (Reikvam et al., 2011; Sampson et al., 2016).

Animal weight and water consumption were monitored every 2–4 days after the start of the antibiotics/vehicle treatment. The care and use of laboratory animals in this study were approved by the Institutional Animal Care and Use Committee at NYMC and conformed to all relevant ethical regulations.

2.2. Intraperitoneal glucose tolerance test (IPGTT)

The assays were performed at 7 weeks of age for all mice ($n > 12$ per group). Animals had access to drinking water (with or without Abx/supplements) at all times. A bolus intraperitoneal (i.p.) glucose injection (2 g/kg body weight) was given after 6 h fasting (Andrikopoulos et al., 2008). Blood glucose levels were measured from tail nick samples using a handheld glucometer (AlphaTrak, Abbott Laboratories, Chicago, Il) before and 15, 30, 60, 90 and 120 min after glucose injection. To ensure minimal blood loss, pressure to the incision was applied briefly after each measurement. At the end of the experiment, food was added to each cage and animals were housed for another week under the specified for each group conditions.

2.3. SCFA supplement

In the experiments with SCFA supplementation, a mixture of the three main dietary fiber fermentation products were added to the drinking water two days after beginning of the Abx or vehicle treatment. The concentrations were similar to endogenously found in the gut acetate (67.5 mM), butyrate (40 mM) and propionate (25.9 mM) as described before (Kiraly et al., 2016; Smith et al., 2013; Braniste et al., 2014).

2.4. Stress test

On the stress test day, all mice (8 weeks old) were transferred to clean cages and fasted for 3 h prior to metabolic stress exposure. Water ± Abx were provided ad libitum. Each experimental group was divided into two subgroups ($n \geq 6$ per group): saline-treated and insulin-treated. Hypoglycemia was induced by insulin (i.p. injection of 2 IU/kg regular human insulin Humulin R; Eli Lilly, Indianapolis, IN) as described before

(Shum et al., 2001; LaGamma et al., 2014; Kudrick et al., 2015; Giri et al., 2019). Equivalent volumes of 0.9% saline were given to corresponding controls under similar conditions. Blood glucose levels were monitored from the tail before and every 30 min after injection. All efforts were made to minimize animal suffering. If animals developed seizures or showed aberrant blood glucose levels, they were rescued and not included in the study. Mice were sacrificed after 90 min (Fig. 1A) with an overdose of ketamine-xylazine cocktail. To avoid fluctuations in the circadian rhythm, all samples were collected at the same time of the day. Tissue samples (caecum, adrenals) were dissected, snap-frozen and stored at −80 °C for further molecular analysis.

2.4.1. Urine collection and epinephrine analysis

Urine was collected at baseline prior to injection, during the treatment period and immediately before sacrifice to measure epinephrine levels. The samples were acidified immediately by addition of an equal volume of 0.01 M HCl and stored at −80 °C for analysis (Wang et al., 2016). Urine epinephrine levels were quantified using commercially available competitive enzyme immunoassay kit (Rocky Mountain Diagnostics, Colorado Springs, CO) and normalized to urinary creatinine concentrations in the same samples by DetectX Urinary Creatinine Kit (Arbor Assays, Ann Arbor, MI) as described (Giri et al., 2019).

2.4.2. Blood collection and serum hormones measurement

Blood was collected by cardiac puncture at sacrifice. All samples for hormonal assays were measured in duplicates. Plasma corticosterone and glucagon concentrations were determined using commercially available immunoassay kits (Arbor Assays, Ann Arbor, MI and Millipore Sigma, Burlington MA, resp.).

2.5. SCFA analysis

Caecal SCFA gas chromatography analysis was performed at the Gnotobiotics, Microbiology and Metagenomics Center (Boston, Massachusetts) as described before (Giri et al., 2019). Briefly, the chromatographic analysis was carried out using an Agilent 7890B system with a flame ionization detector (FID, Agilent Technologies, Santa Clara, CA). A high resolution gas chromatography capillary column 30 m × 0.25 mm coated with 0.25μm film thickness was used (DB-FFAP) for the volatile acids (Agilent Technologies). Nitrogen was used as the carrier gas. The oven temperature was 145 °C and the FID and injection port was set to 225 °C. The injected sample volume was 1 μl and the run time for each analysis was 12 min. Chromatograms and data integration was carried out using the OpenLabChemStation software (Agilent Technologies). A volatile acid mix containing 10 mM of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic and heptanoic acids was used for standard solution (Supelco CRM46975, Bellefonte, PA). An internal standard control (stock solution containing 1% 2-methyl pentanoic acid, Sigma-Aldrich St. Louis, MO) was used for the volatile acid extractions.

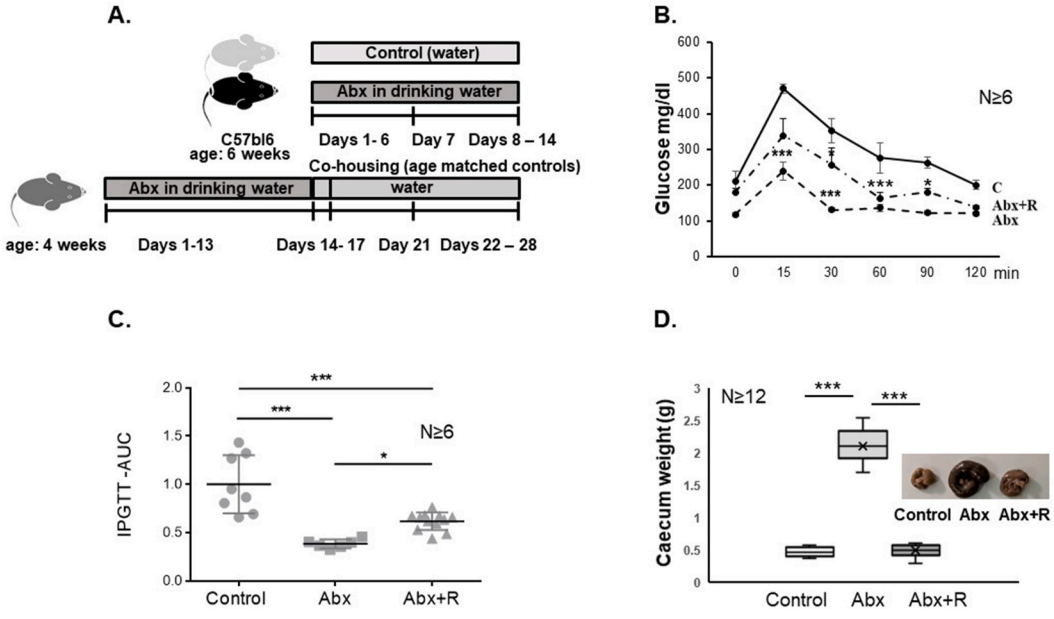

Fig. 1. Oral antibiotic treatment successfully depletes gut microbiome

A: Study design: Animals were divided into 3 major groups: control (normal colonization, drinking regular sterile water); Abx (mice given mixture of non-absorbable broad spectrum antibiotics in the drinking water for 2 weeks) and Abx + R (mice on Abx treatment at 4–6 weeks followed by recolonization by co-housing with age-matched controls for 2 weeks).

