

Randomized, Double-Blind, Placebo-Controlled Study to Assess the Effect of Two Probiotics on the Preterms' Gut Microbiota

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ABSTRACT

Objective: To evaluate the effect of a new probiotic strain combination, *Ligilactobacillus salivarius* subsp. infantis PS11603 and *Bifidobacterium longum* PS10402, on gut bacterial colonization of preterm infants.

Methods: A randomized, double-blind, placebo-controlled study was conducted in preterm infants from 28 weeks + 0 days to 30 weeks + 6 days of gestation. Thirty preterm infants were randomly selected after birth to receive either probiotics or placebo. Stool samples were collected before product intake and then sequentially during the first weeks of their admission. Classical microbiological, metagenomics and multiplex immunological analyses were performed to assess the bacterial and immune profile of the samples.

Results: Twenty-seven infants completed the study (14 vs 13, probiotic and placebo groups). A higher number of participants were colonized by Lactobacilli in the probiotic group than in the placebo group (93% vs 46%; $P=0.013$). Similar results were obtained when analysing bifidobacterial colonization (100% vs 69%; $P=0.041$). Earlier colonization was observed in the probiotics group versus the placebo group, specifically 5 weeks for *Lactobacillus* and 1 week for *Bifidobacterium*. Although no effect was observed in the faecal immunological profile, a decreasing trend could be observed in T_H17 response during the first week of probiotic treatment. None of the adverse events (AEs) registered were related to product intake.

Conclusion: Probiotic supplementation with *L. salivarius* PS11603 and *B. longum* subsp. infantis PS10402 enhanced an earlier colonization of *Lactobacillus* and *Bifidobacterium* in preterm infants' guts in 5 and 1 week, respectively. A higher number of infants were colonized by Lactobacilli with the probiotics' intake at the end of the study.

Key Words: Bifidobacterium, colonization, immune profile, Lactobacillus, Ligilactobacillus

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Premature infants, particularly those with very low birth weight (<1500 g) and born before 32 weeks of gestation, present delayed, abnormal development of intestinal microbiota (1,2).

What Is Known

- The gut colonization pattern in early life is associated with short, middle and long-term health effects on the host.
- Preterm birth has a negative impact on the gut microbiota of preterm infants which has been described as aberrant.
- Some probiotic strains have shown to be safe and beneficial for preterm infants.

What Is New

- In this study, the oral administration of the probiotic strains combination of *Ligilactobacillus salivarius* PS11603 and *Bifidobacterium longum* subsp. infantis PS10402 induced an earlier gut colonization with Lactobacilli and Bifidobacterial.

Differences between at-term and preterm infants include a characteristic lower abundance of *Bifidobacterium*, *Lactobacillus* and *Bacteroides*, and higher levels of *Enterobacteriaceae* and *Enterococcaceae* in the latter (1,3–5). Intestinal dysbiosis has been associated with a high risk of neonatal sepsis and necrotizing enterocolitis (NEC), as well as defects in immunologic and neuronal development (6–8).

Probiotics may inhibit the growth of pathogens and improve intestinal permeability and epithelial resistance, competing with potential pathogens for nutrients and epithelial adhesion sites (1,9,10). A few studies have described the effect of probiotics on intestinal microbiota colonization and showed higher levels of *Bifidobacterium* and *Lactobacillus* and lower levels of pathogens

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such as *Enterococcus*, *Klebsiella* or *Escherichia* in neonates treated with probiotics (11–15).

Most probiotic strains belong to the genera *Lactobacillus* and *Bifidobacterium*. The revision of the *Lactobacillus* genus taxonomy led to the reclassification of some species into 25 genera that comprise phylogenetically related microorganisms (16). Despite that reclassification the generic term ‘lactobacilli’ will remain useful to designate all organisms that were classified as *Lactobacillaceae* until 2020 (16). The main objective of this study was to investigate the effect of the probiotic strains *Ligilactobacillus salivarius* PS11603 (according to the classification update, *Lactobacillus salivarius* was renamed as *Ligilactobacillus salivarius*) and *Bifidobacterium longum* subsp. *infantis* PS10402 on the colonization of the intestinal tract. These two strains were isolated from the vagina and human milk, respectively, of a healthy woman, and belong to the ProbiSearch SL (Tres Cantos, Madrid) strain collection. They were selected because of their sensitivity to antibiotics and their in vitro antimicrobial activity against pathogens normally present in neonatal hospital units, among other characteristics (Text, Supplemental Digital Content (SDC) 1, <http://links.lww.com/MPG/C731>). Secondary objectives were to assess the immunological profile of the gastrointestinal tract, the incidence of NEC and sepsis, and the length of stay in the Neonatal Unit. Safety and anthropometric parameters were recorded during the recruited infants’ participation in the study.

