J Appl Physiol 135: 1348–1359, 2023.
First published October 26, 2023; doi:10.1152/japplphysiol.00368.2023

american physiological society

JOURNAL OF APPLIED PHYSIOLOGY

RESEARCH ARTICLE

Vancomycin modestly attenuates symptom severity during onset of and recovery from exertional heat stroke in mice

Kentaro Oki,[1] Jermaine A. Ward,[1] Shauna M. Ward,[1] Mark L. Plamper,[1] Chloe G. Henderson,[1,2] Thomas A. Mayer,[1] Aaron R. Caldwell,[1,2] and Lisa R. Leon[1]

[1]*Thermal and Mountain Medicine Division, United States Army Research Institute of Environmental Medicine, Natick, Massachusetts, United States and* [2]*Oak Ridge Institute of Science and Education, Oak Ridge, Tennessee, United States*

Abstract

Increased intestinal permeability during exertion and subsequent leakage of bacteria into circulation is hypothesized to accelerate exertional heat stroke (EHS) onset and/or exacerbate EHS severity. To provide proof of concept for this theory, we targeted intestinal microbiota via antibiotic prophylaxis and determined whether vancomycin would delay EHS onset and/or mitigate EHS severity and mortality rates using a mouse model of EHS. Mice were *1*) designated as EHS or Exercise Control (ExC) and *2*) given 7 days of vancomycin (VEHS, VExC) or untreated water (EHS, ExC) before EHS/Exercise. Following EHS/ExC, mice were euthanized immediately (0 h) or returned to their home cage (25°C) and euthanized after 3 h or 24 h. VEHS mice exhibited reduced abundance and altered composition of fecal bacteria (with notable decreases in genera within orders *Clostridiales* and *Bacteroidales*); increased water consumption, lower core temperature (T_C) before and during heating (T_{CMax}), lower circulating markers of organ damage and inflammation at 24 h; and reduced hepatic activation of stress pathways at 0 and 3 h compared with EHS mice. Vancomycin-induced alterations to the intestinal microbiota likely influenced EHS outcomes, but it is unconfirmed whether this is due to attenuated bacterial leakage into circulation or other (in)direct effects on physiology and behavior (e.g., decreased T_C, increased water consumption). To our knowledge, this is the first study quantitating antibiotic effects in conscious/unanesthetized, *exertional* HS animals.

NEW & NOTEWORTHY Vancomycin prophylaxis lowered core temperature before and during EHS, mitigated EHS-associated rise of hepatic biomarkers and cytokines/chemokines in circulation (particularly at 24 h), and corresponded to inhibited phosphorylation of hepatic c-Jun NH_2-terminal kinase on Threonine 183/Tyrosine 185 at 0 and 3 h in conscious, unanesthetized mice. However, vancomycin also induced cecal enlargement suggesting its off-target effects could limit its utility against EHS.

antibiotic; exertional heat stroke; mouse

INTRODUCTION

Exertional heat stroke (EHS) is a serious condition that arises from exertion in a temperate to hot environment. In contrast to classic heat stroke (CHS, also known as passive heat stroke), EHS results from an active component (i.e., exercise/exertion) with high endogenous thermogenesis and insufficient heat dissipation from the body resulting in hyperthermia. Consequently, CHS predominantly affects the very young and the elderly—individuals who cannot easily thermoregulate and/or may have underlying health issues—whereas EHS typically affects young, active individuals who are otherwise healthy (1). Laborers/farm workers, athletes, and military personnel are particularly vulnerable to EHS onset (2–5).

Aside from environmental temperature and exertion, release of intestinal microbiota into circulation may also contribute to EHS risk or severity. This theory, known as the "Dual Pathway Model of Heat Stroke" (6), asserts that hyperthermia and/or exercise disrupt tight junctions between epithelial cells in the intestines, leading to increased intestinal permeability and subsequent release of bacteria into circulation, specifically lipopolysaccharide (LPS; also referred to as endotoxin), a component in the walls of Gram-negative bacteria. Circulating bacteria then initiate an inflammatory response that may accelerate EHS onset and/or exacerbate EHS symptoms. In humans, LPS levels positively correlate with EHS or EHS-associated symptoms (7–11). In animal studies, hyperthermia increases intestinal permeability and damage in anesthetized rats (12) and increases endotoxin leakage in anesthetized primates with the effects most pronounced above 43°C (13). This effect is also present and reversible in an in vitro tissue model (14). Furthermore, prophylactic antibiotics and antagonists directed against intestinal flora or LPS specifically mitigate heat stroke severity in anesthetized or restrained rabbits, rats, and primates (15–19). However, this has not been confirmed in an *unanesthetized/conscious*, *exertional* heat stroke paradigm.

The purpose of this study was to determine whether antibiotic prophylaxis would alter intestinal microbiota and improve outcomes in a previously established unanesthetized

Correspondence: K. Oki (kentaro.oki.civ@health.mil).
Submitted 9 June 2023 / Revised 2 October 2023 / Accepted 19 October 2023

8750-7587/23 Published by the American Physiological Society.

EHS mouse model (20). We tested whether the antibiotic vancomycin would alter EHS onset and severity with comparisons focusing on untreated (EHS) and vancomycin-treated EHS (VEHS) groups. Although LPS originates from Gram-negative bacteria (21) and vancomycin is typically effective against Gram-positive bacteria (22), vancomycin was used to test the underlying principle that independent of specific bacterial serotype, reduced microbiota in intestines/circulation could be effective against EHS. We expected improved outcomes would entail *1*) delayed EHS onset, *2*) faster recovery following EHS, and/or *3*) a less severe EHS phenotype (e.g., circulating biomarkers, mortality). Bacterial compositions were analyzed in fecal samples to measure antibiotic treatment effects on intestinal microbiota. In addition, to understand potential vancomycin effects on EHS severity in organs particularly vulnerable to EHS damage, we measured hepatic c-Jun NH_2-terminal kinase (JNK) phosphorylation in EHS and VEHS mice. JNK is a kinase in the family of mitogen-activated protein kinases (MAPKs) that is active during development and immune responses with a role in regulating cell survival and apoptosis. It is also responsive to stress and is activated by osmotic stress, UV exposure, metabolic stress, and inflammation (23). The liver is heavily impacted by acute and long-term EHS symptoms (24–26), and we have previously observed that hepatic JNK phosphorylation may correspond to heat illness severity (20).

MATERIALS AND METHODS

Ethical Approval

All procedures were approved by the Institutional Animal Care and Use Committee (Protocol 20-26-A; approved 12/21/2020). Investigators adhered to the *Guide for the Care and Use of Laboratory Animals* in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

Animals

Experimental timeline (Fig. 1) for male C57BL/6J mice (6–8 wk old, average 24 ± 1.6 g, Jackson Laboratories, Bar Harbor, ME) and details of their care/housing are similar to what we have previously described (20). Vancomycin-treated mice received antibiotics (2 g/L) in their drinking water for 7 days before testing, consistent with a prior study (27).

Radiotelemetry Transmitter Implantation

As described previously (20, 28), mice were intraperitoneally implanted with a disinfected and saline-rinsed radiotelemetry transmitter (1.1 g, model G2 Emitter; Starr Life Sciences Corp., Inc., Oakmont, PA) under isoflurane anesthesia and a subcutaneous injection of slow-release buprenorphine (0.05 mg/kg). Once implanted, body core temperature (T_C; ±0.1°C) and activity were continuously recorded at 60-s intervals on the VitalView system (Starr Life Sciences Corp., Inc., Oakmont, PA; Fig. 1*A*). After 7–14 days of recovery, mice underwent forced running in motorized running wheels (model 80840; Lafayette Instrument, Lafayette, IN) within an environmental chamber (model 3950; Thermo Forma, Marietta, OH) maintained at 25 ± 2°C, ~30% RH for 1 h starting at 3.0 m/min and increasing 0.5 m/min every 10 min. On completion, mice were returned to their home cages for at least one wash-out day before testing.

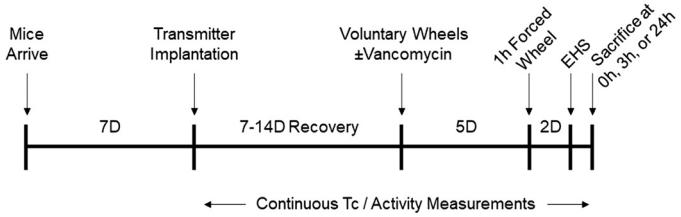


Figure 1. Schematic showing the timeline of procedures beginning from animal arrival to euthanasia. On arrival, mice were allowed to acclimate to their new environment for at least 7 days and then surgically implanted with telemetry transmitters. Once implanted, T_C and activity counts were measured continuously until euthanasia. After 7–14 days of recovery, voluntary wheels were placed into each mouse's home cage, and half of the mice began vancomycin treatment. After 5 days, all mice were placed in the chambers with motorized running wheels at 25°C, subjected to forced running for 1 h, and returned to their home cages for 2 days. Vancomycin treatment continued until the exertional protocol. On the day of the exertional protocol, half of the untreated mice were subjected to exertional heat stroke (EHS) or normothermal exercise (ExC), and half of the vancomycin-treated mice were subjected to EHS (VEHS) or normothermal exercise (VExC). Specimens were subsequently euthanized at 0, 3, and 24 h.

