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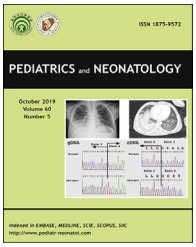

Original Article

Single strain versus multispecies probiotic on necrotizing enterocolitis and faecal IgA levels in very low birth weight preterm neonates: A randomized clinical trial

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Key Words
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Background: According to the literature, probiotics are an attractive alternative to prevent necrotizing enterocolitis (NEC). However, due to differences in probiotic composition, randomized controlled trials are necessary to compare different probiotic mixtures. The objective of this study was to compare single strain (*Lactobacillus acidophilus boucardii*) versus multispecies probiotics on NEC incidence and faecal secretory Immunoglobulin A (sIgA) levels in very low preterm newborns.

Methods: We performed a double-blind randomized trial in 90 newborns. *L. acidophilus boucardii* strain or multispecies probiotics were randomly assigned to preterm newborns. As the primary outcome, we evaluated NEC incidence on the total length of neonatal intensive care unit (NICU) stay. As the secondary outcome, we measured the change in faecal sIgA levels from baseline to 3 weeks following the use of probiotics.

Results: NEC incidence was similar between groups (0% vs. 2.2% for the single strain and multispecies probiotic, respectively). Faecal sIgA levels increased significantly ($p < 0.001$) within groups (31% for single strain and 47% for multispecies probiotic), but this increase was not different between groups. Neonates with a faecal sIgA level increment >0.45 mg/dl showed higher gestational age, birth weight, and weight at the second and third weeks of follow up

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than neonates with a faecal sIgA level increment ≤0.45 mg/dl. No adverse effects were found after probiotics use.
Conclusions: No difference between strains of probiotics used was found on NEC incidence or in the increase of faecal sIgA levels. Faecal sIgA levels were positively related to gestational age and body weight in very low preterm infants. ClinicalTrials.gov/NCT02245815.

1. Introduction

Necrotizing Enterocolitis (NEC) is still a devastating disease in preterm newborns. Factors such as genetical predisposition, immature intestinal barrier, disequilibrium in the microvascular tone, and abnormal microbial gut colonization have been involved in its physiopathology.[1]

Although NEC is not an acute bacterial insult, infants that develop NEC seem to acquire an altered intestinal microbiota, which influences intestinal homeostasis weeks prior to the onset of clinical symptoms.[2] In general, during the first week of life, the intestinal microenvironment of a newborn is widely colonized by *Actinobacteria* (including *Bifidobacterium*), *Proteobacteria, Bacteroides* and fewer *Firmicutes* (including the *Lactobacillus* spp; which dominates vaginal flora).[3] In contrast, newborns with a birth weight <1200 g are primarily colonized by both *Firmicutes* and *Tenericutes*, with lower presence of *Actinobacteria*.[4] Previous studies have suggested that fetal inoculation occurs through the placenta according to the gestational age. Furthermore, bacteria in the meconium, such as *Escherichia coli*, *Enterococcus faecium*, and *Staphylococcus epidermidis*, could be present because of translocation of the intestinal microbiota through blood.[5]

Controlled clinical trials with probiotic bacteria have demonstrated the mechanisms involved in their beneficial effects, such as the improvement of the epithelial barrier function, competitive exclusion of pathogens, direct anti-inflammation effects over the lines of epithelial signalization, and an increase in secretory immunoglobulin A (sIgA) levels. This evidence supports the use of probiotics as a therapeutic strategy for the prevention of NEC in newborns.[6,7]

Furthermore, probiotics have been widely studied in infants; for example, a recent study suggests that probiotics may be beneficial as an adjunct treatment for term infants with hyperbilirubinemia by reducing the duration of hospitalization,[8] and in the prophylaxis and treatment of urinary tract infections in children.[9]

