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Proximal remnant intestinal colonization in newborn infants with enterostomy: Protocol design for a longitudinal study

Colonização do intestino proximal remanescente em recém-nascidos com enterostomia: Desenho do protocolo de um estudo longitudinal

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ABSTRACT

Background: The human microbiota is the collection of microorganisms that is mostly settled in the gastrointestinal tract, predominantly bacteria. This plays a main role in the maintenance of the hosts' health and may be involved in mechanisms for disease development. Exposure to different conditions in early life contributes to distinct "pioneer" bacterial communities, which shape the newborn infants development and influence their later physiological, immunological and neurological homeostasis. Newborn infants with congenital malformations of the gastrointestinal tract (CMGIT), necrotizing enterocolitis (NEC), and spontaneous intestinal perforation (SIP) commonly require abdominal surgery and enterostomy. While intestinal microbiota has been extensively studied in infants with anatomically uninterrupted intestine, the knowledge of longitudinal intestinal colonization in this population is scarce.

Methods: This is an exploratory, observational, and longitudinal prospective study, whose primary aim is to determine longitudinally the microbiota colonization of the proximal remnant intestine, in newborn infants with enterostomy during the first three weeks after surgery by CMGIT, NEC and SIP. The secondary objective is to explore associations between the colonization and the variables: mode of delivery, gestational age, postnatal age, duration of fasting, type of enteric feeding, antimicrobial therapy, H_2-receptor antagonist therapy and length of proximal remnant intestine.

Ethics and legal issues: The study protocol was approved by the Administration Board and Ethics Committee of Centro Hospitalar de Lisboa Central.

Keywords: Congenital malformations of the gastrointestinal tract, Enterostomy, Microbiota, Necrotizing Enterocolitis, Newborn Infants.

RESUMO

Estado de arte: O microbiota humano é o conjunto de microrganismos que reside maioritariamente no trato gastrointestinal, e que são predominantemente bactérias. Desempenha um papel determinante na saúde do indivíduo e também pode estar envolvido no desenvolvimento de diversas patologias. A exposição a condições distintas no período peri-natal contribui para o estabelecimento de um microbiota diferente, que influenciam o desenvolvimento dos recém-nascidos e a sua homeostasia à *posteriori*. Os recém-nascidos com malformação congénita do trato gastrointestinal (MCTG), com enterocolite necrosante (ECN) e perfuração intestinal isolada (PII) normalmente são sujeitos a cirurgia abdominal e enterostomia. Apesar do microbiota intestinal estar amplamente estudado em recém-nascidos com intestino íntegro, o conhecimento longitudinal da colonização intestinal nesta população é escasso.

Métodos: Este estudo exploratório observacional prospetivo, tem como principal objetivo determinar o padrão de microbiota que coloniza o intestino proximal remanescente, em recém-nascidos com enterostomia durante as três semanas após a intervenção cirúrgica por MCTG, ECN e PII. O objetivo secundário consiste na averiguação de associações entre o tipo de colonização e as seguintes variáveis: tipo de parto, idade de gestação, idade pós-natal, duração da pausa alimentar, tipo de nutrição entérica, terapêutica antimicrobiana, medicamentação com antagonistas dos recetores H_2 e a extensão do intestino proximal remanescente.

Questões ético-legais: Este estudo foi aprovado pelo Conselho de Administração e Comissão de Ética do Centro Hospitalar de Lisboa Central.

Palavras-chave: Enterocolite necrosante, Enterostomia, Malformação congénita do trato gastrointestinal Microbiota, Recém-nascido.

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INTRODUCTION

The human microbiota is the collection of microorganisms that live in the human body, mostly settled in the gastrointestinal tract (GIT), predominantly bacteria with higher density in the colon[1-4]. This represents an intestinal symbiosis that plays a principal role in the maintenance of the host's health[5]. The microbiota may be involved in mechanisms for disease development and is increasingly regarded as an "invisible organ"[6]. Microbiota participate, among others functions, in metabolism and nutrient digestion, vitamin synthesis, immune tolerance, intestinal mucosa's maturation, and brain development[7]. Exposure to different conditions in early life contributes to distinct "pioneer" bacterial communities, which shape the newborn infants development[8] and influence their later physiological, immunological and neurological homeostasis[9-14].

