

# The effect of microbiota-based investigational drug RBX2660 on the gut microbiome and resistome revealed by a placebo-controlled clinical trial

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## Research

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# Abstract

Background Intestinal microbiota restoration can be achieved by replacing a subject's perturbed microbiota with that of a healthy donor. Recurrent *Clostridioides difficile* infection (rCDI) is one key application of such treatment. Another application of interest is depletion of antibiotic resistant genes (ARGs) and organisms (AROs). In this study, we investigated fecal specimens from a multicenter, randomized, double-blind, placebo-controlled phase 2b study of microbiota-based investigational drug RBX2660. Patients were administered either placebo, 1 dose of RBX2660 and 1 placebo, or 2 doses of RBX2660 via enema and longitudinally tracked for changes in their microbiome and antibiotic resistome.

Results All patients exhibited significant recovery of gut microbiome diversity and decrease of ARG abundance during the first 7 days post-treatment. However, the microbiome and resistome shifts towards healthier configurations were more significant and longer lasting in RBX2660 recipients compared to placebo. We identified 18 taxa and 21 metabolic functions distinguishing the baseline microbiome of non-transplanted patients, and the majority of features were correlated to intrinsic vancomycin resistance. We also identified 7 patient-specific and 3 RBX2660-specific ARGs and tracked their dynamics post treatment. Whole genome sequencing of AROs cultured from RBX2660 product and patient samples indicate ARO eradication in patients via RBX2660 administration, but also, to a lesser extent, introduction of RBX2660-derived AROs.

Conclusions By including a placebo group, we distinguished the effects of RBX2660 from baseline post-antibiotic microbiome dynamics. Antibiotic discontinuation alone resulted in significant recovery of gut microbial diversity and reduced ARG abundance, but RBX2660 administration more rapidly and completely changed the composition of patients' microbiome, resistome, and ARO colonization by transplanting RBX2660 microbiota into the recipients. Although ARGs and AROs were transmitted through RBX2660, the resistome post-RBX2660 more closely resembled that of the administered product—a proxy for the donor—than an antibiotic perturbed state.

## 1. Background

Intestinal microbiota restoration by microbiota-based therapy, such as fecal microbiota transplantation (FMT) from healthy donors to patients, has been applied as a treatment for disorders caused by intestinal dysbiosis [1]. As the contributions of the gut microbiota to the host immune system, energy metabolism, and central nervous system have been uncovered, the range of potential applications of intestinal microbiota restoration therapy is expanding to various disorders, such as inflammatory bowel disease [2], functional gastrointestinal disorders [3], metabolic syndrome [4, 5], and neuropsychiatric disorders [6, 7]. Accordingly, studies for understanding and refining the action of intestinal microbiota restoration therapies are being actively conducted [8].

*Clostridioides difficile* infection (CDI) is one area where intestinal microbiota restoration therapy has been applied successfully. Although oral administration of antibiotics is the standard first-line therapy for CDI,

antibiotics perturb the commensal gut microbiota and decrease colonization resistance against other pathogens [9, 10]. Approximately 15–30% of CDI patients therefore experience recurrent CDI (rCDI) resulting from either a relapse of the previous CDI or reinfection [11]. Moreover, antibiotic therapies during CDI treatment may promote the expansion of antibiotic resistant organisms (AROs) such as vancomycin resistant Enterococci (VRE) [12, 13]. On the other hand, intestinal microbiota restoration has shown to be effective for CDI treatment as well as the restoration of colonization resistance against *C. difficile* and AROs [14, 15]. Indeed, intestinal microbiota restoration has become a commonly performed investigational therapy for rCDI with high success rates [8, 16–19].

However, due to the transmissible nature of the treatment, microbiota restoration therapy may communicate not only desirable but also undesirable factors derived from donors. For instance, the transmission of antibiotic resistant genes (ARGs) and AROs derived from donor samples is a potential risk of fecal transplantation [20, 21]. AROs are responsible for increasing infection cases each year, and more than 35,000 patients died as a result of ARO infections in the United States in 2017 [22]. Recently, two cases of bacteremia caused by extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* in patients after FMT from the same donor sample have been reported, resulting in the death of one of the patients [21]. Moreover, the dissemination of ARGs and pathogenic AROs in patients hampers effective medical care of infections and results in longer hospitalization and higher medical expenditures [23]. Still, multiple studies report efficient eradication of ARGs and decolonization of AROs through microbiota transplantation [24, 25].

In the current study, we explored the effect of a microbiota-based investigational drug RBX2660, a suspension of healthy donor microbiota [26–29], on the intestinal microbiome and resistome of recipients treated for rCDI. In an international, multicenter, randomized, and blinded phase 2b study, rCDI patients received either placebo (control group), one dose, or two doses of RBX2660 (Fig. 1), with more patients being recurrence-free after either RBX2660 regimen than placebo [26]. Through shotgun metagenomic sequencing, we demonstrate considerable shifts of taxonomic and resistome structures common to both placebo- and RBX2660-treated patients likely from discontinuation of antibiotics, particularly during the first week after treatment. By controlling for placebo effects, we could also distinguish taxonomic and resistome changes specific to RBX2660 treatment. Furthermore, we identified discriminative features strongly correlated with microbiota transplant and demonstrated an overall decrease in AROs as well as introduction of a few AROs by RBX2660.

## 2. Results

### 2.1. Study cohorts and sample collection

Fecal specimens from a total of 66 patients and their corresponding RBX2660 products were collected during a multicenter, randomized, blinded, and placebo-controlled phase 2b study for the treatment of rCDI (Fig. 1) [26]. 21 patients received 2 doses of placebo (14 females, 9 CDI recurrence, median age 63 years), 22 patients received 1 dose of RBX2660 and 1 dose of placebo sequentially (15 females, 5 CDI

recurrence, median age 63 years), and 23 patients received 2 doses of RBX2660 (15 females, 8 CDI recurrence, median age 68 years) [26]. Each RBX2660 dose derives from a single donor, and RBX2660 dose selection was not constrained to ensure a single donor was represented in patients that received two RBX2660 doses (Supplementary Table 1). The first dose of study drug (RBX2660 or placebo) was administered 24–48 hours following completion of antibiotic treatment for CDI, and the second treatment was administered  $7 \pm 3$  days later (Fig. 1). Patients who experienced a new rCDI episode within 60 days after the first dose were moved to open-label treatment and received two additional doses of randomized RBX2660. Patient fecal specimens were collected at selected time points from baseline (day 0) through 365 days after the first dose. AROs from each fecal sample were isolated on selective media plates (Methods, Supplementary Table 2).

## **2.2. RBX2660 shifted taxonomic structures of patients' intestinal microbiome towards those of healthy donors**

A comparison of alpha diversity (Shannon diversity) of the gut microbiota between RBX2660 product and rCDI patients revealed that rCDI patients had significantly lower alpha diversity than donors before the treatment (Fig. 2a) as previously described [29]. Following study drug administration, the alpha diversity of all rCDI patients' microbiota increased to near-RBX2660 levels regardless of the treatment group, with the steepest increase during the first week (Fig. 2b). The taxonomic composition of the gut microbiota of RBX2660 recipients and placebo recipients both changed over time (Fig. S1). Analysis of intra-subject Bray-Curtis dissimilarities indicated that the largest taxonomic shift occurred during the first week in all treatment groups (Fig. S2a).

Prior to treatment, the majority of patients had distinct gut microbiome taxonomic compositions from those of RBX2660 (Fig. 2c). Bray-Curtis dissimilarities between recipient and corresponding RBX2660 product were calculated to assess the level of taxonomic transformation towards that of RBX2660. For placebo recipients, the dissimilarity was measured from a pseudo-donor (DS00) profile calculated from the average species-level taxonomic profile of all RBX2660 products in this study (Fig. 2c). The mean Bray-Curtis dissimilarity of DS00 from RBX2660 products was 0.4926, which was lower than the inter-RBX2660 Bray-Curtis distance of 0.6274. Bray-Curtis dissimilarities between patients and RBX2660 demonstrate that RBX2660 administration effectively changed recipients' microbiome structure towards RBX2660 at a larger magnitude and for a longer duration as compared to placebo (Kruskal-Wallis test,  $P = 0.043$  at day 30,  $P = 0.028$  at day 60, Fig. 2d).

