Universitat de Girona

# Probiotics combination to prevent Necrotizing Enterocolitis in Extremely low birth weight

## A multicenter, randomized, double blind, placebo, parallel-group trial

**FINAL DEGREE PROJECT**

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*I would like to express my gratitude to all the professionals that step by step helped me to improve and carry out this work*

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# 1. LIST OF ABREVIATIONS

| | |
|---|---|
| **BDP** | Bronchopulmonary Dysplasia |
| **BOV** | Bovine milk-based preterm formula |
| **CFU** | Colony-forming unit |
| **CSF** | Cerebrospinal fluid |
| **ELBW** | Extremely low birth weight infants; <1000 grams |
| **HUF** | Human milk fortifier |
| **INE** | Instituto Nacional de Estadística (Spain) |
| **LOS** | Late-onset sepsis |
| **NEC** | Necrotizing Enterocolitis |
| **NICUs** | Neonatal Intensive Care Units |
| **NNTB** | Number needed to treat to benefit |
| **PAF-AF** | Platelet activating factor- acetylhydrolase |
| **PAF** | Platelet- Activating Factor |
| **SEN** | Sociedad Española Neonatología (Spain) |
| **SGA** | Small for gestational age |
| **VLBW** | Very low birth weight infants; <1500 grams |
| **WG** | Weeks gestation |

## 2. ABSTRACT

Background: The Necrotizing Enterocolitis (NEC) is a devastating neonatal gastrointestinal emergency. The incidence is inversely proportional to the gestational age (prematurity) and birth weight. Although the diagnosis and treatment are fast, the best way to treat this pathology is the prevention. A recent meta-analysis shows that supplementing enteral feeding with a combination of probiotics can reduce the incidence of NEC and mortality related with NEC in VLBW (very low birth weight infants; <1500g) but it has not clear results, for lack of sample, in the ELBW (extremely low birth weight infants; <1000g).

Objective: To evaluate the effectiveness of supplementing the feeding with probiotics (*Lactobacillus Acidophilus* and *Bifidobacterium Bifidum*) vs. placebo for the prevention of NEC in ELBW. The secondary objective is to asses the mortality, mortality related to NEC, and late-onset sepsis.

Methods: The design is a multicenter, randomized, double blind, placebo, parallel-group trial. 912 patients will be recluted during 3 years and randomizied into two groups. 456 will receive enteral feeding with probiotic supplementation and 456 will receive enteral feeding without supplementation.

Key words: Probiotics, necrotizing enterocolitis, infant, preterm, extremely low birth weight.

# 3. INTRODUCTION

## 3.1 OVERVIEW OF NECROTIZING ENTEROCOLITIS

Necrotizing Enterocolitis is a devastating neonatal gastrointestinal emergency. It is a disorder characterized by a ischemic necrosis of the intestinal mucosa of new-born.[1]

**The incidence** of NEC in Europe according to "EuroNeoNet", based on weight:

| 1500-1251g. | 1250-1001g. | 1000-751g. | 750-501g. | <501g. |
|---|---|---|---|---|
| 1.4% | 3.4% | 5.1% | 7.5% | 8% |

*Table 1: Incidence of NEC. Adapted from EuroNeoNet.* [2]

In Spain according to "Informe Anual SEN1500", based on weight:

| 1500-1251g. | 1250-1001g. | 1000-751g. | 750-501g. | <501g. |
|---|---|---|---|---|
| 2.8% | 6.6% | 11.6% | 15.4% | 13.3% |

*Table 2: Incidence of NEC. Adapted from Informe Anual SEN1500.* [3]

As noted above, the incidence is inversely proportional to weight. Therefore, the preterm under 1000g are the potential population which would benefit more from probiotic, nevertheless they are the population less studied.

Although the early diagnosis and fast treatment has improved clinical results, nowadays NEC is an important cause of mortality in neonatal intensive care, particularly in VLBW (<1500g) but even more in ELBW (<1000g). As seen in Table 3, mortality related to NEC increases as the weight in the preterm decreases. For this reason, research efforts, especially in this population, are important to reduce the risk of NEC and mortality.

Mortality related to NEC

| 1500 - 1401g. | 1400 - 1301g. | 1300 - 1201g. | 1200 - 1101g. | 1100 - 1001g. | 1000 - 901g. | 900 - 801g. | 800 - 701g. | 700 - 601g. | 600 - 501g. |
|---|---|---|---|---|---|---|---|---|---|
| 2.8% | 1.7% | 4.4% | 5% | 8.9% | 10.4% | 13% | 11.2% | 9.4% | 19% |

*Table 3: Mortality related to NEC. Adapted from Informe Anual SEN1500.* [3]

Another important point is the morbidity associated with NEC. NEC increases the hospital stay, increases the duration of parenteral nutrition and the surgical interventions

with its possible complications (for example infection, the development of short gut or the occurrence of adhesions).[4]

## Pathogenesis

The etiology of NEC is still nowadays unknown. It has been associated with multifactorial factors which through different mechanisms can produce the mucosal injury.

- Prematurity, 90% of the cases take place in premature infant.[5] Preterm infants are predisposed to an increased risk of intestinal damage due to different factors such as intestinal immaturity, microbial colonization, and the hypoxic state of neonatal gut. [4]

- Intestinal immaturity

  Preterm present an immature motility, inadequate local non-specific mucosal defenses (less polymeric IgA, alteration of digestive enzymes and lower gastric acid production). They have an altered regulation of the gastrointestinal inflammatory response, which produces an excessive inflammatory reply against pathologic colonization.[6]

- Microbial colonization

  The establishment of stable and diverse intestinal flora allows an appropriate regulation of the immune response. Premature have a delayed and often inappropriate colonization. A decreased microbial diversity may reduce colonization resistance, and in consequence the new-born can be colonized by pathogenic bacteria. This produces an excessive inflammatory response and the mucosal injury.[5][6]

- Intestinal Ischemia

  The neonatal gut is more susceptible to a hypoxic state; which can produce a mesenteric endothelial dysfunction and increase the vascular permeability, edema formation, and the development of NEC. [7]

Some conditions that decreases blood oxygen content are associated with an increased risk of NEC such as patent ductus arteriosous (reduces the diastolic blood flow), the treatment with Indomethacin or Ibuprofen (decrease intestinal blood flow for the inhibition of cyclooxygenase), peripartum asphyxia, cyanotic congenital heart disease, maternal cocaine abuse and umbilical arterial catheter.[(4)]

- Enteral Feeding

  The intestinal mucosa injury maybe initiated by different mechanism related with enteral feeding. The type of feeding (human milk versus formula), timing of initial feed, the rate of daily feeding volume advancement and the osmolarity of the feeding, are the most important factors which can iniciate the mucosa injury.

- There are some risk factors identified in full-term infants, even that NEC is uncommon in term infants. They include: cyanotic congenital heart disease, perinatal asphyxia, preeclampsia and small size for gestational age. [(4)]

## Diagnosis and treatment of NEC

Clinical signs and symptoms are highly variable. The most typical are: feeding intolerance, abdominal distension, gross blood in stool and bilious gastric residuals. They are nonspecific manifestation. For this reason, the diagnosis is done with the correlation of:

- Clinical signs: in addition to previous signs commented, preterms can also present, bradycardia, apnoea or temperature instability. During an advanced stage of NEC it may appear a more specific sign, a shiny, distended and erythematous abdomen.
- Radiologic signs: pneumatosis intestinalis (presence of gas in the wall of loops of intestine), and free air in the portal vein, are pathognomonic signs. In advanced stages pneumoperitoneum can appear, although it is not a pathognomonic sign.
- Laboratory analysis: data supporting the diagnosis are: anemia, left shift of neutrophilis or neutropenia (in the most severe cases), thrombocytopenia and

metabolic acidosis (arterial pH <7.2, which in the case it continues longer than 4 hours indicates clinical deterioration).

NEC is diagnosed and classified, according to Bell's criteria (1972) modified for Walsh and Kliegman (in 1986) (see in Annex 1). This classification takes into account the clinical and radiologic signs and allows an early diagnosis and fast treatment according to the severity of the classification. (Annex 2). [(1)(4)]

The time of presentation of NEC has a bimodal distribution, with a first pic categorized as early-onset NEC occurring in newborns under 14 days of age (more prevalent for VLBW) and second pic categorized as late-onset NEC occurring over 14 days (more prevalent for ELBW).[(8)]

## Prevention

Despite all advances in medical and surgical management, the best way to reduce morbidity and mortality from NEC remains in the prevention. Preventive strategies try to regulate pathogenesis alteration and interact with the risk factors.

- Human milk

  The human milk has been considered the feeding choice to prevent NEC, for its immunological factors, it contains antibodies such as IgA, and in small amounts IgG, macrophages, lymphocytes, neutrophils, immunoactive proteins such as lysozyme, interferon, epidermal growth factor, components of complement system and lactoferrin).[(4)] An important component is PAF-AF, the enzyme inhibiting PAF. It has been demonstrated that lower levels of PAF-AF and higher of PAF are related to the pathogenesis of the intestinal immaturity. The PAF-AF can have a preventive action.[(9)]

  For all of these benefits, the mother's milk is the best option for feeding the preterm and preventing NEC, although it can be difficult for the mothers of ELBW. Not all mothers produce sufficient milk to meet their infant's needs. In a randomized clinical trial with total of 207 infants, only 30% were able to supply 100% of their ELBW needs.[(10)]

In these cases and in this population (ELBW), the best way to feed is not quite proven. There are different options such as bovine milk-based preterm formula (BOV), donor human milk or human milk-based human milk fortifier (HUF). Two multicenter randomized trials, concluded that preterm formula versus human milk in ELBW have a significantly greater duration of parenteral nutrition, higher incidence of NEC and higher requiring surgical intervention.[(11)(12)]

The best way to feed, has been most studied in preterm (<37 weeks' gestation (WG)) specially VLBW. A systematic review of Cochrane[(13)] tried to determinate the effect of feeding with formula versus donor breast milk in situations that breast milk mother is not available. The primary outcome was growth and neurodevelopment, and a secondary outcome was NEC. Preterm feeding with formula versus donor breast milk, have higher rate of short-term growth, although this outcome should be interpreted with caution. Two trials which compared formula with HUF did not detect statistically significant difference in long-term growth[a] in extremely premature infants[(10)] and ELBW.[(11)] There is also no difference in neurodevelopment. Nevertheless, the preterms feeding with formula have higher risk of feeding intolerance and NEC (total RR: 2.77 (95% CI 1.40-5.46) and NNTB: 25 (95% CI 14 to 50)).[(13)]

- <u>How should feeding begin: Early trophic feeding vs. Enteral fasting</u>

  The optimal time for initial feeding is uncertain. There are potential benefits in trophic feeding conventionally defined as giving small volumes of milk (12 to 24 ml/kg/day) intragastrically starting the first or second day after birth and increasing the volume slowly during the first week postnatally. Early trophic feeding, stimulates the development of inmature gastrointestinal tract and it has endocrine, metabolic and gastrointestinal motility effects. [(14)]

  In a meta-analysis published by Cochrane[(15)] "Early trophic feeding versus enteral fasting for very preterm or VLBW", no statistical difference on the incidence of NEC (RR: 1.07 95% CI 0.67 to 1.70) was detected. Despite the results of the

[a] <u>Long-Term growth:</u> defined as weight, height or head circumference (and/or proportion of infants who remain below the 10th percentile for the index population's distribution) assessed as intervals from six month post-term.

metaanalysis, early trophic feeding due to the demonstrated benefits is used in most NICUs.

