Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This very-well written paper is investigating the differences in the infant gut microbiome associated with mode of delivery (Cesarean section versus vaginal delivery). By using shotgun metagenomics, the authors describe the nicrobiome differences at multiple levels including the taxonomic composition, overall functional potential, and strain-level functional traits. Moreover, the study is supported by immunological assays (cytokine profiling), isolation of LPS from stool and tests in human cell lines. Overall the approach is sound and complete.

The main strengths of the paper are the combination of sequencing and functional profiling and the rigorous computational analysis, whereas the main limitation is the rather small sample size (12 mother-neonate pairs only 4 of which represent vaginal deliveries).

My main comments are listed below.

1. I think the sample size here is a bit small (12 mother-neonate pairs). Other studies of the infant gut microbiome are much larger, in the order of hundreds of infants such as PMID 28112736 and PMID 25974306. The problem of the small sample size is that some statistically significant differences in the functional potential could be driven by some specific strains enriched by chance in one of the considered groups. Can the authors test their conclusions using the data from PMID 28112736 and PMID 25974306? If their hypotheses are confirmed in these other two studies I think that the message would be much more statistically supported.

2. The main point of the paper is that birth mode (Cesarean against natural) influences the infant microbiome. However, other reports such as PMID 25974306 conclude that birth mode plays only a secondary role into the shaping of the gut microbiome. I think it is necessary that the authors directly deal with this partially conflicting theory, especially because this might again be related with the limited sample size.

Reviewer #2 (Remarks to the Author):

In this manuscript, Wampach et al test the hypothesis that microbes from mothers colonize their neonates, and then evaluate the metabolic functions encoded by the microbes that colonize the neonates. They compare the composition of neonates born by C-section vs. Vaginal delivery. The identity LPS, GAG degradation and other glycan degradation genes as being differentially abundant in VD neonates and find that stimulating primary human immune cells with LPS from VD neonate feces induces higher expression of TNF-alpha and IL18. These cytokines are also found to be more abundant in neonates born by VD compared to CSD. It does not appear that the authors controlled for potential confounders in this analysis, such as maternal exposure to antibiotics in the perinatal time period. The authors conclude that C-section influences mother-to-neonate microbe transmission and that this has immune-stimulatory effects with “likely effects” on human physiology later in life.

In general, the authors use appropriate and standard methods for sample collection from this small cohort, as well as appropriate methods for DNA extraction and data analysis. The method to remove “artifactual” reads is unusual based on my reading of work in this space, but as I am not an expert in neonate microbiome analysis, it is difficult for me to comment. Slightly older infant fecal samples typically have more than enough DNA, and thus are not low biomass. The

manuscript is, in general, well written, and the methods are well explained. The figures, though very dense, are of high quality.

Major concerns:

1) Recent manuscripts, notably Chu et al (Aagaard group) have put into question the model that bacterial strains are passed vertically from mother to child. Additionally re-analysis of existing data sets by Katie Pollard's group have done the same. This should be brought up in the introduction as this is a relevant, current, and very active and important debate in this field. Importantly, no data are provided regarding antibiotic exposure for the mothers or neonates. Maternal antibiotic exposure is probably a major confounder in these types of analyses, and should be explored. This should be possible as the authors have stated that they have a very rich and complete set of clinical metadata. Multivariable analysis are likely indicated to evaluate the impact of birth method on the questions at hand, especially as it relates to LPS production, for example.

2) The authors design a method that removes "artifactual" reads - this is useful for low biomass samples, but are fecal samples from neonates truly low biomass? Input DNA amounts for sequencing strategies using the Nextera prep, for example, require only 1ng of input DNA. I think the method the authors developed is reasonable, but it is interesting to note that most of the manuscripts that I have read on the topic of neonatal microbiome sequencing have not treated neonatal fecal samples as "low biomass". Does the removal of these "artifacts" result in dramatically different interpretations of the results?

Also, Regarding the choice of the artifact control sample, I found it interesting and unusual that Caco-2 cells were used - typically blank water controls are used for the controls, as mammalian cell culture likely is bacterially-contaminated. I find this choice rather unconventional.

3) In general, it would be nice if the authors pointed to the tools they use for "binning" (line 74), and identifying "strain-determining variant patterns" (line 75) in the actual manuscript. Also, how did they "reconstruct" genomes - they mention MEGAHIT in the methods, but it would be nice to cite this in the actual manuscript. Many of the references in the bioinformatic analysis section of the main manuscript appear to be incorrect or misplaced to me: for example, Ref 17 on line 74 appears incorrect (this referenced paper is not a binning tool as is implied by the location of the citation). Also, Ref 18 on line 75 appears to be incorrect. PhyloPhlAn is not a strain-determine variant pattern tool, as is implied by the location of the citation. In reading the methods, it is clear that the team engaged an experienced bioinformatician. It would be good if this bioinformatician carefully read over the main manuscript and checked all citations.

4) The inclusion of a significant proportion of infants who were small for gestational age (SGA) may have skewed results substantially. These are not healthy, usual, full-term births and are likely biological outliers. What is the justification/reasoning for inclusion of this group in the study? It was not clear to me.

5) For the LPS extraction, how did the authors account for samples where adequate LPS could not be extracted? Are we to conclude that LPS was absent? Did the authors measure the amount of LPS in the samples using methods such as targeted Mass spec? I am concerned that the size of the cohort studied and the limitations of not being able to extract LPS from all samples limits the generalizability of the findings presented. Also, why would babies born by VD have "higher immunostimulatory potential" than those born by CSD? A discussion of the expected impact of this observation would be helpful. Also, in the methods, it seems that of the 13 neonatal fecal samples, only 11 produced measurable quantities of LPS. In the main manuscript, I read this section as suggesting that 13 samples produced measurable quantities of LPS. Were samples that did not produce LPS from the VD, CSD or CSD+SGA cohorts? Why were only some of the total samples available for LPS extraction?

Given the very small n here, it seems that the results are depicted are of limited strength and may be very susceptible to over-interpretation.

Furthermore, based on Figure 4, it appears that Escherichia is highly abundance in all but one VD individual. Has this been described in other studies? Is this what is driving the LPS abundance in these samples? If so, this is worth discussing in the manuscript and putting into context for the readers. I would read the figure as Escherichia species being highly abundant in the neonatal microbiome of VD infants and that this may have consequences for LPS production. Is the E. coli that is present in these individuals the strain(s) that is from the mother? Based on Figure 3a, it does not appear so, as best as I can tell. So is one to conclude that VD neonates have a high abundance of E. coli that produces immunostimulatory LPS, but that the likely producer of this LPS (E. coli, which is abundant), is not vertically transmitted? If this interpretation is correct, I am not sure how the two main thrusts of the manuscript link to one another. Another area of confusion for me is EDFig 2C. In the neonatal stool from day 3, which I believe was used for the LPS extraction, I do not see many samples that have abundant Escherichia, as would be expected from Figure 4 - in fact, the samples that were chosen from the VD cohort for the LPS study appear to be the only two VD samples that have detectable E. coli. This may be skewing the results.

6) How was the LPS concentration and purity determined for each LPS sample that was extracted from feces determined? The effects that they saw may have to do largely with purity of the LPS as opposed to structural differences / immunogenicity of the LPS. I would be more convinced of the argument they are trying to make if each of the LPS samples had been subjected to mass spectrometry to evaluate purity, for example. I simply worry that the LPS extraction procedure is, in itself, very flawed and subject to error. And with such a small n, the results may not be terribly robust, even if intriguing.

Minor:

1) Not enough detail in the manuscript - how were differentially abundant functional categories from KEGG identified? I appreciate the strict space limitations, but some information regarding method should be included in the manuscript.

2) The authors refer to how the “early microbial functions in VD neonates reflected the mothers’ functional microbiome profiles” on line 86-87, but they have not yet introduced any analysis of the mother’s microbiome. This is confusing to me. Also, are they looking at the maternal vaginal microbiome or the maternal stool microbiome (both of which have been correlated with the neonatal microbiome). Did they look at the neonatal fecal microbiome and the maternal skin microbiome to compare functional potential? This would be suggested by Dominguez-Bello’s paper from PNAS 2010.

3) The methods applied in Supplementary note 3 (to determine if birth method affected the microbiome) are not adequately explained and do not appear to be statistically driven. What is presented is not adequate to conclude that “the different feeding regimes of neonates did not explain the functional differences …” on lines 94-95.
4) It seemed like the LPS biosynthesis gene was “cherry-picked” from a list of significantly differentially abundant genes. Also, what as the FDR cutoff? Why was this gene chosen? What were the most significantly differentially abundant genes?

5) GAG and other glycan metabolism is mentioned in the abstract but is only addressed in the supplement. If this is an important point, it should be included in the main manuscript. If not in the main manuscript, it should be left out of the abstract.

6) When cytokine levels were obtained from 31 neonates, it seems inappropriate to label the 14 additional infants as an “independent validation cohort” if the results are aggregated with the

discovery cohort. This is misleading to me.

7) The reference citation format appears to change at line 404. This is a small typographical issue.

Reviewer #3 (Remarks to the Author):

In this interesting research paper by Wampach et al, authors used a very robust bioinformatics approach to track specific bacterial strains from mothers to neonates during the perinatal period, taking into account levels of genetic diversity using well-established molecular ecology methods. This study has numerous strengths that are original and should be published: it combined taxonomic and functional profiling of the microbiome using reference independent metagenomics analysis, included controls and a bioinformatic approach that accounted for reagent and host derived contaminant DNA and removal of sequencing artifacts, and utilized sequencing data to inform the experimental parameters of an immune assay, which was informative of the potential interactions between LPS and the immune system. However, these strengths are not the main claim of this study. This clinical study was not designed nor was it shown to be powered to determine perinatal microbiome differences driven by mode of birth, and its results should not be interpreted as such. Below is a list of major and minor suggestions that should be incorporated in a new version of this manuscript.

Major comments

1. The sample number in this study is simply too low and there were no power calculations performed a priori to determine if it could identify difference between birth modes. Given that other small studies have failed to show microbiome differences driven by birth mode, it is hard to believe that this study was powered to assess the influence of birth mode, let alone the influence of low birth weight. I recommend that authors present power calculations based on previously published data to determine this. A good resource to perform these calculations for studies with multivariate data is MetSizeR. Clearly, the results from this study are in line with most other studies that have convincingly shown that mode of birth drives temporal neonatal composition and diversity. However, a recent study from the Xavier group (DOI: 10.1126/scitranslmed.aad0917) showed that while most CS births were associated with lower Bacteroides species abundance, about 20% of the VD babies displayed a similar pattern. With an N=4 per group (including 1 set of twins in the CSD groups) with only 2 valid time points (Day 1 only had 2 samples in 2 of the groups, so in my opinion should be excluded for comparisons), there is simply no statistical power to detect appropriate variance of a given population, especially considering the interindividual variability of the gut microbiome during the first days of human life.

2. Although differences between groups were detected using appropriate statistical tests, without confidence intervals and proper statistical tests to deconfound the effect of birth mode from other variables known to influence microbiome composition, it is not possible to properly assess whether birth mode truly drives these differences. Given that authors have access to 20 more samples (6 for which they had 16S data and 14 from a previous cohort that was collected in a similar way), the authors could use the 16S data and PICRUSt, at a minimum, to determine if other variables are also influencing the associations found between birth mode and microbiome composition. An excellent tool for this purpose is MsAsLin (https://huttenhower.sph.harvard.edu/maaslin). Variables to include in this model are gestational age, antibiotic use, dose, duration, time of meconium passage, if babies ingested colostrum, etc. In addition, authors should demonstrate that the samples used for metagenomics analysis are representative of the larger group of samples. For this, they could compare microbiome characteristics (beta or alpha diversity) as well as clinical variables (mode of birth, gestational age, etc.)

3. The results from the immune assay with extracted LPS from different neonatal samples are quite compelling but remain rather preliminary, and could be enhanced in several ways. As it stands, the purity of the LPS was not assessed, it is vaguely assumed that the majority of the cytokine response measured originated from E.coli LPS, and although it is mentioned in the text, it is not known if the differences in cytokine response are due to differences in LPS structure. Addressing at least 2 of these issues will significantly strengthen this paper. LPS purity can be determined by gel electrophoresis (comparing silver, protein and DNA staining, for example), or if available, mass spectrometry analysis. Differences in LPS structure (rough vs smooth, for example) can also be obtained through these methods. LPS purity can also be confirmed functionally by showing that the purified products did not activate Toll-like receptor 2 (TLR2), nuclear oligomerization domain 1 (NOD1), or NOD2 but did activate TLR4.
Importantly, if the authors have more samples from other cohorts available, why not increase the number of samples from this assay?
As for determining the origin of the LPS, could LPS genes not be assigned to the mOTUs given that the information is available? This is important as it will inform which taxa may or may not be relevant in stimulating immune cells during the first days of life.

4. Extended data figure 2. Change colour palette. Too many tones of blues and greens. Don't do colour coordination according to phyla.

Minor comments

1. Re. blinding in Life Sciences reporting summary: "As assigned study groups were predefined prior to delivery, blinding was irrelevant to our study." This is false. Many studies with predefined study groups are blinded and this adds strength to the study design. This study was simply non blinded, please change.

2. FIGURE 2: It is unclear how the DESEQ2 generated results are displayed in Figure 2. Deseq2 compares 2 groups. According to Figure 2a it looks that the comparison was made between VD samples and all CSD samples combined but figure legend and methods section describes two separate comparisons. Were the same 5 pathways differential in both comaprisons or only when VD samples were compared to all CS babies?

3. FIGURE 4: The figure legend does not fully explain 4a. Specifically, if was only after I ready the main text that I understood that the EU amounts were measured in the immune assay. I was confused and initially thought it was the LPS concentration used in the assay.

4. Line 87, vaginal or stool in mothers?

5. Line 94-96 briefly mention what statistical method was used (if any) to rule out the influence of feeding method

6. Lines 99-105 Where there any comparisons done between VD samples and each of the CSD groups. If so, this should be mentioned in this section. If not why separate the CSD groups?

7. Line 144, sources of microbial origin are too speculative, I suggest removing

8. Line 174 I suggest changing "more specifically" for "especially"

9. Line 202-203 Huge stretch here. There is no data on chronic diseases in this super small cohort. Also omit from the summary figure in extended data.

10. Lines 483-487 Unclear why bacterial DNA amount needed to be controlled in the LPS assay.

Please elaborate either here on in the Supplementary notes. These assays conditions will not mick the amount of LPS that interacts with a peripheric immune cells so I do not understand the reasoning behind this. Why not normalize by LPS units only? DNA quantification via qpCR of 18S RNA gene will vary depending on the number of 16S copies per cell so it is not the best way to quantify bacterial load. Further, this method will account for all bacterial cells, not just gram-negative (LPS containing cells). Flawed method.