The microbiota acquisition starts in utero and it the intestinal colonization process is dynamic developing rapidly from birth[6, 15, 16]. At this moment, human intestine is an aerobic environment, which gradually becomes anaerobic over a period of days[8, 9]. Facultative aerobes bacteria are the earliest to colonize the intestine, comprising *Escherichia* and *Enterococcus*, that will consume the oxygen which permits colonization by obligate anaerobes, including Firmicutes such as *Clostridia*, Bacteroidetes, and especially *Bifidobacterium*[8, 9].

In newborn infants, the type and diversity of intestinal microbiota differs widely among individuals and is influenced by several factors, such as the mode of delivery, gestational age, infant's postnatal age, and type of feeding; in neonates under intensive care, fasting, exposure to antibiotics, H_2-receptor antagonists, and surrounding environment are additional factors[5, 6, 17-19].

Interactions of host-microbiome in early life provide a signal for immune system development, and can influence the development of several organs[9, 20, 21]; at intestinal level, these interactions are important for development of barrier function, integrity, and mucosal and systemic immune function[19].

Under physiological conditions, the evolution of the infants' microbiome depends on the initial exposure to microbes in amniotic fluid, colonization through the vaginal canal, feeding own mother's milk, and skin-to-skin contact with the mother[22].

In the neonatal intensive care unit (NICU), infants are usually nursed in high-sanitary incubators, treated with antibiotics, with possible restricted own mothers' milk intake and limited contact with mother's skin[23]. This is determinant for an early life dysbiosis, characterized by a delayed and suboptimal colonization, which has been associated with long-term morbidities, including obesity, inflammatory bowel disease, autism, asthma, and celiac disease[5, 15]. Preterm infants in particular are immunologically immature, turning them particularly sensitive and responsive towards intestinal colonizing bacteria[23]. In this population, the type of intestinal microbiota may influence the development life-threatening morbidities, including late onset sepsis and necrotizing enterocolitis (NEC)[19].

Newborn infants with congenital malformations of the gastrointestinal tract (CMGIT), NEC, and spontaneous intestinal perforation (SIP) commonly require abdominal surgery and enterostomy[24]. While intestinal microbiota has been extensively studied in infants with anatomically uninterrupted intestine[25], to the best of our knowledge only five longitudinal studies or case reports addressing intestinal colonization in infants with enterostomy were published[24, 26-29] (Table 1). The effect of an enteral oil supplementation on the intestinal microbiome was

tested in 32 preterm infants with enterostomy after surgery for CMGIT, NEC and SIP; the control group receiving standard nutritional therapy had relative expansion of many genera from Enterobacteriaceae family, including *Escherichia, Pantoea, Serratia*, and *Citrobacter*[24]. In a case report of two preterm infants with enterostomy after surgery for SIP and NEC, the composition of microbiota was examined according to the length of remnant intestine (colostomy or jejunostomy)[28]. In two other reported cases with ileostomy, respectively of a preterm infant treated for SIP and a term infant with intestinal obstruction of complicated cystic fibrosis, two known probiotic bacteria, Lactobacillus and Bifidobacterium, were found[29]. In a report of four cases[26], early probiotic therapy was evaluated after major surgery in newborn infants, two of them with CMGIT and enterostomy; probiotic therapy induced the equilibrium of intestinal microbiota and consequently the intestinal absorptive functions were activated and severe infections were prevented. In an observational study[27], on 15 infants undergoing intestinal resection before 180 days of age (7 of them with enterostomy), due to CMGIT, NEC and SIP, early differences in the microbiota detected in intra-operative intestinal tissue versus fecal samples was determined; the results addressed the reliance on fecal microbiota as a predictor factor for the developing intestinal microbiota.

It is not known to what extent the surgical interruption affects the developing intestinal ecosystem in newborn infants with an enterostomy with consequent contact with air[29]. An enterostomy represents a “window” for collection of intestine contents and facilitates the study of microbiota; this method was previously reported as an convenient method for studying the proximal intestinal microbiota, in adults[30].

