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Intestinal microbiome disruption in patients in a long-term acute care hospital: a case for development of microbiome disruption indices to improve infection prevention

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Abstract

Background—Composition and diversity of intestinal microbial communities (microbiota) are generally accepted as a risk factor for poor outcomes; however, we cannot yet use this information to prevent adverse outcomes.

Methods—Stool was collected from eight long-term acute care hospital (LTACH) patients experiencing diarrhea and two fecal microbiota transplant donors; 16S rDNA V1-V2 hypervariable regions were sequenced. Composition and diversity of each sample were described. Stool was also tested for Clostridium difficile, vancomycin-resistant enterococci (VRE), and carbapenem-resistant Enterobacteriaceae. Associations between microbiota diversity and demographic and clinical characteristics, including antibiotic use, were analyzed.

Results—Antibiotic exposure and Charlson Comorbidity Index were inversely correlated with diversity (Spearman = −0.7). Two patients were positive for VRE; both had microbiomes dominated by Enterococcus faecium, accounting for 67–84% of their microbiome.

Discussion—Antibiotic exposure correlated with diversity; however, other environmental and host factors not easily obtainable in a clinical setting are also known to impact the microbiota. Therefore, direct measurement of microbiome disruption by sequencing, rather than reliance on surrogate markers, might be most predictive of adverse outcomes.
Conclusions—If and when microbiome characterization becomes a standard diagnostic test, improving our understanding of microbiome dynamics will allow for interpretation of results to improve patient outcomes.

Introduction

In recent years, research on the collective genome of microbial communities, known as the microbiome, living in or on humans has accelerated.\(^1\),\(^2\) A healthy intestinal microbiota assists in digestion and metabolism, and protects against pathogen invasion and overgrowth of pathobionts, which are commensal bacteria that can intermittently reside as minor members of the microbiota and also can act as pathogens when that microbiota becomes disrupted.\(^3\) Loss of microbial diversity or protective species, and overgrowth or dominance by a single organism are characteristic of microbiome disruption.

From birth, environment and host factors impact a person’s microbiota. However, capturing a lifetime of exposures is not feasible. Microbiome disruption is generally accepted as a risk factor for poor outcomes, such as infection and, as recently suggested, sepsis.\(^4\) However, we cannot yet use microbiome status to predict or prevent poor outcomes. One way to translate increasing understanding of the microbiome to the field of infection control is via the development and use of ‘Microbiome Disruption Indices’ (MDIs) (Figure 1). Such indices could become standardized criteria for not only characterizing the status of a patient’s microbiome but also evaluating and communicating the disruptive potential of various drugs, including antibiotics. Applications for MDIs range from improving antibiotic stewardship, infection control, and clinical management of patients, to assigning a risk index to antibiotics and other microbiota disruptive drugs during the drug approval process.

Among the host and environmental factors that lead to microbiota shifts,\(^5\) –\(^8\) antibiotic exposures cause dramatic disruptions, lasting six months or more.\(^9\) Not only do antibiotic-induced disruptions lead to a loss of colonization resistance to MDROs, but once colonization does occur, further disruptions can lead to dominance (defined as a single MDRO constituting ≥30% of the microbiota), which is associated with the occurrence of invasive infection and increased transmission risk through skin and environmental contamination.\(^10\),\(^11\) MDROs, such as Clostridium difficile, vancomycin-resistant enterococci (VRE), and carbapenem-resistant Enterobacteriaceae (CRE), are major public health concerns in healthcare settings, where they are transmitted between patients and can colonize the lower intestine in more individuals than they infect.\(^12\),\(^13\)

Long-term acute care hospital (LTACH) patients are a population with high antibiotic consumption,\(^14\) likely leading to severe intestinal microbiota disruption. In an effort to make a case for the potential impact of MDIs in improving infection control, we describe and compare the microbiomes from LTACH patients with prior antibiotic exposure, when individuals are most susceptible to MDRO colonization, to those of fecal microbiota transplant donors from a small pilot study, described below. We also examine associations between intestinal microbiome diversity, and clinical and demographic characteristics.
METHODS

Study design and participants

The study was a cross-sectional pilot evaluation of the clinical characteristics and intestinal microbiome from a convenience sample of eight LTACH patients with new onset diarrhea and two healthy fecal microbiota transplant donors. The donors had no history of antibiotics in at least the previous 90 days and were not taking any other medications. Neither the LTACH patients nor the donors had histories of Crohn’s disease, ulcerative colitis, or other inflammatory bowel disease.

