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Distinct Changes in Gut Microbiota of Patients With Kidney Graft Rejection

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Background. Kidney graft rejection still represents the major cause of graft loss in kidney transplant recipients. Of growing interest is the bidirectional relationship between gut microbiome and immune system suggesting that gut microbiota can affect allograft outcome. **Methods.** In this cross-sectional case-control study, we characterized the gut microbial profile of adult renal transplant recipients with and without graft rejection to define a cohort-specific microbial fingerprint through 16S ribosomal RNA gene sequencing. We used very strict inclusion and exclusion criteria to address confounder of microbiota composition. **Results.** Different relative abundances in several gut microbial taxa were detectable in control patients compared with patients with kidney allograft rejection. Alpha diversity was lower in the rejection group and beta diversity revealed dissimilarity between patients with and without kidney graft rejection ($P < 0.01$). When the rejection group was stratified according to different types of allograft rejection, major changes were identified between patients with chronic T-cellular–mediated rejection and controls. Changes in alpha diversity within the gut microbiome were related to the probability of chronic T-cellular–mediated rejection ($P < 0.05$). Kidney transplant patients without rejection showed significant enrichment of rather anti-inflammatory taxa whereas in the rejection group bacteria well known for their role in chronic inflammation were increased. For example, amplicon sequence variant (ASV) 362 belonging to the genus *Bacteroides* and ASV 312 belonging to *Tannerellaceae* were enriched in no rejection ($P < 0.001$ and $P < 0.01$), whereas ASV 365 was enriched in patients with allograft rejection ($P = 0.04$). Looking at metagenomic functions, a higher abundance of genes coding for enzymes involved in bacterial multidrug resistance and processing of short-chain fatty acids was found in patients without rejection but an increase in enzymes involved in nicotinamide adenine dinucleotide phosphate production was seen in patients with allograft rejection. **Conclusions.** A distinct microbial fingerprint of patients with allograft rejection might serve as noninvasive biomarker in the future.

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Allograft rejection (AR) represents one of the most common complications after renal transplantation, which can negatively impact long-term graft survival.[1] AR refers to inflammation of the donor organ mediated by the host's immune system, due to the genetic differences between donor and recipient.[2] However, immunologic tolerance after renal transplantation involves complex immunologic pathways which allow the coexistence of the allograft and the host.[3] Tight balance between the activation of proinflammatory pathways, mediated by T helper (Th) 1 and Th17 cluster of differentiation 4 positive cells, and anti-inflammatory pathways, sustained by T regulatory cells, determines tolerance or rejection of the kidney transplant.[4] So far, AR can only be diagnosed/excluded with a kidney biopsy, being an invasive procedure with approximately 3% risk for bleeding, and the development of arteriovenous fistulas or macrohematuria.[5] Therefore, noninvasive diagnostic tools that could help to predict the risk of AR are highly demanded. Despite BANFF classification, there can be significant differences in biopsy interpretation depending on the pathologist, highlighting the urgent need for observer-independent markers for AR.[6] Activity of the immune system is regulated by several endogenous and exogenous mechanisms, among which the gut microbiota is primarily involved through its capability to modulate human immune system.[7] Several studies described the interaction between microbiota and the innate and adaptive immune systems, locally and systemically.[7,8] While the main products of intestinal commensals including short-chain fatty

acids contribute to the maintenance of an anti-inflammatory phenotype.[9,10] A dysbiotic gut bacterial profile sustains proinflammatory pathways though the activation of the inflammasome[11] and nuclear factor kappa-light-chain-enhancer of activated B cells pathway.[12] In the field of solid organ transplantation, few data are available concerning dysbiosis and AR. Furthermore, investigating gut microbial composition of a particular cohort, identifying a disease-associated microbiome signature needs to be evaluated in distinct geographic areas, since "geography" summarizes most of gut microbiota influencing factors such as genetic, birth mode, breastfeeding, socioeconomics, lifestyle, diet, access to healthcare, and environmental exposure to xenobiotic, infections, and pollution.[13] So far, only few studies investigated the gut microbial composition of kidney-transplanted patients with AR.[14-17] All of these cohorts were from China and mostly compared only antibody-mediated rejection (AMR) to control without considering important confounder in microbiome composition.

In our study, we investigated the gut microbial composition of a German cohort of renal transplanted patients with and without AR with very strict inclusion and exclusion criteria with regard to comedication, other diseases, and patient lifestyle to rule out potential confounders effecting gut microbiota. Our aim was to identify a gut microbial fingerprint specific for each group, which could possibly suggest different bacterial functional profiles.

MATERIALS AND METHODS

Study Design, Subject Recruitment, and Study Population

This prospective, cross-sectional case-control study was conducted at the University Hospital of Erlangen, Germany. The study protocol was approved by the Ethics Committee of our center (No. 192_20B) and all participants gave written informed consent. Subject recruitment took place in the outpatient clinic of the Nephrology Department of the University Hospital Erlangen. Between November 2020 and June 2022 outpatient kidney transplants recipients were screened for eligibility according to our inclusion and exclusion criteria.

Basic inclusion criteria were adult age (>18 y), a renal graft function expressed as glomerular filtration rate of at least 15 mL/min and 1 liter of urine output per day, and a transplantation history of at least 6 mo. This last criterion was assigned to rule out dysbiosis associated with perioperative antibiotics and high-dose steroid administration and to minimize the disturbance of the adaptation of the immunosuppression level usually happening during the first months after transplantation.[18] In addition, establishing a minimal time since transplantation allowed us to standardize our patients with regard to medication, since target immunosuppressive levels and prophylactical antimicrobial drugs are reduced/discontinued 6 mo after transplantation in our center.

Specific inclusion criteria assigned to the control group were a stable renal graft function over time, defined as a creatinine fluctuation not >0.3 mg/dL, and a negative history of biopsy-proven AR at any time since transplantation. More detailed inclusion/exclusion criteria are summarized in the Table 1.

In total 76 patients were initially enrolled in the study. From these, 42 patients were included in the AR group and 34 in the no AR group (control group). Diagnosis of AR was performed by renal biopsy and classified according to the 2019 updated BANFF criteria. Patients were recruited shortly before kidney biopsy and had to collect the stool sample before

TABLE 1.

Summary of the inclusion and exclusion criteria

Inclusion criteria for all groups	
Adult age (>18 y old)	
Kidney transplantation longer than 6 mo	
GFR > 15 mL/min and urine output > 1.0 L/d	
Inclusion criteria specific for controls	**Inclusion criteria specific for AR**
Stable graft renal function (fluctuation of the serum creatinine < 0.3 mg/mL)	Biopsy evidence of kidney graft rejection according to BANFF criteria 2019
No history of graft rejection at any time	
No history of BKV infection	
Exclusion criteria for all groups	
Corticosteroid dosing >5 mg/d	
Change of the immunosuppressive regime in the last 30 d	
Kidney graft rejection therapy in the last 30 d	
Systemic antibiotic treatment in the previous 3 mo before providing the fecal sample, with the only exception of cotrimoxazole	
Use of metformin, cyclophosphamide, SSRI, or laxatives in the last 90 d	
Major dietary change during previous month	
Major GI tract surgery in the past 5 y	
Major bowel resection at any time	
Active uncontrolled GI disorders as inflammatory bowel disease, indeterminate colitis, irritable bowel syndrome, persistent infectious gastroenteritis, and colitis or gastritis	
Acute or chronic diarrhea of any etiology	
Clostridium difficile infection	
Helicobacter pylori infection	
Consume of probiotics	
Exclusion criteria specific for controls	**Exclusion criteria specific for the AR group**
Evidence of rejection in prior biopsies	Delivery of the stool sample after initiation of a high-dose corticosteroid therapy
Acute kidney graft dysfunction	Rejection due to lack of immunosuppressant intake

