University of Turin

DOCTORAL SCHOOL IN LIFE AND HEALTH SCIENCES

PhD Programme in Medical Physiopathology

Gut microbiome composition changes during allogenic stem cell transplant and impact of MDR bacteria gut colonization: A single center pilot study

XXXII CYCLE

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A.A. 2018/2019

"Where there's a will there's a way".

To my family

1. Introduction

1.1 Role of microbiome in human health

Two major phyla, *Bacteroidetes and Firmicutes,* dominate the healthy gut microbiota (1-6). Approximately 1.5 kg of bacteria lives in the human intestinal tract, and anaerobes are found at 100–1,000 time that of aerobes and facultative aerobes (1-7). Even if this general profile remains constant, gut microbiota exhibits both temporal and spatial shift in species distribution. From the esophagus distally to the rectum, there is a marked difference in diversity and number of bacteria ranging from 10 per gram of contents in the esophagus and stomach to 10_{12}/gram of contents in the colon and distal gut. The large intestine constitutes over 70% of all microbes and they are generally identified by stool samples (1-9). Microbiota begins to inhabit the human intestinal tract after vaginal birth, with facoltative aerobes as earliest resident, such as *E. coli, Streptococci or Enterococcus* spp. Normal gut microbiota has several functional aspects, such as defense against pathogens, cell proliferation and differentiation, mucus secretion, and barrier function; furthermore it has anti-inflammatory and antioxidative effects and it acts as antimicrobial protection by protecting mucosal barrier function (10-15). In fact, the integrity of the gut barrier is maintained by modulation of expression of proteins required for maintenance of desmosomes at epithelial villus as well as stimulation of microbial cell wall peptidoglycan or transcription of angiogenin factor 3, implicated in the development of intestinal microvasculature. The other mechanism to supervise the overgrowth of pathogenic strains is by inducing local immunoglobulins A through activation of intestinal dendritic cells (16-19).

The composition of human microbiota is easily regulated by many environmental factors, particularly age, diet and antibiotic use (12;17). Although the initially developing microbiota is largely influenced by feeding, several factors during life modify gut microbiota. Gut microbiota changes according to age and

its composition in the elderly differs considerably between individuals, as extensively studied in the ELDERMET cohort (20). In this study, a shift in the microbiota toward a *Bacteroidetes*-predominated population in frailer older patients compared to younger individuals was observed (20). This large variance is probably due to external factors influencing the microbiota, such as diet, exercise and inactivity, and medication (29). Several ongoing studies, such as the NuAge project, will apply multi-omics analysis to determine if microbiota changes are related to or are the cause of health loss and how other physiological processes might influence these changes (21). This will establish any possible impact on aging health status, by or even modulating the microbiota composition and function, the possibility to target microbiota for interventions to promote healthier aging (20-21). A strong body of evidence has now demonstrated that the use of antibiotics has long-term effects on the ecology of gut microbiota, with a reduction of taxonomic diversity and persistence of changes in the majority of individuals (22-29) (**Figure 1**).
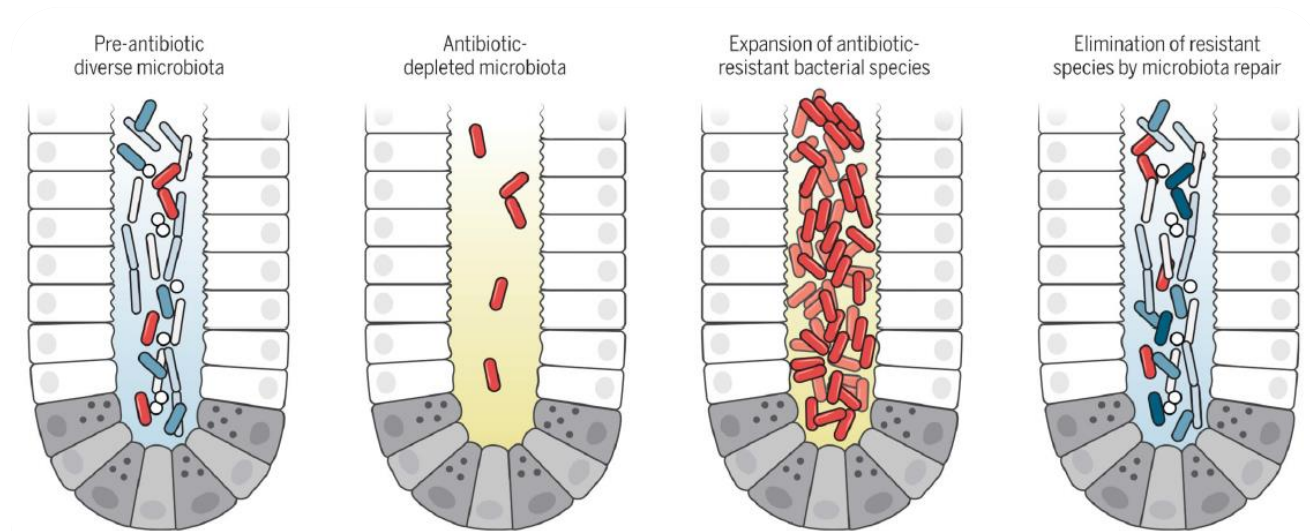

Figure 1. Impact of antibiotic therapies on gut microbiota composition

Data showed that the effect of short-term use of broad-spectrum antibiotics with predominant anaerobic coverage could last up to 2 years, with a persistent depletion of the diversity of *Bacteroides* (22-24).

Different studies have demonstrated that a short course of *H. pylori* eradication results in a dramatic reduction in the diversity of Actinobacteria that may endure over 4-year period, as well as it may lead to an overgrowth of potentially pathogenic organism including multi drug resistant (MDR) Enterobacteriaceae and yeast (30). These data were confirmed in recent studies using molecular techniques based on analysis of 16S rRNA, in which the loss of diversity and changes in different bacteria taxa have been reported in patients treated with prolonged antibiotic therapies (31). Moreover, the antibiotic-induced alterations increase susceptibility to gastrointestinal infections, such as salmonellosis and diarrhea and colitis by *C. difficile* (CDI) (31).

1.2 Gut Microbiota as Reservoir of multi drug resistant organisms (MDR)

During prolonged antibiotic treatments, gut microbiota can be affected by collateral damage, becoming a significant "reservoir" of MDR microorganisms with a "nosocomial profile" of antibiotic resistance (22; 32). Intestinal acquisition of resistant bacteria over the healthy microbiota is well known to be one of the major risk factor for the development of invasive infection by MDR bacteria, such as carbapenemase-producing *K. pneumoniae* (CP-Kp), extended spectrum beta lactamase Enterobacteriaceae (ESBL) or by Candida (32). Modulation of the gut microbiota might be an effective treatment strategy for restoring healthy function; however, the precise effect of antibiotics on the intestinal microbiota and the effect on the acquisition and the overgrowth of MDR bacteria needs to be further investigated.

In fact, MDR bacteria that are not eliminated by antibiotic treatment, can proliferate and reach higher density in the intestinal lumen (33). In CDI the causal role of a dysbiotic microbiota is well known, and it has been suggested that similar alterations may favor intestinal colonization by CP-Kp (18; 34). Intestinal colonization by CP-Kp seems to be one of the most important risk factors for the development of CP-Kp invasive infections and selective digestive tract decontamination (SDD) of colonized patients with non-

absorbed antibiotics has been suggested to reduce transmission and prevent invasive infections, although concerns are rising regarding the risk of resistance in patients who fail to respond to gut decontamination (34;35). In clinical practice, colonized patients pose an epidemiological threat to other hospitalized individuals but are also in danger of developing systemic infections with gut-colonizing microorganisms, with a higher risk in the hematological setting (36). Thus, one of the major concerns in addition to the alteration of the normal microbiota diversity, is that the use of broad spectrum antibiotics can lead to gut colonization by MDR strain of bacteria that could easily overwhelm the bloodstream (37-38). These opportunistic pathogens may translocate across the intestinal barrier or, after fecal contamination of skin and other body sites, cause infections, especially in catheters or intravenous lines (39).

The large reservoir of antibiotic-resistance genes that is present in the gut is called the "resistome" (40). The gut reservoir includes two different resistomes: the resident resistome, which is carried by the commensal bacteria, and the transitory resistome, which is carried by bacteria that are only periodically present in the gut. The latter bacteria can transfer their resistance to the commensal microbiota or become permanent microbiota residents themselves (41). Consequently, there is considerable interest in characterizing the antibiotic resistance gene reservoir of the human gut microbiota and to understand to what extent the antibiotic resistance genes can spread among different members of the gut microbiota, particularly between commensals and opportunistic pathogens (42). The exact mechanism by which specific components of the microbiota influence colonization by other pathogens is not yet fully known. Previous studies have found that particular commensal species inhibit the growth of specific MDR bacteria. This has been shown in mice, where vancomycin resistant enterococci (VRE) did not colonize microbiota containing the obligate anaerobe *Barnesiella* spp. (43-44). Caballero et al. also demonstrated that administration of a defined bacterial consortium containing *Blautia* producta and *Clostridium bolteae* prevented colonization of VRE and cleared persistent VRE in mice (45). In humans, one study reported

that four species of bacteria *Desulfovibrio, Oscillospira, Parabacteroides, and Coprococcus*, where not present in the microbiota of adult patients colonized by ESBL-producing bacteria, whereas these four species were present in the microbiomes of patients not colonized by ESBL-producing bacteria (46).

Little knowledge is available regarding the mechanisms which promote progression from colonization to infection. Martin et al. and Gorrie et al. reported that gastro intestinal carriage of *Klebsiella pneumoniae* in hospitalized patients was associated with a greater risk of developing an infection (47-48). Additionally, gut colonization by MDR bacteria has been described as an important risk factor for severe outcomes in both solid organ and hematopoietic stem cell transplantation (HSCT) (49-51).

1.3 Gut microbiota injuries in allogenic stem cell transplant (allo-HSCT)

Recent studies in mice and humans suggest important relationships between the microbiota and outcomes in allo-HSCT recipients, and several clinical trials targeting the microbiota in allo-HSCT recipients are ongoing. Associations between certain bacteria and allo-HSCT outcomes have been found repeatedly: several studies showed that the commensal microorganisms residing in the intestinal lumen, collectively termed the gut microbiota, play an important role in the pathophysiology of graft versus host diseases (GVHD) (52-54). During the course of an allo-HSCT, the diversity of the intestinal flora significantly decreases (55-56). The changes can be rapid and are thought to be due to the effects of antibiotics, intestinal inflammation and changes in diet and most patients are exposed to prophylactic and therapeutic antibiotics during neutropenia, which occurs in the first weeks after allo-HSCT (57). In a retrospective analysis of faecal specimens collected from 80 allo-HSCT recipients at the time of neutrophil engraftment — a critical time point indicating the establishment of donor haematopoiesis — a low diversity of the faecal flora was associated with significantly increased mortality (52%) compared with a high diversity of the faecal flora (8%) (58). Specifically, a greater abundance of Gammaproteobacteria (including