- Probiotics

  Probiotics are defined as non-pathogenic live bacteria that when administrated in adequate amount, confer health benefits. [16]

  Nowadays, probiotics are extensively studied for preventing intestinal pathology, for example diarrhea, in lacting infants. In the neonatal period it can represent an important prevention measure for NEC. Probiotics can prevent the mucosal injury through different mechanism: they might help modulate intestinal microbiota, modulate the inflammatory response with anti-inflammatory effects in cells preparations[17], prevent the increase of intestinal permeability[18], promote the colonization of beneficial commensal microbiota[19], promote intestinal motility in animal models[20] in newborns of 34 weeks gestational age[21]. Even though none of these mechanisms have been studied directly in VLBW or ELBW.

## 3.2 ACTUAL SITUATION OF PROBIOTICS IN NEC

The feeding supplements with probiotics for preventing NEC are one of the most studied interventions of neonatal medicine in the recent years.[22]

On one hand, a meta-analysis of Pediatrics[23] and a posterior review of Cochrane[22] demonstrate evidence for the use of probiotics for preventing NEC in VLBW. The Cochrane's review, explained a significantly reduced incidence of NEC (stage II or more) (RR: 0.431 (95% CI: 0.31-0.56)), a significantly reduced mortality (RR: 0.66 (95% CI: 0.50-0.81)), NEC related mortality (RR: 0.38 (95% CI: 0.18-0.82)), and no significant effect on culture proven sepsis (RR: 0.92 (95% CI 0.81-1.04)) in VLBW.

On the other hand, only two clinical trials[24][25] study ELBW. This population have the highest incidence and mortality related to NEC, although it can be the population potentially more benefited, there are no evidence of the use of probiotics for preventing NEC. The first study conducted by Al Hosni 2012[24] tried to evaluate the benefits of growth as a primary outcome, while another study, the ProPrems conducted in 2013[25]

studied the reduction of late-onset sepsis. In the two clinical trials, the incidence of NEC was a secondary outcome, and this produced that the sample size did not have enough statistical power to find statistical differences. Therefore, currently, there is no evidence that the administration of prophylactic probiotic could reduce the incidence of NEC, mortality, mortality related to NEC and late-onset sepsis in ELBW.

Another important concept that analyzed the Cochrane's review[22] is the effect of different species of probiotics. Before the Cochrane's review, studies analyzed the results regardless of the specie of probiotic used in the clinical trial. But as demonstrated in animal models, the effect obtained with one bacterial species cannot be extrapolated to another.[20] If we analyze the results between species, we can see that the combination of probiotics would reduced the incidence of NEC (RR: 0.37 (95% CI: 0.25-0.54) and mortality (RR: 0.62 (95% CI: 0.47-0.81*).* Nevertheless, without the combination of species, only *Lactobacillus* species reduce the incidence of NEC (RR: 0.45 (95% CI: 0.27-0.75). The other species studied alone, such as *Bifidobacterium* and *Sacharomyces boulardii*, did not found differences. Without combination, no species, demonstrated a reduction of the mortality.

In regard to sepsis, just three clinical trials[25][26][27] showed that the prophylactic treatment with combination of probiotics can reduce the incidence of late-onset sepsis. However the Cochrane's review[22] did not confirm this association. The administration of *Lactobacillus* species, *Bifidobacterium* species or the combination of probiotics did not reduce the incidence of culture proven sepsis.

### 3.3 WHICH PROBIOTICS COMBINATION IS THE MOST SUITABLE?

Nowadays, literature shows evidence that combining probiotics is better than just using one specie. But, the question raised would be, which is the best combination?
There are no clinical trials which compare different multi-strains or single versus multi-strains probiotics products in preterm neonates. All clinical trials compare a probiotic product versus placebo.

To choose the best strain combination, we base our decision on previous studies which have reported safest and most effective probiotic combination in ELBW and VLBW.

In the Cochrane's review[22], 24 clinical trials where analyzed. Nine of them compared different combinations of probiotics versus placebo for different primary and secondary outcomes. The combination of probiotics, the participants and the results of the 9 clinical trials are summarized in Annex 3.

From the 9 clinical trials, six[25][26][27][28][29][30] demonstrated that the prophylactic treatment with probiotic combination reduced the incidence of NEC in VLBW, four[26][27][28][30] demonstrated that the prophylactic treatment with probiotics combination reduced death, and from these, three of them[27][28][30] death related to NEC in VLBW. Finally, three[25][26][27] demonstrated that the prophylactic treatment with probiotics combination reduce the incidence of late-onset sepsis in VLBW.
From the other clinical trials, Al Hosni 2012[24] evaluated the growth velocity in ELBW, Rougé 2009[31] evaluate a possible faster introduction of enteral feeding. Finally Fernández-Carrocera 2013[32] observed a reduction in the number of cases of NEC in the probiotic group versus the control group, but the difference was not statistically significant.

To choose the optimal combination for our population ELBW, we will take account the previous results in ELBW and VLBW, but before we want know the colonization gut situation in order to know which will be the better strains to introduce to enteral feeding. Another item will be how many strains we can combine and finally the security of probiotics.

Firstly, regarding the colonization gut situation in ELBW, Gewolb et al.[33] showed that the colonization of the gut of ELBW is made up by a paucity of bacterial species. *Bifidobacterium* and *Lactobacillus* where found in <5% infants. These data suggest that this low colonization may serve as predisposing factor in microbial infection. They suggested that the possibility of modifying gut flora could be worthwhile. Furthermore, they suggested that some factors such as the treatment with antibiotics and feeding the infant with formula feed could reduce the diversity of microbial colonization in ELBW.

Secondly, we should ask ourselves, how many strains can we combine? G. Desgpande, et al.[34] in "Evidence-based Guidelines for use probiotics in preterm neonates"

explained that using more than two or three strains, each with an optimal dose, may result in higher risk of bacterial translocation, especially in ELBW. The use of more than 2 or 3 strains not in an optimal dose, could mean an inapropiated colonization.

Finally, security should be taken into account. The use of probiotics was described as safe and well tolerated. None of the clinical trials analyzed in the Cochrane's review[(22)] reported adverse effects. Nonetheless, we propose a preventive action, so it is important that it cannot be harmful.

In the literature review there are three cases of sepsis produced by probiotics. The first one was produces by *Bifidobacterium breve*, in an infant of 37 WG, birth weight of 2060g and treated with surgery four hours after birth for an omphalocele.[(35)] Regarding Lactobacillus, there were two cases published by Kunz et al.[(36)] they described two preterm infant (from 36 and 34 WG) who developed *Lactobacillus* bacteraemia, after beening treated with *Lactobacillus rhamnosus GG* . Both of the preterm infants, had short-gut syndrome. The first one, due to congenital intestinal atresia and the second one to gastroschisis. However, in the last Cochrane review[(22)] more than 2700 preterm where analyzed, where no cases produced by probiotics of sepsis were explaining. Therefore, we can conclude that under normal conditions it is not a harmful treatment.

In conclusion, the best option will be a combination of two strains, *Lactobacillus* specie and *Bifidobacterium* specie, previously used in VLBW or ELBW with safe and positives results related with our objectives.

There are two possible combination of probiotics used in clinical trials with ELBW. Al Hosni 2012[(24)] used *Lactobacillus rihamnosus* and *Bifidobacterium infants*, although the authors said that it could be a safe combination, we reject it, due to the two declared cases of *Lactobacillus rhamnosus GG* sepsis. ProPrems 2013[(25)] used a combination of *Bifidobacterium infantis, Bifidobacterium lactis* and *Streptococcus thermophilus.* They analysed 235 infants in the subgroup of <1000g probiotic supplementation, without a significant conclusion. Therefore, we conclude this combination of strains might not be the optimal choice for our study.

Regarding the combinations used in VLBW, just in two studies, meet all of our objectives, which are reducing the incidence of NEC, mortality and sepsis.

The fisrt one, published by Samanta et al.[26] combined *Bifidobacteria infantis, Bifidobacteria bifidum, Bifidobacteria longum* and *Lactobacillus acidophilus.* As comented above, it is better not to use more than two or three strains to not increase the risk of bacterial translocation.

Finally Lin 2005[27] combined *Lactobacillus Acidophilus* and *Bifidobacterium infantis,* concluding a reduction in the incidence of NEC, mortality and sepsis in VLBW. Therefore we expected similar results in our population, ELBW. Those results were; the NNT to prevent 1 case of NEC is 27 and the NNT to prevent 1 death related to NEC is 31. Incidence of Sepsis (culture proven): 12.2% probiotic group vs 19.3% control group ($p<0.03$).

Concerning the security about this strains, Wagner et al.[37] suggested that *Bifidobacterium* were less pathogenic than *Lactobacillus* and that this *Lactobacillus* strain, *Lactobacillus acidophilus,* appear to be innocuous in his preparations of immunodeficient mice. In addition Lin 2005[27] defines this combination as safe given that no adverse effects were reported.

Lin 2005[27] obtained the probiotic combination from INFLORAN®. It is marketed in Spain by an Italy laboratory. Nowadays, they do not commercialize this preparation. They changed the formula from *Bifidobacterium infantis* to *B.Bifidum,* maintaining *Lactobacillus acidophilus.* This new strain of Bifidobacterium is more common in the gut of healthy infants.[38]

The new combination of INFLORAN® (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) was used by Lin 2008[28] he described that it could reduce the incidence of NEC and the incidence of death or NEC in VLBW. Their results were; NNT to prevent 1 case of NEC is 20 patients, and the NNT to prevent 1 death or NEC is 14 patients. This study did not show that probiotics reduce the incidence of sepsis in VLBW. Although his previous study showed the reduction, the meta-analysis[22] did not confirm

the association. He concluded that although probiotics can have a positive role, they would not prevent late-onset sepsis because of the complexity of the disorder.

In our point of view, this combination of probiotic is the most adequate for our study in ELBW. This combination has positives results related with our objectives in VLBW so we can expect similar results in ELBW. Regard to segurity, it has been used in other clinical trials in children or infants with no adverse effects commented in the Abridged Investigator's Brochure.[39] In addition, another positive aspect is the availability. Although other combinations could be used in our clinical trial by importing them from other countries, if in a future probiotics are used as a prophylactic treatment it would be clinically more suitable a preparation marketed in Spain.

Actually, this preparation of INFLORAN® is used in Australia routinely to prevent NEC. It was impulsed by Dr Deshpande in Nepean Hospital of Sydney, Australia. They administered probiotics to preterms gestation up to 31 weeks and 6 days and VLBW; gestation up to 30 weeks and 6 days irrespective of birth weight; that begin enteral feeding before 48 hours.[40]

## 3.4 OVERVIEW OF LATE-ONSET SEPSIS AND MORTALITY IN ELBW

### Late-onset sepsis

Sepsis can be classified according to her mechanism of transmission, or by the time of acquisition. According to the mechanism of transmission we define sepsis as vertical (reflects usually ascending infections from the maternal genital tract), or nosocomial infection (reflects usually infections by germs located in hospital). According to the onset, sepsis is divided in early-onset sepsis, usually vertical, which appears during the first 72h after birth in ELBW, and late-onset sepsis, usually nosocomial, which appears after the first 72h of birth in ELBW.