AR, allograft rejection; BKV, BK virus; GFR, glomerular filtration rate; GI, gastrointestinal; SSRI, selective serotonin reuptake inhibitor.

corticosteroid therapy was started. As beginning of antirejection therapy was not delayed to obtain the stool sample, 5 patients were excluded due to inadequate timing of stool collection. One patient was excluded because AR was caused by lack of intake of immunosuppressants >3 wk. The total of samples used to perform the AR analysis was 36, of which 13 had an acute T-cellular–mediated rejection (aTCMR), 8 a chronic T-cellular–mediated rejection (cTCMR), 8 an AMR, and 7 a histological pattern of borderline rejection. This last group did not show a change in glomerular filtration rate nor proteinuria and was, therefore, classified as borderline rejection without clinical relevance (BORi). Among a total of 36 patients diagnosed with AR without exclusion criteria, 25 patients had undergone renal biopsy at the time of inclusion into the study. From the remaining 11 patients, diagnosis of AR was based on at least 2 biopsies from the previous 12 mo. Among the control cohort of 34 patients, 3 patients were excluded either due to initiation of a therapy with metformin (2 patients) and citalopram (1 patient) between enrollment and sample collection. Furthermore, 1 patient was excluded due to serological signs of kidney graft dysfunction immediately after enrollment. From our control cohort reduced to 30 participants, we collected a total of 30 fecal specimens (**Figure S1, SDC**, http://links.lww.com/TXD/A612).

Sample Procurement, Sample Processing, and DNA Purification

Stool samples were collected in a DNA stabilizing solution (PSP Spin Stool Collection Tubes with Stool DNA Stabilizer; Invitek Molecular, Berlin, Germany), following a standard protocol provided with the stool collection tubes, including the use of a feces collection article to minimize contamination. Samples had to be transferred to the laboratory within 3 d and were immediately stored at −80°C upon arrival until DNA extraction. DNA extraction from the stool samples was performed with the PSP Spin Stool DNA Basic Kit (Invitek Molecular) following the manufacturer's instructions. After DNA extraction, DNA was quantified with the Qubit 2.0 double stranded DNA HS assay kit (Invitrogen, Schwerte, Germany). Library generation, sequencing, and bioinformatic analysis described below were performed in the Core Unit Microbiome Analysis Center of Erlangen.

Amplification of the V4 Region of the Bacterial 16S Ribosomal RNA Gene and Sequence Analysis

Ten ng of stool genomic DNA was used in polymerase chain reaction (PCR) amplification of genomic 16S ribosomal RNA V4 regions using the prokaryotic primer pair (515F forward primer: 5′-GTGYCAGCMGCCGCGGTAA–3′; 806R reverse primer: 5′-GGACTACNVGGGTWTCTAAT–3′) containing barcodes on the forward primer 515F (https://earthmicrobiome.org/protocols-and-standards/16s/). The NEBNext Q5 Hot Start Hifi PCR Master Mix (New England Biolabs, Frankfurt am Main, Germany) was used in a reaction employing 25 PCR cycles. The resulting PCR products were purified with AMPure XP Beads (Beckmann Coulter GmbH, Krefeld, Germany), pooled in equimolar ratios and analyzed by 2 × 151 paired-end sequencing on an Illumina MiSeq device (Illumina, Inc., San Diego, CA). Raw fastq files were then imported and analyzed in QIIME2 v2022.2 with DADA2 as the method for quality control, dereplication, and amplicon sequence variant (ASV) table generation.[19,20] The SILVA (a comprehensive on-line resource for quality checked and aligned ribosomal RNA sequence data) small subunit database release 138 was used at a 99% similarity cutoff for taxonomic classification. For further analysis, ASV and taxonomic tables were imported into R (version 4.2) as a phyloseq object and the "vegan" (2.6.4) package was used for diversity analysis and ordination. Before calculation of Bray-Curtis and Jaccard (dis)similarities counts were log transformed for variance stabilization. For calculation of alpha diversity indices, rarefaction to the smallest library size (16172) was performed. The glm function in R was used for fitting logistic regression models. Ggplot2 was used for generation of graphical illustrations.

Statistical Analysis

Clinical Data

Demographic characteristics and clinical parameters to compare kidney-transplanted recipients suffering from rejection to controls were evaluated using Pearson's chi-square test for categorical data and Mann-Whitney *U* test (non-normally distributed data) or Welch's *t* test (normally distributed data with unequal variance) for continuous variables using the Statistical Package for Social Sciences (Chicago, IL). A significance level of 0.05 at 2-tailed hypothesis was given to attribute statistic relevance.

Bioinformatics Data

For estimation of the structure gut microbial communities, we calculated Fisher, Faith's phylogenetic diversity, Abundance-based coverage estimators index, and Shannon alpha diversity indices. The Wilcoxon rank-sum test was used for statistical comparison of alpha diversities between groups.

Beta diversity was analyzed based on Bray-Curtis, Jaccard, and Aitchison distance matrices of microbial communities in all samples and visualized using Principal Coordinate Analysis. The adonis2 function (9999 permutations) within vegan 2.6.4 was used for permutational ANOVA. In the case of multigroup comparisons, pairwise Adonis (https://github.com/pmartinezarbizu/pairwiseAdonis) was used. When applicable, results were adjusted using the Benjamini-Hochberg approach. Analysis of Compositions of Microbiomes with Bias Correction was used to identify the differential abundance of genera and ASVs between the groups.

The functional composition of the intestinal microbiota was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) software,[21] based on the Kyoto Encyclopedia of Genes and Genomes (KEGG).[22]

RESULTS

Demographic and Clinical Characteristics of the Kidney Transplant Recipients

Statistical comparison of demographic and clinical characteristics between controls and patients with AR showed no relevant differences in body mass index or time since transplantation, while age at enrollment was significantly lower in the AR group (Table 2). We further looked for correlations between age and AR as well as serum creatinine and AR and could find strong correlations between both parameters. Patients with AR had higher serum creatinine compared with control patients and higher age was less likely to have AR (**Figure S2A** and **B, SDC**, http://links.lww.com/TXD/A612).

TABLE 2.

Comparison of demographic and clinical characteristics between kidney transplant patients with AR and controls

