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# A randomized controlled study protocol of the TOBBI trial: the effect of a 6 weeks intervention with synbiotics on the recovery speed of the gut microbiota after antibiotic treatment in Dutch toddlers

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## Abstract

**Background** Antibiotic-induced disruption of the gut microbiome in the first 1000 days of life is linked to an increased risk of the development of immunological, metabolic, and neurobehavioral childhood-onset conditions. Supporting the recovery of the gut microbial community after it has been perturbed by antibiotics might be a promising strategy to reduce these risks. In this clinical study, the effect of a 6 weeks supplementation with synbiotics (*Bifidobacterium breve* M-16 V, short chain galacto-oligosaccharides and long chain fructo-oligosaccharides) after antibiotic treatment on the recovery speed of the gut microbiota of toddlers will be studied.

**Methods/design** A cohort of 126 Dutch toddlers aged 12 to 36 months old, who receive an amoxicillin or amoxicillin/clavulanic acid treatment, will be followed for 12 weeks. Participants will be randomized into an intervention group, who will consume the study product over a 6 weeks period starting at the last day of the antibiotic treatment or into a control group that will continue their usual eating pattern. Stool samples and their characteristics will be collected weekly by both groups. Stool samples will be analyzed for total microbiota and *Bifidobacterium* spp.. The differences in the proportion of *Bifidobacterium* out of total gut microbiota, composition of species belonging to *Bifidobacterium,* and beta diversity overtime will be compared between the two groups to study the effect of the intervention on the gut microbiota after perturbation. Furthermore, the effect of the treatment will also be studied in terms of the gut microbiota metabolic activity and stool characteristics. Additionally, food intake will be recorded to investigate whether diet, especially dietary fibers, may influence the gut microbiota as well. The findings may highlight a potential intervention strategy to support the recovery of the gut community after it has been perturbed by antibiotics in early life.

**Trial registration** The TOBBI trial was approved by the board of Medical Ethics Review Committee NedMec in June 2022 and registered at https://www.onderzoekmetmensen.nl/en/trial/20358 under the code NL75975.081.20, and at the World Health Organization at https://trialsearch.who.int/Trial2.aspx?TrialID=NL-OMON20358 under NTR-new: NL8996.

**Keywords** Toddler, Microbiota, Antibiotics, Synbiotics, Nutrition, Recovery speed

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## Background

In the human gut a collection of microorganisms, like bacteria, fungi, and viruses can be found. This community plays a crucial role in processes like digestion, and immunity. The so-called gut microbiota is shaped during the developmental trajectory in the first 1000 days of life and has an important role in health related functions such as food digestion, energy metabolism, and development of the immune system [1]. In this key period, the gut microbiota transitions from a sterile prenatal environment to adult-like homeostatic configurations around the third year of life [2, 3]. However, perturbations during the first 1000 days can result in a disturbed gut community and, therefore, may affect the development trajectory; this could be linked to higher risks of developing several diseases like asthma, allergies, obesity, inflammatory bowel disease and aberrant behavior [4–6]. The first 1000 days is, therefore, a critical window in which the effect of perturbations of the gut community needs to be minimized to promote health [7].

In 2022, antibiotics were the most prescribed drugs for Dutch children up to an age of 10. Approximately 17% of the children aged between zero and twelve months received at least one dose of amoxicillin, making it the most commonly prescribed antibiotic for this age group [8]. Across Europe antibiotic use is even more frequent [9]. The use of antibiotics within the first 1000 days is significantly associated with an increased risk of several long-term immunological, metabolic, and neurobehavioral childhood-onset health conditions [5]. Amoxicillin was found to reduce the overall abundance of the *Bifidobacterium* genus [10, 11]. This is thought to be unbeneficial since these bacteria can protect against pathogens, release nutrients from indigestible sources, play a role in the immune system, and its abundance is associated with a positive health status [12]. Considering the prevalent usage of antibiotics in toddlers, the consequential impact on their microbiome, and the associated long-term effects, it is important to mitigate the disruptive effects on the gut microbiome. Interventions to accelerate the recovery of the gut microbiome after a course of antibiotics in the first 1000 days of life might be a promising strategy to decrease its risk on negative health effects for this group of children.

