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Zaborin, Alexander
Smith, Daniel
Garfield, Kevin
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Membership and Behavior of Ultra-Low-Diversity Pathogen Communities Present in the Gut of Humans during Prolonged Critical Illness

Alexander Zaborin,[a] Daniel Smith,[b]* Kevin Garfield,[c] John Quensen,[c] Baddr Shakhsheer,[a] Matthew Kade,[a] Matthew Tirrell,[a] James Tiedje,[c] Jack A. Gilbert,[a,b] Olga Zaborina,[a] John C. Alverdy[a]

University of Chicago, Chicago, Illinois, USA[a]; Argonne National Laboratory, Argonne, Illinois, USA[b]; Michigan State University, East Lansing, Michigan, USA[c]

* Present address: Baylor College of Medicine, Houston, Texas, USA.

O.Z. and J.C.A. are senior coauthors.

ABSTRACT We analyzed the 16S rRNA amplicon composition in fecal samples of selected patients during their prolonged stay in an intensive care unit (ICU) and observed the emergence of ultra-low-diversity communities (1 to 4 bacterial taxa) in 30% of the patients. Bacteria associated with the genera *Enterococcus* and *Staphylococcus* and the family *Enterobacteriaceae* comprised the majority of these communities. The composition of cultured species from stool samples correlated to the 16S rRNA analysis and additionally revealed the emergence of *Candida albicans* and *Candida glabrata* in ~75% of cases. Four of 14 ICU patients harbored 2-member pathogen communities consisting of one *Candida* taxon and one bacterial taxon. Bacterial members displayed a high degree of resistance to multiple antibiotics. The virulence potential of the 2-member communities was examined in *C. elegans* during nutrient deprivation and exposure to opioids in order to mimic local conditions in the gut during critical illness. Under conditions of nutrient deprivation, the bacterial members attenuated the virulence of fungal members, leading to a "commensal lifestyle." However, exposure to opioids led to a breakdown in this commensalism in 2 of the ultra-low-diversity communities. Application of a novel antivirulence agent (phosphate-polyethylene glycol [Pi-PEG]) that creates local phosphate abundance prevented opioid-induced virulence among these pathogen communities, thus rescuing the commensal lifestyle. To conclude, the gut microflora in critically ill patients can consist of ultra-low-diversity communities of multidrug-resistant pathogenic microbes. Local environmental conditions in gut may direct pathogen communities to adapt to either a commensal style or a pathogenic style.

IMPORTANCE During critical illness, the normal gut microbiota becomes disrupted in response to host physiologic stress and antibiotic treatment. Here we demonstrate that the community structure of the gut microbiota during prolonged critical illness is dramatically changed such that in many cases only two-member pathogen communities remain. Most of these ultra-low-membership communities display low virulence when grouped together (i.e., a commensal lifestyle); individually, however, they can express highly harmful behaviors (i.e., a pathogenic lifestyle). The commensal lifestyle of the whole community can be shifted to a pathogenic one in response to host factors such as opioids that are released during physiologic stress and critical illness. This shift can be prevented by using compounds such as Pi-PEG15-20 that interrupt bacterial virulence expression. Taking the data together, this report characterizes the plasticity seen with respect to the choice between a commensal lifestyle and a pathogenic lifestyle among ultra-low-diversity pathogen communities that predominate in the gut during critical illness and offers novel strategies for prevention of sepsis.

The gastrointestinal tract reservoir is the primary site of colonization of health care-associated pathogens and the site from which most pathogens disseminate to cause serious infections (1–5). In addition, the intestinal tract has been considered to be the key site for the emergence of antibiotic resistance and virulence expression among health care-associated pathogens that ultimately cause life-threatening sepsis (6–8). This situation is especially prevalent during prolonged critical illness, when the gut undergoes nearly complete ecological collapse owing to the selective pressures imposed by modern intensive care therapy, including multiple antibiotic exposure, provision of all nutrients exclusively through the intravenous route (total parenteral nutrition), and the use of vasoactive agents that alter the intestinal blood supply, acid-reducing agents, and opioids. As a result, a syndrome of gut-derived sepsis can be observed to occur late in the course of critical illness as microbes struggle to survive. Emergence of anti-
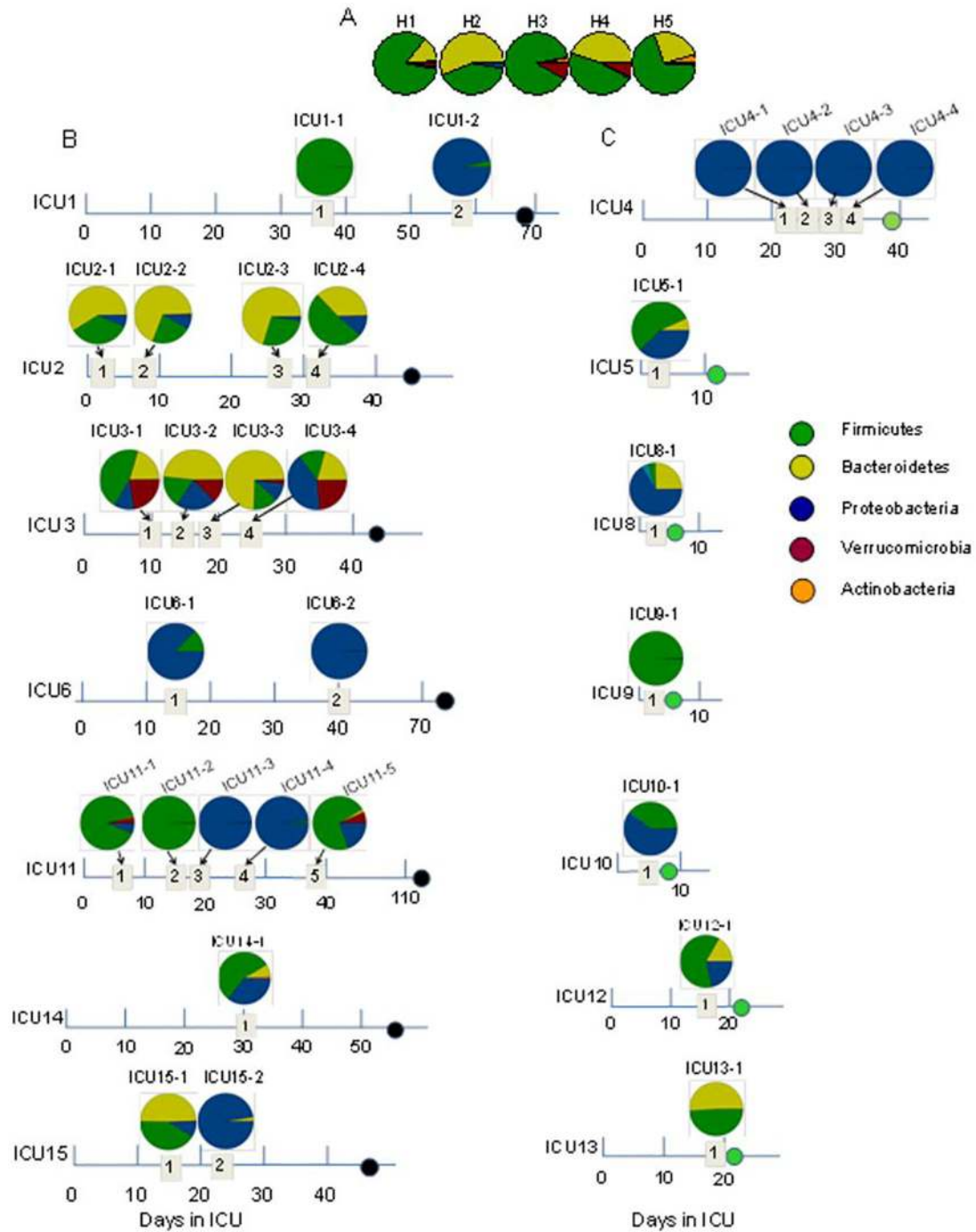
FIG 1 The taxonomic composition of the gut microbiome at the phylum level determined by 16S rRNA analysis of stool samples collected from healthy volunteers (A), ICU patients dying with signs of severe sepsis (as indicated by black circles on the time line) (B), and ICU patients who had recovered (as indicated by green circles on the time line) (C). Dates of stool collection are displayed in numbered quadrants.

biotic resistance under such circumstances is proposed to occur in the gut due to these aggregate selective pressures. Thus, the term "late-onset sepsis" is being used to describe sepsis late in the course of critical illness involving multidrug-resistant (MDR) health care-acquired pathogens (9–14). A better understanding of the ecological perturbations that develop in the microbiota during critical illness, in terms of both composition and function, is cru-

cial to prevent late-onset sepsis during the often-unanticipated prolonged course of recovery from extreme medical interventions such as organ transplant, cancer chemotherapy, and burn injury.

Here we characterized the composition and function of microbial communities from several sets of fecal samples from patients suffering from prolonged critical illness. Our results indicate that prolonged critical illness results in near-complete disruption of

the normal microbiota, the members of which are replaced by ultra-low-diversity communities of highly resistant pathogens whose virulent or nonvirulent behavior is dependent on the interactions between its members and provocative host factors (i.e., opioids). A more complete understanding of the threat of highly virulent and resistant pathobiomes that emerge as members of the normal microbiota disappear over the course of prolonged critical illness is warranted to develop new strategies to prevent late-onset sepsis in hospitalized patients.

