



## Research Paper

# Oral Phage Therapy of Acute Bacterial Diarrhea With Two Coliphage Preparations: A Randomized Trial in Children From Bangladesh



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## ARTICLE INFO

## Article history:

Received 19 November 2015

Received in revised form 23 December 2015

Accepted 27 December 2015

Available online 5 January 2016

## Keywords:

Bacteriophages

Diarrhea

*Escherichia coli*

*Streptococcus*

*Bifidobacterium*

Children

Bangladesh

## ABSTRACT

**Background:** Antibiotic resistance is rising in important bacterial pathogens. Phage therapy (PT), the use of bacterial viruses infecting the pathogen in a species-specific way, is a potential alternative.

**Method:** T4-like coliphages or a commercial Russian coliphage product or placebo was orally given over 4 days to Bangladeshi children hospitalized with acute bacterial diarrhea. Safety of oral phage was assessed clinically and by functional tests; coliphage and *Escherichia coli* titers and enteropathogens were determined in stool and quantitative diarrhea parameters (stool output, stool frequency) were measured. Stool microbiota was studied by 16S rRNA gene sequencing; the genomes of four fecal *Streptococcus* isolates were sequenced.

**Findings:** No adverse events attributable to oral phage application were observed (primary safety outcome). Fecal coliphage was increased in treated over control children, but the titers did not show substantial intestinal phage replication (secondary microbiology outcome). 60% of the children suffered from a microbiologically proven *E. coli* diarrhea; the most frequent diagnosis was ETEC infections. Bacterial co-pathogens were also detected. Half of the patients contained phage-susceptible *E. coli* colonies in the stool. *E. coli* represented less than 5% of fecal bacteria. Stool ETEC titers showed only a short-lived peak and were otherwise close to the replication threshold determined for T4 phage *in vitro*. An interim analysis after the enrollment of 120 patients showed no amelioration in quantitative diarrhea parameter by PT over standard care (tertiary clinical outcome). Stool microbiota was characterized by an overgrowth with *Streptococcus* belonging to the *Streptococcus gallolyticus* and *Streptococcus salivarius* species groups, their abundance correlated with quantitative diarrhea outcome, but genome sequencing did not identify virulence genes.

**Interpretation:** Oral coliphages showed a safe gut transit in children, but failed to achieve intestinal amplification and to improve diarrhea outcome, possibly due to insufficient phage coverage and too low *E. coli* pathogen titers requiring higher oral phage doses. More knowledge is needed on *in vivo* phage–bacterium interaction and the role of *E. coli* in childhood diarrhea for successful PT.

**Funding:** The study was supported by a grant from Nestlé Nutrition and Nestlé Health Science. The trial was registered with Identifier NCT00937274 at [ClinicalTrials.gov](http://ClinicalTrials.gov).

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## 1. Introduction

In view of the growing threat of antibiotic resistance, WHO has warned for a return into a pre-antibiotic era (Martinez, 2012). Alternatives to antibiotics are thus urgently needed (Pidcock, 2012; Stanton, 2013). Phage therapy (PT), the use of bacterial viruses (phages) against pathogens, is a potentially attractive option for the prevention and treatment of some bacterial infections (Sulakvelidze et al., 2001; Brüssow, 2005, 2012). Indeed, a large, randomized and placebo-controlled trial conducted in children from Tbilisi/Republic of Georgia during the 1960s

**Abbreviations:** Cfu, colony forming unit; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; EAEC, enteroaggregative *E. coli*; M, ColiProteus phage cocktail from Microgen; ORS, oral rehydration solution; P, placebo; pfu, plaque forming unit; PT, phage therapy; qPCR, quantitative polymerase chain reaction; RCT, randomized controlled trial; T, T4 phage cocktail from NRC.

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described prevention of *Shigella* dysentery and *Escherichia coli* diarrhea with orally applied *Shigella* phages (Sulakvelidze et al., 2001). PT was practiced in Poland in patients with antibiotic-resistant bacterial infections on a case-by-case basis, apparently with high success (Międzybrodzki et al., 2012). However, randomized controlled trials (RCTs) in humans are needed to rationally assess the potential of PT. So far, a single small RCT documented an effect in carefully selected patients suffering from otitis externa (“swimmer’s ear”) (Wright et al., 2009).

*E. coli* is an important cause of diarrhea in children from developing countries (Qadri et al., 2005), resistant against many antibiotics (Jiang et al., 2002) while efficient *E. coli* diarrhea vaccines are not yet available (Ahmed et al., 2013). Other than zinc and oral rehydration solution, no specific, effective, safe and affordable treatment is available to reduce the severity or duration of illness caused by this bacterial agent. We hypothesize that oral administration of coliphage will be effective in reducing severity of diarrhea in children with proven *E. coli* induced diarrhea. Therefore a RCT of PT with two different coliphage cocktails for which safe use was demonstrated in healthy adults (Sarker et al., 2012; McCallin et al., 2013) was performed in children with *E. coli* diarrhea. The primary endpoint was the safety of oral coliphage in children infected with *E. coli*, the secondary endpoint was the titration of fecal coliphage and *E. coli* pathogen to assess *in vivo* lytic phage activity and the tertiary endpoint was the impact of oral phage on quantitative diarrhea parameters. Oral coliphage reached the intestine, but did not achieve treatment effects over placebo most likely because intestinal *E. coli* titers were low and close to the replication threshold of coliphages. Microbiota analysis revealed a marked outgrowth of fecal streptococci during the acute phase of diarrhea.

## 2. Methods

### 2.1. Participants and Study Design

A prospective, single center, randomized, placebo-controlled, parallel group clinical trial was undertaken to assess the safety and efficacy of T4-like phage cocktail compared to Microgen ColiProteus phage cocktail or placebo in 6–24-month-old male (to obtain stool without urine contamination) children presenting with acute onset of dehydrating diarrhea of less than 48 h duration. The study took place at the Dhaka Hospital of the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) between June 2009 and September 2011. The trial was approved by the Research Review Committee and Ethical Review Committee of icddr,b (protocol #2008-062) and registered with the Identifier NCT00937274 at the [ClinicalTrials.gov](http://www.clinicaltrials.gov) site. The study was performed according to Good Clinical Practice and the Declaration of Helsinki ([http://www.who.int/bulletin/archives/79\(4\)373.pdf](http://www.who.int/bulletin/archives/79(4)373.pdf)). Written informed consent was obtained from parents or legal guardians of the child before study enrollment. The T4 phage cocktail contained eleven T4-like phages (AB2, 4, 6, 11, 46, 50, 55; JS34, 37, 98, D1.4) (Bourdin et al., 2014a,b). The composition of the Microgen ColiProteus phage cocktail was described previously by metagenome sequencing and electron microscopy (McCallin et al., 2013). The placebo was reduced osmolarity oral rehydration solution (ORS) supplemented with zinc, the standard treatment for uncomplicated watery diarrhea at icddr,b. The primary outcome of the safety part of the study was the acceptability of the phage products by the patients as determined by clinical observation and liver, renal and hematology tests described previously (Sarker et al., 2012; McCallin et al., 2013). The secondary endpoint was the determination of coliphage and total *E. coli* titers and their phage susceptibility in stool and the clearance of ETEC from stool. The tertiary outcome was changes in total stool output (expressed as g/kg of body weight per day), frequency of stool (number/day) and ORS need for rehydration (in ml/kg body weight per day) from randomization to resolution of illness.

Children with at least four liquid stools during the previous 24 h, with some degree of dehydration (WHO methodology) were considered for study enrollment after obtaining informed consent.

### 2.2. Randomization

A random permuted block design (with block size of 6) (to equalize the number of patients in the two treatment groups after short intervals) was used for allocation of patients to the study interventions, namely the two active products, *i.e.* T4 coliphage cocktail, Microgen phage cocktail in ORS or placebo (0.9% NaCl) at a 1:1:1 ratio. The icddr,b hospital pharmacist, not associated with the study in any other way, prepared the products and handed it over to the study nurse unaware of the product for dispensing to the patients following a computer-generated list of random numbers developed by an independent biostatistician not associated with the study. Code envelopes were kept by the sponsor and by the investigator for un-blinding in emergency situations and after the blind review meeting. The subjects, principal investigator, hospital staff and laboratory personnel were masked to the treatment assignments. After mixing phage or placebo with ORS powders in 30 ml mineral water (Vittel mineral water, pH 7.6, bicarbonate 384 mg/l) and a food dye, the interventions products were indistinguishable. The study products were given 3 times per day (8 am, noon, 4 pm) for four days.

### 2.3. Power Calculation

We estimated the sample size based on stool output. In a previous trial at icddr,b in children hospitalized with untreated ETEC and EPEC diarrhea we observed a stool output of 176 + 100 ml/kg body weight in a 4-day period (Casswall et al., 2000). With an anticipated 30% reduction in stool volume, we estimated a sample size of 71 at 5% significance level and 80% power. To adjust for a 5% drop-out rate, we decided to enroll 225 children into the trial. Note that the interim analysis was done with 120 patients, 53% of the planned enrollment.

### 2.4. Procedures

Following confirmation of the absence of rotavirus in stool by ELISA and *Vibrio* by dark field microscopy, and sending stool samples for further microbiological assessments, *i.e.* *Salmonella*, *Shigella*, *Aeromonas* and *Campylobacter* according to standard procedures at icddr,b (Harris et al., 2008) and for identification of ETEC, EPEC or EAEC by multiplex PCR assay using specific primers as described (Svenungsson et al., 2000), the enrolled children were randomized to intervention. Children with systemic infection, invasive diarrhea, severe acute malnutrition (weight for height z score < -3SD of WHO median or presence of edema), significant medical abnormalities and who had received or needed antibiotic treatment were excluded. Initial correction of dehydration was performed using hypo-osmolar ORS solution or initial intravenous rehydration solution (for children with severe dehydration) followed by ORS solution, equal to the amount of abnormal (watery or liquid) stool loss, until diarrhea resolves.

Children whose fecal culture identified other bacterial enteric pathogens or no *E. coli* pathogen remained in the study and their data were used for Intention-to-Treat (ITT) analysis. Sub-group analysis was done for confirmed *E. coli* infection. Freshly passed stool was obtained daily at about 4 pm (the time was kept fixed as the first dose was fed exactly at 4 pm) for quantitative colony counts of *E. coli* on EMB and MacConkey agar plates, total bacterial count by qPCR (Nadkarni et al., 2002) and microbiota analysis. Three putative *E. coli* colonies from day 1 stool agar plates were tested for sensitivity to the applied phage cocktails at NRC, Switzerland.

The nursing staff recorded the frequency, consistency and volume of stool and urine passed every six hours (to a sensitivity of 1 g). This was achieved through a combination of placing the subject on a cholera cot and collecting urine in a pediatric urine collection bag. The data collected from this procedure was also used to calculate amounts of ORS solution required to account for fluid losses due to diarrhea. Stool frequency was counted by the study nurse and consistency of every stool passed

was recorded. The passage of the last abnormal (watery) stool prior to formed or soft or no stool produced during two consecutive 6-hour periods were used to estimate the duration of diarrhea. Feeding appropriate for age was introduced early as *ad libitum* breastfeeding (for breast fed children) or formula feeding (for non-breast fed children) or standardized hospital diet to provide approximately 100 kcal per kg body weight per day.