Patients were enrolled sequentially at first diarrheal episode during December 2013 through February 2014 when stool was being collected for C. difficile diagnostic PCR testing (GenExpert, Cepheid). Providers (S.L., J.M.) consented patients (or a family member for patients unable to consent) to have stool collected for microbiome analysis.

Data collected on each patient during retrospective chart review (A.C.) included demographics, proton pump inhibitor use, previous C. difficile infection, comorbidities, and antibiotic use in both the LTACH and acute care settings. Each antibiotic was classified by when it was administered in reference to the date of stool collection: day of or day before, during the seven days before, and during the 30 days before stool collection. Data were used to calculate cumulative antibiotic days and the number of days exposed to any (i.e., ≥ 1) antibiotic. Antibiotics were categorized into classes: carbapenems, cephalosporins (first-generation), cephalosporins (third- and fourth-generation), beta-lactam/beta-lactamase inhibitor (BL/BLI) combinations, fluoroquinolones, glycopeptides (vancomycin), metronidazole, or other antibiotics. For vancomycin, route of administration was documented.

The Emory University Institutional Review Board (IRB) approved this study protocol. No incentives were provided for participation.

MDRO Colonization Status

In addition to C. difficile diagnostic PCR testing, patient stool was cultured for VRE using Spectra VRE chromogenic agar (Remel, Lenexa, KS) and screened for CRE using a selective medium containing ertapenem (http://www.cdc.gov/hai/pdfs/labSettings/Klebsiella_or_Ecoli.pdf). At the time of collection, stool specimens were de-identified; linkage to the clinical specimen was known only to C.K.

Sample processing and next generation sequencing analyses

Because the majority of the microbiota cannot be cultured using current laboratory methods, next generation sequencing analysis to describe the bacteria present was performed on either residual stool (i.e., stool remaining after C. difficile diagnostic PCR testing) or stool collected within 7–10 days of C. difficile diagnostic PCR testing if insufficient residual stool was available. Stool was stored at 4°C and processed within 24 hours. DNA was extracted; the target 16S ribosomal gene hypervariable V1 and V2 regions were PCR amplified and PCR products were sequenced using an Illumina MiSeq instrument (Illumina, San Diego,
Within each sample, any DNA sequences sharing ≥97% similarity were clustered into operational taxonomic units (OTUs) (equivalent to a bacterial species, genus, or family). To assess within-sample microbial composition, or alpha diversity, we calculated values for Shannon Diversity Index and Observed Species, represented by the number of unique OTUs within a single sample. The Shannon Index accounts for both abundance and evenness to measure non-redundancy within a sample. To measure between-sample composition, we completed beta-diversity community analyses, using weighted UniFrac distance, which accounts for OTU abundances and phylogenetic distances. For additional details on sample processing, next generation sequencing, and sequence analyses, see Supplemental Methods.

Data analysis

For univariable analyses, relationships between demographic and clinical variables and alpha diversity were evaluated using nonparametric methods: Spearman’s correlation for continuous variables and Wilcoxon Rank Sum exact for categorical variables. All analyses were performed using SAS 9.3 (Cary, NC) or R statistical software (version 3.0.2).

RESULTS

Study design and participants

Summary demographic and clinical characteristics are in Table 1. Both donors were male, ages 29 and 30 years. Four of eight patients were male, and median age at stool collection was 66.5 (range: 50–75) years. At the time of stool collection, patients had been in the facility for a median of 3 (range: 0–52) days and were hospitalized in a short-stay acute care hospital a median 41.5 (range: 18–85) days before admission to the LTACH. All patients received antibiotics in the 30 days before stool collection. Patients received a median 34 (range: 4–76) cumulative antibiotic days and spent a median of 17.5 (range: 3–30) days on any antibiotic in the 30 days before stool sample collection.