RESULTS

16S rRNA analysis of the bacterial composition in stool samples of ICU patients. (i) Phylum level. Stool samples from healthy volunteers demonstrated dominance of *Firmicutes* and *Bacteroidetes* among the four stool samples (H1, H2, H4, and H5). A predominance of *Firmicutes* and very low levels of *Bacteroidetes* were found in one (H3) stool sample. Proteobacterial abundance remained below 1% in healthy volunteers (Fig. 1A). In 50% of intensive care unit (ICU) patients (ICU1, ICU4, ICU6, ICU9, ICU11, and ICU15), either *Proteobacteria* or *Firmicutes* organisms were totally dominant from at least one time point of stool collection (Fig. 1B and C). In patients ICU1, ICU11, and ICU15, during their course of hospitalization, we observed dramatic changes in microbial composition, with *Firmicutes* being completely replaced by *Proteobacteria*. *Proteobacteria*-dominated communities that persisted during the course of hospitalization were found in the stool samples of patients ICU6 (Fig. 1B) and ICU4 (Fig. 1C). Importantly, *Proteobacteria* and *Firmicutes* dominated in the stool of both deceased (Fig. 1B) and discharged (Fig. 1C) ICU patients.

(ii) Genus level. In the group of healthy volunteers, the microbial communities comprised ~40 bacterial genera (see Fig. S1 in the supplemental material), including *Coprococcus* (phylum *Firmicutes* [p_*Firmicutes*], family *Lachnospiraceae* [f_*Lachnospiraceae*]), *Bacteroides* (p_*Bacteroidetes*, f_*Bacteroidaceae*), *Roseburia* (p_*Firmicutes*, f_*Lachnospiraceae*), *Prevotella* (p_*Bacteroidetes*, f_*Prevotellaceae*), a nonidentified genus of f_*Lachnospiraceae* (p_*Firmicutes*), a nonidentified genus of f_*Coprobacillaceae* (p_*Firmicutes*), and *Blautia* (p_*Firmicutes*, f_*Lachnospiraceae*) as the seven most abundant. The microbial composition in critically ill patients was profoundly different from that in healthy volunteers. We observed decreased diversity in >80% ICU stool samples as seen by Chao1 index values below 50 and extremely low diversity in ~50% of ICU stool samples as seen by Chao1 diversity index values below 10 (Fig. 2). Unusually high abundances of *Pseudomonadaceae* were observed in the stool of ICU2 and ICU3 (10% and 30%, respectively) (see Fig. S2). Decreased diversity and domination by *Escherichia* and *Enterococcus* were found in seven patients (ICU5, ICU8, ICU10, and ICU12 to ICU15) (see Fig. S3).

The emergence of extremely low bacterial diversity (>90% abundance of one bacterial taxon) was determined in samples from five patients (ICU1, ICU4, ICU6, ICU9, and ICU11) (Fig. 3; see also Fig. S4 in the supplemental material). A predominance (>90% abundance) of *Enterococcus* (p_*Firmicutes*, f_*Enterococcaceae*), *Staphylococcus* (p_*Firmicutes*, f_*Staphylococcaceae*), and f_*Enterobacteriaceae* with an absence of p_*Bacteroidetes* was the most striking feature of this group. Another striking feature of this group was fast dynamic replacement of one dominated phylum by another (i.e., *Firmicutes* by *Proteobacteria*). The taxonomic composition in the microbiomes in the members of these groups was
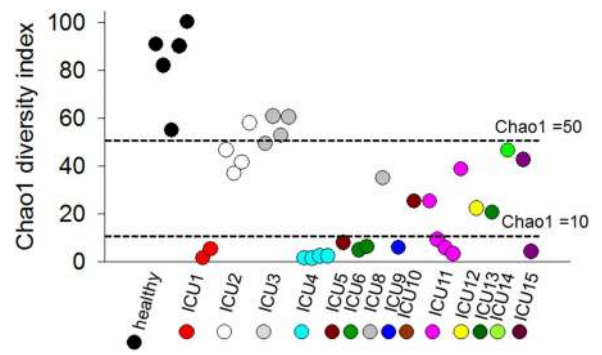

FIG 2 Chao1 diversity index of bacterial composition in fecal samples. Chao1 index values for stool samples of healthy volunteers (black circles) and ICU patients (colored circles) are ordered as follows (left to right): 1 to 5, healthy volunteers; 6 and 7, ICU1-1 and ICU1-2; 8 to 11, ICU2-1 to ICU2-4; 12 to 15, ICU3-1 to ICU3-4; 16 to 19, ICU4-1 to ICU4-4; 20, ICU5-1; 21 and 22, ICU6-1 and ICU6-2; 23, ICU8-1; 24, ICU9-1; 25, ICU10-1; 26 to 30, ICU11-1 to ICU11-5; 31, ICU12-1; 32, ICU13-1; 33, ICU14-1; and 34 and 35, ICU15-1 and ICU15-2. The horizontal dashed lines corresponding to Chao1 index values <50 and <10 represent arbitrary cutoffs. For the samples in this study, 14 had Chao1 index values of <10, 12 had Chao1 index values of 10 to 15, and 9 had Chao1 index values of >50. Mean Chao1 index value, 33; median, 25; range, 1.46 to 100.24.

found to be similar to the composition of cultured bacteria (Fig. 3; see also Fig. S4), a unique situation that may develop only when the microbiome diversity is profoundly decreased. We also noticed the presence of the eukaryotic pathogen *Candida* in four of five patients. One of the five patients (ICU9, who was discharged after 10 days in the ICU) did not harbor *Candida* species. Reads corresponding to *Enterococcus* determined by 16S rRNA amplicon sequencing in the stool of ICU9 comprised 98.82% of all sequence reads and represented 2 cultivable *Enterococcus* species, MDR *E. faecium* and *E. gallinarum* (see Fig. S4). Blood cultures from this patient yielded *Escherichia coli* but were culture negative for *Enterococcus*. The other 4 patients (ICU1, ICU4, ICU6, and ICU11) were admitted to the ICU for longer than a month; for these patients, a temporal series of stool samples were acquired. Thus, we could correlate the 16S rRNA amplicon profiles with the clinical histories, antibiotic therapy, and microbiological culture data from nongastrointestinal sources (i.e., blood, sputum, and urine) and gastrointestinal sources (feces) for each of these patients (Fig. 3).

The 16S rRNA profiling is in agreement with culture analysis of stool samples in ICU patients harboring low-diversity pathogen communities. (i) Patient ICU1 (Fig. 3A). Patient ICU1 was admitted for a liver transplant but died from severe sepsis before undergoing transplantation following 8 weeks of ICU confinement. In this patient's stool sample collected at time point 1 (i.e., ICU1-1), *Enterococcus* reads comprised 99.9% of the 16S rRNA sequenced reads. Culture analysis of the first stool samples (ICU1-1) presented a 2-member community of MDR *E. faecium* and *Candida albicans*. A retrospective analysis of the clinical history of this patient demonstrated that the urine and paracentesis fluid of this patient were culture positive for *C. albicans* and *E. faecium* (Fig. 3A). In the second stool sample (ICU1-2) taken 23 days later, *Enterobacteriaceae* and *Enterococcus* reads comprised 96% and 3.3% of the 16S rRNA reads, respectively. Culture results from the feces demonstrated a 4-member community consisting of MDR *Serratia marcescens*, MDR *Klebsiella oxytoca*, tetracycline-
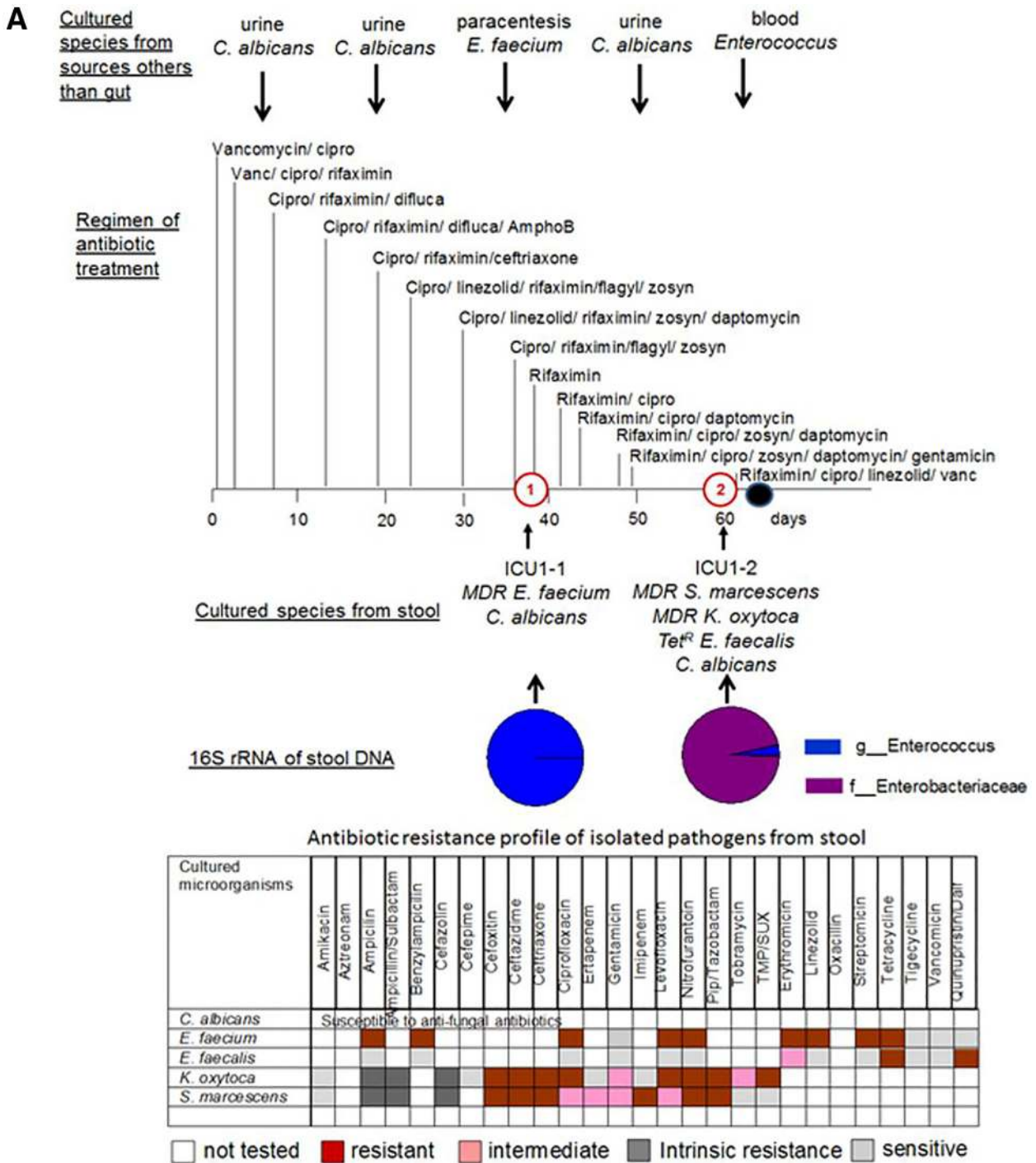
FIG 3 Taxonomic and culturing composition of 2-member pathogen communities in correlation to antibiotic regimen, antibiotic resistance of stool isolates, and microbial data of cultured species from nonintestinal samples. (A) Patient ICU1. (B) Patient ICU6. (C) Patient ICU11. (D) Patient ICU4. Cultured species from sources other than the gut refer to species cultured from extraintestinal sources such as blood, urine, and lung. The arrows indicate the time points along the course of antibiotic exposure at which these organisms were cultured. Vanc, vancomycin; cipro, ciprofloxacin; difluca, fluconazole (Diflucan); AmphoB, amphotericin B; flagyl, metronidazole (Flagyl); Pip, piperacillin; TMP/SUX, trimethoprim/sulfamethoxazole; Quinupristin/dalf, quinupristin/dalfopristin; Staph, Staphylococcus.

