Decreased Intestinal Microbiome Diversity in Pediatric Sepsis: A Conceptual Framework for Intestinal Dysbiosis to Influence Immunometabolic Function

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Supplemental Digital Content

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SUPPLEMENTAL METHODS

A. *Definitions of Pediatric Sepsis:*

- Severe sepsis was defined using published consensus criteria as 1) ≥2 age-based systemic inflammatory response syndrome criteria, 2) confirmed or suspected invasive infection, and 3) cardiovascular dysfunction or ≥2 non-cardiovascular organ system dysfunctions (1).
- Septic shock was defined as the subset of patients with severe sepsis who also had cardiovascular dysfunction, which included hypotension, treatment with a vasoactive medication, or impaired perfusion (1).
- Organ dysfunction was defined using published consensus criteria (1) as follows:
    i. *Cardiovascular:* Treatment with vasoactive infusion or systolic blood pressure <5th percentile for age >2 consecutive measurements, or presence of two of the following: base deficit more negative than 5.0 mEq/L, lactate ≥2.0 mmol/L, urine output <0.5 ml/kg/hr for any 4 hours, capillary refill "flash" or >2 seconds
    ii. *Respiratory:* Treatment with high-flow nasal cannula, continuous/bi-level positive airway pressure, or invasive mechanical ventilation (with need for increase settings for patients with pre-existing or baseline respiratory dysfunction)
    iii. *Kidney*: Serum creatinine ≥2 times upper limit for age/sex or ≥2 times increase from admission
    iv. *Liver*: Total Bilirubin ≥4 mg/dL or alanine aminotransferase (ALT) ≥2 times upper limit of normal for age
    v. *Heme*: Platelet count < 80,000/μL or international normalized ratio (INR) >2.0
    vi. *Neurologic*: Glasgow coma scale score ≤11 or mental status/behavior different from patient's baseline per nurse, physician, or caretaker

B. *Microbiome Sequencing:* Patients stooled into a specimen collector, from which stool was collected at each timepoint using two separate nylon-flocked dry swabs (Copan Diagnostics) while avoiding urine contamination to the extent possible. If stool was soaked into a diaper, a 1x1 cm area of the diaper containing only stool was placed into a 1.5 mL Eppendorf tube. Following storage at -80°C, microbial DNA was isolated from stool using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD), followed by16S rRNA gene sequencing. Barcoded primers containing Illumina adapters were used to amplify the V1-V2 region of the 16S rRNA gene (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 338R: 5'-TGCTGCCTCCCGTAGGAGT-3'). PCR reactions were performed in quadruplicate using Q5 polymerase (New England Biolabs, Ipswich, MA). Each PCR reaction contained 0.5 uM of each primer, 0.34 U Q5 Pol, 1X Buffer, 0.2 mM dNTPs, and 2.5 ul DNA in a total volume of 25 ul. Cycling conditions were as follows: 1 cycle of 98°C for 1 m; 20 cycles of 98°C for 10 s, 56°C for 20 S, and 72°C for 20 sec; 1 cycle of 72°C for 8 m. Samples were pooled in equimolar amounts and then sequenced on the Illumina MiSeq instrument using 2x250 bp chemistry. Extraction blanks and DNA free water were processed and sequenced in parallel. Additionally, eight artificial 16S gene fragments were included as positive sequencing controls. DNA extractions and sequencing were performed by the Children's Hospital of Philadelphia Microbiome Center.

C. *Short-chain fatty acids:* Short chain fatty acids were quantified using a Waters Acquity uPLC System with a HSS T3 1.8 μm 2.1x150 mm column and a Photodiode Detector Array. Plasma samples were diluted (1:4) in ice-cold methanol and centrifuged at 13,000g for 5 minutes. The supernatants were loaded into total recovery vials (Waters, Milford, MA) for analysis. Fecal samples were homogenized in volatile free fatty acid mix (5 μL/mg stool, Sigma Aldrich, St. Louis, MO) and centrifuged twice (13,000g for 5 minutes). The supernatant was filtered using 1.2, 0.65, and 0.22 μm filter plates (Millipore, Billerica, Massachusetts) and the filtrate was loaded into a total recovery vial (Waters,