METHODS

Study Design

This study investigated the efficacy, safety, and tolerance of a new probiotic strain combination of *L. salivarius* PS11603 and *B. longum* subsp. *infantis* PS10402 and was conducted in the Neonatal Unit of the Hospital Universitario La Paz. The study was approved by the Research Ethics Committee of this hospital on May 24, 2018. Infants admitted to the Neonatal Intensive Care Unit (NICU) between November 2018 and November 2019 were eligible to participate if they were born between week 28 + 0 days and week 30 + 6 days of gestation, written informed consent was obtained, 10 mL kg⁻¹ day⁻¹ enteral feeding was tolerated, and they were < 5 days old. Neonatologists involved in the study as investigators were responsible to enrol participants. A randomization list was created using a complete random method for sequence generation of the R package *randomizeR*; subsequently, the quality of this list was verified to assure balanced allocation of participants and blinding of investigators and participant’s parents. This list was used to assign participants to one of the two parallel study groups: the control group with placebo consumption and the probiotic consumption group (1×10^9 CFU of *Lsalivarius* PS11603 and 1×10^8 CFU of *Blongum* subsp. *infantis* PS10402). Placebo appearance, colour, and taste were identical to the probiotic treatment, and both were identically packaged and stored and labelled with a 4-number code. Both products in this study were produced by ZinereoPharma (Porriño, Spain) and provided certificates of analysis confirming viability and purity of finished products. Either probiotic or placebo were orally administered daily until discharge from the Neonatal Unit or until the 36th week post-gestational age, whichever came first. Care providers, investigators and researchers were all blinded in this study. Only the statistician who analysed the clinical and lab data was finally unblinded in this study, and only after primary outcome analysis.

Clinical and Safety Parameters

Demographic characteristics and relevant medical history were recorded after patient inclusion. Investigators assessed safety

and tolerance outcomes throughout the study duration. Adverse events (AEs) were coded as stipulated in the medical dictionary for regulatory activities (MedDRA version 17.1).

Stool Collection and Analysis

The primary outcome, the effect of the probiotic strains on lactobacilli and bifidobacterial colonization in the intestinal tract, was determined from stool samples with culture-dependent techniques at ProbiSearch, SL facilities. Samples were collected before starting product intake (meconium), weekly for the first month and subsequently every two weeks until the end of the study participation. The first deposition sample of the day per infant was collected.

Additionally, to characterize in deep the gastrointestinal bacterial composition and possible changes in infants after the intervention period, sequencing with Illumina technology was performed at the Unidad de Genómica of the Fundación Parque Científico de Madrid (Spain).

In parallel, the concentration of immunoglobulins (Ig); IgA, IgM and 21 cytokines, chemokines and growth factors were determined in meconium and faecal samples at ProbiSearch, SL facilities. A detailed description of the culture dependent and independent techniques is provided together with the immune profile assessment in SDC 1, <http://links.lww.com/MPG/C731>.

Statistical Analysis

Statistical analysis was conducted using R software (3.4.0, R-project, <http://www.R-project.org>). The sample size was estimated with MedCalc statistical software (MedCalc Software Ltd, Belgium). In a pilot study conducted in five infants with a different probiotic strains combination, 20% of the infants had culturable Bifidobacterial whereas the 100% of them had culturable Lactobacilli at the end of the study (Moles, 2015). Considering this prevalence in culturable Lactobacilli and Bifidobacterial in preterm infants during their first few weeks of life, we hypothesized that a 50% of difference in the percentage of participants colonized by Lactobacillus and/or Bifidobacterium should be achieved between groups by the intervention, at least in the first weeks of life. With $\alpha = 0.05$, statistical power of 90%, 15 infants were needed per group to allow for a 10% dropout rate. The random allocation sequence was generated using R software, “*randomizeR*” package (v.1.4.2). Clinical and safety outcomes were evaluated in the intention to treat population, ITT (preterm infants who were randomized). All efficacy analyses were performed in the modified intention to treat population, mITT (preterm infants who finished the treatment). The qualitative results were analysed by chi-square or Fisher tests. The quantitative results were analysed by one-way or multivariate ANOVA (MANOVA) for normal distributed data or by Kruskal-Wallis (KW) test for nonparametric data. Post-hoc pairwise t-test with Bonferroni correction or pairwise Nemenyi test with Holm correction was used where needed. Linear mixed effects models, with treatment allocation and sampling time as fixed effects and the subject as random effect term, with the restricted maximum likelihood (REML) estimation method, were performed to evaluate the effect of probiotic intake over time in lactobacilli and/or bifidobacterial counts in faecal samples of preterm infants during NICU stay using the “nlme” package from R. Generalized linear mixed models (GLMM) were fitted to assess the robustness of the main outcome assumption. To that end, the binary “Yes/No sample colonization” was used as the response and “group of treatment” was used as independent variable. Once the default model was fitted, possible covariables were subsequently included and models were sequentially compared using ANOVA with chi-square test.

