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The establishment of the infant intestinal microbiome is not affected by rotavirus vaccination

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The microbial colonization of the intestine during the first months of life constitutes the most important process for the microbiota-induced host-homeostasis. Alterations in this process may entail a high-risk for disease in later life. However, the potential factors affecting this process in the infant are not well known. Moreover, the potential impact of orally administered vaccines upon the establishing microbiome remains unknown. Here we assessed the intestinal microbiome establishment process and evaluated the impact of rotavirus vaccination upon this process. Metagenomic, PCR-DGGE and faecal short chain fatty acids analyses were performed on faecal samples obtained from three infants before and after the administration of each dose of vaccine. We found a high inter-individual variability in the early life gut microbiota at microbial composition level, but a large similarity between the infants' microbiomes at functional level. Rotavirus vaccination did not show any major effects upon the infant gut microbiota. Thus, the individual microbiome establishment and development process seems to occur in a defined manner during the first stages of life and rotavirus vaccination appears to be inconsequential for this process.

The basis of a healthy intestinal microbiota lies in the early neonatal period with the initial steps of establishment of this complex microbial ecosystem[1]. Microbial colonization of the gut in human newborns is started by facultative anaerobes which contribute to the establishment and development of strict anaerobic bacterial populations by reducing oxygen content[2]. Different factors including mode of delivery, feeding habits, gestational age or use of medication have been reported to affect this process[3,4]. The initial microbial colonization has been shown to constitute the most important moment for the microbiota-induced host-homeostasis. This microbe-host interaction in early life is necessary for a proper maturation of the immune system[5,6], and results essential for a normal host development and physiology[7,8]. Therefore, during this relatively unstable and sensitive initial period any alteration in the microbiota development process may increase the risk of disease in later life[1,9]. After weaning the complexity and diversity of the microbiota increases rapidly and at the age of 2–3 years the infant microbiota reaches an adult-like composition[10].

The delivery mode[11,12], gestational age[13,14,15,16] or antibiotics administration[14,17,18,19] are known to affect the microbiota composition. Nevertheless, the impact of other factors, such as other medical interventions in early life, on the process of establishment of the intestinal microbiota in newborns still remains poorly understood as most of the currently available studies have focused on the adult population[20]. Moreover, most of the studies carried out using modern next-generation-sequencing techniques have applied 16S rRNA gene-sequencing for microbiota analyses whilst few works have assessed the total infant metagenome composition[10,20,21,22]. To this regard, metagenomic analyses have the advantage of providing not only data at microbial composition level, such as 16S rRNA gene data, but also data on the functions present in the metagenome. Furthermore, most reports on the infant microbiome have evaluated the effect of delivery mode, feeding habits, gestational age or disease, whereas the impact of some common early life medical interventions on the establishing intestinal microbiota remains largely unknown.

Nowadays, vaccination is a very common practice in developed countries. Among the different vaccines some are orally administered and contain attenuated microorganisms or viruses, such as the one for rotavirus, a double stranded RNA virus from the family *Reoviridae* which produces gastroenteritis and diarrhoea. Oral vaccines are