Milford, MA) for analysis. The flow rate was 0.25 mL/min, the injection volume was 5 uL, the column temperature was 40°C, the sample temperature was 4°C, and the run time was 25 min per sample. Eluent A was 100 mM sodium phosphate monobasic, pH 2.5; eluent B was methanol; the weak needle wash was 0.1% formic acid in water; the strong needle wash was 0.1% formic acid in acetonitrile, and; the seal wash was 10% acetonitrile in water. The gradient was 100% eluent A for 5 minutes, gradient to 70% eluent B from 5-22 minutes, and then 100% eluent A for 3 minutes. The photodiode array was set to read absorbance at 215 nm with 4.8 nm resolution. Sample concentrations were quantified against standard curves of at least five points run in triplicate, and quality control checks (blanks and standards) were run every eight samples. Two patient replicates at each timepoint were averaged to determine final concentration. The minimal range of detection was ≥1 μmol/g of stool and ≥5 μM in plasma. For measurements below the level of assay detection, we imputed a value of 1 μmol/g for stool SFCA and 5 μM for plasma SCFA to facilitate inclusion of these low levels into the analyses.

D. *Mitochondrial respiration:* Mitochondrial respiration was measured in PBMCs isolated from citrated whole blood by density gradient centrifugation as previously described (2). PBMC cell counts were performed using trypan blue exclusion (Countess II, Life Technologies, Grand Island, NY) with median viability 88% (interquartile range 76-95%). After isolation, the PBMC pellet was re-suspended in Hank's balanced salt solution (pH 7.40) containing 5.5 mM glucose, 1mM pyruvate, and 10 mM HEPES, centrifuged a final time at 100g for 10 minutes at 20°C, and then again re-suspended in the same "respiration buffer". Mitochondrial respiration was measured in 2-4 x 10^6 intact PBMCs at 37°C using a high-resolution oxygraph (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria). Oxygen flux (in pmol O_2/sec/10^6 cells), which is directly proportional to oxygen consumption (respiration), was recorded continuously using DatLab software 4.3 (Oroborus Instruments,

Innsbruck, Austria) as shown below and as previously described (2, 3). Intact cells were utilized in order to maintain the cellular microenvironment such that respiration relied on endogenous substrates. After routine oxygen consumption was recorded for 10-20 minutes, the ATP-synthase inhibitor oligomycin (1 μg/mL) was added to induce a state 4-like respiration independent of mitochondrial ATP production. Under these conditions, respiration was primarily due to leakage of protons across the inner mitochondrial membrane (LEAK). Maximal oxygen consumption through the electron transport system (ETS) was obtained by stepwise titration (1-2 μM) of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) until no further increase in oxygen consumption was detected (ETS_{max}). The ETS_{max} indicates the maximal oxygen consumption possible through the electron transport system after pharmacologically uncoupling oxygen utilization from ATP production in order to assess the integrity of the ETS independent of energy production. Therefore, ETS_{max} reflects only the ability the ETS to use electrons to reduce oxygen to water without the need to add high-energy phosphate bonds to produce ATP, a final step in the energy-production pathway that normally helps to regulate ETS activity. Mitochondrial respiration was then inhibited by adding the ETS complex IV inhibitor, sodium azide, in 5-10 mM increments, followed by the ETS complex III inhibitor, antimycin A, in 5 μL increments until no further decrease in oxygen consumption was observed. The residual oxygen flux reflective of non-mitochondrial respiration was subtracted from other respiration values. Respiration supporting mitochondrial ATP synthesis (ATP-linked respiration) was calculated as routine minus LEAK respiration. Spare respiratory capacity (SRC), calculated as ETS_{max} minus routine respiration, is the mitochondrial bioenergetic reserve available for cells to produce ATP in response to a stress-induced increase in metabolic demand. SRC indicates how close to its bionenergetic limit a cell is operating and is, therefore, an indicator of cell metabolic health (4).

eTable 1: Study Specimens Included in Analyses

Study Measure	Controls	Sepsis (Day 1-2)	Sepsis (Day 3-6)	Sepsis (Day 8-14)
Number eligible	44	43	43	43
Stool, microbiome	44 (100)	17 (40)	12 (28)	8 (19)
Stool, SCFAs	0	17 (40)	15 (35)	12 (28)
Plasma, SCFAs	0	39 (91)	35 (810)	23 (53)
PBMC, mitochondrial respiration	0	36 (84)	29 (67)	16 (37)
LPS-stimulated TNF-α	0	36 (84)	29 (67)	16 (37)