Several strategies have been proposed. Some focus on protecting the gut bacteria by inactivating the active compound of the antibiotic in the gut [13], other strategies focus on supporting the recovery of the gut community after antibiotics-induced dysbiosis through the use of pro- and/or prebiotics [14–17]. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [18]. Supplementation with probiotics to support the recovery of gut bacteria after a course of antibiotics in children seem ineffective [19, 20]. In contrast to probiotics, prebiotics can affect the recovery of gut bacteria following antibiotic-perturbation by enhancing the growth of bifidobacteria and beta-lactam producing taxa [21, 22]. Prebiotics are non-digestible food components that acts as a food source for beneficial microorganisms, that promote their growth and activity to support host health [23]. Galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are two common types of prebiotics. They are naturally found in either (human) milk, fruits and vegetables and are effective in promoting the growth of bifidobacteria in the gut [19, 24, 25]. In this study, we examine the efficacy of a synbiotic: a probiotic combined with prebiotics which provide nutrients for the probiotic, to overcome the challenges that are faced by supplementing probiotics alone, such as its viability in the gut [26]. The beneficial effects of synbiotics on the gut microbiota are shown in various studies. In vitro supplementation with the probiotic *Lactobacillus acidophilus* combined with the prebiotic fructo-oligosaccharides (FOS) resulted in an increase of the abundance in *Bifidobacterium* genus and an increase in volatile fatty acids in an adults [27]. A clinical trial demonstrated that term infants delivered by cesarean section who were supplemented with the probiotic *Bifidobacterium animalis* subsp. lactis and the prebiotics 3'- and 6'-sialyllactose had significantly increased intestinal bifidobacteria counts [28]. Furthermore, another clinical trial showed that cesarean section born infants supplemented with a probiotic combination of *Bifidobacterium lactis Bi-07* and *Lactobacillus rhamnosus HN001* and the prebiotic galacto-oligosccharides (GOS) showed a significant increase in *Lactobacillus* and *Bifidobacterium* genus [29]. An in vivo intervention with GOS and a probiotic mixture of a *Lactobacillus* strain and three *Bifidobacterium* strains was also found to modulate human gut microbiota by increasing the abundance of potentially beneficial microbial species, such as *Lactobacillus* spp., *Ruminococcus* spp., and *Bifidobacterium* spp., that are negatively associated with obesity [30]. Furthermore, another clinical study showed a reduction in asthma-like symptoms in infants one year after the consumption of a *Bifidobacterium* strain combined with GOS and FOS [11]. However, trials that study the effect of synbiotics on the recovery speed of the gut microbiome after antibiotics are rare, especially involving human subjects. In a study, where mice were supplemented with a mixture of probiotic strains 3 weeks prior and during an antibiotic treatment preserved the Lactobacillales and expanded the Verrucomicrobiales and Bifidobacteriales order, but did not prevent the depletion of Bacteroidales and the

short-term proliferation of Enterobacteriales [31]. Unfortunately the study did not focus on the recovery speed of the microbiome.

The TOBBI trial (Toddlers receiving synbiotics after antibiotics), presented here, is designed to study whether an intervention with synbiotics (*Bifidobacterium breve* M-16 V combined with short chain GOS and long chain FOS) can affect the recovery speed of the gut microbiota of toddlers after amoxicillin or amoxicillin/clavulanic acid treatment. Bifidobacteria are susceptible to amoxicillin, therefore we will primarily study the recovery speed in terms of abundance of species belonging to the genus *Bifidobacterium.* We hypothesize that *B. breve* M-16 V may accelerate the recovery of *Bifidobacterium* species after antibiotic disruption as it was found that M-16 V enhances *Bifidobacterium* colonization [32]. Additionally, *B. breve* M-16 V has been found to be safe, may promote intestinal immunity, reduce inflammation, protect against pathogens, utilize wide variety of complex carbohydrates including GOS, FOS, mucin, and releasing byproducts beneficial to other bacteria [33, 34]. This in turn may also indirectly support e.g. through cross-feeding the recovery of other gut bacteria, the metabolic activity of the gut community and its role in stool characteristics [34, 35]. Therefore, our secondary aim is to study these indirect effects. The addition of a GOS/FOS mixture or GOS alone to infant formula is bifidogenic. Additionally the microbiome of formula-fed infants enriched in GOS and FOS prebiotics is more inline with the microbiome of breastfed infants when compared to the microbiome of formula-fed infants without added prebiotics [36]. It was found that a combination of GOS and FOS in the diet is most efficient in increasing the abundance of *Bifidobacterium* in an in vitro study [37]. Furthermore, we are aware that fibers in the regular diet of the participant can affect the gut microbiota composition. Therefore, we will assess the dietary fiber intake of the participants and its relation to the recovery speed of the gut microbiota.