resistant (Tet^r) *E. faecalis*, and *C. albicans*. At that time, the urine and blood of the patient were culture positive for *C. albicans* and *Enterococcus*. The bacterial members of pathogen communities were found to be resistant to multiple antibiotics (Fig. 3A). A retrospective analysis of the clinical history demonstrated that antibiotics were aggressively administered to treat the presumed infection with combinations of up to six antibiotics administered daily. *C. albicans* was sensitive to the fungal antibiotics used, but
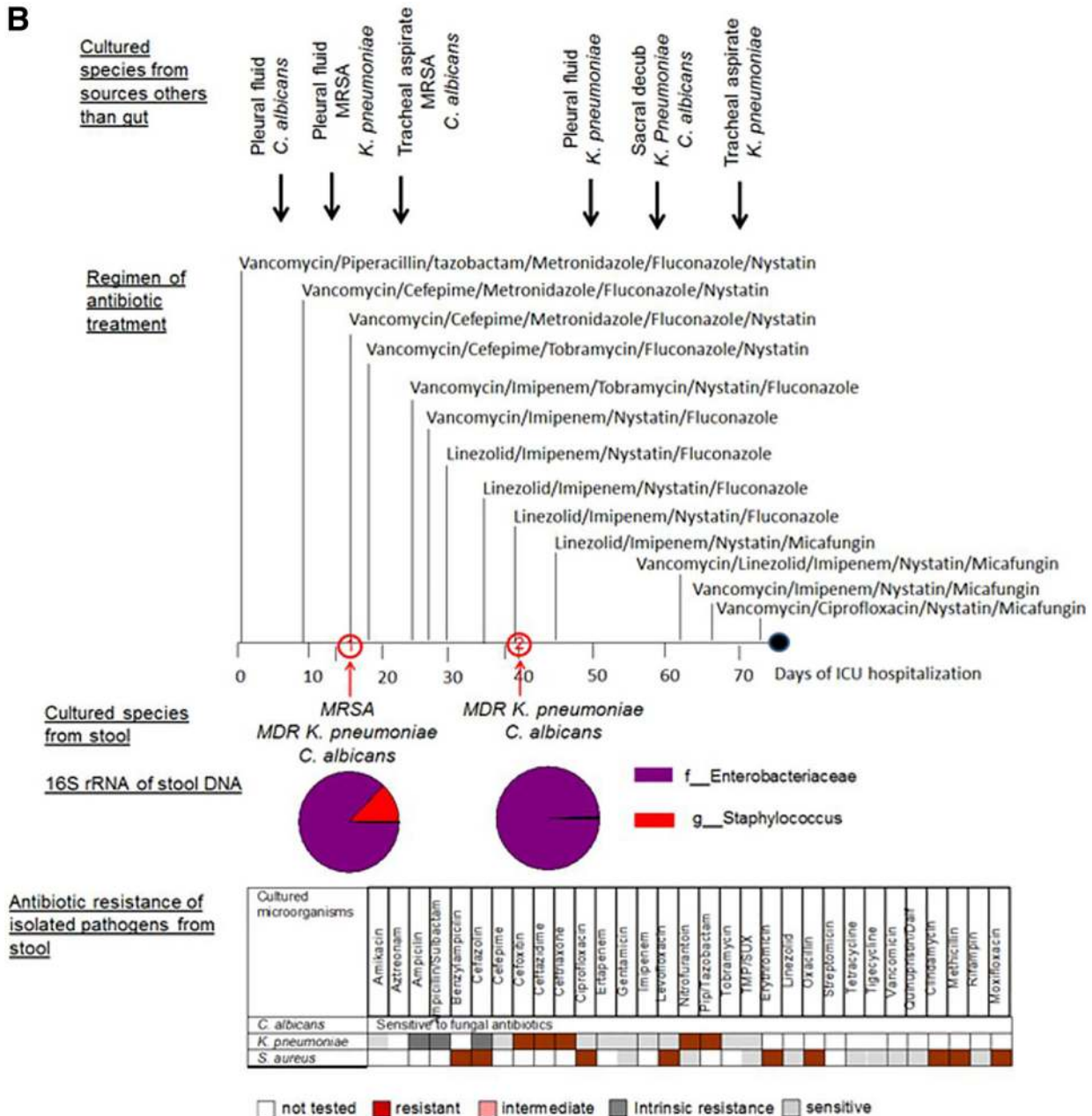
FIG 3 (Continued)

the bacteria present were resistant to several of the antibiotics used, including ciprofloxacin, piperacillin-tazobactam (Zosyn), and linezolid. The observation that microbial pathogens persisted in the gut suggests that they might have been a continuing source of infection.

(ii) Patient ICU6 (Fig. 3B). Patient ICU6 was transferred from an outside hospital, where he was treated for a presumed pneumonia without any improvement. He was suspected of having a postprocedure esophageal perforation. The patient was placed on broad-spectrum antibiotics during his hospital stay because of mediastinitis and bilateral empyema. Throughout his stay, methicillin-resistant *Staphylococcus aureus* (MRSA) was cultured from his sputum as well as from his chest tube sites bilaterally and