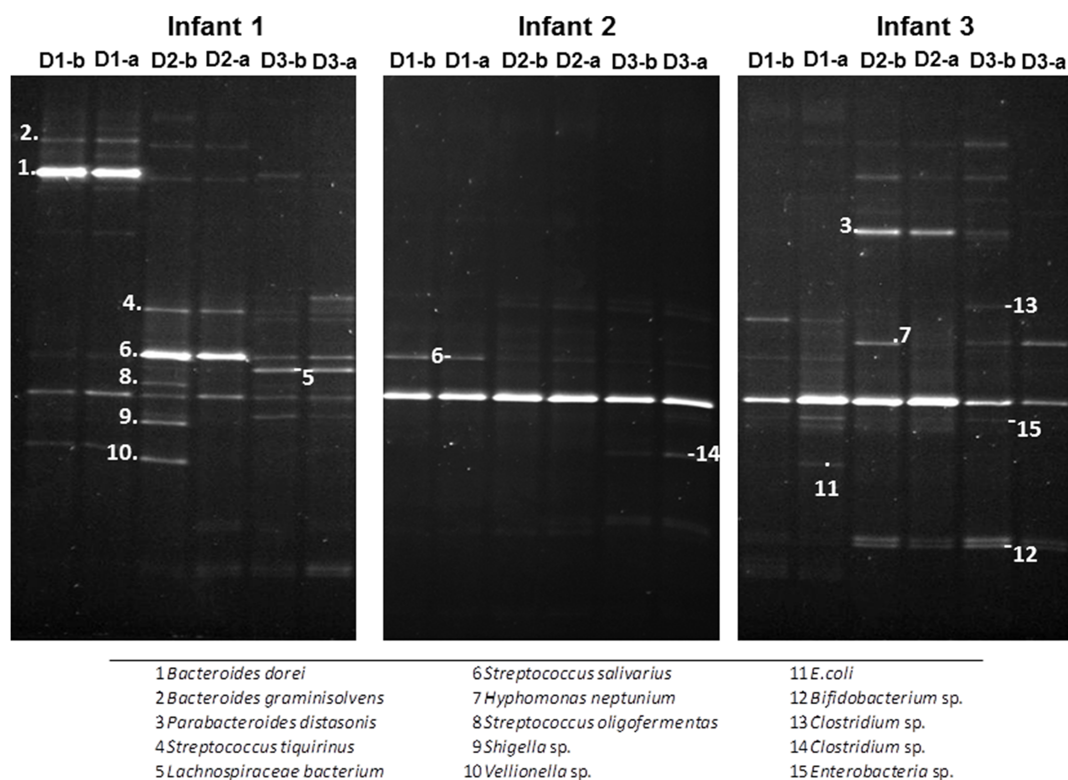

Figure 1 | PCR-DGGE faecal microbiota profiles obtained before and after (b or a) each rotavirus vaccination dose (D1, D2 and D3) from each infant. Numbers indicate the results of the species identification of the corresponding DGGE band.

expected to interact with intestinal immune cells eliciting an immune response at mucosal level. Therefore, such vaccination may have the potential for modifying the intestinal environment, thus altering the establishing infant gut microbiome.

The aim of the present study was to assess the process of establishment of the intestinal microbiome in infants and to evaluate the potential impact of oral rotavirus vaccination upon this process. To this end, total metagenomic analyses, in combination with PCR-DGGE and faecal short chain fatty acids (SCFA) determinations, were performed.

Results

PCR-DGGE. In spite of the changes observed over-time, the PCR-DGGE results showed clearly different profiles for the three infants (Figure 1). In addition, no major changes in the DGGE patterns were evidenced when the samples taken before administration of each rotavirus vaccine dose were compared with those obtained after administration of the vaccine dose.

The DGGE pattern obtained for infant 2 showed less bands than those obtained for the other two infants, suggesting a less complex microbiota (Figure 1). Moreover, the profile of the infant 2 remained largely unchanged during the study with a predominant band that could not be un-ambiguously identified but belonging to the phylum Proteobacteria (the highest homology scores were all obtained with different proteobacteria). Faeces from infant 1 showed initially (first sample, 2 months) a strong band corresponding to *Bacteroides*, with a band identified as *Streptococcus* becoming the strongest one at the second sampling point. The samples of the third infant presented a clear band along the sampling period, likely corresponding to proteobacteria, with bands from anaerobes such as *Bacteroides* and *Bifidobacterium* becoming apparent in the second and third sampling points (4 and 6 months).

Short chain fatty acids. The SCFA profiles were found to be very stable and not affected by the vaccination, with molar proportions of the three major SCFA (acetic, propionic and butyric) showing little variation along time and among individuals (Supplementary Figure S1). The acetic acid was the most abundant SCFA in the three infants along the study, followed by propionate and then butyrate.

Metagenomic analyses. Metagenomic data showed clear differences in microbiota composition among infants, with all the samples from the same infant clustering together and independently of those of the other infants (Figure 2, Supplementary Figure S2). These differences point to the individual as the main factor determining the microbiota composition; clear differences were found, at genus level, among the intestinal microbiota of the three babies, although their respective microbiotas showed limited variability over time and an almost negligible vaccination-effect.

These results indicate that the vaccine had minimal or no effect on the individual microbiota profile. Thus, in order to assess the microbiome evolution during the first months of life in these three babies we calculated, in each infant and sampling point (vaccine dose), the mean of the relative proportion for each bacterial group obtained before and after vaccine. Microbial composition at different taxonomic levels showed clear differences among infants (Figure 3 and Supplementary Figure S3). At genus level *Bacteroides* dominated in the first sample of infant 1 (2 months of age) with *Bifidobacterium* becoming the dominant genus at later sampling points (3 and 4 months of age). The microbiota of infant 2 was dominated by enterobacteria, mainly *Escherichia*, during the whole sampling period whilst in the case of infant 3 enterobacteria dominated at the first two sampling points (up to 4 months of age), with *Bifidobacterium* becoming dominant later on.