If diarrhea continued beyond five days, treatment was judged a failure and the child was transferred to the general ward of the hospital for further management following the hospital's standard protocol. Resolution of illness was defined as passage of formed or soft stools during two consecutive six-hour periods. Patient follow-up visit occurred 21 days after first dose.

Safety evaluation of patients included, in addition to clinical observation, chemistry tests which comprised liver and renal function assays and hematology tests that were done at the admission and discharge from the hospital as described previously (Sarker et al., 2012; McCallin et al., 2013).

### 2.5. Phage Detection

One gram of stool sample was resuspended in TS (8.5 g NaCl and 1 g tryptone/l) to a final volume of 5 ml and centrifuged for 15 min at 14,500 ×g. The cleared supernatant was filtered through a Millex AP20 prefilter followed by a 0.45 µm pore size Minisart filter. The presence of phages was determined on two indicator strains: *E. coli* strain WG5 obtained from J. Jofre, University of Barcelona (Spain), a common indicator strain used in phage ecology studies (Guzmán et al., 2008), and *E. coli* strain K803, a K-12 derivative lacking prophage lambda, obtained from E. Kutter, Evergreen College, Olympia, WA, USA. This strain lacks restriction-modification genes and contains only the core part of the lipopolysaccharide making it susceptible to a wide range of coliphages (Chibani-Chennoufi et al., 2004). The strains were propagated in Hershey broth supplemented with casein-derived amino acids (15 g/l). Phages were enumerated using the double layer plaque assay, with top agar at 7.5 g/l on a bottom layer of 15 g agar/l in broth (Chibani-Chennoufi et al., 2004). Phage dilutions were prepared in TS: 100 µl of stool filtrate and 100 µl of an exponentially growing bacterial culture were mixed together, incubated at room temperature for 15 min and used to inoculate 3 ml of top agar. Plates were incubated over-night at 37 °C and phage plaques were counted. Phage titer was calculated for plaque forming units (pfu) per g stool.

### 2.6. Stool Microbiota Analysis

Total DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN), following the manufacturer's instructions, except for the addition of a series of mechanical disruption steps (11 × 45 s) using a FastPrep apparatus and Lysing Matrix B tubes (MP Biochemicals) as described (Junick and Blaut, 2012).

The 16S variable region V1 to V3 was PCR amplified and sequenced on Roche 454 GS-FLX-Titanium Sequencer as described (Sanchez et al., 2014). Raw sequence data were deposited in the GenBank Short Read Archive (Accession number: SRP067629) and analyzed using Mothur v.1.33.0 and QIIME v.1.8 software packages (Schloss et al., 2009; Caporaso et al., 2010). Pyrosequencing reads were denoised with the Mothur implementation of PyroNoise (Quince et al., 2009) according to the 454 SOP (Schloss and Westcott, 2011). Chimeras were identified using usearch61 in QIIME (Edgar et al., 2011). The sequences were then trimmed as described in the Mothur 454 SOP in order to keep sequences overlapping the same 16S region. OTUs *de novo* picking at 97% identity was performed using uclust in QIIME (Edgar, 2010). Taxonomy assignment of OTU representative sequences used the RDP Classifier in QIIME with confidence threshold of 0.6 on the Greengenes reference database v.13.8 (Simeoni et al., 2015). At species level, the taxonomic assignment was determined by BLAST (Altschul et al., 1990) using the

website <http://blast.ncbi.nlm.nih.gov> with a 97% identity threshold. Diversity analyses were performed in QIIME, Mothur and Calypso (<http://bioinfo.qimr.edu.au/calypso/>). Dirichlet Multinomial Mixture analysis was performed in Mothur as described (Ding and Schloss, 2014). Figures were created with GraphPad Prism version 6.07 for Windows (GraphPad Software, La Jolla California USA) and Microsoft Excel 2010.

### 2.7. Genome Sequencing

Individual bacterial colonies were isolated from feces on a *Streptococcus*-enriching agar, characterized as streptococci by API fermentation gallery (API 20 STREP, bioMérieux, France) and four colonies identified as *Streptococcus* by 16S rDNA sequencing, three derived from different diarrhea patients and one derived from a local age-matched control child, underwent whole genome sequencing.

Genomic DNA was extracted from mid-exponential cultures using Genra DNA Purge kit (Qiagen). 10 Kb libraries were prepared following Pacific Biosciences (PacBio) protocol. Sequencing was performed on the PacBio RS platform using C2/C2 chemistry and four single-molecule-real-time (SMRT) cells were used per strain with a 120 min collection protocol. The sub-reads were *de novo* assembled using the PacBio Hierarchical Genome Assembly Process (HGAP)/Quiver software package (Chin et al., 2013). The HGAP workflow consists of three steps: Preassembly to generate long and highly accurate sequences; assembly of the long and highly accurate sequences using Celera assembler and finally, Consensus Polishing using the Quiver algorithm to derive a highly accurate consensus for the final assembly.

The strains were assembled into one to three contigs. The results of the sequencing and assemblies are summarized in Suppl. Table 1. The genomes were annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) and have been deposited at GenBank (NCBI) under accession numbers CP0113688 and CP013689 for isolates S1 and S5 (1 contig) and LPVQ00000000 and LPVR00000000 for isolates S3 and S6 (2–3 contigs). Plasmid DNA was not sequenced by our approach, but plasmids were not detected in the sequenced strains.

A global alignment of chromosomes was plotted using MUMMER package (nucmer parameters: -maxmatch-c 20-g 0-b 1-l 20-noextend). The KSNP phylogenetic tree was built with KSNP3 tool (Gardner and Hall, 2013) with the following parameters: -k 13-ML.

### 2.8. Genomic Safety Analysis

In order to reduce the complexity, sequence reads for the four streptococcal genomes showing 100% sequence identity were removed from the total reads to generate a set of non-redundant (nr) reads. This set of reads was investigated for virulence factors in three ways. First, the reads were mapped against closely related streptococci with proven (*Streptococcus gallolyticus*) and suspected (*Streptococcus lutetiensis*) virulence genes. Matches were manually investigated by additional BlastX searches to verify the annotations from the deposited and the newly sequenced streptococci. Second, the nr reads were mapped against all genes present in an in-house Database for Undesirable Genes described previously (Denou et al., 2009). Third, the nr reads were screened against two public databases compiling antibiotic resistance genes (ARDB) (Liu and Pop, 2009) and bacterial virulence factor genes (VFDB) (Chen et al., 2012). Only hits having two or more reads aligned and covering more than 30% of the gene were considered as positive hits and were further investigated.

### 2.9. Statistical Analysis

Outcome variables, e.g., stool output (g/kg × d), and ORS Intake (ml/kg × d) were compared among the groups using one-way analysis of variance (ANOVA) and between the groups using student's t test. Confounders, e.g., age, duration of diarrhea before hospitalization, and nutritional status were adjusted using multivariate analysis (multiple

regression or logistic regression as appropriate). The data with skewed distribution were normalized by log transformation, and the t test was applied on transformed data. The Wilcoxon's rank test was used for skewed data that cannot be normalized by transformation.

### 3. Results

Four hundred children hospitalized with acute watery diarrhea were screened for this study; 280 were excluded mainly for rotavirus ( $n = 251$ ) or *Vibrio* diagnosis ( $n = 12$ ); 120 fulfilled the entrance criteria and were randomized: 41 received placebo (saline) with standard treatment (ORS plus zinc) (P), 40 received orally  $1.4 \times 10^9$  pfu of the Microgen ColiProteus (McCallin et al., 2013) cocktail (M) and 39 children received  $3.6 \times 10^8$  pfu of the Nestlé Research Centre (NRC) T4-like coliphage cocktail (T) in addition to standard treatment (Sarker et al., 2012; Bourdin et al., 2014a,b). Fig. 1 shows the study flow chart.

#### 3.1. Safety Analysis

Per protocol a safety analysis was done after enrollment of 75 patients. No observations indicative of a Jarisch–Herxheimer reaction (Yang et al., 2010) were seen (Table 1). Liver, kidney and hematological functions were normal when using standard tests (Sarker et al., 2012; McCallin et al., 2013). One T4 phage and one placebo recipient developed generalized tonic–clonic convulsion a few hours after the initial product application, which was attributed by the clinician to hyponatremia and acidosis associated with diarrhea.

#### 3.2. Lack of Clinical Efficacy of Oral Phage

At enrollment, children in the three groups did not differ for prior duration of diarrhea, stool frequency, or vomiting (Table 2). At an interim analysis with 120 enrolled and 115 evaluated children, no significant difference was seen between the groups when evaluating quantitative diarrhea criteria (stool frequency, stool weight, oral rehydration solution needed to correct dehydration) (Fig. 2A–C) ( $P > 0.1$  for all comparisons, t test). In the acute phase, we observed lower means for stool weight in T compared to M; T also happened to have a higher percentage of *E. coli*-positive cases than M and P (Fig. 1). However, even when limited to proven *E. coli* infections, oral phage had no significant impact

on quantitative diarrhea criteria (Table 3). Four days after admission, 50% of the children were discharged from the hospital (no difference between the groups). The increase in quantitative diarrhea values seen at day 5 in Fig. 2 reflects therefore enrichment for children with more severe diarrhea. At day six, 93, 88, and 84% of patients treated with T, M or P, respectively, had recovered from diarrhea (difference not significant, t test).

#### 3.3. Detection of Fecal Phage

Oral Microgen, and to a lesser extent the T4 phage cocktail, resulted in a significantly higher fecal phage prevalence and titer than seen in placebo recipients likely producing endogenous phage (Chibani-Chennoufi et al., 2004) (Fig. 2 D, E). No patient showed higher fecal output than oral input. Electron microscopy done for the first 56 patients with sensitivity to detect  $10^6$  viral particles per g stool (Goldsmith and Miller, 2009) demonstrated only few phage particles in three patients excluding substantial *in vivo* replication of oral phage (data not shown).

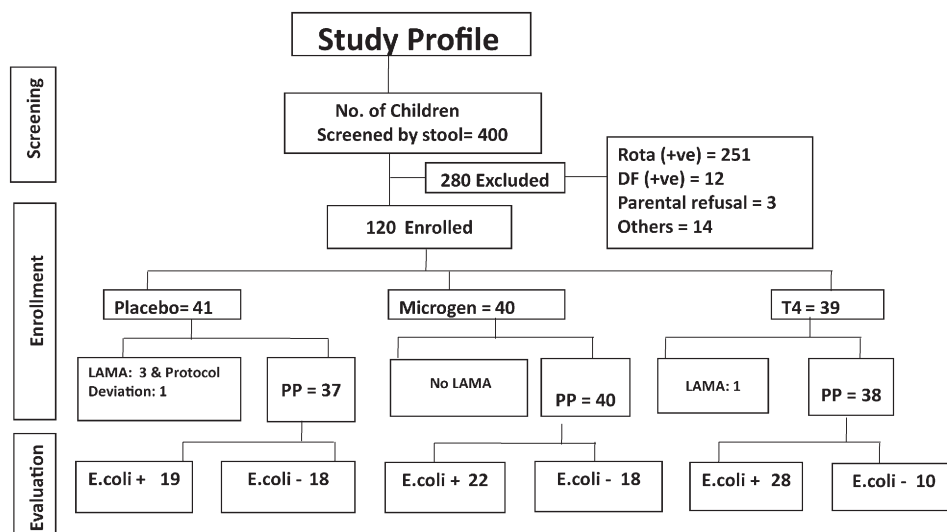
Fecal coliphage titers did not differ significantly between patients with diagnosed ETEC or EPEC/EAEC infection or patients lacking a diarrheagenic *E. coli* (Suppl. Fig. 1A). In addition, fecal phage titers were not higher in patients harboring a phage-sensitive *E. coli* colony in the stool (50% of all patients) compared to patients lacking such colonies (three random colonies were tested per patient, Suppl. Fig. 1B) suggesting passive transit of oral phage through the gut.

