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Randomized control trials

Probiotics and growth in preterm infants: A randomized controlled trial, PREMAPRO study☆

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SUMMARY

Background & aims: Recent studies have suggested that the gut microflora has metabolic effects. We aimed to evaluate postnatal growth in preterm infants who received different probiotic supplements, and to assess the safety of probiotic administration.

Methods: This prospective, randomized, double-blind, controlled trial was performed at three tertiary care neonatal units. Preterm infants were randomly assigned to receive daily supplementation over 4–6 weeks with placebo (group C) or probiotics (group P). Group P comprised three subgroups: P1 received *Bifidobacterium lactis*, P2 received *Bifidobacterium longum*, and P3 received *B. lactis* and *B. longum*. We assessed postnatal growth during the supplementation period and up to a corrected gestational age (GA) of 41 weeks when body composition was assessed using whole-body dual-energy X-ray absorptiometry. Aerobic and anaerobic blood cultures were performed on suspicion of late-onset sepsis.

Results: The study comprised 199 preterm infants with a mean GA of 29.1 ± 1.4 weeks and a mean birth weight of 1173 ± 210 g, who received a placebo (group C, n = 52) or probiotics (group P, n = 147) from the first week of life. At the end of the supplementation period, no statistically significant differences were seen between the groups in relation to the mean body weight (group C = 1906 ± 23 g, group P = 1875 ± 14 g, p = 0.25), length, or head circumference. The incidence rates of necrotizing enterocolitis and late-onset sepsis were similar in the two groups. At the corrected GA of 41 weeks, there were no differences between the groups with respect to anthropometric measurements or body composition analysis.

Conclusions: Preterm infants receiving *Bifidobacterium* supplements did not exhibit better postnatal growth compared with those who received placebo treatment. No adverse effects were associated with probiotic administration.

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Abbreviations: ANCOVA, analysis of covariance; DES, day at the end of supplementation; DEXA, dual energy X-ray absorptiometry; GA, gestational age; HC, head circumference; HM, human milk; ITT, intention to treat; NEC, necrotizing enterocolitis; PCR, polymerase chain reaction; PP, per protocol; SD, standard deviation; TT, theoretical term; VLBW, very low birth weight.

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1. Introduction

Optimal postnatal growth is essential for very low birth weight (VLBW) infants. Indeed, extra-uterine growth restriction is related to complications associated with prematurity and to deficits in nutrient intakes. Recent studies have suggested that aggressive nutritional support can help to reduce weight and length deficits upon discharge from hospital [1].

Probiotics, including *Bifidobacterium*, significantly reduce the incidence of necrotizing enterocolitis (NEC) and mortality rates in VLBW infants [2]. Furthermore, a positive effect on weight gain has been reported in rapidly growing animals [3], which could be associated with the metabolic effects of probiotics [4]. Some authors have even suggested that there might be a relationship between the composition of the gut microflora and a later risk of obesity in adults [5]. In a previous observational study, we reported a relationship between the diversity of the intestinal microbiota and weight gain in VLBW infants [6]. Kitajima et al. suggested that supplementation with *Bifidobacterium breve* might improve gastrointestinal tolerance and weight gain in VLBW infants colonized with *B breve* [7]. *Bifidobacterium lactis* has been shown to evoke a similar effect in term infants [8]. Reports suggest that full enteral feeding might be achievable earlier in preterm infants who receive supplements of *Lactobacillus sporogenes* alone [9], a mixture of *Lactobacillus* GG and bovine lactoferrin [10], a combination of strains of *Bifidobacterium* species [11], or a combination of *Bifidobacterium* and *Lactobacillus acidophilus* [12]. Randomized, controlled trials that consider weight gain as the main outcome are scarce, and none of these studies showed an improvement in weight gain [2]. The probiotic supplements used in these studies included *Lactobacillus* [9,13] and *Saccharomyces boulardii* [14], but not *Bifidobacterium*.

The primary objective of this study was to evaluate the effect of *Bifidobacterium* supplementation on short-term postnatal growth and body composition in VLBW infants, and its secondary objective was to assess the safety of probiotic administration.

2. Subjects and methods

2.1. Study design

This was a multicenter, double-blind, randomized, placebo-controlled trial that compared two groups of patients treated with probiotics or a placebo.

2.2. Population

Preterm infants who were hospitalized at French tertiary care centers in Lyon, Montpellier, and Bron, were eligible to participate in the study if they met the following criteria: a gestational age (GA) at birth of between 25 weeks and 31 weeks, a birth weight of between 700 g and 1600 g that was appropriate for the GA according to Usher's reference growth curves, admission to a participating unit within seven days of life, enteral feeding initiated before the fifth day of life, and the receipt of written parental consent. Infants were not eligible to participate in the study if they presented with NEC at ≥stage 1B [15], a severe malformation or any gastrointestinal malformations, or a severe medical or surgical condition. Furthermore, infants were not eligible to participate if their mothers had not been administered antenatal steroids or if their parents lived at too great a distance from the participating center to attend the follow-up visits. Participating infants were subsequently excluded from the study if any of the following occurred: an interruption of enteral or oral feeding for more than 72 h caused by severe gastrointestinal disorders, including Bell's stage NEC ≥ 2A [15], major gastrointestinal surgery, a confirmed or suspected intolerance to cow's milk, prompting the use of protein-hydrolyzed formula, or the withdrawal of parental consent.

2.3. Interventions

The preterm infants received one capsule daily that contained either probiotics plus maltodextrin if they were in the probiotics group (group P), or maltodextrin alone if they were in the control group (group C). The intervention was blinded, because the powders within the capsules were similar in color and appearance. Three probiotic mixtures were used: group P1 received *B. lactis*, group P2 received *B. longum*, and group P3 received *B. lactis* and *B. longum*. The quantity of probiotics administered was 10^9 colony-forming units/d, which was similar to that used in the first randomized, controlled trial [16]. Each capsule contained 250 mg of powder, which was dissolved in 1 mL of sterile water at the bedside and was administered by nurses at the beginning of the midday feed. Quality control of the capsules was performed every six months during the study. Infants started to receive the supplement before the end of the first week of life, and they continued to receive the supplement for four weeks if their GA at birth was ≥29 weeks or for six weeks if their GA at birth was ≤28 weeks. Duration of intervention depended on GA because we aimed to evaluate probiotic supplementation during a minimal 4 weeks period, and 2 weeks more in younger babies in whom it is relevant to assess postnatal growth over a longer period.

