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Prolonged antibiotic use induces intestinal injury in mice that is repaired after removing antibiotic pressure: implications for empiric antibiotic therapy

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Abstract

Metabolic profiling of urine and fecal extracts, histological investigation of intestinal ilea, and fecal metagenomics analyses were used to investigate effects of prolonged antibiotic use in mice. The study provides insight into the effects of extended empiric antibiotic therapy in humans. Mice were administered a broad-spectrum antibiotic for four consecutive days followed by oral gavage with *Clostridium butyricum*, an opportunistic gram-positive pathogenic bacteria commonly

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isolated in fecal and blood cultures of necrotizing enterocolitis patients. Metagenomics data indicated loss of bacterial diversity after 4 days on antibiotics that was restored after removing antibiotic pressure. Histological analyses indicated damage to ileal villi after antibiotic treatment that underwent repair after lifting antibiotic pressure. Metabolic profiling confirmed intestinal injury in antibiotic-treated mice indicated by increased urinary trans-4-hydroxy-L-proline, a breakdown product of collagen present in connective tissue of ileal villi that may serve as a biomarker for antibiotic-induced injury in at risk populations.

Keywords

Antibiotic; Metabolomics; Mouse model; Necrotizing enterocolitis; NMR; PCA

1 Introduction

Empiric antibiotic therapy is prescribed for many human conditions including urinary tract infections (Aypak et al. 2009), inflammatory bowel disease (Balfour Sartor 2004; Shanahan and Bernstein 2004; Perencevich and Burakoff 2006; Hammer 2011) organ transplants (Hamandi et al. 2009), lyme disease (Klempner et al. 2001; Marques 2008; Wright et al. 2012), cancer patients with febrile neutropenia caused by chemotherapy Kern et al. 2013) and nursing home acquired infections in elderly patients (Xie et al. 2012). Prolonged broad-spectrum empiric antibiotics are also prescribed for serious bacterial infections such as bacteremia, sepsis and pneumonia (Kollef 2008; Iapichino et al. 2008; Siddiqui and Razzak 2012). While antibiotics offer obvious benefits for treating human bacterial infections, even short regimens can have long-lasting impacts on the human gut microbial population (Jernberg et al. 2007; Dethlefsen et al. 2008; Jakobsson et al. 2010) and prolonged broad spectrum antibiotic therapy can significantly alter the normal balance of beneficial microbiota (Jernberg et al. 2007; Dethlefsen et al. 2008; Willing et al. 2011) essential to innate and adaptive immunity, relative infection susceptibilities, immune tolerance, bioavailability of nutrients, and intestinal barrier function (Preidis and Versalovic 2009; Neish 2009; MacDonald and Monteleone 2005). Antibiotic therapy can also give rise to emergent antibiotic-resistant pathogenic bacteria precipitating severe health consequences as in antibiotic associated diarrhea (Doron et al. 2008; Hempel et al. 2012) caused by *Clostridium difficile* leading to pseudomembranous colitis (Faris et al. 2010).

Judicial use of empiric antibiotics is crucial in fragile populations such as neonates and preterm infants. Recent studies have demonstrated a strong correlation between empiric antibiotic treatment of extremely low birth weight (ELBW) preterm infants and higher rates of neonatal necrotizing enterocolitis (NEC) (Kuppala et al. 2011; Cotton et al. 2009), early onset sepsis (Stoll et al. 2002; Cordero and Ayers 2003; Clark et al. 2006), and death (Kuppala et al. 2011; Clark et al. 2006). Nearly all ELBW neonates receive empiric antibiotics in the first days following birth (Cotton et al. 2009) even though many are not actually infected (Clark et al. 2006; Stoll et al. 2005). Empiric antibiotic therapy continues to be standard in neonatal intensive care units for preterm infants considered at risk of sepsis as the benefits are widely perceived to outweigh the risks (McGuire et al. 2004) although there is growing concern about this practice (Tripathi et al. 2012; Tzialla et al. 2012).

The gastrointestinal tract of preterm infants is often colonized initially with relatively few beneficial bacteria compared to full-term newborn infants due to empiric antibiotic therapy. Preterm infants also experience delayed gut colonization with protective bacterial species, such as *Bifidobacteria*, while high levels of *Enterobacteria* and *Clostridia* are commonly found in these same infants with *C. butyricum* and *C. perfringens* most common among *Clostridial* species (Gewolb et al. 1999). This early gut microbiota imbalance may contribute to NEC onset since minor microflora species inhabiting intestines under normal circumstances have the potential to become pathogenic when present in high numbers (Lawrence et al. 1982; Kosloske 1994) and *C. butyricum*, suggested as a potential contributing factor in NEC (Sturm et al. 1980; Howard et al. 1977), is thought to be such an opportunistic pathogen.

Here, in BALB/c mice, we examined the gut microbial community, urine and fecal metabolic profiles, and ileum of the small intestine under extended broad-spectrum antibiotics and following removal of antibiotic pressure and exposure to *C. butyricum*. Fecal metagenomics data, histological analyses of intestinal ilea, and proton (^1H) nuclear magnetic resonance (NMR) spectroscopy-based metabolic profiling of urine and fecal extracts indicated decreased gut microbial diversity and injury to distal intestinal ileal villi under antibiotic pressure that reversed after stopping antibiotic administration indicating an important host/gut microbial symbiosis required for normal gut structure and function. Metabolic profiling data revealed urinary trans-4-hydroxy-L-proline, a breakdown product of collagen present in connective tissue of ileal villi, as a potentially useful biomarker of antibiotic-induced injury in at risk populations receiving empiric or prolonged broad-spectrum antibiotics.

2 Materials and methods

2.1 Animals and treatment

All animal studies were approved by the Miami University Institutional Animal Care and Use Committee. Mice were 4 weeks old at the start of the study, which corresponded to 1 week post weaning. Mice were fed a common rodent liquid diet that comes in powder form was prepared each day. Samples were collected from 24 BALB/c mice (Harlan Laboratories, Indianapolis, IN) held in custom-built metabolism cages (Romick-Rosendale 2011). Modifications were made to cages to limit cross contamination between the urine and feces. Mice were administered 50 ml of PMI micro-stabilized rodent liquid diet LD101 (Test-Diet, Richmond, IN) once daily for four consecutive days. On the fourth day, four mice were sacrificed for histological studies. Beginning on the fifth day, all remaining mice were given the same liquid diet supplemented with 0.4 μl per gram of body mass of enrofloxacin 10 % oral solution (Oxford Veterinary Hospital, Oxford, Ohio) for four consecutive days. Similar dosages of enrofloxacin were previously administered to rodents and shown to eliminate both gram-negative and gram-positive microflora. On the eighth day, four additional mice were sacrificed for histological studies. On the ninth day, mice were administered *C. butyricum* LMG 1217 (Belgian Co-Ordinated Collections of Micro-Organisms, Belgium) by intragastric gavage. All remaining mice were sacrificed on the thirtieth day and subjected to histological analyses. Animal experiments were conducted according to federal and local

guidelines and the animal handling protocols were approved by Miami University Institutional Animal Care and Use Committee.

