ARTICLE

Translational Development of Microbiome-Based Therapeutics: Kinetics of *E. coli* Nissle and Engineered Strains in Humans and Nonhuman Primates

Caroline Kurtz[1], William S. Denney[2], Larry Blankstein[1], Sarah E. Guilmain[1], Suman Machinani[1], Jonathan Kotula[1], Saurabh Saha[3], Paul Miller[1] and Aoife M. Brennan[1,*]

Understanding the pharmacology of microbiome-based therapeutics is required to support the development of new medicines. Strains of *E. coli* Nissle (EcN) were genetically modified and administered to cynomolgus monkeys at doses of 1×10^9 and 1×10^{12} colony-forming units (CFU)/day for 28 days. A clinical study to evaluate the exposure and clearance of EcN in healthy volunteers was also performed. Healthy subjects received oral doses of EcN, 2.5 to 25×10^9 CFU 3 times daily for 28 days or a single day. In cynomolgus monkeys, replicating strains yielded higher fecal concentrations than nonreplicating strains and persisted for longer following cessation of dosing. In the clinical study, all subjects cleared EcN following cessation of dosing with median clearance of 1 week. Quantitative methodology can be applied to microbiome-based therapeutics, and similar kinetics and clearance were observed for EcN in cynomolgus monkeys and humans.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ There are little published data on methods to assess the pharmacology of microbiome-based therapeutics.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ The studies described here were designed to 1) develop methodologies to determine the pharmacology of EcN (a probiotic bacteria) and engineered strains; 2) evaluate the pharmacology of engineered strains in nonhuman primates; and 3) quantitatively assess the pharmacology of EcN in healthy human volunteers.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE
☑ The study demonstrates that it is feasible to assess pharmacology of bacterial therapeutics using specific

PCR-based assays and quantitative pharmacology methodologies. It also demonstrated that nonhuman primates may be an appropriate preclinical model to evaluate pharmacology for EcN and engineered strains.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE
☑ The methods initiated in this article support translation of microbiome-based therapeutics from nonclinical to clinical species. The studies also support the design of first-in-human studies of microbiome-based therapeutics with quantitative methods for assessing kinetics and washout with both unmodified and auxotrophic bacterial therapeutics.

An increasing understanding of the importance of host: microbiome interactions in health and disease has led to the initiation of several clinical studies of microbiome-based interventions. Despite the proliferation of basic science and epidemiological research, translation of discoveries in this area into new therapies is limited by gaps in our ability to evaluate pharmacology for live products that act from within the gut lumen. This leads to difficulty in extrapolating from preclinical disease models and uncertainty in selecting dose and regimen for clinical studies.

Most analyses of microbiome or probiotic-based therapeutics have historically involved culturing bacteria from stool or assessment of the relative abundance of bacterial species in stool using 16S RNA sequences. While these

methodologies can provide information about the presence or absence of broad taxa, their utility in quantitative assessment of microbial load for specific species is limited by specificity of the culture media and variability in expression and copy number of 16S rRNA in bacteria. Further complicating quantitative assessment is the unknown and inconsistent recovery of microbial DNA from stool. Therefore, detecting bacterial strain exposure using sensitive and specific methodologies in humans remains a challenge.

Escherichia coli is one of the earliest probiotic bacterial strains to be developed for human therapeutic use (Nissle A, 1916). Among probiotic *E. coli* strains, *E. coli* Nissle (EcN) 1917 has been the topic of numerous studies in humans since its discovery in the early 1900s, and is marketed in Germany and other countries under the brand name Mutaflor

[1]Synlogic, Cambridge, Massachusetts, USA; [2]Human Predictions, Cambridge, Massachusetts, USA; [3]Atlas Venture, Cambridge, Massachusetts, USA. *Correspondence: Aoife M. Brennan (aoife@synlogictx.com)

(Ardeypharm, Herdecke, Germany).[1] While prior studies have been performed evaluating the clearance of EcN in humans, many gaps remain regarding the quantitative kinetics of EcN, translation from preclinical models, and the contribution of *in vivo* replication.[2–6]

We are developing synthetically modified strains of EcN as therapeutics for rare inborn errors of metabolism such as urea cycle disorders and phenylketonuria. We developed strains of EcN with kanamycin resistance (SYNB975) as well as with combination kanamycin resistance, thymidine auxotrophy, and a modified arginine synthetic pathway to enhance conversion of ammonia to arginine (SYNB1010). In advance of studying these strains in patients, we designed preclinical and clinical studies with the modified strains and the parent strain (EcN) to develop methodologies for quantitative microbial kinetics using specific probes to genomic sequences and quantitative polymerase chain reaction (PCR). The current research comprised a two-part investigation, first to characterize the kinetics and clearance for each strain in cynomolgus monkeys, and second to evaluate the exposure and clearance of EcN in healthy volunteers.