11. Line 124 of Supplementary notes: I would change “corresponded” to correlated

12. The manuscript could use a brief discussion on the choice of adult blood DCs and how it may have differed from neonate peripheric immune cells. Neonatal immune cells are known produce immune responses similar to adults in some aspects but not others.

Reviewer # 1

Overall summary

1.1. This very-well written paper is investigating the differences in the infant gut microbiome associated with mode of delivery (Caesarean section versus vaginal delivery). By using shotgun metagenomics, the authors describe the microbiome differences at multiple levels including the taxonomic composition, overall functional potential, and strain-level functional traits. Moreover, the study is supported by immunological assays (cytokine profiling), isolation of LPS from stool and tests in human cell lines. Overall the approach is sound and complete.

The main strengths of the paper are the combination of sequencing and functional profiling and the rigorous computational analysis, whereas the main limitation is the rather small sample size (12 mother-neonate pairs only 4 of which represent vaginal deliveries).

Response:

We appreciate the reviewer's recognition of the quality and comprehensiveness of the study. In light of the editor's and reviewers' comments, we have analyzed additional 16S rRNA gene amplicon and metagenomic sequencing data from collected samples that cover the first 5 days *postpartum* (please refer to the table below for an overview on the additional data that has been included in the revised manuscript; Supplementary Data 1). Please also refer to our reply to **comment 1.2.** below for a more detailed description of the additional samples collected as well as the additional analyses performed. While the increased number of samples underscores the statistical analyses, we want to emphasize that the main thrust of the manuscript is not solely statistics-driven. Given their high degree of specificity, the presence of transferred strains is relevant only on an individual, sample-to-sample basis in relation to resolving mother-to-neonate transfer. In particular, using our high-quality data, we are able to resolve the transfer of strains and linked functional repertoire on a personalized level at the required level of specificity. This in turn is necessary to address questions of mother-to-neonate transfer. Please also refer to our detailed response to **comment 3.2.** for more information. Important to stress is that the results from the analysis of the additional data support our original conclusions.

	Total number of samples	Total number of neonates	Study group composition
Initial dataset	130	18	7 VD, 6 CSD, 5 CSD + SGA
Updated dataset	**176**	**33**	**15 VD, 13 CSD, 5 CSD + SGA**

Major comments

1.2. I think the sample size here is a bit small (12 mother-neonate pairs). Other studies of the infant gut microbiome are much larger, in the order of hundreds of infants such as PMID 28112736 and PMID 25974306. The problem of the small sample size is

that some statistically significant differences in the functional potential could be driven by some specific strains enriched by chance in one of the considered groups. Can the authors test their conclusions using the data from PMID 28112736 and PMID 25974306? If their hypotheses are confirmed in these other two studies I think that the message would be much more statistically supported.

Response:

We thank the reviewer for the relevant comment. While other studies with a focus on the neonatal gut microbiome have involved apparent larger (albeit uneven for VD and CSD) sample sizes, these studies have not involved the systematic collection and appropriate preservation of paired mother and infant samples along the timeline of our study or have not included very early neonatal samples. However, the collection of such samples is essential to track differences between earliest microbiome colonization in relation to delivery mode and to resolve potential vertical transmission of specific strains and encoded functions from mothers to neonates. In other words, only if the same sample types (i.e. stool) and collection time points (i.e. prior to delivery for the mother and shortly *postpartum* for the neonate) are matched per mother-neonate pair, one can objectively assess the vertical transfer of gut strains from one mother to her respective neonate.

With reference to the studies cited by the reviewer in relation to potentially validating our findings, PMID 28112736 presents only 2 out of 49 mother-infant pairs, for which stool samples were collected from both mother and neonate shortly after birth. In both pairs, infants were delivered vaginally, which makes the assessment of the delivery mode effect on strain transfer impossible. On the other hand, PMID 25974306 presents 26 out of 98 mother-infant pairs for whom maternal stool samples were collected at the day of delivery. In fact, previous analyses by Nayfach *et al.* (PMID 27803195) based on the data from PMID 25974306 were able to detect strain transmission from mother to neonate in case of vaginal delivery. However, they were unable to resolve any strains from the samples collected from CSD mother-neonate pairs. Consequently, the effect of delivery mode in relation to strain transfer could not be assessed properly. For our revised manuscript, our findings were put into context with regards to these relevant previous studies especially within the extended text limits of a *Nature Communications* article.

Introduction, manuscript lines 48 to 51: During vaginal birth, specific bacterial strains are transmitted from mothers to infants[3–6] and differences in microbial colonization in neonates born by CSD have been identified[7–10] as early as 3 days postpartum[7,10].

Introduction, manuscript lines 90 to 99: At the same time, several studies have hypothesized that CSD impedes the vertical transfer of strains from mother to neonate during delivery[3,4,18,25]. In addition, although single nucleotide variants (SNVs) have been tracked over time, no such studies have so far covered the earliest time points after delivery (days 0-5) in well-matched mother-neonate pairs with respect to a direct

comparison of delivery modes. Consequently, there is a strong need for adequate high-resolution metagenomic analyses capable of resolving the vertical transmission of individual-specific strains and encoded functions from mothers to neonates on an individual basis, while also supplementing observed *in silico* findings with further *in vitro* validation experiments.

Discussion, manuscript lines 444 to 454: While previous studies have used analogous analytical approaches (16S rRNA gene amplicon sequencing and metagenomics) to resolve the early neonatal gut microbiome, they have not involved the systematic collection and appropriate preservation of paired mother-neonate samples[11], they did not specifically track vertical strain transfer[11,18], they did not include provisions for the removal of artefactual sequences[3,5,6,11,18], they did not focus on the earliest time points after delivery[3,18], nor did they analyse differences of functional potential according to delivery mode[3,5,6,11,18]. However, consideration of these factors is essential to assess the effect of delivery mode on the earliest transfer of community structure and function, subsequent microbiome colonization patterns and the resulting implications for neonatal physiology.

In summary, the publicly available data is not appropriate for validating our findings. In the vast majority of cases, the sample type and collection time point for both mothers and neonates are not properly matched, which makes the assessment of strain transfer and the linked transfer of functional potential impossible. Additionally, although strain transfer was analyzed in previous studies, no effect of delivery mode could be assessed due to a lack of neonates delivered by C-section.

In order to address the reviewer's valid concern regarding the overall sample size, we have performed analyses of additional 16S rRNA gene amplicon and metagenomic data from additional mother-neonate pairs (see table above under **comment 1.1**; Supplementary Data 1). Moreover, we have performed additional analyses assessing the physiological effects of the observed functional differences between CSD and VD neonates (Fig. 4a; Supplementary Fig. 11 and 12; Supplementary Data 12 and 13). The results of these analyses support our original conclusions, especially with regards to the significantly increased relative abundance of Gram-negative bacteria in early faecal samples of VD neonates as well as the significantly increased functional potential of the earliest VD gut microbiome with respect to LPS biosynthesis.

Results, manuscript lines 252 to 258: To corroborate the apparent higher propensity of the VD microbiome for LPS biosynthesis, we annotated the OTUs resulting from the 16S rRNA gene amplicon sequencing data according to their attributed Gram staining information. Hereby, we observed that the gut microbiomes of VD neonates harboured significantly higher relative abundances of Gram-negative bacteria at days 3 and 5 compared to CSD (±SGA) neonates (Wilcoxon rank-sum test, FDR-adjusted $P = 1.7 \times 10^{-3}$ and $P = 4.0 \times 10^{-3}$ for day 3 and 5 respectively; Supplementary Fig. 3b).

1.3. The main point of the paper is that birth mode (Caesarean against natural) influences the infant microbiome. However, other reports such as PMID 25974306 conclude that birth mode plays only a secondary role into the shaping of the gut microbiome. I think it is necessary that the authors directly deal with this partially conflicting theory, especially because this might again be related with the limited sample size.

Response:

We thank the reviewer for this comment. The results of our study demonstrate that early differences exist in the gut microbiomes of neonates born vaginally or via caesarean section, and that these differences may impact early immune system stimulation. Our findings do not conflict with the results of the study highlighted by the reviewer as the authors of that study found, similar to our own results, that the gut microbiome of vaginally delivered infants exhibits significantly greater resemblance to the mothers' microbiome when compared to infants delivered by C-section (the authors also found that the microbiome is shaped by nutrition; such differences are however only apparent outside of the time window of our study). Our study adds essential new elements to these earlier observations by describing the direct transfer of specific strains from mother to infants in the context of vaginal delivery, the differences in functional potential of the earliest microbiome conferred by these strains and the potential physiological repercussions of these differences during the first days of life. Thereby, our results do not sit at odds with previous observations but expand on these and provide new mechanistic insights into the physiological repercussions of a lack of transfer of specific microbiota from mother to infant in the case of C-section. For the revised manuscript, we have included the reviewer's suggested references in order to properly situate our study and results amongst the currently partially conflicting results from other studies. In this context, we highlight previous work, inconsistencies and general trends in the field of neonatal gut microbiome colonization.

Introduction, manuscript lines 51 to 55: However, due to conflicting results, which principally imply a negligible impact of delivery mode on the colonizing neonatal microbiome in the gut[11], it remains unclear whether disruption of mother-to-infant transmission of microbiota through CSD occurs and whether it affects human physiology early on, with potentially persistent effects in later life.

Introduction, manuscript lines 65 to 76: While the majority of studies so far indicate that delivery mode is the strongest factor determining early neonatal gut microbiome colonization[3,7–10,18], these effects are either extenuated or largely absent in other studies[11,19]. Nevertheless, the possibility of microbial transfer from mother to neonate during vaginal delivery cannot be excluded in studies which have reported a negligible effect due to delivery mode[11]. In this context, it is important to consider that CSD may be performed as a result of underlying maternal or foetal medical

conditions (e.g. multiple gestation, foetal malpresentation or suspected foetal macrosomia)[20] and can co-occur with other microbiome-influencing factors. More specifically, CSD is most often accompanied by the administration of antibiotics to mothers due to local health regulations or hospital practices (e.g. in case of a positive screening of the mother for group B *Streptococcus*)[21].

Reviewer # 2

Overall summary

2.1. In this manuscript, Wampach et al test the hypothesis that microbes from mothers colonize their neonates, and then evaluate the metabolic functions encoded by the microbes that colonize the neonates. They compare the composition of neonates born by C-section vs. Vaginal delivery. The identity LPS, GAG degradation and other glycan degradation genes as being differentially abundant in VD neonates and find that stimulating primary human immune cells with LPS from VD neonate faeces induces higher expression of TNF-alpha and IL18. These cytokines are also found to be more abundant in neonates born by VD compared to CSD. It does not appear that the authors controlled for potential confounders in this analysis, such as maternal exposure to antibiotics in the perinatal time period. The authors conclude that C-section influences mother-to-neonate microbe transmission and that this has immune-stimulatory effects with "likely effects" on human physiology later in life.

In general, the authors use appropriate and standard methods for sample collection from this small cohort, as well as appropriate methods for DNA extraction and data analysis. The method to remove "artefactual" reads is unusual based on my reading of work in this space, but as I am not an expert in neonate microbiome analysis, it is difficult for me to comment. Slightly older infant faecal samples typically have more than enough DNA, and thus are not low biomass. The manuscript is, in general, well written, and the methods are well explained. The figures, though very dense, are of high quality.

Response:

We appreciate the reviewer's recognition of the main message of the study as well as the overall high quality of the manuscript. In relation to the reviewer's comment on potential confounders, we have performed additional multivariate analyses (using the multivariate tool MaAsLin), in relation to maternal antibiotics intake, day of sample collection, gestational age, delivery mode and feeding regime. The results of these additional analyses demonstrate that the maternal intake of antibiotics may have a small effect on the neonatal gut microbiome composition but that the observed fundamental changes in earliest community compositions are first and foremost due to the delivery mode. For further information, please refer as well to our response to **comment 2.2.** below.

Results, manuscript lines 194 to 202: In order to resolve the effect of delivery mode from other potential contributing factors such as maternal antibiotic intake prior to delivery, gestational age, feeding regime and sampling time point, differentially abundant taxa for both 16S rRNA gene amplicon and metagenomic sequencing data were determined separately using a multivariate additive general model approach (MaAsLin[35]). Taking into account the effects of the above-mentioned factors, delivery mode was found to be the dominant driver of neonatal gut microbiome colonization, with other measured factors having considerably less of an effect (Supplementary Note 4; Supplementary Data 9).

Results, manuscript lines 260 to 267: A multivariate analysis (MaAsLin[35]) was performed to compare the functional profiles of CSD (±SGA) to VD neonates and for both generated datasets (i.e. predicted KO functional categories based on 16S rRNA gene amplicon sequencing data and annotated KOs based on metagenomic sequencing data). Results from the multivariate analyses demonstrated that delivery mode was the strongest determining factor in both datasets (i.e. predicted and metagenomic-based KOs) for explaining differentially abundant genes (Supplementary Data 9).

Supplementary Information, Supplementary Note 4: The administration of antibiotics to mothers prior to caesarean section delivery is often (e.g. in Luxembourg) mandatory, resulting in delivery by caesarean section and the maternal intake of antibiotics to be commonly coinciding factors. Additionally, vaginally delivering mothers that are positively screened for group B *Streptococcus* infection receive antibiotics prior to delivery to reduce the risk of infections in neonates. For these reasons, most mothers were administered antibiotics in this study, for both delivery modes. As other factors (e.g. day after delivery, gestational age, feeding regime, etc.) have been suggested to impact neonatal gut microbiome colonization as well, we used a multivariate analysis (MaAsLin[35]) to identify differentially abundant taxa for both metagenomic and 16S rRNA gene amplicon sequencing data (Supplementary Data 9). After correcting for the respective effects of all the above-mentioned variables for the metagenomic sequencing data, three mOTUs were associated with delivery mode (CSD±SGA) and at the same time maternal antibiotics intake, namely *Bacteroides fragilis* (for all conditions: $Q = 8.1 \times 10^{-3}$), *Bacteroides xylanisolvens* (for all conditions: $Q = 8.1 \times 10^{-3}$) and *Parabacteroides merda* (for all conditions: $Q = 8.1 \times 10^{-3}$). In contrast, when correcting the different factors for the larger cohort screened by 16S rRNA gene amplicon sequencing using MaAsLin[35], delivery mode had a distinct driving effect on the earliest microbiome. More specifically, *Bacteroides* was significantly decreased in CSD±SGA neonates ($Q = 2.6 \times 10^{-3}$ and $Q = 2.9 \times 10^{-2}$) as well as Bacteroidaceae (CSD; $Q = 3.9 \times 10^{-2}$). One OTU of *Escherichia-Shigella* was significantly decreased in CSD±SGA neonates (both $Q = 3.6 \times 10^{-2}$). Two OTUs belonging to *Bacteroides* ($Q = 3.6 \times 10^{-2}$ and $Q = 3.9 \times 10^{-2}$) and two OTUs belonging to *Bifidobacterium* ($Q = 1.6 \times 10^{-2}$ and $Q = 4.8 \times 10^{-2}$) were significantly decreased in CSD neonates while one OTU belonging to *Staphylococcus* was significantly increased in CSD neonates relative to VD ($Q = 2.3 \times 10^{-2}$). Feeding regime was found

to be a potential contributing factor of the relative abundances of *Trichococcus* (formula feeding and mixed feeding regime; $Q = 3.6 \times 10^{-3}$ and $Q = 4.6 \times 10^{-2}$), *Escherichia-Shigella* (mixed feeding regime; $Q = 1.3 \times 10^{-2}$) and one OTU blonging to *Rothia* (formula feeding regime; $Q = 2.9 \times 10^{-4}$). The genus *Proteus* ($Q = 1.0 \times 10^{-2}$) was associated with maternal antibiotics intake and multiple genera and OTUs were associated with faecal samples collected at day 1 postpartum. Although we cannot exclude an effect of maternal antibiotic exposure or minor effects of feeding regime or collection time point on the taxonomic composition of the neonatal gut microbiome, the main differences in microbial taxa in both datasets (16S rRNA gene amplicon and metagenomic sequencing data) were clearly due to delivery mode.

