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# ORIGINAL ARTICLE

# Oral probiotics: *Lactobacillus sporogenes* for prevention of necrotizing enterocolitis in very low-birth weight infants: a randomized, controlled trial

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**Background/Objective:** The identification of probiotic species involved in gut homeostasis and their potential therapeutic benefits have led to an interest in their use for preventing necrotizing enterocolitis (NEC). Although bifidobacterium and lactobacilli sp. have been used to reduce the incidence of NEC in clinical trials. *Lactobacillus sporogenes* has not been used in the prevention of NEC in very low-birth weight infants yet. The objective of this study was to evaluate the efficacy of orally administered *L sporogenes* in reducing the incidence and severity of NEC in very low-birth weight (VLBW) infants.
**Subjects/Methods:** A prospective, blinded, randomized controlled trial was conducted in preterm infants with a gestational age of $<33$ weeks or birth weight of $<1500$ g. VLBW infants who survived to start enteral feeding were randomized into two groups The infants in the study group were given *L. sporogenes* with a dose of 350 000 000 c.f.u. added to breast milk or formula, once a day, starting with the first feed until discharged. The infants in the control group were fed without *L. sporogenes* supplementation. The primary outcome measurement was death or NEC (Bell's stage $\geqslant 2$).
**Results:** A total of 221 infants were studied: 110 in the study group and 111 in the control group. There was no significant difference in the incidence of death or NEC between the groups. Feeding intolerance was significantly lower in the probiotics group than in the control group (44.5% (*n*: 49) vs 63.1% (*n*: 70), respectively; $P=0.006$).
**Conclusions:** *L. sporogenes* supplementation at the dose of 350 000 000 c.f.u/day is not effective in reducing the incidence of death or NEC in VLBW infants, however, it could improve the feeding tolerance.
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**Keywords:** necrotizing enterocolitis; preterm infant; probiotics

## Introduction

Necrotizing enterocolitis (NEC) is the most common, serious acquired disease of the gastrointestinal tract in preterm infants. Despite advances in neonatal care, it remains a leading cause of morbidity and mortality in neonatal intensive care units (Alfaleh and Bassler, 2008; Hunter *et al*., 2008).

Colonization by commensal bacteria is required for the normal development and maturation of the newborn intestine, virtually sterile at birth (Dai and Walker, 1998). Lactobacilli and Bifidobacteria are the principal kind of probiotics bacteria that predominate in the normal gut flora of healthy, breast-fed term neonates (Orrhage and Nord, 1999). In contrast, the intestine of the preterm infant tends to be colonized by different microorganisms, predominantly by coliforms, enterococci and by bacteroides species. The combination of an increase in potentially pathogenic microorganisms, together with a decrease 'in normal flora' found in preterm infants, is one of the factors that render these infants at an increased risk of developing NEC (Hall *et al*., 1990; Claud and Walker, 2001).

Identification of probiotics bacterial species involved in gut homeostasis and potential therapeutic benefits of probiotics have led to an interest in their use for preventing NEC (Alfaleh and Bassler, 2008; Embleton and Yates, 2008).

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Probiotics compete with other microbes for binding sites and substrates in the bowel, and produce a wide range of antimicrobial substances, such as bacteriocins, microcins, reuterin, hydrogen peroxide and hydrogen ions (Talarico *et al*., 1988; Reid *et al*., 1990; Juven *et al*., 1991). Bin-Nun *et al*. (2005) and Lin *et al*. (2005 and 2008) showed that orally administered probiotics, such as *Bifidobacterium infantis, Bifidobacterium bifidus, Lactobacillus acidophilus*, reduce the incidence of NEC in very low-birth weight (VLBW) infants. *Lactobacillus sporogenes* has been successfully used in the prevention of antibiotic-associated diarrhea in children (La Rosa *et al*., 2003). Indications for the use of *L. sporogenes* cover all the usual range of probiotics, such as lactose intolerance, gastrointestinal infections, dyspepsia and urinary tract infections (De Vecchi and Drago, 2006). Although bifidobacterium and lactobacilli sp. have been used to reduce the incidence of NEC in clinical trials (Dani *et al*., 2002; Bin-Nun *et al*., 2005; Lin *et al*., 2005, 2008), *L. sporogenes* has not been used in the prevention of NEC in VLBW infants yet.

We, thus, hypothesized that *L. sporogenes* supplementation could reduce the incidence and severity of NEC in VLBW infants. To evaluate this possibility, we planned a prospective, blinded, randomized controlled study.

