Original investigation

Probiotic Supplementation and Development of Preterm Infant Gut Microbiota and Antibiotic Resistome

An Observational Multi-Center Study

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KEY POINTS

Question: Can probiotic supplementation restore gut microbiota composition and the antibiotic resistome in preterm infants?

Findings: In a multi-center, study including 31 extremely preterm infants receiving probiotics and 35 very preterm infants not receiving probiotics, *Bifidobacterium* dominated the gut microbiota short after commencing probiotics. Extremely preterm infants receiving probiotics had much higher antibiotic exposure, but microbial diversity and abundance of antibiotic resistance genes was not different than in the more mature infants at 4 weeks and 4 months.

Meaning: Probiotic supplementation may alleviate harmful effects of antibiotics on gut microbiota composition. A gradual dose increase after birth may be warranted.

ABSTRACT

IMPORTANCE: Gut microbiota dysbiosis is associated with development of necrotizing enterocolitis (NEC) in preterm infants. Probiotic supplementation may reduce rates of NEC, but there is limited data on the impact of probiotics on early development of gut microbiota composition and the antibiotic resistome in extremely preterm infants.

OBJECTIVE: To determine the association between probiotic (bifidobacteria and lactobacilli) supplementation and development of the gut microbiota and the antibiotic resistome in extremely preterm infants, and compare data with very preterm infants not supplemented with probiotics and healthy full-term infants.

DESIGN: Prospective, longitudinal observational multicenter study.

SETTING: Six Norwegian tertiary care neonatal intensive care units.

PARTICIPANTS: Between January and December 2015 we enrolled 76 infants; 31 extremely preterm infants supplemented with probiotics, 35 very preterm infants not supplemented with probiotics and 10 healthy vaginally delivered full-term control infants.

EXPOSURES: Probiotic supplementation and antibiotic therapy.

MAIN OUTCOMES AND MEASURES: Taxonomic composition and antibiotic resistance genes (ARGs) in fecal samples collected at 7 and 28 days and 4 months of age. Extracted DNA was analyzed using shotgun metagenome sequencing.

RESULTS: Mean gestational age/birth weight were 26 weeks/826 grams and 29 weeks/1290 grams in preterm infants exposed and not exposed to probiotics, respectively. At one week of age we found higher median relative abundance of *Bifidobacterium* in probiotic supplemented infants (64.7) compared to non supplemented preterm infants (0.00) and term control infants (43.9). *Lactobacillus* was only detected in small amounts in all groups, but the relative abundance increased up to age 4 months. We detected higher abundance of ARGs in infants receiving broad-spectrum antibiotics compared to narrow-spectrum regimens. Extremely preterm infants receiving probiotics had much higher antibiotic exposure, still overall microbial diversity and abundance of ARGs was not different than in the more mature infants at 4 weeks and 4 months.

CONCLUSIONS AND RELEVANCE: We speculate that probiotic supplementation may induce colonization resistance and thereby partly alleviate harmful effects of antibiotics on the gut microbiota and antibiotic resistome. The early high abundance of *Bifidobacterium* in probiotic-supplemented extremely preterm infants may suggests that a gradual increase in probiotic supplementation is warranted.

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INTRODUCTION

Preterm infants experience unique challenges in establishing their gut microbiota. Cesarean deliveries, extensive antenatal and neonatal antibiotic exposure, parenteral nutrition and residing for long periods in a neonatal intensive care unit (NICU), may cause unpredictable perturbations of the gut microbiota development.[1] Gut microbiota dysbiosis is associated with development of necrotizing enterocolitis (NEC).[2] Probiotic supplementation to preterm infants aims to restore the gut microbiota and to prevent NEC and other complications.[3-5]

Meta-analyses of randomized and observational trials show that probiotic supplementation, mainly with bifidobacteria and/or lactobacilli, reduce rates of NEC.[3,4,6,7] There seems to be strain-specific effects[4] and not all products are efficacious.[8] Still, based on recent evidence[3] and expert opinion[9], many NICUs in Europe, Australia and Canada have implemented routine probiotic supplementation to preterm infants. Probiotics are infrequently used in preterm infants in the US.[10] Risks of probiotic sepsis and contaminations of probiotic products may explain skepticism.[11-14] Some experts recommend waiting for additional studies to confirm the safety and efficacy of an available and reliable product.[15] Moreover, there is a paucity of in-depth knowledge on microbiological effects and effective dose of probiotic therapy.

Antibiotics are the most commonly prescribed medications in the NICU, [16] and prolonged therapy increases the risk for NEC.[17,18] Antibiotics may influence both the physiological gut microbiota composition and the collection of antibiotic resistance genes (ARGs) in the gut, defined as the gut resistome.[19] However, there is limited knowledge on how probiotic supplementation influences the gut resistome in extremely preterm infants.

In Norway probiotic supplementation was implemented as standard of care for extremely preterm infants at high risk for NEC in 2014. In a longitudinal multi-center study, using shotgun-metagenomic sequencing, we set out to evaluate the taxonomy and the antibiotic resistome of the gut microbiota of extremely preterm infants supplemented with probiotics, and compare it to very preterm infants not supplemented with probiotics and a group of healthy, full-term infants.

MATERIALS AND METHODS

Study patients and sampling procedure

We prospectively planned to include two convenient groups of preterm infants from six Norwegian NICUs; one group of extremely preterm infants (gestational age 25-27 weeks and/or birth weight < 1000 g) supplemented with probiotics, and one group of very preterm infants (gestational age 28-31 weeks and/or birth weight 1000-1500 g) not supplemented with probiotics. Exclusion criteria were gestation below 25 weeks and/or an early, life threatening condition leading to high risk of not surviving the first weeks of life. We included a control group of ten healthy, vaginally delivered full-term control (FTC) infants born at the University Hospital of Northern Norway. No formal power calculation was performed, but we expected that around 30 infants in each group of preterm infant would allow us to detect differences in gut microbiota composition up to 4 months of age. The sample size was also adapted to cover the high expenses for shotgun metagenome sequencing. The original protocol[20] focused on taxonomic composition. We decided post hoc to add a resistome analysis.

After careful instructions, fecal samples were collected by a nurse in the NICU at around seven and 28 days of age, and by the parents at home at around four months of age. We used a commercially available sampling kit (OMNIgen GUT kit, DNA Genotek, Ottawa, Canada) allowing storage of samples at ambient temperatures for up to 14 days before DNA extraction (eMethods).[21] We obtained routine clinical data including details on antibiotic exposure.

DNA extraction, library preparation and sequencing

DNA extraction, library preparation and shotgun-metagenomic DNA sequencing (Miseq, Illumina Inc) were performed using standard procedures (eMethods).

Taxonomic profiling and the gut resistome

The relative abundance of bacteria at genus level was calculated using MetaPhlAn 2.0.[22] The prediction of ARGs was performed on the assembled metagenomes, searched against the Comprehensive Antibiotic Resistance Database (CARD).[23] Data are presented as distribution

of ARG classes among the three different groups of infants at three time points. In order to obtain quantitative measures of the putative ARGs in each sample, the quality trimmed reads were analyzed using Short, Better Representative Extract Dataset (ShortBRED)[24] against a formatted CARD database and normalized per total reads in each sample. Data are presented as abundance of ARGs among the three different groups of infants at three time points.

Probiotic supplementation

A consensus-based protocol for probiotic supplementation was implemented in Norway in 2014.[25] After considering the safety profile, a widely used probiotic combination product was selected (Infloran®).[26] One capsule Infloran contained 10^9 *Lactobacillus acidophilus* (ATCC 4356) and 10^9 *B. longum* subspecies *infantis* (ATCC 15697). One half capsule once daily was initiated on day 3-4 and increased to one capsule daily after 4-7 days.

Influence of antibiotic therapy

To quantify changes in the gut microbiota composition and resistome after antibiotic exposure, we stratified four different categories of antibiotic exposure: (i) antenatal exposure, (ii) short (< 72-96 h) versus prolonged (> 72-96 h) exposure in the first week of life, (iii) any exposure after first week of life (yes/no) and (iv) narrow- versus broad-spectrum exposure after first week of life. Potential effects of antenatal exposure and short versus prolonged therapy after birth were only investigated at 7 days of age.

Ethics and statistical analysis

The study was approved by the Norwegian Regional Ethical Committee. Informed written consent was obtained from all parents. Data were analyzed using IBM-SPSS version 22 (IBM, Armonk NY, USA) statistical software, the R statistical framework (version 3.2.4; http://www.r-project.org/), and Statistical Analysis of Metagenomic Profiles (STAMP) software package.[27] We used Mann-Whitney U test or a Kruskal-Wallis test for comparisons between two or multiple independent groups. We used a Poisson generalized linear model to calculate trends in the relative abundance of genera and ARGs in the gut microbiota. Corrections based on multiple comparisons were performed by the Benjamini-Hochberg false discovery rate (FDR).[28] A FDR P value $\leq .10$ was considered significant for any analyses with

multiple comparisons. A standard *P* value ≤.05 was considered significant for all other analyses.

Alpha diversity was assessed by calculating the Shannon Diversity index (MEGAN, v5.10.6).[29] To detect changes in alpha diversity over time, we first performed a normality test and found that the residuals were normally distributed. Therefore, differences in alpha diversity over time between the three different groups were calculated using linear mixed models. The same model was used to calculate the influence of antibiotic exposure on alpha diversity. Multiple beta diversity metrics of samples was performed using non-metrical multidimensional scaling (NMDS) based on a matrix of Bray-Curtis distances calculated using the vegan R package. Differences between groups were tested using permutational multivariate analysis (PerMANOVA) on beta diversity matrices.

RESULTS

Study population and antibiotic exposure

Figure 1 shows study flow. We enrolled 66 preterm infants and 10 healthy full-term control (FTC) infants between January and December 2015. Clinical characteristics, antibiotic and probiotic exposure, duration of parenteral nutrition and enteral nutrition data are reported in Table 1. The "probiotic extremely preterm (PEP)" infants received much more antibiotics than the "non-probiotic very preterm (NPVP)" infants after first week of life.

Taxonomic composition

On day 7, we found higher relative abundance of *Bifidobacterium* and *Lactobacillus* in PEP-infants compared to NPVP-infants (Figure 2a, eTable 1). FTC infants had higher abundance of some genera (*Streptococcus*, *Veilonella* and *Haemophilus*) that were only sparsely present in the two preterm infant groups (Figure 2a). Mode of delivery did not lead to detectable differences in the microbiota composition within the preterm groups on day 7 (data not shown).

On day 28, there was a striking increase in relative abundance of *Escherichia* in the PEP-infants and a similar striking increase in relative abundance of *Bifidobacterium* in

NPVP-infants. FTC infants had significantly higher relative abundance of *Lactobacillus* than NPVP infants. Overall, at 28 days of age the FTC- and NPVP-infants had higher abundance of *Veilonella* and *Streptococcus* than PEP-infants, while both preterm groups had higher relative abundance of *Staphylococcus* and *Enterococcus* than FTC-infants (Figure 2b).

By four months of age, there were no significant differences in taxonomic profile between PEP- and FTC-infants. The NPVP-infants had more *Prevotella* than PEP-infants, but otherwise all three groups were similar (Figure 2c). Duration of parenteral nutrition did not lead to detectable differences in the microbial composition between the preterm group(s) on 28 days and at 4 months of age (data not shown).

Influence of antibiotic exposure on taxonomic composition

We found no significant influence of antenatal antibiotic exposure on the gut microbiota composition on day 7. However, 57/66 (86%) preterm infants also received antibiotic therapy (ampicillin or penicillin + gentamicin) during the first week of life (Table 1) limiting the possibility to detect isolated effects of antenatal exposure. There was no difference in the gut microbiota between those exposed to a short (<72 or 96 hours) compared to a prolonged (>72 or 96 hours) course during first week of life. Broad-spectrum antibiotic therapy after the first week of life was mainly given to PEP-infants. At four months of age there was reduced relative abundance of *Lactobacillus* and *Veilonella* in those exposed to broad-spectrum antibiotics compared to infants exposed to narrow-spectrum therapy (eTable 2-3). Moreover, there was a non-significant trend towards reduced relative abundance of *Bifidobacterium* and increased relative abundance of *Escherichia* among all preterm infants exposed to broad-spectrum antibiotics at both 28 days and 4 months of age (eTable 2-3).