Table 1. Longitudinal studies and case reports addressing colonization of proximal remnant intestine in infants with enterostomy.

Underlying surgical condition	Type of study	Aim	n	Reference
CMGIT, NEC and SIP	Trial	Effect of an enteral oil supplementation on the intestinal microbiome	32 preterm infants	Younge N, *et al*. 2016[24]
SIP and NEC	Case report	Microbiota diversity according to the length of remnant intestine	2 preterm infants	Barrett E, *et al.* 2013[28]
CMGIT and SIP	Case report	Determination of Lactobacillus and Bifidobacterium probiotic strains in the neonatal ileum	2 (1preterm and 1 term infant)	Wall R, *et al.* 2008 [29]
CMGIT	Case report	Effect of early probiotic therapy after surgery	2 (1preterm and 1 term infant)	Kanamori, *et al.* 2013 [26]
CMGIT, NEC and SIP	Observational study	Early differences in microbiota detected in intra-operative intestinal tissue *versus* fecal samples	7 with enterostomy	Romano-Keeler J, *et al*. 2014 [27]

CMGIT - congenital malformation of the gastrointestinal tract; NEC - necrotizing enterocolitis; SIP - spontaneous intestinal perforation

AIMS

The primary aim is to determine longitudinally the microbiota colonization of the proximal remnant intestine, in newborn infants with enterostomy undergoing surgery for CMGIT, NEC and SIP.

The secondary objective is to explore associations between the colonization and the following variables: mode of delivery, gestational age, postnatal age, duration of fasting, type of enteric feeding, antimicrobial therapy, H_2-receptor antagonist therapy and length of proximal remnant intestine.

METHODS

Study design

This is an exploratory, observational, and longitudinal prospective study. The infants will be recruited at the Neonatal Intensive Care Unit of Hospital Dona Estefânia, Centro Hospitalar de Lisboa Central (Lisbon, Portugal), and the laboratory analyses will be performed at the Laboratory of Nutrition and Metabolism of NOVA Medical School | Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, (Lisbon, Portugal).

Ethical and legal issues

The study protocol was approved by the Administration Board and Ethics Committee of Centro Hospitalar de Lisboa Central; major further amendments to the approved protocol shall be submitted for approval. Signed written informed consent is required from parents or legal guardians of the participants, to permit the collection of metadata from the medical records and the enterostomies effluents. Withdrawal of consent, in case parents or legal guardians decide to abandon the study, is contemplated without affecting the quality of care. Parents and legal guardians have access to the additional information provided by the study.

Eligibility, inclusion and exclusion criteria

Eligibility criteria: newborn infants with enterostomy after surgery for CMGIT, NEC or SIP.

Inclusion criteria: consecutive newborn infants assessed up to three weeks after surgery.

Exclusion criteria: newborn infants with diagnosed inborn errors of metabolism and those whose parents or legal guardians will not consent to participate or withdrawn the consent will not be included or will be excluded *a posteriori*.

Samples size estimation

The sample size estimation was based on the prevalence of 11 patients with enterosostomy hospitalized during one year (January 2016 to January 2017) in the NICU of Hospital Dona Estefânia: 8 with NEC and 3 with CMGT. Therefore, in the total study period is estimated to include about 20 neonates.

Study procedures

Clinical variables

Perinatal data: gestational age, antibiotics use during pregnancy and intrapartum, date of birth, mode of delivery (vaginal or caesarean section), sex, weight, length, and head circumference at birth;

Surgical data: underlying surgical condition (CMGIT, NEC and SIP), date of surgery, level of enterostomy (duodenostomy, jejunostomy, ileostomy, or colostomy), length of proximal remnant intestine (if available), type of feeding before surgery;

Postsurgical data: daily record of body weight , enteral feeding (including the fasting period), parenteral nutrition, type feeding (trophic or nutritional feeding), type of feds (mother's milk, donor's milk, or formula), mode of administration (intermittent or continuous), volume administered, central catheter and type, antimicrobial and H_2-receptor antagonists therapy, invasive ventilation, and acute events such as sepsis; enterostomy effluent characteristics, including consistency (watery, with granules or pasty), amount, and color; and date of discharge.

