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Gut colonization by aerobic microorganisms is associated with route and type of nutrition in premature neonates

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ABSTRACT

We hypothesized that the beneficial effects of early enteral compared with parenteral feeding are related to the increased variety of aerobic microorganisms that colonize the gut. Our aim was to describe the relationship, first, between the type of feeding and mucosal colonization and, second, between the type of feeding and the development of late-onset sepsis (LOS) and necrotizing enterocolitis (NEC) in preterm neonates. In total, 159 neonates aged 72 hours or less with risk factors for early-onset sepsis were recruited to a prospective 2-center study. Rectal swabs were collected on admission and twice per week thereafter. The feeding regimen was recorded for the first 7 days and categorized into total parenteral nutrition (TPN) and 2 regimens of enteral nutrition, that is, breast milk containing regimen (BMCR), for which breast milk constituted at least 11% of the enteral diet, or formula. Herein, 70 neonates received formula, 48 received BMCR, and 41 received TPN; 69 cases of LOS and 15 cases of NEC were observed in 50 neonates. A multiple logistic regression analysis indicated that formula and BMCR were associated with 4- to 5-fold increases in colonization by Gram-negative bacteria (odds ratio [OR], 4.52; 1.87-10.95, and OR, 4.95; 1.90-12.87, respectively) and 5 to 9 times higher odds of colonization by Gram-positive microorganisms (OR, 5.75; 1.89-16.72, and OR, 8.61; 2.52-29.36, respectively) compared with TPN. The only difference between BMCR and the other feeding groups was the higher colonization with *Staphylococcus haemolyticus* in the latter (formula—OR, 6.24; 1.73-22.50; TPN—OR, 2.75; 1.08-6.97). Compared with BMCR, TPN was associated with an increased odds of LOS (OR, 3.04; 1.02-9.07) and an increased odds of death (19.75; 3.64-107.12) compared with formula. Although early enteral feeding is associated with a higher odds of colonization with opportunistic microorganisms, it should be preferred over TPN whenever feasible, due to the favorable effect on the prevention of LOS.

Abbreviations: ALV, artificial lung ventilation; AR, ampicillin resistant; BMCR, breast milk containing regimen; BW, birth weight; CI, confidence interval; CoNS, coagulase-negative staphylococci; GA, gestation age; GIT, gastrointestinal tract; IQR, interquartile range; LOS, late-onset sepsis; MRSA, methicillin-resistant staphylococci; NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; OR, odds ratio; PROM, prolonged rupture of membranes; TPN, total parenteral nutrition.

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1. Introduction

Preterm neonates are at great risk of late-onset sepsis (LOS) [1] and necrotizing enterocolitis (NEC) [2] due to the immaturity of the immune system and exposure to risk factors, such as the premature rupture of membranes [3], indwelling catheters, total parenteral nutrition (TPN), prolonged mechanical ventilation, and gut colonization with potentially pathogenic microorganisms [4–6]. Strains that colonize the gut have been observed to genetically relate those that cause bloodstream infections, suggesting that mucosal colonization could be a prerequisite for neonatal sepsis, especially if the mucosal integrity is disturbed [7–10].

Microorganisms enter the gastrointestinal tract (GIT) via food or feeding devices [11,12]. However, the influence of the feeding regimen on intestinal colonization remains controversial. Some studies have found a higher abundance of aerobic bacteria in the GIT of formula-fed babies compared with breast-fed babies [13–15], whereas others have described the opposite finding [16] or no influence of the feeding modality [17]. Very few studies have examined the direct impact of TPN on gut colonization, but the shortening of TPN gastric villi has been shown to cause a loss of mucosal DNA, protein content, and enzymatic activity [18]. Despite the protective effect of breast milk in preventing neonatal sepsis [19–21], several case reports have shown that breast milk contamination by pathogenic microorganisms (group B streptococci, methicillin-resistant staphylococci [MRSA], and Enterobacteriaceae) has lead to LOS in premature babies [22–24]. The contamination of formula leading to invasive infection has also been reported [11,25,26].

