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MAJOR ARTICLE

Protective Factors in the Intestinal Microbiome Against *Clostridium difficile* Infection in Recipients of Allogeneic Hematopoietic Stem Cell Transplantation

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Background. *Clostridium difficile* infection (CDI) is a frequent complication in recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT), who receive intensive treatments that significantly disrupt the intestinal microbiota. In this study, we examined the microbiota composition of allo-HSCT recipients to identify bacterial colonizers that confer protection against CDI after engraftment.

Methods. Feces collected from adult recipients allo-HSCT at engraftment were analyzed; 16S ribosomal RNA genes were sequenced and analyzed from each sample. Bacterial taxa with protective effects against development of CDI were identified by means of linear discriminant analysis effect size analysis and then further assessed with clinical predictors of CDI using survival analysis.

Results. A total of 234 allo-HSCT recipients were studied; postengraftment CDI developed in 53 (22.6%). Within the composition of the microbiota, the presence of 3 distinct bacterial taxa was correlated with protection against CDI: Bacteroidetes, Lachnospiraceae, and Ruminococcaceae. Colonization with these groups at engraftment was associated with a 60% lower risk of CDI, independent of clinical factors.

Conclusions. Colonization with these 3 bacterial groups is associated with a lower risk of CDI. These groups have been shown to be vital components of the intestinal microbiota. Targeted efforts to maintain them may help minimize the risk of CDI in this at-risk population.

Keywords. *Clostridium difficile*; microbiome; Bacteroidetes; Lachnospiraceae; Ruminococcaceae.

Clostridium difficile infection (CDI) is a frequent cause of infectious diarrhea in recipients of hematopoietic stem cell transplantation (HSCT), with reported incidences of approximately 15%–20%, ranging greatly from as low as 6% to upward of 39%, depending on factors such as the transplant population, timing, and testing method [1–8]. Prophylactic and therapeutic antibiotic administration during neutropenia disrupts the stable composition of the intestinal microbiota and is probably an important contributor to the high rate of posttransplant CDI. Longitudinal analyses of fecal microbiota composition in patients undergoing allogeneic HSCT (allo-HSCT) have demonstrated disruption of diversity and marked instability of intestinal microbiota composition [9, 10].

The commensal bacterial species correlated with resistance to CDI are increasingly being defined, through murine models [11–15] or case-control human studies [16–22]. In the current work, we sought to perform a prospective microbiome study of CDI in a patient population experiencing a high incidence of infection: recipients of allo-HSCT.

Kinnebrew et al [7] previously reported on CDI during the early phase of allo-HSCT (ie, within first 35 days after transplantation), observing high rates of prior colonization with *C. difficile*. These cases occurred very early, overlapping with conditioning chemotherapy and stem cell infusion. On the other hand, CDIs occurring later in the course of allo-HSCT were not correlated with peritransplantation factors or early CDI itself. This may suggest that these varieties of CDI are clinically distinct.

In the current study, we focused on CDI occurring after hematopoietic engraftment, examining the intestinal microbiota to identify bacterial groups that might confer resistance to CDI. High rates of infection in this patient population may provide a unique opportunity to prospectively identify microbial factors associated with a decreased risk of subsequent CDI.

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METHODS

Patients and Routine Practices in Transplantation

Adult patients who underwent allo-HSCT at Memorial Sloan Kettering Cancer Center from 1 December 2010 to 30 November 2014 were studied. In general, these patients received routine antibiotic prophylaxis based on institutional practice guidelines, consisting of intravenous vancomycin and ciprofloxacin during times of neutropenia, around the time of stem cell infusion. Patients in whom fever developed during neutropenia were typically treated empirically with piperacillin-tazobactam or cefepime, or aztreonam in patients with penicillin allergy. Prophylaxis for *Pneumocystis carinii/jiroveci* was started at the beginning of allo-HSCT, with aerosolized pentamadine or atovaquone, with a switch to trimethoprim-sulfamethoxazole after stable white blood cell count recovery. Our institution does not typically administer antibiotics for total or selective gut decontamination (eg, metronidazole, neomycin, and rifaximin).