Placebo recipients exhibited similar direction of taxonomic structural changes toward RBX2660, but the change was not as dramatic as that of RBX2660 recipients (Fig. 2e). When comparing groups based on rCDI treatment success, treatment-failure patients who experienced a new rCDI episode within 60 days after treatment and treatment-success patients did not exhibit significant difference regarding taxonomic structural change (Fig. S2b – d).

## **2.3. Taxonomic structures of RBX2660 were transplanted to patients**

To quantify and compare patients' levels of change in microbiome composition, we calculated a transplantation index indicating extent of microbiome convergence of RBX2660 microbiome transplantation. This index was defined as the change in Bray-Curtis distance from donor (first dose) between baseline ( $Distance_{BL}$ ) and selected time point ( $Distance_T$ ), divided by the distance from donor at baseline:  $(Distance_{BL} - Distance_T) / Distance_{BL}$ . DS00 was used for placebo recipients, who were then used to determine taxonomic transplantation success. RBX2660 recipients were categorized as transplanted or non-transplanted based on whether their transplant index was higher (transplanted) or lower (non-transplanted) than the maximum value of the placebo group (Fig. 3a). The transplantation ratio trended higher in two-dose recipients versus one dose recipients; this categorization showed 33.3% and 70.6% transplantation for single and double dose recipients, respectively, by day 7 (Chi-square test,  $P = 0.02752$ ), and 29.4% and 58.3% by day 60 (Chi-square test,  $P = 0.1212$ ). Regardless of dose, non-transplanted patients at day 7 maintained non-transplanted status until day 60. *Veillonella atypica* was the only baseline taxonomic feature determined by Linear discriminant analysis Effect Size (LEfSe) [30] that distinguished patients with successful microbiome transplantation by day 60 from non-transplanted patients in both single and double RBX2660 treatment arms (Fig. 3b).

Although double RBX2660 doses led to more effective transplantation of RBX2660 microbiome structure by day 7, there were 4 double-dose recipients (R2-01, R2-02, R2-03, R2-14) who showed lower transplantation indices than placebo recipients at day 60. Patient R2-14 exhibited a lower transplantation index than placebo patients at day 60 for the first dose RBX2660 (white square, Fig. 3a) but a higher transplantation index for the second dose (Fig. S3b). We determined 18 taxa (Fig. 3c) and 21 functions (Fig. S3a) as features specifically explaining the baseline microbiome of these 4 patients by comparing with other double-dose recipients that showed durable taxonomic transplantation by day 60 using LEfSe [30]. Of these, 4 taxonomic features were fungi, which are intrinsically vancomycin insensitive, and 7 functional features of eukaryote-specific metabolic pathways (Fig. 3a and S3a). We further investigated the predicted vancomycin insensitivity of other taxonomic features and found 8 additional intrinsically vancomycin resistant bacteria including *Pediococcus* strains [31–33], *Lactobacillus* and *Leuconostoc* strains [34–36] as well as gram-negative and fungal strains. *Enterococcus casseliflavus*, which has low level resistance to vancomycin, was also identified [37]. 4 taxa (*Clostridium glycolicum* [38], *Gemella haemolysans* [39], *E. faecalis* [40], and *C. difficile* [41]) are predicted to be vancomycin susceptible. As compared to the transplanted patients, the 4 non-transplanted patients did not exhibit any other distinctive taxonomic characteristics in terms of alpha diversity and composition of Bacteroidetes, Firmicutes, and Proteobacteria phyla (Fig. S3c – g).

## 2.4. Cessation of antibiotics is a major factor for resistome regression

Prior to treatment, rCDI patients showed a similar resistome alpha diversity (Wilcoxon signed-rank test,  $P = 0.18$ , Fig. 4b) but significantly higher ARG abundance (Wilcoxon signed-rank test,  $P < 0.0001$ , Fig. 4a) as well as distinct resistome composition from RBX2660 products (Fig. S4). Specifically, major facilitator superfamily (MFS) and resistance-nodulation-cell division (RND) efflux pumps were the major ARG

families present in rCDI patients before the treatment, whereas CfxA beta-lactamase, tetracycline-resistant ribosomal protection proteins, and Erm 23S rRNA methyltransferases were representative of the RBX2660 resistome (Fig. S4).

Over time, microbiome ARG abundance significantly decreased in all treatment arms including the placebo group (Fig. 4c). We tracked individual changes in resistome composition of each patient for 60 days using t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis [42] and resistome transplantation indices defined analogously to the microbiome transplantation index. rCDI patients showed distinctive resistome compositions as compared to those of RBX2660 prior to the treatment, but over time their resistome compositions converged to become similar to RBX2660 (Fig. 4d). The speed of resistome transformation toward RBX2660-like structures varied by patient, and the convergence toward RBX2660 donor resistome structure showed strong correlation to the taxonomic transplantation irrespective of treatment arm ( $R^2 = 0.406$ ,  $P < 0.0001$ , Fig. 4e). RBX2660 administration led to higher taxonomic and resistome transplantation indices than the placebo (Fig. 4e).

To identify features distinguishing patient and RBX2660 resistomes, we used a Random Forest Classifier (Fig. S5a – b). Of the top 10 features of importance, 7 ARGs, namely MFS efflux pump, RND efflux pump, OXY  $\beta$ -lactamase, Pmr phosphoethanolamine transferase, undecaprenyl pyrophosphate related proteins, ATP-binding cassette (ABC) efflux pump, small multidrug resistance (SMR) efflux pump, and tetracycline resistant ribosomal protein, were specific to patients' baseline resistomes. Class A  $\beta$ -lactamases (CfxA and CblA) and a tetracycline resistance protein, which are frequently identified in otherwise healthy populations [43–46], were classified as RBX2660-specific ARGs (Fig. 5a). Abundances of all selected ARGs were significantly altered in recipients one week after study drug administration. (Fig. 5b – k). The regression of patient-origin ARGs occurred in all patients without statistically significant differences among placebo and RBX2660 recipients (Fig. 5b – h and S5c – i). Administration of RBX2660 increased abundances of RBX2660-origin  $\beta$ -lactamases in a dose-dependent manner (Fig. 5i and 5j), while the abundance of tetracycline resistant ribosomal protection protein increased in all patients irrespective of treatment (Fig. 5k).

## **2.5. RBX2660 effectively cleared AROs compared to placebo but introduced new AROs**

We identified both persisting and newly introduced AROs based on whole genome sequence analyses of isolates from both blind and open-label treatment patients. ARO isolates were *Escherichia coli* ( $n = 104$ ), vancomycin-resistant *Enterococcus* (VRE) ( $n = 25$ ) and other species ( $n = 135$ ). The majority of donor derived AROs were *E. coli* (Fig. 6). We selected *E. coli* and VRE, the plurality of screened AROs, for further analyses based on availability of donor-patient matched pairs and longitudinal samples. Pairwise average nucleotide identity (ANI) was above 97% for all *E. coli* isolates (Fig. S7), with more than 99.43% identity for all VRE (Fig. S6). Core genome phylogeny indicated the *E. coli* were mostly of the B2 and D phylogroups. Isolates not only clustered together based on the patient of origin, but also with their corresponding donor (Fig. S7).