from his pleural fluid, and he eventually died of severe sepsis. Two stool samples (ICU6-1 and ICU6-2) collected 25 days apart were analyzed using 16S rRNA amplicon sequencing. In sample ICU6-1, 86.83% of sequence reads showed the presence of *Enterobacteriaceae*, while 12.9% showed the presence of *Staphylococcus*; these were confirmed by culture to represent *K. pneumoniae* and methicillin-resistant *S. aureus*. In sample ICU6-2, 97.78% of the 16S rRNA reads indicated the presence of *Enterobacteriaceae*, which was in agreement with culture results indicating that the reads exclusively represented *K. pneumoniae*. The eukaryotic pathogen *C. albicans* was cultured in both stool samples (ICU6-1 and ICU6-2). Despite the sensitivity to fluconazole, *C. albicans* persisted in the gut of patient ICU6 throughout treatment with
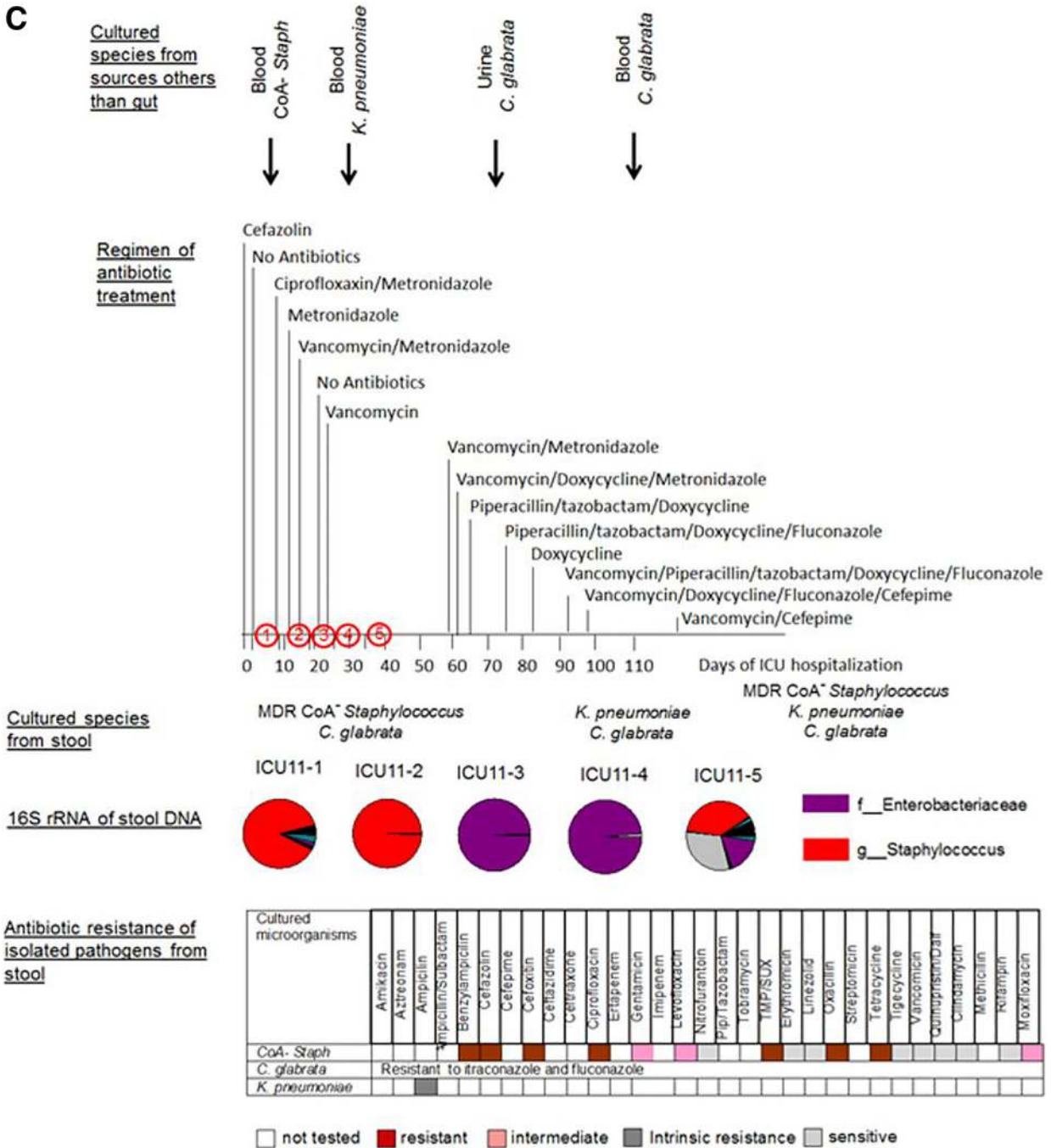
C

Cultured species from sources others than gut

Blood CoA- Staph
Blood K. pneumoniae
Urine C. glabrata
Blood C. glabrata

Regimen of antibiotic treatment

Cefazolin
No Antibiotics
Ciprofloxaxin/Metronidazole
Metronidazole
Vancomycin/Metronidazole
No Antibiotics
Vancomycin
Vancomycin/Metronidazole
Vancomycin/Doxycycline/Metronidazole
Piperacillin/tazobactam/Doxycycline
Piperacillin/tazobactam/Doxycycline/Fluconazole
Doxycycline
Vancomycin/Piperacillin/tazobactam/Doxycycline/Fluconazole
Vancomycin/Doxycycline/Fluconazole/Cefepime
Vancomycin/Cefepime

① ② ③ ④ ⑤

0 10 20 30 40 50 60 70 80 90 100 110   Days of ICU hospitalization

Cultured species from stool

MDR CoA⁻ Staphylococcus
C. glabrata

K. pneumoniae
C. glabrata

MDR CoA⁻ Staphylococcus
K. pneumoniae
C. glabrata

16S rRNA of stool DNA

ICU11-1   ICU11-2   ICU11-3   ICU11-4   ICU11-5

f__Enterobacteriaceae
g__Staphylococcus

Antibiotic resistance of isolated pathogens from stool

Cultured microorganisms: Amikacin, Aztreonam, Ampicillin, Ampicillin/Subactam, Benzylampicillin, Cefazolin, Cefepime, Cefoxitin, Ceftazidime, Ceftriaxone, Ciprofloxacin, Ertapenem, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Pip/Tazobactam, Tobramycin, TMP/SUX, Erythromicin, Linezolid, Oxacillin, Streptomicin, Tetracycline, Tigecycline, Vancomicin, Quinupristin/Dalf, Clindamycin, Methicilin, Rifampin, Moxifloxacin

CoA- Staph
C. glabrata — Resistant to itraconazole and fluconazole
K. pneumoniae

☐ not tested   ☐ resistant   ☐ intermediate   ☐ Intrinsic resistance   ☐ sensitive

FIG 3 (Continued)

multiple fungal antibiotics, including fluconazole. *K. pneumoniae* was found to produce extended-spectrum beta-lactamase, defining it as strain ESBL, and also to be resistant to multiple antibiotics. It was sensitive, however, to tobramycin and imipenem, but despite the patient being treated with these antibiotics, *K. pneumoniae* ESBL persisted in his gut during his ICU stay. *C. albicans*, MRSA, and *K. pneumoniae* were cultured from the pleural fluid and tracheal aspirates over the course of hospitalization in patient ICU6.

(iii) Patient ICU11 (Fig. 3C). Patient ICU11 suffered from intestinal bleeding, requiring several operations to control the bleeding. The patient had several underlying comorbidities, including a nonischemic cardiomyopathy. Following the last operation, the patient developed progressive decompensation and multiorgan system failure and eventually died from severe sepsis after being confined to the ICU for 2 months. Stool samples were collected at 5 time points (Fig. 3C). ICU11-1 and ICU11-2 16S rRNA reads indicated 89.7% and 99.4% levels of *Staphylococcus*, respectively, which was in agreement with culturing analyses yielding only coagulase-negative (CoA⁻) staphylococcus. Stool samples ICU11-3 and ICU11-4 demonstrated the presence of *Enterobacteriaceae*, corresponding to 99.26% and 94.98% of se-
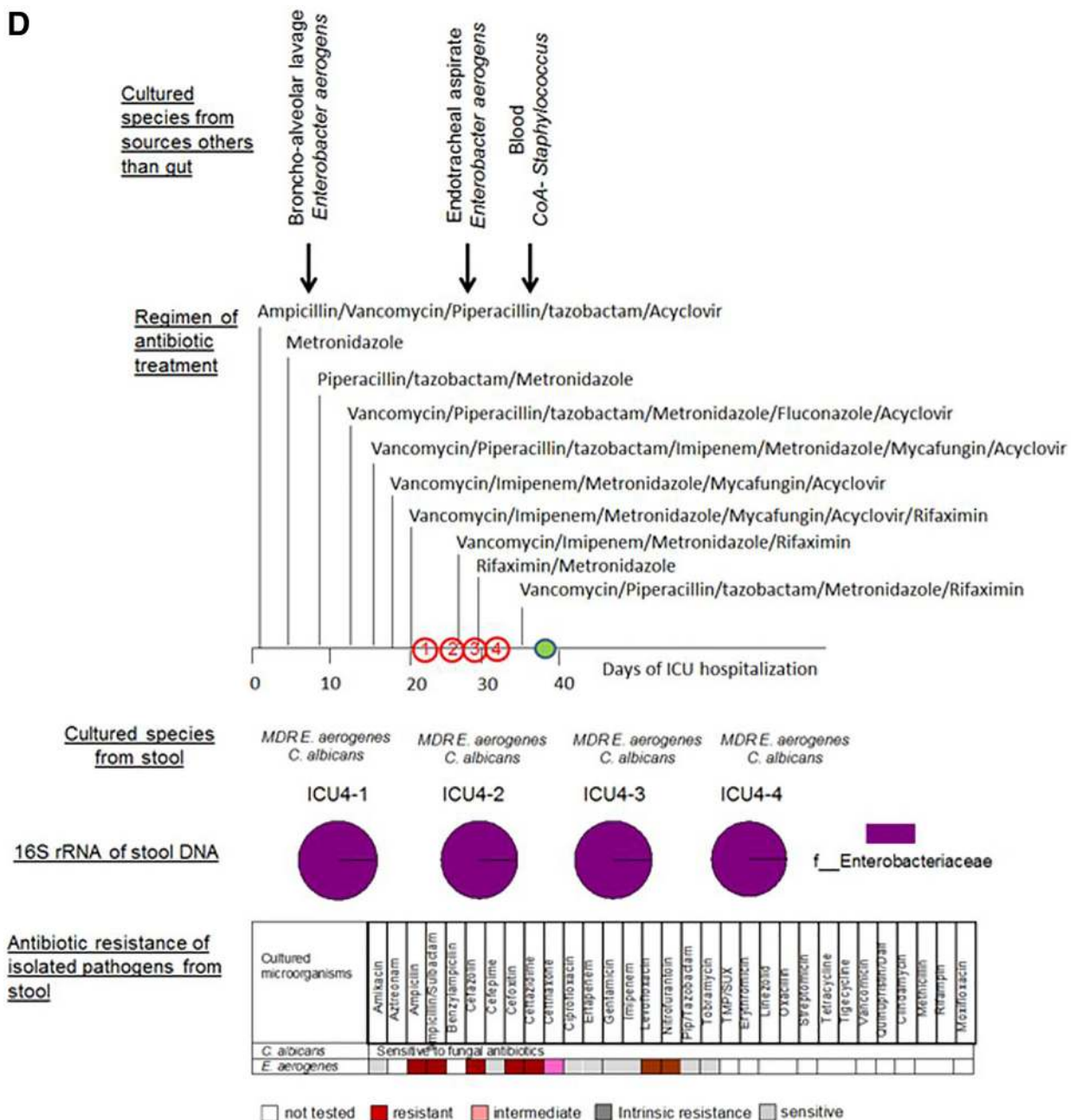
FIG 3 (Continued)

quence reads, respectively, which was in agreement with culture identifying *K. pneumoniae*. Later in the course of the patient's illness (ICU 11-5), coagulase-negative staphylococci emerged again. The eukaryotic pathogen *Candida glabrata* persisted at all points of stool collections from this patient. CoA⁻ *Staphylococcus*, *K. pneumoniae*, and *C. glabrata* were cultured from blood and urine at various times during the course of ICU confinement. Similarly to the cases described above, the gut microbial pathogens persisted regardless of their sensitivity or resistance to antibiotics applied to the patient.