An additional analysis to test for differentially abundant taxa associated with delivery mode was performed using ANCOM[73]. The results further confirmed the significantly decreased relative abundances of *Bacteroides*, *Escherichia-Shigella, Bifidobacterium* and *Parabacteroides* in CSD±SGA neonates, while *Staphylococcus* was significantly increased in CSD±SGA neonate (all these results were based on the statistical analyses based on Wilcoxon rank-sum tests, as well as the multivariate analyses). Our results demonstrate that although several minor trends inside the earliest neonatal gut microbiome were associated with distinct neonatal or maternal factors, the observed fundamental changes in earliest community compositions were first and foremost due to the delivery mode.

Discussion, manuscript lines 477 to 480: Our results based on both 16S rRNA gene amplicon and metagenomic sequencing, and supported by multivariate analyses, demonstrate that early differences exist in the gut microbiomes of neonates and that these differences are predominantly driven by the mode of delivery.

Regarding the study design and employed methods, we would like to emphasize that our study involved the systematic and careful collection of high-quality samples from the first days of life. While indeed not routine, the failure of removing artefactual reads may lead to serious flaws in interpretation, particularly when working on samples containing a low microbial biomass. In the absence of appropriate controls, sequences derived from contaminant taxa in reagents may be relatively prominent, mask actual signals from taxa present and confound results regarding the presence of actual taxa (doi:10.1038/nature.2014.16327; doi:10.1186/s12915-014-0087-z; doi:10.1371/journal.pone.0110808; doi:10.1038/d41586-018-00664-8; our own recent work on contaminant RNA in low-biomass samples: doi:10.1186/s12915-018-0522-7). Failure to remove contaminants may in fact be partially responsible for the conflicting results in earlier studies, especially in relation to apparent mother-to-infant transfer. We are confident of the need for the applied methodological workflow especially in relation to the removal of contaminant sequences and we addressed this more specifically in the discussion of the revised manuscript.

Results, manuscript lines 153 to 180: To account for the presence of artefactual sequences in the metagenomic data, we devised an additional, combined *in vitro* and *in silico* strategy to identify and remove artefactual sequences from the metagenomic data (Fig. 1a). For the *in vitro* part, DNA was extracted from a human gut epithelial cell line using the same procedure as for the neonatal stool samples and diluted to the levels of DNA extractable from the collected low-biomass samples (Methods). The choice of human DNA as a negative control was based on the following criteria: (i) the inability to generate a sequencing library from blank water control samples due to the inherent very low amounts of DNA (these are typically below the threshold for library construction); (ii) the ability to clearly differentiate signal (in the titration series: human sequences) from artefacts (non-human sequences); microbial DNA was not chosen as the homology between contaminant and *bona fide* sequences may have confounded delineation; (iii) the removal of human sequences is common practice when performing metagenomic analyses on human samples and appropriate methods exist to distinguish between human and microbial sequences *in silico*; (iv) the blinding of the variability originating from the laboratory environment or sequencing facility due to the nature of the samples (i.e. human control samples were treated with the exact same reagents as the faecal study samples). Our *in silico* workflow for the identification and removal of artefacts from metagenomic data (Fig. 1b) first clusters[32] contigs from the artefact control samples and the study samples together (Supplementary Fig. 1a). It subsequently removes contigs from study samples that cluster with the artefactual contigs, i.e. that fall into the same bin (Supplementary Note 1). After subsequent filtering steps and the successful removal of artefactual contigs from all study samples, we observed differences in the number of removed reads according to sample type (Supplementary Fig. 1b; Supplementary Data 2). Based on this essential data curation step, sequences from *Achromobacter xylosoxidans* or *Burkholderia* spp. taxa were for example identified and subsequently eliminated from the *bona fide* metagenomic data.

Discussion, manuscript lines 464 to 477: As earliest neonatal gut microbiome samples are naturally of low biomass, the accurate identification and removal of potential artefactual sequences is essential. In the absence of appropriate controls, sequences derived from contaminant taxa in reagents may be relatively prominent, thereby masking actual signals and confounding results regarding in particular the transfer of taxa and functions from mothers to neonates. In our study, adequate controls were included and putative artefactual reads removed based on a combined *in vitro* and *in silico* workflow. In order to reach the required specificity (and thereby resolution) to unambiguously address the question of vertical transmission of microbial community structure and function from mother to neonate, the use of curated, high-resolution metagenomic sequencing data, rather than solely performing 16S rRNA gene amplicon sequencing, is imperative. More specifically, the applied methodological approach, allows the highly specific tracking of individual microbial functions and strains from mother to neonates on an individual basis.

Major comments

2.2. Recent manuscripts, notably Chu et al (Aagaard group) have put into question the model that bacterial strains are passed vertically from mother to child. Additionally re-analysis of existing data sets by Katie Pollard's group have done the same. This should be brought up in the introduction, as this is a relevant, current, and very active and important debate in this field.

Importantly, no data are provided regarding antibiotic exposure for the mothers or neonates. Maternal antibiotic exposure is probably a major confounder in these types of analyses, and should be explored. Multivariable analyses are likely indicated to evaluate the impact of birth method on the questions at hand, especially as it relates to LPS production, for example.

Response:

We thank the reviewer for this point and agree that the field of neonatal colonization is currently actively discussing some contradictory findings. In accordance with the reviewer's suggestion, we have discussed the previous work, inconsistencies and general trends in the introduction of the revised manuscript. Apart from the issues surrounding the impact of contamination on observed early colonization patterns (see also our detailed response to **comment 2.1.** above), another important consideration for inconsistent results regarding potential vertical transmission from mother to infant and early microbial colonization is the fact that the methods used to date (particularly 16S rRNA gene amplicon sequencing) do not afford the required specificity (and thereby resolution) to unambiguously address this question. In contrast, our methodological approach based on metagenomic sequencing allows the highly specific tracking of strains from mother to neonates on an individual basis, which is necessary to unambiguously link specific organisms from specific mother-neonate pairs. Nevertheless, it is important to stress that the work by Chu et al. (highlighted by the reviewer) made observations which go in the same direction as our results, e.g. that meconium samples harbor OTUs that originate from the maternal gut (doi:10.1038/nm.4272). As these observations were based on 16S rRNA gene amplicon sequencing, it is important to highlight that we were able to expand on these previous suggestions and provide detailed results from corresponding high-resolution metagenomic sequencing data. More specifically, we were able to perform strain-tracking from mothers to infants on a case-by-case basis and assess differences at the level of the functional potential.

Introduction, manuscript lines 83 to 88: Apart from confounding factors, the methods and study designs employed over the past years may in part explain some of the conflicting results regarding the effect of delivery mode on the early gut microbiome. Notably, taxonomic profiling based on rRNA gene amplicon sequencing does not offer sufficient resolution to assess the direct effect of the delivery mode at the level of strain transmission, which is expected to be a determinant of succession.

C-section and maternal antibiotics intake are commonly coinciding factors, also in multiple previous studies. In Luxembourg, the maternal administration of antibiotics in case of C-section delivery is mandatory. Additionally, vaginally delivering mothers that were positively screened for group B *Streptococcus* infection receive antibiotics prior to delivery to reduce the risk of neonatal infection. Collectively, this reflects why most study participants were administered antibiotics in the framework of this study and for either delivery mode. According to the additional multivariate analyses, which we have performed using MaAsLin, delivery mode was the most determinant driver of the neonatal gut microbiome composition. However, when considering the metagenomic sequencing data, three mOTUs, namely *Bacteroides fragilis*, *Bacteroides xylanisolvens* and *Parabacteroides merda*, were associated with both factors, delivery mode (CSD±SGA) and maternal antibiotics intake. Although we cannot exclude an effect of maternal antibiotic exposure on the taxonomic composition of the neonatal gut microbiome, the main differences in microbial taxa in both datasets (16S rRNA gene amplicon and metagenomic sequencing data) are clearly due to delivery mode. Please also refer to the manuscript passages highlighted above under **comment 2.1.**

2.3. The authors design a method that removes "artefactual" reads - this is useful for low biomass samples, but are faecal samples from neonates truly low biomass? I think the method the authors developed is reasonable, but it is interesting to note that most of the manuscripts that I have read on the topic of neonatal microbiome sequencing have not treated neonatal faecal samples as "low biomass". Does the removal of these "artefacts" result in dramatically different interpretations of the results?

Also, Regarding the choice of the artefact control sample, I found it interesting and unusual that Caco-2 cells were used - typically blank water controls are used for the controls, as mammalian cell culture likely is bacterially-contaminated. I find this choice rather unconventional.

Response:

We appreciate the reviewer's points on the need for removing artefactual sequences in case of working on low biomass samples. Given the extended length limitations of *Nature Communications* (in contrast to the original *Nature* Letter format), we have expanded on our rationale for the removal of artefactual reads in the revised manuscript. Briefly, according to our earlier work (doi:10.3389/fmicb.2017.00738), neonatal stool samples from days 1-5 contain between 0.0001 - 10 ng of microbial DNA per mg of stool. As discussed in response to the reviewer's **comment 2.1.** above, low microbial biomass samples are prone to overrepresentation of artefactual sequences, which may explain some of the inconsistencies between previous studies. According to our own results, the removal of artefactual sequences is necessary to resolve actual signals. More specifically, in our datasets, sequences from *Achromobacter xylosoxidans* or *Burkholderia* taxa were found to be prominent in

both low biomass samples and negative controls and these stem from contaminated reagents (doi: 10.1186/s12915-014-0087-z; doi: 10.1007/s10096-016-2644-6). Our protocols allow for the diligent removal of such sequences, which is necessary to resolve actual microbial community compositions and track strains from mothers to neonates.

Given that the influence of artefactual sequences depends on the amount of DNA in the original sample, we performed titrations with human DNA to assess the overall complement of contaminant sequences analogous to previous work (doi:10.3389/fmicb.2017.00738). Please also refer to the manuscript passages highlighted above under **comment 2.1.**

The removal of artefactual sequences was performed across all of the analyzed samples in a consistent manner and this removal was independent of the human sequences, which were generated to identify artefactual sequences. We do not fully understand the reviewer's comment about likely contamination of the mammalian cell culture. Nevertheless, we would like to reassure the reviewer by stressing that the Caco-2 cells from which the human DNA was extracted were maintained in DMEM containing pen/strep. Furthermore, the presence of *Mycoplasma* is routinely monitored in our cell culture lab. Based on these facts and as the metagenomic sequencing did not include any *Mycoplasma* sequences, we can confidently exclude a bacterial contamination of the mammalian cell culture and are confident that our protocol is entirely robust for identifying and removing artefactual sequences.

Material and methods, manuscript lines 708 to 712: Given that the Caco-2 cells were cultured in the presence of 1% penicillin–streptomycin, that the routine surveys for *Mycoplasma* were negative, and that the metagenomic sequencing data did not include any *Mycoplasma* sequences, any bacterial contamination of the mammalian cell culture could be confidently excluded.

2.4. In general, it would be nice if the authors pointed to the tools they use for "binning" (line 74), and identifying "strain-determining variant patterns" (line 75) in the actual manuscript. Also, how did they "reconstruct" genomes - they mention MEGAHIT in the methods, but it would be nice to cite this in the actual manuscript. Many of the references in the bioinformatic analysis section of the main manuscript appear to be incorrect or misplaced to me: for example, Ref 17 on line 74 appears incorrect (this referenced paper is not a binning tool as is implied by the location of the citation). Also, Ref 18 on line 75 appears to be incorrect. PhyloPhlAn is not a strain-determine variant pattern tool, as is implied by the location of the citation. In reading the methods, it is clear that the team engaged an experienced bioinformatician. It would be good if this bioinformatician carefully read over the main manuscript and checked all citations.

Response:

We appreciate the reviewer highlighting the importance for providing full methodological details. All the methodological details were outlined in the Materials and Methods section and in accordance with the requirements of *Nature Communications*, a detailed Materials and Methods section now forms an integral part of the manuscript. Also, we incorporated additional methodological steps that are important for the understanding into the revised main text where necessary. There were indeed two wrong citations in the original manuscript in relation to the bioinformatic analyses, which have been corrected in the revised manuscript.

2.5. The inclusion of a significant proportion of infants who were small for gestational age (SGA) may have skewed results substantially. These are not healthy, usual, full-term births and are likely biological outliers. What is the justification/reasoning for inclusion of this group in the study? It was not clear to me.

Response:

We thank the reviewer for the comment on infants that were born small for gestational age (SGA). As detailed in the original manuscript, SGA neonates were included in the study as this condition typically coincides with caesarean delivery. In addition, neonates born SGA have an elevated propensity for developing metabolic disorders during childhood or adulthood, and this elevated risk has been linked to changes in the gut microbiome (doi:10.1186/2049-2618-2-38). Given that CSD may be linked to SGA and that early changes to the microbiome may be at the origin of later-life conditions, SGA infants were explicitly included to compare patterns observed in them against non-SGA CSD neonates. Although the original manuscript included references to the rationale, this was not explicit due to length limitations. In accordance with the reviewer's comment, we have included additional explanations on the rationale for including neonates born SGA in the revised manuscript.

Introduction, manuscript lines 76 to 81: Although CSD is not associated with improved health outcomes, being born small for gestational age (SGA) frequently coincides with CSD (i.e. more than 50% of all SGA neonates)[22,23]. SGA neonates have an elevated propensity for developing metabolic disorders during childhood or adulthood, which has been associated with alterations to the gut microbiome[22,24], and may be linked to the elevated rate of CSD in this population.