Additionally, the Akaike information criterion (AIC) was obtained for each model. Alpha and beta diversity indexes were determined in infants' meconium and faecal microbiota after the treatment period. The relative abundance of bacteria detected in meconium and faecal samples at the end of the treatment was calculated and summarized as median (Q1–Q3) at phylum, genus, and species level. For Type I error control in primary outcome analysis, the α was adjusted to 0.017 ($\alpha = 0.05/3$). No further multiplicity correction was applied to avoid an uncontrolled increase in type II errors, given the high number of variables analysed.

RESULTS

Study Participants

In this study, 30 infants were recruited (ITT), 27 completed the study (mITT) and 3 withdrew. Additionally, three subjects were excluded from the per protocol population (PP) due to protocol deviations (Fig. 1). Baseline characteristics were similar among groups (Table, SDC2, <http://links.lww.com/MPG/C732>). Nevertheless, the number of relevant medical issues during pregnancy were statistically higher in the infants' mothers from the probiotics group than from the placebo group (16 vs 5) ($P = 0.012$). In fact, 6 of 14 infants from the probiotics group were born after a premature rupture of membranes, whereas no such rupture occurred in the placebo group ($P = 0.016$) (Table, SDC2, <http://links.lww.com/MPG/C732>).

Intestinal Tract Colonization

In this study, the number of participants colonized by lactobacilli was higher in the probiotic group than in the placebo

group (93% vs 46%; $P = 0.013$). Similar results were obtained when analysing bifidobacterial colonization (100% vs 69%; $P = 0.041$). Additionally, in the first 4 weeks of the study, differences in colonization by any of both genera were greater than 50% ($P < 0.05$) between the groups, except for the second week where no statistically significant differences ($P = 0.129$) were observed. The bacterial colonization dynamics analysis revealed an earlier colonization in 5 weeks for *Lactobacillus* and 1 week for *Bifidobacterium* in the probiotic group compared with the placebo group (Fig. 2A, B, Figure, SDC3, <http://links.lww.com/MPG/C733>). The dynamics of lactobacilli and/or bifidobacterial counts were assessed in longitudinally collected samples using a linear mixed model regression (Figure SDC3, <http://links.lww.com/MPG/C733> and SDC4, <http://links.lww.com/MPG/C734>). The fit was statistically significant ($P < 0.0001$) with a strong correlation for the fixed effects included in the model ($P = 0.0011$ for treatment and $P < 0.0001$ for time) but also for the interaction between treatment and time, $F(6, 131) = 3.59$, $P = 0.0025$. Therefore, the effect of treatment and time were pairwise analysed, and P values were adjusted using the Bonferroni multiple testing correction method. A statistically significant change in lactobacilli and bifidobacterial counts in the placebo group was detected at week 6 ($df = 12$, $P < 0.001$) but not at week 8 ($df = 6$, $P = 0.650$) probably due to the reduction of participants because of discharge from the NICU. Furthermore, change in lactobacilli and bifidobacterial counts in the probiotic group was significant from week 1 ($P = 0.036$) to week 8 ($df = 5$, $P = 0.626$) where the discharge from NICU made the comparison meaningless. Lactobacilli counts showed a strong correlation with each one of the fixed effects included in the model: $P = 0.001$ and $P < 0.001$ for treatment and time, respectively, but not for the interaction $F(6, 131) = 1.86$, $P = 0.093$. In contrast,