2.2 Metagenomics analysis of bacterial species in feces

Frozen fecal samples were pooled from six mice in each of the four groups and shipped to the Research and Testing Laboratory, LLC (Lubbock, Texas) for metagenomics data collection. The 16sRNA samples were extracted on site by Research and Testing Laboratory, LLC and subjected to tag-encoded FLX amplicon pyrosequencing (Sun et al. 2011) at a 3 K average coverage. Raw metagenomics data was analyzed using Qiime (Caporaso et al. 2010) (<http://www.qiime.org>). The resulting data were subjected to a two-way cluster analysis using Ward's clustering method and the Jaccard similarity coefficient in R (R Development Core Team 2008).

2.3 Intestinal histology

Intestinal ileum biopsies from control mice, antibiotic-treated mice, and *Clostridial*-treated mice were fixed in neutral buffered formalin solution overnight, dehydrated, embedded in paraffin and sectioned at 5 μ m. Staining of intestinal ileum sections was carried out with hematoxylin and eosin. Results are based on assessment of four or more mice per group examined.

2.4 Biofluid sample collection

Control urine and fecal samples were collected for 4 days prior to treatment. Urine and fecal samples were collected once daily during the 4 days that enrofloxacin (Baytril) was given. Once *C. butyricum* was introduced by intragastric gavage, urine and fecal samples were collected every other day for the remainder of the study. All mice were maintained in separate animal cages and transferred to individual metabolic cages for all sample collection. Mice were transferred to the metabolic cages at 6 p.m. and urine and fecal samples were collected at 9 a.m. the following day. All samples were kept at -80°C after collection and prior to NMR analysis.

2.5 Preparation of biological fluids for NMR analysis

Samples were thawed on ice prior to preparation for NMR analysis. A 1-ml aliquot of each urine sample was centrifuged for 10 min at $2,500\times g$, and then 350 μ l of clear urine was pipette into a 1.5 ml microcentrifuge tube. A volume of 350 μ l of buffer (300 mM KH_2PO_4 , 2 mM NaN_3 , 0.01 % Trimethylsilyl propionate (TSP) in 100 % D_2O , pH 7.4) was added to each urine sample. A volume of 600 μ l of the urine/buffer mixture was then pipette into a 5 mm NMR tube (Norell ST500-7). Aqueous fecal extract was prepared by adding a known amount of thawed feces (~ 0.5 g) to two volumes (w/v) of sterile phosphate buffered saline (1.0 mM Na_2HPO_4 , 8.1 mM NaH_2PO_4 , 150 mM NaCl, pH 7.4). The fecal/buffer mixture was then homogenized by vortexing for 1 min per sample. The samples were then centrifuged at 4°C for 1 h at $18,000\times g$. A volume of 350 μ l of the fecal extract was then added to 350 μ l of water containing 20 % D_2O , and 0.02 % TSP in a 1.5 ml microcentrifuge tube. The mixture was then vortexed for 1 min and then centrifuged for 10 min at $10,000\times g$.

A volume of 550 μ l of the clear supernatant was pipette into a 5 mm NMR tube (Norell ST500-7).

2.6 NMR data collection and processing

All data were collected as described in Romick-Rosendale et al. (Romick-Rosendale et al. 2009) at 850 MHz ^1H frequency using a Bruker Avance III NMR spectrometer.

2.7 Multivariate statistical analysis of NMR spectra

Principal component analysis (PCA) and statistical analysis were performed using AMIX (Bruker Biospin, Billerica, MA). NMR spectra were binned into 0.03 ppm wide buckets over the region δ 10.0–0.5 ppm. The region of δ 4.75–4.875 was removed from the analysis to avoid effects of imperfect water suppression. All spectra were normalized and PCA analysis was conducted as described in Watanabe et al. (2011). The Bonferroni-corrected α -values were determined as described by Goodpaster et al. (Goodpaster et al. 2010) for each urine and fecal comparison: Urine Control/Antibiotic-treated: 5.07×10^{-5} , Urine Antibiotic-treated/*Clostridium*-treated: 7.94×10^{-5} , Urine Control/*Clostridium*-treated: 8.01×10^{-5} , Feces Control/Antibiotic-treated: 9.82×10^{-5} , Feces Antibiotic-treated/*Clostridium*-treated: 9.09×10^{-5} , and comparison between Feces Control/*Clostridium*-treated: 9.03×10^{-5} . Changes in bucket intensities and metabolite concentrations, with p values less than the Bonferroni-corrected α -values were considered to be statistically significant. The loadings plot data points were color-coded according to bucket p -values: black ($>\alpha$ -value, i.e., not statistically significant), blue (α -value $> 10^{-5}$), green (10^{-5} – 10^{-6}), yellow (10^{-6} – 10^{-7}), red (10^{-7} –0). Fold-changes were calculated by dividing the antibiotic-treated bucket means by the control bucket means, the *Clostridium*-treated bucket means by the control bucket means, and the clostridium-treated bucket means by the antibiotic-treated bucket means. For fold changes less than 1.0, the negative inverse of the above ratios were calculated and the resulting values were expressed as “negative” fold changes.

2.8 Mahalanobis distance and F value calculations

Mahalanobis distance calculations were performed in MatLab using a method to quantitatively and statistically analyze group separation of NMR data developed by Goodpaster et al. (Goodpaster and Kennedy 2011) Critical F values were calculated using <http://www.danielsooper.com/statcalc/calc04.aspx>.

2.9 Identification of metabolites

Statistical significance analysis was used to identify the bucket frequencies that showed significant changes in the various treatment groups compared to the control, or changes between treatment groups (Goodpaster et al. 2010). Next, a list of tentatively assigned metabolites was generated for further validation. The chemical shifts for each tentatively assigned metabolite were compared against the chemical shifts of candidate metabolites using the ChenomX NMR Suite (ChenomX Inc., Edmonton, Alberta, Canada) and other published data (Bundy et al. 2007). A summary of NMR spectral assignments of metabolites is given in Table S1.