Thus, the selection of the most appropriate early feeding regimen for critically ill neonates remains controversial. A better understanding of how the feeding regimen influences mucosal colonization and, thereby, is associated with invasive infection could contribute to the improved management and outcome of critically ill neonates.

We hypothesized that gut colonization depends on the route and type of early feeding regimen, more specifically, that enteral feeding compared with parenteral feeding leads to faster and more variable gut colonization and that the pattern of colonization depends on enteral feeding regimen (breast milk *vs.* formula). We aimed to characterize the influence of the feeding regimen on the gut colonization and development of LOS and/or NEC in preterm neonates treated at a third level neonatal intensive care unit (NICU).

2. Methods and materials

2.1. Study population and design

This study was a post hoc analysis of data collected in a prospective, cluster-randomized trial that primarily aimed to compare the efficacy of ampicillin with that of penicillin (both combined with gentamicin) in neonates at risk for early-onset sepsis. The study was conducted in two Estonian NICUs designated herein as unit A and unit B. The details of the study are described elsewhere [27,28]. The post hoc analysis included neonates admitted during the first 72 hours of life that met the following criteria: (1) stayed in the unit for more than 72 hours and (2) had a gestation age (GA) 32 weeks or less. Of the 276 neonates enrolled in the parent study, 159 qualified for this analysis. The excluded subjects either had a GA of 33 weeks or more (n = 104) or remained in the unit less than 72 hours (n = 13). The study was approved by the ethics committee of the University of Tartu and registered at ClinicalTrials.gov with the identifier number NCT00487019.

2.2. Data collection

The following demographic and clinical data were collected for each patient: GA, birth weight (BW), sex, artificial lung ventilation (ALV), unit, and study period (divided into first from August 2, 2006, to March 20, 2007, and second from March 21, 2007, to November 30, 2007), duration of NICU stay, empiric antibiotic regimen, use of broad-spectrum antibiotics, indwelling catheters, maternal age, antibiotic and steroid treatment during pregnancy, presence of prolonged rupture of membranes (PROM) for more than 18 hours or chorionamnionitis, multiple births, and mode of delivery.

The feeding regimen was documented on days 3 and 7 and categorized into 3 groups based on the route and character of the food: (1) TPN—at least 90% of daily caloric intake given via the parenteral route; (2) breast milk containing regimen (BMCR)—the mother's own fresh or frozen breast milk constituted at least 11% of enteral feed, and greater than 10% of daily calories were given via the enteral route; and (3) formula feeding—all remaining cases. A ready-made liquid preterm formula providing 82 kcal/100 mL, Nenatal by Nutricia, was used. Breast milk fortification was started only when an enteral volume of 100 mL/kg was reached. Total parenteral nutrition was initiated with glucose (4-6 $g\,kg^{-1}\,d^{-1}$) and amino acids (1 $g\,kg^{-1}\,d^{-1}$) within the first hours of life and increased by 2 $g\,kg^{-1}\,d^{-1}$ for glucose and 1 $g\,kg^{-1}\,d^{-1}$ for amino acids as tolerated. Lipids (0.5-1 $g\,kg^{-1}\,d^{-1}$) were started on the second day of life and advanced by 0.5 to 1 $g\,kg^{-1}\,d^{-1}$ as tolerated.