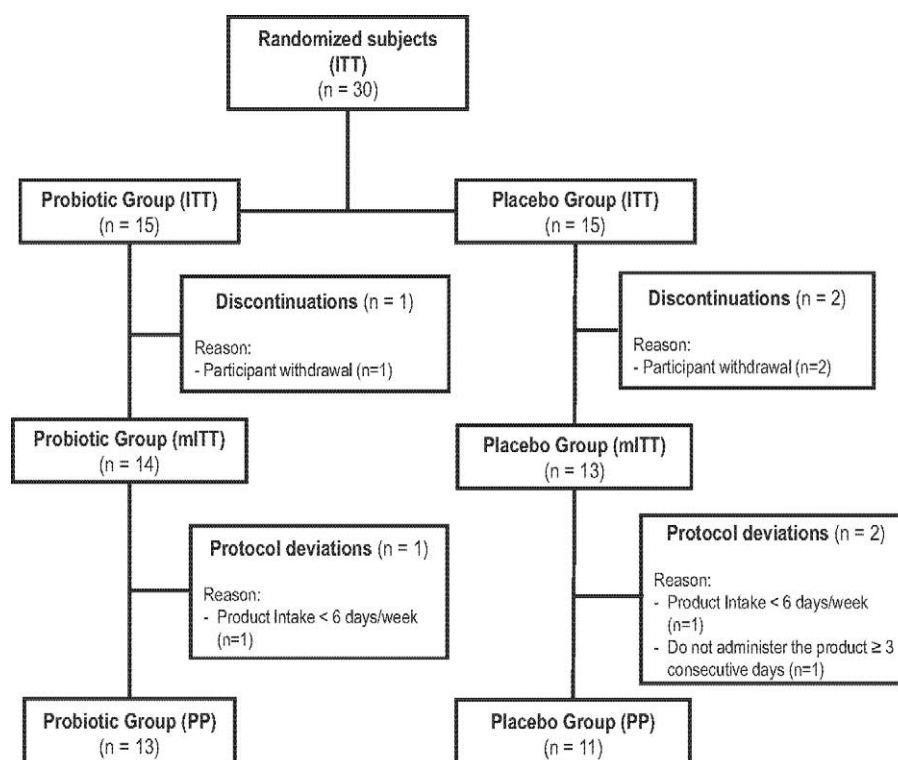


FIGURE 1. Flowchart of the study. The number of participants recruited (intention to treat population) together with the number of participants that completed the study (modified intention to treat population) and the number of participants that accomplished with the protocol of the study (per protocol population) are shown. All the analyses have been performed in the mITT population.

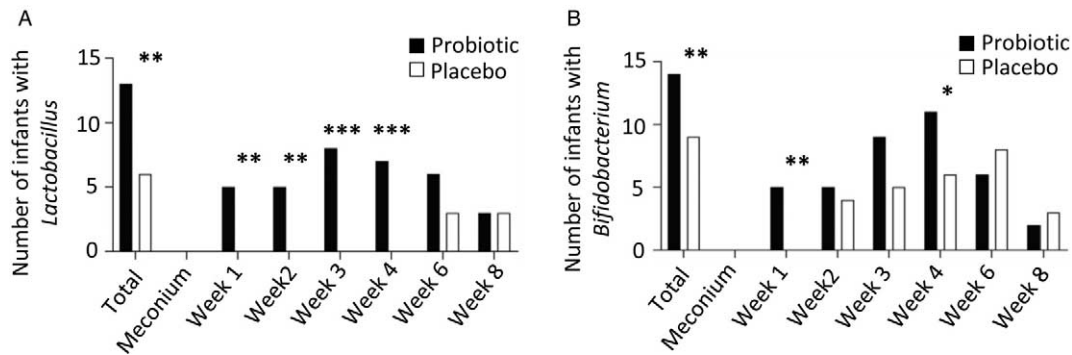


FIGURE 2. Probiotic supplementation induced earlier colonization by beneficial bacteria. (A) Number of infants with positive *Lactobacillus* counts throughout the study. (B) Number of infants with positive *Bifidobacterium* counts throughout the study. Absence, presence contingency tables were obtained for types of bacteria by group and week, then Fisher test were used to analyse the independency (* $P < 0.1$; ** $P < 0.05$; *** $P > 0.01$).

Bifidobacterium counts showed a strong correlation with time $P < 0.001$ and a lack of fit with treatment $P = 0.304$, which was also reflected in the interaction of both factors $F(6,131) = 1.59$, $P = 0.155$. Pairwise comparisons, using paired t -test with Bonferroni correction, showed that mean lactobacilli and bifidobacterial counts were significantly different between placebo and probiotic groups at week 1 ($P = 0.005$) although not at other different time points. The analyses included the quantification of all species of *Lactobacillus* and *Bifidobacterium* and not only the probiotics species (Figure, SDC4, <http://links.lww.com/MPG/C734>).