Diversity of the gut microbiota and influence of antibiotic exposure

We found large intra-individual differences in the gut microbiota composition, in particular at 7 and 28 days of age (Fig 2a-c). The alpha diversity increased significantly with age in both preterm infant groups, but not in FTC-infants (Fig 3a). FTC-infants had significant higher diversity compared to PEP infants at 7 days of age. On day 28 and at 4 months of age, there were no significant differences in alpha diversity between any groups. Significant overall

community (beta diversity) differences were detected at 7 days of age and 28 days of age (Figure 3b-d). However, we found no difference in alpha or beta diversity between different categories of antibiotic exposure at the three sampling time points.

Antibiotic resistome – distribution of ARG classes and abundance of ARGs

In all three groups, we identified putative ARGs conferring resistance to nine different classes of antibiotics, including beta lactams, aminoglycosides, tetracyclines, fosfomycine, sulphonamides, vancomycin, and the macrolide-lincosamide-streptogramin B group. Genes conferring resistance to fluoroquinolones and chloramphenicol were only detected in PEP- and NPVP-infants. Several genes encoding efflux pumps were also identified at all three sampling time points. In total 99 unique ARGs were identified, of which 28 (28%) were located on mobile genetic elements, and these latter were found in more than 80% of all infants (eTable 4).

We found 21 different genes encoding beta-lactamases, including broad-spectrum and extended-spectrum beta lactamases (ESBLs). ESBL-genes were represented at all three time points in NPVP- and FTC-infants, but not detected in PEP-infants. The methicillin resistance gene (*mecA*) was identified at seven days and 28 days of age in 11/35 NPVP-infants and 13/31 PEP-infants, but not at 4 months of age. Only one PEP-infant and four NPVP-infants were persistent fecal carriers of *mecA* at days 7 and 28. Vancomycin ARGs were identified at four months of age in 16 infants, but only four of these had received vancomycin. Many of the ARGs identified, encoded resistance to other antibiotics than those used in the NICUs.

On day 7 NPVP-infants had higher abundance of ARGs from four different ARG classes and PEP-infants higher abundance of ARGs from two other ARG classes (Table 2). Only 24% of ARG-classes changed significantly their abundance during over the three sampling points ($p<0.05$) (Table 2).

On day 7 and at 4 months of age, different antibiotic exposure did not result in significant difference in total abundance of ARGs (eTable 5-8). However, on day 28, we detected significantly higher abundances of four classes of ARGs, including genes encoding

beta-lactam and aminoglycoside resistance, in infants exposed to broad-spectrum antibiotics compared to infants treated with narrow-spectrum regimens (eTable 5).

DISCUSSION

The main aim of this explorative, observational multi-center study was to obtain in-depth knowledge on the impact of probiotic supplementation to extremely preterm infants on gut microbiota and the antibiotic resistome. Previous studies have shown that the gut microbiota in preterm infants differs from term infants with limited diversity and delayed acquisition of a stable profile.[30-32] However, most studies have assessed the gut microbiota composition collapsed at phylum level by sequencing of the 16S ribosomal RNA gene[26,33], and few studies[19] have investigated the association between use of probiotics, antibiotics and gut resistome development using shotgun-metagenomic sequencing.

Bifidobacteria strongly dominated the gut microbiota in extremely preterm infants only few days after commencing probiotic supplementation, in stark contrast to very preterm infants not receiving probiotics who predominantly had *Escherichia*. High levels of probiotic bacteria are not necessarily indicative of colonization, but may represent the passage of DNA from the administered probiotic species through the host.[34] Still, this early bifidobacterial dominance may potentially enhance the risk of translocation to the blood stream, in particular at a very early stage when enteral nutrition with “fuel for bifidobacteria” is not yet fully established.[11,12] Previous studies have shown that the gut microbiota of preterm infants shortly after birth have a high proportion of *Proteobacteria* and that a bloom of *Bifidobacterium* first occurs around 33 weeks of age, in line with our findings in NPVP-infants at 7 and 28 days of age.[35,36]

Lactobacillus was only detected in small amounts in all groups, but relative abundance increased up to four months of age in all three groups. High levels of *Bifidobacterium* and barely detectable levels of *Lactobacillus* have been reported earlier in infants supplemented with equal doses of a probiotic combination of bifidobacteria and lactobacilli.[26] A possible explanation for this observation is the spatial organization of

intestinal bacteria, where lactobacilli are found in intestinal crypts, thus less accessible to collection of luminal contents.[37]

There is no consensus on the optimal dose of probiotics. One study from India compared standard and high-dose probiotic regimens and found no difference in proportion of infants colonized or quantitative colonization rates with probiotic species.[38] Most large randomized trial have used daily doses of 1 x 10^8 - 10^9 CFU.[34,39,40] Some authors suggest that at least 1 x 10^9 CFU is required to achieve a beneficial effect, in line with doses in our study.[41] However, we speculate that the early and very high relative abundance of *Bifidobacterium* in PEP-infants, observed in our study, may not be optimal for the developing gut ecosystem. A more gradual increase in probiotic supplementation concomitantly with increased enteral nutrition may replicate the physiological gut microbiota development, and secure gut growth, digestive maturation and an appropriate response to bacterial colonization.[42 43]

A lower relative abundance of *Bifidobacterium, Lactobacillus and Veilonella*, and a higher relative abundance of *Escherichia*, were observed at day 28 and 4 months of age among infants treated with broad-spectrum compared to narrow-spectrum antibiotic regimens. Reduced abundance of protective anaerobe commensals and higher abundance of *Enterobacteriaceae* after antibiotic exposure has also previously been reported.[44,45] When comparing presence and absence of antibiotic exposure after the first week of life, no differences in diversity or taxonomic composition were found. Previous studies on alpha diversity and influence of antibiotic treatment have shown inconsistent results.[46] However, infants who were most heavily exposed to antibiotic treatment in our study, were also supplemented with probiotics. In animals probiotics may alleviate the potential loss of microbial diversity created by antibiotic treatment.[54] This may explain why PEP-infants, exposed to massive antibiotic pressure, did not have reduced microbial gut diversity compared to other groups. Thus, probiotic supplementation may offer a protective effect partly compensating harmful effects of antibiotics in preterm infants. However, the early low number of taxa in preterm infant stools places constraints on interpreting diversity changes as diversity in a non-complex population may reflect changes in only one taxon.

In line with others, we found that the gut antibiotic resistome of preterm and term infants is established early, independent of antibiotic exposure.[19,47-49] We detected significant higher abundance of ARGs in infants receiving broad-spectrum antibiotics compared to narrow-spectrum regimens. Gibson and co-workers also showed that broad-spectrum antibiotic therapy in preterm infants, was associated with enrichment of specific ARGs.[19] We aimed to investigate how probiotic supplementation can influence the gut antibiotic resistome. Overall there were no differences in distribution of ARG-classes or abundance of ARGs at 28 days and 4 months of age between PEP-infants, exposed to massive antibiotic therapy, and the two other groups with limited or no antibiotic exposure. One possible mechanisms for this finding is that probiotic bacteria can produce bacteriocins that improve mucosal integrity and thereby reduces the pathogenic bacterial population and antibiotic resistance.[50]

Strengths and limitations

At the time of this study, probiotic supplementation to extremely preterm infants was considered standard of care in Norway. We were therefore beyond equipoise to perform a randomized study comparing probiotic to no probiotic supplementation in this population. The NPVP-infant group has limitations as a control group due to maturational differences and the difference in antibiotic exposure compared to the PEP-infants. However, more antibiotic exposure in the PEP-infants would most likely have led to less diversity and higher abundance of ARGs. Still, we found few differences between the two preterm groups at 28 days and 4 months of age, suggesting a protective effect of probiotics in the PEP-infant group. The gut microbiota composition of preterm infants may differ between hospitals [51], but our multi-center approach intended to average local differences and strengthen generalizability. Infants harbor a much lower gut microbial diversity compared to adults. Any variation in the gut microbiota composition caused by storage may thus theoretically have a proportionally greater effect on the composition.[21] We chose a standardized sampling technique in order to avoid potential biases due to freezing of samples at different time points and temperature variation during transport to the laboratory.

Conclusions

We speculate that probiotic supplementation may induce colonization resistance and thereby partly alleviate harmful effects of antibiotics on gut microbiota composition and antibiotic resistome. The high relative abundance of *Bifidobacterium* in probiotic-supplemented extremely preterm infants at one week of age, suggests that a gradual increase in probiotic doses may be warranted.

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Contributor's Statement: Eirin Esaiassen organized all phases of the study, analyzed data, wrote the first version of the manuscript and revised the manuscript. Tanja Pedersen, Jannicke Andresen, Siren Rettedal, Ragnhild Støen and Britt Nakstad were responsible for inclusion of patients at participating centers, data retrieval and revised the manuscript. Erik Hjerde, Jorunn Pauline Cavanagh and Nils P Willassen took part in study design, were responsible for microbiological (JPC) and bioinformatics (EH, NPW) analyses and revised the manuscript. Claus Klingenberg conceptualized and designed the study, directed all phases of the study, and revised the final manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work. Eirin Esaiassen and Claus Klingenberg had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Groer MW, Luciano AA, Dishaw LJ, et al. Development of the preterm infant gut microbiome: a research priority. *Microbiome.* 2014;2:38.
2. Pammi M, Cope J, Tarr PI, et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome.* 2017;5(1):31.
3. AlFaleh K, Anabrees J. Probiotics for prevention of necrotizing enterocolitis in preterm infants. *Cochrane Database of Systematic Reviews.* 2014(4).
4. Aceti A, Gori D, Barone G, et al. Probiotics for prevention of necrotizing enterocolitis in preterm infants: systematic review and meta-analysis. *Ital J Pediatr.* 2015;41:89.
5. Sawh SC, Deshpande S, Jansen S, Reynaert CJ, Jones PM. Prevention of necrotizing enterocolitis with probiotics: a systematic review and meta-analysis. *PeerJ.* 2016;4:e2429.
6. Lau CS, Chamberlain RS. Probiotic administration can prevent necrotizing enterocolitis in preterm infants: A meta-analysis. *J Pediatr Surg.* 2015;50(8):1405-1412.
7. Olsen R, Greisen G, Schroder M, Brok J. Prophylactic Probiotics for Preterm Infants: A Systematic Review and Meta-Analysis of Observational Studies. *Neonatology.* 2016;109(2):105-112.
8. Costeloe K, Bowler U, Brocklehurst P, et al. A randomised controlled trial of the probiotic Bifidobacterium breve BBG-001 in preterm babies to prevent sepsis, necrotising enterocolitis and death: the Probiotics in Preterm infantS (PiPS) trial. *Health Technol Assess.* 2016;20(66):1-194.
9. Tarnow-Mordi W, Soll RF. Probiotic supplementation in preterm infants: it is time to change practice. *J Pediatr.* 2014;164(5):959-960.
10. Viswanathan S, Lau C, Akbari H, Hoyen C, Walsh MC. Survey and evidence based review of probiotics used in very low birth weight preterm infants within the United States. *J Perinatol.* 2016;36(12):1106-1111.
11. Esaiassen E, Cavanagh P, Hjerde E, et al. Bifidobacterium longum Subspecies infantis Bacteremia in 3 Extremely Preterm Infants Receiving Probiotics. *Emerg Infect Dis.* 2016;22(9):1664-1666.
12. Zbinden A, Zbinden R, Berger C, Arlettaz R. Case series of Bifidobacterium longum bacteremia in three preterm infants on probiotic therapy. *Neonatology.* 2015;107(1):56-59.
13. Kolacek S, Hojsak I, Berni Canani R, et al. Commercial Probiotic Products: A Call for Improved Quality Control. A Position Paper by the ESPGHAN Working Group for Probiotics and Prebiotics. *J Pediatr Gastroenterol Nutr.* 2017;65(1):117-124.
14. Vallabhaneni S, Walker TA, Lockhart SR, et al. Notes from the field: Fatal gastrointestinal mucormycosis in a premature infant associated with a contaminated dietary supplement--Connecticut, 2014. *MMWR Morb Mortal Wkly Rep.* 2015;64(6):155-156.