Necrotizing enterocolitis was diagnosed based on the Bell criteria [29] requiring the presence of at least two of the following clinical signs and symptoms without any other recognized reasons: vomiting, abdominal distention, prefeeding residuals, redness of the flanks, or persistent microscopic or gross blood in stools; or one of the following criteria: pneumoperitoneum, pneumatosis intestinalis, or unchanging "rigid" loops of the small bowel [29]. A small GA was defined as a BW below the 10th percentile. Twin percentiles were used for all multiple births. Culture-proven LOS was diagnosed when at least 2 clinical and 2 laboratory criteria of infection (hyper- or hypothermia; apnea or bradycardia spells; increased oxygen requirement; feeding intolerance; abdominal distension; lethargy and hypotonia; hypertension; skin and subcutaneous lesions, such as a petechial rash, abscess, scleroderma; white blood cell count <5 or >20 × 10^9/L; immature to total white blood cell ratio (I/T) >0.2; platelet count <100 × 10^9/L; CRP >10 mg/L) were present after 72 hours of life, and a pathogen (coagulase-negative staphylococci [CoNS] from at least two different specimens) was isolated from a normally sterile body fluid [27]. The outcome (LOS, NEC, and mortality) was recorded on the day of discharge or on day 60, whichever occurred earlier.

2.3. Sample collection and microbiological studies

Rectal samples were collected with transport swabs (Nuova Aptaca, Canelli, Italy) on admission to the NICU and then twice per week until discharge or day 16, whichever occurred first. Although rectal swabs may not necessarily reflect mucosal colonization, they are technically easy to perform and have been mostly used in similar studies [1,30]. Transport swabs were stored at –20°C for a maximum of 1 week and analyzed in batches at the Department of Microbiology, University of Tartu. After thawing, the swabs were directly plated onto blood, MacConkey, and Sabouraud agar. In addition, MacConkey agar containing 16 μg/mL of ampicillin was used to isolate Gram-negative ampicillin-resistant (AR) strains. The blood and MacConkey agar plates were incubated at 37°C for 24 to 48 hours in ambient air, and Sabouraud agar plates were incubated at 25°C for at least 1 week. Each morphologically different colony type was identified on the species and genus levels according to the Clinical and Laboratory Standards Institute criteria [31]. All isolates were stored in skimmed milk at –80°C for further research. Nonfermentative Gram-negative bacteria and staphylococci API commercial kits (API 20E, API 20NE, and API STAF; Biomerieux, Marcy l´Etoile, France) were used for the final identification of enterobacteria, whereas the CHROMagar Candida kit (BD BBL, Heidelberg, Germany) was used for yeasts. *Staphylococcus aureus* strains were tested for the *mecA* gene by polymerase chain reaction as described elsewhere [32,33].

Blood (0.5 mL) or cerebrospinal fluid (0.2 mL) in suspected cases of meningitis was cultured on admission, and symptoms suggestive of neonatal infection [27] were subsequently noted. Samples were processed using a BACTEC 9240 (Bac. Tec. LLC, Lincoln, USA) or VITEC 2 system in the microbiology laboratories of the University Clinics of Tartu or the North-Estonian Medical Center. The microbiological techniques remained unchanged throughout the study.

2.4. Statistical analyses

The software programs Sigma Stat for Windows 2.0 (Jandel Corporation, USA) and R 2.6.2 (A Language and Environment, http://www.r-project.org) were used for the statistical analyses. Differences in categorical values were compared with a χ^2 or Fisher exact test, and continuous variables are compared with a Student t test or Mann-Whitney test as appropriate. Normally distributed data are presented as means with SDs, and nonnormally distributed data are presented as medians with the interquartile range (IQR). Each pairwise comparison at a significance level of $P < .05$ underwent a Bonferroni correction.

To assess the influence of the feeding regimen and cofactors on rectal colonization, the development of NEC, LOS, and death, we applied a univariate logistic regression analysis as described for the data collection. All variables significant at a P value of .05 or less were entered into multiple logistic regression models with backward stepwise removal in order of insignificance. To avoid confounding effects of highly interrelated variables, only one variable was included. The BW and GA ($r = 0.816$) strongly correlated, as did the duration of NICU stay and postnatal age (PNA) ($r = 0.996$). Gestational age was considered a better determinant of immaturity than BW [1,34], and the duration of NICU stay was considered more important than PNA in influencing mucosal colonization.