The incidence of late-onset sepsis (LOS) is inversely associated with birth weight, as seen in table Table 4. So is a relevant problem in our population of the study.

| Weight | Late-onset sepsis % |
|---|---|
| 1500-1251 g. | 13.4% |
| 1250-1001 g. | 28.4% |
| 1000-751 g. | 48.3% |
| 750-501 g. | 57.3% |
| <501 g. | 33.3% |

*Table 4. Late-onset sepsis. Adapted from Informe Anual SEN1500* [3]

LOS is a complexity disorder with multiple factors. Immaturity is one of the most important factors; other risk factors are the long-term use of invasive interventions, such as mechanical ventilation or intravascular catheterisation, a prolonged duration of parenteral nutrition, hospitalization and surgery.[41]

Regarding the etiology, "Grupo de Hospitales Castrillo", published by the SEN, the distribution of germs in nosocomial sepsis in preterms under 1500g the most common are: Coagulase-negative staphylococcocci (CONS), where the *Staphylococcus epidermidis* is the predominant pathogen of LOS (42.3%). Followed by *Candida spp* (15.4%), *E. coli* (5.7%), *Enterococcus* (7.6%) and *Klebsiella* (7.6%).[42]

About the prevention of LOS, hygiene is the most important factor including in it: hand hygiene before touch preterm and use sterile materials such as individual phonendoscope for every preterm. Nevertheless, more preventive actions are required to reduce the incidence. Probiotics is one of the strategies to increase the intestinal mucosal barrier, prevent translocation of bacteria and the competitive exclusion of potential pathogens. Although the potential benefits of probiotics in order to prevent LOS; in the most recent meta-analysis[22] no preventable association is found. This could be explained by the heterogeneity of the different studies analyzed or because LOS includes multiple risk factors. Therefore, more studies are required to determine the effectiveness and safety of Probiotics in preterms.

## Mortality

Low birth weight and prematurity are the main factors to infant mortality, in the developed countries. As seen in Table 5 the mortality increases with the decreasing of the birth weight.

In ELBW, deaths are more frequent during the first and third day of life. The most frequent causes are: extreme immaturity, asphyxia and sepsis. Followed by respiratory distress syndrome and intraventricular haemorrhage.[43]

| Weight | Mortality % |
|---|---|
| 1500-1401 g. | 1.5 % |
| 1400-1301 g. | 3.7 % |
| 1300-1201 g. | 4% |
| 1200-1101 g. | 5.1% |
| 1100-1001 g. | 8.5% |
| 1000-901 g. | 11.2% |
| 900-801 g. | 15.5% |
| 800-701 g. | 30.2% |
| 700-601 g. | 46.2% |
| 600-501 g. | 49.2% |
| 500-401 g. | 77.8% |

*Table 5 Mortality, percentage of the total number of newborns admitted at the NICUs involved in SEN1500.* [3]

## 3.5 JUSTIFICATION OF STUDY

NEC is a well studied emergency for the preterms infants. Nowadays, the best tool for treating NEC is prevention. Currently, human milk is the most important variable controllable to prevent NEC. However, human milk feeding cannot eradicate NEC. In a multicenter clinical trial 3 out of 4 infants included in a probiotics study group and 6 out of 14 infants included in the control group, which were receiving exclusively breast milk feeding, still developed NEC.[28]

Therefore, more strategies for preventing NEC are needed. Probiotics is one of the most studied. Probiotics seems to be a safe, simple and non-invasive treatment that attempt to recreate or anticipate the natural or normal flora. It seems to be effective preventing NEC in VLBW. A recent meta-analysis[22] show that the prophylactic treatment with a combination of probiotics had good results reducing the incidence of NEC and mortality in VLBW. We can expect these results also in ELBW, which is the population with higher incidence of NEC, the population with a possible more benefit but with no definite results about the relation between probiotics and the prevention of NEC.

Knowing the applicability of probiotics in ELBW, it can be an important step for starting to use routinely in the NICUs. So even though we have to do a multicenter study to achieve enough patients to obtain the necessary sample size for get statistically significant results, this study could make a change to the routine clinical practice.

What this study adds:

- This is the first clinical trial that evaluates the reduction of the incidence of NEC as a primary outcome in ELBW.
- This is the first clinical trial that evaluates the effectiveness of supplementing the feeding with a combination of probiotics in Spain and the second in Europe, although it will be the first one that evaluates the reduction of the incidence of NEC as a primary outcome in Europe.
- This study employs a probiotic combination (*Lactobacillus Acidophilus* and *Bifidobacterium Bifidum*) without adverse effects being reported. Used before in a clinical trial with positive results in VLBW, which is nowadays used as a routinely prophylactic treatment in some NICUs from Australia.

## 4. QUESTION, HYPOTESIS AND OBJECTIVES

- **Question:** Supplemening the feeding with a combination of probiotics (*Lactobacillus Acidophilus* and *Bifidobacterium Bifidum*) is more effective than placebo in the prevention of NEC in ELBW?

- **Hypothesis**:

  Primary Hypothesis:

  - Supplementing the feeding with a combination of probiotics (*L. Acidophilus* and *B. Bifidum*) can prevent more cases from NEC than placebo in ELBW.

  Secondary Hypothesis:

  - Supplementing the feeding with a combination of probiotics (*L. Acidophilus* and *B. Bifidum*) can reduce the mortality and/or mortality related to NEC when compared with placebo in ELBW.
  - Supplementing the feeding with a combination of probiotics (*L. Acidophilus* and *B. Bifidum*) can reduce the incidence of late-onset sepsis compared to placebo in ELBW.

- **Objectives:**

  Primary objective:

  - To evaluate the effect of a probiotics combination (*L. Acidophilus* and *B. Bifidum*) compared with placebo in the incidence of NEC in ELBW.

  Secondary objectives

  - To evaluate the effect of a probiotics combination (*L. Acidophilus* and *B. Bifidum*) compared with placebo in the mortality and/or mortality related to NEC in ELBW.
  - To evaluate the effect of combination of probiotics (*L. Acidophilus* and *B. Bifidum*) compared with placebo in the incidence of late-onset sepsis in ELBW.

# 5. METHODS

## 5.1 STUDY DESIGN

A prospective, multicenter, randomized, double blinded, parallel-group trial that compare probiotic combination (*L. Acidophilus* and *B. Bifidum*) versus placebo in the prevention of NEC in ELBW.

## 5.2 STUDY POPULATION

**Inclusion criteria**

Newborns admitted at the NICUs:

- Born <28 completed week gestation and weight <1000g.
- Less than or equal to 7 days of age at the time of enteral feeding initiation.

**Exclusion criteria**

- If they had:
  - Chromosomal anomalies.
  - Cyanotic congenital heart disease.
  - Congenital defects of the bowel, such as: midgut volvulus, omphalocele, gastroschisis, intestinal atresia, diaphragmatic hernia or congenital short bowel.
- If the preterm fed exclusively with formula.
- If the preterm death before the introduction of Probiotic.

**Sample**

Sample size:

Power calculator GRANMO, with the POISSON approximation was used. Accepting an alpha risk of 5% and a beta risk of 20%, with an incidence of NEC of 13.4%, in two-side test. 456 in probiotics supplementation group and 456 in the control group are necessary to recognize as statistically a relative risk greater than or equal to 0.55, with a reason between the samples equal 1 and an anticipated drop-out rate of 5%.

We estimate a relative risk of 0.55 according to the results of previous studies in VLBW. The Cochrane's review[22] show with the combination of probiotics a reduction of the incidence of NEC (RR:0.37 (95% CI: 0.25-0.54). We expect similar results in ELBW.

For the secondary variables: with this sample size, the statistical power is 82% to obtain a statistically significant difference of 20% (46.30% - 37.04%) between probiotic and control group for the incidence of late-onset sepsis. And the statistical power is 83% to obtain a statistically significant difference of 45% (12.60% - 6.93%) between probiotic and control group for the incidence of death related with NEC.

Estimated time of recruitment

There are no data published, about the incidence of ELBW stratified for the different hospitals in Spain. But using the INEbase (www.ine.es) we can know the number of cases of ELBW for the different autonomous communities (Table 6)

| | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|
| Catalonia | 208 | 200 | 213 | 160 |
| Madrid | 196 | 200 | 196 | 160 |
| Andalusia | 187 | 210 | 214 | 179 |
| Valencia | 107 | 111 | 107 | 94 |
| Total in Spain | 1214 | 1219 | 1178 | 1029 |
| Total birth in Spain | 486,537 | 471,994 | 454,648 | 425,715 |

*Table 6. Nº of cases of ELBW for the 4rth autonomous communities with more births.*

If we want study a total of 912 ELBW, we have to design a multicenter study, with the participation of hospitals with NICUs level IIIB from Catalonia, Madrid, Andalusia, and Valencia. With the adequate collaboration and coordination, we can enroll the 912 ELBW in approximately 3 years (according to the number of hospitals participating).

The Hospitals that we will offer to participate to the study are:

- Catalonia: Hospital Josep Trueta, Hospital Vall d'Hebron, H. Sant Joan de Deu.
- Madrid: Hospital La Paz, Hospital 12 de Octubre y Hospital Gregorio Marañon.
- Andalucia: Hospital Virgen de la Macarena de Sevilla.
- Valencia: Hospital La Fe de Valencia.

## 5.3 VARIABLES

**INDEPENDENT**:

- The administration of the **combination of Probiotics:** *Lactobacillus Acidophilus* and *Bifidobacterium Bifidum*.

  It will be measured as a dichotomous nominal qualitative variable (yes or no).

  Equipment required: descriptive analysis.

**DEPENDENT:**

*Primary dependent variable:*

- The **incidence of Necroziting Enterocolitis.**

  Description: The diagnosis of NEC will be done with the modified Bell's classification (Annex 1). A case of NEC is defined as a stage IIA or more in the Bell's classification.

  It will be measured as a dichotomous nominal qualitative variable (yes or no).

  Equipment required: described in "5.4 Measure instruments".

*Secondary dependent variable:*

- **Confirmed Late-onset sepsis**

  Description: we have to differentiate clinical or culture-proven late-onset sepsis.

  *Culture-proven late-onset sepsis*: when we isolate a pathogen from blood, urine or cerebrospinal fluid (CSF), 72h after birth. (CSF: we include it because meningitis in this age group is usually haematogenous, so a positive CSF can be considering as a positive blood culture).

  *Clinical late-onset sepsis*: is defined with at least two signs: (temperature>38ºC or <36ºC, tachycardia>200/min or bradycardia<120/min, apnoea[b], hiperglycemia>140 mg/dl, base excess < -10 mEq/l, increase oxygen requirements (intubation)), and one laboratory sign (C.reactive protein>2 mg/dl, procalcitonine >0.5 ng/ml, platelet count <100/nl) without a pathogen cultured, 72h after birth.[(44)]

[b] Apnoea: is defined as the cessation of respiration for >20s or cessation of respiration of any duration accompanied by bradycardia (<120/min) and/or cianosis.

The two, culture-proven and clinical late-onset sepsis are described as dichotomous nominal qualitative variable (yes or no). Equipment required: described in "5.4 Measure instruments".

- **Mortality:** defined as death occurring after the admission to NICUs and before discharge home. It is a dichotomous nominal qualitative variable (yes or no). Equipment required: descriptive analysis.

- **NEC and mortality**: mortality attributed or related to NEC. When the patient meets the conditions for NEC and mortality. It is a dichotomous nominal qualitative variable (yes or no). Equipment required: descriptive analysis.

**COVARIABLES** All of them are descriptive analysis, obtained from the clinical history, or recorded in the "Case report form" created for the study.