Variable	Controls (n = 30), n (%)	AR (n = 36), n (%)	*P* at *P* < 0.05
Age at enrollment, y	56	50	**0.01**
Male sex	7 (23)	18 (50)	**0.02**
BMI (kg/m^2)	26,8	27,4	0.749 (NS)
Time since kidney transplantation, y	8.6	6.6	0.190 (NS)
Underlying disease			
IgA nephropathy	6 (20)	4 (11)	
ADPKD	6 (20)	1 (3)	
Diabetic nephropathy	2 (6)	3 (8)	
Chronic pyelonephritis due to reflux nephropathy	1 (3)	5 (14)	
Secondary FSGS	2 (6)	1 (3)	
Hereditary kidney diseases	2 (6)	0 (0)	
Interstitial nephritis	2 (6)	2 (6)	
Unknown	6 (20)	9 (25)	
Others	3 (10)	11(30)	
Diabetes	8 (26)	7 (20)	0.486 (NS)
Type 1	2 (6)	2 (6)	
Type 2	3 (10)	2 (6)	
Posttransplant diabetes	3 (10)	3 (8)	
Serum creatinine (mg/dL)	1,35	2,17	**<0.0444**
CKD stage after KDIGO	3b	4	**0.00001**
Medications			
PPI	18 (60)	26 (72)	0.332 (NS)
Cotrimoxazole	9 (30)	18 (50)	0.109 (NS)
Immunosuppression			
Tacrolimus	26 (86)	31 (87)	0.922 (NS)
Cyclosporin A	2 (6)	2 (6)	0.947 (NS)
MPA or MMF	26 (86)	24 (66)	0.053 (NS)
Everolimus or sirolimus	3 (10)	8 (22)	0.204 (NS)
Induction therapy			
Anti-thymoglobuline	11 (37)	12 (33)	0.777 (NS)
Basiliximab	16 (53)	14 (39)	0.240 (NS)
Unknown	3 (10)	10 (28)	
Donor type			
Living donor	9 (30)	9 (25)	0.649 (NS)
Deceased donor	21 (70)	27 (75)	0.649 (NS)
Other transplants	3 (10)	5 (14)	0.629 (NS)
Pancreas	2 (6)	4 (12)	
Liver	1 (3)	1 (3)	
Donor-specific antibody	5 (16)	13 (36)	**0.0378**
MFI <5000	5 (16)	5 (14)	
MFI >5000	0 (0)	8 (22)	
Rejection type			
BORi		7 (19)	
aTCMR		13 (36)	
cTCMR		8 (22)	
AMR		8 (22)	

ADPKD, autosomal dominant polycystic kidney disease; AMR, antibody-mediated rejection; AR, allograft rejection; aTCMR, acute T-cellular–mediated rejection; BMI, body mass index; BORi, borderline rejection without clinical relevance; CKD, chronic kidney disease; cTCMR, chronic T-cellular–mediated rejection; FSGS, focal segmental glomerulosclerosis; KDIGO, Kidney Disease Improving Global Outcomes; MFI, mean fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolic acid; NS, not significant; PPI, proton pump inhibitor.

Male gender was significantly more represented in the group of AR. No relevant differences in the standard immunosuppressive therapy or in the use of proton pump inhibitors (PPIs) and cotrimoxazole were found. As expected, patients with AR had significantly higher level of donor-specific antibody, worse serum creatinine level at the time of sampling and more advanced stage of chronic kidney disease. The presence of comorbidities like diabetes or the coexistence of another transplanted organ was not different between the 2 groups.

Relative Abundance, Alpha Diversity, and Beta Diversity of Gut Microbiota of Patients With Kidney Transplant Rejection and Controls

The relative abundances of different bacterial taxa between kidney transplant rejection and controls was compared at a phylum and family level as shown in Figure 1A by stacked bar plotting. At phylum level, the relative abundance of *Proteobacteria*, *Desulfobacterota*, and *Bacteroidota* was higher in the AR group, whereas controls were characterized by higher

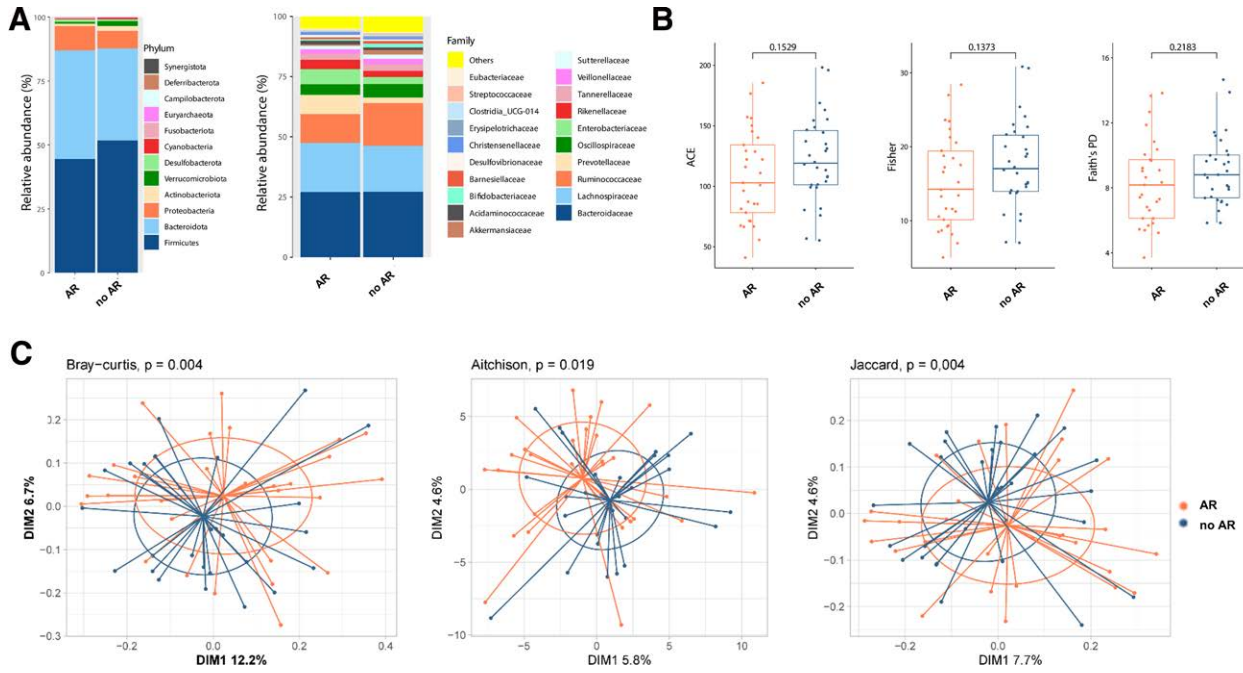

FIGURE 1. Comparison of gut microbiota of AR and control patients shows changes in relative abundances of different bacterial taxa, richness, and beta diversity in AR. A, Relative abundance (%) of gut microbial community structure of the study population at the phylum and family level. A difference >5% was regarded as being relevant. B, Alpha diversity metrics (ACE, Fisher's alpha, and Faith's PD) at ASV level were compared between individuals with and without kidney graft rejection. Wilcoxon rank-sum tests were used for statistical analysis. C, ASV-level gut microbial community variation represented by principal coordinates analysis (Bray-Curtis, Aitchison distance dissimilarity, and Jaccard distance). PERMANOVA corrected for age was used for statistical analysis and *P* values are given above the graphs. The number of control patients was 30 and the number of patients with clinically relevant AR was 29 (36 patients with AR minus 7 patients with BORi). ACE, Abundance-based coverage estimators index; AR, allograft rejection; ASV, amplicon sequence variant; BORi, borderline rejection without clinical relevance; DIM1, dimension 1; DIM2, dimension 2; no, no rejection; PD, phylogenetic diversity; PERMANOVA, permutational ANOVA.

abundance of *Firmicutes*, *Cyanobacteria*, *Verrucomicrobiota*, and *Actinobacteriota*. At family level, the relative abundance of *Enterobacteriaceae* and *Prevotellaceae* were higher in the AR group, whereas a higher relative abundance of *Ruminococcaceae* and *Oscillospiraceae* was found in controls.

Alpha diversity given as ACE index ($P = 0.1529$), Fisher's alpha ($P = 0.1373$), and Faith's phylogenetic diversity ($P = 0.2183$) were not significantly different between microbiota of patients with AR and controls. However, a clear trend of lower richness within samples from patients with AR was identified through these analyses (Figure 1B).

