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TRANSPLANTATION: RESEARCH ARTICLE

Intestinal mycobiota composition and changes in children with thalassemia who underwent allogeneic hematopoietic stem cell transplantation

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Abstract

Background: Allogeneic hematopoietic stem cell transplantation (HSCT) alters the diversity of the intestinal bacterial microbiota. This study aimed to evaluate human mycobiota composition pre-HSCT and post-HSCT in children with thalassemia.

Method: Ten children with thalassemia undergoing allogeneic HSCT were enrolled. The stool samples were collected before the transplantation regimen, before the transplant day, and +15, +30 days, and three months after transplantation. Stool samples were also collected from the donor and the patient's caregivers. Gut mycobiota composition was evaluated with metagenomic analysis.

Results: Pretransplant mycobiota of children with thalassemia (the predominant genus was *Saccharomyces*, 64.1%) has been shown to approximate the diverse mycobiota compositions of healthy adult donors but becomes altered (lower diversity) following transplant procedures. Three months after HSCT, phyla Ascomycota and Basidiomycota were 83.4% and 15.6%, respectively. The predominant species were *Saccaharomyces_uc* and *Saccharomyces cerevisiae* (phylum Ascomycota); we also observed *Malassezia restricta* and *Malassezia globosa* (phylum Basidiomycota) (~13%). On day 90 after HSCT, we observed 65.3% *M. restricta* and 18.4% *M. globosa* predominance at the species level in a four-year-old boy with acute graft-versus-host disease (GVHD) (skin and gut involvement) 19 days after transplantation included.

Conclusion: The mycobiota composition of children with thalassemia altered after HSCT. We observed *Malassezia* predominance in a child with GVHD. Further studies in children with GVHD will identify this situation.

KEYWORDS
allogeneic hematopoietic stem cell transplantation, graft-versus-host disease, GVHD, HSCT, microbiota, mycobiota, thalassemia

Abbreviations: GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; VOD, veno-occlusive disease.

1 | INTRODUCTION

The human gut has a complex microbial ecosystem, including bacteria, fungi, archaea, and viruses, and is a key player in host health.[1–4] Mycobiota is the community of fungi (0.01%-0.1% of the human gut microbiome) that lives in symbiosis with a given host.[5,6] The colonization of mycobiota varies according to different body sites (lungs, vaginal tract, urinary tract, oral cavity, and intestines, as well as on the skin, and in breast milk).[7–10] In stools of healthy individuals, *Candida*, *Penicillium*, *Wallemia*, *Cladosporium*, and *Saccharomyces* are reported to be the most prevalent genera.[4,11] Similar to bacteria, fungi can also primo-colonize the intestine at birth and during breastfeeding; later, they can be affected by consumed food, the respiratory tract, or contact between mouth and skin.[3,10,12,13] In addition, several factors including the host's age, gender, diet, host genetics and immunity, geographical environment, medication such as antibiotics, and the bacterial microbiome through interkingdom interactions determine gut mycobiota composition.[2,14] The role of fungal gut microbiota in host homeostasis and several physiopathologic settings is now clearly identified, and findings claim that the gut mycobiota can influence the host immune system.[1,3,4,9] Dysbiotic states of the intestinal mycobiota are characterized by overgrowth (domination) of specific fungal taxa and loss of diversity, and accumulating evidence shows that many diseases are inextricably linked to mycobiota.[7,15]

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative modality for both treating high-risk hematological malignancies and nonmalignant conditions, such as thalassemia.[16] However, it is believed that HSCT leads to dysbiosis and disruption of intestinal homeostasis due to conditioning regimen, use of broad-spectrum antibiotics, alterations in nutrition, intestinal inflammation, and donor cell–derived immune reconstitution. In recent years, an increasing body of evidence indicates that the role of the deviation of the posttransplant microbiota and their metabolites is closely associated with transplant outcomes, including infectious complications, graft-versus-host disease (GVHD), disease relapse, and that mortality after allo-HSCT has been increasing.[17,18]

Previous studies have mainly evaluated gut bacterial microbiota compositions, and Anderman et al.[18] proposed the next steps to investigate the microbiome-host relationship in allo-HSCT and highlighted the potential role of the mycobiome in HSCT outcomes. However, there are no data on the composition of the human mycobiome in patients undergoing allo-HSCT and no data for patients with thalassemia with or without undergoing HSCT. High-throughput sequencing for the gastrointestinal mycobiome has become the decisive step in evaluating fungal symbiosis in the gut.[19] We used next-generation sequence analysis to characterize intestinal mycobiota compositions in a pilot prospective cohort of allo-HSCT pediatric recipients with thalassemia, donors, and caregivers.

2 | MATERIALS AND METHODS

Ten patients with thalassemia major underwent allo-HSCT with busulfan-based conditioning regimens at Hacettepe University Faculty of Medicine and Ankara Children's Hematology-Oncology Hospital. To analyze patients with similar risk factors, class II patients (the presence of one or two of "hepatomegaly or portal fibrosis or chelation history (regular/irregular)") were included in the study.[20] The demographic data and transplantation characteristics of the children with thalassemia were prospectively collected. Table 1 shows the pretransplant and transplantation characteristics of the patients.

2.1 | Donor characteristics and sources of stem cells

The donors' median age was 18 years (range, 6-47.3); three donors (30%) were males. The donors of six cases were HLA-matched siblings (Table 1). The source of stem cells was bone marrow in eight patients. All patients received unmanipulated stem cells.