METHODS
Nonhuman primate study
A study was conducted in the cynomolgus monkey to explore tolerability and evaluate the pharmacokinetics of two EcN strains: SYN975 (kanamycin-resistant EcN) and SYNB1010 (kanamycin-resistant, arginine-producing, and thymidine auxotroph); see **Supplementary Methods, Table S1** for a comparison of the genetic modifications of each bacterial strain. Bacteria were diluted in phosphate-buffered saline and administered by nasogastric gavage once daily for 28 days to groups of three female cynomolgus monkeys. SYN975 or SYNB1010 were administered at dose levels of 1×10^9 and 1×10^{12} colony-forming units (CFU)/day. Assessments included daily clinical observations and weekly body weights and blood samples for hematology and clinical chemistry. Fecal samples or fecal swabs (when no feces were produced) were collected at baseline, during dosing, and during washout. No terminal necropsies were performed and animals were returned to the stock colony upon completion of the study. Fecal samples from Day –5 (predose), Days 2, 21, and 28 (during dosing), and Days 30 and 35 (during washout) were analyzed by quantitative PCR using EcN-specific primers.

Clinical study
A prospective, open-label, single-center study was performed in healthy subjects who received oral doses of EcN in the form of Mutaflor capsules. The study included two cohorts: a multiple-day cohort in which subjects received EcN 3 times daily with meals for 28 days and a single-day cohort in which subjects received EcN 3 times with meals in a single day. The primary objective of the study was to evaluate EcN clearance in the gastrointestinal tract, including the percentage of subjects with positive samples at 24 weeks after treatment initiation as well time to no detection of EcN in feces in two consecutive samples. Secondary objectives included evaluation of safety, tolerability, and changes in gastrointestinal symptoms.

Eligible subjects were males or females aged 18–55 years with no preexisting medical conditions, a body mass index of 18.5–30 kg/m^2, between four and 14 bowel motions per week, and all subjects had to be negative for EcN at baseline based on the results of a fecal EcN qualitative PCR assay. Subjects agreed to maintain a stable diet with no major dietary changes during the treatment phase of the study and until 2 weeks after the last treatment administration.

Following completion of informed consent and confirmation of eligibility, subjects initiated treatment with dispensed investigational product refrigerated at their homes and self-administered orally on a prespecified schedule. Subjects were instructed to take one capsule of EcN (Mutaflor) with meals three times daily for 28 days (multiple-day cohort) or in a single day (single-day cohort). Each capsule nominally contained 2.5 to 25 $\times 10^9$ bacteria. During the dosing period, subjects maintained diaries to record treatment compliance and were continuously monitored for adverse events (AEs), concomitant medications, and gastrointestinal tolerability. Blood cultures were performed at baseline in both cohorts and on Days 4 and 14 in the multiple-day cohort. Blood samples for hematology and biochemistry were collected at baseline and end of study in both cohorts. Following completion of dosing, subjects were followed until two consecutive fecal samples were negative for EcN or until at least 6 months (multiple-day cohort) or 12 weeks (single-day cohort) after the last EcN administration.

This study was conducted in accordance with the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Good Clinical Practices, including collection of written informed consent from all subjects prior to study participation. The study protocol and related materials were approved by local regulatory authorities and a site-specific Ethics Committee prior to study initiation.

Assessments
Treatment compliance was assessed through patient diaries, and AEs were recorded and reviewed by the investigator at each visit. Fecal samples were collected by subjects in sterile plastic containers at their homes on protocol-specified days, stored at 4°C, and returned to the phase I unit the following day for processing. During dosing in the multiple-day cohort, fecal samples were provided for qualitative and quantitative PCR at baseline (Day 0), Days 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, and 28 and at Weeks 5, 6, 7, 8, 9, 12, 16, 24, 32, 40, and 48. In the single-day cohort, fecal samples were provided for qualitative and quantitative PCR at screening, before the start of treatment (Day 0), on Days 1, 2, 3, 4, 5, 7, 14, 21, and 28 and at Weeks 5, 6, 7, 8, 9, 10, 11, and 12.

PCR methods and primers
PCR primers for the qualitative and quantitative assays were generated by performing comparative sequence analysis on 49 *E. coli* genomes including Nissle to identify sequences

Table 1 PCR primers

Qualitative[a] primer set (primer name)	Sequence	Study	Used for
N1 (B - 2F)	gcaactggcccgtaattatcc	Clinical study	Screening of subjects and determining clearance post treatment
N1 (B - 2R)	acgcatcgcacgtaggttt		
N2 (B - 3F)	tgccgaaaggctaaacaggt		
N2 (B - 3R)	tgctctattctgaccgtgcc		

Quantitative[b] primer set and probe	Sequence	Study	Used for
16075F	ggcgcggcgctacac	Clinical and nonclinical studies	Quantification of *E. coli* Nissle sequences and determination of clearance (nonclinical)
16075R	tgcaattcgaatcatcttctcatc		
16075 Probe	ggcgcggcgctacac		
16073F	ggcccgtaattatccatagctgt	Nonclinical study	Quantification of *E. coli* Nissle Sequences and determination of clearance (nonclinical)
16073R	gctcgccatcattgcgtt		
16073 Probe	tgatcactcggtccgaatt		

[a]Syber Green Methodology.
[b]TaqMan Methodolgy.

unique to Nissle among *E. coli* and also not found in other bacterial species (see **Supplementary Methods**). This process generated multiple primer pairs, from which two were selected for validation in an SYBR Green assay and primer/probe sets were identified for the quantitative (Taqman format) assay (**Table 1**).