Fecal Specimen Collection

Fecal specimens were collected longitudinally over the course of each patient's initial hospitalization for transplantation, using an institutional fecal biospecimen collection protocol, described elsewhere [9]. In the current study, we focused on the fecal specimen collected after hematopoietic engraftment (absolute neutrophil count, ≥500/mL for 3 consecutive days), and analyzed it for the ability to predict subsequent CDI. The engraftment sample consisted of the first sample collected after engraftment, not to exceed hospital discharge.

For each subject's engraftment specimen, DNA was extracted and purified, and the V4-V5 region of the 16S ribosomal RNA (rRNA) gene was amplified with polymerase chain reaction using modified universal bacterial primers. The sequence library was sequenced on an Illumina Miseq platform [23] to obtain paired-end reads. These reads were assembled, processed, and grouped into operational taxonomic units of 97% similarity using the UPARSE pipeline [24]. Taxonomic assignment to species level was performed using an algorithm incorporating nucleotide BLAST (Basic Local Alignment Search Tool) [25], with the National Center for Biotechnology Information Sequence Read Archive [26] as the reference training set. These methods are described in further detail in the Supplemental Methods.

Assessment of CDI

CDI was defined as lower gastrointestinal symptoms associated with a positive toxigenic *C. difficile* assay, which consisted of polymerase chain reaction detection of *C. difficile* toxin B gene (Xpert *C. difficile* assay; Cepheid GeneXpert) for the entire study period. Clinical testing was performed at the discretion of the patient's treating physician. The primary clinical end point we studied was postengraftment CDI, which we defined as CDI within 1 year after stem cell engraftment. CDI severity was defined as low (outpatient illness or uncomplicated inpatient management without need for imaging), medium (colitis at imaging), or severe (associated with clinical sepsis, intensive care unit admission, death due to colitis, or pseudomembranous colitis) [27]. CDI recurrence was defined as symptomatic recurrence ≥14 days after the initial episode. All CDI cases were reviewed independently by 2 physicians (Y. J. L. and E. P. A.).

Analysis

The outcome of interest was CDI after hematopoietic engraftment. Patients were followed up for up to 1 year after engraftment; observations were censored if death occurred, or if the patient underwent a second stem cell transplant. To assess whether microbial taxa from the engraftment samples were positively or negatively predictive of subsequent CDI, we compared the microbial composition of subjects with or without subsequent CDI. These comparisons were performed using linear discriminant analysis (LDA) effect size analysis [28], where conditioning regimen intensity was used for subclass comparisons. We used a logarithmic LDA cutoff of 2.0, and an α value of .05 for Kruskal–Wallis testing among classes and for Wilcoxon testing between subclasses.

Bacterial taxa that were associated with protection against postengraftment CDI were further analyzed using survival analysis, along with clinical risk factors. A combined relative abundance of 0.001 was used as the cutoff. Detection of *C. difficile* in the postengraftment 16S rRNA sequences was also assessed as a predictor for subsequent clinical CDI. Clinical variables analyzed included age, sex, underlying disease, pretransplantation conditioning regimen intensity, stem cell graft source, HLA matching of the graft, time to engraftment, and clinical diagnosis of CDI during hospitalization for transplantation before engraftment. Cox proportional hazard modeling was used to examine these clinical and microbiota predictors.

Variables were first analyzed individually by univariate analysis. Predictors with a univariate P value ≤.30 were analyzed in a multivariate model. Kaplan-Meier plots were used to further estimate the impact of the identified bacterial taxa on subsequent postengraftment CDI; the log-rank test was used to determine differences in cumulative risk. A heat map of relative abundances at the species level was generated to show further detail of the microbial composition at engraftment. All statistical analyses were performed using R software, version 3.2 (R Development Core Team). Data from this study are stored in the National Center for Biotechnology Information Sequence Read Archive (http://www.ncbi.nlm. nih.gov/sra; accession No. PRJNA356739).