2.2 | Conditioning regimen and graft-versus-host disease prophylaxis

All patients received busulfan, cyclophosphamide, and thiotepa as conditioning regimen (Table 1). All patients received cyclosporine A and short-course methotrexate for prophylaxis. Demographic data of patients and their donors, HSCT data as the number of stem cells given to patients, neutrophil and platelet recovery times, transplantation-related complications (acute and chronic GVHD, infections, veno-occlusive disease [VOD], engraftment syndrome), graft failure status, and survival were noted.

2.3 | Supportive treatment

All patients were hospitalized in HEPA-filtered single rooms. All patients received antiviral, antibacterial, and antifungal prophylaxis (acyclovir, ciprofloxacin, metronidazole, trimethoprim-sulfamethoxazole, and fluconazole, respectively). Cytomegalovirus infection was routinely monitored with real-time PCR assays. At the first documentation of fever ($T_{max} > 38°C$) broad-spectrum antibiotics were initiated. Glutamine, ursodeoxycholic acid, low-molecular-weight heparin, and vitamin E were started for VOD prophylaxis.

2.4 | Definitions

Neutrophil engraftment was specified as the first day of three consecutive days with a neutrophil count over 0.5×10^9/L. Platelet

TABLE 1 Pretransplant and transplantation characteristics of the patients

Age at transplantation [median (range)] (years)	7.6 (2.9-14.1)
Gender (male/female)	7/3
Pretransplant ferritin level [median (range)] (mg/dL)	1159 (523-2611)
Donor characteristics HLA-matched sibling HLA-matched other relatives HLA 1 antigen–mismatched relative	6 3 1
Gender (donor/recipient) M/M F/M F/F	3 4 3
Source of stem cells Bone marrow Peripheral blood	8 2
Cell dose ($CD34^+$ cells/kg), [median (range)]	3.5×10^6/kg (1-8.3)
Conditioning regimen	Busulfan (85-95 mg/L × h AUC; initial doses were 0.8 to 1.2 mg/kg/dose qid, according to weight of the patient for four days) Cyclophosphamide (50 mg/kg/day for four days) Thiotepa (10 mg/kg/day for one day)
GVHD prophylaxis	Cyclosporine A Short-course (+1, +3, +6) methotrexate

engraftment was described as a platelet count over 20×10^9/L without platelet transfusion for at least seven days. Complete donor chimerism was defined as ≥95% donor cells in peripheral blood; however, 10%-95% donor cells were accepted as mixed chimerism. Acute and chronic GVHD were classified according to the Seattle Criteria.[21,22] VOD was defined and grouped according to the Seattle Criteria.[23]

2.5 | Stool samples

Stool samples were collected before the transplantation regimen (1), within 72 hours before the transplant day (2), +15 (3), +30 days (4), and three months after transplantation (5). All participants provided a minimum of 5 mL of fresh stool. All samples were collected in a sterile Falcon tube and were quickly transferred to −80°C for upright storage until DNA extraction.

Stool samples were collected once before the transplant from the donor (6) and on the day of transplantation from the patient's caregivers (7) and stored similarly. The QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to extract total DNA of fecal samples. Extracted samples were shipped on dry ice for further metagenomic analysis. DNA was quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA) and 1 ng of DNA from each sample, with a concentration of 0.2 ng/μL, was used for shot gun library preparation with the Nextera DNA Flex Library Prep kit (Illumina, Inc., San Diego, CA, USA) following the manufacturers' protocol. Sequencing was carried out on a NextSeq 500 sequencing system (Illumina) with 2 × 150-bp paired-end chemistry.

2.6 | Sequence bioinformatics analysis

For analysis of the raw output files in .fastq format, an initial module for preprocessing was applied. It included the trimming and quality control using prinseq-lite-0.20.4[24] with the following parameters: minimum number of allowed ambiguous bases (N) (NS-MAX_P = 1), minimal allowed length of the reads (MIN_LEN = 50), minimal average quality base (MIN_QUAL_MEAN = 25), quality threshold score for quality trimming from the 3′ end (TRIM_QUAL_RIGHT = 25), type of quality score calculation to use (TRIM_QUAL_TYPE = min), window size for quality trimming (TRIM_QUAL_WINDOW = 15), step size used to move the sliding window (TRIM_QUAL_STEP = 15), and the threshold value used to filter sequences by sequence complexity (LC_THRESHOLD = 0.70). Next, the forward and reverse reads were joined with FLASH-1.2.11, and reads of human origin were mapped against the reference human genome database (GRCh38.p13) using bowtie 2-2.3.5.1 in very sensitive mode, in order to map and remove more potential contaminant human reads.[25,26] For the unaligned reads, taxonomic annotation was implemented by the classifier Kaiju v1.7.0 with the following parameters: MIN-MATCH_LENGHT = 20, MAX_MISMATCHES = 2, and MAX-EVALUE = 1e−10, and using its database "kaiju-db-nr-euk.fmi."[27] Using the lowest common

ancestor strategy, the free statistical package R selected the best hits at the lowest supported taxonomic level and was used for counting the taxa abundance for each sample and creating a taxonomic abundance table for all samples.[28] This matrix served for analyses of composition, abundance, and diversity analyses using scripts from the QIIME pipeline v1.9.0.[29] For this study, only taxa taxonomically assigned as fungi were selected for downstream analyses. For each fungal taxon, composition—measured as presence/absence in the set of samples—was analyzed at different taxonomic ranks (from the hierarchically more global phylum level, to the more specific species level, with intermediate family and genus levels) to detect their distribution across samples. Statistics such as mean, standard deviation, median, minimum, and maximum were calculated. Additionally, the relative abundance of the different taxa was computed all samples and for groups of them to determine which fungi were more abundant.