Testing Groups and Protocol

Procedures done and equipment used were as previously described (20). In brief, once baseline T_C was ≤36.5°C between 0600 and 1000 h, EHS mice were removed from their home cages, weighed, and placed in motorized running wheels inside of incubators. Mice rested in the motorized wheels at 30% humidity whereas T_{Env} inside the incubator reached 37.5 ± 0.2°C (~45 min), at which point, the forced running protocol was initiated. Starting at 3.0 m/min, wheel speed was increased by 0.5 m/min every 10 min; once wheel speed reached 8.5 m/min, this speed was maintained until mice collapsed. EHS mice were then removed from the wheel and placed in another compartment of the chamber at 37.5°C for 20 min. This was an operational step to increase symptom severity and mortality rate to ~25%; previous studies lacking this step resulted in 100% survival, which prevented evaluation of treatment or intervention effects on mortality during heat illnesses. Each EHS mouse was paired to an Exercise Control (ExC) mouse that underwent an identical protocol at T_{Env} = 25 ± 2°C. Total numbers for treatment groups were as follows: EHS = 48; VEHS = 47; ExC = 36; VExC = 39. Due to euthanasia at three different time points, some mortality, and to ensure comparisons were adequately powered, relatively high numbers of animals were tested. Mice were either euthanized immediately (0 h) or allowed to recover undisturbed at T_{Env} = 25 ± 2°C and provided food and water ad libitum in their home cages until sample collection at 3 or 24 h postprotocol. Premature mortality was defined as not surviving to the designated euthanasia timepoint. Combining all specimens for the three separate timepoints of euthanasia, 5/47 VEHS and 10/48 EHS specimens died before planned euthanasia; none of the ExC (0/36) and VExC (0/39) mice died before planned euthanasia.

Blood and Organ Collection

Under isoflurane anesthesia, blood was collected via cardiac puncture and immediately transferred to 500-μL ethylenediaminetetraacetic acid (EDTA)-coated and 200-μL lithium heparin-coated microcentrifuge tubes and placed on ice.

Liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST), metabolic indicators of renal health blood urea nitrogen (BUN) and creatinine, and a potential indicator of hepatic and renal health albumin (ALB) were determined using a Vetscan VS2 Chemistry Analyzer (Abaxis, Union City, CA). Following exsanguination, animals were euthanized, and the liver was excised, rinsed with cold 0.9% saline, snap-frozen in liquid nitrogen, and stored at −80°C.

Plasma Cytokine and Chemokine Measurements

Plasma was separated by centrifugation (4°C; 10 min, 1,000 *g*) and stored at −80°C until analysis. Cytokines—interleukins-1 β (IL-1β), -6 (IL-6), -10 (IL-10), and -12 subunit p40 (IL-12p40), granulocyte-colony-stimulating factor (G-CSF), and tumor necrosis factor α (TNF-α)—and chemokines—interferon γ-induced protein 10 (IP-10 or CXCL10), keratinocytes-derived chemokine (KC or CXCL1), macrophage inflammatory proteins 1-α (MIP-1α or CCL3), 1-β (MIP-1β or CCL4), and 2 (MIP-2 or CXCL2), and regulated on activation, normal T cell expressed and secreted (RANTES or CCL5)—were determined using a MILLIPLEX MAP Mouse Cytokine/Chemokine Panel 12-Plex (Millipore, Burlington, MA) on a Bio-Plex 200 system (Bio-Rad, Hercules, CA; 7–14 mice/group).

Plasma ELISA Assays

Plasma ELISAs measured intestinal fatty acid-binding protein 2 (iFABP2) and D-dimer (both Cloud-Clone, Katy, TX) and lipoteichoic acid (LTA; MyBioSource, San Diego, CA) in mouse plasma samples. Circulating iFABP2 indicates intestinal enterocyte damage, D-dimer indicates a major coagulation event, and LTA is a component of Gram-positive bacteria.

Lipopolysaccharide

Plasma samples were processed by Associates of Cape Cod Incorporated to measure LPS content in EHS mice exhibiting the most severe symptoms.

Western Blotting

Livers were homogenized and prepared as previously described (20). Equal amounts of protein from lysates as determined by BCA assay were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and confirmed using MemCode protein stain (Thermo Fisher). Membranes were blocked at room temperature (RT) and incubated in primary (1:1,000 in TBST + 1% BSA) and secondary antibodies (LI-COR donkey anti-rabbit 926–32213—1:10 K in TBST). Protein bands were visualized using fluorescence imaging (LI-COR Odyssey, Lincoln, NE) and quantitated via densitometry on ImageJ (NIH, Bethesda, MD). Results were expressed as bands normalized to pre-block MemCode staining. Phosphorylated protein intensities were expressed with total protein signal as a covariate. Primary antibodies were directed against phosphorylated [Threonine (Thr)183/Tyrosine (Tyr)185] and total c-Jun NH_2-terminal kinase (JNK; Cell Signaling Technology—CST—Danvers, MA: 9252 and 4668). Due to no mortalities in ExC and VExC mice, hepatic JNK signal analysis focused on EHS compared with VEHS.

Calculations

Percent dehydration was calculated as previously described (29). In addition, water consumption from the night before EHS/ExC was calculated for a subset of specimens. Maximum T_C (T_{CMax}) and minimum T_C (T_{CMin}) in EHS mice were typically observed during passive heating and ~3 h post-EHS, respectively. Thermal load (°C·min; measured as thermal area) was also calculated as previously described (29). Hypothermia duration was defined as total time (min) when $T_C < 34.5$°C (30).

Intestinal Microbiota

Fecal samples ($n = 7$/group, all 4 groups) were collected on completion of the exertional protocol (though not controlled for when mice passed the sample during exertion) and stored at −80°C until processed and analyzed by ZymoBIOMICS (Zymo Research, Irvine, CA). DNA was extracted using a 96 MagBead DNA Kit (Zymo Research). Bacterial 16S ribosomal RNA gene amplicon sequencing was performed using custom-designed primers targeting the V3–V4 region of the 16S rRNA gene. Fungal ITS gene targeted sequencing was performed using the Quick-16S NGS Library Prep Kit with custom ITS2 primers substituted for 16S primers. PCR products were quantified and pooled based on equal molarity. The final pooled library was cleaned with the Select-a-Size DNA Clean & Concentrator (Zymo Research), then quantified with TapeStation (Agilent Technologies, Santa Clara, CA) and Qubit (Thermo Fisher Scientific, Waltham, WA). The ZymoBIOMICS Microbial Community Standard and Microbial Community DNA Standard were used as positive controls for each DNA extraction and each Targeted Library Preparation, respectively. Negative controls included blank extraction controls and a blank library preparation control. The final library of 300 bp paired-end reads was sequenced on Illumina MiSeq with a v3 reagent kit (600 cycles) and 10% PhiX spike-in.

Potential sequencing errors and chimeric sequences were removed and unique amplicon sequences variants (ASVs) were inferred from raw reads using the DADA2 pipeline (31). Taxonomy assignment was performed using Uclust from Qiime v.1.9.1 with the Zymo Research Database (32). All analyses were performed with Qiime v.1.9.1 (32). To determine absolute abundance, a quantitative real-time PCR was set up with a standard curve made with plasmid DNA containing one copy of the 16S gene prepared in 10-fold serial dilutions and the same primers used in the Targeted Library Preparation. The equation for the plasmid DNA standard curve was used to calculate the number of gene copies in the reaction for each sample, and the PCR input volume was used to calculate the number of gene copies per microliter in each DNA sample. Genera with significant differences in relative abundance between EHS and VEHS were identified by Linear Discriminant Analysis of Effect Size (LEfSe) (33) using default settings at http://galaxy.biobakery.org/.

STATISTICS AND PRIOR DATA PRESENTATION

Adequacy of group sizes was determined using a two-sample *t* test power calculation estimating ~1°C reduction in post-EHS hypothermia and accounting for ~30% attrition due to mortality or technical issues with telemeters. All analyses were completed using the R statistical computing language

(version 4.1.2). Thermoregulatory variables were compared using a Wilcoxon rank-sum test (EHS vs. VEHS). For biomarker and cytokine/chemokine analyses, due to significant skew of the distributions visually confirmed through plots of the residuals, biomarkers measured in the plasma and organs were Box-Cox transformed to normalize distributions, and the residuals were visually inspected to confirm appropriate model fit. Time and treatment main effects and interaction effects were determined. Biomarker comparisons were then made using an ordinary least squares linear model (i.e., ANOVA). Comparisons between EHS and VEHS conditions were made with specific contrasts using the "emmeans" R package. To control for multiple comparisons, a Holm-Bonferroni correction was applied to the pairwise comparisons. Statistical significance was determined at $\alpha < 0.05$. Data are expressed as means ± SD, unless otherwise indicated. Vancomycin had no effects on outcomes in ExC versus VExC. Accordingly, the primary comparison and data visualizations were made between EHS and VEHS as our primary objective was to infer whether prior antibiotics mitigated EHS severity.

EHS data for biomarkers and cyto-/chemokines as well as 0 h EHS liver samples for analyzing JNK phosphorylation have been published in a prior publication (20). However, the current study made novel comparisons across different treatment groups (i.e., EHS and VEHS) and provides insight on whether antibiotic treatment can mitigate EHS severity.