## Patients and methods

A prospective, blinded, randomized controlled trial was conducted in the neonatal intensive care unit (NICU) of Zekai Tahir Burak Maternity Training Hospital in Turkey between October 2008 and June 2009. Preterm neonates with a gestational age $<33$ weeks or birth weight $<1500$ g, who survived to feed enterally, were eligible for the study. Criteria for exclusion were major congenital malformations and lack of parental consent.

The infants were randomly assigned to one of two groups prospectively. Randomization was performed by using sequential numbers generated at the computer center of the NICU. The allocations were contained in opaque, sequentially numbered sealed envelopes. The study group was fed with *L. sporogenes* (DMG ITALIA SRL, Rome, Italy) with a dose of 350 000 000 c.f.u. once a day with breast milk or mixed feeding (breast milk and formula) Milupa Prematil Formula (Milupa AG, Friedrichsdorf, Germany) was used in the study, starting with the first feed until discharged. The dose of *L. sporogenes* supplementation was empirically decided regarding the results of a previous study performed in children (La Rosa *et al*., 2003). The control group was fed with breast milk or formula without the addition of *L. sporogenes*. *L. sporogenes* was kept in a dry place at room temperature, away from light and humidity, in tightly closed container, and mixed with breast milk or formula before feeding. *L. sporogenes* was prepared using a sterile technique utilizing a suspension of freeze-dried powder in breast milk or formula to obtain 350 000 000 c.f.u./ml. Immediately after the preparation, 1 ml suspension was added to the breast milk or formula. This supplementation did not change the physical appearance of the milk and the formula. Fresh suspension of supplements were prepared by personnel in the breast-milk team who were not involved in the care of the infant and who followed instructions from the sealed envelope. Thus, the only personnel who knew of the infants' group assignments were the investigators and those in the breast-milk team who were not involved in the care of the study infants.

Feeding was started when the infant had stable vital signs, had active bowel sound without abdominal distention and had no bile or blood from the nasogastric tube. Feeding consisted of breast milk or formula, and was started at 10–20 ml/kg depending on the gestational age of the infant. The amount of feeding was advanced if tolerated with no more than a 20 ml/kg per day. If there were $\geqslant 2$ signs of feeding intolerance (gastric residuals in the amount that was more than half of the previous feeding, abdominal distention or heme-positive stools) feeding was stopped and was withheld as long as the signs of feeding intolerance continued. Infants who weighed $<1000$ g, received total parenteral nutrition until half of the calories were supplied by the oral route.

Whenever an infant was suspected to have NEC, the infant was evaluated by two senior-attending neonatologists who did not know the group assignment of the infant. Clinical signs and abdominal radiographs were reviewed and NEC was categorized by modified Bell's classification. The cases of NEC that had reached Bell's stage 2 or higher were considered (Walsh *et al*., 1988).

The main action of *Lactobacillus* sp. seems to consist in stimulating and in regulating the intestine's host-defense mechanisms (Isolauri, 1999). However, this effect may not occur immediately, therefore, in this study the cases of NEC or deaths occurring after 7 days of *L. sporogenes* supplementation have been taken into account.

Demographic and clinical variables that are risk factors for NEC, including prenatal steroid use, small for gestational age, prolonged rupture of amniotic membranes, chorioamnionitis, asphyxia, the indications for surfactant and ibuprofen, intraventricular hemorrhage and sepsis were prospectively recorded. Duration of mechanical ventilation, antibiotic treatment, umbilical venous catheterization, parenteral nutrition, type of feeding and age at the beginning of feeding and the duration of *L. sporogenes* were also recorded.

Primary outcome of present study was death or stage $\geqslant 2$ NEC. Secondary outcomes were culture-proven sepsis without NEC, grades 3–4 intraventricular hemorrhage, feeding intolerance (number and length of the episodes intolerance), feeding amount per week, days to reach full enteral feeding and weight gain per week. Adverse effects, including culture-proven sepsis attributable to *L. sporogenes*, flatulence and diarrhea were also recorded.