Data are presented as n (%)
SCFAs, short-chain fatty acids; PBMC, peripheral blood mononuclear cells; LPS, lipopolysaccharide; TNF, tumor necrosis factor

eTable 2: Patient Characteristics

Variable[a]	Controls	Sepsis	P-value
N	44	43	
Age, years	6.1 (4.4-11.8)	11.2 (6.7-15.0)	0.003
Sex, male	19 (43)	26 (61)	0.08
Race			0.12
White	32 (73)	24 (56)	
Black	8 (18)	10 (23)	
Asian	1 (2)	0	
Other	3 (7)	9 (21)	
Previously healthy	44 (100)	7 (16)	<0.001
Cancer	0	6 (14)	0.01
Inflammatory bowel disease	0	2 (5)	0.24
PRISM-III score	--	10 (5-15)	
PELOD score, PICU admission	--	11 (10-12)	
VIS score, first blood draw	--	14 (5-25)	
Site of infection			
Bacteremia, primary	--	5 (12)	
Respiratory	--	16 (37)	
Abdominal	--	4 (9)	
Genitourinary	--	4 (9)	
Central nervous system	--	1 (2)	
Skin or soft tissue	--	2 (5)	
Other	--	3 (7)	
Unknown	--	8 (19)	
Type of infection			
None	--		
Bacterial	--	17 (40)	
Viral	--	8 (19)	
Unknown	--	18 (42)	
Antibiotic therapy			
Vancomycin	0	36 (84)	
Beta-lactam	0	18 (42)	
Cephalosporin	0	35 (81)	
Carbapenem	0	4 (9)	
Aminoglycoside	0	3 (7)	
Fluoroquinolone	0	7 (16)	
Azithromycin	0	5 (12)	
Clindamycin	0	11 (26)	
Metronidazole	0	8 (19)	
Sulfa	0	1 (2)	

"--" indicates not applicable; PRISM, pediatric risk of mortality; PELOD, pediatric logistic organ dysfunction; PICU, pediatric intensive care unit; VIS, vasoactive-inotrope score
[a]Data presented as median (interquartile range) or n (%)

eTable 3: Association of Abundance of Taxa with Stool Short-Chain Fatty Acids

SCFA	Taxa	β Estimate[a]	P-Value[b]	Significant
Acetic acid	p__Bacteroidetes f__Rikenellaceae	-0.2623	4.719e-14	*
Acetic acid	p__Bacteroidetes g__Bacteroides	-0.2256	9.917e-08	*
Acetic acid	p__Bacteroidetes g__Parabacteroides	-0.1703	0.0004808	*
Acetic acid	p__Proteobacteria g__Bilophila	-0.1597	0.0004808	*
Acetic acid	p__Firmicutes g__Acidaminococcus	-0.1087	0.0005888	*
Acetic acid	p__Firmicutes f__Christensenellaceae	-0.1274	0.0007932	*
Acetic acid	p__Bacteroidetes g__Odoribacter	-0.1135	0.004818	*
Acetic acid	p__Bacteroidetes f__[Barnesiellaceae]	-0.07408	0.007729	*
Acetic acid	p__Fusobacteria g__Fusobacterium	-0.1076	0.01396	*
Acetic acid	p__Firmicutes g__Faecalibacterium	-0.09538	0.01396	*
Acetic acid	p__Proteobacteria g__Sutterella	-0.08545	0.02841	*
Acetic acid	p__Firmicutes g__[Eubacterium]	-0.07944	0.02841	*
Acetic acid	p__Firmicutes g__Blautia	-0.09495	0.03872	*
Acetic acid	p__Actinobacteria g__Corynebacterium	-0.08967	0.03872	*
Acetic acid	p__Firmicutes g__Oscillospira	-0.08509	0.03872	*
Acetic acid	p__Firmicutes g__Clostridium	-0.07806	0.04037	*
Acetic acid	p__Firmicutes g__[Ruminococcus]	-0.07937	0.0499	*
Acetic acid	p__Firmicutes g__Turicibacter	-0.06313	0.0499	*
Acetic acid	p__Firmicutes g__Ruminococcus	-0.07814	0.05393	
Acetic acid	p__Firmicutes g__Oribacterium	-0.05993	0.05393	
Acetic acid	p__Proteobacteria g__Pseudomonas	-0.06678	0.06143	
Acetic acid	p__Firmicutes g__Lactobacillus	-0.08139	0.06143	
Acetic acid	p__Firmicutes g__Holdemania	-0.06132	0.06143	
Acetic acid	p__Proteobacteria g__Pelomonas	-0.0591	0.06394	
Acetic acid	p__Firmicutes f__Ruminococcaceae	-0.07789	0.08195	
Acetic acid	p__Proteobacteria g__Ehrlichia	-0.02989	0.08388	
Acetic acid	p__Firmicutes f__Erysipelotrichaceae	-0.06727	0.08876	
Acetic acid	p__Firmicutes g__Enterococcus	-0.07469	0.08876	
Acetic acid	p__Proteobacteria g__Klebsiella	0.07416	0.08876	
Acetic acid	k__Bacteria	-0.07427	0.08876	
Butyric acid	p__Firmicutes f__Christensenellaceae	0.01039	1.684e-06	*
Butyric acid	p__Proteobacteria g__Bilophila	0.009597	0.001356	*
Butyric acid	p__Firmicutes g__Blautia	0.009176	0.001356	*
Butyric acid	p__Firmicutes g__Phascolarctobacterium	0.004353	0.002129	*
Butyric acid	p__Bacteroidetes g__Paraprevotella	0.003929	0.005345	*
Butyric acid	p__Firmicutes g__Dialister	0.006892	0.02499	*
Butyric acid	p__Firmicutes g__Catenibacterium	0.004093	0.02534	*