**Prenatal:**

- Prenatal steroids for long maturation: defined as a mother receiving 2 doses of steroids (betamethasone or dexamethasone) given ≥ 24h before delivery.
  It is a dichotomous nominal qualitative variable (yes or no).
- Prolonged rupture of amniotic membrane: defined as rupture of the amniotic membrane >18h before delivery. It is a dichotomous nominal qualitative variable (yes or no).
- Chorioamnionitis: defined as maternal fever (37.5ºC axilary measure, with thermometer), smelling amniotic fluid, leukocytosis and left shift. Confirmed by the obstetrician. It is a dichotomous nominal qualitative variable (yes or no).
- Preeclampsia: defined as have high blood pressure (140/90 mmHg) and one of more of the following complications after the 20$^{th}$ week of pregnancy:
  - Protein in urine (>30 mg/dl urine strip or >300 mg/dl urine samples taken over 24h), new decrease of platelet count, fluid in the lungs (radiologic changes that reflect pulmonary edema), new onset headaches or visual disturbances. Confirmed by the obstetrician.

  It is a dichotomous nominal qualitative variable (yes or no).
- Maternal cocaine abuse: is a dichotomous nominal qualitative variable (yes or no).

**Demographic:**

- Birth weight. It is a continuous quantitative variable and will be expressed in grams.
- Gestational age: will be calculated from the best obstetric estimate based on early prenatal ultrasound and obstetric examination.
  It is a discrete quantitative variable (weeks of gestation)
- Small for gestational age (SGA): defined as infants with birth weight >2 standard deviation respect the weight that corresponds for her gestational age. (Using Carrascosa's table). It is a dichotomous nominal qualitative variable (yes or no).
- Male: is a dichotomous nominal qualitative variable. (yes or no)
- Race: is a nominal qualitative variable (Black, White, Other)
  (Male and Male and black have more risk of NEC-associated death, defined by Holman and associates [45] in an epidemiologic study)

**Clinics:**

- Asphyxia: defined by the following criteria: I) umbilical pH≤ 7. II) Apgar score of 0-6 at 5 minutes. III) Hypoxic-ischemia Encephalopathy. IV) Other organ failure.
  It is a dichotomous nominal qualitative variable (yes or no).
  The Hypoxic-ischemia Encephalopathy will be classified using the Sarnat classification, evaluating the difficulty for initiate and maintain respiration, consciousness, muscle tone, reflex, difficulties in feeding times and convulsions (EEG). It is an ordinal qualitative variable (mild, moderate, severe)
- Anemia: defined as Hb<10 g/dl or haematocrit <30%. It is a dichotomous nominal qualitative variable (yes or no)
- Surfactant: used for respiratory distress syndrome within 2 hours after birth in cases of ventilated infants needing oxygen supplementation and showing radiologic changes typical of respiratory distress syndrome. (It is an indirect measure of hypoxia). It is a dichotomous nominal qualitative variable (yes or no).
- Patent ductus arteriosous: defined as connection between the aorta and the pulmonary artery, when a left to right shunt is proven by echocardiography.
  It is a dichotomous nominal qualitative variable (yes or no).
- Treatment with Ibuprofen: is the treatment of patent ductus arteriosous.
  It is a dichotomous nominal qualitative variable (yes or no).

- Type of feeding: is a nominal qualitative variable, composed of the following categories:
    - Exclusive mother milk.
    - Mother milk + donor human milk.
    - Mother milk + donor human milk fortifier.
    - Mother milk + formula.
    - Exclusive donor human milk.
- Timing of initial feed: defined as delayed time between birth and initiation of enteral feeding. It is a discrete quantitative variable (days of life).
- Days to establish full enteral feeds: it is a discrete quantitative variable (days of life)
- Suspension of enteral feeding: defined as days that the preterm are not feed enterally. It is a discrete quantitative variable (number of days).
- Courses and days of antibiotic treatment: they are discrete quantitative variables (number of courses) and (number of days).
  (The two variables can alter the composition of the intestinal flora).
- Days of total parenteral nutrition: is a discrete quantitative variable (num. of days).
- Umbilical artery catheter, umbilical venous catheter, central venous catheter: they are discrete quantitative variables; they will be measured with number of days, for each one.
- Surgery: is a dichotomous nominal qualitative variable (yes or no).
- Use of invasive ventilation: is a discrete quantitative variable (number of days).

**Follow up of study:**

- Bronchopulmonary Dysplasia(BDP): defined as oxygen treatment and/or respiratory support at 28 days of life and persistent radiographic changes that reflect chronic pulmonary damage. It is a dichotomous nominal qualitative variable (yes or no).
- Retinopathy of prematurity: diagnosed by the examination of fundus of the eye (with ophthalmoscope or RETCAM) in the screening premature control, and classified in stages according to The International Classification of Retinopathy of Prematurity (ICROP). It is an ordinal qualitative variable (stage 1, stage 2, stage 3, stage 4).

- Intraventricular Hemorrhage: diagnosed with transfontanellar echography. And classified with the classification of Papile from Grade I to Grade IV. It is an ordinal qualitative variable (Grade I, Grade II, Grade III, Grade IV).
- Sepsis: the same definition than late-onset sepsis, but without the exclusion criteria from >72h after birth.
- NEC.

### 5.4 MEASURE INSTRUMENTS

All the measures will be done routinely in NICUs in the ELBW. With the objective of homogenize the diagnostic attitudes between hospitals, the following diagnostic patterns are proposed:

- **Diagnosis of NEC:**

  With the clinic suspicion of NEC, we realize: plain abdominal radiography (anteroposterior and left lateral decubitus (to assess the presence of air-fluid levels and free intraperitoneal gas)) every 8h during acute phase of suspected NEC.

  Also will be necessary every 6-12h, complete blood count (impedance), platelet count (impedance), leukocytes count (flow cytometry), blood gas measurement for assessment of the acid-base status (with potentiometric sensors we determine pH, $pCO_2$, Na, K, Ca, and with electrode amperometric we determine $pO_2$, glucose and lactate), and blood cultures (collect 0.5 ml in BACTET peds plus and if Becton Dickson detect that it is positive, cultivate in Blood agar, Blood agar enriched and Chocolate agar).

  Other equipment, for the clinic suspicion of NEC: continuous cardiorespiratory monitoring, thermometer, incubator.

- **Diagnosis of Late-onset sepsis**

  With the diagnosis of clinic late-onset sepsis, we will do 2 blood cultures from different pathways separated 30'-8h, 1 urine culture (suprapubic aspiration; cultivate in Blood agar, Mac Conkey agar, and Cled agar) and 1 cerebrospinal fluid culture (lumbar puncture; cultivate in Blood agar, Chocolate agar, and Thioglycolate broth).

We consider culture-proven late-onset sepsis, when in a 1 blood culture; we isolate a germ gram negative, fungus or gram positive different than *Coagulase-negative staphylococci* (CONS), in this case it will be necessary 2 blood cultures positives. (It is a skin commensal germ in the preterm; it can contaminate the blood at the time of extraction). A sepsis induced for *Lactobacillus* or *Bifidubacterium,* it will be isolated in blood culture or CSF.

Other equipment required: continuous cardiorespiratory monitoring, thermometer, incubator, blood gas measurement, glucometer (enzymatic method using hexokinase), PCR (turbidimetry enhanced by particles), quantitative procalcitonine (electrochemiluminescence or ECL) and platelet count (impedance).

## 5.5 STUDY INTERVENTION

### 5.5.1 Randomization

All of the newborns under 28 WG with the birth weight under 1000g admitted in the NICUs from the hospitals adhered to our clinical trial, that meet the criteria for enter to the study, whose parents after been well informed (patient information sheet, Annex 4) will accept the informed parental consents (Annex 5), will be eligible for the trial.

The statistical specialist, build a randomization sequence using a statistical software. This will provide perform a probability sampling, a simple random sampling. From each patient we will obtain a code. It will provide to the pharmacist department and to the nurse unrelated to childcare. This schedule will let them know in which group is every child allocated, to be able to prepare properly the enteral feeding. Apart from the pharmacist and the nurse all the neonatologist staff and parents are blinded to the randomization allocation.

### 5.5.2 The administration of probiotics combination

Delivery: The Pharmacy department of each center, will prepare the dose, using INFLORAN® (part of Abridged Investigator's Brochure, Annex 6), they will prepare the dose and store in closed capsules. Capsules will be kept in the department of pharmacy; stored in a refrigerator at 2ºC to 8ºC.

Before administrating to the infants, a nurse unrelated to childcare will open the capsule and will introduce it in the enteral nutrition. The enteral nutrition preparation will be labeled with the name and identification number of patient, with no indication of study group assignment.

Characteristics of the preparation

- Diluent: breast milk either from the infant's own mother or from a breast milk bank.[34]
- Volume for diluent: minimum 1 to 1.5 ml per dose.[34]
- Osmotic neonatal milk feed: solution should be diluted to keep a range of osmolarity below 246 to 320 mOsm/l.[46]
- Shelf life: is a manipulated product, so the shelf life is the 25% (6 months) of the shelf life documented in the Abridged Investigator's Brochure (24 months).

Dose: daily dose of $1.5x\ 10^9$ cfu/day. When they reach enteral feeds of 50 to 60 ml/kg/day, daily dose of $3x10^9$ cfu/day.[34] *(An author opinion based on the lower clinically effective doses used in the trials of meta-analysis*[23])

When to start: At the time of first enteral feeding.[22]

How long to continue: Until 35 weeks corrected age.[22] Probiotic supplementation will finish if the infant develops NEC.

When to stop? The administration of the probiotic supplementation will be stopped during the periods when the enteral feeding is stopped. The common indications to stop the feeding are: sign of feeding intolerance (defined as the presence of gastric aspirate in the amount that is more than half of the previous feeding), abdominal distension or blood in the stool. Also during sepsis, NEC or critical illness. Probiotic supplementation could be reintroduced 24 hours after restarting feeding, except after an episode of NEC.

### 5.5.3 The feeding

Probiotic combination will be added to the breast milk, for a nurse unrelated to childcare. We will have two types of preparations. On one hand, we will provide breast milk with probiotic combination for the probiotic group. On the other hand, breast milk without probiotic for the control group.

It is complicated to propose a common strict protocol about feeding for all the centers which will participate in the study. There is no consensuses about a definite feeding pattern for ELBW, it is related to multiple factors, so we propose some rules to homogenize the characteristic of enteral nutrition between the different hospitals.

When a hospital accepts participate in the study, they will accept this rules.

- The time for initial enteral feed, will be decided by the neonatologist of each center. The preterm will start the enteral feeding, when he/she has hemodynamic stability, active bowel sounds without abdominal distension and no bile in nasogastric tube. It will be initiated, if it is possible, the first-second day of live. In any case, it will be always begun during the first week (otherwise the preterm do not meet one of the inclusion criteria).
- According to the weight and gestational age, we will give a certain amount of breast milk. The objective will be to increase the volume of enteral feeding slowly. The increase will not exceed 20 ml/kg/day.
- Suspend parenteral nutrition, when the preterm tolerate a volume of enteral nutrition from 120 ml/kg/day.

### 5.5.4 The diagnosis of NEC

The diagnoses maybe clear, but in other cases it could be more doubtful. It will be made by two neonatologists who are blinded to which group the infants are assigned, probiotics or control group. If they disagree, then the senior neonatologist in each center will be consulted to make the final diagnosis.

### 5.5.5 The diagnosis of late-onset sepsis

The diagnosis of late-onset sepsis maybe clear or doubtful. It will be done by multidisciplinary team. It will be done by the team that controls nosocomial infections formed by neonatologist, preventive specialist, microbiologist and infectologist.

## 5.6 DATA COLLECTION

The data collection will be prospectively abstracted from the medical records. We will create, for the development of the study a Case report form (CRF) (Annex 7).