RESULTS

Patient Characteristics

During the study period, 234 allo-HSCT recipients were studied (233 patients; 1 patient underwent a second allo-HSCT

procedure for secondary graft failure); subjects participated provided fecal biospecimen samples at the time of engraftment. Among the 234 allo-HSCT recipients, postengraftment CDI developed in 53 (22.6%). Of these CDI cases, 48 (90.6%) were classified as low severity, 3 (5.7%) as medium severity, and 2 (3.8%) as severe. After the 53 incident cases of postengraftment CDI, 15 (20.3%) had ≥1 recurrence within the 1-year study period. Baseline characteristics were similar between patients with and those without postengraftment CDI (Table 1); the 2 groups were approximately similar in terms of demographics, disease, type of transplant, and subsequent graft-vs-host disease (GVHD).

Analysis of Microbial Composition

Fecal samples collected from each of the 234 subjects after engraftment were sequenced. After sequencing error filtering, a total of 3 247 640 16S rRNA reads (mean, 13 879 per sample) were used for analysis. From these sequences, we identified a total of 1076 operational taxonomic units (OTUs). Of these OTUs, 358 (33.3%) were unique to single subjects, whereas 7 (0.7%) were found in more than half of the subjects. Microbial diversity of the samples varied greatly, with inverse Simpson indices ranging from 1.0 to 20.1 (median, 1.90). Repeated analysis of UPARSE without the singleton removal step (also known as UPARSE+1) revealed similar results; inverse Simpson diversity metrics differed by a median of 0.001.

Analysis of microbial composition of engraftment samples for association with clinical CDI using LDA effect size is shown in Figure 1. We identified 3 distinct groups of bacteria, all obligate anaerobic, that were significantly correlated with protection against CDI: Bacteroidetes phylum, Lachnospiraceae family, and Ruminococcaceae family. The Bacteroidia class and Bacteroidales order, which represent deeper levels within the Bacteroidetes phylum but are largely similar in composition, were also identified as protective. Bacterial taxa that correlated with risk of CDI consisted of *Enterococcus faecalis*, at various rank designations; these are shown in Supplemental Table 1.

Survival Analysis of Protective Bacteria and Clinical Factors

Having identified Bacteroidetes, Lachnospiraceae, and Ruminococcaceae as bacterial groups that are associated with protection against CDI, we further analyzed the presence of these taxa as predictors of CDI in a Cox proportional hazard model, using a combined relative abundance of 0.001 as a threshold (Table 2). Univariate analysis showed that presence of the protective groups was associated with a 60% lower risk of CDI. Detection of *C. difficile* among the 16S rRNA gene sequences was not correlated with subsequent postengraftment CDI. Clinical factors that had some association with the risk of CDI included higher disease risk status and HLA-mismatched

Table 1. Clinical Characteristics in 234 Allogeneic Hematopoietic Stem Cell Transplant Recipients With or Without Postengraftment CDI