MDRO colonization status

Patient-07 had a positive C. difficile PCR diagnostic test. Two of eight patient stools were positive for VRE by culture (Patient-09, 10), and one was positive for CRE (Patient-09) (Figure 2).

Next generation sequencing

Family-level microbial community composition for each sample is depicted in Figure 2. Seven of the patient microbiomes examined were dominated (≥30%) by a single OTU. Patient-04 was dominated by Bacteroides fragilis (32%), two patients were dominated by Enterococcus faecium (Patient-09, 67%; Patient-10, 84%), and five were dominated by other OTUs: Patient-06, 88% Pseudomonas aeruginosa; Patient-07, 98% Alistipes finegoldii; Patient-08, 48% Faecalibacterium prausnitzii; Patient-11, 98% Lactobacillus OTUs (57% Lactobacillus salivarius, 41% Lactobacillus rhamnosus). No single OTU dominated the microbiome of Patient-05.

To assess internal validity of our findings, we compared next generation sequencing results and clinical microbiology results. C. difficile sequences (<1%) were identified in Patient-07.
stool, who had a positive *C. difficile* diagnostic PCR. Patient-08 and Patient-09 stools contained OTU sequences matching *C. difficile* (2%, 9%, respectively), although *C. difficile* diagnostic PCR performed approximately one week before stool was collected for next generation sequencing was negative. No subsequent *C. difficile* infections were documented in these two patients. *E. faecium* and *E. faecalis* sequences were identified in both patient stools that were culture positive for VRE (Patient-09, 10). Because the genus or species was not identified during CRE screening, we could only confirm whether the clinical microbiology results matched the next generation sequencing results at the family-level. Sequences matching several members of the Enterobacteriaceae family were identified in the CRE-positive patient (Patient-09), including *Escherichia coli, Serratia marcescens*, and *Salmonella enterica*.

**Microbiome Composition: Diversity**

Median Shannon Index was 3.1 (range: 0.5–6.1) overall, 2.3 (range: 0.5–5.1) among patient samples, and 6.0 (range: 6.0–6.1) among donor samples (Table 1, Figure 2). Median Observed Species (OTU count) overall was 339.2 (range: 119.5–901.6), 242.55 (range: 119.5–827.0) among patient samples, and 829.8 (range: 757.9–901.6) among donor samples.

When all individuals were included, the number of Observed Species was inversely correlated with cumulative antibiotic days in the previous 30 days (Spearman correlation coefficient = −0.8, *P* = 0.009), days exposed to antibiotics in the previous 30 days (Spearman = −0.7, *P* = 0.02), cumulative antibiotic days in the previous seven days (Spearman = −0.6, *P* = 0.05), days exposed to antibiotics in the previous seven days (Spearman = −0.6, *P* = 0.05), and Charlson Comorbidity Index Score (Spearman = −0.8, *P* = 0.008) (Table 2). Shannon Index correlated inversely with cumulative antibiotic days in the previous 30 days (Spearman = −0.8, *P* = 0.002), days exposed to antibiotics in the previous 30 days (Spearman = −0.8, *P* = 0.01), and Charlson Comorbidity Index Score (Spearman = −0.9, *P* = 0.002).

Among patients only, alpha diversity measures only correlated with cumulative antibiotic days in the previous 30 days although not significant (Shannon Index: Spearman = −0.7, *P* = 0.06; Observed Species: Spearman = −0.6, *P* = 0.08), and Charlson Comorbidity Index Score (Shannon Index: Spearman = −0.7, *P* = 0.05; Observed Species: Spearman = −0.7, *P* = 0.08). Alpha diversity was significantly lower among patients who had received third or fourth-generation cephalosporins during the 30 days before stool collection (Shannon Index: 0.68 versus 3.75; Wilcoxon, *P* = 0.04; Observed Species: 122.0 versus 404.8; Wilcoxon, *P* = 0.04). The number of Observed Species was also lower among patients who had received carbapenems in the past 30 days (122.0 versus 404.8, *P* = 0.07) (Table 2).

