Maternal immunoglobulins differentially bind a diverse bacterial community in human colostrum and the stool of breastfed neonates.

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Abstract

If the main source of the gut microbiota bacteria in newborns is controversial; the successional establishment of the primordial strains is another interesting topic of research where the mother's immunoglobulins IgA1, IgA2, IgM and IgG appear to be critical. In this work, the functional role of the maternal immunoglobulins which differentially bind a diverse bacterial community in human colostrum and the intestine of breastfed neonates was explored. We observed that in our human colostrum samples, the bacteria are bound by maternal immunoglobulins, where IgA2 and IgM bind alfa and beta Proteobacteria which could stimulate the neonatal immune system developing in the gut. Other immunoglobulins like IgG predominantly bind facultative anaerobes belonging to the Firmicutes phylum, reported as part of human milk microbiota and pioneer colonizers of the neonatal gut. It was also observed that the maternal immunoglobulins also bind a wide diversity of bacteria in the neonatal

stool. For instance, IgA2 and IgM bound more members of the phylum Bacteroidetes in comparison to IgG, these Bacteroidetes and some firmicutes have been reported as late colonizers of the neonatal gut, and their presence is important due to their ability to produce important short chain fatty acids like propionate and butyrate. Our results support the current view that microbial and immunoglobulin transference is crucial for developing the neonate's immune system and individual gut microbiota.

Keywords: Human colostrum; gut microbiota; maternal immunoglobulins; neonates; FACS; NGS.

1 Introduction

Human colostrum contains a complex composition with multiple biologically active molecules necessary for newborn development. Through colostrum, the neonate receives maternal cells, immunomodulatory molecules such as immunoglobulins, and microorganisms, which cooperate to maintain healthy host-microbe interactions in the early days [1]. The bacterial community found in human milk also known as microbiota, is highly diverse and complex, yet the concentration of bacteria is low. In Colostrum, several dozens of genera and more than 200 species have been identified [2]. Human milk is a source of commensal bacteria that may colonize the infant, linking maternal and infant microbiomes [3]. The microbiota has a major role in the development of the infant's immune system, influencing the health of the individual over the long term, including the risk of allergies, diabetes, and infections [4]. An aberrant gut colonization or a disruption in the relationship between the gut microbes and the newborn may influence the health later in life, especially regarding metabolic and immune-mediated disease [5].

The immune system interacts with the gut microbiota through different mechanisms. In the early days, maternal immunoglobulins are essential for sustaining a balanced gut environment by influencing the interaction between the host and the microbiome. In newborns, these immunoglobulins are mainly supplied by colostrum, a rich source of immunoglobulins (Igs), such as secretory IgA (SIgA) and in smaller proportions secretory IgM (SIgM), and IgG [6]; since Infants cannot synthesise Igs during the first four weeks of life [7]. Human milk antibodies are involved with multiple aspects of a newborn's mucosal immunity, such as providing protection from infection, selecting beneficial bacteria for seeding the gut, and helping the neonate to build up a tolerance to mucosal antigens during infancy [8]. Secretory IgA (SIgA) in HM comes in two different forms: IgA1 and IgA2, the latter being the more abundant in colostrum [9]. IgA subtypes bind diverse bacteria in the colostrum [10], probably helping the microbiota to establish itself in the gut by allowing microbial adherence to the epithelial cells and biofilm formation [1]. Similarly, the abundance of IgA2 is higher in the large intestine and more resistant to bacterial proteases than IgA1 [11].

Although IgA is the most common type of antibody found in colostrum and gut, IgM and IgG are also present in smaller quantities [6]. Generally, in the gut IgA and IgM are produced in response to microbial epitopes, recognizing similar patterns, whereas IgG is produced in response to antigens that must pass through the epithelial barrier [12]. So far, most research done on how the immune system interacts with the microbiota in early life has been done using animals, data from human studies remains scarce, and little information is available on the bacteria covered by immunoglobulins in human milk due to the lack of research in this area. To gain a better understanding of bacterial immune recognition by the maternal immunoglobulins IgA, IgG, and IgM in colostrum and the effect of the coating of gut microbiota during microbiome development, we characterized the bacteria in colostrum and the stool of neonates fed with the colostrum that was coated with maternal immunoglobulins within the first 48 hours postpartum.