In the plaque assay T4-like and T7-like coliphages can be differentiated by plaque size (Suppl. Fig. 2A–C). If a differential survival or replication occurred for these phages in the gut, one would expect a shift in relative proportion from the 1:1 ratio seen for T4 and T7 phages of the M cocktail (McCallin et al., 2013) in the stool of recipients of this cocktail. No such shift was observed (Suppl. Fig. 2D).

#### 3.4. Etiology of Diarrhea

Stool series from the first 56 patients underwent detailed clinical microbiology analysis (Harris et al., 2008; Svenungsson et al., 2000): 77% yielded an *E. coli* pathogen by culture and subsequent pathotype analysis (ETEC: 23; EAEC: 10; enteropathogenic (EPEC): 2; mixed *E. coli*: 8 patients).

The presence of *E. coli* virulence genes was corroborated for these 56 patients by PCR (Vidal et al., 2005) on whole stool DNA. ETEC-specific



**Fig. 1.** Study flow chart. The numbers indicate the children with diarrhea who were screened, enrolled and evaluated. Rota (+ve), patients with rotavirus positive stool in ELISA; DF (+ve), patients with *V. cholerae* positive stool in dark field microscopy; others, patients meeting other exclusion criteria; Microgen, commercial ColiProteus phage cocktail from Microgen, Russia; T4, phage cocktail consisting of T4-like phages from NRC; LAMA, left the study against medical advice; PP, per protocol, *E. coli* +, patient displaying a pathogenic *E. coli* in the stool; *E. coli* - patient without a pathogenic *E. coli* in the stool.

**Table 1**  
Physical examinations in children hospitalized with diarrhea at admission and after four days of treatment with the allotted phage or placebo product.

Treatment	T4-phage		Microgen phage		Placebo	
	Adm	Day 4	Adm	Day 4	Adm	Day 4
n	25	23	26	20	27	23
Age, month	10 ± 3.2 (8.8–11.3)		12 ± 4.1 (10.4–13.6)		13 ± 4.8 (11.2–14.8)	
Weight, kg	8.1 ± 1.3 (7.6–8.6)	8.0 ± 1.3 (7.5–8.5)	8.4 ± 1.4 (7.9–8.9)	8.3 ± 1.5 (7.6–9.0)	8.4 ± 1.5 (7.8–9.0)	8.2 ± 1.7 (7.5–8.9)
Temp, °C	36.9 ± 0.5 (36.7–37.1)	36.7 ± 0.5 (36.5–36.9)	36.7 ± 0.6 (36.5–36.7)	36.5 ± 0.4 (36.3–36.7)	36.9 ± 0.8 (36.6–37.2)	36.8 ± 0.7 (36.5–37.1)
Pulse/min	119 ± 7 (116–122)	118 ± 4 (116–120)	121 ± 5 (119–123)	118 ± 5 (116–120)	120 ± 6 (118–122)	119 ± 5 (117–121)
Respiration/min	33 ± 6 (31–35)	31 ± 2 (30–32)	32 ± 5 (30–34)	30 ± 3 (29–31)	34 ± 3 (33–35)	32 ± 2 (31–33)
BP systolic (mmHg)	89 ± 6 (87–91)	86 ± 5 (84–88)	89 ± 6 (87–91)	87 ± 5 (85–89)	88 ± 5 (86–90)	87 ± 5 (85–89)
BP diastolic	54 ± 5 (52–56)	53 ± 4 (51–55)	56 ± 6 (54–58)	52 ± 3 (51–53)	57 ± 8 (54–60)	55 ± 4 (53–57)

n, number of children per investigated group. Values are means ± standard deviations (95% CI).

No significant differences were seen between clinical parameters at admission and 4 days of phage or placebo treatment in diarrhea patients in paired t-tests. However, T4-phage treated children were significantly younger than placebo recipients ( $P = 0.01$  in t-test), but did not differ from Microgen phage treated children.

genes were detected in 28 (23 *elt+ est+*; 3 *elt+*; 2 *est+*); EPEC in 5 (4 *eae+ bfp+*; 1 *eae+*); EAEC (*daaD+*) in 7 patients for at least 2 successive stool samples. Stool samples were also cultured for other bacterial pathogens (Harris et al., 2008). *Campylobacter jejuni*, *Campylobacter coli*, *Aeromonas*, *Vibrio cholerae*, *Salmonella*, and *Shigella flexneri* were detected in 9, 2, 2, 2, 1 and 1 patients, respectively. All but two also harbored an *E. coli* pathogen. Electron microscopy of putative viral pellets from these 56 stools identified one patient infected with rotavirus, which apparently escaped ELISA detection of group A rotavirus.

### 3.5. Fecal *E. coli* Titers

Total fecal bacterial counts as determined by real time PCR (Nadkarni et al., 2002) were significantly lower in patients than in controls ( $P < 0.001$  in Dumm test) (Fig. 3A). Viable *E. coli* colony counts represented only 5% of all fecal bacteria in diarrhea patients (Fig. 3B) and were at most 10-fold higher in *E. coli* as compared to non-*E. coli* diarrhea patients (median viable stool *E. coli* titers were  $5 \times 10^8$  cfu/g stool for ETEC,  $2 \times 10^8$  for EAEC/EPEC patients and  $5 \times 10^7$  for patients without *E. coli* diarrhea diagnosis).

The titer of heat-stable (Fig. 3C) and heat-labile (Fig. 3D) enterotoxin genes were investigated over the course of diarrhea by real time PCR (Guion et al., 2008). By calibration against an *elt+ est+ E. coli* strain of known titer, the data were expressed as cfu ETEC per g stool. Median titers were low, but the titers were widely spread. When limited to microbiologically confirmed ETEC infections, peak median titers of  $3 \times 10^7$  ETEC cfu/g stool were observed on day 2 of hospitalization with rapid decline over the next days (Fig. 3C, D). Most children with microbiologically defined EPEC or EAEC infection and healthy controls had enterotoxin gene counts below the detection limit of the test, confirming the specificity of the test. No significant decrease was seen for ETEC titers as assessed by real time PCR of heat-labile and heat-stable enterotoxin genes in phage-treated patients compared to the placebo recipients ( $P > 0.1$ , t test) (Fig. 4).

### 3.6. Fecal Microbiota Composition by 16S rDNA Sequencing: Transient *Streptococcus* Overgrowth

We determined the microbiota composition in daily collected stool samples for the first 56 patients enrolled into the trial by 454

sequencing of PCR-amplified 16S rDNA using universal bacterial primers (Sanchez et al., 2014) and compared it with the microbiota composition in stools sampled at the same time period from 20 age-matched healthy control children (median age 11 months) living in the same city under comparable socioeconomic conditions. During the first day of hospitalization, fecal *Streptococcus* represented a significantly higher abundance in diarrhea patients than in healthy controls ( $P = 0.001$ , Fisher's exact test); *Escherichia* was higher in patients than in controls, but not significantly so ( $P = 0.06$ ) (Fig. 5A). Control children showed a higher stool abundance of *Bifidobacterium* than diarrhea patients in the acute phase ( $P = 0.003$ ). Over the next days of hospitalization, a gradual increase in *Bifidobacterium* and a concomitant decrease in *Streptococcus* abundance were observed (Fig. 5A,  $P = 0.008$ ). This shift from *Streptococcus* to *Bifidobacterium* during the hospital stay was seen whether the sequence analysis was based on mean (Fig. 5A) or median abundance (Fig. 6A–C) or whether the analysis was based on the V123 (all presented data in the current report) or on the V456 regions (data not shown) of the 16S rRNA gene. When further differentiated for the diarrhea pathogen, the decrease in fecal *Streptococcus* abundance during hospitalization was clearer for patients with EPEC and EAEC infections or diarrhea without *E. coli* pathogen than for ETEC patients (Fig. 6D, E). Subjects with elevated *Escherichia* abundance had ETEC, but not EPEC/EAEC infections (Fig. 6C, F). Children with non-*E. coli* diarrhea (Fig. 6F) and healthy controls (Fig. 6C) showed with few exceptions  $< 1\%$  *Escherichia* abundance.

The heat map of correlation between bacterial genera showed a strong anti-correlation between *Bifidobacterium* and *Streptococcus* while only a weak anti-correlation between *Bifidobacterium* and *Escherichia* was observed (Fig. 7A). No significant difference was observed for microbiota composition and temporal development of the stool microbiota between placebo and phage recipients when all patients were considered (data not shown). Even when limited to patients with proven ETEC infection, only a difference of borderline statistical difference was seen for microbiota composition (not involving *Escherichia*) between placebo and T, but not M phage recipients (Suppl. Table 2).

