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Original Article

Gut microbiome alterations precede graft rejection in kidney transplantation patients

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Abbreviations: ABMR, antibody-mediated rejection; *acK*, acetate kinase; *bcD*, butyryl-CoA dehydrogenase; *buT*, butyryl-CoA:acetate CoA-transferase; CKD, chronic kidney disease; DZIF, German Center of Infectious Diseases; KEGG, Kyoto Encyclopedia of Genes and Genomes; KT, kidney transplantation; *mmdA*, methylmalonyl-CoA decarboxylase; MPA, mycophenolic acid; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; PICRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; SCFA, short-chain fatty acid; TCMR, T cell–mediated rejection; Treg, regulatory T cells; Tx, transplant.

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ABSTRACT

Kidney transplantation (KT) is the best treatment for end-stage kidney disease, with graft survival critically affected by the recipient's immune response. The role of the gut microbiome in modulating this immune response remains underexplored. Our study investigates how microbiome alterations might be associated with allograft rejection by analyzing the gut microbiome using 16S rRNA gene amplicon sequencing of a multicenter prospective study involving 562 samples from 245 individuals of whom 217 received KT. Overall, gut microbiome composition showed gradual recovery post-KT, mirroring chronic kidney disease (CKD)-to-health transition as indicated by an increase in Shannon diversity. Prior to graft rejection, we observed a decrease in microbial diversity and short-chain fatty acid–producing taxa. Functional analysis highlighted a decreased potential for short-chain fatty acid production in patients preceding the rejection event, validated by quantitative PCR for the production potential of propionate and butyrate. Postrejection analysis revealed normalization of these microbiome features. Comparison to published microbiome signatures from CKD patients demonstrated a partial overlap of the microbiome alterations preceding graft rejection with the alterations typically found in CKD. Our findings suggest that alterations in gut microbiome composition and function may precede and influence KT rejection, suggesting potential implications as biomarkers or for early therapeutic microbiome-targeting interventions.

1. Introduction

Kidney transplantation (KT) represents the best treatment option for patients with advanced kidney failure (chronic kidney disease [CKD] G5).[1] Despite ongoing efforts to prevent the progression of CKD and related comorbidities, successful KT and graft survival, especially the prevention of graft rejection, is of utmost importance. Graft rejection is the medium- and long-term complication with the highest impact on graft and recipient survival.[2] Whether a patient experiences graft rejection or not is largely dependent on immune mechanisms, the modifiers of which still remain poorly understood.[3]

Of late, the gut microbiome gained attention as a key modulator of immunity in both health and disease conditions.[4] Microbiota, their metabolites, and associated molecules interact with the host, including mucosa-associated and systemic immune cells, and thereby shape host immunity.[5] Patients with CKD exhibit marked alterations to their gut microbiome composition and subsequent dysregulation of metabolite abundance.[6] Mainly, a switch from saccharolytic to proteolytic fermentation can be observed,[7] leading to lower levels of antiinflammatory[8] short-chain fatty acids (SCFA),[9] and increased levels of proinflammatory metabolites, such as tryptophan-derived indoxyl sulfate.[9]

After KT, CKD-related microbiome alterations can persist, presumably supported by additional factors such as immunosuppression.[10] Recent studies demonstrated an association between lower gut microbial diversity in patients with organ transplantation and increased mortality.[11,12] Experimental studies implicate the gut microbiota in alloimmunity and graft rejection, as germ-free and antibiotic-treated mice show prolonged skin graft survival compared to conventional mice.[13] In line with this, studies in mice showed that prebiotic and postbiotic SCFA augmentation mediates donor-specific tolerance to kidney allografts through the induction of regulatory T cells (Treg).[14] Clinical trials are underway, aiming to improve CKD-associated microbiome alterations by prebiotics[15] or at improving immune regulation by supplementation of Treg.[16]

In this study, we analyzed longitudinal changes in the composition and function of the gut microbiome of KT recipients enrolled in the transplant (Tx) cohort of the German Center of Infectious Diseases (DZIF)[17] and connected them with graft rejection. In a propensity score–matched subcohort we identify microbial alterations preceding graft rejection and Tx dysfunction.

2. Materials and methods

2.1. Study population and design

All patients analyzed in this study were part of the Tx cohort of the DZIF, which is a multicenter prospective cohort study conducted at 4 German Tx centers (University Hospitals in Heidelberg, Munich (Technical University and Ludwig Maximilian University) and Tübingen).[17] Together, these centers cover over 20% of solid organ Tx in Germany[18] providing a representative picture of post-Tx courses in Germany.

For the present study, we analyzed data from all KT recipients who consented to participate in accordance with the Declaration of Helsinki and received a KT between 2014 and 2021. Patients

receiving multiorgan transplantation, patients who underwent previous solid organ transplantation, and participants without fecal samples (or less than 2 fecal samples in nonrejection patients) were excluded from our analysis (Supplementary Fig. S1). Ethics approval was obtained from all participating centers (Heidelberg #S-585/2013, TU Munich #5926/13, LMU Munich #380-15, Tübingen #327/2014BO1), and all participants provided written informed consent. The experimental and computational analysis of fecal samples was approved by the ethics board of Charité – Universitätsmedizin Berlin (EA2/208/21). The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the "Declaration of Istanbul on Organ Trafficking and Transplant Tourism."

The study design, including biomaterial collection, has been described previously.[17] Fecal samples were collected in DNA stabilizing buffer (STRATEC). Study visits were performed immediately before KT, at months 3, 6, 9, and 12 after KT, and when infections or rejection events were detected. Clinical and laboratory data were collected at each study visit. Patients with histologically proven rejection events were assessed according to Banff classification as T cell–mediated rejection (TCMR, Banff category 4), suspicious for acute TCMR (borderline, Banff category 3), and antibody-mediated rejection (ABMR, Banff category 2). For the statistical matching of rejection and nonrejection patients in subgroup analyses, only patients with available fecal samples before the rejection event (TCMR and borderline) were considered, because samples from only 1 patient with ABMR were available. Detailed information on clinical data preparation (Supplementary Table 1, variables included in statistical analysis), the standard of care treatment, matching of rejection/nonrejection patients, sample collection, 16S amplicon sequencing, SCFA production gene-targeting assay (qPCR, primer sequences in Supplementary Tables 2 and 3), and reanalysis of the CKD dataset[19] can be found in Supplementary Methods.

2.2. Statistical analysis

Continuous variables were expressed as medians and interquartile ranges. Categorical variables were presented as numbers and percentages. All statistical analyses were conducted using R (4.2.3). Multivariate comparisons were performed using PERMANOVA testing (adonis2, v.2.6-4). Nonhypothesis-driven univariate testing of longitudinal data was performed using LongDat (v.1.1.2) and of cross-sectional data using metadeconfoundR (v.0.2.8). We analyzed functional microbial capacities predicted from taxonomic data using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), analyzed by Mann-Whitney U test with Benjamini-Hochberg false discovery rate correction. Diversity parameters shown in boxplots, qPCR, and individual bacterial taxa displayed as boxplots were compared using the Mann-Whitney U test, and $P < .05$ was considered statistically significant. A full description of the statistical analysis can be obtained from Supplementary Methods.