Butyric acid	p__Bacteroidetes g__Parabacteroides	0.008014	0.02536	*
Butyric acid	p__Bacteroidetes g__Butyricimonas	0.003893	0.02782	*
Butyric acid	p__Firmicutes f__Erysipelotrichaceae	0.00611	0.03948	*
Butyric acid	p__Bacteroidetes g__Odoribacter	0.004769	0.1175	
Butyric acid	p__Firmicutes g__Megasphaera	0.004279	0.1212	
Butyric acid	p__Firmicutes g__Turicibacter	0.003436	0.1348	
Isobutyric acid	p__Proteobacteria g__Ehrlichia	0.1492	9.405e-19	*
Isobutyric acid	p__Bacteroidetes f__Rikenellaceae	0.2253	9.405e-19	*
Isobutyric acid	p__Bacteroidetes f__[Barnesiellaceae]	0.1286	9.405e-19	*
Isobutyric acid	p__Proteobacteria g__Sutterella	0.1661	1.907e-15	*
Isobutyric acid	p__Bacteroidetes g__Bacteroides	0.2166	1.46e-11	*
Isobutyric acid	p__Firmicutes g__Faecalibacterium	0.1495	5.915e-10	*
Isobutyric acid	p__Firmicutes g__[Eubacterium]	0.1348	1.739e-09	*
Isobutyric acid	p__Bacteroidetes o__Bacteroidales	0.1415	2.385e-08	*
Isobutyric acid	p__Firmicutes g__Holdemania	0.1144	1.265e-07	*
Isobutyric acid	p__Firmicutes g__Clostridium	0.1332	5.836e-07	*
Isobutyric acid	p__Firmicutes f__Christensenellaceae	0.141	5.836e-07	*
Isobutyric acid	p__Firmicutes g__Ruminococcus	0.137	1.81e-06	*
Isobutyric acid	p__Bacteroidetes g__Odoribacter	0.1323	3.206e-06	*
Isobutyric acid	p__Firmicutes g__Oscillospira	0.1346	5.201e-06	*
Isobutyric acid	p__Firmicutes f__Ruminococcaceae	0.1438	6.805e-06	*
Isobutyric acid	p__Firmicutes g__Turicibacter	0.0945	1.874e-05	*
Isobutyric acid	p__Firmicutes g__Dialister	0.1346	2.853e-05	*
Isobutyric acid	p__Proteobacteria g__Bilophila	0.1419	3.957e-05	*
Isobutyric acid	p__Fusobacteria g__Fusobacterium	0.1229	4.79e-05	*
Isobutyric acid	p__Firmicutes g__[Ruminococcus]	0.1155	4.79e-05	*
Isobutyric acid	p__Actinobacteria g__Eggerthella	0.1154	5.755e-05	*
Isobutyric acid	p__Bacteroidetes g__Parabacteroides	0.1484	0.0001863	*
Isobutyric acid	p__Proteobacteria f__Enterobacteriaceae	0.1169	0.003508	*
Isobutyric acid	p__Verrucomicrobia g__Akkermansia	0.0936	0.004812	*
Isobutyric acid	p__Actinobacteria g__Atopobium	0.08822	0.004919	*
Isobutyric acid	p__Actinobacteria g__Corynebacterium	0.08423	0.005871	*
Isobutyric acid	p__Firmicutes g__Granulicatella	0.0936	0.006057	*
Isobutyric acid	p__Firmicutes g__Oribacterium	0.06412	0.006659	*
Isobutyric acid	p__Firmicutes o__Clostridiales	0.07501	0.006932	*
Isobutyric acid	p__Firmicutes g__Veillonella	0.09842	0.007904	*
Isobutyric acid	p__Firmicutes g__Parvimonas	0.07568	0.00865	*
Isobutyric acid	k__Bacteria	0.09393	0.009522	*
Isobutyric acid	p__Firmicutes g__Coprococcus	0.07214	0.02065	*