15. Frost BL, Modi BP, Jaksic T, Caplan MS. New Medical and Surgical Insights Into Neonatal Necrotizing Enterocolitis: A Review. *JAMA Pediatr.* 2017;171(1):83-88.
16. Hsieh EM, Hornik CP, Clark RH, et al. Medication use in the neonatal intensive care unit. *Am J Perinatol.* 2014;31(9):811-821.
17. Esaiassen E, Fjalstad JW, Juvet LK, van den Anker JN, Klingenberg C. Antibiotic exposure in neonates and early adverse outcomes: a systematic review and meta-analysis. *J Antimicrob Chemother.* 2017.
18. Cotten CM, Taylor S, Stoll B, et al. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. *Pediatrics.* 2009;123(1):58-66.
19. Gibson MK, Wang B, Ahmadi S, et al. Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nat Microbiol.* 2016;1:16024.
20. Klingenberg C. Preterm Infant Gut (PINGU) - a Norwegian Multi Centre Study (PINGU). https://clinicaltrials.gov/ct2/show/NCT02197468. Accessed October, 2017.
21. Hill CJ, Brown JR, Lynch DB, et al. Effect of room temperature transport vials on DNA quality and phylogenetic composition of faecal microbiota of elderly adults and infants. *Microbiome.* 2016;4(1):19.
22. Truong DT, Franzosa EA, Tickle TL, et al. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat Methods.* 2015;12(10):902-903.
23. McArthur AG, Waglechner N, Nizam F, et al. The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother.* 2013;57(7):3348-3357.
24. Kaminski J, Gibson MK, Franzosa EA, et al. High-Specificity Targeted Functional Profiling in Microbial Communities with ShortBRED. *PLoS Comput Biol.* 2015;11(12):e1004557.
25. Klingenberg C. *Manual of neonatology (Metodebok i Nyfødtmedisin).* 5 ed: Barne-og ungdomsavdelingen, Universitetsykehuset Nord-Norge; 2017.
26. Abdulkadir B, Nelson A, Skeath T, et al. Routine Use of Probiotics in Preterm Infants: Longitudinal Impact on the Microbiome and Metabolome. *Neonatology.* 2016;109(4):239-247.
27. Parks DH, Tyson GW, Hugenholtz P, Beiko RG. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics.* 2014;30(21):3123-3124.
28. Benjamini YH Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *The Journal of the Royal Statistical Society Series B.* 1995;57:289-300.
29. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. *Genome Res.* 2007;17(3):377-386.
30. Weng M, Walker WA. The role of gut microbiota in programming the immune phenotype. *J Dev Orig Health Dis.* 2013;4(3):203-214.
31. Hill CJ, Lynch DB, Murphy K, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome.* 2017;5(1):4.

32. Arboleya S, Binetti A, Salazar N, et al. Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiol Ecol.* 2012;79(3):763-772.
33. Stewart CJ, Embleton ND, Marrs EC, et al. Temporal bacterial and metabolic development of the preterm gut reveals specific signatures in health and disease. *Microbiome.* 2016;4(1):67.
34. Jacobs SE, Tobin JM, Opie GF, et al. Probiotic effects on late-onset sepsis in very preterm infants: a randomized controlled trial. *Pediatrics.* 2013;132(6):1055-1062.
35. Wang Y, Hoenig JD, Malin KJ, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *Isme j.* 2009;3(8):944-954.
36. Butel MJ, Suau A, Campeotto F, et al. Conditions of bifidobacterial colonization in preterm infants: a prospective analysis. *J Pediatr Gastroenterol Nutr.* 2007;44(5):577-582.
37. Swidsinski A, Loening-Baucke V, Lochs H, Hale LP. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. *World J Gastroenterol.* 2005;11(8):1131-1140.
38. Dutta S, Ray P, Narang A. Comparison of stool colonization in premature infants by three dose regimes of a probiotic combination: a randomized controlled trial. *Am J Perinatol.* 2015;32(8):733-740.
39. Costeloe K, Hardy P, Juszczak E, Wilks M, Millar MR. Bifidobacterium breve BBG-001 in very preterm infants: a randomised controlled phase 3 trial. *Lancet.* 2016;387(10019):649-660.
40. Gritz EC, Bhandari V. The human neonatal gut microbiome: a brief review. *Front Pediatr.* 2015;3:17.
41. Deshpande GC, Rao SC, Keil AD, Patole SK. Evidence-based guidelines for use of probiotics in preterm neonates. *BMC Med.* 2011;9:92.
42. Siggers RH, Siggers J, Thymann T, Boye M, Sangild PT. Nutritional modulation of the gut microbiota and immune system in preterm neonates susceptible to necrotizing enterocolitis. *J Nutr Biochem.* 2011;22(6):511-521.
43. Cilieborg MS, Boye M, Sangild PT. Bacterial colonization and gut development in preterm neonates. *Early Hum Dev.* 2012;88 Suppl 1:S41-49.
44. Hall MA, Cole CB, Smith SL, Fuller R, Rolles CJ. Factors influencing the presence of faecal lactobacilli in early infancy. *Archives of Disease in Childhood.* 1990;65(2):185-188.
45. Fouhy F, Guinane CM, Hussey S, et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob Agents Chemother.* 2012;56(11):5811-5820.
46. Rose G, Shaw AG, Sim K, et al. Antibiotic resistance potential of the healthy preterm infant gut microbiome. *PeerJ.* 2017;5:e2928.
47. Fouhy F, Ogilvie LA, Jones BV, et al. Identification of aminoglycoside and beta-lactam resistance genes from within an infant gut functional metagenomic library. *PLoS One.* 2014;9(9):e108016.

48. von Wintersdorff CJ, Wolffs PF, Savelkoul PH, et al. The gut resistome is highly dynamic during the first months of life. *Future Microbiol.* 2016;11(4):501-510.
49. Moore AM, Patel S, Forsberg KJ, et al. Pediatric fecal microbiota harbor diverse and novel antibiotic resistance genes. *PLoS One.* 2013;8(11):e78822.
50. Pamer EG. Resurrecting the intestinal microbiota to combat antibiotic-resistant pathogens. *Science.* 2016;352(6285):535-538.
51. Ravi A, Estensmo ELF, Abee-Lund TML, et al. Association of the gut microbiota mobilome with hospital location and birth weight in preterm infants. *Pediatr Res.* 2017.

Figure legends

Figure 1: Study flow diagram

Figure 2 a-c. Relative abundance of dominant taxa (> 0.5%) at genus level.

Figure 2a. Relative abundance at 7 days

Figure 2b. Relative abundance at 28 days

Figure 2c. Relative abundance at 4 months

Figure 3 a-d. Alpha diversity calculated by Shannon diversity index and beta diversity calculated by non-metrical multidimensional scaling (NMDS) based on a matrix of Bray-Curtis distances.

Figure 3a. Shannon diversity index of three groups of infants at three sampling points. *The inside bar represent median, the outer horizontal line of the box represents the 25th and the 75th percentile. Error bars represent the standard error. Differences between groups at a given time point and at different time points were tested with linear mixed model.*

Figure 3b. Beta diversity (NMDS) at 7 days

Figure 3c. Beta diversity (NMDS) at 28 days

Figure 3d. Beta diversity (NMDS) at 4 months

559 **Table 1.** Clinical background data
560

	Probiotic Extremely Preterm (PEP) Infants (n= 31)	**Non-Probiotic Very Preterm (NPVP) Infants (n=35)**	**Full Term Control (FTC) Infants (n=10)**
Birth weight, g, mean (SD)	825 (178)	1290 (220)	3651 (463)
Gestational age at birth, weeks, mean (SD)	26 (1)	29 (1)	40(1)
Gender; male/female	13/18	20/15	(3/7)
Route of delivery; Caesarean/vaginal	21/10	20/15	0/10
CRIB score, mean (SD)	11 (2)	5 (2)	-
Antenatal antibiotic exposure, *n*	8/31	12/35	0
Antibiotic exposure* first week of life, days, median (IQR), *n*	6 (4-7), 30	4 (3-5), 27	-
Antibiotic exposure after first week of life, days, median (IQR), *n*	6.5 (2.75-13), 22	10 (5.5-14), 5	
Ampicillin or Penicillin + Gentamicin after first week, median (IQR), *n*	6 (3-12), 16	9.5 (6-10), 4	-
Third-generation cephalosporin, median (IQR) ,*n*	7 (6-7), 7	6,1	-
Vancomycin, median (IQR) ,*n*	7 (7-14), 7	4 (4.5-4.5), 2	-
Meropenem, median (IQR), *n*	1	13, 1	-
Total days of antibiotic exposure, median (IQR), *n*	9.5 (6-18), 30	4 (3-6), 27	-
Probiotic supplementation, days, median (IQR)	46 (40-57)	-	-
Parenteral nutrition, days, median (IQR), *n*	9 (6-13), 31	5 (3.25-8), 16	-
Exclusive human milk nutrition until discharge	17/31	16/35	

561 * Only ampicillin or penicillin + gentamicin in first week of life

562

Table 2. Median abundance of antibiotic resistance genes among infants in each group

Antibiotic resistance genes encoding	**7 days (n=60 samples)**					**28 days (n=64 samples)**					**4 months (n=60 samples)**				
	PEP (n=20)	**NPVP (n=30)**	**FTC (n=10)**	**P**	**FDR P**	**PEP (n=24)**	**NPVP (n=31)**	**FTC (n=9)**	**P**	**FDR P**	**PEP (n=24)**	**NPVP (n=29)**	**FTC (n=7)**	**P**	***FDR P***
Class A Beta lactamase	0.61	4.2*	0.00*	**0.001**	**0.020**	0.00	0.00	0.00	0.080	0.586	1.43	1.0	0.00	0.443	1.327
Class C Beta lactamase	0.00	0.00	0.20	0.126	0.229	0.98	0.22	0.00	0.492	0.812	9.1	12.7	9.5	0.605	1.134
Aminoglycoside acetyltransferase	0.00	0.00	0.00	0.202	0.311	-	-	-	-	-	-	-	-	-	-
Aminoglycoside phosphotransferase	0.00	0.00	0.00	0.590	0.653	0.00	0.16	0.00	0.114	0.497	-	-	-	-	-
Aminoglycoside nucleotidyltransferase	0.00	0.00	0.00	0.765	0.765	0.00	0.00	0.00	0.296	0.426	0.00	0.00	0.00	0.584	0.814
Tetracycline efflux	0.00	0.00*	0.00	**0.015**	**0.050**	0.00	0.00	0.00	0.173	0.423	0.00	0.00	0.00	0.174	1.949
Tetracycline ribosomal protection	0.00	0.26	4.4*	**0.047**	0.118	0.52	3.7	1.77	0.397	0.615	6.4	23.4	23.4	0.407	1.041
Quinolone resistance†	9.0	21.6	5.3	0.062	0.138	9.81	7.6	0.77	0.133	0.470	9.2	9.4	7.1	0.501	1.186
Macrolide/MLS resistance	0.00	0.00	0.00	0.757	0.797	-	-	-	-	-	-	-	-	-	-
ABC efflux pump†	0.13	1.15	0.25	0.206	0.294	1.06	1.35	0.06*	0.013	0.414	0.70	0.96	0.83	0.766	0.887
RND antibiotic efflux	5.2	41.9*	38.4	**0.034**	**0.097**	37.7	53.7	4.1	0.170	0.683	94.0	116.7	90.3	0.674	0.936
MFS antibiotic efflux	1.16	113.3	29.0	0.339	0.342	85.8	119.1	16.0	0.056	0.489	105.2	119.5	84.7	0.614	0.839
Multidrug efflux pump activity	0.00	24.6	1.92	0.337	0.449	20.9	21.7	4.9	0.346	0.478	10.0	14.0	8.1	0.616	1.552
Multidrug resistance efflux pump	0.00	0.00	0.00	0.668	0.742	0.00	0.00	0.00	0.603	0.678	0.18	0.00	0.60	0.496	0.819
Gene modulating antibiotic efflux	5.6	41.0**	0.76	**0.012**	**0.060**	14.7	20.1	0.34	0.163	0.376	19.7	27.7	27.5	0.645	0.871
SMR antibiotic efflux	-	1.2	-	-	-	0.00	0.00	0.00	0.914	0.932	-	-	-	-	-
Chloramphenicol acetyltransferase	0.00	0.00	0.00	**0.071**	0.142	-	-	-	-	-	-	-	-	-	-
Antibiotic target†	0.48	0.00	0.00**	**0.013**	**0.052**	0.00	0.00	0.00	0.266	0.396	0.00	0.00	0.00	0.720	0.768
Gene modulating resistance	53.5	8.1**	39.2	**0.003**	**0.030**	37.6	27.8	44.6	0.419	0.419	37.5	45.8	46.2	0.678	1.286
rRNA methyltransferase†	0.00	10.6	10.6	0.128	0.213	6.0	8.8	1.72	0.008	0.464	4.1	5.4	4.4	0.665	0.887
Other ARG†	5.3	16.7**	2.02	**0.011**	**0.073**	7.3	8.4	0.26	0.132	0.413	7.2	10.5	6.3	0.613	