Enterobacteriaceae) was associated with higher mortality, whereas a greater abundance of Lachnospiraceae and Actinomycetaceae was associated with a more favourable outcome. In allo-HSCT, simultaneous use of prophylactic antibiotics and antibiotic treatment of febrile neutropenia affect microbial diversity, which in turn increases susceptibility to infections (**Figure 2**).
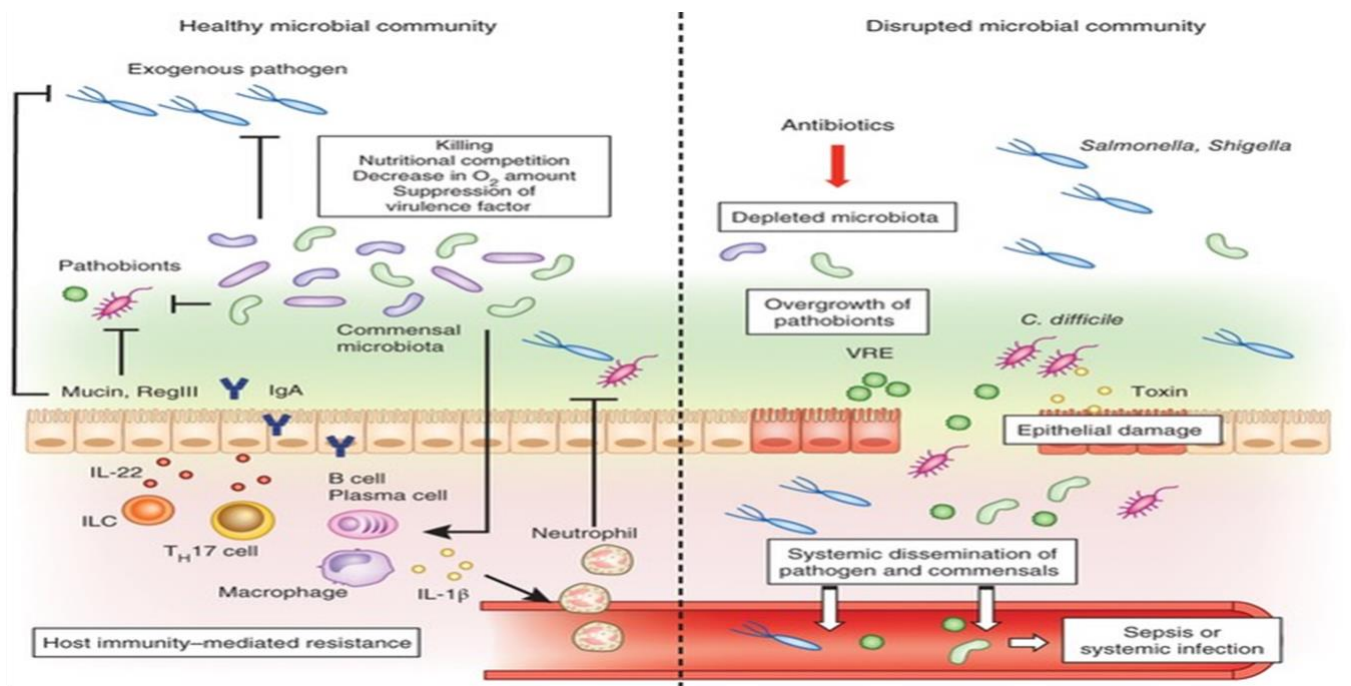

Figure 2 Impact of antibiotics on gut microbiota composition

Whereas a healthy individual usually carries on the order of 1,000 different species, near dominance of the intestinal flora, particularly by a single species of Proteobacteria, *Enterococcus* or *Streptococcus*, is frequently observed after allo-HSCT (55). In particular, exposure to the antibiotic vancomycin or metronidazole is associated with *Enterococcus* domination (19,27). Importantly, domination of *Enterococcus* or Proteobacteria was correlated with the increased risk of developing bacteremia with VRE or Gram-negative bacteria, respectively (55).

Moreover, intestinal domination by Gammaproteobacteria was identified as a significant predictor of pulmonary complications (including infections) after allo-HSCT in a single-center observational study irrespective of whether a patient received an antibiotic or had a pretransplant comorbidity (59).

These results are consistent with a hypothesis that antibiotic-induced elimination of commensal microbiota can result in expansion of pathogenic facultative bacteria in the gut consortium (60). Major microbiota injuries observed in studies of patients after allo-HSCT are summarized in **Figure 3**.
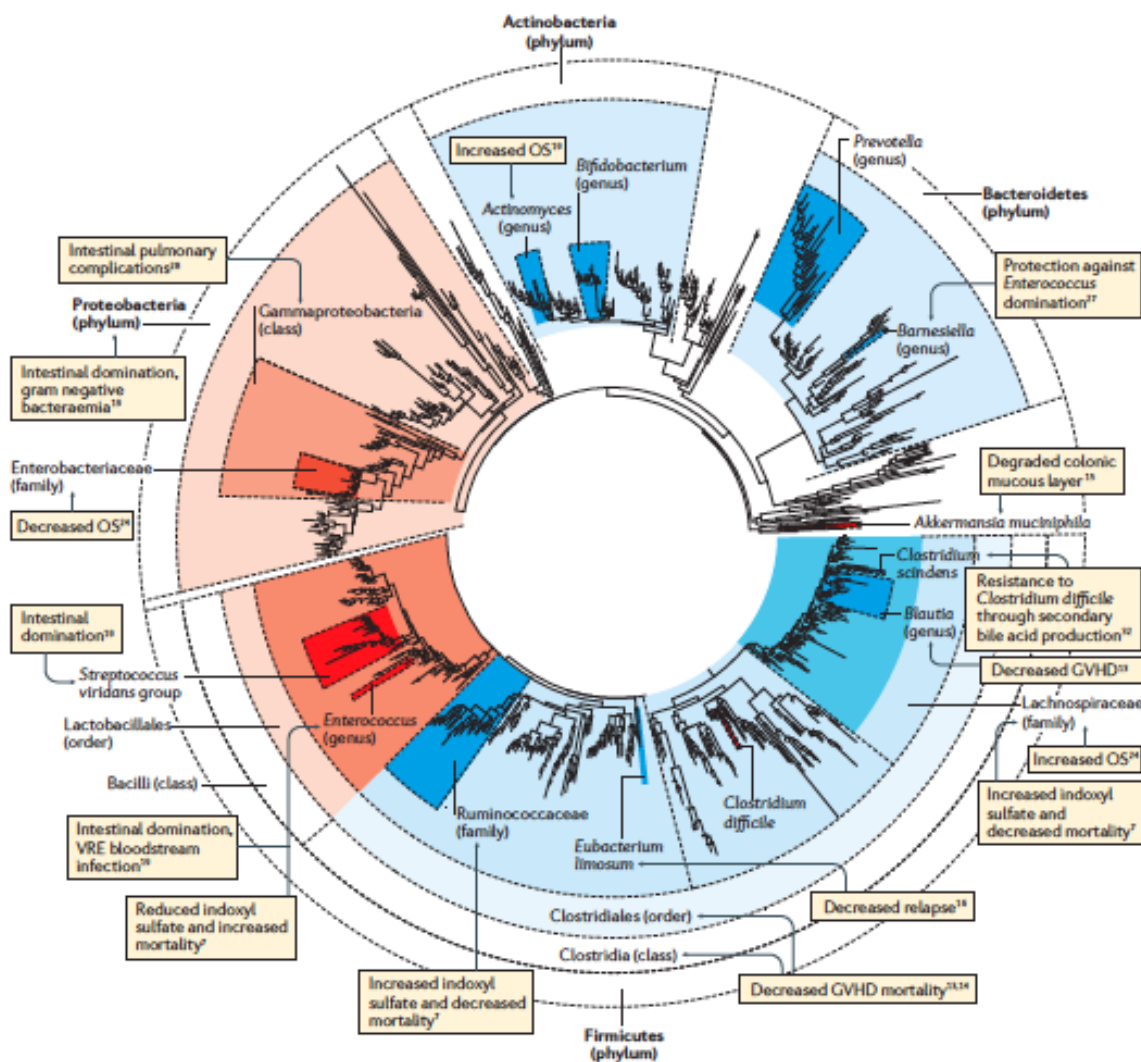

Figure 3. Studies on microbiota injuries in allo-HSCT recipients (60).

2. Aim of the study

Considering the above presented data, we investigate the changes in gut microbiota composition during allo-HSCT and we evaluated if the acquisition of MDR colonization affects the microbiota.

The primary objective of the study was to explore the changes in microbiome composition during allo-HSCT.

The secondary aims were:

1) Investigate if the acquisition of MDR colonization may affect gut microbiome diversity

2) Evaluate the impact of carbapenems treatment on gut microbiota

3) Identify a "critical time" for dysbiosis and a "time to recovery" during allo-HSCT

3. Materials & Methods

Patients recruitment and samples collection

This was a single center prospective pilot study. All patients consecutively admitted to the Haematological Unit of City of Health and Science, Molinette Hospital in Turin, Italy a tertiary referral Hospital and undergoing allo-HSCT between August 2017 to August 2019 were enrolled in the study.

Exclusion criteria: HIV infection with CD4 count less than 200 cells/mcL, ulcerative colitis, IBD, esophageal varices, and unwillingness or inability to consent.

Patients did not receive any antibiotic prophylaxis for neutropenia. Antiviral prophylaxis was administered in all patients with oral acyclovir 400 mg *bid.* Antifungal prophylaxis was administered with azoles or micafungin according to the prediction of neutropenia duration and haematological diagnosis.

Febrile neutropenia was defined as an oral temperature >38.3°C or two consecutive readings >38.0°C for 2 hours and an absolute neutrophil count <$0.5 \times 10_9$/L or a count expected to fall below $0.5 \times 10_9$/L (62). Febrile neutropenia was empirically treated with cefepime plus amikacin, with the exception of baseline colonized patients by CP-Kp, in which empirical therapy with piperacillin/tazobactam plus tigecycline was administered. GVHD prophylaxis was administered to all patients. Acute GVHD (aGVHD) was defined as clinical symptoms typically (maculopapular erythematous rash, gastrointestinal symptoms, or cholestatic hepatitis) occurring within 100 days after HCT or donor leukocyte infusion (61). Only GRADE 2-4 of aGVHD was registered.

Bloodstream infections (BSI) were defined as positive blood culture positive, either central or peripheral, with the same organism(s) isolated from two or more blood cultures drawn on separate occasions within a 48 hour period in a patient with at least one of the following signs and symptoms: fever (>38˚C), chills, rigors, hypotension. Pneumonia was defined as clinical and radiological respiratory findings with or without positive culture from bronchoalveolar lavage or sputum.

Study Procedures

For each patient enrolled in the study stool sample and rectal swab were collected every seven days from hospital admission until 28 days after allo-HSCT. Patients were followed up until 12 months after transplant and stool samples and rectal swabs were prospectively collected at day 100, 180 and 1 year after allo-HSCT (**Table 1**).

	Baseline	**Day 7**	**Day 14**	**Day 21**	**Day 28**	**Day 100**	**Day 180**	**1 year**
	Hospital admission	From HSCT	From HSCT	From HSCT	From HSCT	From HSCT	From HSCT	From HSCT
Enrollement	X							
Baseline Stool Culture	X							
Baseline Rectal swab	X							
Stool culture		X	X	X	X	X	X	X
Rectal swab		X	X	X	X	**X**	X	X
Assessment medications and illnesses	X	X	X	X	X	X	X	X
Clinical Data Collection	X	X	X	X	X	X	X	X

Microbiological samples. A proactive surveillance program to detect intestinal colonization by CRE, VRE and ESBL has been adopted in onco-hematology wards at time of admission on a weekly basis. Rectal swabs were collected using the Fecal Swab™system (Copan,Brescia, Italy) with liquid transport medium, containing 2 ml of Modified Amies Medium that was suitable for culture. Specimens were processed by automated direct plating using Wasp® instrument (Copan, Brescia, Italy). Ten μL of the FecalSwab™ liquid medium were inoculated on Brilliance CRE medium (Oxoid Ltd, Hampshire, United Kingdom), BBL Chromagar ESBL (Becton Dickinson GmbH, Heidelberg, Germany), and VRE Brilliance agar (Oxoid), for carbapenemase resistant Gram-negative (GN), extended spectrum beta-lactamases producing GN, and vancomycin resistant *Enterococcus* isolation, respectively. Inoculated plates were incubated for 18-24 hours at 35±2°C. The grown colonies were identified by MALDI-TOF analysis

(Bruker DALTONIK GmbH, Bremen, Germany) and their antimicrobial susceptibility was determined by a commercial microdilution method (Microscan Walkaway 96 Plus- Beckman Coulter). Antimicrobial susceptibility testing for Gram negative and *Enterococcus sp*. isolates were performed using Panel 83 (Microscan) which included ESBL confirmation test and Panel 33 which included vancomycin and teicoplanin testing. Carbapenemase activity was evidenced in *Enterobacterales* isolates using combination disc diffusion test "Mastdiscs® combi Carba plus" (Mast Group Ltd, UK) and confirmed by genotypic assay "Xpert Carba-R*"* on GeneXpert platform (Cepheid, Sunnyvale, CA)

The Cepheid Xpert Carba-R assay (Cepheid, Sunnyvale, CA, USA) is an automated *in vitro* diagnostic test for the qualitative detection of the *bla*KPC, *bla*NDM, *bla*VIM, *bla*OXA-48, and *bla*IMP genes. The assay is performed on the GeneXpert instrument systems and can be performed on rectal swab specimens (a validated U.S. *in vitro* diagnostics [US-IVD] and European Union *in vitro* diagnostics [EU-IVD] method) or on pure cultures of carbapenem-nonsusceptible bacterial isolates (a validated US-IVD method).