3. Results

3.1. Gut microbiota composition recovers gradually post-KT

We used clinical data and fecal samples from the DZIF Tx cohort.[17] This multicenter cohort consists of nearly 2400 patients undergoing solid organ transplantation. Here, we focused on fecal samples from patients undergoing KT (n = 562 samples from n = 245 individuals), including kidney donors (n = 28) and, if available, pre-Tx samples (n = 26). Baseline characteristics of the cohort can be found in Table 1 and individual sampling timelines in Supplementary Figure S2.

We analyzed the microbiome composition using 16S rRNA gene amplicon sequencing. For longitudinal analysis post-KT, samples were grouped according to sampling time (Supplementary Fig. S1). Principal coordinates analysis (PCoA) shows time-dependent alterations to the microbiome composition post-KT, with a distinct composition compared to healthy donors and pre-KT groups (Fig. 1A). This significant shift post-KT persists when analyzing without pre-KT and healthy donors (Supplementary Fig. S3A). Using linear mixed-effect models, we observed a significant increase in the alpha diversity over time as quantified by the number of observed operational taxonomic units (OTUs) (Fig. 1B) and Shannon diversity index (Fig. 1C). For comparison, kidney donors and pre-KT samples are shown. We observed a similar trend for Simpson evenness (Fig. 1D). Of note, these time-dependent effects were not confounded by therapeutic antibiotic use (confounders tested are shown in Supplementary Table 1).

We performed a longitudinal analysis[20] to identify bacterial taxa regulated over time post-KT. Post-KT, typical SCFA-producing genera like *Coprococcus*,[21] *Roseburia*,[22] *Faecalibacteria*,[23] *Ruminococcoccus* torques group,[24] and an unknown genus belonging to the *Lachnospiraceae* family,[25] increased significantly over time (Fig. 1E), suggesting an improvement of CKD-associated microbiome alterations, specifically the impaired production of SCFA as one of its hallmarks.[6,9] Furthermore, we observed a decrease in *Streptococcus*, which was recently linked with subclinical atherosclerosis[26] (Fig. 1E). In summary, our analysis demonstrates a dynamic regeneration of the microbiome over time after KT toward a more physiological state after 3 years or more post-KT.

3.2. Kidney Tx rejection profoundly impacts microbiome composition

Because we observed dynamic microbiome changes over time in KT patients, we aimed to understand how Tx rejection events influence this process and, vice versa, how microbiome alterations may impact allograft immunity and rejection. Therefore, we identified fecal samples (n = 157) from patients (n = 76) with biopsy-proven rejection events at any time after KT and compared them to samples (n = 377) from patients (n = 141) with

Table 1
Clinical baseline characteristics total cohort. KT donors, pre-KT CKD patients, and post-KT CKD patients from the transplant cohort of the DZIF were analyzed. Data are presented as median and interquartile range or absolute values and percentages as appropriate. Primary graft dysfunction: the patient still required dialysis 3 months after KT. Delayed graft function: the patient required up to 3 times dialysis after KT.

Characteristic	Donors	Pre-KT	Post-KT	N
Individuals (N)	28	26	217	
Samples	28	26	508	
Samples per participant	1	1	2 (2-3)	
Age (y)	53 (46-68)	52 (34-60)	54 (40-63)	28, 26, 213
Female	20 (71%)	6 (25%)	70 (33%)	28, 24, 211
BMI (kg/m^2)	26.1 (24.0-28.3)	25.1 (21.6-27.3)	24.6 (21.6-28.0)	22, 26, 210
Kidney diagnosis				26, 213
Cystic	NA	4 (15%)	43 (20%)	
Diabetic	NA	2 (8%)	14 (7%)	
Glomerular	NA	10 (38%)	71 (33%)	
Hereditary/congenital	NA	6 (23%)	25 (12%)	
Hypertensive	NA	0	16 (8%)	
Other/unknown	NA	4 (15%)	37 (17%)	
Tubulointerstitial	NA	0	7 (3%)	
Living donor	NA	NA	81 (38%)	212
ABO-incompatible KT	NA	NA	18 (9%)	204
In-patient length of stay	NA	NA	19 (14-26)	210
Delayed graft function	NA	NA	38 (18%)	218
Primary graft dysfunction	NA	NA	8 (4%)	209
Positive PRA	NA	NA	44 (23%)	194
Donor-specific antibodies (of PRA+)	NA	NA	6 (18%)	34
Positive cross-match	NA	NA	5 (3%)	179
Patients with viral infections	NA	NA	87 (41%)	218
Number of viral infections			149	
BK-Virus	NA	NA	59 (40%)	149
Epstein-Barr-Virus	NA	NA	6 (4%)	149
Cytomegalo-Virus	NA	NA	37 (25%)	149
Others	NA	NA	47 (32%)	149
Rejection	NA	NA	76 (35%)	213
Antibody-mediated	NA	NA	1 (1%)	76
T cell–mediated	NA	NA	32 (42%)	76
Borderline	NA	NA	43 (57%)	76
Induction therapy	NA	NA		
Steroid, MPA, tacrolimus			184 (87%)	212
Steroid, MPA, ciclosporin			23 (11%)	212
Other combination			4 (1.9%)	212
Additional basiliximab			83 (39%)	212
Additional ATG			8 (3.8%)	212

(*continued on next page*)

Table 1 (*continued*)

Characteristic	Donors	Pre-KT	Post-KT	N
Maintenance immune suppression	NA	NA		
Steroid (>6 mo)			206 (99%)	207
MPA			210 (99%)	212
Tacrolimus			205 (97%)	212
Ciclosporin			38 (18%)	212
Everolimus			21 (10%)	212
Azathioprine			9 (4.3%)	212
Belatacept			8 (3.8%)	212
ATG			7 (3.3%)	212
Rituximab			5 (2.4%)	212
Sirolimus			3 (1.4%)	212
Eculizumab			1 (0.9%)	212

ATG, antithymocyte globulin; BMI, body mass index; CKD, chronic kidney disease; DZIF, German Center of Infectious Diseases; KT, kidney transplantation; MPA, mycophenolic acid; PRA, panel-reactive antibodies.

