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Efficacy of *Bifidobacterium breve* and *Lactobacillus casei* oral supplementation on necrotizing enterocolitis in very-low-birth-weight preterm infants: a double-blind, randomized, controlled trial[1–3]

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ABSTRACT

Background: Probiotics are used for the prevention of necrotizing enterocolitis (NEC) because of their positive effects on intestinal motor function, modulation of inflammatory response, and mucosal barrier function.

Objective: The objective was to assess whether the combined use of *Lactobacillus casei* and *Bifidobacterium breve* may prevent the occurrence of NEC stage $\geq$2 by the criteria of Bell in very-low-birth-weight preterm infants.

Design: A double-blind, randomized, controlled clinical trial was conducted in 231 preterm infants weighing from 750 to 1499 g at birth. The intervention group was composed of 119 infants who received human milk with probiotic supplementation (*B. breve* and *L. casei*) and a control group of 112 infants who received human milk containing no probiotics. The primary outcome was the occurrence of NEC stage $\geq$2 as defined by Bell's modified criteria.

Results: Four confirmed cases of NEC stage $\geq$2 by Bell's criteria occurred only in the control group.

Conclusions: Oral supplementation of *B. breve* and *L. casei* reduced the occurrence of NEC (Bell's stage $\geq$2). It was considered that an improvement in intestinal motility might have contributed to this result. This trial was registered at www.isrctin.org as number 67165178 (International Standard Randomized Controlled Trial). *Am J Clin Nutr* 2011;93:81–6.

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most serious emergency condition related to the digestive tract in preterm infants. It is a multifactorial disease in which the functional immaturity of the intestine plays an important role in its pathogenesis (1–4). Many authors have defended the use of probiotics for its prevention (5–20).

Probiotics are microbial supplements that have the capacity to produce beneficial effects in the host when administered in adequate quantities. Studies with animals have suggested that probiotic effects may be obtained by nonviable bacteria or by bacterial DNA (21, 22). The most frequently reported mechanisms that seem to justify the benefits of probiotics are as follows: a resistance to colonization, immunomodulation, and nutritional contribution (2–4, 21–26). More recently, the role of probiotics in intestinal motor function has also been similarly highlighted (27–29).

Deshpande et al (30), in a meta-analysis, found a significant reduction in the risk of NEC in preterm infants with the use of probiotics, and 3 of the analyzed clinical trials also showed a shorter time to reach full enteral feeding. The effect on intestinal motility may explain the effect of the use of probiotics in the prevention of NEC (28, 29).

The action attributed to probiotics is species specific (28). Although *Bifidobacterium* belongs to a dominant microbiota from the human digestive tract (31), few randomized clinical trials in human newborns receiving *Bifidobacterium* analyzed the prevention of NEC as a primary outcome (1, 32). It is believed that modulation of the immune response may be obtained when one or more probiotics are consumed concomitantly and act synergistically (5, 20). The objective of this study was to evaluate whether the combined use of *Bifidobacterium breve* and *Lactobacillus casei* prevent the occurrence of NEC (Bell's stage $\geq$2) in very-low-birth-weight preterm infants.

SUBJECTS AND METHODS

Study design

A prospective, double-blind, randomized controlled trial was conducted in which the treatment group received the probiotics during their first month of life, and the control group received no treatment. The primary outcome was the occurrence of confirmed NEC, classified in accordance with Bell's criteria, modified by Walsh and Kliegman (33).

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Study population

The study was conducted at the Instituto de Medicina Integral Professor Fernando Figueira in the city of Recife (capital of Pernambuco State, Northeast Brazil), where the maternity unit is a reference center for high-risk pregnancies for the whole state. All infants included in this study were born locally and admitted to the Neonatal Intensive Care Unit (NICU) with a birth weight from 750 to 1499 g. None of the infants had major congenital malformations, previously diagnosed life-threatening chromosomal alterations, and/or congenital infections diagnosed at birth. The infants were weighed during their first hour of life by the neonatologist in the delivery room (model BP no. 620/95 electronic scales with a 15-kg capacity; Filizola, São Paulo, Brazil), and the gestational age was estimated through clinical evaluation by using the Ballard method (34) during the first 24 h of life.

Once eligibility was established, mothers were informed about the study procedures, and their written informed consent was obtained for each infant before randomization. The mothers were interviewed by using a form containing a closed, precoded questionnaire regarding morbidity and details of antenatal and delivery care. All mothers were informed of the confidential nature of the data and were told that they were free to withdraw their children from the study at any time. The research was approved by the Research Ethics Committee at Instituto de Medicina Integral Professor Fernando Figueira (number 812). The External Study Committee evaluated all the results every 6 mo.