As for the diversity within samples, an analysis was carried out with 1,000 replicates of randomly chosen subsets of 250 reads per sample with replacement, and the Chao1 richness estimator and Shannon diversity index were calculated. Boxplots were created using the R statistical package and significant differences were determined using two-tailed *t* tests. The diversity between samples was analyzed using Bray-Curtis dissimilarity index matrices obtained with the QIIME pipeline for principal coordinates analysis. The metagenome data sets from this study are available in the EBI Short Read Archive under the study accession number PRJEB46003, with accession numbers (ERS6630850-ERS6630911).

3 | RESULTS

We evaluated 48 fecal samples from 10 children (collected five times for 10 patients; two fecal samples are discarded for technical reasons). Sixteen stool samples were collected once before the transplant from the donor and on the day of transplantation from the patient's caregivers. The donor and patient's caregivers were the same person for four patients. There were 148 fungal taxa observations, the total fungal read count was 327 064, and the table density (fraction of nonzero values) was 0.15. Fungal read counts per sample ranged between a minimum of four and a maximum of 66 293 (mean: 4037; standard deviation: 10 026; median: 1847). We detected at least one fungal read from fecal samples. The rate of contamination of human sequences ranged between 0 % and 3.11%, with an average of 0.14%. No significant difference among subjects was observed in the fungal community, whereas there are some differences in the same subject over time. Baseline mycobiota composition does not differ between HSCT recipients and healthy donors (for Shannon Index, Chao1 index), and principal coordinates analysis was generated from unweighted UniFrac distance matrices in QIIME). The Shannon index was low, as expected considering previous gut mycobiota studies, and similar between patients (before transplantation regimen) and donors/caregivers, after HSCT, but significantly lower at days 15 and 30 after transplantation.

3.1 | Gut mycobiota composition in children with thalassemia before transplant regimen

The fungal taxa were ascribed as phylum Ascomycota (67%), followed by phylum Mucoromycota (32%). The gut mycobiota composition of the children before the transplant regimen was similar to that of the donors (Ascomycota 63.8% and Mucoromycota 35.3%). Before the transplant regimen, the most abundant taxa at the family level in children was Saccharomycetaceae (65.2%), followed by Mucoromycota_uc and Mucoraceae, similar to donor mycobiota. At the genus level, it was *Saccharomyces* (64.1%), followed by *Mucoromycota_uc* and *Mucor* (Figure 1).

3.2 | Gut mycobiota composition in children with thalassemia before transplant day and after transplantation

Gut mycobiota composition of children with thalassemia before transplant day and on days 15 and 30 after HSCT was similar. At the phylum level, approximately all reads were Ascomycota (98.4%, 96.9%, and 99.1%, respectively). Three months after HSCT, we observed that Ascomycota was 83.4% and Basidiomycota 15.6%. Upon further evaluation, we found that this Basidiomycota represented 13.8% of *Malassezia* at the genus level. At the species level, although the predominant taxa were *Saccaharomyces_uc* and *Saccharomyces cerevisiae*, we also observed *Malassezia restricta* and *Malassezia globosa* (approximately 13%) (Figures 1 and 2). When we compared gut mycobiota composition of children with thalassemia before transplant day, and three months after HSCT, *Rhizophagus sp. MUCL 43196* and *Mucor ambigus* were significantly higher at before transplant day ($P < 0.05$ for both) and significantly lower three months after HSCT. *Rhizophagus sp. MUCL 43196* and *Mucor ambigus* were also significantly lower in children with thalassemia three months after HSCT compared with donor's samples ($P < 0.01$). Fifteen days and 30 days after HSCT, *Aspergillus sclerotionger* were significantly higher compared with before transplant day ($P < 0.001$). Three months after HSCT, *Aspergillus sclerotionger and Saitoella complicata* were significantly higher compared with before transplant day in children ($P < 0.05$ for both).

When we evaluated the gut mycobiota composition of each child at day 90 after HSCT for *Malassezia* dominance, we observed, at the species level, 65.3% *Malassezia restricta* and 18.4% *Malassezia globosa* in patient 4. There was no *Malassezia* species abundance in other fecal samples for patient 4, and *M. restricta* was 6.4% of the donor's gut mycobiota composition. We learned from the medical records that patient 4 was a four-year-old boy with grade 2 skin and gut GVHD 19 days after the engraftment, treated with methylprednisolone (Figure 2). In our three-month follow-up, we observed GVHD in three cases and VOD in two cases. We did not observe gut mycobiota alterations in other children aside from patient 4 (Figures 2 and 3).

