UNIVERSIDAD AUTONOMA DE MADRID

Facultad de Medicina

Departamento de Pediatría

Programa de Doctorado en Ciencias de la Salud

Microbiota, probióticos y morbilidad en recién nacidos de muy bajo peso

Tesis doctoral presentada por

Esperanza Escribano Palomino

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Director: Dr. Miguel Saenz de Pipaón Marcos

Madrid 2019

A mis padres, a mi hermana, a mi marido y a mis hijos

INFORME DEL DIRECTOR DE TESIS PARA LA AUTORIZACIÓN DE DEFENSA DE TESIS DOCTORAL

Don Miguel Sáenz de Pipaón Marcos

Director de la tesis doctoral de Dª Esperanza Escribano Palomino

Informa favorablemente la solicitud de autorización de defensa de la tesis doctoral con el Título: **Microbiota, probióticos y morbilidad en recién nacidos de muy bajo peso.**

Presentada por dicha doctoranda.

Programa de Doctorado: Medicina y Cirugía. Plan 641.

La tesis se presenta como compendio de publicaciones.

La tesis no está sometida a procesos de confidencialidad.

Resultados y valoración *(Results and assessment):*

ANTECEDENTES DE LA CUESTIÓN Y OBJETIVOS PROPUESTOS *(Background of the field and main goals)* *La enterocolitis necrotizante y la sepsis son enfermedades graves en el recién nacido prematuro. Las bacterias intestinales juegan un papel protector o aumentan el riesgo de estas enfermedades. Sin embargo el efecto del ambiente epidémico sobre la microbiota, el papel protector de determinados probioticos en niños extremadamente prematuros o la interacción entre bacterias probioticos y el huésped no son conocidas.*
DESARROLLO DEL TRABAJO Y METODOLOGÍA *(Procedure and Development of the general matter and methodology)* *Análisis secuencial de la adquisición de microbiota fecal en recién nacidos prematuros de bajo peso durante las tres primeras semanas de vida en dos escenarios epidemiológicos diferentes en la misma unidad.* *Estudio retrospectivo ecológico en una cohorte de recién nacidos extremadamente prematuros, nacidos entre la 23 y la 27 semanas de edad gestacional, que explora los efectos de la administración de Infloran®.* *Estudio piloto para elucidar si la administración de dos cepas probioticas aisladas de leche humana aparecen en las heces del recién nacido y la evolución de distintos parámetros inmunológicos.*
APORTACIONES DE CARÁCTER GENÉRICO O EXPERIMENTAL *(Contribution to the experimental and general field)* *El establecimiento de la microbiota se ve influida por el ambiente epidemiológico hospitalario. La epidemia de Serratia influye sobre la adquisición de microbiota.* *La administración de Infloran se asocia con un aumento en la incidencia de enterocolitis. En todas las heces de los recién nacidos se asilaron las bacterias probioticas extraídas de la leche materna. Se observó una disminución de calprotectina en heces.*

INFORME DEL DIRECTOR DE TESIS PARA LA AUTORIZACIÓN DE DEFENSA DE TESIS DOCTORAL

PUBLICACIONES A QUE HAYA DADO LUGAR
(Publications appeared already)

1: Moles L, Escribano E, de Andrés J, Montes MT, Rodríguez JM, Jiménez E, Sáenz de Pipaón M, Espinosa-Martos I. Administration of Bifidobacterium breve PS12929 and Lactobacillus salivarius PS12934, two strains isolated from human milk, to very low and extremely low birth weight preterm infants: a pilot study. J Immunol Res. 2015;2015:538171. doi: 10.1155/2015/538171. Epub 2015 Feb 22. PubMed PMID: 25759843; PubMed Central PMCID: PMC4352454.

2: Escribano E, Zozaya C, Madero R, Sánchez L, van Goudoever J, Rodríguez JM, de Pipaon MS. Increased incidence of necrotizing enterocolitis associated with routine administration of Infloran™ in extremely preterm infants. Benef Microbes. 2018 Sep 18;9(5):683-690. doi: 10.3920/BM2017.0098. Epub 2018 Jun 11. PubMed PMID: 29888655.

3: 10 de mayo 2019: PONE-D-18-32393R1
Influence of a Serratia marcescens Outbreak on the Gut Microbiota Establishment Process in Low-Weight Preterm Neonates
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VALORACIÓN GLOBAL
(Final evaluation)
La Tesis doctoral realizada por Dª Esperanza Escribano Palomino reúne los requisitos de interés y calidad científica exigidos, incluyendo diseño metodológico y resultados, aportando datos relevantes para la comunidad científica internacional. Ha efectuado un trabajo impecable, participando de forma activa en cuantos análisis, comentarios, sugerencias y acciones se han ido derivando desde la obtención de los resultados iniciales.

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(Signature):

(En el caso de que se trate de directores no vinculados al programa de doctorado cursado por el doctorando, se incluirá a continuación la ratificación razonada del tutor)
(In case of Thesis Supervisors not related to the doctoral program followed by the PhD Candidate, a Thesis Advisor's ratification must be submitted)

PRESENTACIÓN DE LA TESIS DOCTORAL

Esta Tesis ha sido estructurada según la normativa de presentación en la modalidad de compendio de publicaciones que fue aprobada por la Comisión de Doctorado de la Facultad de Medicina. La componen tres artículos relacionados y enmarcados en la temática de la tesis, los tres han sido publicados en revistas indexadas en bases de datos internacionales de reconocido prestigio:

1. Influence of a *Serratia marcescens* Outbreak on the Gut Microbiota Establishment Process in Low-Weight Preterm Neonates

Escribano E, Saralegui C, Moles L, Montes MT, Alba C, Alarcón T, Lázaro-Perona F, Rodríguez JM, Sáenz de Pipaón M, del Campo R. Plos One. 2019 May 14(5), e0216581. https://doi.org/10.1371/journal.pone.0216581. PMID: 31112570.

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2. Increased incidence of necrotizing enterocolitis associated with routine administration of Infloran™ in extremely preterm infants.

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3. Administration of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934, two strains isolated from human milk, to very low and extremely low birth weight preterm infants: a pilot study.

Moles L[1*], **Escribano E[2*]**, de Andrés J[3*], Montes MT, Rodríguez JM, Jiménez E, Sáenz de Pipaón M, Espinosa-Martos I. J ImmunolRes. 2015; 2015:538171. doi: 10.1155/2015/538171. PMID: 25759843.

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“El papel de lo infinitamente pequeño en la naturaleza es infinitamente grande”

Louis Pasteur

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INTRODUCCIÓN

INTRODUCCIÓN

EL MICROBIOMA HUMANO

El cuerpo humano aloja una multitud de bacterias, arqueas, virus y microbios eucariotas, que se distribuyen a lo largo de las mucosas y epitelios de diversos órganos y que, globalmente conforman nuestra microbiota[1]. El término microbioma, es mucho más amplio y hace referencia al conjunto de esas comunidades microbianas incluyendo sus genes y metabolitos, así como las condiciones ambientales que lo rodean[2]. El papel del microbioma humano es crucial para la salud y el bienestar. Desde hace siglos se sabe que el ser humano es portador de muchos microorganismos. En los últimos años, se han identificado y caracterizado los microorganismos que se encuentran asociados al ser humano, permitiendo explorar la relación entre cambios en el microbioma humano y su estado de salud y enfermedad[3]. Gracias a las tecnologías independientes del cultivo y herramientas bioinformáticas como la secuenciación de biomarcadores, el gen 16S del RNAribosomal, metagenómica, metatranscriptómica, metaproteómica y metabolómica se ha mostrado en el microbioma humano una enorme diversidad, capacidad funcional y cambios según la edad [4].

En el 2008 surge el Proyecto Microbioma Humano (HMP) como iniciativa del National Institute of Health con el objetivo de desarrollo de un conjunto de referencia de secuencias de genomas microbianos y realización de la caracterización preliminar del microbioma humano[5]. Estos estudios permiten estimar que en nuestro cuerpo habitan más de 10000 especies bacterianas diferentes, de las que menos del 1% pueden ser potencialmente patógenas. La ratio entre bacterias y células humanas es casi 1:1, con las últimas mediciones más precisas, ya que previamente se indicaban en la literatura valores de 10:1 o 100:1[6].

En cuanto a la diversidad microbiana se observa que la composición varía en cada persona y va variando influenciada por muchos factores a lo largo de la vida. En los primeros meses de vida, la diversidad microbiana es baja, inestable y fácilmente modificable (alimentación, vía de parto…), conforme el niño va creciendo la microbiota va también madurando y aumenta su diversidad y continua siendo susceptible a cambios (alimentación, medicamentos, cambios hormonales…). En el adulto, la microbiota es más estable y díficil de modificar, es más diversa y diferente entre individuos y ya en la tercera edad la diversidad disminuye y la microbiota se hace similar entre individuos. Además de la edad en la adquisición de la microbiota influyen la genética, el sexo, la localización geográfica, la interacción con otros individuos o la ocupación[7].

En la adquisición de la microbiota se configuran “ecosistemas microbianos” dentro del cuerpo humano, éstos están compuestos por el predominio de determinadas especies bacterianas, unas pocas especies muy abundantes y frecuentes, junto con muchas bacterias distintas representadas en pequeño número.

Al comparar los distintos “ecosistemas microbianos” en el humano, entre ellos el tracto gastrointestinal, genitourinario, respiratorio, cavidad oral y la piel, es el tracto gastrointestinal el más complejo, ya que es el de mayor densidad celular y además contiene virus y dominios Archaea, Bacteria y Eukarya, además de ser de difícil acceso [8,9].

MICROBIOTA DEL TRACTO INTESTINAL

En el tracto gastrointestinal, la gran mayoría de bacterias residen en el colon, más del 90%, y estas concentraciones no se modifican de forma significativa a lo largo del tiempo[10]. Sin embargo, la microbiota intestinal no está aislada, sino que se halla en una compleja y constante interacción bioquímica-inmunológica-neurológica con el resto de células y sustancias que habitan en el intestino, ésto juega un papel muy importante en el mantenimiento de la homeostasis intestinal e inmunológica.

Determinadas enfermedades gastrointestinales[11,12], como la enfermedad inflamatoria intestinal, el intestino irritable y la gastroenteritis infecciosa; enfermedades hepáticas, metabólicas, neurológicas (trastorno del espectro autista, depresión, ansiedad y esquizofrenia), asma, enfermedades cardiovasculares y artritis se han asociado con alteraciones en la composición del microbioma intestinal. El microbioma intestinal es considerado un nuevo órgano en el cuerpo humano.

La microbiota intestinal también influye en el desarrollo y maduración de la barrera del epitelio intestinal y del sistema inmune del huésped.

La microbiota modula el grosor de la capa mucosa, que cubre enterocitos y las células epitaliales especializadas de las vellosidades intestinales y es sobre la capa de moco el lugar donde interactúan antígenos, microorganismos y medio externo[13]. La microbiota también interviene en la expresión de receptores de nutrientes e influye en el desarrollo de estructuras linfáticas[14]. El sistema linfático está constituido por una red de vasos linfáticos que conectan los órganos linfoides primarios (timo y médula ósea) y secundarios (ganglios

linfáticos, placas de Peyer intestinales) y tiene como funciones principales la recirculación de líquido intersticial y el transporte de linfocitos y células presentadoras de antígeno.

El sistema inmune interviene en la adecuada discriminación por parte del huésped entre aquellos microorganismos patógenos y comensales, activando una respuesta inmune controlada para mantener un estado de tolerancia frente a microorganismos comensales. Los microorganismos instaurados en la luz intestinal además de ocupar su espacio compiten con los patógenos por los nutrientes disponibles, favorecen condiciones de pH concretas y estimulan a nivel del epitelio intestinal la síntesis y liberación de proteínas antibacterianas, entre las que se incluyen defensinas, catelicidinas, lectinas tipo C y lipocalina 1 además de balancear la producción de IgA y modular la diferenciación de determinadas poblaciones linfoides. La microbiota intestinal a través de receptores extracelulares de los enterocitos tipo "*Toll-like receptors*" (TLR) e intracelulares "*Nucleotide-binding oligomerization domain (NOD)-like Receptor"* (NLR) envían estas señales bacterianas que regulan en parte la expresión de estas proteínas antibacterianas a través de la activación de factores de transcripción (NF-κB, JNK y MAPKs)[15]. Estas proteínas secretadas por el epitelio son retenidas en la capa de moco limitando el acceso de los microorganismos patógenos de la luz intestinal al torrente sanguíneo.

El balance entre una adecuada respuesta e inmunotolerancia es necesaria para prevenir enfermedades mediadas por el sistema inmune y también a través de los TLR. La microbiota interviene en el desarrollo y maduración de la respuesta inmune porque contribuyen al balance de los linfoctios Th1:Th2, y a su vez las células reguladoras T promueven la tolerancia y el sobrecrecimiento de microbiota[16].

Otras funciones de la microbiota intestinal son derivadas de la diversidad del genoma microbiano intestinal, que proporciona una gran variedad de enzimas y vías bioquímicas distintas de los recursos propios del huésped. Entre la funciones metabólicas de la microbiota intestinal están la fermentación de hidratos de carbono no digeribles por el huésped[17] (vía de degradación de polisacáridos vegetales), favoreciendo la producción de ácidos grasos de cadena corta, fuente de energía para la bacterias que habitan en el colon, pero además estos ácidos grasos son absorbidos y a través del torrente sanguíneo realizan variedad de funciones a distancia en el huésped como antiinflamatoria y antitumoral[18]. Otras funciones metabólicas son la deconjugación de sales biliares[19], la degradación determinadas toxinas y el metabolismo de medicamentos orales[20], producción de vitaminas, cofactores...

ADQUISICIÓN DE LA MICROBIOTA INTESTINAL

La colonización bacteriana del tracto gastrointestinal se inicia durante la etapa uterina y tras el nacimiento, se intensifica gracias al contacto del neonato con microorganismos procedentes de la microbiota vaginal, intestinal y/o mamaria de la madre y del medio ambiente que le rodea[21, 22]. Clásicamente se consideraba estéril la placenta, el cordón umblical, el líquido amniótico y el feto, sin embargo, estudios han mostrado la presencia de microorganismos, en placenta, cordón umbilical y meconio humanos de embarazos sanos[23,24]. Las bacterias han sido aisladas en escasa cantidad en el meconio con métodos de cultivo tradicionales y moleculares[25]. La ruta enteromamaria es un mecanismo de colonización por el que la misma microbiota que proviene del intestino materno se detecta en la placenta y leche materna[26].

En consecuencia, distintos factores influyen en la composición del microbioma del recién nacido como son la microbiota materna, influenciada por antibioterapia materna[27], la rotura prematura de membranas o la presencia de corioamnionitis durante el embarazo[28], la modificación de la microbiota durante las últimas semanas del embarazo[29], la edad gestacional, tipo de parto, cesárea versus parto vaginal dado que los nacidos por vía vaginal presentan una microbiota similar a la microbiota vaginal e intestinal materna)[30,31,32], la medicación recibida al nacer, en particular la antibioterapia que reduce la diversidad de microorganismos, a mayor duración del tratamiento mayor impacto sobre la microbiota[33,34], la alimentación, diferenciando entre lactancia materna versus fórmula [35], sexo, características genéticas del huésped, respuesta inmune, infecciones, uso de tratamientos inmunosupresores, el estilo de vida… Todos estos factores juegan un papel clave en un proceso del que dependen funciones tan importantes como la absorción de nutrientes, la formación de una barrera frente a patógenos, el neurodesarrollo o la maduración del sistema inmune[36, 37].

La microbiota de los niños nacidos a término por parto vaginal sin medicación y alimentados exclusivamente con leche materna de la propia madre se considera el prototipo de la microbiota ideal para esta edad.

Un proceso importante, por tanto, en la vida del ser humano es la colonización microbiana del tracto gastrointestinal en las primeras etapas de la vida puesto que cada vez resulta más evidente que las interacciones iniciales microbiota-huésped tienen consecuencias importantes para la salud a corto, medio y largo plazo.

MICROBIOTA Y PREMATURIDAD

Los niños prematuros, aquellos que nacen con una edad gestacional menor a 37 semanas, que supone entre el 5-13% de los nacimientos, tienen una experiencia postnatal muy distinta y más desfavorable a mayor inmadurez, en general, que la de aquellos nacidos a término. La prematuridad está asociada a una mayor morbilidad y mortalidad relacionada con patologías como enterocolitis necrotizante y sepsis.

El estudio de la microbiota en recién nacidos de muy bajo peso y de intervenciones moduladoras de esta microbiota como son la administración rutinaria de probióticos o el ambiente epidemiológico, pueden prevenir o favorecer la aparición de morbilidad en esta población tan vulnerable.

Los recién nacidos pretérmino nacen a un ambiente extrauterino hospitalario tras rotura prematura o prolongada de membranas, la madre suele recibir antibioterapia durante periodos relativamente prolongados, tienen retrasado el inicio de la alimentación enteral y cuando se inicia puede no ser con leche de la propia madre, poseen una barrera gástrica alterada y unos órganos inmaduros. Todos estos factores conducen a una alteración en el establecimiento de la microbiota intestinal que se considera aberrante[38], con aumento en la prevalencia de colonización de bacterias potencialmente patógenas y una colonización más lenta con menor diversidad microbiana según se incrementa la estancia en las unidades de cuidados intensivos neonatales (UCIN) con respecto a los recién nacidos a término[39]. La microbiota de los niños prematuros se suele caracterizar por una disminución de lactobacilos y de anaerobios estrictos, como *Bifidobacterium* y *Bacteroides*, y un aumento de bacterias de la familia *Enterobacteriaceae*[40,41], el filo *Proteobacteria* predomina durante su

primer mes de vida[39]. No obstante, cada unidad de cuidados intensivos posee su propia microbiota[42]. La colonización intestinal del pretérmino es dependiente del ambiente en el que se encuentra, del propio centro. El intestino pasa a ser reservorio de microorganismos patógenos[43] y esto puede estar relacionado con los brotes epidémicos de sepsis y enterocolitis necrotizante (NEC; del inglés, necrotizing enterocolitis) agrupadas que ocurren en las UCIN[44,45]. La disbiosis en los recién nacidos prematuros parece jugar un papel importante en la morbilidad de éstos.

La NEC es una de las patologías más devastadoras que afectan a los recién nacidos prematuros y se estudia conocer mejor su etiopatogénia para poder prevenir y tratar esta enfermedad. Se caracteriza por presentar afectación del intestino que varía desde pequeñas lesiones en la mucosa hasta la necrosis con perforación intestinal. La edad de presentación suele ser entre la segunda y tercera semana de vida. Los principales factores implicados en su patogénia son la inmadurez intestinal, la alimentación enteral, microbioma intestinal, inflamación y daño de isquemia-reperfusión intestinal[46].

En las UCIN al aumentar la supervivencia de los recién nacidos prematuros el número de niños con riesgo de desarrollar NEC aumenta[47]. La incidencia de NEC varía en función de la zona geográfica y aumenta con la inmadurez. En una revisión sistemática reciente encontró una diferencia de incidencia de NEC entre el 2 y el 7% en menores de 32 semanas y entre el 5 y el 22% en menores de 1000g según las distintas naciones[48]. En Estados Unidos afecta a más de 4000 niños al año con una elevada morbilidad y una mortalidad cercana al 33%. Los costes para el sistema de salud superan el billón de dólares anuales[49].