The lower prevalence of protective *Lactobacillus* or *Bifidobacterium* species in preterm infants compared to term infants makes these species of probiotics a target for the prevention of NEC.[10] A systematic review to assess the efficacy and safety of probiotics for the prevention of NEC in preterm infants found that severe NEC was significantly reduced in trials where patients received probiotics. In addition, the combination of 2 probiotics (*Lactobacillus acidophilus* with *Bifidobacterium bifidum*) seemed to produce the greatest benefits.[11] This has been reinforced by a recent meta-analysis reporting that multiple strains of probiotics seemed to be the most effective way to prevent NEC and reduce mortality in preterm infants of ≤34 weeks' gestation or of a birth weight ≤ 1500 g.[12] However, due to the differences in probiotic components and administration, it would be necessary to perform randomized controlled trials comparing different probiotic mixtures. Therefore, the purpose of this study was to compare *L. acidophilus boucardii* strain versus multispecies probiotic on NEC incidence and faecal secretory IgA levels in very low birth weight preterm neonates.

2. Methods

We performed a double-blind randomized clinical trial in 90 preterm newborns in a tertiary healthcare inpatient unit from January 2014 to May 2015. The protocol was registered at the US National Institutes of Health, ClinicalTrials.gov/NCT02245815. Newborns were selected for consecutive cases that met the inclusion criteria to complete the sample size. Then, through a random-number-table, they were individually randomized to one of the two parallel groups with a 1:1 allocation ratio. Health care providers and data collectors were blinded to the intervention group.

Preterm newborns between 700 and 1500 g from less than 33 gestational weeks were included in the study, with no counter indication for enteral nourishment in the first 7 days of extrauterine life. They were recruited the day they started enteral nourishment according to the decision of their physicians.

We excluded preterm patients with >1500 g, Apgar < 6 at 5 min, gastrointestinal malformations, patent ductus arteriosus with hemodynamic alteration and septic shock.

2.1. Ethical considerations

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the local ethical committee R-2013-1002-7. Written informed consent was required from the parents in order for their children to be included in the study.

2.2. Intervention

Newborns randomly assigned received either 1×10^9 [CFU] of *L. acidophilus boucardi* strain or multispecies probiotic which contains 1×10^9 [CFU] of *L. acidophilus*, 4.4×10^8 [CFU] of *Lactobacillus rhamnosus*, 1.0×10^9 [CFU] *of Lactobacillus casei*, 1.76×10^8 [CFU] of

Lactobacillus plantroom, 2.76 × 10^7 [CFU] of *Bifidobacterium infantis, and* 6.6 × 10^5 [CFU] of *Streptococcus thermophilus*.

Both probiotics in powder presentation form were mixed in the feed each 24 h during three weeks and no color/odor of the milk given to the infants changed. Nurses who were blinded to the group assignment individually prepared fresh supplement suspensions. They were not directly involved in the routine care of patients using *L. acidophilus boucardi* strain (Carnot® laboratories, scientific products, S.A. de C.V., registered by the Ministry of Health in Mexico: 274M91 SSA) or the multispecies probiotic (ITALMEX laboratories, scientific products, S.A. de C.V., registered by the Ministry of Health in Mexico: 107M96 and 106M96). We sought the commercially available product in Mexico that best reached bacterial concentration and strain combination as suggested by Timmerman HM et al.,[13] for example not including *Bacteroides* or *Enterobacteriaceae* considering they have high activities of xenobiotic metabolizing enzymes like β-glucuronidase. Both probiotics were preserved at room temperature. Quality control records were available at any time from the producer and we reviewed them twice during the study period.

Before the study intervention, all newborns were given enteral trophic stimulation through 5 days, followed by increments of 20 ml/kg by day of breast milk or premature formula, according to the guidelines of clinical practice for enteral nourishment in premature newborns. Probiotics were started on day 5 in both groups. The diagnosis of NEC was performed by their treating physician according to Bell's criteria. Evolution, treatment, complication, and motive for departure were monitored.

2.3. Following

Alimentation was interrupted if there were any signs of intolerance such as vomiting, the presence of biliary or bloody content by the orogastric probe, abdominal distension or evacuations with blood. They all received parenteral nutrition until they arrived at 100 ml/k/day of enteral nourishment. Presence of sepsis was monitored according to clinical signs of systemic inflammatory response and positive blood culture.