All Fridays and Mondays a neonatologist (one for each center) will fill the CRF. Every Friday the data from Monday until Friday will be collected and every Monday will be collected the data from Saturday and Sunday. It will be done it every week since the

corrected age of 37 WG for every child. Every Monday, the neonatologist from each hospital, will send by email the CRF of all the week, to the study coordinator. He will send to the statistical specialist, who will introduce the data in a database. The data will be classified for centers, to be able to analyze the results for each hospital independently. (There can be a possible bias for a difference between hospitals).

## 5.7 OTHER ACTIVITIES FROM THE STUDY

### 5.7.1 Pilot experiment

Before starting the clinical trial, a pilot experiment will be conducted for two months to evaluate the CRF, the possible difficulties for obtaining the data and analyzing the correct coordination between hospitals.

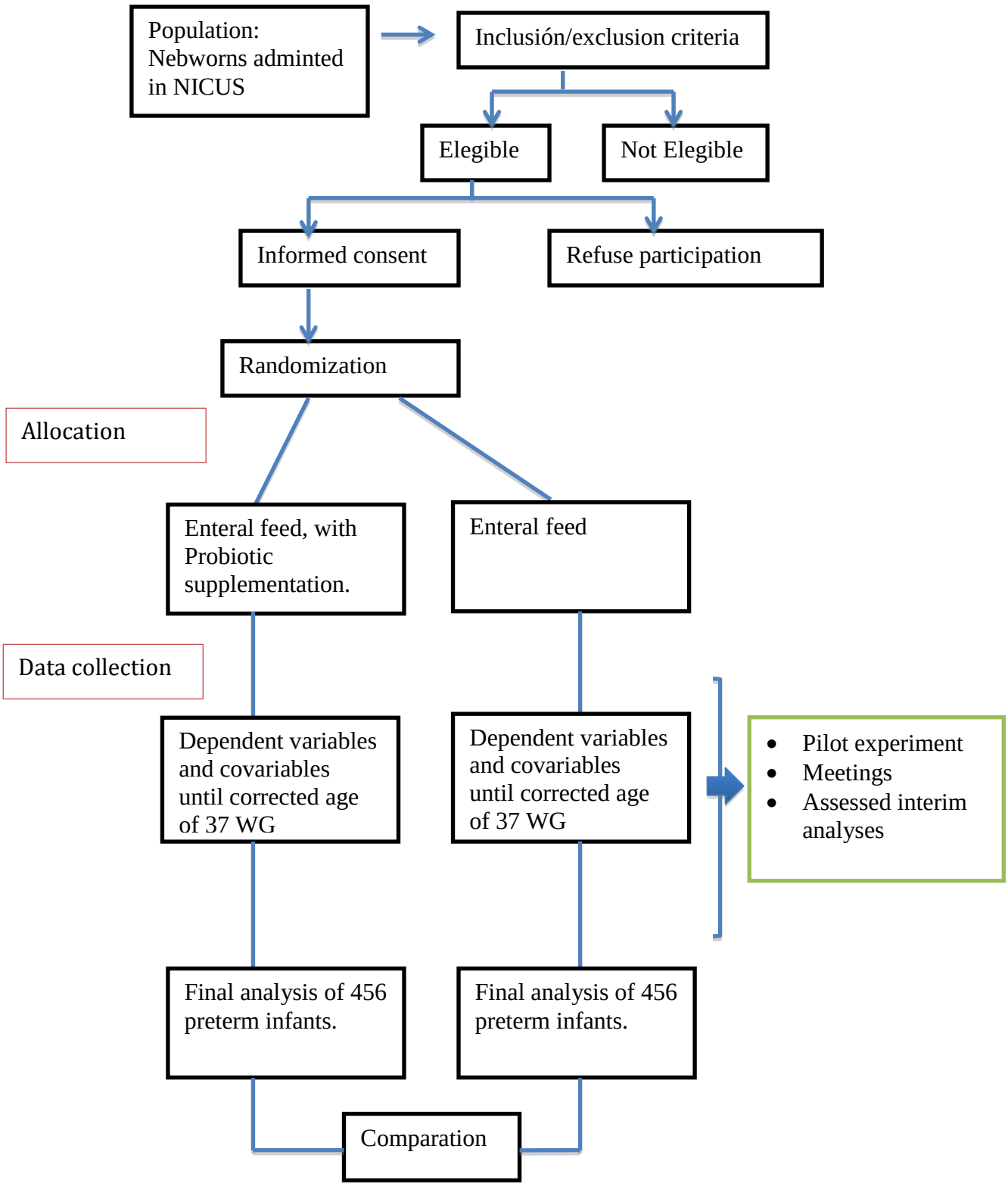
### 5.7.2 Meetings

We will organize 5 meetings with the neonatologist from each hospital, the study coordinator and the statistical specialist. The meetings will be done in Barcelona. The aim of the meetings (before and after the pilot experiment, at the end of the first year, and at the end of second year of data collection) will be to ensure the correct operation of the study (we specify the specific objectives of each meeting in the work plan). At the end of the study, another meeting will be done to analyze and interpret results. We ensure a permanent communication between hospitals and the study coordinator.

### 5.7.3 Assessed interim analyses

At the 100, 200, 400, 600, enrolled infants, the statistical specialist will analyze the data obtained from the outcomes (NEC, mortality, morality related to NEC, late-onset sepsis and possible adverse events) with the objective to stop the study if he find differences clinically relevant between the groups. Finally he will realize the statistical analysis from all the data, when we enrolled 912 infants.

## 5.8 SUMMARY OF THE PROGRESS OF THE SUBJECTS



## 5.9 TASK AND RESEARCH TEAM

- Pharmacy Technician: (1 for hospital = 8).
    - He/she will prepare the dose of probiotics combination and will store them in closed capsules.
- Pharmacy: (1 for hospital = 8).
    - He/she will carry out the protocol that follow the pharmacy technician, to perform the dose preparations of probiotics. (Just 1 pharmacy department, the other centres use the same protocol).
    - At the end of every production of probiotics capsules with the desired dose, the pharmacy specialist will check them. They will also do a control of the final osmolarity of the feeding after adding the probiotic capsule. (The Pharmacy specialist of each hospital)
- Nurse unrelated to childcare: (1 for hospital = 8).
    - Prepare the enteral nutrition: on one hand, mix the probiotic capsule with the enteral nutrition for the probiotic group. On the other hand, prepare the enteral nutrition without probiotic for the control group. In addition he/she will ensure that the enteral nutrition arrives to the correct child. This work is important for ensure the double blinded, just the nurse and pharmacy knows in which group are each patient.
- Neonatologists: (1 for hospital =8).
    - Will fill the CRF, and send every Monday the data from the week to the study coordinator.
    - Interpret and analyze results.
- The Statistical specialist: 1.
    - Design randomization program.
    - Assessed interim analysis at the 100-200-400-600 enrolled infants.
    - Statistical analysis.
- The study coordinator: 1.
    - He/she is the responsible for the correct operation of the study, the coordination and to get and convey the data from the hospitals to the statistical specialist.
    - Interpret and analyze results.

## 6. STATISTICAL ANALYSIS

In the **univariate analysis**, we will define variables as categorical or continuous.

- Categorical variable will be described as percentages and proportions.
- Quantitative variables will be described, with means ± standard deviation (the variables with normal distribution) and with median and interquartile range (25-75) (the variables with skewed distribution).

In the **bivariate analysis**, the independent and primary and secondary dependent variables are categorical. So the comparison between the independent and dependent variables will be carried out with Chi-Square test.

In the **multivariate analysis**, a logistic regression test will be used to estimate odds ratio and 95% confidence intervals. It will be used to assess the relationship between different outcome measures and the effect of probiotics, after adjustment for the potentially confounding effects. For each dependent variable, the potentially confounding effects are:

- NEC: birth weight, gestational age, prenatal steroids, prolonged rupture of amniotic membrane, chorioamnionitis, preeclampsia, maternal cocaine abuse, SGA, male, male and black, asphyxia, anemia, surfactant, patent ductus arteriosous, treatment with Ibuprofen, type of feeding, timing of initial feed, days to establish full enteral feed, suspension of enteral feeding, course and days of antibiotic treatment, days of parenteral nutrition, umbilical artery and venous catheter.
- Late-onset sepsis: birth weight, gestational age, SGA, days of total parenteral nutrition, umbilical artery, venous or central catheter, surgery and use of invasive ventilation.
- Death: birth weight, gestational age, SGA, asphyxia, surfactant, BDP, Retinopathy of prematurity, intraventricular hemorrhage, sepsis and NEC.

All statistics analysis will be carried out with the Statistical Package for Social Science (SPSS). To manage computed data, Microsoft Excel tool will be used. P value of $<0.05$ will be considered to indicate statistical significance. Analysis will be done in intention to treat and the results may be stratified for the different hospitals.

## 7. LIMITATION

- The incidence of NEC is higher in ELBW than VLBW, but there are more VLBW than ELBW, therefore to be able to achieve the sample needed, we will do a multicenter study. This involves:
    - Differences between hospitals, for example in the enteral feeding protocols, due to this, we propose common rules that can be adapted within the different protocols with the aim to homogenize. The data it maybe analyzed for the different hospitals separately, because we can evaluate if it is necessary a possible interaction.
    - Difficult coordination between hospitals: We will do 5 meetings, to put in contact the different hospitals.
- The diagnosis of NEC and late-onset sepsis can be clear, or in other cases doubtful, for this reason it will be always done for more than one person.
- It is important to maintain the blindness (just the nurse unrelated to child care and the pharmacy team from all the clinical team knows in which group are each infant). It is important for avoid an observer bias (information bias, during data collection).
- There are different variables that can increase the incidence of NEC. Although with the randomization this variables will be distributed in the two groups, we analyze them in multivariate analysis for adjust the results for the confusion factors.
- It is a clinical trial, so by definition the study has a higher cost. Especially with the population that we analyze. The infants have a higher insurance cost. Even so it is the best design to answer our hypothesis.
- The study may be an important step for start to use probiotics routinely in the NICUs. Even so, we will have to consider that there are no data for the population that are involved in the exclusion criteria (chromosomal anomalies, cyanotic congenital heart disease, congenital defects of the bowel), the effectiveness and safety is not proven in this population.
- The study delivers results for a determinate combination of probiotics. There are no studies comparing different strains or combinations, so other combination it can be adequate. Further studies must be performed to identify the most effectiveness combination.

# 8. ETHICAL ASPECTS

This clinical trial follows the declaration of Helsinki involving ethical principles for Medical Research involving Human subjects (last actualization October 2013).

It must be approved by the Clinical Research Ethics Committee (CEIC) of all the centers participating in the study.

The research project will be performed according the Spanish laws related to clinical trials: "Ley 29/2006 de 26 de Julio, de garantías y uso racional de los medicamentos y productos sanitarios

- RD 223/2004 de 6 de febrero: ensayos clínicos con medicamentos
- RD 1591/2009 de 16 de octubre y 1616/2009 de 26 de octubre: investigación con productos sanitarios"

It will be registered in AEMPS web page with the EudraCT application.

All the legal guardians of the subjects participating in the clinical trial it will be informed properly before sign the informed consent. All the data collected from each patient will be treated and used anonymously, preserving the confidentiality of the patient according to the "Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal".

All the investigators will have to declare no conflict of interest.

In this clinical trial, ethic principles are respected:

- INFLORAN® is compared with placebo, because there is no evidence about the effectiveness of probiotics in ELBW, even the positive results in VLBW.
  If we studied population of VLBW we would have to compare probiotic combination with other probiotic combination.
- We have planned assessed interim analyses, for stop the trial in case of detects differences clinically relevant between groups.