La relación entre la NEC y la microbiota es muy interesante para conocer la relación etiopatogénica entre microbiota y enfermedad[50]. Un mecanismo potencial sugiere que la colonización bacteriana anómala (disbiosis) induce un estado proinflamatorio que permitiría la traslocación de patógenos a través de la mucosa intestinal[51].

Hay estudios que describen un cambio en la colonización intestinal previo al inicio de NEC con predominio de Proteobacteria (*Enterobacter cloacae* y *Escherichia coli*) y con disminución de estreptococo y estafilococo[52].

En otro estudio se objetivaba una disminución de Firmicutes y aumento de Proteobacteria una semana previa al desarrollo de NEC[53]. Los recién nacidos que desarrollan NEC pueden presentar hemocultivos o cultivos de líquido peritoneal positivos.

La inmadurez del sistema inmune intestinal implica un aumento de la translocación bacteriana y a su vez el desarrollo del sistema inmune depende de una colonización bacteriana favorable.

Las bifidobacterias y los lactobacilos en la luz intestinal, de los que se coloniza el el intestino de los recién nacidos a término en condiciones normales, influyen en la maduración del sistema inmune intestinal. El aumento de secreción de IgA y de los receptores IgA, el aumento de las uniones epiteliales intestinales, la disminución del pH intraluminal a través de la fermentación ácida y la modulación de la respuesta inflamatoria intestinal a través de la estimulación de linfocitos T helper son mecanismos de disminución de la translocación bacteriana[54].

El uso prolongado y precoz de antibioterapia en los recién nacidos prematuros disminuye la diversidad bacteriana, favoreciendo la colonización por enterobacterias que favorecen un estado proinflamatorio. En esta situación aumenta la producción de óxido nítrico y radicales de superóxido que forman nitratos y pueden ser

fermentados por enterobacterias pero no por especies anaerobias como bifidobacterias.
La colonización aberrante intestinal del prematuro puede ser, por tanto, un evento etiológico y/o un marcador de enfermedad.

Por otro lado, el hecho de nacer prematuro aumenta el riesgo de padecer infecciones neonatales[55]. La sepsis tardía, definida como la infección sanguínea que ocurre entre las 72 horas de vida y el alta hospitalaria es una causa muy importante de mortalidad y morbilidad del recién nacido prematuro [56]. La incidencia de sepsis tardía es variable, y es la causa más frecuente de mortalidad neonatal en los países en desarrollo, representa el 30-50% del total de muertes neonatales[55].
Si bien la sepsis precoz es causada por gérmenes presentes en el líquido amniótico o en el canal del parto [57], el origen de la sepsis tardía parece provenir de la piel a través de catéteres o el paso a la sangre desde el intestino que alberga patógenos causantes de sepsis[58]. La colonización intestinal aberrante se asocia a sepsis tardía en prematuros como describe Mai et al[59], al comparar que en los casos de sepsis predominaban estafilococos y una disminución de bifidobacterias, frente a los controles.

Tras introducir el papel de la colonización intestinal y la morbilidad del recién nacido pretérmino cabe exponer qué intervenciones se pueden realizar sobre esta población con el fin de disminuir su morbilidad influenciando en la adquisición de la microbiota intestinal.

ESTRATEGIAS PARA MODULAR MICROBIOTA EN EL PRETÉRMINO

Hay distintas estrategias para prevenir la disbiosis del recién nacido prematuro desde el embarazo, incluso previo al embarazo, a través de la dieta y la modificación de los hábitos de vida y tras el nacimiento mediante la administración de probióticos, leche humana, prebióticos y polifenoles, entre otros.

Los probióticos según la Organización Mundial de la Salud se definen como microorganismos vivos que confieren un beneficio a la salud del huésped cuando se administran en cantidades adecuadas, aunque las bacterias muertas y componentes moleculares bacterianos pueden mostrar propiedades probióticas[60]. En la actualidad es un área de investigación activa. Las especies más frecuentemente usadas son lactobacilos y bifidobacterias, también levaduras (*Saccharomyces boulardii* ha mostrado beneficios para la salud del huésped).

Los mecanismos de acción de los probióticos son el aumento de la capacidad de aumentar la barrera intestinal (favorecen producción de mucinas, restauran la pared intestinal inflamada,…), aumento de adhesión a la mucosa intestinal, exclusión de microorganismos patógenos, favorecen la producción de sustancias antimicrobianas y toxinas, modulan la inflamación y regulan la expresión de genes de proteínas de la unión restaurando la integridad de la mucosa intestinal [61].

Los resultados de análisis basados en la evidencia de estudios humanos y modelos animales han mostrado el potencial de los probióticos frente a distintas enfermedades, como diarrea, intolerancia a la lactosa y complicaciones postoperatorias[62].

La evidencia muestra que la suplementación nutricional de probióticos

es eficaz para prevenir enterocolitis necrotizante[63]. Un metaanálisis reciente concluye que el uso de múltiples cepas es más efectivo que el uso de una única cepa en disminuir la mortalidad y la enterocolitis en recién nacidos prematuros de muy bajo peso[64].

En la actualidad, la suplementación con probióticos es segura en los recién nacidos de muy bajo peso, aunque escasos estudios evalúan específicamente la población menor de 28 semanas. Un trabajo reciente por un grupo español no muestra protección de los probioticos en niños menores o iguales de 27 semanas[65] .

A pesar de que en algunas UCIN se emplean de forma rutinaria los probióticos, aún existen dudas en lo que respecta a la calidad de los preparados y efectos adversos. Hasta el momento la regulación de los probióticos no está regulada como producto farmacéutico, sino como suplemento nutricional.

Los distintos estudios evalúan distintos microorganismos, dosis, inicio, duración… El efecto de un probiótico depende de la cepa concreta y actualmente, se desconoce qué mezclas de probióticos son más eficaces y, por tanto, son necesarios más estudios para conocer el probiótico óptimo o la mezcla optima de probióticos así como la eficacia y seguridad del uso de probióticos en recién nacidos con peso extremadamente bajo al nacimiento.

Hace unos años un metaanálisis mostraba los efectos beneficiosos del uso rutinario de probióticos[66] y esta evidencia hizo incorporarlos como medida rutinaria en algunas unidades neonatales.

Recientemente, un estudio retrospectivo observacional de cohortes en niños con un peso al nacimiento inferior a 1500g reveló que la suplementación rutinaria con *Lactobacillus rhamnosus GG* no se asociaba con factor protector frente a la NEC[67].

La evaluación del uso de probióticos en la práctica rutinaria fuera de un

ensayo clínico permite ver los beneficios y riesgos.
La alimentación con leche materna influye en la microbiota del recién nacido ingresado en las unidades de cuidados intensivos neonatales[68,69].

La leche materna es un líquido biológico complejo, posee un papel inmunomodulador a través de componentes como inmunoglobulinas, ácidos grasos, poliaminas, oligosacáridos, lisozima, lactoferrina, péptidos antimicrobianos e interviene en la microbiota intestinal a través de microorganismos propios de la leche materna y además de los oligosacáridos de la leche humana (HMO), hidratos de carbono complejos), la membrana del glóbulo de grasa derivada del epitelio de la glandula mamaria y los ácidos grasos de cadena larga.

La leche materna tradicionalmente se considerada estéril y, sin embargo, una de sus características principales es que posee una microbiota específica[70]. Estos microorganismos son los que en primer lugar colonizan el intestino humano[71] y pueden modular, dirigir la colonización intestinal. Existen numerosos estudios que identifican los microorganismos por métodos independientes de cultivo. Cada mililitro de leche humana puede contener entre 10^3-10^4 UFC[72]. Los géneros *Streptococcus*, *Lactobacillus*, *Weissella* y *Staphylococcus* fueron aislados en el 50% de todos los estudios. *Streptococcus* y *Staphylococcus* son más frecuentes inmediatamente tras el parto.

En los últimos años, investigaciones de diferentes grupos sugieren que el origen de las bacterias en la leche humana proviene en parte del tracto gastrointestinal materno desde el cuál pueden translocarse y migrar a la glándula mamaria[73], implicándose en el transporte de estos microorganismos macrófagos o células dendríticas[74].

Los recién nacidos pretérmino de menos de 32 semanas de edad gestacional no son capaces de succionar y por tanto la microbiota oral del prematuro no puede influir en la microbiota de la leche materna.

Factores como el índice de masa corporal materno[75], la raza materna, el sexo del recién nacido, el tipo de parto (vaginal versus cesárea), entre otros, influyen en la microbiota de la leche humana y por tanto en la microbiota gastrointestinal inicial del prematuro[76]y cuanto más precoz sea mayor es su influencia[77].

En los últimos años, gracias a la existencia de bancos de leche materna donada, muchos de los recién nacidos prematuros son alimentados a base de ella. La leche materna es sometida a procesos como la congelación y la pasteurización que alteran los micronutrientes y macronutrientes perdiendo factores bioactivos, microorganismos… No obstante, la leche materna donada es la mejor opción frente a la leche de fórmula porque se mantienen importantes propiedades inmunológicas de la leche materna propia, objetivándose menores tasas de NEC en pacientes alimentados con leche materna donada, lo que ofrece la oportunidad de mejorar la nutrición de todos aquellos recién nacidos pretérminos que no pueden disponer de la leche de su madre. Si bien, hay estudios de la microbiota gastrointestinal en pretérminos alimentados con leche de materna donada en los que ésta, se asemeja a la microbiota de los pretérminos los alimentados con fórmula de prematuros[78]. Hay que tener en cuenta cómo el ambiente microbiológico de las UCIN influye en la colonización del tracto gastrointestinal de los recién nacidos pretérmino ingresados durante largo periodo de tiempo.

JUSTIFICACIÓN

JUSTIFICACIÓN

Entidades tan devastadoras como la enterocolitis necrotizante y la sepsis en los grandes prematuros justifican este estudio que busca aportar la influencia en esta población de la colonización intestinal por microorganismos bajo la influencia del ambiente de las unidades de cuidados intensivos neonatales y las intervenciones nutricionales como uso probióticos y la administración selectiva de cepas probióticas provenientes de leche humana.

HIPÓTESIS

HIPÓTESIS

La hipótesis del estudio es que puesto que la microbiota es un "órgano" esencial en el desarrollo y metabolismo del ser humano se postula que:

El estudio de la microbiota en recién nacidos de muy bajo peso y de intervenciones moduladoras de esta microbiota como son la administración rutinaria de probiótios o el ambiente epidemiológico, pueden prevenir o favorecer la aparicion de morbilidad en esta población tan vulnerable.

OBJETIVOS

OBJETIVOS

Los objetivos de las publicaciones que componen esta Tesis Doctoral serían los siguientes:

P1. Conocer cómo el ambiente epidemiológico en las Unidades neonatales influye sobre la colonización temprana del recién nacido prematuro.

P2. Conocer si la administración oral profiláctica de una mezcla comercial de probióticos, *Lactobacillus acidophilus* y *Lactobacillus bifidum* (Infloran®) es segura y disminuye la incidencia de enterocolitis necrotizante y sepsis en aquellos niños más vulnerbles, nacidos a una edad gestacional inferior a 28 semanas.

P3. Conocer si dos probióticos extraídos de la leche materna (*Bifidobacterium breve* PS12929 y *Lactobacillus salivarius* PS12934), con características in vitro prometedoras, administrados a recién prematuros colonizan el intestino de los niños y modulan la respuesta inmunitaria local y sistémica y se relacionan con la evolución clinica.

PUBLICACIONES QUE COMPONEN ESTA TESIS DOCTORAL

PLOS ONE

RESEARCH ARTICLE

Influence of a *Serratia marcescens* outbreak on the gut microbiota establishment process in low-weight preterm neonates

Esperanza Escribano[1☯], Claudia Saralegui[2☯], Laura Moles[2,3], María Teresa Montes[1], Claudio Alba[4], Teresa Alarcón[4], Fernando Lázaro-Perona[5], Juan Miguel Rodríguez[3], Miguel Sáenz de Pipaón[1,6], Rosa del Campo[2]*

1 Servicio de Neonatología, Hospital Universitario La Paz, and Universidad Autónoma de Madrid, Madrid, Spain, **2** Servicio de Microbiología y Parasitología, Hospital Universitario Ramón y Cajal, and Instituto Ramón y Cajal de Investigaciones Sanitarias (IRYCIS), Madrid, Spain, **3** Departamento de Bromatología, Facultad de Veterinaria Nutrición y Ciencia de los Alimentos, Universidad Complutense de Madrid, Madrid, Spain, **4** Servicio de Microbiología, Hospital Universitario La Princesa, and Universidad Autónoma de Madrid, Madrid, Spain, **5** Servicio de Microbiología, Hospital Universitario La Paz, Madrid, Spain, **6** Red de Salud Materno Infantil y del Desarrollo, Instituto de Salud Carlos III, Madrid, Spain

☯ These authors contributed equally to this work.
* rosacampo@yahoo.com

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Data Availability Statement: The clinical data have been uploaded as a Supporting Information file. Additionally, the genome sequences are deposited in the European Nucleotide Archive database with the accession numbers QYRU00000000 and QYSA00000000 for Clone A (submission numbers SUB4493714 and SUB4514092); and QYRV00000000 and QYSB00000000 for Clone B (submission numbers SUB4510090 and SUB4525283). Fasta files are deposited in the NCBI

Abstract

Adequate gut microbiota establishment is important for lifelong health. The aim was to sequentially analyze the gut microbiota establishment in low-birth-weight preterm neonates admitted to a single neonatal intensive care unit during their first 3 weeks of life, comparing two epidemiological scenarios. Seven control infants were recruited, and another 12 during a severe *S. marcescens* outbreak. Meconium and feces from days 7, 14, and 21 of life were collected. Gut microbiota composition was determined by 16S rDNA massive sequencing. Cultivable isolates were genotyped by pulsed-field gel electrophoresis, with four *S. marcescens* submitted for whole-genome sequencing. The expected bacterial ecosystem expansion after birth is delayed, possibly related to antibiotic exposure. The *Proteobacteria phylum* dominates, although with marked interindividual variability. The outbreak group considerably differed from the control group, with higher densities of *Escherichia coli* and *Serratia* to the detriment of *Enterococcus* and other *Firmicutes*. Curiously, obligate predators were only detected in meconium and at very low concentrations. Genotyping of cultivable bacteria demonstrated the high bacterial horizontal transmission rate that was confirmed with whole-genome sequencing for *S. marcescens*. Preterm infants admitted at NICU are initially colonized by homogeneous microbial communities, most of them from the nosocomial environment, which subsequently evolve according to the individual conditions. Our results demonstrate the hospital epidemiology pressure, particularly during outbreak situations, on the gut microbiota establishing process.

web site under the Bioproject PRJNA510235 reference.

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Introduction

The relationship between our human cells and the microbial communities living inside us can be classified as mutualistic, commensal, or pathogenic. This consideration delimits the fine barrier that distinguishes colonization from infection, and can fluctuate over time due to influence from the host or from microbial and environmental factors. The influence of the intestinal microbiota on global human health has been confirmed [1–3], forming new research perspectives aimed to optimize the composition and the functionality of this bionetwork.

Adequate microbiota establishment in newborns is a process particularly relevant for their lifelong health [4], and its management represents a scientific challenge. Human bacterial colonization might start *in utero*, but the critical step begins with the exposure to maternal bacteria at birth and during the early postnatal period [5–6]. Bacterial populations fluctuate considerably during the first months of life, until a stable ecosystem is established when the infant is approximately 2–3 years of age [7–8]. Universal criteria defining a "normal" or "healthy" gut microbiota have not yet been established, which should be characterized by a high diversity, marked inter-individual variability, and conserved intraindividual stability. However, the composition of this ecosystem is influenced by numerous factors [9–10], such as the gestational age at which the neonate is born [11–12].

Preterm birth is the main cause of perinatal morbidity and mortality, as well as an important risk factor for death in the first 5 years of life [13]. A considerable increase in preterm birth rates over the past two decades has been reported worldwide, in both developed and developing countries [14]. In Spain, the preterm birth rate of all live births increased from 7.1% in 1996 to 8.2% in 2008, which is one of the highest rates in Europe [15]. This global tendency can be explained by several factors, including an early or advanced mother's age, a small gap between pregnancies, low body mass index, multiple pregnancy, history of infectious diseases, stress, alcohol consumption, and periodontal disease [13]. Nevertheless, approximately half of the spontaneous preterm births have an unidentified cause, and it has been suggested that the composition of the maternal microbiota could play a relevant triggering role [7,16].

In low-birth-weight preterm infants (<2500 g), the gut microbiota composition and their biodiversity are aberrant, given the bacterial establishment is delayed by their prolonged hospital stay and their intense exposure to antimicrobials [12,17–19]. This fact explains, at least partially, why preterm infants have a very immature immune system and typically experience infectious complications [20–22]. In this context, *Serratia marcescens* is one of the most relevant emerging pathogens causing severe outbreaks in this population [23–24]. Pathogenic gut colonization during nosocomial outbreaks has frequently been reported; to our knowledge, however, the influence of an outbreak on the microbiota establishment process has not thus far been studied.

The aim of the present study was to sequentially analyze the gut microbiota establishment of low-birth-weight preterm neonates admitted to a single neonatal intensive care unit (NICU) during their first 3 weeks of life, comparing two epidemiological scenarios: a normal period and a period with a nosocomial *S. marcescens* outbreak.

Materials and methods

Preterm neonate inclusion criteria and sampling procedure

La Paz University Hospital (Madrid, Spain) has a 23-bed level III NICU, from which 19 low-birth-weight preterm neonates (<32 weeks gestational age) were recruited in two separate periods: (A) during an epidemiologically normal period in 2015 (control group, n = 7); and (B) during a severe *S. marcescens* outbreak from December 2016 to March 2017 (outbreak

group, n = 12). Despite the different sampling periods, there were no significant changes in the NICU. It is important to note the data lack in the control group about antibiotic consumption, and clinical data, as a limitation of our work. From each preterm infant, four fecal samples were collected after birth: meconium, and feces from 7, 14, and 21 days of life. Although our initial aim was to extend the recruitment period, logistical limitations limited the study to the first three weeks. The samples were directly recovered from the diaper using a sterile plastic stick and immediately stored at -80˚C. Although our intention was to collect fecal samples immediately after deposition, we cannot rule out the possible contact and contamination with urine. However, the contribution of the urinary microbiota should be insignificant.

For the control group, only DNA from fecal samples was available, whereas for the outbreak group bacterial growth was obtained by culture-dependent techniques in addition to DNA. The ethics committee "Comité Ético de Investigación Clínica del Hospital Universiatrio La Paz" approved the study (reference HULP3551), and the data of all the neonates were obtained from their clinical chart. The infants were categorized according to four variables: 1) epidemiological situation (normal or *S. marcescens* outbreak); 2) delivery mode (vaginal or C-section); 3) gestational age (extremely preterm: <28 weeks; very preterm: 28–30 weeks; or moderately preterm: 30–32 weeks); and 4) birth weight (<1000 g; 1000–1500 g; or >1500 g).