	Patients, No. (%)	
Clinical Variable	No CDI (n=181)	CDI (n=53)
Age, y		
≤29	9 (5.0)	6 (11.3)
30–39	27 (14.9)	4 (7.5)
40–49	29 (16.0)	6 (11.3)
50–59	61 (33.7)	18 (34.0)
≥60	55 (30.4)	19 (35.8)
Sex		
Female	69 (38.1)	20 (37.7)
Male	112 (61.9)	33 (62.3)
Underlying disease		
Leukemia	91 (50.3)	27 (50.9)
Lymphoma/CLL	37 (20.4)	14 (26.4)
Multiple myeloma	18 (9.9)	5 (9.4)
Myelodysplastic syndrome	28 (15.5)	6 (11.3)
Myeloproliferative disorder	6 (3.3)	1 (1.9)
Nonmalignant hematologic disorders	1 (0.6)	0 (0.0)
Disease risk[a]		
High	34 (18.8)	12 (22.6)
Intermediate	43 (23.8)	17 (32.1)
Low	96 (53.0)	23 (43.4)
Not applicable	8 (4.4)	1 (1.9)
Conditioning intensity		
Myeloablative	98 (54.1)	28 (52.8)
Nonmyeloablative	19 (10.5)	7 (13.2)
Reduced intensity	64 (35.4)	18 (34.0)
Stem source		
Matched related	54 (29.8)	14 (26.4)
Related nonidentical	1 (0.6)	0 (0.0)
Unrelated identical	61 (33.7)	16 (30.2)
Unrelated nonidentical	20 (11.0)	9 (17.0)
Double cord	27 (14.9)	11 (20.8)
Double cord plus haploidentical	18 (9.9)	3 (5.7)
Stem cell manipulation		
Unmodified	99 (54.7)	29 (54.7)
T-cell depleted (ex vivo)	82 (45.3)	24 (45.3)
Time to engraftment, d		
<14	138 (76.2)	41 (77.4)
≥14	43 (23.8)	12 (22.6)
Pre-engraftment CDI		
No	160 (88.4)	46 (86.8)
Yes	21 (11.6)	7 (13.2)
GVHD overall grade		
0 (none)	111 (61.3)	35 (66.0)
1	11 (6.1)	2 (3.8)
2	40 (22.1)	15 (28.3)
3	11 (6.1)	1 (1.9)
4	7 (3.9)	0 (0.0)
Not evaluable	1 (0.6)	0 (0.0)

Abbreviations: CDI, *Clostridium difficile* infection; CLL, chronic lymphocytic leukemia; GVHD, graft-versus-host disease.

[a]Classification based on American Society for Blood and Marrow Transplantation Request for Information Form.