# 9. WORK PLAN AND CHRONOGRAM

The research team will be composed by: one study coordinator (**SC**), neonatologist (**Neo**, one for center), nurse unrelated to child care (**Nuc**, one for center), pharmacy technician (**Ptch**, one for center), pharmacy (**P**, one for center) and one statistical specialist (**SS**).

The duration of the clinical trial it will be 4.5 years, and it will be organized according the following steps:

1. Design the protocol (3 months) it will be do it for the SC.
2. Coordination of the centers and members of the study (3 months): selection of the centers that will participate in the study, every center will select the Neo, Nuc, Ptch and P. The protocol it will be discussed with the members of the study to make sure that all the centers agree with the procedures. Finally the Neo of each hospital will have to obtain the ethical approval of the protocol from the CEIC from each center.
3. Meeting 1: before starting the pilot experiment, the first meeting will be done to solve all the questions and check that the protocol and the CRF have been understood in order to be followed by all of them. (SC, Neo from the different hospitals, SS).
4. Pilot experiment (2 months): to detect problems, mistakes of the CRF and possible failure of coordination. (SC, Neo, Nuc, Pth, P).
5. Meeting 2: the aim will be to discusse if there are any improvement of the CRF and coordination, before starting the data collection. (SC, Neo from the different hospitals, SS).
6. Study or data collection (36 months): patients recruiting, randomization in the two study groups and data collection. The duration of the study will be 3 years (It can be modified; the objective is to enroll 912 ELBW). During this time, we will do the following activities:
    a. Ptch and P: he/she has to supply the probiotics capsules preparations.
    b. Nur: he/she has to mix the probiotic with enteral feeding in the probiotic group and supply the enteral feeding in the two groups.

   c. Neo: fill the CRF.

7. Meeting 3 (12 months after initiate data collection) and Meeting 4 (24 months after initiate data collection). The aim will be to solve problems, check the quality of the data collected in each center, and to check that the protocol has been followed. Another activity will be interpreting the preliminary results from the interim analyses and quantifying the number of patients with the objective to determine the real duration of the study. (SC, Neo from the different hospitals, SS).
8. Statistical Analysis, it will be done by the SS.
   a. Preparation randomization. Design statistical software to carry out the probability sampling (simple random sampling).
   b. Assessed interim analysis, in the 100, 200, 400, 600, enrolled infants with the finality to stop the clinical trial if the probiotics or placebo show clinically relevant results.
   c. Finally statistical analysis (6 months). Data will be analyzed, with the 912 ELBW.
9. Meeting 5 (after statistical analysis) with the objective to interpret and discuss the results from the statistical analysis. (SC, Neo from the different hospitals, SS).
10. Publication (SC, Neo, SS) and dissemination (SC) of the research findings (6 months). Write and edit the results to publish them and assist to conference to disseminate the findings.

## CHRONOGRAM SCHEME

| Activities | Personnel | 2014 | 2015 | | | | 2016 | | | 2017 | | | 2018 | | | 2019 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 11-12 | 1 | 2-4 | 5-6 | 7-12 | 1-4 | 5-8 | 9-12 | 1-4 | 5-8 | 9-12 | 1-4 | 5-10 | 11-12 | 1 | 2-4 |
| 1. Design protocol | SC | ■ | ■ | | | | | | | | | | | | | | |
| 2. Coordination hospitals | SC, Neo | | | ■ | | | | | | | | | | | | | |
| 3. Meeting 1 | SC, Neo, SS | | | ● | | | | | | | | | | | | | |
| 4. Pilot experiment | SC, Neo, Nuc, P, Ptch | | | | ■ | | | | | | | | | | | | |
| 5. Meeting 2 | SC, Neo, SS | | | | | ● | | | | | | | | | | | |
| 6. Data collection | SC, Neo, Nuc, P, Ptch | | | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | | | |
| 7. Meeting 3 Meeting 4 | SC, Neo, SS | | | | | | | ● | | | ● | | | | | | |
| 8. Statistical Analysis<br>• Preparation randomization<br>• Assessed interim analysis<br>• Finally statistical analysis | SS | | | ● | | ● | | ● | | ● | | ● | | ■ | | | |
| 9. Meeting 5 | SC, Neo, SS | | | | | | | | | | | | | | ● | | |
| 10. Publication results | SC, Neo, SS | | | | | | | | | | | | | | ■ | ■ | ■ |
| 11. Dissemination the findings | SC | | | | | | | | | | | | | | | | ■ |

## 10.FEASIBILITY

The research study will be proposed to perform in 8 hospitals from Spain. All the procedures that we evaluate will be done routinely. Except the supplementation the enteral feeding with probiotic, which will be registered in the budget.

The hospitals will provide personal salaries, instruments and the cost of the techniques to carry out the diagnosis, the cost of enteral feedings, computers to send CRF and fridges for storing the capsules of probiotic.

We have estimated that the duration of the data collection will be 3 years. This will be possible with the participation of the most representative hospitals from the 4 communities with more premature births. Although the data in Table 6 shows a decrease of ELBW, we can conclude that the premature rate has not stopped increasing in the past 20 years and parallel, the trend of ELBW rate is maintained or even increased. Using published data in www.ine.es, we can observe, the trend of premature birth rate and ELBW rate, for the total births in Spain for each year.

| Year | 1996 | 2006 | 2012 | 2013 |
|---|---|---|---|---|
| % of preterm birhts | 5.84 % | 6.84 % | 7.72 % | 7.6 % |
| % of ELBW births | 0.170 % | 0.241 % | 0.272 % | 0.254 % |

The possible reasons of this increase maybe the delayed maternity, the increase of assisted reproductive technology, the increase of multiple birth, work stress and maternal problems (such as diabetes or high blood pressure).

We know that is difficult to enroll 912 ELBW for our study, but the relevance of the study and the possible impact of the results, justifies the efforts. The preterms are a population more vulnerable and increasingly present in our society.

## 11.BUDGET

For perform the budget, we will do the following estimates:

- The majority of preterms are from 27-28 WG (we use 27 for the estimation) and they will be treated with probiotics until the 35 corrected WG. A total of **8 weeks,** 56 days.
- It will be necessary a total of **104 capsules** of $1.5x10^9$ cfu/day for each child.

  Explication: In normal conditions, the enteral feeding, start at the second day with 10 ml/kg/day. They will be treated with dose of $1.5x10^9$ cfu/day until the seventh day, when in normal conditions the preterm receives 65 ml/kg/day. In this moment the preterm receives $3x10^9$ cfu/day and we maintain it until the 35 corrected WG (a total of 49 days).

  In conclusion: the first day without enteral feeding, 6 days with a supplementation of $1.5x10^9$ cfu/day, and 49 days with a supplementation of $3x10^9$ cfu/day.
  (6+ 49x2 = 104 capsules of 1.5 $10^9$ cfu/day).

| | **Description** | **Total cost** |
|---|---|---|
| **STAFF** | | |
| - Pharmacy Technician | 13.89€/100 capsules x 475 operations (A)<br>(0.2315€/min x 60' of work) | 6,597.75€ |
| - Pharmacy | | |
| ○ Protocol | 19.99€ (0.3333€/min x 60' of work) | 19.99€ |
| ○ Control | 3.33€ (0.3333€/min x10') x 475 operations | 1,583.17€ |
| - Nurse unrelated to Childcare | 200€/year. For 8 hospitals, and 3 years (B) | 4,800€ |
| - Neonatologist | 350€/year. For 8 hospitals, and 3 years (C) | 8,400€ |
| - Statistical Specialist | 40€/h x 70h | 2,800€ |
| - Study coordinator | 350€/year. For 4.5 years (D) | 1,575€ |
| **PROBIOTICS** | | |
| - INFLORAN® | 3.9 units x 456 preterms x 8.56€ for unit (E) | 15,223.10€ |
| **MATERIAL** | | |
| - Capsules | 0.00515726€/unit x 47,424 capsules | 244.57€ |
| - Fungible (CRF, and form) | | 700€ |
| **MEETINGS** | | |
| - Coordination meetings | 50€/person. 9 persons. 4 meetings | 1,800€ |
| - Analyze the results | 50€/person. 9 persons. 1 meeting | 450€ |
| **INSURANCE** | CONFIDE estimation, for the 912 infants and 3 years. | 30,000€ |

| | | |
|---|---|---|
| **PUBLICATION and DISSEMINATION**<br>- Cost of publication<br>- National journey to disseminate the findings<br>- International journey to disseminate the findings | | <br>2,500€<br>1,000€<br><br>2,000€ |
| | **Total cost** | 79,693.58€ |

(A) Every patient treated with probiotic need 104 capsules, for the 456 patient from the probiotic combination group, makes a total of 47,424 capsules; it will be made in groups of 100, in 475 operations.

(B) The work will be in part-time. One more step (add the probiotics and ensure the blindness) to a work that is performed in the NICUs.

(C) The work will be in part-time.

(D) The work will be in part-time. The study coordinator, it will be one of the Neonatologists from the hospitals that participate in the study.

(E) In a package of INFLORAN®, we have 20 capsules of $2x10^9$ cfu/day, and we can obtain 26.66 capsules of $1.5x10^9$ cfu/day for every package.
We need 104 capsules, and we have 26.66 capsules in a package, so we need 3.9 units of INFLORAN® for each child.

## 12.CONFLICT OF INTEREST

The authors declare no conflict of interest.

## 13.IMPACT OF THE PROJECT

If the results are relevant and our hypotheses are validated, probiotics could start to be used routinely as preventive treatment of NEC. It can be a change in the medical practice.

On one hand, it will be a positive change, because the preventive treatment with probiotic is cheap and it seems to have good results in VLBW. Therefore, if these results are similar in ELBW, we can reduce the incidence of NEC, and this would reduce the expenditures in the NICUs.

On the other hand, the economic burden of NEC is substantial, owing to prolonged hospital stays, surgical interventions and the possible complications (such as death, infection, the development of short gut, or the occurrence of adhesions). There are no studies from the economic impact of NEC in Spain, but we have it from USA. J.Bisquera estimate in a case-control study [(47)] that if an infant which develops a NEC, survives reciving medical treatment, he/she will be hospitalized for an additional 22 days and incur additional hospital charges of $73,700. Moreover, if he/she develops a NEC and receives surgical treatment, he/she will be hospitalized for an additional 2 months and incur additional hospital charges of $186,200. Finally, it was estimated that the economic cost of NEC is approximately 19% of neonatal expenditures in the United States.

At last, a recent paper of “Anales de Pediatría” from “Grupo de Nutrición y Metabolismo Neonatal de la SEN”[(48)] considers that the use of probiotic should be taken into account in preterms <32 WG and/or <1500g, including <1000g, although there is insufficient evidence about the efficacy of probiotics in this subgroup. We consider, that now is the most appropriate time for carring out the study and consequently according to the results, implement or not the routine use of probiotics to prevent NEC in the Spanish NICUs.