Discussion, manuscript lines 428 to 432: Based on all analyses, no differences in taxonomical compositions or functional potentials were apparent when comparing CSD and CSD+SGA neonates, suggesting that the impact of delivery mode was a stronger determinant for neonatal gut microbiome colonization than the SGA status.

2.6. For the LPS extraction, how did the authors account for samples where adequate LPS could not be extracted? Are we to conclude that LPS was absent? Did the authors

measure the amount of LPS in the samples using methods such as targeted Mass spec? I am concerned that the size of the cohort studied and the limitations of not being able to extract LPS from all samples limits the generalizability of the findings presented. Also, why would babies born by VD have "higher immunostimulatory potential" than those born by CSD? A discussion of the expected impact of this observation would be helpful. Also, in the methods, it seems that of the 13 neonatal faecal samples, only 11 produced measurable quantities of LPS. In the main manuscript, I read this section as suggesting that 13 samples produced measurable quantities of LPS. Were samples that did not produce LPS from the VD, CSD or CSD+SGA cohorts? Why were only some of the total samples available for LPS extraction?

Response:

We appreciate the reviewer's reflections on the isolated LPS fractions and thank the reviewer for the suggested additional experiments to strengthen the study. The fact that sufficient LPS could not be isolated from samples can be explained by the limited amounts of faecal samples that were obtainable. This does not mean that LPS was absent in the samples collected from these neonates at the specific time points. Given that the amounts of sample material which can be collected from neonates on days 1-5 were very limited and that large parts of the limited sample material was used for DNA extraction, sufficient sample material was simply not left for LPS extraction (the method requires 150 mg of material to result in sufficient LPS for downstream analyses). Consequently, no data was obtainable for these samples. The amount of LPS was quantified using state-of-the-art methods and we have now included additional information on the characterization of the LPS fractions in the revised manuscript (see also our detailed responses to **comment 3.4.** below). Although we investigated analysis of LPS by mass spectrometry as per the reviewer's suggestion, such an analysis was not possible due to the very high amount of LPS needed, which is not easily obtainable for early neonatal stool samples. Given the fact that LPS moieties are highly diverse, such analyses would have not provided any conclusive qualitative information and accurate quantification via chromatography coupled to mass spectrometry would have also not been possible. In contrast, we have applied additional, in our opinion more relevant, assays involving agarose gel electrophoresis and reporter cell lines to further characterize the obtained LPS fractions (see also our detailed response to **comment 2.9.** below).

In order to address the reviewer's concern regarding the generalizability of the results, we have included data from additional samples in the revised manuscript, which, along with the more detailed characterizations of the LPS fractions, support our original conclusions. Collectively, we now present isolated LPS mixtures from faecal samples collected at day 3 postpartum from a total of 16 neonates (7 VD, 7 CSD, 2 CSD + SGA). The apparent "higher immunostimulatory potential" of the early microbiome in VD neonates is, according to the results in the original manuscript and supported by the new data, due to the higher numbers of Gram-negative bacteria and the enrichment of the LPS biosynthesis pathway in the gut microbiome of VD

neonates. In order to clarify this point, we have included an additional section in the revised manuscript. The reason for only some samples producing measurable quantities of LPS has been described and clarified in the revised manuscript. Please also refer to revised text passages cited under **comment 1.2.**

Results, manuscript lines 298 to 303*:* Notably, in the case of vaginal delivery, multiple strains of Gram-positive bacteria (e.g. *Bifidobacterium*) were transferred from mother to neonate (Fig. 3a; transmission in 71% of all VD neonates, 0% in CSD ± SGA on days 3 and 5), as well as Gram-negative bacteria (e.g. Bacteroidetes; Fig. 3a; transmission in 79% of all VD neonates, 0% in CSD and 20% in CSD+SGA on days 3 and 5).

Results, manuscript lines 353 to 362: As LPS forms part of the outer membrane of Gram-negative bacteria, the attributed Gram staining information of microorganisms directly corresponds to their propensity to synthetize LPS. Importantly, LPS is a highly potent innate immune activator that is recognized by the Toll-like receptor (TLR) 4. The earliest VD gut microbiome exhibited an enrichment in the microbial LPS biosynthesis pathway (Fig. 2a and Fig. 3h) as well as in Gram-negative taxa, which were frequently transmitted from the mother (Fig. 3a). This observation is supported by the 16S rRNA gene amplicon sequencing data (Supplementary Fig. 3b). Consequently, an apparent higher microbial synthesis of LPS likely results in an increased immunostimulatory potential of the developing gut microbiome.

Discussion, manuscript lines 502 to 512: Independent of the precise mechanism of strain transfer, we observed that several functional pathways were significantly under-represented in CSD neonates, while these were in turn enriched in VD neonates and linked to vertically transmitted strains, in particular the LPS biosynthesis pathway (Fig. 2a). LPS, an outer surface membrane component of Gram-negative bacteria, promotes the secretion of pro-inflammatory cytokines and thereby sits at the interface of the earliest gut microbiome colonization and neonatal immune priming. Following the apparent enrichments in LPS biosynthesis in VD neonates due to higher amounts of Gram-negative bacteria, the subsequent extraction and quantification of LPS from neonatal stool and stimulation of primary human immune cells therewith demonstrated a reduced immunostimulatory potential of the earliest gut microbiome in CSD neonates.

2.7. Given the very small n here, it seems that the results are depicted are of limited strength and may be very susceptible to over-interpretation.

Response:

We analyzed the data from faecal samples from additional samples and have included the results in the revised manuscript. The analyses from the additional samples support the original results and conclusions. Please also refer to **comment 1.2.** for

additional details.

2.8. Furthermore, based on Figure 4, it appears that *Escherichia* is highly abundance in all but one VD individual. Has this been described in other studies? Is this what is driving the LPS abundance in these samples? If so, this is worth discussing in the manuscript and putting into context for the readers. I would read the figure as *Escherichia* species being highly abundant in the neonatal microbiome of VD infants and that this may have consequences for LPS production. Is the *E. coli* that is present in these individuals the strain(s) that is from the mother? Based on Figure 3a, it does not appear so, as best as I can tell. So is one to conclude that VD neonates have a high abundance of *E. coli* that produces immunostimulatory LPS, but that the likely producer of this LPS (*E. coli*, which is abundant), is not vertically transmitted? If this interpretation is correct, I am not sure how the two main thrusts of the manuscript link to one another. Another area of confusion for me is Fig2C. In the neonatal stool from day 3, which I believe was used for the LPS extraction, I do not see many samples that have abundant *Escherichia*, as would be expected from Figure 4 - in fact, the samples that were chosen from the VD cohort for the LPS study appear to be the only two VD samples that have detectable *E. coli*. This may be skewing the results.

Response:

We thank the reviewer for this detailed comment. The apparent difference in the abundance of *Escherichia coli* across the different VD neonates (Figure 4) was highlighted in the original manuscript. According to our results, *E. coli* must indeed be important for early LPS-based immunostimulation in many neonates but not all. According to our results (Figure 3a), other Gram-negative bacteria, which contribute to the isolated LPS mixtures and were transmitted from mothers to VD neonates, may occupy the same role (Figure 3h). For the revision of the manuscript, we have performed additional measurements, in particular qPCR analyses to quantify *E. coli* abundances (see paragraph below) that allow us to validate these findings. The additional data has been included in the manuscript to clarify this point. In the revised manuscript, we have discussed the apparent importance of *E. coli* as well as other Gram-negative bacteria with respect to LPS-mediated immune stimulation early on in life. Accordingly, we have included a supplementary note on the potential effect of E. coli abundance and linked immunostimulatory potential in the revised manuscript.

Supplementary Information, Supplementary note 6: LPS from VD neonate C117, in whom the microbiome displayed lower presence of Gram-negative bacteria based on 16S rRNA gene amplicon sequencing data and the lowest amounts of *E. coli* according to qPCR measurements, also had the lowest immunostimulatory potential among all VD neonates, and triggered a negligible cytokine response. Although a higher *E. coli* abundance appeared to coincide with an increased immunostimulatory potential of the isolated LPS, the proportion of *E. coli* alone was not sufficient to explain the lack of immunostimulative effects in CSD (±SGA) neonates. More

specifically, in five cases of faecal samples collected from CSD±SGA, the absolute abundance of *E. coli* was at least 25-fold increased when compared to the faecal sample collected from VD neonate C007, which triggered an immune response (Fig. 4a). However, the isolated LPS from these five samples did not result in any considerable immune response in terms of TNF-α production. Additionally, in CSD neonate C121, the microbiome was depleted of *E. coli*, while the extracted LPS fraction still triggered an immune response. At the same time, CSD+SGA neonate C119 was depleted of *E. coli* but had a high proportion of Gram negative bacteria overall, while the LPS extract only triggered a minimal immune response in some of the MoDCs from adult donors. Collectively, these observations indicate that the composition of the isolated LPS fractions has an important role in their activity as well.

As Figure 4 was based on samples for which no metagenomic data was available at that time, the proportion of *E. coli* was derived from the 16S rRNA gene amplicon sequencing data and not metagenomic sequencing data. We have now performed additional qPCR analyses for assessing the actual proportions of *E. coli* per sample, which are included in the Figure 4 of the revised manuscript. According to the additional data, we have 7 faecal samples from VD neonates collected on day 3 for which 5 out of 7 include an increased proportion of *E. coli* (resolved based on mOTU analysis as well). Therefore, the presence of *E. coli* in samples collected from VD neonates are unlikely to skew the results but represent a common trait in the gut microbiome of VD neonates at day 3. These results have been included and discussed in the revised manuscript.

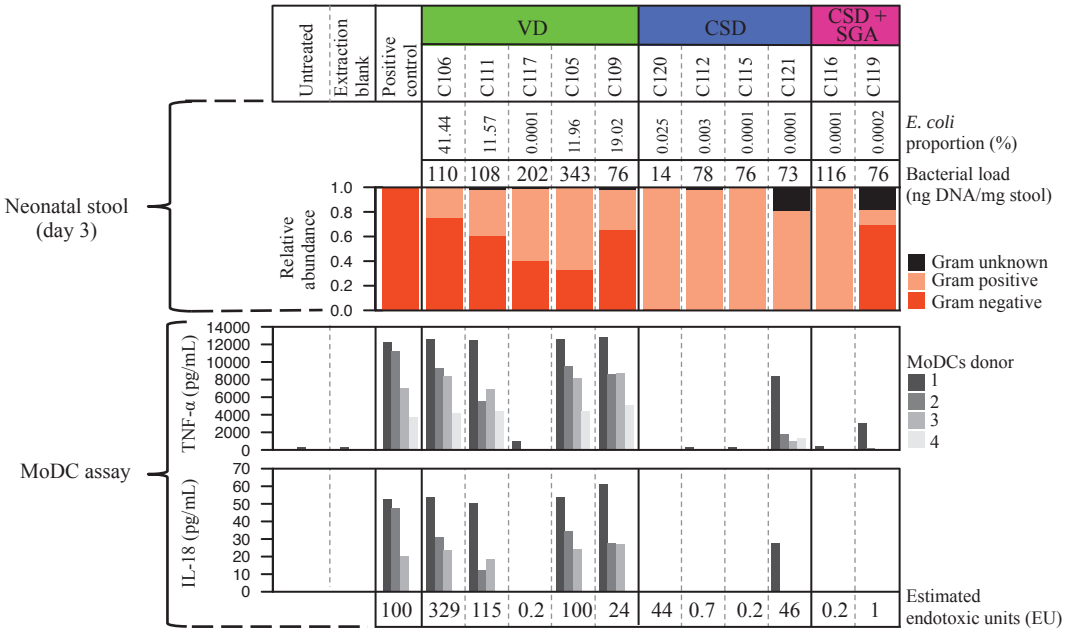


Figure 4 | Cytokine measurements in monocyte-derived dendritic cells after stimulation with isolated LPS from neonatal stool and in neonatal plasma. a,

Lipopolysaccharide (LPS) was isolated from faecal samples collected on day 3 postpartum from neonates in vaginal delivery (VD), caesarean-section delivery (CSD) and CSD with small for gestational age (SGA) status (CSD+SGA) groups, and incubated for 24 h with human monocyte-derived dendritic cells (MoDCs) isolated from a total of 12 adult donors. Exact numbers of donors used per sample are given in the plot. Positive control: isolated LPS from *E. coli* overnight culture. Neonates C115 and C116 are twins. **b,** Plasma levels of TNF-α and IL-18 in samples collected at day 3 after birth from VD and CSD (± SGA) neonates. Comparison by Wilcoxon rank-sum test with multiple testing adjustment; *false discovery rate (FDR)-adjusted P <0.05 and **(FDR)-adjusted P <0.01. Circles correspond to neonates with metagenomic data, crosses represent neonates without metagenomic data.

2.9. How was the LPS concentration and purity determined for each LPS sample that was extracted from faeces determined? The effects that they saw may have to do largely with purity of the LPS as opposed to structural differences / immunogenicity of the LPS. I would be more convinced of the argument they are trying to make if each of the LPS samples had been subjected to mass spectrometry to evaluate purity, for example. I simply worry that the LPS extraction procedure is, in itself, very flawed and subject to error. And with such a small n, the results may not be terribly robust, even if intriguing.

Response:

We appreciate the reviewer's concern regarding the purity of the extracted LPS fractions. As already partially discussed in response to **comment 2.6.**, chromatography followed by mass spectrometric analysis could not be performed as high amounts of LPS are needed which we could not obtain because neonatal faecal samples are low biomass. Additionally, such analyses would not provide any conclusive qualitative information and accurate quantification via chromatography coupled to mass spectrometry would also not be possible. Nevertheless, to address the reviewer's concerns, we have performed additional quantitative and qualitative characterizations of the LPS fractions (including from samples from additional neonates) using the assays detailed below. In accordance to the reviewers' comment and **comment 3.4.**, we have included the results of additional analyses in the manuscript.