2 Materials and Methods

2.1 Study Design and Participants

In this work, an analytical, observational, and cross-sectional study was performed. Donor recruitment was performed at the Hospital Regional de Alta Especialidad de Ixtapaluca (HRAEI) (19°19'07" N 98°52'56" W), and the Hospital General José María Rodríguez (HGJMR) (19°36′35″ N, 99°03′36″ W), both hospitals located in the State of Mexico. All participants signed a written consent for the use of biological samples and acquiring data in according with the Declaration of Helsinki, and the research protocol was approved by the ethics and research committee of the HRAEI Hospital (NR-CEI-HRAEI-06-2021) and HGJMR Hospital (217B560002018006). Samples were collected from 10 mothers-neonate pairs. Clinical and Sociodemographic data of the participants are detailed in (Table 1). For the study, only lactating healthy participants were included with spontaneous vaginal delivery or non-elective C-section, and full-term neonates (37-41 post-natal weeks) exclusively breastfed or mostly breastfed. As exclusion criteria women who declared metabolic diseases such as diabetes, hypertension, metabolic syndrome, and obesity. Women under hormonal treatment or the use of alcohol, cigarettes, and drugs during pregnancy were excluded. The sample size was determined by a non-probabilistic convenience sampling, considering new delivery mothers who met with inclusion criteria between July 2021 and December 2021.

2.2 Sample Collection

500-3000 µL of human colostrum and 0.2-0.5 mg of neonate stool were collected from each mother-neonate dyad after 22-40 hours post-partum and immediately processed after sample collection. Colostrum was obtained by manual expression within the first 48 hours post-partum using sterile gloves, and a protease inhibitor was added. Fecal samples were collected as previously reported by Sanchez-Salguero [10].

2.3 Sample pretreatment

300 mg of fecal samples were suspended in 3 ml of sterile PBS phosphate buffer saline (PBS) (Cat# P4417-100TAB, Sigma-Aldrich®, St Louis, Missouri, USA) and filtered through a 40-mm sterile nylon cell strainer (falcon, 352340) filter into a new sterile tube. The buffer was sterilized and passed through sterile 0.22-µm filters before use. The fecal suspension was centrifuged (400 x g, 4° C for 10 minutes) to pellet large debris, and the supernatant was recovered in a new tube. Bacteria were pelleted by centrifugation (8000 x g, 4° C for 5 minutes). For the colostrum samples, the methodology applied in this work was previously reported by Sanchez-Salguero [10], which consists of centrifugation and fat removal steps. Fecal and colostrum bacteria were washed three times in 1X-PBS.

2.4 Separation of bacteria into Immunoglobulin-bound fractions

For each sample (colostrum or feces) bacteria were separated based on their association with maternal immunoglobulins, such as IgA subclasses (IgA1, IgA2), IgM, and IgG by Fluorescence Associated Cell Sorting (FACS), we also studied the bacterial content of the complete sample (total). With this purpose, bacterial pellets were incubated for 30 minutes at room temperature with 500 ul of PBS pH 7.45 solution containing 0.25 % BSA, 5 % FCS, and 2 mM EDTA, followed by a washing process with PBS and finally resuspended in 1 mL of PBS. The whole sample volume was equally divided into five sterile 1.5 mL polypropylene tubes for the total sample and each immunoglobulin fraction. IgA1-coated bacterial staining was performed using 1:500 diluted anti-human IgA1 labeled with PE (ABCAM, # ab99797), and 1:250 diluted anti-human IgA2-FITC (Southern Biotech, #9140-02).

Biotinylated mouse anti-human IgM (ABCAM, #AB99745) and IgG (ABCAM, #AB201842) were added for 30 minutes at 37° C. For IgG and IgM-coated bacteria, samples were washed and resuspended in PBS with streptavidin-APC-Cy7 (BioLegend, #405208) and streptavidin-PerCP (BioLegend, #405213) respectively at 1:500 dilution in both cases. Flow cytometry sorting was made in the SH800S Cell Sorter (SONY™) using the light sources 488 nm (blue) and 635 nm (red) lasers. Default cytometer settings and lower 40 psi pressure on the 100 µm nozzle were used, with a low FSC and SSC threshold that was set to a logarithmic scale to allow bacterial detection. Sorted bacteria were collected in sterile PBS at 4° C, and centrifuged at 8,000 ×g, 10 min, at 4° C to pellet bacteria, and stored at −20° C until DNA extraction. After sorting and collection, the purity of each bacterial fraction was verified by flow cytometry.

2.5 DNA extraction, library preparation, and sequencing

DNA extraction from total samples and all immunoglobulin fractions was carried out using FavorPrep Milk Bacterial DNA Extraction Kit (Cat.: FAMBD001, Favorgen, Biotech Corp, Taiwan) following the manufacturer's instructions. Extraction of negative controls was carried out to assess the possibility of reagent contamination. The DNA concentration of all samples was measured at 260/280 nm absorbance using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). For library preparation, ~281 bp amplicons of the V3 region of the 16S rRNA gene were generated by PCR using primers, reagents, and PCR conditions previously reported [13] Free DNA template negative controls were used for PCR, with no amplicon detection. These reactions were processed through the sequencing pipeline used to analyze the samples. Equal mass amounts of each 1 to 100 barcoded amplicons quantified by gel densitometry were pooled for Ion Torrent. The mixture was purified using an E-Gel iBase Power System (Invitrogen) the downstream protocol for the Ion Torrent PGM sequencing was as previously described [13,14].