The event rate for sample size calculation was based on unpublished data of NICU of Zekai Tahir Burak Maternity Training Hospital database (2006–2007). Our recent event

rate for death or NEC (stage $\geqslant 2$) for VLBW infants was ~32% in our NICU. With the $\alpha$-error set at 0.05 and the $\beta$-error set at 0.2, and an absolute reduction in the incidence of NEC or death of 50%, the number needed to verify our hypothesis was 111 infants for each arm. Statistical analyses were performed with SPSS for Windows, version 15.0 (SPSS, Chicago, IL, USA). The relative risk and risk difference with its confidence interval were calculated for primary outcome. The $\chi^2$-test was used to analyze the categorical data, along with Fischer's exact test when applicable. The Student's *t*-test was used for continuous data. The level of significance was set up at $P<0.05$.

## Results

There were 268 VLBW infants admitted to our NICU during the study period. Among these infants, 26 infants were excluded because of major congenital malformations ($n=4$) and lack of parental consent ($n=22$). A total of 242 infants were enrolled in the study; three infants in the study group and one in the control group dropped out of the program because of parent's wish to withdraw the infants from the trial. One infant in the control group was excluded because of spontaneous intestinal perforation. A total of 16 infants died during the first 7-day period after enrollment and were excluded. A total of 221 infants completed the study protocol. There were 110 infants in the study group and 111 infants in the control group. Figure 1 shows the flowchart of the participants.

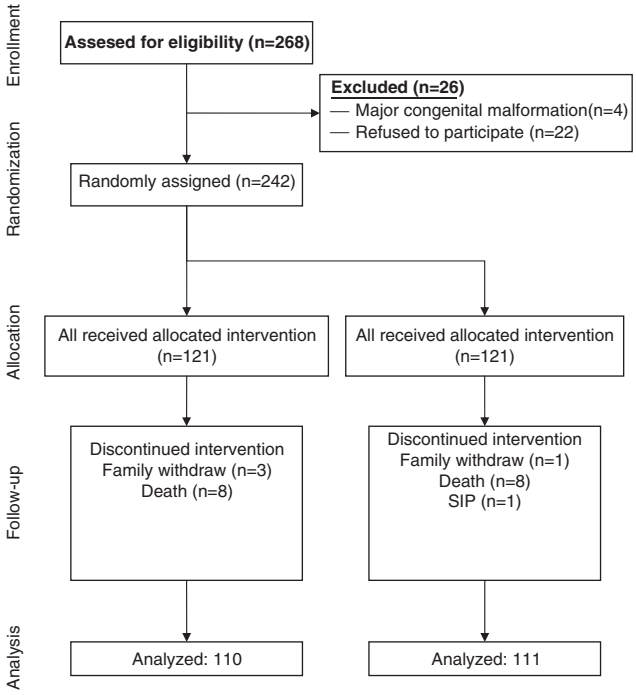



**Figure 1** Participants' flowchart.

The mothers' clinical and infants' demographic and clinical characteristics did not differ between the two groups. Although the rate of prenatal steroids use in this study is very low, there was no significant difference between the groups (Table 1). None of the infants with asphyxia had NEC. The infants' clinical variables also did not differ between the two groups except for longer duration of umbilical venous catheterization in the probiotics group (Table 2).

**Table 1** Mothers' clinical and infant's demographic and clinical characteristics

| *Characteristics* | *Study group (n = 110)* | *Control group (n = 111)* |
|---|---|---|
| Prolonged rupture of amniotic membrane, *n* (%) | 12 (10.9) | 13 (11.7) |
| Preeclampsia, *n* (%) | 8 (7.3) | 12 (10.8) |
| Prenatal steroid, *n* (%) | 31 (28.2) | 41 (36.9) |
| Cesarean section, *n* (%) | 74 (67.3) | 84 (75.7) |
| Multipregnancy, *n* (%) | 37 (33.6) | 42 (37.8) |
| Chorioamnionitis, *n* (%) | 1 (0.9) | 1 (0.9) |
| Men, *n* (%) | 60 (54.5) | 62 (55.9) |
| Small for gestational age, *n* (%) | 10 (9.1) | 6 (5.4) |
| Gestation, week | 29.5 ± 2.4* | 29.7 ± 2.4* |
| Birth weight, g | 1231 ± 262* | 1278 ± 282* |
| Asphyxia, *n* (%) | 1 (0.9) | 2 (1.8) |

None of the differences are statistically significant ($P>0.05$).
*Values are shown as mean ± s.d.