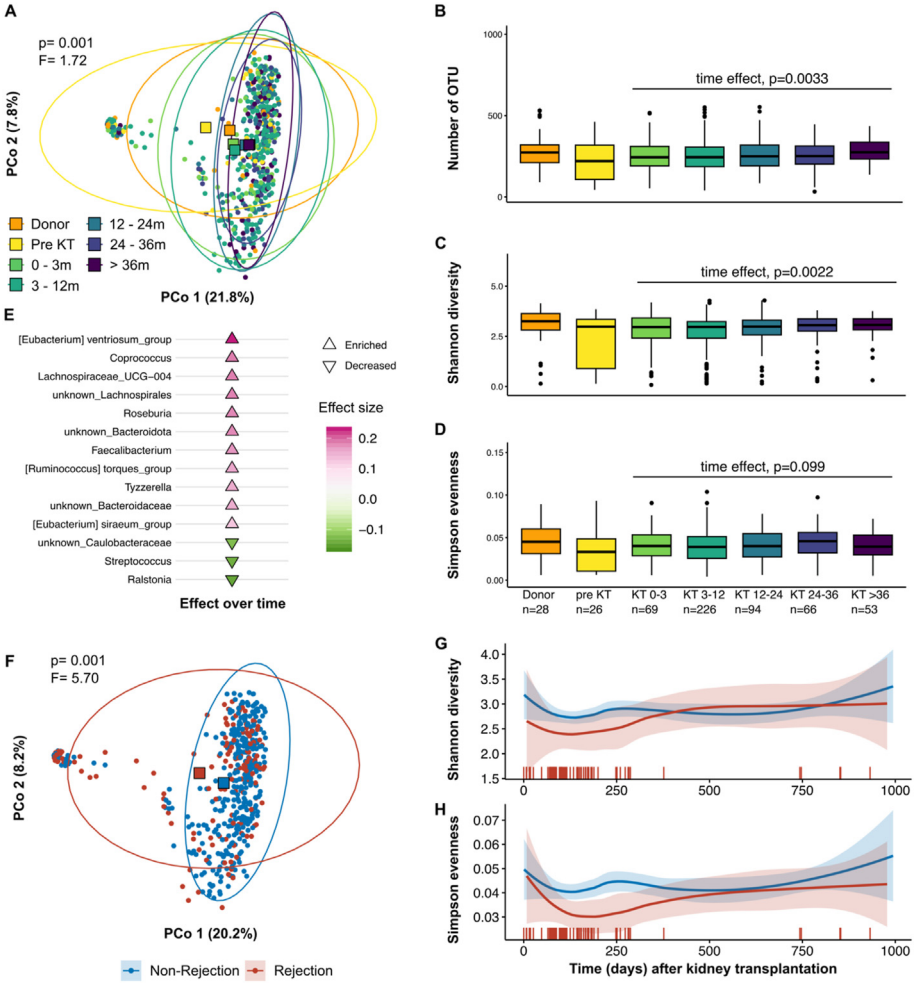

Figure 1. Longitudinal changes to the gut microbiome after kidney transplantation and impact on allograft rejection. 16S rRNA gene amplicon sequencing from fecal material from kidney transplantation (KT)-related samples from the transplant cohort of the German Center of Infectious Diseases. Samples were grouped according to healthy kidney donors, pre-KT, 0 to 3 months post-KT, 3 to 12 months, 12 to 24 months, and over 24 months. (A) Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity; the squares mark the centroids of each group. *P* value and F value from statistical comparison by PERMANOVA. Quantification of the (B) number of detected operational taxonomic units (OTUs), (C) Shannon diversity, and (D) Simpson evenness, *P* values for the time from linear mixed-effect models (patient as the random effect, time since transplantation as the fixed effect). (E) Cuneiform plot displaying significantly altered bacteria posttransplant over time as assessed by LongDatR. Microbial diversity in kidney transplant recipients with and without graft rejection at any point is shown in F-H. (F) PCoA based on Bray-Curtis dissimilarity. The squares mark the respective centroids. (G, H) Longitudinal analysis of Shannon diversity and Simpson evenness. The shaded area represents the 95% CI. Vertical red bars indicate individual rejection events over time, coinciding with alterations in microbial diversity.

no reported rejection event (Table 1). The PCoA showed clustering of patients experiencing graft rejection compared to KT patients with no reported rejection (Fig. 1F). Although the number of detected OTUs was not altered (Supplementary Fig. S3B), Shannon diversity and Simpson evenness were reduced during the first year post-KT in rejection patients (Fig. 1G, H), which was coincident with the allograft rejection. Lastly, we performed univariate analyses at the genus level using metadeconfoundR[27]

accounting for the repeated samples per patient. Corresponding to the altered composition in the beta-diversity analysis, we found a large number of differentially abundant genera (Supplementary Fig. S4). Interestingly, most observed effects related to rejection showed a contrasting trend in correlation with the duration between sampling and kidney Tx rejection event (Supplementary Fig. S3). This correlation indicates either microbiome alteration preceding the rejection event, changes in the microbiome postrejection, or both. Of note, our analysis indicates that many effects were confounded by patient age and distance to KT.

Taken together, patients who experience KT rejection have an altered gut microbiome composition. We hypothesize that these changes might precede Tx rejection as the alterations are associated with the time between sampling and the KT rejection event.

3.3. Microbiome alterations precede kidney Tx rejection

To overcome the unequally distributed age, sex, and distance to KT of rejection and nonrejection patients and to further understand which alterations to the microbiome precede KT rejection, we performed propensity score matching of patients with available fecal samples before KT rejection to patients without KT rejection. Matched prerejection and nonrejection groups were comparable in age, sex, donor type, underlying CKD disease category, and time between KT and sample (Supplementary Fig. S5). Baseline characteristics of this subcohort are shown in Table 2.

Rejection patients exhibited impaired renal function as compared to nonrejection patients as displayed by an increase in plasma creatinine within the first year post-KT, which remained the case during follow-up (Fig. 2A). This corresponds to most rejections in our cohort occurring within the first year post-KT (Tables 1 and 2). However, both rejection and normal progress patients reached a similar minimal creatinine as well as time to minimal creatinine (Fig. 2B), indicating that the initial graft function was comparable. After the rejection event, KT showed sustained graft dysfunction as indicated by higher minimal detected creatinine values after their rejection event, as well as higher last recorded creatinine (Fig. 2B). Of note, HLA mismatch grade, rate of ABO incompatibility, and delayed graft function, were comparable between both groups (Table 2).

Microbiome analysis indicated alterations prior to the rejection event. Prerejection microbiome was characterized by a distinct composition indicated by different clustering in the PCoA (Fig. 2C), and lower alpha diversity, reaching significance for Simpson evenness (Fig. 2D). Next, we tested for differentially abundant genera using confounder-aware analysis incorporating antibiotics, viral infection, creatinine, and other routine laboratory work as well as patient characteristics like age and sex (Supplementary Table 1). Patients who rejected the transplanted kidney had lower levels of known SCFA-producing genera, like *Blautia*, *Clostridia*, or *Ruminococcus* torques group.[24] Interestingly, we observed an increase in bacteria typically found in CKD patients, like *Fusobacterium*,[9] and disease-associated genera,[28] such as *Strepcoccus*[26] (Fig. 2E).

Despite our efforts to match the rejection and nonrejection groups, a higher rate of viral infections occurred in the rejection group. We, therefore, carefully assessed the use of immunosuppressants and antiinfective medication (Supplementary Figs. S6-S8), pointing out 2 main differences, namely, a lower number of patients on mycophenolic acid (MPA) at the sampling date—likely due to the viral complications—and a lower number of patients with basiliximab for induction in the rejection group. Beta-diversity analysis revealed no difference in microbiome composition in rejection patients with or without basiliximab induction (Supplementary Fig. S9), but MPA withdrawal showed a nonsignificant shift in microbiome composition. Therefore, we performed differential abundance analysis, including and excluding patients with MPA discontinuation, which was closely correlated ($R = 0.98$, $P < .001$) (Supplementary Fig. S10). This indicates that the observed differences were independent of basiliximab induction and MPA discontinuation.