Sample size

The basis for calculating sample size was the incidence of confirmed NEC (Bell´s stage $\geq$2) of 10% at the study unit over the previous 2 y. On the basis of an α error of 0.05, a study power of 80%, and an estimated reduction of 60% in the incidence of confirmed NEC (1), the required minimum sample size was 282 infants in each study group. According to the hospital bed occupancy rate, the estimated duration of data collection was set at 2 y. One year after the intervention began, the External Study Committee observed a major benefit in one of the groups and recommended that the study be interrupted; at this time there were a total of 231 participants (119 in the probiotics group and 112 in the control group). Initially, 243 children were randomly assigned to 1 of the 2 groups; however, some newborns were admitted in very serious condition, which resulted in 3 deaths in the probiotics group and 9 deaths in the control group before the intervention began (**Figure 1**).

Randomization

Randomization was carried out in blocks of 10, and the list of random numbers was generated by the subprogram Epitable from Epi-Info 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Two trained external personnel from the NICU were responsible for obtaining this list and for randomly assigning the infants to the probiotics and control groups. A sealed envelope with the identification number in ascending order, containing information about which group they belonged to, was provided for each infant and sent to the hospital's nutritional center. Three previously trained research assistants, who were not part of the neonatal team, interviewed the mothers, recruited the infants, and sent the envelopes to the nutritional unit. The supplement (fractionation of human milk from the milk bank with added probiotics) was prepared by 2 nutrition assistants, who were supervised by a nutritionist who had no contact with either the team or the patients from the NICU.

Intervention and outcome

The intervention was initiated on the second day of life of all infants and was maintained until 30 d of life, a diagnosis of NEC, discharge from the hospital, or death, whichever occurred first. At 0900, the infants from the probiotics group received 3 mL human milk from the bank milk to which *L. casei* and *B. breve* had been added [half an envelope of Yakult LB (São Paulo, Brazil) providing 3.5×10^7 to 3.5×10^9 CFU; the infants in the control group received the same volume of human milk without

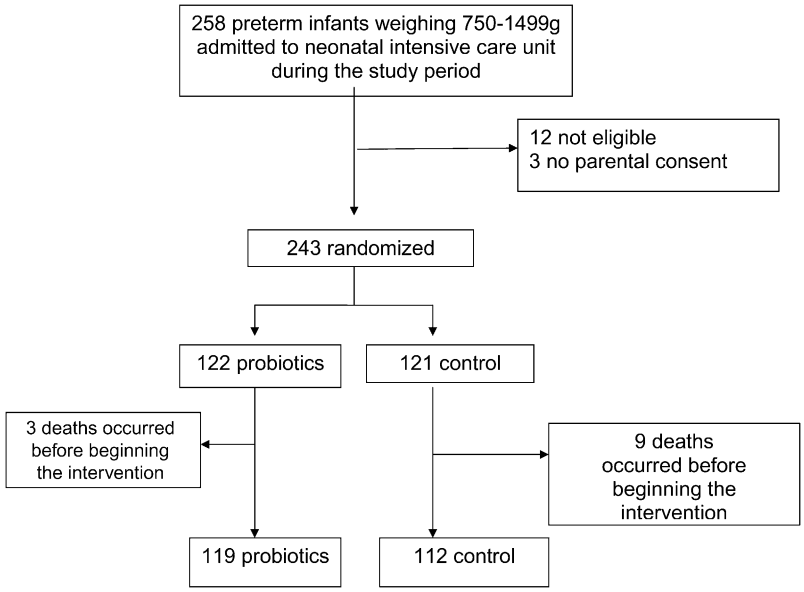

FIGURE 1. Clinical trial profile.

probiotics]. The probiotics and/or human milk were offered in glass receptacles, identified with the infants' respective names, between 30 and 60 min after preparation, even if the diet had been suspended. In infants using an orogastric tube, the tube was kept closed for 1 h after the probiotics plus human milk or human milk only were administered. Neither the medical and nursing staff responsible for monitoring the infants nor the researchers were aware of which group the infants were allocated to. The primary outcome was the occurrence of NEC, as defined by Bell's criteria and modified by Walsh and Kliegman as stage $\geq$2 (33).