Weighted UniFrac beta diversity analysis results are plotted in Figure 3. The VRE positive patients (LTACH-09, 10) were closest (weighted UniFrac distance=0.26), followed by LTACH-10, 11 (0.53), and the two healthy donors (0.65). Small distances indicates similarity between microbial communities from different individuals.
Discussion

We describe intestinal microbial communities among LTACH patients with diarrhea, in an effort to highlight the potential impact of MDIs on infection control and prevention. In addition to Charlson Comorbidity Index Score, we found that antibiotic exposure in the previous month was inversely correlated with diversity measures, further strengthening the conclusion that cumulative antibiotic exposure is a major driver of microbiome disruption. Disruption could result in poor future outcomes, including infections and transmission (Figure 1). Additionally, although we did not assess outcomes, the significant inverse correlation with the Charlson Comorbidity Index further supports our hypothesis that microbiota characterization might also predict poor future outcomes. Before microbiome characterization or MDIs can become a standard diagnostic test that drives improved patient outcomes, our understanding of the dynamics of microbiome composition needs to be advanced.

The spectrum of microbiome composition among patients ranged from resembling healthy donors, such as in Patient-04 which had a weighted UniFrac distance of 0.81 from the healthy donors), to partial loss of Bacteroidetes (Patient-05) in a patient receiving only inhaled tobramycin, to dominance by \textit{E. faecium} (Patient-09) and virtual mono-dominance by \textit{E. faecium} (Patient-10), \textit{Lactobacillus} (Patient-11), or Rikenellaceae (Patient-07) (Figures 2, 3). Although dominance by \textit{Enterococcus} in LTACH patients has not been previously described, dominance by \textit{Enterococcus} has been reported in patients undergoing allogeneic hematopoietic stem cell transplantation and is associated with increased risk for bacteremia.\textsuperscript{10}

The impact of mono-dominance by other bacteria on morbidity and mortality is not well-understood and might vary by patient population. For example, \textit{Lactobacillus} species are typically thought of as a non-dominant part of the commensal microbiome, including along the digestive tract; however, we found \textit{L. rhamnosus} and \textit{L. salivarius} dominated the microbiome of Patient-11. \textit{Lactobacillus} are frequently resistant to vancomycin (10%–27% susceptible),\textsuperscript{21} and Patient-11 received oral and intravenous vancomycin before stool collection, which might have enabled overgrowth of \textit{Lactobacillus}. Intravenous vancomycin has been identified as an independent risk factor for \textit{C. difficile} infection,\textsuperscript{22} demonstrating its disruptive potential. Although \textit{Lactobacillus} can cause infection in sterile sites among highly debilitated patients, they cause infection much less frequently than well-recognized pathobiont MDROs, such as VRE. Consequently, it will be important to gain increased understanding of how dominance by low virulence commensals, such as \textit{Lactobacillus}, impact outcomes. The genus or species dominating the intestine, and the metabolic functions performed by these dominating bacteria (e.g., \textit{Enterococcus} versus \textit{Bacteroides}) may be impacting overall health of the host and could be translated into a MDI. Determining how often dominance by different bacteria leads to infection in specific patient populations will help us better elucidate the impact of mono-dominance by bacteria not generally considered pathogens.

Recent exposures to third- or fourth-generation cephalosporins and carbapenemams were common in microbiomes that tended towards lower diversity. This is biologically plausible...
for carbapenems, which have broad spectrum and distribution in tissues and feces,
as well as for intravenous ceftiraxone, which is excreted in stool at high concentrations. Receipt of third- and fourth-generation cephalosporins has also been identified as an important and widespread risk factor for \textit{C. difficile}-associated diarrhea. The inverse correlation between antibiotic exposures and microbiome disruption reiterates the value and importance of implementing and maintaining an antimicrobial stewardship program to improve prescribing practices, in particular optimizing duration.

This study had several limitations, including that the design was a pilot study in a convenience sample, which prevents us from drawing major, generalizable conclusions, establishing whether \textit{C. difficile} or MDRO colonization or infection preceded disruption, or performing multivariable analyses controlling for factors previously identified as impacting microbial diversity. However, the intent of the study was to allow us to present the concept of the importance of development of microbiome disruption indices and their potential implications on infection control and prevention efforts. In addition, by targeting patients with diarrhea, we selected for individuals likely to have more disrupted intestinal microbial communities than asymptomatic patients. Donors were younger than patients. Compared to younger adults, intestinal microbial communities vary widely in older populations; however, this may be due to increasing exposure to antibiotics over time.