3 Results

3.1 Metagenomics analysis of fecal bacteria

Metagenomics analysis of feces (1) prior to antibiotics, (2) 4 days after continuous antibiotics, (3) 3 weeks after stopping antibiotics and administration of oral gavage with *C. butyricum*, and (4) 6 weeks after stopping antibiotics and 3 weeks after oral gavage with *B. infantis* indicated 76 operational taxonomic units (OTUs) with 18 OTUs present in all four samples, 13 OTUs present in three samples, 16 OTUs present in two samples and 29 OTUs present in one sample (Fig. S1). A redistribution of bacterial taxa occurred under antibiotic pressure that largely reversed after removing antibiotics, however, differences in the bacterial taxa profile remained even after 6 weeks. Examination of the twenty most abundant bacterial taxa in each group (Fig. 1a) revealed changes in relative abundance of bacterial taxa. Prior to antibiotics, the most abundant bacterial taxa had comparable relative abundance, whereas *Akkermansia muciniphila* dominated under antibiotic pressure. Among *Clostridial* species (Fig. 1b) *C. tepidiprofundum* dominated *Clostridial* species prior to antibiotics, diminished relative to other *Clostridial* species under antibiotic pressure, and again dominated *Clostridial* species after removing antibiotics.

3.2 Metabolic profiling of mice urine and fecal extracts indicated similarity between control mice and mice exposed to *C. butyricum* after removal of antibiotic pressure

NMR data were collected on samples from control mice (C), under antibiotic pressure (A), and following removal of antibiotics and exposure to *C. butyricum* (CB). Representative urine and fecal extract NMR spectra from each group are shown in Fig. S2. Spectral differences between groups were evident from visual inspection and validated by statistical significance analysis. Unsupervised principal components analysis (PCA) was carried out on spectra from all three groups. PCA scores plots of urine samples (Fig. 2a) revealed that all groups formed distinct clusters indicating unique urinary metabolic profiles. Magnitudes and statistical significance of cluster separations were evaluated by calculating the Mahalanobis distance (D_M) between cluster centroids and comparing F-scores to critical F values (Fig. 2a, Supplementary Table S2). The D_M between C and A group centroids was 2.063 (F value of >20) indicating statistically significant separation of these groups. Antibiotic-treated mice clustered separately from CB mice, with a D_M of 3.103 (F value of >44). Surprisingly, urine spectra of CB mice appeared to cluster with C mice, however the small D_M of 1.579 (F-score of >11), indicated the group separation remained statistically significant. Similar cluster patterns were observed in fecal sample PCA scores plots (Fig. 2b), however, cluster separations were larger. For example, the D_M was 4.152 between the cluster centroids of C and A groups (F value > 60). Likewise, D_M was 4.938 between cluster centroids of A and CB groups, whereas D_M was 1.276 between C and CB group clusters.

PCA scores plots of pair-wise comparisons of Control/Antibiotic-treated (C vs A), Antibiotic-treated/C. *butyricum*-treated (A vs CB), Control/C. *butyricum*-treated (C vs CB) urine and fecal samples are shown in Fig. 3 and the D_M values and F values are given in Table S2. The PCA indicated all groups separated into statistically significant distinct clusters. The PCA loadings plots for each pair-wise comparison are shown in Fig. S3. Eight metabolites (trans-4-hydroxy-L-proline, betaine, taurine, ornithine, hippurate, 3-

indoxylsulfate, allantoin, and 2-oxobutyrate) differed in urines of C and A groups (Table S3) whereas 12 metabolites (creatinine, creatine, β -alanine, succinate, 3-hydroxyisovalerate, 3-methyl-2-oxovalerate, formate, fumarate, trans-4-hydroxy-L-proline, glycolate, betaine, and sarcosine) differed between CB and A groups (Table S4). Only two metabolites (methionine and uracil) differed between C and CB groups (Table S5). More metabolite changes occurred in comparisons of fecal samples than urine samples. Five metabolites (galactose, glucose, choline, glutamine, and propionate) differed between fecal extracts of C and A groups (Table S6) whereas nine metabolites (succinate, propionate, β -alanine, valine, isoleucine, galactose, glucose, glutamine, and choline) differed between fecal extracts from CB and A groups (Table S7). Twelve metabolites (phenylalanine, tyrosine, creatinine, creatine, aspartate, methionine, β -alanine, isoleucine, valine, leucine, propionate, and butyrate) changed between C and CB groups (Table S8).

3.3 Histological analyses of mice distal intestinal ilea revealed villi damage under antibiotic pressure that was repaired after removal of antibiotic pressure

Histological cross-sections of distal intestinal ilea from C, A and CB mice are shown in Fig. 4. Characteristic features in C mice (Fig. 4a–c) included well-shaped, elongated villi lined with columnar epithelia having regularly-spaced dark-staining nuclei and evenly distributed dark-staining nuclei in lamina propria defining the villi core, composed of arteries, veins, capillaries, lymphatic vessels, and connective tissue. Figure 4d–f show cross-sections of distal intestinal ilea from A mice, revealing loss of regular villi structure, lack of regularly distributed columnar epithelial cell nuclei lining villi, shortened and irregular villi, in one case ruptured villi (Fig. 4e), and concentration of dark-staining nuclei near the villi base (Fig. 4d–f). These data revealed damage to distal ileal villi associated with prolonged antibiotic treatment. Moreover, two mice died following antibiotic treatment and the histological investigation showed bowel perforation in these mice. Sections of distal intestinal ilea of mice sacrificed 2 weeks after *C. butyricum* administration are shown in Fig. 4g–i revealing villi resembling those of C mice. These data indicated removal of antibiotic pressure and restored gut bacterial colonization resulted in repair of antibiotic-associated damage to ileal villi.

3.4 Metabolic evidence of collagen breakdown in distal ileal villi in antibiotic-treated mice and repair following removal of antibiotic pressure

Trans-4-hydroxy-L-proline (T4HP) was absent in urines of C and CB mice but present in large amounts in A mice. Moreover, T4HP experienced the largest change in the C versus A group comparison exhibiting an average +2.1 fold change for the 15 T4HP-associated buckets, i.e. an average >100 % increase in peak intensity in the A Group compared to the C group. Correspondingly, the p-value for 14 out of the 15 T4HP-associated buckets were 10^{-4} or smaller. T4HP is a breakdown product of collagen (Näntö-Salonen et al. 1984) found largely in connective tissue (Udenfriend 1966). The villi lamina propria core is mainly composed of connective tissue (Udenfriend 1966); therefore damage to ileal villi and its connective tissue could result in collagen leakage into ileal lumina and degradation into constituent components like T4HP. Breakdown of collagen would explain T4HP in urine of A mice consistent with rat and human studies reporting increased urinary T4HP in collagen breakdown from other causes (Näntö-Salonen et al. 1984, Prockop and Sjoerdsma 1961;

Anasuya and Rao 1970). T4HP may serve as a useful marker of intestinal necrosis in NEC in preterm infants and in other childhood inflammatory bowel diseases.

