Supplemental Online Content

Louie T, Golan Y, Khanna S, et al. VE303, a defined bacterial consortium, vs placebo for the prevention of recurrent *Clostridioides difficile* infection: a randomized clinical trial. *JAMA*. doi:10.1001/jama.2023.4314

This supplemental material has been provided by the authors to give readers additional information about their work.

eMethods

Pharmacokinetics and Pharmacodynamics

Stool samples were collected longitudinally from each participant in Study VE303-002, at screening and on days 1, 7, 14, 28, 56, and 168, to determine the prevalence and abundance of the VE303 consortium strains during and after treatment with VE303 and the association of this treatment with presence or absence of CDI recurrence. Descriptions of stool sample processing, metagenomic sequencing, and bioinformatics algorithm are given below. The datasets presented here, reflecting samples collected from 78 participants enrolled in study VE303-002, confirm that the VE303 strains colonized robustly and durably in participants who had received antibiotic treatment for a qualifying CDI episode, with significantly greater colonization in the high-dose VE303 group compared with the low-dose VE303 group.

Sample Processing and Metagenomic Sequencing

Stool samples were collected fresh, and an aliquot of each stool sample was transferred to an OMNIgene-GUT tube (DNAgenotek, Ottawa, Canada) and resuspended in the preservation buffer according to manufacturer's instructions. An aliquot of approximately 250 µL of each sample was extracted using the Qiagen PowerSoil Pro DNA isolation kit (Qiagen, Valencia, California, USA). Libraries were prepared using the Illumina Nextera XT DNA Library Prep kit (Illumina Inc., San Diego, California, USA). Libraries were whole-genome sequenced using 1×100 single-end reads, with a target median read depth of 4.3M reads per sample, and sequenced on the Illumina NovaSeq platform at Diversigen (Minneapolis, Minnesota, USA).

Stool metagenomic sequences (or reads) were assigned to taxonomy using the k-mer–based One Codex platform (https://www.onecodex.com/). The estimated relative abundance of endogenous bacterial species in the microbial community was determined using the standard One Codex algorithm from quality filtered metagenomic reads after removal of unclassified reads and reads that map to the human host.

A specialized bioinformatics assay was developed for sensitive and specific detection of VE303 from Illumina metagenomic sequencing datasets based on the detection and frequency of highly curated genomic marker regions, as previously described in [15].

Modeling VE303 Colonization in VE303 Versus Placebo Groups

To examine differences in early (at day 14) colonization of VE303 between the VE303-dosed and placebo groups, we conducted a Wilcoxon rank-sum test on microbiome relative abundance data and on total VE303 proportion values. The Wilcoxon test was chosen because it assumes no underlying distribution, is robust to outliers, and is appropriate for ordinal data such as total strain proportion. We excluded all samples collected after the onset of a CDI recurrence event *or* during the dosing of antibiotics, whichever occurred first. **eTable 3** shows the statistical summary with false discovery rate control for comparison of total VE303 abundance and proportion across cohorts on day 14.

Exposure–Response Analysis in VE303-Treated Participants

To model the probability of recurrence-free survival as a function of exposure to VE303, we calculated Kaplan-Meier curves for VE303-dosed subjects (N = 56) with "High Colonization" and "Low

Colonization." For this analysis, the two active treatment arms were combined. We conducted a cut-point analysis to determine meaningful "High" vs "Low" colonization categories using the proportion of detected VE303 strains per subject at day 14. Kaplan-Meier curves and log-rank p-values were computed for all possible cut-points from 0 to 8 (**eTable 4**).

In high- versus low-colonized subjects, event-free probability was calculated as a function of total VE303 colonization. Using colonization as a covariate, we fit a Cox proportional hazard model (Therneau T.M. & Grambsch, P.M., (2000). The Cox model. In *Modeling survival data: extending the Cox model* (pp. 39-77). Springer, New York. "A Package for Survival Analysis in R." R package version 3.2-11); the comparative probability of recurrence in the "Low" vs "High" groups was computed over the duration of the study and represented by the hazard ratio (i.e., the exponential of the model estimate). We noted that "High" colonized subjects had a significantly lower probability of recurrence for two of the models: when n > 4 strains colonized, p = 0.08; and when n > 5 strains colonized, p = 0.05. The remaining models were not significant (p >= 0.2, **eTable 4**). This finding may reflect the minimal colonization required for clinical efficacy (at least 5 strains colonizing). Furthermore, we observed that all subjects with > 5 strains colonizing were non-recurrent. Therefore, lack of significance in models requiring n > 6 and n > 7 strains in the "High" colonized group may be due to low N and/or unbalanced group numbers (N subjects "High" < N subjects "Low").