To evaluate the robustness of the main outcome, a general regression model was fitted including the premature rupture of membranes (PRM), antibiotics administered to the infant after birth (concomitant antibiotics), mother's antibiotic therapy and weekly mother's milk feeding as fixed effects. Neither PRM, nor mother's antibiotics showed an effect on the default model when sequential ANOVA with chi-square test was performed ($P > 0.05$). In contrast, concomitant antibiotics ($P = 0.041$) and mother's milk feeding showed an improvement of the correlation ($P < 0.001$), which supports the relationship between colonization and the use of probiotics.

A total of 54 samples were sequenced with a metagenomic approach, including 27 meconium samples and 27 faecal samples collected at the end of the intervention period from 14 patients in the probiotics group and 13 in the placebo group. It was not possible to obtain enough DNA quantity and quality from four meconium samples. A mean of 95,323 ($\pm 13,205$) reads were obtained for every sample and an average of 91.66% of the reads passed the quality filter. Results showed that meconium samples had higher bacterial diversity than faecal samples collected at the end of treatment in both groups. No statistically significant differences were observed between groups by alpha diversity indices at species level. In contrast, the beta diversity Bray-Curtis index, which considers abundance or count data, showed a significantly higher beta diversity in infants from placebo group at the end of the treatment ($P = 0.027$), while Jaccard and Sorensen indices, which consider only presence/absence were equal at the end of the treatment (Figure, SDC5, <http://links.lww.com/MPG/C735>, which shows the beta diversity of meconium and faecal samples at species level). This change may be due to the loss of the mother-derived species, mainly associated with an adult microbiome. The metagenomic results analysis revealed a different bacterial profile at the end of the treatment. Probiotics group was significantly dominated by *Actinobacteria* compared to placebo group ($P = 0.036$) (Fig. 3) (Table, SDC6, <http://links.lww.com/MPG/C736>, which shows the

intestinal microbiota composition at phylum level). In contrast, *Proteobacteria* was the most abundant phylum in the placebo group (Fig. 3) (Table, SDC6, <http://links.lww.com/MPG/C736>). The genera *Bifidobacterium* (a genus that belongs to Actinobacteria phylum), *Lactobacillus* (a genus that belongs to Firmicutes phylum) and *Actinomyces* increased significantly ($P = 0.037$; $P = 0.037$ and $P = 0.033$, respectively) in the probiotics group whereas *Enterococcus* and *Coprococcus* decreased significantly ($P = 0.020$ and $P = 0.028$) (Table, SDC7, <http://links.lww.com/MPG/C737>, which shows the intestinal microbiota composition at genus level). Similar results were observed at the species level. *B. longum* and *Lsalivarius* were statistically more abundant in infants from the probiotics group (mean relative frequencies from 4.09 to 60.40 and from 0.81 to 2.39) than from the placebo group (mean relative frequencies from 1.38 to 19.09 and from 0.28 to 0.02) ($P = 0.026$ and $P = 0.003$, respectively) (Figure, SDC8, <http://links.lww.com/MPG/C738>, which shows the species detected in infants from the probiotics and placebo groups). Other potentially pathogenic species, such as *Enterococcus faecalis*, *Staphylococcus devriesei* and *Cutibacterium acnes*, were more abundant in infants from the placebo group ($P = 0.013$, $P = 0.020$ and $P = 0.012$, respectively) (Figure, SDC8, <http://links.lww.com/MPG/C738>).

Faecal Immunological Profile

The immunological profile of meconium and faeces were studied at the first week and at the end of treatment (Table, SDC9, <http://links.lww.com/MPG/C739>, which shows the immunological profile of faecal samples). Regarding the meconium immune profile, data revealed significant differences between both treatment groups in G-CSF, which was higher in the placebo group (Table, SDC9, <http://links.lww.com/MPG/C739>). Specific immune responses were estimated by summative contributions of several cytokines. After one week of treatment, data showed an association between a lower specific T_H17 response ($P = 0.050$) and a trend for T_H2 and growth factors ($P = 0.073$, $P = 0.109$, respectively) with the probiotic intake by preterm infants (Fig. 4A). Specifically, a trend to decreased of pro-inflammatory cytokines interleukin (IL)-17 ($P = 0.050$) and IL-22 ($P = 0.074$) were associated to the probiotics group. Although at the end of treatment there were no differences in cytokines concentrations, a trend in the reduction of the innate response ($P = 0.061$) and in the increase of Treg response ($P = 0.099$) were observed in the probiotics group (Fig. 4B). Finally, the analysis of the paired changes of immunoglobulins levels showed a different behaviour for IgA, specifically,