Isobutyric acid	p__Firmicutes g__Anaerococcus	0.0602	0.02473	*
Isobutyric acid	p__Firmicutes g__Enterococcus	0.07885	0.02513	*
Isobutyric acid	p__Firmicutes f__Streptococcaceae	0.06763	0.0252	*
Isobutyric acid	p__Bacteroidetes g__Prevotella	0.06607	0.04781	*
Caproic acid	p__Proteobacteria g__Ehrlichia	0.01825	4.707e-18	*
Caproic acid	p__Bacteroidetes f__[Barnesiellaceae]	0.0202	3.511e-15	*
Caproic acid	p__Proteobacteria g__Sutterella	0.02642	1.493e-13	*
Caproic acid	p__Bacteroidetes f__Rikenellaceae	0.03042	6.039e-09	*
Caproic acid	p__Firmicutes g__Faecalibacterium	0.02397	8.09e-09	*
Caproic acid	p__Firmicutes g__[Eubacterium]	0.02168	1.968e-08	*
Caproic acid	p__Bacteroidetes o__Bacteroidales	0.0246	3.924e-08	*
Caproic acid	p__Firmicutes g__Holdemania	0.01907	2.308e-07	*
Caproic acid	p__Firmicutes g__Clostridium	0.02276	7.361e-07	*
Caproic acid	p__Bacteroidetes g__Bacteroides	0.02887	1.266e-06	*
Caproic acid	p__Firmicutes g__Turicibacter	0.01774	1.641e-06	*
Caproic acid	p__Firmicutes g__Ruminococcus	0.0237	1.674e-06	*
Caproic acid	p__Firmicutes f__Christensenellaceae	0.02359	1.88e-06	*
Caproic acid	p__Firmicutes g__Oscillospira	0.02381	2.459e-06	*
Caproic acid	p__Firmicutes f__Ruminococcaceae	0.02537	3.95e-06	*
Caproic acid	p__Bacteroidetes g__Odoribacter	0.02241	4.29e-06	*
Caproic acid	p__Firmicutes g__[Ruminococcus]	0.0204	4.255e-05	*
Caproic acid	p__Actinobacteria g__Eggerthella	0.02074	4.812e-05	*
Caproic acid	p__Firmicutes g__Dialister	0.02242	0.0001464	*
Caproic acid	p__Verrucomicrobia g__Akkermansia	0.02146	0.0003701	*
Caproic acid	p__Proteobacteria g__Bilophila	0.02274	0.0003701	*
Caproic acid	p__Bacteroidetes g__Parabacteroides	0.025	0.0005357	*
Caproic acid	k__Bacteria	0.02107	0.001693	*
Caproic acid	p__Fusobacteria g__Fusobacterium	0.01879	0.003222	*
Caproic acid	p__Proteobacteria f__Enterobacteriaceae	0.022	0.003363	*
Caproic acid	p__Firmicutes g__Enterococcus	0.01452	0.04565	*
Caproic acid	p__Firmicutes g__Anaerococcus	0.0108	0.07957	
Caproic acid	p__Actinobacteria g__Corynebacterium	0.01214	0.1106	

SCFA, short-chain fatty acid

[a]A negative β estimate indicates that a lower taxa abundance is associated with a higher level of the short-chain fatty acid. A positive β estimate indicates that a lower taxa abundance is associated with a lower level of the short-chain fatty acid.

[b]P-values corrected for multiple comparisons with significance if corrected $p<0.05$.

eTable 4: Intestinal Microbiome Diversity and PBMC Mitochondrial Respiration

Mitochondrial Respiration	Alpha-Diversity				Beta-Diversity			
	Richness		Shannon		Unweighted		Weighted	
	β	P-value[a]	β	P-value[a]	R^2	P-value[a]	R^2	P-value[a]
Basal	-3.8	0.14	-0.07	0.22	0.05	0.006	0.14	0.002
ATP-linked	-4.9	0.09	-0.06	0.38	0.05	0.008	0.16	0.001
LEAK	2.4	0.8	-0.27	0.17	0.04	0.03	0.04	0.12
ETS_{max}	-1.1	0.13	-0.04	0.03	0.06	0.002	0.04	0.11
SRC	-1.2	0.17	-0.04	0.03	0.06	0.002	0.02	0.30

ATP, adenosine triphosphate; ETS_{max}, maximal uncoupled respiration through the electron transport system; SRC, spare respiratory capacity

[a]P-value after correction for multiple comparisons using the Benjamin-Hochberg false discovery rate method

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