3. Results

3.1. Study population

The characteristics of the study population are presented in Table 1. On day 7 of life, 44% of babies were fed formula, 30.2% were fed BMCR (30 of 48 or 62.5% receiving exclusively breast milk), and 25.8% received TPN. The latter group was the most immature (75.6% with GA ≤28 weeks and 73.2% with BW ≤1000 g) and had a higher prevalence of LOS and mortality than those in the enteral feeding groups (Table 1). The number of subjects receiving TPN was significantly greater in unit A than in unit B (odds ratio [OR], 47.40; 95% confidence interval [CI], 6.31-356.35); accordingly, the number of subjects receiving BMCR was smaller in unit A than in unit B (OR, 0.10; 95% CI, 0.05-0.22). Nevertheless, the wide confidence intervals should be noted.

A total of 69 culture-proven LOS cases in 50 neonates occurred during the study. Most of these cases (60%) was due to Gram-positive organisms, whereas Gram-negative bacteria and *Candida* spp. accounted for 34% and 6% of cases, respectively. Twenty-one culture-proven LOS cases (9 caused by Gram negatives) occurred during the first 7 days of life. Almost half (48%) of these neonates received TPN for the first 7 days of life, and the frequency of formula (24%) or BMCR (28%) use was similar. The distribution of causative agents of LOS in different feeding groups is presented in Table 2. Fifteen neonates had NEC, 7 of whom received TPN, 5 received formula, and 3 received BMCR. A total of 7 neonates were small for GA, 1 in TPN and 3 in the BMCR and formula groups each. Three of these patients had LOS (1 in TPN and 2 in the formula group), and 1 formula-fed neonate had NEC.

A total of 19 neonates died during the NICU stay, and 3 additional neonates died during the hospital stay. Only 1 of them received BMCR in the first week of life. The others were given TPN (n = 19) or formula (n = 2). Eight deaths occurred during the first 7 days of life; all were fed by TPN. Importantly, one third received broad-spectrum antibiotics (β-lactam + betalactamase inhibitors; III and IV generation cephalosporines; and carbapenems) in addition to an early empiric antibiotic regimen, but antibiotic administration did not differ between feeding groups.

3.2. Prevalence and dynamics of GIT colonization by aerobic and facultative anaerobic microorganisms

By day 16, approximately 90% of subjects were colonized by Gram-positive bacteria. Coagulase-negative staphylococci predominated the flora, accounting for 94% of Gram-positive isolates. Approximately 60% of the patients were colonized by Gram-negative microorganisms (Table 3). Enterally fed neonates were more frequently colonized by Gram-negative bacteria, including AR microorganisms, than those receiving TPN. Patients receiving BMCR were more frequently colonized by *Escherichia coli* than those receiving TPN, although the statistical significance of this difference was marginal.