**Table 2** Clinical variables in study infants

| *Variables* | *Study group (n = 110)* | *Control group (n = 111)* | P-*value* |
|---|---|---|---|
| Age at enrollment, days | 2 | 2 | 0.546 |
| Use of surfactant, *n* (%) | 26 (23.4) | 29 (26.4) | 0.613 |
| Use of ibuprofen, *n* (%) | 22 (20) | 30 (27) | 0.218 |
| Umbilical arterial catheter, days[a] | 3 | 3 | 0.856 |
| Umbilical venous catheter, days[a] | 7 | 3 | **0.034** |
| Intermittent mandatory ventilation, days[a] | 3 | 4 | 0.707 |
| $O_2$, days[a] | 6 | 7 | 0.703 |
| Duration of first-course antibiotic, days | 9 | 10 | 0.602 |
| Duration of total antibiotic treatment, days | 11.5 | 10 | 0.268 |
| Use of dopamine, *n* (%) | 57 (52.8) | 65 (59.6) | 0.309 |
| Nothing per ora, days[a,b] | 1 | 2 | 0.438 |
| Total parental nutrition, days[a,c] | 12 | 11 | 0.681 |
| Age onset of NEC, days[a] | 15 | 12.5 | 0.384 |
| Intraventricular hemorrhage, grades 3–4, *n* (%) | 11 (10) | 10 (9) | 0.983 |
| Sepsis (culture proven), *n* (%) | 29 (26.4) | 26 (23.4) | 0.613 |
| Sepsis, Gram-negative, *n* (%) | 9 (8.2) | 9(8.1) | 1.000 |
| Sepsis, Gram-positive, *n* (%) | 17 (15.5) | 16(14.4) | 0.978 |
| Sepsis, fungus, *n* (%) | 3 (2.7) | 1 (0.9) | 0.369 |
| Exclusive breast milk feeding, *n* (%) | 26 (23.8) | 36 (32.8) | 0.454 |
| Mixed feeding, *n* (%) | 74 (76.2) | 64 (67.2) | 0.759 |
| NICU stay, days[a] | 34.5 | 30 | 0.919 |

Abbreviations: NEC, necrotizing enterocolitis; NICU, neonatal intensive care units.
[a]Values are shown as median.
[b]Days from birth to initiation of enteral feeding, median.
[c]Duration of parenteral nutrition, median.
Bold value indicates statistical significance.

Table 3 shows the primary outcomes of the study. The incidence of NEC was not significantly lower in the probiotics group than in the control group (5.8 vs 9%, respectively; $P=0.447$). The incidence of death or NEC was also not significantly lower in the probiotics group when compared with the control group (8.2 vs 11.7%, respectively; $P=0.515$). Relative risk and risk difference for death and stage $\geqslant 2$ NEC were 0.70 (95% confidence interval: 0.32 to 1.53) and −0.04 (95% confidence interval: −0.12 to 0.05), respectively. There were two cases of severe NEC (Bell stage 3) in the probiotics group and three cases in the control group ($P=1.0$). Only one patient with severe NEC had surgery in both probiotics and control groups. There was no difference between the study group and control group in the incidence of death attributable to NEC (0 of 110 infants vs 1 of 111 infants; $P=1.0$).

The mean age at diagnosis of NEC was 17.2 ± 9.4 days in infants of the probiotics group and 13.3 ± 5.4 days in infants of the control group ($P=0.428$). Infants who developed NEC in the study group were fed with breast milk or formula with *L. sporogenes* supplementation for 16.3 ± 9.4 days before the onset of NEC. Infants who developed NEC in control group were fed with breast milk or formula for 13.7 ± 5.3 days before the onset of NEC ($P=0.635$).

The incidence of culture-proven sepsis was not significantly lower in the probiotics group when compared with the control group (26.4 vs 23.4%, respectively; $P=0.613$). The pathogens were most often related to catheter-related infections in both the groups. None of the positive blood cultures grew *L. sporogenes*. The other adverse effects attributed to the use of the *L. sporogenes* administration (that is, flatulence or diarrhea) were also not observed during the study.

For the secondary outcomes, beside the sepsis, there was also no significant difference between the two groups with respect to severe intraventricular hemorrhage ($P=0.983$).

Infants with at least one episode of feeding intolerance were significantly lower in the probiotics group when compared with control group (44.5 vs 63.1%, respectively; $P=0.006$). Feeding intolerance periods of the infants in both groups were less than 72 h, and only 7 (6.4%) infants in probiotics group and 10 (9%) infants in the control group had more than three episodes of feeding intolerance. Table 4 shows that the feeding amount, age of attainment of full feeding and weight gain at various ages were similar between the two groups.