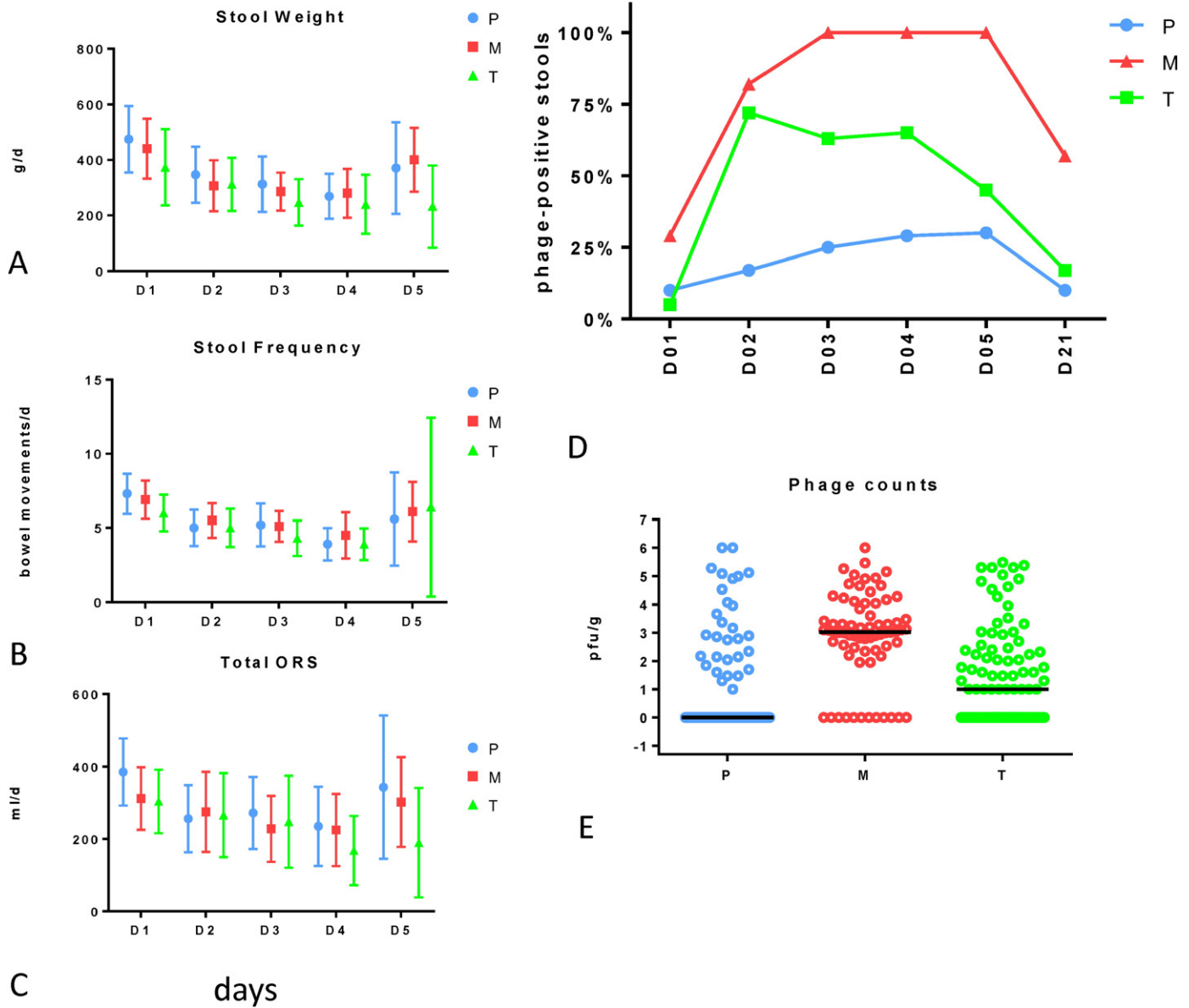
When the 16S rRNA gene sequence data were analyzed at the species level (Fig. 5B), fecal streptococci (van den Bogert et al., 2013) were categorized into two groups, namely the *S. gallolyticus/lutetiensis* (Schlegel et al., 2003) and the *Streptococcus salivarius* groups, with very few *S. infantis* calls. These were the same streptococcal species

**Table 2**  
Diarrhea patients' characteristics before first dose of allotted treatment (baseline).

Group	Number of patients	Admission weight kg	Diarrhea duration before 1st dose (hours)	Stool frequency/day before 1st dose	No of vomiting/day before 1st dose
Placebo	41	8.70 ± 1.7 (8.2–9.2)	32.1 ± 10.3 (29.0–35.2)	16.0 ± 7.1 (13.8–18.2)	5.9 ± 4.7 (4.5–7.3)
Microgen phages	40	8.43 ± 1.4 (8.0–8.9)	33.1 ± 10.4 (32.5–33.7)	14.6 ± 6.5 (12.6–16.6)	6.4 ± 5.7 (4.6–8.2)
T4-like phages	39	8.36 ± 1.3 (8.0–8.8)	32.6 ± 10.2 (29.4–35.8)	16.0 ± 7.6 (13.6–18.4)	7.7 ± 7.2 (5.4–10.0)

Values are means ± standard deviation (numbers in parenthesis denote 95% CI).

Two-tailed P values in unpaired Student t-tests  $> 0.19$  for all comparisons.



**Fig. 2.** Oral phages reach the intestine in children hospitalized with acute bacterial diarrhea, but show no impact on quantitative clinical diarrhea outcomes. Left: Mean and standard deviation for stool weight (measured without urine contamination) in g/day (A), stool frequency per day (B), and need for oral rehydration solution in ml/day to correct dehydration (C) for the specified day of hospitalization in children enrolled into the three treatment groups identified by the color code. No significant difference was detected between the treatment groups. Right, D: Prevalence of phage positive stools ( $\geq 10$  pfu/g stool on *E. coli* indicator strain K-12) at the indicated time points (D01–05: days 1 to 5 of hospitalization; D21: re-convalescent visit 21 days after hospital admission) in the three treatment groups; green: NRC T4-like phage cocktail (T), red: Russian Microgen phage cocktail (M), blue: placebo (P). E: Titer distribution and median fecal phage titer in  $\log_{10}$  pfu/g stool for the indicated treatment groups.

which we found, albeit with different proportions, in healthy control children. Likewise, the same *Bifidobacterium* groups, namely *B. longum*, *B. kashiwanohense*, *B. bifidum* and *B. catenulatum* group, were detected both in diarrhea patients and healthy controls (Fig. 5B).

### 3.7. Streptococcal Abundance Correlates With Diarrhea Symptoms

Two observations associated *Streptococcus* abundance with diarrhea symptoms in the enrolled patients. First, an increase in *Streptococcus* abundance was observed in patients at day 5 of hospitalization (Fig. 5A). Since 50% of the initially enrolled patients were discharged from the hospital at day 4, these subjects represented more severely affected patients as also seen for quantitative diarrhea parameters at day 5 (Fig. 2C). Interestingly, a fecal microbiota difference was already

observed in the early phase of hospitalization between patients later staying for 4 (short hospitalization) or 5 days (long hospitalization) in the hospital: children with an earlier diarrhea resolution also showed an earlier normalization of the *Bifidobacterium*/*Streptococcus* ratio and a lower *Escherichia* percentage than children with a later diarrhea resolution (day 5 or later) (Fig. 5C).

Second, when we plotted the diarrhea stool output of a patient during hospitalization against the *Streptococcus* abundance in the stool microbiota, we observed a positive correlation. This correlation was significant when all patients, only ETEC patients (Fig. 7B) or diarrhea patients without an *E. coli* infection were analyzed. Notably, *Bifidobacterium* stool abundance showed a significant negative correlation with diarrhea stool output (Fig. 7C). *Escherichia* abundance did not correlate with stool output (data not shown).

**Table 3**  
Quantitative diarrhea parameters in patients with microbiologically confirmed *E. coli* diarrhea treated with Microgen or T4-like phage cocktail or placebo.

Confirmed <i>E. coli</i> infections (n = 69)	Day 1	Day 2	Day 3	Day 4
<i>Stool frequency:</i>				
Placebo (n = 19)	7.5 ± 3.4 (6.0–9.0)	6.0 ± 3.1 (4.6–7.4)	6.3 ± 4.5 (4.3–8.3)	4.8 ± 3.9 (3.0–6.6)
Microgen (n = 22)	6.3 ± 4.1 (4.6–8.0)	5.3 ± 2.9 (4.1–6.5)	4.2 ± 2.5* (3.2–5.2)	4.6 ± 5.1 (2.5–6.7)
T4-like phage (n = 28)	6.6 ± 4.1 (5.1–8.1)	5.8 ± 4.3 (4.2–7.4)	5.0 ± 3.9 (3.6–6.4)	4.3 ± 3.2 (3.1–5.5)
<i>Stool weight:</i>				
Placebo (n = 19)	514 ± 444 (314–714)	422 ± 360 (260–584)	350 ± 352 (192–508)	316 ± 280 (190–442)
Microgen (n = 22)	433 ± 265 (322–544)	276 ± 196 (194–358)	278 ± 213 (189–367)	293 ± 279 (176–410)
T4-like phage (n = 28)	406 ± 480 (228–584)	344 ± 321 (225–463)	280 ± 284 (175–385)	280 ± 345 (152–408)
<i>ORS need:</i>				
Placebo (n = 19)	393 ± 261 (276–510)	341 ± 341 (188–494)	333 ± 319 (190–476)	283 ± 373 (115–451)
Microgen (n = 22)	328 ± 220 (236–420)	252 ± 294 (129–375)	192 ± 256 (85–299)	216 ± 280 (99–333)
T4-like phage (n = 28)	330 ± 289 (223–437)	281 ± 368 (137–425)	293 ± 438 (131–455)	201 ± 312 (85–317)

Values are means ± standard deviation (95% CI, confidence interval).

Days 1–4 of treatment.

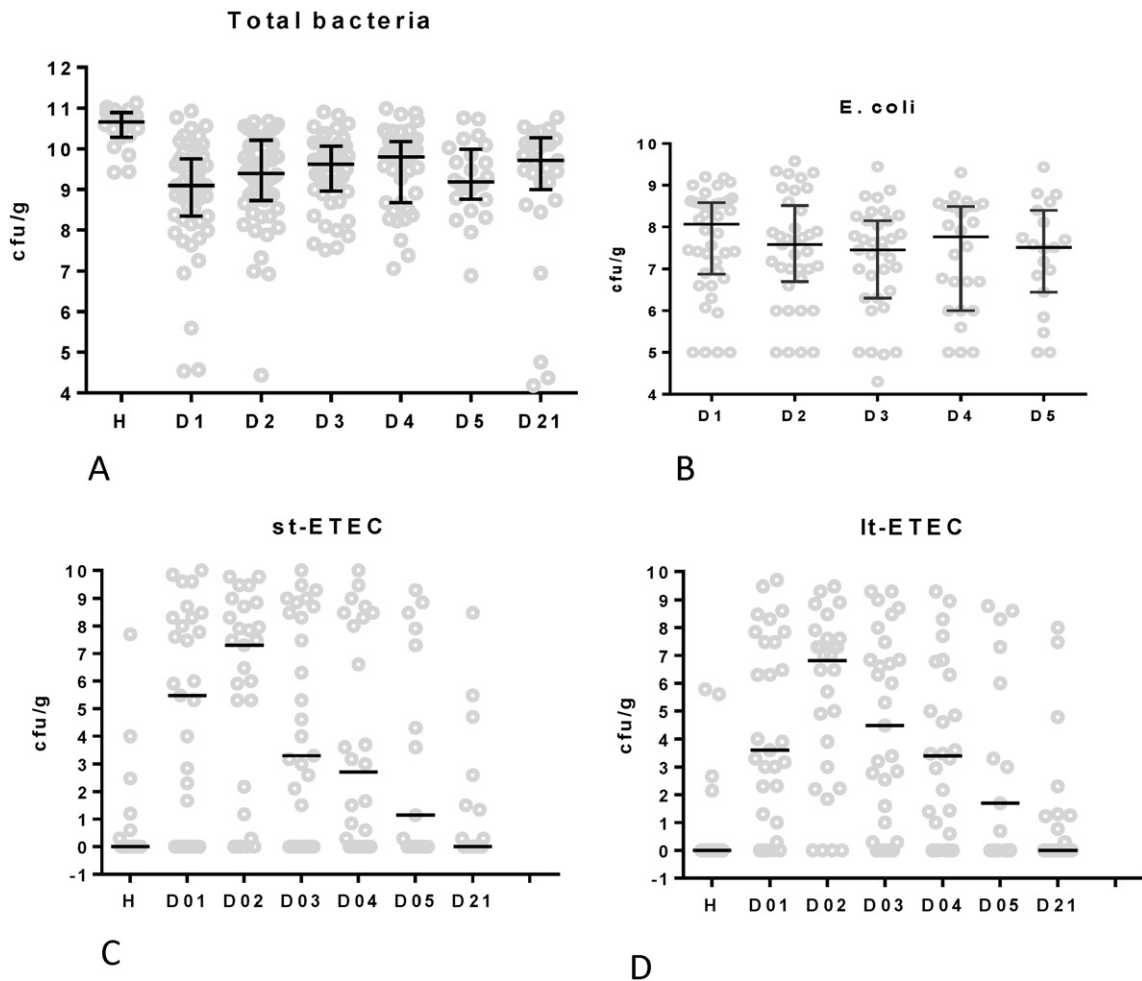
Stool frequency: number of bowel movements per day for the indicated day of hospitalization.