Assay development
For the qualitative PCR assay, a standard was generated using EcN DNA that had been prepared from a Mutaflor capsule using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD) with DNA eluted in 100 μL of elution buffer. Good linearity of signal was observed for the dilution series between 5×10^0 and 5×10^6 CFU/PCR reaction. The limit of detection was ≤ 5 CFU/PCR for both the N1 and N2 primer sets corresponding to ≤ 500 CFU/mL heat-treated stool sample. The N1 and N2 primer pairs also showed no crossreactivity when tested against purified DNA from 34 different bacterial species frequently isolated from human stool samples, demonstrating good specificity. For the quantitative PCR assay, two target sequences were identified for detection by primer pairs and probes that were designated assays 16073 and 16075 (**Table 1**). A standard curve was generated using the selected conditions (see **Supplementary Methods** for additional details) to determine the assay range, specificity, linearity, limit of detection, and quantitation over a range between 1×10^1 to 1×10^8 copies of a DNA fragment containing the target sequences. At a threshold setting of 0.2, the limit of detection for assays 16073 and 16075 were estimated to be 40 copies and 20 copies, respectively. Specificity was confirmed using DNA extracted from yeast tRNA, Lambda DNA, cynomolgus monkey genomic DNA and total RNA, mouse genomic DNA and total RNA, and human placental DNA and RNA. No indication of nonspecific amplification was observed with any of these substrates. The qualitative and quantitative PCR methods were qualified against samples of human and monkey stool samples spiked with

known concentrations of EcN DNA and found to be sensitive and valid (see **Supplementary Methods**). The 16073 primer set demonstrated a limit of quantitation of 40 copies, while the 16075 set had a limit of quantitation of 20 copies.

Fecal sample preparation and DNA Extraction
Qualitative assay. Approximately 200–400 mg of native stool material was mixed with 2 mL H_2O (high-performance liquid chromatography grade) and vortexed until a homogeneous solution was obtained. The solution was incubated for 10 min at 100°C in a heating block. After cooling, the samples were centrifuged at $3500 \times$ g for 10 min. DNA was extracted from 200 μL of heat-treated stool sample supernatants using the QIAamp DNA Mini Kit according to the manufacturer's instructions.

Quantitative assay. Fecal samples were diluted 1:1 with phosphate-buffered saline (pH 7.4) and mixed by vortexing. Approximately 250 μL were homogenized using the PowerLyzer PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's instructions. Purity and concentration determinations of the extracted DNA were performed on the Nanodrop 8000 with PowerLyzer DNA elution buffer as the blanking buffer.

Analysis of stool samples. Stool samples from the cynomolgus monkey or human were analyzed by qualitative and quantitative PCR. For the qualitative assay, PCR reactions were carried out using QuantiTect SYBR Green PCR Kit (Qiagen), with separate reactions for the N1 and N2 primer pairs (see **Supplementary Methods**). For the quantitative assay, assessments were performed using the QuantStudio 7 Flex Real-Time PCR System with QuantStudio Real-Time PCR Software (see **Supplementary Methods**). Primers were used in the qualitative PCR protocol in a presence/absence assay. If a sample yielded a positive result for both primer pairs, it was scored as "Nissle-positive." If a sample was negative for both primers, it was

scored as "Nissle-negative." Samples that were positive for one primer but negative for the other were reevaluated. Samples that continued to produce a mixed result after retesting were considered negative.

Statistical analysis

In the nonhuman primate study, relative abundance was estimated by a log-scale linear model of fecal concentration using data at the top dose on dosing days, and clearance was determined by first measurement below the limit of quantification. Statistical analyses were performed using R (v. 3.3.2, Vienna, Austria) for descriptive statistics with the PKNCA (v. 0.8.1) library for assessing time to steady state and the rstanarm (v. 2.14.1) library to assess relative abundance.[8–10]

No formal sample size calculations were conducted in the clinical study. Safety analyses were performed for all subjects who received at least one dose of EcN, and kinetic analyses were performed for all subjects who completed treatment per protocol, with no imputations for missing data. Descriptive statistics were used to summarize the baseline characteristics of both cohorts. The time to steady state and time to clearance were calculated based on the quantitative and qualitative PCR data.