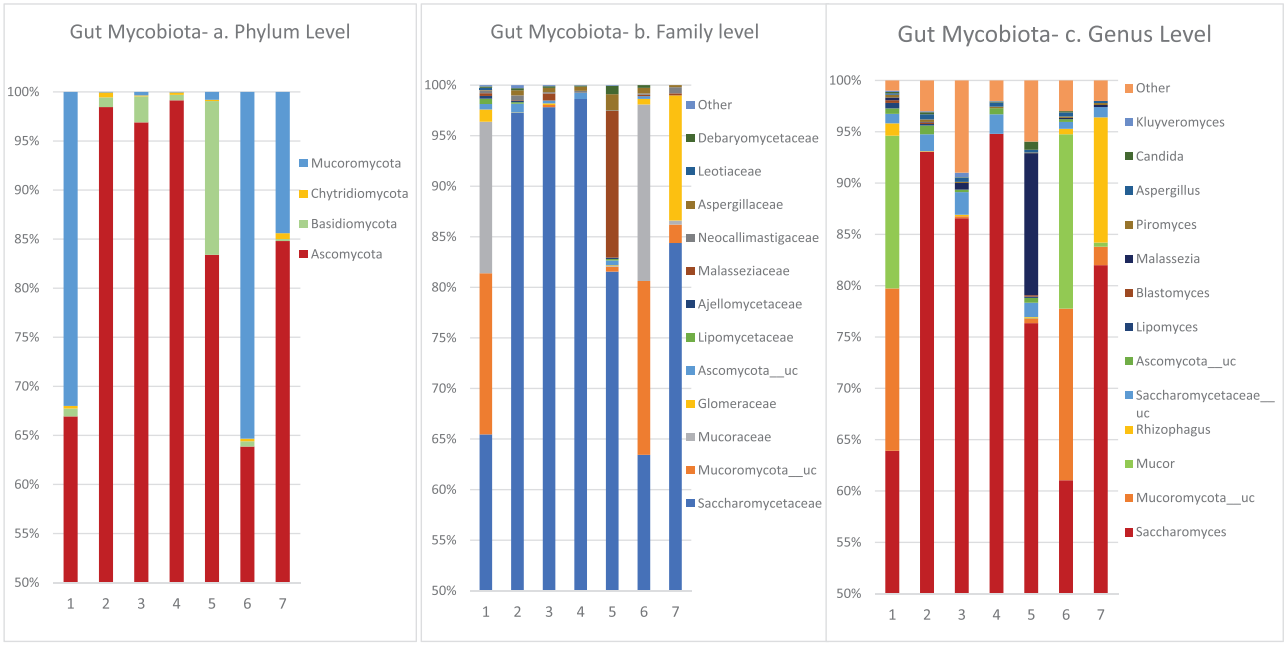

FIGURE 1 Gut mycobiota composition and abundance of children with thalassemia undergoing allo-HSCT, donors and caregivers, at phylum level (a), family (b), and genus level (c). *Stool samples were collected before the transplantation regimen (1), within 72 hours before the transplant day (2), +15 (3), +30 days (4), and three months after transplantation (5), from the donor (6) and from the patient's caregivers (7)

3.3 | Gut mycobiota composition of healthy adult donors/caregivers

Among 15 previously healthy adults, we found 51 different genera. *Saccharomyces*, *Mucor*, and *Rhizophagus* were detected in 87.5% of all samples. At the phylum level, Ascomycota was the predominant taxon (ubiquitously present across the samples), followed by Mucoromycota. At the genus level, *Saccharomyces* was predominant among donors and caregivers, followed by *Mucoromycota_uc* and *Mucor* in donors and *Rhizophagus* in caregivers (Figure 4).

4 | DISCUSSION

This is the first longitudinal study in children with thalassemia undergoing allo-HSCT. We showed that pretransplant mycobiota of children with thalassemia approximated the diverse mycobiota compositions of healthy adult donors but became altered (lower diversity) following transplant procedures. We also observed long-term characterization (approximately three months) of individual variation in the mycobiota composition and recovery after allo-HSCT.

Mycobiome composition and diversity are influenced by age, gender, nutrition, diurnal cycles, host genetics, diabetes and obesity, anorexia nervosa, variances among body sites, and geographical regions.[30,31] In our study, the population was children with thalassemia; there are no studies of intestinal mycobiota composition among children with thalassemia. An experimental iron-overloaded study in thalassemia mice revealed high fecal gram-negative bacteria (*Bacteroides spp.*) and increased gut leakage, endotoxemia, and serum inflammatory cytokines.[32] Therefore, iron overload might result in intestinal-permeability disturbance, gut translocation of microorganism or microorganism particles, systemic inflammation and then sepsis. In our study, the most abundant taxa at the family level in children with thalassemia was Saccharomycetaceae (65.2%), followed by Mucoromycota_uc and Mucoraceae, similar to donor mycobiota. We did not observe any difference in mycobiota between children with thalassemia and donors/caregivers. Schloss et al.[33] analyzed gut microbiome (bacterial) profiles between family members and unrelated subjects and found similar profiles among people sharing the same life history and environment.

Microbiota composition has been previously performed in allo-HSCT patients, mainly for bacterial composition. Changes in bacterial microbiota composition occur in many patients undergoing allo-HSCT, likely due to conditioning regimens involving chemotherapy and radiotherapy, prophylactic and therapeutic antibiotics, and major dietary changes during the peritransplant period.[34] Morjaria et al.[35] showed prominent shifts in bacterial microbiota composition from stem cell infusion to restructuring healthy immune cells in 272 longitudinal stool samples from 18 patients undergoing HSCT. There are no published data on gut mycobiota composition after HSCT in children. Although the evidence suggests that fungi are strongly associated with human health and disease, they have often been neglected.[36]

Our study showed similar gut mycobiota composition in children with thalassemia before transplant day and on days 15 and 30 after HSCT. At the phylum level, approximately all reads were *Ascomycota* (97%-99%). Three months after HSCT, Ascomycota was