(iv) Patient ICU4 (Fig. 3D). Patient ICU4 was the only patient of the four with ultra-low-diversity pathogen communities who

survived and was discharged after 40 days in the ICU. This patient was admitted to the ICU with a history of anaplastic astrocytoma, including 2 weeks of decreased oral intake, altered mental status, and fevers. The analysis of four stool collections at various time points (Fig. 3D) demonstrated the persistence of the same composition microbial community dominated by *Enterobacteriaceae*, comprising 99.8% to 99.9% of the 16S rRNA sequences, which was in agreement with the culture results showing the presence of *Enterobacter aerogenes* resistant to multiple antibiotics. Additionally, the eukaryotic pathogen *C. albicans* was persistently cultured from feces of patient ICU4 during the ICU stay. *C. albicans* was found to be sensitive to fluconazole and *E. aerogenes* to be sensitive
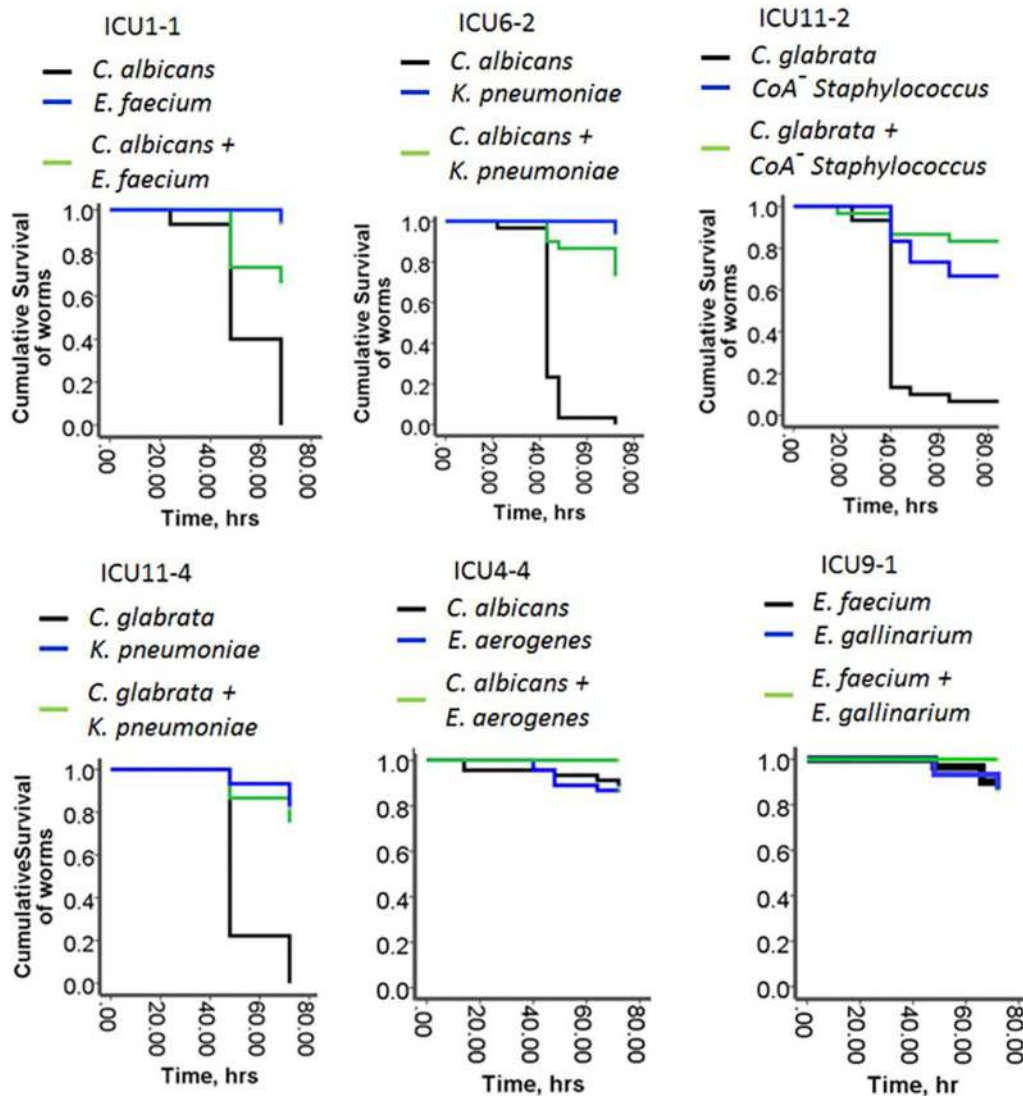
FIG 4 The commensal behavior of the 2-member pathogen communities. Data represent Kaplan-Meier survival curves of *C. elegans* fed on 2-member pathogen communities and their individual members ($n = 10$/plate, 3 plates/group). Data represent the cumulative results of 2 experiments.

to piperacillin-tazobactam and imipenem—antibiotics with which the patient was treated. Despite the sensitivity to these antibiotics, the community comprising *C. albicans* plus *E. aerogenes* persisted during this course of treatment (Fig. 3D). *E. aerogenes* was recovered from the bronchoalveolar lavage samples and endotracheal aspirates of this patient.

To conclude, in all cases listed above (ICU1, ICU4, ICU6, and ICU11), there were similar striking features that included a strong agreement of 16S rRNA amplicon sequencing profiles with the bacterial composition shown by culture, an agreement of the results determined using microbes cultured from intestinal and nonintestinal sources, and a failure of aggressive antibiotic treatment to eliminate these pathogens regardless of their sensitivities to administered antibiotics. Antibiotic susceptibilities of all the cultured microbes from the patients in this study are displayed in Table S1 in the supplemental material. As might be predicted, a high frequency of antibiotic-resistant microorganisms colonizing the gut of critically ill patients was observed.

***Ex vivo* analysis of potential virulence of low-diversity intestinal communities.** Given the close correlation of 16S rRNA amplicon sequencing profiles to microbial composition revealed by culture, we decided to determine the virulence potential of these pathogen communities by reassembling them *ex vivo* and allowing *C. elegans* to feed on them. *C. elegans* normally feeds on bacteria as a food source, and therefore its response to pathogen communities can be viewed as a proxy for the virulence potential of the bacterium. Six communities were included in the analysis: ICU1-1 (*C. albicans*: *Enterococcus faecium*), ICU6-2 (*C. albicans*: *Klebsiella pneumoniae*), ICU4-4 (*C. albicans*: *Enterobacter aerogenes*), ICU11-2 (*C. glabrata*: CoA⁻ *Staphylococcus*), ICU11-4 (*C. glabrata*: *K. pneumoniae*), and ICU9-1 (*Enterococcus faecium*: *Enterococcus gallinarum*). Based on results of *C. elegans* experiments, we made several important observations recorded below.

(i) Commensal lifestyle of 2-member pathogen communities. In the first round of the experiments, to mimic the nutrient-limited environment typical of the gut of a critically ill host, we
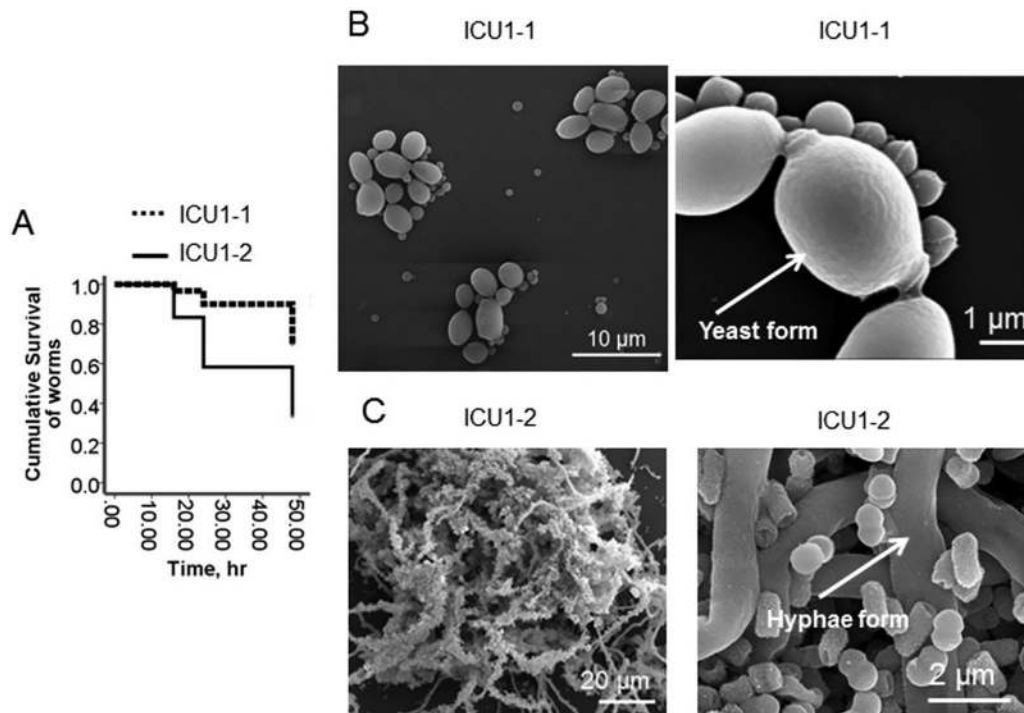
FIG 5 Replacement of bacterial comember switches the commensal behavior to pathogenic. (A) Kaplan-Meier survival curves of *C. elegans* fed on ICU1-1 and ICU1-2 pathogen communities (*n* = 10/plate, 3 plates/group). Data represent the cumulative results of 2 experiments. (B and C) Scanning electron microscopy images of ICU1-1 (B) and ICU1-2 (C) grown for 20 h in 0.1× TY.