RESULTS
Nonhuman primate results

In nonhuman primates, both modified EcN strains tested (SYN975 (kanamycin-resistant) and SYNB1010 (thymidine auxotroph, kanamycin-resistant, and arginine-producer)) were well tolerated, with no clinical observations or hematology or clinical chemistry findings. Due to limited kinetic sampling in the study, relative abundance (i.e., C_{max} ratio) and clearance were the only parameters calculated for the study. SYN975 yielded higher fecal concentrations than SYNB1010, with an estimated 6.9-fold higher abundance in feces when compared with the same dose level of SYNB1010 (**Figure 1**). SYNB1010 appeared to clear faster than SYN975, with 1×10^9 CFU of SYNB1010 clearing in two of three animals on Day 30 and in three of three animals on Day 35, compared with clearing of 1×10^9 CFU of SYN975 in zero of three and two of three animals, respectively. Three of three animals cleared on Day 35 for 1×10^{12} CFU of SYNB1010 compared with two of three animals for SYN975.

Clinical study results
Subject disposition

Between September 2016 and April 2017, 83 healthy adult male and female subjects were screened for study inclusion at a single site in Bulgaria, including 55 subjects who were enrolled and received treatment in either the single-day ($n = 10$) or multiple-day ($n = 45$) cohort (**Figure 2**). All subjects enrolled in the single-day cohort completed the intervention and are included in the safety and kinetics analyses. Of the 45 subjects enrolled in the multiple-day cohort, two subjects discontinued dosing prematurely by subject request and were excluded from kinetics analyses. Baseline characteristics for both cohorts are summarized in **Table 2**.
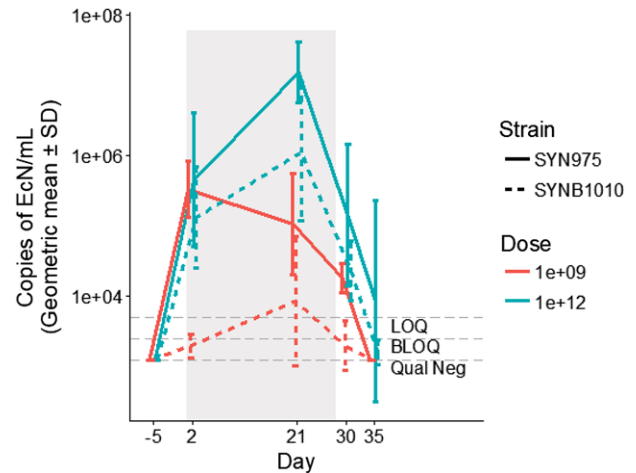

Figure 1 Quantitation of *E. coli* Nissle-derived strains in cynomolgus monkeys using quantitative PCR. The red color indicates a dose of 1×10^9 CFU and the green color indicates a dose of 1×10^{12} CFU; the colored solid lines indicate the geometric means for animals administered SYN975, while the colored dashed lines indicate animals administered SYNB1010; the gray background indicates the dosing period; the gray dashed lines indicate the LOQ, BLOQ, and Qual Neg. BLOQ is plotted as LOQ/2, and Qual Neg is plotted as LOQ/4. BLOQ, below the limit of quantitation with quantitative PCR but positive with qualitative PCR; EcN, *E. coli* Nissle; LOQ, limit of quantitation estimated as the lowest measured copies of EcN/mL; Qual Neg, one or both qualitative PCR primers were negative; SD, standard deviation.

Compliance and safety

Subjects were compliant with dosing and fecal collection, with more than 98% of subjects consuming all prescribed doses and 99% of fecal samples provided per protocol.

No SAEs were reported during the study. Seven treatment-emergent AEs were reported in seven subjects (16%) in the multiple-day cohort. Six subjects (13%) experienced events of headache (moderate intensity), five of which were considered not related to EcN administration and one of which was considered unlikely related. All headache events resolved completely within 2 days. One subject (2%) experienced an AE of increased hepatic enzymes (mild intensity) that was detected at the final study visit and considered not related to EcN administration. Overall, EcN was well tolerated, with minimal changes in gastrointestinal symptoms over the study period (data not shown).