B: Intraperitoneal glucose tolerance test (IPGTT) performed at 7 weeks of age for all groups after 6 h fasting period. Control (solid line); Abx (broken line); Abx + R (dashdotted line). Blood glucose levels were recorded before (0 time point) and at indicated time points after injecting glucose load (2 g/kg body weight). The results are presented as mean ± SEM ($n \geq 6$). The Abx group displayed significantly lower blood glucose levels, compared to the water controls: at 15, 30 and 60 min time point (***$P \leq 0.001$) and *$P \leq 0.05$ at 90 min; and Abx + R group had increased glucose levels at 30 min as compared to Abx group (at *$P \leq 0.05$). Analyses were done using Two Way Repeated Measures ANOVA and all pairwise multiple comparisons - Bonferroni *t*-test.

C: The IPGTT results were also expressed as areas under the curves (AUC) to estimate the extent of the glucose tolerance impairment. Data are normalized to the value in control group (taken as 1). One Way Analysis of Variance followed by all pairwise multiple comparison procedures (Holm-Sidak method): *$P \leq 0.05$, (Abx vs. Abx + R, $t = 2.614$); ***$P \leq 0.001$ (both, Control vs. Abx, $t = 6.383$; and Control vs. Abx + R, $t = 4.690$) and $F = 21.793$; $DF = 2$, ($n \geq 6$). 1. Symbols indicate individual samples in each experimental group.

D: At the time of sacrifice, each caecum was dissected and weight was determined using a precision scale for all groups. Data are presented as median with IQR and min/max values as error bars, $n = 12$. One Way Analysis of Variance (Kruskal-Wallis) followed by Holm-Sidak multiple comparisons test was performed. Marked increase in caecal weights was observed in Abx group (***$P \leq 0.001$, DF 2; $F = 382.29$, $t = 24.099$, compared to control).

2.6. Whole-genome shotgun sequencing (WGSS) and metagenomic bioinformatics analyses

Fecal samples from each experimental group were collected the last two days before the stress test and sent out for analysis (CosmosID Inc., Rockville MD). DNA isolation (Zymobiomics Miniprep Kit) and libraries preparation (Illumina Nextera XT library preparation kit) were done according to the manufacturer's protocols. Libraries were sequenced on Illumina HiSeq platform 2 × 150bp. Unassembled sequencing reads were directly analyzed by CosmosID bioinformatics platform described elsewhere (Ottesen et al., 2016; Ponnusamy et al., 2016; Hasan et al., 2014; Lax et al., 2014) for multi-kingdom microbiome analysis and quantification of organism's relative abundance. The resultant taxa abundance tables are used to calculate observed and expected species richness, alpha diversity indices and beta diversity distance matrices. Principle coordinate analysis (PCoA) was performed to cluster samples based on abundance Bray-Curtis distance matrix (community structure).

Functional profiling of metagenomics data was done at CosmosID. Briefly, paired-end reads of the sequencing data were trimmed using BBDuk (https://jdi.doe.gov/data-and-tolls/bbtools) with atributing minlen = 25 parameter. Next, MegaHit (Li et al., 2015) was used to construct the assemblies from the filtered raw reads of metagenomes using 77, 99, 127 values for K-mer size parameter. Each assembled metagenome was sent to Prokka (Seemann, 2014) in order to predict ORF based protein coding genes and assigning functions to the predicted genes in text and GenBank formats. Sequencies of the genes, In Fasta format, were used to map to the raw FastQ files using BBmap and allowing FPKM (fragments per kilobase of gene per million) parameter, which produced sample-wise output in text format having qualified values of every gene in FKPM format. Sequences of predicted protein coding genes, identified in the Fasta format from the Prokka were sent to InterProScan for assignment of KEGG patway or GO processes. FPKM values of every protein/gene assigned to each of the KEGG pathway or GO process were derived from mapping the gene to the FKPM list of genes identified in the previous step. In order to get the abundance of each KEGG pathway and GO process, the FKPM values of belonging genes in a pathway or process were summed up and considered as the abundance of the pathway and process respectively.

2.7. Statistics

All parametric data sets were expressed as means ± standard error of

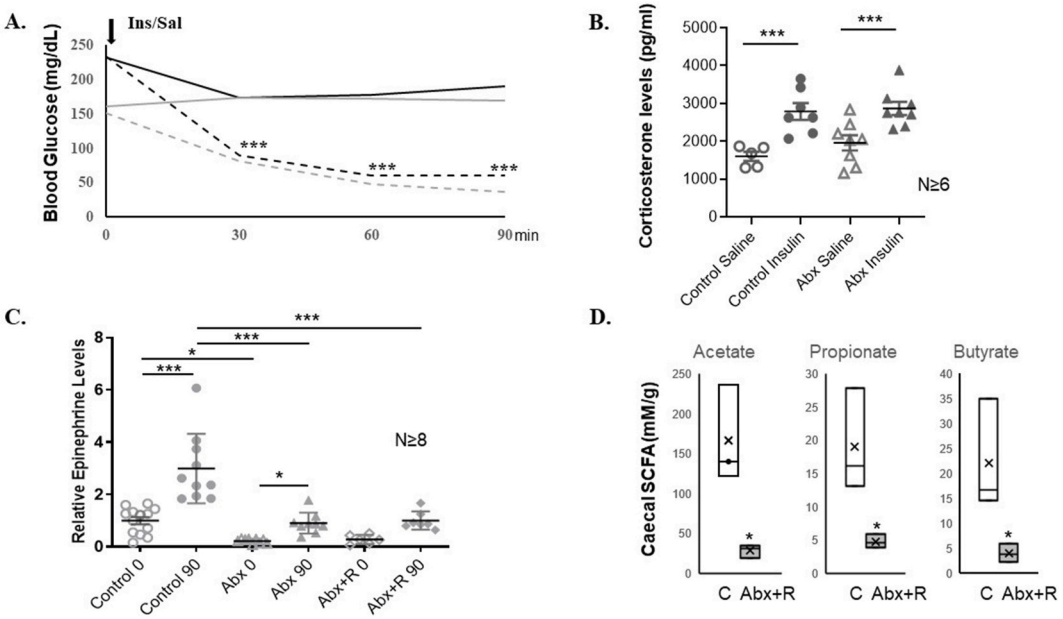

Fig. 2. Mice with gut microbiome depletion have impaired epinephrine responses to metabolic stress

A: The magnitude of insulin-induced hypoglycemia was similar between control (black lines) and Abx-treated (grey lines) mice. Blood glucose concentrations following insulin/saline treatment were measured in mice after 3 h fast as described in Methods. The values for saline (solid lines) and insulin-treated (broken lines) groups are shown as mean ± SEM, n ≥ 6. Data are summarized from 4 independent experiments and analyzed by Two Way Repeated Measures ANOVA, followed by Bonferroni *t*-test: Control Saline/Insulin was significantly different from Abx Saline/Insulin only at 0 time point (**P < 0.002, t = 3.964, and ***P < 0.001, t = 4.599 resp.). Control Sal vs Control Ins differed at 30, 60 and 90 min (***P < 0.001, t = 4.848; ***P < 0.001, t = 6.853, and ***P < 0.001, t = 7.600 resp.); and Abx Sal differed from Abx Ins for the same time points (P < 0.001, t = 5.210; P < 0.001, t = 6.955 and P < 0.001, t = 7.476, resp.).