Stool weight: stool weight in g per patient and the indicated 24 h period.

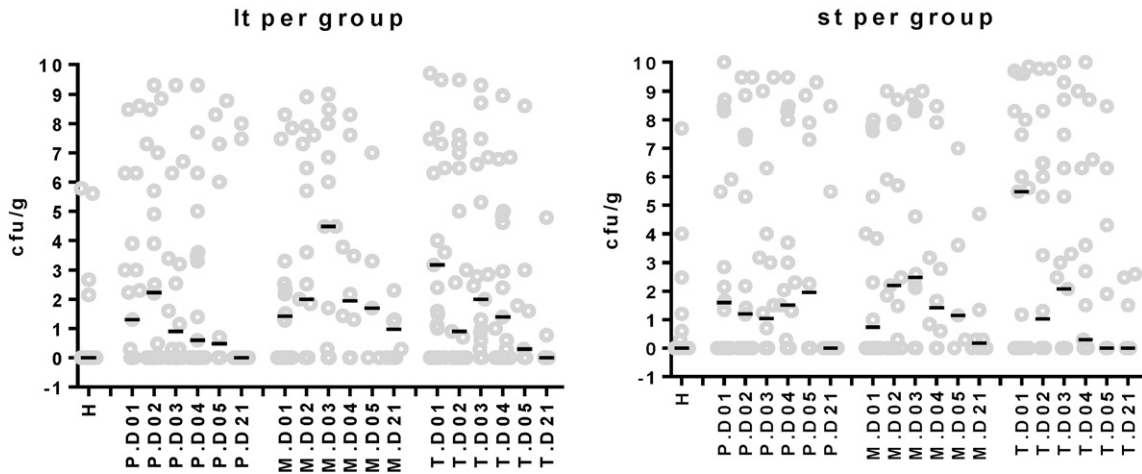
ORS: volume of ORS in ml needed per indicated day to correct dehydration.

n = number of patients per specified group.

\* P = 0.07 in unpaired Student t-test, for all other comparisons P > 0.1.



**Fig. 3.** Total bacteria, *E. coli* and enterotoxigenic *E. coli* titer development in serial stool samples of hospitalized children during an episode of acute diarrhea. A. Median titer with interquartile range for total stool bacteria determined by real time qPCR with universal bacterial 16S rDNA primers; expressed as  $\log_{10}$  cfu/g stool equivalents for healthy local control children (H) and diarrhea patients at the indicated day after hospital admission. Only H is significantly different from the other time points (Dunn's multiple comparisons tests). B. Median with interquartile range of viable *E. coli* counts on MacConkey agar determined as  $\log_{10}$  cfu/g fresh stool (ordinate) for the indicated day of hospitalization of diarrhea patients (no significant difference). C and D. Titer distribution and median titers for heat-stable (st-EETEC, C) and heat-labile (It-EETEC, D) enterotoxin-carrying bacteria in the stool of diarrhea patients hospitalized with a microbiologically confirmed ETEC infection. Titers determined by real time PCR are for the indicated days of hospitalization and are compared to healthy control children (H). The titers are expressed as  $\log_{10}$  median titers in ETEC cfu equivalents.

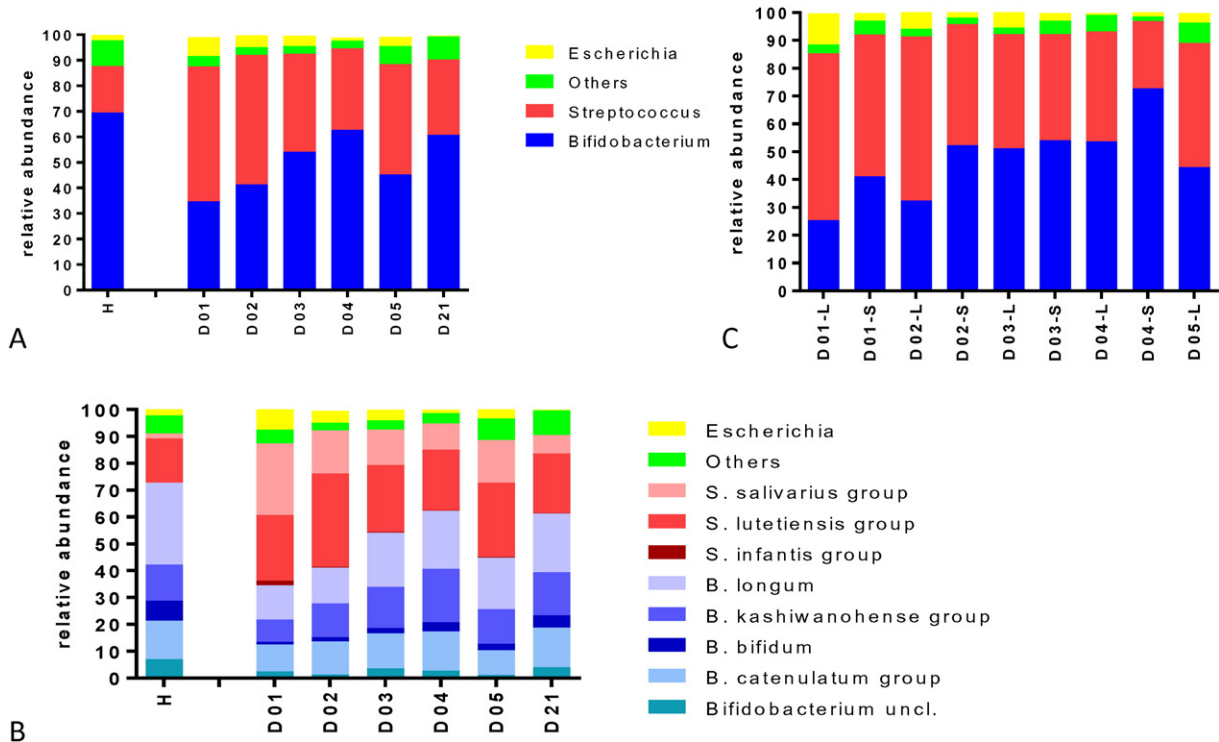


**Fig. 4.** No oral phage treatment effect on fecal ETEC titers. Titer distribution and median titers for heat-labile (lt, left) and heat-stable (st, right) enterotoxin gene-carrying ETEC bacteria in the stool of diarrhea patients treated with placebo (P), Microgen phage (M) or NRC T4 phage (T). Titers were determined by real time PCR for the indicated day of hospitalization (D01 to D05) and the return visit (D21). Titers in healthy control children (H) are given for comparison. The titers are expressed as log<sub>10</sub> titers in ETEC cfu equivalents.

### 3.8. Streptococcal Genomics

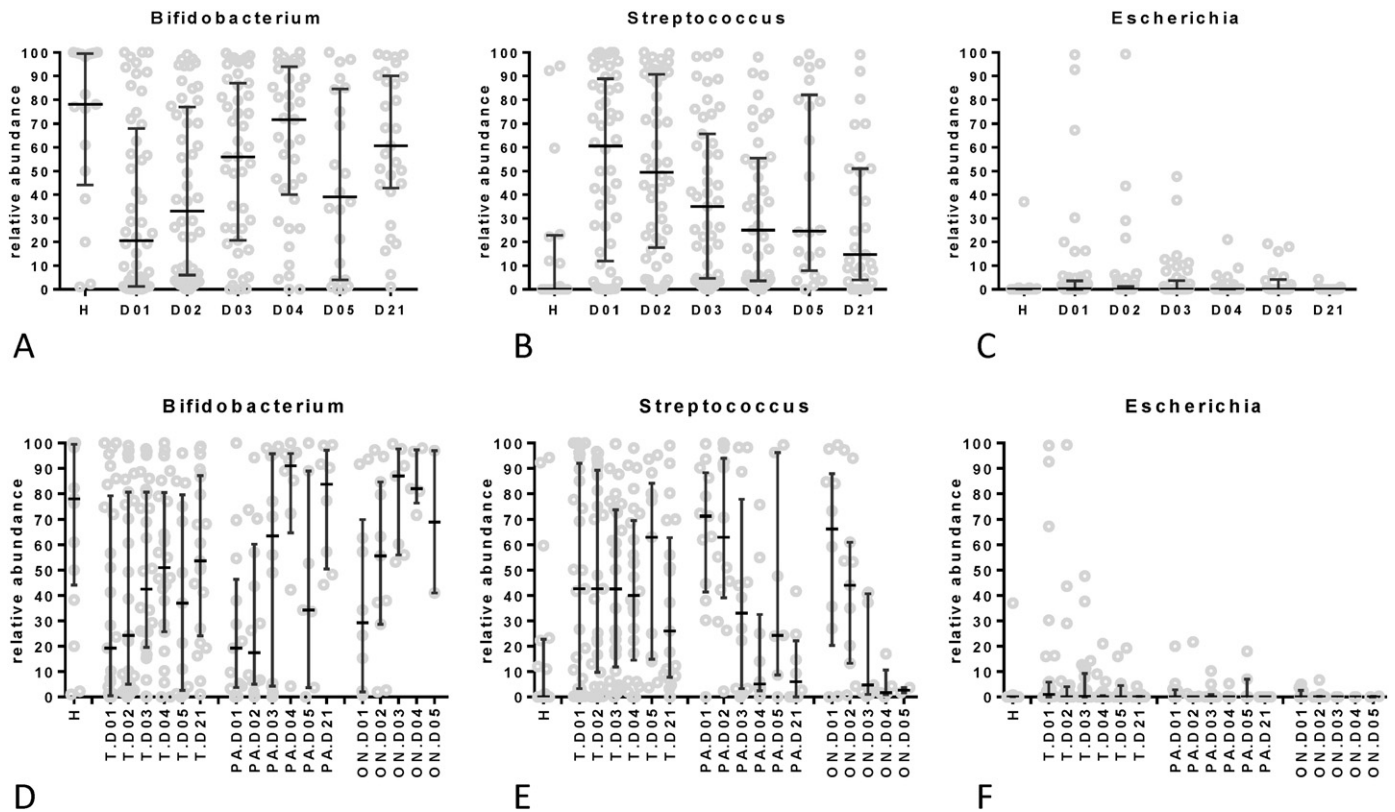
Streptococci were isolated from the stools of three diarrhea patients and identified by API fermentation gallery and 16S rDNA sequencing as *S. gallolyticus* (strains S1, S3) or *S. infantarius* (strain S5). Their genome length was 1.8 to 2.05 Mb and represented by a single (S1, S5) or 2 contigs (S3) (Suppl. Table 1). Their genomes were closely related to each other as well as to the sequenced *Streptococcus* strain S6 isolated

from a healthy control child (Fig. 8A). These genomes were closely related to sequenced strains of the *S. bovis/S. equinus* species complex to which also belong food-associated species (*S. pasteurianus*, *S. macedonicus*), commensal species (*S. infantarius*, *S. equinus*) and facultative pathogens (*S. gallolyticus*) (Fig. 8B). By phylogenetic tree analysis based on single nucleotide polymorphisms of whole sequenced genomes (Gardner and Hall, 2013) S5 and S6 were placed close to *S. infantarius*, S1 associated with *S. macedonicus*, S3 with *S. gallolyticus* (Fig. 8C). More distantly related