3.4. Lower SCFA production potential characterizes the prerejection microbiome

Metabolites produced by microbiota are known to influence and modulate host immune responses. To identify potential candidates, we analyzed functional capacities predicted from taxonomic data using PICRUSt2.[29] To identify functional pathways in the significantly altered Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologues, we used GOmixer.[30,31] We observed an overall clustering of rejection and nonrejection patients in the principal component analysis (Fig. 3A). We found enrichment in proteolytic fermentation, reactive nitrogen and oxygen species, and ammonia pathways in the rejection group (Fig. 3B, C). Conversely, overall sugar and polysaccharide utilization and mucus degradation were enriched in microbiomes from nonrejection controls (Fig. 3B, C). Matching to the reduced number of SCFA-producing genera, we found a reduction in butyrate and acetate fermentation pathways (Fig. 3B, C), again highlighting the reduction of SCFA production in stool samples preceding KT rejection (Fig. 3B, C). Because SCFA and regulatory immune functions are closely linked, we aimed to confirm the reduction of SCFA production using qPCR measuring key enzymes for butyrate, propionate, and acetate production. Overall, we found a significant reduction of butyryl-CoA:acetate CoA-transferase (*buT*), a key enzyme for butyrate production, and methylmalonyl-CoA decarboxylase (*mmdA*), a key enzyme for propionate production (Fig. 4A, B). Another central enzyme for butyrate synthesis, butyryl-CoA dehydrogenase (*bcD*), and acetate kinase (*acK*), facilitating the last reaction during bacterial acetate synthesis, were reduced without reaching significance (Fig. 4A, B). Taken together, a key feature of the gut microbiome in samples from patients preceding KT rejection is a marked reduction of the potential to produce SCFA.

3.5. Microbiome alterations normalize postrejection

As our initial analysis indicated a potential shift postrejection, we analyzed longitudinal samples from our matched cohort within a timeframe of 90 to 1000 days after the first sample ($n = 21$ rejection and $n = 54$ nonrejection patients). The PCoA shows that the microbiome composition becomes more similar to the nonrejection control group in postrejection samples (Fig. 5A).

Table 2
Clinical baseline characteristics of the propensity score–matched cohort. Samples were obtained after KT, but prior to rejection (rejection group). The nonrejection group was propensity score matched. Data are presented as median and interquartile range or absolute values and percentages as appropriate. Primary graft dysfunction: the patient still required dialysis 3 months after KT. Delayed graft function: the patient required up to 3 times dialysis after KT.

Characteristic	Nonrejection	Rejection	All	N
Individuals (N)	60	32	92	
Age (y)	55 (44-63)	58 (47-62)	56 (45-63)	92
Female	22 (37%)	11 (34%)	33 (36%)	92
BMI (kg/m^2)	23.4 (20.8-27.6)	24.6 (21.5-27.2)	24.1 (21.1-27.5)	92
Sample days after KT	111 (85-175)	114 (84-192)	112 (85-181)	92
Rejection days after KT	NA	173 (132-282)	NA	92
Rejection	NA		NA	
T cell–mediated	NA	15 (47%)	NA	32
Borderline	NA	17 (53%)	NA	32
Living donor	20 (33%)	10 (31%)	30 (33%)	92
ABO-incompatible KT	5 (8%)	1 (3%)	6 (7%)	92
HLA mismatches (HLA-A, HLA-B, HLA-DR)	2 (2-3)	3 (2-4)	3 (2-4)	79
Positive panel-reactive antibodies (PRA)	14 (25.5%)	7 (25.9%)	21 (25.6%)	82
Donor-specific antibodies (of PRA+)	4 (33.33%)	1 (33.33%)	7 (33.33%)	15
Positive cross-match	0 (0%)	2 (8.3%)	2 (2.7%)	75
Primary graft dysfunction	0	0	0	88
Delayed graft function	9 (15%)	2 (6%)	11 (12%)	92
In-patient length of stay (d)	19 (14-24)	18 (14-26)	19 (14-24)	91
Recipient CMV$^+$	26 (46%)	14 (47%)	40 (46%)	87
Recipient EBV$^+$	35 (83%)	18 (90%)	53 (85%)	62
No. of bacterial infections before the sample	32	20	52	92
Patients with bacterial infection before the sample	17 (28%)	20 (34%)	37 (40%)	92
No. of viral infections before the sample	12	23	35	N/A
BK-Virus	7 (58%)	9 (39%)	16 (46%)	35
Epstein-Barr-Virus	0	2 (9%)	2 (6%)	35
Cytomegalo-Virus	4 (33%)	7 (30%)	11 (31%)	35
Others	1 (8%)	5 (22%)	6 (17%)	35
Patients with a viral infection before the sample	9 (15%)	14 (44%)	23 (25%)	92

BMI, body mass index; HLA, humal leukocyte antigen; KT, kidney transplantation; PRA, panel-reactive antibodies.

Over a similar time frame, the microbiome composition of nonrejection controls was stable. The same trend can be observed for the number of detected OTUs, Shannon diversity, and Simpson evenness (Fig. 5B). Next, we analyzed microbiome alterations postrejection at the genus level (Fig. 5C). Although the nonrejection control showed no significant alterations, most genera dysregulated in preceding rejection were significantly changed in the opposite direction after the KT rejection event (Fig. 5C). Especially known genera of SCFA production like *Blautia* and *Faecalibacterium* increased, whereas disease-associated genera like *Fusobacterium* and *Streptococcus* decreased (Fig. 5D).

Taken together, we observe a normalization of the pre-rejection microbiome toward the normal KT signature in a longitudinal follow-up analysis. One could speculate that a lack of normalization of the microbiome favors chronic rejections. Due to insufficient sample size, we could not further test this hypothesis in this study.

3.6. The microbiome signature in kidney Tx rejection is a prolonged CKD signature

Lastly, we aimed to contextualize the microbiome alteration preceding KT rejection. We observed a partial overlap with