B: Corticosterone levels in the control and Abx-treated mice after saline or insulin injection are similar. The corticosterone levels were measured in plasma samples from saline and insulin treated mice (shown for control and Abx groups). Data are summarized from two independent experiments, n ≥ 6 animals per group. Results for 90 min time point are presented as mean ± SEM, and normalized to the levels in saline injected control group, taken for 1. Samples were analyzed by Two Way Analysis of Variance and Holm-Sidak test: Control Saline vs. Control Insulin ***P ≤ 0.001, t = 4.317, Abx Saline vs. Abx Ins. ***P ≤ 0.001, t = 3.622. Symbols indicate individual samples in each experimental group.

C: Urinary epinephrine output: Epinephrine levels were analyzed in urinary samples collected before (0′ time point) and after the hypoglycemic episode (90 min) for each experimental group. Catecholamine levels were normalized to creatinine content in each sample, with 0′ time point control values taken as 1. Results are presented as mean ± SEM, n ≥ 8. Statistics: Two Way Analysis of Variance and Holm-Sidak all pairwise multiple comparison method. Control 0′ vs. 90' ***P 0.001, t = 7.136; Abx 0′ vs. 90' *P ≤ 0.034, t = 2.177; Abx + R 0′ vs. 90′ P = 0.056, t = 1.957; Control 0′ vs. Abx 0′ P < 0.027, t = 2.715; Control 90′ vs. Abx 90′ P < 0.001, t = 6.861; Control 90′ vs. Abx + R 90′ P < 0.001, t = 6.094.

D: SCFA content (mM/g caecal tissue) was determined by gas chromatography analysis as described in Methods. SCFA were not detected in the Abx group. Data for Control and Abx + R are summarized from randomly selected individual samples (n = 3) for each experimental group and compared between groups by unpaired two-tailed *t*-test: Abx + R group displayed significantly lower Acetate (DF = 4; t stat = 3.861, *P < 0.018); Propionate (DF = 4, t stat = 3.127, *P < 0.035) and Butyrate (DF = 4, t stat = 2.776, *P < 0.05) levels.

the mean (SEM) and significance was set at $P < 0.05$. A single value per group outside the mean ± 2SD range was excluded as an outlier (e.g. Fig. 2C; control). Normality test (Shapiro-Wilk) and Equal Variance test (Brown-Forsythe) were run for each data set before choosing the appropriate statistical tool (SigmaPlot version 14.5 software). Statistical analyses of blood glucose levels after bolus glucose injection (IPGTT tests, Fig. 1B) and Insulin/Saline treatments (Figs. 2A and 3C) were performed using two-way repeated measures ANOVA followed by pairwise comparisons (Bonferroni's post hoc test). Area under the curve data after IPGTT (Figs. 1C and 3B), and caecum weights (Fig. 1D) were analyzed using One Way Analysis of Variance and all pairwise multiple comparison procedures (Holm-Sidak method). Two way ANOVA was applied to analyze hormonal data sets (2B, 2C and 3D) followed by Holm-Sidak procedure. Caecal SCFA content was compared by unpaired two-tailed *t*-test for individual SCFA in each condition (Fig. 2D).

Analyses of the microbiome sequencing data included generation of a heatmap (Fig. 4A) based on the relative abundance of each microorganism (%) in each sample using the NMF R software package and taxonomic matrices from the CosmosID company (Gaujoux and Seoighe, 2010). The taxonomic profiles obtained with compositional analysis of the sequences were used to calculate species alpha diversity indices (Fig. S1), Kruskal-Wallis test was used to determine the significance of differences between groups. Relative abundance of bacterial species in the fecal microbiome of each experimental group were also analyzed via principal coordinate analysis using the Bray-Curtis distance measure (Fig. S2). F-value was calculated based on permutation-based ANOVA (PERMANOVA) test from the Vegan package. Differential organism abundance between cohorts was calculated using three methods: Kruskal-Wallis sum-rank test for statistical significance, alpha value of 0.05; Wilcoxin rank-sum test for biological consistence, alpha value of 0.05; and Linear Discriminant Analysis (LDA, log10 score threshold of 2.0), Fig. 4B and C (for predictive functional annotation of bacterial taxa).

3. Results

3.1. Oral antibiotics induce reversible gut microbiome depletion

To determine if depletion of gut microbiome in young adult mice can influence the response to metabolic stress, male C57BlN mice received a cocktail of non-absorbable antibiotics (Abx) in the drinking water for 2 weeks according to a previously published protocol (Reikvam et al., 2011; Sampson et al., 2016). Two additional cohorts were included in the study: controls, who received water; and mice given Abx treatment for 2 weeks followed by a complex recolonization (co-housing with age-matched controls (Buffington et al., 2016), - Abx + R group (Fig. 1A). Daily fluid intake and body weights over the course of the experiment did not differ significantly between groups (data not shown).

To confirm the efficacy of the treatment protocols, we took advantage of the established fact that gut microbiota modulates adiposity and glucose metabolism (Schroeder and Backhed, 2016). All mice were subjected to intraperitoneal glucose tolerance test (IPGTT) at 7 weeks of age after 6 h fast (Andrikopoulos et al., 2008), Fig. 1B. As compared to age-matched vehicle treated controls, mice receiving Abx in the drinking

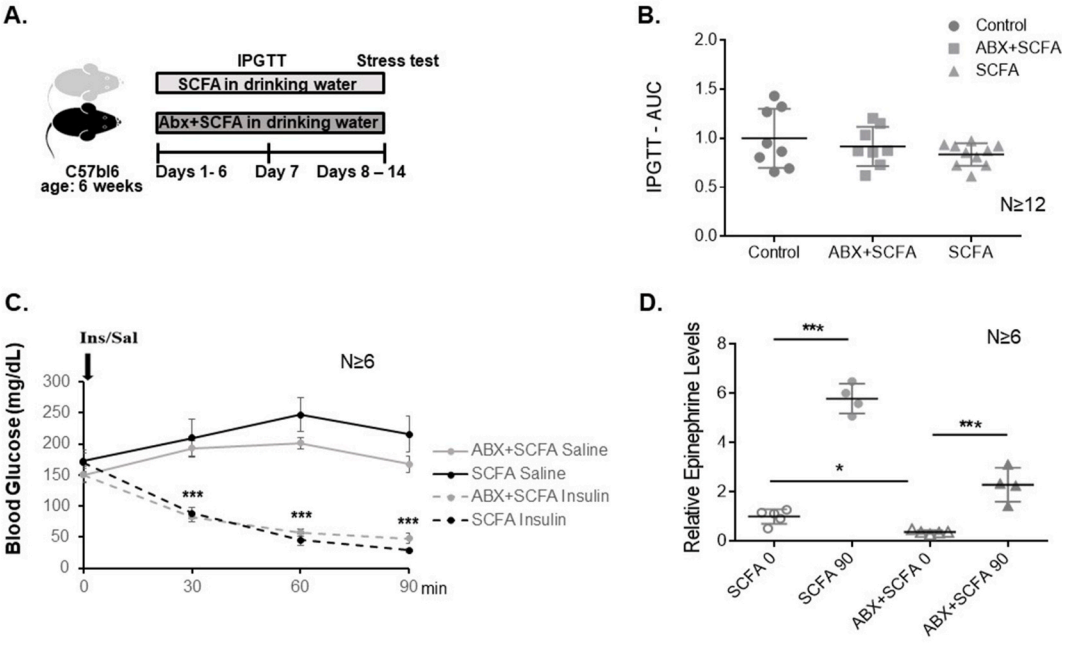

Fig. 3. SCFA supplement improves basal – and hypoglycemia induced urinary epinephrine levels in Abx mice

A: Experimental design: animals were divided randomly into two groups, receiving Abx + SCFA or SCFA alone in the drinking water for 2 weeks.