Enterostomy effluent sampling and storage

The samples will be collected by a nurse and delivered to the investigator. The collection will be done using a sterile syringe directly from the ostomy bag to a sterile collection tube. The previously coded tube will be stored at 4 °C for a maximum of 24-hours period in a thermal bag with a thermoregulator. Subsequently, the tubes will be stored at the Laboratory of Nutrition and Metabolism, conserved at -80 °C prior to the samples analysis. All samples will be anonymised and labelled by study number.

The first sample will be collected as close as possible to the surgical procedure, after the placement of the ostomy bag. From that day, a new collection will be done every 3 days, until the 21st day after the surgical intervention (Figure 1). If the onset of enteral nutrition is greatly delayed, the time limit may be increased.

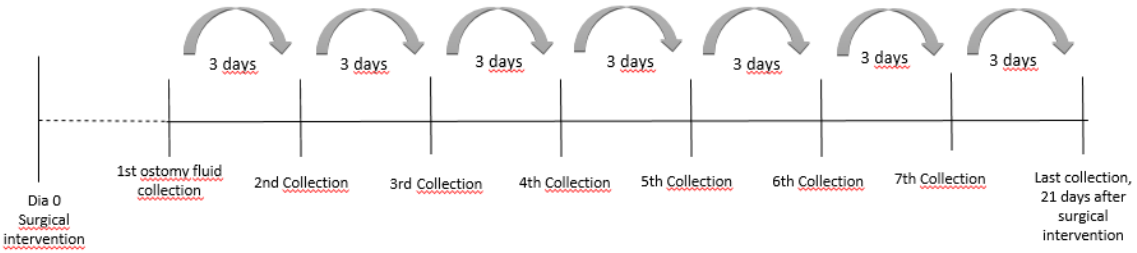

Figure 1. Timeline of the study

Procedures for enterostomy management

In the NICU of Hospital Dona Estefânia, only staff and direct family or legal guardians have contact with newborn infants, and they are taught on adequate hygienic and aseptic procedures.

By routine the ostomy bag is changed every 3 days, and every day the content is aspirated every 3 hours. Attempting to assure a sufficient amount of sample for analysis, the previously scheduled aspiration to the collection will be suspended.

Microbiota analyzes - DNA extraction

DNA will be extracted directly from intestinal content samples using a NZY Tissue gDNA Isolation Kit, as previously described by Marques *et al*[31], with some modifications adapted from a protocol described by Zoetendal *et al* [32]. The principal modifications encompass the utilization of 5-10 g of intestinal content, and before performing pre-lysis with 1.4 ml of buffer NT1, samples will be mixed with 5 ml of PSB, vortexing during 30 seconds and centrifuged at 3000 x *g* for 1 min. After these steps, the mixture will be incubated, in the water bath with shaking at 80°C for 15 min. Samples will be centrifuged at 3000 x *g* for 1 min. Then, 25 µL of proteinase K were added to the supernatant for incubation at 70°C for 15 min. The following steps are the manufacturer's instructions, but the quantity of ethanol added is based in the proportion 210 µL of ethanol to 200 µL of final supernatant, and must repeat the step of passing 600 µL of the mixture through the column of 2 ml NZYSpin Tissue about 15-20 times or until there is no mixture left. DNA quantification will be assessed with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Microbiota analyzes - Quantitative analysis by PCR

Real-time PCR will be performed as described by Marques *et al*[31]. PCR reactions mixtures (total of 10 mL) contain 5 µL of 2xFaststart SYBR Green (Roche Diagnostics Ltd), 0.2 µL of each primer (final concentration of 0.2 µM), 3.6 µL of water and 1 µL of DNA (equilibrated to 20 ng). Previously published primers will be used for the identification of microorganisms and the conditions for PCR amplification reactions are reported in Table 2. The analysis of data will be done by using the LightCycler software (Roche Applied Science).