The concentration of LPS was determined using a state-of-art ELISA-based endotoxin detection assay (Endolisa; # 609033, Hyglos GmbH, Germany). Detailed qualitative assessments of the LPS fractions were performed using standard agarose gel electrophoresis (to exclude the presence of DNA contamination) combined with Coomassie (to exclude protein contamination) as well as silver staining (to visualize LPS). To assess the immunogenicity as well as the purity of the LPS fractions, we have included the results of additional analyses in the revised manuscript including: (i) stimulation of primary dendritic cells with additional purified LPS, and (ii)

stimulation of specific HEK blue reporter cell lines overexpressing, either hTLR4 (#HKB-HTLR4, InvivoGen), hTLR2 (#HKB-HTLR2, InvivoGen) or hNOD1 (#HKB-HNOD1, InvivoGen) or hNOD2 (#HKB-HNOD2, InvivoGen). The results of these additional experiments demonstrate that: (i) the protocol for LPS extraction and purification results in pure LPS fractions when compared to commercially available, pure LPS; (ii) no immunological stimulation of dendritic cells was observed which would be attributable to DNA; (iii) if LPS was used at a defined concentration for all samples, only hTLR4 was activated and not hTLR2, nor hNOD1 or hNOD2; (iv) if isolated LPS was used at sample-specific concentrations to mimic realistic *in vivo* conditions, LPS mixtures were still relatively pure and comparable to commercially available LPS (Sigma) as hNOD1 and hNOD2 were in both cases not activated. In samples that were highly enriched in LPS also hTLR2 was activated, which was also the case for commercially available, pure LPS. Finally, in case a minimal amount of endotoxin unit (EU) of LPS was used to stimulate dendritic cells, the TNF-α response did not correlate with a linearly increasing amount of both LPS and TNF-α, suggesting that indeed the composition of the different isolated LPS mixtures does play a role in the immune response. Based on the immunogenicity of the purified LPS fractions, additional factors may be at play, however the detailed elucidation thereof goes beyond the scope of this study. In this context, we would like to stress again that the present work represents the first study to show a difference in immunostimmulatory potential of the earliest gut microbiome between VD and CSD neonates and during a critical window of opportunity for immune system priming. More detailed future work will be necessary to elucidate the different molecular factors involved in immune system priming. With regards to the reviewers' comments, we have now included an additional supplementary note and figures on all additional experiments that were conducted to properly assess the purity and immunogenicity of the isolated LPS mixtures from neonatal stool samples.

Material and methods, manuscript lines 972 to 1013: In order to verify the purity of the extracted LPS fractions, HEK-Blue™ reporter cell lines over-expressing one of the receptors hTLR2, hTLR4, NOD1 or NOD2 (InvivoGen, France), were stimulated with LPS extracted from five selected neonatal faecal samples (three VD and two CSD), which presented sufficient amounts of extractable LPS. HEK-Blue™ TLR and NOD cells are designed to detect stimulants of the human receptors by induction of secreted embryonic alkaline phosphatase (SEAP). For all the cell lines, the levels of SEAP were determined with HEK-Blue™ Detection (InvivoGen, France), a cell culture medium that allows for real-time detection of SEAP.

While the hTLR4 receptor only recognizes LPS, hTLR2 recognizes peptidoglycan, lipoteichoic acid and lipoprotein from gram-positive bacteria, lipoarabinomannan from mycobacteria, and zymosan from the yeast cell wall, the receptor NOD1 binds to bacterial molecules containing the D-glutamyl-meso-diaminopimelic acid (iE-DAP) moiety and NOD2 recognizes bacterial molecules (peptidoglycans) and stimulates an immune reaction. HEK-Blue™ cells were grown and maintained in DMEM (4.5 g/L

glucose, L-glutamine, Sigma-Aldrich, Belgium), supplemented with 10% foetal bovine serum (Thermo Fisher Scientific), 1% penicillin–streptomycin (Sigma-Aldrich, Belgium), 100 µg/ml Normocin (InvivoGen, France) and respective selective antibiotics according to the user's manual.

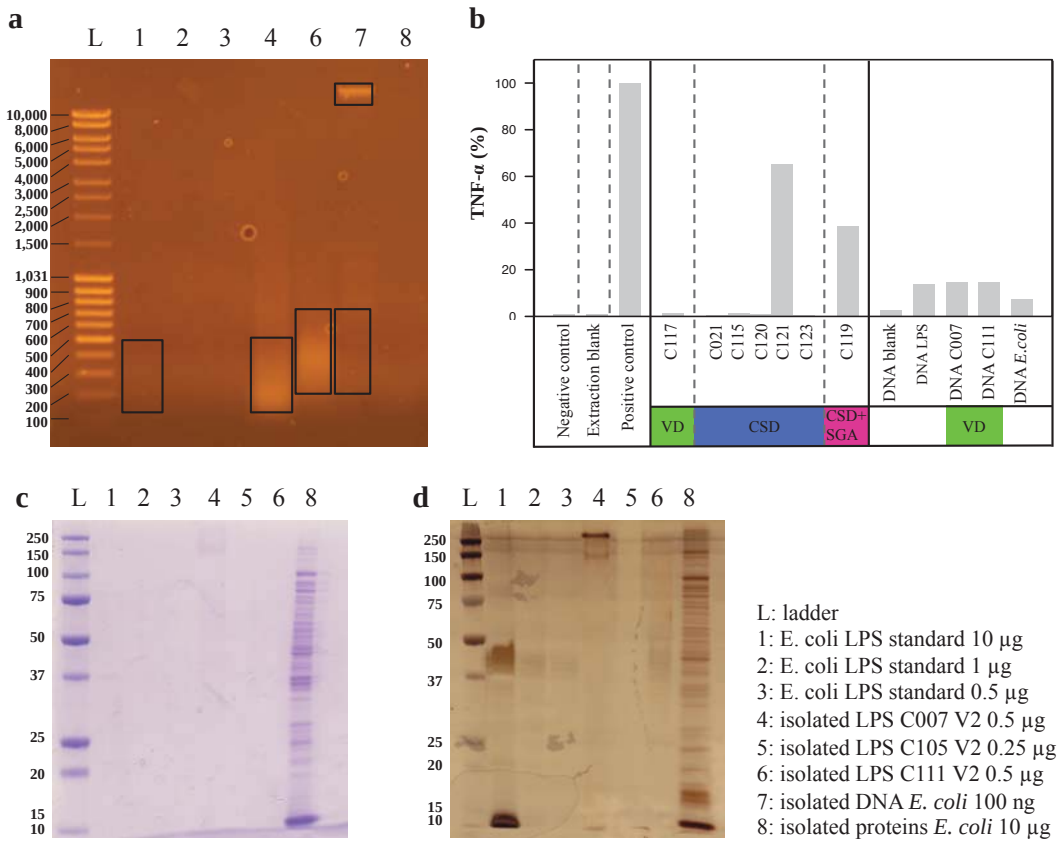
To monitor the activation of NF-κB, HEK-Blue™ cells were seeded according to the user's manual in HEK-Blue™ Detection medium (InvivoGen, France), in flat-bottom 96-well plates and stimulated for 22 hours with LPS samples. We used two conditions: first, using the same concentration of LPS, where 1 µl of extracted LPS (0.01 ng/µl) was added per well, and second, using the same volume of LPS, where 7.5 µl extracted LPS was added to 10^5 HEK-Blue™ cells. To convert endotoxin activity (EU) into mass (ng), we considered that around 10 EU are equivalent to 1 ng endotoxin[81]. For positive controls, HEK-Blue™ NOD1 cells were stimulated with 1 µl TriDAP (10 µg/µl, InvivoGen, France), HEK-Blue™ NOD2 cells with 1 µl Murabutide (10 µg/µl, InvivoGen, France), HEK-Blue™ hTLR2 cells with 1 µl of Pam3CSK4 (1 µg/µl; InvivoGen, France) and HEK-Blue™ hTLR4 cells with 1 µl ultrapure LPS (5 µg/µl, source strain: ATCC 12014; CDC 5624-50 [NCTC 9701], InvivoGen, France). In addition, all cell lines were treated with 1 µl ultrapure LPS (5 µg/µl, InvivoGen, France) and 1 µl ultrapure LPS (0.01 ng/µl, InvivoGen, France) as well as with commercially available LPS (standard LPS; *Escherichia coli* O55:B5, gel-filtration chromatography; Sigma-Aldrich, Belgium): 1 µl of 5 µg/µl and 1 µl of 0.01 ng/µl. For the negative control, HEK-Blue™ cells were incubated with 1 µl of endotoxin-free H_2O (InvivoGen, France). All conditions were performed in duplicates and SEAP expression was monitored using a microplate reader at 655 nm (Biotek instruments, Germany) except for LPS isolated from C117 where only 7.5 µl extracted LPS/10^5 HEK-Blue™ cells was added to the cells and tested in duplicates.

Results, manuscript lines 368 to 388: We isolated LPS from faecal samples with sufficient biomass collected on day 3 postpartum from 16 neonates (7 VD, 7 CSD, 2 CSD+SGA; Supplementary Data 12; Methods) and used several approaches to assess the purity of the isolated LPS fractions (Supplementary Note 5; Methods). Using agarose and polyacrylamide gel electrophoresis, we successfully visualized the isolated LPS and did not find traces of protein contamination but observed minor traces of fragmented DNA. However, this DNA did not contribute considerably to the immunostimulatory effect of the LPS fractions (Supplementary Fig. 11). No contamination with peptidoglycan or other bacterial molecules containing the D-glutamyl-meso-diaminopimelic acid moiety were detected in the isolated LPS fractions using the highly sensitive HEK-Blue™ reporter cells overexpressing the receptors hTLR2, hNOD1 and hNOD2, respectively (Supplementary Fig. 12; Methods). Some microbial products detected by hTLR2 (e.g. lipoteichoic acid, lipoprotein from Gram-positive bacteria, lipoarabinomannan from Mycobacteria or zymosan from yeast cell walls) were likely present in LPS samples for which high amounts of LPS were obtained from faecal samples. Conclusively, the isolated LPS fractions were assessed to be of high purity based on the HEK-Blue™ cell assays,

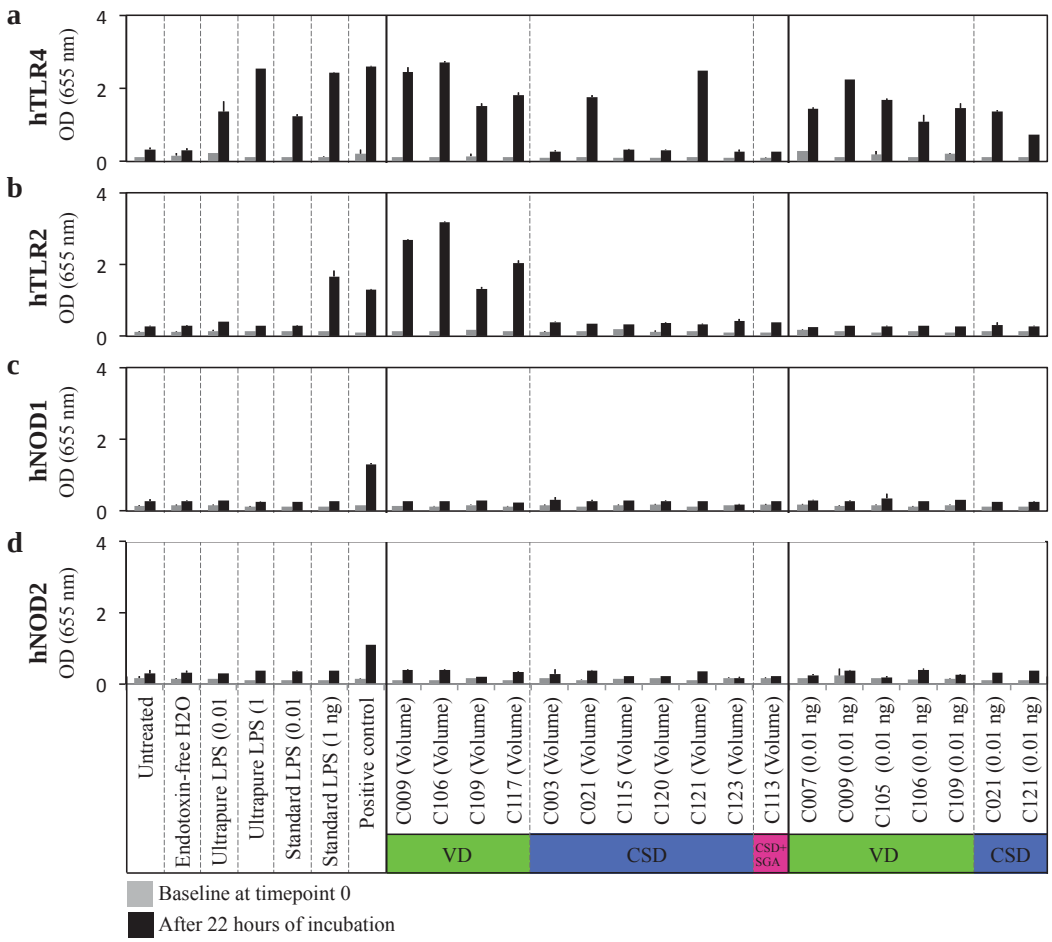
although some unknown microbial products may play a stimulatory role in the high yield LPS fractions (1 ng of standard LPS and an average of 2.9 ng of LPS isolated from VD neonates; Supplementary Fig. 12). Consequently, the composition of the different isolated LPS fractions played an important role in the subsequently triggered immune response.

Supplementary Information, Supplementary Note 5: To assess the LPS purity, we performed detailed characterizations of the obtained LPS fractions. First, we used agarose gel electrophoresis to exclude the presence of DNA contamination (Supplementary Fig. 11a) with the subsequent extraction of DNA from excised agarose bands and measurement of TNF-α in the supernatant of MoDCs upon stimulation (Supplementary Fig. 11b). Our results confirmed that DNA contamination did not considerably contribute to the observed immune activation of MoDCs. Second, we used polyacrylamide gel electrophoresis combined with Coomassie staining in order to successfully exclude the presence of proteins (Supplementary Fig. 11c). Third, we visualized LPS using polyacrylamide gel electrophoresis followed by silver staining (Supplementary Fig. 11d).

To further assess the immunogenicity as well as the purity of the LPS fractions, we performed additional stimulation assays using specific HEK-Blue™ reporter cell lines which overexpressed either hTLR4, hTLR2, hNOD1 or hNOD2 (Supplementary Fig. 12a to d). In summary, when LPS was used at a defined concentration for all samples, only hTLR4 was activated and not hTLR2, nor hNOD1 or hNOD2; if isolated LPS was used at sample-specific concentrations to mimic realistic *in vivo* conditions, LPS mixtures were still relatively pure and comparable to commercially available LPS (Sigma) as hNOD1 and hNOD2 were in both cases not activated. In samples that were highly enriched in LPS also hTLR2 was activated, which was also the case for the commercially available, pure LPS. Finally, in case a minimal amount of endotoxin unit (EU) of LPS was used to stimulate dendritic cells, the TNF-α response did not correlate with a linearly increasing amount of both LPS and TNF-α, suggesting that indeed the composition of the different isolated LPS mixtures does play the dominant role in the observed immune responses.