Sample processing

Fecal samples from the *S. marcescens* outbreak group were slowly defrosted at -20˚C for 24 h and 4˚C for another 24 h, in order to avoid bacterial death. Portions between 0.3–0.5 g of each sample were inoculated into Brain Heart Infusion (BHI) broth (Difco, Detroit, Michigan) and incubated at 37˚C for 24 h as a bacterial pre-enrichment. Cultivable bacteria were isolated in selective and nonselective agar media from the BHI tube, including agar plates of M-*Enterococcus*; De Man, Rogosa and Sharpe (MRS); mannitol salt; McConkey; and Columbia, with 5% sheep blood. The culture media were purchased from Difco, and the plates were incubated at 37˚C for 24–48 h, including 5% CO_2 for the blood agar plates, and anaerobic conditions for the MRS plates. Colony identification was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker, Germany), and all the isolates were conserved at -80˚C in semi-skimmed milk. In parallel to bacterial cultures, total DNA was obtained from fecal aliquots of 0.3–0.5 g with the QiaAMP kit (Qiagen, Germany), determining their concentration and quality by Qubit fluorometer (Invitrogen, USA).

16S rDNA next-generation sequencing

The fecal DNA samples were sent to FISABIO (Valencia, Spain) for massive Mi-Seq 2 × 300 bp paired-end Illumina 16S rDNA sequencing (Cod. 15044223 Rev. A) from the V3 and V4 regions, which were amplified with the following primers: (Forward Primer: `5′-TCGTCGGCAG CGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG`), (Reverse Primer: `5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG GGTATCTAATCC`) [25]. Shannon and Chao1 indexes were used for alpha bacterial diversity estimation and were calculated, eliminating taxa with fewer than three lectures. Taxonomic affiliations were assigned using the Ribosomal Database Project (RDP) classifier, and reads with an RDP score below 0.8 were assigned to the upper taxonomic rank, leaving the last rank as unidentified. Sequence quality was measured according to the following parameters: minimum length, 250 bp; trimming quality measure type, mean; trimming quality number from 3' extreme, 30; trimming quality window, 10 bp. Relative abundance and contingency tables included singletons and very low-represented taxons. The statistical analysis was performed using R statistical software and several open source libraries. The quantitative data of the reads were homogenized using their

relative percentage from the total reads of each sample to facilitate the comparison between samples. Finally, the Galaxy Huttenhower Platform (http://huttenhower.sph.harvard.edu/galaxy) was used in order to calculate the Linear Discriminant Effect Size Analysis (LEfSe) algorithm and to obtain cladograms in which microbial taxa that explain significant differences among groups of samples were represented. A free software platform was used according to paper instructions [26]. For the statistical analysis, samples with fewer than 1,000 reads were dismissed, and all the samples from patient 7B were excluded due to a lack of some demographic and perinatal information. Fasta files are deposited in the NCBI web site under the Bioproject PRJNA510235 reference.

Pulse-field gel electrophoresis typing

Cultivable bacterial isolates were genotyped by pulse-field gel electrophoresis (PFGE), using the habitual particular settings for the PFGE protocol and also for the restriction enzymes (*Sma*I for staphylococci and enterococci, *Xba*I for *Escherichia coli*, and finally *Spe*I for *Serratia* and *Klebsiella*). The PFGE pattern analysis was made with Phoretix 5.0 software (TotalLab, Newcastle upon Tyne, UK), and the representation of the results was made based on Dice coefficients and the unweighted pair group method with arithmetic mean algorithm.

Whole genome sequencing

Four *S. marcescens* isolates from different infants were submitted to whole genome sequencing (WGS) by MiSeq technology (Illumina), and the genetic relationships were analyzed in the Galaxy Huttenhower Platform. The genome sequences are deposited in the European Nucleotide Archive database with the accession numbers QYRU00000000 and QYSA00000000 for Clone A, and QYRV00000000 and QYSB00000000 for Clone B.

Fungi identification

The internal transcribed spacer (ITS)-1 region was amplified from the total fecal DNA using polymerase chain reaction (PCR) in order to analyze fungi diversity, using the primers ITS1-F (`5′-CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGGCTTGGTCATTTAGAGGA AGTAA-3′`) and ITS1-R (`5′-TCCTCCGCTTATTGATATGC-3′`) [27]. Afterward, the amplicons were separated with denaturing gradient gel electrophoresis (DGGE), using the D-CODE system (BioRad Laboratories, USA). The gel bands were cut, reamplified, and sequenced in an AQBI Prism 7000 apparatus.

Statistical analysis

The Kruskal-Wallis index was used for differences between two groups of samples (comparing medians) for each variable of study, and Dunn's test was used when more than two groups were defined regarding a variable of study. The principal component analysis was applied for the multivariant analysis regarding taxonomical data in order to see differences between groups according to the variables of study. Statistical significance was adjusted to $p < .005$.

Results

Characteristics of the preterm infants

The demographic and clinical characteristics of both newborn groups are shown in Table 1 and in S1 Dataset. The most relevant differences were that the control infants presented a higher weight at birth and a lower incidence of sepsis. One neonate from the control group (14.3%) suffered from early-onset sepsis, whereas late-onset sepsis was microbiologically or

Table 1. Clinical and demographic characteristics of the preterm infants of both groups.

Characteristic	Control Group (7 infants)	Outbreak Group (12 infants)	*p* value
Weight at birth (g)	1462 (720–1890)[a]	971 (600–1537)[a]	0.009
Gestational Age (weeks)	30 (25–31)[a]	28 (25–31)[a]	0.26
Vaginal delivery (n, %)	5, 71.4%	3, 25%	0.04
Male sex (n, %)	5, 71.4%	2, 16.6%	0.0001
Sepsis (n, %)	1, 14.3%	6, 50%	0.04
Length of stay (days)	14 (5–140)[a]	55 (7–89)[a]	0.1

[a]Values expressed as the median value and the range (between parentheses).

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clinically diagnosed in five neonates from the outbreak group (41.7%), being *Staphylococcus epidermidis* and *S. marcescens* the microorganisms implicated. Data on antimicrobial therapy were only available for the outbreak group and were included the administration of prophylactic cefazolin during labor (10 infants), empiric treatment based on ampicillin, gentamicin, and clarithromycin during the first week of life (8 infants), and treatment that included vancomycin (7 infants), cefotaxime (2 infants) piperacillin/tazobactam (2 infants), meropenem (1 infant), and amikacin (3 infants) for the second and third weeks of life.

Gut microbiota establishment by next-generation sequencing

The number of operational taxonomic units (OTUs) and the alpha diversity indexes of the meconium samples were similar to the further fecal samples (Fig 1), indicating a delayed bacterial establishment process. LEfSe analysis allowed us to explore the differences in microbiota composition between the control and the outbreak groups for the four variables stated above (epidemiological situation, delivery mode, gestational age, and birth weight).

Significant differences in the gut microbiota composition of the control and outbreak groups were detected in meconium and in the day 21 samples and were more relevant in those found at day 21 ($p = .0024$ at the genus level and $p = .073$ at the *phylum* level) (Fig 2). The outbreak group was characterized by a higher proportion of γ-*Proteobacteria*, related to a higher density of *Serratia*, and with lower proportions of the *Firmicutes* and *Fusobacteria phyla*.

In relation to the delivery mode, differences between vaginal delivery and C-section delivery were only significantly different at day 0 ($p = .0066$ at the genus level and $p = .0363$ at the *phylum* level) (Fig 3). Significant differences among bacterial communities regarding gestational age and birth weight were not detected.

Taking into account all 55 samples, the predominant *phylum* during the first weeks of life of the low-weight preterm infants was *Proteobacteria* (median±SD 70.1% ± 26.9%, range 0.3%–99.4%), followed by *Firmicutes* (median±SD 22.1% ± 26.8%; range 0.05–99.4); and although up to another 23 *phyla* were detected, their contribution was nearly anecdotic (Fig 4A). At the genus level, the abundance of *Escherichia/Shigella sp*. increased over the studied period while that of *Enterococcus sp*. and *Staphylococcus sp*. decreased (Fig 4B).

Reads accounting for the predator bacteria *Bdellovibrio* (2 infants), *Peredibacter* (1 infant), and *Vampirovibrio* (1 infant) were found in meconium samples from the outbreak (3 infants) and the control (1 infant) groups. The relative abundance of predatory species was extremely low (0.004%–0.11%), and none could be detected in the subsequent fecal samples.

Serratia was detected in the meconium samples from all the patients in the outbreak group (median value, 5558 OTUs), whereas this genus was considerably less abundant among the meconium samples from the control group (median value, 180 OTUs) (Table 2). Also noticeable was

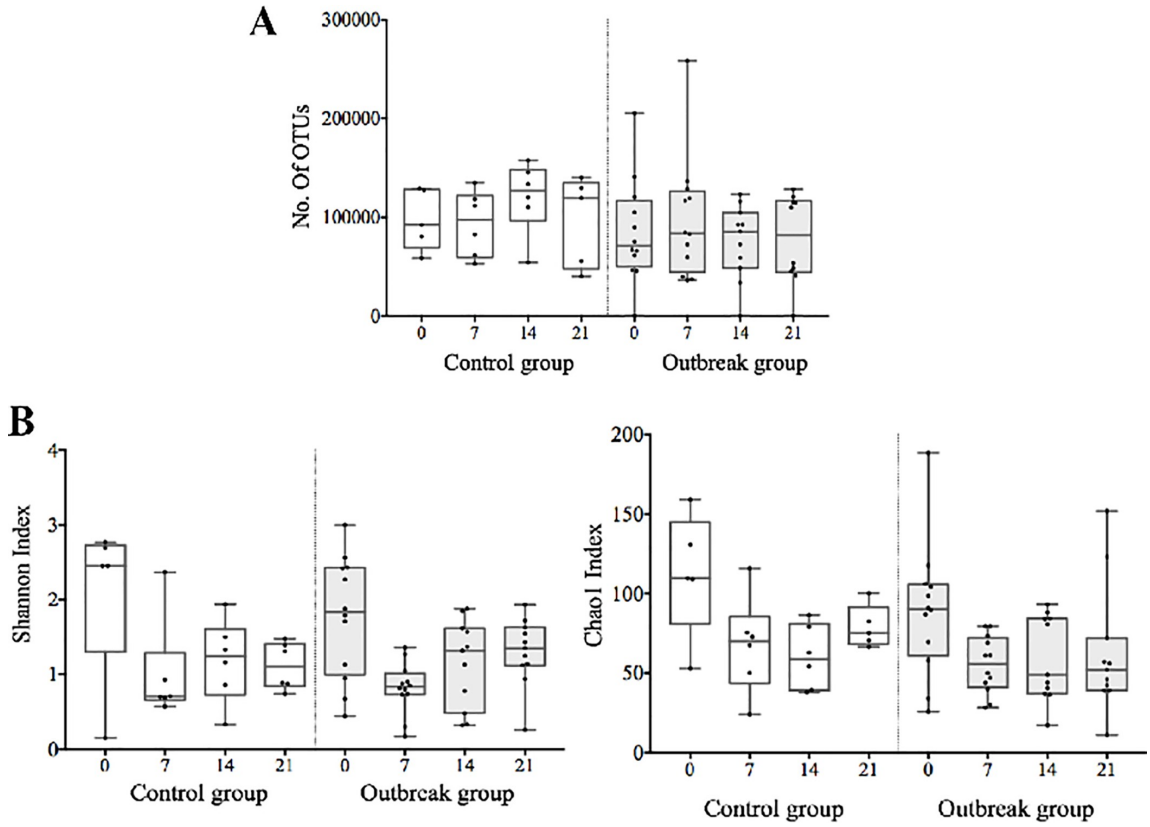

Fig 1. Number of operational taxonomic units (OTUs) **(A)**, and alpha diversity measured by the Chao1 index **(B)** in all samples studied.

https://doi.org/10.1371/journal.pone.0216581.g001

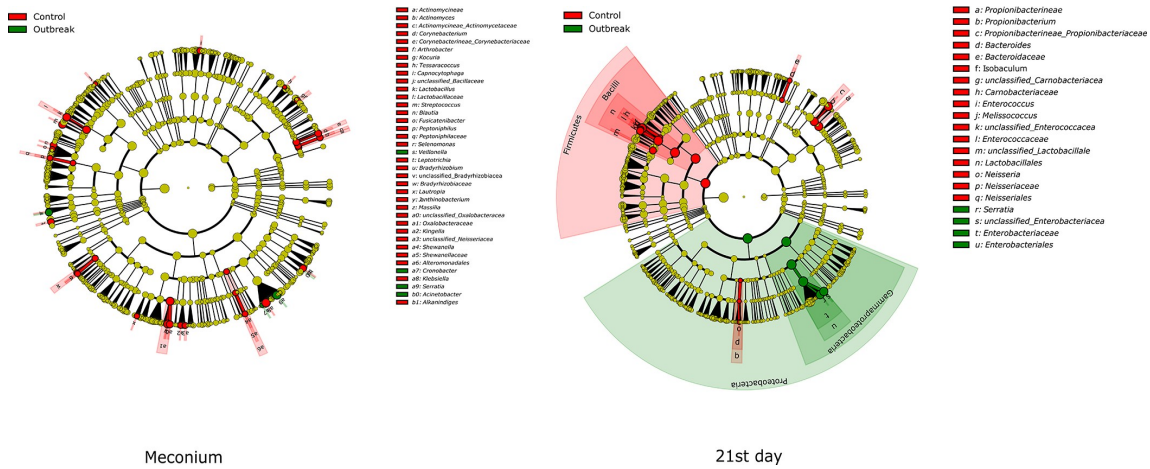

Fig 2. Cladograms showing the significant differences of gut microbiota composition in meconium and 21 days feces between control and outbreak groups.

https://doi.org/10.1371/journal.pone.0216581.g002

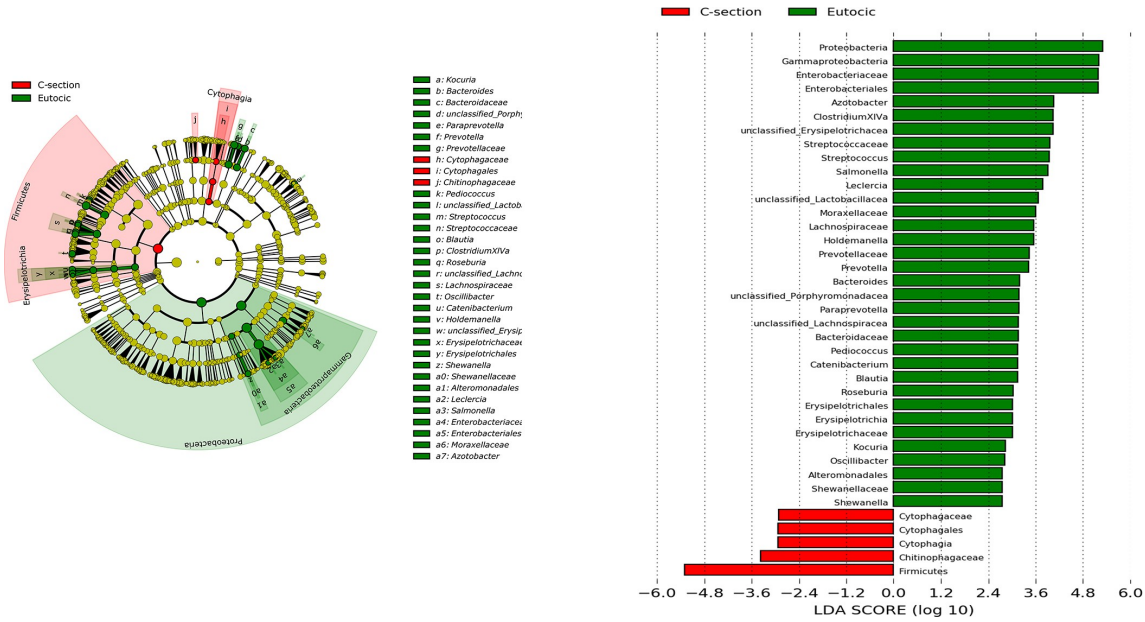

Fig 3. Significant differences in the gut microbiota of meconium by the delivery mode.

https://doi.org/10.1371/journal.pone.0216581.g003

that the *Serratia* reads considerably increased just before the diagnosis of *Serratia* sepsis in some patients from the outbreak group, and particularly in infant O11, who finally died from *S. marcescens* sepsis at day 10 after birth. The gut enrichment of *Serratia* sequences in this infant was manifest, reaching levels of 95% of the total intestinal microbiota at day 7 (Table 2).

Characterization of cultivable isolates from the outbreak group

A total of 16 *E. coli* (8 infants), 35 *Enterococcus faecalis* (11 infants), 2 *Enterococcus faecium* (1 infant), 12 *Klebsiella oxytoca* (7 infants), 6 *Klebsiella pneumoniae* (5 infants), 32 *S. epidermidis* (all 12 infants), 1 *Serratia liquefaciens* and 14 *S. marcescens* (8 infants) isolates were recovered. Regarding the meconium samples, the cultivable microorganisms were *S. epidermidis* (7 infants), *E. faecalis* (3 infants), and *S. marcescens* (2 infants). Only four meconium samples (33.3%) did not yield viable microorganisms. PFGE analysis showed that isolates recovered from different samples from the same infant were identical or closely related (Fig 5).

Similarly, a single *E. faecalis* pulsotype was detected, colonizing three infants and two major clones of *K. pneumoniae* in two infants. Among the *Serratia* isolates, two genetically unrelated clones were detected, affecting four and five infants each. The relative abundance of *Serratia* in the gut microbiota of each infant is presented in Table 2, which compares both next-generation sequencing (NGS) and microbiological culture techniques. It shows the high counts of *Serratia* in all samples from the outbreak group, confirming the results previously observed in the NGS analysis.