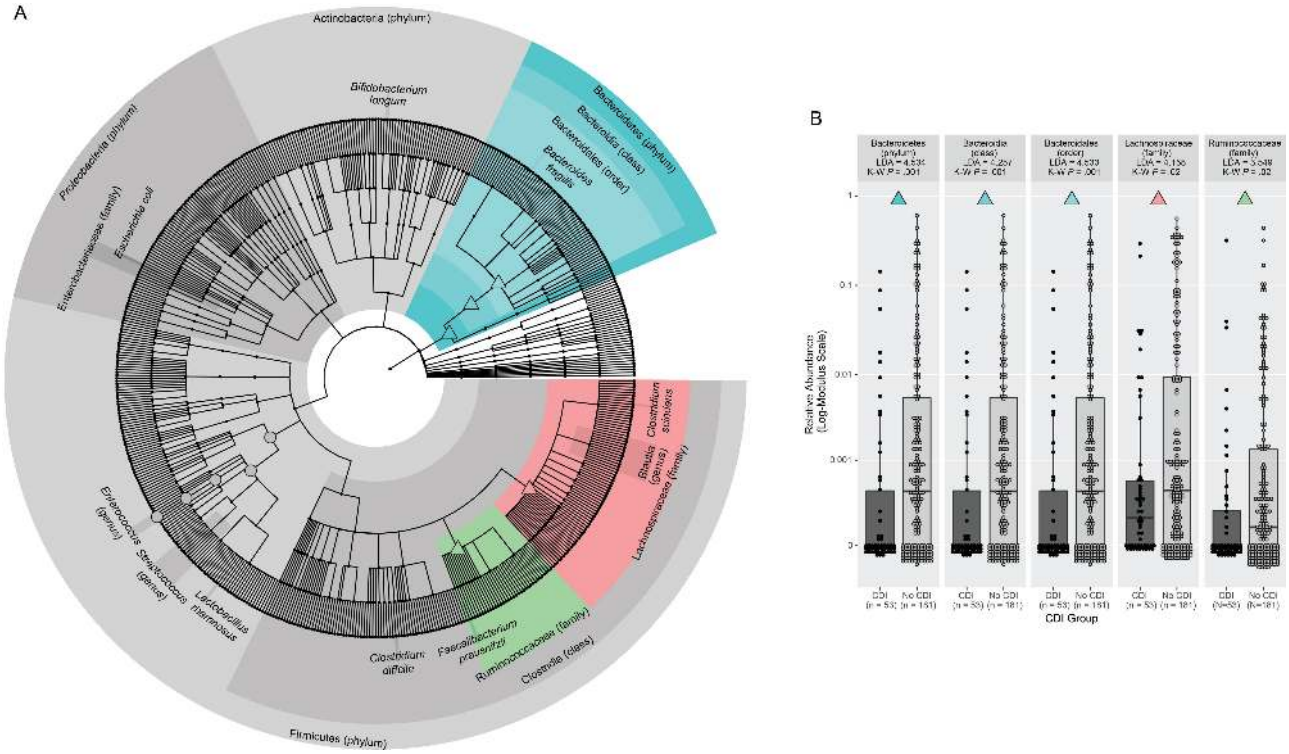

Figure 1. Identification of bacterial taxa associated with increased or decreased risk of *Clostridium difficile* infection (CDI), by linear discriminant analysis (LDA) effect size. *A,* Cladogram depicting all observed bacterial taxa (1010 nodes total). Five bacterial taxa (*open colored triangles*) were associated with reduced risk of subsequent CDI, and 5 (*open gray circles*) were associated with increased risk of CDI. All other taxa (*small black circles*) were not significantly associated. *B,* Relative abundances (*circles*) are shown for each of the 5 taxonomic groups associated with protection against CDI (*colored triangles corresponding to cladogram*), grouped by whether or not CDI occurred. Data are accompanied by box plots showing median and interquartile range (IQR); whiskers represent 1.5 times IQR. Note that Bacteroidia (class) and Bacteroidales (order) are equivalent taxonomic designations.

graft. After adjustment for these clinical factors, presence of the 3 protective bacterial groups remained as a significant protective factor against subsequent postengraftment CDI. Kaplan-Meier estimates for these 3 bacterial groups are shown in Figure 2; protective effects can also be seen here, though Ruminococcaceae was not significant by log-rank test.

To determine whether microbial diversity could be used as a surrogate measure of the microbiota, rather than analysis of specific taxonomic groups, we performed the same survival analysis but instead used the inverse Simpson diversity index as a predictor of postengraftment CDI; we found that diversity was not significantly predictive of subsequent development of CDI (Supplemental Figure 1). We also used Cox modeling to examine for the association between CDI and subsequent development of GVHD, and we found no association (Supplemental Table 2).

Species Composition

The composition of the 3 protective bacterial groups is depicted at the genus level in Figure 3, and individual species-level identification of OTUs is listed in Supplemental Table 3. Of the subjects who were colonized with bacteria belonging to the 3 protective groups, cocolonization occurred frequently. Evidence of this could also be seen in the Cox model if the 3 protective groups were analyzed as separate predictors. Bacteroidetes, Lachnospiraceae, and Ruminococcaceae were each separately associated with protection against CDI but did not demonstrate independent protection in a simultaneous model (Supplemental Table 4).

DISCUSSION

In this study, we examined the impact of the intestinal microbiota composition on CDI in recipients of allo-HSCT and identified 3 distinct bacterial groups associated with protection against CDI after hematopoietic recovery. Prior studies have examined the microbiota in the setting of CDI using similar sequencing methods. However, most of those studies used samples collected after infection had already occurred and compared them with samples from healthy controls or asymptomatically colonized individuals [16, 17, 20–22]. One prior study was able to examine stool samples before the onset of symptoms by using a nested case-control design but nonetheless derived its subjects by selecting patients with or without CDI [18, 19]. Our study is unique in that it examined the effect of the microbiota on CDI prospectively, starting from a defined time point in a single cohort of patients. This design allowed for the calculation of

Table 2. Clinical and Microbiota Risk Factors for CDI After Stem Cell Engraftment