The participants, care providers, and those assessing patient outcomes were blinded to the interventions administered.

2.4. Number of subjects

The sample size was calculated to permit the detection of a 150 g difference in body weight at the end of the intervention period between the placebo and the pooled probiotics groups, which is less than the 200 g difference in body weight reported by Kitajima et al. [7], with a randomization ratio of 1:3, a power of 90%, and an α-error of 5%. Assuming an early termination rate of 10%, we planned to include 50 subjects in group C and 150 subjects in group P. With an early termination rate of 20%, the sample size allowed the detection of a 180 g difference in body weight between the placebo and pooled probiotics groups, with a power of 90%.

2.5. Randomization and group allocation

Infants were assigned to their treatment groups according to a pre-established randomization list that was stratified according to the investigating center and the GA at birth (≤28 weeks or ≥29 weeks), with a block size of four. Two treatment groups were defined, the control group (group C) and the probiotics group (group P), with the latter being composed of three subgroups (P1, P2, and P3). Each patient was randomized to one of the four treatment groups (C, P1, P2, or P3) with a 1:1:1:1 ratio within each center and stratum, leading to a 1:3 randomization in relation to group C and group P, which formed the focus of the primary analysis. The 1:3 randomization ratio in favor of group P was justified by the known protective effect of probiotics against NEC in preterm infants [16]. The randomization sequence was generated by CK using PROC PLAN with SAS® version 9.1 (SAS Institute Inc., Cary, NC, USA). Patients were allocated to receive the different treatments using consecutively numbered, sealed, opaque envelopes for each center and stratum.

2.6. Feeding regimen

Feeding practices were standardized across the centers. Parenteral feeding was provided until an enteral intake of 100–120 mL/kg·d Pasteurized human milk (HM) (donor milk or own mother's milk), was administered until the infant weighed 1500 g. Human milk was enriched with 4 g of Eoprotine® powder (Milupa, France) per 100 mL of HM. If the mother did not produce sufficient milk, a preterm formula (Pré Nidal, Nestle France, Marne-la-Vallee, France) was provided, either in addition to the HM or alone. The preterm formula provided 80 kcal and 2.3 g protein per 100 mL, and was packaged in ready-to-use 90 mL bottles that were delivered to the hospital. When the infants were discharged from hospital, Pré Nidal was provided to the families until the study ended at a corrected GA of 41 weeks.

2.7. Study schedule and outcome assessment

Visits were planned on the day of inclusion into the study (day 1), on day 21, on the day at the end of the supplementation period (DES), which was day 28 or day 42, and at the end of the study's theoretical term (TT).

2.7.1. Growth and body composition

The primary efficacy variable was the body weight measured on the DES. Growth was also assessed by calculating the increase in body weight (g/kg·d), length (cm/wk), and head circumference (HC) (cm/wk) during this period. Weight for length, weight for age, length for age, and HC for age Z-scores, and changes from baseline Z-scores were calculated at birth, upon study inclusion, on the DES, at discharge, and at the end of the study's TT, using Olsen's reference curves [17]. At a corrected GA of 41 weeks, a whole-body dual-energy X-ray absorptiometry (DEXA) scan (Hologic QDR 4500A, Infant WB software; Hologic Inc., Waltham, MA, USA) was performed to assess bone mineral content, soft tissue composition. As the study took place in three different units, quality control of the bone mineral content and soft tissue measurements was carried out using our all-solid DEXA phantoms [18,19].

2.7.2. Nutrient intakes and gastrointestinal tolerance

Enteral and parenteral intakes were prospectively recorded daily, allowing the calculation of total daily energy (kcal/kg·d) and protein (g/kg·d) intakes. The occurrence of NEC at Bell's stage $\geq$2 was recorded. A gastrointestinal tolerance score, based on daily records of regurgitations, vomiting, gastric residuals, numbers of stools, and abdominal distension, was calculated as previously described [6].

2.7.3. Stool analyses

On day 21 of the supplementation schedule, the composition of the gut microbiota was determined for all infants using the polymerase chain reaction (PCR) temporal temperature gradient gel electrophoresis method. This method, which uses a non-selective PCR amplification of the V3 hypervariable region of the 16S ribosomal ribonucleic acid gene, which is conserved among eubacteria, to determine the number and nature of the different taxonomic units present in a sample, has been compared previously with a conventional culture method for the analysis of gut microbiota in neonates [20]. The number of different taxonomic units, or the diversity index, reflects the diversity of the microbiota in each patient [6]. We measured fecal calprotectin on day 21, using an enzyme-linked immunosorbent assay (Bühlmann Laboratories AG, Schönenbuch, Switzerland).

2.7.4. Blood cultures

Whenever late-onset sepsis was suspected, two blood samples (1 mL) were drawn for aerobic and anaerobic blood cultures to assess the safety of the probiotic supplementation.

2.8. Data collection and management

Nurses collecting the data, the doctors involved in the follow-up, and attending physicians were blinded with respect to which intervention groups the infants had been allocated. Parental characteristics and antenatal data were extracted from the infants' medical records. Body weights were recorded daily, and crown-heel lengths and HC were recorded from the time the infants were included in the study to the time of their discharge from the hospital at the TT using an electronic diary.