Table 1 – Demographic and clinical characteristics of the study population

	TPN	Formula	BMCR
Number of patients	41	70	48
Neonatal factors			
Duration of NICU days, median (IQR)	13.3 (7.05-38)	11.4 (5.9-21.2)	14.55 (7.25-24.85)
% of patients in unit A vs unit B, %	97.6 vs 2.4 **	41.4 vs 58.6	22.9 vs 77.1
GA, week, means (SD)	26.27 (2.48) **	28.87 (2.46)	28.46 (2.48)
(min; max)	(23; 32)	(24; 32)	(22; 32)
≤28 weeks, no. (%)	31 (75.6) ***	28 (40)	25 (52.1)
BW, g, means (SD)	908.42 (262.57) **	1291.37 (408.58)	1229.06 (466.86)
≤1000 g, no. (%)	30 (73.2) **	21 (30)	17 (35.4)
Small for GA, no. (%)	1 (2.4)	3 (4.3)	3 (6.3)
Male vs female, no.	20 vs 21	37 vs 33	32 vs 16
Empirical antibiotics: pen vs amp, no.	16 vs 25	32 vs 38	24 vs 24
Duration of empirical AB in hours: pen/amp, median (IQR)	72 (48-102.6)/60 (55.5-126)	60.5 (49-102)/60 (60-120)	96.5 (60-144)/84.5 (60.5-153)
Additional broad spectrum AB, %	51.2	48.6	45.8
Neonates with sepsis, no. (%)	23 (56.1) **	20 (28.6)	14 (29.2)
EOS, no. (%)	1 (2.4)	3 (4.3)	3 (6.3)
LOS, no. (%)	22 (53.7) **	17 (24.3)	11 (22.9)
LOS-GN, no. (%)	9 (22)	9 (12.9)	5 (10.4)
Mechanical ventilation, no. (%)	39 (95.1) ***	52 (74.3)	42 (87.5)
NEC II, III, no. (%)	7 (17.1)	5 (7.1)	3 (6.2)
Death in NICU, no. (%)	17 (41.5) **	2 (2.9)	0
Death in hospital, no. (%)	19 (46.3) **	2 (2.9)	1 (2.1)
Central venous catheters, no. (%)	38 (92.7)	63 (90)	43 (89.6)
Arterial catheters, no. (%)	36 (87.8)	59 (84.3)	42 (87.5)
Maternal factors			
Multiple birth, no. (%)	10 (24.4)	21 (30)	10 (20.8)
Caesarian section, no. (%)	19 (46.3)	47 (67.1)	30 (52.5)
Antenatal steroids, no. (%)	34 (82.9) *	50 (71.4)	24 (50)
Antenatal antibiotics, no. (%)	6 (14.6)	15 (21.4)	16 (33.3)
Maternal chorionaminionitis, no. (%)	13 (31.7)	10 (14.3)	14 (29.2)
PROM >18 h, no. (%)	9 (22)	16 (22.9)	12 (25)
Mother's age, median (min; max)	29 (18; 43)	29 (19; 42)	30 (16; 44)

Statistically significant differences between study groups is calculated by using χ^2 or Mann–Whitney test and corrected with Bonferroni. Difference in the TPN group is compared against other groups.
Additional broad spectrum antibiotics included β-lactam + betalactamase inhibitors, III and IV generation cephalosporines, and carbapenems.
Abbreviations: AB, antibiotics; Amp, ampicillin; BW, bright weight; EOS, early onset sepsis; LOS-GN; late onset sepsis caused by Gram negatives; Pen, penicillin.
* $P < 0.016$ vs BMCR.
** $P < 0.016$ vs BMCR and formula.
*** $P < 0.016$ vs formula.

Staphylococcus haemolyticus was less frequent in those who received BMCR than those who received TPN or formula (Table 3).

3.3. *Association between feeding regimen, mucosal colonization, and development of LOS, NEC, and death*

As demonstrated in Table 3, a multiple logistic regression analysis showed that both enteral feeding regimens (BMCR and formula) were associated with 4 to 9 times higher odds of colonization by Gram-positive and Gram-negative microorganisms, including Gram-negative AR strains, than TPN. On a species level, enteral feeding was associated with enterococci. The only independent difference between BMCR and formula was a greater colonization by *S. haemolyticus* in the latter regimen compared with the former regimen (Table 4).

In terms of the influence of the feeding regimen on the development of LOS and NEC, we found that TPN during the first 7 days of life was associated with increased odds of LOS and in-hospital death, but not increased odds of NEC, compared with BMCR or formula feeding (Table 5).