Fecal DNA extraction. Nucleic acid was extracted from the feces collected. Total DNA from the samples was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples, with an incubation of 1h at 37 °C. DNA was quantifed using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 5ng/µL.

16S rRNA amplicon target sequencing. DNA directly extracted from fecal samples was used to assess the microbiota by the amplification of the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. (63) PCR amplicons were cleaned using Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc. San Diego. CA) according to the manufacturer's instructions. After the 2nd purification step, amplicons products were quantified using a QUBIT dsDNA Assay kit (Life Technologies).

Subsequently, equal amounts of amplicons from different samples were pooled. The pooled sample was run on an Experion workstation (Biorad, Milan, Italy) for quality analysis prior to sequencing. The sample pool (4 nM) was denatured with 0.2N NaOH, diluted to 12 pM, and combined with 20% (vol/vol) denatured 12 pM PhiX, prepared according to Illumina guidelines. The sequencing was performed with a MiSeq Illumina instrument (Illumina) with V3 chemistry and generated 250bp paired-end reads according to the manufacturer's instructions.

Bioinformatics analysis. Paired-end reads were first assembled using FLASH software (64) with default parameters. Joint reads were further quality filtered (aat Phred <Q20) using QIIME 1.9.0 software (65) and short reads (<250 bp) were discarded through Prinseq (66). Chimera filtering was performed through USEARCH software version 8.1 (67). Operational Taxonomic Units (OTUs) were picked at 97% of similarity threshold by UCLUST algorithms (68) and centroids sequences of each cluster were matched to the Greengenes 16S rRNA gene database version 2013. After sequencing, a total of 2,100,009 raw reads (2×250 bp) were obtained. After joining, a total of 1,919,311 reads passed the filters applied with QIIME, with an average value of 23,406±31,535 reads/sample and a sequence length of 457 bp. The rarefaction analysis and Good's coverage, expressed as percentages, indicated that there was satisfactory coverage for all the samples (Good's coverage average, 92%). In order to avoid biases due to the different sequencing depth, OTU tables were referred to the lowest number of sequences *per sample* (4078 reads/sample). The OTU table displays the higher taxonomy resolution that was reached; when the taxonomy assignment was not able to reach the genus, family name was displayed. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict abundances of KEGG orthologs (KO) based on 16S-based structure of the microbiota (69). The KO abundance table was then collapsed at level 3 of the KEGG annotations in order to display the inferred metabolic pathways and the table was imported in gage Bioconductor package (70) in order to carry out

pathway enrichment analysis to identify biological pathways overrepresented or underrepresented between samples.

Oligotyping analysis. In order to identify sub-OTUs populations, reads assigned to genera within Ruminococcaceae and Lachnospiraceae were extracted and entropy analysis and oligotyping were carried out (71). Briefy, the extracted reads were then used to identify nucleotide positions that will explain the maximum amount of biological diversity across the samples utilizes Shannon entropy in order to identify positional variation to facilitate the identification of nucleotide positions of interest (71). To reduce the noise, each oligotype was required to appear in at least 10 samples, occur in more than 1.0% of the reads for at least one sample, represent a minimum of 500 reads in all samples combined, and have a most abundant unique sequence with a minimum abundance of 100. BLASTn was used to query the representative oligotype sequences against the NCBI nr database, and the top hit was considered for taxonomic assignment.

To increase the number of samples due to the prospective nature of the study, in some cases, T_6 and T_7 samples were represented together in the Results section.

Statistical analysis. Gut microbiota α-diversity was assessed by Chao1 index, estimating the number of diferent taxa, and by Shannon diversity index, evaluating the taxa richness and evenness calculated using the diversity function of the vegan package (72) in R environment (http://www.r-project.org). OTU table was used to build a principal-component analysis (PCA) as a function of the sampling time by using the made4 package of R. ADONIS and ANOSIM statistical test was used to detect significant differences in the overall microbial community by using the Weighted UniFrac distance matrices and the OTU table. Not-normally distributed variables were presented as median (range interquartile). The individual differences between end of the study values minus baseline values were calculated (deltas). The delta
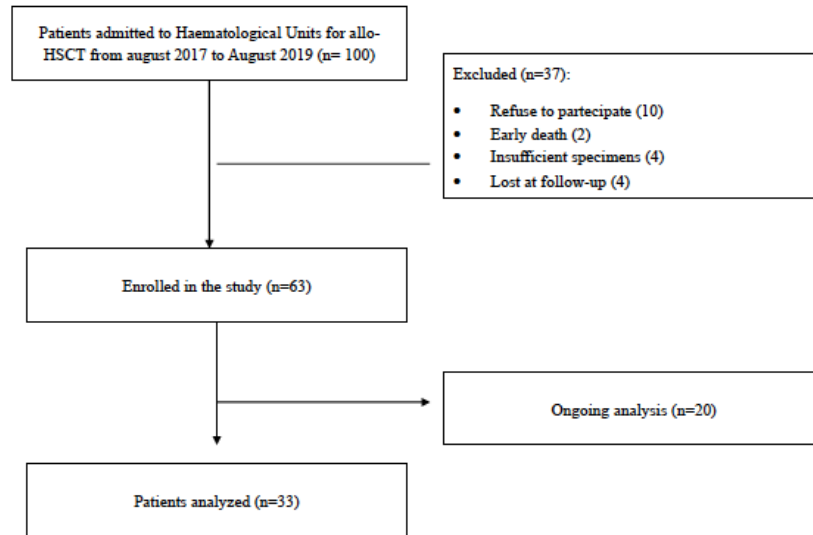
median values were reported. Within-participant differences in bacterial richness at different time points were evaluated by paired-sample t-test, or Wilcoxon matched pairs test, as appropriate. Differences between categorical variables were computed by chi-square test. Differences in gut microbiota or oligotypes were calculated by t-Student test or Mann-Whitney test. Box plots represented the interquartile range between the first and the third quartile, with the error bars showing the lowest and the highest value. The correlation plots were visualized in R using the corrplot package of R. Bonferroni's correction for multiple comparisons was applied; a P value of 0.002 or lower was considered as statistically significant.

Ethics approval and consent to participate. Each participant gave her written informed consent to participate in the study. The study protocol was approved by the Ethical Committee of the "Città della Salute e della Scienza" Hospital of Turin (Prot. N.0038691; August 2017). This study was supported by the Ethical Committee funds for no-profit studies of the "Città della Salute e della Scienza" Hospital of Turin.

4. Results

4.1. Clinical characteristics of overall population

Sixty-three patients were enrolled in the study until August 2019. Of them ten patients were excluded for early death, insufficient specimens or lost at follow-up. One hundred-thirty five samples from 33 patients were analyzed (**Diagram 1**).

Patients admitted to Haematological Units for allo-HSCT from august 2017 to August 2019 (n= 100)

Excluded (n=37):
- Refuse to partecipate (10)
- Early death (2)
- Insufficient specimens (4)
- Lost at follow-up (4)

Enrolled in the study (n=63)

Ongoing analysis (n=20)

Patients analyzed (n=33)

Clinical and demographic characteristics of enrolled patients were described in **Table 1**.

Variable	N (%) N=33
Age (years, SDV)	54 (±10)
Indication for allo-HSCT:	
-AML	11 (33)
-NHL/DLBCL	5 (15)
- RAEB	6 (18)
- others*	6 (18)
*LLC, CML, AA, MM	
Condition intensity	
- myeloablative	25 (75.7)
- RIC	8 (24.4)
Early GHVD (within 28 days)	9 (27)
Neutropenia (mean, SDV; days)	20.4 (±8.3)
Antifungal Prophylaxis	
- micafungin	7 (21)
- mold active	2 (6)
- fluconazole	22 (66.7)
Febrile neutropenia	30 (90)
Carbapenems treatment (either empiric or targeted)	14 (45)

Table 1. Clinical and demographic characteristics of allo-HSCT recipients

In the majority of cases, patients underwent allo-HSCT due to AML, and 75% of patients received a myeloablative conditioning regimen. At baseline 13 patients (39%) were colonized by MDR bacteria, mostly ESBL (27%) (**Table 2).** During allo-HSCT, four patients had a positive rectal swab for MDR and four patients lost the colonization during the hospital stay (**Table 3**).

Variable	N(%) n=33
Baseline positive rectal swab	13 (39)
- ESBL	9 (27.3)
- CRE	1 (3)
-VRE	3 (9)
Positive rectal swab during allo-HSCT	4 (12)
- ESBL	2 (6)
- CRE	1(3)
- VRE	1 (3)

Table 2. Colonization status

Baseline colonization	Colonization status during allo-HSCT	N of patients
+	+	9
+	-	4
-	-	16
-	+	4

Table 3. Changes in colonization status during allo-HSCT

Ninety percent of patients developed febrile neutropenia (90%) and were treated with a combination therapy, usually including ceftazidime plus amikacin, with the exception of colonized patients who were treated accordingly to local guidelines described in the methods section. The most frequent antibiotic administered are describe in **Figure 4**. Fourty-five percent of patients received carbapenems as empiric or targeted therapy during the hospital stay.
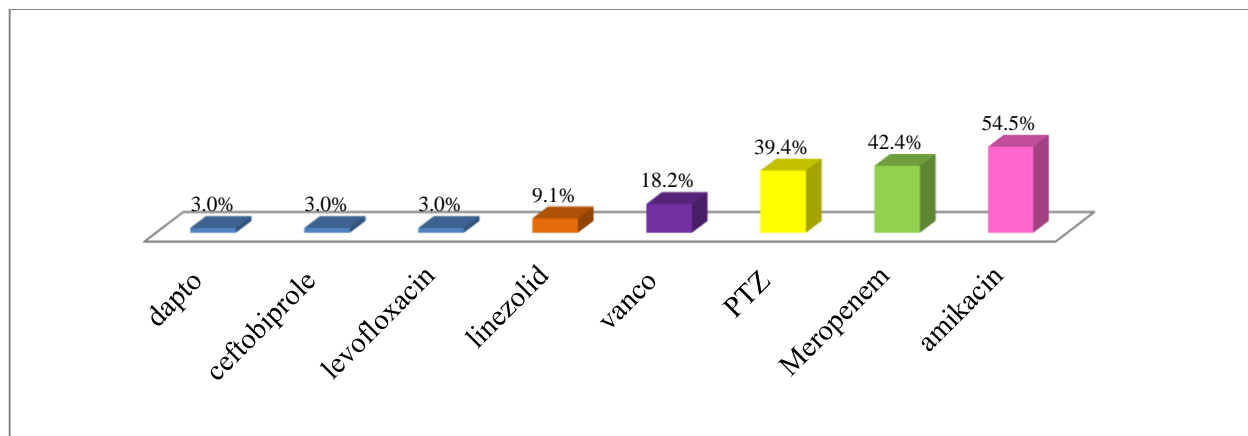
Figure 4. Antibiotics for the empiric treatment of febrile neutropenia