2.6 Bioinformatics analysis

After sequencing, reads were filtered by the PGM software to exclude low-quality and polyclonal sequences. Demultiplexed read quality was verified using FastQC and the sequences were trimmed to 200 nt length using Trimmomatic v0.36. Trimmed sequences were imported to the QIIME2 pipeline (Quantitative Insights Into Microbial Ecology 2) software version 2022.8 for processing [15]. Denoising was performed with the DADA2 plugin using default settings following standard quality filtering and classifying sequences into amplicon sequence variants (ASVs). Taxonomy was assigned to each ASV using the qiime feature-classifier classify-consensus-blast plugin with 97% identity using the Greengenes as reference database v13.8. To minimize the effect of contaminant sequences we used the decontam R package (Version 1.10.0) [16] to identify contaminant features using the frequency (based on DNA concentration) and prevalence method which rely on the presence/absence of a particular feature in the sample compared with the prevalence in negative controls. Also, the contaminant features previously reported in the salter list [17] were removed if present in only one sorted fraction and absent from paired fractions. Sequences and metadata used for the analysis in this work were deposited in the National Center for Biotechnology Information Database (Accession Number: PRJNA985669) and can be accessed at https://www.ncbi.nlm.nih.gov/sra/PRJNA985669.

2.7 Microbial Abundance and Diversity

The relative abundance of bacterial communities in the samples at phylum and family taxonomic levels was calculated with QIIME2 and plotted with ggplot2., The linear discriminant analysis effect size (LEfSe v1.0) was performed using the Galaxy module from The Huttenhower Lab [18] to detect statistical differences in the relative abundance of bacterial taxa among the bacterial fractions and the

total community. For beta diversity calculations, Weighted UniFrac analysis was performed using a rarefied sample at 10,000 reads per sample to even sequencing depth and discarding the single sequences (singletons). Permutational multivariate analysis of variance (PERMANOVA) was used to evaluate the significance among the groups. A three-dimensional scatter plot was generated using principal coordinate analysis (PCoA) and visualization was created with the Emperor software of QIIME2. Alpha diversity was estimated through the calculation of the richness index chao1 and the diversity and dominance indices Shannon and Simpson with Phyloseq 1.4.0 and graphs were created with ggplot2 packages in R environment (v4.1.2).

2.8 Source Tracking Analysis

To predict the possible origin of the bacteria in each neonate stool sample source tracking analysis was performed. As a source, we use immunoglobulin-bound bacterial fractions of colostrum and neonate stool as a sink. The analysis was conducted with sourcetracker2 gibs plugin QIIME2 using the *p-no-loo* parameter [19]. The analysis shows the proportion of ASV present in the copro of neonates that may come from colostrum bacteria bound to the different immunoglobulins, as well as "unknown" which indicates other possible sources not evaluated in the work.

2.9 Metabolic Pathways Prediction

We used PICRUST2 (Phylogenetics Investigation of Communities by Reconstruction of Unobserved States) v2.4.1 [20] to predict the metabolic function of the metagenomes from 16S rRNA gene data set, with the Metacyst classification database. Statistical Analysis of Taxonomic and Function software (STAMP v2.1.3) with Weach's t-test was used to determine significant differences in the abundance of ASVs and metabolic pathways.

2.10 Statistical Analyses

Data are presented as the mean ± SD for graphics representations, with p-values < 0.05 considered significant. The Shapiro-Wilk test was used to establish the normality of variables. Differences for multiple groups comparison with non-normal distribution data were determined by no parametric Kruskal Wallis and post hoc test. Bonferroni p-value correction was applied for beta diversity comparisons, and Differences in beta diversity between groups were assessed with an analysis of similarities (ANOSIM). Data were analyzed by SPSS 20 software and Graph Prism 5.0.

3 Results

3.1 Mothers in the binomina were young and in good health condition at delivery

The ten studied binomial were characterized by women with an average age of 23 years, and newborns close to 26 hours of age fed exclusively with human milk. Seven participant women were living in Ecatepec, an overcrowded municipality area of 8,860 inhabitants/km², and Ixtapaluca, a semirural area of 1,704 inhabitants/km² of the Estado–de–México, a state located in the central part of Mexico. Most women were housewives, with at least one child, and had received the SARS-CoV-2 vaccine at the time of the study. For the Neonates, most of them were males, born by vaginal delivery or non-elective C-section, with weight and size within normal parameters for Mexican newborns (Table 1).