Morbidities, procedures, and diet

Infants were monitored daily for 30 d. Their weight, information regarding all care provided, and their nutritional routine were recorded. Precoded forms were used to record any prescribed medication, use of oxygen therapy, type of venous access, and parenteral nutrition. A strict feeding protocol was followed for all infants in this study: 1–2 mL milk from the human milk bank or their mother's breast milk began when infants had stable vital signs; an increase in the daily diet was not to exceed 20 mL $\cdot$ kg^{-1} $\cdot$ d^{-1}. The diet was to be interrupted if there were signs of intolerance, defined as the presence of gastric residuals exceeding 25% of the volume offered within the previous 6 h, abdominal distension, or blood in the stool.

From the third week of life, for infants whose mothers did not have a sufficient breast-milk volume, a diet of human milk from the milk bank was alternated with specific formulas for preterm infants, according to the nutritional assessment. Parenteral supply of amino acids was initiated for all children with a birth weight $<$1300 g within the first 24 h of life, and total parenteral nutrition was maintained until it reached 100 mL $\cdot$ kg^{-1} $\cdot$ d^{-1} by enteral feeding.

Statistical analysis

The questionnaires were precoded and checked daily for consistency, accuracy, and completeness. The data were entered independently by 2 personnel using Epi-Info version 6.04 and the data entry was checked by the validating program.

Statistical analysis was conducted by using the Statistical Package for the Social Sciences (version 12.0; SPSS Inc, Chicago, IL). Student's *t* test or the Mann-Whitney *U* test was used to compare continuous variables, and the chi-square test was used to compare categorical variables. Fisher's exact test was used when appropriate. The Kaplan-Meier survival probability method was used to evaluate the time to achieving the complete transition of orogastric tube feeding to breastfeeding; the log-rank test was used to compare differences between groups. Statistical significance was set at $P \leq 0.05$.

RESULTS

A total of 231 newborn infants took part in the study between May 2007 and April 2008; 119 were allocated to the probiotics group and 112 to the control group. The baseline characteristics of the newborns were similar in both groups and are presented in **Table 1**. Only 4 confirmed cases of NEC (Bell's stage $\geq$2) were observed in the control group (**Table 2**).

Although no differences were observed between the groups in relation to the beginning of enteral feeding and advancements for enteral feeding volume, infants in the probiotics group achieved full enteral feeding faster than did those in the control group ($P = 0.02$; **Table 3**). The survival curve, determined with the Kaplan-Meier method to evaluate the complete transition time of orogastric feeding tube to breastfeeding, showed a shorter time in the probiotics group ($P = 0.03$; **Figure 2**).

DISCUSSION

Many meta-analyses have shown the protective effects of probiotics in preventing NEC, although differences were observed between the types of microorganisms, the dosage, and the time of use (30, 35, 36). Only a small number of randomized clinical trials to evaluate the prevention of NEC have evaluated the association of lactobacillus and bifidobacterium (1, 32). Different responses reported among the various probiotic bacteria, whether in the adhesive capacity of intestinal mucous, in the control of inflammatory cytokine production (9, 21, 22, 37), or in normalizing the intestinal motor function (28). Germ-free animal studies have shown the effects on intestinal function observed through determined species of bacteria, which could not be

TABLE 1
Baseline characteristics of very-low-birth-weight preterm infants in the probiotics and control groups[1]

	Probiotics group (n = 119)	Control group (n = 112)	P value
Use of antenatal corticoid [n (%)]	96 (82.0)	92 (82.1)	0.87[2]
Rupture of membranes >12 h [n (%)]	35 (29.4)	28 (25.0)	0.54[2]
Cesarean delivery [n (%)]	64 (53.8)	55 (49.1)	0.56[2]
Twins [n (%)]	19 (15.9)	25 (22.3)	0.23[2]
Apgar score at 1 min	5.7 $\pm$ 2.4[3]	6.1 $\pm$ 2.3	0.39[4]
Apgar score at 5 min	7.9 $\pm$ 1.5	8.1 $\pm$ 1.3	0.39[4]
Male sex [n (%)]	58 (48.7)	55 (49.1)	0.93[2]
Birth weight (g)	1194.7 $\pm$ 206.3	1151.4 $\pm$ 224.9	0.13[4]
GA (wk)	29.5 $\pm$ 2.5	29.2 $\pm$ 2.6	0.25[4]
Birth weight <10th percentile for GA [n (%)]	26 (21.8)	20 (17.9)	0.55[2]

[1] GA, gestational age.
[2] Chi-square test.
[3] Mean $\pm$ SD (all such values).
[4] Student's *t* test.