To preserve balanced analysis subgroups for this manuscript, we reported the model in which "High" colonized subjects had n detected strains above the median across all VE303-dosed subjects (i.e., 5 to 8 VE303 strains), and "Low" colonized subjects had n detected strains below the median across all VE303-dosed subjects (i.e., 0 to 4 VE303 strains; Figure 3C, **eTable 5**). High-colonization participants showed lower probability for CDI recurrence than low-colonization participants, regardless of the dose of VE303 that they received (p=0.08, log rank test), with a low:high hazard ratio of 5.32. Event-free times were not significantly different (p>0.2) across colonization categories when defined using exposure to total VE303 (sum abundance across all 8 strains). This finding may be due to an inability to detect the effect with a small sample size (N=34 dosed participants) or the greater importance of a subset of the live biotherapeutic product strains to positively affect the gut ecosystem.

Modeling Diversity in VE303 Versus Placebo Groups

Species diversity in subject stool samples was determined using the Shannon Index, a measure of the number of species in a habitat (richness) and their relative abundance (evenness). Diversity was calculated as

$$D = -\sum_{i=0}^{n} p_i log(p_i)$$

where p_i is the relative abundance of species i, and n is the total number of species in the sample.

To examine early (i.e., through day 14) diversity and associations with recurrence between the VE303-dosed and placebo groups, we used a linear mixed effects (LME) model [lmer R package] on log-transformed microbiome relative abundance data. The diversity differences between treatment groups per timepoint, diversity change from Screening to Day 14, and delta diversity (recurrent minus non-recurrent) per treatment group were assessed by fitting additional models that encoded an interaction

between treatment and Visit, or treatment and recurrence status. Treatment group, standard-of-care antibiotic type, recurrence status, and sequencing batch were included as covariates in the model; repeated sampling per participant was handled as a random effect, following the equation:

Diversity ~ Treatment (High Dose/Low Dose/Placebo) + Antibiotic (Vancomycin/Fidaxomicin) + Recurrence (No/Yes) + Time + 1|Participant

The results of the LME model are presented in **eTable 6**. Models were fit to examine the differences in diversity due to:

Treatment group: VE303-dosed vs placebo participants across all timepoints (Contrasts = Treatment) at day 14 only (Contrasts = Timepoint: Treatment).

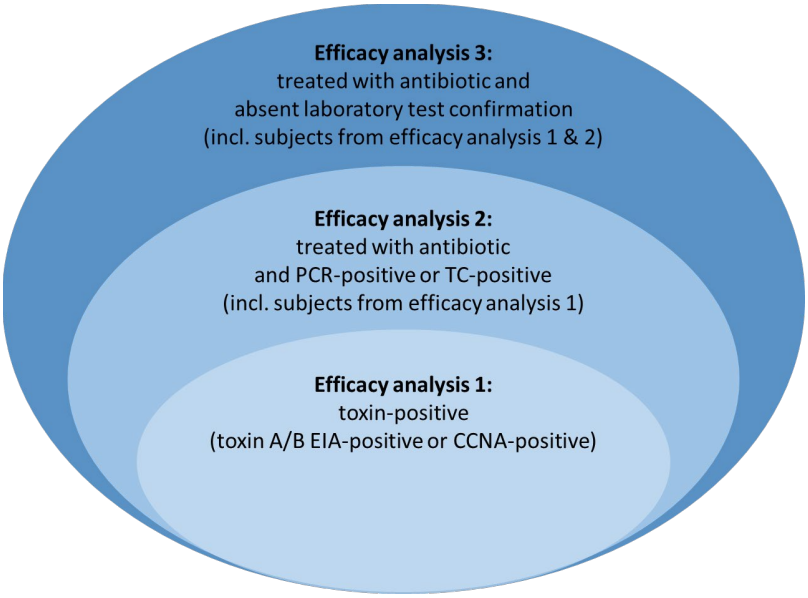
Response: non-recurrent vs recurrent participants regardless of treatment group (Contrasts = Response) and per treatment group (Contrasts = Treatment: Response).