Results of fecal E. coli Nissle kinetics in humans

Results from the primary end-point analyses indicated no subjects with fecal samples positive for EcN at 24 weeks after treatment discontinuation. The median time to clearance following the final administration of EcN using the qualitative PCR assay was ~4 days (range 3–55 days) in the single-day cohort and 13 days (range 0–142 days) in the multiple-day cohort (**Figure 3**). Because of the requirement for two negative samples for declaration of clearance, a comparison of duration of clearance between the two dosing regimens is limited by disparate postdose sampling schedules. The quantitative assay was highly correlated with the qualitative assay; 71% of samples that tested negative with the qualitative PCR assay were below the lower limit of
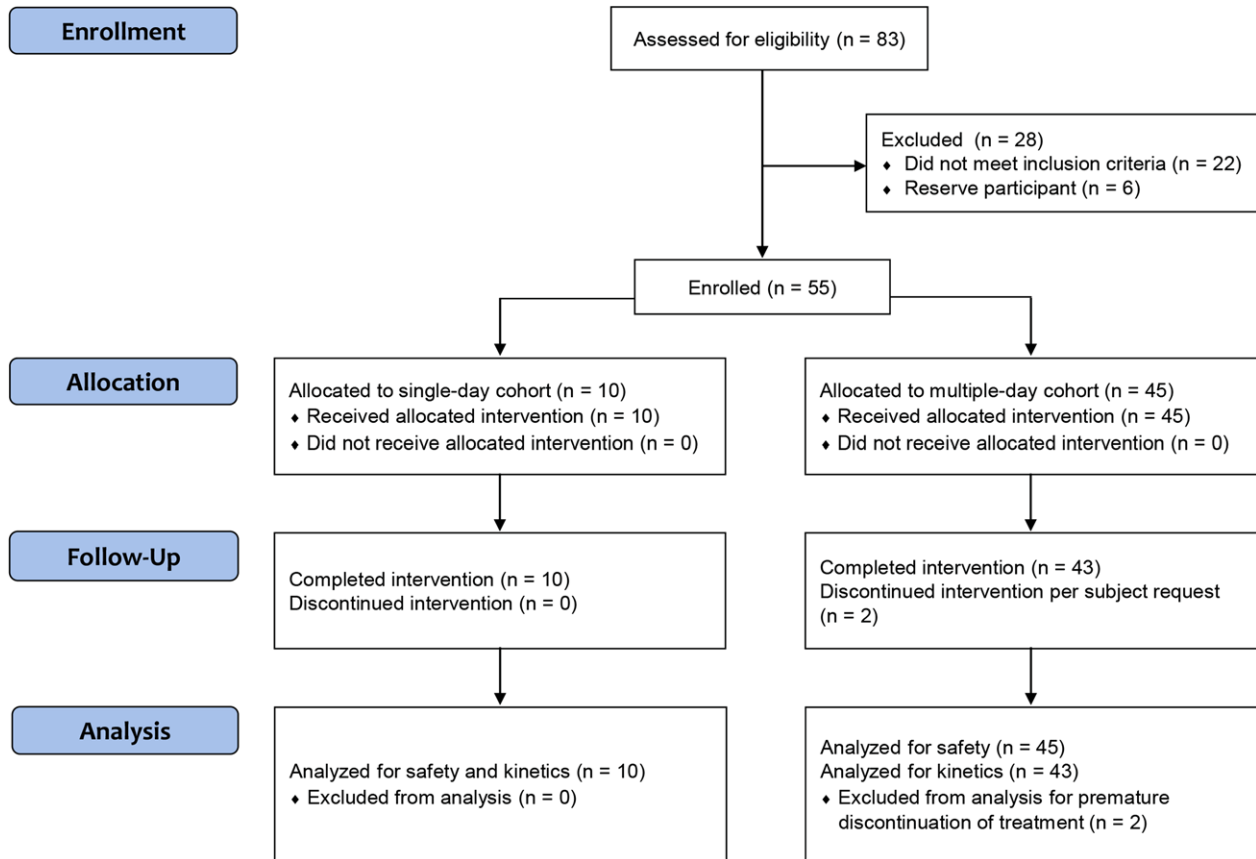
Figure 2 Consort diagram for the clinical study.

Table 2 Baseline characteristics by cohort (Safety Analysis Set)

Characteristic	Statistic	Multiple-day cohort (*n* = 45)	Single-day cohort (*n* = 10)
Male:female (n)		14:31	6:4
Caucasian (%)		100	100
Age (years)	Mean ± SD	36.4 ± 9.1	35.9 ± 6.1
Height (cm)	Mean ± SD	169.1 (7.7)	174.6 (10.9)
Weight (kg)	Mean ± SD	71.9 (12.3)	74.3 (14.0)
BMI (kg/cm^2)	Mean ± SD	25.1 (3.4)	24.2 (2.9)

n, number of subjects; SD, standard deviation.

quantification, while 90% of samples that tested positive in the qualitative assay were above the lower limit of quantification.

Analysis of the noncompartmental parameters for quantitative PCR (**Table 3**, **Figure 4**) indicated that the median time to maximum observed quantitative PCR concentration (T_{max}) with single-day dosing was on Day 2, while with multiple-day dosing T_{max} was on Day 6. The high variability of day-to-day measurements indicated that the maximum observed concentration (C_{max}) does not define the accumulation ratio directly, and other methods for assessing accumulation were required (described below).

While day-to-day measurements were variable, the data were sufficient to estimate an approximate time to steady state with multiple dose administration. The time to steady state was rapid; it was estimated at or before the first measurement in the study, 2.2 or 4 days, when estimated with

either a monoexponential time to steady state or linearity test; the times to steady state align with the observation of median T_{max} on Day 6 with multiple dosing.[11] Using quantitative PCR from fecal samples, results from 28 days of dosing with EcN indicate accumulation of ~1.6-fold copies/mL compared with single-day levels.