Permutational ANOVA analysis corrected for age based on Aitchison distance, Bray-Curtis dissimilarity, and Jaccard distance revealed differences in microbial beta diversity between stool samples from controls and patients with AR with a *P* value respectively of $P = 0.019$, $P = 0.004$, and $P = 0.004$, as shown in the Figure 1C.

As age is known to influence gut microbiome diversity, we included a multiple linear regression analysis for alpha and beta diversity of our data. However, age had no significant effect on alpha diversity of gut microbiota in our study (data not shown). Even though age had a small influence on beta diversity of gut microbiota the effect was much less than that of rejection. There was no statistic interaction between rejection and age when using Bray-Curtis and Jaccard analysis.

Relative Abundance, Alpha Diversity, and Beta Diversity of Gut Microbiota of Patients With Different Subtypes of Kidney Transplant Rejection and Controls

The relative abundances of different bacterial taxa according to type of rejection are shown in Figure 2A. *Bacteroidetes* was the most predominant phylum in the cTCMR group, followed by *Firmicutes* and *Proteobacteria*. In the aTCMR, AMR, BORi, and control groups, the prevailing phylum was *Firmicutes*, followed by *Bacteroidota* and *Proteobacteria*. A higher relative abundance of *Proteobacteria* was seen in the AMR group, whereas the lowest in the cTCMR group. *Cyanobacteria*, *Fusobacteriota*, *Verrucomicrobiota*, *Actinobacteriota*, and *Desulfobacterota* constituted the next most predominant phyla. *Verrucomicrobiota* and *Actinobacteriota* were most expressed in the control group and lowest found in cTCMR. *Bacteroidaceae*, *Lachnospiraceae*, and *Ruminococcaceae* were the most abundant families in each subgroup of rejection as well in controls. The highest abundance of *Bacteroidaceae* was seen in samples from patients with cTCMR, the highest abundance of *Lachnospiraceae* was seen in the BORi group, whereas the highest abundance of *Ruminococcaceae* between controls. *Prevotellaceae* were most represented in the aTCMR and cTCMR groups. *Enterobacteriaceae* were highly abundant in the AMR group and underrepresented in controls. *Oscillospiraceae*, *Rikenellaceae*, *Tannerellaceae*, *Veillonellaceae*, *Sutterellaceae*, *Acidaminococcaceae*, and *Desulfovibrionaceae* were the next most predominant families.

When splitting the AR group according to the 4 types of AR, major differences in alpha diversity measures were identified between patients with cTCMR and controls. Patients without AR showed higher bacterial richness ($P < 0.05$). Indeed, Abundance-based coverage estimators index ($P = 0.023$) and Fisher ($P = 0.023$) were significantly lower in the cTCMR group as shown in the Figure 2B. Furthermore, trends in these differences were also detected between cTCMR and AMR (however, they did not reach statistical significance).

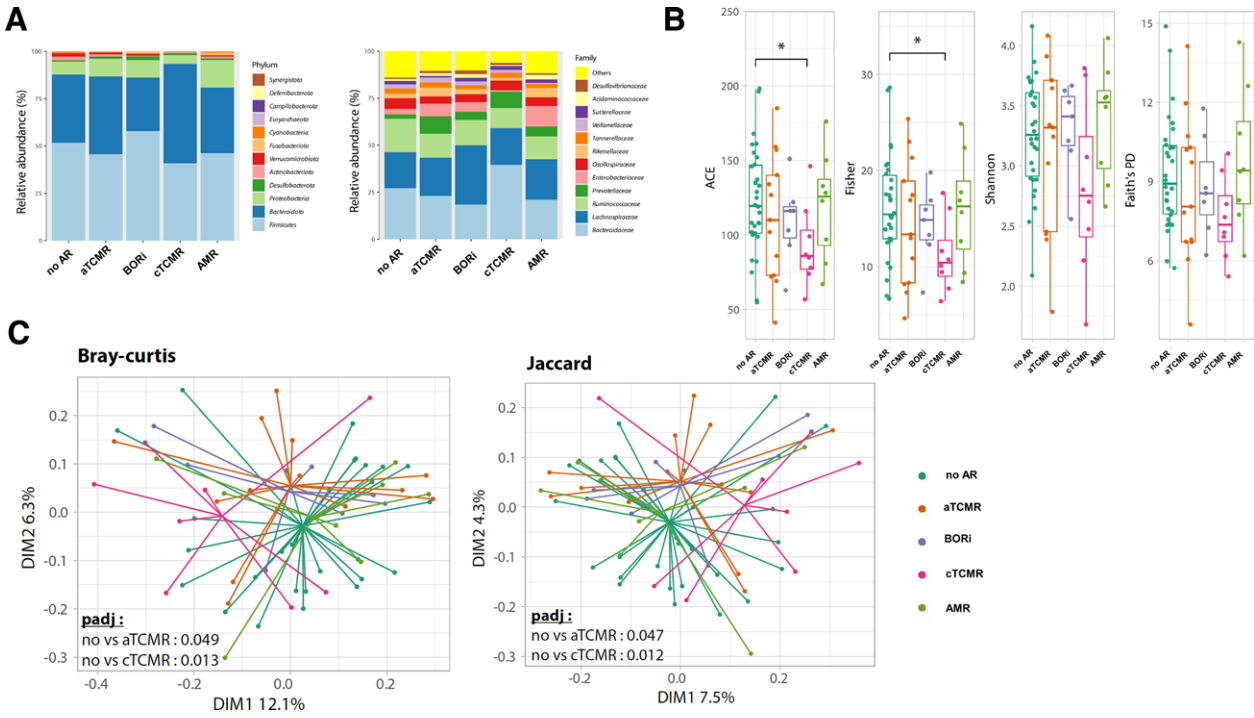

FIGURE 2. Comparison of gut microbiota between different types of AR and control reveals differences in relative abundance of microbiota, alpha diversity, and beta diversity. A, Stacked bar plots representing relative abundance (%) of gut bacterial composition at phylum and family level of kidney-transplanted patients stratified as indicated. B, Alpha diversity metrics (ACE, Fisher's alpha, Shannon, and Faith's PD) were used for comparison of patient cohorts. Wilcoxon rank-sum tests were used for statistical analysis. C, ASV-level gut microbial community variation represented by principal coordinate analysis (Bray-Curtis dissimilarity and Jaccard distance). PERMANOVA followed by Benjamini-Hochberg correction was used for statistical analysis. The number of control patients included in the analysis was 30. Patients with biopsy-proven AR were 36, of which 13 had an aTCMR, 8 a cTCMR, 8 an AMR, and 7 a histological pattern of borderline rejection but no change in GFR (BORi). *P < 0.05. ACE, Abundance-based coverage estimators; AMR, antibody-mediated rejection; AR, allograft rejection; ASV, amplicon sequence variant; aTCMR, acute T-cellular–mediated rejection; BORi, borderline rejection without clinical relevance; cTCMR, chronic T-cellular–mediated rejection; DIM1, dimension 1; DIM2, dimension 2; GFR, glomerular filtration rate; no, no rejection; padj, adjusted P-value; PD, phylogenetic diversity; PERMANOVA, permutational ANOVA.

Beta diversity analysis comparing the (dis)similarity of the microbial composition of controls to the 4 AR subgroups revealed statistically significant differences between samples derived from controls and those from patients with cTCMR with an adjusted P-value of $P = 0.013$ based on Bray-Curtis and of $P = 0.012$ based on Jaccard distance. Patients with aTCMR showed significant differences in beta diversity compared with controls, with an adjusted P-value of $P = 0.049$ based on Bray-Curtis and of $P = 0.047$ based on Jaccard distance (Figure 2C).