Previous research showed a relationship between presence of specific obligate anaerobes, \textit{Barnesiella} species and \textit{C. scindens}, and absence of VRE and \textit{C. difficile}, respectively. In our sample, only the healthy donors, as well as Patient-04 and Patient-08 had \textit{Barnesiella} species (Figure 2). As predicted, these individuals simultaneously lacked any \textit{Enterococcus} sequences in their intestinal microbiomes and may be protected from colonization if transmission were to occur. \textit{C. scindens} was not identified in any of the microbiomes analyzed. In addition, \textit{Faecalibacterium prausnitzii}, identified as an anti-inflammatory intestinal community member, was also found in both healthy donors and three LTACH patients.

Our cross-sectional pilot highlighted several potential MDIs beyond diversity metrics: relative absence of healthy components of the microbial flora, loss of keystone components that fill an ecologic niche and prevent colonization by a MDRO, and presence or dominance by microbial community components that are indicators of microbiome disruption. One example of the need for multiple MDIs, interpreted in context, is Donor-02, whose microbiome had a high proportion of \textit{Bacteroides vulgatus} (33%), which previous research has suggested can be pro-inflammatory when present at levels higher than observed in the healthy intestinal microbiomes. However, this individual’s microbiome also had an alpha diversity score higher than any of the other dominated microbiomes (Figure 2) and contained sequences matching bacteria previously identified as protective and anti-inflammatory (\textit{Barnesiella} species and \textit{F. prausnitzii}, Figure 2), indicating that a single measure of the microbial composition is likely insufficient for determining risk for poor outcomes.

There are many host and environmental drivers of microbial composition, including some not easily captured or described, such as antibiotics received early in life, whether breastfed or not during infancy, mode of delivery at birth, diet, co-morbidity history, a history of...
appendectomy or other gastrointestinal surgery, receipt of certain drugs (other than antibiotics), and the intestinal microbial composition of close contacts. Because the selection of MDRO genetic determinants within a bacterial species is a relatively rare event following genetic mutation, rearrangement, or translocation, the more common widespread effect of antibiotics on MDRO transmission is mediated via microbial community disruption. Even when compared to a reliable, comprehensive antibiotic exposure history dating back several years, MDIs are the more proximate and therefore comprehensive indicators of risk for not only infection, but also transmission. MDIs determined for patients at critical points throughout their course of care hold promise for guiding infection control and clinical management (Figure 1).

We anticipate that MDIs will someday be used to predict the degree and type of disruption that typically result from administration of a specific antibiotic (Figure 1: Footnote 1), as well as describe the risk status of a patient at a given point in time for subsequent colonization (Figure 1: Footnote 2), dominance (Figure 1: Footnote 3), transmission and infection (Figure 1: Footnote 4) by a MDRO. As microbiome analyses become fiscally feasible, interpretation of patients’ MDIs at admission or during care could help identify modifiable patient-, unit- or facility-level factors, including informing infection control measures, such as appropriate use of cohorting, antibiotic selection, and treatments to help patients with poor MDIs return to a more healthy state (e.g., fecal microbiota transplant).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

We would like to thank Duncan MacCannell for early input on bioinformatics tool selection, Jessica Ingersoll and Deborah Abdul-Ali for assistance in the laboratory.

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**References**


Figure 1.
Causal Pathway from health to disease: Microbiome Disruption Indices (MDI)
1. Antibiotic MDI indicates the potential an antibiotic has for disrupting the intestinal microbiome
2. Disrupted microbiome status MDI characterizes the degree and type of disruption in the intestinal microbiome, as well as the susceptibility to colonization by a MDRO
3. MDRO colonization MDI indicates susceptibility to overgrowth and dominance by a MDRO
4. MDI characterizing overgrowth and dominance by a MDRO indicates susceptibility for infection with a MDRO, and the potential for transmission to others through skin/environment contamination.
Figure 2.
OTUs identified in each intestinal microbiota were aggregated at the Family taxonomic level and those that constituted ≥1% of sequences in the dataset are plotted. Each color represents a different Family. Sequences present at <1% were grouped into “Others”. The bottom portion of the figure aligns the demographic and clinical data of each patient with their intestinal microbiota.
*Third and fourth generation cephalosporins only.
Figure 3.
Weighted UniFrac Beta diversity results. 'D' indicates a donor microbiome. 'L' indicates an LTACH patient microbiome.
Table 1.