Episodes of infections during allo-HSCT

Eleven infections were observed during the hospital stay. In the majority of cases infections were bacteremia (BSI; 7; 63%) or pneumonia (3; 27%) (**Figure 5**). The most frequent isolates were gram positive bacteria (methicillin resistant *S. aureus* and Enterococci) causing BSI.
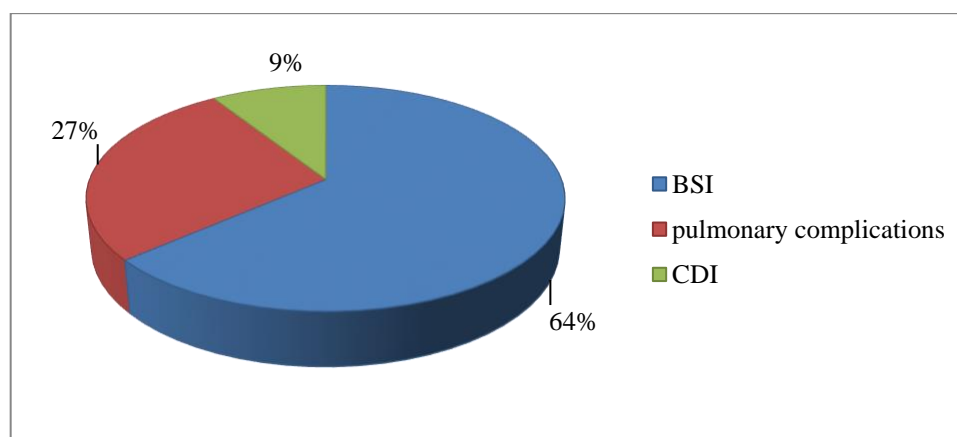

Figure 5 Episodes of infections during allo-HSCT

Among BSI, we described one CP-Kp BSI in one patient colonized by CP-Kp and two cases of ESBL-BSI, of which one occurred in a patient previously colonized by ESBL (**Figure 6**).
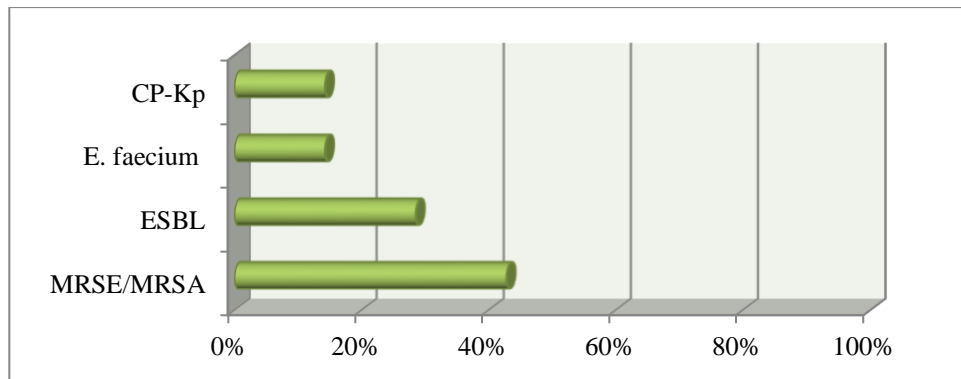
Figure 6 Etiology of BSI

4.2 Changes in gut microbiome composition during allo HSCT

The analysis of microbiota taxa abundance at genus level on overall population during allo-HSCT, from admission to 1 year after transplant, is described in **Figure 7a and 7b**. Only the statistical significant changes in microbiome composition were displayed. Boxplot at genus level revealed a significant reduction in the abundance of *Dorea* and *Blautia* especially from T_0 to T_5. A significant reduction in the abundance of *Akkermansia, Bifidobacterium, Ruminococcus*, and a significant increase of *Enterococcus spp* was observed from T_0 to T_6. The most significant reduction of alpha diversity was observed for all species at T_3, which represent 15 days after allo-HSCT.
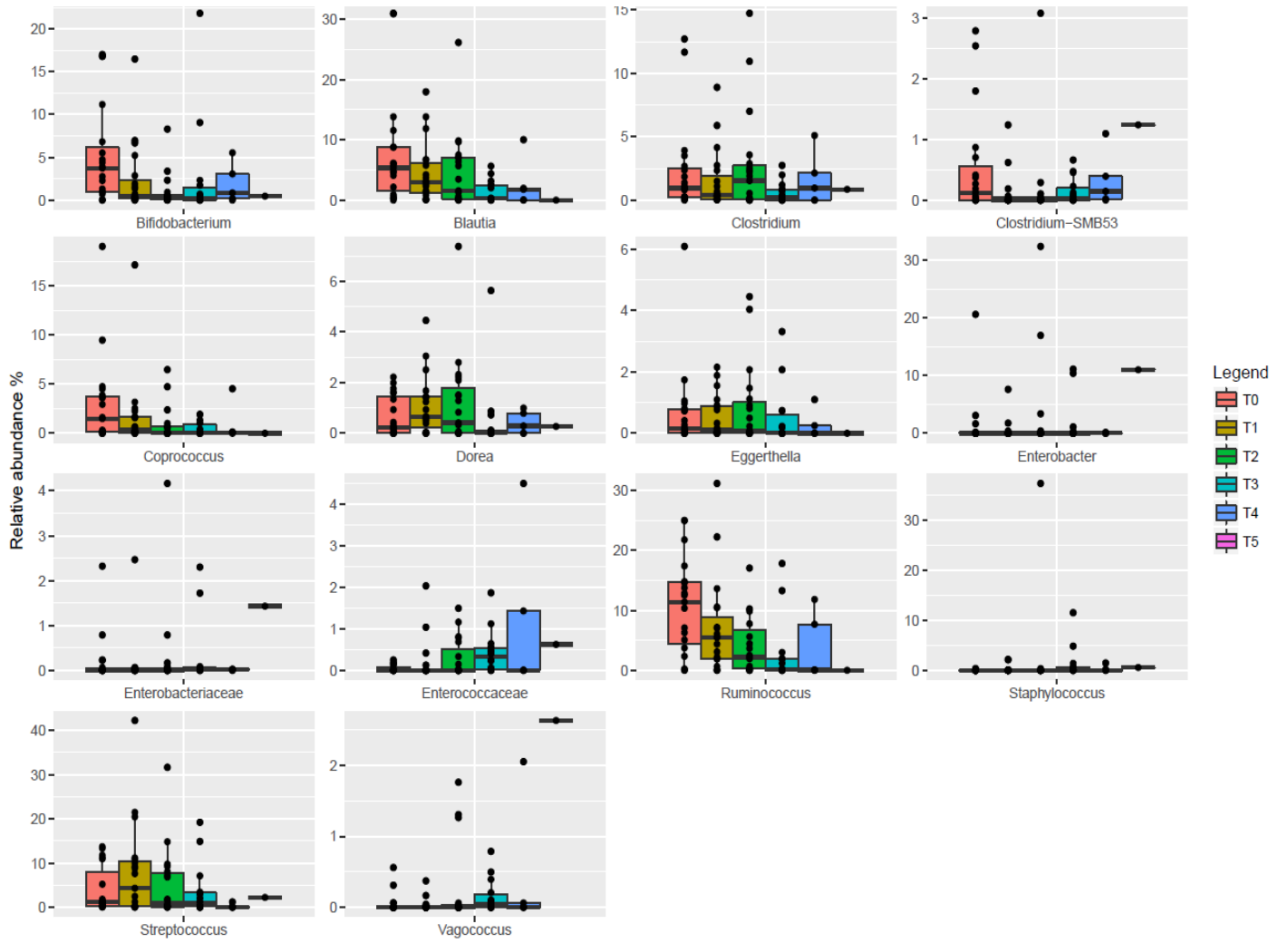
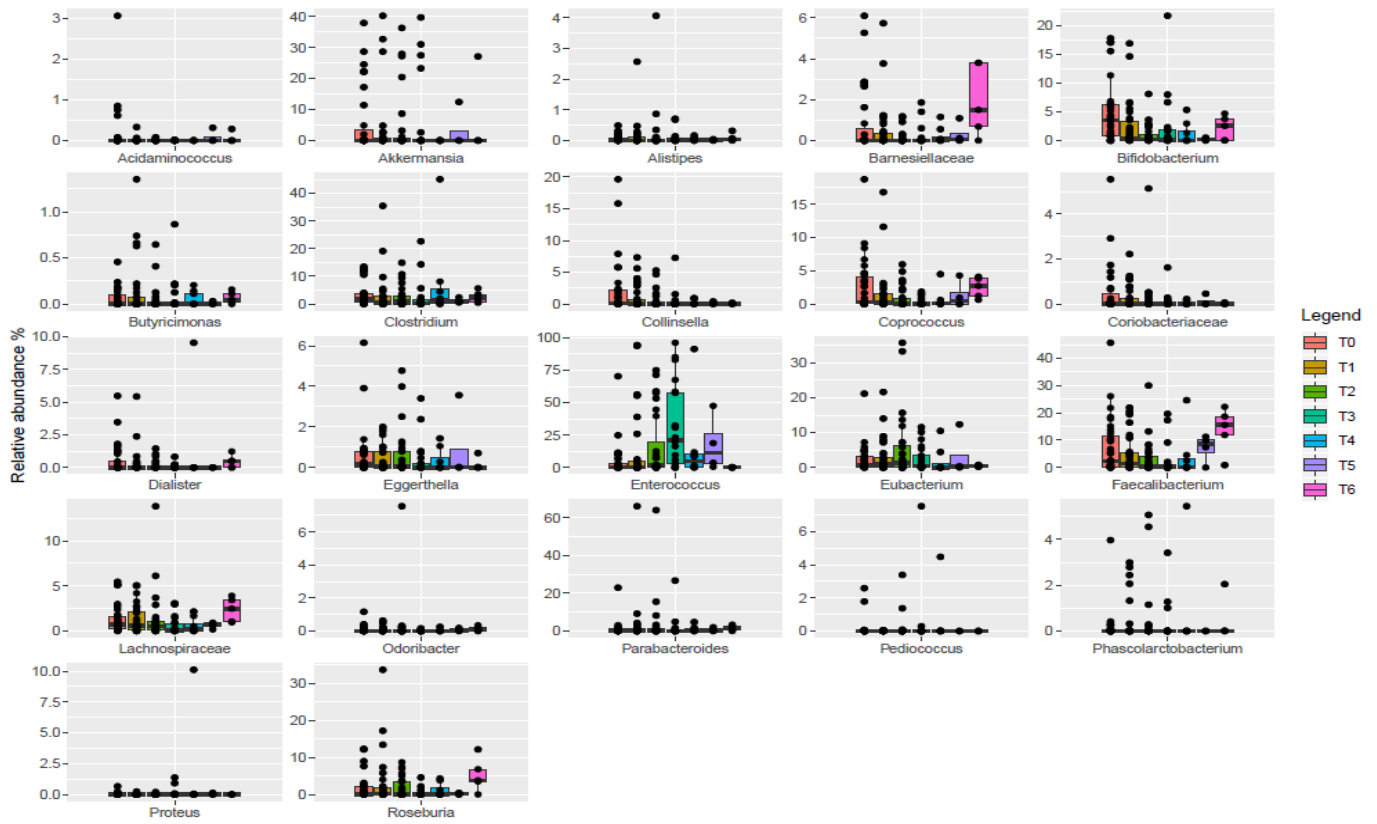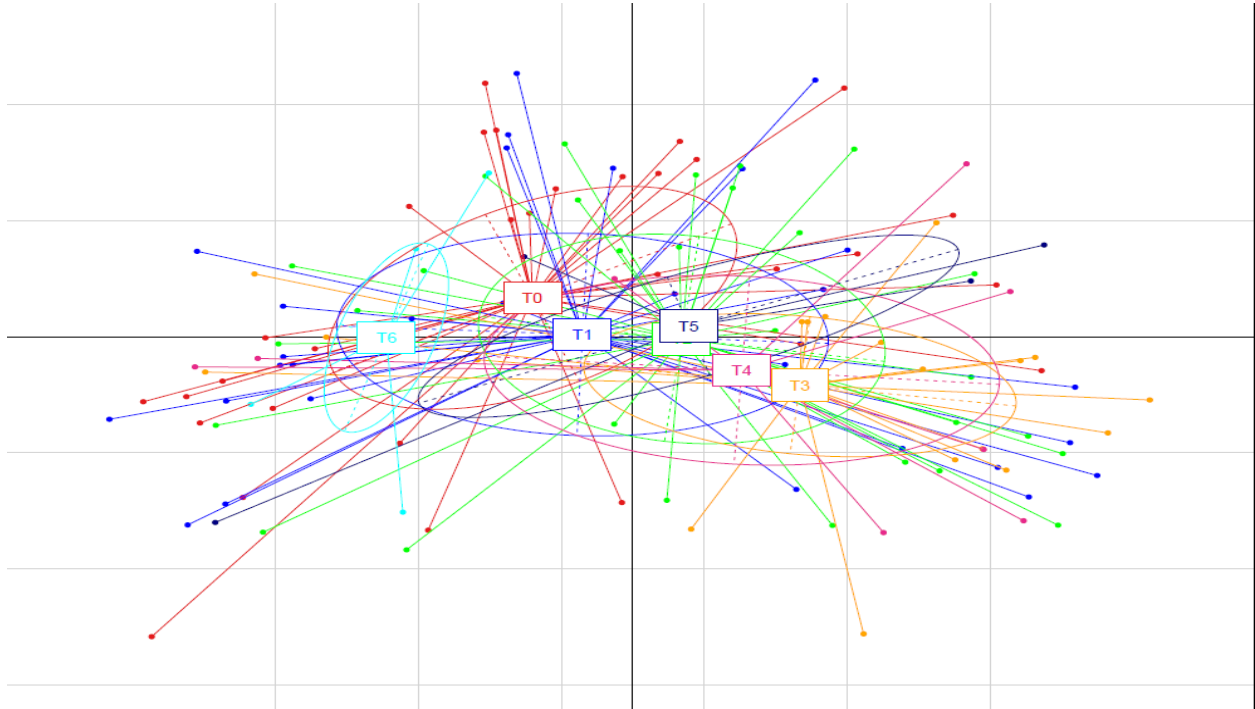*Figure 7 (A and b). Changes in gut microbiota composition during allo-HSCT from T_0 to T_5*