grew the individual members of the communities as well as assembled whole communities in nutrient-poor 0.1× TY liquid media (tryptone, 1 g/liter; yeast extract, 0.5 g/liter, potassium phosphate buffer, 0.1 mM [pH 6.0]) (referred to here as 0.1× TY). After 4 h of growth in 0.1× TY, prestarved and antibiotic-treated worms were transferred to the microbial cultures. Antibiotic treatment was used to eliminate all residual bacteria from the intestinal tubes of the worms. We found that all but one *Candida* species (*C. albicans* ICU4-4) caused high levels of mortality in *C. elegans* (Fig. 4). None of the individual bacteria demonstrated a high killing capacity, and they were able to attenuate the *C. elegans* mortality caused by the *Candida* species, resulting in a commensal assemblage.

(ii) Replacement of a comember can switch commensal organisms to display pathogenic behavior. As seen in Fig. 5, the killing ability of the ICU1-2 community significantly exceeded the killing ability of the ICU1-1 community. This correlated with the morphology of *C. albicans* inside the community, with the yeast form being prevalent in the ICU1-1 sample and the hyphal form being prevalent in the ICU1-2 sample. These data confirm our previous demonstration that the ICU1-2 community consisting of *C. albicans* plus *S. marcescens* plus *K. oxytoca* plus *E. faecalis* induces a high level of mortality in *C. elegans* and mouse models (15). Therefore, the sudden replacement of a comember may provoke pathogenic behavior in a newly created community.

(iii) Critical care therapy can break commensalism. As was previously demonstrated, critical care factors used for treatment of critically ill patients (i.e., steroids, hormones, and opioids) can induce bacterial virulence via interkingdom signaling through their interaction with quorum-sensing signaling systems (15–23). Opioids can also be released in the gut during physiological stress

such as that seen following ischemia, which commonly occurs during critical illness (16). To determine if host-derived factors can affect the commensalism, we included a synthetic opioid (U-50,488) in the growth media. We have previously shown that this potent ligand of the kappa-opioid receptor acts similarly to the endogenous kappa-opioid dynorphin (16), which is released during intestinal ischemia. *C. elegans* was allowed to feed on bacteria grown under conditions of nutrient limitation (0.1× TY) and exposure to opioids (50 μM of U-50,488). In two communities, ICU6-2 and ICU11-2, the addition of 50 μM U-50,488 significantly increased the killing capacity of bacterial members *K. pneumoniae* ICU6-2 and CoA⁻ *Staphylococcus* ICU11-2; accordingly, the virulence of each assemblage was shifted from a commensal behavior to a pathogenic one (Fig. 6). However, not all the pathogen communities responded with enhanced killing capacity during opioid exposure. There was no significant increase in the mortality of worms fed on ICU1-1, ICU11-4, ICU4-4, and ICU9-1 communities (Fig. 6; see also Fig. S5 in the supplemental material), a result similar to the baseline control of mortality seen with *C. elegans* fed on a nonpathogen food source such as *E. coli* OP 50 (see Fig. S6).

We have summarized the community compositions, their behavior in the *C. elegans* model, and the outcomes of patients in Table 1. Although this study was not powered to determine if *C. elegans* mortality readouts can predict patient outcome, there appears to be an association between whether a community displays commensal versus pathogenic behavior and an outcome. For example, community ICU1-1, which displayed commensal behavior, became replaced by community ICU1-2, which displayed a pathogenic behavior, and this patient died. The prote-
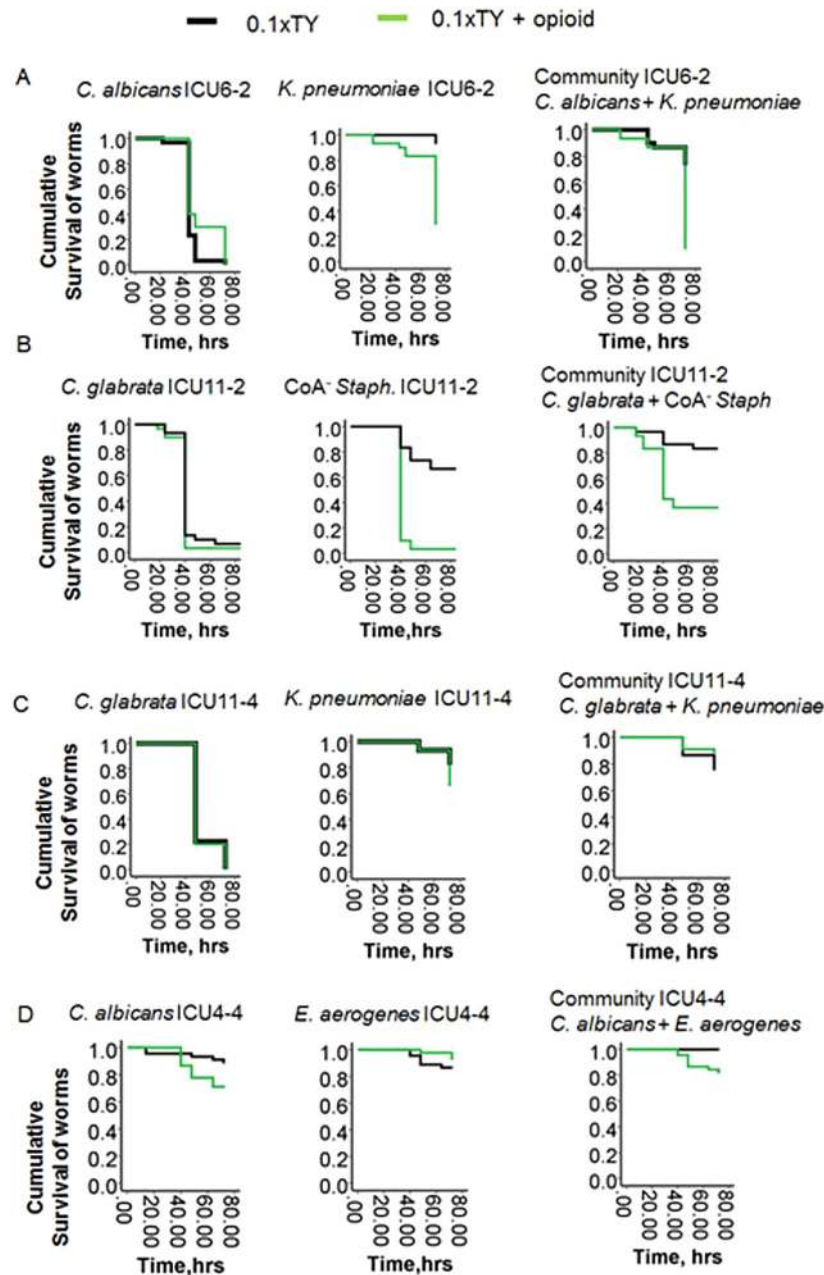
FIG 6 Opioids can break the commensalism of 2-member pathogen communities. Kaplan-Meier survival curves of *C. elegans* fed on ICU6-2 (A), ICU11-2 (B), ICU11-4 (C), and ICU4-4 (D) 2-member communities in the presence of an opioid (50 μM U-50,488) (*n* = 10/plate, 3 plates/group; *P* < 0.01). Data represent the cumulative results of 2 experiments. Exposure to opioids broke the commensalism in the ICU6-2 and ICU11-2 communities.

odominant ICU4-4 community displayed commensal behavior even under provocative conditions (i.e., opioid exposure), and the patient recovered and was discharged. In patient ICU11, pathogenic community ICU11-2 (CoA⁻ *Staphylococcus* plus *C. glabrata*) was replaced by a nonpathogenic community, ICU11-4 (*K. pneumoniae* plus *C. glabrata*); however, reemergence of CoA⁻ *Staphylococcus* was observed later, at time point 5 (ICU11-5), and this patient died. These data (although not powered) do suggest that the development of sepsis may not necessarily be a function of the specific taxa present but rather of whether the remaining species behave commensally or pathogenically as a community.