Characteristics of population and health status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of subjects</th>
<th>No. (%) LTACH patients</th>
<th>No. (%) Stool donors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>10</td>
<td>8 (80)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Median age at collection (range)</td>
<td>61.5 (29–75)</td>
<td>66.5 (50–75)</td>
<td>29.5 (29–30)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>6 (60)</td>
<td>4 (50)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Female</td>
<td>4 (40)</td>
<td>4 (50)</td>
<td></td>
</tr>
<tr>
<td><strong>Proton pump inhibitor use</strong></td>
<td>7 (70)</td>
<td>7 (87.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Acid reducer (H2 blocker) use</strong></td>
<td>1 (10)</td>
<td>1 (12.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Steroid use</strong></td>
<td>3 (30)</td>
<td>3 (37.5)</td>
<td></td>
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<tr>
<td><strong>Feeding tube</strong></td>
<td>8 (80)</td>
<td>8 (100)</td>
<td></td>
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<tr>
<td><strong>Charlson Comorbidity Index Score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2 (20)</td>
<td>0 (0)</td>
<td>2 (100)</td>
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<td>1–3</td>
<td>1 (10)</td>
<td>1 (12.5)</td>
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<tr>
<td>4–6</td>
<td>4 (40)</td>
<td>4 (50)</td>
<td></td>
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<tr>
<td>7–9</td>
<td>3 (30)</td>
<td>3 (37.5)</td>
<td></td>
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<tr>
<td><strong>Number of observed species (OTUs), Median (range)</strong></td>
<td>339.2 (119.5–901.6)</td>
<td>242.55 (119.5–827)</td>
<td>829.75 (757.9–901.6)</td>
</tr>
<tr>
<td><strong>Shannon Diversity Index, Median (range)</strong></td>
<td>3.08 (0.51–6.05)</td>
<td>2.3 (0.51–5.11)</td>
<td>5.99 (5.95–6.05)</td>
</tr>
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</table>
Table 2.

Correlation between demographic and clinical data, and diversity measures

<table>
<thead>
<tr>
<th>Population</th>
<th>Spectrum Correlation (P-value)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>All individuals</td>
</tr>
<tr>
<td>Observed Species (OTUs)</td>
<td>−0.77 (0.009)</td>
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<tr>
<td>Shannon Diversity Index</td>
<td>−0.84 (0.002)</td>
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<td></td>
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<tr>
<td></td>
<td>Cumulative Antibiotics (30 days)</td>
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<td></td>
<td>Days exposed (30 days)</td>
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<tr>
<td></td>
<td>−0.72 (0.02)</td>
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<td>−0.77 (0.01)</td>
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<tr>
<td></td>
<td>Cumulative Antibiotics (7 days)</td>
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<tr>
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<td>Days exposed (7 days)</td>
</tr>
<tr>
<td></td>
<td>−0.64 (0.05)</td>
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<td>−0.54 (0.1)</td>
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<tr>
<td></td>
<td>Age (years)</td>
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<tr>
<td></td>
<td>−0.62 (0.05)</td>
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<td>−0.51 (0.1)</td>
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<tr>
<td></td>
<td>Charlson Comorbidity Index Score</td>
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<td>−0.44 (0.2)</td>
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<td>−0.35 (0.3)</td>
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<td></td>
<td>Days hospitalized before LTACH admission</td>
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<td>−0.78 (0.008)</td>
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<td>−0.85 (0.002)</td>
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<td>−0.58 (0.08)</td>
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<td>ITACH patients only</td>
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<tr>
<td>Observed Species (OTUs)</td>
<td>−0.65 (0.08)</td>
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<tr>
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<td>0.29 (0.5)</td>
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<td>−0.7 (0.05)</td>
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<td>−0.19 (0.7)</td>
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