Supplementary Figure 11 | Purity assessment of isolated LPS fractions from neonatal stool. a, Agarose gel electrophoresis with Ethidium bromide staining to visualize the presence of DNA contamination in LPS fractions that were isolated from neonatal faecal samples. Agarose bands at the highlighted sizes were cut and DNA was isolated. **b,** Stimulation of human monocyte-derived dendritic cells (MoDCs) from adult donor 9 with extracted DNA samples obtained from **(a)**. Immune reaction was measured by levels of TNF-α in the supernatant. Untreated MoDCs were used as negative control, while MoDC stimulation with 15 endotoxin units (EU) of LPS isolated from an overnight culture of *Escherichia coli* strain K-12 (sub-strain MG1655) were used as positive control. **c,** Poly-acrylamide gel electrophoresis combined with Coomassie staining to visualize protein contamination. d, Poly-acrylamide gel electrophoresis combined with silver staining to visualize LPS presence.



Supplementary Figure 12 | Immune stimulation assays of reporter cell lines after stimulation with isolated LPS from neonatal stool. Read-outs of HEK-Blue cell lines overexpressing respectively one of the membrane receptors hTLR4 **(a)**, hTLR2 **(b)**, NOD1 **(c)** or NOD2 **(d)** were stimulated with LPS fractions isolated from neonatal faecal samples. **a,** The hTLR4 receptor only recognizes LPS. Positive control: ultrapure LPS (5 µg). **b,** The hTLR2 receptor recognizes peptidoglycan, lipoteichoic acid and lipoprotein from gram-positive bacteria, lipoarabinomannan from mycobacteria, and zymosan from yeast cell wall. Positive control: Pam3CSK4 (1 µg). **c,** The NOD1 receptor binds to bacterial molecules containing the D-glutamyl-meso-diaminopimelic acid (iE-DAP) moiety. Positive control: TriDAP (10 µg). **d,** The NOD2 receptor recognizes bacterial molecules (peptidoglycans) and stimulates an immune reaction. Positive control: Murabutide (10 µg). OD: optical density. Technical duplicates were done per condition and error bars reflect the standard deviation between duplicates.

Minor comments

We thank the reviewer for the valuable input. We address the important minor comments in the following sections but all the reviewer's minor comments have been comprehensively addressed in the revised version of the manuscript.

2.10. Not enough detail in the manuscript - how were differentially abundant

functional categories from KEGG identified? I appreciate the strict space limitations, but some information regarding method should be included in the manuscript.

Response:

We appreciate the reviewer's concern over the apparent lack of methodological details outside the material and methods section of the manuscript. Differentially abundant functional categories from KEGG were identified using the Deseq2 package using the R statistical package, while differentially abundant pathways were detected through pathway enrichment analysis using a custom R script according to doi:10.1038/nmicrobiol.2016.180. Due to previous space limitations, we refrained from adding too many details on the methods into the main text of the manuscript. However, we have now included as much methodological information as needed in the revised main text.

2.11. The authors refer to how the "early microbial functions in VD neonates reflected the mothers' functional microbiome profiles" on line 86-87, but they have not yet introduced any analysis of the mother's microbiome. This is confusing to me. Also, are they looking at the maternal vaginal microbiome or the maternal stool microbiome (both of which have been correlated with the neonatal microbiome). Did they look at the neonatal faecal microbiome and the maternal skin microbiome to compare functional potential? This would be suggested by Dominguez-Bello's paper from PNAS 2010.

Response:

The analysis of maternal gut microbiome was first mentioned in the introductory paragraph, but we agree with the reviewer that an additional mention of the different maternal samples that were analyzed should be added earlier in the main text. In our study, we assessed the maternal vaginal and stool microbiome through metagenomic sequencing, however the earliest gut functional potential of VD neonates resembled significantly more the functional potential of the maternal gut than vaginal microbiome, which is why Figure 2 only includes the maternal gut microbiome data. We have referred to this this more precisely in the revised manuscript.

Results, manuscript lines 206 to 224: To assess whether the apparent taxonomic differences between the gut microbiomes of VD and CSD neonates are reflected at the level of functional potential, we used the metagenomic sequencing data to compare functional profiles of all neonates to the gut microbial potential of their respective mothers. We also compared the CSD (±SGA) microbiota at day 3 and day 5 postpartum to those of VD neonates. The correlations of the functional profiles of the neonatal gut microbiome to the respective maternal vaginal microbiome were lower (median rho 0.06 for day 1, 0.37 for day 3 and 0.44 for day 5) than the correlation between neonatal and maternal gut microbiomes (median rho 0.29 for day 1, 0.59 for day 3 and 0.62 for day 5). While the correlations of the functional profiles of the

neonatal gut microbiome and the maternal vaginal microbiome did not differ significantly between delivery modes, early microbial functions in VD neonates better reflected the mothers' functional gut microbiome profiles compared to CSD (± SGA) neonates (Fig. 2a; Wilcoxon rank-sum test, FDR-adjusted $P = 4.1 \times 10^{-3}$ for day 3). CSD (± SGA) neonates lacked most functions at day 3 compared to VD neonates (Supplementary Fig. 4-9), wile some appeared at day 5 (Fig. 2a). Notably, neonatal-maternal correlations between community-wide functional potentials of the gut microbiomes at days 1, 3 and 5 postpartum were higher for VD than for CSD (± SGA) (Fig. 2b; Wilcoxon rank-sum test, FDR-adjusted $P = 6.0 \times 10^{-3}$ for day 3 and $P = 1.8 \times 10^{-2}$ for day 5).

As the composition and diversity of the skin microbiome is highly dependent on body site, it would be improbable to get a complete assessment of skin-to-neonate strain transfer, which is why we focused on the two microbially rich body sites that are known for taking an important role in neonatal gut colonization (i.e. maternal gut and vaginal microbiomes). With regards to the reviewer's comment, we have included our reasoning in the revised manuscript.

Results, manuscript lines 136 to 139: For each mother-neonate pair, we sampled microbiomes of maternal body sites, which are indicated to be important in relation to neonatal gut colonization (collection of stool and vaginal swabs; Methods) less than 24 h before delivery.

2.12. The methods applied in Supplementary note 3 (to determine if birth method affected the microbiome) are not adequately explained and do not appear to be statistically driven. What is presented is not adequate to conclude that "the different feeding regimes of neonates did not explain the functional differences …" on lines 94-95.

Response:
We thank the reviewer for the valuable comment. Although it may not be completely excluded that the milk feeding regimen had an effect on the neonatal microbiome at day 5 after birth, according to the multivariate statistical analyses, delivery mode was the main factor at the origin of observed effects at day 3 after birth. These additional results are discussed in the revised manuscript.

Supplementary Information, Supplementary Note 4: Feeding regime was found to be a potential contributing factor of the relative abundances of *Trichococcus* (formula feeding and mixed feeding regime; $Q = 3.6 \times 10^{-3}$ and $Q = 4.6 \times 10^{-2}$), *Escherichia-Shigella* (mixed feeding regime; $Q = 1.3 \times 10^{-2}$) and one OTU blonging to *Rothia* (formula feeding regime; $Q = 2.9 \times 10^{-4}$).

2.13. It seemed like the LPS biosynthesis gene was "cherry-picked" from a list of

significantly differentially abundant genes. Also, what as the FDR cutoff? Why was this gene chosen? What were the most significantly differentially abundant genes?

Response:

The enriched microbial pathway 'LPS biosynthesis' comprises several genes, which are statistically significantly different between CSD and VD. The pathway of LPS biosynthesis was specifically chosen for further validation because (i) this pathway harbors the potential to be closely involved in the earliest priming of the neonatal immune system during the crucial window of opportunity in early neonatal life and early exposure to LPS may have persisting effects on the later health status. (ii) Established methods exist for isolating LPS and performing informative experiments. In the revised manuscript, we have included a supplementary note on the other differentially abundant functional pathways.

Results, manuscript lines 240 to 250: Other important microbial metabolic pathways, which were enriched with differentially abundant genes between VD and CSD, included flagellar assembly (Fig. 2a; hypergeometric test, FDR-adjusted $P = 4.9 \times 10^{-12}$), bacterial chemotaxis (Fig. 2a; hypergeometric test, FDR-adjusted $P = 1.5 \times 10^{-2}$), cationic antimicrobial peptide (CAMP) resistance (Fig. 2a; hypergeometric test, FDR-adjusted $P = 4.0 \times 10^{-3}$), two-component system (Fig. 2a; hypergeometric test, FDR-adjusted $P = 2.5 \times 10^{-5}$) and ABC transporters (Fig. 2a; hypergeometric test, FDR-adjusted $P = 1.3 \times 10^{-4}$). Notably, all pathways also showed higher relative gene abundances in VD compared to CSD (± SGA) neonates except for the ABC transporter pathway (Fig. 2c; Wilcoxon rank-sum test, FDR-adjusted $P = 4.1 \times 10^{-3}$, 3.8×10^{-2}, 2.2×10^{-4}, 2.1×10^{-2}, respectively).

Discussion, manuscript lines 544 to 573: Apart form LPS biosynthesis, other pathways that were significantly enriched in the gut microbiome of VD neonates included genes involved in membrane transport, i.e. ATP-binding cassette (ABC) transporters. On the one hand this may reflect the adaptation of the colonizing microbiome of VD neonates to the gut environment through enhanced nutrient intake. On the other hand, associated ABC transporter proteins for both Gram-positive and Gram-negative bacteria were previously shown to be immunogenic[51], which could suggest an implication in the activation of the neonatal immune system. Additionally, enrichments in pathways relating to bacterial motility were observed. This included the two-component system pathway, which is an important mediator of signal transduction, flagellar assembly and bacterial chemotaxis. More specifically, these pathways are essential for bacterial motility in response to external stimuli and consequently competition with other members of the gut microbiome[52]. Additionally, flagellin, the main structural component of the flagellum, is an effective stimulator of innate immunity[53] and promotes mucosal immunity through the activation of TLR5 (ref 54). Another functional pathway that is potentially interacting with the human immune system early on is resistance to cationic antimicrobial peptides (CAMP). While resistance to antimicrobial peptides has been found in all major commensal

phyla of the human gut and across all members of the phylum Bacteroidetes, this pathway is essential to evade detection by the human immune system through the modification of the microbial LPS structure[55]. In the context of our study, an enrichment in CAMP resistance may prevent the predominantly colonizing gut bacteria (i.e. Bacteroidetes) from being recognized by the immune system and subsequently removed from the VD neonatal gut. Future studies are needed to assess whether the gut microbiome of VD neonates harbours more modified LPS moieties linked to CAMP resistance and what the subsequent immunostimulatory effects of altered LPS structures may be on the neonatal immune system. In accordance with the observation of an apparent enrichment in flagellar biosynthesis, bacterial chemotaxis, CAMP resistance, other microbiota-derived molecular factors, apart from LPS, may be involved in immune system priming.

The FDR cut-off was set to 0.05. We have additionally included the statistics per KO in the supplementary data of the revised manuscript (Supplementary Data 10).

2.14. GAG and other glycan metabolism is mentioned in the abstract but is only addressed in the supplement. If this is an important point, it should be included in the main manuscript. If not in the main manuscript, it should be left out of the abstract.

Response:

We appreciate the reviewer's observation and agree that the section on GAG and other glycans was referred to in the abstract but not discussed in the main text due to the space limitations for the original format (*Nature* Letter format). Following the analysis of additional metagenomic sequencing data, we now find additional microbial pathways that are enriched in VD neonates compared to CSD and that could also be highly relevant for the neonatal host organism. We have revised the abstract as well as reported and discussed these pathways in the revised manuscript. For more information, please also refer to the reply to **comment 2.13.** above.

2.15. When cytokine levels were obtained from 31 neonates, it seems inappropriate to label the 14 additional infants as an "independent validation cohort" if the results are aggregated with the discovery cohort. This is misleading to me.

Response:

We agree with the reviewer and have changed this accordingly in the revised manuscript.

2.16. The reference citation format appears to change at line 404. This is a small typographical issue.

Response:

To avoid any confusion when adding a reference number to a number in the text (as for software version numbers), we decided to use the chosen style. However, in order to stick to a uniform reference style, we have changed the citation style throughout the revised manuscript.

Reviewer #3

Overall summary

3.1. In this interesting research paper by Wampach et al, authors used a very robust bioinformatics approach to track specific bacterial strains from mothers to neonates during the perinatal period, taking into account levels of genetic diversity using well-established molecular ecology methods. This study has numerous strengths that are original and should be published: it combined taxonomic and functional profiling of the microbiome using reference independent metagenomics analysis, included controls and a bioinformatic approach that accounted for reagent and host derived contaminant DNA and removal of sequencing artefacts, and utilized sequencing data to inform the experimental parameters of an immune assay, which was informative of the potential interactions between LPS and the immune system. However, these strengths are not the main claim of this study. This clinical study was not designed nor was it shown to be powered to determine perinatal microbiome differences driven by mode of birth, and its results should not be interpreted as such. Below is a list of major and minor suggestions that should be incorporated in a new version of this manuscript.

Response:

We appreciate the reviewer's assessment of the quality and importance of the work. As discussed above and in the following comment (see responses to **comments 1.2.** and **3.2.**), we have included results from additional samples from additional individuals in the revised version of the manuscript. The results from the additional analyses support the conclusions in the original manuscript.

Major comments

3.2. The sample number in this study is simply too low and there were no power calculations performed a priori to determine if it could identify difference between birth modes. Given that other small studies have failed to show microbiome differences driven by birth mode, it is hard to believe that this study was powered to assess the influence of birth mode, let alone the influence of low birth weight. I recommend that authors present power calculations based on previously published data to determine this. A good resource to perform these calculations for studies with multivariate data is MetSizeR. Clearly, the results from this study are in line with most other studies that have convincingly shown that mode of birth drives temporal neonatal composition and diversity. However, a recent study from the Xavier group (DOI: 10.1126/scitranslmed.aad0917) showed that while most CS births were

associated with lower Bacteroides species abundance, about 20% of the VD babies displayed a similar pattern. With an N=4 per group (including 1 set of twins in the CSD groups) with only 2 valid time points (Day 1 only had 2 samples in 2 of the groups, so in my opinion should be excluded for comparisons), there is simply no statistical power to detect appropriate variance of a given population, especially considering the interindividual variability of the gut microbiome during the first days of human life.

Response:

We agree with the reviewer that the number of study participants is comparatively low in relation to previous studies (see response to **comment 1.2.**). Nevertheless, it is important to emphasize important unique aspects of our study: (i) paired mother-infant samples, (ii) samples from the earliest time points, and (iii) high-resolution, contaminant-free metagenomic data. We want to stress again that the main point of the manuscript is not statistics-driven, as the presence of strains, given their high degree of specificity, is valid only on an individual, sample-to-sample basis. Single nucleotide variants are highly specific and the presence of the exact same gut strains in mothers and their vaginally delivered neonates demonstrates that transfer of strains happens from mothers to neonates, with vaginal delivery being the most probable mode of vertical transfer. The fact that considerably less maternal enteric strains were found in neonates that were delivered by C-section, provides evidence that delivery through C-section impedes this vertical transfer. Our high-resolution approach of strain tracking in addition to statistical tests on the functional profiles (which are statistically significant; see also our response to **comment 3.3.** below) is more robust and to date unprecedented with regards to the earliest neonatal gut microbiome. Nevertheless, we have included additional metagenomic sequencing data, as well as 16S rRNA gene sequencing data from additional neonates, which support our high-resolution, strain-specific tracking results. Please also refer to **comment 1.2.** for additional details.