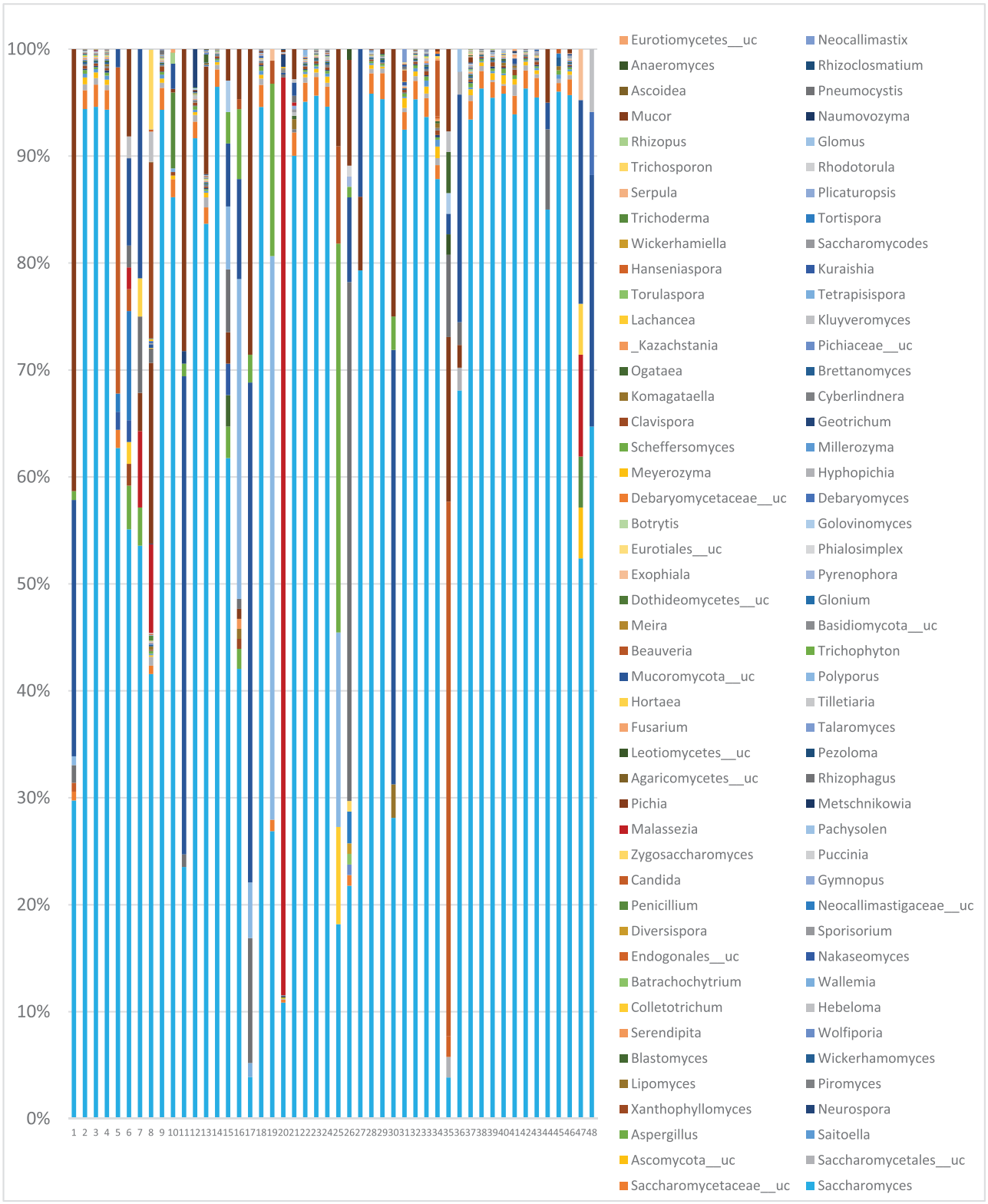

FIGURE 2 Gut mycobiota composition and abundance of each child with thalassemia undergoing allo-HSCT, at genus level in different five time points. *Patient 1: 1-5, patient 2: 6-10, patient 3: 11-15, patient 4: 16-20, patient 5: 21-24; patient 6: 25-29, patient 7: 29-34, patient 8: 35-38, patient 9: 39-43, patient 10: 44-48