Table 1. Sociodemographic and clinical data of the study population

Maternal Data		**n (%)**
Age in years*		23.1 ± 4.14
Gestational Age		39 ±1
Birthplace		
	Ixtapaluca (State-of-Mexico)	3 (30)
	Ecatepec (State-of-Mexico	7 (70)
Main Activity		
	Housewife	6 (60)
	Student	2 (20)
	General employee	2 (20)
Parity		
	Uniparous	4 (40)
	Multiparous	6 (60)
Delivery mode		
	Vaginal	8 (80)
	C-Section	2 (20)
SARS-CoV-2 vaccine		
	AstraZeneca/Sputnik/Pfizer	6 (60)
	None	3 (30)
Neonatal Data		**n (%)**
Age in hours		25.88 ± 12.32
Weight (g)		3091.44 ±422.85
Size (cm)		49.44±1.62
Sex		
	Female	4 (40)
	Male	6 (60)

* Expressed as mean ± standard deviation, n— sample number

3.2 Bacteria in human colostrum are differentially bound by maternal immunoglobulins

The ion semiconductor massive sequencing of the V3-16S rRNA gene libraries was satisfactory for all samples, where most of them had > 10,000 reads (Table S1). The human colostrum contains commensal bacteria bound to maternal immunoglobulins IgA1, IgA2, IgM, and IgG in homeostatic conditions. The analysis of the sequencing data revealed several ASVs shared among the studied fractions; however, it is noticed that each immunoglobulin could select a discrete group of ASVs being IgG the immunoglobulin with the larger number (Fig. 1A). In general, at the family level, the apparent relative abundances of the taxa bound to each immunoglobulin are different to the relative abundance observed for the taxa in the total sample, while the diversity of the bound taxa among the immunoglobulins are different (Fig. 1B). However, at phylum level significant differences were observed for Firmicutes and Proteobacteria for the same samples (Table S2). The alfa diversity for the data, indicates a tendency for higher diversity in the total sample in comparison to the fractions (Fig 1C), although there is no statistically significant difference (Table S3).

3.3 Maternal IgA2 and IgM bind predominantly alpha and beta proteobacteria in human colostrum

The beta diversity analysis of the sequencing data shows that the bacteria bound by IgA2 and IgM are different from the bacteria present in the total sample (Fig. 2A and 2B). The comparative analyses of the relative abundances support that IgA2 ($p = 0.007$) and IgM ($p = 0.046$) fractions are enriched in

Proteobacteria to the total sample (Fig. 2C). Further analysis indicated a trend of enrichment of alpha, beta, and gamma proteobacteria in the immunoglobulin fractions compared to the total sample (Fig 2D). A more detailed LefSe analysis showed that the families *Sphingomonadaceae* and *Xanthobacteraceae* are enriched in the IgA2 and IgM fractions, while the orders *Burkholderiales*, and Ellin329 are enriched in the IgA2, and the family *Comamonadaceae* are enriched in the IgM fraction (Fig. 2E and 2F). The predicted metabolome of the bacteria bound by IgA2 and IgM shows relevant metabolic pathways related to the biosynthesis of saturated and unsaturated long-chain fatty acids, and ubiquinol biosynthesis as well (Fig. 3A and 3B). These contrast with the metabolic pathways found in the total fraction of bacteria which are characterized by galactose degradation and peptidoglycan biosynthesis (Fig. 3A and 3B).

3.4 Maternal IgG and IgA2 bind different groups of bacteria in human colostrum

In addition to differences in bacterial taxa abundances for each immunoglobulin concerning the total bacteria in colostrum, the possibility of relevant differences between pairs of immunoglobulin fractions was explored. The analysis of the beta diversity revealed that the population of bacteria selected by IgG and IgA2 differ in their composition. The principal coordinate plot of data shows two clusters with statistical significance according to the unweighted UniFrac distance metric using the Anosim statistical test (Fig. 4A). A linear discriminant analysis (LEfSe) disclosed that the differences between IgG and IgA2 are explained by distinct abundance of specific taxa. For instance, the families *Propionibacteriaceae, Corynebacteriaceae, Moraxellaceae, Halomonadaceae, Aurantimonadaceae, and Lactobacillaceae*, are the ones characterizing IgG; while for IgA2, the taxa belong to bacteria of the order *Burkholderiales*, and the families *Sphingomonadaceae, Rhizobiaceae*, and *Pseudomonadaceae* (Fig. 4B). The predicted metabolome for the bacteria bound by IgG, shows relevant metabolic pathways mainly related to biosynthesis and degradation of nucleotides, and carbohydrate metabolism, while the bacteria bound by IgA2 features metabolic pathways related to fatty acid metabolism (Fig. 4C).