Numbers are presented as median total reads normalized by the total number of reads in each sample.
Antibiotic resistance genes analyzed using ShortBRED.
PEP, probiotic extremely preterm infants; NPVP, non-probiotic very preterm infants; FTC, full-term control; FDR, false discovery rate
Comparisons between all three treatment groups by nonparametric Kruskal-Wallis test
Post hoc comparisons by non-parametric Mann Whitney U-test (versus PEP) (***P<0.001, ** P<0.01, * P<0.05)
Comparison between different time points by generalized linear model with a Poisson family (†P<0.05)
Genes modulating antibiotic efflux: *norA, baeR, marA, phoQ, ramA, soxR.* Genes modulating resistance: *WblE, WhiB.* Other ARG: *bacA*

Fig. 1

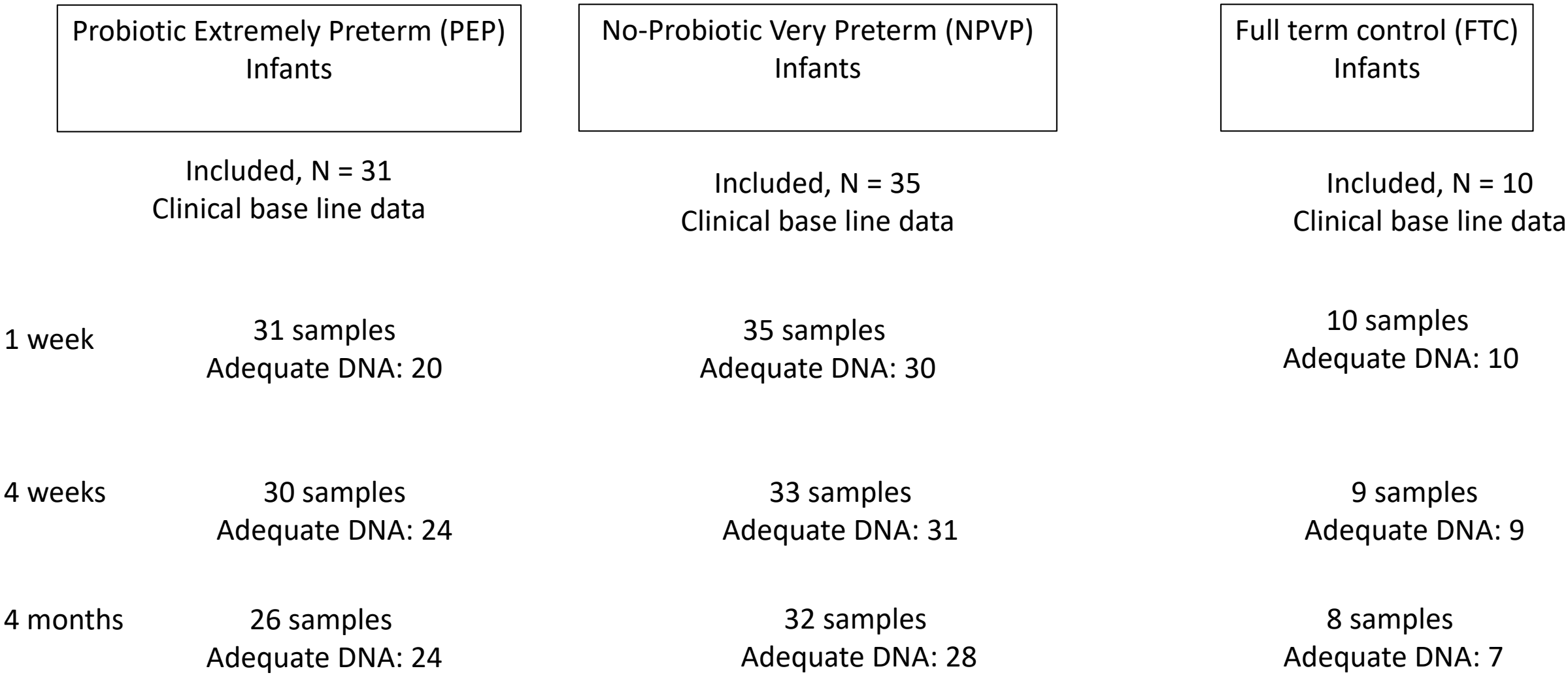

Probiotic Extremely Preterm (PEP) Infants
No-Probiotic Very Preterm (NPVP) Infants
Full term control (FTC) Infants
Included, N = 31
Clinical base line data
Included, N = 35
Clinical base line data
Included, N = 10
Clinical base line data
1 week
31 samples
Adequate DNA: 20
35 samples
Adequate DNA: 30
10 samples
Adequate DNA: 10
4 weeks
30 samples
Adequate DNA: 24
33 samples
Adequate DNA: 31
9 samples
Adequate DNA: 9
4 months
26 samples
Adequate DNA: 24
32 samples
Adequate DNA: 28
8 samples
Adequate DNA: 7

Fig. 2a

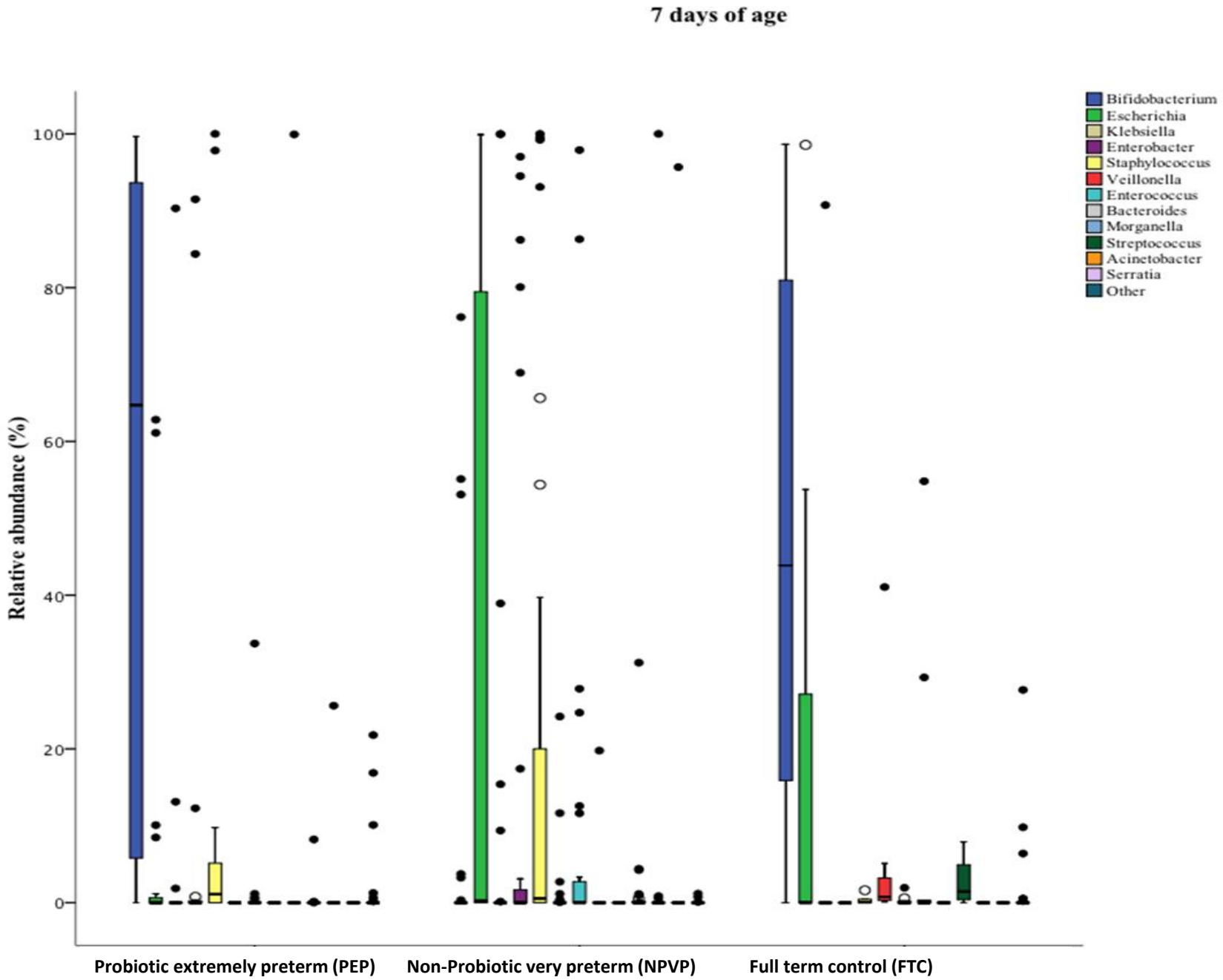

7 days of age
Relative abundance (%)
100
80
60
40
20
0
Bifidobacterium
Escherichia
Klebsiella
Enterobacter
Staphylococcus
Veillonella
Enterococcus
Bacteroides
Morganella
Streptococcus
Acinetobacter
Serratia
Other
Probiotic extremely preterm (PEP)
Non-Probiotic very preterm (NPVP)
Full term control (FTC)

Fig. 2b

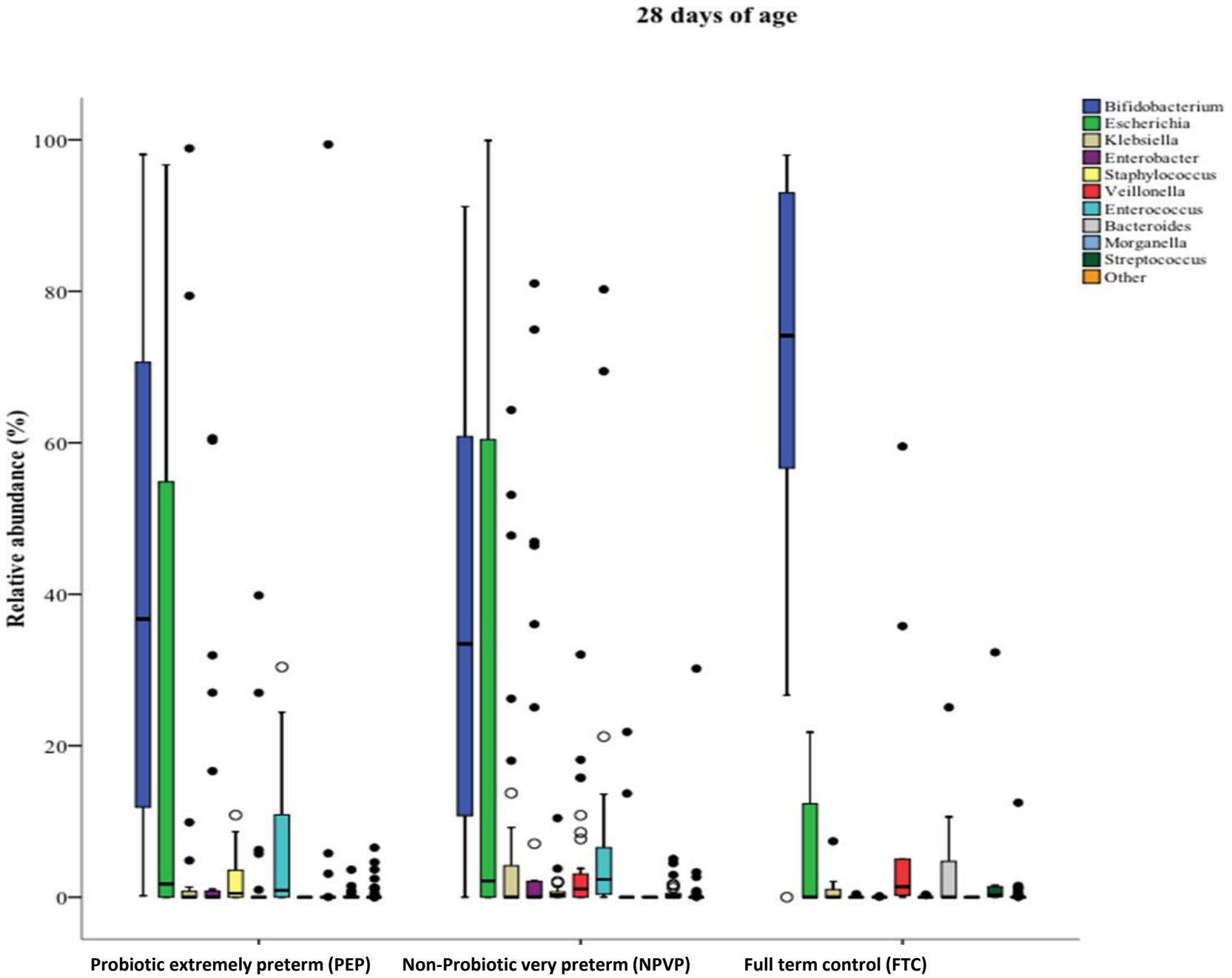

28 days of age
Relative abundance (%)
100
80
60
40
20
0
Bifidobacterium
Escherichia
Klebsiella
Enterobacter
Staphylococcus
Veillonella
Enterococcus
Bacteroides
Morganella
Streptococcus
Other
Probiotic extremely preterm (PEP)
Non-Probiotic very preterm (NPVP)
Full term control (FTC)