RESULTS

Thermal and Hydration Data

Compared with EHS mice, VEHS mice had lower T_{CMax} (42.3 ± 0.4°C vs. 42.6 ± 0.4°C; $P = 0.005$; $n = 47–48$/group) and Dehydration Percentage (11.7 ± 1.9% vs. 12.8 ± 2.0% dehydration; $P = 0.005$; $n = 47–48$/group) following exertional heating. VEHS mice had lower T_C in cage during daytime/resting phase (35.9 ± 0.2 vs. 36.3 ± 0.2°C) and nighttime/active phase (36.6 ± 0.2 vs. 37.0 ± 0.2°C) before exertional heating when compared with EHS mice that consumed only water ($P < 0.001$; $n = 46–48$/group for both phases). VEHS mice also drank more water on the day before exertional heating when compared with EHS mice (6.5 ± 0.9 mL vs. 5.6 ± 0.9 mL; $P < 0.001$; $n = 47–48$/group). Similar vancomycin effects on preexertional T_C were also observed in VExC mice compared with ExC mice (data not shown). No differences were observed for Thermal Load, Hypothermia Duration, or T_{CMin} (Table 1).

Blood Biomarkers

There was an interaction of treatment and time leading to reduced levels of ALB, AST, and ALT in VEHS compared with EHS mice. ALB (Fig. 2*A*) was lower at 0 h ($P = 0.04$; $n = 11–12$/group) and 3 h ($P = 0.002$; $n = 6–12$/group) in VEHS. ALT was lower at 24 h (Fig. 2*D*; $P = 0.008$; $n = 8–10$/group) in the vancomycin group. There was also a trend after the Holm-Bonferroni correction for lower AST in VEHS at 24 h (Fig. 2*C*; $P = 0.053$; $n = 8–10$/group). There was a main effect of treatment ($P = 0.028$; $n = 26–35$/treatment) for creatinine, which was lower in VEHS versus EHS mice (Fig. 2*B*). Other measured biomarkers (not shown; see data repository for more information) did not exhibit any differences.

Cytokines: IL-6, G-CSF, and IL-10

There was an interaction of treatment and time leading to reduced IL-6 in VEHS compared with EHS mice (Fig. 3*A*; $P = 0.023$; $n = 35–38$/treatment), specifically at 24 h (Fig. 3*A*; $P = 0.002$; $n = 11$/group). For G-CSF (Fig. 3*B*; $n = 35–38$/treatment) and IL-10 (Fig. 3*C*; 29–32/treatment), there were individual main effects of treatment (G-CSF $P = 0.022$; IL-10 $P = 0.018$) with lower values in both cytokines in the vancomycin group. There were no differences for other cytokines in VEHS compared with EHS (data not shown; see data repository for more information).

Chemokines: MIP-1α/CCL3, MIP-1β/CCL4, KC/CXCL1, and MIP-2/CXCL2

There was an interaction of treatment and time leading to reduced levels of MIP-1α ($P = 0.033$), MIP-1β ($P = 0.025$), and KC ($P = 0.027$) in VEHS compared with EHS mice. MIP-1α was lower at 3 h (Fig. 3*D*; $P = 0.038$; $n = 12–14$/group), MIP-1β was lower at 24 h (Fig. 3*E*; $P < 0.001$; $n = 11$/

Table 1. *Thermoregulatory responses during heat exposure and recovery*

	EHS[1]	VEHS[1]	*P* Value[2]
Pre-EHS			
T_C resting phase (morning)	36.3 ± 0.2 ($n = 48$)	35.9 ± 0.2 ($n = 47$)	<0.001
T_C active phase (night)	37.0 ± 0.2 ($n = 48$)	36.6 ± 0.2 ($n = 47$)	<0.001
Water consumed (night before)	5.6 ± 0.9 ($n = 48$)	6.5 ± 0.9 ($n = 47$)	<0.001
Heat exposure			
Time to collapse, min	246.8 ± 44.3 ($n = 47$)	237.7 ± 34.7 ($n = 47$)	0.3
T_C at collapse, °C	42.3 ± 0.2 ($n = 48$)	42.1 ± 0.2 ($n = 47$)	<0.001
T_{CMax}, °C	42.6 ± 0.4 ($n = 47$)	42.3 ± 0.4 ($n = 47$)	0.005
Thermal load, °C·min	781.4 ± 139.0 ($n = 26$)	732.2 ± 113.3 ($n = 27$)	0.2
Dehydration, %	12.8 ± 2.0 ($n = 48$)	11.7 ± 1.9 ($n = 47$)	0.005
Recovery			
Time to T_{CMin}, min	194.9 ± 84.1 ($n = 11$)	148.8 ± 31.3 ($n = 11$)	0.11
T_{CMin}, °C	30 ± 1.5 ($n = 11$)	30.1 ± 0.8 ($n = 11$)	0.9
Hypothermia duration, min	397.3 ± 219.5 ($n = 9$)	473.9 ± 149.9 ($n = 11$)	0.4
Survival, %	79.2	89.4	0.17

[1]Welch's two-sample *t* test. Values are presented as means ± SD. [2]Wilcoxon rank-sum exact test. Vancomycin treatment for 1 wk lowered core temperature (T_C) during the resting phase (morning) and active phase (night) before exertional heat stroke (EHS). Mice treated with vancomycin also consumed more water than untreated mice the night before EHS. Following exertional heating, T_C at collapse, maximal core temperature (T_{CMax}), and dehydration percentage were lower in vancomycin-treated EHS (VEHS) compared with EHS mice.

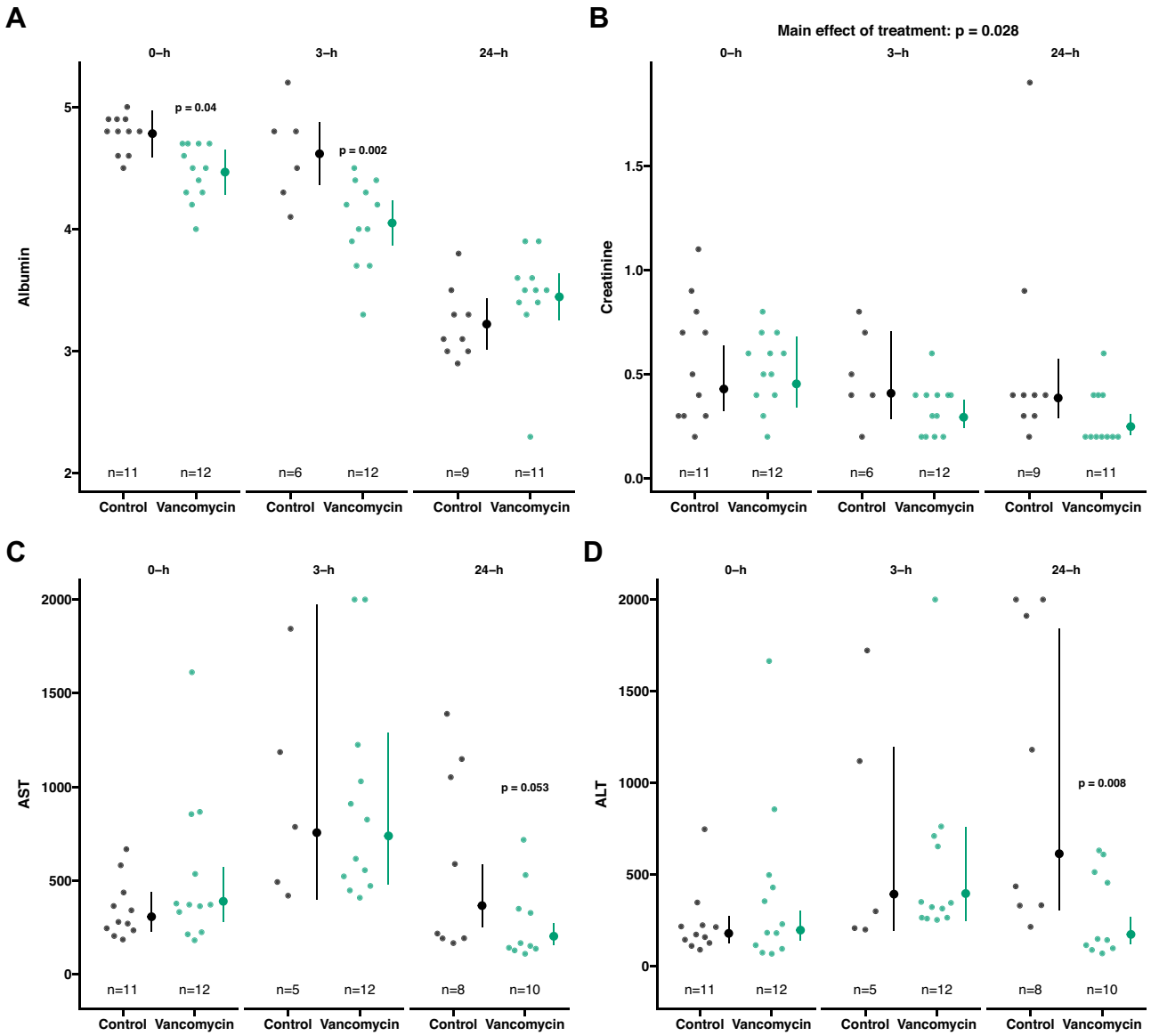


Figure 2. Circulating biomarkers in exertional heat stroke (EHS) and vancomycin-treated EHS (VEHS) mice at 0, 3, and 24 h. *A*: albumin was decreased in VEHS compared with EHS mice at 0 h ($P = 0.04$; $n = 11$–12/group) and 3 h ($P = 0.002$; $n = 6$–12/group). *B*: there was a main effect of treatment with creatinine, with VEHS having overall lower levels of creatinine ($P = 0.028$). *C*: there were also no differences in aspartate transaminase (AST) levels between EHS and VEHS at any timepoint, although AST tended to be lower in VEHS compared with EHS at 24 h ($P = 0.053$; $n = 8$–10/group). *D*: there was a main effect of treatment across all timepoints for alanine transaminase (ALT) to be lower in VEHS compared with EHS ($P = 0.028$; $n = 24$–34/group across all timepoints). Specifically, ALT was lower in VEHS compared with EHS at 24 h ($P = 0.008$; $n = 8$–10/group).