DISCUSSION

Studies of the human microbiome in health and disease have highlighted a potential therapeutic value of live bacteria as an approach to combat complex and intractable disease states that are associated with microbial dysbiosis or have a potential site of action in the gut.[12] Treatment with probiotic bacteria may have potential to restore microbiome balance, and numerous studies and meta-analyses have been conducted reviewing their effectiveness in different disease states.[13] The
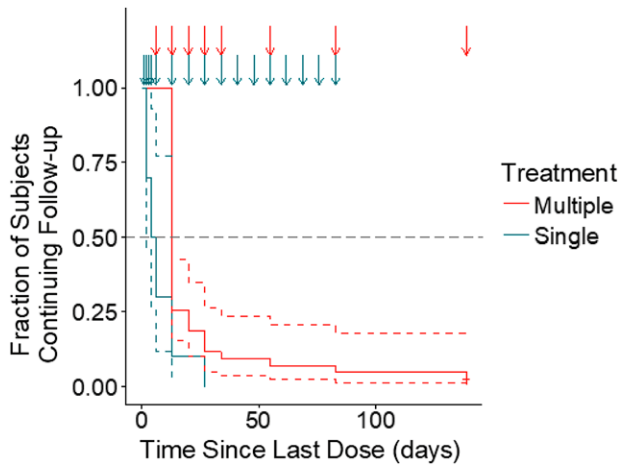
Figure 3 *E. coli* Nissle clearance via qualitative assay with two negative results required to define clearance. The horizontal axis represents days since the last dose, and the vertical axis is the fraction of subjects continuing follow-up (i.e., had not yet cleared *E. coli* Nissle). The solid and dashed lines indicate the Kaplan–Meier estimates for time to clearance and its 95% confidence interval; the arrows at the top indicate times when monitoring occurred with color indicating dosing regimen.

Table 3 Clinical kinetic parameters (Kinetics Analysis Set)

Regimen	C_{max} (CFU/mL feces)	T_{max} (days)
Multiple-day cohort ($n = 45$)	1260000 [444]	6 [0, 28]
Single-day cohort ($n = 10$)	179000 [4950]	2 [0, 14]

CFU, colony-forming unit(s); C_{max}, maximum observed concentration; n, number of subjects; T_{max}, time of C_{max}.
C_{max} values are geometric mean [geometric coefficient of variation]; T_{max} values are median [range].
For both single- and multiple-day data, parameters were calculated from Day 1 to Day 28; due to additional measurements with multiple-day data, the ratio of C_{max} values is not comparable as the accumulation ratio.

quality of the clinical evidence for probiotics is variable, with mostly small and open-label trials, the majority of which do not report data on exposure in either the active or placebo group. For probiotic therapies to be rigorously developed as therapeutic treatments, a better understanding of microbial colonization and kinetics in the gastrointestinal tract is needed. Previous studies with probiotic bacteria (including EcN 1917) used various methodologies to detect colonization of the human intestinal tract, including plating on selective media and PCR detection from stool samples.[2,14,15] In one of the best examples of such a study, the excretion of EcN in the stool of healthy volunteers was followed out to 48 weeks following a 7-day dosing period with EcN (Mutaflor) in the presence or absence of mesalamine using PCR with Nissle-specific primers.[2] The authors report some subjects still being positive at 48 weeks; however, when evaluating the data from this study, there are several limitations to the interpretability of the results. Neither the sensitivity and specificity of the PCR primers nor the quantification of levels of the bacteria found in stools were reported. These analyses are complicated by the challenges of poor recovery of stool, extraction efficiency of bacteria from stool, and specificity of the methodology for detecting EcN in the stool. Traditional methods of plating on selective media are limited by the inability
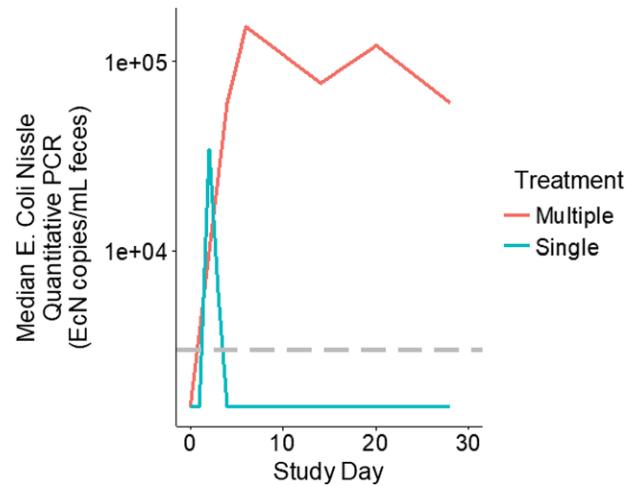

Figure 4 Time course of median *E. coli* Nissle quantitative PCR for single and multiple dose regimens. EcN, *E. coli* Nissle; PCR, polymerase chain reaction.

to accurately distinguish EcN from other enterobacteriaceae from stool cultures. Quantitative PCR methodologies require identification of unique primers and probes within the EcN genome. Recently, the complete genome sequence of EcN has been obtained and published, allowing the use of bioinformatic approaches to identify unique sequences found in EcN but no other sequenced *E. coli* or enterobacteriaceae species.[16,17]

Results from the current investigation demonstrated the feasibility of applying quantitative methodology to the assessment of EcN kinetic parameters in stool in both preclinical species and human subjects.