Fig. 2c

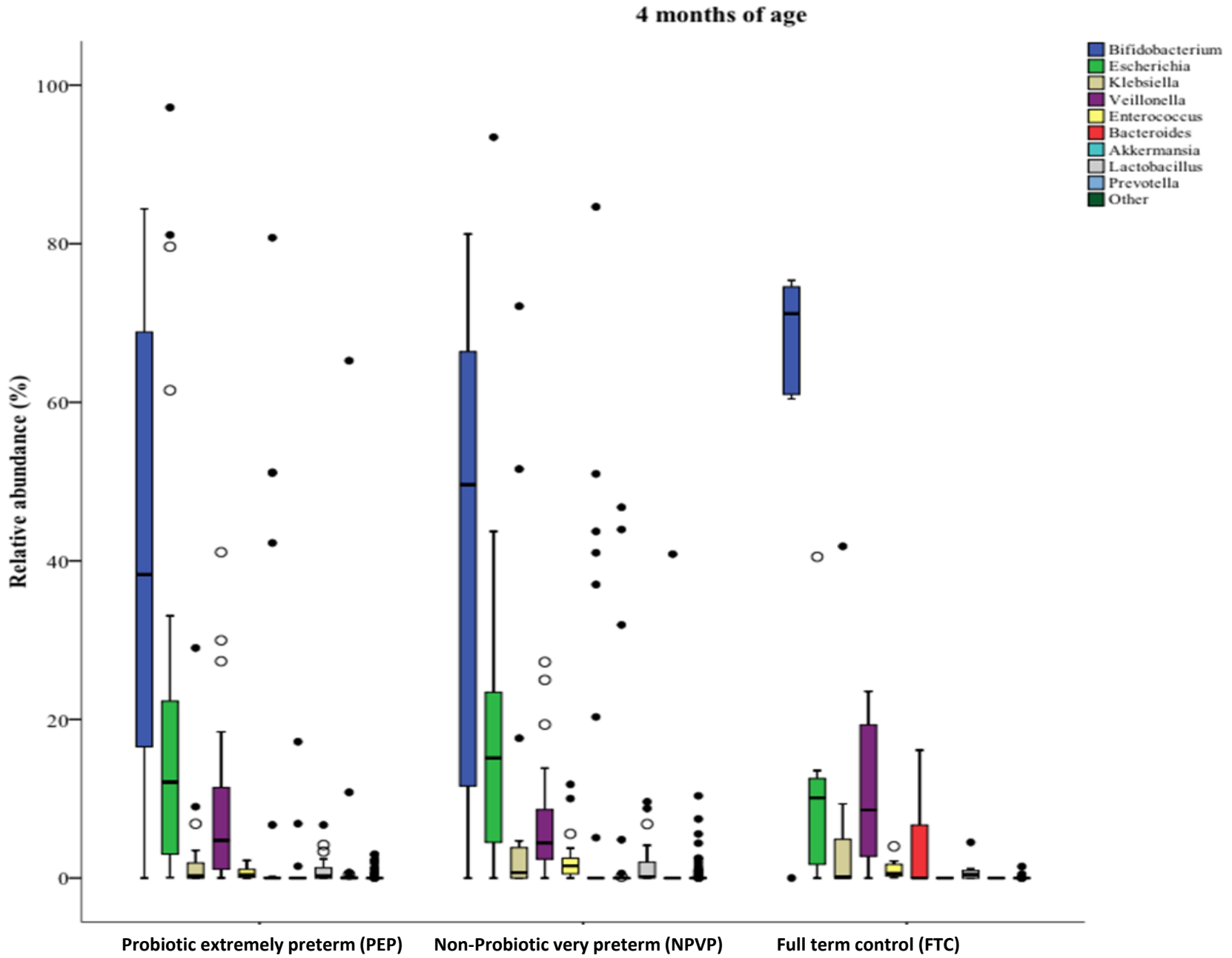

4 months of age
Bifidobacterium
Escherichia
Klebsiella
Veillonella
Enterococcus
Bacteroides
Akkermansia
Lactobacillus
Prevotella
Other
Relative abundance (%)
100
80
60
40
20
0
Probiotic extremely preterm (PEP)
Non-Probiotic very preterm (NPVP)
Full term control (FTC)

Fig. 3a

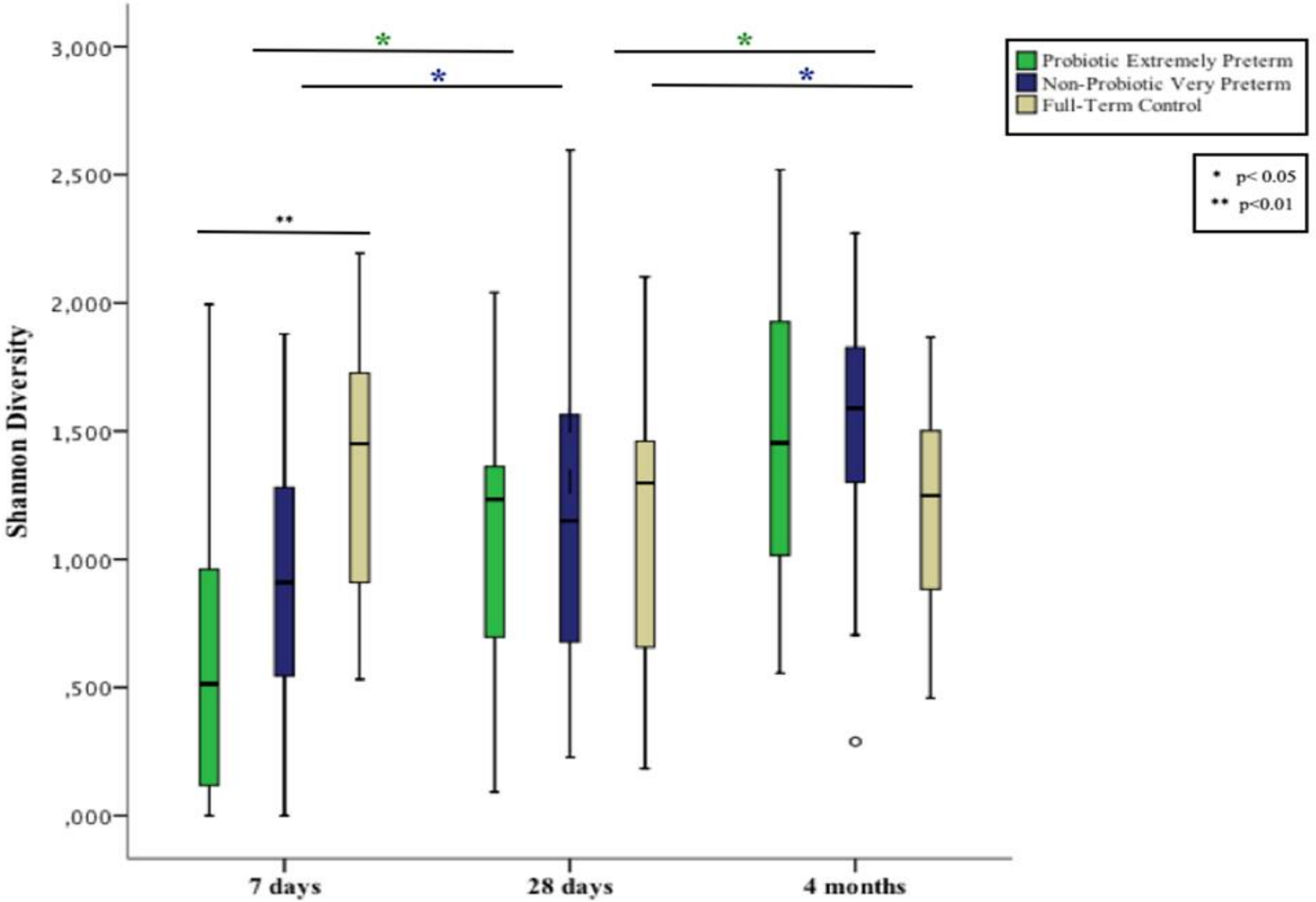

Probiotic Extremely Preterm
Non-Probiotic Very Preterm
Full-Term Control
* p< 0.05
** p<0.01
Shannon Diversity
3,000
2,500
2,000
1,500
1,000
,500
,000
7 days
28 days
4 months