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# 15. ANNEX

## 15.1 Annex 1-Modified Bell's staging criteria for diagnosis NEC [4]

| Stage | Classification | Systemic Signs | Intestinal Signs | Radiologic Signs |
|---|---|---|---|---|
| IA | Suspected NEC | Temperature instability, apnea, bradycardia | Elevated pregavage residuals, mild abdominal distention, occult blood in stool | Normal or mild ileus |
| IB | Suspected NEC | Same as IA | Same as IA, plus gross blood in stool | Same as IA |
| IIA: Mildly ill | Definite NEC | Same as IA | Same as I, plus absent bowel sounds, abdominal tenderness | Ileus, pneumatosis intestinalis |
| IIB: Moderately ill | Definite NEC | Same as I, plus mild metabolic acidosis, mild thrombocytopenia | Same as I, plus absent bowel sounds, definite abdominal tenderness, abdominal cellulitis, right lower quadrant mass | Same as IIA, plus portal vein gas, with or without ascites |
| IIIA: Severely ill, bowel intact | Advanced NEC | Same as IIB, plus hypotension, bradycardia, respiratory acidosis, metabolic acidosis, disseminated intravascular coagulation, neutropenia | Same as I and II, plus signs of generalized peritonitis, marked tenderness, and distention of abdomen | Same as IIB, plus definite ascites |
| IIIB: Severely ill: bowel perforated | Advanced NEC | Same as IIIA | Same as IIIA | Same as IIB, plus pneumoperitoneum |

## 15.2 Annex 2- Treatment according Bell's classification

| Stage | Treatment Strategy |
|---|---|
| IA- IB<br><br>Suspected NEC | Close clinical observation for increased abdominal distension and feeding intolerance.<br><br>Consideration of bowel decompression and brief discontinuation of feeding (24h); abdominal radiograph (anteroposterior and left lateral decubitus); monitoring of white-cell and platelet counts (sudden decreases suggest progression of disease); consideration of blood cultures and short course of intravenous antibiotics. |
| IIA- IIB<br><br>Confirmed NEC | Bowel decompression and discontinuation of enteral feeding for approximately 7-10 days.<br><br>Close monitoring of white-cell and platelet counts (sudden decreases suggest progression of disease); blood culture and intravenous antibiotics for 7-10 days; close monitoring of abdominal radiographs (anteroposterior and left lateral decubitus);<br>notification of surgical team. |
| IIIA- IIIB<br><br>Definite NEC | Exploratory laparotomy for resection necrotic gut and preserve the vital intestine.<br><br>Percutaneous drain: in unstable patients, to stabilize the patient before surgery. |

*Annex 2. Treatment of NEC. Adapted: NEC: The New England Journal of Medicine* [1]

## 15.3 Annex 3- Results of the 9 clinical trials with Probiotics combination

| Probiotics combination | Participants | Results | Study |
|---|---|---|---|
| *Lactobacillus rihamnosus* and *Bifidobacterium infants* | 101 infants with birth weight <1000g<br><br>(50 PS vs. 51C)<br>No difference between groups<br><br>PS: probiotic supplementation<br><br>C: control | Primary outcome:<br>Feeding with PS improves growth velocity in ELBW in the first 28 days, but it has no difference in the percentage of preterms <10$^{th}$ percentile weight at 34 weeks postmenstrual age.<br><br>Secondary outcome:<br>This PS combination is safe, no difference PS vs. placebo in sepsis, intraventricular hemorrhage, chronic lung disease and retinopathy of prematurity.<br><br>No difference in NEC or mortality (for the small number of subjects enrolled) | Al Hosni 2012 [24] |
| *Bifidobacterium infantis* *Bifidobacterium lactis* and *Streptococcus thermophilus* | 1099 infants <32 WG and <1500g<br><br>(548 PS vs. 551C)<br>No difference between groups.<br><br>Subgroup <28 WG and <1000g<br><br>(235 PS vs. 239C)<br>No difference | Primary outcome:<br>PS combination produce a reduction in late-onset sepsis in subgroup of >28 WG, but not for those <28 WG.<br><br>Secondary outcome:<br>Reduce the incidence of NEC, but not in the subgroup of <1000g (the study was not powered to detect a differential NEC reduction and they have a small number of affected infants in this subgroup).<br><br>PS combination is safe, without significant adverse effects. | ProPrems 2013 [25] |
| *Bifidobacteria infantis*, *Bifidobacteria bifidum*, *Bifidobacteria longum* and *Lactobacillus acidophilus* | 186 infants <32 WG and <1500 g<br><br>(91 PS vs. 95 C)<br>No difference | Primary outcomes:<br>The number of days required to reach full enteral feed, , the incidence of NEC and the death rate was significantly low in PS compared to the control group<br><br>Secondary outcomes:<br>The duration of hospital stay and the incidence of sepsis was significantly low in PS group compared to the control group. | Samanta 2009 [26] |

| | | | |
|---|---|---|---|
| *Lactobacillus Acidophilus* and *Bifidobacterium infantis* | 367 infants <1500g<br><br>(180 PS vs. 187C)<br>No difference | Primary outcome:<br>The combination of probiotic, reduce significantly the incidence of NEC in VLBW infants. Also found a lower incidence of death and death or NEC.<br><br>Secondary outcome:<br>They found a significantly lower incidence of sepsis (culture proven). | Lin 2005 [27] |
| *Bifidobacterim bifidum* and *Lactobacillus acidophilus* | 454 infants <34 WG and <15000g<br><br>(217 PS vs. 217C)<br>Difference in low birth weight (p:0,03)<br><br>Stratification for the different weight groups:<br><br>1500-1001g:<br>(115 PS vs. 138C)<br><br>1000-751g:<br>(69 PS vs. 61C)<br><br>750-500g:<br>(33 PS vs. 18C) | Primary outcome:<br>This combination of Probiotics, feeding enterally to VLBW for 6 weeks, reduced the incidence of NEC and death or NEC. Incidence of NEC was significantly lower in infants weighted 1001-1500g and 500-750g , but not in 751-1000g.<br><br>Limitations:<br>The number of infants was limited for infants weighing <750g (33 in PS study group and 18 in control group. (They neglected to stratify according to birth weight)).<br>Without the stratification by weight, the combination of Probiotics reduce the incidence of NEC and death or NEC<br><br>Secondary outcome:<br>The incidence of sepsis did not differ between study and control groups for infants in the different weight groups. | Lin 2008 [28] |
| *Bifidobacterium breve* and *Lactobacillus casei* | 231 infants <1500g<br><br>(119 PS vs.112C)<br>No difference | Primary outcome:<br>Oral supplementation of these probiotics reduced the incidence of NEC.<br><br>Secondary outcome:<br>No difference in sepsis, duration of birth weight recovery and hospital stay.<br><br>PS group achieve time to full enteral feeds faster than control group. | Braga 2011 [29] |
| *Bifidobacteria infantis, Streptococcus thermophilus* and | 145 infants <1500g<br>(72 PS vs. 73C)<br>No difference | Primary outcome:<br>The PS reduced the incidence and severity of NEC. | Bin- Nun 2005 [30] |

| | | | |
|---|---|---|---|
| *Bifidobacteria bifidus* | | Secondary outcome:<br>No difference in sepsis,days to full feeds and days till total parenteral nutrition stopped,<br><br>They found a lower rate of mortality in PS group front C group but it was not statistical difference. Althought when they combined incidence of NEC and/or mortality, they found statistical difference between PS and C group. | |
| *Bifidobacterium longum and Lactobacillus rhamnosus* | 94 infants <32 WG and <1500 g<br><br>(45 PS vs. 49 C)<br>No difference | They stop the inclusions after the fourth sequential analysis, when they showed no statistically significant difference in his primary outcome, the percentage of infants receiving >50% of their overall nutritional needs enterally at a post-natal age of 14 days.<br>(The simple size was estimated with the expected rate to detect a 20% increase in proportion of infants receiving >50% of nutritional needs in enteral administration, in probiotics group compared control group).<br><br>No differences in others secondary outcomes as nosocomial infections, sepsis, duration of antibiotic use, NEC, duration of hospital stay and death. | Rougé 2009 [31] |
| *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus casei, Lactobacillus plantarum, Bifidobacterium infantis*, and *Streptococcus thermophilus* | 150 infants <1500g<br><br>(75 PS vs. 75 C)<br>No differences | Primary outcome:<br>No differences in term of NEC risk reduction (RR: 0, 54 95%CI 0,21 to 1,39) although they observe a clear trend in reduction of NEC in PS group, 6 (8%) versus the control group, 12 (16%).<br><br>(The sample size was estimated for reduce the incidence of NEC on 20% in subjects without treatment and 5% with treatment)<br><br>Secondary outcomes:<br>No difference in sepsis, apnea, anemia, patent ductus arteriosous and death between PS and control group | Fernández-Carrocera 2013 [32] |

Abbreviations: PS Probiotics-supplementation; C control; WG weeks gestation.

## 15.4 Annex 4- Patient information sheet

**HOJA DE INFORMACIÓN AL PACIENTE**

**TÍTULO DEL ENSAYO CLÍNICO:** Uso de la combinación de probióticos para prevenir la Enterocolitis Necrotizante en los Extremos Bajo peso al nacer: Ensayo clínico, multicéntrico, aleatorizado, con doble ciego, placebo y diseño en paralelo.

**INTRODUCCION**

Nos dirigimos a usted para informarle sobre un estudio de investigación en el que se le invita a participar. El estudio ha sido aprobado por el Comité Ético de Investigación Clínica del Hospital Universitari de Girona Dr.Josep Trueta y la Agencia Española del Medicamento y Productos Sanitarios (AEMPS), de acuerdo a la legislación vigente, el Real Decreto 223/2004, de 6 de febrero, por el que se regulan los ensayos clínicos con medicamentos.

Nuestra intención es tan solo que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación. Además, puede consultar con las personas que considere oportuno.

**PARTICIPACIÓN VOLUNTARIA**

Debe saber que su participación en este estudio es voluntaria y que puede decidir no participar o cambiar su decisión y retirar el consentimiento en cualquier momento, sin que ello altere la relación con su médico ni se produzca perjuicio alguno en su tratamiento.

**DESCRIPCIÓN DEL ESTUDIO**

La enterocolitis necrotizante es una emergencia gastrointestinal. Su incidencia está inversamente relacionada con la edad gestacional y el peso de nacimiento. Aunque se realiza un diagnóstico temprano e un tratamiento rápido, actualmente es una causa importante de morbilidad y mortalidad en las UCIs neonatales, particularmente en los

Bajo peso al nacer (<1500 g) y sobre todo, en los extremo bajo peso al nacer (<1000 g). En los últimos años, se han estudiado diferentes intervenciones preventivas para disminuir la incidencia de enterocolitis, una de ella es la suplementación de la alimentación con probióticos. Estos han demostrado reducir la incidencia de enterocolitis en los bajo peso al nacer (<1500 g). El objetivo de nuestro estudio es estudiar la eficacia de los probióticos versus placebo en la prevención de la enterocolitis en los extremo bajo peso al nacer (<1000 g). Además se estudiará la eficacia de los probióticos versus placebo en la reducción de la mortalidad, mortalidad relacionada con enterocolitis y sepsis tardía (aparece >72h).

Para ello se ha diseñado este ensayo clínico donde se administrará de forma aleatoria la combinación de probióticos o placebo. Al ser un proceso aleatorizado todos los pacientes tienen las mismas posibilidades de recibir probióticos o placebo.

**PROCEDIMIENTOS DEL ENSAYO**

Los probióticos se administrarán junto la leche materna o de banco, en el grupo placebo se administrará la leche materna o de banco sin la suplementación.

La combinación de probióticos se obtendrá de un preparado comercializado, INFLORAN®. Se trata de una combinación de *Lactobacillus acidophilus* y *Bifidobacterium Bifidum*. Se administrará a una dosis de 1.5 $x10^9$ ufc/día hasta que tolere 50-60 ml/kg/día en ese momento se administrarán $3x10^9$ ufc/día.

**BENEFICIOS Y RIESGOS DERIVADOS DE SU PARTICIPACIÓN EN EL ESTUDIO**

Este estudio pretende ser una referencia, para iniciar el tratamiento profiláctico con probióticos en los recién nacidos de extremo bajo peso al nacer.

Se realizarán análisis evaluadores internos durante el transcurso del estudio, para asegurar que no hay diferencias clínicamente relevantes entre los dos grupos de estudio.