3.5 Maternal immunoglobulins bind low-frequency intestinal taxa in the neonate stool

The relative abundance of bacteria bound for each immunoglobulin in the neonatal feces, shows an enrichment of taxa in comparison to the bacteria present in the total sample. In the total sample there was a dominance of *Bifidobacteriaceae, Clostridiaceae*, and *Enterobacteraceae*; while a dominance was not observed for the bacteria bound by IgA1, IgA2, IgM, and IgG. In this case, an increase in the abundance of taxa like *Alcaligenaceae, Propionibacteraceae*, and *Rhizobiaceae* was observed (Fig. 5A). The diversity indexes Chao1, Shannon, and Simpson confirm the change in the diversity of the bacterial taxa selected by the immunoglobulins (Fig. 5B), with statistical significance (S4 Table). The analysis of the beta diversity agrees with the results; here the principal coordinate plot of data shows two clusters when the bacteria in the total sample, was compared to each of the bacteria bound by IgA1, IgA2, IgM, and IgG (Fig. 5C).

3.6 Maternal IgA2 and IgM bind more butyrate-producing bacteria than IgG in the neonate stool

The relative abundance of the main phyla in the total sample found in the neonate stool shows that within the first 48 h, *Proteobacteria* and *Firmicutes* are the more abundant phyla in the total samples, followed by *Actinobacteria* and *Bacteroidetes* (S2 Table). There is a slight increase in the abundance of *Proteobacteria* over *Firmicutes* when IgA1, IgA2, IgM, and IgG bind and select the bacteria; however, only for *Bacteroidetes*, there is a statistically significant difference in the bacteria bound by the different immunoglobulins (S2 Table). As occurred for *Proteobacteria* in the colostrum samples, the *Bacteroidetes* abundance in the IgA2 ($p = 0.045$) and IgM ($p = 0.018$) fractions were enriched in

comparison to the total sample (Fig. 6A). The analysis LEfSe, of the bacteria bound by each immunoglobulin versus the total sample uncovered several common enriched bacteria (Table S2), (Fig S1). The same analysis revealed interesting differences for the bacteria bound between the IgA2 versus IgG (Fig. 6B), where *Firmicutes* like *Clostridiales*, and *Ruminococcus*, and *Bacteroidetes* like S24_7 and Odoribacter characterize IgA2, while *Firmicutes* like *Planococcaceae*, and *Proteobacteria* like *Sphingomonadaceae* characterize the IgG bound bacteria (Fig. 6B). A different result was observed for the analysis of IgG versus IgM. In this case, *Firmicutes* like *Clostridiales*, *Lachnospiraceae*, *Ruminococcus*, and *Bacteroidetes* like *Odoribacter*, S24_7, and *Bacteroides* characterize IgM; no bacteria were revealed for IgG (Fig. 6C). The PICRUSt analysis revealed a predicted metabolome for the bacteria bound by IgA2, where succinate fermentation, mannan degradation, and glycol metabolisms and degradation were the relevant metabolic pathways (Fig. 6D). For the case of IgM and IgG, succinate fermentation and palmitate biosynthesis characterized IgM, while nucleotide biosynthesis characterized IgG (Fig. 6E).

3.7 Ig-bacteria from colostrum are an important source of microbiota for the newborn

SourceTracker, an application based on Bayesian probability, was employed to conduct a more extensive examination of the contributions of various microbial sources to the neonatal intestinal community. The results revealed that colostrum IgG-microbiota constituted the main potential bacteria source, contributing 36.93% to the neonatal stool microbiota, and was prevalent in all samples (100% prevalence). Among other potential bacterial sources, IgA2, IgM, and IgA1 were identified, being the IgA1-bound bacteria the minor contributor source. Nonetheless, other unknown sources accounted for 27% (ranging from 1% to 98%) of the potential sources suggesting the presence of other environmental sources [13] that could have contributed to the neonatal bacterial microbiota (Fig. 7A). The colostrum Ig-bacteria played a significant role in shaping the neonatal intestine's bacterial composition, exhibiting varying contributions depending on the immunoglobulin fraction. IgA2 was found to primarily contribute to the family *Enterobacteriaceae*, representing nearly 50% of the relative abundance. Conversely, IgG, IgM, and IgA1 exhibited greater involvement with bacterial taxa such as *Clostridium* and *Bifidobacterium*, among others (Fig. 7B).