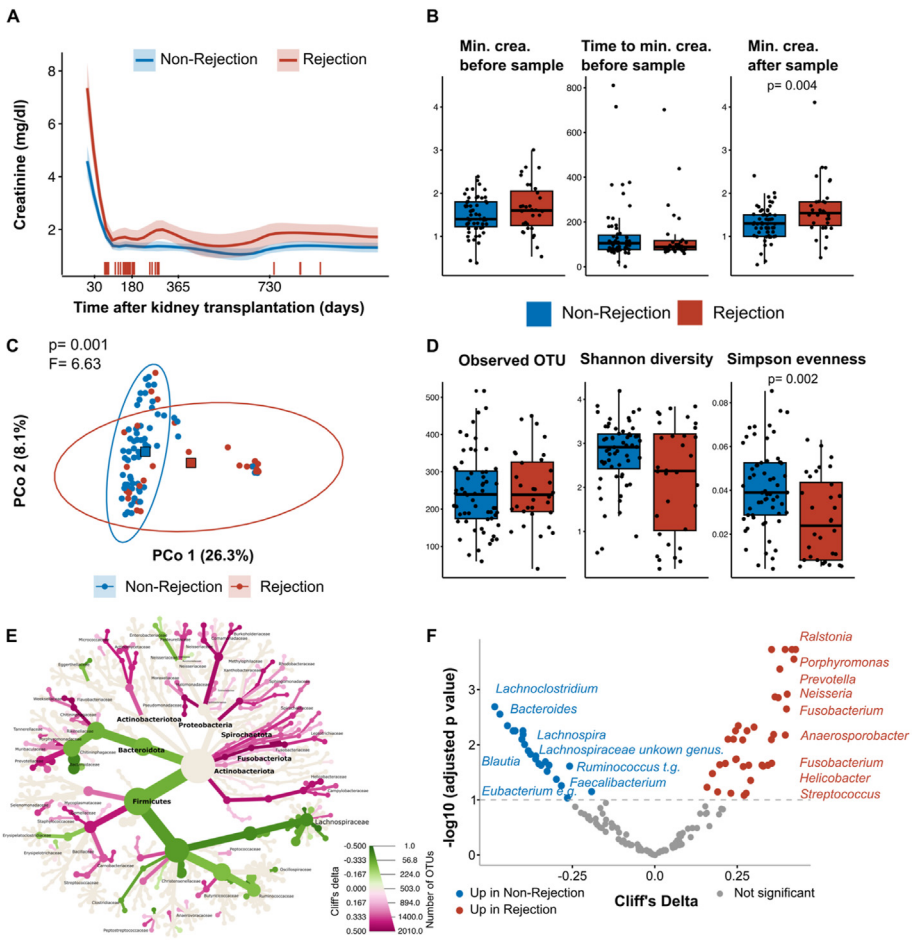

Figure 2. Microbiome alteration precedes kidney transplant rejection. Patients with available fecal samples prior to kidney rejection (rejection group) for propensity score matched (1:2) to patients without kidney rejection (nonrejection group). (A) Plasma creatinine levels are depicted over time. The shaded area represents the 95% CI. Red bars indicate individual rejection events over time. (B) Analysis of minimum creatinine levels before and after sampling, along with the time taken to reach the first. (C) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity; the squares mark the centroids of each group. *P* value and F value from statistical comparison by PERMANOVA. (D) Number of detected operational taxonomic units (OTUs), Shannon diversity, and Simpson evenness for the propensity score–matched cohort. (E) Differential abundance analysis for bacterial taxa. Bacteria are shown in a phylogenetic tree where green lineages represent bacteria depleted and pink bacteria enriched in patients with kidney rejection (F) Differential abundance analysis of bacterial genera, x-axis indicates Cliff's Delta and y-axis false discovery rate–corrected *P* value.

microbiome features of pediatric CKD patients in the report that was recently published by us.[9] Therefore, we hypothesized that the microbiome features preceding KT rejection, in part, reflect a prolonged CKD signature after KT. Therefore, we reanalyzed a recently published 16S rRNA gene sequencing dataset from CKD patients (n = 217) and healthy controls (n = 479).[19] In line with our hypothesis, the KT rejection signature correlated with the CKD signature in the dataset from Ren et al[19] (for the 120 common genera, R= 0.19, *P* = .033) (Fig. 6A). In particular, the overlap of both datasets also held true for the reduction of important SCFA producers like *Blautia* and *Faecalibacterium*, as well as for the increase in *Streptococcus* and *Fusobacterium*, with the latter not reaching significance in the CKD-healthy controls comparison (Fig. 6A, B). Lastly, we performed a targeted analysis of bacterial taxa captured by our *but*, *bcd*, and *mmdA* assays. Overall, the abundance of butyrate and propionate producing taxa was lower in CKD patients mirroring the effect we observed in microbiome samples preceding KT rejection (Fig. 6C). In aggregate, our data indicate that the prerejection signature we observed in our cohort might, in part, be a sustained CKD signature. Especially the lack of fiber fermenting, SCFA-producing bacteria is a key feature found in both disease states.

4. Discussion

This study investigates the gut microbiome in KT recipients and its relationship with allograft rejection. We describe compositional and functional differences in the microbiome in a representative cohort of 217 Tx patients with and without rejection. Our analysis of longitudinally collected fecal samples unveils a dynamic trajectory of microbiome recovery post-KT, which undergoes a gradual shift toward a more stable and healthier microbiota composition (such as an increase in *Roseburia* and *Faecalibacterium*),[11,28] mirroring CKD-to-health transition. These regenerative microbiome shifts post-KT are significantly perturbed in the case of graft rejection. Preceding the rejection event, we observed profound alterations in microbiome composition, characterized by a diminished diversity and underrepresentation of SCFA-producing bacterial populations. We consider these observations to be of potential functional relevance for allograft immunity due to the known immunomodulatory properties of SCFA.[8,32] Our results suggest that the microbiome is an important modulator of immunologic events post-KT—an observation that may also have prognostic significance for the prevention of KT rejection and graft survival.