Vaccination showed no effects on α-diversity or gene richness (data not shown). In general, infant 1 displayed higher α-diversity than infants 2 and 3 (Table 1). Diversity and gene richness increased

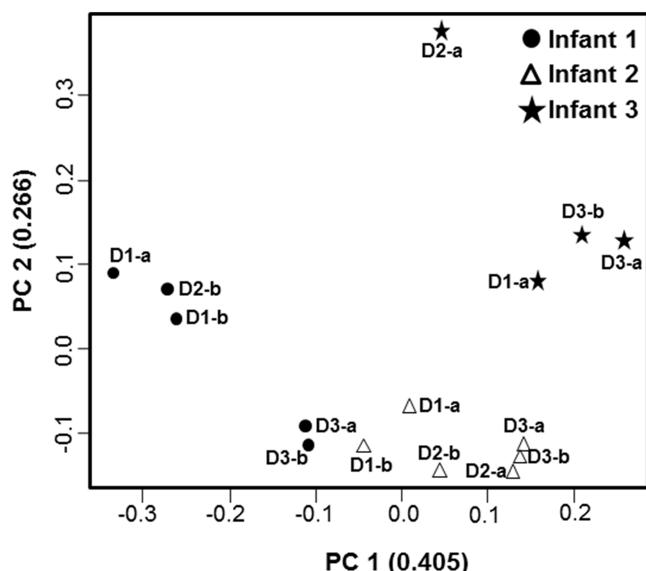

Figure 2 | PCoA analysis of the microbiota composition at genus level obtained from faecal samples before and after (b or a) each rotavirus vaccination dose (D1, D2 and D3) from each infant. PCoA was performed with Spearman correlation distance.

over time in infants 1 and 2 but remained almost unchanged in infant 3 (Table 1).

Regarding the functional features of the infants microbiome the KEGG level B categories "enzyme families", "membrane transport", "carbohydrate metabolism" and "amino acid metabolism" were the most represented ones in all the infants and samples analysed (Figure 3).The metagenomes showed higher stability and lower variability at functional level than at microbial composition (genus) level (Figure 3 and Supplementary Figure S2). Nevertheless, PCoA analysis of functional data still clustered together the different samples belonging to each infant (Supplementary Figure S4). When spearman correlation distances were calculated using either KEGG (levels B or C) or composition at genus level, it became clear that the distances among samples were lower for KEGG data (Figure 4) than for the genera data. This corroborated the greater similarity of infant metagenomes at functional than at microbial composition level.

Discussion

The process of intestinal colonization by microorganisms during the postnatal period is very important for later health[8,9,23]. Therefore, controlling or minimizing the impact of early life medical interventions on the establishing gut microbiome may have a large influence in later health. Unfortunately, our knowledge on the gut microbiome establishment process in the neonatal gut and the effect of perinatal medical interventions upon this process is still limited.

Some vaccines such as the one for rotavirus are administered orally and will interact with the intestinal mucosa which may modify the intestinal environment and, therefore, may affect the intestinal microbiome establishment process. To this regard, norovirus infection in adults did not show major effects on the intestinal microbiota in the majority of patients, although in a minority of cases microbiota alterations were observed[24]. Nevertheless, the impact of other viruses causing enteric infections or that of orally administered vaccines against them remains largely unknown. García-López and co-workers[25] used 16S rRNA gene-based analysis to compare the microbiotas of rotavirus vaccinated and unvaccinated children after one year of age (12–15 months) without observing any long-term microbiota differences. In the present study we have used total metagenome analyses to test whether vaccination against rotavirus affected the process of establishment of the intestinal microbiota in infants at the time of vaccination.

Although the limited number of infants does not allow establishing firm conclusions, our PCR-DGGE and metagenomic analyses of the intestinal microbiota of the three infants participating in the study suggest that rotavirus vaccination has no significant effect upon the establishing gut microbiome. In general, our results on microbial composition are in the range of previously reported data for healthy neonates[26–30]. A significant presence of bifidobacteria (*Actinobacteria*) was evidenced in the faeces of the two breast-fed infants participating in the study whilst the levels of this microbial group were lower in the formula-fed infant, who presented higher levels of *Proteobacteria* than the breast-fed babies during the first months of life. This is in contrast to the dominance of *Bacteroidetes* and *Firmicutes* occurring in adults[31–34]. In spite of the microbiota changes over time we found clearly differentiated individual profiles, with the samples obtained at different times for each infant clustering together. This suggests a predominant role of the individual over the age on determining the gut microbiome during the first months of life and supports data previously obtained by 16S rDNA sequencing showing high inter-individual microbiota variability in neonates[29].