In general, RBX2660 recipients demonstrated faster clearance of AROs as compared to placebo recipients (Fig. 6). Simultaneously, new AROs from RBX2660, mostly *E. coli*, were introduced to corresponding patients. Calculation of single nucleotide polymorphism (SNP) distances (Methods 5.7) revealed many of these AROs were likely clonal, with a median of 6 SNPs for all pairwise distances indicating near-identical genomes (Supplementary Table 3). We sorted post-treatment ARO *E. coli* into RBX2660-origin or patient-origin strains and determined clonal persistence following RBX2660 intervention. In some cases, we observed clonal persistence of patient AROs (e.g., patients R1-05 and R2-18), while in some we observed strain replacement by donor-derived AROs (e.g., patient R2-16). Interestingly, patients receiving the same donor stool did not display identical trends. Patient R2-21 received the same donor stool as R2-18 yet only R2-21 engrafted the donor ARO (Fig. 6).

Isolate ARGs did not indicate a changing resistance profile for these ARO lineages over time. For instance, *E. coli* isolates exhibited an average of 60 predicted ARGs, and these numbers remained stable throughout the time frame of this investigation (Supplementary Table 4). The 15 RBX2660-origin AROs which were engrafted to corresponding recipients harbored beta-lactamase genes such as AmpC (12 AROs), TEM-1 (8), CARB (3, one each of CARB-17, 19, and 20) or CTX-M-14 (1) (Supplementary Table 4). Antibiotic susceptibility testing (AST) corroborated these findings on the phenotypic level with all introduced AROs being resistant to ciprofloxacin and levofloxacin, and 60% (9/15) resistant to ampicillin (Fig. S8). Approximately half were resistant or intermediate to trimethoprim-sulfamethoxazole (7) and doxycycline (7), and a few were resistant to ampicillin-sulbactam (3) and cefazolin (4), while all were susceptible to cefotetan, ceftazidime, meropenem, imipenem, piperacillin-tazobactam, ceftazidime-avibactam, amikacin, aztreonam, tigecycline, and nitrofurantoin (Supplementary Table 2). The introduced AROs were Enterobacteriaceae and resistant to a median of 4 antibiotics, which was less than that of the patient-derived Enterobacteriaceae AROs (median resistance to 7 antibiotics, Supplementary Table 2). The most resistant isolate introduced from RBX2660 was an *E. coli* strain which was engrafted into patient R1-09. It was retrieved at 5 subsequent time points (final stool collected at 12 months, all < 20 SNPs, Fig. 6, Supplementary Table 2). This isolate was resistant to ceftriaxone and cefepime and classified as an ESBL-producing *E. coli* (DI11, Supplementary Table 2).

### 3. Discussion

In this study, we investigated factors underlying changes in the microbiome derived from RBX2660 in a randomized, placebo-controlled clinical trial [26]. Consistent with a previous evaluation using 16S rRNA methods [29] but in higher resolution with shotgun metagenomic sequencing, we demonstrated that not only RBX2660 recipients but also placebo recipients exhibited noticeable changes toward a healthier microbiome. All patients increased alpha diversity (Fig. 2b), shifted taxonomic structure (Fig. 2e and S2a), and decreased ARG burden (Fig. 4c) regardless of treatment. Based on the shifts observed among placebo-recipients, some of these changes could be accredited to the natural trajectory of recovery after antibiotic discontinuation, as has been observed [10, 47]. Nonetheless, we distinguished RBX2660-derived effects from placebo effects through a simple yet novel metric, the transplantation index. The transplantation index accounts for long-term changes in the microbiome while controlling for individual

variation in baseline composition. With this index, we found that RBX2660 recipients exhibited stronger and longer-lasting changes than placebo recipients.

During the first week, both single and double RBX2660 treatments caused notable taxonomic transformation toward RBX2660, while double RBX2660 resulted in an increase in the ratio of transplanted patients compared to a single dose (Fig. 3a). Despite their apparent difference in transplantation indices, one and two RBX2660 doses showed equivalent clinical efficacy [26], so further exploration is needed to clarify the impact of transplantation efficiency on clinical efficacy. Likewise, although early-stage transplantation by day 7 appeared to be an important factor determining durable transplant by day 60, it did not always secure successful prevention of additional CDI recurrences and vice versa (Fig. 3a). Taxonomic changes of the non-transplanted patients toward corresponding donors were not distinguishable from those of placebo patients (Fig. 3a). 1 single-dose recipient (R1-04) and 3 double-dose recipients (R2-01, R2-03, and R2-14) failed to maintain their transplanted state at day 7 until day 60 and eventually reverted to below the taxonomic transplantation threshold. The cause for this instability is unknown, but may reflect multifactorial microbial interactions [48].

In an effort to predict transplantation success, we identified baseline taxonomic features that had strong correlations with taxonomic non-transplantation. Species with intrinsic vancomycin resistance were discriminative baseline features of the 4 patients who failed to acquire or maintain transplantation by double RBX2660 administration by day 60 (R2-01, R2-02, R2-03, and R2-14). Prior to treatment, 94% of all patients (64/68) including the 4 patients had received vancomycin, with the remainder receiving metronidazole or fidaxomicin prior to study drug (Fig. 1). Previously reported microbiome signatures of vancomycin administration include lower diversity, lower Firmicutes, and higher Proteobacteria abundances [10, 49, 50], but these characteristics could not distinguish the 4 non-transplanted patients from transplanted patients (Fig. S3c – g). Thus, the specific abundance of intrinsically vancomycin-resistant species could be an indicator of severe microbiome disturbance derived from vancomycin use. Interestingly, the baseline abundance of *V. atypica* was significantly and positively correlated with durable taxonomic transplantation of RBX2660 microbiome in both the single and double administration arms (Fig. 3b). *V. atypica* can build metabolomic networks based on its metabolic function in the host gut—converting lactate to propionate [51]—and interspecies communication with lactic acid bacteria [52, 53]. Further studies with metabolomic analyses as well as microbiome analyses are necessary to uncover the mechanism underlying the positive role of *V. atypica* in the durable microbiota transplantation by RBX2660.

Consistent with a previous report for FMT [24], rCDI patients exhibited higher abundance of total ARGs than RBX2660 products prior to study drug administration (Fig. 4b). However, alpha diversity of the baseline resistome of rCDI patients was not greater than those of RBX2660 donors (Fig. 4a) when ARGs were grouped into ARG families based on the organizational structure in CARD [54]. A random forests classifier distinguished between patient baseline and RBX2660 resistomes with a precision score of 0.95 and AUC (area under the curve) score of 1 (Fig. S5b). This high performance of the classifier indicates distinct resistomes of the perturbed and healthy state. Of the top 10 ARGs of importance, 7 were specific

to rCDI patients' resistome, while 3 were more abundant in RBX2660 products. In particular, antibiotic efflux pumps dominated the patient baseline resistome, while tetracycline resistant ribosomal protection protein was the most enriched ARG in RBX2660 (Fig. 5k and S4). Tetracycline resistant mechanisms have previously been reported to be enriched in healthy donor stool in FMT trials [20]. Total ARG load and resistome structure were recovered in all the three treatment arms (Fig. 4c and S4). Moreover, the abundance of the 7 patient-specific ARGs decreased irrespective of the dose of RBX2660. The decrease in the 7 ARGs was steepest during the first week of the clinical trial (Fig. 5 and S8c – i). These outcomes suggest that antibiotic discontinuation and recovery of the microbiota post-antibiotics [47] could be the drivers of the changes in resistome during this clinical trial. The patient-specific 7 ARGs were discriminative features of the resistome disturbed by antibiotic treatment for CDI. As efflux pumps and ribosomal protection proteins are ubiquitous in commensal microbiota [55], further studies comparing ARGs and their functional consequences are required for better defining microbiome health and disturbance.