2.9. Statistical analysis

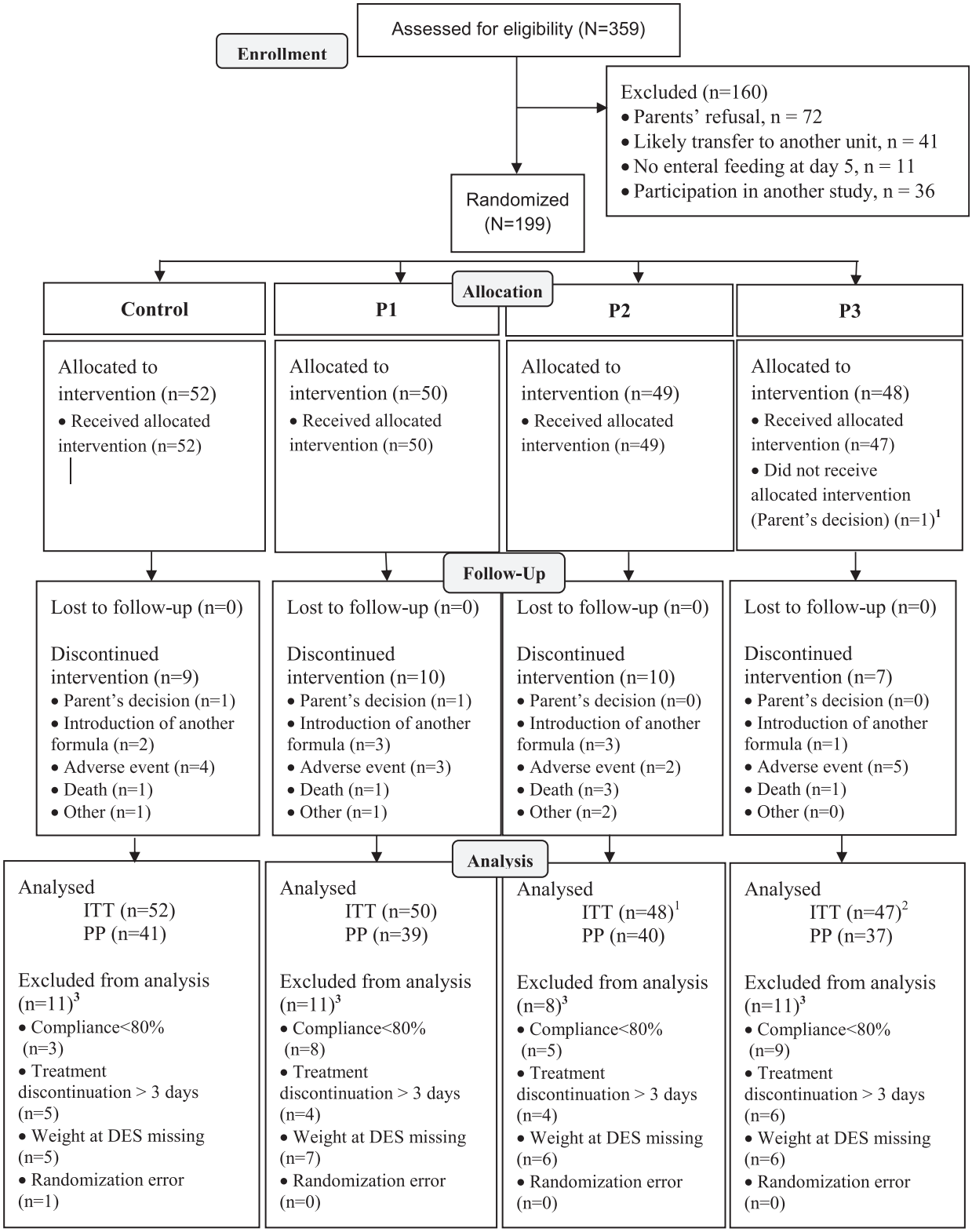
Three populations were defined for analysis. The population in which the primary efficacy analysis was undertaken was the intention-to-treat (ITT) population, which was defined as the group of patients who were administered at least one dose of the placebo or probiotic and were assessed at least once while they were being treated. The confirmatory per protocol (PP) population was defined as the subset of the ITT population that was not subject to any major protocol deviations. Major deviations, defined *a priori* before the study was unblinded, comprised the administration of any probiotic before study inclusion, any randomization error or error in the administration of treatment, treatment compliance that was $\leq$80% (i.e. study capsules not administered for at least four days to patients with a GA at birth of $\leq$28 weeks or for at least six days to patients with a GA at birth of $\geq$29 weeks), probiotics not being administered for more than three consecutive days, and no weight assessment on the DES. Any other protocol deviation was considered to be minor. The third analysis set was the safety population, which was defined as infants who had received at least one dose of the placebo or probiotic and from whom safety data were collected.

The results were analyzed by a statistician who was blinded to the treatment allocation, using SAS 9.2 (SAS Institute Inc., Cary, NC, USA) for Windows. Statistical tests were two-sided and conducted at the 5% level. Bonferroni corrections for multiplicity were planned in case of a significant global treatment effect conditioning analyses of the effect of the three subgroups of probiotics as planned in the protocol. It was emphasized, subsequently to the design of this study, that probiotics cannot be studied as a whole and that any positive or negative effects on the selected outcome should be investigated in relation to each probiotic species [21]. Hence, we provide descriptive results for each of the three probiotic subgroups (i.e. P1, P2, and P3) even if no global treatment effect was found.

Anthropometric measurements (weight, height, and HC) were analyzed using fixed-effect analyses of covariance (ANCOVAs) with the baseline values of the parameters of interest, namely, the mean daily protein intakes, mean daily energy intakes, and gender, as covariates, and gender, the randomization stratum, that is, a GA of $\leq$28 weeks or a GA of $\geq$29 weeks, and the treatment group (placebo or pooled probiotics) as fixed effects (ANCOVA Model 1).

A confirmatory analysis was undertaken on the PP population. Post-hoc confirmatory ANCOVAs were performed, which accounted for the intake of HM and the number of days on which antibiotic treatment was administered during the supplementation period (ANCOVA Model 2).