4 Discussion

The interaction between the microbiota and the host is regulated by the immune system. In the initial stages of life, the immune system plays a significant role in the establishment of a diverse bacterial population in the neonatal gut. The pioneer bacteria in the newborn primarily originate from maternal sources, like the vaginal canal, maternal gut, maternal skin, and human milk. Human milk is a crucial supplier of bacteria and immunoglobulins that actively aid in the proper establishment of the microbiota in the neonate's intestines during the early days of life. This research explores the taxonomic patterns of bacteria bound to maternal immunoglobulins in human colostrum. These Ig-bacteria play a crucial role as a significant source of microorganisms for the neonatal gut in the early postnatal period. The findings presented here add to the expanding understanding of how maternal bacteria are transferred and established in the neonatal gut during the early period after birth. Based on the results for the studied sample, maternal IgA2 and IgM bind predominantly to *Proteobacteria*, which have highly lipidic metabolic pathways. In contrast, IgG binds bacteria which are the first to colonize the gut and possess the ability to break down milk carbohydrates in colostrum.

In human colostrum, our analysis detected Proteobacteria as one of the primary phyla in the samples. This finding aligns with previous reports from women of rural or low-income populations, including Indian [21], Ethiopian [22], Mexican [13], and Mayan populations [23]. These populations have also

reported alpha and beta Proteobacteria as major constituents of bacteria in human milk. We were able to identify a core group of human milk bacteria coated with IgA2 and IgM, including the family *Sphingomonadaceae*, and the genera *Xanthobacter* and *Burkholderia*. This finding agrees with the SIgA-coated bacterial fractions isolated from fecal samples of healthy human volunteers, where IgA also coated these specific groups of Proteobacteria. In this report, the coating of commensal bacteria with IgA may be attributed to the ongoing stimulation of the immune system by these particular taxa [24]. The significance of Proteobacteria abundance in human milk during the perinatal period has been underscored by prior findings that highlight a rise in proteobacteria levels in pregnant women during the third trimester in contrast to the first trimester [25,26]. Furthermore, the fecal samples of mothers in the third trimester of pregnancy, collected from the same population examined in this study, revealed the presence of both alpha and beta proteobacteria [27].

It has been documented that specific gut commensal bacteria residing in the maternal gut stimulate IgA production in the Peyer's patches (PP), with subsequent transference to human milk via the maternal entero-mammary pathway. This mechanism aligns the antibody repertoire in human milk with the maternal microbiota composition, providing comparable mucosal barrier protection to neonates and an immune response against encountered microorganisms [28,29]. This observation potentially elucidates why IgA2 shows a propensity for selectively binding *Sphingomonas* and other members of the Proteobacteria group within the colostrum since these bacteria are naturally resident members of the microbiota in healthy mammary gland tissue and the maternal intestine [30,31]. These microorganisms might induce an immune response through their structural antigens or via the synthesis of metabolites. For example, the sphingolipids, a special antigen produced by *Sphingomonas*, along with specific members of the Bacteroidetes phylum found within the microbiota, play a significant role in the maintenance of the mucosal homeostasis, influencing the immune function of invariant natural killer T cells (iNKT) in the early postnatal period [32,33]. Studies on murine models reveal that sphingolipids regulate excessive host Colonic iNKT cell proliferation during neonatal development and the expression of antigenic glycosphingolipid products also plays a role in iNKT cell activation [33,34]. Moreover, early exposure to these bacteria is critical for establishing mucosal iNKT tolerance to subsequent environmental stimuli [35]. Sphingolipids have also been linked to the differentiation of naive B cells into IgA cells within Peyer's patches, as well as facilitating the movement of IgA+ cells into the lamina propria [36]. Furthermore, the production of metabolites by the commensal bacteria recognized by immunoglobulins could also contribute to the host immunity. For example, our analysis of metabolic predictions reveals increased metabolic pathways associated with long-chain fatty acids such as palmitoleate, gondoate, and oleate. Notably, specific fatty acids, including palmitic acid, have demonstrated effects on the production of intestinal IgA antibodies [36,37].

Concerning *Staphylococcus, Streptococcus,* and *Propionibacterium,* which are bacteria frequently identified as integral constituents of the colostrum's "core" microbiota and early colonizers of the gut in neonates [38,39], their recognition by IgA2 within the colostrum environment was notably limited. These microorganisms were predominantly identified in the total fraction, existing either uncoated or covered by other immunoglobulins, such as IgG. In contrast to IgA2, IgG demonstrated recognition of facultative anaerobes, including *Lactobacillus* and *Propionibacterium*. We observed that IgG-associated bacteria exhibited elevated levels of predicted metabolic pathways associated with carbohydrate degradation. These bacteria are recognized as lactate producers and glycolytic microorganisms, contributing to the breakdown of lactose and glucose from human milk [40,41]. For this reason, IgG-bound bacteria could be a source of first colonizers of the neonatal gut since they can metabolize human milk carbohydrates. Another characteristic of this group of microorganisms is an increased predicted nucleotide biosynthesis metabolism, this could be related to the capacity of this bacteria to expand and colonize the infant’s gut. It has been reported that nucleotide biosynthesis

pathways have strong links with the virulence of opportunistic bacteria and a role in the colonization of the mouse gastrointestinal tract [42]. In addition, the bacterial pyrimidine ribonucleotide biosynthesis is enriched in the first week of the life of infants [43].