RBX2660-derived effects were also observed. Patients gained RBX2660-origin beta-lactamases in a dose-dependent manner while simultaneously losing patient-specific ARGs (Fig. 5i and 5j). At the isolate level, RBX2660 simultaneously introduced and eradicated AROs in patients during the process of transplantation (Fig. 6). Previous studies have also demonstrated some efficacy of FMT for eradicating AROs [56], but to our knowledge this is the first to comprehensively track clonality for patient-derived ARO isolates. Most introduced AROs were antibiotic resistant *E. coli* that are commonly present in a healthy population [57, 58] (Fig. 6). The introduced AROs were found in patients longitudinally for up to one year post-treatment (Fig. 6 and Supplementary Table 2). In some patients, the RBX2660 ARO replaced that of the patient, while the patient strain persisted in others. Persisting AROs derived from patients R1-05 and R2-18 showed higher phenotypic resistance than their corresponding RBX2660-derived isolates, which failed to engraft. On the other hand, patient R2-21 received the same RBX2660 product as R2-18 but the RBX2660 ARO was engrafted. Patient R2-21 lacked baseline AROs and perhaps provided a “clean slate” for the ARO engraftment.

We identified one ESBL-producing *E. coli* strain from a donor carrying AmpC and CTX-M-14, whose RBX2660 product was administered to one patient, R1-09. The patient was a single-dose recipient, with recorded treatment success (i.e. no recurrence of CDI and absence of diarrhea for 8 weeks post-treatment) and no known clinical disease resulted from the trial. ESBL-producing *E. coli* are not inherently more virulent than other strains but can pose a therapeutic challenge if infection occurs [59]. Of note, this trial enrolled patients from December 2014 to November 2015, prior to recognition of ESBL as an important aspect of donor screening. At that time, donor stools were screened for carbapenem-resistant Enterobacteriaceae (CRE) but not ESBL, whereas Rebiotix now screens all donor stools for CRE and ESBL. Moreover, to date there have been no adverse infection events due to bacterial transmission from RBX2660 in any clinical trials. In light of a recent death caused by ESBL-producing *E. coli* bacteremia in an immunocompromised patient after FMT [21], our findings highlight the importance of a controlled and regulated donor screening program as well as mandatory, monitored safety reporting. Likewise, our

findings prompt a general consideration of risk factors for infections from intestinal microorganisms in any life biotherapeutic investigational product.

## 4. Conclusions

We thoroughly examined the impact of RBX2660 on the taxonomic structure, resistome, and ARO colonization of recipients during a randomized and placebo-controlled clinical trial. This study is based on samples from a completed placebo-controlled clinical trial of intestinal microbiota restoration, which enabled us to determine microbiome effects of the microbiota-based drug. Significant changes in both taxonomic and resistome structures following placebo administration, implicating microbiota recovery following cessation of antibiotic treatment as a major contributor to microbiota recovery. Still, RBX2660 administration transplanted healthy microbiota in the recipients in a dose-dependent manner. *V. atypica* and intrinsic vancomycin resistant species were discriminative features of patients showing long-lasting microbiota transplantation and resisting microbiota transplantation, respectively. RBX2660 led to more dynamic transformation of the resistome than placebo, but antibiotic discontinuation was the major driving force behind the reduction of patient-origin ARGs, while RBX2660 simultaneously introduced RBX2660-origin ARGs in a dose-dependent manner. RBX2660 more efficiently decolonized AROs than placebo but simultaneously introduced new AROs. Genomic outcomes of intestinal microbiota restoration with RBX2660 in the current study show both latent limitations of microbiota transplantation as well as its potential benefits and highlight the importance of the design and quality control of microbiota-based drugs.

## 5. Methods

### 5.1. Study cohort, drug, and specimen

Subjects were recruited from among 17 centers in the United States and Canada from 10 December 2014 through 13 November 2015. Subjects were adults with recurrent CDI who have had either i) at least two recurrences after a primary episode (total three CDI episodes) and had completed at least two rounds of oral antibiotic therapy or ii) had at least two episodes of severe CDI resulting in hospitalization. They were randomly assigned to one of three treatment groups: placebo, single, or double doses of RBX2660. All treatments were blinded and delivered by enema [26]. The second dose was administered approximately 7 days after the first dose. For patients that received two RBX2660 doses, donor selection was random and not constrained to provide a single representative donor per patient.

The selection and screening of donors for RBX2660 were performed as previously described [27,28]. The placebo composed of normal saline and formulation solution including cryoprotectant in the same proportions used for the RBX2660 preparation. RBX2660 and placebo were stored frozen after preparation until administration. They were thawed for 24 hours in a refrigerator and administered within 48 hours after thawing. AROs were isolated from patient stools and RBX2660 products on selective agar media plates, chromID VRE (bioMerieux, Marcy-l'Etoile, France), MacConkey with Cefotaxime (Hardy

Diagnostics, Santa Maria, CA), MacConkey with Ciprofloxacin, (Hardy Diagnostics), and HardyCHROM™ ESBL (Hardy Diagnostics), at 35°C in air. The remaining stools were stored frozen at -80°C until metagenomic DNA extraction. Isolate colonies were sub-cultured to trypticase soy agar with 5% sheep blood (Becton Dickinson, Franklin Lakes, NJ) and identified using VITEK MS matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) system [60,61]. Each isolate was frozen in tryptic soy broth with glycerol at -80°C.

## 5.2. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed through Kirby Bauer disk diffusion, and the resulting zone sizes were interpreted according to the M100 document from the Clinical and Laboratory Standards Institute [62].

## 5.3. DNA extraction and sequencing

Metagenomic DNA was extracted from approximately 100 mg of stool samples using DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol excepting the stool lysis step: stool samples were lysed by 2 rounds of bead beating for 2 min (total 4 min) at 2,500 oscillations/min using a Mini-Beadbeater-24 (Biospec Products). Samples were chilled on ice for 2 min between the two bead beating rounds. Extracted DNA was quantified using a Qubit fluorometer dsDNA HS Assay (Invitrogen) and stored at -20°C until the library preparation. Metagenomic DNA was diluted to 0.5 ng/μL before preparing the sequencing library. Libraries were prepared using the Nextera DNA Library Prep Kit (Illumina) as previously described [63]. The libraries then were purified through the Agencourt AMPure XP system (Beckman Coulter) and quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) before sequencing. Approximately 70 library samples were pooled in an equimolar manner at the final concentration of 5 nM for each sequencing lane. Prepared pools were submitted for 2 × 150 bp paired-end sequencing on an Illumina NextSeq High-Output platform at the Center for Genome Sciences and Systems Biology at Washington University in St. Louis with a target sequencing depth of approximately 5.5 million reads per sample.

Isolate genomic DNA was extracted using QIAmp BiOstic Bacteremia DNA Kit (Qiagen). Libraries for whole genome sequencing of isolates were prepared from diluted genomic DNA (0.5 ng/μL) as described above. About 180 libraries were pooled together in an equimolar manner at the final concentration of 5 nM for each sequencing lane. Prepared pools were submitted for 2 × 150 bp paired-end sequencing on an Illumina NextSeq High-Output platform at the Center for Genome Sciences and Systems Biology at Washington University in St. Louis with a target sequencing depth of approximately 2 million reads per sample.

## 5.4. Data processing and genome assembly

Sequence reads were binned by index sequence. Adapter and index sequences were trimmed using Trimmomatic v.0.38 [64] using the following parameters: java -Xms2048m -Xmx2048m -jar trimmomatic-0.38.jar PE -phred33 ILLUMINACLIP: NexteraPE-PE.fa:2:30:10:1:true SLIDINGWINDOW:4:15 LEADING:10 TRAILING:10 MINLEN:60. Human sequence contamination was eliminated using Deconseq [65], and the qualities of resulting reads were verified by FastQC (<https://github.com/s-andrews/FastQC>).

Isolate genomes were assembled, assessed, and annotated using SPAdes [66], QUAST [67], and Prokka [68], respectively. Average nucleotide identity between *E. coli* and VRE isolate pairs were calculated using dnadiff [69]. Within-species pan genomes and core genome alignments were obtained with Roary [70] with default parameters, using 24 and 4 NCBI reference strains (Supplementary Table 5) for *E. coli* and VRE, respectively, with additional *Escherichia fergusonii* and general *Enterobacter faecalis* as outgroups. Alignments were converted via FastTree [71] and visualized on iTOL v4 [72].