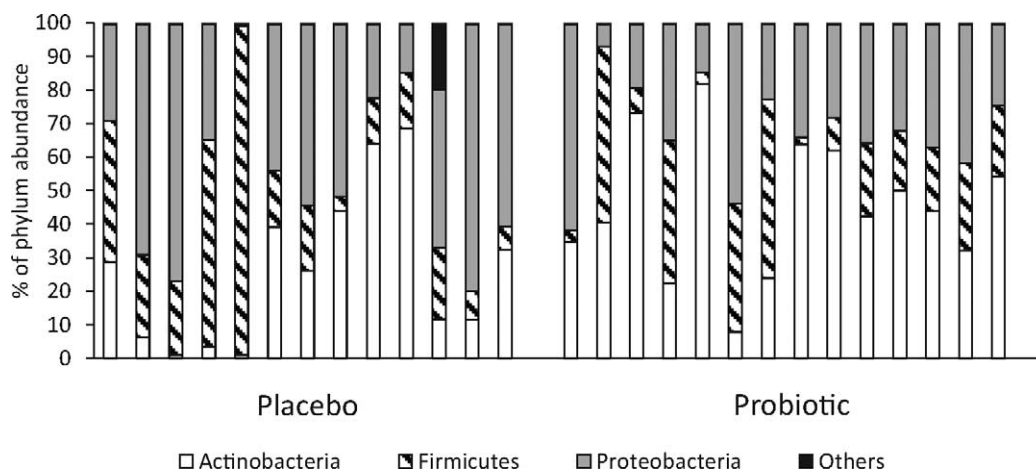


FIGURE 3. Percentage of phylum abundance in all faecal samples at the end of the treatment.

a trend to increase in the probiotic group and to decreased in the placebo group ($P = 0.109$) whereas IgM increased in both groups throughout the study; however, the IgM levels in the first week of the study were greater in placebo, so the changes observed lead to equalize the IgM levels in both groups (Fig. 4C). To gain insights about associations between the use of these probiotics and immune system modulation further studies with larger number of participants are needed.

Safety and Tolerance Evaluation

One hundred and three AEs were reported during the intervention period, 47 from the probiotics group and 56 from the placebo group (Table, SDC10, <http://links.lww.com/MPG/C740>, which shows the number and percentage of subjects and duration of AEs and SAEs) with no significant differences between groups. None of the AEs reported by hospital investigators were related to