4. Discussion

This study of premature neonates admitted to a third-level NICU demonstrated that early enteral feeding both via BMCR or formula is an independent risk factor of increased colonization by Gram-positive and Gram-negative microorganisms, especially by enterococci and AR strains, compared with TPN. Thus, we have proven one of our hypotheses, which stated that gut colonization is associated with the route of early feeding, but reject our hypotheses that this colonization is associated with the type of enteral feeding. Compared with TPN, early enteral feeding accelerates the acquisition of a larger variety of colonizing microorganisms with only a minimal difference arising from the type of

Table 2 – Distribution of causative agents of LOS in different feeding groups

	TPN	Formula	BMCR
Number of LOS cases	33	23	13
CoNS (%)	36.4	47.8	30.8
S. aureus (%)	9.1	8.7	23.1
Enterococci, staphylococci (%)	12.1	4.3	7.7
Enterobacteriaceae (%)	18.2	26.1	30.7
Nonfermentative (%)	12.1	13.0	7.7
Yeasts (%)	12.1	0	0

Statistically significant differences between study groups were not found by using χ^2 test and corrected with Bonferroni
Abbreviation: GN, Gram negative.

Table 3 – Percentage of patients with rectal colonization by selected microorganisms by day 16

Microbes	TPN	Formula	BMCR
Gram negative	36.5**	65.7	70.8
Gram-negative AR	17.1**	42.9	58.3
K. pneumonia	19.5	20	10.4
K. oxytoca	4.9	11.4	18.8
E. cloacae	14.6	28.6	25
E. coli	2.4●	8.6	18.8
Serratia spp.	2.4	5.7	14.6
Acinetobacter spp.	7.3	15.7	10.4
Stenotrophomonas spp.	2.4	1.4	2.1
Pseudomonas spp.	2.4	0	6.3
Gram positive	80.5[a]	91.4	95.8
S. aureus	7.3	10	20.8
MRSA	2.4	2.9°	16.7
CoNS in total	80.5	91.4	79.2
S. haemolyticus	48.8*	38.6*	8.3
S. epidermidis	41.5	52.9	60.4
S. hominis	22	12.9	4.2
Streptococcus spp.	0	14.3	4.2
Enterococcus spp.	26.8	40	47.9
C. albicans	22	11.4	10.4
Other *Candida* spp.	2.4	8.6	2.1

Values are percentages.
Gram negatives—ampicillin resistance; *S. aureus*—MRSA.
Statistically significant differences between study groups is calculated by using χ^2 test and correlated with Bonferroni.
*$P < 0.016$ vs BMCR (●$P = 0.018$; °$P = 0.021$; [a]$P = 0.04$).
**$P < 0.016$ vs BMCR and formula.

enteral feeds. The BMCR regimen prevents colonization by *S. haemolyticus* compared with formula or TPN. Thus, we suggest that the character of the feed is essential to gut colonization rather than the route of feeding. Despite greater mucosal colonization by potentially pathogenic microorganisms (eg, Enterobactriaceae and *Enterococcus* spp.), both enteral feeding regimens were associated with a lower odds of LOS and mortality in premature neonates compared with TPN, as also demonstrated in previous studies [19,21].

Previous studies have shown that in addition to preterm birth [35] and inappropriate initial microbial colonization [36], specifically by Enterobacteriaceae [37], enteral feeding may be a risk factor for NEC when associated with a rapid advancement of volume or high osmotic strength formula feeding [35,37]. Other studies have contradicted these results, and early enteral feeding has been suggested to decrease the risk of NEC [38]. Breast milk has also been suggested to play a role in reducing NEC [39]. In this study, which examined a limited number of NEC cases (n = 15), we did not observe an association between the feeding regimen and NEC, although numerically, most NEC cases (n = 7) occurred in the TPN group. Notably, patients with known risk factors for NEC (eg, low GA and BW) also predominated in the TPN group [35].