3.5 Further metabolic evidence of intestinal and kidney injury in antibiotic-treated mice

Creatinine, creatine and 3-indoxysulfate decreased and taurine and ornithine increased in A mice urines. Excretion of creatinine and creatine into urine is regulated by kidneys and glomerular filtration rate (Luft et al. 1978). Decreased glomerular filtration associated with kidney injury could lead to decreased excretion of these metabolites. Removal of antibiotic pressure and exposure to *C. butyricum* restored these metabolites to levels observed in C mice urines. Gut flora can also regulate urine levels of creatinine and creatine since their degradation is enzymatically driven in bacteria unlike in vertebrates (Wyss and Kaddurah-Daouk 2000). Lower urinary levels of 3-indoxysulfate in A mice compared to CB mice were likely associated with reduced excretion, since individuals suffering kidney disease often experience uremia in which metabolites such as 3-indoxysulfate accumulate in blood and are not excreted as waste products in urine (Zgoda-Pols et al. 2011).

Increased urinary levels of taurine, ornithine and betaine in A mice indicated reduced reabsorption in renal proximal tubules as observed in cases of renal damage (Merheb et al. 2007). Taurine has protective properties (Kerai et al. 2001) and hosts may increase taurine production to counteract negative renal and intestinal effects associated with antibiotic treatment. Increased excretion of ornithine occurs in kidney damage and other diseases, such as cystinuria (Fjellstedt et al. 2003). Betaine, increased in urine of A mice compared to C and CB groups, is synthesized from choline in vertebrates (Craig 2004) and may be increased in urine of A mice due to increased urinary choline. Researchers have suggested that betaine is synthesized by renal tubular cells to protect kidney cells against osmotic stress (Chambers and Kunin 1985).

Fecal butyrate and glutamine changed in mice with suspected intestinal injury. Butyrate is a major fermentation product of many bacterial species (Miller and Wolin 1996), especially *C. butyricum*; however, butyrate produced after colonization of mice with *Clostridial* species was likely consumed during ileal villi epithelia repair. Butyrate is a chief energy source for intestinal epithelial cells (Roediger 1980), which could explain its decrease in feces of CB mice compared to C mice since ileal epithelial cells would consume butyrate to repair damage associated with antibiotic treatment. Elevated glutamine in A mice feces was correlated with ileal villi damage. Glutamine is metabolized in the small intestine as key respiratory fuel for enterocytes (Hartmann and Plauth 1989); however, intestinal injury caused by antibiotics likely hindered uptake resulting in increased fecal excretion.

3.6 Changes in gut bacteria metabolism

Urinary fumarate, sarcosine, and glycolate decreased in CB mice compared to A mice likely due to loss of microbial enzymes. Succinate is normally converted to fumarate (King et al. 2006), however, absent certain bacterial enzymes the reaction does not take place in the gut. Consequently, succinate accumulation in CB mice indicated absence of key bacterial enzymes for conversion to fumarate. Sarcosine and glycolate are in the creatine/creatinine pathway and creatine is converted to sarcosine by microflora. However, loss of these

microbial enzymes could cause elevated creatine in CB mice compared to A mice (Wyss and Kaddurah-Daouk 2000). Glycolate is a bacterial degradation product of creatinine (Wyss and Kaddurah-Daouk 2000) and without key bacterial enzymes for degradation, creatinine would be higher in CB mice urines.

Hippurate, allantoin, 2-oxobutyrate and β -alanine decreased in urine of A mice compared to C mice. Hippurate is produced from benzoate (Beliveau and Brusilow 1987); however, altered gut metabolism could lead to reduced benzoate production and ultimately hippurate synthesis (Williams et al. 2010; Liu et al. 2011). Allantoin decreased in A mice urine due to reduced protein break-down into purine and pyrimidine bases by gut microflora, reduced absorption through the intestinal wall, and reduced allantoin end product excreted in urine (Chen et al. 1992; Condon and Hatfield 1970). A similar scenario could explain decreased urinary 2-oxobutyrate in A mice since it is produced from metabolism of amino acids such as threonine and methionine (Paxton et al. 1986) and loss of microbial species would reduce these breakdown products. β -alanine is produced from incomplete oxidation of pantothenate (Gojković et al. 2001) and from digestion of proteins by gut microbial species (West 2011). Decreased β -alanine in A mice indicated reduced microbial species to perform these metabolic processes. Similar findings for β -alanine were found in fecal extracts.

Uracil decreased in urine of CB mice compared to C mice. Uracil catabolism is performed by bacteria using a reductive pathway to convert uracil to β -alanine (West 2011). Here, removing antibiotic pressure and treatment with *C. butyricum* resulted in gut microflora more efficient at catabolizing pyrimidine bases such as uracil, resulting in higher urinary uracil levels in C mice. Methionine increased in urine of CB mice compared to C mice due to more efficient conversion of homocysteine to methionine by CB mouse gut microbes (Shapiro et al. 1964). S-adenosylmethionine is needed for homocysteine conversion; normal gut flora apparently generate an abundance of this metabolite (Salem and Foster 1972) and methionine increased in fecal extracts of CB mice compared to C mice.

Tyrosine, creatinine, creatine and aspartate increased in fecal extracts of CB mice compared to C mice. Intestinal microflora metabolize these metabolites; consequently, their accumulation in feces would result from reduced catabolism by re-colonized gut flora compared to C mice gut bacteria (Wyss and Kaddurah-Daouk 2000; Sparnins and Chapman 1976). Branched-chain amino acids (BCAAs) isoleucine, leucine and valine increased in feces of CB mice compared to C and A groups. BCAAs are Krebs cycle intermediates produced from breakdown of proteins by microbial enzymes (Salter and Fulford 1974; Freundlich et al. 1962). Reduced gut microbial enzymes in A mice and less efficient breakdown of proteins in C mice compared to CB mice may explain these findings.