Mean daily energy and protein intakes during the supplementation period were analyzed using fixed-effect ANCOVA models using the subjects' weights at baseline as covariates, and with



1 A subject received one dose of the allocated treatment but never assessed on treatment, excluded from the ITT analysis set

2 A subject never received the allocated treatment (parents 'decision), excluded from the ITT analysis set.

3 Patients could be excluded from the PP analysis for more than one reason.

Fig. 1. Study flow chart. The probiotics subgroup P1 was administered *Bifidobacterium lactis*, P2 was administered *Bifidobacterium longum*, and P3 was administered *B. lactis* and *B. longum*. ITT, Intention to treat; PP, Per protocol; DES, Day at the end of supplementation.

gender, the randomization stratum, and the treatment group as fixed effects. It was emphasized subsequent to the design of this study, that probiotics cannot be studied as a whole and that any positive or negative effects on the selected outcome should be investigated in relation to each probiotic species [21]. Hence, we provide descriptive results for each of the three probiotic subgroups (i.e. P1, P2, and P3).

2.10. Ethics

The study was conducted in accordance with French regulations and the Helsinki Declaration of 1975 that was revised in 1983. The protocol was approved by the lead center's ethics committee (*CPP Sud Méditerranée IV*) and by the French National Agency for Medicines and Health Products Safety, and it was registered on ClinicalTrial.gov (NCT01379417).

3. Results

Between November 2007 and June 2010, 199 infants with a mean ± standard deviation (SD) age of 6.4 ± 1.5 days were included in the study (Montpellier: 78, Lyon: 65, and Bron: 56). Forty (20.3%) subjects discontinued the study prematurely and, of these, 11 (21.2%) infants were in group C and 29 (20%) infants were in group P (Fig. 1).

The mean duration of hospitalization was 50.4 ± 17.4 days, which was similar for both treatment groups. The reasons for discharge from the participating centers were similar for the two treatment groups: 63.3% of infants were discharged to their homes, 24.2% were transferred to other hospital, and six (3.0%) infants died. The remaining 19 (9.5%) subjects stayed in hospital when the study ended at a corrected GA of 41 weeks. Subjects in group C received a mean of 32.3 ± 7.0 capsules and subjects in group P received a mean of 31.0 ± 9.9 capsules, which corresponded to a mean rate of compliance of 94.5 ± 12.7% and 90.4 ± 23.3%, respectively.

The population characteristics of groups C and P were similar (Table 1). Half of the neonates were very immature with a GA of ≤28 weeks.

Table 1
Characteristics of the very low birth weight infants (n = 197, representing the intention-to-treat population) who received either a placebo or a probiotic supplement.

	Control group (n = 52)	Probiotic group (n = 145)
Pregnancy/Delivery		
Cesarean section (%)	75	79.3
Per-partum antibiotics (%)	53.8	39.6
At birth		
GA (wk)	29.4 (27.9; 30.6)	29.0 (28.1; 30.1)
BW (g)	1170 (1055; 1370)	1170 (1000; 1320)
Male gender (%)	67.3	45.8
Z-score for BW	−0.36 (−0.88; 0.09)	−0.38 (−0.90; 0.05)
Since birth		
Surfactant (%)	63.5	59.3
PDA requiring treatment (%)	19.2	13.8
Assisted ventilation (%)	50	52.4
IVH or PVL (%)	15.4	9
At study inclusion		
Postnatal age (days)	7.0 (5; 8)	7.0 (6; 8)
Body weight (g)	1085 (968; 1295)	1090 (930; 1196)
Assisted ventilation (%)	9.6	11
Antibiotics (%)	9.6	9.7

The data are presented as % for categorical parameters and as medians (Q1; Q3) for continuous variables. BW: birth weight; GA: gestational age; IVH: intraventricular hemorrhage; PDA: patent ductus arteriosus; PVL: periventricular hemorrhage.

Parenteral nutrition was administered to 81.2% of the subjects with a mean volume of 91.6 ± 33.2 mL/kg·d administered at inclusion, which corresponded to a mean intake of 11.3 ± 4.5 g/kg·d of carbohydrates, 1.4 ± 0.8 g/kg·d of lipids, and 2.5 ± 1.1 g/kg·d of protein. Before the infants were included in the study, enteral nutrition consisted of HM exclusively for 70.1% of the subjects, HM with preterm formula for 29.4% of the subjects, and preterm formula exclusively for one (0.5%) infant. At this time, the mean daily enteral intake was 77.3 ± 40.7 mL/kg·d. There were no differences in HM, energy and protein intakes between the groups during the supplementation period (Table 2).

Gastrointestinal tolerance and the incidence of NEC were similar in the two groups (Table 2), with the mean gastrointestinal tolerance score being 1.05 ± 0.36 in group C and 1.03 ± 0.39 in group P. Poor gastrointestinal tolerance, which corresponded to a score of at least 3, lasted for a mean period of 1.2 ± 1.7 days or 3.3 ± 4.5% of the supplementation period in group C, and 0.9 ± 1.6 days or 3.0 ± 5.8% of the supplementation period in group P ($p = 0.21$ for the number of days; $p = 0.26$ for the percentage of days). The mean age of the infants at which full enteral feeding was achieved was very similar in Group C and Group P (16.6 ± 9.7 days and 15.8 ± 9.3 days, respectively, $p = 0.67$).