B: Area under the curves following glucose tolerance test at 7 weeks was determined as described (data are normalized to water control values, taken as 1). The results are presented as mean ± SEM; $n \geq 12$; no significant difference between groups - One Way Analysis of Variance ($DF = 2$, $F = 1.344$; $P = 0.280$).

C: Blood glucose levels during insulin-induced hypoglycemia. Stress test was performed at 8 weeks after 3 h fast. SCFA controls (black lines); Abx + SCFA – grey lines; saline injected – solid lines; insulin treated – broken lines. The blood glucose values (mg/dL) are shown as mean ± SEM, $n \geq 6$ for each group and treatment. Data were analyzed by Two Way Repeated Measures ANOVA, followed by Bonferroni *t*-test. Glucose levels in SCFA Sal controls were not different from Abx + SCFA Sal at any time point, as well as in SCFA Insulin vs. Abx + SCFA Insulin. Injection of Insulin in both, SCFA and Abx + SCFA groups caused significant drop in blood glucose levels at 30, 60 and 90′ compared to 0′ time point (***$P < 0.001$, $t = 4.988$, 7.610 and 8.652; and ***$P < 0.001$, $t = 4.228$. 5.919 and 6.336 resp.).

D: Relative urinary epinephrine levels were determined before (0 min) and after the stress test (90 min) in each experimental group. Results are normalized to the 0′ time point in control (SCFA alone) values (taken as 1) and presented as mean ± SEM, $n \geq 6$. Data were analyzed by Two Way ANOVA followed by all pairwise multiple comparison procedures (Holm-Sidak): SCFA 0′ vs.90 ***$P < 0.001$, $t = 17.346$; Abx + SCFA 0′ vs 90’ ***$P < 0.001$, $t = 7.273$; SCFA vs Abx + SCFA at 0’ *$P < 0.038$, $t = 2.267$; SCFA vs. Abx + SCFA at 90’ ***$P < 0.001$, $t = 12.340$. Symbols indicate individual samples in each experimental group.

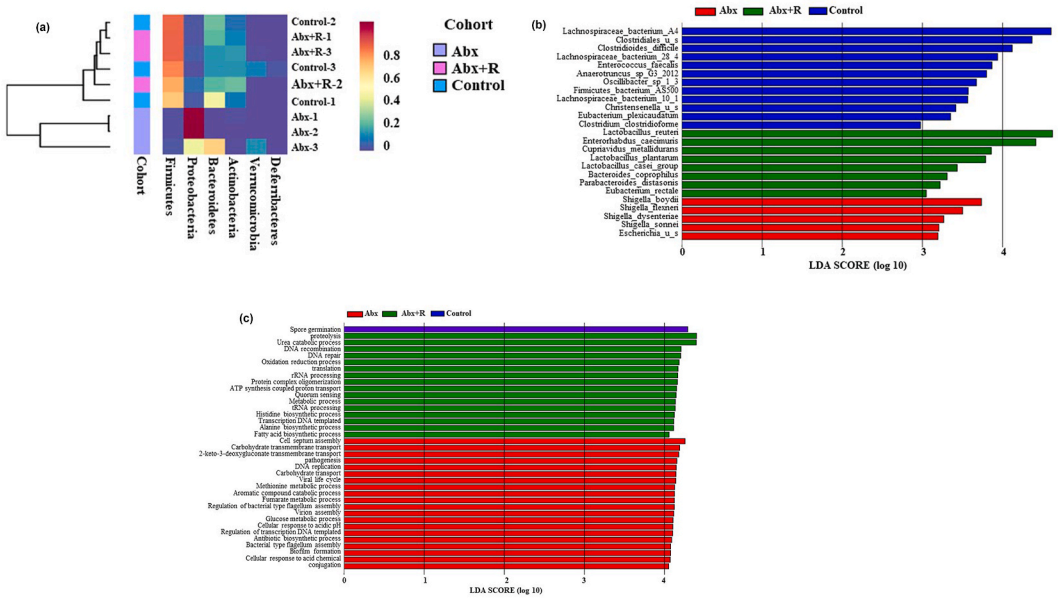

Fig. 4. Fecal microbiome metagenomics – comparison analyses

A: Hierarchically clustered heatmap displaying the relative abundance of bacterial taxa, with 3 pooled samples in each cohort. Bacterial taxa (phylum) are indicated on the bottom, the color gradient key and cohorts are shown on the right of the figure. The heatmap was created using the NMF R package, based on the taxonomic matrices from the CosmosID (Gaujoux and Seoighe, 2010).

B: Differentially abundant taxa (species level) that showed significant differences in relative abundance of bacterial communities in control (blue), Abx (red) and Abx + R (green) cohorts as identified by linear discriminant analysis (LDA) assessed by effect size analysis (LefSe) algorithm. The figure was generated using LefSe tool from Huttenhower lab, based on species matrices from CosmosID analysis. The differentially abundant and biologically relevant features were ranked by effect size after undergoing linear discriminant analysis (LDA, log10 score treshold of 2.0).

C: Predictive functional annotation of bacterial taxa - differential features abundance using GO biological processes as a reference was calculated for control (blue), Abx (red) and Abx + R (green) cohorts. For each bar, the name is the enriched feature and all features shown meet $p \leq 0.05$ for Kruskal-Wallis and Wilcoxin tests, and have an LDA score $\geq$2.0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

water exhibited lower basal glucose levels and faster glucose load clearance (area under the curve AUC 62% lower than in controls, Fig. 1C, ***P $\leq$ 0.001). Of note, even one week of recolonization increased basal blood glucose and the AUC in Abx + R experimental group, but remained 38% lower than control values.

In addition, quantitative measurement of caecal weights at sacrifice (macroscopic hallmark for gut microbiome depletion (Smith et al., 2007) demonstrated a marked increase in animals treated with Abx (Fig. 1D, ***P $\leq$ 0.001). In contrast, following recolonization there was no significant difference between the mean caecal weight values in C and Abx + R group, suggesting microbial community recovery (Fig. 1D).