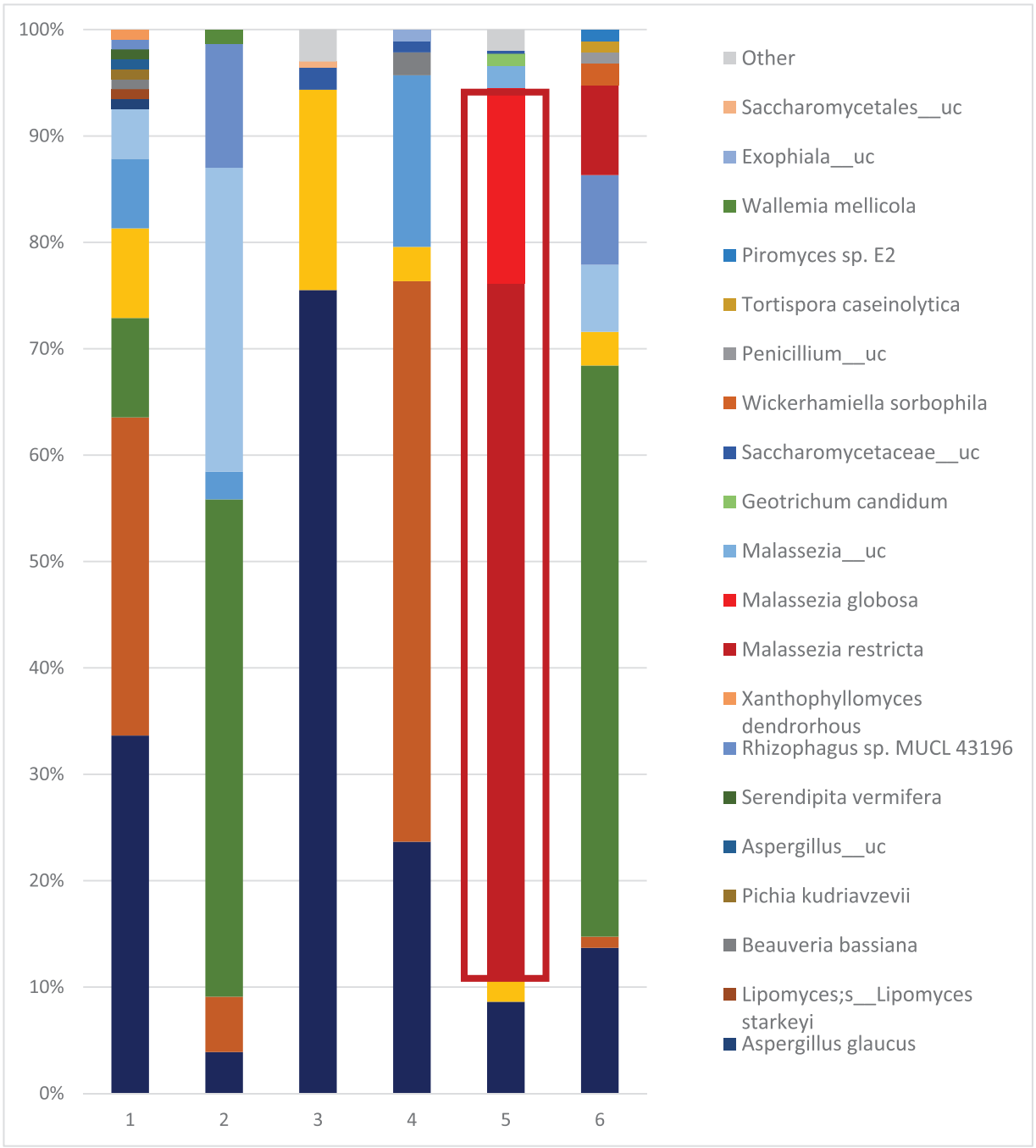

FIGURE 3 *Malassezia* dominance in gut mycobiota composition at day 90 after allo-HSCT. *Stool samples were collected before the transplantation regimen (1), within 72 hours before the transplant day (2), +15 (3), +30 days (4), and three months after transplantation (5), from the donor (6)

predominant (83.4%), and we also observed Basidiomycota (*Malassezia* at the genus level). At the species level, the predominant taxa were *Saccaharomyces_uc* and *S. cerevisiae*; lower percentages of *M. restricta* and *M. globosa* were also detected. In our study, in addition to a standard conditioning regimen, antiviral, antibacterial, and antifungal prophylaxis were commenced in all patients (acyclovir, ciprofloxacin, metronidazole trimethoprim-sulfamethoxazole, and fluconazole, respectively). Patients also received empirical or culture-driven antibiotics for the treatment of febrile neutropenia. Although broad-spectrum antibiotics have caused a dramatic improvement in infection-related mortality, antibiotics result in considerable microbiota disruption. Morjaria et al.[35] showed that piperacillin-tazobactam caused the most severe decrease in obligate anaerobes. Patients who started antibiotics before transplantation revealed significantly more microbiome disruption than those who began antibiotics on or following day 0 or who did not take antibiotics.[19] Suppression of the bacterial microbiota during treatment with antibiotics has been shown to result in dysbiosis and the overgrowth of the gut mycobiota likely due