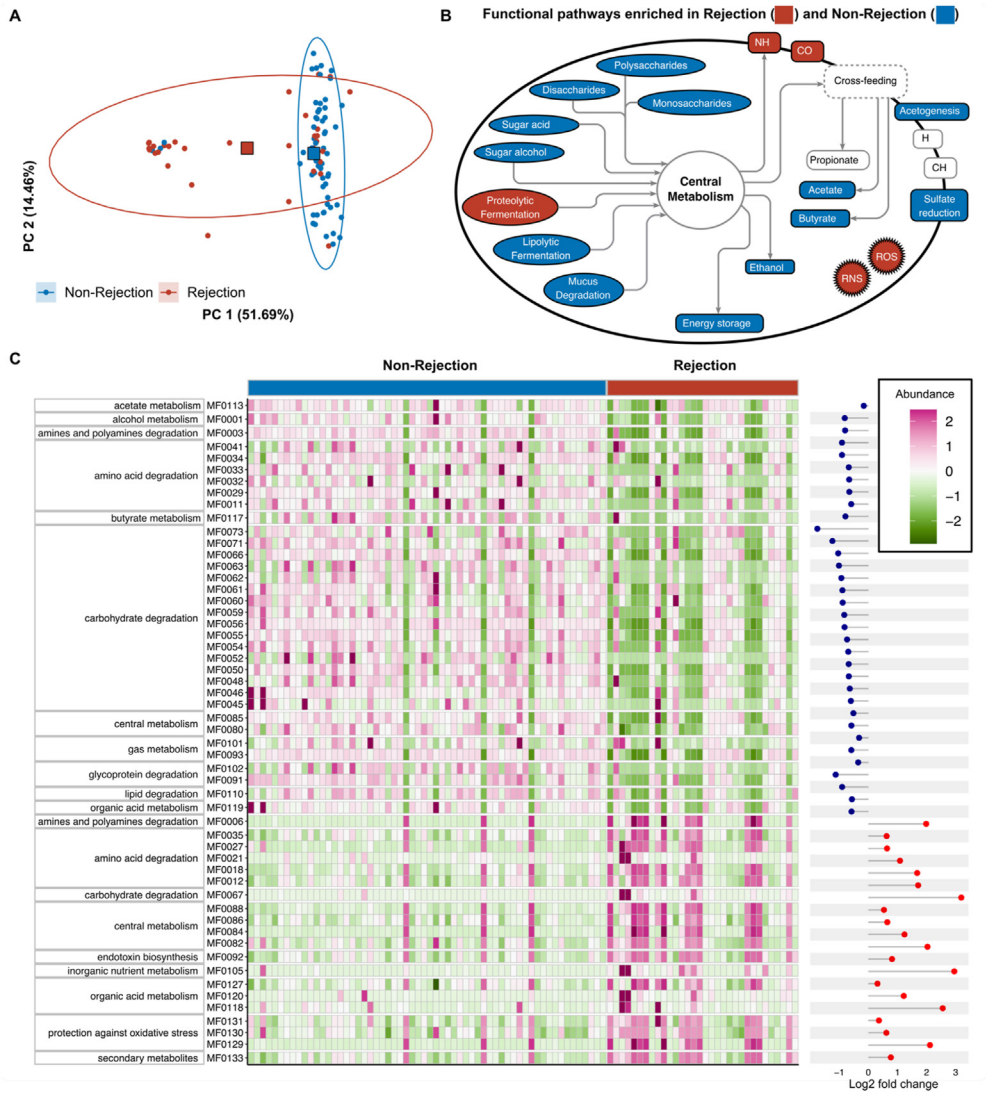

Figure 3. Fecal short-chain fatty acid production potential is reduced before kidney transplant rejection. Patients with available fecal samples prior to kidney rejection (rejection group) for propensity score matched (1:2) to patients without kidney rejection (nonrejection group). Inference of functional data from taxonomic data using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States and mapping to pathways using GOmixer. (A) Clustering of kidney transplant recipients based on the presence (red circles) or absence (blue circles) of graft rejection, illustrating differences in microbiome-derived functional potential. Squares represent centroids. *P* value and F value from statistical comparison by PERMANOVA. (B) Overview of dysregulated functional pathways enriched (red) or depleted (blue) in patients who experienced graft rejection. (C) Heatmap shows all 54 significant GOmixer modules, with pink indicating high predicted abundances and green representing low abundances across the 92 patients, divided into nonrejection and rejection. The dot plot to the side indicates log2 fold changes. Enrichment in rejection is shown in red, and in nonrejection in blue. PCA, principal component analysis; RNS, reactive nitrogen species; ROS, reactive oxygen species.

The observed gradual normalization of the microbiome composition after KT for several bacterial taxa aligns with the normalization of known alterations in the gut microbiome of CKD patients. The gut microbiome of CKD patients is relatively well investigated. It is characterized by a lower diversity and a shift in metabolic output.[6,9,19,33] Although the production of SCFA metabolites, recognized for their antiinflammatory effects,[8] is reduced,[9] we and others have observed an increase in the production of microbiome-derived uremic toxins like TMA,[34,35] p-cresols[36] and indoles.[9,37] Of note, the microbiome of CKD patients is characterized by an increase of bacterial taxa frequently linked to health-to-disease transition in large representative metagenomic studies.[28,38] In the present study, we observe a decrease in known pathogenic bacteria and an increase in beneficial commensals over the course of more than 3 years post-KT. Thus, the observed changes are reminiscent of a CKD-to-health transition in the microbiome.

Our data is in line with the results reported from the analysis of 1300 patients after liver and kidney transplantation within the TransplantLines cohort.[10-12] Swarte et al[12] demonstrated that lower gut microbial diversity in Tx recipients is associated with an increased overall mortality. Of note, there is a considerable overlap of genera reported to be dysregulated in solid organ Tx recipients with taxa altered over time in our data, like an increase in *Streptococci* or a decrease in *Faecalibacteria*.[10] However, Swarte et al[10] focus more on the comparison to a healthy microbiome, resulting in descriptions of alterations in KT recipients. In contrast, we interpret KT more as a first step toward a "reset to health" state in terms of microbiome composition, hence focusing primarily on the resolution of CKD-specific microbiome alterations post-KT. In this context, similarities and discrepancies between both cohorts have to be interpreted carefully, as they are likely to be influenced by the availability of longitudinal samples and the pre-Tx microbiome state, which might be influenced by the rate of preemptive transplantations and duration of kidney replacement therapy.

We further demonstrate that alterations of the gut microbiome, including a reduced microbial diversity, and alterations in the abundance of more than 50 bacterial taxa, occur before graft rejection. We observed an increase in *Streptococcus*, which was recently correlated to subclinical atherosclerotic lesions in a cohort of nearly 9000 patients.[26] Another genus found to be

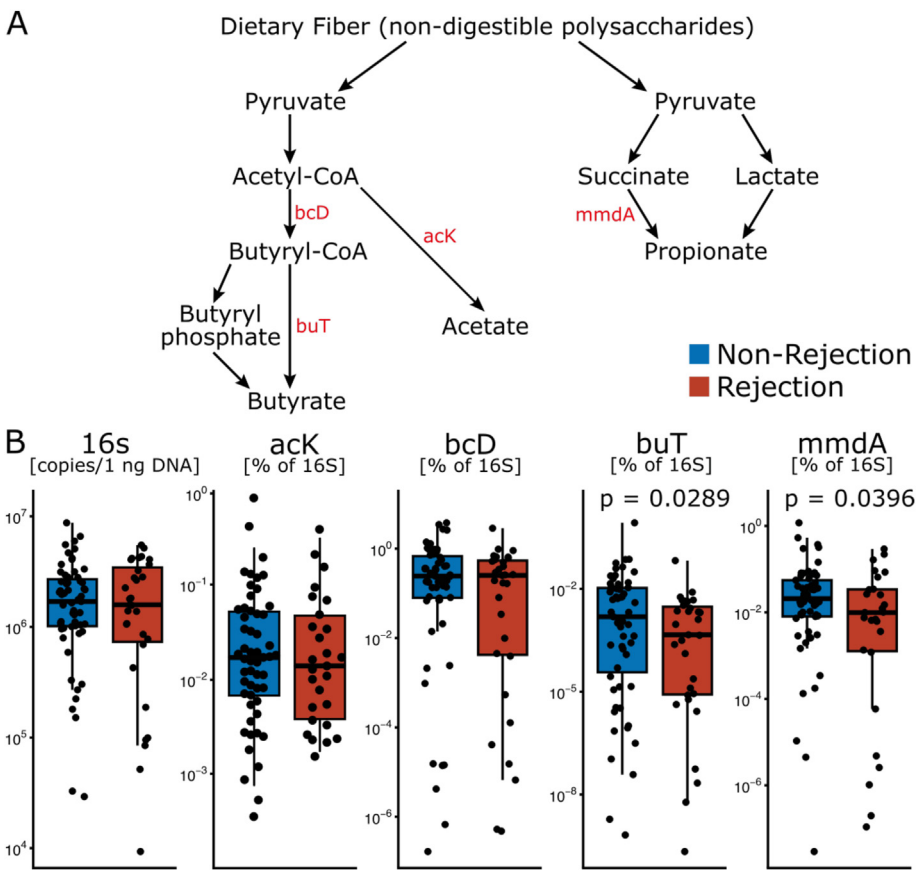

Figure 4. Decreased abundance of short-chain fatty acid producing enzymes in the microbiome before kidney transplant rejection. Patients with available fecal samples prior to kidney rejection (rejection group) for propensity score matched (1:2) to patients without kidney rejection (nonrejection group). (A) Bacterial nutrient metabolism leading to SCFA, specifically butyrate and propionate, production. Key bacterial enzymes are highlighted in red. (B) The abundance of bacterial enzymes for SCFA production was assessed by qPCR from fecal DNA and normalized to 16S content. Although butyryl-CoA dehydrogenase (*bcd*) and acetate kinase (*acK*) were not significantly altered, butyryl-CoA:acetate CoA-transferase (*but*) and methylmalonyl-CoA decarboxylase (*mmdA*) showed lower abundance in fecal samples from patients with subsequent kidney rejection compared to those without rejection.

upregulated in rejection and CKD (although not reaching significance in the cohort we reanalyzed[19] as compared to other studies[9]) was *Fusobacterium*. *Fusobacterium nucleatum* is a driver of uremic toxins production and CKD progression,[33] again underscoring the notion of a prolonged CKD signature in the microbiome as a potential risk factor for graft rejection. Of note, this phenotype seems to be independent of kidney function prior to the rejection event, as rejection and nonrejection patients had a comparable course post-Tx (lowest creatinine value prior to rejection as well as a similar time to reach the lowest creatine after transplantation). Additionally, in our confounder-aware analysis, creatinine was not picked up as a potential confounder of this disease signature.