Time: Day 14 vs screening per treatment group (Contrasts = Treatment: Timepoint)

A positive coefficient indicates increased diversity in dosed (compared to placebo)/non-recurrent (compared to recurrent)/day 14 (compared with screening) groups.

eFIGURES

eFigure 1. Prespecified Efficacy Analyses

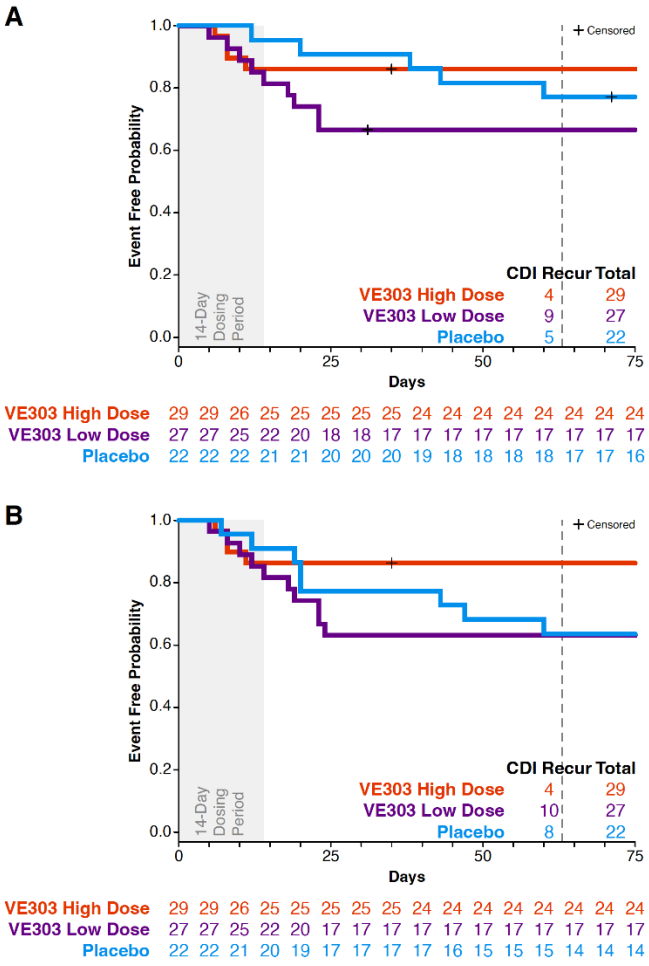


Efficacy was analyzed with 3 prespecified analyses: (1) An episode of diarrhea consistent with CDI that included a toxin-positive stool sample (EIA for toxin A/B or CCNA); (2) an episode of diarrhea consistent with CDI that included a positive PCR or TC test, followed by treatment with standard-of-care antibiotic; and (3) an episode of diarrhea consistent with CDI in the absence of laboratory confirmation, followed by treatment with standard-of-care antibiotic. These efficacy analyses were conducted in a cumulative approach, meaning that all participants who were included in efficacy analysis 1 were also included in analyses 2 and 3; similarly, all participants included in efficacy analysis 2 were automatically included in efficacy analysis 3. The VE303 arms were compared with the pooled placebo group.

Abbreviations: CCNA, cell cytotoxicity neutralization assay; EIA, enzyme immunoassay; PCR, polymerase chain reaction; SoC, standard of care; TC; toxigenic culture.

eFigure 2. Recurrence Rates of *Clostridioides difficile* Infection Through Week 8

Kaplan–Meier estimates for A) toxin-positive (efficacy analysis 1), and B) toxin-, PCR-, or toxigenic culture-positive (efficacy analysis 2) on-study CDI recurrences.