Fig. 3b

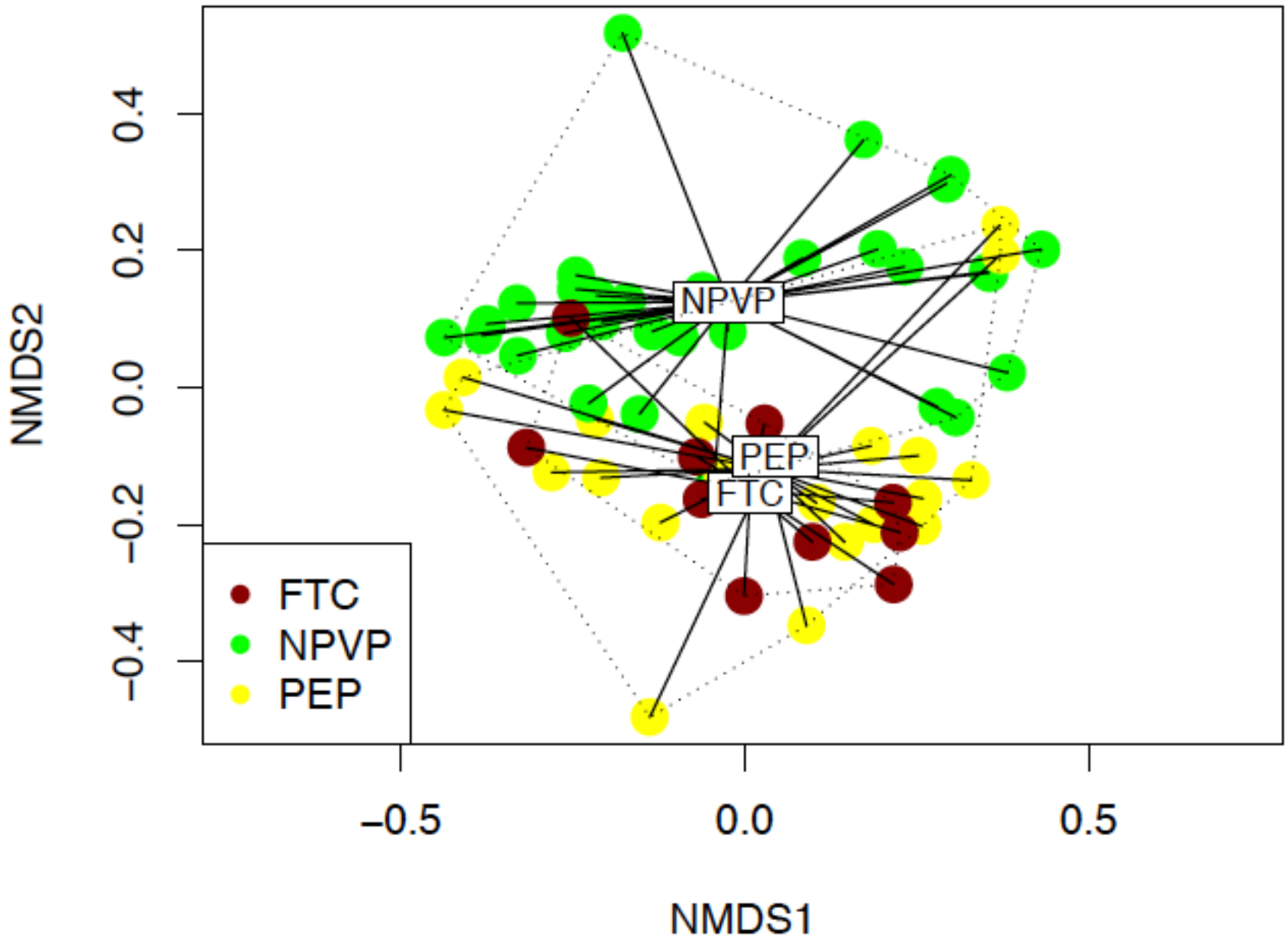


Fig. 3c

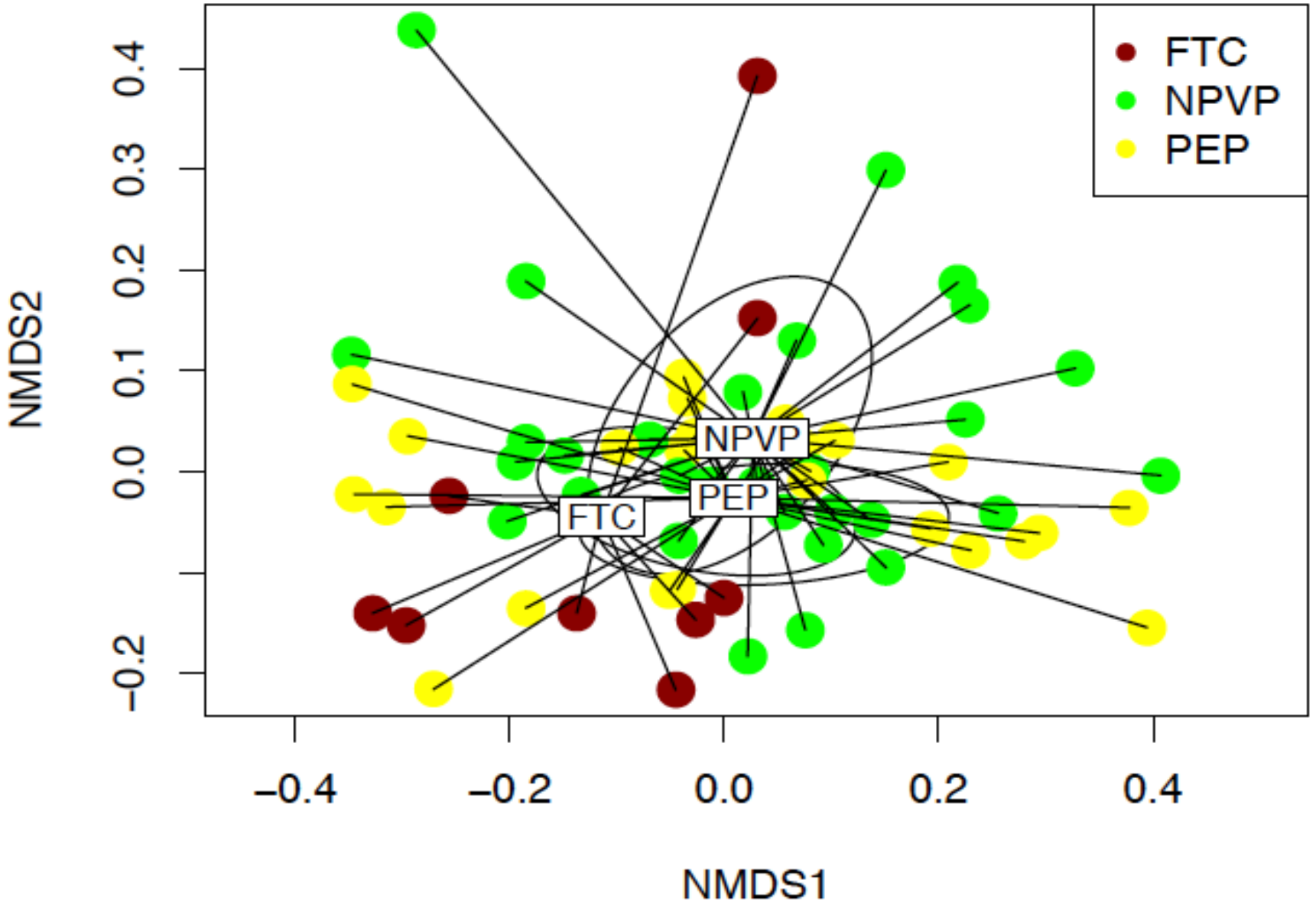
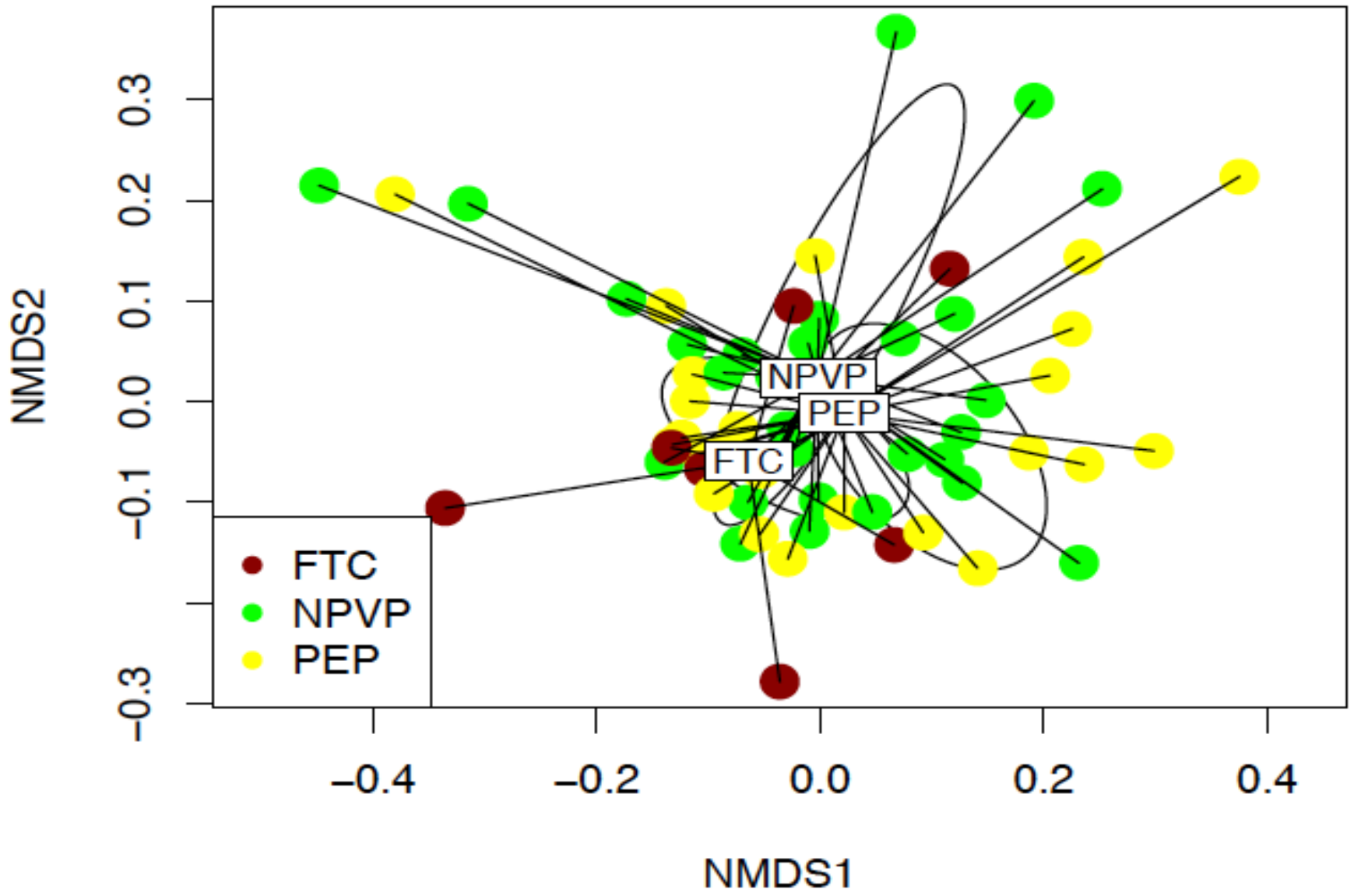

FTC
NPVP
PEP
NPVP
PEP
FTC
NMDS2
NMDS1
0.4
0.3
0.2
0.1
0.0
−0.2
−0.4
−0.2
0.0
0.2
0.4

Fig. 3d


NMDS2
0.3
0.2
0.1
0.0
−0.1
−0.3
−0.4
−0.2
0.0
0.2
0.4
NMDS1
NPVP
PEP
FTC
FTC
NPVP
PEP

Online-Only Supplements

Probiotic Supplementation and Development of Preterm Infant Gut Microbiota and Antibiotic Resistome
An Observational Multi-Center Study

Esaiassen E et al.

eMethods

Sampling procedure

We performed a pilot test where we compared the commercial fecal sampling kit (OMNIgen GUT kit, DNA Genotek, Ottawa, Canada) with a standard fecal sampling procedure using sterile Eppendorf tubes which were frozen at -70 C° immediately after fecal collection. We measured the quality of extracted DNA and the taxonomic composition after sequencing with paired samples obtained with both sampling methods. To further assess the preservative ability of the stabilization buffer we arranged a cocktail of different bacterial species and evaluated the microbial composition after various times of storage. The bacterial composition in the cocktail was based on a representative selection of Gram positive and Gram negative bacteria commonly found in the human gut microbiota of infants. Samples were analysed by metagenome sequencing using the Illumina sequencer (Miseq, Illumina Inc). Results showed that both sampling procedures displayed good concordance. Furthermore, the microbial composition was independent of the length of sample storage. Ease of use and the possibility of storage at ambient temperature for 7-14 days offered an important solution to logistical issues in our trial. Samples were transported to the laboratory for DNA extraction which was carried out preferentially within one week. Recently, two studies reported similar beneficial characteristics of the same sample kit as used in our study.[1,2]

DNA extraction, library preparation, sequencing and assembly

Total metagenomic DNA was extracted using the NorDiag Arrow Stool DNA Extraction kit (NorDiag, Oslo, Norway). An extra beadbeating step was added to facilitate cell lysis as studies have shown that this can increase extraction of DNA from Gram positive bacteria.[3] DNA was quantified using the Nanodrop 1000 and Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) along with the Qubit® dsDNA HR assay kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA was then stored at -70°C.

The indexed paired-end libraries were prepared for whol genome sequencing using the Nextera XT Kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions.[4] Fifty nanogram genomic DNA was tagmented at 55°C for 10 min. The tagmented DNA was amplified with two primers from Nextera DNA sample preparation Index Kit. PCR products were cleaned using Agencourt AMPure XP beads (Beckman Coulter, Indiana, USA). Purified PCR products were quantified using the Qubit® 2.0 (Invitrogen, Carlsbad, CA, USA), along with the Qubit® dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The fragment size distribution (500-1000 bp) was analyzed using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Waldbronn, Germany). The samples were pooled at concentration of 4nM per sample. Eight to twelve samples were pooled per each sequencing run. Pooled samples was denatured with 0.2N NaOH, then diluted to 10pM with hybridization buffer. Subsequently, samples were submitted for v3 reagents with 2 × 300 cycles paired-end sequencing using the Illumina Miseq platform, according to the manufacturer's instructions. In total, 184 samples were sequenced to an average (range) sequence depth of 4.8 (1.8-12.6) million reads per sample for microbiota and functional analysis. Prior to all downstream data analysis the sequence quality was calculated using FastQC (v0.11.3)[5]. All samples were screened for human contamination using Deconseq[6] with default parameters and build up 38 of the human genome as reference. Quality filtering of the read was performed using Trimmomatic v0.36 [7] with LEADING:3, TRAILING:3, MINLEN:75 as parameter settings. Assemblies were performed on the trimmed reads using MEGAHIT.[8] Functional annotation was added using an in-house genome annotation pipeline, the META-pipe (Department of Chemistry, University of Tromsø, Norway [https://arxiv.org/abs/1604.04103]).

Calculating the relative abundance of species from shotgun-metagenomic sequencing

The relative abundance of species was calculated from the trimmed reads using MetaPhlAn 2.0.[9] Relative abundance tables for each individual sample were merged. From the total samples, all genera with a lower average relative abundance than 0.5% were omitted from further analysis. To calculate longitudinal changes, sequences were reconstructed using the Lowest Common Ancestor (LCA) classifier.[10]

Calculating the relative abundance classes of antibiotic resistance genes (ARGs) and absolute reads of antibiotic resistance genes (ARGs) from shotgun-metagenomic sequencin

The prediction of genes presumed to confer antibiotic resistance was performed on the assembled metagenomes using Abricate [https://github.com/tseemann/abricate] against the resistance gene identifier in the Comprehensive Antibiotic Resistance Database (CARD; version 1.1.1; Department of Biochemistry and Biomedical Science, McMaster University, Canada [https://card.mcmaster.ca/home])[11] with the minimum identity threshold set to 75%. Because of the fragmented nature of the metagenome assemblies, and therefore presence of fragmented genes, multiple hits against the same antibiotic resistance gene were regarded as one hit. For all samples, this yielded a presence/absence table (eTable).