Serum creatinine correlated with the probability of aTCMR, cTCMR, and AMR but not BORi in our study group (Figure 3). Using Fisher's alpha diversity index, we looked for a correlation between alpha diversity of gut microbiota and subtypes of kidney transplant rejection. Only cTCMR was correlated with alpha diversity (Figure 3F). The lower the alpha diversity of gut microbiota the higher was the probability of cTCMR.

Specific Taxa Associated With Kidney Graft Rejection and Controls

Kidney transplant patients without AR showed significant enrichment of rather anti-inflammatory taxa, whereas in the AR group bacteria well known for their role in chronic inflammation were increased. Among others, ASV 362 belonging to the genus *Bacteroides*, ASV 312 belonging to the family *Tannerellaceae*, and ASV 422 belonging to the genus *Alloprevotella* were enriched in the group with no AR (fold change 2.1; $P < 0.001$, fold change 1.96; $P < 0.01$, and fold change 1.3; $P < 0.001$). In contrary, ASV 365 was depleted in no AR (fold change –2,54; $P = 0.04$) (Figure 4A and B).

Next, we looked for ASV that were differently abundant between no AR and different types of AR. Comparing aTCMR to no AR, the aTCMR group was characterized by a higher abundance of ASVs representing *Bacteroides* and *Eubacterium* genera (ASV 365, fold change 3.3, $P = 0.02$ and ASV 968, fold change 2.3, $P = 0.03$) (Figure 5A). ASV belonging to ASF 356 (ASV 899) and *Alloprevotella* (ASV 422) were decreased in borderline rejection compared to no AR (fold change –1.1, $P = 0.04$ and fold change –1.2, $P = 0.04$) (Figure 5B). ASV 603 belonging to *Faecalibacterium*, and ASV 312 belonging to *Tannerellaceae* were decreased in cTCMR compared with no AR (fold change –4.3, $P < 0.001$ and fold change –2.2, $P < 0.001$), whereas ASV 1207 belonging to *Lachnoclostridium* was significantly enriched in cTCMR versus no AR (fold change 3.2, $P = 0.003$) (Figure 5C). ASV 45 (*Escherichia-Shigella*) was increased in the AMR group compared with no AR (fold change 3.6, $P = 0.002$), whereas ASV 362 belonging to *Bacteroides* was decreased in AMR (fold change –2.1, $P < 0.001$) (Figure 5D). A list of fold changes and P values comparing all detected ASV in the stool microbiome of no AR to the different types of AR can also be found in the supplements (**Table S1, SDC**, http://links.lww.com/TXD/A612).

Predicted Functional Pathways Associated With Kidney Graft Rejection and Controls

PICRUSt2-based prediction analysis of changed gene ontology groups and KEGG pathways revealed that among others, the microbiome of controls was characterized by higher expression of cytolethal distending toxin subunits, dimethylsulfone monooxygenase, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine transaminase, DNA-directed RNA polymerase subunit, 2-methylcitrate dehydratase, and 2-methylcitrate synthase. Microbiome of AR was characterized by higher abundance of dTDP-6-deoxy-L-talose 4-dehydrogenase (Figure 6A and B).

DISCUSSION

AR is the most important reason for kidney graft failure in renal transplant recipients. However, it is not that easy to diagnose, based on laboratory parameters, and the characterization of AR type usually requires a kidney graft biopsy. So far, no noninvasive marker can sufficiently replace the information contents of a biopsy result. However, in many clinical situations with an increased risk for bleeding complications (ie, patients under anticoagulation therapy), it would be helpful to have additional tools to estimate the general risk of AR. In the last years, specific interactions between the host immune system and the intestinal microbiome became more and more evident since different microbial community structures can have a distinct impact on graft outcome, from promoting, to inhibiting or being neutral to transplant survival.[23] Here, we studied stool microbiome from transplant patients with and without AR. We found that the gut microbial composition of patients with AR was significantly different from that of controls, characterized by a lower alpha diversity and higher abundance of inflammation-sustaining taxa.

In contrast, the control group was characterized by a higher alpha diversity and an eubiotic, immunomodulating microbial fingerprint.

Beta diversity analysis revealed statistically relevant differences in the microbial composition between controls and patients with AR with major differences between patients with cTCMR and controls. Furthermore, the lower the alpha diversity the higher also the probability for cTCMR. The predicted functional analysis of microbial communities showed a greater, statistically significant variety of enzymes and metabolic pathways in the control group than in the AR cohort.