With specific regards to the study from the Xavier group, we want to stress that their conclusions on the low relative abundance levels of *Bacteroides* were (i) based on a disproportionate distribution of study participants regarding delivery mode (4 C-section delivered against 35 vaginally delivered infants), which compromises their statistical power, (ii) were based on 16S rRNA gene amplicon sequencing, (iii) did not include paired mother-neonate pairs, and (iv) did not focus on earliest time points after birth. In our case, as the tracking of highly specific strains from mother to neonate is considered on a case-by-case basis, our main conclusions are valid in light of inter-individual variability of the gut microbiome during the first days of human life.

3.3. Although differences between groups were detected using appropriate statistical tests, without confidence intervals and proper statistical tests to deconfound the effect

of birth mode from other variables known to influence microbiome composition, it is not possible to properly assess whether birth mode truly drives these differences. Given that authors have access to 20 more samples (6 for which they had 16S data and 14 from a previous cohort that was collected in a similar way), the authors could use the 16S data and PICRUSt, at a minimum, to determine if other variables are also influencing the associations found between birth mode and microbiome composition. An excellent tool for this purpose is MsAsLin (https://huttenhower.sph.harvard.edu/maaslin). Variables to include in this model are gestational age, antibiotic use, dose, duration, time of meconium passage, if babies ingested colostrum, etc. In addition, authors should demonstrate that the samples used for metagenomics analysis are representative of the larger group of samples. For this, they could compare microbiome characteristics (beta or alpha diversity) as well as clinical variables (mode of birth, gestational age, etc.)

Response:

We thank the reviewer for this constructive comment. In accordance with the reviewer's suggestion, we have included additional 16S rRNA gene amplicon sequencing data in the revised manuscript (see also our response to **comment 1.2.**). Furthermore, in accordance with the reviewer's suggestion to determine whether other variables are potentially influencing the associations found between delivery mode and microbiome composition, we have indeed used the tool MaAsLin by including variables such as delivery mode, feeding regime, gestational age and maternal antibiotics intake. The major trends that were highlighted in the initial manuscript were thereby still explained by delivery mode [i.e. higher relative abundance of *Staphylococcus* or lower levels in *Bacteroides* in CSD (± SGA) neonates]. Please also refer to our reply to **comment 2.1.**

In accordance with the reviewer's suggestion to infer functional profiles based on the extensive 16S rRNA gene amplicon data, we also used the tool PanFP (https://github.com/srjun/PanFP) and observed similar trends to the pathways that we detected based on metagenomic sequencing. In order to predict the functional profiles of microbial communities based on our 16S rRNA gene amplicon data, we have included additional analyses in the revised manuscript that are based on the tool PanFP (doi:10.1186/s13104-015-1462-8). While PICRUSt presumes a closed-reference OTU picking strategy, which results in a strong dependency on the completeness of the reference database, PanFP is highly compatible with the open-reference strategy of NG-Tax (doi:10.12688/f1000research.9227.1) that we used for processing the 16S rRNA gene amplicon data. An additional paragraph on the outcome of these analyses has been included in the revised manuscript.

Results, manuscript lines 258 to 276: Additionally, the relative abundances of 7,000 KO functional categories were predicted using PanFP[37] based on the extensive 16S rRNA gene amplicon data (Supplementary Data 11). A multivariate analysis (MaAsLin[35]) was performed to compare the functional profiles of CSD (±SGA) to

VD neonates and for both generated datasets (i.e. predicted KO functional categories based on 16S rRNA gene amplicon sequencing data and annotated KOs based on metagenomic sequencing data). Results from the multivariate analyses demonstrated that delivery mode was the strongest determining factor in both datasets (i.e. predicted and metagenomic-based KOs) for explaining the differentially abundant genes (Supplementary Data 9). Whilst not statistically significant, the trends for the predicted microbial pathways obtained with PanFP were largely concordant with the enriched pathways in VD neonates based on the differential analysis of the metagenomic data. Nevertheless, predictions of functional potentials based on 16S rRNA gene amplicon sequencing data are likely unreliable as a significant fraction of the gut microbiome (i.e. up to 40%) is represented by microorganisms without a sequenced isolate genome[38]. In contrast, the metagenomic data, through resolving the actual functional gene complement, allows a detailed comparison of the functional potential of the earliest gut microbiomes, as well as the tracking of individual-specific single-nucleotide variants (SNVs).

In order to assess whether the samples used for metagenomic analysis were representative of the larger group of samples in the initial manuscript, we included a supplementary note and supplementary figures on specific microbiome characteristics, including beta and alpha diversity, in the original manuscript. According to this information, the presented metagenomic data is representative of the larger group of samples.

Results, manuscript lines 186 to 194: The 16S rRNA gene amplicon and the metagenomic sequencing data, which was generated for a subset of mother-neonate pairs, showed highly similar succession trends in terms of diversity, evenness and richness measures (Supplementary Fig. 2a&b; Supplementary Note 2). The taxonomic profiles derived from the 16S rRNA gene amplicon and metagenomic sequencing were highly correlated (Supplementary Fig. 3a). The differences in taxonomic profiles according to delivery mode reflected results from previous studies, notably the higher relative abundance in *Bacteroides* and *Parabacteroides* and lower levels in *Staphylococcus* in VD neonates at days 3 and 5 postpartum[7,10] (Supplementary Data 6 to 8; Supplementary Note 3).

Supplementary Information, Supplementary Note 3: To identify differences between the birth modes (and SGA status) and the different collection time points postpartum, we performed Wilcoxon rank-sum tests (without multiple-alignment adjustments) on the sum-normalized relative abundances of metagenomic and 16S rRNA gene amplicon sequencing data (Supplementary Data 7 and 8). Although several statistically significant differences were found, we only considered genera and OTUs that showed the same trends for both CSD and CSD+SGA neonates and had a P-value below 0.05. In VD neonates, compared to both CSD and CSD+SGA, the metagenomic data resolved a higher relative abundance of the species *Bacteroides dorei/vulgatus* (day 5 VD vs CSD, $P = 2.5 \times 10^{-2}$ and VD vs CSD+SGA, $P = 1.2 \times$

10^{-2}), as well as the specific *Bacteroides dorei/vulgatus* mOTU (day 5 VD vs CSD, $P = 1.4 \times 10^{-2}$ and VD vs CSD+SGA, $P = 6.7 \times 10^{-3}$). Based on the 16S rRNA gene amplicon sequencing data, significantly higher relative abundances of the genus *Bacteroides* were observed in VD neonates at days 3 and 5 (day 3, $P = 7.8 \times 10^{-3}$ and $P = 1.2 \times 10^{-2}$; day 5, $P = 1.2 \times 10^{-3}$ and $P = 5.1 \times 10^{-3}$, respectively). The genus *Parabacteroides* was significantly increased ($P = 4.2 \times 10^{-3}$ and $P = 2.8 \times 10^{-2}$), while *Rothia* was found to be significantly decreased in VD neonates ($P = 1.8 \times 10^{-2}$ and $P = 2.2 \times 10^{-4}$). A total of 10 OTUs assigned to the genus *Staphylococcus* were significantly increased in CSD±SGA neonates, while OTU 243 assigned to *Bacteroides* was significantly increased in VD neonates at days 3 and 5 (day 3, $P = 2.3 \times 10^{-2}$ and $P = 2.5 \times 10^{-2}$; day 5, $P = 2.5 \times 10^{-3}$ and $P = 9.4 \times 10^{-3}$, respectively).

3.4. The results from the immune assay with extracted LPS from different neonatal samples are quite compelling but remain rather preliminary, and could be enhanced in several ways. As it stands, the purity of the LPS was not assessed, it is vaguely assumed that the majority of the cytokine response measured originated from *E. coli* LPS, and although it is mentioned in the text, it is not known if the differences in cytokine response are due to differences in LPS structure. Addressing at least 2 of these issues will significantly strengthen this paper. LPS purity can be determined by gel electrophoresis (comparing silver, protein and DNA staining, for example), or if available, mass spectrometry analysis. Differences in LPS structure (rough vs smooth, for example) can also be obtained through these methods. LPS purity can also be confirmed functionally by showing that the purified products did not activate Toll-like receptor 2 (TLR2), nuclear oligomerization domain 1 (NOD1), or NOD2 but did activate TLR4.

Importantly, if the authors have more samples from other cohorts available, why not increase the number of samples from this assay?

As for determining the origin of the LPS, could LPS genes not be assigned to the mOTUs given that the information is available? This is important as it will inform which taxa may or may not be relevant in stimulating immune cells during the first days of life.

Response:

We appreciate the reviewer's recognition of the compelling nature of the immunological data presented and thank him/her for the suggested additional experiments to strengthen the study. To address the reviewer's comments, we have now conducted additional experiments including additional LPS extractions from additional samples from early neonates. More specifically, in the revised manuscript we have included results reflecting the purity of the isolated LPS and on the specificity of immunological responses. Please also refer to our detailed response to **comment 2.9.** above. Bearing in mind these additional experiments, we have addressed both suggestions of the reviewer regarding assessment of LPS purity and immunogenicity. The results of these additional analyses underscore our earlier

results and thereby further strengthen our original conclusions.

For determining the origin of LPS, we annotated the OTUs resulting from the 16S rRNA gene sequencing data according to their Gram staining characteristics using the recorded microbial attributes from NCBI (http://www-ab2.informatik.uni-tuebingen.de/megan/taxonomy/microbialattributes.zip). More precisely, the Gram staining information of microorganisms directly corresponds to their propensity of producing LPS, therefore the direct assignment of OTUs being either Gram positive or negative is robust, while identifying LPS gene functions in the complement of reconstructed genomes provides comprehensive results only for the most dominant taxa, i.e. those with almost complete genome reconstructions. On a genome-scale level, we identified which taxa may be relevant in stimulating immune cells during the first days of life in the initial manuscript and this information was represented by the colored spokes in the Circos plots in Figure 3. Based on these observations, we suggested that strains that were transferred from mother to VD but not CSD neonate were significantly enriched in genes that are involved in LPS biosynthesis. To clarify these points, we have included additional text passages in the revised manuscript. Please refer to the cited text passages given under **comment 1.2.** and **2.6.**

3.5. Extended data figure 2. Change color palette. Too many tones of blues and greens. Don't do color coordination according to phyla.

Response:

We thank the reviewer for this constructive comment. We have tried several color combinations for this supplementary figure but concluded that the current color palette based on phyla assignment was the most informative for the visual assessment of the neonatal gut microbiome composition. For a more detailed evaluation, the complete mOTU table obtained by metagenomic sequencing is provided in the supplementary data (Supplementary Data 3).

Minor comments

We thank the reviewer for their valuable input. We comprehensively addressed all the reviewer's minor comments in the revised version of the manuscript.

3.6. Blinding in Life Sciences reporting summary: "As assigned study groups were predefined prior to delivery, blinding was irrelevant to our study." This is false. Many studies with predefined study groups are blinded and this adds strength to the study design. This study was simply non-blinded, please change.

Response:

We thank the reviewer and agree with this comment. We have made a corresponding change to the reporting summary.

3.7. FIGURE 2: It is unclear how the DESEQ2 generated results are displayed in Figure 2. Deseq2 compares 2 groups. According to Figure 2a it looks that the comparison was made between VD samples and all CSD samples combined but figure legend and methods section describes two separate comparisons. Were the same 5 pathways differential in both comparisons or only when VD samples were compared to all CS babies?

Response:

We thank the reviewer for this relevant comment. Indeed, as DESeq2 compares two groups, we made comparisons based on VD vs CSD and VD vs CSD+SGA. Subsequently, we combined all significantly differential KOs from both tests for which the FDR-adjusted *P* value of the Wald test was <0.05 for at least one comparison and for which the directionality of change in both comparisons was the same. We have made corresponding changes to the revised manuscript.

Material and methods, manuscript lines 836 to 844: Differential analysis of KO abundance, comparing VD to CSD and VD to CSD+SGA with a linear model, which considered the different collection time points containing at least 1,000 KOs (days 3 and 5) as covariates, was performed with the R package 'DESeq2' version 1.10.1[36]. KOs were considered significantly differentially abundant in VD and CSD (± SGA) if the FDR-adjusted *P* value of the Wald test was <0.05 for at least one comparison (CSD versus VD or CSD+SGA versus VD) and the directionality of change in both comparisons was the same. Differentially abundant pathways were detected through pathway enrichment analysis using a custom R script[59].

Results, manuscript lines 226 to 232: We detected a total of 1,697 functional categories from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) database that were differentially abundant in the combined comparisons of the gut microbiome of CSD and CSD+SGA neonates to VD neonates and were presenting the same directionality of log fold change. We used the R package 'DESeq2'[36] with a linear model, which considered the different collection time points containing at least 1,000 KOs (days 3 and 5) as covariates (Fig. 2a; Supplementary Data 10).

Figure legend, manuscript lines 1320 to 1332: Heatmap of relative abundance of gut microbial orthologous gene groups with significant differential abundances in neonates born by vaginal delivery (VD) compared to either caesarean-section delivery (CSD) or CSD with small for gestational age (SGA) status (CSD+SGA) groups and having the same direction of log2 fold change (calculated with the R package 'DESeq2'[36] and indicated by the color key; false-discovery-rate (FDR)-adjusted *P* <0.05).

3.8. FIGURE 4: The figure legend does not fully explain 4a. Specifically, it was only

after I read the main text that I understood that the EU amounts were measured in the immune assay. I was confused and initially thought it was the LPS concentration used in the assay.

Response:

We thank the reviewer for pointing this out. In fact, the endotoxin units (EU) given in Figure 4a (now Supplementary Fig. 13 in the revised manuscript) were estimated based on the quantification of the LPS samples prior to the immunostimulatory assay. No LPS amounts were measured in the immune assay. We have revised the manuscript and figure legends to make this clearer.

Figure legend, manuscript lines 1367 to 1380: Lipopolysaccharide (LPS) was isolated from faecal samples collected on day 3 postpartum from neonates in groups of vaginal delivery (VD), caesarean-section delivery (CSD) and CSD with small for gestational age (SGA) status (CSD+SGA), and incubated for 24 h with human monocyte-derived dendritic cells (MoDCs) isolated from a total of 12 adult donors. MoDCs were stimulated the with the exact same LPS volume that was extractable from the same initial amount of faecal material from each neonate sample (Methods).

3.9. Line 87, vaginal or stool in mothers?

Response:

We thank the reviewer for pointing this out. We clarified this part in the revised manuscript.