Table 2. Primer sequences and real-time PCR conditions used for microbiota analysis

Target group	Primer sequence (5'-3')	Genomic DNA Standard	AT	Reference
Actinobacteria	F: TACGGCCGCAAGGCTA R: TCRTCCCCACCTTCCTCCG	*Bifidobacterium longum subsp. Infantis* ATCC 15697	61.5	(33)
Bifidobacterium	F:CGC GTC YGG TGT GAA AG R: CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum subsp. Infantis* ATCC 15697	60	(31)
Firmicutes	F:ATG TGG TTT AAT TCG AAG CA R:AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri* ATCC 33323	60	(31)
Lactobacillus	F: GAG GCA GCA GTA GGG AAT CTT C R:GGC CAG TTA CTA CCT CTA TCC TTC TTC	*Lactobacillus gasseri* ATCC 33323	60	(31)
Staphylococcus	F: GAT GTG CGA AAG CGT GGG GAT R: GAA CTG AGA ACA ACT TTA TGG GA	*S. aureus* ATCC 12600	60	(34)
S. *aureus*	F: AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG R: CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	*S. aureus* ATCC 12600	56	(35)
S. *epidermis*	F: ATC AAA AAG TTG GCG AAC CTT TTC A R: CAA AAG AGC GTG GAG AAA AGT ATC A	*S. epidermidis* ATCC 14990	56	(35)
Clostridium	F: AAAGGAAGATTAATACCGCATAA R: ATCTTGCGACCGTACTCCCC	*C. perfringens* AF316589	54	(36)
Enterococcus	F: CCC TTA TTG TTA GTT GCC ATC ATT R: ACT CGT TGT ACT TCC CT TGT	*Enterococcus gilvus* ATCC BAA-350	61	(31)
B. *fragilis*	F: TCRGGAAGAAAGCTTGCT R: CATCCTTTACCGGAATCCT	*B. fragilis* ATCC 25285	60	(37)
Protobacteria	F: TCGTCAGCTCGTGTYGTGA R: CGTAAGGGCCATGATG	*E. coli* ATCC 25922	61.5	(33)
E. *coli*	F: GTTAATACCTTTGCTCATTGA R:ACCAGGGTATCTAATCCTGTT	*E. coli* ATCC 25922	60	(38)
K. pneumoniae	F: CCTGGATCTGACCCTGCAGTA R: CCGTCGCCGTTCTGTTTC	*K. pneumoniae* ATCC 13883	60	(37)
Serratia	F: CCGGCATCGGCAAAGTCT R: ATCTGGCCCGGCTCGTAGCC	*Serratia ficaria* ATCC 33105	55	(39)
Candida	F: TTGGTGGAGTGAT TTGTCTGCT R:TCTAAGGGCATCACAGACCT G	*C. albians* ATCC10231	60	(40)

AT- Anneling Temperature (ºC)

Outcomes

Primary outcomes

To describe longitudinally the postsurgical microbiota colonization of the proximal remnant intestine, specifically in each underlying condition (CMGIT, NEC or SIP).

Secondary outcomes

To determine associations of types of microorganism identified with mode of delivery, gestational age, postnatal age, duration of fasting, type of enteric feeding, antimicrobial therapy, H_2-receptor antagonist therapy and length of proximal remnant intestine.

Rationale for outcomes

The NICU environment has major influence for intestinal dysbiosis and pathogenic colonization[9], with short- and long-term negative consequences for health[10, 11]. The intestinal bacteria acquired by newborn infants under intensive care have been found to be the same that appear on the hospital surfaces and equipment[22]. The intestinal microbiota becomes more similar among inpatient newborn infants as the duration of hospitalization increases[10, 41].

The importance of this study is grounded in some unexplored aspects. Infants with enterostomy have specific factors that may influence the development of intestinal microbiota, including the interruption of the intestine and the intraluminal contact with air through the stoma[42, 43]. Pathogenic bacteria that can be identified may contribute to understand their possible role in neonatal intensive care related-infection and thus be useful to plan preventive and therapeutic approaches. Enterostomy is a convenient window to collect samples and an opportunity to study the microbiota of the remnant intestine[28]. The knowledge on longitudinal intestinal colonization in newborn infants with enterostomy, including by potentially pathogenic microorganisms, is scarce (Table 1)[24, 26-29].