WGS of *Serratia* clones

Four *Serratia* isolates representing the two dominant PFGE clones associated with sepsis in the outbreak group were submitted to WGS (Fig 6). The genome analysis confirmed two

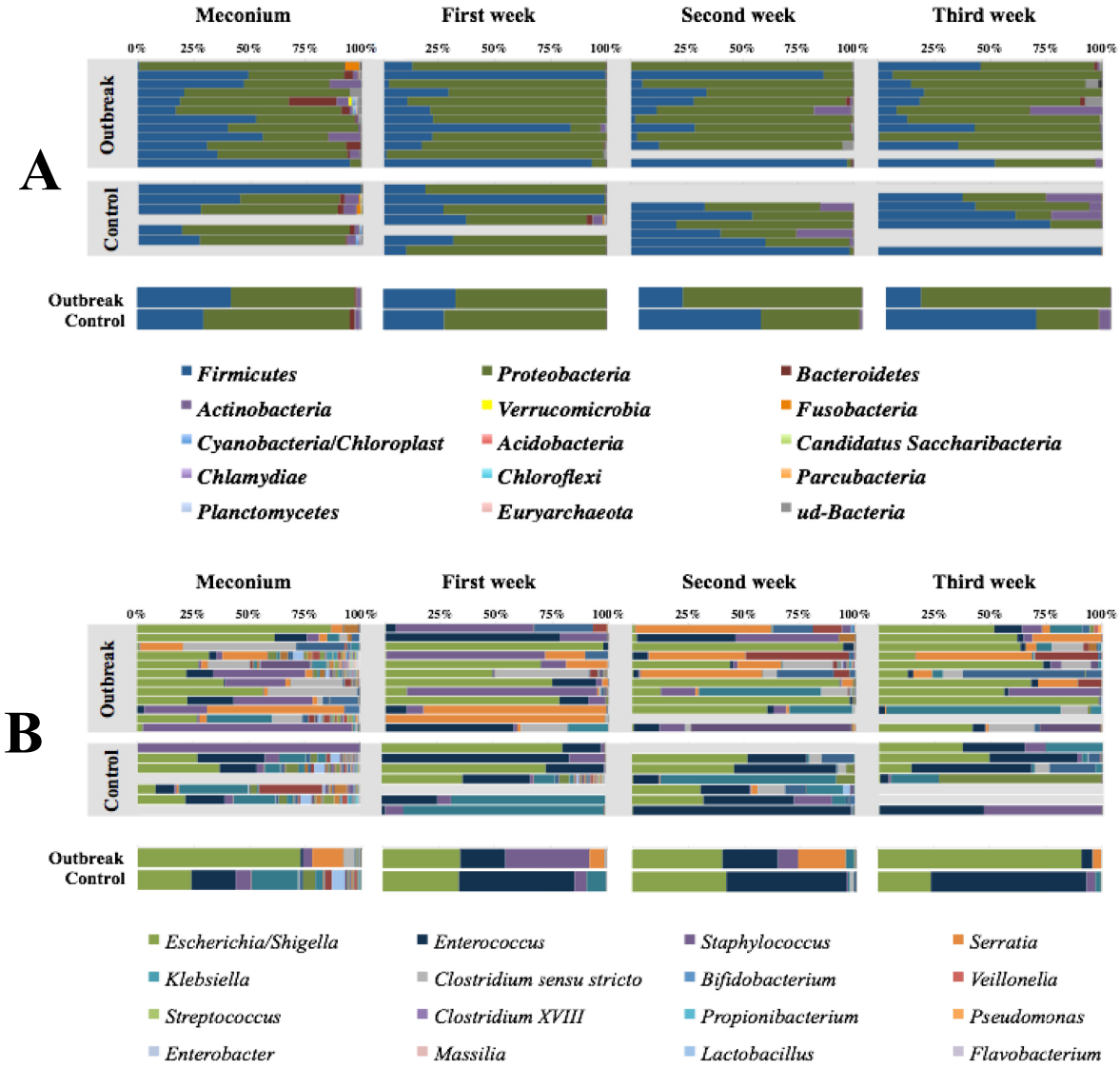

Fig 4. **(A)** *Phyla* percentage in each sample and infant, and summary of both groups expressed as the medians values. **(B).** Genera percentage in each sample and infant, and summary of both groups expressed as the median values. The 16 most abundant genera are highlighted in the figure, although up to 215 genera were detected in the samples analyzed in this study.

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phylogenic linages unrelated to others previously published. It is important to note that the isolate causing the sepsis and death of infant O11 was identical to the isolate obtained from infant O7, who had a satisfactory clinical evolution. WGS allowed the characterization of the resistome (*aac*(6')-*Ic*-1, $bla_{SRT-2-1}$, $bla_{SRT-1-1}$, and *qnr*E) and the virulome (*che*B, *che*R, *che*W, *che*Y, *flg*B, *flg*C, *flg*G, *flg*I, *flh*A, *flh*C, *flh*D, *fli*A, *fli*G, *fli*I, *fli*M, *fli*N, *fli*P, *fli*Q, and *fli*Z), which

Table 2. *Serratia* abundance detected by molecular tools and distribution of the two major clones detected in the outbreak group. The underlined isolates were submitted to whole genome sequencing. High abundance of *Serratia* by NGS is marked in light grey color, whereas the dark grey means a clear dominance of the *Serratia* genera.

	INFANT	Meconium		Day 7		Day 14		Day 21	
		Cultivable	NGS (%)	Cultivable	NGS (%)	Cultivable	NGS (%)	Cultivable	NGS (%)
OUTBREAK GROUP	O1	Clone A	5.4		0.002		12.1		1.0
	O2		12.4		0.003		0.2		1.7
	O3		2.5	Clone B	17.3		19.0		
	O4		2.4	Clone B	2.4	Clone B	4.8		16.9
	O5		1.8		0.003		0.04	Clone A	28.8
	O6		11.8	Clone B	5.1	Clone B	26.5	Clone B	37.4
	O7		2.9	Clone B	0.2		35.5		4.9
	O8		1.9		0.7		1.3		0.02
	O9		2.0		0.004		0.004		0.006
	O10	Clone A	59.5	Clone A	78.5	Clone A	0.8	Clone A	0.4
	O11		3.1	Clone B	94.5				
	O12		0.3	Clone A	1.3		0.1		1.2
CONTROL GROUP	C1		0.004		0.005				0.008
	C2		0.006		0.001		0.001		0.006
	C3		0.1		0.0007		0		0
	C4				0.06		0.2		0.01
	C5		0.8				3.1		
	C6		0.06		0		0		
	C7				0.0008		0		0.002

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was uniform in all four isolates. Nevertheless, our isolates remained susceptible to most antibiotics, given the resistant genes detected were located on the chromosome and not transferable.

Fungi detection

PCR-DGGE yielded positive amplifications in infant 6 (days 21 and 28) and infant 8 (days 14 and 21), and the nucleotide sequences of these amplicons corresponded to *Candida albicans*.

Discussion

In the present study, we have described the succession of the gut microbiota composition from meconium to the first 3 weeks of life of low-weight preterm infants, comparing two epidemiological scenarios from the same NICU. The primary result of our work is related to the *S. marcensens* outbreak influence on infant gut microbiota patterns, including meconium, which might have been modified even before birth in relation to the hospital admission of mothers to prevent premature birth. Although there is marked inter-individual variability, the gut microbiota of low-weight premature infants is dominated by *Proteobacteria phylum*, particularly *E. coli*. Moreover, the expected bacterial ecosystem expansion after birth appears to be delayed, probably in relation to antibiotic exposition. In that sense it is important to remark the data lack about antibiotic prescription on the control group, that represent an important limitation of our work.

Regarding to the outbreak group, all but two infants received empiric antibiotics immediately after birth, and most required culture-guided antibiotic treatments during their admittance, including up to three different antimicrobial families. However, the high incidence of infectious complications among preterm neonates is the main argument used to justify the

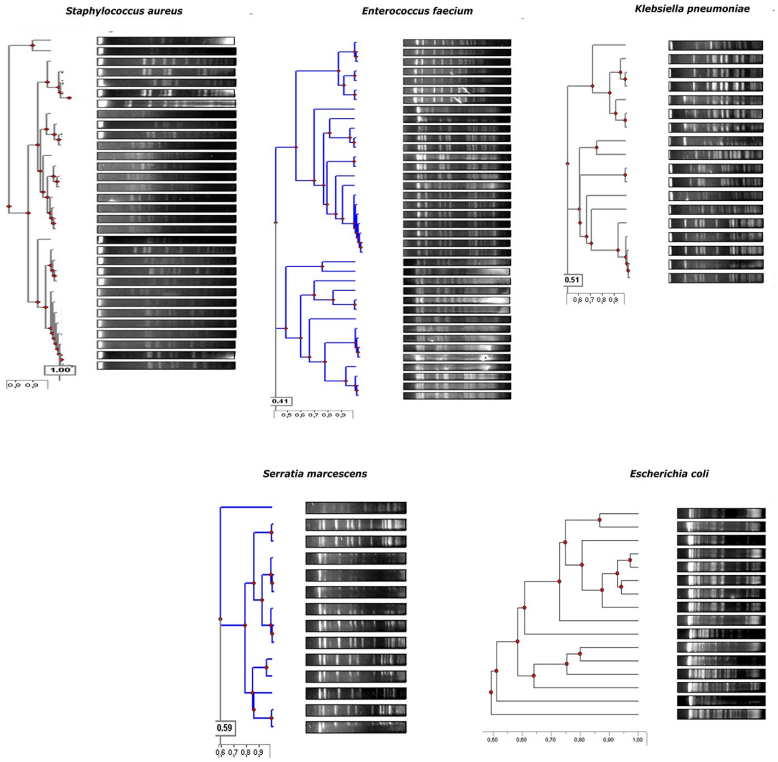

Fig 5. Dendrograme showing the genetic relationship among the cultivable isolates based on the Dice's coefficient.

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wide empiric and culture-guided use of antimicrobials in preterm infants, particularly in those with low birth weight, as in our case. Early biomarkers of preterm sepsis, together with the development of microbiome-based approaches, are urgently required to reduce antibiotic use in NICUs [28–29].

Prematurity is the main cause of neonatal morbidity and mortality, and the establishment of an adequate gut microbiota appears to be one of the most promising strategies to improve preterm infants' health and to reduce the impact of sequelae later in life [30–31]. Preterm infants admitted to an NICU have a high risk of infection, and *S. marcescens* is one of the most relevant nosocomial pathogens;[23] their intestinal carriage has been identified as a potential reservoir [32].

Our results demonstrated considerable *Proteobacteria* enrichment in both preterm infant groups, although the enrichment was significantly higher in the outbreak group from the second week of life, in concordance with other authors [33–36]. Previous studies found that *Proteobacteria* (mostly *Enterobacter* and *Photorhabdus*), *Firmicutes* (mostly *Enterococcus* and *Lactobacillus*), and *Actinobacteria* (*Bifidobacterium*) dominated the microbial composition of meconium [37]. *E. coli*, *Staphylococcus sp*., *Klebsiella sp*., and a high rate of facultative anaerobes also commonly appear in the meconium of preterm neonates [38–39]. Our study demonstrated significant differences between the control group dominated by *E. coli* and *Enterococcus* and the outbreak group with higher densities of *E. coli* and *Serratia*. Recent data have shown that current 16S rDNA technology is not applicable for the gut ecosystem of premature infants [40]; this is an important limitation of our work, probably showing lower

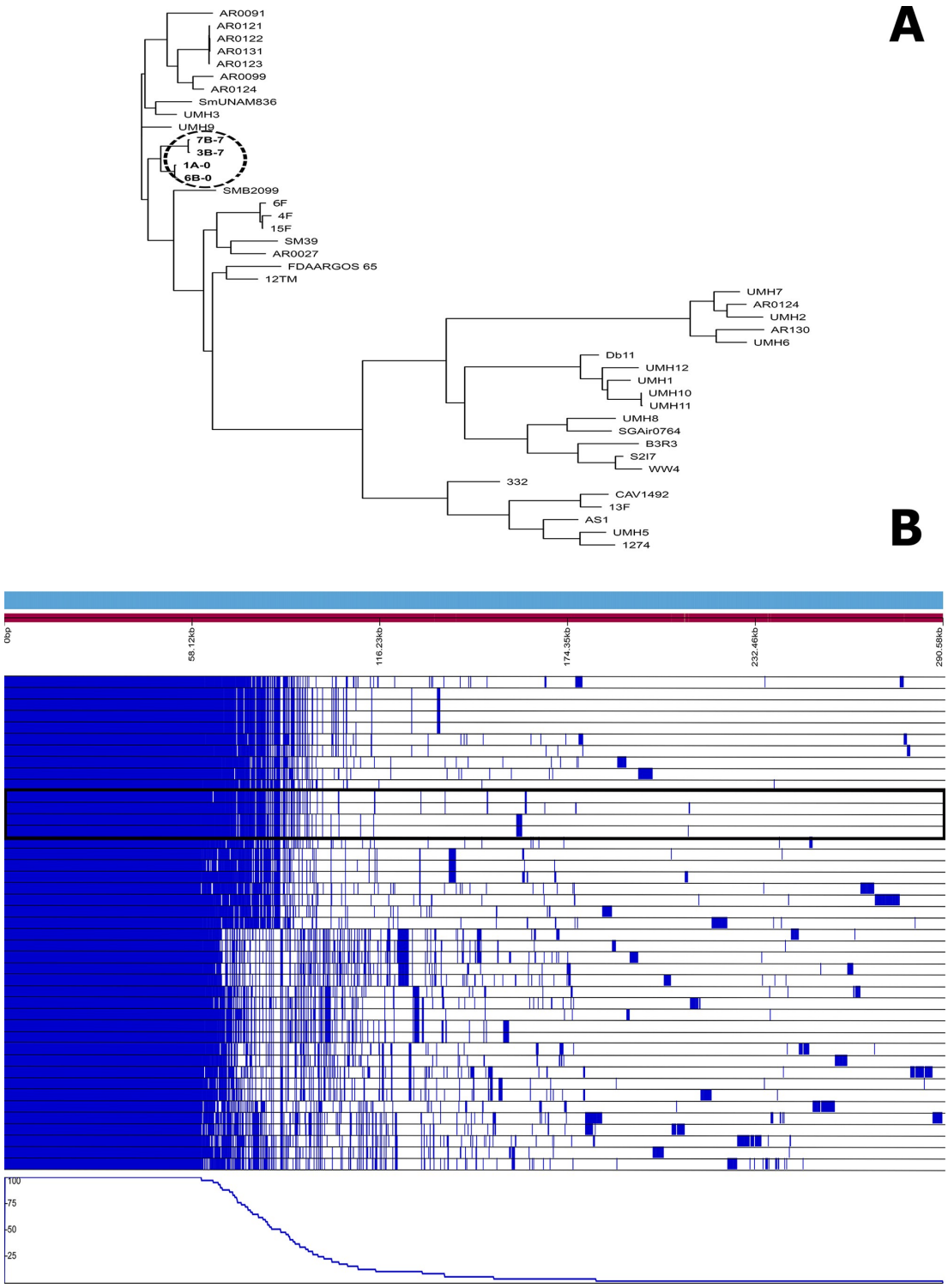
A
AR0091
AR0121
AR0122
AR0131
AR0123
AR0099
AR0124
SmUNAM836
UMH3
UMH9
7B-7
3B-7
1A-0
6B-0
SMB2099
6F
4F
15F
SM39
AR0027
FDAARGOS 65
12TM
UMH7
AR0124
UMH2
AR130
UMH6
Db11
UMH12
UMH1
UMH10
UMH11
UMH8
SGAir0764
B3R3
S2I7
WW4
332
CAV1492
13F
AS1
UMH5
1274
B
0bp
58.12kb
116.23kb
174.35kb
232.46kb
290.58kb
100
75
50
25

Fig 6. Phylogenic representation of the 4 *S.marcescens* genomes sequenced in this study and comparison with other 32 public *S. marcescens* genomes **(A).** The second part of the figure **(B)** represents the common core of all 36 genomes versus the isolate-specific genes. Our 4 isolates grouped together in both analysis and are marked.

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detection of the *Bifidobacterium* population. A recent work also demonstrated that *Bifidobacterium* density is related to the gestational age [12].

Curiously, the meconium samples from both groups were characterized by high interindividual variability and similar alpha diversity as the subsequent fecal samples, pointing to a marked delay in the establishment of the ecosystem. Similar results have been reported in very-low-birth-weight preterm infants [17]. Other authors have described an increase of fecal microbiota complexity during the NICU stay [41].

Although intrauterine fetal gut colonization is still a controversial issue [6–8], the detection of the *Serratia* microorganism in meconium samples suggests the possibility that its preterm colonization could start before birth. Most of the mothers in our study (7 of 12) were admitted to prevent a premature birth by various causes for a median period of 9 days before birth, with a range from 1 to 49 days. In contrast, the mother whose infant died from *S. marcescens* sepsis was admitted only on the day before delivery. Whereas this microorganism was scarcely represented in the meconium, it was very dominant at day 7, a fact that probably preceded the blood translocation and the sepsis episode that occurred at day 10. Therefore, a systematic routine exploration for potential enrichment of specific gut bacterial populations could possibly contribute to prevention of bacteremia in susceptible groups of patients, such as preterm infants. Recently, a novel functional methodology using volatile organic compounds as biomarkers for early detection of gut bacterial enrichment was reported [42]. Our results also demonstrated that some epidemic microorganisms, such as *S. marcescens*, are able to colonize and eventually infect preterm neonates even when state-of-the-art preventive measures have been applied. The PFGE analysis grouped isolates colonizing the 12 infants in the outbreak group into two major clones, whereas the WGS revealed a close relationship between them, suggesting the existence of a common ancestor. These molecular techniques also revealed that the virulome of the strain causing bacteremia and death was identical to other strains with clinical successful evolution, reinforcing the hypothesis that the unclear barrier delimiting colonization from infection is influenced by numerous factors.

Some OTUs assigned to the mandatory predator bacteria *Bdellovibrio*, *Vampirovibrio*, and *Peredibacter* were detected in the three meconium samples analyzed in this study. Such bacteria need to predate other bacteria to grow and reproduce and are considered to be important ecological balancers of the microbial communities [43]. Few studies have focused on these bacteria in human ecosystems; however, their presence in meconium samples suggests that they might not be infrequent in the gut microbiota. A predator's inoculation could represent an ecological tool to modulate bacterial communities, taking into account predator-prey specificity [44].

The preterm nutrition policy of the hospital specifies neonates be fed maternal milk, although this is typically combined with human milk from donors and with preterm-adapted formulas. All the participating preterm infants received all three types of milk during the study, and although such data are not detailed, we are aware that this factor also influences the gut microbiota establishment. Maternal milk can reshape the infant gut microbiota [9], contributing its own site-specific microbiota [45–47], but also promoting the increase of a precise population by its prebiotic action [48]. Thus, it would be suitable to include in the microbiota profiling scheme the differentiation between living and dead bacteria in order to identify real colonizers from casual bacterial passengers associated with food intake [49].

Globally, our results indicate that, regardless of their perinatal settings, preterm neonates admitted to the same NICU are initially colonized by similar microbial communities that later evolve according to individual conditions. A *Serratia* outbreak influence on the establishment of the gut microbiota appears to be universal from the first days of admission; however, our results might also be applied to outbreaks caused by other microorganisms. This highlights the importance of the environment regarding the pattern of gut colonization of hospitalized preterm infants.

Supporting information

S1 Dataset. Supplementary clinical data.
(XLSX)

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Author Contributions

Conceptualization: Esperanza Escribano, Claudia Saralegui, Juan Miguel Rodríguez, Miguel Sáenz de Pipaón, Rosa del Campo.

Data curation: Laura Moles.

Formal analysis: Claudia Saralegui, María Teresa Montes, Claudio Alba, Teresa Alarcón, Fernando Lázaro-Perona.

Funding acquisition: Miguel Sáenz de Pipaón.

Investigation: Esperanza Escribano, Claudia Saralegui, Laura Moles, María Teresa Montes.

Methodology: Claudio Alba, Teresa Alarcón, Rosa del Campo.

Project administration: Juan Miguel Rodríguez.

Supervision: Miguel Sáenz de Pipaón, Rosa del Campo.

Validation: Teresa Alarcón, Fernando Lázaro-Perona.

Writing – original draft: Rosa del Campo.

Writing – review & editing: Juan Miguel Rodríguez, Miguel Sáenz de Pipaón.