3.2. *Antibiotics-triggered microbial imbalance causes persistent epinephrine hypo-responsiveness to stress*

Next we investigated the effect of targeted gut microbiome depletion on the counter-regulatory responses to insulin – induced hypoglycemia. Each experimental group was randomly divided into two subgroups: insulin treated and saline injected. The blood glucose levels were monitored during the experiment as described (see methods). Abx group had significantly lower blood glucose levels at baseline, compared to water controls (***P $\leq$ 0.001, Fig. 2A). As expected, injection of saline did not significantly affect the blood glucose levels throughout the experiment in all groups. Following insulin injection, blood glucose levels decreased rapidly in all groups. There was also no significant difference between the magnitude of hypoglycemia achieved in control, Abx and Abx + R (Fig. 2A, data shown for C and Abx).

Plasma corticosterone (hypothalamic/adrenal cortex) and glucagon (parasympathetic reflex arc) concentrations were similar across the experimental groups at 90 min after injecting saline or insulin (Fig. 2B, data shown for Corticosterone in C and Abx). In contrast, the relative urinary epinephrine levels (sympathetic reflex arc) at baseline and 90 min after injecting the insulin were significantly lower in Abx mice than the corresponding values in control animals (Fig. 2C, *P $\leq$ 0.027 and ***P $\leq$ 0.001 resp.), similar to our results in GF mice (Giri et al., 2019). Two weeks recolonization were not sufficient to restore the phenotype (Fig. 2C, Control vs. Abx + R group at 90', ***P $\leq$ 0.001).

Gut microbial depletion includes shifts in microbial composition and production of microbial-derived metabolites such as SCFA (Zarrinpar et al., 2018). The persistent hypo-responsiveness of the sympatoadrenal system to hypoglycemia may stem from an altered microbial ecosystem functionality. We measured the caecal SCFA in the 3 cohorts as an index of the microbiome metabolic activity (Fig. 2D). As expected, Abx group had no detectable caecal SCFA, consistent with microbiome depletion. Recolonization partially recovered the production of acetate, butyrate and propionate, but remained significantly lower compared to control values (*P $\leq$ 0.018; 0.05 and 0.035 resp.).

3.3. *SCFA supplementation during the Abx treatment improves steady state and insulin-induced urinary epinephrine levels*

We next sought to test the hypothesis that oral SCFA supplementation will alleviate the effect of Abx treatment on sympathoadrenal stress responses. In this experiment, mice received Abx cocktail in the drinking water. A mixture of the three principle SCFA (acetate, butyrate and propionate) was added two days after initiation of Abx treatment (Abx + SCFA) as described in methods. Control animals received only SCFA (Fig. 3A). Addition of SCFA to the drinking water of vehicle and Abx-treated animals did not affect daily fluid intake and body weights over the course of the experiment (data not shown). We evaluated the effect of SCFA supplement (with and without Abx) on glucose homeostasis after one week (at 7 weeks of age, Fig. 3B). The area under the curve for both experimental groups (SCFA and Abx + SCFA) group was similar and not significantly different from the water control group. Only fasting

baseline glucose levels were lower in the Abx + SCFA, compared to both, SCFA alone and to water controls (Morrison and Preston, 2016).

After the IPGT test the animals were returned to cages and their assigned treatments continued for another week. The stress test was performed as described before. No significant differences in blood glucose levels were observed between the groups at each time point for saline or for insulin treated mice. The magnitude of insulin-induced hypoglycemia was similar between mice given SCFA alone and animals exposed to both, Abx and SCFA (Fig. 3C). Of note, mice that received Abx + SCFA (microbiome depletion) exhibited significantly larger caecums at sacrifice compared to SCFA alone group (intact gut microbiome) as expected (not shown). Exposure to hypoglycemia resulted in potentiated epinephrine response in the SCFA group (Fig. 3D), compared to water-consuming controls (see Fig. 2C and Table 1, a 6 fold vs. 3 fold induction by stress resp.). Animals receiving Abx + SCFA displayed improved steady state (0.372 vs. 0.217 in Abx, relative to their respective control's values), and insulin-induced urinary epinephrine levels (compared to Abx alone group, Fig. 2C, Table 1 – a 6.4 vs. 4.1 fold increase resp.). These results suggest that gut derived SCFA play a role in regulating stress responsivity in peripheral catecholaminergic pathways – specifically the adrenal medulla as the sole source of circulating epinephrine. It should be noted that our data do not exclude possible involvement of other bacterial metabolites (rev. in (Cryan and Dinan, 2012).

3.4. Antibiotics affect gut microbial composition

To reveal which bacteria are making the molecular messages affecting peripheral epinephrine responses to stress, we performed whole genome shotgun sequence profiling of pooled fecal samples from control, Abx and Abx + R cohorts (see methods). Sample relative abundances for all bacterial phyla, genera, species and strains detected in this study are presented in Supplementary Table 1. As reported by others (Reikvam et al., 2011; Kiraly et al., 2016; Erny et al., 2015; Zarrinpar et al., 2018) treatment of young adult mice with an antibiotic cocktail for two weeks disrupted gut microbial composition. Comparison analysis revealed decreased alpha diversity in Abx cohort as indicated by Shannon index (a measure of species evenness and richness) compared to control and Abx + R group (Supplemental Fig. 1A). The reduction in bacterial diversity in Abx mice was mainly attributed to enrichment of Proteobacteria (common marker of gut microbiome depletion (Shin et al., 2015), with a parallel reduction in the abundance of Firmicutes and Bacteroidetes thus confirming the efficacy of antibiotic treatment (Fig. 4A). Abx treatment also altered fecal bacteria beta diversity (Bray-Curtis, Supplemental Fig. 1B) as reported before. PCoA (principal coordinate analysis) demonstrated clear separation of bacterial communities after 2 weeks Abx treatment. Of note, no differences were found between control and Abx + R cohorts. Shown on Fig. 4B is the Linear Discriminant Analysis Effect Size (LefSe) for all three cohorts. At the bacterial species level, the gut microbiome changes in Abx mice were associated with significantly increased relative abundance of members of the Proteobacteria - from genus Shigella (Shigella boydii, Shigella flexnei, Sigella dysenteriae and Shigella sonnei) and Escherichia. In control group significantly more abundant were 12 species, belonging to phylum Firmicutes, class Clostridia (10 – Lachnospiraceae bacterium A4; Clostridiales u_s; Clostridioies difficile; Lachnospiraceae bacterium 28_4; Anaerotruncus G3_2012; Oscillibacter 1_3; Lachnospiraceae bacterium 10_1; Christensenella u_s; Eubacterium plexicaudatum; Clostridium clostridioforme), class Bacilli (Enterococcus faecalis) and class Firmicutes (Firmicutes bacterium AS500). When Abx treatment was followed by recolonization (Abx + R cohort), 8 species were most abundant including Lactobacillus reuteri; Lactobacillus plantarum and Lactobacillus casei (Firmicutes, class Bacilli); Eubacterium rectale (Firmicutes, Clostridia); Bacteroides coprophilus and Parabacteroides distasonis (Bacteroidetes, Bacteroidia); Enterorhabdus caecimuris (Actinobacteria, Coriobacteria) and Cupriavidus metallidurans (Proteobacteria, Betaproteobacteria).