Blood cultures with gram-positive, negative and anaerobic cards were taken if there was data of systemic inflammatory response and NEC EII using BacT/ALERT® [REF] 259794 20ml Pediatric (Biomereiux, Francia) INC. Durham, NS 27704.

Faeces (10 g) were taken at baseline and at the end of the third week of probiotic administration. The samples were processed and frozen to −70°c, the concentration of IgAs was quantified using an immune enzyme test: Calbiotech Secretory IgA ELISA (Calbiotech, SC221A, Spring Valley, CA).

2.4. Sample size and statistical analysis

The sample size was calculated according to the desired difference of 20% of the incidence of severe NEC after comparing both probiotics according to $\alpha = 0.05$ and $\beta = 0.20$ values. A total of 45 patients by group was obtained and intent-to-treat analysis was performed. Two sample t test or Mann—Whitney test was carried out to evaluate differences in continuous variables between both study groups; Chi-squared test was used for differences in mortality and complications. Statistical significance was considered when $p < 0.05$.

3. Results

Ninety-two newborns were included in the study. Two patients were excluded for coarctation of the aorta and intestinal atresia, leaving the group randomly assigned to *L. acidophilus boucardii* strain (Group A) or to multispecies probiotics (Group B) with 45 patients each (Fig. 1).

The characteristics of the study population are shown in Table 1. No difference was found in birth weight, gestational age, Apgar scores or delivery method between groups. During the 3 weeks of study period, 78 children received antibiotics (group A: 38; group B: 40).

At the end of the study, no difference in body weight between groups was found.

Newborns were fed by breast milk in 60% vs. 80% ($p = 0.04$), by mixed nourishment (22 vs. 16%; $p = 0.47$) or by formula (18 vs. 4%; $p = 0.03$) in group A and B, respectively.

Two patients in group B developed feed intolerance after starting the intervention and probiotic was interrupted for 3 days, but it was restarted without any other adverse effect.

Only one patient assigned to multispecies developed NEC stadium IIA between the first and second week of life. No patient died during this study.

Faecal sIgA levels increased significantly ($p < 0.001$) within groups (31% for single strain and 47% for multispecies probiotic), but this increase was not different between groups ($p = 0.14$) (Fig. 2).

Baseline and final faecal sIgA levels were higher in group B than in group A (Table 1).

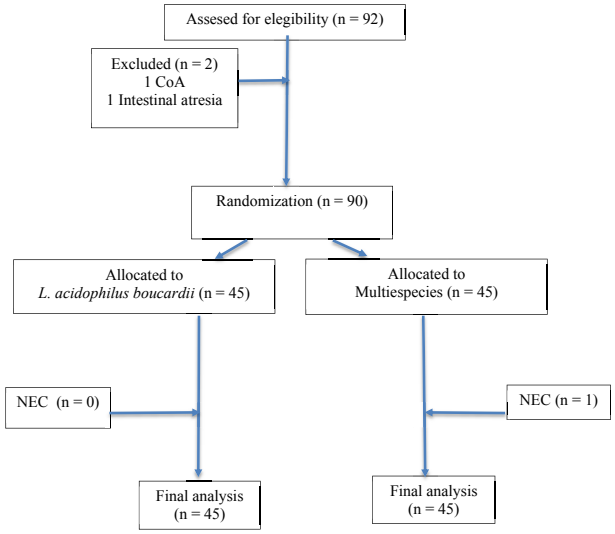

Figure 1 Enrollment, randomization, and follow-up of study patients.

Table 1 Clinical characteristics and faecal sIgA levels in study patients.