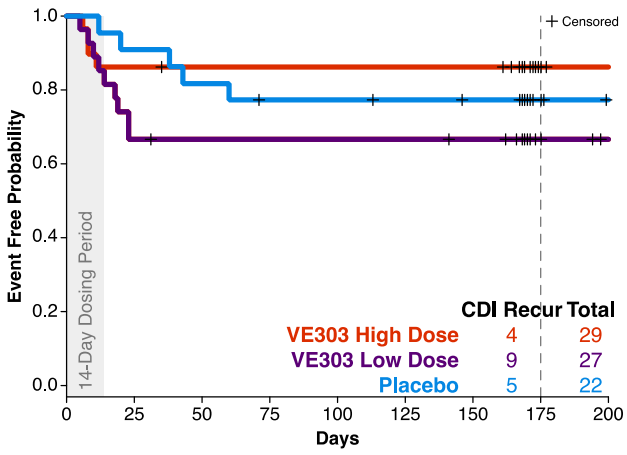
Per the prespecified statistical analysis plan, week-8 CDI recurrences included those with onset up to day 63.

eFigure 3. Recurrence Rates of *Clostridioides difficile* Infection Through Week 24

Kaplan–Meier estimates for A) toxin-positive (efficacy analysis 1), B) toxin-, PCR-, or toxigenic culture-positive (efficacy analysis 2), and C) toxin-positive, PCR- or toxigenic culture-positive, and absence of laboratory confirmation (efficacy analysis 3) on-study CDI recurrences.

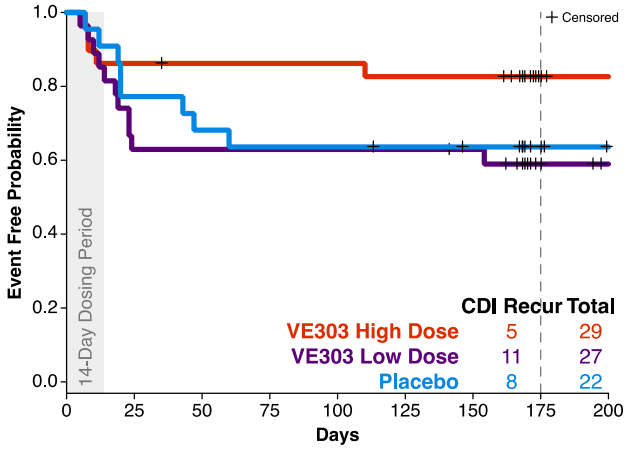
Efficacy is described in **eFigure 2**. In follow-up through week 24, two additional CDI recurrences were reported: 1 in the high-dose VE303 group on day 110 and 1 in the low-dose VE303 group on day 154 (**B**). Two participants assigned to placebo, with CDI recurrences on day 28 and day 44, were included in efficacy analysis 3 only; in both instances, the on-study recurrences could not be confirmed through laboratory tests due to delayed collection and logistical issues with the stool samples (**C**). Per the prespecified statistical analysis plan, week-24 CDI recurrences included those with onset up to day 175.

A



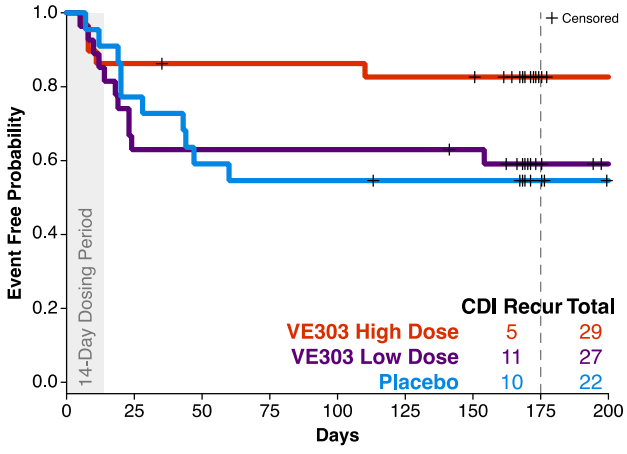
VE303 High Dose	29	25	24	24	24	24	24	3	0
VE303 Low Dose	27	18	17	17	17	17	16	3	0
Placebo	22	20	18	16	16	15	14	3	0