group), and KC (Fig. 3*F*; $P = 0.026$) was lower at 24 h ($n = 11$/group). There was a main effect of treatment ($P < 0.001$) for MIP-2, indicating lower levels in VEHS compared with EHS mice (Fig. 3*G*).

iFABP2, D-Dimer, LPS, and LTA

No differences were observed between EHS versus VEHS for circulating iFABP2 or D-dimer levels at any of the timepoints investigated. In addition, LTA levels at 0 h did not differ between EHS and VEHS mice (Supplemental Fig. S1; https://www.doi.org/10.17605/OSF.IO/YKPMZ). LPS was not detected in EHS mouse samples ($n = 9$ across 0, 3, and 24 h), but it was detected in serum from septic mice from a previous study that served as positive controls (data not shown) indicating LPS levels were below the detection threshold in our EHS model.

Liver JNK Phosphorylation

There was a main effect of treatment on liver JNK phosphorylation at Thr183/Tyr185 at 0 and 3 h with VEHS lower than EHS (Fig. 4; $P = 0.004$). Validation for antibody specificity and MemCode images used as loading controls provided in Supplemental Fig. S2.

Intestinal Microbiota

Vancomycin resulted in a 10^2–10^4-fold reduction in total fecal bacterial populations in VEHS and VExC specimens compared with EHS and ExC specimens (Fig. 5*A*;

data plotted on a $\log_{10}$ scale) and pronounced shifts in community composition (Fig. 5*B*). The most salient difference in microbiota was that genera within orders *Clostridiales* and *Bacteroidales* comprised a combined ~94%–95% of the bacteria in untreated specimens but only ~1%–4% in vancomycin-treated specimens. Relative abundance of an unidentified taxa within the order *Bacteroidales* and the genus *Lachnoclostridium* and unidentified genera within families *Lachnospiraceae* and *Ruminococcaceae* (all belonging to order *Clostridiales*) comprised the majority of microbiota in EHS and ExC specimens. In contrast, the dominant genera varied across vancomycin-treated specimens although *Bacillus* and *Curtobacterium* were the predominant genera in half of the VEHS and VExC specimens. In the other half of VEHS and VExC specimens, the microbiota were dominated by a single genus that differed across specimens: *Weisella*, *Alcaligenes*, and *Pediococcus* in 2, 1, and 1 VEHS specimens, respectively, and *Kerstersia*, *Burkholderia-Burkholderia-Paraburkholderia*, and *Cupriavidus* in 1 VExC specimen each (Fig. 5*B*). Focusing on differences between VEHS and EHS mice, LEfSe analysis indicated relative abundance of bacteria of genera *Weissella*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Arundo-Oryza meyeriana*, *Thermoactinomyces*, *Curtobacterium*, and *Saccharopolyspora* was increased in VEHS mice. In contrast, bacteria of genera *Oscillibacter*, *Lachnoclostridium*, and unidentified genera in orders *Clostridiales* and *Bacteroidales* and in families *Ruminococcaceae* and *Lachnospiraceae* were increased in EHS mice (Fig. 5*C*). Bray–Curtis dissimilarity analysis shows separation of EHS and VEHS mice by principal coordinates (Supplemental Fig. S3).

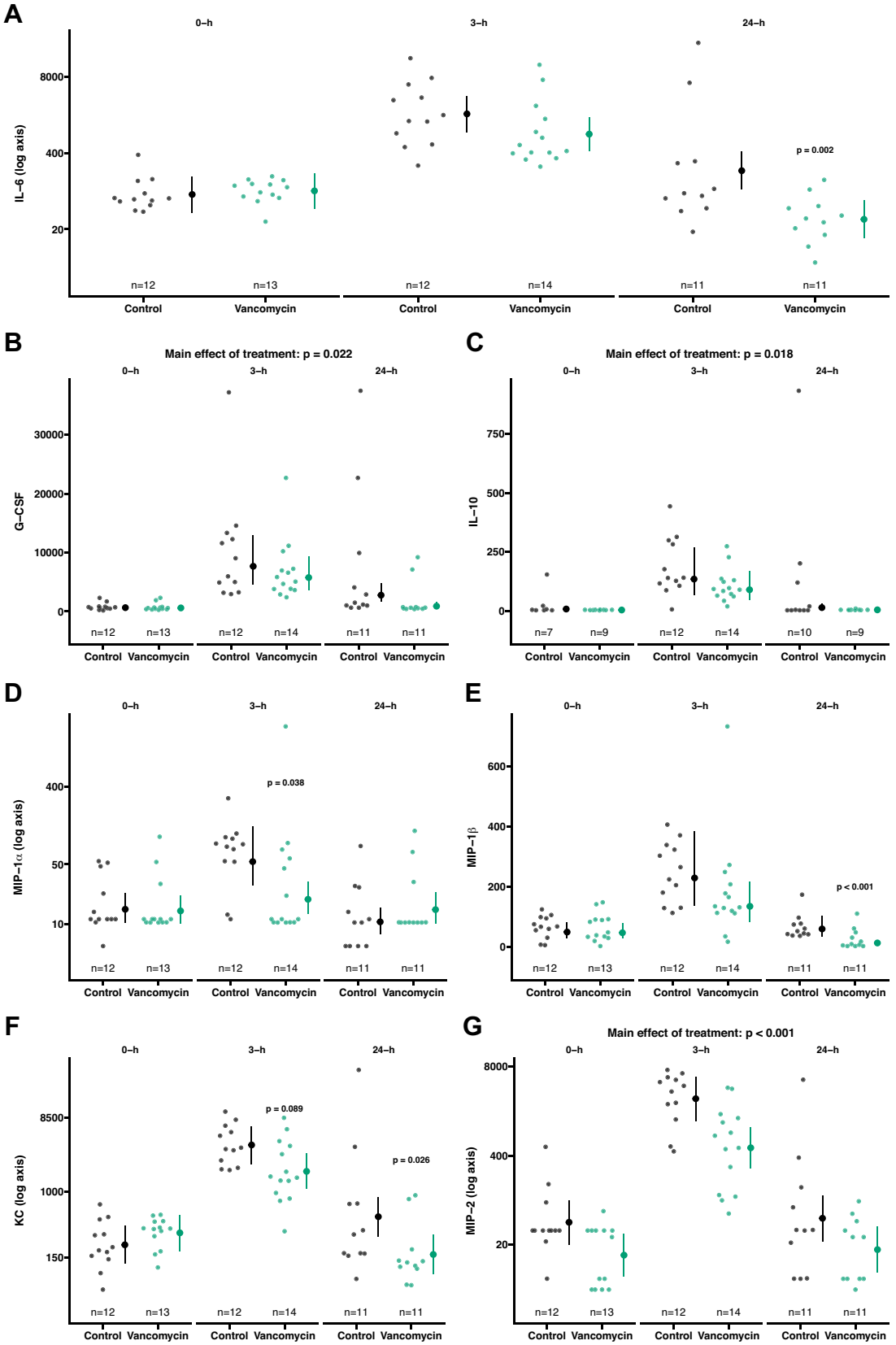
DISCUSSION

VEHS mice exhibited modest improvements compared with EHS mice, reflected by lower circulating levels of markers for organ damage and inflammation (Figs. 2 and 3). VEHS mice also tended to have lower mortality (5/47) compared with EHS mice (10/48), although this was not significant ($P = 0.17$). However, vancomycin in the drinking water may have also prolonged survival. When mortalities occurred, EHS mice typically died during or just after passive heating, closest to 0 h in 9/10 cases. However, in the five VEHS deaths, two died closest to 0 h, two died ~1.5 h post-EHS between 0 and 3 h, and one died ~4.5 h post-EHS. Evidence for vancomycin action was reflected in lowered abundance and altered composition of fecal bacteria in treated animals (Fig. 5) with consistent effects across VEHS and VExC mice. Specifically, genera within orders *Clostridiales* and *Bacteroidales* comprised a combined ~94%–95% of the microbiota in untreated specimens (EHS and ExC), but vancomycin reduced those orders to ~1%–4% of the microbiota in VEHS and VExC mice. VEHS mice also exhibited decreased markers of organ damage and inflammation at 3 and 24 h post-EHS and decreased phosphorylation of JNK in the liver at 0 and 3 h post-EHS.