Figure 7b. Changes in gut microbiota composition during allo-HSCT from T_0 to T_6

Moreover, Principal component analysis (PCA) based on microbiota composition revealed a significant similiarity between T_0 and T_6 (**Figure 8**).

Changes in the a-diversity was also observed according to the duration of neutropenia. In patients with ≥15 days of neutropenia, we described a significant reduction of *Blautia, Dorea, Ruminococcus, Bifidobacterium and Lactospiracea* (**Figure 9a**) from T_0 to T_6, with the strongest depletion at T_3 (**Figure 9b**).
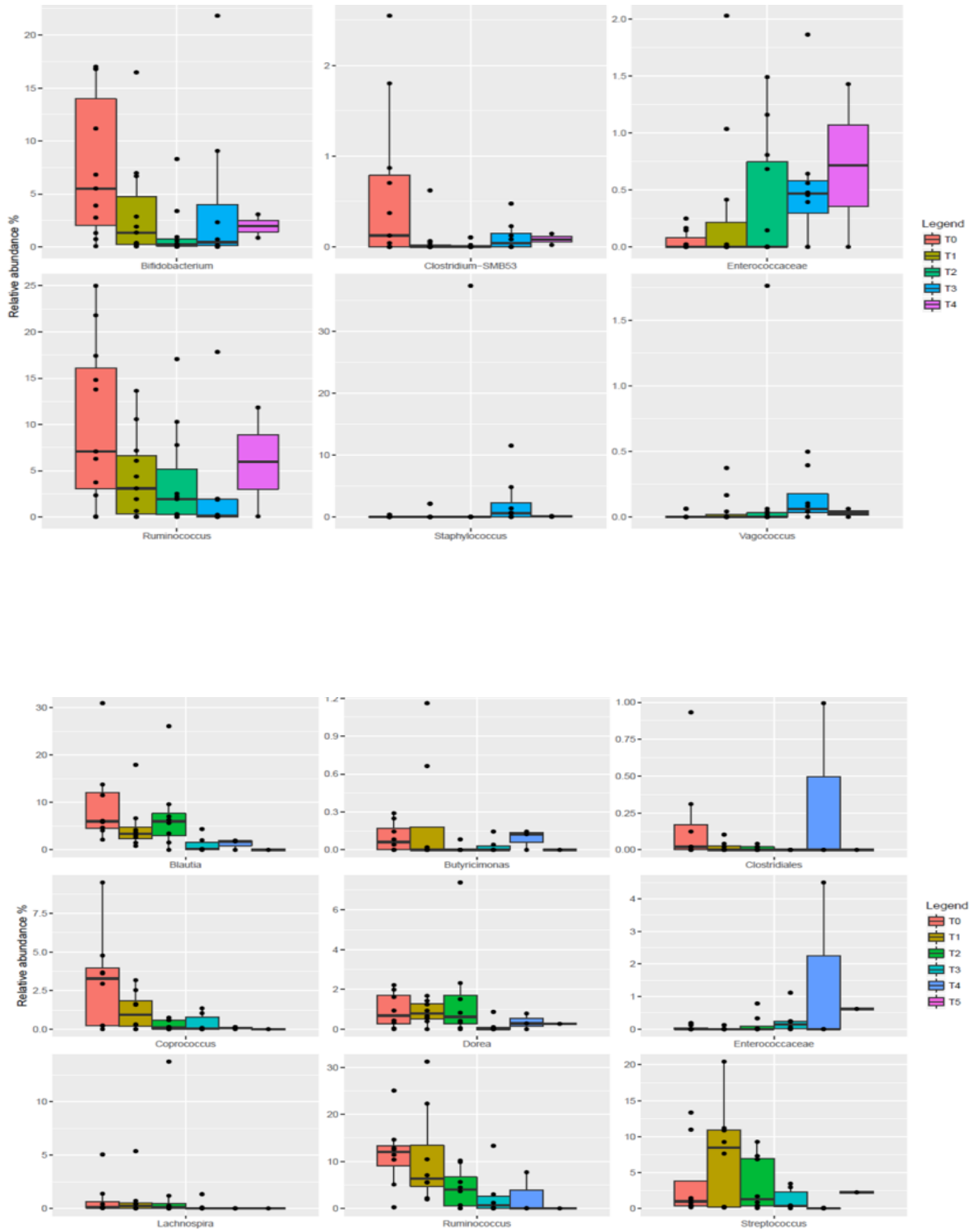
Figure 9 (a and b) Figure a: changes in neutropenia < 15 days; fig. 9b: changes in neutropenia > 15 days

Nine out of 33 (27%) patients developed an aGVHD, mostly involving gastrointestinal tract. In these patients we observed a reduction of *Clostridiales* and *Bifidobacterium*, especially at baseline and at the early days after transplant (**Figure 10**).
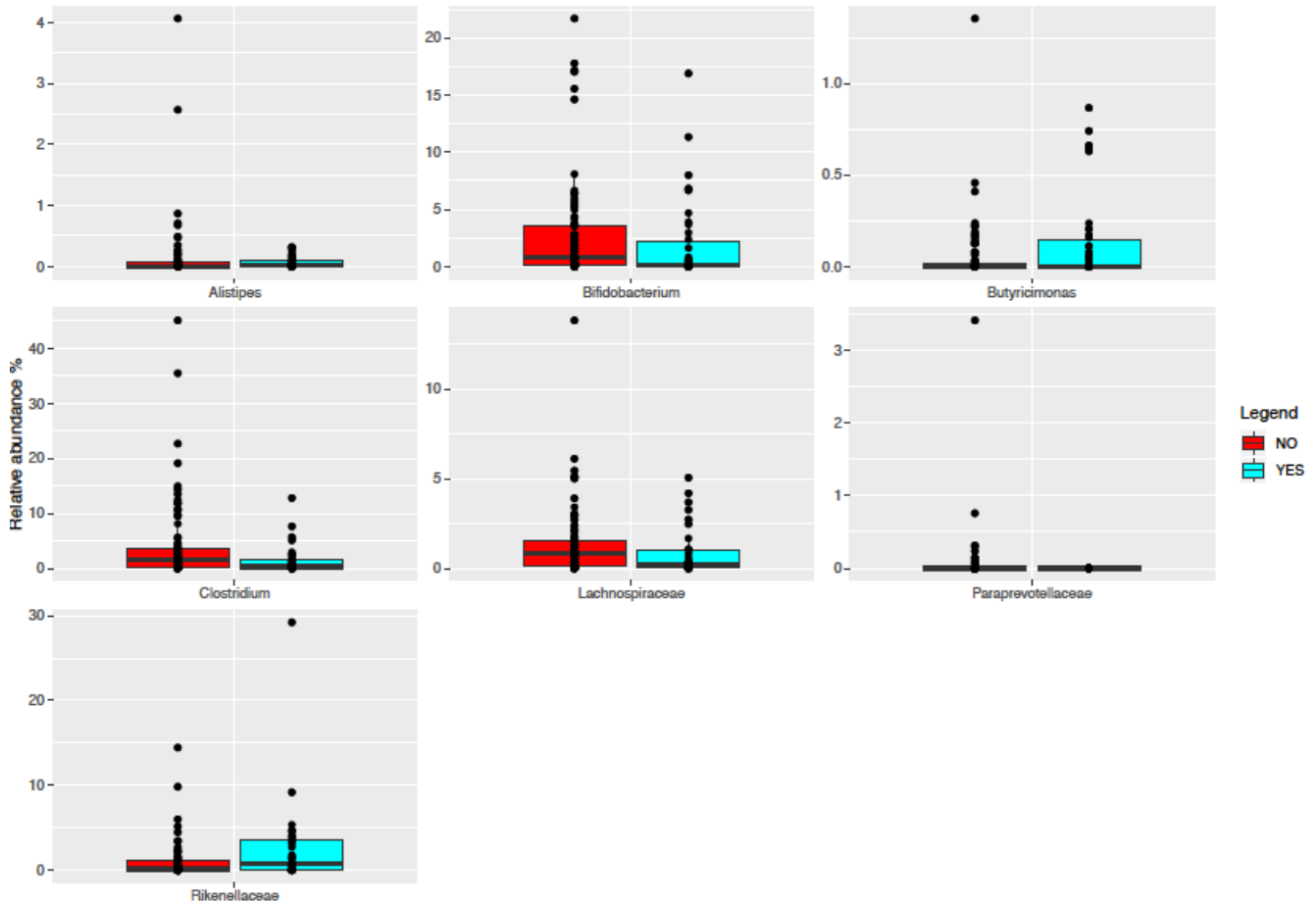

Figure 10 Gut microbiota changes according to aGVHD

4.3. Differences in gut microbiome composition according to MDR colonization

We further evaluated the microbiota diversity between patients colonized by MDR at baseline (T_0; n=13) and patients who were not. We observed a significant decrease in *Granulicatella, Prevotella and Gemellacea* in baseline colonized patients (**Figure 11**).
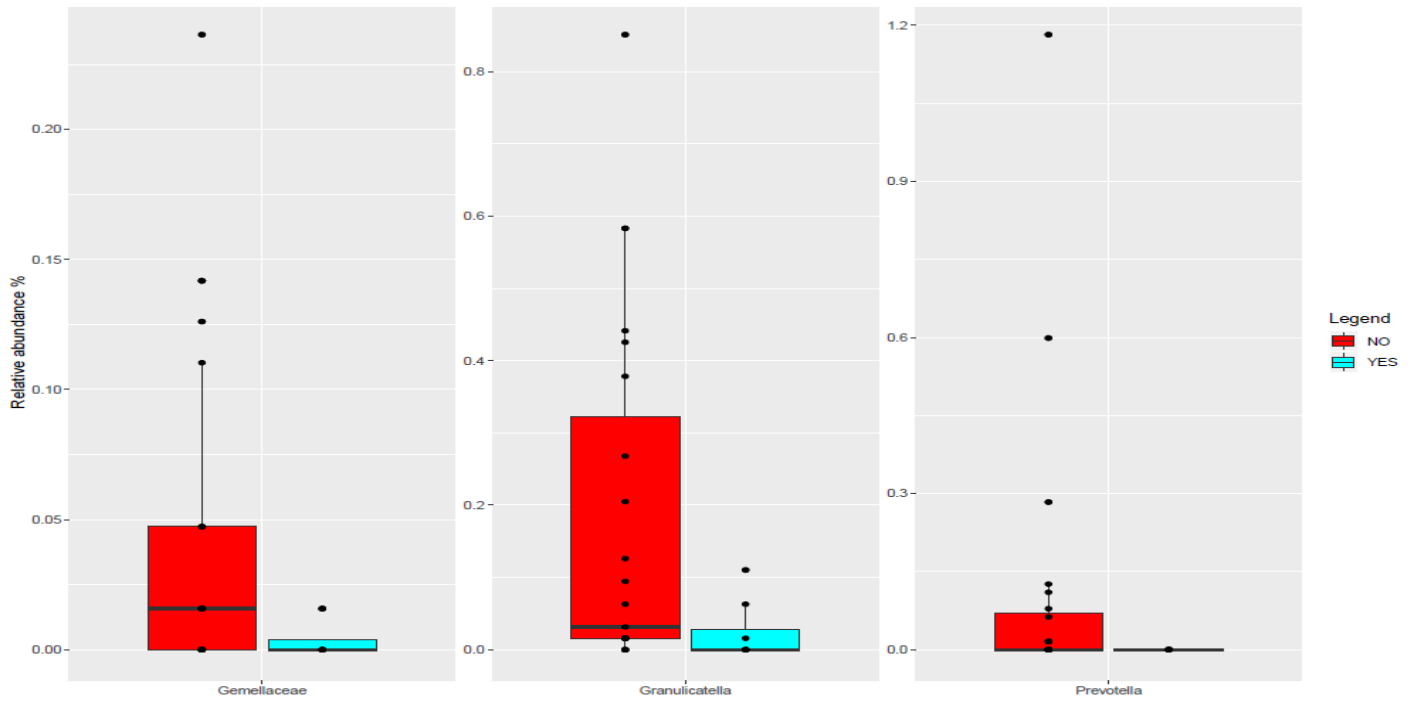
Figure 11 Differences in patients colonized by MDR at baseline (red: not colonized; blue: colonized)