In this area, most of the studies performed so far have used 16S rRNA gene-based analyses of the intestinal microbiota of full-term newborns but only a few works have assessed the total metagenome composition in these infants[10,20,21]. To this regard, our results shed some light on the still limited knowledge of the gut microbiome establishment process, indicating that the inter-individual microbiome shows higher similarity at functional than at microbial composition level. This is also supported by the limited inter-individual variability in faecal metabolites such as SCFA.

To sum up, although the limited sample size precludes establishing definitive conclusions, the present work underlines a high inter-individual variability in the gut microbiota composition and evolution at early life. However, this relatively large microbial community diversity renders few differences between the infants' microbiomes at functional level. Moreover, the individual microbiome establishment and development process seems to occur in a defined manner during the first stages of life and it is not affected by oral rotavirus vaccination.

Methods

Volunteers. The study was approved by the Regional Ethical Committee of Asturias Public Health Service (SESPA) and carried out in accordance with the approved guidelines of the Ethics Committee. Written informed consent was obtained from the parents. The study included three Caucasian male infants. Infant 2 was delivered by caesarean section and received mixed feeding (breast-milk and infant formula) whilst the other two infants (1 and 3) were vaginally delivered and exclusively breast-fed. The three infants received the rotavirus vaccine "RotaTeq®" (Sanofi Pasteur MSD, Lyon, France) which contains attenuated viruses and it is orally administered in three doses during the first six months of life. Infants 1 and 2 received the three doses at 7–8, 11–12 and 15–16 weeks of life, whereas infant 3 received them at 8, 16 and 24 weeks of life.

Faecal Sample Collection and DNA extraction. Faecal samples were collected the day before and the day after (between 24 and 48 hours) the administration of each dose of vaccine. Fresh faecal samples were immediately frozen. For DNA extraction faecal samples were weighed, diluted 1/10 in sterile PBS solution, and homogenized in a LabBlender 400 stomacher (Seward Medical, London, UK) at full-speed for 4 min. The homogenate (1 mL) was then centrifuged and the supernatant obtained was filtered and frozen at −20°C for SCFA analyses. The DNA was extracted from the faecal pellet using the QIAamp DNA stool kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's specifications as previously described[14]. The extracted DNA was kept frozen (−70°C) until analysis.

SCFA Analysis. The analysis of SCFA was carried out in a chromatographic system composed of a 6890N GC (Agilent Technologies Inc., Palo Alto, CA, USA) connected to a FID and a MS 5973N detector as described previously[14].

Denaturing Gradient Gel electrophoresis (DGGE). The profile of the dominant microbial populations in faeces at the different sampling points was determined by PCR-DGGE. PCR-DGGE reaction mixture, conditions and universal primers

Figure 3 | Main faecal microbial groups at genus level (A) and gene functional annotations at KEGG level 2 (B) determined from the metagenomic analyses of the samples obtained at different time points from each infant.

previously described (357F; TACGGGAGGCAGCAG and 518R; ATTACCGCGGCTGCTGG) were used[35]. PCR products were separated by DGGE in a DCode system (BioRad Laboratories) in a 30% to 60% gradient of urea-formamide in Tris-Acetate-EDTA (TAE) buffer (pH8). Selected bands were excised from gels and submitted to a new PCR reaction with the same PCR-DGGE primers without the GC clamp. After purification, the amplified PCR products were sequenced in a capillar ABI3730XL DNA Analyzer (Macrogen Europe, Amsterdam, Netherland) and partially identified by comparison (BLAST) with data held in the GenBank database[35].

Metagenomic analyses. *DNA Sequencing*. The extracted faecal DNA (5 μg) was precipitated by standard sodium acetate/ethanol precipitation, submitted to Zhejiang California International Nanosystems Institute (ZCNI, Hangzhou, Zhejiang, China) for library processing and sequenced at BGI (Shezhen, China). Three out of the eighteen samples did not render enough DNA, for the other fifteen samples the libraries were constructed (500 bp insert size, with adapter) and a 2 × 101 Pair-End sequencing strategy was carried out in a Hiseq 2000 sequencing platform (Illumina). CASAVA-1.8.2 was used for base calling with default parameters except –mismatches 1 -mask y100n, I6n, Y10n – adapter-sequence.