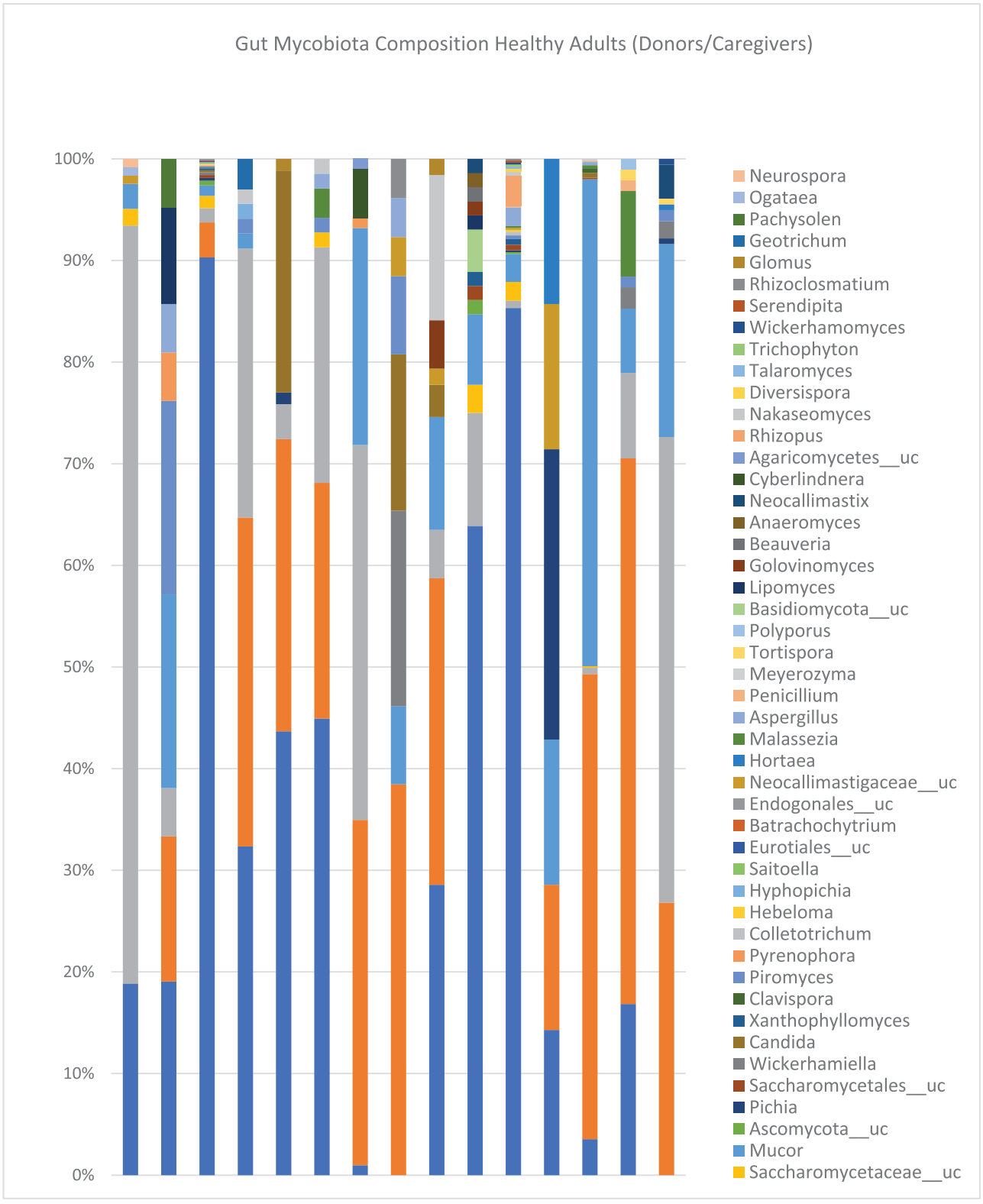

FIGURE 4 Gut mycobiota composition of healthy adults (donors and caregivers) at genus level

to reduced ecological competition.[37,38] Therefore, commensal fungi, including *S. cerevisiae* or *C. albicans*, can replace intestinal bacterial flora following antibiotic therapy.[2] In addition to antimicrobial agents, the microbiota is also affected by chemotherapeutics. Cyclophosphamide, which is frequently given for both treatment, and conditioning regimen of allo-HSCT can alter the microbiota composition. However, a causal relationship between conditioning regimens and the diversity of intestinal bacteria cannot be asserted due to other confounding factors. Nevertheless, antifungal drugs might have limited activity when attempting to target specific gut mycobiota members.[6] Although prolonged delivery of antifungal drugs can partially overcome this barrier, undesired outcomes on commensal microbiota and inflammatory disease outcomes might occur. The HSCT patients received antifungal prophylaxis, which also affects gut mycobiota composition. Previous studies on children and adults undergoing HSCT have been performed on patients with hematological malignancies. These children have received long-term chemotherapy and antimicrobial prophylaxis/treatment, not similar to our cohort of children with thalassemia. Because dietary factors affect the gastrointestinal mycobiota throughout life,[39] our study population received a neutropenic diet after the procedure. Dietary and nutritional differences in cohorts could elucidate some of the interindividual variabilities.

Acute GVHD (aGVHD) is found in about 30%-50% of patients and usually develops within 100 days after transplantation; the skin, lungs, gastrointestinal tract, and liver are the main target organs. Low diversity of gut microbiota in the early phase after bone marrow transplantation increases the risk of GVHD and mortality.[40,41] Our study observed GVHD in three children, but there are limited data on the relationship between the gut mycobiome and GVHD. In the only study in this area, Van der Velden et al.[42] reported that colonization with *Candida spp*. developed significantly more grade II-IV acute GVHD and more gastrointestinal GVHD compared with noncolonized patients in retrospective analysis on 153 adult patients with HSCT. Gastrointestinal GVHD incidence was lower in patients receiving fluconazole compared with those who did not receive fluconazole. This indicates a role for the mycobiome in the pathogenesis of GVHD and suggests that altering the mycobiome through antifungal drugs can improve gastrointestinal GVHD.

Perturbation of innate mucosal immunity due to cytotoxic therapy or gene polymorphisms, with changes in the abundance and content of gastrointestinal microbiota (dysbiosis), can exacerbate inflammation of the intestinal mucosal barriers and stimulate alloreactive T-cell responses, inducing GVHD. On the other hand, GVHD triggers gut damage and alters the host response such as T cell–mediated Paneth cell damage and decreased α-defensin release. These changes are followed by dysbiosis and increased risk for bacteremia, fungemia, and continuation of GVHD.[42] We did not observe *Candida* predominance in our cohort with or without GVHD. When we evaluated the gut mycobiota composition of each child at day 90 after HSCT, we observed *Malassezia* genus predominance in patient 4; *Malassezia restricta* (65.3%) and *M. globosa* (18.4%) were prominently observed at the species level. There was no *Malassezia* species abundance in other fecal samples from patient 4. Liu et al.[43] investigated the dissimilarity in microbiota compositions between the donor and HSCT recipient for the mechanism of acute gastrointestinal GVHD and the donor's microbiota may also influence GVHD through mechanisms independent of mismatch with the recipient's microbiota. Our case was four years old and had grade 2 skin and gut GVHD 19 days after the engraftment and *M. restricta* constitutes 6.4% of the donor's (only child donor of our study) gut mycobiota composition. *Malassezia*, which is by far the most abundant component of the skin mycobiota, has been implicated in some skin diseases such as atopic dermatitis, seborrheic dermatitis, pityriasis versicolor, and psoriasis.[3,4,38,44] *Malassezia* has also been detected in significant abundance in fecal samples and may have a role in the gut health.[45] In some studies, the *Malassezia* genus has been reported to be the second most abundant genus among all human stools, reaching up to 4%, whereas *M. restricta* and *M. globosa* represent the most abundant species.[3,31,45] However, in our patient with GVHD, approximately 84% of gut mycobiota composition is *Malassezia spp*. Malassezia can be associated with human gut–related disease. Although the mechanisms by which Malassezia cells use to trigger such diseases are not yet clearly defined, the current hypothesis is that the diseases can be induced either directly by tissue invasion by fungal filaments or indirectly through yeast-induced immunological and metabolic mechanisms.[3] *Malassezia* was observed to increase globally during inflammatory bowel disease flare, and *M. restricta* was reported to be increased in the mycobiota-associated mucosa of Crohn's disease patients.[46] Our patient has skin and gut GVHD, and the predominance of *Malassezia spp*. in his gut mycobiota composition, mainly *Malassezia restricta*, might be related to GVHD (skin and gut involvement). It is unknown whether they translocate from the skin or whether these cells are primed locally in response to fungal dysbiosis. However, the other two patients with GVHD had no mycobiome alterations. Future studies that analyze the status of *Malassezia spp*. in patients with GVHD may lead to improve our knowledge and therapeutic approaches that target this specific genus. In addition, recent studies revealed that allo-HSCT is associated with major metabolomics variations (reduced production of tryptophan-derived metabolites, reduced production of plasmalogens together with the increased level of bile acids and polyunsaturated acids).[47] There are no published studies about the gut mycobiome and metabolomics in patients with GVHD.