Classes of antibiotic resistance genes in the CARD database and the specific genes included in each class are listed below

- Beta lactamase: *blaMIR, blaZ, blaACT, blaTEM, blaCMY, blaLEN, blaADC, blaACI, blaOXA, blaOXY, blaSHV, blaDHA, blaOKP, blaACC, blaSED, blaMOR, blaCMG, blaCFE, cfiA, cepA, cfxA*
- Methicillin resistance*: mecA*
- Aminoglycosides: *aac(6´)-aph(2), aac(6´)-Ic, aac(6´)-Im, aadA, aadB, aadD, aadE, ant(6)-Ia, aph(2)-Ib, aph(3)-Ia, aph(3)-III, spc, str, strA ,strB*
- Tetracyclines: *tet(A), tet(B), tet(M), tet(K), tet(X), tet(O), tet(L), tet(U), tet(Q), tet(W), tet(S), tet(32), tet(34), tet(35), tet(37), tet(40), tet(41), Otr(A)*
- Fluoroquinolones: *QnrB, QnrD*
- MLS; Macrolide: *erm(A), erm(B), erm(C), erm(F),erm(G), erm(T), erm(X), mph(A), mph(C);* Lincosamide: *lnu(B), lnu(C*); Streptogranin: *vat(B), vat(F)*
- ABC efflux: *lsa(A),lsa(B), lsa(C), msr(A), mrs(C), msr(D), ole(B), car(A)*

- RND efflux pumps: *oqxA*
- Efflux pumps: *vga(A), mef(A)*
- Multidrug efflux pumps: *norA*
- Chloramphenicol: *cat, catA, catB, catS, cmlA, cml*
- Fosfomycin*: fos(A)*
- Sulfonamides: *sul1, sul2*
- Antibiotic target: *dfrA, dfrG*
- Vancomycin: *VanC, VanS, VanT, VanR, VanY*
- Metronidazole: *nimB*

In order to obtain quantitative measures of the potential ARGs in each sample, the quality trimmed reads were analysed using Short, Better Representative Extract Dataset (ShortBRED)[12] against a formatted CARD database. ARGs with a total number of reads less than ten across all samples were omitted from further analysis. The identified absolute reads against ARGs were used for further analysis. Using (ShortBRED we identified the antibiotic resistance gene classes and genes listed below:

- Class A Beta lactamase
- Class C Beta lactamase
- Aminoglycoside acetyltransferase
- Aminoglycoside phosphotransferase
- Aminoglycoside nucleotidyltransferase
- Tetracycline efflux
- Tetracycline ribosomal protection

- Quinolone resistance
- Macrolide/MLS resistance
- Adenosine triphosphate (ATP)-binding cassette (ABC) efflux pump
- Resistance/nodulation/division (RND) antibiotic efflux
- Major facilitator superfamily (MFS) antibiotic efflux
- Multidrug efflux pump activity
- Multidrug resistance efflux pump
- Genes modulating antibiotic efflux: *norA, baeR, marA, phoQ, ramA, soxR*
- Small multidrug resistance (SMR) antibiotic efflux
- Chloramphenicol acetyltransferase
- Antibiotic target
- Genes modulating resistance: *WblE, WhiB*
- rRNA methyltransferase
- Other ARG: *bacA*

Antibiotic therapy; broad- versus narrow-spectrum regimen

We defined regimens including third-generation cephalosporins or carbapenems as a broad-spectrum regimens when compared to regimens containing aminoglycosides for coverage against Gram-negative bacteria. This definition was based on previous reports indicating that empiric therapy containing a third-generation cephalosporin for Gram-negative coverage induces significantly more resistance than a regimen containing an aminoglycoside.[13]

eReferences

1. Hill CJ, Brown JR, Lynch DB, et al. Effect of room temperature transport vials on DNA quality and phylogenetic composition of faecal microbiota of elderly adults and infants. *Microbiome.* 2016;4(1):19. doi: 10.1186/s40168-016-0164-3.

2. Choo JM, Leong LE, Rogers GB. Sample storage conditions significantly influence faecal microbiome profiles. *Sci Rep.* 2015;5:16350. doi: 10.1038/srep16350.

3. Knudsen BE, Bergmark L, Munk P, et al. Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. *mSystems.* 2016;1(5). doi: 10.1128/mSystems.00095-16.

4. Illumina. *Nextera® DNA Library Prep Reference Guide.* USA: Illumina; 2016.

5. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010; https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

6. Schmieder R, Edwards R. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. *PLoS One.* 2011;6(3):e17288. doi: 10.1371/journal.pone.0017288.

7. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114-2120. doi: 10.1093/bioinformatics/btu170.

8. Li D, Luo R, Liu CM, et al. MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices. *Methods.* 2016;102:3-11. doi: 10.1016/j.ymeth.2016.02.020.

9. Truong DT, Franzosa EA, Tickle TL, et al. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat Methods.* 2015;12(10):902-903. doi: 10.1038/nmeth.3589.

10. Lanzen A, Jorgensen SL, Huson DH, et al. CREST--classification resources for environmental sequence tags. *PLoS One.* 2012;7(11):e49334. doi: 10.1371/journal.pone.0049334.

11. McArthur AG, Waglechner N, Nizam F, et al. The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother.* 2013;57(7):3348-3357. doi: 10.1128/aac.00419-13.

12. Kaminski J, Gibson MK, Franzosa EA, Segata N, Dantas G, Huttenhower C. High-Specificity Targeted Functional Profiling in Microbial Communities with ShortBRED. *PLoS Comput Biol.* 2015;11(12):e1004557. doi: 10.1371/journal.pcbi.1004557.

13. de Man P, Verhoeven BA, Verbrugh HA, Vos MC, van den Anker JN. An antibiotic policy to prevent emergence of resistant bacilli. *Lancet.* 2000;355(9208):973-978.

Online-Only Tables

eTable 1. Median relative abundance (%) of dominant genera in infant gut microbiota at 7 days, 28 days and 4 months of age

	7 days (n=60 samples)					28 days (n=64 samples)					4 months (n=60 samples)				
Genus	**PEP (n=20)**	**NPVP (n=30)**	**FTC (n=10)**	**P**	**FDR P**	**PEP (n=24)**	**NPVP (n=31)**	**FTC (n=9)**	**p-value**	**FDR P**	**PEP (n=24)**	**NPVP (n=29)**	**FTC (n=7)**	**p-value**	**FDR *P***
Bifidobacterium	64.7	0.00***	43.9	**<.001**	**<.001**	36.7	33.5	74.1	0.088	0.156	38.3	49.6	71.2	0.243	0.555
Escherichia	0.00	0.27	0.02	0.107	0.245	1.76	2.10	0.00	0.351	0.511	12.1	15.2	10.10	0.377	0.754
Klebsiella	0.00	0.00	0.00	0.737	0.786	0.00	0.00	0.00	0.663	0.816	0.25	0.67	0.11	0.738	1.0
Enterobacter	0.00	0.00	0.00	0.125	0.222	0.00	0.00	0.00	0.225	0.360	0.00	0.00	0.00	0.110	0.440
Staphylococcus †	1.10	0.54	0.05	0.230	0.368	0.51	0.23	0.01*	**0.038**	**0.076**	0.00	0.00	0.00	0.472	0.839
Veilonella †	0.00	0.00*	0.75***	**<.001**	**<.001**	0.00	1.09*	1.38*	**0.018**	**0.072**	4.75	4.44	8.59	0.812	1.0
Enterococcus †	0.00	0.01	0.00	0.118	0.236	0.90	2.35	0.00*	**0.003**	**0.016**	0.39	1.53**	0.58	0.019	0.152
Bacteroides †	0.00	0.00	0.00	**0.005**	**0.013**	0.00	0.00	0.00	**0.001**	**0.008**	0.00	0.00	0.00	0.996	1.0
Morganella	0.00	0.00	0.00	0.368	0.535	0.00	0.00*	0.00	**0.030**	**0.069**	0.00	0.00	0.00	0.098	0.523
Streptococcus	0.00	0.00	1.45***	**<.001**	**<.001**	0.00	0.06*	0.26*	**0.018**	**0.058**	0.15	0.14	0.06	0.149	0.477
Akkermansia	0.00	0.00	0.00	1.0	1.0	0.00	0.00	0.00	1.00	1.0	0.00	0.00	0.00	0.171	0.456
Lactobacillus	0.00	0.00*	0.23	**0.004**	**0.013**	0.00	0.00	0.23	**0.019**	**0.051**	0.26	0.18	0.42	0.682	1.0
Prevotella †	0.00	0.00	0.00	0.716	0.818	0.00	0.00	0.00	0.435	0.580	0.00	0.00**	0.00	**0.001**	0.016
Acinetobacter	0.00	0.00	0.00	0.525	0.70	0.00	0.00	0.00	0.834	0.953	0.00	0.00	0.00	1.000	1.0
Haemophilus	0.00	0.00	0.14*	**<.001**	**<.001**	0.00	0.00	0.07**	**<0.001**	**<0.001**	0.00	0.00	0.00	0.996	1.0
Serratia	0.00	0.00	0.00	0.607	0.747	0.00	0.00	0.00	0.834	0.890	0.00	0.00	0.00	1.000	1.0

PEP, probiotic extremely preterm; NPVP, non-probiotic very preterm; FTC, full term control; FDR, false discovery rate

Dominant taxa have an overall median relative abundance > 0.5 % at 7 days, 28 days and 4 months of age.

Overall comparison by all three treatment groups by non-parametric Kruskal-Wallis test

Post hoc comparisons by non-parametric Mann Whitney U-test (NPVP or FTC versus PEP) (****P*<0.001, ** *P*<0.01, * *P*<0.05).

†Comparison between different time points by generalized linear model with a Poisson family (†*P*<0.05)

eTable 2. Influence of antibiotic exposure (broad versus narrow after first week of life*) on taxonomic composition in all preterm infants

Bacterial genera	Microbiota at 28 days Median relative abundance			Microbiota at 4 months Median relative abundance			
	Broad (*n*=7)	**Narrow (*n*=15)**	***P***	**Broad (*n*=9)**	**Narrow (*n*=13)**	***P***	***P* FDR**
Bifidobacterium	14.4	28.9	0.783	14.3	41.5	0.096	0.512
Escherichia	44.5	1.40	0.368	17.4	9.9	0.209	0.669
Klebsiella	0.00	0.00	0.680	0.25	0.57	0.845	0.623
Enterobacter	0.00	0.45	0.123	0.00	0.00	0.235	0.627
Staphylococcus	0.42	0.08	0.783	0.00	0.00	1.00	1.00
Veilonella	0.00	0.00	0.945	1.25	6.01	**0.001**	**0.016**
Enterococcus	2.73	0.68	0.783	0.64	0.39	0.647	1.00
Streptococcus	0.00	0.00	0.630	0.07	0.18	0.126	0.504
Lactobacillus	0.00	0.00	0.891	0.00	0.87	0.071	0.568

Median relative abundance of *Bacteroides*, *Morganella*, *Akkermansia*, *Prevotella*, *Acinetobacter*, *Haemophilus* and *Serratia* were < 0.001 at 28 days and four months of age and there were no statistical difference between groups.
Bold indicate significant difference between broad and narrow antibiotic exposure.
PEP, probiotic preterm; NPVP, non-probiotic preterm;
*We defined regimens including third-generation cephalosporins or carbapenems as a broad-spectrum regimen
FDR, false discovery rate

eTable 3. Influence of antibiotic exposure (broad versus narrow narrow after first week of life*) on taxonomic composition in probiotic supplemented extremely preterm infants

Bacterial genera	Microbiota at 28 days Median relative abundance			Microbiota at 4 months Median relative abundance			
	Broad (*n*=5)	**Narrow (*n*=12)**	***P***	**Broad (*n*=7)**	**Narrow (*n*=11)**	***P***	***P* FDR**
Bifidobacterium	14.39	32.50	0.574	14.31	45.96	**0.035**	0.187
Escherichia	44.54	0.69	0.160	33.06	9.88	0.179	0.477
Klebsiella	0.00	0.00	0.721	0.26	0.57	1.000	1.00
Enterobacter	0.00	0.52	0.195	0.00	0.00	0.143	0.572
Staphylococcus	0.42	0.36	0.879	0.00	0.00	1.000	1.000
Veilonella	0.00	0.00	0.506	0.96	6.01	**0.004**	**0.064**
Enterococcus	2.73	0.15	0.506	0.33	0.40	0.536	0.858
Streptococcus	0.54	0.00	0.442	0.07	0.14	0.285	0.651
Lactobacillus	0.00	0.00	0.959	0.00	1.21	**0.004**	**0.032**