B



VE303 High Dose	29	25	24	24	24	23	23	2	0
VE303 Low Dose	27	17	17	17	17	17	16	3	0
Placebo	22	17	15	14	14	13	12	3	0

C



VE303 High Dose	29	25	24	24	24	23	23	2	0
VE303 Low Dose	27	17	17	17	17	17	16	3	0
Placebo	22	17	13	12	12	11	11	3	0

eTable 1. VE303 Strain Identity

Strain	Cluster Designation	Closest Relative as Determined by Whole-Genome Sequencing[a]
VE303-01	XIVa	*Enterocloster bolteae*
VE303-02	IV	*Anaerotruncus colihominis*
VE303-03	XIVa	*Sellimonas intestinalis*
VE303-04	XIVa	Clostridium_Q *symbiosum*
VE303-05	XIVa	*Blautia* sp001304935
VE303-06	XIVa	Dorea_A *longicatena*
VE303-07	XVII	Clostridium_AQ *innocuum*
VE303-08	IV	*Flavonifractor plautii*

[a] Whole-genome sequence assignment according to genome taxonomy database release 207 (https://gtdb.ecogenomic.org/; April 2022).

eTable 2. Summary of Study Protocol Changes

Amendment no. Protocol Version Date	Summary of Major Changes
Original Version 1.0 21 June 2018	No participants were enrolled under the original protocol
Amendment 1 Version 2.0 15 November 2018	One (1) participant was enrolled under Amendment 1, Version 2.0 • Changed trial design from an adaptive, dose-finding design to a double-blind, placebo-controlled design, which required a reworking of the statistical methods and analyses • Revised timing of efficacy endpoints • Modified sample size from up to 160 to approximately 132 • Updated randomization from 1:1:1:1 to 2:1:2:1 • Modified the study treatment doses for evaluation • Added criteria around inclusion of women of childbearing potential • Added specificity to exclusion criteria for history of diarrhea • Added exclusion criteria around gastrointestinal disorders
Amendment 2 Version 3.0 25 January 2019	One (1) participant was enrolled under Amendment 2, Version 3.0 • Modified study design to include SSR based on results of an IA • Updated that DMC will be unblinded for the IA

Amendment no. Protocol Version Date	Summary of Major Changes
	• Updated participant numbers for randomization to approximately 146 to 300, depending on the outcome of the IA and SSR, for an estimated 124 to 255 evaluable participants • Updated timepoints for PROMIS® questionnaire to be completed weekly on days 1,7, and 14, prior to administration of study treatment • Removed requirement for pregnancies of participants' partners to be reported to the treating physician and the sponsor, as well as follow-up of these pregnancies • Updated criteria around participants with compromised immune systems
Amendment 3 Version 4.0 06 May 2019	Six (6) participants were enrolled under Amendment 3, Version 4.0 • Updated IA timing to be conducted when safety and efficacy data were available up to and including the week 8/day 56 follow-up visit for ≥62 evaluable participants, rather than approximately 57 participants • Added a pre-screening informed consent • Removed *C. difficile* testing from the day 56 and day 168 assessments • Removed unacceptable AE(s) or failure to tolerate study treatment administration from discontinuation of study treatment criteria and replaced with participant experiencing related Grade 3 or higher AE and/or any related SAE • Added criteria and categories for action taken with study treatment for AEs and outcomes of AEs
Amendment 4 Version 5.0 02 July 2019	Ten (10) participants were enrolled under Amendment 4, Version 5.0 • Expanded study population to include participants with pCDI-hr • Updated tests, requirements for local versus central laboratories, and timeframe for stool sample testing • Updated study-specific definitions • Revised power calculations • Added inclusion criteria around a "qualifying episode" of CDI • Added criteria that participants should be clinically stable related to CDI at randomization • Added exclusion around prior treatments, including FMT • Removed exclusion around planned PPI use • Added additional IAs other than the IA for dose selection and SSR to inform business and development strategy in general