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Increased incidence of necrotizing enterocolitis associated with routine administration of Infloran™ in extremely preterm infants

E. Escribano[1], C. Zozaya[1], R. Madero[2,3], L. Sánchez[1], J. van Goudoever[4], J.M. Rodríguez[5] and M. Sáenz de Pipaon[1,3*]

[1]Department of Neonatology-Pediatrics, La Paz University Hospital, Autonomus University of Madrid, Paseo de la Castellana 261, Madrid 28046, Spain; [2]Biostatistics, La Paz University Hospital, Madrid, Spain; [3]Institute of Health Carlos III, Maternal and Infant Health and Development Network-SAMID, Madrid, Spain; [4]Department of Pediatrics, VU University Medical Center, Amsterdam, the Netherlands; Department of Pediatrics, Emma Children's Hospital, AMC, Amsterdam, the Netherlands; [5]Department of Nutrition, Food Science and Food Technology, Complutense University of Madrid, Madrid, Spain; msaenz.hulp@salud.madrid.org

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RESEARCH ARTICLE

Abstract

We aimed to evaluate the isolation of strains contained in the Infloran™ probiotic preparation in blood cultures and its efficacy in reducing necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) in extremely preterm infants. Routine use of probiotics was implemented in 2008. Infants born at <28 weeks gestational age were prospectively followed and compared with historical controls (HC) born between 2005 and 2008. Data on sepsis due to any of the two probiotic strains contained in Infloran and rates of LOS and NEC were analysed. A total of 516 infants were included. During the probiotic period (PC), none of the strains included in the administered probiotic product were isolated from blood cultures. Probiotic administration was associated with an increase in NEC stage II or higher (HC 10/170 [5.9%]; PC 46/346 [13.3%]; *P*=0.010). Surgical NEC was 12.1% in PC (42/346) versus 5.9% (10/170) in HC (*P*=0.029). Adjusting for confounders (sex, gestational age, antenatal steroids and human milk) did not change those trends (*P*=0.019). Overall, clinical LOS and the incidence of staphylococcal sepsis were lower in PC (172/342, 50.3, and 37%, respectively) compared with HC (102/169, 60.3 and 50.9%, respectively) (*P*=0.038 and *P*=0.003, respectively). No episodes of sepsis attributable to the probiotic product were recorded. The period of probiotic administration was associated with an increased incidence of NEC after adjusting for neonatal factors, but also with a reduction in the LOS rate.

Keywords: necrotizing enterocolitis, probiotics, sepsis, extremely low gestational age

1. Introduction

Necrotizing enterocolitis (NEC) is a gastrointestinal emergency in preterm infants. Various strategies have been applied to reduce its incidence; among them, the use of probiotics has gained attention because this condition is characterised by an alteration of the gut microbiota (Grishin *et al.*, 2013; Sim *et al.*, 2015). Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014). Four recent meta-analyses examined the impact of probiotics on NEC, sepsis and overall mortality rates (AlFaleh and Anabrees, 2014; Lau and Chamberlain, 2015; Olsen *et al.*, 2016; Rao *et al.*, 2016). The risk of NEC, overall mortality and late onset sepsis were reduced among infants receiving probiotics. However, these results must be interpreted with caution because the beneficial effects of probiotics depend on several factors, including the target population investigated in each assay. Therefore, given the lack of specific data on extremely preterm (<28 weeks' gestation) infants, evaluation of the benefits of probiotics in extremely preterm infants is difficult.

Although some neonatologists urge the routine use of probiotics in neonatal intensive care units (NICUs), others express some concern in relation to product quality and

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potential adverse effects (Neu, 2011). Probiotics are often regulated as dietary supplements rather than as pharmaceutical products; thus, there is no legal requirement to demonstrate safety, purity and/or concentration before marketing them. This can lead to significant inconsistencies between the stated and actual contents of probiotic preparations (Boyle *et al.*, 2006). The most important area of concern with probiotic use in preterm neonates is the risk of sepsis (Hempel *et al.*, 2011).

In the NICU of La Paz University Hospital (Madrid, Spain), a probiotic product (Infloran™, Laboratorio Farmaceutico, Mede, Italy), containing a strain of *Lactobacillus acidophilus* and a strain of *Bifidobacterium longum* subsp. *infantis,* both at ~10^9 cfu/capsule, has been administered to all preterm babies born at ≤29 weeks' gestation and/or weighing ≤1,250 g as a standard of care since 2008 until 2016. Evidence at that time urged clinicians to introduce this prophylactic approach (Alfaleh and Bassler, 2008). Routine probiotic prophylaxis outside the rigid framework of randomised clinical trials was expected to provide data on real-life outcomes, including both potential benefits and adverse effects of this intervention. This ecological observational study aimed to investigate the effect of Infloran™ on the NEC incidence in extremely low gestational age neonates after implementation in our NICU, when compared with a historical control cohort (HC). The secondary aim was to assess the influence of this probiotic mixture on the rates of surgical NEC, late-onset sepsis (LOS) and mortality. Finally, another objective was to evaluate the presence of the probiotic strains in culture-proven sepsis.

2. Patients and methods

Probiotics and enteral feedings

From 2008, all infants with ≤29 weeks of gestational age and/or birth weight ≤1,250 g admitted to our NICU received Infloran. The doses (twice daily) were ~10^9 cfu of each strain per kg in infants with a birth weight <1000 g, and ~10^9 cfu of each strain (1 capsule) in those with a birth weight >1000 g. Capsules were opened, and the content was mixed with 2 ml of glucose 5% immediately before administration to infants. Administration of Infloran started at the time of the first feed, between 2 and 5 days of life, and continued for 6 weeks or until discharge, whichever occurred first. Infloran was not used in infants with gut malformations and its use was discontinued when enteral feeding was withheld. Nutritional policy during these periods relied on the administration of own mother's milk and supplementation with preterm-adapted formula (PF); at 120 ml/kg/day approximately 70% of enteral feeding intake was human milk (HM). Fortification of human milk was identical between the two cohorts, with a bovine milk fortifier started as soon as enteral feeding reached 100 ml/kg/day. The standardised feeding protocol followed in our NICU consisted on starting enteral feeding within 2 days of life, unless hypotension that necessitated use of a vasopressor agent. The initial volume was 20-25 ml/kg/day. When the infants were stable, feeding volume was gradually advanced by 20 ml/kg/day when gastric residuals did not exceed half of the current feeding volume. Feedings were withheld when there was hypotension, abdominal examination changes, or when gastric residual volumes exceeded the current feeding volume. Parenteral nutrition was maintained until the infant tolerated 120 ml/kg/day. The median days on parenteral nutrition was 17 days (range 11-25 days). The median age of reaching 120 ml/kg/day of enteral feeding was 16 days (range 12-24 days).

Study design and eligibility

We conducted a retrospective ecological analysis of prospectively collected data from a cohort of extremely low gestational age neonates to explore the effects of Infloran in infants with ≥23 and <28 weeks of gestational age. Data from infants receiving Infloran (probiotic cohort [PC]; January 2009-December 2015) were compared with those from an HC that did not receive probiotics (January 2005-December 2007). Patients who died within the first 48 h after birth and patients who were admitted to NICU after 48 h of life, were excluded from the study.

Data collection

Data were retrieved from the database collected prospectively from infants with birth weight <1,500 g admitted to our NICU from January 2005 to December 2015. Data between January 2008 and December 2008 were not collected, assuming that adhesion to the protocol was fully operative 1 year after active implementation (Ament *et al.*, 2015).

Demographic and basic clinical parameters

Definitions

Administration of maternal antenatal corticosteroid therapy consists of administration of two doses of intramuscular betamethasone 24 h apart. A patent ductus arteriosus (PDA) was defined as the presence of a hemodynamically significant PDA based on echocardiographic and clinical criteria. Chronic lung disease (BPD) was defined as oxygen dependence at 36 weeks postmenstrual age. Intraventricular haemorrhage was graded according to Volpe (1995).

Neonatal outcome

The primary outcome parameter was proven NEC (stage IIA and above), according to modified Bell's stages (Bell *et al.*, 1978). Other outcome parameters were death before discharge and LOS. LOS was defined as systemic

infection with clinical and haematologic evidence only or bacteriologically-proven systemic infection occurring after 72 h of age. Specifically, LOS due to *Staphylococcus* sp. was recorded. The cultivability of the two strains included in Infloran was confirmed by the microbiology department of the hospital. Blood cultures were incubated in the BACTEC automated blood culture device (Becton Dickinson, Franklin Lakes, NJ, USA) and BacT/ALERT (bioMérieux, Marcy l'Etoile, France) blood culture bottle systems (Hirvonen *et al.*, 2012). All positive blood cultures were routinely sub-cultivated on three agar plates: sheep blood agar, chocolate blood agar and *Brucella* blood agar and they were incubated overnight.

From January 2012, all patients admitted to our NICU were included in the program of implementation of measures to prevent central venous catheter (CVC)-related bacteremia (training for health care personnel and introduction of specific measures related to the insertion and care of CVC, bacteremia zero [BZ] protocol). Accordingly, the PC was separated into two subgroups (before vs after BZ).

Statistics

The statistical analysis was performed using Fisher's exact test for categorical data and Student's t-test for continuous variables. To quantify the association between probiotic use and outcomes (NEC, LOS, mortality), univariate analyses were performed. A multivariable analysis was performed to assess the independent effect of probiotic supplementation on NEC that included the confounding covariates (sex, gestational age in 7-day intervals, antenatal corticosteroids and human milk intake). *P*-values <0.05 were regarded as statistically significant. The impact of probiotics on LOS and late-onset sepsis due to *Staphylococcus* sp. was controlled for the influence of the BZ protocol in a model of multivariable analysis that included sex, gestational age in 7-day intervals and birth weight in 100 g intervals. The same model was used for mortality. The statistical analysis was performed with STATA 13.1 (statistical software Stata Corp, TX, USA).

Ethics and registration

The ethics committee of La Paz University Hospital approved the study design (reference code 3551). Informed consent was not considered necessary by the hospital ethics board since the probiotic product was introduced for routine therapy and the controls were analysed retrospectively.

3. Results

Screening

A total of 1,519 very low birth weight (VLBW) infants who were admitted to the tertiary NICU of La Paz University Hospital from January 2005 to December 2007 and from January 2009 to December 2015 were screened for eligibility. Among them, 516 were born with ≥23 and <28 weeks of gestational age, and 67 infants were excluded because of death in the first 48 h after birth and admission in our NICU after 48 h of life. Therefore, data retrieved from 516 infants (HC: n=170; PC: n=346) were finally analysed (Figure 1).

Demographic and basic clinical parameters

Baseline characteristics of the infants were not different between the two cohorts (Table 1). Treatments with both antenatal and postnatal corticosteroids were more frequent among infants in the PC (+12%, *P*=0.003 and +4%; *P*=0.006, respectively) than in the HC.

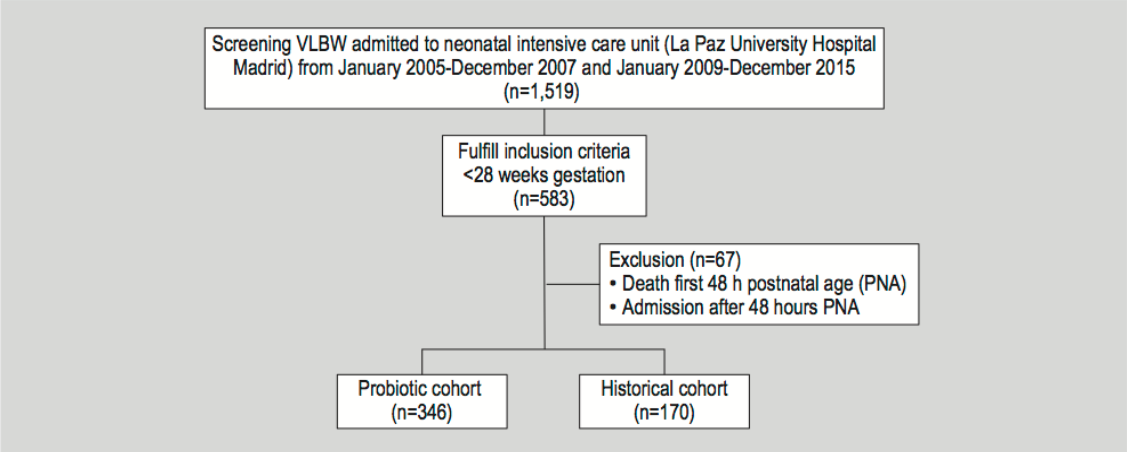

Figure 1. Flow chart showing the screening of <28 weeks' gestation for study eligibility. VLBW = very low birth weight.

Table 1. Demographic and basic clinical parameters of the studied population.[1]

	Probiotic cohort (n=346)	Historical cohort (n=170)	*P*-value[2]
Gestational age[3] (weeks)	26±1	26±1	0.54
Birth weight[3], g	827.1±177	822.7±161.8	0.78
Male sex, %	53	55	0.57
Multiple delivery, %	27.2	22.9	0.34
Caesarean section, %	71	66	0.26
Apgar, 5 min <5, %	8.3	4.7	0.14
CRIB score	5.1	5.5	0.25
Antenatal corticosteroids, %	76.3	63.5	**0.003**
Postnatal corticosteroids, <14 days, %	3.76	0	**<0.01**
PDA, %	69.1	63.5	0.23
HMD, %	81.5	80.6	0.81
Bronchopulmonary dysplasia, %	49.4	35.4	**0.012**
Grade III IVH and /or PHI, %	20.8	17.6	0.48
Cystic periventricular leukomalacia, %	4.65	2.35	0.34

[1] CRIB = clinical risk index for babies; PDA = patent ductus arteriosus; HMD = hyaline membrane disease; IVH = intraventricularhaemorrhage; PHI = periventricular haemorrhagic infarction.
[2] *P*-values <0.05 were considered statistically significant and are printed in bold letters.
[3] Mean ± standard deviation.

Neonatal outcome parameters

We found a higher NEC incidence with the probiotic era (13.3%) when compared with the HC period (5.8%), *P*=0.01 (Table 2). NEC incidence was also higher in the PC after controlling for confounding variables (*P*=0.019, Table 3). In addition, in infants with NEC, those that needed acute surgical treatment, surgical NEC, rates were higher in the PC than in the HC, *P*=0.03, Table 2.

Mortality rates were not significantly different between the PC (22%) and the HC (16%) (*P*=0.13), even after controlling for confounding variables. The LOS rate in the PC (50.3%) was significantly lower than that found in the HC (60.4%) (*P*=0.038, Table 2). Within infants with LOS, those due to *Staphylococcus* sp. in the PC (37.1%) was also significantly lower than in the HC (50.9%) (*P*=0.003, Table 2). After controlling for confounding variables only probiotics and BZ together decreased the LOS rate. More specifically in the PC, the LOS odds ratio (95% CI) in the pre-BZ period was 0.77 (0.50-1.21, *P*=0.26) vs 0.56 (0.36-0.89, *P*=0.012) in the post-BZ period compared with the HC (Table 4). Regarding LOS due to *Staphylococcus* sp., incidence was lower in both periods, pre-BZ and post-BZ, than in HC, after controlling for confounding variables (Table 4).

Table 2. Comparison of neonatal outcomes in the studied cohorts.[1]

	Probiotic cohort (n=346)	Historical cohort (n=170)	*P*-value[2]
NEC Stage 2-3 n (%)[3]	46 (13.3 %)[a]	10 (5.9%)	0.01
Surgical NEC[3]	42 (12.1%)	10 (5.9%)	0.03
LOS[3]	172 (50.3%)	102 (60.3%)	**0.038**
Staphylococcus sp. LOS[3]	127 (37.1%)	86 (50.9%)	**0.003**
Mortality (%)[3]	75 (21.7%)	27 (15.9%)	0.13
Postnatal age *at exitus* (days)[4]	39±59	22±26	0.16

[1] NEC = necrotizing enterocolitis; LOS = late-onset sepsis.
[2] *P*-values <0.05 were considered statistically significant and are printed in bold letters.
[3] Numbers with percentage in parentheses.
[4] Mean ± standard deviation.

Table 3. Multivariable analysis for assessing the independent effect of probiotic supplementation on necrotizing enterocolitis (NEC) and surgical NEC that included the confounding covariates (sex, gestational age in 7-day intervals, antenatal corticosteroids, and human milk intake).[1]

	NEC			Surgical NEC		
	OR	95% CI	*P*-value[2]	OR	95% CI	*P*-value[2]
Probiotics	2.76	1.182-6.447	**0.019**	2.39	1.009-5.674	**0.048**
GA (7-d intervals)	0.88	0.666-1.166	0.375	0.76	0.570-1.027	0.074
Sex (female)	0.95	0.494-1.833	0.881	0.79	0.393-1.600	0.517
Antenatal corticosteroids	1.48	0.652-3.367	0.348	1.32	0.570-3.051	0.517
Human milk	0.75	0.353-1.618	0.471	0.71	0.309-1.634	0.421

[1] OR = odds ratio; CI = confidence interval; GA = gestational age.
[2] *P*-values <0.05 were considered statistically significant and are printed in bold letters.

Table 4. Multivariable analysis to assess the impact of probiotics with or without the influence of the bacteremia zero (BZ) protocol on late-onset sepsis and late-onset sepsis due to *Staphylococcus* sp. that included the confounding covariates (sex, gestational age in 7-day intervals and birth weight in 100-g intervals).[1]

		Late-onset sepsis			*Staphylococcus* sp. sepsis		
		OR	95% CI	*P*-value[2]	OR	95% CI	*P*-value[2]
Period	Probiotics	0.76	0.498-1.209	0.262	0.61	0.393-0.947	**0.028**
	Probiotics + BZ	0.57	0.365-0.885	**0.012**	0.53	0.339-0.821	**0.005**
GA (7 day intervals)		0.77	0.646-0.929	**0.006**	0.83	0.691-0.990	**0.039**
Sex (female)		0.95	0.673-1.411	0.890	0.97	0.669-1.406	0.873
Birth weight (100 g intervals)		0.87	0.764-0.990	**0.035**	0.89	0.777-1.010	0.070

[1] OR = odds ratio; CI = confidence interval; GA = gestational age.
[2] *P*-values <0.05 were considered statistically significant and are printed in bold letters.

No cases of sepsis due to any of the strains included in the probiotic product occurred during the study period.

4. Discussion

This study revealed that the administration of Infloran to a cohort of extremely preterm infants was associated with an increased incidence of NEC, but not with mortality rates. The use of this probiotic product was associated with a lower LOS incidence when an infection prevention bundle was implemented. *Staphylococcus* sp. sepsis was lower in the probiotic period even before implementation of measures to prevent CVC-related bacteremia started. Furthermore, this study found that infections with the probiotic product itself seem to be exceptional.

These results need further consideration due to their important findings. The risk of developing NEC is inversely related to gestational age at birth (Morgan *et al.*, 2011). Only two studies included in the meta-analyses provided data on the subgroup of extremely low gestational age neonates (AlFaleh and Anabrees, 2014; Olsen *et al.*, 2016). Both studies showed no decrease of NEC in those born before 28 weeks' gestation (AlFaleh and Anabrees, 2014; Olsen *et al.*, 2016). Therefore, we included only infants with a gestational age <28 weeks in the analysis.

Due to gastrointestinal maturation, treatment with antenatal corticosteroids halves the risk of NEC. The intestinal barrier is presumably strengthened. Although there were differences in antenatal and postnatal steroid use between our two cohorts, the effect of probiotics is clarified in our study when both factors are taken into consideration. Probiotics provide a benefit by improving the mucosal barrier, inhibiting gut colonisation with pathogenic bacteria and decreasing the nuclear transcription factor Kβ-mediated inflammatory response (Bermudez-Brito *et al.*, 2012). Probiotics might not be able to adhere to the

intestinal wall in extremely low gestational age neonates; thus, their efficacy would be reduced.