The mechanisms underlying the surgical conditions of infants with enterostomy are quite diverse. For instance, intestinal inflammation and ischemia are present in NEC and SIP[44, 45], but not in CMGIT. These factors are likely to influence the

microbiota existent previously to surgery and in the postoperative period. Despite a single microbial “cause” for NEC is not evident, a decrease in Firmicutes and an increase in Proteobacteria phyla were described prior to diagnosis[5, 19]. In addition, a low diversity of bacteria and of potential pathogenic microorganisms such as *Clostridium spp*., *E. coli*, *Klebsiella pneumoniae, torovirus, astrovirus, cytomegalovirus, and Candidia spp.* were described in NEC[23, 46].

Several general factors are likely to influence intestinal microbiota in newborn infants under intensive care (mode of delivery, gestational age, postnatal age, enteric feeding, antimicrobial therapy) and particular factors in newborn infants with enterosmomy (H_2-receptor antagonists, length of remnant intestine). The associations of these factors with the type of intestinal colonization have not been sufficiently studied in this last group and the rationale to evaluate those associations is described below.

- Mode of delivery: while vaginally delivered infants are colonized with *Lactobacillus* and *Prevotella* species, in infants born by cesarean section the microbiota is dominated by species such *Clostridium, Staphylococcus, Propionobacterium,* and *Corynebacterium,* and characterized by deficiency of anaerobes like *Bacteroides* and *Bifidobacterium*[6, 47, 48].
- Gestational age and postnatal age: prematurity affects microbiota composition, which is different between preterm and full-term infants[11, 21]. Preterm infants have a low diversity, with a delayed *Bifidobacterium* microbiota colonization, a high prevalence of *Enterobacteriaceae, Staphylococcus, Enterococcaceae* and other lactic acid bacteria as the genus *Lactobacillus* and *Weissella*[21]. In addition, preterm neonates have a greater number of pathogenic bacteria, such as *Escherichia coli* and *Clostridium difficile*[14].

- Fasting and type of enteric nutrition: feeding is one of the most important modulator of microbiota diversity and the first postnatal external component in contact with the neonatal intestinal tract[23, 48, 49]. In the early postoperative period the neonates may have variable periods of fasting; thereafter, the neonate diet can vary according to the condition and evolution, including own mother milk, donor milk, fortified human milk, or formula[5]. Breastfeeding induces intestinal enrichment in *Bifidobacterium spp., Lactobacillus* and *Bacteroide*s. In contrast, formula-fed neonates have an abundance of *Clostridia* and Staphylococci[48], and higher proportion of proinflammatory Gammaproteobacteria which delay intestinal maturation[50].
- Antimicrobial therapy: antibiotic treatment, used for pathogenic bacteria, also affects non-pathogenic bacteria[22]. Perinatal exposure to antibiotics, even prenatal, is an independent risk factor for NEC occurrence[22, 48], with consequences for later health[9, 11]. Infants, whose mothers have received antibiotics during labor, have decreased *Bifidobacterium* after birth and reduced *Bacteroides* at 3 months, changes that may persist until the first year of age[51]. Postnatal antibiotic treatment reduces *Bifidobacterium* spp., *Lactobacillus* and Clostridia and overgrowth of *Enterococcus spp*., *Staphylococcus* and Enterobacteriaceae compared with untreated[11, 22, 48, 51, 52], and diversity of microbiota have an inverse correlation with the duration of antibiotics treatment[52].
- H_2-receptor antagonist therapy: physiologically, human stomach produce acid decreasing the passage of viable pathogens into the distal intestine[53]. In some intestinal resections in neonates, H_2-receptor antagonists are used to reduce the effect of gastrin hyper-secretion[54]. Therapeutically inhibited gastric acid

production influence the microbiota[5, 55]. The use of H_2-receptor antagonists in preterm infants is associated with increased phylum Proteobacteria, which contains Enterobacteriaceae[41].