## 5.5. Microbiome composition and comparison

Microbiome taxonomic composition was predicted by MetaPhlAn v2.0 [73] and controlled for relative abundance. Genus-level composition plots were obtained by grouping together genus present in less than 50% of samples as “Other.” DS00 pseudo-donor microbiome was obtained by averaging the species-level taxonomic profiles of all donor microbiomes. Bray-Curtis distances were calculated using the vegan package [74] and visualized as PCoA plots via the ape package [75] in R 3.5.3. LEfSe [30] identified baseline taxonomic and metabolic features distinguishing transplanted and non-transplanted patients (alpha value for the factorial Kruskal-Wallis test = 0.05, threshold on the logarithmic LDA score = 2). HUMAnN2 [76] was employed for metabolic pathway prediction.

## 5.6. Resistome identification and random forest classifier

ARGs in the microbiome were identified using ShortBRED [77] with CARD [54]. Isolate ARGs were identified with RGI and CARD [54,78]. The resulting genes were manually curated into more general ARG families (n = 64). A subset of 70% of available resistomes were then used to train a random forest classifier distinguishing patient baseline and donor stool resistomes (training set n=103), which was then tested on the remaining samples (test set n=45). The random forest classifier was built with the package scikit-learn (<https://scikit-learn.org/stable/index.html>) on Python 3.7.3, with trees averaging 12 nodes and a maximum depth of 4.

## 5.7. ARO tracking and SNP calling

SNPs were called using Bowtie2 [79], SAMtools and BCFtools [80], with the first isolate from the patient or donor used as the reference genome. Reads from subsequent isolates of the same species were aligned against the reference with Bowtie2 (-X 2000 -no-mixed -very-sensitive -n-ceil 0,0.01). BAM files were obtained and sorted with SAMtools (view and sort), which were then converted to pileup files (mpileup). BCFtools view generated VCF files, and variants were called, with the following criteria: minimum coverage of 10 reads per SNP, major allele frequency above 95%, and FQ-score of -85 or less. Indels were excluded. VCF files for each patient were compiled with BCFtools merge, after which SNPs were parsed and counted using custom python and R scripts.

## List Of Abbreviations

**FMT**, fecal microbiota transplantation; **ARG**, antibiotic resistant gene; **ARO**, antibiotic resistant organism; **CDI**, *C. difficile* infection; **rCDI**, recurrent *C. difficile* infection; **VRE**, vancomycin-resistant *Enterobacter*; **SNP**, single nucleotide polymorphism.

## Declarations

- **Ethics approval and consent to participate**

Participants of the Rebiotix Phase 2b study were enrolled at 21 centers in the United States and Canada from 10 December 2014 through 13 November 2015. The study protocol received institutional review board approval at each center. All participants provided written informed consent [26].

- **Consent for publication**

Not applicable

- **Availability of data and material**

The metagenomic sequencing data are uploaded to NCBI under BioProject PRJNA606075 (<https://www.ncbi.nlm.nih.gov/bioproject/606075>). The isolate genome sequences and assemblies are uploaded to NCBI under BioProject PRJNA606074 (<https://www.ncbi.nlm.nih.gov/bioproject/606074>).

- **Competing interests**

Rebiotix provided access to study specimens and data, and reviewed the manuscript prior to submission, but was not involved in this study's design, specimen processing, data analysis, or interpretation. Erik R. Dubberke is a consultant for Sanofi, Pfizer, Synthetic Biologics, BioK+, and Rebiotix, and has a grant from Pfizer.

- **Funding**

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- **Authors' contributions**

S.K., J.C., E.R.D., and G.D. conceived the study design, experiments, and analyses. E.R.D. assembled the cohorts. T.H., K.A.R., K.B., and C.J. oversaw the collection of samples and clinical metadata. T.H. and C.A.B. performed stool cultures, isolate screening and identification. T.H. and M.H.B. performed isolate genomic DNA extraction. S.K. and X.S. extracted metagenomic DNA from stools. S.K. prepared sequencing libraries. S.K. and J.C. analyzed clinical metadata, shotgun metagenomic sequencing data, and isolate genome sequencing data with advice from C.A.B., E.R.D., and G.D. S.K. and J.C. drafted the manuscript and figures with input from E.R.D. and G.D. All authors reviewed the manuscript.