Variable	Group A N = 45	Group B N = 45	P value
Gender F/M	24/21	23/22	0.835
Cesarean section, n (%)	33 (73.3)	36 (80)	0.652
Gestational age (weeks)	30.3 ± 1.83	31.1 ± 2.3	0.053
Small for gestational age, n (%)	15 (33)	13 (28)	0.607
1 min Apgar	6.6 ± 1.4	7.1 ± 1.0	0.103
5 min Apgar	7.8 ± 1.1	8.2 ± 0.9	0.064
Birth weight (g)	1175 ± 21	1214 ± 24	0.433
Antenatal steroid use, n (%)	25 (55.5)	26 (57.7)	0.833
Surfactant use, n (%)	17 (37)	16 (35)	0.843
Days of antibiotic, median (min–max)	12 (0–21)	13 (0–23)	0.338
Days of parenteral nutrition, median (min–max)	14 (4–28)	15 (0–34)	0.347
Days to reach total feeding, median (min–max)	18 (0–56)	15 (0–39)	0.092
Days of mechanical ventilation, median (min–max)	5 (0–26)	4 (0–29)	0.436
Days of continuous pressure of breathing, median (min–max)	6 (0–33)	7 (0–54)	0.687
Days of study, median (min–max)	28 (8–32)	26 (3–45)	0.524
Baseline sIgA levels (mg/dl), mean (95% CI)	0.67 (0.53–0.81)	1.5 (1.2–1.7)	0.001
Final sIgA levels (mg/dl), mean (95% CI)	0.97 (0.85–1.1)	2.0 (1.9–2.2)	0.001

Group A *Lactobacillus acidophilus boucardii* strain.
Group B *Multispecies* strain.

We found that neonates who increased faecal sIgA levels >0.45 mg/dl (the mean value) in the entire group showed higher gestational age and weight at baseline, at 2 and at 3 weeks of follow up than neonates who increased faecal sIgA levels ≤0.45 mg/dl (Table 2).

4. Discussion

This essay shows the tolerance of very low birth weight preterm neonates to two tested probiotics. No patient developed sepsis for these strains or alterations in evacuation characteristics. In contrast, cholangitis and bacteremia have been reported in preterm newborns who received probiotics.[14,15]

More group B patients received an exclusively breastmilk diet compared to group A patients. This is relevant because it has been reported that breastmilk bifidobacteria promote intestinal acidification and are linked to levels of faecal secretory IgA and lysozyme. Furthermore, bifidobacteria have been shown to constrain the implementation and development of intestinal microorganisms such as *E. coli*,

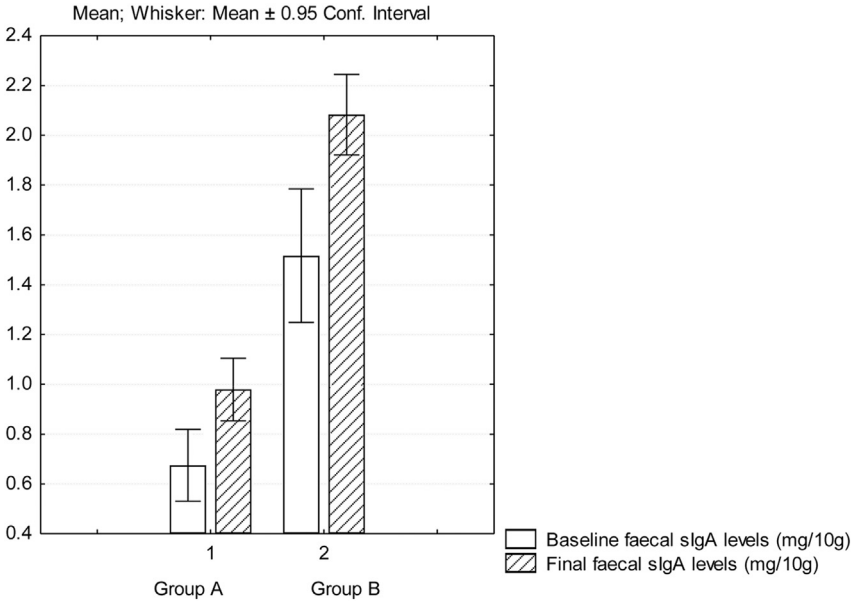

Figure 2 Comparison of change in faecal sIgA levels between groups (p = 0.14). Group A = *L.acidophilus boucardii* strain, Group B = Multispecies.