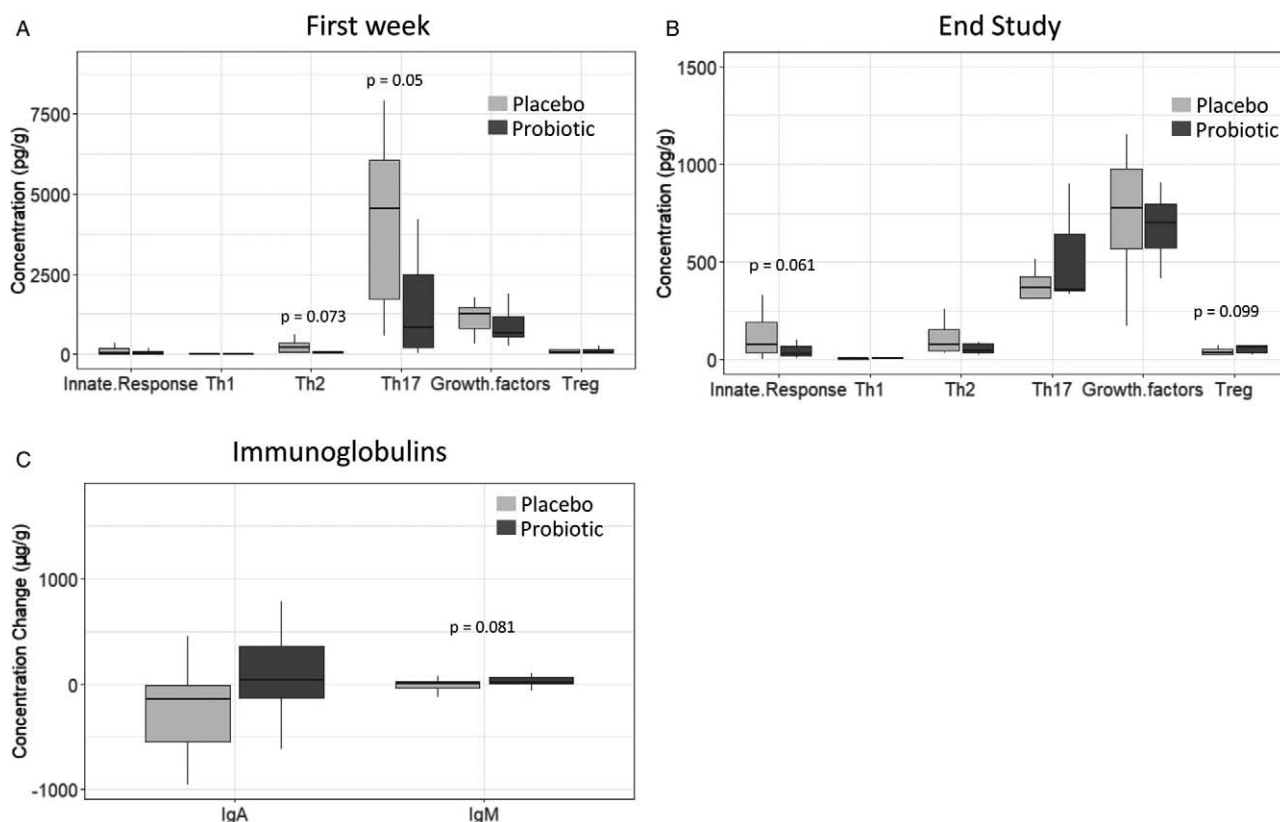


FIGURE 4. Immunomodulatory effect of probiotic supplementation in preterm infants. Levels of major immune responses after 1 week of probiotic treatment (A) and at the end of the treatment (B). (C) Change of the immunoglobulin A levels throughout the study. Data were tested by Kruskal-Wallis test.

the investigational product intake. Secondary variables did not differ between treatment groups at the end of the study, (Table SDC11, <http://links.lww.com/MPG/C741> shows secondary clinical variables at the end of the study). The type of feeding provided to infants was recorded throughout the study period and summarized as the main type of feeding given weekly. No differences were found among groups (Table, SDC12, <http://links.lww.com/MPG/C742> shows type of feeding by group of treatment and study week). The use of concomitant antibiotics was recorded, and no differences were found between groups, neither in the number of concomitant antibiotics administered (k) nor in the number of subjects that received concomitant antibiotic (n) for each one of the antibiotics used, except for the median days of vancomycin and ampicillin duration, which were greater in the probiotic group ($P = 0.035$ and $P = 0.056$, respectively) (Table, SDC13, <http://links.lww.com/MPG/C743>: summary of the concomitant antibiotics). Finally, our results showed no differences in anthropometric parameters throughout the study (length, weight, and head circumference).

DISCUSSION

This study investigated the effect of the probiotic strains *L. salivarius* PS11603 and *B. longum* subsp. infantis PS10402 on the gut colonization of preterm infants by lactobacilli and bifidobacterial. The results obtained by culture dependent techniques and 16 s rRNA sequencing showed a significant higher abundance of *Lactobacillus* and *Bifidobacterium* in the gastrointestinal tract of preterm infants treated with probiotic supplementation compared with control infants. Additionally, an earlier intestinal colonization by these bacteria was detected from the first week of treatment, whereas in control group bifidobacterial and lactobacilli did not appear until the second and sixth week of treatment, respectively. The high rates of human milk feeding during the study may have led to early colonization by bifidobacterial in both groups as seen in previous observations (17). In this study, bifidobacterial were detected in week 2 in three infants from the placebo group who only received their own mother's milk from the beginning of their lives. Preterm infants are routinely tube fed with a mixed diet regimen alternating own mother's milk, donor human milk and formula milk until they are physiologically ready for the coordination of sucking, swallowing, and breathing (18). Human milk is an important source of different bacteria for the infant gut, especially lactic acid bacteria and bifidobacterial, which play an important role in infant-gut microbiota development, thereby improving the neonatal health in both the short and long term (19,20). Our results showed that supplementation with two probiotic strains, *L. salivarius* PS11603 and *B. longum* subsp. infantis PS10402 improved *Lactobacillus* and *Bifidobacterium* colonization regardless of feeding type.

The robustness of the results related to the main outcome of the study were tested by modelling a general regression model for binary outcomes where possible covariables such as PRM, concomitant antibiotics, mother's antibiotic therapy and weekly mother's milk feeding were included as fixed effects. The related models showed how concomitant antibiotics and mother's milk feeding had an effect, improving the fit of the default model (ANOVA chi-squared test), whereas PRM and mother's antibiotics did not.