**Table 3** Primary outcomes of the study

| *Characteristics* | *Study group (n = 110)* | *Control group (n = 111)* | P-*value* |
|---|---|---|---|
| Death or NEC, *n* (%) | 9 (8.2) | 13 (11.7) | 0.515 |
| NEC stage 2, *n* (%) | 4 (3.6) | 7 (6.3) | 0.546 |
| NEC stage 3, *n* (%) | 2 (1.8) | 3 (2.7) | 1.000 |
| NEC stage $\geqslant 2$, *n* (%) | 6 (5.5) | 10 (9) | 0.447 |
| Death attributable to NEC, *n* (%) | 0 | 1 (0.9) | 1.000 |
| Death not attributable to NEC, *n* (%) | 3 (2.7) | 3 (2.7) | 1.000 |

Abbreviation: NEC, necrotizing enterocolitis.
None of the differences are statistically significant ($P>0.05$).

**Table 4** Feeding amount and weight gain outcomes with probiotics

| *Variables* | *Study group (n = 110)* | *Control group (n = 111)* | P-*value* |
|---|---|---|---|
| *Feeding amount*[a,b] | | | |
| 14 days[b] | 115 ± 47 | 119 ± 41 | 0.539 |
| 28 days[b] | 135 ± 40 | 146 ± 36 | 0.099 |
| 42 days[b] | 147 ± 42 | 152 ± 27 | 0.468 |
| Full feeding day[b] | 17.3 ± 8.7 | 18.3 ± 9.8 | 0.438 |
| Feeding intolerance, *n* (%) | 49 (44.5) | 70 (63.1) | 0.006 |
| $\geqslant 3$ episodes of feeding intolerance, *n* (%) | 7 (6.4) | 10 (9) | 0.627 |
| *Weight gain*[b,c] | | | |
| 14 days[b] | 3.7 ± 7.1 | 3.7 ± 6.0 | 0.977 |
| 28 days[b] | 10.0 ± 5.1 | 10.5 ± 5.2 | 0.555 |
| 42 days[b] | 12.6 ± 4.3 | 12.3 ± 5.0 | 0.769 |

[a]ml/kg per day, mean ± s.d.
[b]Values are shown as mean ± s.d.
[c]g/kg per day, mean ± s.d.

## Discussion

This is the first randomized controlled study that investigates the efficacy of orally administered *L. sporogenes* in reducing the incidence and severity of NEC in VLBW infants. Our study showed that *L. sporogenes* supplementation at the dose of 350 000 000 c.f.u./day significantly lowered the feeding intolerance; however, it was not effective in reducing the incidence and severity of NEC.

Intestinal microbiological flora is an important factor in the host-defense mechanism against bacterial infections. Colonization of the intestine with pathogenic microorganisms may serve as a predisposing factor in development of NEC (Lin *et al*., 2005). It has been suggested that the growth of pathogens might be prevented by inducing the colonization of the intestine non-pathogenic bacteria (probiotics) of species normally resident in the gut of preterm and term infants (Dani *et al*., 2002). Studies have shown that orally administered non-enteropathogenic bacteria can decrease the incidence of NEC (Hoyos, 1999; Lin *et al*., 2005, 2008).

In a multicenter double-blind study, preterm infants with a gestational age of <33 weeks or birth weight of <1500 g, who survived >2 weeks, were randomized to receive either placebo or *L. rhamnosus GG* once a day, starting with the first fed until discharged. The incidence of urinary tract infection, bacterial sepsis and NEC were examined as outcome measures. There were no significant differences between

the probiotics and placebo groups with regard to any of the outcome variables (Dani *et al*., 2002).

A randomized controlled trial found that infants whose feed was supplemented with *Bifidobacterium breve* had higher rates of fecal bifidobacterial colonization at 2 weeks of age (73 vs 12%), improved weight gain and had feeding tolerance. However, the incidence and severity of NEC were not reported in this study (Kitajima *et al*., 1997).