Amendment no. Protocol Version Date	Summary of Major Changes
Amendment 5 Version 6.0 18 September 2019	Sixty-one (61) participants were enrolled under Amendment 5, Version 6.0 • Updated sample testing for participant enrollment • Added guidance for study treatment discontinuation for suspected on-study CDI recurrence • Added a supplemental analysis for CDI recurrences • Updated criteria around "qualifying episodes" of CDI
Amendment 6 Version 7.0 15 July 2021	No participants were enrolled under Amendment 6, Version 7.0 • Adjusted sample size to 60 to 80 participants • Changed final analysis to descriptive statistics for safety and efficacy • Removed SSR • Formalized the addition of 3 interim analyses informally added in version 5.0 based on earlier correspondence and agreement with FDA • Modified personnel and groups to be unblinded for IAs as consistent with the unblinding plan • Adjusted statistical section to account for sample size and IA changes

Abbreviations: AEs, adverse events; CDI, *Clostridioides difficile* infection; FDA, Food and Drug Administration; FMT, fecal microbiota transplant; IA, interim analysis; pCDI-hr, primary CDI at high-risk; PPI, proton-pump inhibitors; SAE, serious adverse event; SSR, sample size re-estimation.

eTable 3. Model Summary: Comparison of Total VE303 Abundance and Proportion Across Dosed and Placebo Groups

Comparison	P-adjust Abundance	Effect Size	P-adjust Proportion	Effect Size
VE303 low dose vs high dose	0.09	−0.30	0.02	−0.25
Placebo vs VE303 high dose	0.0000002	−3.5276607	0.0000002	−0.63
Placebo vs VE303 low dose	0.0000002	−2.9204017	0.0000002	−0.38

eTable 4. Cut-point Models Summary: Recurrence-free Probability in All Possible High vs Low Colonization Categories

Cut-point	Estimate	Hazard Ratio = exp(estimate)	logrank p-value
Low = N colonize = 0	0.301175124	1.351445991	0.8
Low = N colonize =< 1	-0.537162585	0.584404099	0.6
Low = N colonize =< 2	-1.085972205	0.337573436	0.3
Low = N colonize =< 3	0.885541872	2.424297694	0.2
Low = N colonize =< 4	1.672102953	5.323350791	0.08
Low = N colonize =< 5	3.91	49.89895197	0.05
Low = N colonize =< 6	2.02	7.538324934	0.2
Low = N colonize =< 7	0.42	1.521961556	0.5

A hazard ratio > 1 indicates higher recurrence probability in low colonized vs high colonized subjects.

eTable 5. Cox Model Summary: Comparison of Recurrence-Free Probability in High vs Low Colonizing Participants

Covariate	Coefficient	Std error	Statistic	P value	Hazard ratio = exp(estimate)
Low colonize vs high colonize (VE303 proportion)	1.67	1.06	1.56	0.08	5.32
Low colonize vs high colonize (VE303 abundance)	−0.58	0.73	−0.79	0.4	0.56
Treatment (high dose vs low dose, log rank test)	9.34	NA	NA	0.002	NA

A hazard ratio > 1 indicates higher recurrence probability in low-colonized vs high-colonized subjects.

eTable 6. Linear Mixed Model Summary: Comparison of Diversity Across Treatment and Response Groups over Time

Contrasts	Covariate	Coefficient	Std. Error	P value
Treatment	VE303 low dose vs placebo	0.076	0.221	0.73
	VE303 high dose vs placebo	0.266	0.213	0.22
Response	non-recurrent vs recurrent	0.443	0.195	0.02
Treatment:Response	Placebo: non-recurrent vs recurrent	0.337	0.321	0.30
	VE303 low dose: non-recurrent vs recurrent	0.272	0.316	0.39
	VE303 high dose: non-recurrent vs recurrent	0.810	0.457	0.08
Treatment:Timepoint	Placebo: day 14 vs screening	-0.204	0.245	0.41
	VE303 low dose: day 14 vs screening	0.175	0.226	0.44
	VE303 high dose: day 14 vs screening	0.654	0.226	0.004
Timepoint:Treatment	Day 14: VE303 low dose vs placebo	0.277	0.312	0.38
	Day 14: VE303 high dose vs placebo	0.705	0.306	0.02

eAppendix. List of Investigators

Investigators in the United States of America (USA)

Principal Investigator	Site name	Site Location
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