	Univariate Analysis		Multivariate Analysis	
Predictor	Hazard Ratio	*P* Value	Hazard Ratio	*P* Value
Age (y)	1.00 (0.98–1.03)	.67	…	…
Sex (female)	0.99 (0.56–1.70)	.96	…	…
Underlying disease (leukemia vs other)	0.96 (0.56–1.66)	.89	…	…
Disease risk (intermediate/high vs low)[a]	1.46 (0.85–2.54)	.17	1.54 (0.88–2.73)	.13
Conditioning intensity (myeloablative vs other)	0.72 (0.35–1.74)	.44	…	…
HLA-mismatched graft	1.34 (0.77–2.30)	.29	1.22 (0.69–2.14)	.48
T-cell depleted graft (ex vivo)	0.92 (0.53–1.58)	.77	…	…
Cord blood graft (vs noncord)	1.17 (0.61–2.11)	.61	…	…
Time to engraftment (≥14 d)	1.05 (0.53–1.93)	.88	…	…
Pre-engraftment CDI	1.06 (0.44–2.20)	.88	…	…
Presence of *Clostridium difficile*[b]	1.25 (0.52–2.60)	.59	…	…
Presence of Bacteroidetes, Lachnospiraceae, or Ruminococcaceae[b]	0.43 (0.24–0.75)	.003	0.41 (0.23–0.71)	.001

Abbreviation: CDI, *Clostridium difficile* infection.

[a]Classification based on American Society for Blood and Marrow Transplantation Request for Information Form.

[b]Total relative abundance ≥0.001 at engraftment.

incidence and use of survival methods such as Kaplan-Meier and Cox proportional hazards. In case-control studies, calculation of incidence is not possible.

In most patient populations, a prospective microbiota study of CDI may be difficult to perform. CDI rates in general hospitalized patients are estimated to be approximately 9 per 1000 hospitalizations [29]; thus, a study seeking to collect fecal samples before the onset of CDI would need to collect ≥111 fecal samples to observe a single case of CDI. However, the incidence of CDI among recipients of allo-HSCT is much higher than in the general population, as shown in the current study, in which CDI developed in one-fifth of subjects during the study period, making prospective study of the microbiome on risk of CDI more feasible. The high CDI rates we observed here were similar to those in past studies examining CDI in the setting of allo-HSCT [1–5].

In the current study, we identified 3 taxonomic groups of bacteria that were associated with protection against CDI: Bacteroidetes, Lachnospiraceae, and Ruminococcaceae. All of these groups consist of obligate anaerobic bacterial species recognized as common members of a healthy flora, with increasing evidence of association with human health.

Bacteria from the Bacteroidetes phylum are well adapted to the gut and contribute to development of the host mucosal immune system [30–32]. Prior case-control studies of intestinal microflora and CDI, using either culture-based or molecular-based approaches, have correlated loss of the Bacteroidetes phylum with CDI [16, 19–22, 33, 34]. In studies of fecal microbiota transplantation, Bacteroidetes was shown to be restored in patients cured of recurrent CDI [35–38].

Lachnospiraceae and Ruminococcaceae, also designated as *Clostridium* clusters XIVa and IV, respectively [39, 40], have also been shown to promote intestinal homeostasis [41] and are often missing in case-control studies of CDI [17, 21]. Other studies have also observed potential protection from these bacterial taxa, though at higher classification levels, such as Firmicutes (phylum), Clostridia (class), or Clostridiales (order) [18–20]. These higher levels of classification do not necessarily comprise purely beneficial microbes, because they include potential pathogens such as *Staphylococcus*, *Enterococcus*, *Clostridium perfringens*, and even *C. difficile* itself. Lachnospiraceae have specifically been shown to have protective effects against CDI in several preclinical studies. In a study of murine CDI, an isolated member of Lachnospiraceae was protective against infection [11]. In another study, *Clostridium scindens*, a member of Lachnospiraceae, was also protective against CDI in a secondary bile acid–dependent manner [42] In the allo-HSCT population, Lachnospiraceae may confer benefits to clinical end points other than CDI. In a previous report, Taur et al [43] noted that Lachnospiraceae were associated with reduced transplant-related mortality. *Blautia,* a genus within Lachnospiraceae, was observed to be associated with protection against GVHD [44].