Most experts recommend breast milk (either the mother's own or donor milk) as the first food for premature infants [18,40]. However, mothers of very premature babies often have insufficient amount of breast milk, are too sick to breast feed, or are not in the same hospital/ward as the NICU during the first weeks after birth [19]. Hospitals with pasteurization facilities can use donor milk, but those without such facilities have to use special preterm formula. Although the protective role of human milk over formula in avoiding episodes of LOS in preterm infants has been demonstrated [19,21], the data remain inconclusive [41]. In our study, the only independent difference between BMCR and formula was a greater mucosal colonization by *S. haemolyticus* in the formula- than in the BMCR-fed babies. This finding is not surprising because *S. haemolyticus* is almost never found in breast milk [42,43] and is likely acquired from the hospital environment rather than from breast milk, similar to most other microorganisms [6]. The ability of breast milk feeding to reduce bloodstream infections caused by *S. haemolyticus* is less clear.

Breast milk is not a sterile environment. It almost always contains staphylococci and streptococci, but the presence of Enterobacteriaceae is not unusual either [42]. Therefore, the fact that breast milk is an important source for gut colonization is not surprising. Except in cases when formula is contaminated during the preparation process, formula is generally sterile. Despite this sterility, the microbial colonization rates with BMCR and formula in our study were similar, with very few exceptions. Microorganisms are believed to originate from the mouth as well as the nasogastric tubes during formula feeding because more than 80% of tubes are contaminated by various microorganisms, including Enterobacteriaceae, irrespective of the feeding regimen [11].

The most important issue in terms of colonization is its relevance to the development of invasive disease. Presently, several studies have demonstrated a genetic similarity between microorganisms that colonize the GIT (Enterobacteriaceae and CoNS) and those that cause bloodstream infections, suggesting that the two are directly connected [8,10]. In addition, bloodstream infections have been reported in neonates fed contaminated breast milk [22]. However, we and others have demonstrated that TPN is associated with an increased rate of invasive infections and mortality compared with breast milk and/or formula feeding, despite decreasing the rates of gut colonization [20]. Thus, whether mother's milk should be subject to a microbiological investigations and processed before being given to extremely premature babies remains open to interpretation. Further studies are needed before general guidance can be provided to neonatologists.

Enterococci are common colonizers of the GIT and are increasingly associated with neonatal sepsis [44]. A multivariate analysis showed that enterococcal colonization was

Table 4 – Association between nutrition habits with rectal colonization by selected microorganisms

Microorganism	Factor	Univariate logistic regression		Multivariate logistic regression	
		OR	95% CI	OR	95% CI
Gram-negative	BMCR vs TPN	**4.21**	**1.73-10.25**	**4.95**	**1.90-12.87**
	Formula vs TPN	**3.32**	**1.49-7.43**	**4.52**	**1.87-10.95**
Gram-negative AR	Formula vs TPN	**3.64**	**1.42-9.34**	**5.75**	**1.98-16.72**
	BMCR vs TPN	**6.80**	**2.21-18.40**	**8.61**	**2.52-29.36**
E. coli	BMCR vs TPN	**9.23**	**1.12-76.33**	4.24	0.32-55.26
Gram-positive	BMCR vs TPN	**5.58**	**1.11-27.96**	**8.76**	**1.31-58.57**
	Formula vs TPN	2.59	0.83-8.08	**5.20**	**1.23-22.07**
MRSA	BMCR vs formula	**6.80**	**1.38-33.61**	3.36	0.59-19.02
Enterococcus spp.	BMCR vs TPN	**2.51**	**1.03-6.13**	**3.52**	**1.30-9.56**
	Formula vs TPN	1.82	0.78-4.21	**3.04**	**1.16-7.91**
S. haemolyticus	Formula vs BMCR	**6.91**	**1.23-21.40**	**6.24**	**1.73-22.50**
	TPN vs BMCR	**10.48**	**3.18-34.53**	**2.75**	**1.08-6.97**
S. hominis	TPN vs BMCR	**6.47**	**1.31-31.94**	3.03	0.41-22.65

In univariate and multivariate logistic regression analysis, all feeding regimens and cofactors: GA, sex, ALV, ward and study period, duration of NICU stay, empiric antibiotic regimen, use of indwelling catheters, maternal age, antibiotic and steroid treatment during pregnancy, presence of PROM for more than 18 hours or chorionamnionitis, multiple birth, and mode of delivery, were applied. Statistical important differences for feeding regimen are presented in bold.

significantly more frequent with enteral feeding than with TPN. In vitro data suggest that GIT enterococci play a beneficial role because some strains of *Enterococcus fecalis* could suppress the proliferation of intestinal pathogens and thus prevent infection and the induction of inflammation [45]. Conversely, *Candida albicans* colonization was more frequent in neonates receiving TPN than in neonates receiving enteral feeds, a finding that was similar to previous reports [41,46], but this difference was not significant.