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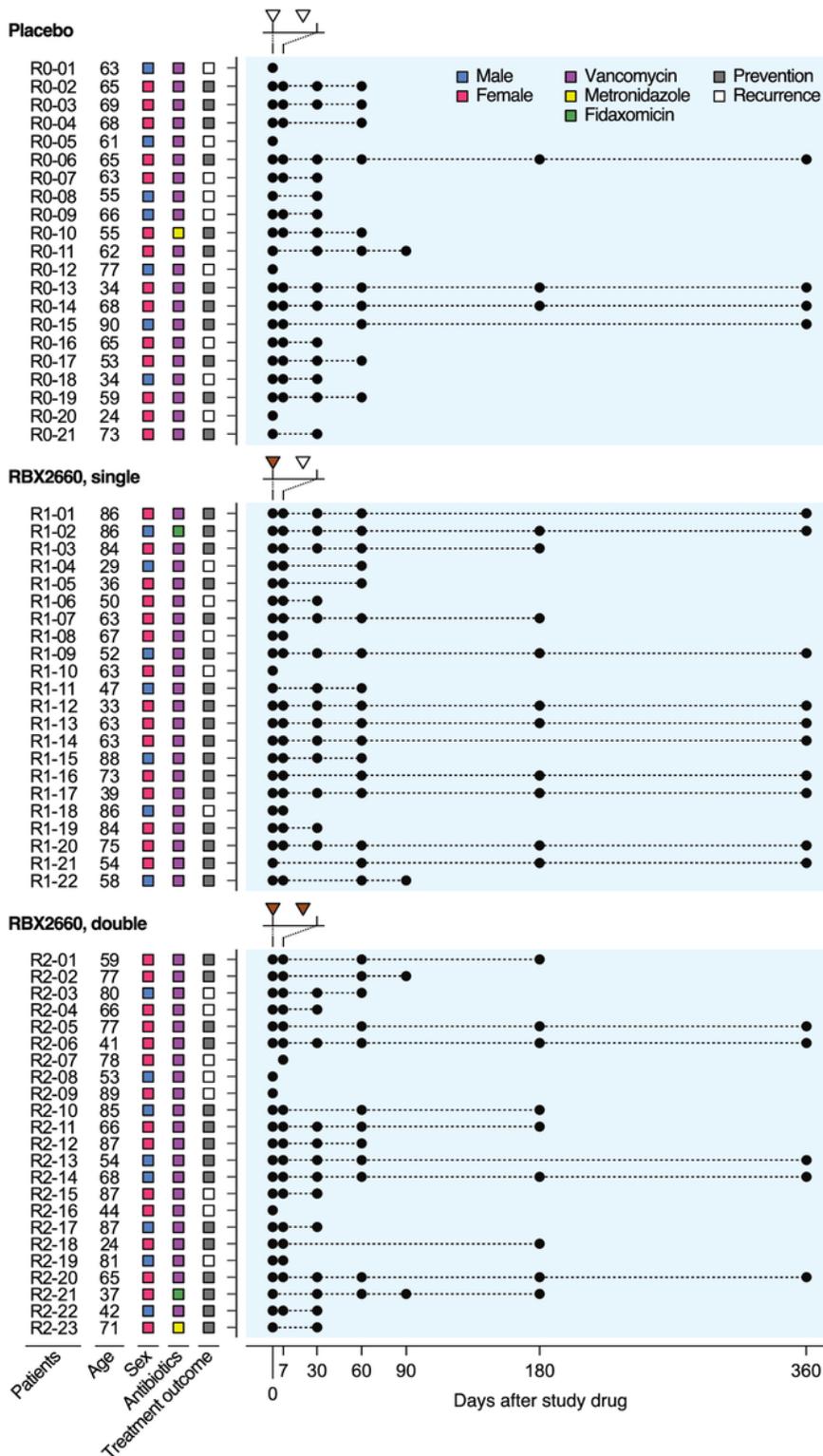
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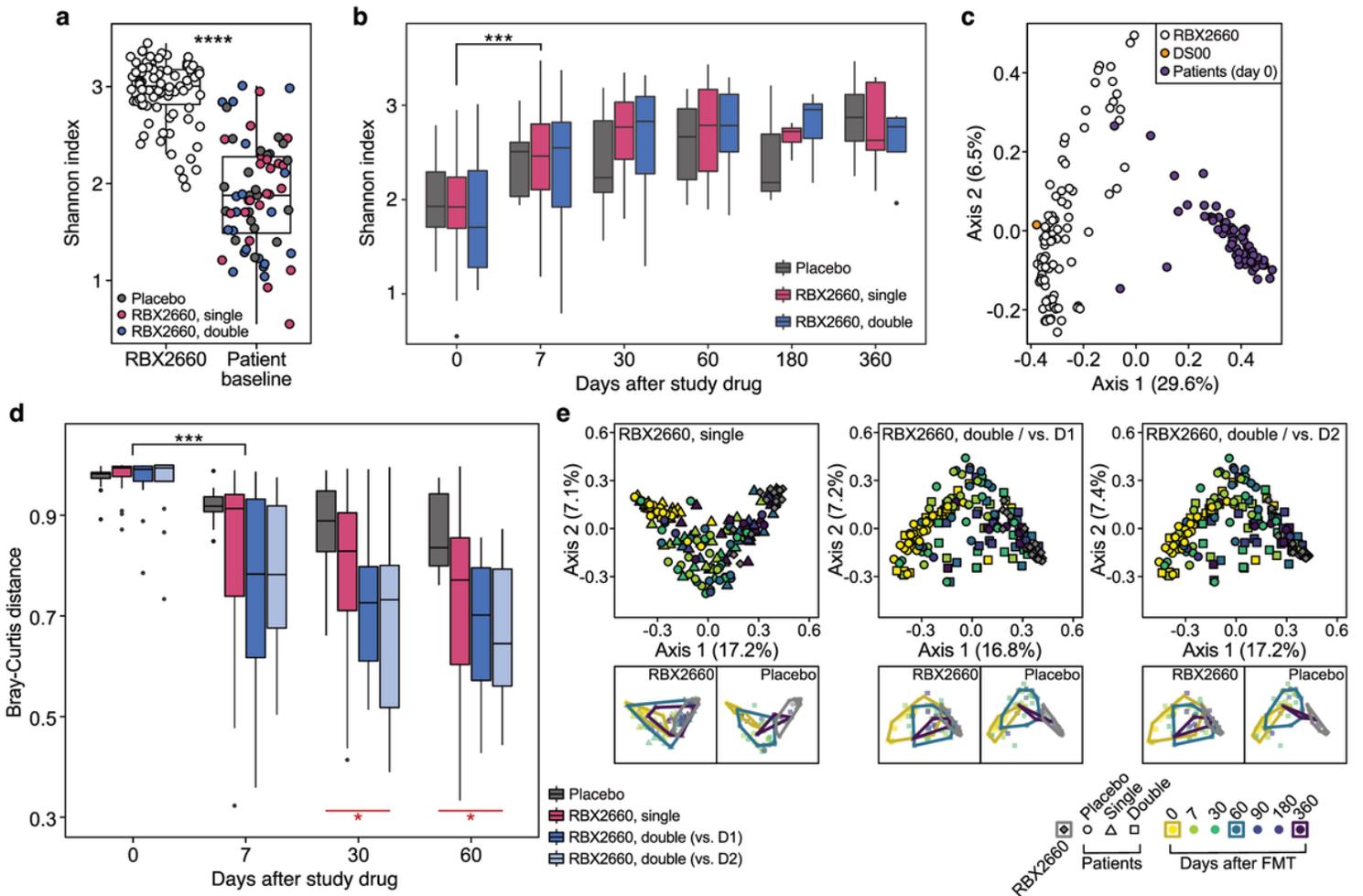
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## Figures



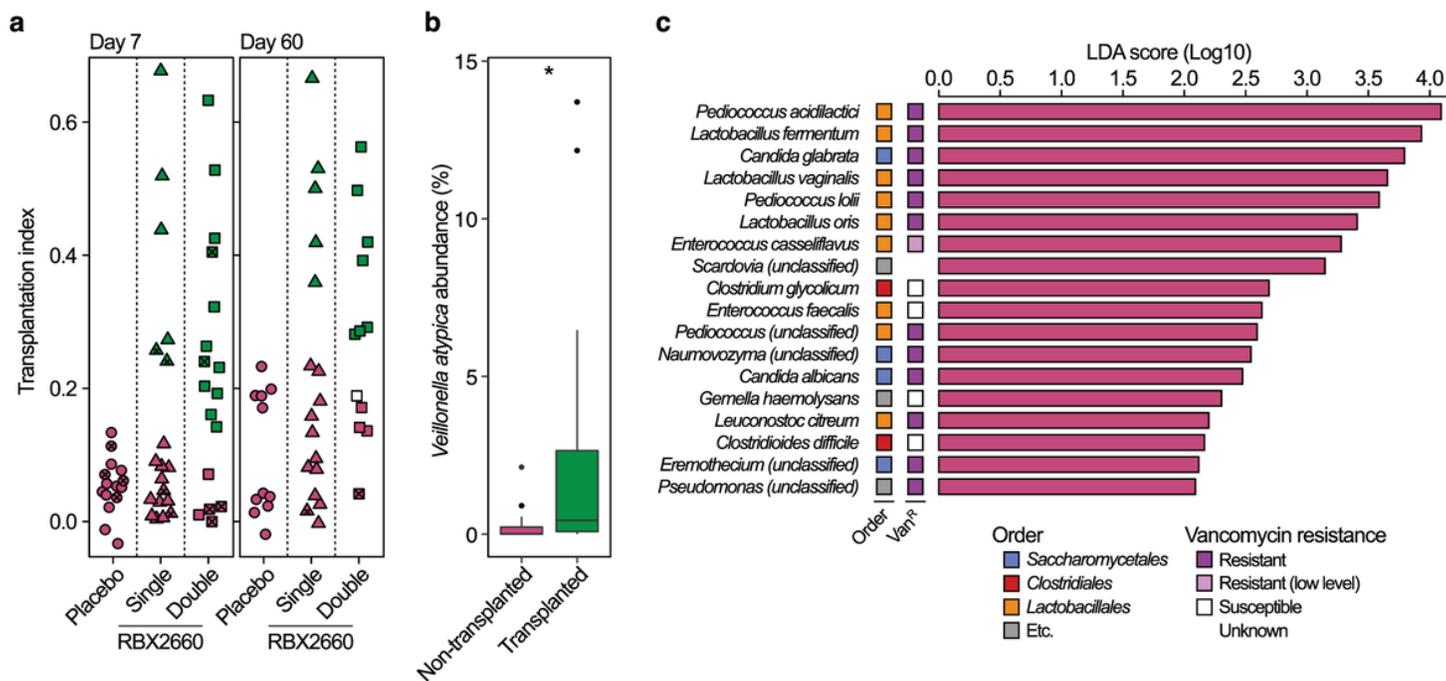
**Figure 1**

Study design for the use of RBX2660 to prevent recurrent *Clostridioides difficile* infection (rCDI). Total of 66 patients with a history of rCDI were treated with RBX2660 in a randomized and blinded manner. Placebo (white triangle) and RBX2660 (brown triangle) were administered and fecal samples (black circle) were collected at the indicated time points. Patients who were declared a new episode of rCDI within 60 days (white square) were moved to open-label treatment.