## Methods/design

### Study design and population

The TOBBI trial is a single-center, on-going, intervention study, investigating the effect of synbiotics on the recovery speed of the gut microbiota after antibiotic treatment in a Dutch toddlers cohort in a randomized controlled, but not blinded setting. In this study we compare standard post-antibiotic care to a standard post-antibiotic care with synbiotic supplementation, therefore, blinding is not feasible. The privacy of participants is safeguarded using unique study IDs. This ID is generated in Castor, an online software tool designed for clinical trials [23]. The study consists of a screening visit, a 6-week intervention period or no-intervention period and an additional 6-week run-off period as shown in Fig. 1. This study was approved by the Medical Ethics Review Committee NedMec and is being performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

#### *Participant enrolment*

Parents or guardians (hereafter "caregivers") of 12—36 month old children with a prescription for antibiotics are informed about the study at participating pharmacies in the Netherlands. The investigators contact the interested caregivers and arrange further enrolment of eligible participants to the study during the course of antibiotics. Screening takes place after both caretakers signed the informed consent. In order to be eligible to participate in this study, a participant must meet all of the following inclusion criteria: (1) written informed consent obtained from both caregivers; (2) caregivers are willing to comply with the requirements of the study; (3) aged 12 to 36 months old at the time of enrollment; (4) drinks cow's milk (non-fat, semi-skimmed or full fat) regularly; and (5) received a prescription for amoxicillin or amoxicillin/clavulanic acid for non-gastrointestinal infections. There are no restrictions on the use of any (other) antibiotics earlier in life. We aim to enroll participants that receive antibiotics for lung, ear or bladder infections, hospitalized children are not eligible to enroll. A potential participant will be excluded if he/she does not meet the inclusion criteria. Other exclusion criteria includes: (1) any GI complaints, known structural GI abnormalities, or previous GI surgery; (2) known clinically significant cardiac, vascular, liver, pulmonary, psychiatric disorders, severe renal insufficiency, human immunodeficiency virus infection, acquired immunodeficiency syndrome, hepatitis B or C or abnormalities of haematology, urinalysis, or blood biochemistry; (3) known to have an allergy or intolerance to any of the ingredients in the study product, including lactose and cow's milk protein; and (4) is receiving breastmilk, or has received breastmilk in the last 7 days before start of antibiotic treatment.

#### *Baseline characteristics*

During a home visit, prior to the start of the study which is the last day of the antibiotic, the following baseline characteristics are collected: age in months; sex; antibiotic treatment duration in days; use of pre-and probiotics before enrolment; mode of delivery; gestational age at birth in weeks; number of antibiotic treatments before enrollment and breastfeeding duration.