We developed a sensitive and specific quantitative PCR assay based on the identification of two unique sets of primers and probes based on sequences found within EcN genomic DNA that are not found in other *E. coli* species or other enterobacteriaceae sequences. This method has a limit of detection of 20 and 40 copies of Nissle DNA per μg of extracted stool DNA, for the two primer sets 16075 and 16073, respectively.

In the nonclinical study in cynomolgus monkeys, 10^9 or 10^{12} CFU of EcN-derived strains were very well tolerated during 28 days of treatment, despite the fact that the microbial load given was 40-fold above the highest human dose given in the clinical study (2.5×10^{10} CFU) for an animal that is $1/17^{th}$ the weight of a healthy human (4 kg vs. 70 kg) and an approximately similar fraction of gastrointestinal volume.[18] There was some dose-dependency on the clearance, but both dose levels cleared rapidly following cessation of dosing and fell below the limit of quantification by 7 days postdosing.

Subjects treated in the clinical study demonstrated good compliance with stool collection in an outpatient setting, and the resulting data permitted the calculation of pharmacokinetic parameters. The administration of EcN capsules for 1 and 28 days was well tolerated, with no safety signals identified and no growth on blood culture, consistent with the known safety profile of EcN.[19] The clinical study suggested that EcN reached an approximate steady state

over 2–4 days and cleared relatively rapidly following discontinuation. Median clearance occurred within 1 week, and over 80% of subjects had cleared EcN within 3 weeks. The clearance in this study confirms prior reports that EcN does not colonize the gut in adult subjects.[2] Recovery of bacterial genetic material from the feces also indicated good exposure in the colon. Assuming 100 g of fecal material per day and uniform distribution, 10^6 CFU of the estimated 3×10^9 CFU administered daily was recovered in the feces at steady state.[20]

Data from the study in the cynomolgus monkey indicate that *in vivo* replication contributes to kinetics, as bacteria that were engineered to be incapable of replicating *in vivo* through deletion of an essential gene, thymidylate synthase, demonstrated more rapid clearance and lower steady-state concentrations compared with replication-competent strains. Kinetic parameters were consistent between cynomolgus monkeys and healthy human volunteers; both the EcN and the kanamycin-resistant EcN had similar clearance in both species, with a median clearance of <2 weeks across species, suggesting that cynomolgus monkeys may be an appropriate preclinical model. The kinetic similarities further suggest that translation of kinetic parameters for time to steady state and time to clearance may be directly comparable between cynomolgus monkeys and humans, and additional work is warranted to compare kinetics more quantitatively. Microbiome profiling has demonstrated species-specific patterns.[21] In addition, differences have been described in host–microbiome interactions between nonhuman primates and humans, including differences in adaptations to dietary changes and diversity.[22] Despite these observations, for approaches that do not aim to change the ecology of the gut, data in nonhuman primates may help identify appropriate doses by providing data regarding *in vivo* kinetics prior to studies in humans.

Limitations of the nonclinical study include variability in stool output (when stool samples were not available, fecal swabs were taken), and fewer sampling points for quantitative PCR during the dosing and postdosing periods and a small number of animals per group ($n = 3$). Limitations of the clinical study include the outpatient treatment administration and fecal collection, which may have contributed to variability in the quantitative assessments. The random fecal sampling performed by each subject, as well as potential differences in the methods and storage of fecal samples, may have influenced the fecal PCR data. While prior publications suggest that bacteria may not be uniformly distributed within feces, median values are considered instructive, particularly as there was consistency between the single and multiple-day cohorts and clearance kinetics were consistent with prior publications.[2,23] Initial results suggest concordance between cynomolgus monkeys and humans, but further investigation of the nonclinical to clinical microbe kinetic translation are warranted to confirm the initial findings reported here and to increase the ability to quantitatively translate nonclinical to clinical microbiome therapeutics. The absence of clinical data on the auxotrophic strains warrant further investigation to assess both the microbial kinetics and pharmacokinetic/pharmacodynamic relationship of engineered bacteria in a clinical study setting. Despite these limitations,

these data demonstrate the feasibility of applying quantitative methodology to the study of microbiome-based therapeutics and provide a tool that can be used in the development of both colonizing and noncolonizing bacteria, including engineered strains in humans.