- Length of proximal remnant intestine: number and composition of microbes varies along the GIT[29]; and its concentration increases with proximity of the colon due to its relatively high pH and low peristaltism in comparison with the upper GIT[4]. To the best of our knowledge, only one study evaluated the microbiota composition according to the length of remnant intestine in newborn infants with enterostomy[28]; in infants with colostomy, there was a predominance of phyla Actinobacteria and Firmicutes, and *Bifidobacterium* and *Clostridium* at genus level[28]; in infants with ileostomy, there was a predominance of the phylum Proteobacteria, and *Bifidobacterium.* This last genera was initially dominant and decreased over the time, with an increase in *Streptococcus* and Enterobacteriaceae[28].

Primary outcome assessment

The microorganisms colonizing the proximal remnant intestine will be identified and quantified by using a precise technique culture-independent, real-time PCR analytical method that targets the bacterial 16S[56] and fungi 18S ribosomal RNA gene[57]. This is particularly sensitive, given its greater hybridization potential for primers on conserved regions, which target regions close to hypervariable regions[58]. In addition, it permits a quick analysis and it is not a very expensive technique. This method, based on the microorganisms DNA identification, has advantages providing information on viable and not-viable microorganisms. Therefore, it allows the quantification of microorganisms that become not-available with the contact with oxygen through the ostomy and during its stay within the

ostomy bag. Specifics primers allow precise quantification of each known species, and the use of SYBR Green permits the real-time monitoring of amplification of a targeted DNA molecule during the PCR.

The primers selected are representative of the principal phyla for a first general characterization. These selected primers are also specific for bacteria and fungi most commonly colonizing the intestine of newborn infants, as well as potentially pathogenic microorganisms identified in newborn infants cared in NICU.

The microbiota is collected longitudinally from the enterostomy's effluent samples. The periodicity of every 3 day change of ostomy bags determines the same periodicity for sample collection. The collection will be made within the first day after of the bag change, to minimize the bias of potential contamination by growth of microorganisms that may remain in the bag after aspiration.

The period scheduled for the study is 21 days after enterostomy is based on the average of stay of 16 days reported for newborn infants with corrective surgery of major CMGIT[59]. This period is much shorter than the reported stay of preterm infants with NEC needing surgery[60].

DISCUSSION AND CONCLUSION

While general factors affecting the microbiota in preterm and full-term newborn infants have been widely addressed in several studies[5, 6, 17-19], specific factors involved in particular conditions have less explored. It is still unclear which type of colonization occurs in newborn infants with enterostomy cared for in intensive care unit, influenced by specific factors such as postoperative fasting, exposure to antibiotics, H_2-receptor antagonists, length of remnant intestine, and contact with air through the ostomy. The knowledge about longitudinal intestinal colonization in in this population is scarce[24, 26-29].

This observational study is likely to have the following interests in infants with enterostomy: 1) to determine the pattern of colonization in three underlying conditions: CMGIT, NEC or SIP; 2) to identify pathogenic microorganisms with potential role in nosocomial infections, contributing to plan preventive and therapeutic measures.

Strength of this study should be acknowledged. This is the first longitudinal colonization assessment of the proximal remnant intestine in three distinct surgical conditions, during the first three weeks after enterostomy; this assessment is based on a sensitive technique to identify viable and not viable cells.

The limitations of our study should also be acknowledged. Firstly, in this single centre study, it may difficult to achieve a sufficient sample size to test certain outcomes - some aforementioned associations. Secondly, the contact with air through the ostomy may affect the colonization by anaerobe bacteria. Thirdly, in case the collection will not be made within the first day after of the bag change, the potential proliferation of bacteria in ostomy bag may be a bias. Fourthly, the bacterial identification will not be made by the more refined whole-genome analysis techniques, since non-availability of required specialist bioinformatics and other logistic support.

This exploratory study is promising for future research in the studied population, as it may serve as basis for interventional studies evaluating the effect of modulation (*eg,* prebiotics and probiotics) of the intestinal microbiota on short and long-term health.

To the best of our knowledge, this is the first protocol for a longitudinal study assessing the intestinal colonization in newborn infants with enterostomy.

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