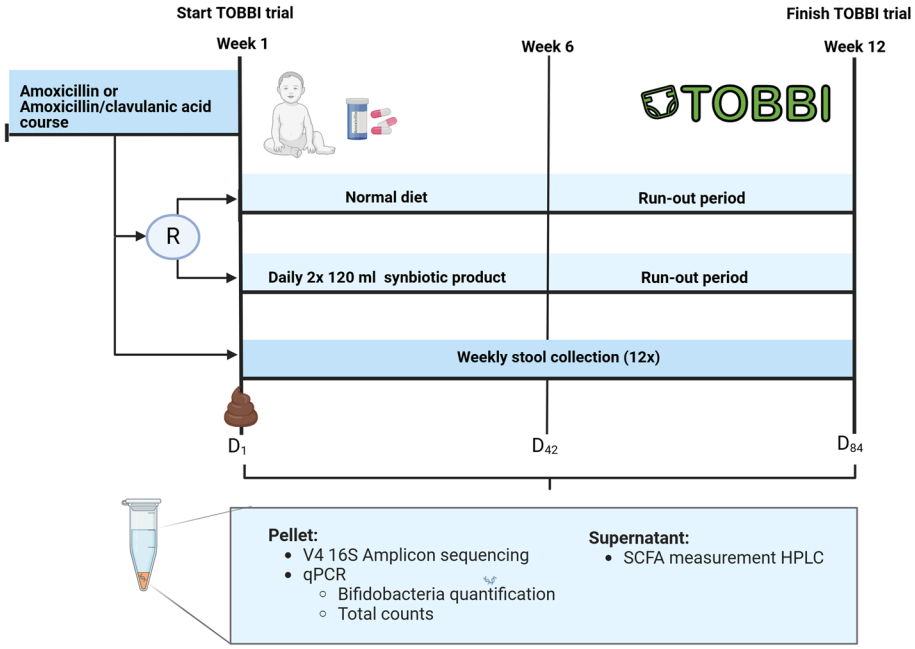


**Fig. 1** Schematic figure of the TOBBI Trial 12-week study set-up. During the course of antibiotics, parents of potential participants will be informed about the trial. After receiving informed consent eligible children will be enrolled to the study. Caretakers are asked to collect the first fecal sample at the last day of the course of antibiotics ($D_1$). Participants will be randomized (R) to either a 6-week intervention period or no intervention. In the intervention period participants consume twice a day 120 ml of the synbiotic product. The control group will not change their current feeding pattern. Both groups collect one fecal sample weekly. Created with BioRender.com

### *Study arms*

During the antibiotic treatment period, the participants will be randomly assigned to either the intervention or the control group by Castor at the moment of inclusion.

### *Study design*

On the last day of the antibiotic treatment, participants in the intervention group will start consuming 125 mL of the study product twice a day for 6 weeks, followed by a run-out period with no intervention of another six weeks. Participants can enroll anytime during the antibiotic course, but the intervention starts on the last day of the antibiotic course to ensure a common starting point for all participants. The study product is cow's-milk-based formula (CESAR biotik), fortified with 0.8 g/100 mL scGOS and lcFOS (9:1), and 3.00×10^9 CFU/g of *Bifidobacterium* breve M-16 V. This amount of study product consumption, as indicated by findings from the SMILE study [17] and the GUMLI study [27], was found adequate to anticipate an effect among healthy children without disrupted gut communities. Consequently, we expect that this amount is also sufficient to affect the recovery of gut microbiota following a course of antibiotics.

To monitor adherence, caregivers will report if the participant consumed more, less or between 100–500 mL study product daily during the intervention period. This range is used since participants may not consume all of the prepared milk, or parents may use more product than advised. In addition to the study product provided, other milk- and milk products may be consumed. The intervention can be discontinued on participant request.

The control group will continue with standard care, meaning no intervention or changes in diet or behavior for the total study period. No distinction or requirements will be made on the type of cow's milk consumed in this group, thus this can be normal cow's milk or formula milk. The type of milk used and consumed during the study in both groups will be registered as some formula milk nowadays may contain prebiotics with or without probiotics.

### *Fecal sample collection, stool characteristics and GI symptoms*

Upon enrolment that takes place somewhere between the start and the end of the antibiotic course caregivers are asked to collect one fecal sample as soon as possible, and one sample on the last day of the antibiotic treatment. If enrolment is just before the last day of antibiotics, only a fecal sample on the last day of the treatment will be collected. Fecal sample collection will then continue on a weekly basis until week 12. Asking for a baseline sample before the start of the antibiotic treatment is unfeasible,

since that would mean to postpone the start of the antibiotic treatment while the child is ill. Stool samples of an age-matched group, children of the same age who did not receive any broad-spectrum antibiotics in the past 6 months, will be collected and used as a reference. These children will be recruited via posters in public places and daycares around the participating pharmacies. After signing informed consent by both caretakers, fecal samples will be collected along with a short questionnaire to record baseline characteristics as previously described.

Fecal samples are collected with a scoop attached to the lid of a sampling tube. Caretakers are asked to fill the tube with a minimum of one scoop of feces and a maximum of 2/3 of the 30 ml tube. Collected samples will be stored in a coolbag at -20 $^0$C in a freezer at home. At the end of the study all samples will be collected by the investigator and transferred on ice to the investigational site to -80 $^0$C for later analysis. Caregivers also report the fecal characteristics of the collected samples according to the modified Bristol Stool Form Scale, which is a 5-point scale ranging from 1 (hard) to 5 (diarrhea) [38] at the day of sampling. In case the participant had multiple defecations on the day of sampling, the fecal characteristics up to 5 defecations will be reported. Furthermore, caregivers provide weekly reports on whether participants display any of the following gastrointestinal symptoms: vomiting, abdominal pain, flatulence, diarrhea, or constipation, as part of monitoring the safety of the participants. Suspected adverse event (AEs) will be communicated to the responsible physician, who will assess whether the AEs could be associated with the study product. No serious adverse events are expected from the administration of the synbiotic product. Nevertheless, in case of a suspected adverse event, the coordinating investigator will immediately notify the research teams, manufactures of the product and the involved METC committee.