TABLE 2
Selected outcomes of very-low-birth-weight preterm infants in the probiotics and control groups[1]

	Probiotics group (n = 119)	Control group (n = 112)	RR (95% CI)
Use of umbilical catheter [n (%)]	90 (75.6)	87 (77.7)	0.97 (0.84,1.12)
Use of exogenous surfactant [n (%)]	56 (47.1)	48 (42.9)	1.10 (0.82,1.46)
Use of vasoactive amines [n (%)]	26 (21.8)	27 (24.1)	0.91 (0.56,1.45)
Use of theophylline [n (%)]	48 (40.3)	41 (36.6)	1.10 (0.79,1.53)
Use of dexamethasone [n (%)]	11 (9.2)	16 (14.3)	0.65 (0.31,1.33)
Use of parenteral nutrition [n (%)]	84 (70.6)	84 (75.0)	0.94 (0.80,1.10)
Use of antibiotics [n (%)]	99 (83.2)	101 (90.2)	0.92 (0.83,1.02)
Use of oxygen therapy [n (%)]	114 (95.8)	110 (98.2)	0.98 (0.93,1.02)
NEC, Bell's stage $\geq$2 [n (%)]	0 (0.0)	4 (3.6)	0.00[2]
Sepsis [n (%)]	40 (33.6)	42 (37.5)	0.90 (0.63,1.27)
Death [n (%)]	26 (21.8)	27 (24.1)	0.91 (0.56,1.45)

[1] NEC, necrotizing enterocolitis; RR, relative risk.
[2] 95% CI unable to be calculated (P = 0.05, Fisher's exact test).

extrapolated to others (28). Within the *bifidus* group, *B. breve* is the predominant species in preterm infants and appears to have more affinity with the immature intestine (6).

The probiotic used in this trial was made of *L. casei* and *B. breve,* and the confirmed cases of NEC occurred only in the control group, which suggests that the use of probiotics may prevent the occurrence of this outcome. Although some studies have evaluated the overall incidence of NEC, it should be highlighted that, because of the criteria used in the present study, these confirmed cases were only those classified according to Bell's criteria as equal to or above stage 2. In the initial stages, morbid conditions occur that can lead to a distended abdomen and may have a distinct pathogenesis from NEC.

The use of probiotics in clinical practice has been described as an attempt to mimic natural microbiota to obtain its beneficial effects, which, according to most researchers, helps improve the mucosal barrier function against the colonization of enteropathogenesis, immunostimulation, and immunomodulation (29, 38). However, research based on molecular biology concerning the role of intestinal microbiota has indicated that normal colonization with commensal bacteria modifies the expression of the genes involved not only in the mucosal barrier immunity and function but also in motility and neurotransmission (28). The present study analyzed the time taken to reach full enteral feeding, considered to be a volume of 150 mL $\cdot$ kg^{-1} $\cdot$ d^{-1}, as an indirect indicator of intestinal motility. A time reduction was observed in the probiotics group. Despite this small mean time difference for the acquisition of a full diet in infants in the probiotics group, we observed no difference in the gestational age (29.5 compared with 29.2 wk), progression rate, or type of diet between groups. Possibly, the use of probiotics could justify this difference, which agrees with the findings of previous studies (1, 6, 19, 30).

It is known that the pathogenesis of NEC encompasses a series of factors, among which prematurity is considered the main risk factor. Currently, it is believed that animal models used to investigate NEC reproduce situations that would explain the pathogenesis of this condition in term infants; however, they are not suitable for preterm infants. Unlike term infants, in whom ischemia and hypoxic injury play a predominant role in its pathogenesis, the cascade of events that culminate in intestinal injury in preterm infants is probably explained by the presence of food substrate in a functionally immature and inadequately colonized intestine (29).

Research on humans and animals has shown that the development of gastrointestinal motility begins in the second semester of pregnancy and develops until term. Gastric emptying is slower in preterm than in term infants. The propulsion of waves through the intestine is not observed before 33 or 34 wk of gestation. The increase in gastric residual volume, as a consequence of slower emptying and delayed intestinal transit, associated with deficient digestive functioning may expose the immature intestine to harmful substances (39–41).