Several limitations of this study should be noted. First, this study was a post hoc analysis of data rather than a prospective comparative study evaluating the effect of different feeding regimens on gut colonization. Therefore, significant differences between study groups (eg, a greater number of patients receiving TPN in unit A than unit B or more vulnerable babies in the TPN group than the enteral feeding group) should be noted. However, comparative studies may no longer be feasible because early enteral feeding has become common practice. Second, the study included only patients who required level 3 neonatal intensive care. Thus, all patients had received at least one antibiotic regimen, but many had received several antibiotics; antibiotic administration did not differ between study groups. Antibiotics are well known to interfere with the development of the gut microflora. However, antibiotic-naive patients are extremely rare in a third-level NICU that admits critically ill patients [47]. Because the distribution of additional antibiotic therapy (in terms of frequency and choice of drugs) was similar between all feeding groups, we believe, that our conclusions on the impact of feeding regimen are not affected. We acknowledge, that the results are applicable to a specific clinical scenario, still, one that applies to the majority of extremely preterm neonates. Third, we only included potentially pathogenic microorganisms but acknowledge that the feeding regimen will also influence the colonization of beneficial bacteria, such as bifidobacteria and lactobacilli [48]. Nevertheless, we believe that our study population well reflects those hospitalized in a third-level NICU and should add important knowledge to everyday practice. Finally, the grouping of feeding regimens was an important drawback because most premature neonates receive a mixed regimen in the first week of life rather than pure breast milk or formula. We considered neonates receiving more than 10% of enteral calories from breast milk to belong to the BMCR group because even small amounts of microorganisms present in breast milk may influence gut colonization. Thus, the very small differences between the BMCR and formula groups may be partly due to the lack of clear differences between these two study groups. We believe that these limitations were adequately considered in the interpretations of our results.

In conclusion, although enteral feeding (especially breast milk) was associated with greater mucosal colonization by opportunistic microorganisms than TPN feeding, enteral foods appeared to prevent the development of LOS and in-hospital mortality in critically ill babies admitted to a third-level NICU. These data once again underline the benefits of the early introduction of enteral feeding either via breast milk, if available, or via specialized formula when breast milk cannot be given.

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Table 5 – Association between feeding regimens with development of NEC, LOS, and death

Complications	Factor	Univariate logistic regression		Multivariate logistic regression	
		OR	95% CI	OR	95% CI
LOS	TPN vs BMCR	**3.10**	**1.29-7.45**	**3.04**	**1.02-9.07**
	TPN vs formula	**3.19**	**1.43-7.15**	2.11	0.76-5.84
Death in NICU	TPN vs formula	**24.08**	**5.18-112.04**	**19.75**	**3.64-107.12**
Death in hospital	TPN vs BMCR	**40.59**	**5.10-322.83**	**43.25**	**2.98-628.50**
	TPN vs formula	**29.36**	**6.33-136.19**	**26.53**	**4.71-149.30**

In univariate and multivariate logistic regression analysis, all feeding regimens and cofactors: GA, sex, ALV, ward and study period, duration of NICU stay, empiric antibiotic regimen, use of indwelling catheters, maternal age, antibiotic and steroid treatment during pregnancy, presence of PROM for more than 18 hours or chorionamnionitis, multiple birth, and mode of delivery, were applied. Statistical important differences for feeding regimen are presented in bold.

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