**Fig. 5.** Fecal microbiota analysis by 16S rRNA gene sequencing in serial stool samples from children hospitalized with acute bacterial diarrhea. A. Bacterial community structure profiles for fecal samples from 20 healthy control children (H) and 56 diarrhea patients at the indicated day of hospitalization (D01–D21). The panel gives the mean relative abundance in percent for the bacterial genera identified by the color code at the right. B. Bacterial community structure at species level. Healthy controls (H) and diarrhea patients at the specified day of hospitalization are displayed for relative abundance at species level defined by the color code at the right. *S. salivarius* and *S. vestibularis* are members of the *S. salivarius* group. *S. lutetiensis*, *S. infantarius*, *S. equinus*, *S. pasteurianus*, *S. gallolyticus*, *S. macedonicus*, *S. alactolyticus* and *S. porcorum* are members of the *S. gallolyticus* group. *B. catenulatum*, *B. pseudocatenulatum*, *B. angulatum*, *B. ruminantium*, *B. merycicum*, *B. adolescentis*, *B. dentium*, and *B. stercoris* are members of the *B. catenulatum* group. *B. kashiwanohense* and *B. breve* are members of the *B. kashiwanohense* group. C. Bacterial community structure for 56 diarrhea patients that were hospitalized for 4 day (S—short stay) or 5 days (L—long stay) at the specified day of hospitalization. The panel gives the mean relative abundance in percent for the bacterial genera identified by the same color code as in panel A.





**Fig. 6.** Succession of the three major bacterial genera in the gut microbiota during recovery from diarrhea. Median percentage with interquartile range and distribution for the individual patients with respect to *Bifidobacterium* (A and D), *Streptococcus* (B and E) and *Escherichia* (D and F) contribution to the stool microbiota at the indicated day of hospitalization. In A to C data are presented for all investigated diarrhea patients; in D–F, patients were separated with respect to etiology (ON: other bacterial pathogens than *E. coli* and no pathogens detected; PA: EPEC and EAEC; T: ETEC infections) compared to healthy local controls (H).

streptococci belonged to the *S. salivarius* complex comprising oral commensals (*S. salivarius*) and dairy strains (*S. thermophilus*) and *S. pyogenes*, a human pathogen (Fig. 8B, C).

The S1 chromosome is with a length of 2'052 kb 298 kb shorter than the clinical *S. gallolyticus* isolate UCN34 isolated from an endocarditis patient (Danne et al., 2011). When using BlastN 80% of S1 chromosome aligns to UCN34 chromosome with an identity of 80% bp identity or more. On the dotplot (Suppl. Fig. 4A), we can see 2 large loci that are absent from S1 chromosome. Locus 1 is approximately 47 kb long (56 prophage genes), and Locus 2 is approximately 60 kb long (71 genes) and comprises the pilus genes which were biologically proven virulence genes of this endocarditis isolate (Danne et al., 2011). Locus 3 is specific to S1 chromosome and is 53.5 kb long.

Our isolates were also closely related to *S. lutetiensis* strain 0033 (Suppl. Fig. 4B) identified in Chinese children hospitalized with diarrhea for which no known enteropathogen could be identified (Jin et al., 2013). The Chinese researchers proposed numerous virulence factors in their isolate, some were shared by our isolates (Suppl. Table 3). However, upon reinvestigation, alternative annotations were found for them that question their role as virulence genes.

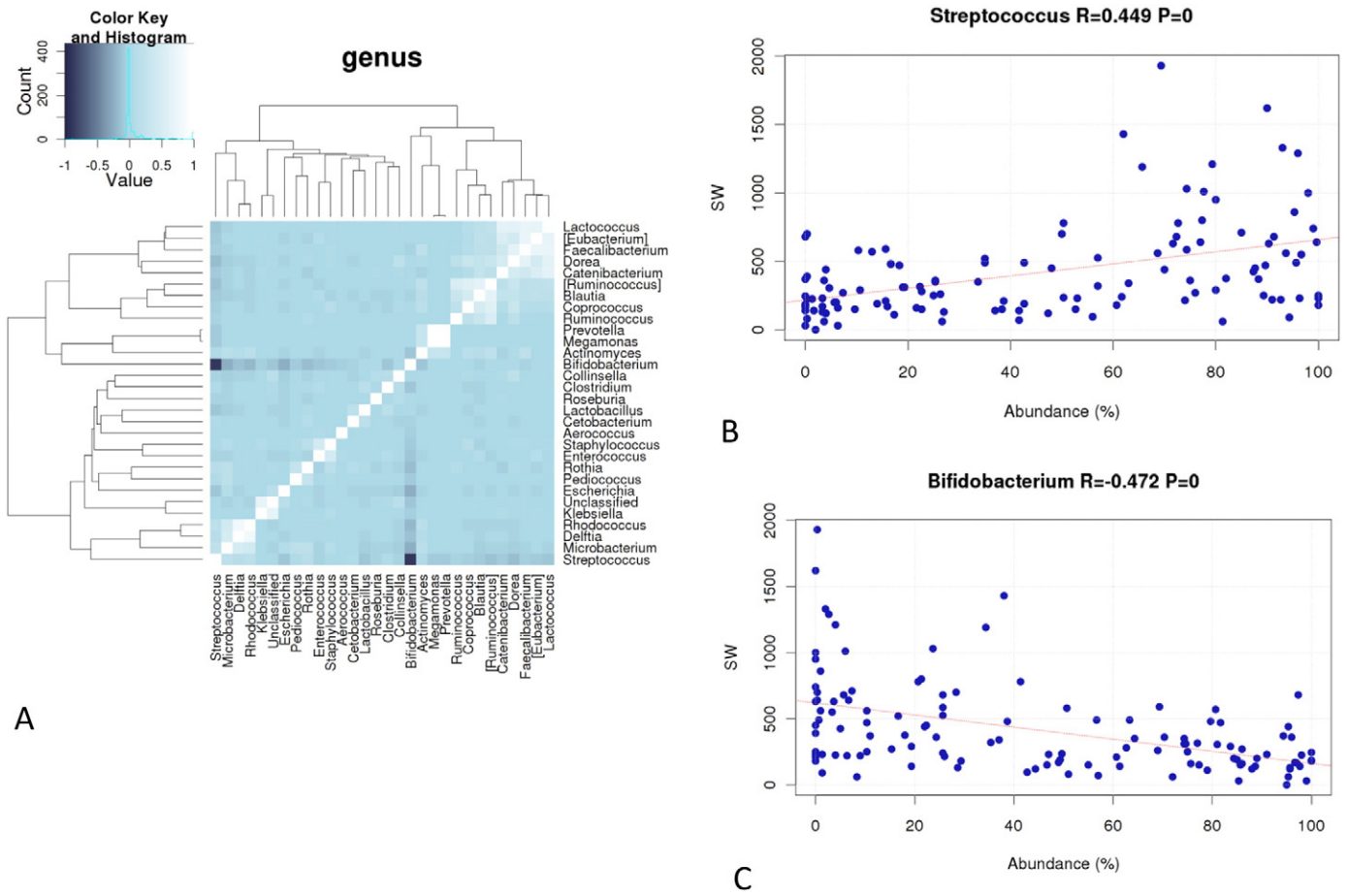
### 3.9. Fecal Microbiota Association With Diarrhea

When the three major fecal bacterial genera were analyzed for the transition period from acute diarrhea into recovery, neither *Escherichia* nor *Streptococcus* showed a clear association with this transition. We found low abundance of *Escherichia* in the acute phase of EPEC/EAEC diarrhea patients and a high *Streptococcus* persistence into the recovery phase in ETEC patients. A high *Streptococcus* abundance was also seen in some healthy controls (Suppl. Fig. 4). The Dirichlet Multinomial Mixture modeling (Holmes et al., 2012) classified the investigated stool samples in four distinct partitions (“fecal community types”)

displayed as a bubble plot in Fig. 9A and described in Suppl. Table 4. The genera *Bifidobacterium* and *Streptococcus* are by far the most important contributors to the differences in community structures of the partitions (37 and 31%, respectively), followed by *Escherichia* and *Dorea* (4% each) (Suppl. Table 5). Partition 3, which is characterized by a very high abundance of *Bifidobacterium* (mean = 90.6%) and a low abundance of *Streptococcus* (4%), was the dominant community type in control children. This community type was lower in diarrhea patients during the acute phase with a trend for increase with recovery (Fig. 9B). Partition 4, which is the most variable partition (Suppl. Table 4) is characterized by a high abundance of *Streptococcus* (68.5%) combined with either elevated amounts of *Escherichia* (4.9%) or a reduced amount of *Bifidobacterium* (16.0%). Compared to controls partition 4 was increased in all diarrhea patients over the hospitalization period (Fig. 9B), and this was independent of diarrhea etiology (data not shown). Partitions 2 and 1 showed medium *Bifidobacterium* (58.2 and 46.6%, respectively), elevated *Streptococcus* (34.4 and 16.0%), and small, but widely distributed *Dorea* (in partition 1 = 7.7%) and very low *Escherichia* (0.8 and 2.3%, respectively) abundance – this combined constellation was found with comparable abundance in both diarrhea patients and controls while partition P1 on its own increased with recovery from diarrhea (Fig. 9B). Notably, partition 4 gradually decreased with recovery from diarrhea (Fig. 9B), as if fecal streptococci develop a pathological potential only in the presence of *Escherichia* or if not counter-acted by bifidobacteria.

## 4. Discussion

In the current PT trial, T4-like phages were chosen for safety concerns (Sarker et al., 2012). T4-like coliphages are obligate lytic viruses, their genomes are well characterized and devoid of bacterial virulence genes and they were propagated on a safe production strain (*E. coli* K-



**Fig. 7.** Anti-correlation between *Streptococcus* and *Bifidobacterium* abundance in the feces and their association with stool output in diarrhea. Heat map of correlation between the indicated bacterial genera identified in the stool of diarrhea patients (A). The strength for correlation and anti-correlation is given in the color key at the top left. Correlation between percentage of *Streptococcus* (B) or *Bifidobacterium* (C) in fecal microbiota (abscissa) and stool weight in g/day (ordinate) for patients hospitalized with ETEC diarrhea. Correlation coefficients and their P value are given at the top of the panels.