3.7 Changes in gut bacteria fermentation

Urinary alanine, succinate and 3-hydroxyisovalerate increased in CB mice compared to A mice, potentially due to fermentation by *Clostridial* species. Alanine and succinate are common products of sugar (Ravot et al. 1996) and carbohydrate fermentation (Scheifinger and Wolin 1973), respectively. 3-hydroxyisovalerate is produced by leucine and valine fermentation (Freundlich et al. 1962).

Phenylalanine increased in CB mice feces compared to C mice, likely due to higher tyrosine fermentation in C mice (Smith and Macfarlane 1997). Propionate increased in C and CB mice feces compared to A mice. Propionate, like succinate, is derived from carbohydrate fermentation (Scheifinger and Wolin 1973; Stams et al. 1984) using transcarboxylase (Stams et al. 1984). Loss of this microbial enzyme in A mice would result in decreased propionate production. Galactose and glucose increased in A mice feces compared to C and CB mice. Fermentation of sugars by microbial species is well documented (Schaub and Lentze 1973; Koser and Saunders 1933) and sugars are an ideal carbon source for many bacterial strains (Brückner and Titgemeyer 2002). Glucose, catabolized by the Embden-Meyerhof-Parnas pathway, may be diminished in antibiotic-treated mice because of loss of important microbial enzymes (Macy et al. 1978).

3.8 Changes in bacterial growth factors

Formate decreased in CB mice urine compared to A mice. Formate is consumed by many bacteria including methanogens (Kosaka et al. 2008; Cselovszky et al. 1992). Formate is a growth substrate consumed by intestinal bacteria (Kosaka et al. 2008). Increased urinary formate in A mice indicated reduced gut microbial growth.

Choline, increased in feces of A mice compared to C and CB mice, is converted to trimethylamine by intestinal microflora (De La Huerga and Popper 1952); however, reduced gut microbes in A mice resulted in accumulation of choline in feces. Decreased choline in feces of CB compared to A mice was correlated with increased gut microbial colonization resulting in increased choline utilization in cell membrane synthesis, phospholipid metabolism and cholesterol integration (Martin et al. 2008; Mosser and Tomasz 1970).

4 Conclusions

Empiric antibiotic treatment of preterm infants has been implicated as a potential causative factor of NEC, late onset sepsis, and even death and there is growing debate as to whether prolonged administration of prophylactic empirical antibiotic therapy for preterm infants at risk to infection causes more harm than benefit (Kuppala et al. 2011). Our mouse study indicated distal intestinal ileal villi injury associated with prolonged antibiotic pressure. Metagenomics analysis indicated a significant reduction of gut bacteria diversity under antibiotic pressure and increased abundance of the commensal bacterium, *A. muciniphila*. Overcolonization of mucin-degrading bacteria may be a causative factor in development of NEC-like symptoms and associated intestinal injury, since intestinal mucosa is not only utilized as an energy source by, but also is an important habitat for, intestinal flora (Hooper et al. 2002; Derrien et al. 2004).

Prolonged and empiric antibiotics has potentially far reaching consequences for diseases other than NEC. For example, disruption of the mucosal barrier has been implicated in the inflammatory bowel disorders Crohn's disease and ulcerative colitis (UC). More specifically, the mucosal layer in the inflamed region of the intestine in UC patients was found to be thinner (Pullan et al. 1994), leading to the hypothesis that reduced mucus may correlated with elevated abundance of mucin-degrading bacteria, as observed here. In our case, once antibiotic pressure was removed, metagenomics analysis indicated that the

diversity of gut bacterial species returned to pre-antibiotic-treatment levels, however, the composition changed significantly upon re-colonization. These observations indicated that prolonged empirical antibiotic therapy could have potentially negative effects on the immature gut of the premature human infant and potentially contribute to NEC incidence in human preterm infants.

Rupturing of intestinal villi can lead to decreased intestinal surface area and reduced nutrient absorption into the blood stream through the intestinal wall. Consistent with our histological findings, the most striking change in the metabolic profiles of urine samples from mice under antibiotic pressure was the appearance of T4HP. If prolonged antibiotic administration also leads to ileal villi damage in human preterm infants, then urinary T4HP could serve as a potential early marker for ileal damage preceding localized gut perforation in preterm infant patients receiving empiric antibiotic therapy.

Although NEC etiology remains unknown, gut colonization with specific microbial organisms has been observed and suggested as potential causative factors in NEC initiation and progression. Our results indicated antibiotic-associated damage to distal intestinal ileal villi that repaired after re-colonization of the gut with diverse microflora suggesting a symbiotic benefit of bacterial colonization and diversity of the ileal villi that outweighed any pathogenic effect of *Clostridial* species detected in the gut microflora of control mice. Consequently, gut microflora may provide ileal villi with an important proximal source of nutrients from bacterial metabolism that is depleted under antibiotic pressure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCAAs	Branched-chain amino acids
D_M	Mahalanobis distance
ELBW	Extremely low birth weight
NEC	Neonatal necrotizing enterocolitis
NMR	Nuclear magnetic resonance
OTU	Operational taxonomic units
PCA	Principal components analysis

T4HP	Trans-4-hydroxy-L-proline
TSP	Trimethylsilyl propionate
UC	Ulcerative colitis