Growth during the supplementation period was similar in the two groups (Table 3). Overall, there was no statistically significant difference between group C and group P in relation to the median weight on the DES (1903 [1742–2140] g vs. 1810 [1610–1995] g, respectively, $p = 0.27$), but there were significant effects associated with the randomization stratum ($p < 0.0001$) and the weight at inclusion ($p < 0.0001$). Similar results were obtained when the intake of HM and the number of days on which antibiotic treatment was administered during the supplementation period were considered. Hence, there was no statistically significant difference between group C and group P ($p = 0.545$), but there were significant effects associated with the randomization stratum ($p < 0.0001$) and the weight at inclusion ($p < 0.0001$), in addition to the mean intake of human milk ($p = 0.005$) and the mean protein intake ($p = 0.04$) during the supplementation period (Table 4). No statistically significant difference was seen between groups C and P with respect to the daily weight gain when it was averaged over the supplementation period, either in the primary analysis (16.6 ± 3.1 g/kg·d vs. 15.9 ± 4.1 g/kg·d, $p = 0.17$) or in the adjusted analysis taking the HM intake and the number of days of antibiotic treatment into account ($p = 0.35$). Similar results were obtained for the PP population. Likewise, there were no significant differences between the groups in relation to the crown-heel length or HC on the DES. The confirmatory analyses performed on the PP population generated similar results.

At a corrected GA of 41 weeks, there were no statistically significant differences between the groups with regard to the anthropometric data (Table 3), whole body mineralization, or fat mass (Table 3). Analysis of the PP population gave similar results.

After three weeks of treatment, the most frequently identified microbiological families detected in the stools were, by decreasing frequency, *Staphylococcus* spp., Clostridiales, Enterobacteriaceae, and *Enterococcus* spp., with no differences between group C and group P. *Bifidobacterium* spp. were more frequently identified in subjects in group P (30.1%) than in subjects in group C (13.0%) ($p = 0.04$), particularly in infants who received *B. lactis* either alone (34.8%) ($p = 0.03$ compared with group C), or in combination with *B. longum* (32.6%) ($p = 0.04$ compared with group C) (Table 5). The mean diversity scores were very similar in group C (3.4 ± 1.8) and group P (3.4 ± 1.3) ($p = 0.75$). No statistically significant effect of the diversity index on daily weight gain was seen between the day of inclusion and day 21 with the weight at inclusion, mean energy and

Table 2
Nutrition, antibiotics, and gastrointestinal tolerance during the supplementation period in very low birth weight infants (n = 197, representing the intention-to-treat population) who received either a placebo or a probiotic supplement.

	Control group (n = 52)	Probiotics group P (n = 145)	Group P1 (n = 50)	Group P2 (n = 48)	Group P3 (n = 47)
Antibiotics, n (%)	6 (11.5)	20 (13.8)	8 (16.0)	3 (6.3)	9 (19.1)
Human milk intake (mL/kg·d)	110 (80; 130)	124 (104; 137)	126 (109; 131)	123 (109; 138)	117 (94; 143)
Days with gastrointestinal intolerance[a]	7 (4; 11)	7 (3; 11)	6 (3; 10)	7 (3; 11)	7 (3; 11)
NEC, n (%)	3 (5.8)	8 (5.5)	2 (4.0)	1 (2.0)	5 (10.6)

Probiotics subgroup P1 was administered *Bifidobacterium lactis*, P2 was administered *Bifidobacterium longum*, and P3 was administered *B. lactis* and *B. longum*.
The data are presented as numbers (%) or medians (Q1; Q3). NEC: necrotizing enterocolitis.
There were no statistically significant differences between the intervention groups for any of the parameters.
[a] Gastrointestinal intolerance: gastrointestinal tolerance score ≥2.

Table 3
Growth (z-scores for body weight, length and head circumference), and body composition of very low birth weight infants (n = 197, representing the intention-to-treat population) who received either a placebo (Control) or a probiotic supplement (P).

	Control (n = 52)	Probiotics (P) (n = 145)	P1 (n = 50)	P2 (n = 48)	P3 (n = 47)
W/A z-score					
A, sd	−0.36[−0.88; 0.09]	−0.38[−0.90; 0.05]	−0.40[−0.74;−0.07]	−0.40[−0.91; 0.02]	−0.29[−1.04; 0.21]
B, sd	−1.22[−1.66;−0.97]	−1.31[−1.63;−0.96]	−1.31[−1.54;−1.11]	−1.36[−1.68;−0.95]	−1.18[−1.65;−0.70]
C, sd	−1.14[−1.63;−0.84]	−1.35[−1.72;−0.98]	−1.37[−1.67;−1.11]	−1.44[−1.78;−1.09]	−1.22[−1.62;−0.84]
D, sd	−0.23[−0.82; 0.35]	−0.42[−1.01; 0.24]	−0.41[−0.99; 0.31]	−0.51[−0.93; 0.24]	−0.37[−1.04; 0.20]
L/A z-score					
A, sd	−0.46[−0.89; 0.10]	−0.32[−0.72; 0.24]	−0.25[−0.49; 0.27]	−0.32[−0.86; 0.24]	−0.57[−0.90; 0.20]
B, sd	−0.85[−1.26;−0.23]	−0.80[−1.12;−0.30]	−0.81[−1.04;−0.40]	−0.82[−1.30;−0.30]	−0.74[−1.14;−0.15]
C, sd	−1.57[−2.25;−1.08]	−1.72[−2.14;−1.28]	−1.74[−2.04;−1.29]	−1.59[−2.12;−1.05]	−1.78[−2.62;−1.32]
D, sd	−1.55[−1.89;−1.08]	−1.58[−1.93;−1.20]	−1.50[−1.93;−1.23]	−1.65[−2.03;−1.23]	−1.56[−1.93;−0.93]
HC/A z-score					
A, sd	−0.08[−0.70; 0.51]	−0.14[−0.62; 0.31]	−0.10[−0.42; 0.30]	0.02[−0.74; 0.29]	−0.18[−0.62; 0.36]
B, sd	−0.68[−1.36;−0.14]	−0.76[−1.24;−0.34]	−0.94[−1.27;−0.42]	−0.73[−1.19;−0.41]	−0.61[−1.24;−0.23]
C, sd	−0.97[−1.41;−0.58]	−1.25[−1.68;−0.75]	−1.30[−1.69;−0.71]	−1.31[−1.70;−0.78]	−1.19[−1.59;−0.69]
D, sd	−0.38[−1.07; 0.12]	−0.62[−1.36; 0.00]	−0.72[−1.53;−0.10]	−0.65[−1.50; 0.07]	−0.29[−0.86; 0.24]
DEXA					
Fat, %	14.5[12.3; 16.2]	14.6[10.4; 18.6]	14.3[10.8; 18.8]	14.6[8.1; 17.0]	15.1[10.6; 18.1]
BMC, g	45.6[40.4; 50.9]	47.6[40.9; 51.8]	48.4[42.6; 54.0]	47.7[42.1; 50.0]	46.6[37.3; 51.3]