We and others have shown that SCFA are relevant bacterial metabolites for immune homeostasis in CKD as well as for other chronic diseases.[9,32,39-41] Using computational prediction of functional microbiome properties (PICRUSt2[29]), we show a significantly reduced potential of the gut microbiome to produce SCFA in KT patients prior to rejection. PICRUSt2 predicts microbiome functions based on the available genomic data and the inferred presence of genes from closely related taxa.[29] PICRUSt accuracy significantly decreases for microbes with fewer close relatives in reference databases.[42] Acknowledging this limitation, we validated the downregulation of several key enzymes for SCFA synthesis using gene-targeting qPCR assays, confirming a reduced potential for SCFA production in fecal samples from KT patients with subsequent graft rejection.

SCFA have been shown to influence the function and differentiation of Treg both through G-protein–coupled receptor signaling and histone deacetylase inhibition.[8,43] A recent meta-analysis concluded that immune cell therapies, including transfer of Treg are a useful approach to reduce immunosuppression during KT.[16] Experimental transplantation models demonstrate the efficacy of SCFA treatments through the induction of Treg. Kidney Txs showed prolonged survival in animals fed a high-fiber diet or directly the SCFA acetate.[14] Thus, the rapid attainment of a healthy SCFA production potential could be relevant for Treg function and the prevention of rejection events as well as other comorbidities found after KT.[44] Interestingly, similar effects of the SCFA-Treg-axis have been described for both graft-versus-host disease[45] and autoimmunity.[46] In the context of multiple sclerosis, the first promising data on disease modulation through oral SCFA treatment and improved Treg function have been published.[47] In summary, these data support the idea of targeting the SCFA-Treg axis to prevent allograft rejection and, thereby, improve transplantation-related outcomes.

Three other studies similarly suggest alterations of the microbiome in patients with rejection. Although the first study is severely limited by sample size (n = 3 patients),[48] the second study (n = 24)—albeit focusing on ABMR compared to our focus on TCMR—describes on a broad scale similar features like a reduced alpha diversity.[49] Despite describing a lower gut microbiome diversity, Visconti et al[50] show a lower abundance of genes coding for enzymes involved in SCFA production in line

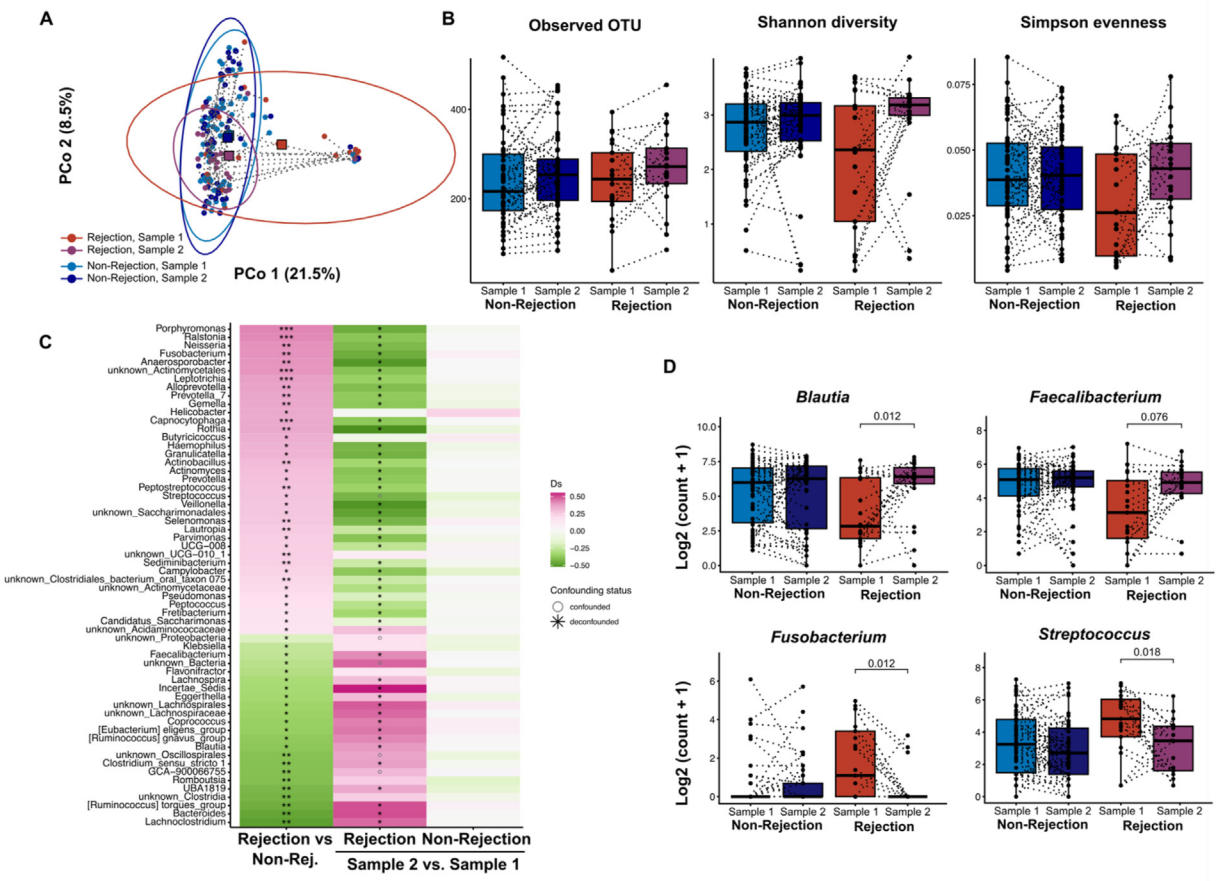

Figure 5. Microbiome composition normalizes post–kidney transplant rejection. Patients with available fecal samples (sample 1) prior to kidney rejection (rejection group) and 90 to 1000 days postrejection (sample 2) were analyzed. The propensity score–matched patients without kidney rejection (non-rejection group) with 2 comparable samples were included as controls. (A) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity, squares mark the centroids of each group. (B) Microbial diversity is measured by the number of detected operational taxonomic unit (OTU), Shannon diversity, and Simpson evenness. (C) Heatmap comparison of genus-level alterations between prerejection and postrejection samples reveals significant directional changes in microbial populations after a rejection event, contrasting with the stable profiles observed in nonrejection controls. Prerejection comparison is taken from Figure 3F. Asterisks indicate significance levels: false discovery rate (FDR) <0.1*; FDR <0.01**; FDR <0.001***. Confounded signals are shown as circles. Effect size is shown as Cliff's delta with pink enriched and green depleted. (D) Boxplots show shifts at the genus level, of short-chain fatty acid producers, *Blautia* and *Faecalibacterium,* and disease-associated genera, *Fusobacterium* and *Streptococcus*.

with our findings. However, the study is severely limited in sample size especially as it includes patients with TCMR and ABMR.[50]

We carefully consider drug-microbiota interactions throughout our analysis as potential confounders of our findings. However, we cannot exclude with absolute certainty a potential influence of a lower exposition toward immunosuppressive drugs in patients with rejections, which might be a consequence of the increased burden of viral infections. In addition, the evolving field of pharmaco-microbiomics explores how an altered gut microbiome may also directly impact the microbial metabolism of immunosuppressive drugs.[51,52] Microbial enzymes such as beta-glucuronidase have been shown to modify mycophenolate mofetil plasma levels through enhanced enterohepatic recirculation and increased therapeutic efficacy as well as side effects.[53] To support further investigation into this field, we have made our data publicly available, enabling researchers to explore these mechanistic pathways as computational tools advance.