References

- Anasuya A, Rao BS. Relationship between body collagen and urinary hydroxyproline excretion in young rats fed on a low-protein or low-calorie diet. *The British Journal of Nutrition*. 1970; 24:97–107. [PubMed: 5424274]
- Aypak C, Altunsoy A, en Düzgün N. Empiric antibiotic therapy in acute uncomplicated urinary tract infections and fluoroquinolone resistance: a prospective observational study. *Annals of Clinical Microbiology and Antimicrobials*. 2009; 8:27–34. [PubMed: 19852849]
- Balfour Sartor R. Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology*. 2004; 126:1620–1633. [PubMed: 15168372]
- Beliveau GP, Brusilow SW. Glycine availability limits maximum hippurate synthesis in growing rats. *The Journal of Nutrition*. 1987; 117:36–41. [PubMed: 3102706]
- Brückner R, Titgemeyer F. Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. *FEMS Microbiology Letters*. 2002; 209:141–148. [PubMed: 12007797]
- Bundy JG, Papp B, Harmston R, Browne RA, Clayson EM, Burton N, et al. Evaluation of predicted network modules in yeast metabolism using NMR-based metabolite profiling. *Genome Research*. 2007; 17:510–519. [PubMed: 17339370]
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. Qiime allows analysis of high-throughput community sequencing data. *Nature Methods*. 2010; 7:335–336. [PubMed: 20383131]
- Chambers S, Kunin CM. The osmoprotective properties of urine for bacteria: the protective effect of betaine and human urine against low pH and high concentrations of electrolytes, sugars, and urea. *The Journal of Infectious Diseases*. 1985; 152:1308–1316. [PubMed: 3905988]
- Chen XB, Chowdhury SA, Hovell FD, Orskov ER, Kyle DJ. Endogenous allantoin excretion in response to changes in protein supply in sheep. *The Journal of Nutrition*. 1992; 122:2226–2232. [PubMed: 1432263]
- Clark RH, Bloom BT, Spitzer AR, Gerstmann DR. Empiric use of ampicillin and cefotaxime, compared with ampicillin and gentamicin, for neonates at risk for sepsis is associated with an increased risk of neonatal death. *Pediatrics*. 2006; 117:67–74. [PubMed: 16396862]
- Condon R, Hatfield E. Metabolism of abomasally infused ribonucleic acid by sheep. *Journal of Animal Science*. 1970; 31:1037.
- Cordero L, Ayers LW. Duration of empiric antibiotics for suspected early-onset sepsis in extremely low birth weight infants. *Infection Control and Hospital Epidemiology*. 2003; 24:662–666. [PubMed: 14510248]
- Cotton CM, Taylor S, Stoll B, Goldberg RN, Hansen NI, Sánchez PJ, et al. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. *Pediatrics*. 2009; 123:58–66. [PubMed: 19117861]
- Craig SA. Betaine in human nutrition. *American Journal of Clinical Nutrition*. 2004; 80:539–549. [PubMed: 15321791]
- Cselovszky J, Wolf G, Hammes W. Production of formate, acetate, and succinate by anaerobic fermentation of *Lactobacillus pentosus* in the presence of citrate. *Applied Microbiology and Biotechnology*. 1992; 37:94–97.
- De La Huerga J, Popper H. Factors influencing choline absorption in the intestinal tract. *The Journal of Clinical Investigation*. 1952; 31:598–603. [PubMed: 14938439]

- Derrien M, Vaughan EE, Plugge CM, de Vos WM. Akkermansia muciniphila gen. nov. sp. nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*. 2004; 54:1469–1476. [PubMed: 15388697]
- Dethlefsen L, Huse S, Sogin ML, Relman DA. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *Public Library of Science Biology*. 2008; 6:2383–2400.
- Doron SI, Hibberd PL, Gorbach SL. Probiotics for prevention of antibiotic-associated diarrhea. *Journal of Clinical Gastroenterology*. 2008; 42:S58–S63. [PubMed: 18542041]
- Faris B, Blackmore A, Haboubi N. Review of medical and surgical management of Clostridium difficile infection. *Techniques in Coloproctology*. 2010; 14:97–105. [PubMed: 20454824]
- Fjellstedt E, Harnevik L, Jeppsson JO, Tiselius HG, Söderkvist P, Denneberg T. Urinary excretion of total cystine and the dibasic amino acids arginine, lysine and ornithine in relation to genetic findings in patients with cystinuria treated with sulfhydryl compounds. *Urological Research*. 2003; 31:417–425. [PubMed: 14586528]
- Freundlich M, Burns RO, Umbarger HE. Control of isoleucine, valine, and leucine biosynthesis. I. Multivalent repression. *Proceedings of the National Academy of Science USA*. 1962; 48:1804–1808.
- Gewolb IH, Schwalbe RS, Taciak VL, Harrison TS, Panigrahi P. Stool microflora in extremely low birth-weight infants. *Archives of Disease in Childhood. Fetal and Neonatal Edition*. 1999; 80:F167–F173. [PubMed: 10212075]
- Gojkovi Z, Sandrini MP, Piskur J. Eukaryotic beta-alanine synthases are functionally related but have a high degree of structural diversity. *Genetics*. 2001; 158:999–1011. [PubMed: 11454750]
- Goodpaster AM, Kennedy MA. Quantification and statistical significance analysis of group separation in NMR-based metabolomics studies. *Chemometrics and Intelligent Laboratory Systems*. 2011; 9:162–170. [PubMed: 26246647]
- Goodpaster AM, Romick-Rosendale LE, Kennedy MA. Statistical significance analysis of nuclear magnetic resonance-based metabolomics data. *Analytical Biochemistry*. 2010; 401:134–143. [PubMed: 20159006]
- Hamandi B, Holbrook AM, Humar A, Brunton J, Papadimitropoulos EA, Wong GG, et al. Delay of adequate empiric antibiotic therapy is associated with increased mortality among solid-organ transplant patients. *American Journal of Transplantation*. 2009; 9:1657–1665. [PubMed: 19459798]
- Hammer HF. Gut microbiota and inflammatory bowel disease. *Digestive Disease*. 2011; 29:550–553.
- Hartmann F, Plauth M. Intestinal glutamine metabolism. *Metabolism*. 1989; 38:18–24. [PubMed: 2668701]
- Hempel S, Newberry SJ, Maher AR, Wang Z, Miles JN, Shanman R, et al. Probiotics for the prevention and treatment of antibiotic-associated diarrhea. A systematic review and meta-analysis. *Journal of the American Medical Association*. 2012; 307:1959–1969. [PubMed: 22570464]
- Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual Reviews of Nutrition*. 2002; 22:283–307.
- Howard F, Bradley J, Flynn D, Noone P, Szawatkowski M. Outbreak of necrotising enterocolitis caused by clostridium butyricum. *The Lancet*. 1977; 310:1099–1102.
- Iapichino G, Callegari ML, Marzorati S, Cigada M, Corbella D, Ferrari S, et al. Impact of antibiotics on the gut microbiota of critically ill patients. *Journal of Medical Microbiology*. 2008; 57:1007–1014. [PubMed: 18628503]
- Jakobsson HE, Jernberg C, Andersson AF, Sjölund-Karlsson M, Jansson JK, Engstrand L. Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *Public Library of Science ONE*. 2010; 5:1–12.
- Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. *The ISME Journal*. 2007; 1:56–66. [PubMed: 18043614]
- Kerai MD, Waterfield CJ, Kenyon SH, Asker DS, Timbrell JA. The effect of taurine depletion by beta-alanine treatment on the susceptibility to ethanol-induced hepatic dysfunction in rats. *Alcohol and Alcoholism*. 2001; 36:29–38. [PubMed: 11139413]