Table 1 | α-diversity and gene richness obtained at the different time points analysed for the three infants studied. α-diversity and gene richness were calculated based on genus composition and gene abundance, respectively

Infant	Sample	α-diversity			Gene Richness
		Shannon	*Simpson*	*Chao1*	
1	*1*	0.87	0.43	89	43951
	2	1.48	0.75	120	40418
	3	1.44	0.64	147	50516
2	*1*	0.74	0.34	103	29781
	2	0.74	0.35	98	32038
	3	1.14	0.52	107	38032
3	*1*	0.98	0.51	130	43159
	2	0.92	0.44	99	39589
	3	0.88	0.42	128	43023

Raw Data Processing. The quality control on the pair-end sequenced reads was conducted using the following criteria; i) reads with more than 3 ambiguous bases (N) were removed, ii) reads with adapters' sequences at both ends were discarded, iii) if in one read more than 50 bases presented low quality (Q2) the read was discarded, iv) no more than 15 bases at 3′ end of reads would be trimmed if ambiguous bases or bases with low quality (Q2) occurred and v) when one of the paired-end reads did not passed the control, although its mate may have passed, then the whole pair was discarded.

Assembly, Gene prediction, gene set construct and annotation. We use Soap *De novo* version 2.04 to perform a genome assembly[36] and Glimmer 3.02 to predict genes[37]. To construct a non-redundant gene set the method introduced by Qin and co-workers[32] was used. Non-redundant genes were annotated by KAAS system with SBH mode[38].

Abundance profiles of organisms and genes. SOAP aligner version 2.21 was used for aligning the pair-end reads against reference genomes and non-redundant genes (parameters "-r 2 -M 4 -m 100 -x 2000"). Then, the methods described by Arumugan and co-workers[33] were applied to generate abundance profiles of microorganisms and genes.

KEGG Function abundance profile. Each gene was assigned into only one KEGG orthologous group, then the abundance of genes was accounted as abundance of their common unique KEGG orthologous. When a KEGG orthologous belonged to different KEGG functional features at B level or C level its abundance was added to all the relative KEGG functional categories to which the orthologous belonged.

α-diversity and gene richness calculations. Chao1, Shannon and Simpson indices were determined with the *R* program package "vegan" (Oksanen *et al.* 2013, Vegan: Community Ecology Package 2.0-10. http://cran.r-project.org/web/packages/vegan/index.html). To calculate gene richness we parsed the gene abundance profile and then counted number of observed genes as gene richness.

β-diversity calculations. We used Spearman correlation distance as our β-diversity determination, first we calculated samples's spearman coefficient S, then distance matrix M was calculated by 1 - S. This was determined with the following command

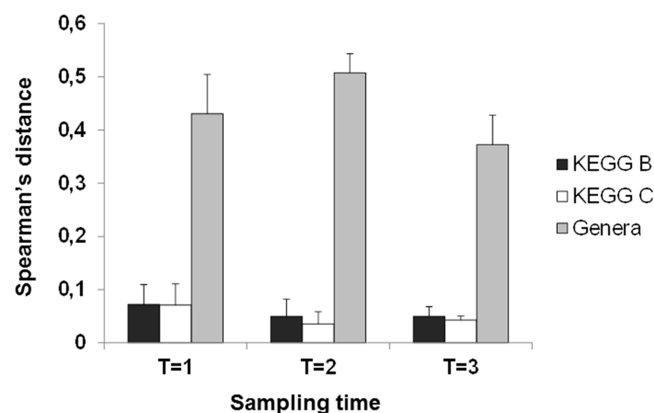

Figure 4 | Spearman's distances (mean and sd) obtained by comparing data at functional KEGG level B, functional KEGG level C and microbial composition (genus level) from all the samples from the different infants at each time point.

"<- 1 – cor (X, method = "spearman")" in *R*, where X is profile of KEGG or Genera composition. We used Wilcoxon rank sum to test if two distance sets differs from each other. PCoA analysis was performed by *R* library "ade4"[39].

Nucleotide sequence accession numbers. The raw sequences reported in this article have been deposited in the EMBL European Nucleotide Archive (accession number PRJEB6972).

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Author contributions

G.S., C.G.R.-G. and M.G. designed the study. G.S. and M.S. recruited the volunteers and collected the samples. L.A., S.A., G.L., Y.C. and Q.N. conducted the metagenomic, PCR-DGGE and SCFA determinations. L.A. and M.G. evaluated the results and wrote the manuscript. All authors discussed the results, reviewed and accepted the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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