#### *Food intake assessment*

In the last week of the study the habitual dietary intake of the participants over the last month will be assessed using a children Food Frequency Questionnaire (FFQ) that is developed with the Dutch FFQ-TOOLTM [39]. This FFQ is tailored to assess the intake of energy, macronutrients, micronutrients, such as calcium, iron, vitamin B1, B2, B6 and B12, and dietary fibers for Dutch children aged 1 to 3 years old. Food items queried in the FFQ are selected based on their contribution to total intake and explained variance in intake of energy, macronutrients and micronutrients as assessed in the Dutch National Food Consumption Survey (DNFCS) of 2006. Nutrient intakes will be calculated using the Dutch food composition database of 2021.

#### Sample size calculation

The sample size was calculated per study arm: intervention and control. We used the following sample size formula: $n = 2 \text{ x } (Z\alpha + Z\beta)^2 \text{ x } (SD/D)^2$. Where α is the accepted probability of type I error, β is the accepted probability of type II error, SD the standard deviation and D the expected difference between the means of the two groups. Here we used α of 0.05 (z-score = 1.96) and a power (1-ß) of 80% (z-score = 0.842), the formula simplifies to $n = 2 \times 7.9 \text{ x } (SD/D)^2$ per group. The expected difference between the two groups in the mean change from baseline to 12 weeks in relative abundance of the genus *Bifidobacterium* out of total bacterial community is 7.5% and an CI of 14%, based on the SMILE study [40]. In the TOBBI trial we expect to see a similar effect already at 6 weeks, as the participants will have received antibiotics before they start consuming the study product, which we expect to affect their relative abundance of the genus *Bifidobacterium*. Including a drop-out rate of 15% a total of 126 children will be included, 63 per study arm.

In the Child Health and Resident Microbes (CHaRM) study, group sizes of 24 participants were large enough to show the small difference in microbiota composition between two groups of toddlers [26]. Therefore, we expect that a group size of 30 for the age-matched group will be large enough to use as reference of a healthy gut microbiota of toddlers.

Participants or caregivers can withdraw from the study at any time for any reason if they wish to do so without any consequences. Participants may be withdrawn from the study by the principal investigator if they or their caregivers do not comply with the rules and regulations of the study. Participants may be withdrawn from the study by the responsible physician in case of reported serious adverse events or in case of other medical/social/psychological events as evaluated by the responsible physician and discussed with the principal investigator. Participants will not be replaced after withdrawal. Participants will not be followed-up after withdrawal, except in case of adverse events. All data collected so far, will be used for analysis.

## Methods

### Microbiota composition analysis

16S rRNA gene amplicon sequencing will be used to assess the microbiota composition. For microbiome analysis bacterial DNA from the fecal samples will be isolated, PCR-amplified, purified, quantified and annotated. Quantities of 0.25 g of feces will be weighed into a 2 mL screw-cap tube filled with 0.5 g of 1.0 mm zirconia and five 2.5 mm glass beads. Screw-cap tubes with beads, but without feces will be used as negative control. Next, 700

µL of Stool Transport and Recovery Buffer (STAR buffer; Roche Diagnostics Corporation, US) will be added. The samples will be bead-beaten (FastPrep-24 5G, MP Biomedicals, US) three times at 5.5 m/s for 60 s with 15 s pause on ice. Furthermore, to enhance cell lysis samples will be incubated for 15 min at 95 °C at 100 rpm, after which they will be centrifuged at 4 °C for 5 min at 15 000 rpm, as recommend by Salonen et al. [41]. The supernatant will temporally be stored at 4 °C. The process will then be repeated with fresh 300 µL STAR buffer. Samples will be incubated and centrifuged as before. The resulting supernatant will be pooled with the first supernatant of which 250 µl of recovered supernatant will be used for DNA extraction with Maxwell RSC Custom Total Nucleic Acid Purification Kit, AX4660 (Promega, The Netherlands) according to the manufacture's protocol. DNA concentration will be measured using QubitTM dsDNA BR Assay kit (Invitrogen by Thermo Fisher Scientific) and a DeNovix DS-11 Fluorometer (DeNovix Inc., United States). Isolated DNA will be diluted to 20 ng/µl. A one-step PCR reaction will follow, where 1 ul of the diluted DNA will be added to a PCR mastermix containing 10 µl 5×HF buffer (green) ( Thermo Fisher Scientific, Lithuania), 1 µl of 10 µM barcoded V4 primers 515F [42] and 10 µM 806R [43] (Eurogentec, Belgium), 1 µl of 10 mM dNTP's (Thermo Fisher Scientific), 0.5 µl of Phusion Hot start II DNA polymerase (Fisher Scientific) and 36.5 µl Nuclease free water (Promega, USA). Additional two MOCK communities with known composition will be used as positive control [44]. The PCR conditions will be 30 s at 95 °C, 25 cycles of 10 s at 98 °C, 10 s at 50 °C, and 10 s at 72 °C, followed by 7 min at 72 °C using a Labcycler 48 (sensoquest, Germany). PCR product will be purified using the CleanPCR kit (GCbiotech, The Netherlands) according to the manufacturer's protocol. Next, DNA will be quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, US) on the Qubit fluorometer according to the manufacture's protocol. Samples will be pooled into libraries at an equimolar concentration of 200 ng and will be purified again with the CleanPCR kit. The Illumina NovaSeq 6000 Platform will be used for sequencing. The resulting data will be annotated with the Qiime2 pipeline with the newest available SILVA database [45].