Considering these findings, IgG-bound bacteria could hold significance as a potential source of the neonatal gut's pioneers, due to their capability to metabolize carbohydrates found in human milk. Another characteristic of this group of microorganisms is their increased nucleotide biosynthesis metabolism pathways in the predicted metabolome, which may be linked to their capacity to proliferate and establish residence within the infant's gastrointestinal tract [42]. Likewise, the enrichment of bacterial pyrimidine ribonucleotide biosynthesis has been observed during the first week of infant life, underscoring its potential significance in early colonization processes [43]. Our hypothesis suggests that IgG plays a role in binding potential bacterial colonizers or pathobionts, thereby potentially regulating their dissemination within the neonatal gastrointestinal tract. In murine models, anti-commensal IgG antibodies have been found to recognize shared features among commensal microbes, such as *lactobacillus*, which cross-react with pathogens, conferring a protective effect [44]. Additionally, these IgG antibodies have been reported to contribute to the regulation of responses to translocated microbes, achieving a dual function of discouraging bacterial association with the intestinal epithelium while preventing immune activation against commensal bacteria [29,44].

On the other hand, within neonatal stool samples, we identified microbiota coated by maternal immunoglobulins when neonates were fed with human colostrum. Alongside IgA2-bound bacteria, the recognition of bacteria bound to IgA1, IgM, and IgG was also observed in this context. Ig-bound bacteria in neonatal stool consisted predominantly of low-frequency bacterial strains, exhibiting an underrepresentation when compared to the prevailing bacteria present in the entire bacterial population. These findings align with reports made in IgA-associated fecal bacteria of healthy adults [24]. The prevalence of these low-frequency taxa within the Ig-bound microbiota was evident in the increased diversity, manifested as higher richness and diversity compared to the total bacterial population within neonatal samples. The neonatal stool microbiota was primarily characterized by a substantial dominance of *Enterobacteria*, *Bifidobacteria*, and *Clostridium*—three well-documented genera frequently observed in the infant microbiota during the initial days of life [45]. The dominance of the most abundant taxa could be masking the presence of the low-frequency groups. But even when the frequency of the bacteria is low, their presence is important since they are active players of human microbiota and in strict interaction with the immune system. Species in low abundance have a major role in their respective community, for example exerting metabolic functions that can impact the local microbial habitats [46].

As a remarkable observation, Bacteroidetes were more abundant in the IgA2 and IgM fraction than in the IgG and total bacteria. The presence of Bacteroidetes in the neonatal microbiome is scarce, but important since members of the Bacteroidetes phyla provide most of the acetate, succinate, and propionate to the intestinal environment [47]. Also, as part of the IgA2-bound and IgM-bound bacteria in the stool of these neonates, important firmicutes reported as butyrate producers were found with more abundance than in the IgG fraction. Some of these bacteria could ferment succinate to butyrate as was shown in the metabolome prediction, for example, the order Clostridiales [47]. Other bacteria such as *Ruminococcaceae* and *Lachnospiraceae* families can also participate in butyrate production using other precursors such as acetate or lactate [48]. Certain of these butyrate-producer bacteria are endospore-forming taxa (as members of Clostridiaceae and Lachnospiraceae), which are vertically transmitted from the mother during birth and colonizing of the infant during the first months or even days, persisting during the first year. These anaerobes can tolerate oxygen to various extents but are present with low diversity and abundance [49,50].

Even if the incidence of these bacteria is low in the neonatal gut, the binding with IgA2 and IgM could highlight its relevance for neonate health, probably serving as the first bacterial seed for the infant's gut. Both IgA2 and IgM could contribute to anchor late Firmicutes and Bacteroidetes colonizers, to the infant mucus. In a similar way to the results obtained in our work, previous work reported dual coating by SIgA and SIgM of these bacteria, especially to Firmicutes members like *Lachnospiraceae* and *Ruminococcaceae*, and a special binding of SIgM to Bacteroides in the intestine of adult's volunteers [51]. These immunoglobulins recognized mainly the mucus-embedded community, suggesting that the commensal coating with IgM may not only help IgA2 to increase the diversity of the bacteria coated in the intestine but also is an auxiliary in the retention of important commensal to the intestinal mucus, given the mucus-binding properties of the secretory component [51,52]. It has been observed *in vitro* that SCFA producers, as members of clostridia cluster XIVa cluster colonize the mucus layer to be closer to the epithelium, which may help to improve immunoregulatory effects and enhance butyrate bioavailability to the epithelial cells [53]. For this reason, the acquisition of these taxa is important, being key to establishing a host-microbiota symbiosis [54]. Additional advantages provided by the establishment of these bacteria in the neonate's gut have been described, as the colonization resistance to invading pathogens. It has been shown that some members of the microbiota as *Bacteroides* and *Clostridium* play a beneficial role in mediating colonization resistance, especially to *Salmonella typhimurium* in mice models [55]. Succinate enhances the colonization in the neonatal gut of clostridium cluster IV and XIVa and concomitantly excludes salmonella [56]. Similarly, through propionate production, Clostridium inhibits the growth of pathogens [57].