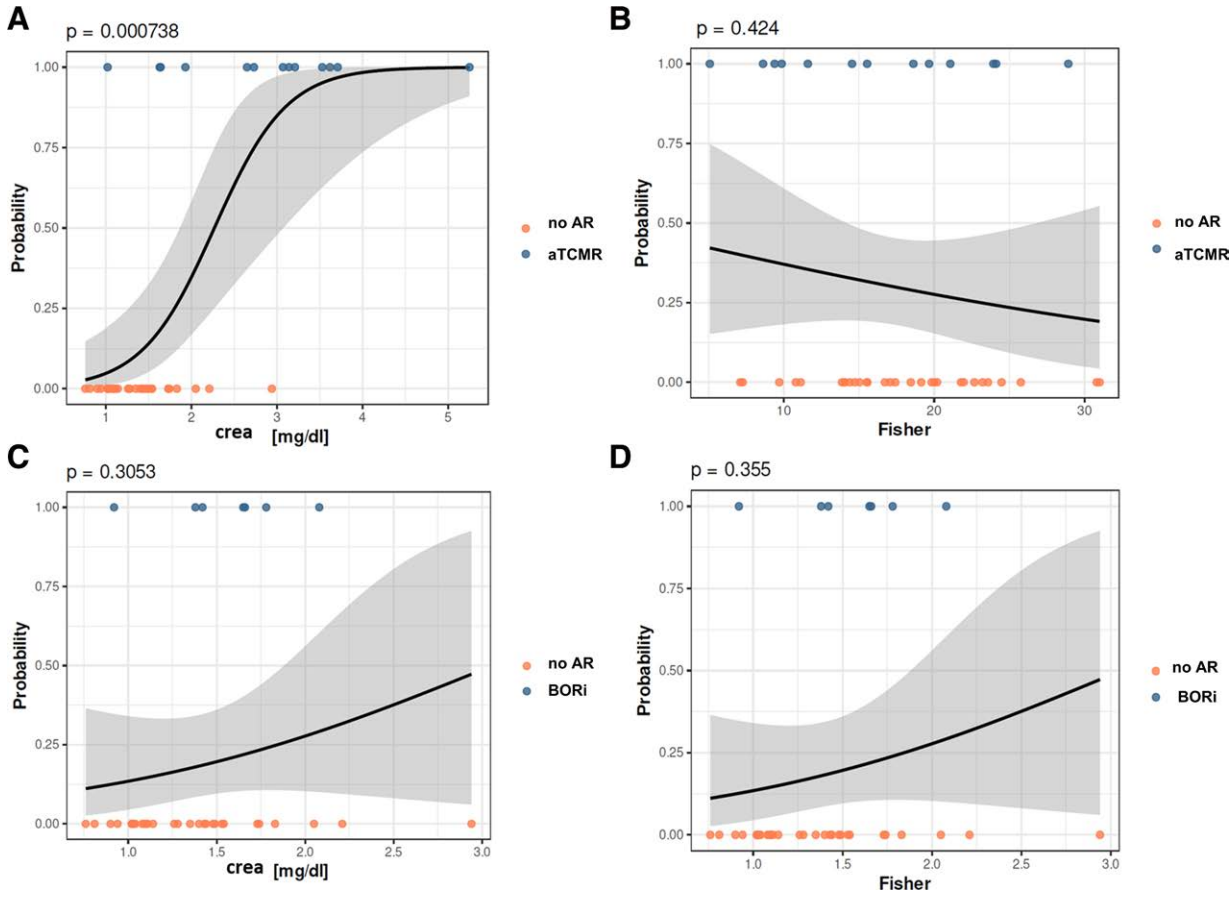

FIGURE 3. Serum creatinine correlates with the portability of aTCMR, cTCMR, AMR, and the lower the alpha diversity of gut microbiome the higher was the probability of cTCMR. Logistic regression analysis between serum creatinine (crea) and probability of aTCMR (A), BORi (C), cTCMR (E), and AMR (G) as well as between Fisher's alpha diversity of gut microbiome (Fisher) and probability of aTCMR (B), BORi (D), cTCMR (F), and AMR (H). Statistical analysis of the model is given as *P* values above the graphs. The number of control patients included in the analysis was 30. Patients with biopsy-proven AR were 36, of which 13 had an aTCMR, 8 a cTCMR, 8 an AMR, and 7 a histological pattern of borderline rejection but no change in GFR (BORi). AMR, antibody-mediated rejection; AR, allograft rejection; aTCMR, acute T-cellular–mediated rejection; BORi, borderline rejection without clinical relevance; cTCMR, chronic T-cellular–mediated rejection; GFR, glomerular filtration rate; no, no rejection.

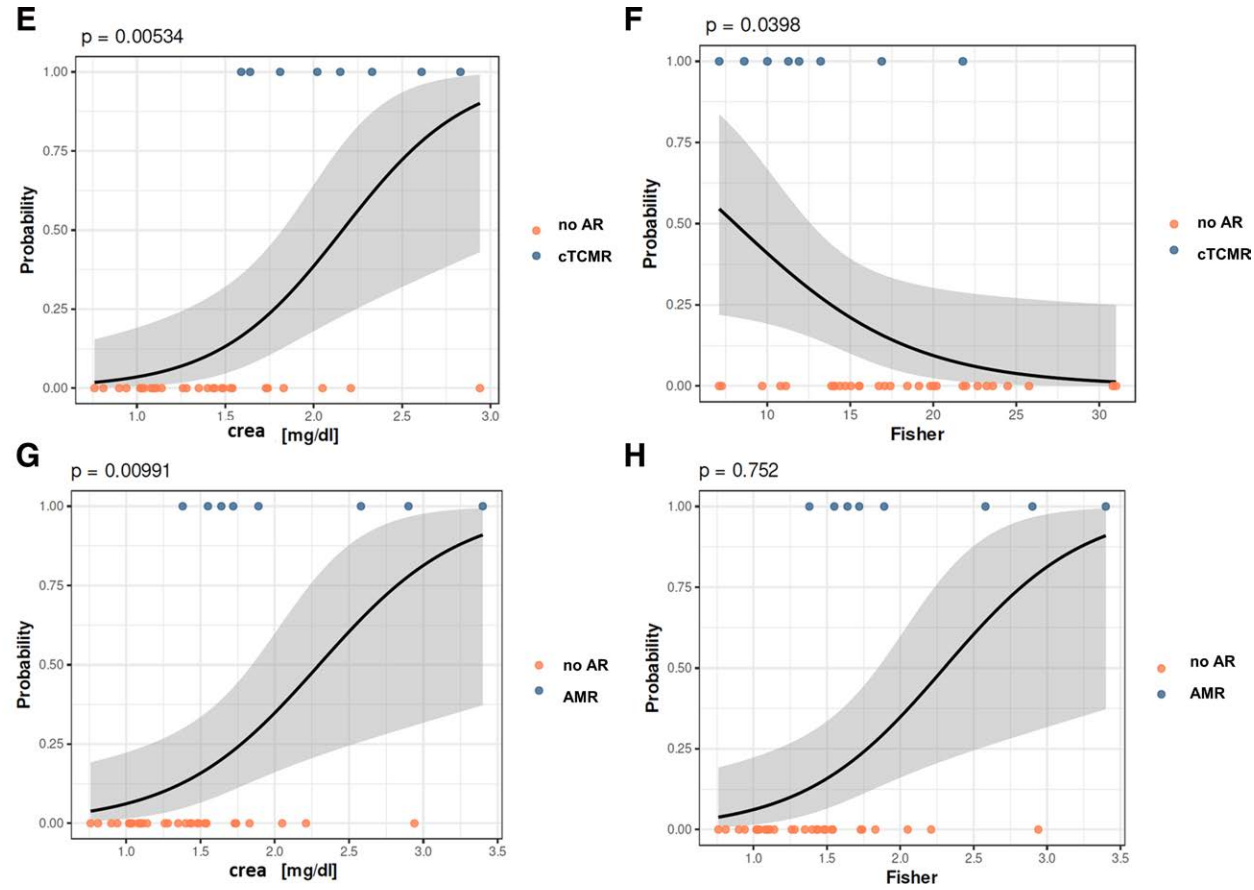

FIGURE 3. Continued

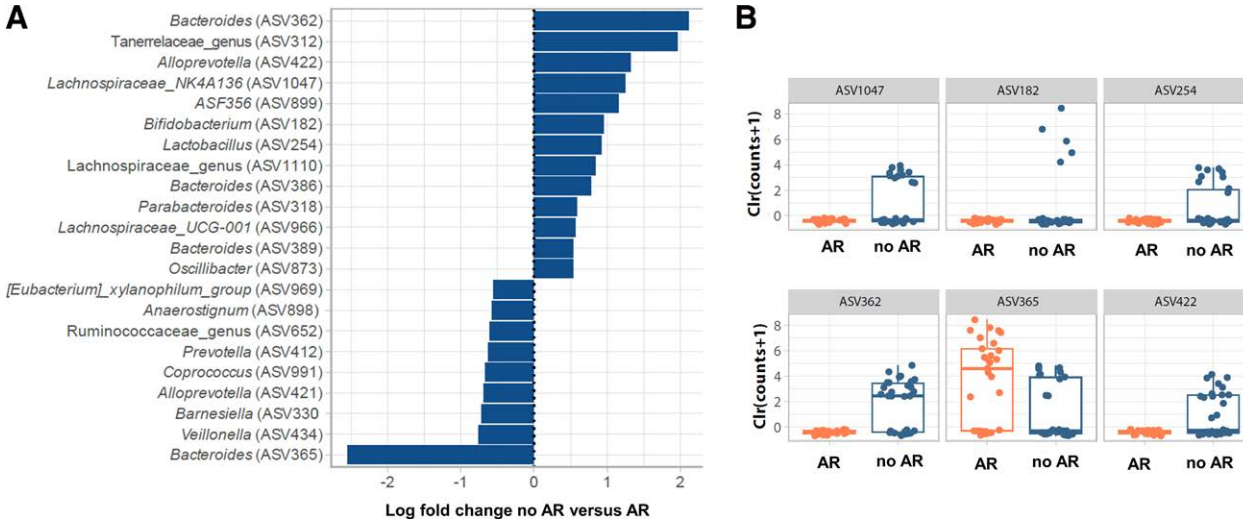

FIGURE 4. Distribution of taxa between control and AR showing differences in ASVs between both groups. A, AnCOM-BC analysis showing differentially abundant ASVs between controls (no AR) and patients with kidney graft rejection (AR). Length of the horizontal bar represents the log2 transformed fold changes from AnCOM-BC analysis, indicated by vertical dotted lines. B, Selected differentially abundant ASVs were compared no AR and AR before plotting pseudocount addition and centered log ratio transformation was applied to the table of ASV counts. AnCOM-BC, Analysis of Compositions of Microbiomes with Bias Correction; AR, allograft rejection; ASV, amplicon sequence variant; no, no rejection.