To explore further the compositional bacterial changes, potentially affecting the peripheral epinephrine responses to stress, we performed a significant difference comparison between control and Abx + R cohorts (Fig. 5A). Out of nine microbes enriched in the control cohort, seven belonged to phylum Firmicutes (Lachnospiraceae bacterium A4, 28–4 and 10–1; Christensenella, Anaerotruncus, Clostridium clostridioforme and Firmicutes bacterium ASF300), and two to Bacteroidetes (Bacteroides xylanisolvens and Bacteroides fragilis). Most of them produce butyrate/SCFA (rev. in (Vacca et al., 2020; Daniel et al., 2017; Chassard et al., 2008; Despres et al., 2016; Waters and Ley, 2019).

Given that in microbial ecosystems the link between community taxonomic composition and metabolic response is not direct (Moya and Ferrer, 2016) we also screened for changes in overall predicted functional output in our experimental groups (Fig. 4C) and more specifically between control and Abx + R cohorts (5B). Shown are the LefSe calculated results for GO "biological processes". Of note, the listed enriched features in Abx cohort (see names of the bars) are characteristic only for mice with depleted gut microbiome (Fig. 4C), while 10 biological processes in the Abx + R cohort (Fig. 5B) were significantly different from the control and Abx group.

4. Discussion

We demonstrated that oral Abx-induced gut microbiome depletion specifically impairs tonic and stress-induced epinephrine release (via the SAM system) in young adult mice without affecting glucocorticoid secretion (HPA axis) or parasympathetic responses (glucagon). This suggests that ongoing interactions along the gut-brain axis are essential for peripheral (and perhaps central) catecholaminergic functions in response to acute hypoglycemic stress whether in GF (Giri et al., 2019) or mature colonized animals. While recolonization by co-housing with age-matched controls increased the bacterial diversity and richness to control values, the SAM stress axis remained hyporesponsive along with reduced caecal butyrate, propionate and acetate levels. Although the precise mechanism(s) of how gut microbiota affects the

Table 1
SCFA supplement potentiated the epinephrine responses to hypoglycemia in control and Abx mice.

Group/Treatment	Control		Abx		Control + SCFA		Abx + SCFA	
	Baseline Ins		Baseline Ins		BaBaseline Ins		Baseline Ins	
Time (Min)	0′	90′	0′	90″	0′	90′	0′	90′
Epi **(Mean + SEM)**	1.0 ± 0.14	**3.0** ± 0.42	**0.22** ± 0.03	**0.9** ± 0.13	**1.0** ± 0.10	**5.8** ± 0.23	**0.37** ± 0.04	**2.39** ± 0.28
Fold Increase:		3.0x		4.1x		5.8x		6.5x

Mean ± SEM of relative urinary Epinephrine levels are presented for the four experimental groups: Control; Abx; SCFA and Abx + SCFA, (n ≥ 6 per group). The samples were collected before (0 time point, baseline) and 90 min after injecting insulin (2 IU/kg regular human insulin Humulin R, Eli Lilly). Catecholamine levels were normalized to creatinine content in each sample, and 0 time point of respective control values taken as 1. Statistics: Two Way Analysis of Variance and Holm-Sidak all pairwise multiple comparison method were used: Control 0′ vs. 90' ***P 0.001, t = 7.136; Abx 0′ vs. 90' *P ≤ 0.034, t = 2.177; Control 0′ vs. Abx 0' *P < 0.027, t = 2.715; Control 90′ vs. Abx 90′ P < 0.001, t = 6.861 (see Fig. 2C); SCFA 0′ vs. 90 ***P < 0.001, t = 17.346; Abx + SCFA 0 vs 90' ***P < 0.001, t = 7.273; SCFA vs Abx + SCFA at 0' *P < 0.038, t = 2.267; SCFA vs. Abx + SCFA at 90' ***P < 0.001, t = 12.340 (Fig. 3D).

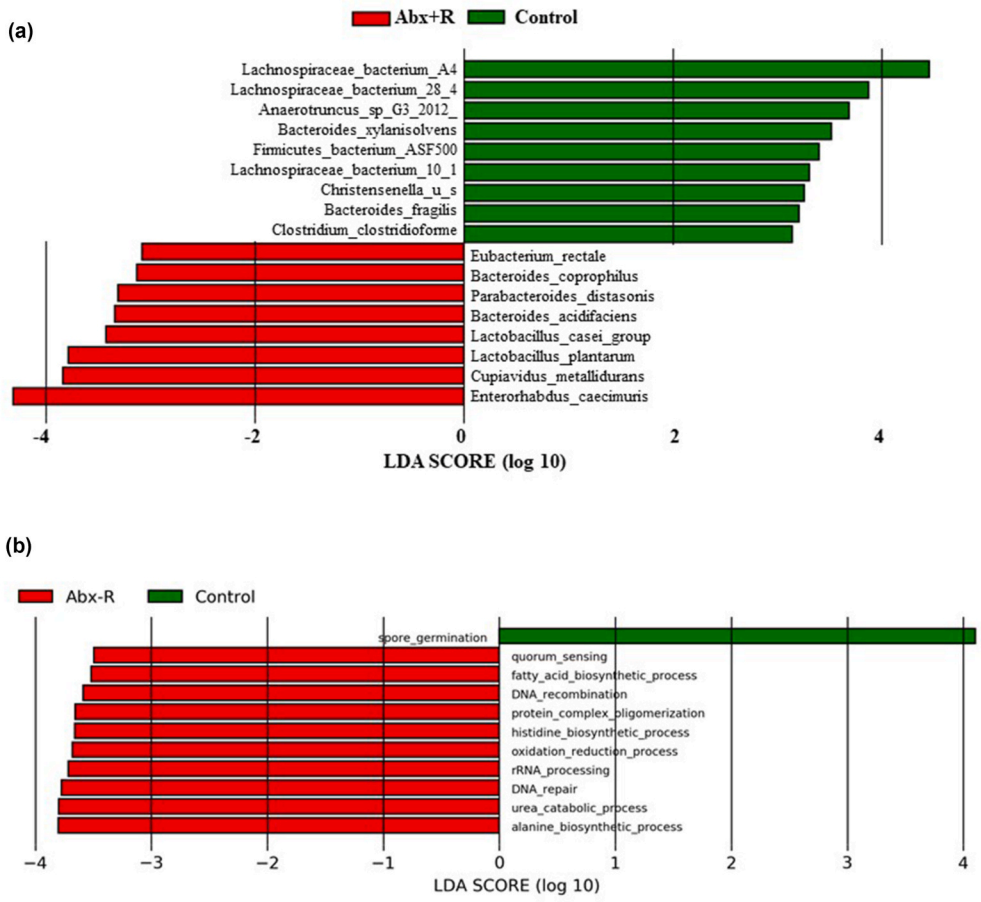

Fig. 5. Co-housing does not restore gut bacterial community on species level
A: Differentially abundant taxa associated with stress response (species level) that showed significance in relative abundance between control (green) and Abx + R (red) cohorts, as identified by LefSe.
B: Differential features abundance (GO biological processes) calculated for control (green) and Abx + R (red) cohorts. For each bar, the name is the enriched feature and all features shown meet $p \leq 0.05$ for Kruskal-Wallis and Wilcoxin tests, and have an LDA score ≥ 2.0 or ≤ -2.0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

sympathoadrenal responses to stress remain to be proven, we demonstrated here that supplementation with bacterial fermentation products (SCFAs: acetate, propionate, butyrate) partially restored the phenotypes (baseline and stress-induced epinephrine release) even when bacteria-derived SCFAs were absent. Furthermore, we identified several bacterial taxa with significantly decreased relative abundance in the Abx + R cohort, which belong to phyla Firmicutes and Bacteroidetes - producers of SCFAs (Lachnospiraceae bacterium A4, 28–4 and 10–1; Christensenella u.s., Anaerotruncus sp. G3 2012, Clostridium clostridioforme, Firmicutes bacterium ASF500, Bacteroides xylanisolvens and Bacteroides fragilis). These species were also missing in the Abx only group implicating them and their SCFA metabolites in the attenuated SAM-specific neurohumoral response.