Finally, human milk provides bacteria coated with immunoglobulins to the neonatal gut, a significant proportion of neonates' microbiota comes from human milk [58,59]. In the studied neonate stool, we observed a high abundance of *Enterobacteria*-bound to IgA2 came from human colostrum. In mice, during the first week of life, the IgA response against Enterobacteriaceae is crucial in the regulation of the maturation of the gut microbiota, mainly limiting Proteobacteria expansion. The presence of IgA contributes to regulating the presence of Proteobacteria in newborns as they are gradually replaced by Bacteroides and Firmicutes [60]. The presence of Enterobacteria in the intestinal environment is important due to the high levels of luminal oxygen in the neonatal gut in the first days. For this reason, facultative anaerobes such as *Lactobacillaceae* and *Enterobacteriaceae* families could colonize and decrease the oxygen present in the gut, allowing the posterior establishment of anaerobes [61,62]. In our results, we observe other important facultative anaerobes as pseudomonas in the neonate were provided by the human colostrum.

The obtained results from this exploratory work provide information about human colostrum microbiota and its relationship with the immune system through immunoglobulin binding. To the extent of what is publicly reported, this is the first study exploring the bacterial binding by the IgA subtypes, IgM, and IgG in human colostrum. Even when this work only identifies by amplicon sequencing the bacteria that are recognized by maternal immunoglobulins in human colostrum and the neonate stool during the first days of life, we could know more about the interaction of the microbiota and the maternal immune system as a first approach. Further studies are needed to evaluate the metabolism of the coated bacteria to a better understanding of the bacteria activity and function in the host-microbiota relationship and its implication in newborn health. The interpretation of the results presented in this study must be carefully considered, since there exists a high intra-sample variability, a major number of samples could help to find better patterns in the microbial community coating.

5 Conclusion

Human colostrum contains bacteria that are bound by maternal immunoglobulins. The IgA2 and IgM type bind mainly alfa and beta Proteobacteria in the colostrum which could stimulate the immune system in the neonatal gut. On the other hand, IgG mainly binds facultative anaerobes from Firmicutes phylum that were widely reported as part of human milk microbiota and as first colonizers of the neonatal gut. Maternal immunoglobulins also bind a wide diversity of bacteria in the neonatal stool. IgA2 and IgM bound more Bacteroidetes in comparison with IgG, these Bacteroidetes and some firmicutes have been reported as late colonizers of the neonatal gut, and their presence is important due to their ability to produce SCFA as propionate and butyrate. The microbial and immunoglobulin transference is crucial for the neonate's immune system and for the development of its microbiota.

6 Appendices

Fig. S1 LEFSe Analysis

Table. S1 Sequencing Summary

Table. S2 Relative abundance of phyla among groups

Table. S3 Alpha diversity indexes for colostrum fractions

Table. S4 Alpha diversity indexes for neonate stool fractions

7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Author Contributions

Conceptualization—KC-C, ES-S, LS-A, and JG-M.; Methodology—KC-C, ES-S, AP-E, LS-A, and JG-M.; Formal analysis—KC-C, AP-E, and JG-M.; Investigation—KC-C, ES-S, AP-E, MNR-C, TR-L, GA-A, MS-M, LS-A, AK, NSL-P and JG-M.; Resources—MNR-C, TR-L, GA-A, MS-M, LS-A, and JG-M.; Data curation—KC-C, MNR-C, and JG-M.; Writing original draft preparation—KC-C, and JG-M.; Writing review and editing—KC-C, ES-S, AP-E, PBZ-S, MNR-C, TR-L, GA-A, MS-M, LS-A, AK, NSL-P and JG-M.; Supervision—AP-E, PBZ-S, MNR-C, TR-L, GA-A, MS-M, LS-A, and JG-M.; Project administration—GA-A, MS-M, LS-A, and JG-M.; Funding acquisition—MNR-C, GA-A, MS-M, LS-A, and JG-M. All authors have read and agreed to the published version of the manuscript.

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