Comparing microbiota composition according to the type of MDR colonization, several differences have been detected: an impressive increase of *Akkermansia* was observed in ESBL colonized patients, whilst in CP-KP there was a predominance of *Butyricimonas*. Among VRE carriers, an increase of Streptococcus spp was observed (**Figure 12**).

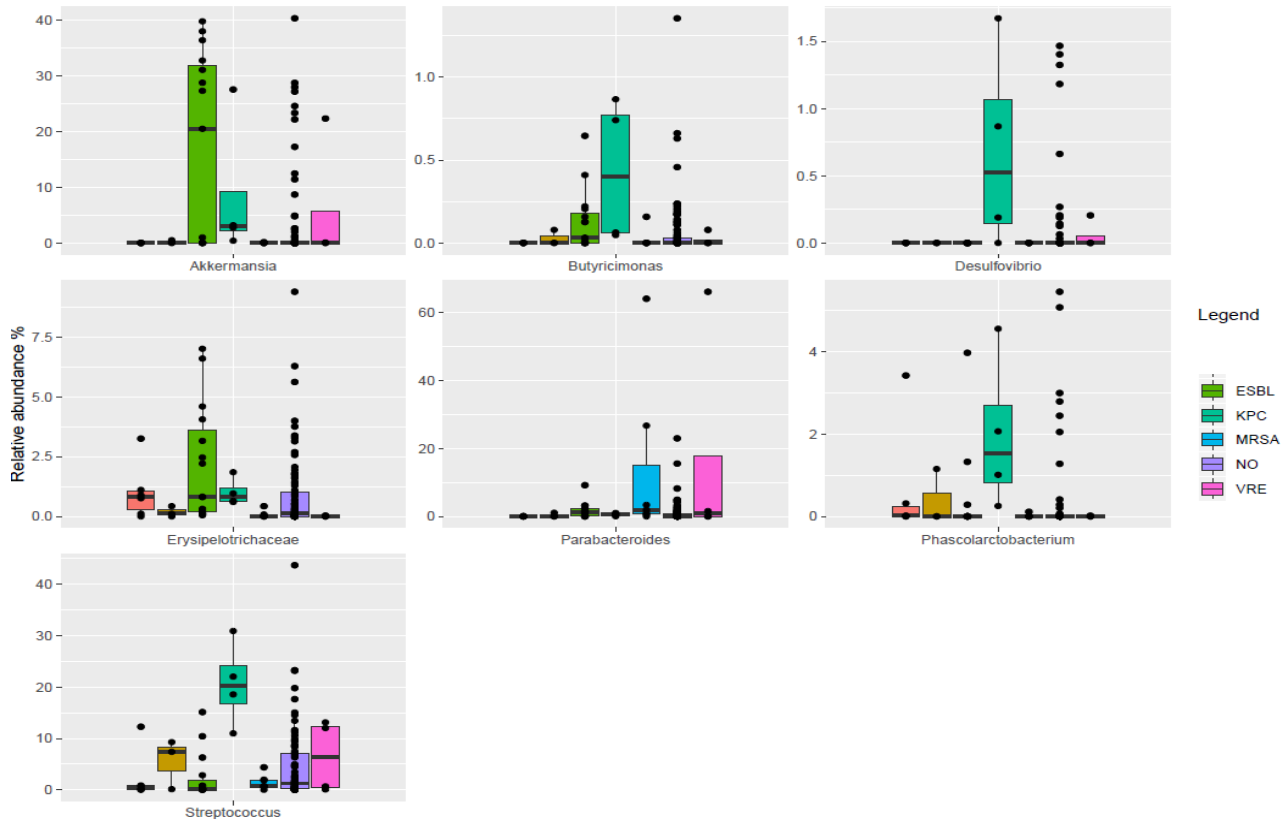*Figure 12 Differences in gut microbiota composition according to the type of MDR colonization*

Since ESBL colonization was predominant in our cohort of patients, we further analyzed the shift of microbiome composition from T_0 to T_6 in this group of patients. We observed a significant reduction of *Bifidobacterium* and *Clostridiales* in ESBL+ patients (**Figure 13**) through the time of observation.
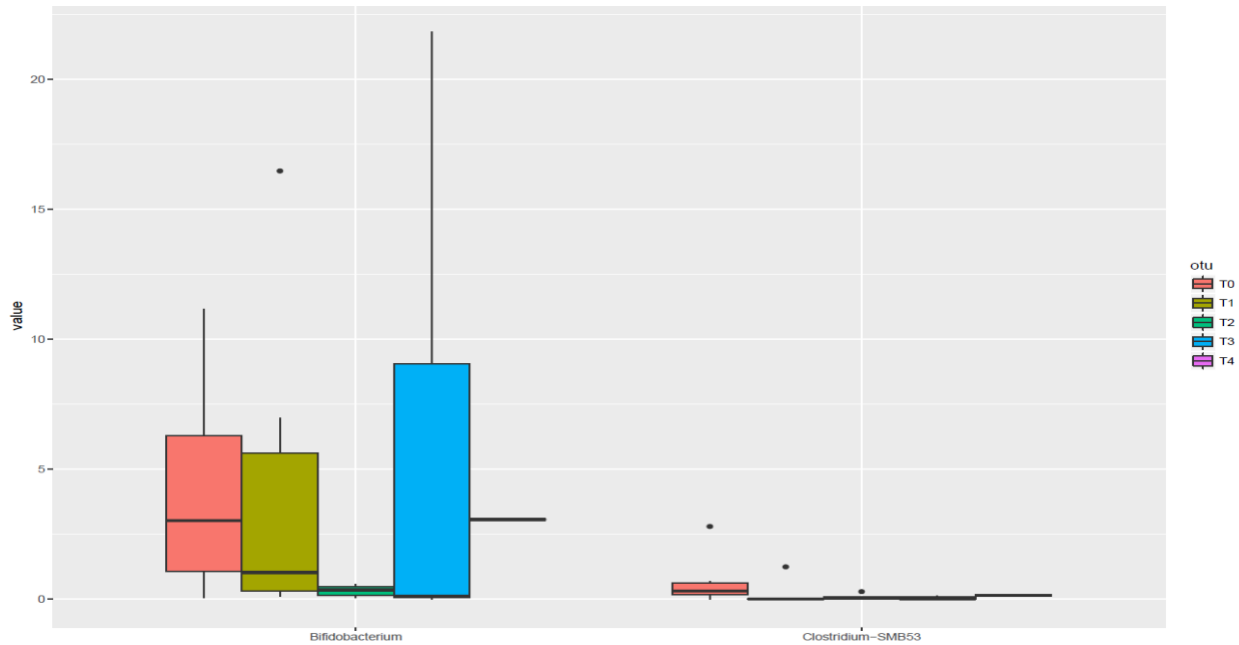
Figure 13 gut microbiota in ESBL colonized patients

4.4. Association between the use of carbapenems and gut microbiota composition

In our cohort, 45% of patients were treated with carbapenems, mostly meropenem, as empiric or targeted therapy during febrile neutropenia, usually as a second line treatment. In patients treated with carbapenems, a significant strong reduction of *Blautia, Ruminococcus* and *Bifidobacterium* was observed, as well as an increase of *Enterococcus*. The shift in microbiome composition was more relevant at T_3 while the restoration of microbiome diversity was observed at T_5-T_6 (**Figure 14**).

Figure 14 Gut microbiota in patients treated with carbapenems
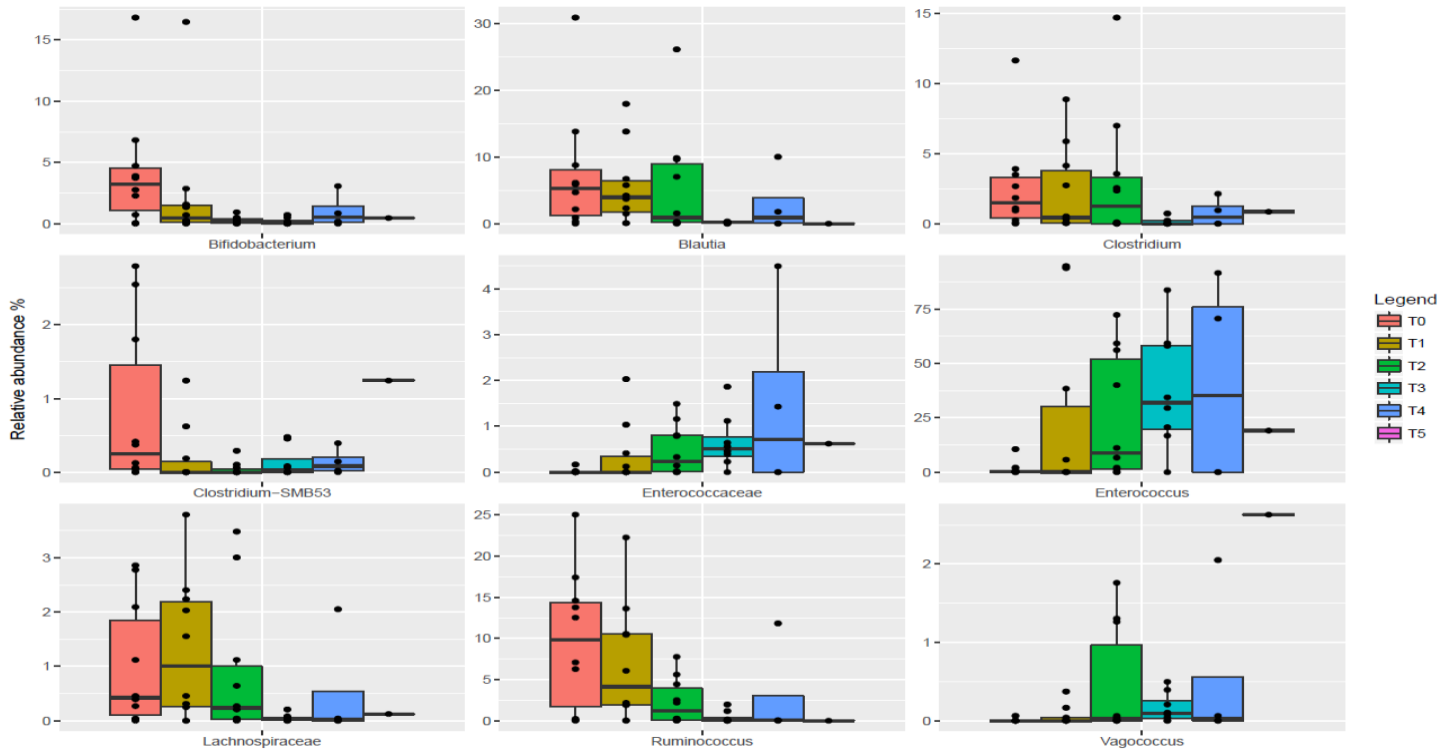
5. Discussion

Infections caused by MDR bacteria are associated with a high mortality rate, especially in the haematological setting. It's well known that gut colonization by MDR bacteria is one of the main risk factors for the development of invasive infections and several factors (i.e. chemotherapy, prolonged antibiotic therapies) may alter the taxonomic diversity of gut microbiota leading to expansion, proliferation and the translocation of bacteria with a nosocomial profile of resistance, as CDI, CP-Kp or ESBL (32; 72-74).