While this is the first study to directly examine the role of the gut microbiome in peripheral SAM hypoglycemic stress-responses in mature animals, the use of antibiotics to disrupt the microbial composition in adult animals and systemic physiology has been described before (Frohlich et al., 2016; Bercik et al., 2011; Gacias et al., 2016; Mohle et al., 2016; Guida et al., 2018). To separate altered microbiome effects from potential confounding exposures to handling stress of daily forced feeding, injections or other systemic influences, Abx were provided in the drinking water. This approach allowed for maintaining normal environmental conditions for all animals as reflected in typical gains in body mass (weight) and similar water consumption (Kiraly et al., 2016). We choose a broad-spectrum Abx cocktail given for two weeks to achieve an overall microbiome depletion based on prior reports (Kiraly et al., 2016; Sampson et al., 2016; Reikvam et al., 2011) before attempting to identify possible molecular mechanism(s) affecting peripheral responses to stress. We also validated successful microbiome depletion using several independent criteria:

1) Fecal WGSS data (Fig. 4A) demonstrated severely disrupted microbial composition and diminished taxonomic diversity following exposure to Abx.
2) We quantified caecal weights at sacrifice which were significantly enlarged following Abx exposure (Fig. 1D); a macroscopic hallmark for gut microbiome depletion and a pathognomonic phenotype of GF mice, (Kiraly et al., 2016; Reikvam et al., 2011; Simpson et al., 2020; Erny et al., 2015; Smith et al., 2007).
3) We examined production of microbiome-derived metabolites and found no SCFAs detected in the caecum of Abx mice (Fig. 2D) as reported before by others (Zarrinpar et al., 2018).
4) An intraperitoneal glucose tolerance test was performed at 7 weeks for all experimental groups, which revealed significantly altered glucose homeostasis in Abx mice (Fig. 1B and C, (Zarrinpar et al., 2018, see Schroeder and Backhed, 2016).

The effects of acute metabolic stress such as insulin-induced hypoglycemia are well documented in animal models and in humans (Kvetnansky et al., 2009). Falling blood glucose levels are detected in the central nervous system, which triggers a hormonal and autonomic nervous system-mediated release of counter-regulatory hormones (i.e. epinephrine, glucagon, cortisol and growth hormone) to restore euglycemia (Verberne et al., 2014). Similar to our recently published results in GF mice (Giri et al., 2019) we did not find altered function of the HPA stress axis in microbiome-depleted mice as there were no significant differences in hypoglycemia-induced plasma corticosterone levels across the experimental groups (Fig. 2B). In contrast, under different experimental conditions - acute (Vagnerova et al., 2019; Sudo et al., 2004) or chronic restraint stress (Huo et al., 2017), GF mice displayed exaggerated corticosterone responses to stress. Of note, exposure to

ether stimulus did not result in significant differences in plasma ACTH or corticosterone between the experimental groups (Sudo et al., 2004). The data are consistent with the hypothesis that the dysregulation of the HPA axis (corticosterone responses) in the absence of microbiome is stressor-specific: evident during restraint stress (Vagnerova et al., 2019; Sudo et al., 2004; Huo et al., 2017) but not during hypoglycemia (Giri et al., 2019, current report) or exposure to ether (Sudo et al., 2004).

Our data are in agreement with other studies utilizing a prolonged course of antibiotics in the drinking water to deplete the microbiome without a significant effect on peak (corticosterone) stress hormone levels in plasma (Kiraly et al., 2016). Lack of a microbiome did not alter the release of glucagon in GF mice (Giri et al., 2019) or in the current study, consistent with intact parasympathetic signaling. In contrast to HPA and parasympathetic effects, gut microbiome depletion significantly reduced baseline and insulin-stimulated urinary epinephrine levels (Fig. 2C) in young adult animals, similar to our previously reported data in GF mice (Giri et al., 2019). Taken together, these results underscore the specificity of gut microbiome depletion in selectively altering the SAM stress axis.

Importantly, even after two weeks recolonization (Abx + R), epinephrine responses remained impaired offering the possibility of a worrisome repercussion that similar effects could occur in humans after antibiotic exposure. Support for these concerns arises from failed physiologic recovery subsequent to prolonged antibiotics for a range of systemic processes (i.e. adiposity, insulin resistance, cognitive function (Cho et al., 2012; Cox et al., 2014; Hwang et al., 2015; Frohlich et al., 2016). The overarching issue is that prolonged antibiotic treatment has the potential to effect a sustained alteration in adaptive physiology well beyond its therapeutic use arising from an indolent persistence of intestinal microbiome alteration as reported in other animal studies (Buffie et al., 2012), in "humanized" flora mouse models (Ng et al., 2019) and in humans (Jernberg et al., 2007).

Given that the gut microbiome produces a large number of diffusible small molecules which affect their host and/or neighboring microbes, we measured caecal SCFA concentration as an index of bacterial metabolic activity in each group (Fig. 2D). In agreement with other reports (Zarrinpar et al., 2018), Abx treatment resulted in a complete disappearance of SCFA from the caecal samples indicating successful gut sterilization (Fig. 2D). Curiously, when compared to control mice, animals who underwent two weeks of recolonization (Abx + R) still had significantly reduced acetate, propionate and butyrate levels, attesting altered metabolic activity following a sustained specific modification of the bacterial community.

Next, we endeavored to carry out a series of experiments supplementing SCFA in the drinking water of antibiotic-treated animals (Fig. 3A). SCFAs acetate, propionate, and butyrate are the primary bacterial breakdown products of non-digestible dietary fibers in the large intestine and serve as key mediators of the beneficial effects of the gut microbiome (Chambers et al., 2018). Addition of SCFA to the drinking water during the Abx treatment (Abx + SCFA) reversed the effect of microbiome depletion in the IPGTT test (Fig. 3B) with no difference in the glucose area under the curve values compared to water control and SCFA alone groups; all suggestive of a role for SCFAs in recovery of glucose homeostasis (rev. in Chambers et al., 2018; Chambers et al., 2015). Importantly, SCFA supplement significantly improved the epinephrine responses to hypoglycemia in both, SCFA alone and Abx + SCFA experimental groups (Fig. 3D, Table 1). These results support the contention, that SCFAs are a class of small molecule physiological mediators of microbiota-gut-brain axis crosstalk (rev. in (Dalile et al., 2019; Dalile et al., 2020) affecting not only certain aspects of the HPA axis (Sudo et al., 2004; Kiraly et al., 2016) but also SAM responses to stress (current study, Giri et al., 2019).