Our results are different from the randomised clinical trials (RCTs) that used Infloran (Lin *et al.*, 2005; Lin *et al.*, 2008). In fact, studies reporting the use of Infloran have provided contradictory results. Lin *et al.* (2005) showed a reduction in the incidence of sepsis in a VLBW infant cohort, whereas a subsequent study from the same group found no difference in sepsis but a 75% reduction in NEC rates (Lin *et al.*, 2008). Hoyos (1999) reported a beneficial effect of Infloran on NEC incidence (3% in the probiotic cohort vs 7% in a historical cohort), but only 8% of infants in the study group had birth weight below 1,500 g. Later, Repa *et al.* (2015) showed no reduction of NEC in a smaller cohort than ours, of more mature VLBW infants, also based on routine use and compared with a historic control. More recently, a retrospective observational cohort study of VLBW infants, more mature than the ones included in our study, revealed that routine supplementation with a different probiotic strain, *Lactobacillus rhamnosus* GG was associated with a higher risk of NEC(Kane *et al.*, 2018).

The negative effect of Infloran on NEC in our population cannot be explained on the basis of a low NEC incidence given the baseline incidence in the HC of our study (5.9%) was similar to that (6.5%) of the cohort studied by Lin *et al.* (2008). In relation to the cited study, our inclusion criteria included a lower gestational age, and the infant birth weight of our cohort was also lower. In addition, the study by Lin *et al.* (2008) was conducted in Taiwan, whereas the population cared for at our hospital is mainly white; therefore, we cannot exclude the possibility that the susceptibilities of the study populations were different. Results showing a different efficacy of the same probiotic product on NEC rates depending on the population have also been reported, although the babies in their study were larger and more mature than those analysed here, and the control incidence of NEC was 17% in their study (Bin-Nun *et al.*, 2005).

The dosage of Infloran used in different studies does not seem to be directly correlated with its effect, the highest doses (4×10^9 cfu) were associated with no efficacy (Repa *et al.*, 2015) while the lowest doses (2×10^9 cfu/kg in Lin *et al.*, 2008; 0.25×10^9 cfu in Hoyos, 1999) were reported to have a beneficial effect. The time of administration in the various RCTs ranges from at birth with no effect to the seventh day of life with a protective effect (Desphande *et al.*, 2010; Repa *et al.*, 2015). Globally, data are too scarce to draw any conclusion on the best dosage or age of initiation of supplementation with this probiotic preparation.

Animal models have shown that certain probiotic strains can even increase NEC rates at a dose of 3×10^9 cfu every 3 h (to a total of 2.4×10^{10} cfu/day) (Cilieborg *et al.*, 2011); comparable doses resulted in decreased sepsis in preterm infants (Samanta *et al.*, 2009). Higher doses in another study in piglets have shown reduced NEC severity (Siggers *et al.*, 2008). Hence, the total dose cannot explain the observed adverse effects. In the study of Cilieborg *et al.* (2011) the NEC incidence in the control group was low, whereas in the recent UK trial (the largest RCT to date, with a population like ours regarding immaturity) no beneficial effect was seen (Costeloe *et al.*, 2016). Cross-colonisation with administered probiotics is likely to modify the gut microbiota of infants other than those for whom it is prescribed and is the major limitation of randomised trials without cluster design (Costeloe *et al.*, 2016). Our study design overcomes this limitation. The clinical effects of probiotics show wide variations depending on the strain (Deshpande *et al.*, 2010).

Taking into account the special vulnerability of this population, a careful evaluation of the potential risks associated with probiotics administration in preterm infants is required. This study showed that oral administration of Infloran was not associated with probiotic-associated sepsis, which is also in agreement with previous studies (Bin-Nun *et al.*, 2005).

There are potential mechanisms by which probiotics might influence preterm infant health. Among them, intestinal barrier maintenance is of particular interest. The efficacy of this barrier depends on several factors, including tight junctions' integrity, intestinal peristalsis, quality and quantity of mucin production, secretory immunoglobulin A, gastric acid production, T-cell immunity and macrophage phagocytosis, all of which can be impaired in preterm, even more in the most immature infants (Arboleya *et al.*, 2012). The safety and efficacy of probiotics in the most immature neonates has not yet been proven. In a recent randomised trial no colonisation by probiotic strains (*B. longum* BB536 and *L. rhamnosus* GG) was detected in infants who weighed ≤1000 g (Rougé *et al.*, 2009).

There is some reluctance to adopt probiotic prophylaxis to reduce the risk of NEC in preterm infants because of the heterogeneity in the strains and populations assayed in the various RCTs (Neu, 2014). Rigorous designs and recommendations are therefore essential to evaluate probiotic efficacy, particularly in extremely low gestational age infants. LOS is a major cause of morbidity. In this study, oral supplementation with Infloran was associated with a reduction in the incidence of *Staphylococcus sp.* sepsis and a reduction of LOS when it was combined with training and CVC care measurements. Probiotic microorganisms are expected to colonise the gut, compete with pathogens, improve gut barrier function and permeability, and modulate immune function. The gastrointestinal tract is reported to be the main reservoir of *Staphylococcus* sp., the most frequent organism responsible for LOS in extremely preterm infants (Rønnestad *et al.*, 2005) Such an

improvement is consistent with an earlier report (Lin *et al.*, 2005) and with the conclusion from a meta-analysis (Rao *et al.*, 2016). In addition to reducing the incidence of LOS and its subsequent high antibiotic therapy use, probiotics could colonise the gut and encourage LOS prevention. The quantity and type of antibiotics administered to the infants before or during Infloran administration may have exerted a relevant influence on their gut microbiota and in the outcomes assessed in this work. However, such influence is unknown, and this can be considered a limitation of this study.

An increased incidence of BPD has been found in the probiotic group. A recent meta-analysis concluded that probiotic supplementation in preterm infants does not affect the risk of BPD (Villaamor-Martínez *et al.*, 2017). These data are of great importance for clinicians. Safety is a major concern when the use of probiotics is considered for fragile populations. We conclude that in infants below 28 weeks' gestation Infloran is associated with an increased NEC incidence, despite an association with a reduced risk of LOS while specific infection prevention bundles are implemented. Further research in the field is essential in order to address the knowledge gaps concerning microbial roles in protecting preterm health, particularly safety and efficacy in extremely preterm infants.

Conflicts of interest

The authors state that there are no financial ties to products used in the study or potential/perceived conflicts of interest.

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Research Article

Administration of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934, Two Strains Isolated from Human Milk, to Very Low and Extremely Low Birth Weight Preterm Infants: A Pilot Study

Laura Moles,[1] Esperanza Escribano,[2] Javier de Andrés,[1,3] María Teresa Montes,[2] Juan M. Rodríguez,[1,3] Esther Jiménez,[3] Miguel Sáenz de Pipaón,[2] and Irene Espinosa-Martos[3]

[1]*Departamento Nutrición, Bromatología y Tecnología de los Alimentos, Universidad Complutense de Madrid, 28040 Madrid, Spain*
[2]*Servicio de Neonatología, Hospital Universitario La Paz, 28046 Madrid, Spain*
[3]*Probisearch, Tres Cantos, 28760 Madrid, Spain*

Correspondence should be addressed to Irene Espinosa-Martos; irene.espinosa@probisearch.com

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The preterm infant gut has been described as immature and colonized by an aberrant microbiota. Therefore, the use of probiotics is an attractive practice in hospitals to try to reduce morbidity and mortality in this population. The objective of this pilot study was to elucidate if administration of two probiotic strains isolated from human milk to preterm infants led to their presence in feces. In addition, the evolution of a wide spectrum of immunological compounds, including the inflammatory biomarker calprotectin, in both blood and fecal samples was also assessed. For this purpose, five preterm infants received two daily doses (~10^9 CFU) of a 1 : 1 mixture of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934. Bacterial growth was detected by culture-dependent techniques in all the fecal samples. The phylum *Firmicutes* dominated in nearly all fecal samples while *L. salivarius* PS12934 was detected in all the infants at numerous sample collection points and *B. breve* PS12929 appeared in five fecal samples. Finally, a noticeable decrease in the fecal calprotectin levels was observed along time.

1. Introduction

The gut microbiota of preterm infants is usually described as aberrant when compared to that of healthy term infants. Very often, the former is characterized by a notably lower bacterial diversity, a lower presence of bifidobacteria, and a higher concentration of potentially pathogenic bacteria [1–7]. This may have short-, medium-, and long-term health consequences since early colonizing organisms interact with the intestinal mucosa to shape the developing immune system [8, 9].

In fact, interactions with different components of the microbiota are crucial to the establishment and development of T-cell subsets, including NK, Treg, and Th17 cells, in the appropriate proportions to achieve homeostasis [10].

Many preterm infants lack an important part of transplacental transfer of maternal antibodies since this process occurs mainly in the last third of pregnancy; in addition, they have an impaired pattern-recognition receptor function and a reduced leukocyte endothelial adhesion and extracellular bacterial elimination [11]. Together, these alterations in the microbial colonization pattern and in the maturation of immune system, together with their stay in a hospital environment and other factors, predispose preterm infants to infections and/or to diseases such as necrotizing enterocolitis (NEC) [12–15].

The administration of probiotics to preterm neonates often leads to a decrease in the morbidity and mortality rates, in those of NEC and, in some cases, even in those of sepsis [16–22]. Additional benefits associated with probiotic supplementation in preterm neonates include earlier achievement of full enteral feeding [22], a lower colonization by *Enterobacteriaceae* [23], and a better neurological and immunological evolution [22, 24]. For these reasons, the number of institutions including probiotic supplementation in routine preterm care is increasing rapidly although the safety of probiotics in very low and extremely low birth weight infants is still a matter of debate [25], the mechanisms backing such effects are not well known yet [10], and global conclusions are difficult to establish because different studies usually make use of different probiotic strains, dosages, and/or treatment period.

Human milk is acknowledged as the best feeding option to preterm infants [26, 27] because its use decreases the incidence of many negative outcomes of prematurity, such as late onset sepsis or NEC [28–30]. In addition, human milk seems to be an important source of potentially beneficial bacteria to the infant gut and some strains may find future applications as probiotics for preterm infants [31–36]. In this context, the objective of this exploratory study was to assess early gut colonization in a short cohort of preterm neonates receiving a combination of two probiotic strains isolated from human milk. Furthermore, a wide variety of blood and fecal immunological parameters were assessed in order to elucidate their utility in future studies involving a larger cohort.

2. Materials and Methods

2.1. Study Design and Sampling. Five preterm infants were enrolled in this study within 2 days after their birth. All of them met the following inclusion criteria: birth weight < 1,300 g, gestational age at birth < 29 weeks, and absence of any malformation or metabolic disease at birth. The most relevant demographic and clinical variables from mother-infant pairs were compiled by the Medical Staff of the Service of Neonatology of the Hospital Universitario La Paz (Madrid, Spain). The Ethical Committee on Clinical Research of the Hospital Universitario La Paz of Madrid approved all study protocols (code number: 3551). Samples and clinical information were obtained after written informed consent by the infants' parents. This trial is registered with ClinicalTrials.gov identifier NCT02192996.

After spontaneous meconium expulsion (between the second and the fourth days of life), a mixture of *Bifidobacterium breve* PS12929 and *Lactobacillus salivarius* PS12934, containing ~1 × 10^9 colony-forming units (CFU) of each strain, was suspended in a sterile saline solution and administered twice a day to the infants through an enteral feeding system. Meconium samples were collected prior to probiotic administration and, later, fecal (n = 14) and blood (n = 10) samples were collected weekly for up to 28 days. Fecal samples were aliquoted and stored at −80°C or −20°C until microbiological or immunological analysis, respectively. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes; subsequently, the plasma was obtained within 4 h after extraction and stored at −20°C until analysis.

2.2. Microbiological Analysis. Adequate dilutions of five meconium and fourteen stool samples were spread onto Kanamycin Aesculin Azide Agar (KAA; Oxoid) for *Enterococcus* species isolation; de Man, Rogosa and Sharpe (MRS; Oxoid, Basingstoke, UK) supplemented with L-cysteine (0.5 g/L) (Sigma, St. Louis, USA) (MRScys) for isolation of lactic acid bacteria; MacConkey (MCK; BioMérieux, Marcy l'Etoile, France) for isolation of *Enterobacteriaceae*; Sabouraud Dextrose Chloramphenicol (SDC, BioMérieux) for isolation of yeasts; TOS-Propionate (TOS; Merck, NJ, USA) for isolation of bifidobacteria; and Columbia Nalidixic Acid Agar (CNA, BioMérieux) as a general medium for isolation of other bacterial groups. Plates were aerobically incubated at 37°C for up to 48 h, with the exception of MRScys and TOS plates that were anaerobically incubated (85% nitrogen, 10% hydrogen, and 5% carbon dioxide) in an anaerobic workstation (Mini-MACS Don Whitley Scientific Limited, Shipley, UK) at 37°C for 48 h. Bacterial counts were recorded as the CFU/g of meconium or feces and transformed to $\log_{10}$ values before statistical analysis.

At least one representative of each different colony type obtained from each sample was isolated. Approximately 140 isolates were analyzed by optical microscopy and identified by MALDI-TOF mass spectrometry in a Vitek-MS instrument (BioMérieux, Marcy l'Etoile, France) in the facilities of Probisearch S. L. (Tres Cantos, Spain).

Pulsed-field gel electrophoresis (PFGE) genotyping of all the isolates identified as *L. salivarius* or *B. breve* was carried following a protocol previously described [37]. The profiles were compared to those of *L. salivarius* PS12934 and *B. breve* PS12929, respectively.

2.3. Immunological Analysis. The concentration of 18 cytokines, chemokines, and growth factors, including interleukin (IL) IL-1$_\beta$, IL-6, IL-12 (p70), interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), IL-2, IL-4, IL-10, IL-13, IL-17, IL-8, growth related oncogene-α (GRO-α), macrophage-monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1$_\beta$ (MIP-1$_\beta$), IL-5, IL-7, granulocyte colony stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF), was determined in 5 meconium, 14 feces, and 10 plasma samples by using a Bio-Plex 200 system instrument (Bio-Rad, Hercules, CA) and the Bio-Plex Pro Human Cytokine, Chemokine and Growth Factor Assays (Bio-Rad). Parallel, the concentration of immunoglobulin (Ig) IgG_1, IgG_2, IgG_3, IgG_4, IgM, and IgA was determined using the Bio-Plex Pro Human Isotyping Assay Kit (Bio-Rad).

Before analysis, 0.1 g of meconium and fecal samples was diluted in 0.9 mL of peptone water, homogenized, and centrifuged for 15 min at 14,000 ×g at 4°C; then, supernatants (≥200 μL) were collected. Plasma samples were defrosted and properly diluted immediately before the immunological assay. Analyses were carried out in duplicate following the

manufacturer's protocol and standard curves were performed for each analyte. Lower limit of quantification (LLOQ) was different for each one of the parameters, ranging from 0.02 to 11.74 ng/L for cytokines and from 0.01 to 2 ng/L for immunoglobulins.

Additionally, calprotectin levels (LLOQ: 8 ng/L) were determined in 5 meconium, 14 feces, and 8 plasma samples using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Calpro, Lysaker, Norway) according to the manufacturer's instructions. The standard curve of calprotectin was obtained from triplicates of each assayed concentration and fit to a 4-parameter curve model.

2.4. Statistical Analysis. The statistical analysis was performed using R 2.15.3 (R-project, http://www.r-project.org). When data were not normally distributed, medians and interquartile ranges (Q1 and Q3) were calculated for all sampling times, and means and 95% confidence interval (95% CI) were used for normally distributed data. The richness and diversity of meconium and fecal microbiota were determined by calculating the Shannon-Weaver diversity index, which takes into account the number and evenness of the bacterial species. The Kruskal-Wallis test for nonnormal data or one-way ANOVA test, when data were normally distributed, was used to evaluate the differences between sampling times, in all measured variables, in plasma samples and for the comparison of immunological variables between plasma and fecal samples. The nonparametric Friedman test or one-way ANOVA test, when data were normally distributed, was used in fecal samples to evaluate the differences between sampling times in all measured variables. In all cases, P values of <0.05 were considered to be significant. Redundancy analysis (RDA) was used for exploration of whole data sets and evaluation of the possible relationship between gut colonization and immunological parameters with the clinical status of the participants. Finally, heatmaps of plasma and fecal samples were plotted. To do this, calculation of Kendall's correlation coefficients was performed and Ward agglomeration methods were used to obtain the clustering of the variables and cases matrix.

3. Results

3.1. Demographic and Clinical Characteristics of the Participants. The clinical and demographic data of the mothers and infants who participate in this study are summarized in Table 1. Although five preterm infants were included in this study, there were 2 sets of twins (infants 1 and 2; infants 3 and 4) and, therefore, data were collected from three mothers (Table 1).

All the infants were female and were born by Cesarean section with a mean gestational age of 28 weeks and 2 days. The mean birth weight was 1,020.4 g and the mean height and head circumference were 34.5 cm and 25.0 cm, respectively. These parameters showed Z-scores < 0. Infants stayed in the NICU a mean time of 30.6 days with a mean age at discharge of 65.4 days, which represented a mean corrected gestational age of 37 weeks and 5 days (Table 1). Additional information of clinical features is provided as supplemental information (Supplemental Information 1; see Table S1 of the Supplementary Material available online at http://dx.doi.org/10.1155/2015/538171).

3.2. Microbiological Analysis. Bacterial growth was detected in one meconium sample and in all the fecal samples. Differences in the bacterial counts of fecal samples were evaluated by nonparametric Friedman test on days 7, 14, 21, and 28 (data not shown).

Globally, the phylum *Firmicutes* predominated in all the fecal samples except in those belonging to infant 5 where *Proteobacteria* was present in a similar proportion (Figure 1(a)). On the other hand, *Proteobacteria* dominated at the 14th day of intervention in fecal samples of the siblings 3 and 4. The phylum *Actinobacteria*, mainly represented by the genus *Bifidobacterium*, was isolated from day 7 although not in all the fecal samples (Figure 1(a)).

Among the *Firmicutes*, the genera *Enterococcus* and *Lactobacillus* were isolated from all the fecal samples except in that of infant 2 at day 21 where *Lactobacillus* could not be detected. The bacterial counts of *Enterococcus* decreased significantly from day 7 to day 21 of treatment ($P = 0.043$) from 10.00 to 8.30 log CFU/g. In contrast, *Lactobacillus* counts increased from 6.60 log CFU/g after 7 days of probiotic treatment to 8.32 log CFU/g at the end of the intervention; in this case, the differences were not statistically significant due to both the individual variability and the small cohort. The genus *Staphylococcus* was mainly isolated in the first weeks of the study from meconium and 7-day fecal samples (Figure 1(b)) with median counts of 4.30 and 9.44 log CFU/g, respectively.