Although each of these taxonomic groups was associated with protection against CDI, we also saw that cocolonization was frequent, so the precise contribution of each bacterial taxon to protection against CDI remains unclear. It could be that all 3 confer protection individually and independently, but it is also possible that the causal protective link exists only with 1 group (with the other 2 as merely associated biomarkers).

It was interesting to note that microbial diversity was not a significant predictor of CDI in this study. This suggests that the presence of the 3 bacterial groups is not merely a marker for diversity or presence of a different member outside of the groups and that specific community composition can be a better predictor of CDI than overall diversity. In contrast to this finding, prior microbiome studies have observed decreased diversity in association with CDI [16, 17, 20, 22]; however, in those studies, fecal samples were collected and examined at or after CDI diagnosis, making it difficult to separate the effects of the disease process and antibiotic treatment from factors that confer protection before development of CDI.

In a prior study, Kinnebrew et al [7] examined CDI during early allo-HCT, before engraftment. That study found CDI to be frequent but relatively mild and difficult to distinguish from other causes of diarrhea, such as toxicity related to conditioning; also pre-engraftment CDI was not associated with subsequent postengraftment CDI. In the current study, we focused

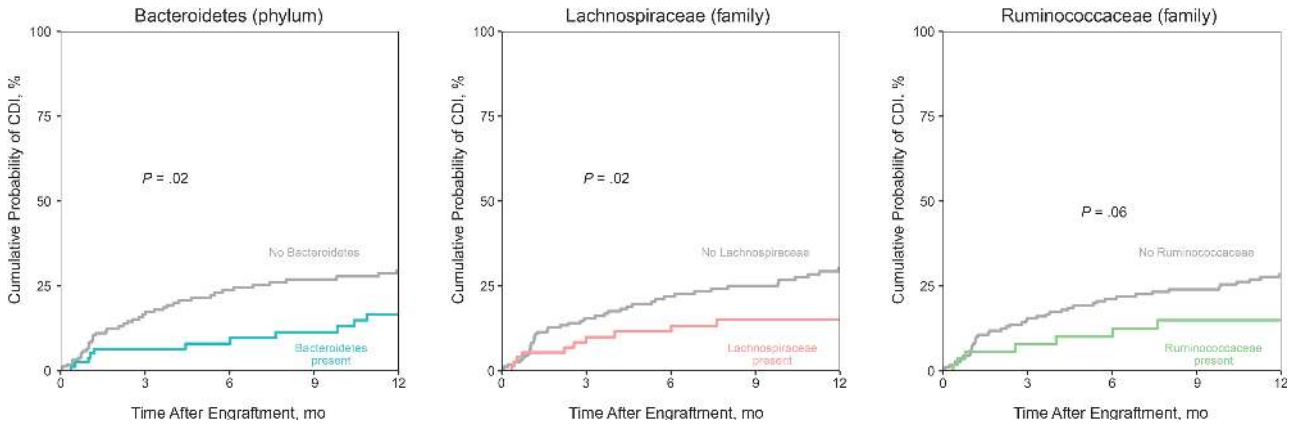

Figure 2. Impact of colonization by protective bacterial taxa on risk of subsequent *Clostridium difficile* infection (CDI). Kaplan-Meier estimates of CDI are plotted for colonization by each of the 3 protective taxonomic groups. Presence or absence within the microbiome was defined as a relative abundance of ≥0.001 in the postengraftment sample. (*P* values determined with log-rank test).

on postengraftment CDI, in a separate cohort of patients. We again found that pre-engraftment CDI was not correlated with postengraftment CDI. Furthermore, the clinical risk factor profiles also differed between pre- and postengraftment CDI, suggesting that these entities are distinct. In another analysis, we also reexamined the impact of CDI on subsequent GVHD analysis and again found no association, similar to prior findings [7].