INFLORAN® no describe efectos secundarios. Aún así el estudio se realiza en prematuros de extremo bajo peso al nacer, por lo que no se pueden garantizar la ausencia de efectos adversos. Los pacientes que lo reciban así como el grupo control recibirán monitorización para detectar posibles efectos secundarios, como mala tolerancia alimentaria. En los diferentes estudios, respecto el uso de probióticos se han descrito 3 casos de sepsis inducida por su suplementación. En todos ellos los prematuros presentaban defectos congénitos intestinales motivo por el cual es un criterio de exclusión

**SEGURO**

Según lo establecido en el RD 223/2004, el promotor del ensayo ha contratado una póliza de responsabilidad civil con la compañía CONFIDE que cubre los posibles daños y perjuicios que le pudiera ocasionar su participación en el ensayo clínico.

**COMPENSACIÓN ECONÓMICA**

Su participación en el estudio no le supondrá ningún gasto. Usted no tendrá que pagar por los probiótcios ni recibirá una compensación económica

**CONFIDENCIALIDAD**

El tratamiento, la comunicación y la cesión de los datos de carácter personal de todos los sujetos participantes se ajustará a lo dispuesto en la Ley Orgánica 15/1999, de 13 de diciembre de protección de datos de carácter personal. De acuerdo a lo que establece la legislación mencionada, usted o su hijo podrán ejercer los derechos de acceso, modificación, oposición y cancelación de datos, para lo cual deberán dirigirse a su médico del estudio. Los datos recogidos para el estudio estarán identificados mediante un código y solo su médico del estudio/colaboradores podrán relacionar dichos datos con su hijo y con su historia clínica.

Sólo se transmitirán a terceros y a otros países los datos recogidos para el estudio que en ningún caso contendrán información que le pueda identificar directamente, como nombre y apellidos, dirección, nº de la seguridad social, etc. En el caso de que se produzca esta cesión, será para los mismos fines del estudio descrito y garantizando la confidencialidad como mínimo con el nivel de protección de la legislación vigente en

nuestro país. El acceso a su información personal quedará restringido al médico del estudio/colaboradores, autoridades sanitarias (Agencia Española del Medicamento y Productos Sanitarios), al Comité Ético de Investigación Clínica y personal autorizado por el promotor, cuando lo precisen para comprobar los datos y procedimientos del estudio, pero siempre manteniendo la confidencialidad de los mismos de acuerdo a la legislación vigente.

Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos y puede exigir la destrucción de todas las muestras identificables previamente retenidas para evitar la realización de nuevos análisis.

## 15.5 Annex 5- **Informed consent**

**CONSENTIMIENTO INFORMADO DEL FAMILIAR RESPONSABLE O REPRESENTANTE LEGAL**

Yo (nombre y apellidos) ……..... en calidad de ……..... ..... (relación con el participante) de ..... .....……. (nombre y apellidos del participante)

He leído la hoja de información que se me ha entregado.
He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.
He hablado con:
.....
(nombre del investigador)
Comprendo que la participación del paciente es voluntaria.
Comprendo que puede retirarse del estudio:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en sus cuidados médicos.

Presto mi conformidad para que ……...……(nombre del participante) participe en este estudio y doy mi consentimiento para el acceso y utilización de los datos en las condiciones detalladas en la hoja de información.

Firma familiar o testigo:
Nombre:
Fecha:

Firma del investigador:
Nombre:
Fecha:

**CONSENTIMIENTO INFORMADO ANTE TESTIGO**

Yo (nombre y apellidos del testigo) ……..... declaro bajo mi responsabilidad que …….....(nombre y apellidos del participante) ha leído (o se le ha leído, en caso de que no pueda leer, la hoja de consentimiento que se le ha entregado)

Ha leído la hoja de información que se me ha entregado.
Ha podido hacer preguntas sobre el estudio.
Ha recibido suficiente información sobre el estudio.
Ha hablado con:
.....
(nombre del investigador)
Comprendo que la participación del paciente es voluntaria.
Comprendo que puede retirarse del estudio:

1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en sus cuidados médicos.

Presta su conformidad para participar en este estudio y da su consentimiento para el acceso y utilización de los datos en las condiciones detalladas en la hoja de información.

| | |
|---|---|
| Firma testigo: | Firma del investigador: |
| Nombre: | Nombre: |
| Fecha: | Fecha: |

## 15.6 Annex 6- INFLORAN®. Part of Abridged Investigator's Brochure

**Note of the Author:** We provide, from the Abridged Investigator's Brochure, the Prescribing information. If some one wants the Abridged Investigator's Brochure completed, contact in this email: u1909195@campus.udg.edu

### Prescribing information:

**Summary of Product Characteristics INFLORAN**

**1. NAME OF THE MEDICINAL PRODUCT**

INFLORAN

**2. QUALITATIVE AND QUANTITATIVE COMPOSITION**

Every 250 mg capsule contains:

Active substances:

- Live lyophilised Bifidobacterium bifidum: not less than 1,000 million
- Live lyophilised Lactobacillus acidophilus: not less than 1,000 million

**3. PHARMACEUTICAL FORM**

Rigid capsules.

**4. CLINICAL PARTICULARS**

4.1 **Therapeutic indications**

*Prophylaxis:* Prevention of gastrointestinal dysmicrobism syndromes.

*Treatment:* Diarrhoeic and dyspeptic syndromes caused by altered bacterial flora (diarrhoea, non specific enteritis in adults and infants, colitis). Dysmicrobism (microbial disorder) caused by antibiotics. Digestive disorders in the formula fed infant. Adjuvant in the elimination of pathogenic chemobiotic-resistant enterobacteria.

4.2 **Posology and Method of Administration**

For children and adults: swallow one capsule with some warm liquid thrice a day before meals. For infants: pour the content of the capsule into a little warm liquid or sugared water. Do not take more than the advised dosage.

4.3 **Contraindications**

Hypersensitivity to the components of the product or to chemically closely related substances.

4.4 **Special warnings and precautions for use**

Do not use for prolonged treatments. After a short treatment period without evident results, seek medical advice. There might be batch-related colour differences between lyophilised products due to the excipients contained in the preparation. The product contains sucrose: this must be considered for diabetics and patients following a low calorie diet.

4.5 **Interaction with other medicinal products and other forms of interaction**

None reported.

4.6 **Pregnancy and Lactation**

Product administration is allowed.

4.7 **Effects on the ability to drive and use machinery**

None.

4.8 **Undesirable effects**

No undesirable effects have been reported at the recommended doses.

4.9 **Overdose**

No overdose reactions have been reported.

**5. PHARMACOLOGICAL PROPERTIES**

5.1 **Pharmacodynamic properties**

Eubiotic saprophytes of the human intestine. They balance and restore intestinal bacterial flora, inhibit colonisation by pathogens through antibiosis mechanisms, and are effective against intestinal dysmicrobism caused by chemobiotics.

5.2 **Pharmacokinetic properties**

Rapidly colonising components of normal intestinal flora that activate physiological functions and survive in the bowel.

5.3 **Preclinical safety data**

Infloran is provenly safe: its tolerability is confirmed by long-term clinical use for several years.

**6. PHARMACEUTICAL INFORMATION**

6.1 **List of excipients**

Sucrose, jelly, magnesium stearate, lactose.

6.2 **Incompatibilities**

None reported.

6.3 **Shelf life**

24 months with the package intact.

6.4 **Special precautions for storage**

Store at a temperature range of + 2°C to + 8°C, away from the light.

6.5 **Nature and contents of container**

PVC/PVDC/aluminium blisters in package containing 20 capsules.

6.6 **Special precautions for disposal and handling**

None.

**7. Marketing Authorisation Holder**

Laboratorio Farmaceutico S.I.T. Specialità Igienico Terapeutiche S.r.l. - Via Cavour 70 - Mede (PV).

**8. Marketing Authorisation Number**

024037018

**9. Date of first authorisation / renewal of authorisation:**

01 June 2005.

**10. Date of (partial) text revision:**

April 2002.

## 15.7 Annex 7- Case Report Form

# CASE REPORT FORM:

**Project:** Probiotic combination for prevent Necrotizing Enterocolitis in ELBW:

A multicenter, randomized, double blind, placebo, parallel-group trial.

| Hospital:_____ | Patient's identification number |
|---|---|
| Dr name:_____ | |
| Date:___/___/___ | Date of birth:___/___/___ |

**Example** ~~Yes~~ | No or write the answer in "_____"

**Prenatal:**

| | | |
|---|---|---|
| • Prenatal steroids for long maturation | Yes | No |
| • Prolonged rupture membrane | Yes | No |
| • Chorioamnionitis | Yes | No |
| • Preeclampsia | Yes | No |
| • Maternal cocaine abuse | Yes | No |

**Demographic:**

| | | | |
|---|---|---|---|
| • Birth weight | _____ g | | |
| • Gestational age | ____ WG | | |
| • Small for gestational age | Yes | No | |
| • Male | Yes | No | |
| • Race | Black | White | Other |

**Clinics:**

- Asphyxia

| Yes | No |
|---|---|

- Hypoxic-ischemia Encefalopathy

| Mild | Moderate | Severe |
|---|---|---|

- Anemia

| Yes | No |
|---|---|

- Surfactant

| Yes | No |
|---|---|

- Patent ductus arteriosus

| Yes | No |
|---|---|

- Treatment with Ibuprofen

| Yes | No |
|---|---|

- Type of feeding

| |
|---|
| o 0) Suspension of enteral feeding |
| o 1) Exclusive mother milk |
| o 2) Mother milk + donor human milk |
| o 3) Mother milk + donor human milk fortifier |
| o 4) Mother milk + formula |
| o 5) Exclusive donor human milk |

Write for the different days, 0 1 2 3 4 or 5 in the following table according to the type of feeding:

| Monday | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|
| | | | | | | |

If the infant stop the enteral feeding, explain the cause:_____

If the infant supplement the feeding with donor human milk fortified, write the commercial preparation:_____

Velocity of initial enteral feed:

| Monday ml/kg/day | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|
| _____ | _____ | _____ | _____ | _____ | _____ | _____ |

- Timing of initial enteral feed ___ day of life
- Days of establish full enteral feeds ___ day of life
- Courses of antibiotic treatment

| |
|---|
| New courses:___ Data:_____ |
| Type of antibiotic: _____ |

- Days of antibiotic treatment

| Monday | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|

- Umbilical artery catheter

| Monday | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|

- Umbilical venous catheter

| Monday | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|

- Central venous catheter

| Monday | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|

- Surgery Type of surgery: _____ Yes | No
- Use of invasive ventilation

| Monday | Tuesday | Wedn. | Thursday | Friday | Saturday | Sunday |
|---|---|---|---|---|---|---|

**Follow up of the study:**

- Bronchopulmonary Dysplasia Yes | No
- Retinopathy of prematurity

| Stage 1 | Stage 2 | Stage 3 | Stage 4 |
|---|---|---|---|

- Intraventricular haemorrhage

| Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|---|---|---|---|

- Sepsis

| Yes | No |
|---|---|

**Dependent variables:**

- Necrotizing Enterocolitis

| Yes | No |
|---|---|

| |
|---|
| I A |
| I B |
| II A |
| II B |
| IIIA |
| III B |

Comments:

- Confirmed late-onset sepsis

| Clinical late-onset sepsis | | Culture-proven late-onset sepsis | |
|---|---|---|---|
| Yes | No | Yes | No |

Pathogen isolated: _____

Comments:

- Mortality

| Yes | No |
|---|---|

- NEC and mortality

| Yes | No |
|---|---|