- Kern WV, Marchetti O, Drgona L, Akan H, Aoun M, Akova M, et al. Oral antibiotics for fever in low-risk neutropenic patients with cancer: A double-blind, randomized, multicenter trial comparing single daily moxifloxacin with twice daily ciprofloxacin plus amoxicillin/clavulanic acid combination therapy-EORTC infectious diseases group trial XV. *Journal of Clinical Oncology*. 2013
- King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene*. 2006; 25:4675–4682. [PubMed: 16892081]
- Klempner MS, Hu LT, Evans J, Schmid CH, Johnson GM, Trevino RP, et al. Two controlled trials of antibiotic treatment in patients with persistent symptoms and a history of Lyme disease. *New England Journal of Medicine*. 2001; 345:85–92. [PubMed: 11450676]
- Kollef MH. Broad-spectrum antimicrobials and the treatment of serious bacterial infections: getting it right up front. *Clinical Infectious Diseases*. 2008; 47:S3–S13. [PubMed: 18713047]
- Kosaka T, Kato S, Shimoyama T, Ishii S, Abe T, Watanabe K. The genome of *Pelotomaculum thermopropionicum* reveals niche-associated evolution in anaerobic microbiota. *Genome Research*. 2008; 18:442–448. [PubMed: 18218977]
- Koser SA, Saunders F. The utilization of certain sugars and their derivatives by bacteria. *Journal of Bacteriology*. 1933; 26:475–488. [PubMed: 16559671]
- Kosloske AM. Epidemiology of necrotizing enterocolitis. *Acta Paediatrica. Supplement*. 1994; 396:2–7.
- Kuppala VS, Meinen-Derr J, Morrow AL, Schibler KR. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. *Journal of Pediatrics*. 2011; 159:720–725. [PubMed: 21784435]
- Lawrence G, Bates J, Gaul A. Pathogenesis of neonatal necrotising enterocolitis. *Lancet*. 1982; 1:137–139. [PubMed: 6119515]
- Liu G, Wang Y, Wang Z, Cai J, Lv X, Zhou A. Metabolomic studies on the biochemical profile of urine from rats with acute cysteamine supplementation. *Metabolomics*. 2011; 7:536–541.
- Luft FC, Bloch R, Sloan RS, Yum MN, Costello R, Maxwell DR. Comparative nephrotoxicity of amino-glycoside antibiotics in rats. *The Journal of Infectious Diseases*. 1978; 138:541–545. [PubMed: 712113]
- MacDonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. *Science*. 2005; 307:1920–1925. [PubMed: 15790845]
- Macy J, Ljungdahl L, Gottschalk G. Pathway of succinate and propionate formation in *Bacteroides fragilis*. *Journal of Bacteriology*. 1978; 134:84–91. [PubMed: 148460]
- Marques A. Chronic lyme disease: an appraisal. *Infectious Disease Clinics of North America*. 2008; 22:341–360.
- Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, et al. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Molecular Systems Biology*. 2008; 4:1–15.
- McGuire W, Clerihew L, Fowlie PW. Infection in the preterm infant. *BMJ*. 2004; 329:1277–1280. [PubMed: 15564261]
- Merheb M, Daher RT, Nasrallah M, Sabra R, Ziyadeh FN, Barada K. Taurine intestinal absorption and renal excretion test in diabetic patients: a pilot study. *Diabetes Care*. 2007; 30:2652–2654. [PubMed: 17666467]
- Miller T, Wolin M. Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Applied and Environmental Microbiology*. 1996; 62:1589–1592. [PubMed: 8633856]
- Mosser JL, Tomasz A. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. *The Journal of Biological Chemistry*. 1970; 245:287–298. [PubMed: 4391619]
- Näntö-Salonen K, Autio S, Härö E, Kivimäki T, Koskela SL, Nantö V, et al. Metabolism of collagen in aspartylglycosaminuria: urinary excretion of hydroxyproline. *Journal of Inherited Metabolic Disease*. 1984; 7:117–121. [PubMed: 6438393]
- Neish AS. Microbes in gastrointestinal health and disease. *Gastroenterology*. 2009; 136:65–80. [PubMed: 19026645]

- Paxton R, Scislawski PW, Davis EJ, Harris RA. Role of branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase in 2-oxobutyrate metabolism. *Biochemical Journal*. 1986; 234:295–303.
- Perencevich M, Burakoff R. Use of antibiotics in the treatment of inflammatory bowel disease. *Inflammatory Bowel Disease*. 2006; 12:651–664.
- Preidis GA, Versalovic J. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: Gastroenterology enters the metagenomics era. *Gastroenterology*. 2009; 136:2015–2031. [PubMed: 19462507]
- Prockop DJ, Sjoerdsma A. Significance of urinary hydroxyproline in man. *The Journal of Clinical Investigation*. 1961; 40:843–849. [PubMed: 13738132]
- Pullan RD, Thomas GA, Rhodes M, Newcombe RG, Williams GT, Allen A, et al. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut*. 1994; 35:353–359. [PubMed: 8150346]
- R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2008. <http://www.R-project.org>. [Accessed February 2012]
- Ravot G, Ollivier B, Fardeau ML, Patel BK, Andrews KT, Magot M, et al. L-Alanine production from glucose fermentation by hyperthermophilic members of the domains bacteria and archaea: A remnant of an ancestral metabolism? *Applied and Environmental Microbiology*. 1996; 62:2657–2659. [PubMed: 8779604]
- Roediger WE. Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut*. 1980; 21:793–798. [PubMed: 7429343]
- Romick-Rosendale, LE. Use of NMR-based metabonomics to study animal models and human disease. Miami University, OhioLink: Doctor of Philosophy; 2011.
- Romick-Rosendale LE, Goodpaster AM, Hanwright PJ, Patel NB, Wheeler ET, Chona DL, et al. NMR-based metabonomics analysis of mouse urine and fecal extracts following oral treatment with the broad-spectrum antibiotic enrofloxacin (Baytril). *Magnetic Resonance in Chemistry*. 2009; 47(S1):S36–S46. [PubMed: 19768747]
- Salem AR, Foster MA. The microbial biosynthesis of methionine. *Biochemical Journal*. 1972; 127:845–853. [PubMed: 4627687]
- Salter DN, Fulford RJ. The influence of the gut microflora on the digestion of dietary and endogenous proteins: studies of the amino acid composition of the excreta of germ-free and conventional chicks. *The British Journal of Nutrition*. 1974; 32:625–637. [PubMed: 4433506]
- Schaub J, Lentze M. Sugars, lactic acid and pH in feces of children. A useful diagnostic approach for gastrointestinal disorders? *Zeitschrift für Kinderheilkunde*. 1973; 115:141–153. [PubMed: 4744209]
- Scheifinger CC, Wolin M. Propionate formation from cellulose and soluble sugars by combined cultures of bacteroides succinogens and selenomonas ruminantium. *Applied Microbiology*. 1973; 26:789–795. [PubMed: 4796955]
- Shanahan F, Bernstein CN. Antibiotics as a first-line therapy for Crohn's disease. *Inflammatory Bowel Disease*. 2004; 10:324–326.
- Shapiro SK, Yphantis DA, Almenas A. Biosynthesis of methionine in *Saccharomyces cerevisiae*. Partial purification and properties of S-adenosylmethionine: Homocysteine methyltransferase. *Journal of Biological Chemistry*. 1964; 239:1551–1556. [PubMed: 14189892]
- Siddiqui S, Razzak J. Early versus late pre-intensive care unit admission broad spectrum antibiotics for severe sepsis in adults (review). *The Cochrane Library*. 2012; 5:1–15.
- Smith EA, Macfarlane GT. Formation of phenolic and indolic compounds by anaerobic bacteria in the human large intestine. *Microbial Ecology*. 1997; 33:180–188. [PubMed: 9115181]
- Sparnins VL, Chapman PJ. Catabolism of l-tyrosine by the homoprotocatechuate pathway in gram-positive bacteria. *Journal of Bacteriology*. 1976; 127:362–366. [PubMed: 931949]
- Stams A, Kremer D, Nicolay K, Weenk G, Hansen T. Pathway of propionate formation in *Desulfobulbus propionicus*. *Archives of Microbiology*. 1984; 139:167–173.
- Stoll BJ, Hansen N, Fanaroff AA, Wright LL, Carlo WA, Ehrenkranz RA, et al. Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. *New England Journal of Medicine*. 2002; 347:240–247. [PubMed: 12140299]