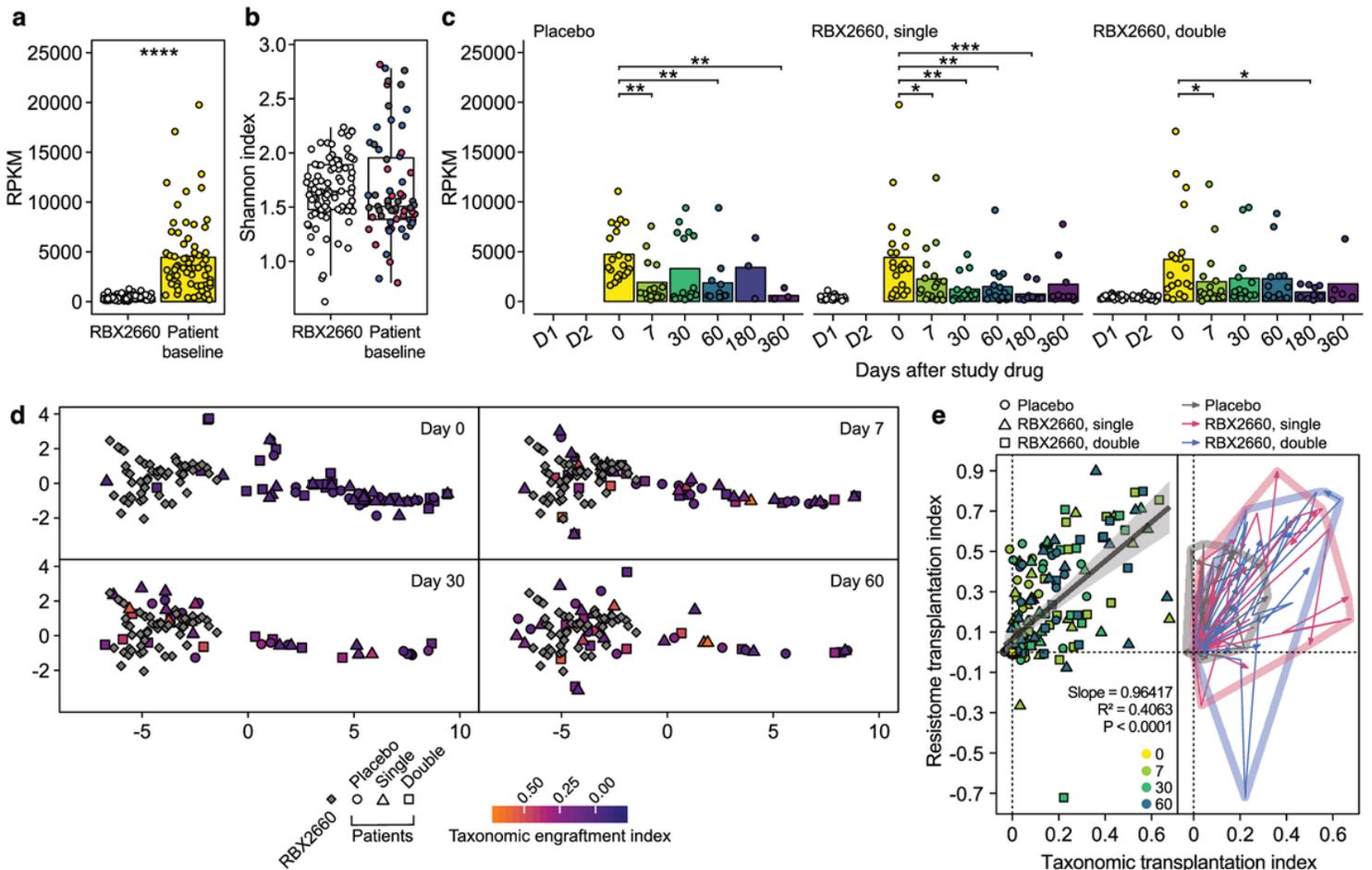
**Figure 2**

RBX2660 shifted taxonomic structures of the gut microbiome of recipients towards a healthy state. a RBX2660 products exhibited significantly higher alpha diversity than patient samples before treatment (Wilcoxon signed-rank test) based on the metagenomic taxonomic profiling data. b Alpha diversity of all patients including placebo recipients increased similarly after treatment. Changes in alpha diversity were significant for the first week after treatment, but there was no statistically significant difference among treatment groups (Kruskal-Wallis test). c Principal coordinates analysis (PCoA) showed a species level clustering of RBX2660 (white) and pseudo-donor sample DS00 (yellow) distinct from patient baseline samples (purple). d Bray-Curtis distance between taxonomic structures of patients and corresponding RBX2660. D1 indicates the first dose, and D2 indicates the second. DS00 was used for calculating the Bray-Curtis distance of placebo recipients. The decrease in Bray-Curtis distances was steepest during the first week after treatment (black, Wilcoxon signed-rank test). RBX2660 recipients showed a more dynamic decrease in Bray-Curtis distances than placebo recipients by day 60 (red, Kruskal-Wallis test). e PCoA describing the direction of changes in taxonomic structures of RBX2660 recipients. Corresponding RBX2660 products and all placebo recipients were included (upper). RBX2660 similarly yet more dynamically transformed taxonomic structures than placebo (lower). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$