We also observed significant interaction between depletion of gut microbiome in young adult conventional mice and the ability to respond to metabolic stress. The drastic changes in bacterial composition in Abx mice were primarily a shift to Proteobacteria (a common marker of microbiome depletion) and a significant reduction in the abundance of Firmicutes and Bacteroidetes (Fig. 4A, Shin et al., 2015). Lower abundance of these last two phyla was associated with a previously recognized decrease in circulating short-chain fatty acids (Fig. 2D, Louis and Flint, 2017). Importantly, our metagenomics analysis identified differences in several other bacterial species with reduced relative abundance in the Abx + R cohort (Figs. 4B and 5A) compared to either controls or Abx alone. All differences in microbiome composition resided in strict anaerobes and belonged to the same two major microbial phyla: Firmicutes (class Clostridia – 6 members, and class Firmicutes −1) and Bacteroidetes. The reduced relative abundance of Clostridia species is significant as these organisms ferment enteral polysaccharides to SCFA (butyrate – essential fuel for colonocytes, and acetate) and ethanol (Lopetuso et al., 2013). Gastrointestinal Bacteroidetes species produce succinic acid, acetic acid, and in some cases propionic acid, as major end-products of fermentation (Chassard et al., 2008; Despres et al., 2016).

Other microbiome changes included an antibiotic-induced reduction of members of the Lachnospiraceae family (L. bacterium A4, 28–4 and 10–1) which is of interest as they are functionally linked to obesity (Turnbaugh et al., 2006, 2009), and protection from colon cancer in humans (Meehan and Beiko, 2014; Daniel et al., 2017). The recently described bacterial family Christensenellaceae is one of the five taxa considered a signature of healthy gut and its relative abundance in the normal gut is associated with human longevity, and inversely correlated with body mass index and metabolic syndrome (rev. in Waters and Ley, 2019). Firmicutes bacterium ASF500 are among the bacterial strains able to cause robust induction of T helper 17 cells (Atarashi et al., 2015), critical in protecting mucosal surfaces against microbial pathogens, in concert with other immune cells (Omenetti and Pizarro, 2015), and in protecting the integrity of intestinal barrier to bacteria (Stockinger and Omenetti, 2017).

Testing every potential mechanism of how various gut microbiome/microbial candidate metabolites specifically affect the SAM stress axis was beyond the scope of this proof-of-principle study. However, the physiologic relevance of these observations in mature animals is amplified by our prior work revealing that the diet-derived SCFA butyrate and structurally related small molecules (having the motif CH3–CH2-R) increase tyrosine hydroxylase mRNA (TH) and TH protein, either *in vitro* (Mally et al., 2004; Patel et al., 2005; Shah et al., 2006; D'Souza et al., 2009) or *in vivo* (Nankova et al., 2014). In addition to its well-recognized ability to cause chromatin remodeling and histone hyperacetylation, the transcriptional activation of TH by butyrate is mediated by several mechanisms. These include activation of PKA/cyclic AMP second messenger and MAP Kinase systems (Decastro et al., 2005; Shah et al., 2006) and induction of transcription factors, interacting with the CRE and a novel enhancer (5′GCCTGGC., Patel et al., 2005) in the promoter of TH gene. Among the pleiotropic effects of butyrate is also its ability to regulate post-transcriptionally the decay of specific mRNAs (including TH mRNA, Parab et al., 2007; Aranyi et al., 2007) in a concentration-dependent manner. Based on this and prior results (Giri et al., 2019), we propose an evolutionary role for neonatal acquisition of the maternal microbiome and fermentation of carbohydrate-rich breast milk in postnatal adaptation (Nankova et al., 2014). Augmented catecholamine availability would ensure postnatal survival by aiding in responses to hypoxia, hypoglycemia, cold, blood loss or even maternal bonding (Slotkin and Seidler, 1988) that now appears to be relevant even in mature animals. Further evidence for this augmented survival premise is the long recognized fact that SCFAs not only serve as a local energy substrate for colonocytes, but also directly modulate host health through a range of tissue-specific mechanisms related to gut (Peng et al., 2009) and brain barrier function (Brahe et al., 2013; Koh et al., 2016; Braniste et al., 2014), glucose homeostasis (Greiner and Backhed, 2011; De Vadder et al., 2014), immunomodulation, appetite regulation and obesity (Byrne et al., 2015).

While the goal of this study was to test for proof-of-concept evidence,

several limitations of the report should be considered:

1) Two weeks recolonization may be too short a time for microbiome recovery to normal bacterial composition after Abx treatment to reestablish physiological functions;
2) Mice were not cannulated, and glucose levels were not clamped during hypoglycemia thus increasing variability in the counter regulatory hormone signal – nevertheless, we observed identical overall responses as in prior work;
3) Our study was a "snapshot" with only one time point examined (90 min after insulin injection), which may not be optimal for evaluating maximal changes in plasma responses for corticosterone and glucagon;
4) Combination of the three most abundant SCFAs were given as a supplement – the effect of individual SCFAs or other microbial metabolites was not examined. Future studies will necessarily require more detailed metabolomic analysis of specific gut byproducts;
5) Due to the size of the animals, we experienced a limited quantity of samples so pooled data per cohort for whole genome sequencing and functional output were analyzed.

5. Conclusions

The marked gut microbiome depletion generated by antimicrobial exposure revealed a crucial role of gut commensal microbes and their metabolites SCFA in sympathoadrenal epinephrine response to acute hypoglycemia in adult mice. Although a restoration of microbial diversity and richness was observed after recolonization, the tonic and stress-induced catecholaminergic responses remained attenuated, associated with significant differences in particular microbial relative abundances and reduced caecal acetate and butyrate. The beneficial effect of oral SCFA supplementation supports their likely contribution to stress-induced epinephrine release probably by a variety of mechanisms including the reported enhancing effects on catecholamine biosynthesis.

Failed recovery of endogenous catecholaminergic stress-responsiveness after prolonged antibiotic exposure raises concern that sustained microbiome depletion will interfere with homeostatic adaptive responses in patients exposed to common intensive care stressors (e.g. hypoxemia, hypotension/hypovolemia, hypoglycemia, cold, sepsis) thus inadvertently contributing to morbidity and mortality. Dietary supplement with microbial-derived metabolites may preserve the functional phenotype and improve clinical outcomes.

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CRediT author contribution statement

Edmund LaGamma designed the study and revised the manuscript with input from all authors. **Furong Hu** conducted the animal experiments, performed data acquisition and hormonal analyses. **Fernando Pena Cruz** performed data acquisition and hormonal analyses. **Philip Bouchev** performed hormonal analyses. **Bistra Nankova** designed the study, conducted animal experiments, analyzed the data, prepared figures and wrote the manuscript with input from all authors.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

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