Median relative abundance of *Bacteroides*, *Morganella*, *Akkermansia*, *Prevotella*, *Acinetobacter*, *Haemophilus* and *Serratia* were < 0.001 at 28 days and four months of age and there were no statistical difference between groups.
*We defined regimens including third-generation cephalosporins or carbapenems as a broad-spectrum regimen
FDR, false discovery rate

eTable 4. Distribution of classes of antibiotic resistance gene among infants in each group

Antibiotic group or resistance mechanisms*	7 days			28 days			4 months		
	PEP *n*=20	NPVP *n*=30	FTC *n*=10	PEP *n*=24	NPVP *n*=31	FTC *n*=9	PEP *n*=24	NPVP *n*=29	FTC *n*=7
Beta lactamases	10/20	24/30	3/10	19/24	22/31	6/9	18/24	25/29	4/7
MecA gene	9/20	11/30	-	5/24	5/31	-	-	-	-
Aminoglycoside	8/20	14/30	3/10	11/24	16/31	2/9	12/24	16/29	2/7
Tetracycline	9/20	22/30	8/10	17/24	30/31	9/9	23/24	29/29	7/7
Fluoroquinolones	-	1/30	-	1/24	-	-	3/24	4/29	-
Macrolides	7/20	5/30	2/10	6/24	2/31	-	2/24	-	-
MLS	3/20	9/30	3/10	4/24	11/31	3/9	8/24	15/29	4/7
ABC efflux pumps	6/20	7/30	-	16/24	24/31	4/9	17/24	23/29	7/7
RND efflux pumps	7/20	12/30	2/10	12/24	18/24	4/9	12/24	19/24	5/7
Efflux pumps	3/20	3/30	8/10	2/24	4/31	2/9	6/24	8/24	3/7
Multidrug Efflux pump	9/20	14/30	1/10	11/24	7/31	1/9	-	-	-
Chloramphenicol	3/30	9/30	-	6/24	7/31	-	9/24	3/29	-
Fosfomycine	18/20	21/30	3/10	22/24	25/31	5/9	20/24	27/29	4/7
Sulfonamides	2/20	3/30	-	6/24	7/31	-	10/24	9/29	2/7
Antibiotic target	1/20	1/30	-	4/24	4/31	-	6/24	3/29	3/7
Antibiotic inactivation	-	2/30	1/10	1/24	1/31	-	6/24	7/29	2/7
Vancomycin	-	-	-	-	-	-	5/24	8/29	3/7
Metronidazole	-	-	-	-	-	-	-	1/29	-

PEP, probiotic extremely preterm; NPVP, non-probiotic very preterm; FTC, full term control;

*See eMethods for further explanation of which antibiotic resistance genes that are included in these groups

eTable 5. Influence of antibiotic exposure (broad versus narrow after first week of life) on abundance of antibiotic resistance genes (ARGs) in all preterm infants

Antibiotic resistance gene (ARG) classes*	ARGs at 28 days Absolute counts/total abundance				ARGs at 4 months Total abundance			
	Broad (*n*=7)	**Narrow (*n*=15)**	***P***	***P* FDR**	**Broad (*n*=9)**	**Narrow (*n*=13)**	***P***	***P* FDR**
Class A Beta Lactamase	0.00	0.00	0.447	0.731	5.00	3.01	0.324	0.864
Class C Beta Lactamase	44.96	0.00	**0.021**	**0.095**	9.11	8.16	0.235	0.752
Aminoglycoside phosphotransferase	6.14	0.00	0.078	0.281	-	-	-	-
Aminoglycoside nucleotidyltransferase	0.93	0.00	**0.008**	**0.072**	0.00	0.00	0.794	0.851
Tetracycline efflux	52.29	0.00	**0.014**	**0.084**	7.92	0.00	0.235	0.94
Tetracycline ribosomal protection	5.97	0.00	0.210	0.540	11.68	2.17	0.393	0.886
Quinolone Resistance	29.75	9.43	0.298	0.671	9.40	8.34	0.357	0.816
ABC efflux pump	3.23	1.07	0.392	0.784	0.70	0.64	0.471	0.814
RND antibiotic efflux	312.10	37.73	0.875	0.875	94.00	84.96	0.393	0.63
MFS antibiotic efflux	272.36	117.02	0.490	0.68	119.50	107.51	0.404	0.59
Multidrug efflux pump activity	22.08	26.53	0.581	0.70	19.08	13.63	0.647	0.69
Multidrug resistance efflux pump	0.00	0.00	0.162	0.486	3.02	0.00	**0.017**	0.272
Gene modulating antibiotic efflux	75.30	15.53	0.490	0.73	19.65	20.86	0.393	0.63
SMR antibiotic efflux	0.00	0.00	0.447	0.805	-	-	-	-
Antibiotic target	1.70	0.00	**0.002**	**0.030**	2.36	0.00	0.096	0.512
Gene modulating resistance	16.25	22.83	0.535	0.69	9.68	39.10	**0.043**	0.344
rRNA methyltransferase	8.59	9.07	0.581	0.65	8.41	5.56	0.601	0.67
Other ARG	24.40	12.15	0.680	0.72	7.21	7.36	0.601	0.74

FDR, false discovery rate

*See eMethods for further explanation of which antibiotic resistance genes that are included in these groups

eTable 6. Influence of antibiotic exposure (broad versus narrow after first week of life) on abundance of antibiotic resistance genes (ARGs) in probiotic supplemented extremely preterm (PEP) infants

Antibiotic resistance genes (ARGs) classes*	ARGs at 28 days				ARGs at 4 months			
	Broad (*n*=5)	Narrow (*n*=12)	*P*	*P* FDR	Broad (*n*=7)	Narrow (*n*=11)	*P*	*P* FDR
Class A Beta Lactamase	0.00	0.00	0.799	0.846	1.43	3.01	0.596	0.867
Class C Beta Lactamase	45,96	0.00	**0.009**	0.162	9.11	9.52	0.328	0.875
Aminoglycoside Phosphotransferase	6.14	0.00	0.082	0.369	-	-	-	-
Aminoglycoside Nucleotidyltransferase	0.93	0.00	0.104	0.312	0.00	0.00	0.860	
Tetracycline Efflux	29.55	0.00	**0.019**	0.171	7.92	7.92	0.375	0.857
Tetracycline Ribosomal Protection	6.49	0.00	0.082	0.369	11.68	28.48	0.246	0.787
Quinolone Resistance	29.75	7.08	0.506	0.828	9.40	9.40	0.425	0.85
ABC efflux pump	3.23	0.43	0.279	0.628	0.70	1.10	0.479	0.852
RND Antibiotic Efflux	312.10	19.81	0.799	0.900	94.00	93.09	0.536	0.858
MFS Antibiotic Efflux	272.36	79.67	0.506	0.759	70.92	111.28	0.860	0.917
Multidrug Efflux Pump Activity	22.08	24.71	0.879	0.879	19.08	6.55	0.647	0.863
Multidrug Resistance Efflux Pump	0.00	0.00	0.234	0.602	3.02	3.02	0.069	0.368
Gene Modulating antibiotic efflux	75.30	13.81	0.328	0.656	19.65	24.88	**0.008**	0.128
SMR Antibiotic Efflux	0.00	0.00	0.506	0.759	-	-	-	-
Antibiotic Target	1.70	0.00	0.064	0.030	2.36	0.00	0.151	0.604
Gene Modulating Resistance	16.25	33.15	0.442	0.756	9.68	60.81	**0.043**	0.344
rRNA Methyltransferase	5.15	6.23	0.799	0.846	8.41	2.85	0.930	0.930
Other ARG	24.40	7.31	0.506	0.700	7.21	7.21	0.724	0.891

Aminoglycoside acetyltransferase, Macrolide resistance genes, Chlorampehicol acetyltransferase were only present at 7 days of age.
FDR, false discovery rate
*See eMethods for further explanation of which antibiotic resistance genes that are included in these groups

eTable 7. Influence of antibiotic exposure (yes versus no after first week of life) on abundance of antibiotic resistance genes in all preterm infants

Antibiotic resistance genes (ARGs) classes	ARGs at 28 days				ARGs at 4 months			
	Yes (*n*=22)	No (*n*=33)	*P*	*P* FDR	Yes (*n*=22)	No (*n*=31)	*P*	*P* FDR
Class A Beta Lactamase	0.00	0.00	0.128	0.576	4.01	0.56	0.786	1
Class C Beta Lactamase	4.37	0.13	0.459	0.826	8.81	12.19	0.829	1
Aminoglycoside Phosphotransferase	0.00	0.00	0.216	0.648	-	-	-	-
Aminoglycoside Nucleotidyltransferase	0.00	0.00	**0.019**	0.342	0.00	0.00	0.408	1
Tetracycline Efflux	0.00	0.00	**0.034**	0.306	0.00	0.00	**0.037**	0.592
Tetracycline Ribosomal Protection	0.37	3.03	0.128	0.576	5.35	25.76	0.213	1
Quinolone Resistance	12.09	6.46	0.171	0.616	8.87	9.24	0.914	1
ABC efflux pump	1.10	1.40	0.705	0.846	0.67	0.91	0.957	0.957
RND Antibiotic Efflux	49.55	53.63	0.655	0.91	89.47	111.20	0.928	1
MFS Antibiotic Efflux	133.57	114.97	0.693	0.891	109.40	90.19	0.357	1
Multidrug Efflux Pump Activity	25.20	26.53	0.399	1	14.23	11.09	0.448	1
Multidrug Resistance Efflux Pump	0.00	0.00	0.806	0.91	0.45	0.00	0.144	1
Gene Modulating antibiotic efflux	17.89	17.66	0.447	0.894	20.25	24.63	0.829	1
SMR Antibiotic Efflux	0.00	0.00	0.869	0.92	-	-	-	-
Antibiotic Target	0.00	0.00	0.939	0.939	0.00	0.00	0.594	1
Gene Modulating Resistance	22.28	29.18	0.525	0.86	19.42	24.63	0.357	1
rRNA Methyltransferase	8.83	7.71	0.612	0.918	5.93	5.01	0.570	1
Other ARG	12.32	8.32	0.418	0.94	7.28	10.37	0.914	1

FDR, false discovery rate

*See eMethods for further explanation of which antibiotic resistance genes that are included in these groups

eTable 8. Influence of antibiotic exposure (yes versus no after first week of life) on abundance of antibiotic resistance genes in probiotic supplemented extremely preterm infants

Antibiotic resistance genes (ARGs) classes*	ARGs at 28 days			ARGs at 4 months			
	Yes (*n*=17)	No (*n*=7)	*P*	Yes (*n*=18)	No (*n*=6)	*P*	*P* FDR
Class A Beta Lactamase	0.00	0.00	0.534	2.22	2.00	0.820	0.875
Class C Beta Lactamase	0.98	16.08	0.576	9.32	6.63	0.581	0.845
Aminoglycoside Phosphotransferase	0.00	0.00	0.455	-	-	-	-
Aminoglycoside Nucleotidyltransferase	0.00	0.00	0.383	0.00	0.00	0.581	0.775
Tetracycline Efflux	0.00	0.00	0.576	0.64	0.00	0.199	0.637
Tetracycline Ribosomal Protection	0.00	2.53	0.318	5.56	30.78	0.626	0.786
Quinolone Resistance	9.43	12.40	0.576	9.46	3.63	0.224	0.597
ABC efflux pump	0.78	1.91	0.288	0.90	0.33	0.280	0.560
RND Antibiotic Efflux	37.73	154.33	0.664	104.33	53.24	0.280	0.630
MFS Antibiotic Efflux	85.74	99.33	1.0	109.40	40.02	**0.033**	0.264
Multidrug Efflux Pump Activity	22.89	26.53	0.260	14.23	4.73	**0.022**	0.352
Multidrug Resistance Efflux Pump	0.00	0.00	0.901	0.45	0.00	0.415	0.664
Gene Modulating antibiotic efflux	14.66	25.21	0.951	22.27	11.44	0.280	0.498
SMR Antibiotic Efflux	0.00	0.00	0.494	-	-	-	-
Antibiotic Target	1.70	0.00	0.534	0.00	0.00	0.770	0.880
Gene Modulating Resistance	28.73	50.26	0.349	29.54	33.83	0.871	0.871
rRNA Methyltransferase	6.79	5.97	0.951	5.93	1.70	**0.040**	0.213
Other ARG	7.33	11.17	0.951	7.28	3.36	0.119	0.476

Aminoglycoside acetyltransferase, Macrolide resistance genes, Chloramphenicol acetyltransferase were only present at 7 days of age.
FDR, false discovery rate
*See eMethods for further explanation of which antibiotic resistance genes that are included in these groups