In relation to *Proteobacteria*, the genus *Enterobacter* was isolated from all the fecal samples except from two from infant 2 (days 7 and 21) and from one of infant 3 at day 28 (Figure 1(b)). Similarly, the genus *Klebsiella* was isolated from all fecal samples except from two collected at day 7 (siblings 3 and 4) and one at day 21 (infant 2). Bacterial counts of these two genera were significantly different at every sampling day ($P = 0.007$ and 0.046 for *Enterobacter* and *Klebsiella*, resp.) and a decrease was observed in *Klebsiella* median counts (from 10.19 log CFU/g at day 7 to 8.48 log CFU/g at day 28).

Finally, the *Bifidobacterium* median counts oscillated between 7.98 and 9.98 log CFU/g in the 6 fecal samples where this genus was detected (Figure 1(b)).

The SDI of the fecal samples fluctuated during the study probably due to the different antibiotic treatments that the infants received (Figure 1(c)).

In order to detect the presence of *L. salivarius* PS12934 and *B. breve* PS12929 in fecal samples, all the fecal isolates belonging to such species were PFGE genotyped. This technique revealed that *L. salivarius* PS12934 was present in all the infants at numerous sampling points while *B. breve* PS12929 could be detected after day 14.

The heatmap obtained from the fecal samples at different sampling times of all the infants is shown in Figure S1. The dendrogram resulted after Kendall correlation coefficient calculation highlights the similar species profile of fecal

Table 1: Epidemiological and clinical relevant data from the mother-infant pairs of this study.

Mothers	1		2		3
Age (years)	30		18		28
Fever	No		Yes		No
Leukocytosis (>15,000 leukocytes/μL)	No		Yes		Yes
C-reactive protein (mg/L)	26		7.6		40
Antenatal antibiotics treatment	Yes		Yes		Yes
Antenatal corticosteroids treatment	Complete		Uncomplete		Complete
Chorioamnionitis	No		Yes		Yes
Type of delivery	C-section		C-section		C-section
Multiple delivery	Yes		Yes		No
Infants	1	2	3	4	5
Rupture of fetal membranes (h)	672	0	0	0	432
Twin position	1	2	2	1	1
Sex	F	F	F	F	F
Gestational age (wk)	28 + 5	28 + 5	28 + 6	28 + 6	27 + 2
Birth weight (g) (Z-score)	1070 (−0.71)	980 (1.01)	1082 (−0.66)	1200 (−0.26)	770 (−1.02)
Birth height (cm) (Z-score)	36 (−1.3)	36 (−1.3)	36 (−1.3)	36 (−1.3)	32 (−1.8)
Birth head circumference (cm) (Z-score)	26 (−0.8)	26 (−0.8)	25.5 (−1.1)	26 (−0.8)	24 (−0.8)
Apgar score at 1 min	8	9	8	5	7
Apgar score at 5 min	9	9	9	7	8
Revival	Ventilation	No	Ventilation	Ventilation	Ventilation
PDA	Yes	No	Yes	Yes	No
Meconium spontaneous expulsion	Yes	Yes	Yes	Yes	Yes
Meconium expulsion (h)	24	9	48	36	14
Probiotic starting age (d)	2	2	2	2	4
Probiotic treatment length (d)	18	18	31	19	25
NICU stay (d)	18	8	14	64	49
Age at discharge (d)	51	51	60	64	101
Corrected gestational age at discharge (wk)	36	36	37	38	42
Death	No	No	No	Yes	No

PDA: patent ductus arteriosus; NICU: neonatal intensive care unit.
Antenatal corticosteroid treatment was uncompleted or complete when mother received one or two doses of betamethasone, respectively, within one week and 24 h before delivery.
Apgar test ranged from 1 to 10: less than 5 means risk; up to 7 means normal.
Twin position means the position at birth, 1 being the infant who was nearest to the cervix.

samples of infant 2 at different sampling times and the almost identical species profile of fecal samples from days 7 and 14 of twins 3 and 4.

3.3. Immunological Analysis. A wide range of immune compounds were analyzed in plasma and fecal samples of the preterm infants throughout the study. An exploratory screening, using a principal component analysis (PCA) to detect outliers, revealed that the 7th day fecal sample from infant 4 was very different from the rest of the sample sets (data not shown). This infant was suffering a gastric bleeding at this sampling time and, therefore, this sample was excluded from the results of data sets.

Median values of the immune compounds concentrations in meconium and, also, in fecal samples at 7th and 14th days of probiotic supplementation are shown in Table 2. In general, the values obtained for all the immune factors showed a high interindividual variability in both detection frequencies and amounts. The levels of some immune compounds changed throughout the study; those of IgG_2 and MCP-1 decreased progressively ($P = 0.074$ and $P = 0.076$, resp.) while that of IgA increased (>50 times) from meconium to fecal samples obtained at day 7 after birth ($P = 0.074$) (Table 2). However, only the inflammatory biomarker calprotectin decreased significantly along sampling time ($P = 0.041$).

Plasma concentrations of the immune compounds are shown in Table 3 and, as it can be observed, no significant changes were found. Globally, chemokines and proinflammatory compounds tended to decrease, with the exception of IL-12 and TNF-α. The levels of the latter and those of the anti-inflammatory compounds remained very constant along time. Plasma immunoglobulins also showed a high individual variability although all decreased, with the exception of IgG4 and IgM (Table 3).

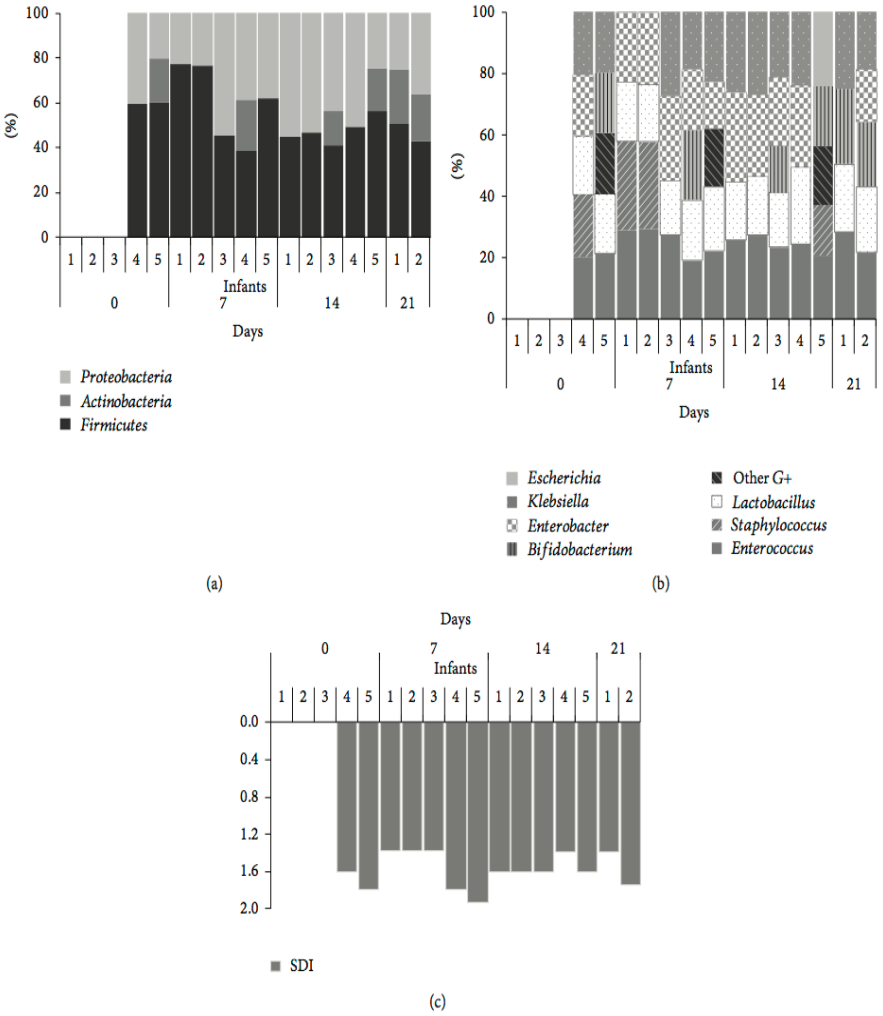

FIGURE 1: Phyla (a), genera (b), and bacterial diversity assessment by the SDI (c) of the microbiota of the meconium and fecal samples analyzed in this study. The relative contributions of the phyla and genera to the microbiota of the infant's gut and the SDI values were labeled per case and sampling time.

The plasma concentrations of the different immune compounds were compared with their respective fecal values. All the immunoglobulins, with the exception of IgA, were significantly different in both types of samples. Among the remaining immune parameters, calprotectin, IL-10, GRO-α, and GM-CSF were significantly higher in feces ($P = 0.000$, $P = 0.045$, $P = 0.048$, and $P = 0.000$, resp.) while IL-8, MCP-1, and MIP-1_β were more abundant in plasma ($P = 0.012$, $P = 0.000$, and $P = 0.001$, resp.) (Table S2).

3.4. Multivariate Analysis of the Studied Population. A multivariate analysis was performed for investigating the possible relationship between clinical features and the immunological and microbiological profiles of fecal and plasma samples. The clinical variables considered were the following: antibiotherapy (Antibiotics); air way resume (AWResume) including ventilation, caffeine, and surfactant treatment; C-RP; hemoglobin amounts (Hb); hematocrit percentage (Hcte); ibuprofen treatment (Ibu.T); ibuprofen doses (Ibu.doses); number of stools per day (N°.stools); nutrition resuming the median feeding type (Nutrition); patent ductus arteriosus (PDA); Sepsis; spontaneous stools (Spont.stools); Transfusion; and Weight.

The redundancy analysis (RDA) of the above-mentioned variables for fecal samples is shown in Figure 2. The obtained model explains the 33% of the variability and the ANOVA test of the model was statistically significant ($P = 0.020$). The meconium samples were located opposite to microbial growth and in coincidence with the constrained antibiotic

Table 2: Frequency and concentration of immune compounds in fecal samples ($N = 14$) along time.

	Day 0 ($N = 5$)		Day 7 ($N = 4$)		Day 14 ($N = 5$)		P value*
	n (%)	Median (IQR) (mg/kg)	n (%)	Median (IQR) (mg/kg)	n (%)	Median (IQR) (mg/kg)	
Immunoglobulins							
IgG_1	5 (100)	3.95 (1.23–6.36)	4 (100)	0.45 (0.23–0.80)	5 (100)	1.26 (0.47–2.43)	0.819
IgG_2	5 (100)	23.82 (23.19–24.17)	4 (100)	2.98 (2.46–3.97)	5 (100)	2.66 (2.60–3.62)	0.074
IgG_3	4 (80)	0.02 (0.01–0.02)	1 (25)	0.01 (0.01–0.01)	2 (40)	0.22 (0.11–0.32)	0.424
IgG_4	5 (100)	0.03 (0.02–0.14)	4 (100)	0.02 (0.01–0.03)	5 (100)	0.03 (0.00–0.06)	0.449
IgM	4 (80)	2.72 (0.19–8.73)	3 (75)	1.10 (0.87–6.00)	5 (100)	2.79 (0.44–10.02)	0.819
IgA	5 (100)	3.57 (0.88–21.73)	4 (100)	201.23 (35.09–356.74)	5 (100)	7.49 (2.96–7.78)	0.074
		(ng/kg)		(ng/kg)		(ng/kg)	
Proinflammatory							
Calprotectin†	5 (100)	309.50 (282.00–343.90)	4 (100)	144.80 (132.30–180.40)	5 (100)	38.42 (34.16–63.96)	0.041
IL-1_β‡	1 (20)	31.47	3 (75)	41.34 (8.00–74.68)	3 (60)	39.20 (−36.24–114.64)	0.937
IL-2	1 (20)	8.47	1 (25)	8.18	0 (0)	—	0.368
IL-6	0 (0)	—	0 (0)	—	1 (20)	27.44	0.368
IL-12 (p70)	2 (40)	29.07 (28.82–29.32)	2 (50)	37.13 (36.38–37.89)	1 (20)	82.98	0.926
IL-17	2 (40)	72.94 (62.76–83.11)	2 (50)	66.08 (64.46–67.71)	2 (40)	69.31 (65.15–73.48)	1.000
IFN-γ	4 (80)	214.90 (190.40–238.30)	4 (100)	299.80 (255.40–320.80)	4 (80)	248.10 (215.80–265.50)	0.449
TNF-α	1 (20)	20.87	0 (0)	—	0 (0)	—	0.368
		(ng/kg)		(ng/kg)		(ng/kg)	
Anti-inflammatory							
IL-4	3 (60)	2.74 (2.43–3.48)	4 (100)	2.63 (2.49–2.85)	3 (60)	2.12 (2.06–2.26)	0.268
IL-5	0 (0)	—	0 (0)	—	0 (0)	—	—
IL-10	1 (20)	25.62	2 (50)	37.21 (35.85–38.57)	3 (60)	39.20 (38.66–53.87)	0.319
IL-13	0 (0)	—	0 (0)	—	0 (0)	—	—
		(ng/kg)		(ng/kg)		(ng/kg)	
Chemokines							
IL-8	4 (80)	20.94 (19.00–23.82)	3 (75)	16.16 (15.56–17.20)	2 (40)	17.05 (16.45–17.64)	0.128
GRO-α‡	5 (100)	206.30 (117.04–295.57)	3 (75)	222.10 (−77.61–521.80)	4 (80)	263.50 (261.05–265.88)	0.763
MCP-1	5 (100)	20.08 (15.02–28.89)	2 (50)	18.37 (16.82–19.93)	3 (60)	16.98 (14.21–17.34)	0.076
MIP-1_β	5 (100)	53.79 (52.03–68.66)	4 (100)	58.16 (46.46–66.42)	4 (80)	49.60 (35.16–69.89)	0.449
		(ng/kg)		(ng/kg)		(ng/kg)	
Haematopoietic stimuli							
IL-7	0 (0)	—	0 (0)	—	0 (0)	—	—
G-CSF	1 (20)	28.99	0 (0)	—	0 (0)	—	0.368
GM-CSF	5 (100)	1729.00 (1086.00–2312.00)	4 (100)	1830.00 (1648.00–2010.00)	4 (80)	1879.00 (1783.00–1920.00)	0.819

Levels of immune compounds were expressed as median and interquartile range (IQR) when data were not normally distributed and as mean and 95% confidence interval (95% CI) when they were. *Friedman test was used to determine the differences between fecal samples along time when data were not normally distributed and one-way ANOVA when they were. †Concentration was expressed as ng/Kg of feces for all the proinflammatory parameters with the exception of calprotectin whose units were mg/Kg. ‡Normally distributed.

vector. Although the rest of fecal samples showed a less clear separation, the evolution of microbial colonization can be observed along the RDA1 axis in coincidence with the constrained vectors for AWResume, Nutrition, Spont.stools, PDA, and Transfusion and in opposite not only with the antibiotics and C-RP vectors, but also with the coordinates of proinflammatory compounds, such as calprotectin, MCP-1, MIP-1_β, TNF-α, and IL-8 (Figure 2).

The RDA of plasma samples (Figure 3) explains the 70% of the variability and the ANOVA test of the model was statistically significant ($P = 0.010$). The bidimensional plot shows two points clearly separated from the others: infant 4 at day 19 and infant 5 at day 7. Three different situations were observed; on the one hand coordinates from infants 1, 2, and 3 did not change among sampling times, while on the other infant 5 showed a normalization far away of proinflammatory variables and hematological parameters coordinates; and finally infant 4 that initially was close to her corresponding twin and the rest of participants appeared at day 19, in the positive RDA1 and RDA2 coordinates, related to

Table 3: Frequency and concentration of immune compounds in plasma samples ($N = 8$) along time.

	Day 7 ($N = 3$)		Day 14 ($N = 5$)		P value*
	n (%)	Median (IQR) (mg/L)	n (%)	Median (IQR) (mg/L)	
Immunoglobulins					
IgG_1	3 (100)	2159.80 (2075.95–2174.30)	5 (100)	1727.30 (1205.50–2029.60)	0.297
IgG_2	3 (100)	1135.20 (796.03–1147.50)	5 (100)	741.24 (683.84–930.95)	0.456
IgG_3	3 (100)	52.54 (46.75–64.53)	5 (100)	43.91 (41.35–48.14)	0.297
IgG_4	3 (100)	23.25 (22.02–67.30)	5 (100)	44.66 (10.59–49.08)	0.655
IgM	3 (100)	263.75 (176.71–934.18)	5 (100)	335.18 (261.41–366.78)	0.882
IgA	3 (100)	27.03 (18.20–40.41)	5 (100)	4.44 (4.00–14.31)	0.101
		(ng/L)		(ng/L)	
Proinflammatory					
Calprotectin†	3 (100)	0.86 (0.47–1.11)	5 (100)	0.37 (0.37–0.63)	0.456
IL-1_β‡	1 (33)	15.81	0 (0)	—	—
IL-2	2 (67)	35.41 (19.34–51.48)	3 (60)	9.70 (6.54–11.23)	1.000
IL-6	3 (100)	24.14 (15.99–65.65)	5 (100)	17.06 (10.10–19.24)	0.297
IL-12 (p70)	3 (100)	27.55 (19.89–91.22)	5 (100)	28.35 (22.71–29.16)	0.882
IL-17	1 (33)	167.20	2 (40)	35.66 (34.65–36.67)	0.221
IFN-γ	2 (67)	670.07 (371.30–968.83)	4 (80)	150.06 (67.73–225.91)	0.643
TNF-α	3 (100)	15.06 (11.35–66.01)	5 (100)	13.14 (11.83–20.40)	0.764
		(ng/L)		(ng/L)	
Anti-inflammatory					
IL-4	3 (100)	1.95 (1.57–7.96)	5 (100)	1.99 (1.69–2.90)	0.882
IL-5	1 (33)	39.43	1 (20)	9.65	0.317
IL-10	3 (100)	11.80 (11.10–69.56)	3 (60)	20.11 (16.02–22.68)	0.513
IL-13	1 (33)	11.27	1 (20)	5.06	0.317
		(ng/L)		(ng/L)	
Chemokines					
IL-8	3 (100)	31.69 (24.45–85.28)	5 (100)	29.76 (22.37–30.79)	0.655
GRO-α‡	2 (67)	204.44 (−1859.94–2268.82)	3 (60)	55.42 (45.11–65.73)	0.306
MCP-1	3 (100)	193.91 (123.14–204.59)	5 (100)	88.62 (60.77–192.54)	0.456
MIP-1_β	3 (100)	234.90 (210.30–292.20)	5 (100)	174.60 (150.00–250.80)	0.297
		(ng/L)		(ng/L)	
Haematopoietic stimuli					
IL-7	2 (67)	28.14 (17.72–38.57)	3 (60)	10.48 (8.84–12.50)	0.564
G-CSF	3 (100)	30.89 (23.21–96.81)	5 (100)	47.27 (41.84–51.46)	0.655
GM-CSF	3 (100)	248.80 (191.00–299.30)	4 (80)	132.35 (114.97–151.83)	0.157

Levels of immune compounds were expressed as median and interquartile range (IQR) when data were not normally distributed and as mean and 95% confidence interval (95% CI) when they were. *Kruskal-Wallis test was used to determine the differences between blood samples along time when data were not normally distributed and one-way ANOVA test when they were. †Concentration was expressed as ng/L of plasma for all the proinflammatory parameters with the exception of calprotectin whose units were mg/L. ‡Normally distributed.

constrained variables vectors corresponding to C-RP, Sepsis, and PDA reflecting the clinical worsening of this infant at this moment.