Although a healthy, indigenous gut microbiota likely provides resistance to colonization by enteric MDRO, exposure to antibiotics and other clinical factors can disrupt gut microbial community homeostasis and reduce "colonization resistance," thus increasing MDRO colonization risk (40-42).

Patients undergoing allo-HSCT are subjected to chemotherapy, radiation, antibiotics and these perturbations can lead to a severe dysbiosis with a profound shift in the intestinal microbiota during transplant (27; 56-58). Several data from literature described the association of these changes with the development of severe complication after allo-HSCT as GVHD, relapse of haematological disease or bloodstream infections (75). Moreover, low diversity is an indipendent predictor of mortality for infectious and non-infectious causes and enteric domination >30% by single pathogens seems to be relate to the bacteriemic episodes (27). Infections are one of the major threats in haematological setting, and in recent years the incidence of colonization and infections by MDRO in this setting are more frequently reported. Invasive infections caused by MDRO are usually associated with a high mortality rate, especially in allo-HSCT recipients (76-77).

In this prospective pilot study we evaluated the changes of microbiota composition in patients undergoing allo-HSCT and we further investigated if the acquisition of colonization by MDR bacteria may affect the

gut microbiome. As reported in previous studies, during the course of allo-HSCT, the diversity of the intestinal flora significantly decrease, with a significant reduction of *Akkermansia, Ruminococcus and Blautia*. The decrease of these species is associated with reduction of bile acids products and degraded mucous layer, usually associated with a "healthy "gut microbiota condition (75).

According to our data, the low diversity observed during allo-HSCT is changing from T_0 to T_6, and a peak of loss of diversity was observed at 15 days after transplant (T_3). This might be easily explain with the fact that this time point reflect the time of more severe neutropenia, frequently associated with fever and empiric antibiotics administration. Therefore, it can be identify as the "critical time for dysbiosis", in which the profound changes in microbiome may promote the gastrointestinal overgrowth of bacteria with a nosocomial profile of resistance and may increase the risk of invasive infections.

Interestingly, PCA analysis showed that only one year after transplant there was a restoration of microbiota composition in allo-HSCT recipients, suggesting that this time frame could be a reasonable time for the gastrointestinal tract to recover from the prolonged insults occurred during allo-HSCT.

Regarding GVHD, loss of diversity, including expansion of Enterobacteriales and loss of Clostridiales, was observed in animal models of GVHD (78-79). Other experiments have demonstrated that a decrease intestinal microbiota-derived short-chain fatty acids (SCFAs) in intestinal epithelial cells is associated with GVHD after allo-HSCT, whereas supplementation with Clostridia, which highly produce SCFAs, could mitigate GVHD (79). In contrast, recent studies have indicated that at the time of engraftment decreased Clostridia are associated with subsequent acute GVHD post-transplantation (78;80). With regards to the mechanism of these microbiota members in GVHD, animal experiments have suggested that Clostridia metabolites could mitigate intestinal barrier damage and induce immune tolerance (79; 81-

82), which depend on the levels of SCFAs and histone acetylation that is influenced by SCFAs (81;83-85).

These findings were confirmed also in our study, in which patients with acute GVHD had a significant reduction of *Clostridiales* and anti inflammatory bacteria as *Ruminococcus*.

We further analyzed the microbial diversity according to baseline MDR colonization. Interestingly, we observed a reduction of microbiome richness in MDR carriers, and several differences have been described classifying patients according to the type of colonization. In patients with ESBL + there was an increase of *Akkermansia* richness, whilst in CP-Kp patients *Butyricimonas* were predominant.

This is the first report describing these species predominance in colonized patients: Akkermansia species have immunoregulatory effects by converting mucins into SCFAs, however they could have a role in degradying the mucous layer and promoting inflammation. Butyricimonas species have a immunomodulatory effects, but so far no date have been published about the association of these species and colonization. Of course these findings need to be confirmed in a larger studies, since the low number of patients and the statistical power of this pilot study could have affected the results.

Lastly we focus our attention on the impact of carbapenems therapy on microbiome changes. Although changes in the intestinal microflora have been observed in patients receiving meropenem and imipenem, they were deemed relatively minor compared with those caused by other carbapenems, presumably due to very low concentrations of both agents observed in the faeces (86-87). In our study, patients treated with meropenem had a significant reduction of anti inflammatory bacteria as *Blautia, Ruminococcus* and *Bifidobacterium*. These findings were similar to those published by Moriaria et al., in which meropenem was administered to 18 patients undergoing allo or auto HSCT: authors described as most detrimental impact on obligate anaerobes was observed in patients treated with piperacillin/tazobactam and

meropenem (88). These data support the relevance of carbapenem sparing strategies when feasible, also in the haematological setting, in order to protect the gut from the severe depletion of anaerobes which may contribute to dysbiosis and development of infections.

This study had several limitations: first we did not investigate the previous antibiotic exposure within 3 months before allo-HSCT which may have a relevant role in baseline composition of gut microbiota as well as on the baseline gut colonization. Second, we did not evaluate the difference in patients who acquired and lost MDR colonization, which may give some extreme interesting information about the relevant changes that may promote acquisition or loss of colonization. Third a shot gun sequences analysis aiming at identifying carriers of resistant genes should be performed to identify the persistence of MDR genes in allo-HSCT recipients.

6. Conclusion & Future perspective

In conclusion, this is the first paper describing the association between microbiome diversity and intestinal colonization by MDR bacteria in allo-HSCT recipients. Preliminary studies support the use of novel strategies to improve outcomes in haematological patients colonized by MDR bacteria, including microbiota analysis to predict the risk of infections. Future projects will aim at study the fecal metabolome (SCFA and bile salt acids production), with the identification of enteric markers which may predict the risk of adverse clinical outcomes in allo-HSCT.

Moreover, data on metagenomics and metabolomics will help to identify patients at high risk for MDR colonization, and based on their metabolite profile, patients could be treated with tailored antibiotic therapies, reducing broad spectrum antibiotic treatments and the risk of infections by MDR.

This should lead to a better use of antibiotic therapy, saving broad spectrum antibiotic treatment for patients with a less favourable metabolite profile.

7. References

1) Rinninella E et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microrganisms 2019; 16-22

2) Tuddenham, S. & Sears, C. L. The intestinal microbiome and health. *Curr. Opin. Infect. Dis.* 28, 464–470 (2015).

3) Pamer, E. G. Resurrecting the intestinal microbiota to combat antibiotic-resistant pathogens. *Science* 352, 535–8 (2016).

4) Dave, M., Higgins, P. D., Middha, S. & Rioux, K. P. The human gut microbiome: current knowledge, challenges, and future directions. *Transl. Res.* 160, 246–57 (2012).

5) Brüssow, H. Microbiota and healthy ageing: observational and nutritional intervention studies. *Microb. Biotechnol.* 6, 326–34 (2013).

6) Khanna, S. & Tosh, P. K. A clinician's primer on the role of the microbiome in human health and disease. *Mayo Clin. Proc.* 89, 107–14 (2014).

7) Sekirov, I., Russell, S. L., Antunes, L. C. M. & Finlay, B. B. Gut microbiota in health and disease. *Physiol. Rev.* 90, 859–904 (2010).

8) Moeller, A. H. The shrinking human gut microbiome. *Curr. Opin. Microbiol.* 38, 30–35 (2017).

9) Castanys-Muñoz, E., Martin, M. J. & Vazquez, E. Building a Beneficial Microbiome from Birth. *Adv. Nutr.* 7, 323–30 (2016).

10) Jernberg, C., Löfmark, S., Edlund, C. & Jansson, J. K. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 156, 3216–23 (2010).

11) Fishman, J. A. From the classic concepts to modern practice. *Clin. Microbiol. Infect.* 20, 4–9 (2014).

12) Stripling, J. *et al.* Loss of Vancomycin-Resistant Enterococcus Fecal Dominance in an Organ Transplant Patient With Clostridium difficile Colitis After Fecal Microbiota Transplant. *Open forum Infect. Dis.* 2, ofv078 (2015).

13) Lynch, S. V. & Pedersen, O. The Human Intestinal Microbiome in Health and Disease. *N. Engl. J. Med.* 375, 2369–2379 (2016).

14) Sheflin, A. M., Melby, C. L., Carbonero, F. & Weir, T. L. Linking dietary patterns with gut microbial composition and function. *Gut Microbes* 8, 113–129 (2017).

15) Million, M., Lagier, J. C., Yahav, D. & Paul, M. Gut bacterial microbiota and obesity. *Clin. Microbiol. Infect.* 19, 305–313 (2013).

16) Oever, J. ten & Netea, M. G. The bacteriome-mycobiome interaction and antifungal host defense. *Eur. J. Immunol.* 44, 3182–3191 (2014).

17) Jakobsson, H. E. *et al.* Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* 5, e9836 (2010).

18) Lankelma, J. M. *et al.* Critically ill patients demonstrate large interpersonal variation in intestinal microbiota dysregulation: a pilot study. *Intensive Care Med.* 1–10 (2016). doi:10.1007/s00134-016-4613-z

19) Gregory, K. E. *et al.* Influence of maternal breast milk ingestion on acquisition of the intestinal microbiome in preterm infants. *Microbiome* 4, 68 (2016).

20) Cusack, S., O'Toole, P. W. & ELDERMET consortium. Challenges and implications for biomedical research and intervention studies in older populations: insights from the ELDERMET study. *Gerontology* 59, 114–21 (2013)

21) Colonon M et al. The Impact of Diet and Lifestyle on Gut Microbiota and Human HealthNutrients. 2015 Jan; 7(1): 17–44.

22) Shin, J. H. & Warren, C. A. Collateral damage during antibiotic treatment of C. difficile infection in the aged host: insights into why recurrent disease happens. *Gut Microbes* 0 (2017). doi:10.1080/19490976.2017.1323616

23) Goudarzi, M. *et al.* Clostridium difficile Infection: Epidemiology, Pathogenesis, Risk Factors, and Therapeutic Options, Clostridium difficile Infection: Epidemiology, Pathogenesis, Risk Factors, and Therapeutic Options. *Sci. Sci.* 2014, 2014, e916826 (2014).

24) De Rosa, F. G., Corcione, S., Pagani, N. & Di Perri, G. From ESKAPE to ESCAPE, From KPC to CCC. *Clin. Infect. Dis.* 60, (2015).

25) Guastalegname, M., Russo, A., Falcone, M., Giuliano, S. & Venditti, M. Candidemia subsequent to severe infection due to Clostridium difficile: is there a link? *Clin. Infect. Dis.* 57, 772–4 (2013).

26) Donskey, C. J. *et al.* Effect of antibiotic therapy on the density of vancomycin-resistant enterococci in the stool of colonized patients. *N. Engl. J. Med.* 343, 1925–32 (2000).

27) Taur, Y. *et al.* Intestinal domination and the risk of bacteremia in patients undergoing allogeneic

hematopoietic stem cell transplantation. *Clin. Infect. Dis.* 55, 905–14 (2012).

28) Caballero, S. *et al.* Distinct but Spatially Overlapping Intestinal Niches for Vancomycin-Resistant Enterococcus faecium and Carbapenem-Resistant Klebsiella pneumoniae. *PLoS Pathog.* 11, e1005132 (2015).