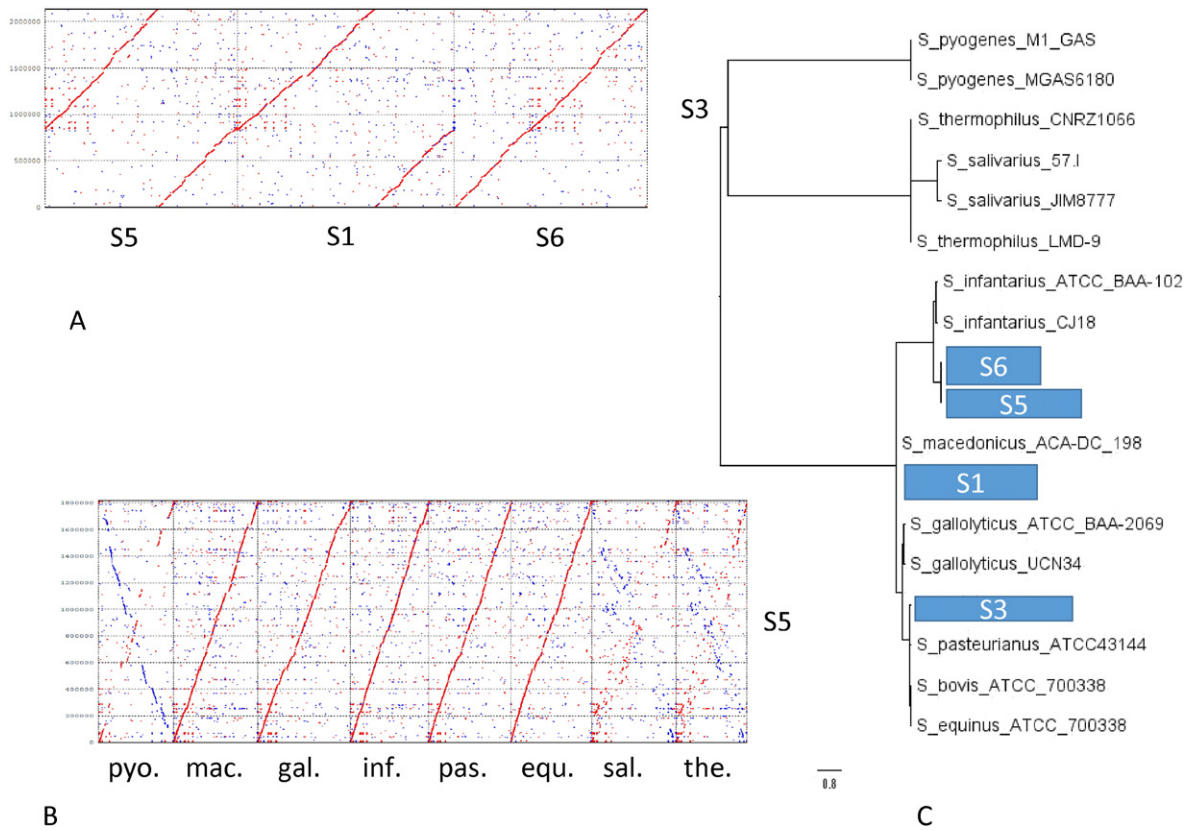
12, itself devoid of virulence genes and inducible prophages) (Denou et al., 2009). However, the *in vivo* replication potential of T4 was previously only explored in mice where it was found to be inferior to T7 phage (Weiss et al., 2009). Whether the prolonged T7 phage excretion is an advantage for PT over the shorter elimination time of T4 phage (Bruttin and Brüßow, 2005) is currently unclear. The commercial Russian phage cocktail consisted of at least 17 different phage types including T7 phage (McCallin et al., 2013). The Microgen recipients showed indeed a higher fecal phage titer than T4 recipients, but they did not show a higher prevalence of T7 over T4 in the stool. The higher fecal output in the M recipients is partly explained by a higher oral phage input.

In acute cases of *E. coli* diarrhea, the barrier function of the gut mucosa in the small intestine is compromised. When the pathogen is lysed by phage, *in vivo* released endotoxins and enterotoxins can thus potentially cross into the circulation. However, no systemic reaction to endotoxin was observed (Yang et al., 2010) which is not surprising since no significant *in vivo* replication of the phage was seen, hence no significant lysis of cells with concomitant toxin release had occurred. While oral coliphages as virions are safe in diarrhea patients, it is still unclear whether oral phages are safe when acting as infectious virus lysing the *E. coli* target cell in the gut of diarrhea patients.

Neither the T4-like nor the Russian coliphage cocktail showed a clinical benefit over standard care in terms of stool output, stool frequency or rehydration. This observation should be interpreted with caution since it could still represent a false-negative outcome because at interim analysis only half of the planned patients were enrolled. The reported trial was thus not powered to detect a treatment effect, but only to

assess safety issues and to gain a mechanistic insight into PT. Notably, we did not observe a substantial *in vivo* replication of the orally applied phage which is a basic requirement for PT. Despite a careful screening, only 60% of the 120 enrolled patients harbored an *E. coli* pathogen in the stool. *E. coli* is thus a less frequent cause of diarrhea (18% of all screened patients) than we had anticipated for Bangladeshi children (36% of all screened patients) (Albert et al., 1999). Furthermore, even in microbiologically defined *E. coli* diarrhea less than 5% of the total fecal bacteria were *E. coli*. When only ETEC pathogens were counted, a median titer of  $10^5$  cfu ETEC per g stool was detected. With an estimated *in vivo* burst size of  $10^6$  (Brüßow, 2013), no phage amplification beyond  $10^6$  pfu/g stool was therefore expected, which is close to the observed peak fecal phage titers. That a microbiologically defined *E. coli* diarrhea was not characterized by high intestinal pathogen titers can also be deduced from the observation that ETEC and EAEC infections in the present study showed only 10-fold higher fecal *E. coli* titers than diarrhea patients lacking an *E. coli* pathogen. Likewise, careful quantitative PCR analysis in stools from Peruvian children with EPEC diarrhea showed titers of  $3 \times 10^5$  EPEC/g stool which were just 10-fold higher than EPEC titers in healthy controls (Barletta et al., 2011). Since the *in vitro* replication threshold for T4 phage on a laboratory indicator *E. coli* strain was determined to  $10^3$  cfu/ml (Wiggins and Alexander, 1985), the pathogenic *E. coli* titer in confirmed cases of *E. coli* diarrhea might simply not be high enough to sustain phage replication *in vivo*.

From our data it is impossible to predict what percentage of the diarrheal episodes is caused by *E. coli*. Only a minority of the screened children had an *E. coli* diarrhea by standard tests. Even less showed a high fecal abundance of *E. coli* by molecular tests and stool titrations.



**Fig. 8.** Genome comparisons for the isolated and sequenced fecal streptococci. A: Dotplot alignment of the genome from *S. galloyticus* fecal isolate S3 (y-axis) against *S. infantarius* isolate S5, *S. galloyticus* isolate S1, and *S. infantarius* isolate S6 (x-axis) obtained from three different patients and one control (S6) at the DNA sequence level. B: Dotplot alignment of the genome from the fecal *S. infantarius* isolate S5 (y-axis) against streptococcal strains of the database at the DNA sequence level. The following genomes were chosen for comparison (from left to right on the x-axis): *S. pyogenes* (human pathogen), *S. macedonicus* (dairy strain), *S. galloyticus* (human pathogen), *S. infantarius* (dairy strain), *S. pasteurianus* (human gut commensal), *S. equinus* (horse gut commensal), *S. salivarius* (oral commensal), and *S. thermophilus* (dairy strain) (from left to right). C: Phylogenetic tree analysis based on single nucleotide polymorphisms of the indicated sequenced genomes with the kSNPv2 software (Gardner and Hall, 2013). The position of the four sequenced fecal streptococci is indicated by the boxes S1, 3, 4, and 5 next to representative streptococci marked with their identifier.

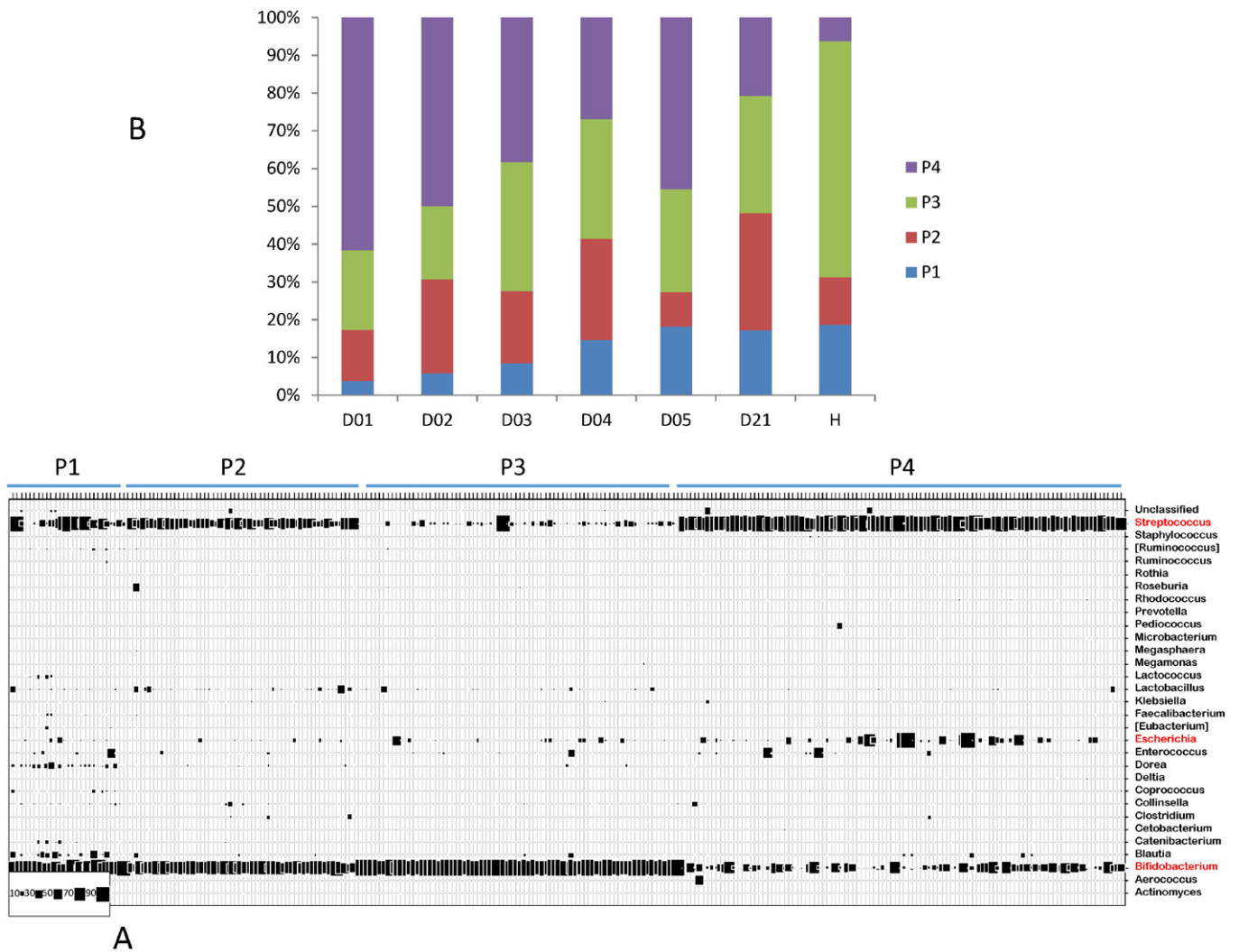
We cannot deduce from our data whether a low abundance bacterial group could be responsible for diarrhea in children. However, to overcome limitations for future phage therapy trials, we strongly recommend to control first and foremost whether the targeted pathogen occurs with sufficient titers to support *in vivo* phage replication.