Lin *et al*. (2005) reported a decrease in NEC, NEC plus mortality and severity of NEC, following probiotics *L. acidophilus* and *B. infantis* (Infloran), prophylaxis in a prospective, randomized blinded study. They also recently reported a multicenter-blinded trial regarding VLBW infants who were randomized to receive *Bifidobacterium bifidum* and *L. acidophilus* for 6 weeks. The results showed a significant reduction in the incidence of death or NEC and no adverse effect, such as sepsis, flatulence or diarrhea (Lin *et al*., 2008). Similarly, Hoyos (1999) reported a significant reduction in the incidence of NEC and NEC-associated death in infants in the NICU after the prophylactic administration of probiotics in the form of Infloran-supplemented enteral feeding. However, infants were more mature and generally had higher birth weights; it is not a blinded trial and comparison was made with historical controls.

Our results suggest a trend toward lower incidence of NEC and, death or NEC, although the difference was not statistically significant. None of the *L. sporogenes*-supplemented fed infants died from NEC; we could not find significant difference in severity of NEC or in mortality rate attributable to NEC between the probiotics and control groups. The use of a single probiotics agent rather than two agents and utility of a relatively low dose of *L. sporogenes* may explain, at least in part, the smaller treatment effect in our study. Longer duration of umbilical venous catheterization in probiotics group also may be another cause in the lesser effect of *L. sporogenes* on NEC prevention. Finally, baseline event rate that has been used to calculate the required sample size was above the actual numbers attained in our study, which in turn make the study underpowered to detect small differences. This may also explain why we failed to detect a significant difference in outcomes between the two groups.

Although some of the studies (Lin *et al*., 2005; Matsumoto *et al*., 2008; Stoll and Hansen, 2008) predicated that probiotics may reduce the incidence of sepsis; literature did not confirm this association (Schanler, 2006; Deshpande *et al*., 2007). Our study did not show that *L. sporogenes* reduced the incidence of sepsis in VLBW infants. We analyzed the frequency of sepsis according to Gram-positive, Gram-negative and fungal infections, and found that pathogens were most often related to catheter-related infections in both groups. This may indicate that changing the intestinal microflora by *L. sporogenes* could not prevent Gram-positive sepsis. On the other hand, sepsis has a complex pathogenesis that is favored by many factors (that is, immune deficiencies of preterm infants, type and frequency of invasive procedures and so on) that cannot be influenced by *L. sporogenes*. The main effect of orally administered *L. sporogenes* is in the gastrointestinal tract, and so *L. sporogenes* alone cannot overcome the invasive procedures including infection.

Lactobacilli and Bifidobacteria are generally regarded as non-pathogenic, except a few reported cases of *Lactobacillus bacteremia* that seemed to occur in immunocompromised or extremely sick infants receiving high doses of Lactobacillus (Land *et al*., 2005). Kunz *et al*. (2004) described *L. bacteremia* in two premature infants who received *L. rhamnosus GG*, and both of those infants had short-gut syndrome. The other authors did not observe sepsis attributable to probiotics in the studies (Dani *et al*., 2002; Kunz *et al*., 2004; Land *et al*., 2005). We observed no cases of sepsis or other adverse effects, such as diarrhea, flatulence attributable to *L. sporogenes* administration.

Our study showed that *L. sporogenes* supplementation significantly lowered the feeding intolerance. It is suggested that the mechanisms of improved feeding tolerance may be regulation of intestinal motility, stimulation of intestinal mucosal lactase activity and lowering the intestinal pH (Gupta and Garg, 2009). *L. sporogenes* produces acids but no gas from fermentation of maltose, mannitol, raffinose, sucrose and trehalose, so this characteristic of the probiotic may be also effective in improving feeding tolerance (De Vecchi and Drago, 2006). Major difference was not observed in other secondary outcomes including weight gain, time to reach full feeds and length of stay or sepsis. Very few infants had more than three episodes of feeding intolerance and once developed, the episodes usually lasted less than 72 h. Second, parenteral nutrition was started immediately for infants who developed feeding intolerance. We suggest that these factors may explain the disagreement between feeding intolerance rates and other secondary outcomes.

To our knowledge, this is the first trial in VLBW infants that studied the efficacy and safety of *L. sporogenes* as a probiotic. In our opinion, improved feeding tolerance in *L. sporogenes*-supplemented group is noteworthy. Also *L. sporogenes* present important advantages over other probiotic strains, such as low cost of production processes, ease of preparation and resistance to production process (De Vecchi and Drago, 2006). So the results and conclusions of this study should be seen as preliminary, and further studies should be performed to identify the ideal strain, single or multiple probiotics supplementation, optimal dose and length of treatment required to prevent NEC.

## Conflict of interest

The authors declare no conflict of interest.

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