29) Singh, R. *et al.* Donor feces infusion for eradication of Extended Spectrum beta-Lactamase producing Escherichia coli in a patient with end stage renal disease. *Clin. Microbiol. Infect.* 20, O977-8 (2014).

30) Corcione S et al. The gastrointestinal microbiome and the enteropathogenetic syndromes: An infectious diseases perspective. Annals of Infections 2017

31) Koh A. Potential for Monitoring Gut Microbiota for Diagnosing Infections and Graft-versus-Host Disease in Cancer and Stem Cell Transplant Patients Clin Chem 2017; 63: 1685-1694

32) Corcione S, Angilletta R, Raviolo S, Filippini C, Fossati L, Di Perri G, Cavallo R, De Rosa FG. Epidemiology and risk factors for mortality in bloodstream infection by CP-Kp, ESBL-E, Candida and CDI: A single center retrospective study.Eur J Intern Med. 2018;48:44-49.

33) Manges, A. R., Steiner, T. S. & Wright, A. J. Fecal microbiota transplantation for the intestinal decolonization of extensively antimicrobial-resistant opportunistic pathogens: a review. *Infect. Dis. (London, England)* 48, 587–92 (2016).

34) Gargiullo et. al Gut Microbiota Modulation for Multidrug-Resistant Organism Decolonization: Present and Future Perspectives Frontiers Microbiology 2019; 10: 1704.

35) Halaby T., Al Naiemi N., Kluytmans J., van der Palen J., Vandenbroucke-Grauls C. M. J. E. (2013). Emergence of colistin resistance in *Enterobacteriaceae* after the introduction of selective digestive tract decontamination in an intensive care unit. *Antimicrob. Agents Chemother.* 57 3224–3229.

36) Battipaglia G., Malard F., Rubio M. T., Ruggeri A., Mamez A. C., Brissot E., et al. (2019). Fecal microbiota transplantation before or after allogeneic hematopoietic transplantation in patients with hematological malignancies carrying multidrug-resistance bacteria. *Haematologica* 10.3324/haematol.2018.198549

37) Biliński J., Grzesiowski P., Muszyński J., Wróblewska M., Mądry K., Robak K., et al. (2016). Fecal microbiota transplantation inhibits multidrug-resistant gut pathogens: preliminary report performed in an immunocompromised host. *Arch. Immunol. Ther. Exp.* 64 255–258.

38) Rice L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J. Infect. Dis.* 197 1079–1081.

39) van Schaik W. (2015). The human gut resistome. *Philos. Trans. R. Soc. B Biol.Sci.* 370:20140087.

40) Casals-Pascual C., Vergara A., Vila J. (2018). Intestinal microbiota and antibiotic resistance: perspectives and solutions. *Hum. Microbiome J.* 9 11–15.

41) Baron S. A., Diene S. M., Rolain J.-M. (2018). Human microbiomes and antibiotic resistance. *Hum. Microbiome J.* 10 43–52.

42) Penders, J., Stobberingh, E. E., Savelkoul, P. H. M., and Wolffs, P. F. G. (2013). The human microbiome as a reservoir of antimicrobial resistance. Front. Microbiol. 4:87

43) Ubeda, C., Bucci, V., Caballero, S., Djukovic, A., Toussaint, N. C., Equinda, M., et al. (2013). Intestinal microbiota containing Barnesiella species cures vancomycin-resistant Enterococcus faecium colonization. Infect. Immun. 81, 965–973

44) Mahieu, R., Cassisa, V., Hilliquin, D., Coron, N., Pailhoriès, H., Kempf, M., et al. (2017). Impact of faecal microbiota transplantation on mouse digestive colonization with two extensively resistant bacteria. J. Infect. 75, 75–77

45) Caballero, S., Kim, S., Carter, R. A., Leiner, I. M., Sušac, B., Miller, L., et al. (2017). Cooperating commensals restore colonization resistance to vancomycinresistant Enterococcus faecium. Cell Host Microbe 21, 592.e4–602.e4

46) Gosalbes, M. J., Vázquez-Castellanos, J. F., Angebault, C., Woerther, P.-L., Ruppé, E., Ferrús, M. L., et al. (2015). Carriage of enterobacteria producing extended-spectrum β-Lactamases and composition of the gut microbiota in an amerindian community. Antimicrob. Agents Chemother. 60, 507–514.

47) Martin, R. M., Cao, J., Brisse, S., Passet, V., Wu, W., Zhao, L., et al. (2016). Molecular epidemiology of colonizing and infecting isolates of Klebsiella pneumoniae. mSphere 1:e00261-16

48) Gorrie, C. L., Mirceta, M., Wick, R. R., Edwards, D. J., Thomson, N. R., Strugnell, R. A., et al. (2017). Gastrointestinal carriage is a major reservoir of Klebsiella pneumoniae infection in intensive care patients. Clin. Infect. Dis. 65, 208–215.

49) Patriarca, F., Cigana, C., Massimo, D., Lazzarotto, D., Geromin, A., Isola, M., et al. (2017). Risk factors and outcomes of infections by multidrugresistant gram-negative bacteria in patients undergoing hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. 2

50) Peric, Z., Vranjes, V. R., Durakovic, N., Desnica, L., Marekovic, I., ServentiSeiwerth, R., et al. (2017). Gut colonization by multidrug-resistant gramnegative bacteria is an independent risk factor for development of intestinal acute graft-versus-host disease. Biol. Blood Marrow Transplant. 23, 1221–1222

51) Macesic, N., Gomez-Simmonds, A., Sullivan, S. B., Giddins, M. J., Ferguson, S. A., Korakavi, G., et al. (2018). Genomic surveillance reveals diversity of multidrugresistant organism colonization and infection: a prospective cohort study in liver transplant recipients. Clin. Infect. Dis. 67, 905–912.

52) Jones, J. M., Wilson, R. & Bealmear, P. M. Mortality and gross pathology of secondary disease in germfree mouse radiation chimeras. Radiat. Res. 45, 577–588 (1971).

53) van Bekkum, D. W., Roodenburg, J., Heidt, P. J. & van der Waaij, D. Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. J. Natl Cancer Institute. 52, 401–404 (1974).

54) Storb, R. et al. Graft-versus-host disease and survival in patients with aplastic anemia treated by marrow grafts from HLA-identical siblings. Beneficial effect of a protective environment. N. Engl. J. Med. 308, 302–307 (1983)

55) Taur, Y. et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin. Infect. Dis. 55, 905–914 (2012).

56) Taur, Y., Jenq, R. R., Ubeda, C., van den Brink, M. & Pamer, E. G. Role of intestinal microbiota in transplantation outcomes. Best Pract. Res. Clin. Haematol. 28, 155–161 (2015)

57) Shono, Y., Docampo, M. D., Peled, J. U., Perobelli, S. M. & Jenq, R. R. Intestinal microbiotarelated effects on graft-versus-host disease. Int. J. Hematol. 101, 428–437 (2015).

58) Taur, Y. et al. The effects of intestinal tract bacterial diversity on mortality following allogeneic hematopoietic stem cell transplantation. Blood 124, 1174–1182 (2014).

59) Harris, B. et al. Gut microbiota predict pulmonary infiltrates after allogeneic hematopoietic cell transplantation. Am. J. Respir. Crit. Care Med. 194, 450–463 (2016).

60) Taur, Y. Intestinal microbiome changes and stem cell transplantation: lessons learned. Virulence 7, 930–938 (2016)

61) Sullivan KM. Graft-versus-host disease. In: Thomas ED, Blume KG, Forman SJ, editors. Hematopoietic Cell Transplantation. Malden, MA: Blackwell Sciences; 1999. pp. 515–536.

62) Freifeld AG, Bow EJ, Sepkowitz KA, et al. Clinical practice guideline for the use of antimicrobial agents in neutropenic patients with cancer: 2010 update by the Infectious Diseases Society of America. Clin Infect Dis. 2011;52:56–93.

63) Klindworth A, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013;41.

64) Magoc T, Salzberg SL. FLASH: Fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011;27:2957–2963.

65) Caporaso JG, et al. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 2010;7:335–336. doi: 10.1038/nmeth.f.303.

66) Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 2011;27:863–864

67) Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011;27:2194–2200.

68) Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26:2460–2461.

69) Langille MGI, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 2013;31:814–821.

70) Luo W, Friedman MS, Shedden K, Hankenson KD, Woolf PJ. GAGE: generally applicable gene set enrichment for pathway analysis. BMC Bioinformatics. 2009;10:161.

71) Eren AM, et al. Oligotyping: Differentiating between closely related microbial taxa using 16S rRNA gene data. Methods Ecol. Evol. 2013;4:1111–1119.

72) Dixon P. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 2003;14:927–930.

73) Ruppé E, Burdet C, Grall N, et al. Impact of antibiotics on the intestinal microbiota needs to be re-defined to optimize antibiotic usage. *Clin Microbiol Infect.* 2018;24:3-5.

74) Zhang L, Dong D, Jiang C,et al. Insight into alteration of gut microbiota in Clostridium difficile infection and asymptomatic C. difficile colonization. *Anaerobe.* 2015;34:1-7.

75) De Rosa FG, Corcione S, Raviolo S, et al. Candidemia, and infections by Clostridium difficile and carbapenemase-producing Enterobacteriaceae: new enteropathogenetic opportunistic syndromes? *Infez Med.* 2015;23:105-1.

76) Shono Y, van den Brink MRM. Gut microbiota injury in allogeneic haematopoietic stem cell transplantation. Nat Rev Cancer. 2018 May;18(5):283-295.

77) Heidenreich D, Kreil S, Nolte F, Hofmann WK, Miethke T, Klein SA. Multidrug-resistant organisms in allogeneic hematopoietic cell transplantation. Eur J Haematol. 2017 ;98(5):485-492.

78) Scheich S, Lindner S, Koenig R, Reinheimer C, Wichelhaus TA, Hogardt M, Besier S, Kempf VAJ, Kessel J, Martin H, Wilke AC, Serve H, Bug G, Steffen B. Clinical impact of colonization with multidrug-resistant organisms on outcome after allogeneic stem cell transplantation in patients with acute myeloid leukemia. Cancer. 2018; 124(2):286-296.

79) Simms-Waldrip TR, Sunkersett G, Coughlin LA, Savani MR, Arana C, Kim J, et al. Antibiotic-induced depletion of anti-inflammatory clostridia is associated with the development of graft-versus-host disease in pediatric stem cell transplantation patients. Biol Blood Marrow Transplant (2017) 23(5):820–9

80) Mathewson ND, Jenq R, Mathew AV, Koenigsknecht M, Hanash A, Toubai T, et al. Gut microbiome-derived metabolites modulate intestinal epithelial cell damage and mitigate graft-versus-host disease. Nat Immunol (2016) 17(5):505–13.

81) Golob JL, Pergam SA, Srinivasan S, Fiedler TL, Liu C, Garcia K, et al. The stool microbiota at neutrophil recovery is predictive for severe acute graft versus host disease after hematopoietic cell transplantation. Clin Infect Dis (2017) 65(12):1984–9.

82) Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, et al. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science (2013) 341(6145):569–73.

83) Riwes M, Reddy P. Microbial metabolites and graft versus host disease. Am J Transplant (2017) 18(1):23–9.10.

84) 19. Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, et al. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal Immunol (2015) 8(1):80–93.

85) 20. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature (2013) 500(7461):232–6.

86) 21. Choi S, Reddy P. HDAC inhibition and graft versus host disease. Mol Med (2011) 17(5–6):404–16.

87) Bhalodi AA, van Engelen TSR, Virk HS, Wiersinga WJ. Impact of antimicrobial therapy on the gut mcirobiome. J Antimicrob Chemother. 2019 Jan 1;74(Supplement_1):i6-i15.

88) Morjaria S, Schluter J, Taylor BP, Littmann ER, Carter RA, Fontana E, Peled JU, van den Brink MRM, Xavier JB, Taur Y. Antibiotic-induced shifts in fecal microbiota densitiy and composition during hematopoietic stem cell transplant. Infect Immun. 2019; 21;87(9).