In order to measure absolute abundance of total bacteria, qPCR will be performed. The quantity of the isolated bacteria (16S rRNA region) will be assessed using a SYBR-based real-time qPCR. The following primer sequences will be used to quantify the total bacteria target the 16S rRNA gene: BACT_1369F: CGG TGA ATA CGT TCY CGG; PROK_1492R: GGW TAC CTT GTT ACG ACT T using the parameters previously described [46]. Furthermore, the bacteria belonging to the genus *Bifidobacterium* will be quantified using primer sequences that target the genus *Bifidobacterium* specific region of the bacterial 16S rRNA gene: g-Bifid-F: CTC CTG GAA ACG GGT GG; g-Bifid-R: GGT GTT CTT CCC GAT ATC TAC, using the parameters previously described [47]. Specifically also the quantity of *Bifidobacterium* breve will be assed using F: TCA TCA CGG CAA GGT CAA GA and R: GGC CAG AAC AGC TGG AAC AA, with the parameters as described by Kim et al. [48].

Since the 16S amplicon sequencing with Illumina rarely provides species information, the species composition of *Bifidobacterium* in the fecal samples are planned to be asses using Internal Transcribe Spacer (ITS) sequencing as previously described by Milani et al. [49]. In brief, ITS sequences of *Bifidobacterium* will be amplified from the extracted DNA using the primers Probio-bif_Uni:CTKTTGGGYYCCCKGRYYG and Probio-bif_Rev CGCGTCCACTMTCCAGTTCTC. These primers are able to identify our probiotic *B. breve* among other *Bifidobacterium* species. The PCR conditions that will be used are 5 min at 95 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C, followed by 10 min at 72 °C. Amplification will be carried out using a Verity Thermocycler (Applied Biosystems). Amplified PCR product will be purified. Emulsion PCR will be carried out using the Ion OneTouchTM 200 Template Kit v2 DL (Life Technologies) according to the manufacturer's instructions. Additionally sequencing of the amplicon libraries will be carried out on a 314 chip using the Ion Torrent PGM system and employing the Ion Sequencing 200 kit (Life Technologies) according to the supplier's instructions. Individual sequence reads will be filtered by PMG software.

#### Functional properties of microbiome

Lactate and short-chain fatty acids (SCFA) levels, namely acetate, propionate, succinate, formate and butyrate, of fecal samples collected at t = 0, t = 21, t = 42 and t = 84 days will be measured using a Shimadzu LC_2030C (HPLC) equipped with a refractive index detector and a Shodex SH1011 column. Samples will be centrifuged for 5 min on full speed to separate the liquid fraction from the solid parts of the stool. Thereafter a Carrez sample clarification was used on 500 ul of the supernatant to remove proteins from the samples, as described by Endika et al. [50]. The external standards will include the indicated SCFA levels and lactate. Two runs will be performed for each sample using an oven temperature of 45 °C and 75 °C with a pump flow of 1.0 mL/min and 0.9 mL/min, respectively. For both runs 0.01N $H_2SO_4$ will be used as eluent. All samples and standards (10 µl) will run for 20 min. The concentrations of the standards will range between 2.5 mM and 60 mM.

The pH of diluted (1:10, deionized water) and homogenized stool samples collected at t = 0, t = 21, t = 42 and t = 84 days will be measured using the Proline pH meter B210.