(iv) Phosphate abundance in the form of Pi-PEG can promote commensalism. Recent work from our laboratory demonstrated that extracellular phosphate (Pi) regulates bacterial virulence across a wide variety of pathogens (24). When phosphate is abundant, pathogen virulence is suppressed even in the presence of opioids; conversely, when phosphate is depleted, virulence expression is enhanced (17, 25). In order to preserve phosphate abundance in the gut during physiologic stress in a durable and bioavailable form, we covalently bound phosphate onto a high-molecular-weight polyethylene glycol (PEG) carrier compound to create Pi-PEG (15). We recently reported the efficacy and mechanism by which Pi-PEG can attenuate the virulence and lethality

TABLE 1 Compositions of microbial communities, their behavior in the *C. elegans* model, and patient outcomes

Patient	Time point of feces collection	Cultured community	Behavior in *C. elegans* model	Patient outcome
ICU1	1	*E. faecium* ICU1-1, *C. albicans* ICU1-1	Commensal	Deceased
ICU1	2	*E. faecalis* ICU1-2, *C. albicans* ICU1-2, *S. marcescens* ICU1-2, *K. oxytoca* ICU1-2	Pathogenic	
ICU4	1	*E. aerogenes* ICU4-1, *C. albicans* ICU4-1	NT[b]	Discharged
ICU4	2	*E. aerogenes* ICU4-2, *C. albicans* ICU4-2	NT	
ICU4	3	*E. aerogenes* ICU4-3, *C. albicans* ICU4-3	NT	
ICU4	4	*E. aerogenes* ICU4-4, *C. albicans* ICU4-4	Commensal	
ICU6	1	*S. aureus* ICU6-1, *K. pneumoniae* ICU6-1, *C. albicans* ICU6-1	NT	Deceased
ICU6	2	*K. pneumoniae* ICU6-2, *C. albicans* ICU6-2	Pathogenic	
ICU9	1	*E. faecium* ICU9-1, *E. gallinarum* ICU9-1	Commensal	Discharged
ICU11	1	CoA⁻ *Staphylococcus* ICU11-1, *C. glabrata* ICU11-1	NT	Deceased
ICU11	2	CoA⁻ *Staphylococcus* ICU11-2, *C. glabrata* ICU11-2	Pathogenic	
ICU11	3	*K. pneumoniae* ICU11-3, *C. glabrata* ICU11-3	NT	
ICU11	4	*K. pneumoniae* ICU11-4, *C. glabrata* ICU11-4	Commensal	
ICU11	5	CoA⁻ *Staphylococcus* ICU11-5,[a] *K. pneumoniae* ICU11-5, *C. glabrata* ICU11-5	NT	

[a] Note the reemergence of CoA⁻ *Staphylococcus* at time point 5 of stool collection.
[b] NT, not tested.

of highly resistant and virulent intestinal pathogens recovered from critically ill patients (15). In the current study, synthesized Pi-PEG15-20 was observed to prevent *C. elegans* mortality when *C. elegans* was feeding on whole communities ICU6-2 and ICU11-2 during exposure to the opioid compound U-50,488 (Fig. 7).

DISCUSSION

The composition, community structure, and behavior of microbes that colonize the human gut during prolonged critical illness remain poorly characterized. Most patients who enter an intensive care unit remain there, on average, 2 to 3 days until they are stabilized, transferred to the recovery floor, and discharged to home. However, the members of the gut microbiota of a patient

FIG 7 Pi-PEG15-20 stabilizes the commensal behavior of 2-member communities. Kaplan-Meier survival curves of *C. elegans* fed on ICU6-2 (left panel) and ICU11-2 (right panel) 2-member communities in the presence of an opioid (50 μM U-50,488) with or without 10% Pi-PEG15-20 ($n = 10$/plate, 3 plates/group; $P < 0.01$). Data represent cumulative results of 2 experiments.

confined for a prolonged period in the ICU may be subjected to unprecedented environmental circumstances where they have to compete for limited resources and survive under harsh and hostile conditions. Microbial diversity can be lost and resistance genes and virulence traits can be selected for as the remaining pathogenic communities struggle to survive conditions of nutrient deprivation and exposure to drugs and host compensatory signals. There exists little information regarding which members of the pathobiota remain in the gut following prolonged critical illness and how they function in this hostile microenvironment. Advantages in next-generation sequencing provide exciting opportunities to determine the composition of the gut microbiome in individual patients as a "microbial fingerprint." The challenge, however, will be to predict the behavior of a given pathogen community in the context of altering the clinical course of the patient.

Here we found that critically ill patients subjected to prolonged confinement in the ICU can harbor multidrug-resistant ultra-low-diversity microbial communities consisting of 2 to 4 pathogens whose individual virulence capacities are dependent on their composition and ability to respond to compensatory host factors released during stress. *Candida albicans* was the most prevalent and most stable member in 2-member pathogen communities despite patients being treated with a variety of antifungals to which it was susceptible. Infections due to *C. albicans* are known to originate from the gut during critical illness and are a major cause of late-onset sepsis and mortality in the ICU (26–28). The *C. albicans* virulence in the dual communities was found to be suppressed by the presence of bacterial comembers such as *E. faecium*, *K. pneumoniae*, and CoA⁻ *Staphylococcus*. Such commensal-like behavior has been recently described by others for *C. albicans* and *E. faecalis* (29, 30), and yet we show that *C. albicans*-bacterium commensalism can be disrupted when the organisms are exposed to opioids, which raises the possibility that the degree of host stress may play a role in shifting communities toward a more pathogenic state. We show for the first time that 2-member communities of *C. albicans* plus bacteria can superdominate the normal gut microbiota during prolonged critical
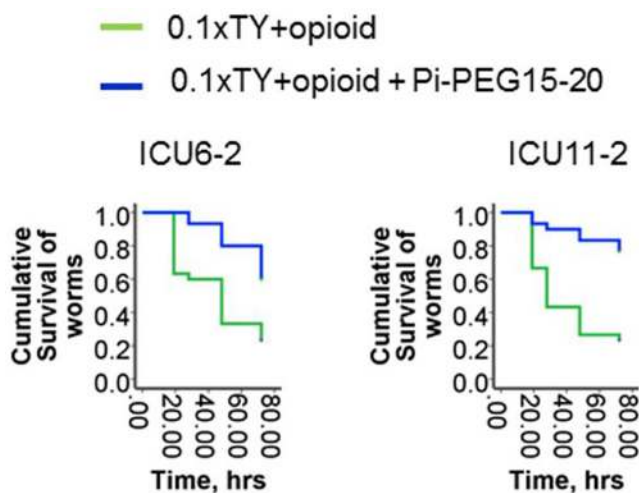
illness. There was strong agreement between 16S rRNA amplicon profiling and the community composition identified on the basis of culture, which provided confidence that the cultured communities could be used *ex vivo* to understand virulence potential by using *C. elegans* models. Previous work in our laboratory has demonstrated that opioids can enter the cytoplasm of bacteria such as *P. aeruginosa* and activate their quorum-sensing signaling system, leading to enhanced virulence (16, 18). Opioids were assessed in this study because they represent major stress-mediated signaling compounds in the gut (16, 31–35). The molecular details of the means by which opioids disrupt commensalism between *C. albicans* and other bacteria that persist in the gut during critical illness remain to be elucidated and are likely complex.

We have previously reported the efficacy of Pi-PEG in suppression of the virulence and lethality of the ICU1-2 community via mechanisms that involve phosphate-regulated pathways of virulence expression and maintenance of the normal indigenous microbiota (15). Studies were carried out both in *C. elegans* and in a mouse model of lethal gut-derived sepsis. In both *C. elegans* and mice, Pi-PEG was highly protective against mortality, and that protection was due in part to its capacity to create local phosphate abundance, which attenuates virulence. We had previously shown that phosphate abundance could prevent virulence of *C. albicans* and override the virulence-inducing effect of opioids in *P. aeruginosa* (17, 25). The present study confirmed that this effect of Pi-PEG is preserved among multidrug-resistant ultra-low-diversity pathogen communities.

A major challenge in treating critically ill patients is the overuse of antibiotics, a practice that is often unavoidable with patients exposed to multiple invasive procedures and extreme physiologic stress. A strategy to promote commensalism among the remaining pathogens by the use of agents such as Pi-PEG, which itself does not affect bacterial growth, may be useful. For example, data from the present study suggest that loss of the bacterial partner from a *C. albicans* community can derepress its virulence, a possible unanticipated consequence of aggressive antibiotic therapy applied to ICU patients. Our methods and results suggest that such a loss of these core commensal organisms can be deleterious, especially when critically ill patients harbor ultra-low-diversity pathogens. Use of stabilizing agents such as Pi-PEG may offer a mechanism to preserve commensalism in the gut when aggressive antibiotics are needed to treat extraintestinal infections.

It is curious that communities that were otherwise seemingly avirulent (i.e., *C. albicans* plus *Enterobacter aerogenes* in patient ICU4 and *E. faecium* plus *E. gallinarum* in patient ICU9) were present in the feces of patients who were ultimately discharged from the hospital. These communities could not be induced to express virulence against *C. elegans* even under provocative conditions. In fact, we have recently reported that *C. albicans* isolated from patient ICU4 did not respond to phosphate limitation with enhanced virulence compared to that seen with *C. albicans* isolated from patients ICU1 and ICU6 (25). The nonvirulent behavior of *C. albicans* isolated from patient ICU4 was again seen in the present study. A study in which dynamic and repeated fecal microbial analysis is coupled to physiologic variables in the host might more accurately inform outcome measure under the highly oscillatory conditions of critical illness.