In conclusion, our study shows that intestinal colonization with bacteria from 3 distinct bacterial taxa, Bacteroidetes, Lachnospiraceae, and Ruminococcaceae, is associated with protection against subsequent CDI. We made these observations after engraftment in recipients of allo-HSCT, a population at high risk for CDI, and found that these protective effects were independent of other clinical risk factors. Currently, the mechanism of benefit conferred by these groups remains unresolved. Given these observations, further study of these beneficial bacteria should be undertaken to define more specifically how these microbial elements work to provide protection. A more sophisticated understanding will help us devise

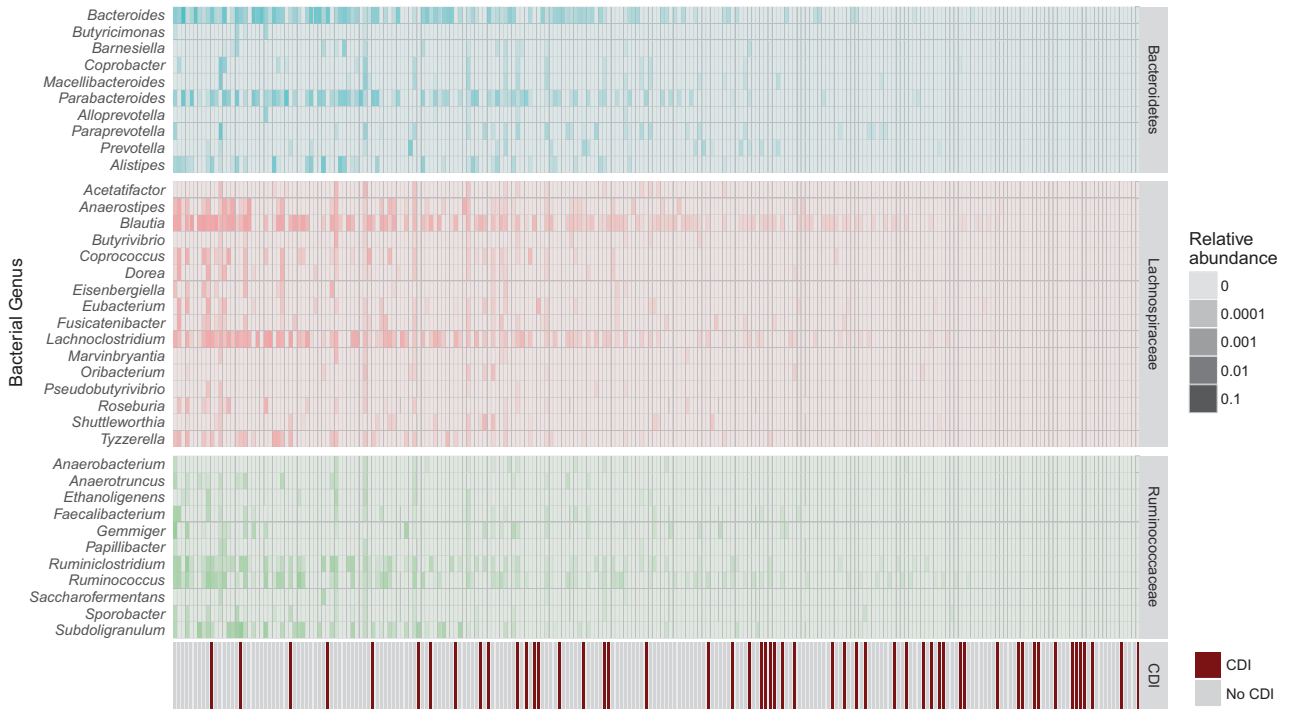

Figure 3. Microbial composition breakdown within the protective groups, by genus level. A heat map shows relative abundances of bacteria at the genus level (*rows*) for each subject (*columns*); shading represents relative abundance. Bottom strip shows whether or not *Clostridium difficile* infection (CDI) developed.

intelligent strategies to prevent CDI, either by preserving a stable microbiota through careful selection of antibiotics that avoid disruption of these valuable members or by repairing disrupted communities through targeted replenishment of specific groups.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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