Vancomycin lowered T_C in treated (VEHS and VExC) mice compared with untreated (EHS and ExC) mice (Table 1) before exertion, which may have contributed to the improved outcomes in VEHS mice. However, despite the lower T_C before exertional heating, this cooling effect did not delay heat-induced collapse or prolong time of running/heating and simply resulted in lower T_C at collapse in VEHS mice when compared with EHS mice. It is uncertain whether lower T_C was due to a direct effect of vancomycin or an indirect effect. VEHS mice consumed more water than EHS mice, and vancomycin may have indirectly influenced T_C via increased hydration. It is also possible that vancomycin effects on the gut microbiota directly influenced T_C. One possibility is that altered GI microbiota may have impacted intestinal nutrient absorption and whole body metabolism, as we observed that several vancomycin-treated mice had loose stool compared with untreated mice (not shown). In addition, vancomycin-treated mice exhibited severe reductions in bacteria of orders *Bacteroidales* and *Clostridiales* (Fig. 5) compared with untreated mice. Although some of these taxa were unidentified in *Bacteroidales*, several families/genera in both orders are believed to contribute to metabolism/fermentation of plant saccharides and amino acids, producing short-chain fatty acids (e.g., 34, 35). Interestingly, some members of these orders are also believed to become pathogenic when translocated from the gastrointestinal tract to other body regions (36), and targeting them may have mitigated symptomatology in VEHS mice. Taken together, we speculate that vancomycin may have reduced key microbes necessary to metabolize and absorb nutrients in the intestines, likely leading to mild malnutrition. In turn, this resulted in deficiencies in substrates/compounds necessary to maintain normal metabolic processes, and the consequent lower whole body metabolism may have been reflected in lower T_C in vancomycin-treated mice. Conceptually, these changes could have also affected the gut-brain axis to affect thermal sensation/behavior in the hypothalamus as evidence in broiler chickens indicates neurochemical (e.g., serotonin) synthesis and metabolism are linked to gut microbes (37).

Vancomycin had a main effect of treatment in lowering creatinine, a metabolite indicative of renal function, across all investigated timepoints. Vancomycin also attenuated rises in circulating levels of liver enzymes indicative of damage; ALT was lower in VEHS compared with EHS mice with a similar trend in AST ($P = 0.053$) at 24 h. Hepatic complications are especially common in severe EHS cases in humans (24, 38, 39), but our results suggest vancomycin conferred some protective effects against EHS-associated hepatic toxicity and damage.

Similar to the biomarker results, vancomycin reduced circulating cytokines and chemokines in VEHS compared with EHS mice at 24 h, a few differences occurring at 3 h; no cytokine or chemokine differed across two separate timepoints when comparing EHS and VEHS (e.g., IL-6 was lower in VEHS compared with EHS at 24 h, but not at 3 h). Most differences were observed at 24 h when IL-6, KC/CXCL1, and MIP-1β/CCL4 were lower in VEHS compared with EHS. All three factors are implicated during LPS response, a key event following intestinal permeability according to the Dual Pathway Hypothesis (6), and are considered proinflammatory: MIP-1β and IL-6 in humans (40) and KC in mouse macrophage cells (41). CXCL1 attracts neutrophils to sites of microbial pathogenesis resulting in inflammatory and immune responses (42), CCL4 is a crucial mediator for monocyte/macrophage recruitment during inflammatory response (43), and IL-6 has pleiotropic effects during inflammatory and immune responses including induction of B-cell differentiation and rapid induction of acute



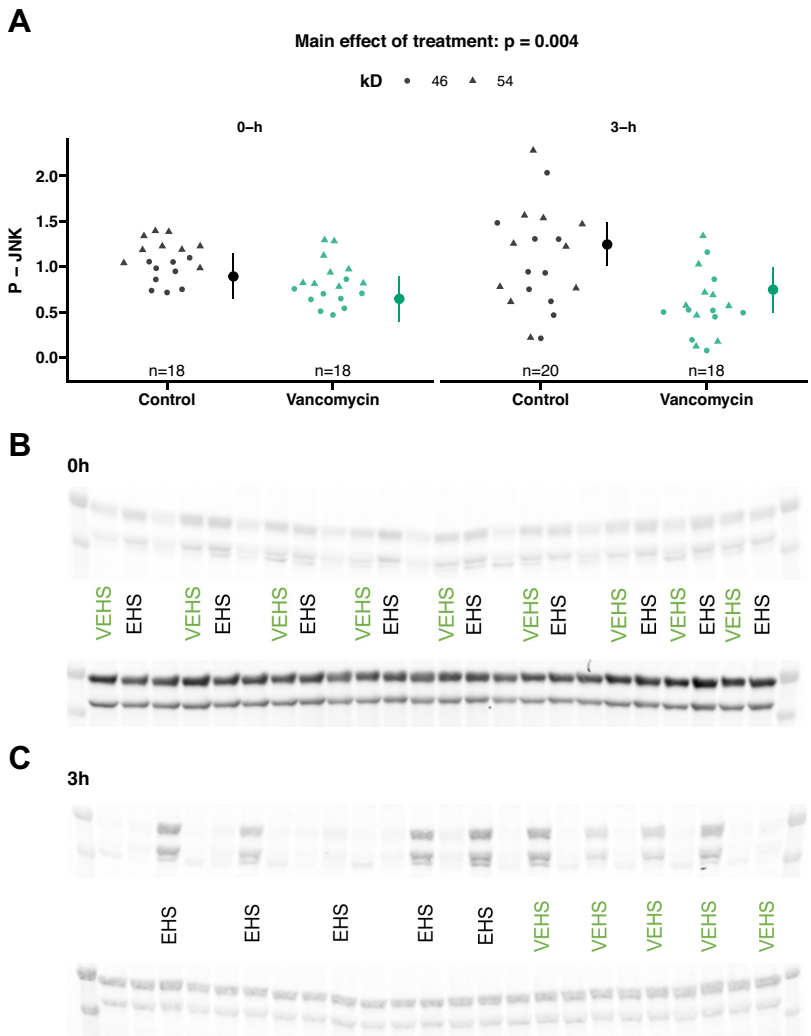


Figure 4. Western blotting for phosphorylated and total hepatic c-Jun NH_2-terminal kinase (JNK). *A*: there was a main effect of vancomycin treatment on phosphorylated liver JNK at Threonine (Thr) 183/Tyrosine (Tyr) 185 at 0 and 3 h ($P = 0.004$; $n = 36$–38 observations/group across all time points). Blots at 0 h (*B*) and 3 h (*C*) suggest vancomycin-treated exertional heat stroke (VEHS) liver exhibited less JNK phosphorylation (upper blots for *B* and *C*) in most, but not all, instances compared with EHS with no effect on total JNK abundance (lower blots for *B* and *C*). Unlabeled lanes include exercise controls (ExC), vancomycin-treated ExC (VExC), and/or specimens that experienced a moderate heat illness with no mortalities (exertional heat injury—EHI).

phase proteins (44). As these were all suppressed in VEHS mice, it is possible that vancomycin prophylaxis suppressed the inflammatory/immune responses elicited by circulating gut microbiota. However, circulating LPS at 0 h post-EHS was undetectable in our current study, and other chemokines (e.g., CXCL2, CCL3) implicated during LPS response (40, 41) were not different with vancomycin treatment.

As LPS is typically associated with Gram-negative bacteria and vancomycin primarily targets Gram-positive bacteria, we also investigated whether LTA, the Gram-positive analogue of LPS implicated in Gram-positive sepsis (45–47), could contribute to EHS severity. This comparison was conducted at 0 h post-EHS as most prominent effects on EHS severity would be obvious at this timepoint. Unlike LPS, LTA was detectable in our mice, but there were no statistical differences in blood LTA levels between EHS and VEHS, suggesting that *1*) LPS/LTA contributes little to EHS pathophysiology in mice, *2*) other uninvestigated/unidentified bacterial components may influence EHS symptoms, and/or *3*) antibiotic-induced increases in water intake resulted in higher levels of hydration to mitigate EHS symptoms. Taken together, VEHS mice exhibited modestly attenuated inflammatory responses at 3 and 24 h compared with EHS mice.

Reduced levels of circulating biomarkers and cytokines/chemokines with vancomycin at 24 h suggested mitigated organ damage and suppressed inflammatory response in VEHS compared with EHS mice. However, all EHS/VEHS fatalities due to

Figure 3. Circulating cytokines (*A*–*C*) and chemokines (*D*–*G*) in exertional heat stroke (EHS) and vancomycin-treated EHS (VEHS) mice at 0, 3, and 24 h. *A*: interleukin (IL)-6 (log scale) was lower in VEHS compared with EHS mice at 24 h ($P = 0.002$; $n = 11$/group). *B*: there was a main effect of treatment ($P = 0.022$; $n = 35$–38/group across all timepoints) to reduce granulocyte-colony-stimulating factor (G-CSF) between EHS and VEHS mice across all timepoints. *C*: there was a main effect of treatment ($P = 0.018$; $n = 35$–38/group across all timepoints) to reduce IL-10 between EHS and VEHS mice across all timepoints. *D*: there was an interaction effect of treatment and time to reduce macrophage inflammatory protein (MIP)-1α levels in VEHS compared with EHS mice. MIP-1α (log scale) was lower in VEHS compared with EHS at 3 h ($P = 0.038$; $n = 12$–14/group). *E*: MIP-1β was lower in VEHS compared with EHS at 24 h ($P < 0.001$; $n = 11$/group). *F*: keratinocyte chemoattractant (KC) was lower in VEHS compared with EHS mice at 24 h ($P = 0.026$; $n = 10$/group). *G*: there was a main effect of treatment for MIP-2 (log scale) to be lower in VEHS compared with EHS mice ($P < 0.001$; $n = 35$–38/group across all time points).