Wang et al[16] analyzed fecal microbiome of kidney transplantation recipients with AMR and controls. They found that bacterial richness significantly decreased in the AMR group similarly to our findings.[16]

To exclude that alterations of diversity indices in our study were simply due to differences in age between the cohorts we performed regression analysis and could exclude that age had an effect on alpha or beta diversity of gut microbiota in our

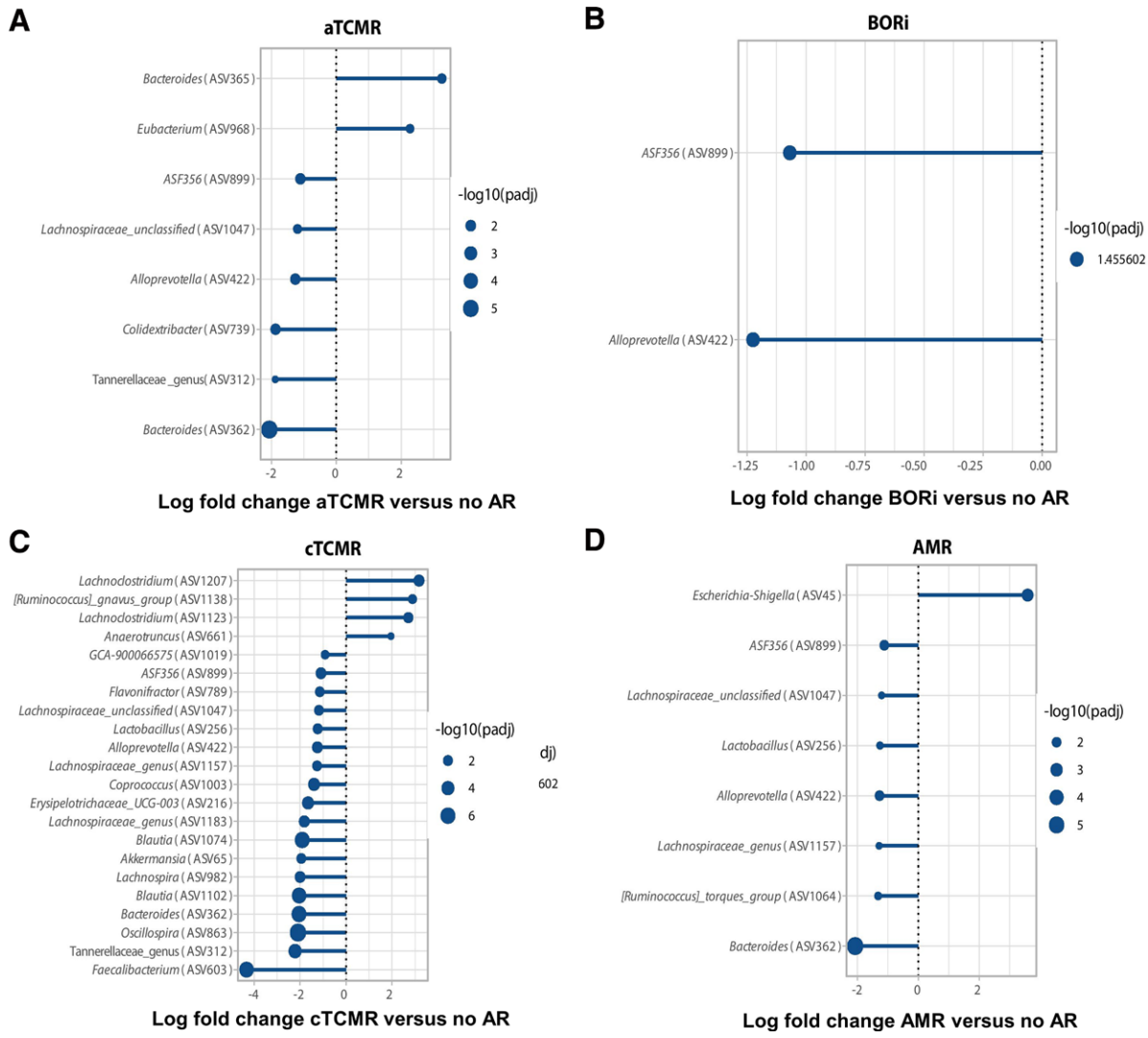

FIGURE 5. Significant changes in distribution of ASVs between subgroups of AR. AnCOM-BC analysis showing differentially abundant genera between patients with kidney graft rejection according to rejection subgroups compared with controls: (A) aTCMR, (B) BORi, (C) cTCMR, (D) AMR, and no. The number of control patients included in the analysis was 30. Patients with biopsy-proven AR were 36 of which 13 had an aTCMR, 8 a cTCMR, 8 an AMR, and 7 a histological pattern of borderline rejection but no change in GFR (BORi). X-axis gives log fold change of genera abundance of control (no) vs kidney graft rejection subtype. AMR, antibody-mediated rejection; AnCOM-BC, Analysis of Compositions of Microbiomes with Bias Correction; AR, allograft rejection; ASV, amplicon sequence variant; aTCMR, acute T-cellular–mediated rejection; BORi, borderline rejection without clinical relevance; cTCMR, chronic T-cellular–mediated rejection; GFR, glomerular filtration rate; no, no rejection; padj, adjusted *P*-value.

study. Among the taxa significantly enriched in the AR cohort, most are notable for their proinflammatory interaction with our immune system. The *Escherichia-Shigella* group is well known for excessively activate the gut mucosal immunity mediated by NOD-like receptor protein 3 inflammasome.[24] High abundance of this group has been observed in several inflammation-mediated diseases, including brain amyloidosis,[25] inflammatory bowel disease,[26] and nonalcoholic fatty liver disease.[27] *Ruminococcus gnavus* was found significantly enriched in the gut of patients with several inflammatory diseases including spondyloarthritis[28] and inflammatory bowel disease.[29] Members of this genus are able to produce an inflammatory polysaccharide, which induces the secretion of the proinflammatory cytokine TNF-α by dendritic cells.[30] *Anaerotruncus* has been associated with Parkinson's disease[31] and hepatocellular carcinoma.[32] On the other hand, the gut microbial fingerprint of controls was characterized by high abundance of *Bifidobacterium* and *Lactobacillus*, well known gut commensals, whose beneficial properties led to the integration of some species of these genera into probiotic supplements.[33,34] Several strains of *Lactobacillus* have a remarkable capability to induce cluster of differentiation 4 and forkhead box P3 positive regulatory T cells, which express high levels of interleukin-10 and transforming growth factor-β and downregulate Th1, Th2, and Th17 cytokines,[35-37] conferring immune-regulating properties to this genus.