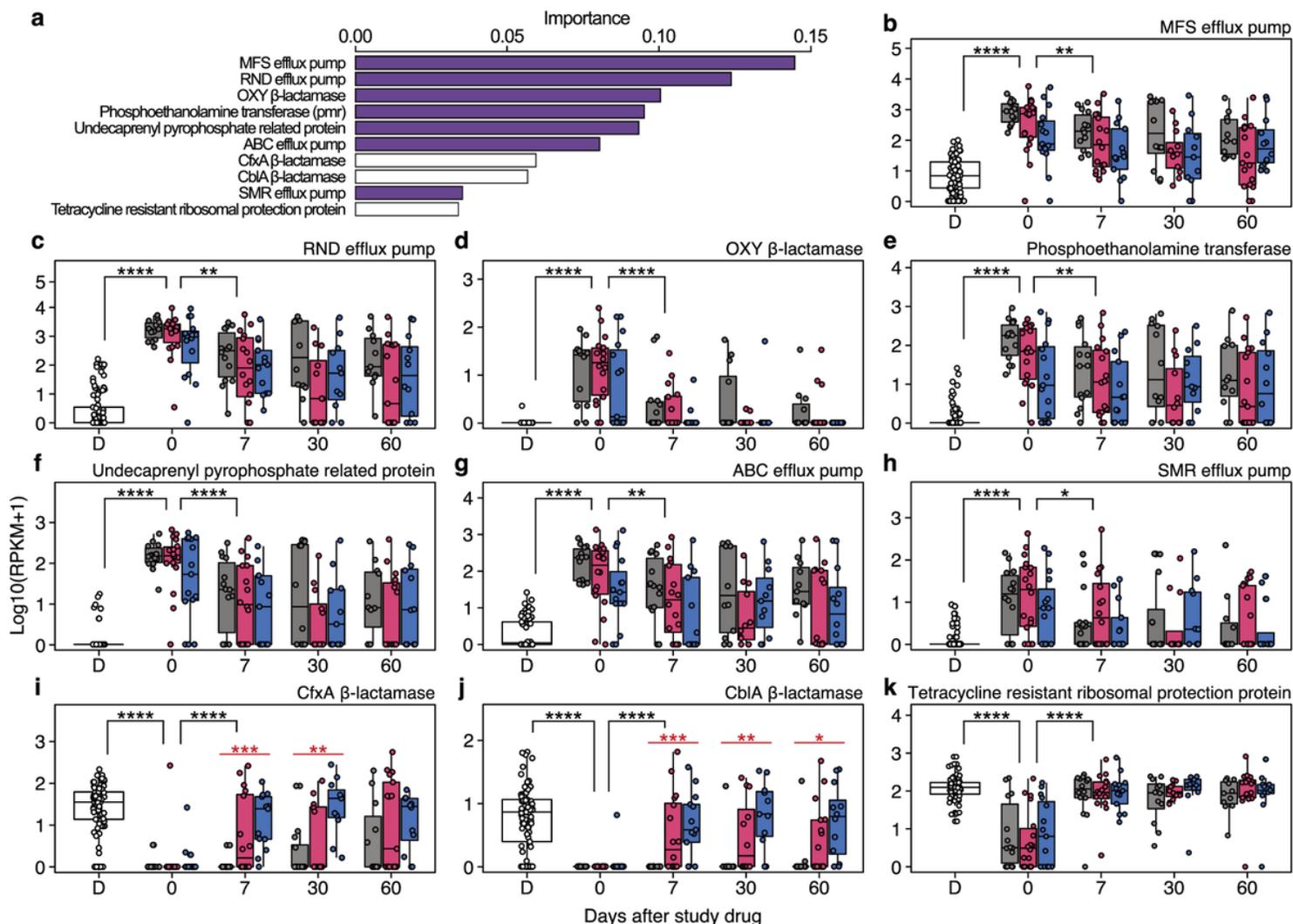
**Figure 3**

Linear discriminant analysis Effect Size (LEfSe) determined discriminative taxonomic features of RBX2660 transplantation. a Transplantation index of patients on day 7 and 60. We defined the taxonomic transplantation as a state showing a higher transplantation index than that of all placebo recipients (green). The patients who were declared rCDI during within 60 days were marked (x). b Higher baseline abundances of *Veillonella atypica* in patients who showed durable taxonomic transplantation by day 60 in both single and double RBX2660 treatment groups (Wilcoxon signed-rank test,  $P = 0.027$ ). c LEfSe determined Taxonomic features of the obstinate non-transplanted patients who exhibited lower transplantation indices than placebo recipients at day 60 after double RBX2660 treatment. 13 species among 18 taxonomic features were intrinsically vancomycin resistant (violet square, including *E. casseliflavus* of low resistance). There was no taxonomic feature specific to transplanted patients determined by LEfSe.



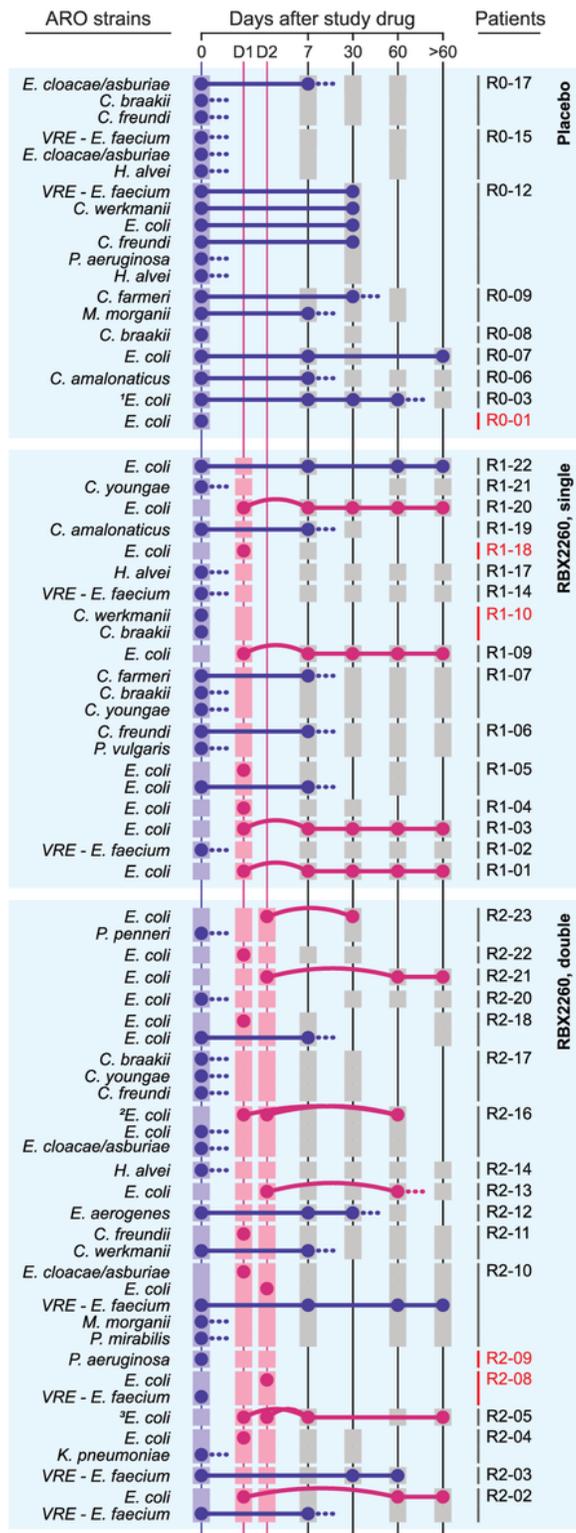
**Figure 4**

RBX2660 fluctuated resistome structures of patients due to the taxonomic transplantation. a Patients had a greater antibiotic resistant gene (ARG) reads per kilobase per million sample reads (RPKM) before treatment (Wilcoxon signed-rank test), b while their resistome alpha diversity was comparable to that of RBX2660 ( $P = 0.18$ ). c Significant decrease in ARG RPKM was observed over time in all treatment groups (Wilcoxon signed-rank test with Benjamini-Hochberg FDR correction,  $FDR < 0.05$ ). Bars indicate mean of individual ARG abundances. D1, the first dose; D2, the second dose. d Patients and RBX2660 products were clustered separately in t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis of resistome structures at day 0. Patient resistome became similar to RBX2660 over time, but the speed of change varied for each patient regardless of RBX2660 dose and taxonomic transplantation index. e RBX2660 simultaneously fluctuated both taxonomic and resistome structures more dynamically as compared to placebo. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$



**Figure 5**

Recipients adopted a resistome profile similar to that of donors. a 10 most important patient-specific (purple) and RBX2660-specific (white) antibiotic resistant gene (ARG) families were identified through Random Forest Classifier. b-k Abundance of the selected 10 ARGs in RBX2660 (D) and patients who received placebo (gray), single RBX2660 (red), and double RBX2660 (blue). Abundance of patient-specific ARGs decreased over time in all patients without statistically significant difference among treatment arms (b-h). Abundance of the two RBX2660-specific beta-lactamases in patients increased by RBX2660 administration in a dose-dependent manner (i-j, red, Kruskal-Wallis test). Tetracycline resistant ribosomal protection protein was a RBX2660-specific ARG, but its abundance in placebo recipients also increased after the treatment (k). These changes were significant during the first week after the treatment (black, Wilcoxon signed-rank test). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$



**Figure 6**

RBX2660 effectively cleared antibiotic resistant organisms (AROs) compared to placebo and simultaneously introduced new AROs. We specifically tracked patient-derived (blue dot) and RBX2660-derived AROs (red dot). Patients with no ARO detected from both the baseline sample and corresponding RBX2660 were excluded. Persistency (solid line), disappearance (dash line), and introduction (curved line) of the AROs were determined by genomic comparison of AROs (Methods 5.7). Squares indicate the

sample availability (blue, patient baseline samples; red, RBX2660; gray, patient samples after RBX2660 administration). Patients with no samples after day 7 were marked with red. 1R0-03 showed 2–3 separate lineages of E. coli prior to day 30, which were reduced to 1 lineage by day 60. 2Patient R2-16 received the same RBX2660 product twice. 3Although the two RBX2660 products for patient R2-05 were prepared from different donor samples, ARO E. coli strains screened from those appeared to be clonal (distance = 8 SNPs).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [200304RBX2bSuppFigurev9JCclean.docx](#)
- [SupplementaryTable4ARGdatav2.xlsx](#)
- [SupplementaryTable1PatientsDrugIdentifiersv2.xlsx](#)
- [SupplementaryTable5NCBIreferences.xlsx](#)
- [SupplementaryTable2ASTResultsv6.xlsx](#)
- [SupplementaryTable3SNPdistancesv3.xlsx](#)