Probiotics subgroup P1 was administered *Bifidobacterium lactis*, P2 was administered *Bifidobacterium longum*, and P3 was administered *B. lactis* and *B. longum*.
A, at birth; B, at inclusion; C: at the end of supplementation; D, at theoretical term (41 wks corrected age); sd, standard deviation, W/A z-score, body weight-for-age z-score; L/A z-score, length-for-age z-score, HC/A z-score, head circumference-for-age z-score; DEXA, dual-energy-x-ray absorptiometry; BMC, bone mineral content.
The data are presented as numbers (%) or medians (Q1; Q3).
There were no statistically significant differences between the intervention groups for any of the parameters.

protein intakes, and diversity index as covariates, and gender, the randomization stratum, and treatment group as independent variables. Fecal calprotectin concentrations were similar among the different treatment groups (Control: 183[94; 268] μg/g, P: 221[104; 275] μg/g, P1: 200[126; 264] μg/g, P2: 226[91; 300] μg/g, P3: 232 [99; 275] μg/g, NS).

The incidence of bloodstream infections - assessed using both aerobic and anaerobic blood cultures - was similar in group P and group C (p = 0.912). There were no differences in the types of microorganisms isolated from peripheral blood samples (Table 6).

During the supplementation period, 10 (19.2%) subjects in group C experienced 15 adverse events and 35 (24.0%) subjects in group P experienced 45 adverse events. Possibly related adverse events affected one (1.9%) subject in group C and five (3.4%) subjects in group P. Two (1.4%) subjects in group P and 11 (7.5%) subjects in group C experienced serious adverse events during the supplementation period. None of these serious adverse events were considered to be related to the study treatment. One (1.9%) subject in group C and four (2.7%) subjects in group P died during the supplementation period. Another subject in group P died after the supplementation period.

4. Discussion

In this population of high-risk preterm infants born in the post-surfactant era, we did not observe any beneficial effects associated with probiotic supplementation in relation to postnatal weight gain, neither any side-effect related to probiotic administration.

We expected that better gastrointestinal tolerance might improve weight gain as shown by Kitajima et al. [7]. Since the study by Kitajima et al. [7], three other studies have reported earlier achievement of full enteral feeding [2], but this did not occur in our study. The first of these studies compared the effect of bovine lactoferrin alone with bovine lactoferrin plus *Lactobacillus* GG [15]. The second study was conducted exclusively in VLBW infants who were fed breast milk that was or was not supplemented with a mixture of probiotics [12]. The third study used *L sporogenes*, a strain that does not appear to have any beneficial effects [9], which is in contrast to *B. lactis* [2,22,23].

We previously reported a relationship between the diversity of gut microbiota and postnatal weight gain [6], which was not observed in our study. The proportion of infants with *Bifidobacterium* in their stools was only about three-times higher in infants

Table 4
Results of the analysis of covariance (ANCOVA) and extended ANCOVA for weight at the end of the intervention period in very low birth weight infants (n = 197, representing the intention-to-treat population) who received either a placebo or a probiotic supplement.

	ANCOVA Model 1	ANCOVA Model 2
	p value	p value
Intervention group	0.265	0.545
GA at birth	<0.0001	<0.0001
Birth weight (BW)	<0.0001	<0.0001
BW*RS	0.057	0.210
Mean intake during intervention:		
Energy (kcal/kg·d)	0.527	0.095
Protein (g/kg·d)	0.222	0.041
Human milk (mL/kg·d)	–	0.005
Gender	0.093	0.271
Days antibiotics used during intervention, n	–	0.583
Weight difference at the end of intervention probiotics-control mean, (95%CI)	−30.49 (−84.33, 23.66)	−16.68 (−70.84, 37.57)

CI: confidence interval.
Results are for fixed-effect analysis of covariance (ANCOVA) models with birth weight, the mean daily intake of proteins, the mean daily energy intake, and gender as covariates, and gender, randomization stratum, defined as a gestational age of ≤28 weeks or ≥ 29 weeks, and intervention group (placebo or probiotics) as fixed effects (Model 1). Model 2 additionally included human milk intakes and the number of days antibiotics were administered during the intervention as covariates.

who received the probiotic supplements compared with those who did not receive a probiotic supplement, and this difference might have been insufficient to observe a beneficial effect on growth. From our data it is not possible to explain why the proportion of infants with BF in stools was not higher than 30% in the probiotic-supplemented group. Further investigations are needed to evaluate the effects of different strains and greater quantities of *Bifidobacterium* and probiotic mixtures using other strains such as lactobacillus on growth. As large randomized studies remain a necessity to ascertain the positive effect of probiotics on NEC incidence [21], growth should be systematically evaluated in these studies.

The absence of any significant differences between the groups in relation to short-term growth could also be attributed to the use of strains of microorganisms that are inappropriate for attaining positive metabolic effects in these infants. Recent data suggest that each strain exerts a distinct effect, with some strains being effective at preventing NEC in preterm infants [22], while others are effective at preventing and managing colic [24] or atopic diseases [25]. *B. lactis* reduces NEC incidence in association with other probiotics [2,22]. A beneficial effect of *B. lactis* on the growth of infants born to human immunodeficiency virus-positive mothers has also been suggested [8]. In preterm infants, an improvement in weight gain that was associated with *B breve* supplementation was reported in a subgroup of subjects [7]. Another study reported an increased

Table 5
Gut microflora expressed as the proportion of infants with at least one of the 13 different species, in very low birth weight infants (n = 197, representing the intention-to-treat population) who received either a placebo or a probiotic supplement.