#### Study outcomes and statistical analysis

In the TOBBI trial we will study the effect of an intervention with synbiotics after antibiotic treatment in toddlers on fecal microbiota composition, metabolic activity, stool characteristics and gastrointestinal symptoms. For this purpose, fecal bacterial composition will be determined by 16S rRNA amplicon, qPCR and ITS sequencing for *Bifidobacterium* spp in feces of several timepoints from participants in the control group and participants in the intervention group who are compliant with their study product consumption. A study participant in this group is compliant when between 100–500 mL study product per day is consumed during at least 80% of the days of the intervention period (34 out of 42 days). The missing days might or might not be consecutive.

Microbiota data analysis will be assessed with the programming language [36], using a variety of in-house R-scripts and public available packages such as phyloseq [37], microbiome [38], vegan [39]. The most up to date software version at the time of analysis will be used. Bacterial composition will be presented as relative abundance and absolute abundance. The absolute abundance of microbial taxa will be calculated for each sample by multiplying the qPCR count of total 16S rRNA gene copies of the sample with the relative abundance of taxa, following the approach of quantitative microbiota profiling [51]. Other numerical data will be shown as mean ± standard deviation for normally distributed continuous data or median [interquartile range] when the continuous data is skewed. Categorical variables will be presented as percentages. Normality of distribution will be verified by the Kolmogorov–Smirnov test. Variables that are not normally distributed, will be transformed using a log and/or a CLR transformation in case the statistical test assumes a normal distribution. The significance of differences will be assessed using a Wilcoxon rank sum test, unless stated otherwise. Obtained *p*-values will be corrected for multiple testing by false discover rate (FDR) following Benjamini–Hochberg procedure. A corrected p-value of $\leq 0.05$ will be considered significant. Additionally, linear mixed models (LMM[i]s) taking into account the effects of repeated measurements will also be used to assess the significance of the differences in the changes of microbiome composition.

Primarily we will investigate potential differences in composition of species classified under the genus *Bifidobacterium* within subjects and between groups for timepoints T1, T21, T42, T84. We will specifically compute the differences, also known as deltas, in absolute abundance, and alpha diversity, within a subject for species classified under the genus *Bifidobacterium* between two timepoints. Alpha diversity will be assessed for each timepoint using different indices such as Shannon, Inverse Simpson, Simpson, Gini, and Chao1. These deltas will be compared between groups. Furthermore, we will calculate the dissimilarity using weighted UniFrac distance between T1 and T21, between T21 and T42, and between T42 and T84 within a subject. Here statistical difference will be assessed using PERMANOVA. These indices will be compared between groups. In order to estimate statistical significance of the covariates, such as breastfeeding duration or mode of delivery, in explaining the variation in microbiome composition, a distance based redundancy analysis (dbRDA), will be used. Other parameters used as explanatory variables in RDA model will include: timepoint, age, gestational age, sex and the number of antibiotic treatments. The purpose of the antibiotic treatment will not be taken into account for the data analysis. Afterwards the significant variables will be also included in LMM.

For the secondary outcomes, we investigate differences over time in fecal microbiota composition of the total gut community; metabolic activity, and stool characteristics. These differences will be compared between timepoints within a subject and between groups at each timepoint using statistical tests and LMM as indicated above. The difference in composition and diversity will be assed as indicated in the primary analysis, however here we plan to compare these difference for the total community. If necessary we will also compare these differences for other timepoints. The metabolic activity of the gut microbiota will as be compared by measuring the concentration of the metabolites acetate, propionate, succinate, formate, butyrate and lactate and the pH of the fecal samples at timepoints T1, T21 T42 and T82. Furthermore, we will assess whether there is an association between groups and stool characteristics at each timepoint using a Chi square test. We'll compute the average number of stools per timepoint within a group and then compare these between groups. Additionally, we will use LMM to study the influence of confounding factors such as the daily intake of fibers on the recovery speed of the gut microbiota after antibiotic exposure for both the control and the intervention group. Individual subjects will be treated as random effects, whereas week and study groups as the fixed effects.