There are still other limitations which have to be considered when interpreting the negative clinical outcome of the present study. Only half of the patients contained phage-sensitive *E. coli* colonies in the stool. The Russian phage cocktail was obviously not customized to the regional patients and we had to adapt our T4 phage cocktail to the ecological situation in Bangladesh to achieve a reasonable coverage (Bourdin et al., 2014b). Since the phages were isolated several years before the start of the trial, a constant adaptation of the phage cocktail to the prevailing ecological situation is needed. However, it is not clear whether phage coverage was indeed the limiting factor in the current trial since the phage susceptibility of the stool isolates did not correlate with fecal phage titers. Both phage preparations were given without antacid. Buffering of gastric acidity by a slightly alkaline mineral water was perhaps too weak and an additional dose of bicarbonate as recommended by the commercial supplier might have increased phage transit through the stomach. Since it would also increase the risk of nosocomial cross-infection in a diarrhea hospital, such a treatment was excluded for ethical reasons. However, we do not believe that pharmaceutical formulation of the phage was a limiting factor in the current trial since children have a higher (more alkaline) stomach pH than adults. Indeed, in previous trials sensitive biologicals were given to children with diarrhea (antibodies, probiotics), which were recovered intact from the feces (Hilpert et al., 1987) or achieved clinical effects in Bangladesh (Sarker

et al., 1998, 2005). However, a higher oral phage dose might be desirable since children in Bangladesh showed a less good fecal bioavailability of phage than adults from Switzerland (Bruttin and Brüssow, 2005) suggesting some loss during gastrointestinal passage.

Regarding the lack of *in vivo* susceptibility of *E. coli* to coliphages, several factors need consideration. Prominent are physical barriers for phages to reach *E. coli* (increased peristalsis in diarrhea, reduced phage diffusion rate in mucus layer, pathogen protection in biofilms) or slow growth rate of bacterial cells in the stationary phase (Brüssow, 2013). It will be important to study *E. coli*-phage interaction *in vivo* with animal experiments. However, *E. coli* does not induce diarrhea in small laboratory animals and infections with *Citrobacter rodentium* in mice, commonly used as a surrogate, are not a suitable model system since it induces a colitis (Collins et al., 2014). Experiments in animals like piglets or calves which suffer naturally from *E. coli* infection in the small intestine and diarrhea (Fairbrother et al., 2005) are clearly needed.

Another important limitation of our study is the question whether our observations in the fecal samples represent what is happening within the gut. From an analytical viewpoint it would be desirable to analyze bacteria and phages in samples from the upper small intestine. ETEC and EPEC infections occur in the upper parts of the gut and the bacteria excreted with stool might not any longer be in the same growth phase as in the small intestine (stationary vs. logarithmic growth). However, such sampling is invasive and ethically difficult to justify in a normally self-limiting disease in children. Again, dissection experiments in experimentally infected and phage-treated pigs might provide the needed insight. In fact, data from pig have supported the “kill-the-winner” model



**Fig. 9.** Fecal Community Type analysis in the diarrhea patients and healthy controls. A. A Dirichlet Multinomial Mixture modeling defined four partitions P1 to P4 (“fecal community types”) in the investigated stool samples. Each vertical line of the bubble plot represents one individual stool sample, each horizontal line represents a bacterial genus identified at the right ordinate. The size of the squares indicates the percentage of 16S rRNA gene sequences represented by this genus in the given stool sample (code at the lower left corner). B. The relative percentage of partitions P1 to P4 is given for all 56 investigated diarrhea patients for the indicated day of hospitalization (day D01 to 05) and the return visit (day D21) in comparison with age-matched local healthy controls (H).

of phage–bacterium population dynamics also for the gut ecosystem (Allen et al., 2011) suggesting that high titers of target bacteria are needed for PT.

ETEC showed only a short-lived stool peak titer which corresponds to recent reports describing in adult cholera patients from Bangladesh an even shorter peak titer of *V. cholerae* maintained for just 6 h (Hsiao et al., 2014). Curiously, the peak titer of ETEC occurred on the second day of hospitalization as if ETEC was not the cause for hospital admission. In fact, the role of *E. coli* as a pathogen has recently been challenged for its low pathogenicity index. Only certain *E. coli* pathotypes in selected age groups of children and geographical areas were by this criterion significantly linked with diarrhea (Taniuchi et al., 2013; Liu et al., 2014; Kotloff et al., 2013). In fact, *E. coli* diarrhea in children from developing countries is increasingly considered as a polymicrobial infection (Taniuchi et al., 2013; Kotloff et al., 2013) as was also seen in the present study.

Microbiota analysis by 16S rRNA gene sequencing confirmed that only a small fraction of the stool microbiota from children hospitalized with a microbiologically diagnosed *E. coli* diarrhea were represented by *Escherichia*. At admission, the dominant stool bacteria were fecal streptococci independently whether ETEC, EAEC or non-*E. coli* diarrhea cases were considered. Comparison with healthy local controls suggests

a marked streptococcal dysbiosis in the acute phase of diarrhea. Notably, the relative abundance of streptococci was positively correlated with stool output, it predicted the length of diarrhea duration and in EAEC or non-*E. coli* diarrhea it decreased with the resolution of diarrhea. Is a fecal streptococcal dysbiosis thus a potential biomarker for diarrhea? Since all these observations apply to the average of the studied population and not to individuals, who showed great variation, this marker is of limited use for the clinical microbiologist. However, fecal streptococci diagnosed as *S. salivarius* also represented a prominent part of the acute diarrhea microbiota in adult cholera patients from Bangladesh (Hsiao et al., 2014). *E. coli* diarrhea in adults and children from Bangladesh showed an ordered gut microbiota succession and fecal streptococci belonged to this sequence (David et al., 2015). In the GEMS survey children from Bangladesh displayed a significant increase in fecal streptococci during diarrhea. This was, although at lower level, also observed in African children (Pop et al., 2014). Are fecal streptococci therefore candidate new enteropathogens as suggested by Chinese researchers (Jin et al., 2013)? This is unlikely for four reasons: first, using effluents from ileostoma patients, fecal streptococci of the *S. bovis* complex have been identified as typical small intestine commensal in humans (Booijink et al., 2010). Fecal streptococci are fast-growing, efficient fermenters of simple carbohydrates (van den Bogert et al., 2013) possibly

allowing a transient outgrowth when competing gut bacteria are purged from the gut by increased peristalsis in diarrhea. Second, no diarrhea-relevant virulence genes were detected in the three sequenced genomes of fecal streptococci isolated from diarrhea patients. Notably, a biologically proven virulence (pilus) gene identified in a *S. gallolyticus* isolated from an endocarditis patient (Danne et al., 2011) was not detected in the stool isolates. Third, an increased abundance of *S. gallolyticus* was also seen in colon cancer patients (Boleij and Tjalsma, 2013). Bacteria of the *S. gallolyticus* species complex might adhere to the chronically (colon cancer) or transiently (diarrhea) injured gut mucosa. Interestingly, the replacement of the streptococci by fecal bifidobacteria occurs with the same kinetics as the repair of the gut mucosa with newly synthesized enterocytes. Finally, the analysis of the fecal microbial community types showed that elevated fecal *Streptococcus* abundance had no pathogenic potential as long as they are counterbalanced by an elevated *Bifidobacterium* abundance. This could mean that our understanding of diarrhea etiology will have to change in the future: not only need quantitative pathogen titers to be determined, pathogen constellations and pathogen interactions with commensal bacteria need to be considered for the understanding of diarrhea. In our dataset, loss of bifidobacteria was in fact the best biomarker for diarrhea.

Based on these considerations and the presented microbiological and clinical observations, we decided to stop the PT trial at interim analysis. In summary, while phage therapy is still one very important alternative tool against antibiotic resistance, we need to understand in detail the *in vivo* interactions between phage and its host bacterium to judge the reliability and efficacy of such approaches. At the moment it is risky to conclude whether phage therapy is or is not a viable option against antibiotic-resistant bacterial pathogens.

### Competing Interests

The authors listed under institutions b and c are paid employees of the two specified research centers of Nestec Ltd., but have otherwise no financial interest in phage therapy.

The sponsor (Nestlé Nutrition and Nestlé Health Science) was not involved in data evaluation.

### Author Contribution

SAS is the PI of the clinical study responsible for study design, protocol writing, for defense of the protocol to institutional review boards, data collection tool development, data analysis, interpretation and manuscript writing; SS is the Co-PI of the clinical study responsible for defending the protocol at IRB, data collection tool development and data collection; GR did stool phage counting and streptococcal isolation; DM did library preparation, the sequencing and the assembly of the Strep genomes; PD did design the *de novo* genome assembly and data interpretation; FC did the phage susceptibility testing of fecal *E. coli* isolates; GB did the T4-like coliphages cocktail development, GMP production and release; SMC did the PCR detection of *E. coli* virulence genes in stool DNA; CN-B contributed to the bioinformatic analysis of the microbiota and the streptococcal genomes; TN did the statistical analysis; MA did the viable *E. coli* counts in fresh stools; SH collected the clinical data; FQ contributed to study design, data interpretation and supervised the etiology analysis in the clinical microbiology lab; KT did clinical microbiology data collection; MK did streptococcal genome data submission and annotation; MD did the qPCR analysis of *elt* and *est* genes; CL did the qPCR of total stool bacteria; YD searched the streptococcal genomes for virulence genes; SEA did the Dirichlet Multinomial Mixtures analysis; BB did acquisition, analysis and interpretation of microbiota data, and contributed to the writing of manuscript; HB designed with the PI the clinical protocol, coordinated and supervised the project and wrote the manuscript.

### Acknowledgments

We thank our colleagues Lionel Philippe (Clinical Development Unit); Thierry von der Weid and Nelly Conus (Nestlé Nutrition) and Martinas Kuslys (Nestlé Health Science) for their help in the execution of the clinical trial. The Nestlé Research Centre, Lausanne, Switzerland, provides support to icddr,b for its operations and research. icddr,b gratefully acknowledges their support and commitment to icddr,b's research efforts. Our heartfelt thanks also go to the nurses and research assistants from icddr,b for their support and help.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.12.023>.

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