	Placebo group (n = 52)	Probiotics group (n = 145)	Group P1 (n = 50)	Group P2 (n = 48)	Group P3 (n = 47)
Staphylococcus spp., n (%)	29 (63.0)	88 (66.2)	31 (67.4)	29 (65.9)	28 (65.1)
Clostridiales, n (%)	20 (43.5)	60 (45.1)	17 (37.0)	22 (50.0)	21 (48.8)
Enterobacteriaceae, n (%)	18 (39.1)	43 (32.3)	15 (32.6)	13 (29.5)	15 (34.9)
Enterococcus spp., n (%)	16 (34.8)	45 (33.8)	15 (32.6)	11 (25.0)	19 (44.2)
Bifidobacterium spp., n (%)	6 (13.0)	40 (30.1)[a]	16 (34.8)[a]	10 (22.7)	14 (32.6)[a]
Acidaminococcaceae, n (%)	6 (13.0)	18 (13.5)	6 (13.0)	6 (13.6)	6 (14.0)
Streptococcus spp., n (%)	2 (4.3)	15 (11.3)	6 (13.0)	4 (9.1)	5 (11.6)
Anaerobes (various), n (%)	4 (8.7)	6 (4.5)	3 (6.5)	1 (2.3)	2 (4.7)
Burkholderiales, n (%)	2 (4.3)	6 (4.5)	1 (2.2)	3 (6.8)	2 (4.7)
Corynebacterium spp., n (%)	0	3 (2.3)	1 (2.2)	2 (4.5)	0
Micrococaceae, n (%)	1 (2.2)	1 (0.8)	0	0	1 (2.3)
Neisseria spp., n (%)	0	1 (0.8)	0	0	1 (2.3)
Pseudomonas spp., n (%)	1 (2.2)	0 (0.0)	0	0	0

Probiotics subgroup P1 was administered *Bifidobacterium lactis*, P2 was administered *Bifidobacterium longum*, and P3 was administered *B. lactis* and *B. longum*.
The data are presented as numbers (%).
[a] $p < 0.05$, chi-squared test (vs placebo).

Table 6
Bloodstream infections in very low birth weight infants (n = 197, representing the intention-to-treat population) who received either a placebo or a probiotic supplement.

	Placebo group (n = 52)	Probiotics group (n = 145)	Group P1 (n = 50)	Group P2 (n = 48)	Group P3 (n = 47)
Proportion of subjects with ≥1 infection (%)	19 (9, 30)	17 (11, 23)	18 (7, 29)	16.7 (6, 27)	17 (6, 28)
Microorganisms (%)					
Coagulase-negative staphylococci, (%)	80 (55, 100)	56 (37, 76)	67 (36, 98)	38 (4, 72)	63 (30, 97)
Staphylococcus aureus, (%)	0 (0, 0)	28 (10, 46)	11 (0, 31)	50 (15, 85)	25 (0, 55)
Candida spp., (%)	10 (0, 29)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)
Other, (%)	10 (0, 28)	16 (2, 30)	22 (0, 49)	13 (0, 36)	13 (0, 36)

Probiotics subgroup P1 was administered *Bifidobacterium lactis*, P2 was administered *Bifidobacterium longum*, and P3 was administered *B. lactis* and *B. longum*.
The data are presented as % (95% confidence intervals).

weight gain related to *B. lactis* supplementation, but only in a subgroup of antibiotic-treated preterm infants [26]. With the exception of our study, only four randomized, controlled studies have analyzed weight gain as an outcome, and invariably as a secondary outcome criterion [9,13,14]. Furthermore, two of these studies [13] were performed over 20 years ago and on such small numbers of infants that they cannot be considered to contribute to the body of evidence. The other study [14] used a highly specific probiotic (*S boulardii*) that cannot be used anymore because of the reported cases of fungemia related to this microorganism [27]. The use of *L sporogenes* as a probiotic has been reported, but it failed to decrease the incidence of death, reduce the incidence of stage $\geq$2 NEC (the main outcome criterion), or to improve weight gain [9]. In this last study, the weight gain was surprisingly low in both the control and the probiotic groups at about 10 g/kg·d at 28 days of life and about 12 g/kg·d at 42 days of life, and it was less than the fetal growth rate [9]. Our study is the first controlled randomized, double-blind study that used weight gain as the main outcome criterion. The effects of probiotics on weight gain may vary depending on the species and the strains of microorganisms employed [28].

A positive effect was expected in these rapidly growing VLBW infants. This was based on previous reports of the effects of probiotic supplementation on metabolism and growth in both animal studies and clinical trials. In poultry, an improved weight gain was reported in birds that received probiotic supplements during growth [3]. The gut microbiota participates in the regulation of energy metabolism in the host. Microbial components target multiple systems and play key roles in metabolism, including nutrient absorption, the maintenance of the integrity of the gut barrier, gut hormone regulation, and fat metabolism [4]. A metabolic effect associated with the gut microbiota has also been reported, and a relationship between the composition of the flora and later obesity has been suggested [5]. In our population we did not observe improved postnatal weight gain in infants who received probiotic supplements compared with the control group. Our study was not designed to evaluate the long-term effects of probiotics on growth, but to assess their impact a few weeks after supplementation had ended, that is, at a corrected GA of 41 weeks. There were no differences between the groups in relation to growth that were determined using anthropometric parameters or body composition, as assessed by whole-body DEXA, a reference method for fat mass assessments in neonates [29].

The absence of any significant differences between the groups in relation to short-term growth in our study might reflect a lack of statistical power. However, this is unlikely because the number of subjects was calculated to detect a clinically relevant [7] difference with a power of 90%. Furthermore, we accounted for the low level of *Bifidobacterium* implantation. Lastly, the number of infants included in our study approached or even exceeded the numbers included in previous nutritional studies that reported positive effects on postnatal weight gain. Our population was representative of a typical population of preterm infants, because we also included very immature infants who are at risk of severe growth restrictions.