## Discussion

The TOBBI trial is a clinical study researching the effect of administration of synbiotics after antibiotic treatment on the recovery speed of the gut microbiota of

12 to 36 months old toddlers. Perturbations of the gut microbiota in the first 1000 days of life are linked to the onset of undesired health conditions [4–6]. Therefore, we aim to reduce this associated effect of perturbation by increasing the recovery speed of the gut microbiota after perturbation by antibiotics using synbiotics. However, this trial has a few limitations. First, participants do not provide a baseline sample. This is not feasible, since we cannot ask the caregivers to wait with the antibiotic administration until a baseline sample is collected. Therefore, we cannot determine if and when the microbiota composition is fully recovered. However, we do collect fecal samples from an age-matched healthy group who have not received antibiotics in the last 6 months before fecal donation. This provides a reference sample of the gut bacterial community at baseline. This community differs per person and might, therefore, not fully represent the gut community of the participants before antibiotic treatment. However, we hypothesize that the induced differences in microbiota composition resulting from antibiotic usage exceed individual differences in microbiota composition. Additionally, participants receive antibiotics according to their medical condition, which may lead to differences in the bacterial community between the age-matched reference group and other participants. Second, the quality of the stool samples strongly depends on how the caretakers collect and store them. Samples that are not handled as instructed can differ in quality, affecting microbiome analysis results. Third, the collection of data about the stool characteristics and food intake relies on compliance of the caretakers. For example, questionnaires about the stool characteristics need to be filled out weekly in order to study the effect of the treatment on stool characteristics. On three separate days caretakers will report on the food and drink intake precisely to study the influence of diet on the recovery speeds after antibiotics. Diet recording might not be precise, and the precision may differ between caretakers.

On the other hand, this study is strengthened by its study design. In this study design, we have taken confounding variables into account, thereby enhancing the overall quality of our research. These variables include the mode of birth, gestational age at birth, sex, breastfeeding duration, consumption of formula milk, number of antibiotic courses and the consumption of dietary fibers. Furthermore, the control group in our study may consist of children who regularly consume cow's milk or formula milk with or without prebiotics. This setup allows us to investigate whether supplementing with the synbiotic product offers greater benefits after antibiotic treatment compared to current standard practices. Currently, there is a lack of clear guidance on the use of pro- and prebiotics following antibiotic treatment, despite the increasing availability of probiotic products. Therefore, with this study design we aim to provide scientifically validated advice on the use of synbiotic products as post-antibiotics care for children, when positive outcomes are validated. This guidance could benefit not only parents in supporting their child's health, but also Medical professionals who advise parents, and potentially shape a new guide lines of care post-antibiotic treatment in children, beneficial for overall public health.

#### Abbreviations

| | |
|---|---|
| FDR | False Discovery Rate |
| GI | Gastrointestinal |
| LMM | Linear Mixed Model |
| lcFOS | Long chain Fructo-oligosaccharides |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative Polymerase chain reaction |
| RDA | Redundancy Analysis |
| rRNA | Ribosomal RNA |
| scGOS | Short chain Galacto-Oligosaccharides |
| Ti | Timepoint i , i in i = 0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84 days |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12887-025-05405-1.

Supplementary Material 1: Appendix A.

Supplementary Material 2: Appendix B.

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#### Authors' contributions

M.A. and C.B. were involved in the conceptualization and design of the study. C.B. obtained funding and is the principle investigator. K.K., M.A. and C.B. supervised the study. K.K., M.A. were involved in the provision of administrative, tech-nical or material support. K.K is the investigators of fieldwork. K.K. drafted the manuscript. C.B. and A. M. critically re-vised the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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#### Data availability

No datasets were generated or analysed during the current study.

## Declarations

### Ethics approval and consent to participate

The study is designed in accordance with the Declaration of Helsinki. The TOBBI trial research protocol version 1.3 May 17th, 2022 was approved by the board of Medical Ethics Review Committee NedMec in June 2022 and registered under the code NL75975.081.20, METC number: 21–214/G. Protocol amendments will we communicated to the journal. The sponsor of the study is Wageningen University & Research, Laboratory of Microbiology, P.O. Box 8033 6700 EH Wageningen. Recruitment started in September 2022. Informed consent was obtained from both parents or legal guardian of all subjects involved in the study by the researcher. The parents or legal guardian had the option to provide additional consent provisions for the use of the participant data and biological specimens in additional studies. This consent was not mandatory to participate in the TOBBI trial.

### Consent for publication

Not applicable.

### Competing interests

The trial is sponsored though TKI_agrifood AF18005 with financial support of Danone Research & Innovation. J. K. is an employee of Danone Research & Innovation. Danone Research & Innovation develops and sells nutritional products for infants and toddlers. Danone Research & Innovation had not been involved in design of the study or in the writing of this manuscript. In addition, it will not have any role during its execution and in the acquisition, analyses and interpretation of the data of the decision to submit results to scientific journals. The other authors have no competing interests to declare.

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## Publisher's Note