Despite the isolation of bifidobacterial in both groups from the beginning of the study, the metagenomic 16 s rRNA sequencing of the faecal samples collected after the intervention period showed an *Actinobacteria* dominated microbiome only in preterm infants from the probiotics group. *Actinobacteria*, the phylum which *Bifidobacterium* belongs to, has been associated with healthy

full-term breastfed infants' gut (12,19). Infants from the placebo group presented an intestinal microbiome dominated by *Proteobacteria*, which is associated to dysbiosis and higher risk of diseases such as NEC and sepsis as previously described (7,21,22).

One of the proposed mechanisms by which probiotic bacteria protect premature infants is the normalization of the intestinal microbiota composition (23). Previous studies have shown the ability of the probiotic species *L. salivarius* and *B. longum* in the colonization of the gastrointestinal tract and the modification of the microbiota composition (20,24,25). Notably, in our study the probiotic strains *L. salivarius* PS11603 and *B. longum* subsp. infantis PS10402 were isolated from infants of the probiotics group indicating their ability to survive and colonize the gastrointestinal tract. Also in our study, a higher level of both probiotic species was found in the probiotic group indicating a possible colonization by the two probiotic strains. Importantly, our results showed not only the colonization of gastrointestinal tract by *L. salivarius* and *B. longum* but also the modulation of the microbiota composition during early life after probiotic supplementation.

Regarding the immunomodulatory effect exerted by the two probiotic strains, previous studies have shown that different species of *Lactobacillus* and *Bifidobacterium* are able to modulate the host's immune system through various mechanisms, such as the induction of anti-inflammatory interleukins, the increase of immunoglobulin levels, natural killer and monocytes, as well as the expression of binding proteins that promote adequate intestinal permeability (24,26,27).

In our study, we observed a greater T_H17 response at the first week and a trend towards increasing innate response at the end of treatment in the placebo group, which reflects a more pro-inflammatory state of the immune system compared to the probiotics group. Similarly, we observed a trend of enhancing Treg response and increasing the immunoglobulins levels in the probiotics group at the end of the study. These data might indicate an immunomodulatory effect of probiotics supplementation during the first weeks of life in these infants born preterm. These observations should be confirmed in larger clinical trials with a higher number of participants.

Randomized clinical trials have evaluated the effect of different probiotic strains on the development of allergic diseases such as asthma, wheezing or atopic dermatitis (28). Our data showed lower levels of T_H2 cytokines in the probiotics group after 1 week of treatment, whereas T_H1 cytokines levels remained stable. High levels of T_H1/T_H2 cytokines during the first weeks of life have been associated with the development of intolerances and allergic diseases (29). Moreover, this anti-allergic effect of probiotic treatment can be reinforced by the changes in the levels of anti-inflammatory cytokines secreted by Tregs and immunoglobulins IgA and IgM at the end of treatment. Previous studies have described higher levels of IgA in non-allergic children suggesting a protective role, and IgA faecal levels have been related with better prognosis in preterm infants (30,31). Thus, these findings suggest that probiotic supplementation with *L. salivarius* PS11603 and *B. longum* subsp. infantis PS10402 during the first weeks of life may decrease the probability of suffering allergic pathologies in the future. The follow-up of these patients could shed light on the longterm effect of probiotic supplementation in allergic diseases.

Several cases of sepsis associated with probiotic treatment have been reported in different studies (32). No such adverse event related with the probiotic administration has been documented in the present work.

The main limitation of this pilot study is the low number of participants, which has hampered the analysis and evaluation of the effects of probiotic supplementation on NEC and sepsis incidence and length of stay. Moreover, data showed a higher number of

premature rupture of membranes in the probiotics group (6/14) versus control group (0/13). Premature rupture of membranes is associated with the use of antibiotics and worse neonatal outcomes (33,34). The robustness of the main outcome of the study was tested by modelling a general regression model for binary outcomes where possible covariables such as PRM, concomitant antibiotics (antibiotics), mother's antibiotic therapy (Mother.AB) and weekly mother's milk feeding (Mother.Milk) were included as fixed effects. The related models showed how concomitant antibiotics and mother's milk feeding have an effect, improving the fit of the default model (ANOVA chi-squared test), while PRM and mother's antibiotics did not, so the differences in these characteristics might underestimate our results.

In conclusion, probiotic supplementation with *L salivarius* PS11603 and *B longum* subsp. infantis PS10402 induce early gut colonization by healthy microbiota. This study provides data that support the use of new probiotic tools to modify the intestinal microbiota, contributing to immune system development and decreasing inflammation.

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