Table 2 Comparison of weight and gestational age in neonates who increased faecal sIgA levels ≤ or >0.45 mg/dl.

Variables	sIgA ≤0.45 mg/dl	sIgA >0.45 mg/dl	P value
Gestational age (weeks)	30.2 ± 2.0	31.4 ± 2.1	0.012
Birth weight	1105 ± 250	1264 ± 183	0.002
Bodyweight at week 1	1176 ± 266	1325 ± 211	0.009
Bodyweight at week 2	1384 ± 286	1534 ± 243	0.010
Bodyweight at week 3	1575 ± 269	1705 ± 206	0.022

Shigella, amoebas, and others.[16] However, other studies have found contrasting results.[17]

Severe NEC was detected in only one newborn; this could suggest that both probiotics decrease its incidence, supported this by similar results previously reported for other strains of probiotics.[18,19] However, there is no strong evidence because of the lack of control group in our study.

A systematic review pooling 3521 newborns found that probiotics reduced NEC incidence (RR 0.39; 95% CI: 0.26—0.57) and mortality (RR 0.70; 95% CI: 0.52—0.93) with no difference to placebo regarding late-onset sepsis (RR 0.91; 95% CI: 0.78—1.06). In the analysis of the different strains, the combination of 2 probiotics (*L. acidophilus* with *B. bifidum*) seemed to produce the greatest benefits.[11] This is concordant with a recent meta-analysis reporting that multiple strain probiotics seemed to be the most effective way to prevent NEC and reduce mortality in preterm infants of ≤34 weeks' gestation or of a birth weight ≤ 1500 g.[12] However, in another meta-analysis, the incidence of severe NEC was significantly reduced in infants receiving *Lactobacillus* (8 trials) -RR 0.61 95% CI [0.40—0.95], *Bifidobacterium* (6 trials) -RR 0.37 95% CI [0.14—0.97], or multispecies (two or more) supplement (18 trials) -RR 0.41 95% CI [0.29—0.56] without mentioning any difference between strains.[20]

We also observed an increase in faecal sIgA levels in both groups. Mohan et al.[21] observed a 45% increase in the total concentration of faecal sIgA with probiotic supplementation. However, the increasing faecal sIgA might only occur because of increasing of age, not probiotics.

We believe that the higher baseline sIgA levels observed in group B are due to differences in diet and gestational age between the two groups. However, 12% patients (8% vs. 4% in groups A and B, respectively) who did not receive breast milk also had increased faecal sIgA levels.

In the entire group, patients with lower birth weight, lower weight during follow up, and lower gestational age showed lower faecal sIgA levels. In term infants, Koutras and Vigorita found that, during the first 8 weeks of extrauterine life, faecal sIgA increased in breastfed infants versus standard formula-fed infants. This effect could be caused by the presence of IgA in breast milk and by a stimulatory effect of breast milk on the gastrointestinal humoral immunologic development.[22] However, in preterm infants, a fermented formula with two probiotic strains, *Bifidobacterium breve* C50 and *S. thermophilus* 065, showed that secretory IgA increased with both mother's milk and the fermented formula.[17] No studies have so far related faecal sIgA levels with gestational age and birth weight in preterm newborns. We consider that these two factors have to be taken into account for future research in probiotics use.

A limitation of our study is that the evaluation of breast milk administered to each newborn was imprecise because of the lack of a milk bank. Also, a number of patients received antibiotics, creating a possible source of bias. Finally, we did not include a control group without any interference to evaluate NEC incidence and change in faecal sIgA levels in preterm neonates in our study population.

In conclusion, we found that NEC incidence was similar in preterm neonates using *L. acidophilus boucardii* strain and multispecies probiotic supplementation.

In both groups, faecal sIgA levels increased between baseline and final evaluation and its levels were positively related to gestational age and body weight.

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Conflict of interest

The authors have no conflicts of interest relevant to this article.

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