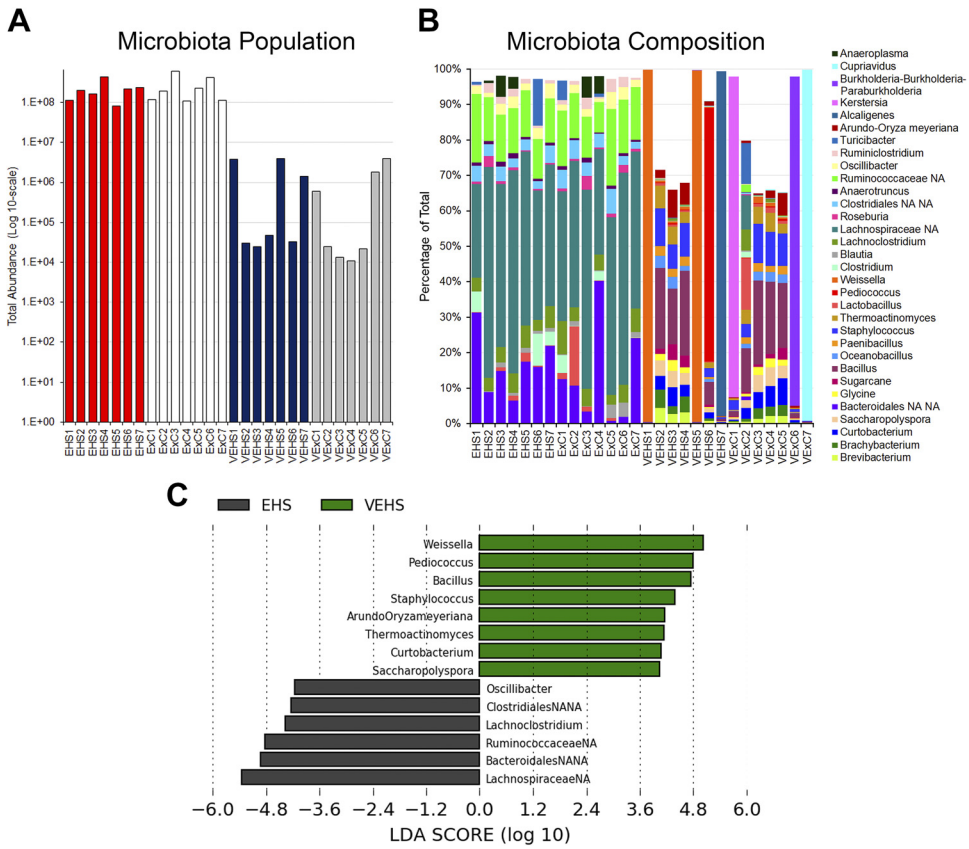


Figure 5. *A*: total bacterial abundance in each specimen plotted on a $\log_{10}$-scale indicated fewer fecal bacteria in vancomycin-treated exertional heat stroke (VEHS) and vancomycin-treated Exercise Control (VExC) mice compared with untreated EHS/ExC mice. *B*: intestinal microbiota composition measured at the end of the exertional protocol (listed by genus name without full taxonomical classification) is shown for all genera that constituted >1% of total. A genus within family *Lachnospiraceae*, a genus within the order *Bacteroidales*, and a genus within family *Ruminococcaceae* were the dominant bacterial genera in EHS and ExC mice. In contrast, the dominant genera in vancomycin-treated mice were varied across specimens, and in half of VEHS and VExC specimens, there was a single predominant genus composing the majority of the fecal microbiota. NA indicates genera that were not identified. Combining genera within orders *Clostridiales* (NA NA, *Ruminococcaceae* NA, *Oscillibacter*, *Lachnospiraceae* NA, *Lachnoclostridium*, *Clostridium*) and *Bacteroidales* (NA NA) accounted for ~94%–95% of the bacteria in EHS and ExC samples; these orders accounted for only ~1%–4% of bacteria in VEHS and VExC samples. *C*: linear discriminant analysis Effect Size (LEfSe) indicates the bacterial genera that differed by relative abundance between EHS and VEHS mice. "NA" indicates unidentified taxonomic classes; in such cases, the "NA" label was used until the name of the lowest taxonomic class identified was provided. EHS, exertional heat stroke; ExC, exercise controls.

exertional heating occurred closer to 0 and 3 h post-EHS rather than at 24 h. Consequently, we focused on elucidating rapidly activated signaling pathways post-EHS via western blotting to determine whether *1*) they may correspond to EHS severity/mortality and *2*) vancomycin or increased water intake affects these pathways at 0 and 3 h to underlie improved outcomes at later timepoints. We have previously demonstrated that hepatic JNK phosphorylation on the Thr183/Tyr185 residues proximal to the collapse of EHS specimens corresponds with, and may modulate, heat illness severity in our mouse model (20). Consistent with that interpretation, JNK phosphorylation was modestly decreased (on average) in VEHS compared with EHS liver despite no alterations in total JNK levels (Fig. 4). There was a main effect of vancomycin to decrease JNK phosphorylation at 0 and 3 h, although there were no significant differences at the individual timepoints. Interestingly, JNK phosphorylation pattern was similar across both timepoints although 0 h is more proximal to T_{CMax} and 3 h is during the hypothermic phase proximal to T_{CMin}. Consequently, we conclude that JNK activation is not *solely* dependent on or directly corresponding to hyperthermia.

Consistent with previous results indicating increased JNK phosphorylation may positively correlate with heat illness severity (20), the current results suggest that inhibited or decreased JNK phosphorylation may correspond to diminished EHS severity. Furthermore, both vancomycin-induced improvements during EHS and the overall reduction of JNK phosphorylation were modest, supporting the concept that JNK and its signaling may be crucial to EHS pathophysiology and could serve as a therapeutic or countermeasure target against EHS. However, there are caveats to this interpretation: vancomycin is not known to be a direct JNK inhibitor, and VEHS mice also had lower T_C values and higher water intake before exertional heating compared with EHS mice. Consequently, it is still unclear *how* vancomycin impacted hepatic JNK phosphorylation and EHS severity and whether this was due to direct effects on the intestinal microbiota and/or due to decreased T_C and increased hydration due to increased water intake. Nonetheless, our past and current findings indicate future work should target JNK to ascertain its role during EHS and EHS-associated hepatic issues.

Limitations

Although vancomycin induced some beneficial effects, it likely also had several off-target effects not analyzed in the current study. VEHS mice had enlarged ceca compared with EHS mice (also observed in VExC mice during organ collection), which suggests there may have also been alterations in gastrointestinal function and structure due to vancomycin. This observation is consistent with results from other studies examining antibiotic-treated mice (48–50), and some speculate this could be due to water retention by intestinal cells during short-term treatment (48). Accordingly, cecal enlargement could be associated with the increased water consumption we observed (Table 1), and increased water intake in turn could have led to increased hydration and lower T_C to improve EHS outcomes. Although a lower vancomycin dose could have minimized off-target or adverse microbiota effects following antibiotic prophylaxis (e.g., cecal enlargement), the current high dose only induced modest changes in EHS outcomes. Accordingly, we speculate that although a lower vancomycin dose would have induced less adverse or off-target effects, it would have also negated any beneficial effects due to antibiotics on EHS outcomes. In addition, although vancomycin reduced EHS severity (e.g., reduced biomarkers and inflammation markers in circulation), antibiotics could have other independent deleterious effects such as increasing antimicrobial resistance in bacterial pathogens (51) and causing or exacerbating diarrhea and dehydration (52). Taken together and despite modest benefits, antibiotics are impractical for EHS prophylaxis.

Although cecal enlargement due to vancomycin could have resulted in discomfort to the mice, there were no abnormal elevations of markers for organ damage or inflammation in the VExC mice, vancomycin did not exacerbate EHS symptomatology, and the EHS protocol was arguably the most stressful condition for tested specimens. There may have been a censoring effect with timed euthanasia (i.e., 0 h euthanasia could have masked the true mortality rate of EHS). However, the time of euthanasia for each animal was determined a priori, and we did not shift this based on EHS phenotype at collapse, thus minimizing end point bias. Furthermore, most of the instances when an animal euthanized at 0 h was noted to have been a possible censored mortality occurred in EHS rather than VEHS mice, suggesting if there was a censoring effect, a significant vancomycin effect on overall post-EHS survival may have been obscured. However, this study was never intended to be solely focused on post-EHS survival, and we assert that our current approach enabled us to characterize antibiotic effects on EHS outcomes while reducing the number of animals required for testing.