Comparable to our results, an increase in *Proteobacteria*, *Bacteroidota*, and *Enterobacteriaceae* as well as decrease in *Firmicutes* and *Ruminococcaceae* have been shown before in acute rejection.[16] Furthermore, increased *Faecalibacterium* and *Clostridia* in controls compared with rejections were also seen.[16]

PICRUSt2-based prediction analysis of changed gene ontology groups and KEGG pathway analysis revealed a significant increase in enzymes responsible for responsible for a broad-spectrum of multidrug resistance (paired small multidrug

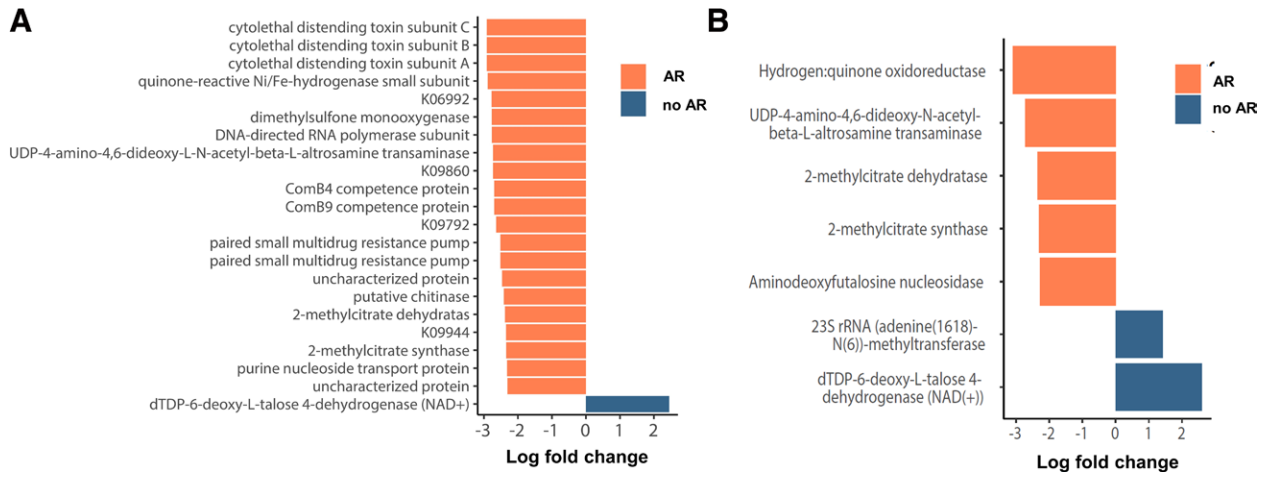

FIGURE 6. Pathway analysis of microbiota of patients with AR and controls shows higher expression of cytolethal distending toxin subunits and distinct monooxygenases, transaminases, and dehydratases in AR. PICRUSt2-based prediction analysis of changed gene ontology groups (A) and KEGG pathways (B) between patients with AR and controls. AnCOM-BC was used for differential abundance analysis. Only groups significantly different ($P < 0.05$) after Benjamini-Hochberg correction were shown. AnCOM-BC, Analysis of Compositions of Microbiomes with Bias Correction; AR, allograft rejection; KEGG, Kyoto Encyclopedia of Genes and Genomes; no, no rejection; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States.rRNA, ribosomal RNA.

resistance pump),[38,39] for degradation of the dimethyl sulfide into the bacterial preferred source of sulphur, namely sulphite (dimethylsulfone monooxygenase), and for the metabolisms of short-chain fatty acids (2-methylcitrate dehydratase and 2-methylcitrate synthase)[40,41] in control patients. In contrast, enzymes for the assembly of "O-antigen" of the lipopolysaccharides (dTDP-6-deoxy-L-talose 4-dehydrogenase) and 23S ribosomal RNA (rRNA) (adenine^{1618}-N^{6})-methyltransferase were increased in AR.

Our study has some limitations: Sequencing of stool bacteria was performed using bacterial 16S rRNA sequencing method, which allows to identify bacterial taxa up to the genus level, leaving the species undefined. For example, the 16S gene sequences of the group "*Escherichia-Shigella*" are indistinguishable with the 16S rRNA sequencing because this group is phylogenetically and genetically highly related.[42] Furthermore, the 16S rRNA gene sequencing only allows to predict the function of the gut microbiota. To reach the species level and also to be able to perform further and deeper taxonomic and functional analysis, other methods such as shotgun metagenomics or shotgun metatranscriptomics would be required.[9] However, 16S rRNA sequencing is a cost-efficient method to provide first screening information of the microbiota.

The second limitation of this study regards the lack of information about the causality or casuality of the differences found in the microbiota between the 2 groups.

Third, the sample size of this study is moderate, nevertheless comparable to the majority of the studies on human microbiota from the past. Wang et al[16] analyzed fecal metabolome of 24 kidney transplantation recipients with AMR and 29 controls. Another study by Lee et al[14] only included 26 kidney transplant recipients and also did not consider the same strict inclusion criteria we used. Clearly, to be able to establish an ASV-based biomarker for AR a larger size trial would be required.

We aimed to validate our results with that of published cohorts. To compare our ASVs with those of Wang et al,[16] we extracted the V4 region of the dataset of Wang et al,[16] who employed 2 × 300 bp sequencing with primers for detection of the V3–V4 region. However, typical technical limitations of the 2 × 300 bp sequencing approach (lower read quality at library ends) quality prevented direct comparison of our data with the Wang et al[16] dataset on the ASV level. Furthermore, comparing both studies is also challenging in itself as Wang et al[16] used a cohort from China, only include AMR as rejection type and had much weaker exclusion criteria compared with our cohort from Germany that included TCMR and AMR and used very strict exclusion criteria to keep other factors that might influence gut microbial composition as similar as possible between rejection group and control. Furthermore, Wang et al[16] also used a group with patients suffering from chronic kidney disease as a control for their transplanted patients with AMR, which is not accurate in our mind due to multiple confounding differences in these groups (immunosuppression, other medication, kidney function, etc.).[16]

On the other hand, our study also has several strengths: We had a rigorous protocol for patient's selection, which has been elaborately designed, and adhered to. Indeed, our strict inclusion and exclusion criteria enabled us to eliminate microbiota-disturbing factors such medications as selective serotonin reuptake inhibitors, antibiotics, metformin, or immunosuppressant at high dosage, while allowing the presence of microbiota-disturbing medications like PPIs, under the condition that a subanalysis matching PPI users to nonusers would investigate whether that could be a relevant interfering factor. We also considered age and serum creatinine the analysis.

Furthermore, this was a monocentric study, in which just 1 medical doctor was responsible for the patient's enrollment, reducing the risk of interpersonal biases.

Another strength of our study was the performance of bioinformatics analysis with different statistical analysis with most updated microbiome tools and statistical tests. Furthermore, the use of the ASV clustering method will allow other researchers to compare their results with ours in the future.

If confirmed by a study of major sample size, a noninvasive and cost-effective, ASVs-based biomarker for AR (or non-AR), could have a great clinical impact in the practice of

transplant monitoring. For example, performing "protocol" biopsies, such as the one usually made 1 y after transplantation to recognize subclinical AR, could become a more targeted procedure, addressed to those patients with AR-related ASVs, or to those without non-AR–associated ASVs. Furthermore, an ASV-based score could be integrated as standard parameter to stratify patients into AR-risk categories. Since no similar studies in Germany nor in Europe have been conducted so far, the current study offers new insight into gut dysbiosis occurring during AR in a German population cohort considering important factors that can influence gut microbiota in the inclusion/exclusion criteria. An open question remains whether the different microbiota signatures are a co-cause or a consequence of AR. Hence, we suggest to propose future prospective trials to investigate this.

CONCLUSIONS

Considering multiple influencing factors of gut microbiota, a distinct microbial fingerprint was detectable in stool samples from patients with AR, which might either facilitate immunologic dysbalance toward kidney graft or might be a result of it. In the future, specific subgroups of stool ASV might serve as a noninvasive biomarker for AR.

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