The circumstance that fecal samples were not consistently available from all patients at all time points in our multicenter cohort is a limitation of our study. However, in nonrejection patients, a minimum sample number of 2 stool samples per patient was not undercut, which enabled the longitudinal character of our study. As a consequence, we investigated a subset of patients of the whole Tx cohort, which inflated the relative number of rejection events and potentially introduced a risk for selection bias. A further limitation is the lack of metabolomic measurements in our study, which we replaced with specific quantifications of the microbial enzyme composition, mirroring metabolic alterations.[35] We performed 16S rRNA amplicon sequencing showing results at the OTU level; however, mapping to amplicon sequence variants showed similar results in our data set (Supplementary Fig. S11). Finally, the study design does not allow causal statements on the mechanistic significance of microbiome changes for rejection. However, our conclusions regarding the role of SCFA in the Treg function are supported by published experimental data. Future studies should consider functional data on immune cells and their relationship to microbiome and rejection to validate our conclusions.

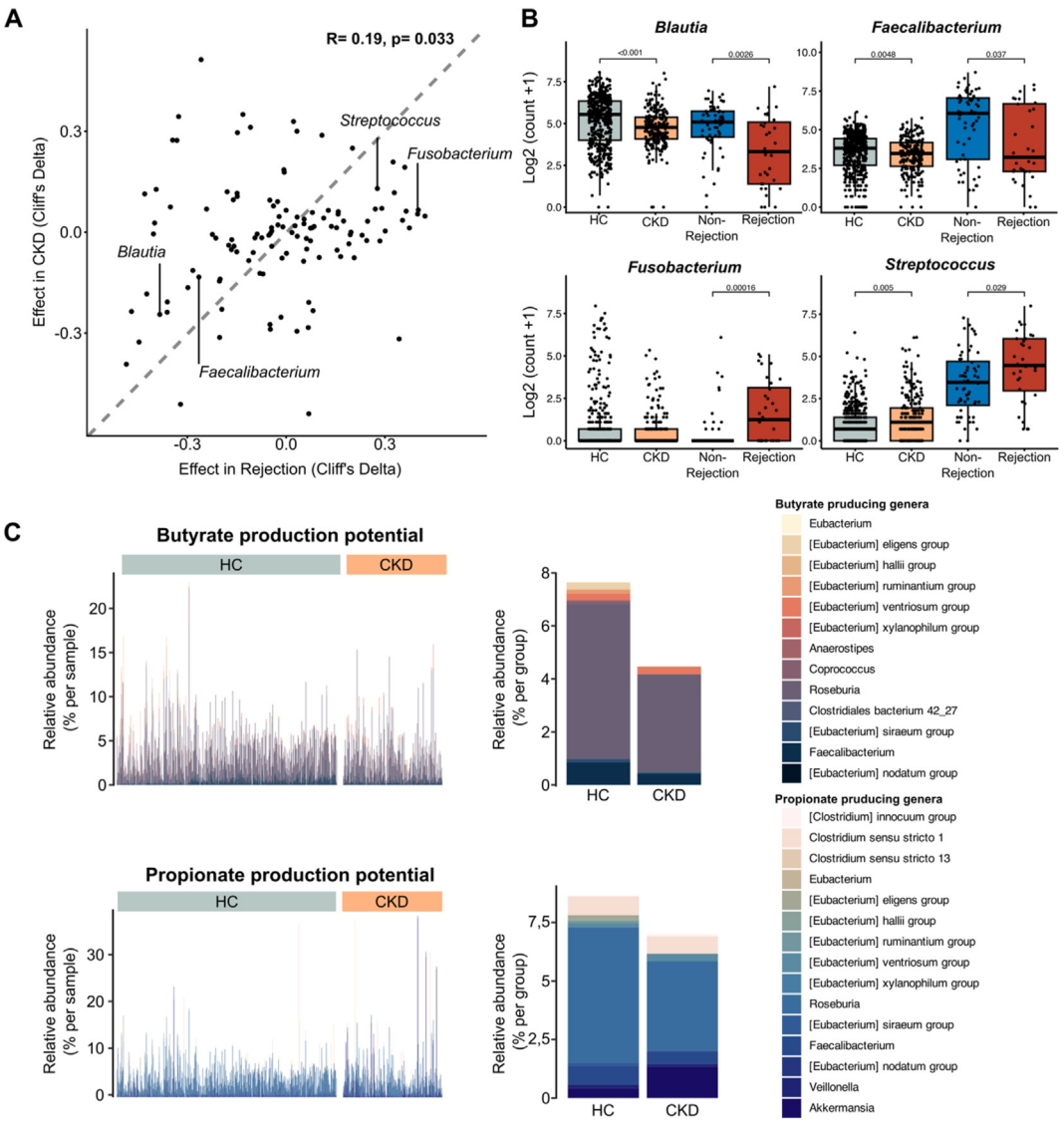

Figure 6. Microbiome composition in kidney rejection partly mirrors chronic kidney disease (CKD)-related microbiome alterations. Correlation between CKD microbial signatures and kidney transplant rejection preceding signatures. (A) Scatter plot indicating the correlation between the microbial signatures observed before kidney transplant rejections and those seen in CKD patients, suggesting shared alterations. The dashed line indicates a perfect overlap of effect sizes. (B) The log-transformed rarefied abundance of key short-chain fatty acid–producing genera (*Blautia* and *Faecalibacterium*) and disease-associated genera (*Streptococcus* and *Fusobacterium*) across healthy controls (HC), CKD patients, and kidney transplant recipients preceding graft rejection or without graft rejection (propensity score matched). (C) Abundance of bacterial taxa involved in butyrate and propionate production between HC and CKD patients, reflecting a decrease in these beneficial taxa in CKD.

Taken together, we demonstrate a disrupted microbiome recovery post-KT as a novel modifying factor in graft rejection. This is, to our knowledge, the first study indicating that microbiome alterations and perturbation of microbial metabolism precede graft rejection. More studies are needed to decipher the interaction of SCFA and Treg in KT patients and to test the potential of microbiome-targeting interventions before and after KT to improve long-term graft survival.

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Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose as described by *American Journal of Transplantation*.

Data availability

Deidentified metagenomic sequencing data for samples of the Transplant Cohort of the DZIF can be accessed from the European Nucleotide Archive under accession number PRJNA1106540 (https://www.ebi.ac.uk/ena/browser/view/PRJNA1106540). Access to pseudonymized phenotype data requires approval from the scientific steering committee of the DZIF Transplant cohort. The source code and the summary data underlying all figures used to generate the results for the analysis are available at https://github.com/rosareitmeir/DZIF-Tx-Cohort-Data-Cleaning-and-Statistical-Analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2025.02.010.

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