Conclusions

Vancomycin prophylaxis targeted gastrointestinal microbiota and modestly mitigated EHS severity, as assessed by decreased JNK phosphorylation, attenuated rises in biomarker levels, and a trend for decreased/delayed mortality. Consequently, our data suggest that the gut microbiota may contribute to EHS pathophysiology in a conscious, unrestrained animal model. This possibility warrants further investigation as other studies could also more conclusively elucidate effects of targeting Gram-negative bacteria, altering timing/duration of antibiotic treatment, if there are other bacterial components besides LPS and LTA that can influence EHS symptomatology, or probiotic prophylaxis to decrease inflammatory responses and increase barrier function in intestines (53). In addition, evidence from this study and prior work (20) indicates future research could directly target JNK action(s) and determine its involvement in the onset and severity of heat casualty. Although it is uncertain whether JNK has a protective or pathological role during EHS, more fully characterizing the role of this node and its signaling pathway (i.e., downstream targets) could enable more specific or targeted interventions (chemical, dietary, training/acclimation, or a combination of the three) to prevent, treat, or provide supportive therapy for EHS cases.

DATA AVAILABILITY

The data that support the findings and interpretations for this study are openly available in an OSF Repository at https://www.doi.org/10.17605/OSF.IO/YKPMZ.

SUPPLEMENTAL DATA

Supplemental Figs. S1–S3: https://www.doi.org/10.17605/OSF.IO/YKPMZ.

ACKNOWLEDGMENTS

We thank the USARIEM Veterinary Support & Oversight Branch for their assistance during experiments. We also thank J Philip Karl, PhD, for his helpful comments during the drafting of this manuscript.

GRANTS

This project was funded by a grant from the Military Operational Medicine Research Program (MO210052).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

DISCLAIMERS

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

AUTHOR CONTRIBUTIONS

K.O., J.A.W., S.M.W., M.L.P., T.A.M., A.R.C., and L.R.L. conceived and designed research; K.O., J.A.W., S.M.W., M.L.P., C.G.H., and T.A.M. performed experiments; K.O., J.A.W., S.M.W., M.L.P., C.G.H., T.A.M., A.R.C., and L.R.L. analyzed data; K.O., J.A.W., S.M.W., M.L.P., C.G.H., T.A.M., A.R.C., and L.R.L. interpreted results of experiments; K.O., J.A.W., S.M.W., M.L.P., C.G.H., A.R.C., and L.R.L. prepared figures; K.O., J.A.W., S.M.W., M.L.P., C.G.H., T.A.M., A.R.C., and L.R.L. drafted manuscript; K.O., J.A.W., S.M.W., M.L.P., C.G.H., T.A.M., A.R.C., and L.R.L. edited and revised manuscript; K.O., J.A.W., S.M.W., M.L.P., C.G.H., T.A.M., A.R.C., and L.R.L. approved final version of manuscript.

REFERENCES

1. **Leon LR**, **Bouchama A.** Heat stroke. *Compr Physiol* 5: 611–647, 2015. doi:10.1002/cphy.c140017.
2. **Casa DJ**, **DeMartini JK**, **Bergeron MF**, **Csillan D**, **Eichner ER**, **Lopez RM**, **Ferrara MS**, **Miller KC**, **O'Connor F**, **Sawka MN**, **Yeargin SW.** National Athletic Trainers' Association position statement: exertional heat illnesses. *J Athl Train* 50: 986–1000, 2015 [Erratum in *J Athl Train* 52: 401, 2017]. doi:10.4085/1062-6050-50.9.07.
3. **Gubernot DM**, **Anderson GB**, **Hunting KL.** Characterizing occupational heat-related mortality in the United States, 2000-2010: an analysis using the Census of Fatal Occupational Injuries database. *Am J Ind Med* 58: 203–211, 2015. doi:10.1002/ajim.22381.
4. **Williams VF**, **Oh GT.** Update: heat illness, active component, U.S. Armed Forces, 2021. *MSMR* 29: 8–14, 2022.
5. **Department of the Army.** *Technical Bulletin Medical 507: Heat Stress Control and Heat Casualty Management*. Fort Belvoir, VA: Army Publishing Directorate, 2022. https://armypubs.army.mil/epubs/DR_pubs/DR_a/ARN35159-TB_MED_507-000-WEB-1.pdf.
6. **Lim CL**, **Mackinnon LT.** The roles of exercise-induced immune system disturbances in the pathology of heat stroke: the dual pathway model of heat stroke. *Sports Med* 36: 39–64, 2006. doi:10.2165/00007256-200636010-00004.
7. **Bosenberg AT**, **Brock-Utne JG**, **Gaffin SL**, **Wells MT**, **Blake GT.** Strenuous exercise causes systemic endotoxemia. *J Appl Physiol (1985)* 65: 106–108, 1988. doi:10.1152/jappl.1988.65.1.106.
8. **Brock-Utne JG**, **Gaffin SL**, **Wells MT**, **Gathiram P**, **Sohar E**, **James MF**, **Morrell DF**, **Norman RJ.** Endotoxaemia in exhausted runners after a long-distance race. *S Afr Med J* 73: 533–536, 1988.
9. **Graber CD**, **Reinhold RB**, **Breman JG**, **Harley RA**, **Hennigar GR.** Fatal heat stroke. Circulating endotoxin and gram-negative sepsis as complications. *JAMA* 216: 1195–1196, 1971. doi:10.1001/jama.216.7.1195.
10. **Hashim IA**, **Al-Zeer A**, **Al-Shohaib S**, **Al-Ahwal M**, **Shenkin A.** Cytokine changes in patients with heatstroke during pilgrimage to Makkah. *Mediators Inflamm* 6: 135–139, 1997. doi:10.1080/09629359791839.
11. **Selkirk GA**, **McLellan TM**, **Wright HE**, **Rhind SG.** Mild endotoxemia, NF-kappaB translocation, and cytokine increase during exertional heat stress in trained and untrained individuals. *Am J Physiol Regul Integr Comp Physiol* 295: R611–R623, 2008. doi:10.1152/ajpregu.00917.2007.
12. **Lambert GP**, **Gisolfi CV**, **Berg DJ**, **Moseley PL**, **Oberley LW**, **Kregel KC.** Selected contribution: hyperthermia-induced intestinal permeability and the role of oxidative and nitrosative stress. *J Appl Physiol (1985)* 92: 1750–1761, 2002. doi:10.1152/japplphysiol.00787.2001.
13. **Gathiram P**, **Gaffin SL**, **Brock-Utne JG**, **Wells MT.** Time course of endotoxemia and cardiovascular changes in heat-stressed primates. *Aviat Space Environ Med* 58: 1071–1074, 1987.
14. **Dokladny K**, **Moseley PL**, **Ma TY.** Physiologically relevant increase in temperature causes an increase in intestinal epithelial tight junction permeability. *Am J Physiol Gastrointest Liver Physiol* 290: G204–G212, 2006. doi:10.1152/ajpgi.00401.2005.
15. **Gathiram P**, **Wells MT**, **Brock-Utne JG**, **Gaffin SL.** Antilipopolysaccharide improves survival in primates subjected to heat stroke. *Circ Shock* 23: 157–164, 1987.
16. **Gathiram P**, **Wells MT**, **Brock-Utne JG**, **Wessels BC**, **Gaffin SL.** Prevention of endotoxaemia by non-absorbable antibiotics in heat stress. *J Clin Pathol* 40: 1364–1368, 1987. doi:10.1136/jcp.40.11.1364.
17. **Gathiram P**, **Wells MT**, **Brock-Utne JG**, **Wessels BC**, **Gaffin SL.** Oral administered nonabsorbable antibiotics prevent endotoxemia in primates following intestinal ischemia. *J Surg Res* 45: 187–193, 1988. doi:10.1016/0022-4804(88)90064-9.
18. **Butkow N**, **Mitchell D**, **Laburn H**, **Kenedi E.** Heat stroke and endotoxemia in rabbits. In: *Thermal Physiology*, edited by Hales JRS. New York: Raven Press, 1984, p. 511–514.
19. **DuBose DA**, **Basamania K**, **Maglione L**, **Rowlands J.** Role of bacterial endotoxins of intestinal origin in rat heat stress mortality. *J Appl Physiol Respir Environ Exerc Physiol* 54: 31–36, 1983. doi:10.1152/jappl.1983.54.1.31.
20. **Oki K**, **Henderson CG**, **Ward SM**, **Ward JA**, **Plamper ML**, **Mayer TA**, **Caldwell AR**, **Leon LR.** Identification of therapeutic targets in a murine model of severe exertional heat stroke. *Am J Physiol Regul Integr Comp Physiol* 323: R935–R950, 2022. doi:10.1152/ajpregu.00150.2022.
21. **Maldonado RF**, **Sá-Correia I**, **Valvano MA.** Lipopolysaccharide modification in Gram-negative bacteria during chronic infection. *FEMS Microbiol Rev* 40: 480–493, 2016. doi:10.1093/femsre/fuw007.
22. **Rubinstein E**, **Keynan Y.** Vancomycin revisited - 60 years later. *Front Public Health* 2: 217, 2014. doi:10.3389/fpubh.2014.00217.
23. **Ip YT**, **Davis RJ.** Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. *Curr Opin Cell Biol* 10: 205–219, 1998. doi:10.1016/s0955-0674(98)80143-9.
24. **Berger J**, **Hart J**, **Millis M**, **Baker AL.** Fulminant hepatic failure from heat stroke requiring liver transplantation. *J Clin Gastroenterol* 30: 429–431, 2000. doi:10.1097/00004836-200006000-00015.
25. **Ichai P**, **Laurent-Bellue A**, **Camus C**, **Moreau D**, **Boutonnet M**, **Saliba F**, **Peron JM**, **Ichai C**, **Gregoire E**, **Aigle L**, **Cousty J**, **Quinart A**, **Pons B**, **Boudon M**, **André S**, **Coilly A**, **Antonini T**, **Guettier C**, **Samuel D.** Liver transplantation in patients with liver failure related to exertional heatstroke. *J Hepatol* 70: 431–439, 2019. doi:10.1016/j.jhep.2018.11.024.
26. **Wallace RF**, **Kriebel D**, **Punnett L**, **Wegman DH**, **Amoroso PJ.** Prior heat illness hospitalization and risk of early death. *Environ Res* 104: 290–295, 2007. doi:10.1016/j.envres.2007.01.003.
27. **Mu Q**, **Edwards MR**, **Swartwout BK**, **Cabana Puig X**, **Mao J**, **Zhu J**, **Grieco J**, **Cecere TE**, **Prakash M**, **Reilly CM**, **Puglisi C**, **Bachali P**, **Grammer AC**, **Lipsky PE**, **Luo XM.** Gut microbiota and bacterial DNA suppress autoimmunity by stimulating regulatory b cells in a murine model of lupus. *Front Immunol* 11: 593353, 2020. doi:10.3389/fimmu.2020.593353.
28. **Leon LR**, **Dineen S**, **Blaha MD**, **Rodriguez-Fernandez M**, **Clarke DC.** Attenuated thermoregulatory, metabolic, and liver acute phase protein response to heat stroke in TNF receptor knockout mice. *Am J Physiol Regul Integr Comp Physiol* 305: R1421–R1432, 2013. doi:10.1152/ajpregu.00127.2013.
29. **Caldwell AR**, **Oki K**, **Ward SM**, **Ward JA**, **Mayer TA**, **Plamper ML**, **King MA**, **Leon LR.** Impact of successive exertional heat injuries on thermoregulatory and systemic inflammatory responses in mice. *J Appl Physiol (1985)* 131: 1469–1485, 2021. doi:10.1152/japplphysiol.00160.2021.
30. **Leon LR**, **DuBose DA**, **Mason CW.** Heat stress induces a biphasic thermoregulatory response in mice. *Am J Physiol Regul Integr Comp Physiol* 288: R197–R204, 2005. doi:10.1152/ajpregu.00046.2004.
31. **Callahan BJ**, **McMurdie PJ**, **Rosen MJ**, **Han AW**, **Johnson AJ**, **Holmes SP.** DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 13: 581–583, 2016. doi:10.1038/nmeth.3869.
32. **Caporaso JG**, **Kuczynski J**, **Stombaugh J**, **Bittinger K**, **Bushman FD**, **Costello EK**, **Fierer N**, **Peña AG**, **Goodrich JK**, **Gordon JI**, **Huttley GA**, **Kelley ST**, **Knights D**, **Koenig JE**, **Ley RE**, **Lozupone CA**, **McDonald D**, **Muegge BD**, **Pirrung M**, **Reeder J**, **Sevinsky JR**, **Turnbaugh PJ**, **Walters WA**, **Widmann J**, **Yatsunenko T**, **Zaneveld J**, **Knight R.** QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7: 335–336, 2010. doi:10.1038/nmeth.f.303.
33. **Segata N**, **Izard J**, **Waldron L**, **Gevers D**, **Miropolsky L**, **Garrett WS**, **Huttenhower C.** Metagenomic biomarker discovery and explanation. *Genome Biol* 12: R60, 2011. doi:10.1186/gb-2011-12-6-r60.
34. **Lopetuso LR**, **Scaldaferri F**, **Petito V**, **Gasbarrini A.** Commensal Clostridia: leading players in the maintenance of gut homeostasis. *Gut Pathog* 5: 23, 2013. doi:10.1186/1757-4749-5-23.
35. **Ormerod KL**, **Wood DL**, **Lachner N**, **Gellatly SL**, **Daly JN**, **Parsons JD**, **Dal'Molin CG**, **Palfreyman RW**, **Nielsen LK**, **Cooper MA**, **Morrison M**, **Hansbro PM**, **Hugenholtz P.** Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals. *Microbiome* 4: 36, 2016. doi:10.1186/s40168-016-0181-2.
36. **Zafar H**, **Saier MH Jr.** Gut Bacteroides species in health and disease. *Gut Microbes* 13: 1–20, 2021. doi:10.1080/19490976.2020.1848158.
37. **Cao C**, **Chowdhury VS**, **Cline MA**, **Gilbert ER.** The microbiota-gut-brain axis during heat stress in chickens: a review. *Front Physiol* 12: 752265, 2021. doi:10.3389/fphys.2021.752265.
38. **Carvalho AS**, **Rodeia SC**, **Silvestre J**, **Póvoa P.** Exertional heat stroke and acute liver failure: a late dysfunction. *BMJ Case Rep* 2016: bcr2016214434, 2016. doi:10.1136/bcr-2016-214434.
39. **Giercksky T**, **Boberg KM**, **Farstad IN**, **Halvorsen S**, **Schrumpf E.** Severe liver failure in exertional heat stroke. *Scand J Gastroenterol* 34: 824–827, 1999. doi:10.1080/003655299750025778.
40. **O'Grady NP**, **Tropea M**, **Preas HL 2nd**, **Reda D**, **Vandivier RW**, **Banks SM**, **Suffredini AF.** Detection of macrophage inflammatory protein (MIP)-1alpha and MIP-1beta during experimental endotoxemia and human sepsis. *J Infect Dis* 179: 136–141, 1999. doi:10.1086/314559.