The absence of effect of probiotics on short-term growth could also be related to an insufficient effect on gut microbiota in the participating neonatal units. The proportion of infants with bifidobacteria in stools has tripled but the basal level was low as it was close to 10%, in agreement with our previous findings and data from other authors [6,30]. Some authors reported higher proportions [31,32]. It is very unlikely that we underestimated the presence of bifidobacteria as the method used for stool analysis was previously validated in neonates [20]. Our current practices (mode of delivery, antibiotics, nutrition …) could have negatively affected the proportion of infants with bifidobacteria in stools. However these practices were similar in all infants included in the study and we were able to show a significant - although insufficient-effect of probiotic supplementation on gut microbiota. In another setting, such an effect might be sufficient to observe an effect on growth.

Three different combinations of probiotics were tested because - when the study was designed - there were concerns about the different strains and mixtures of strains of probiotics. The primary analysis was a comparison of the placebo group with the pooled probiotics and the power calculation took into account the 1/3 randomization ratio in favor of the Probiotics. Pairwise comparisons were only planned in case of a significant global effect which was not the case. So the group Probiotic was considered as a whole. Since we designed our study, the effect of the different strains on NEC and death prevention was studied by different authors. Some of them suggest that there are different effects when different strains are used [21], others found no difference at all [33] or suggested different effects when infants are supplemented with a single or with combined strains [34]. Our *a priori* choice of comparing pooled data of infants receiving different probiotic strains to evaluate the global effect of adding probiotics when compared to infants receiving placebo appears still legitimate.

Another limitation of this study is that any potential beneficial effects of the probiotics on growth might have been blunted by the higher energy and protein intakes in group C. We carefully collected data on actual energy and protein intakes during the supplementation period and took these intakes into account during the analyses, which is rarely undertaken in studies of this type. Almost all infants received human milk, but the human milk composition was not measured in our study, which could have introduced an error. Human milk was assumed to contain 64 kcal/100 mL and 1.4 g of protein/100 mL, which are commonly used average values. By encoding carefully and prospectively the real amount of parenteral fluids intake and of milk intake during the study period, we minimized the risk of error in nutrient's intakes calculations. However, such an error is randomly distributed in all the feeding groups and it is very unlikely that it could have significant influenced our results.

The duration of probiotic supplementation, was 4 or 6 weeks depending on gestational age at birth to get high-quality data over a relatively early in-hospital period. We aimed to evaluate whether or not probiotic supplementation could support optimization of postnatal growth which is required as soon as possible after birth in very preterm infants. When the study was designed a 4 weeks-period was shown to be efficient on NEC and mortality rates and a 6 weeks period was considered as helpful to assess a potential effect in the more immature subjects. A recent Cochrane review showed that there is no difference in the effect on severe NEC, culture proven sepsis and mortality rates between studies using a period of supplementation comprised between 4 and 6 weeks [2]. This review also suggests that probiotic supplementation until discharge could be more efficient than shorter period of supplementation. Further studies on probiotics and growth in preterm infants should evaluate such prolonged supplementation.

The optimal probiotic strains or mixtures of strains to be used in preterm infants are not well defined. One might expect that a mixture of probiotics could help achieve beneficial health effects [2]. In our study, we did not observe any benefits in the group that received a combination of probiotics, and there was even a tendency towards an increased prevalence of NEC in this group. However, this difference was not statistically significant possibly due to a lack of statistical power, because our study was not designed to investigate the effect of probiotics on NEC incidence. Cilieborg et al. reported that preterm pigs that received a mixture of probiotics showed an increased incidence and severity of NEC that was associated with reductions in the mucosal integrity and

digestive function, and an increased expression of pro-inflammatory mediators [35]. In our study, fecal calprotectin, a marker of inflammation, did not show an increased expression in the infants who received a mixture of two probiotics, which raises the possibility that the higher incidence of NEC in this group might have been caused by a random effect. However, our results emphasize the need for careful evaluations of different probiotic strategies in relation to strains and doses, before they are routinely used in preterm infants.

In conclusion, probiotics did not have a significant effect on gastrointestinal tolerance and short-term postnatal growth in this study. There was no significant difference between the two different probiotics tested separately and no clear benefit associated with using a mixture of these two probiotics. No sepsis occurred that was related to the probiotics used for supplementation. Further studies could evaluate other strains or other symbiotic organisms and, perhaps, different dosages to facilitate the definition of the optimal probiotic mixture for NEC prevention and growth promotion.

Authors' contributions

Jean-Charles Picaud (JCP), Guy Putet, Olivier Claris (OC), and Evelyne Decullier designed the study. Stéphane Hays (SH), Aurélien Jacquot, Anne Beissel, Odile Pidoux, Hélène Gauthier, Emmanuelle Lachambre, Laurence Beck, Gilles Cambonie, JCP, and OC conducted the research. Estelle Jumas-Bilak contributed to the design of the study and provided essential materials. Christian Kempf (CK) and JCP analyzed the data. JCP, SH, and CK wrote the paper. JCP had primary responsibility for the paper's final content. All authors read and approved the final manuscript.

Conflict of interest

Stéphane Hays, Aurélien Jacquot, Hélène Gauthier, Christian Kempf, Anne Beissel, Odile Pidoux, Estelle Jumas-Bilak, Evelyne Decullier, Gilles Cambonie, Guy Putet, Olivier Claris, and Jean-Charles Picaud have no conflicts of interest to declare. Emmanuelle Lachambre and Laurence Beck are employees of Nestlé France, the study sponsor. Nestlé supported the research by providing the placebo and probiotics capsules, and by providing financial support for the employment of research nurses during the study period. Nestlé was not involved in either the study design, the analysis, or the interpretation of the results.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2015.06.006.

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