Those clinical categorical variables explained by the fecal and plasma RDAs were used, together with the microbiological, immunological, and clinical parameters, to create two heatmaps, one for each type of samples (Figure 4). The results from all the available fecal samples of the 5 infants were used to perform the heatmap showed in Figure 4(a). The samples' dendrogram shows two arms which clearly separate meconium and feces. The variables' dendrogram, obtained after samples clustering, shows two principal arms. The lower one is divided into two: the first of them that included clinical variables, some bacterial genera such as *Escherichia*, *Staphylococcus*, *Bifidobacterium*, and *Paenibacillus*, immunoglobulins IgG_3 and IgG_4, and cytokines IL-4, IL-13, and IL-2 and the second one that included antibiotherapy, IgG_1, IL-5, IL-6, and IL-7. The upper arm is also divided and included the rest of the bacterial genera and immunological parameters together with the weight of the infants. The results obtained for all the

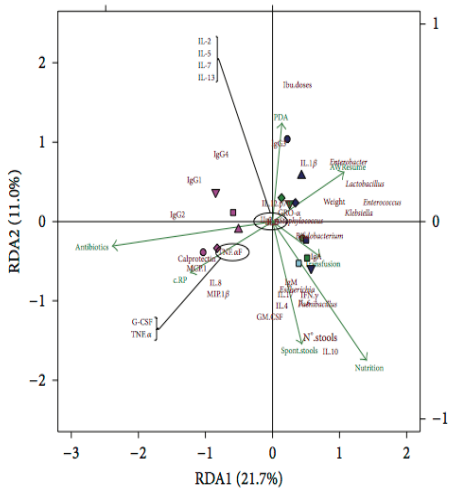

FIGURE 2: Redundancy analysis of the fecal samples obtained at different sampling times from the preterm infants. Cases were represented with points and then labeled per infant (1: circle, 2: square, 3: diamond, 4: triangle, and 5: inverted triangle) and sampling time (0: medium violet red, 7: green, 14: midnight blue, and 21: sky blue) Quantitative variables matrix, including the hematological and immunological parameters, ibuprofen doses (Ibu.doses), number of stools per day (N°.stools), and weight, was represented with each variable name or abbreviator in dark red color; clinical categorized observations vectors matrixes were used as constrained variables (airway resume (AWResume), antibiotherapy (Antibiotics), C-RP, ibuprofen treatment (Ibu treatment), nutrition type (Nutrition), patent ductus arteriosus (PDA), Sepsis, spontaneous stools (Spont.stools), and Transfusion) and represented as vectors in green color. The bidimensional RDA plot explains the 33% of the variability and showed a P value of 0.020 after 299 permutations when ANOVA test of the model was performed.

available plasma samples from the 5 participants were used to perform the heatmap showed in Figure 4(b). The plasma samples' dendrogram shows two groups, in one of them 2 samples of the infant 2 cluster together with her twin at day 14 and samples of infant 5 clusters together with sample of infant 1 at day 7. In the second arm, siblings 3 and 4 at day 14 of probiotic supplementation initiate the clustering, which ends with sample of day 7 of infant 5 and sample of day 19 of infant 4 as previously observed in Figure 3. The dendrogram related to variables, obtained after infants clustering, showed two principal arms: one of them included clinical variables, hematological parameters, calprotectin, IL-1_β, IL-4, IL-13, immunoglobulins IgA and IgG_3, ibuprofen doses, and Hb and the second principal arm also divided including most of the cytokines, chemokines, and growth factors, the rest of the immunoglobulins, the birth weight, and the Hcte.

4. Discussion

In this pilot study, the bacterial composition of fecal samples obtained from five preterm infants supplemented with a probiotic mixture of two strains isolated from human milk during their earlier days of life at the NICU was assessed. In addition, a wide range of cytokines, chemokines, growth factors, and immunoglobulins were determined in all plasma, meconium, and fecal samples in order to describe their immunological profiles, their changes over time, and their potential relationship with bacterial colonization and clinical features.

The results obtained in this study suggest that the administration of *B. breve* PS12929 and *L. salivarius* PS12934 to preterm infants may increase the levels of *Lactobacillus* and *Bifidobacterium* in their feces. In fact, *L. salivarius* PS12934 could be isolated from the fecal samples of the preterm infants from day 7 of intervention and its presence remained constant throughout the study. *B. breve* PS12929 was also isolated from fecal samples after day 14 of intervention and, since then, it had increasing presence in the fecal samples. The higher frequency and concentration of *Lactobacillus* and *Bifidobacterium* in the feces analyzed should be considered a positive outcome of this study because the pattern of gut colonization in this specific infant population is usually characterized by a dominance of opportunistic pathogens and a reduced (or even absent) population of lactobacilli and bifidobacteria [7, 15, 38]. In fact, the SDI values of the fecal samples were higher than those previously described in a similar cohort that did not receive probiotics [7]. The intensive use of antibiotics at the NICU has been related to a dramatic reduction in microbial diversity and to increased presence of *Enterobacter* [39]; however, the administration of the probiotic strains in this study seemed to, somehow, compensate the antibiotic side effects.

Up to the present, there has been a complete lack of studies focused on fecal immunological parameters among preterm infants. As a consequence, there are no reference values for this population and, therefore, this study may constitute a starting point for future investigations. Although scarce, there are some studies dealing with blood immune

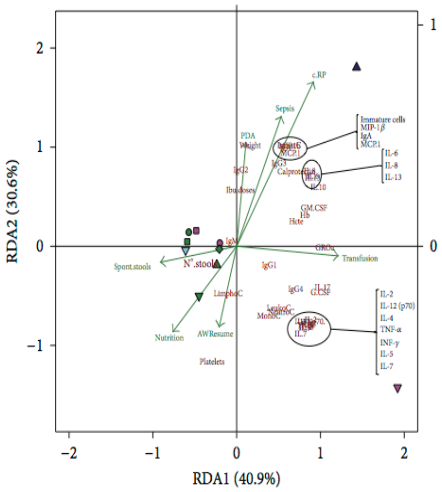

FIGURE 3: Redundancy analysis of the blood samples obtained at different sampling times from the preterm infants. Cases were represented with points and then labeled per infant (1: circle, 2: square, 3: diamond, 4: triangle, and 5: inverted triangle) and sampling time (0: medium violet red, 7: green, 14: midnight blue, and 21: sky blue) Quantitative variables matrix, including the hematological and immunological parameters, ibuprofen doses (Ibu.doses), number of stools per day (N°.stools), and weight, was represented with each variable name or abbreviator in dark red color; clinical categorized observations vectors matrixes were used as constrained variables (airway resume (AWResume), antibiotherapy (Antibiotics), C-RP, ibuprofen treatment (Ibu treatment), nutrition type (Nutrition), patent ductus arteriosus (PDA), Sepsis, spontaneous stools (Spont.stools), and Transfusion) and represented as vectors in green color. The bidimensional RDA plot explains the 71% of the variability and showed a P value of 0.010 after 199 permutations when ANOVA test of the model was performed.

compounds in preterm babies. Globally, they show that there are differences in the blood immune profiles depending on the infant gestational age [40–42]. It is important to note that the volume of the blood samples that are usually extracted from preterm neonates for clinical purposes is usually very low. Therefore, multiplex technologies, as the one used in this study, are required in order to be able to simultaneously analyze a high number of immune compounds [42, 43].

The results obtained in this study must be interpreted with caution due to three relevant limitations: the absence of a control group, a very small population size, and the scarcity of previous studies dealing with the immunological features of very low or extremely low weight birth infants and how they may be affected after a probiotic treatment. In this context, the levels of IL-8 found in a previous work focused on term neonates [44] were lower than those obtained in this study while those of IL-4 and IL-6 were similar; in contrast, the values of the remaining immunological parameters were higher in all the sampling times. This may illustrate the immune immaturity of these preterm infants. Similarly, levels of IL-2, IL-6, IL-8, IL-10, IL-13, IL-17, TNF-α, IFN-γ, and MCP-1 were lower in preterm infants born at 30–32 weeks than in those born after 36 weeks, indicating a lower stimulation or activation of Th1 cells and antigen-presenting cells in preterm babies as the gestational age decreases [42]. In the present work, the concentrations of the chemokines IL-8 and MCP-1 and those of the cytokines IL-4, IL-10, and IL-13, which are related to anti-inflammatory processes, were higher than those reported for preterm neonates born at 30–32 weeks and similar to those found in older infants (>36 weeks) [42]. This suggests that the administration of the probiotic strains may exert a modulatory effect on the immune system of these infants.

In addition, very low or extremely low weight birth infants usually require a strong and highly individualized medical intervention (antibiotics, oxygen, corticoids, ibuprofen, transfusions, etc.) for, at least, the first days of life due to a wide variety of life-threatening conditions. Such conditions, together with their corresponding treatments, may alter the microbial gut colonization process and, also, the infants' immune responses. Therefore, it is very difficult to obtain a homogeneous VLBW or ELBW infant population even in cohorts with a high number of infants. This is another important limitation that interventional studies, such as probiotic administration, must face when dealing with such infant subpopulations.

Despite all the limitations cited above, it is also true that a significant reduction of the inflammatory marker calprotectin in feces was observed throughout the probiotic treatment, which is in agreement with previous studies [3, 45, 46]. This is a promising outcome that must be reassessed in the future in a placebo-controlled intervention involving a large cohort.

The increase in IgA observed at day 7 may be due to the microorganisms colonizing the preterm gut, which triggers the production of this Ig by the gut-associated lymphoid tissue (GALT) [47]. IgA has the ability to penetrate the gut mucosal surface in conjunction with antigens and, as a

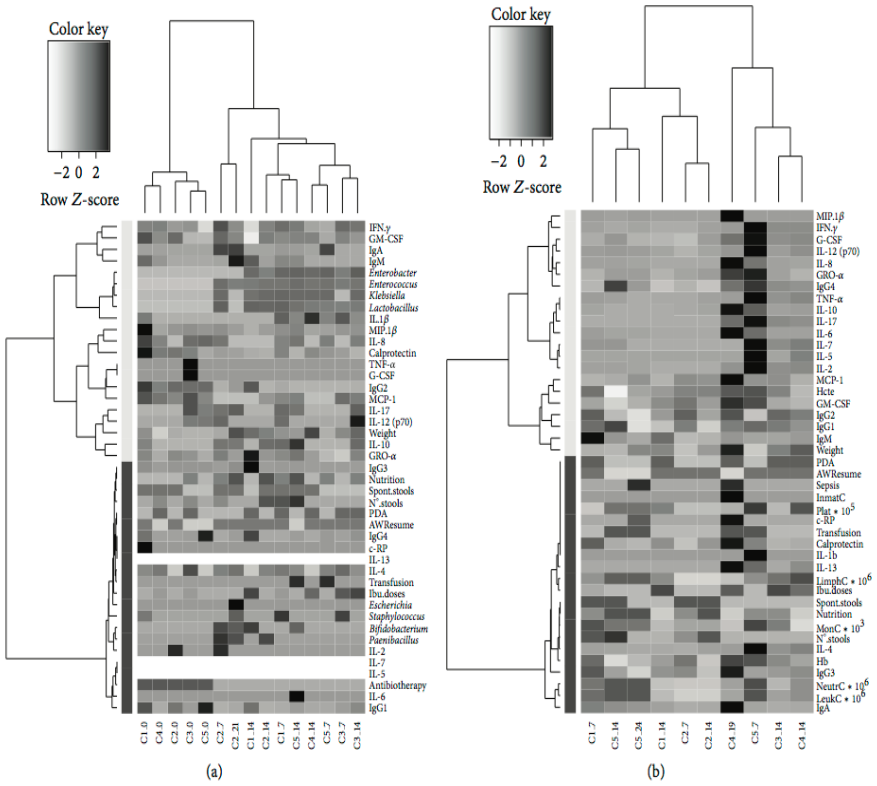

FIGURE 4: Heatmaps of fecal (a) and plasma (b) samples matrixes, considering all the quantitative variables measured and the categorized variables that were explained in the correspondent RDA, were performed. Clustering functions were applied to samples and variables after scaling the whole data set. In order to represent as much information as possible in the plot, the heatmaps were plotted using the measured data matrix scaled per variable and columns were labeled per infant and sampling time.

consequence, to induce effector immune responses, playing a key role in the maintenance of intestinal microbiota and immune homeostasis [48].

The multivariate analysis applied to all the available plasma and fecal samples from the five preterm infants revealed a clear relation between the parameters assessed in this work and the clinical evolution of the infants. In the fecal-related RDA, microbial colonization acted as the principal agent opposed to the levels of certain proinflammatory immunocompounds and in agreement with the clinical variables associated with an improvement of the infants' health. Since bacterial species coordinate coefficients had positive values in the RDA1 axis, calprotectin and other proinflammatory parameters, such as IL-8, MIP-1_β, MCP-1, G-CSF, or TNF-α, showed negative values. RDA1 axis coordinate coefficients for IgG_1, IgG_2, and IgG_4 were negative while those for the secretory IgA and IgM immunoglobulins were positive. Although these findings must be taken with caution due to the inherent limitations of this work and to the high number of potential interactions and confusing factors, it should be noted that an abnormal gut microbial colonization predisposes the neonatal intestine to inflammation and to a cascade of proinflammatory and anti-inflammatory cytokines responses [49]. On the other hand, the evolution of the infants' microbiota was different than that observed in other preterm infants devoid of probiotic treatment [7] but similar to that of preterm neonates that received probiotics [23].

Finally, the dendrograms obtained for samples and variables represented in the heatmaps (Figure 4) seem to reinforce the hypothesis that probiotic strains may contribute to the development of a normal gut bacterial colonization and that this process is essential to reduce the health burden associated with prematurity [50, 51]. Although the present cohort was very small, a promising influence of the probiotic supplementation on gut colonization was observed, including an increase in bacterial diversity and in the presence of lactobacilli and bifidobacteria at relatively high levels.

Although multicenter, randomized clinical trials involving bigger cohorts and longer intervention times with

these strains will be required to determine their efficacy in the prevention of sepsis or NEC, the results of this work may provide useful information for future studies dealing with probiotic gut colonization and, particularly, with the detection and quantification of fecal and blood immunocompounds in preterm infants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Laura Moles, Esperanza Escribano, and Javier de Andrés contributed equally to this work.

Acknowledgments

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CONCLUSIONES

CONCLUSIONES

1. El conocimiento del ambiente epidemiológico en las unidades de Neonatología influye sobre la colonización temprana del recién nacido pretérmino. Así, los recién nacidos prematuros ingresados en la misma UCIN son colonizados inicialmente de forma casi homogénea por comunidades microbianas que predominan en la unidad y más adelante, según las condiciones individuales, esta colonización inicial puede modificarse.
2. La influencia de un brote de *Serratia* spp. en la adquisición de la microbiota intestinal afecta al global de los ingresados desde los primeros días del ingreso y estos hallazgos podrían aplicarse a brotes causados por otros microorganismos.
3. Es importante tener en cuenta el ambiente epidemiológico en relación al patrón de colonización intestinal de los recién nacidos prematuros hospitalizados.
4. La administración oral profiláctica de una mezcla comercial de probióticos, *Lactobacillus acidophilus* y *Lactobacillus bifidum* (Infloran®), a prematuros nacidos a una edad gestacional inferior a 28 semanas no se asocia con crecimiento dell probiótico en cultivos rutinarios y no se han comunicado episodios de sepsis atribuibles a los probióticos.
5. El uso de la mezcla presente en Infloran® aumenta el número prematuros extremos (menores de 28 semanas de edad gestacional) diagnosticados de enterocolitis necrotizante.
6. El uso de Infloran® en esta población se asocia con disminución de la sepsis por *S. Epidermidis* y junto con el protocolo de manejo de vías se asocia con disminución de sepsis nosocomial por cualquier microorganismo.
7. En cuanto a la administración de dos cepas probióticas extraídas de leche materna, *Bifidobacterium breve* PS12929 y *Lactobacillus salivarius* PS12934, se puede evidenciar su crecimiento, mediante técnicas dependientes de cultivo en todas las muestras fecales de los recién nacidos muy prematuros que las recibieron.

8. La administración de estas cepas probióticas se asocia a una reducción del filo *Proteobacteria* y la presencia de DNA de *Lactobacillus* una semana después de su administración mediante técnicas moleculares.
9. Respecto a la respuesta inmunitaria local y sistémica
    a. Los prematuros que recibireron estas cepas presentaron una disminución a lo largo del tiempo en la concentración de calprotectina fecal.
    b. Se objetivó una asociación entre el hallazgo de las cepas probióticas en heces y los mediadores inmunomoduladores y anti-inflamatorios locales y sistemicos.
    c. Respecto a la evolución clínica, se encontró asociación entre la microbiota y la respuesta inmune del huésped con la evolución clínica.

APLICABILIDAD CLÍNICA

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La afectación de la microbiota por el ambiente epidemiológico tiene gran interés en salud pública, prevención y epidemiologia. En las unidades de cuidados intensivos neonatales se han descrito epidemias de infección por *Serratia spp*. Este estudio destaca los cambios precoces que ocurren en el microbioma de los recién nacidos en los episodios epidémicos de infección por *Serratia* que pueden ser de gran valor para minimizar esta enfermedad. Los individuos no son islas aisladas. Los resultados sugieren que las epidemias se acompañan de cambios importantes en la microbiota. Estos datos pueden ser útiles para diseñar un sistema de alerta epidemiológico y probioticos más eficientes que disminuyan el impacto de la epidemia.

La colonización del recién nacido extremadamente prematuro se ve influenciada por distintos factores. La composición de la microbiota intestinal influye sobre el sistema inmune como hemos mostrado en este trabajo. Este subgrupo de población es extremadamente vulnerable. Hemos caracterizado en este trabajo los cambios que ocurren en esta población precozmente. La incidencia de sepsis y enterocolitis en esta población es la mayor de todos los recién nacidos. La evaluación de la eficacia de los probióticos en la población extremadamente prematura es prácticamente inexistente, pudiendo la respuesta a su administración ser diferente a lo que ocurre en los niños mayores de 1000g. Nuestro estudio muestra un riesgo mayor de enterocolitis necrotizante con el uso de una fórmula comercial de probióticos, la más utilizada en España. Nuestro trabajo, necesario, puede ser crucial para la prevención de una enfermedad devastadora como la enterocolitis necrotizante.

La práctica actual en la nutrición del recién nacido prematuro es la administración de leche humana. Los microorganismos presentes en la leche materna son los primeros que colonizan el intestino del recién nacido. Entre los lactobacilos y bifidobacterias aisladas de leche materna se encuentran *Bifidobacterium breve* y *Lactobacillus salivarius*, que son potenciales agentes probióticos (según la EFSA y la FDA). Áreas claves de investigación son la evaluación de mezclas de especies probioticas óptimas y su interacción con la respuesta inmune local y sistémica en el huésped como hemos mostrado en esta tesis. Hemos mostrado la importancia de la administración de *Bifidobacterium breve* y *Lactobacillus salivarius,* la capacidad de colonizar precozmente el intestino, su interacción con el sistema inmune y la correlación con buenos resultados clínicos preliminares. Todo ello pone de manifiesto el efecto de la microbiota intestinal sobre el estado de salud y enfermedad.

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