- Stoll BJ, Hansen NI, Higgins RD, Fanaroff AA, Duara S, Goldberg R, et al. National institute of child health and human development. Very low birth weight preterm infants with early onset neonatal sepsis: the predominance of gram-negative infections continues in the national institute of child health and human development neonatal research network, 2002–2003. *The Pediatric Infectious Disease Journal*. 2005; 24:635–639. [PubMed: 15999007]
- Sturm R, Stanek JL, Stauffer LR, Neblett WW. Neonatal necrotizing enterocolitis associated with penicillin-resistant, toxigenic *Clostridium butyricum*. *Pediatrics*. 1980; 66:928–931. [PubMed: 6905971]
- Sun Y, Wolcott RD, Dowd SE. Tag-encoded FLX amplicon pyrosequencing for the elucidation of microbial and functional gene diversity in any environment. *Methods in Molecular Biology*. 2011; 733:129–141. [PubMed: 21431767]
- Tripathi N, Cotton CM, Smith PB. Antibiotic use and misuse in the neonatal intensive care unit. *Clinical Perinatology*. 2012; 39:61–68.
- Tzialla C, Borghesi A, Perotti GF, Garofoli F, Manzoni P, Stronati M. Use and misuse of antibiotics in the neonatal intensive care unit. *The Journal of Maternal-Fetal & Neonatal Medicine*. 2012; 25(Suppl 4):35–37. [PubMed: 22958010]
- Udenfriend S. Formation of hydroxyproline in collagen. *Science*. 1966; 152:1335–1340. [PubMed: 5327887]
- Watanabe M, Sherriff S, Ramelot TA, Kadeer N, Cho J, Lewis KB, et al. NMR based metabonomics study of DAG treatment in a C2C12 mouse skeletal muscle cell line myotube model of burn-injury. *International Journal of Peptide Research and Therapeutics*. 2011; 17:1–19.
- West TP. Pyrimidine base catabolism in species of *Pseudomonas* and *Burkholderia*. *Research Journal of Microbiology*. 2011; 6:172–181.
- Williams HR, Cox IJ, Walker DG, Cobbold JF, Taylor-Robinson SD, Marshall SE, et al. Differences in gut microbial metabolism are responsible for reduced hippurate synthesis in Crohn's disease. *BMC Gastroenterology*. 2010; 10:108. [PubMed: 20849615]
- Willing BP, Russell SL, Finlay BB. Shifting the balance: antibiotic effects on host-microbiota mutualism. *Nature Reviews Microbiology*. 2011; 9:233–243. [PubMed: 21358670]
- Wright WF, Reidel DJ, Talwani R. Diagnosis and management of Lyme disease. *American Family Physician*. 2012; 85:1086–1093. [PubMed: 22962880]
- Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiological Reviews*. 2000; 80:1107–1213. [PubMed: 10893433]
- Xie C, Taylor DM, Howden BP, Charles PG. Comparison of the bacterial isolates and antibiotic resistance patterns of elderly nursing home and general community patients. *Internal Medicine Journal*. 2012; 42:e157–e164. [PubMed: 21241444]
- Zgoda-Pols JR, Chowdhury S, Wirth M, Milburn MV, Alexander DC, Alton KB. Metabolomics analysis reveals elevation of 3-indoxyl sulfate in plasma and brain during chemically-induced acute kidney injury in mice: Investigation of nicotinic acid receptor agonists. *Toxicology and Applied Pharmacology*. 2011; 255:48–56. [PubMed: 21640743]

Fig. 1. Metagenomics analysis of fecal extracts of mice. **a** Analysis of the relative abundance of the 20 most abundant bacterial taxa found across the four groups of mice. **b** Analysis of the relative abundance of *Clostridial* species across the four groups of mice

Fig. 2.

PCA of mice urine and fecal extracts before and during antibiotic treatment. PC1 versus PC2 scores plot of mouse urine (a) and fecal (b) samples for control (black), antibiotic-treated (red), and after stopping antibiotic treatment and administration of *C. butyricum* (green). The 95 % confidence interval for the three clusters of data points were indicated with dotted oval lines (Color figure online)

Fig. 3.

Pairwise PCA of mice urine and fecal extracts. PC1 versus PC2 scores plot are shown for comparisons of urine (**a–c**) and fecal (**d–f**) samples of control versus antibiotic-treated (**a, d**), antibiotic-treated versus *C. butyricum*-treated (**b, e**), and control versus *C. butyricum*-treated (**c, f**). Control (*black*), antibiotic-treated (*red*), and *C. butyricum*-treated (*green*) spectra are represented (Color figure online)

Fig. 4. Histological sections of distal ilea of control mice, antibiotic-treated mice and mice after stopping antibiotic treatment and administration of *C. butyricum*. The 5-micron H & E stained sections show typical ileal villi of control mice (**a–c**), mice after 4 days of continuous antibiotics (**d–f**), and 3 weeks after stopping antibiotics and administration of *C. butyricum*