41. **De Filippo K**, **Dudeck A**, **Hasenberg M**, **Nye E**, **van Rooijen N**, **Hartmann K**, **Gunzer M**, **Roers A**, **Hogg N.** Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. *Blood* 121: 4930–4937, 2013. doi:10.1182/blood-2013-02-486217.
42. **Sawant KV**, **Poluri KM**, **Dutta AK**, **Sepuru KM**, **Troshkina A**, **Garofalo RP**, **Rajarathnam K.** Chemokine CXCL1 mediated neutrophil recruitment: role of glycosaminoglycan interactions. *Sci Rep* 6: 33123, 2016. doi:10.1038/srep33123.
43. **Maurer M**, **von Stebut E.** Macrophage inflammatory protein-1. *Int J Biochem Cell Biol* 36: 1882–1886, 2004. doi:10.1016/j.biocel.2003.10.019.
44. **Tanaka T**, **Narazaki M**, **Kishimoto T.** IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol* 6: a016295, 2014. doi:10.1101/cshperspect.a016295.
45. **De Kimpe SJ**, **Kengatharan M**, **Thiemermann C**, **Vane JR.** The cell wall components peptidoglycan and lipoteichoic acid from Staphylococcus aureus act in synergy to cause shock and multiple organ failure. *Proc Natl Acad Sci USA* 92: 10359–10363, 1995. doi:10.1073/pnas.92.22.10359.
46. **Ginsburg I.** Role of lipoteichoic acid in infection and inflammation. *Lancet Infect Dis* 2: 171–179, 2002. doi:10.1016/s1473-3099(02)00226-8.
47. **Wang JE**, **Dahle MK**, **McDonald M**, **Foster SJ**, **Aasen AO**, **Thiemermann C.** Peptidoglycan and lipoteichoic acid in gram-positive bacterial sepsis: receptors, signal transduction, biological effects, and synergism. *Shock* 20: 402–414, 2003. doi:10.1097/01.shk.0000092268.01859.0d.
48. **Savage DC**, **Dubos R.** Alterations in the mouse cecum and its flora produced by antibacterial drugs. *J Exp Med* 128: 97–110, 1968. doi:10.1084/jem.128.1.97.
49. **Ge X**, **Ding C**, **Zhao W**, **Xu L**, **Tian H**, **Gong J**, **Zhu M**, **Li J**, **Li N.** Antibiotics-induced depletion of mice microbiota induces changes in host serotonin biosynthesis and intestinal motility. *J Transl Med* 15: 13, 2017. doi:10.1186/s12967-016-1105-4.
50. **Puhl NJ**, **Uwiera RR**, **Yanke LJ**, **Selinger LB**, **Inglis GD.** Antibiotics conspicuously affect community profiles and richness, but not the density of bacterial cells associated with mucosa in the large and small intestines of mice. *Anaerobe* 18: 67–75, 2012. doi:10.1016/j.anaerobe.2011.12.007.
51. **Blair JM**, **Webber MA**, **Baylay AJ**, **Ogbolu DO**, **Piddock LJ.** Molecular mechanisms of antibiotic resistance. *Nat Rev Microbiol* 13: 42–51, 2015. doi:10.1038/nrmicro3380.
52. **Bartlett JG.** Antibiotic-associated diarrhea. *Clin Infect Dis* 15: 573–581, 1992. doi:10.1093/clind/15.4.573.
53. **Madsen K**, **Cornish A**, **Soper P**, **McKaigney C**, **Jijon H**, **Yachimec C**, **Doyle J**, **Jewell L**, **De Simone C.** Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 121: 580–591, 2001. doi:10.1053/gast.2001.27224.