A recent meta-analysis indicated the probable effect of probiotics on the maturation of the gastrointestinal tract (30). It is known that the intestinal microflora contributes to the maintenance

TABLE 3
Characteristics of enteral feeding of very-low-birth-weight preterm infants in the probiotics and control groups

	Probiotics group (n = 119)	Control group (n = 112)	P value
Age when enteral feeding began (d)	2.7 $\pm$ 1.9[1]	2.6 $\pm$ 1.1	0.46[2]
Duration of exclusive and/or predominant human milk (d)	24.5 $\pm$ 6.7	24.1 $\pm$ 5.7	0.65[2]
Rate of feeding increment (mL $\cdot$ kg^{-1} $\cdot$ d^{-1})	12.3 (6.12–17.8)[3]	11.8 (5.9–17.8)	0.88[4]
Time to reach full enteral feeding (d)	15.2 $\pm$ 5.2	17.4 $\pm$ 5.7	0.02[2]
Birth weight recovery up to 14 d of life [n (%)]	50 (42.0)	51 (45.5)	0.82[5]

[1] Mean $\pm$ SD (all such values).
[2] Student's t test.
[3] Median; interquartile range in parentheses (all such values).
[4] Mann-Whitney U test.
[5] Chi-square test.

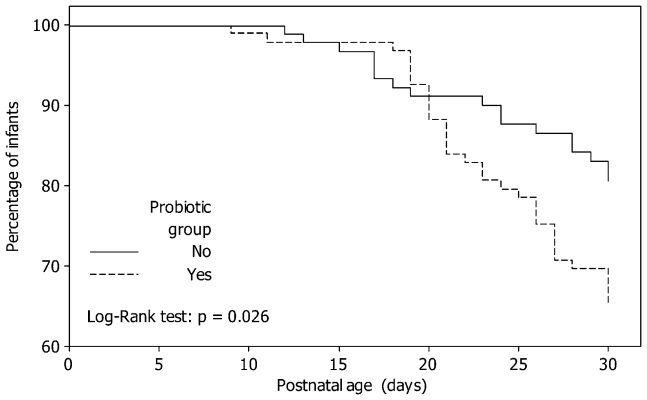

FIGURE 2. Kaplan-Meier survival curves showing the probability of not achieving the complete transition time of orogastric tube feeding to breastfeeding in the probiotics and control groups.

of the motor function of the gastrointestinal tract by releasing bacterial substances. The lymphoid tissue associated with the intestine modulates several digestive functions through the mediators of the immune cells, and the intestinal microflora is essential to the development of this tissue. Indrio et al (42) showed an increase in intestinal motility and gastric emptying using *Lactobacillus reuteri* in preterm infants. A recent study, conducted by Rougé et al (17) showed an improvement in the time taken to reach full enteral feeding in infants born with a weight >1000 g with the use of probiotics. It is possible that the protective effect of probiotics in the prevention of NEC is owed to its ability to bring about an improvement in intestinal motility.

This study considers the transition time of orogastric tube feeding to breastfeeding as a further indicator of the maturation of the digestive tract motor function in newborns. It was possible to perform the transition from one form of administration to another much earlier in the probiotics group than in the control group. The routine for this transition is based on the individualized evaluation of breathing with sucking and swallowing. This study did not identify clinical trials that used probiotics to evaluate the transition time between the means of administering feeding, but the benefits of anticipating breastfeeding in a population subjected to so many risks suggests that this outcome should be studied further. Similarly to previous clinical trials (1, 2, 4, 6, 7, 13–16, 32), the present study observed no complications related to the use of probiotics, although the study was not designed with this objective. Ohishi et al (43) reported a case of sepsis caused by *B. breve* administered as probiotic therapy. Probiotics can be a potential cause of an invasive disease and should be used with care in vulnerable patients. We observed no difference in the occurrence of sepsis or in the number of deaths between the probiotics and control group.

Deshpande et al (44) updated the systematic review published in 2007 by adding 4 new studies, and they concluded that further clinical trials are unnecessary because of the evident benefits of probiotics in preventing NEC. However, aspects related to the individualized effect of probiotics for each species, as well as the quality and safety of the used products, still to be better evaluated.

In summary, this study evaluated the effect of a combined supplementation of *B. breve* and *L. casei* in preterm infants with very low birth weight on the occurrence of NEC as a primary outcome. The use of probiotics seems to have had a beneficial effect on the occurrence of NEC at stage ≥2 according to Bell's criteria and was associated with an improvement in intestinal motility, evaluated on the basis of the time taken to reach full enteral feeding. The number of studies published that have evaluated the role of *Lactobacillus* and *Bifidobacteria* in the prevention of NEC is extremely low. Considering that the effects of probiotics are species specific, it remains to be determined whether there are any probiotic strains more suitable for preventing NEC in preterm infants.

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