In summary, here we demonstrate that the intestinal microbiome in critically ill patients can be considered a "damaged organ" given that its main cellular mass, the normal microbiota, is disrupted and dominated by pathobiota which may be an ever-threatening source for disseminating pathogens. The emergence of ultra-low-diversity pathogen communities and the activation of virulence in the response to host cues released during physiologic stress are characteristic features of this organ in some critically ill patients. Dynamic assessment of the composition and virulence potential of the intestinal microbiota or pathobiota over the course of a critical illness may allow a more calibrated use of antibiotics or, alternatively, the development of novel strategies to preserve the core intestinal microbiome when multiple antibiotics are needed to treat life-threatening extraintestinal infections.

MATERIALS AND METHODS

Stool sample collection. Stool sampling was performed in accordance with the Institutional Review Board (IRB) protocol approved by University of Chicago. In order to establish baseline normal controls, samples were collected from five healthy volunteers who were not treated with any antibiotics for 1 year prior to sample collection. We next consecutively selected 14 patients admitted to the intensive care units (ICUs) at the University of Chicago hospitals from whom consent could be obtained for stool collection. All patients signed consent forms to participate in the study. The 14 patients represented a diverse group of ICU patients with various lengths of stay, including those who were resident for less than 10 days (3 of 14 patients), those who were resident for between 20 and 30 days (3 of 14), and those who were resident for >30 days (6 of 14). When possible, stool samples were collected at several time points during the ICU course.

Demographic and clinical history. Demographic and clinical histories of ICU patients, including past medical history, admitting diagnosis, hospital course, therapy, especially antibiotic therapy, and discharge summary, were available in the patients' charts in accordance with the IRB protocol. Among the 14 ICU patients, 10 were admitted to the ICU for a period of 20 days or longer. Of these 10 patients, 7 died despite continuous intensive care. The patients who died all died with signs of severe sepsis, although there was considerable variability in their comorbidities and underlying illnesses.

16S rRNA analysis of stool samples. The microbial composition of stool samples was analyzed by 16S rRNA V4 iTAG amplicon sequencing of DNA recovered from stool samples. A BiOstic Bacteremia DNA isolation kit from MoBio Laboratories was used to extract DNA. DNA was analyzed using next-generation Illumina sequencing. Reads containing low-confidence base calls (phred value, <20) were dropped, and the remaining 150-bp-long sequences were clustered into 97%-similarity operational taxonomic units (OTUs) using QIIME 1.6.0's pick_otus.py script with default parameters (36, 37). OTUs composed of fewer than 10 sequences were discarded, and the remaining OTUs were assigned taxonomic annotations by searching representative members of each cluster against the Greengenes 12_10 (38) database using the RDP classifier (39). Sequencing depth among samples was normalized by rarefaction to 1,200 sequences per sample prior to calculating taxonomic relative abundances. Microbial-community diversity was calculated for each sample using the Chao1 alpha diversity metric, as implemented by the QIIME alpha_rarefaction.py script.

The samples isolated from ICU patients (patients ICU1-1, ICU1-2, ICU2-1, ICU2-2, ICU3-1, ICU3-2, ICU4-1, ICU4-2, ICU4-3, and ICU4-4) were independently processed for 16S rRNA analysis at Michigan State University. DNA was extracted from the stool samples using a PowerSoil DNA isolation kit from MoBio Laboratories following the manufacturer's instructions with the exception that Inhibitor Removal Solution from MoBio's Ultra-Clean Fecal DNA isolation kit was added to the PowerBead Tubes along with solution C1. PCR amplification and purification were similar to those described by Rosenzweig et al. (40). The amplification and purification procedures differed in that samples were first amplified for 15 cycles using primers 577f and 927r without bar codes or adaptors, and then 2 μl of each reaction mixture was amplified for 15

more cycles with primers containing the bar codes and Roche adaptors. The thermocycler program for the first PCR was 95°C for 3 min and then 15 cycles of 95°C for 45 s, an annealing temperature of 44°C (determined from optimization experiments) for 45 s, and 72°C for 90 s, followed by elongation at 72°C for 7 min before the reaction mixture was held at 4°C. The thermocycler program for the second PCR was 95°C for 3 min and then 15 cycles of 95°C for 45 s, 56°C for 45 s, and 72°C for 90 s, followed by elongation at 72°C for 7 min before the reaction mixture was held at 4°C. Equal mass amounts of the amplicon libraries were mixed and submitted for sequencing to the Research Technology Support Facility at Michigan State University.

The sequence results were obtained and processed using the tools on the Ribosomal Database Project's Pyrosequencing Pipeline at http://py-ro.cme.msu.edu/. Initial processing parameters were a forward primer maximum edit distance of 2, a reverse primer maximum edit distance of 0, a maximum number of N's of 0, a minimum read Q score of 20, and a minimum sequence length of 300. Trimmed sequences averaging 331 bp were aligned and clustered at a distance of 3%, and the cluster results were reformatted to be read into R (41) as an OTU table. Representative sequences for each OTU were submitted to the RDP Classifier for identification and to the Decipher Find Chimeras tool of the University of Wisconsin—Madison at http://decipher.cee.wisc.edu/ using the Short-Length Sequences option. OTUs identified as chimeric sequences were removed from the data. Further analysis was done using the BioDiversityR (42) package within the R program.

The 16S rRNA analysis of the aliquots of stool samples from the same patients was performed by the Argonne National Laboratories and Michigan State University with almost identical results, confirming extremely low diversity of the stool samples from patients ICU1 and ICU4.

Isolation, identification, and antibiotic resistance of cultured microorganisms. Microbial species were isolated by plating bacterial communities on plates selective for Gram-negative ($-$) bacteria (MacConkey II), for Gram-positive ($+$) bacteria (Columbia CNA agar with 5% sheep's blood [SB]) (Becton and Dickinson), for *Pseudomonas aeruginosa* (*Pseudomonas* isolation agar [PIA]), and for fungi (saturated dextrose agar with chloramphenicol and gentamicin) and on Trypticase soy agar with 5% sheep blood (TSA II 5% SB) for cultivating fastidious microorganisms. Gram ($-$) bacteria were identified by the use of a Vitek2 system (bioMérieux, Inc., Durham, NC). Gram ($+$) bacteria were identified by standard manual methods. *Enterococcus* species identification was performed by the use of a Vitek2 system. A positive germ tube test identified *Candida albicans*. Susceptibility testing was performed by testing on a Vitek2 system or by disk diffusion. Susceptibility testing of Gram-negative bacilli was performed by the use of a Vitek2 system or by disk diffusion. Susceptibility testing of MRSA and enterococci was performed by the use of a Vitek2 system. Susceptibility testing of the other Gram-positive cocci was performed using a combination of disk diffusion and E test strips. Susceptibility testing of *Candida* was performed using a Sensititre YeastOne MIC panel (TREK Diagnostic Systems Inc., Cleveland, OH). Susceptibility results were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines.

***Caenorhabditis elegans* killing assay.** The 2-member whole communities were compared to their individual members for killing capacity by assessing mortality of *C. elegans* transferred into liquid microbial cultures as in our previously described experiments (15, 17). Nematodes of *C. elegans* N2 provided by the Caenorhabditis Genetic Center (CGC), University of Minnesota, were used in all experiments. Nematodes were prepared as follows: synchronized nematodes (larval stage 4 [L4] to young adults) were transferred from *E. coli* OP 50 stock plates onto plain agarized plates, followed by a second transfer onto new plain agarized plates (Falcon) (60-mm diameter). Next, 1 ml of 100 μg/ml kanamycin was poured on the agar surface; after 3 h, worms were retransferred into experimental microbial cultures.

Microbial cultures were prepared as follows: microbes from frozen individual stocks were plated on Trypticase soy broth (TSB) solid plates

and incubated at 37°C overnight. Cells grown overnight were used to prepare stock microbial suspensions in 0.1$\times$ TY liquid media (tryptone, 1 g/liter; yeast extract, 0.5 g/liter, potassium phosphate buffer, 0.1 mM [pH 6.0]) (referred to here as 0.1$\times$ TY) to reach an absorbance of 1.0 at an optical density of 600 (OD_{600}). Stock microbial suspensions were diluted in fresh 0.1$\times$ TY to an OD_{600} of 0.1 to prepare individual microbial cultures. To prepare community cultures, the respective stocks were mixed to reach an OD_{600} of 0.1 for each individual microbe in the mixture. In the experiments performed with Pi-PEG15-20, the stock microbial suspensions and final microbial cultures were prepared in 0.1$\times$ TY supplemented with 10% Pi-PEG15-20, and the pH was adjusted with KOH to that of 0.1$\times$ TY (pH 5.8). Microbial cultures were grown individually and as a mixture for 5 h at 37°C with shaking at 200 rpm. Then, 2 ml of the microbial culture was poured into a 35-mm-diameter empty sterile plate and adjusted to room temperature, and 5 to 10 worms were transferred into each plate. For each variant, 3 to 5 plates were prepared. *C. elegans* were fed on microbial cultures for up to 72 h under static conditions at room temperature (~23°C). The mortality of worms was followed dynamically.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.01361-14/-/DCSupplemental.

Figure S1, PDF file, 0.04 MB.
Figure S2, PDF file, 0.2 MB.
Figure S3, PDF file, 0.05 MB.
Figure S4, PDF file, 0.04 MB.
Figure S5, PDF file, 0.1 MB.
Figure S6, PDF file, 0.04 MB.
Table S1, DOCX file, 0.04 MB.

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