1. Wassenaar, T.M. Insights from 100 years of research with probiotic *E. coli. Eur. J. Microbiol. Immunol.* **6**, 147–161 (2016).
2. Joeres-Nguyen-Xuan, T.H., Boehm, S.K., Joeres, L., Schulze, J. & Kruis, W. Survival of the probiotic Escherichia coli Nissle 1917 (EcN) in the gastrointestinal tract given in combination with oral mesalamine to healthy volunteers. *Inflamm. Bowel Dis.* **16**, 256–262 (2010).
3. Lata, J. *et al.* Effect of administration of Escherichia coli Nissle (Mutaflor) on intestinal colonization, endotoxemia, liver function and minimal hepatic encephalopathy in patients with liver cirrhosis. *Vnitr. Lek.* **52**, 215–219 (2006).
4. Wassenaar, T.M., Beimfohr, C., Geske, T. & Zimmermann, K. Voluntarily exposure to a single, high dose of probiotic Escherichia coli results in prolonged colonisation. *Benef. Microbes* **5**, 367–375 (2014).
5. Cukrowska, B., Lodlnová-Zádnlková, R., Enders, C., Sonnenborn, U., Schulze, J. & Tlaskalová-Hogenová, H. Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic E. coli strain Nissle 1917. *Scand. J. Immunol.* **55**, 204–209 (2002).
6. Prilassnig, M., Wenisch, C., Daxboeck, F. & Feierl, G. Are probiotics detectable in human feces after oral uptake by healthy volunteers? *Wien. Klin. Wochenschr.* **119**, 456–462 (2007).
7. Mutaflor Kaps. Prescribing Information, Ardeypharm GmbH, August 2015. (English translation from German: Mutaflor Kaps. Fachinformation, Ardeypharm GmbH, August 2015).
8. R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: https://www.R-project.org.
9. Denney, W.S., Duvvuri, S. & Buckeridge, C. Simple, automatic noncompartmental analysis: the PKNCA R package. *J. Pharmacokinet. Pharmacodyn.* **42**, 11–107, S65. R package version 0.8.1. Available at: <https://github.com/billdenney/pknca>. (2015).
10. Stan Development Team. rstanarm: Bayesian applied regression modeling via Stan. R package version 2.14.1. Available at: http://mc-stan.org (2017).
11. DeSesso, J. & Williams, A. Chapter 21. Contrasting the gastrointestinal tracts of mammals: factors that influence absorption. *Annu. Rep. Med. Chem.* **43**, 353–371 (2008).
12. Morgan, P. *et al.* Can the flow of medicines be improved? Fundamental pharmacokinetic and pharmacological principles toward improving Phase II survival. *Drug Discov. Today* **17**, 419–424 (2012).
13. Rondadelli, M., Faliva, M.A., Perna, S., Giacosa, A., Peroni, G. & Castellazzi, A.M. Using probiotics in clinical practice: where are we now? A review of existing meta-analyses. *Gut Microbes* **22**, 1–23 (2017).
14. Kukkonen, K. *et al.* Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: a randomized, double-blind, placebo-controlled trial. *J. Allergy Clin. Immunol.* **119**, 192–198 (2007).
15. Venturi, A. *et al.* Impact on the composition of the faecal flora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis. *Aliment. Pharmacol. Ther.* **13**, 1103–1108 (1999).
16. Reister, M. *et al.* Complete genome sequence of the gram-negative probiotic Escherichia coli strain Nissle 1917. *J. Biotechnol.* **10**, 106–107 (2014).

17. Cress, B.F., Linhardt, R.J. & Koffas, M.A.G. Draft genome sequence of Escherichia coli strain Nissle 1917 (serovar O6:K5:H1). *Genome Announc.* **1**, e00047-13 (2013).

18. Mordenti, J. Man versus beast: pharmacokinetic scaling in mammals. *J. Pharm. Sci.* **75**, 1028–1040 (1986).

19. Krammer, H.J. *et al.* [Probiotic drug therapy with E. coli strain Nissle 1917 (EcN): results of a prospective study of the records of 3,807 patients]. *Z. Gastroenterol.* **44**, 651–656 (2006).

20. Rose, C., Parker, A., Jefferson, B. & Cartmell, E. The characterization of feces and urine: a review of the literature to inform advanced treatment technology. *Crit. Rev. Environ. Sci. Technol.*, **45**, 1827–1879 (2015).

21. McCord, A.I. *et al.* Fecal microbiomes of non-human primates in Western Uganda reveal species-specific communities largely resistant to habitat perturbation. *Am. J. Primatol.* **76**, 347–354 (2014).

22. Amato, K.R. *et al.* Variable responses of human and non-human primate gut microbiomes to a Western diet. *Microbiome* **3**, 53 (2015).

23. Gorzelak, M.A., Gill, S.K., Tasnim, N., Ahmadi-Vand, Z., Jay, M. & Gibson, D.L. Methods for improving human gut microbiome data by reducing variability through sample processing and storage of stool. *PLoS One* **10**(8),e0134802 (2015).

Supplementary information accompanies this paper on the *Clinical and Translational Science* website. (www.cts-journal.com)