There is no consensus on the definition of a "healthy mycobiome" due to numerous factors, such as low abundance and diversity of fungi in the gut and dynamic instability of the mycobiome in the development stages.[2] The formation of the human mycobiota begins very early in life; infants and children have higher fungal richness than adults.[8,14] There are temporal and spatial changes in the composition and distribution of gut mycobiota. It has been known that *Debaryomyces hansenii* and *Rhodotorula mucilaginosa* are the most abundant fungi from 10-day- to 3-month-old infants, whereas the composition of gut mycobiota changes after 1-2 years, and *S. cerevisiae* come to be the most abundant fungus while *Candida spp*. starts to decline.[48,49] Among adults, in terms of phyla, Ascomycota is suggested to be the most predominant phylum in the gut, followed by Zygomycota and Basidiomycota.[2] Raimondi et al.[45] found that in fecal mycobiome of healthy adults over 12 months, the biodiversity of fungal communities was lower and characterized by

greater disparity; one or two fungal genera dominated most samples. Studies on the composition of gut mycobiota identified tens of fungal genera, most of which were attributed to *Candida*, *Aspergillus*, *Cladosporium*, *Clavispora*, *Cyberlindnera*, *Malassezia*, *Debaryomyces*, *Galactomyces*, *Penicillium*, *Pichia*, *Rhodotorula*, and *Saccharomyces*.[45] Geographic residence and diet habits of the volunteers probably accounted for the differences observed. Subject age, gender, geographic locations, underlying conditions such as diabetes and obesity, together with fungal diversity, have all been examined in mycobiome studies.[50,51] In our study cohort, we first evaluated gut mycobiota composition in adults (healthy donors and caregivers) in Turkey. Among 15 previously healthy adults, Ascomycota was the predominant taxon at the phylum level (ubiquitously present across the samples), followed by Mucoromycota. At the genus level, *Saccharomyces* was predominant among donors and caregivers. Based on the present metagenomics analysis, *S. cerevisiae* was one of the most abundant and frequent species. The genus *Candida* was identified by a metagenomics study in most samples, occasionally occurring at remarkably high amounts. Contrary to Nash et al.,[31] Raimondi et al.[45] did not detect *C. albicans* similar to our study. The disparity in the composition of gut mycobiota resulted in great differences both among subjects and over time within the same subject, like children with thalassemia and adult donors/caregivers.

Although the influence of the bacterial microbiota on host physiology is relatively well defined, there is limited knowledge about interactions between mycobiota and host.[38] Evidence suggests that fungi may stimulate the host's intestinal immune system.[19] Changes in the composition of gut mycobiota have been associated with irritable bowel syndrome, inflammatory bowel disease, hepatitis B, HIV, obesity, diabetes, atherosclerosis, alcohol-induced liver disease, oropharyngeal candidiasis, colorectal carcinoma, cystic fibrosis, asthma, and neurological disorders (both psychiatric and nonpsychiatric disorders, autism spectrum disorders, Rett syndrome, bipolar disorder, schizophrenia).[2,3,7,10] Crosstalk between the gut mycobiome and the host immune system may alter the outcome of some diseases; however, crosstalk between the immune system and fungi can modulate gut bacteria and vice versa.[2]

The current study has some limitations. It is a "proof of concept study," and the sample size is small. Further validation with a large-scale and prospective study is warranted. In addition, due to the limited number of patients in our cohort, we did not evaluate age and gender effects. In this study, we observed *M. restricta* predominance in one child with GVHD, and further studies are needed for GVHD and mycobiome relationship. Also, we could not evaluate the effects of diet (neutropenic diet, total parenteral nutrition, etc.) on gut mycobiota composition due to the small sample size.

Despite accounting for a minor part of the gut, the gut mycobiome clearly plays an indisputable role in host homeostasis and disease development. Furthermore, the interkingdom interactions of diverse fungal species with other gut microbiota components (bacteria, parasites, and viruses) and how these interactions may influence humans need to be investigated further.[2] Immunological alteration by the gut microbiota during the early stages of allo-HSCT could influence later GVHD development; changing or repairing the gut microbiota could be a treatment option for GVHD. It is critical to find biomarkers with high prognostic and predictive value when discussing patient risks and choosing the best treatment options.

AUTHOR CONTRIBUTIONS

SSY, BBK, and NYO conceptualized and designed the study; BBY, TA, and NYO recruited participants; SSY, BBY, TA, MC collected samples; ECD drafted the manuscript; VPB, AM, and ECD conducted and interpreted the analyses; and all the authors critically reviewed the manuscript and agreed to the published version of the manuscript.

INSTITUTIONAL REVIEW BOARD STATEMENT

This study was approved by the Hacettepe University Faculty of Medicine Local Ethical Committee (GO 18/592-34; June 21, 2018). All procedures performed in this trial were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects/parents involved in the study.

DATA AVAILABILITY STATEMENT

The metagenome data sets from this study are available in the EBI Short Read Archive under the study accession number PRJEB46003, with accession numbers (ERS6630850-ERS6630911).

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CONFLICTS OF INTEREST

The authors have indicated they have no potential conflict of interest to disclose.

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