Results, manuscript lines 206 to 219: To assess whether the apparent taxonomic differences between the gut microbiomes of VD and CSD neonates are reflected at the level of functional potential, we used the metagenomic sequencing data to compare functional profiles of all neonates to the gut microbial potential of their respective mothers. We also compared the CSD (±SGA) microbiota at day 3 and day 5 postpartum to those of VD neonates. The correlations of the functional profiles of the neonatal gut microbiome to the respective maternal vaginal microbiome were lower (median rho 0.06 for day 1, 0.37 for day 3 and 0.44 for day 5) than the correlation between neonatal and maternal gut microbiomes (median rho 0.29 for day 1, 0.59 for day 3 and 0.62 for day 5). While the correlations of the functional profiles of the neonatal gut microbiome and the maternal vaginal microbiome did not differ significantly between delivery modes, early microbial functions in VD neonates better reflected the mothers' functional gut microbiome profiles compared to CSD (± SGA) neonates (Fig. 2a; Wilcoxon rank-sum test, FDR-adjusted $P = 4.1 \times 10^{-3}$ for day 3).

3.10. Line 94-96 briefly mention what statistical method was used (if any) to rule out the influence of feeding method

Response:
We thank the reviewer for this comment. In the revised manuscript, we have included a detailed multivariate analysis using MaAsLin. For more details, please refer to our reply to **comments 2.1.** and **2.2.**

3.11. Lines 99-105 Where there any comparisons done between VD samples and each of the CSD groups. If so, this should be mentioned in this section. If not why separate the CSD groups?

Response:
We thank the reviewer for this comment. Indeed, comparisons between VD and CSD and VD and CSD+SGA were done and were largely matching independent of the SGA status. As no discernible differences were observed based on Fig. 4a between CSD and CSD+SGA, we chose to combine both groups in order to increase the statistical power.

Results, manuscript lines 237 to 240: In order to increase the statistical power and as comparisons between VD and CSD and VD and CSD+SGA were largely matching independent of additional SGA status, we combined both groups (CSD and CSD+SGA; Fig. 2b and c).

3.12. Line 144, sources of microbial origin are too speculative, I suggest removing

Response:
We thank the reviewer for the suggestion. We have revised the manuscript and cited previous studies that observed or suggested strain transfer from other environments to the neonate.

Discussion, manuscript lines 485 to 487: Consequently, the gut of CSD neonates is most likely colonized by strains derived from other sources, such as breast milk, skin or saliva, as suggested in previous studies[44–46].

3.13. Line 174 I suggest changing “more specifically” for “especially”

Response:
We thank the reviewer for the suggestion and we have revised the manuscript accordingly.

Results, manuscript lines 401 to 404: The levels of all measured cytokines, and especially of TNF-α and IL-18, were higher in culture supernatants from MoDCs treated with LPS from VD neonates (Supplementary Figure 13; Supplementary Data 16; Supplementary Note 6).

3.14. Line 202-203 Huge stretch here. There is no data on chronic diseases in this super small cohort. Also omit from the summary figure in extended data.

Response:

We thank the reviewer for pointing this out. We agree that the previous statement was too suggestive in the given context. We have now included a separate paragraph based on previous studies, which support our results. We have also removed the summary figure.

Discussion, manuscript lines 522 to 542: Our study highlights differences in immunostimulatory potential of the earliest gut microbiome according to delivery mode. This occurs during a critical window of immune system priming. Notably, alterations to early immune system stimulation may be linked to the higher propensity of CSD infants to develop chronic diseases in later life[2]. For example, previous studies focusing on environmental exposure in early life have suggested that the exposure to Gram-negative bacteria and/or environmental endotoxins (such as LPS) could confer protective effects towards allergy development[48,49]. In this context, the LPS biosynthesis pathway harbours the potential to be closely involved in the priming of the neonatal immune system and the subsequent tolerance towards the colonizing gut microbiome during a most critical window in early neonatal life[12–14], with potential persisting effects on the later health status. Using a mouse model, it has been shown that strongly immunostimulatory LPS can contribute to the protection from immune-mediated diseases such as diabetes[50] and that disruption of host-commensal interactions in early-life can lead to persistent defects in the development of specific immune subsets[12]. Based on additional cytokine measurements in neonatal plasma, VD neonates displayed higher levels of IL-18 and TNF-α, thereby indicating a link between the immunostimulatory potential of microbial LPS in the gut and the overall immune status of the neonatal host early on. Investigations of the longer-term consequences of these differences between CSD and VD neonates will be necessary to assess their possible impact on the development of chronic diseases in later life.

3.6 Lines 483-487 Unclear why bacterial DNA amount needed to be controlled in the LPS assay. Please elaborate either here on in the Supplementary notes. These assays conditions will not mick the amount of LPS that interacts with a peripheric immune cells so I do not understand the reasoning behind this. Why not normalize by LPS units only? DNA quantification via qPCR of 18S RNA gene will vary depending on the number of 16S copies per cell so it is not the best way to quantify bacterial load. Further, this method will account for all bacterial cells, not just gram-negative (LPS containing cells). Flawed method.

Response:

The rationale for using the amount of bacterial DNA was to compare the same bacterial load for each sample and, thus, assess if a distinct sample will induce a

distinct immune response. As the bacterial loads in early meconium samples were in general lower in CSD compared to VD, we thereby aimed to minimize the likelihood that an observed effect is not due to a lower bacterial presence in the CSD meconium. However, we also agree with the reviewer that the current normalization does not reflect the real situation. Therefore, we have included additional immunological assays in the revised manuscript in which we stimulate primary immune cells with the exact same "LPS volume" extracted from the exact same starting faecal mass for each individual sample. Using both methods, we observed the same effects (Fig. 4a and Supplementary Fig. 13). With respect to additional samples that were used, as well as the specific reporter cell lines (**comments 2.6., 2.9. and 3.4.**), we thus show that the immunological stimulatory potential of neonatal faecal samples is much higher in case of vaginal delivery compared to C-section delivery.

Results, manuscript lines 392 to 401: In order to reflect the *in vivo* situation as closely as possible, we stimulated the MoDCs with the exact same LPS volume that was obtainable from the same initial amount of faecal material from each neonate sample and subsequently measured levels of the LPS-inducible cytokine TNF-α in the supernatants using an ELISA assay (Fig. 4a; Supplementary Data 13). In parallel, a panel of additional cytokines was measured using an approach for quantifying and normalizing the employed LPS fractions (Methods). This was based on a maximum stimulation of MoDCs with 100 Endotoxin Units (EU) of LPS in order to mimic the amount of LPS an immune cell may encounter within a given neonatal sample (Supplementary Figure 13; Supplementary Data 14 and 15).

11. Line 124 of Supplementary notes: I would change "corresponded" to correlated

Response:
We agree with the reviewer and have adapted the manuscript accordingly.

Supplementary Information, supplementary note 6: The observed levels of TNF-α mostly correlated with the relative abundance of Gram-negative bacteria (Fig. 4a, Supplementary Fig.3b).

12. The manuscript could use a brief discussion on the choice of adult blood DCs and how it may have differed from neonate peripheric immune cells. Neonatal immune cells are known produce immune responses similar to adults in some aspects but not others.

Response:
We thank the reviewer for this constructive comment. We included details on the choice of adult blood DCs to the revised manuscript.

Material and methods, manuscript lines 879 to 885: Human neonatal dendritic cells (DCs) were previously shown to be competent in MHC class I antigen processing and presentation to the same extent than adult DCs[79]. Most importantly, the NF-kB-dependent pathway in TLR-4 signalling is intact in neonatal MoDCs as they produce pro-inflammatory cytokines upon LPS stimulation, while adult and neonatal DCs are both able to produce comparable levels of TNF-α, IL-6 and IL-8 in response to LPS[80].

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors performed a substantial amount of work and additional experiments for this revised manuscript. The new data is strong and all the crucial issues pointed out on the original submission seem to be fixed. One of the strongest points of the work is the increased vertical transmission of microbes in vaginally delivered infants compared to delivery via C-section.

I still have some remarks for the authors.

1 - The paper is convincing on the fact that vaginal deliver impacts the infant microbiome. The authors state that this was quite controversial, but I partially disagree with this. What was controversial is whether the effect on the microbiome of vaginal versus C-section delivery is long-lasting and whether it is stronger than other conditions including breast-feeding versus formula-feeding. Because in this study the infants are not followed for months and breast feeding is not discussed in depth, these questions cannot be answered. I think the authors should be a bit more careful when discussing the effect of delivery mode on the microbiome, because there are not really many controls for other conditions (again feeding regime).

2 - The introduction now is very comprehensive. However, it is also redundant and repeated in several passages. I feel the introduction should be shortened a bit and repeated concepts minimized. Also the discussion is very verbose.

3 - Is multiple hypothesis testing correction applied on the identification of 1,697 differential KEGG KOs at line 226?

4 - Figure 3A. It is difficult to see the vertical transmission events because of the use of a color gradient (shade of orange). More distinct colors should be adopted here, and in general the figure is not very intuitive. In the legend, it is not clear what "taxon without link" means

5 - line 212-220: microbiomes from different body sites of the mother and infants are here compared using correlation. Giving the large differences in the overall structure of the microbiome across body sites and hosts, I don't think correlation is the correct statistical tool to use.

Reviewer #2 (Remarks to the Author):

In this manuscript, Wampach et al collected stool samples from mothers and matched neonates. They perform shotgun sequencing and analyze the gene-level differences between the stool microbiomes of vaginally born babies vs. C-section born babies. They find that genes encoding LPS synthesis are enriched in the vaginally born babies - and that the LPS extracted from these two stool types, when tested on primary human immune cells, elicits differences in cytokine release. The LPS extracted from the Vaginally born babies appears to be more immunostimulatory. Thus, the authors conclude that C-section disrupts transfer of specific microbial strains that are highly immunostimulatory, and that this may impact “neonatal immune system priming”.

Overall, the manuscript is much improved from its prior version. A notable strength of

this manuscript is the collection and evaluation of very early post-natal samples (day 0-5) and the strong effort to characterize the functional impact of LPS on the immune system. The manuscript, as it stands, is a bit lengthy (especially in the discussion section), but is well carried out and interesting. I offer the following suggestions for improvement.

Major suggestions:

#. The authors now allude to the existing controversy in evaluating the impact of birth-mode on microbial "transmission". I propose that it might be better for the authors to steer clear of the transmission question and to simply report the results in the context of vaginally born and CS born babies having different microbiomes (which may be the consequence of confounding factors other than just birth mode - e.g. antibiotic exposure).

#. The title suggests that birth mode is causal of strain-conferred gut microbiome functions and immunostimulatory potential - I think the title should be adjusted to reflect the fact that the authors identify an association, as opposed to a causal link between birth mode and these outcomes.

#. Are there any other notable differences between the VD and CSD babies? Or were these all elective CSD babies - which would be the best comparator.

#. I find the LPS aspect of the paper the most novel and exciting - but it is only briefly focused on in the manuscript. Also, is there a reason that only a subset of the total number of subjects was included in the LPS part of the study? Lastly, is there a reason the day 3 sample was selected (as opposed to the day 5 sample, which may have been higher biomass)?

Minor suggestions:

#. Would remove the phrase "After data curation" in the abstract - it is vague.

#. line 76 - I am not exactly sure what the authors are referring to when they state "Although CSD is not associated with improved health outcomes…". Also - are the a

#. Line 86 - would call this 16S rRNA gene amplicon sequencing.

#. Line 132 - for what proportion of samples could sufficient DNA be extracted for downstream metagenomic analysis?

#. The majority of the analyses seem to focus on CSD +/- SGA compared to VD; I applaud the authors for including CSD -SGA controls - but am surprised that they chose not to analyze the three groups (CSD -SGA, CSD + SGA and VD) separately, as this would be a much stronger analysis that lacked the likely confounding factors contributed by SGA.

#. Line 422, 458 - calling the approach they employed "artefact-free" seems hyperbolic.

#. line 490 - typo - "form" instead of "from"

#. Only 16 mother/neonate pairs were collected; yet, additional neonates were studied - the methods section could be more clear as to how those additional neonates were recruited to the study.

#. I presume the authors used standard V4 primers for 16S sequencing. Please state this, if so.

#. What is the “optimized low-quantity DNA library preparation kit” that was used?

#. Why were some of the metagenomic libraries (C105, 109, 110 and 119) prepared using another (presumably) kit?

#. The method for removing artifactual reads relies on contain assembly and alignment. What was the contig size cutoff used for this method? If the contig size cutoff is >read length, this might remove low abundance contaminants that are not present at a high enough abundance to assemble into contigs - thus compromising the effectiveness of this contamination removal effort.

#. line 854 - Please provide some additional detail in text regarding the “further purification” that was used to obtain the LPS.

#. Line 856 - why was LPS from the three aliquots of stool pooled - presumably because each extraction resulted in a very small amount of LPS. I would have very much liked to see this assay done in replicate (starting with the stool), as I am not familiar with the variability of LPS extraction efficiency.

#. What proportion of all metagenomic reads were assigned a potential function? I ask because it seems surprising that 2% of all metagenomic reads are classified as part of a 2-component system, for example. That seems rather high.

#. Figure 3 is very complex and does not communicate a strong, focused story. I wonder if the authors could simplify the figure (by, perhaps, moving some of the panels to the supplement)?

#. It is interesting (Fig 4) that one of the CSD+SGA neonates has a high a high proportion of Gram neg organisms and a very low amount of TNF-alpha release in the MoDC assay. What do the authors make of this? Perhaps this was in the discussion and I missed it?

Reviewer #3 (Remarks to the Author):

After reviewing this manuscript again, I am quite impressed with the amount of attention and added work that the authors have given to the reviewers’ comments and suggestions. While the previous manuscript was already strong, the authors acknowledged the rightful arguments made by all reviewers and submitted a revised paper that appropriately addressed all of the major points. The authors: added samples to the study (for microbiome analysis and immune assays), controlled for confounding variables known to influence microbiome structure, confirmed purity of LPS, performed the LPS assay under alternative conditions that normalized LPS concentrations across samples, provided added evidence that samples used for metagenomics were representative of all cohort samples, and limited the discussion to their findings, without associating to future health outcomes that were not assessed in their study. Truly, the authors should be commended for their effort. The result is an outstanding contribution to the field that in my view, confirms that mode of delivery impacts the infant microbiome, both taxonomically, functionally and in relation to the immune consequence to the infant host. I would seriously question who would continue to debate on this

issue after the findings presented by Wampach et al.

One comment I would like to leave with the authors, which does not impact this paper but may be helpful in future studies of a similar nature, is in reference to the answer in their rebuttal letter
(Lines 1126-1228):

“We want to stress again that the main point of the manuscript is not statistics-driven, as the presence of strains, given their high degree of specificity, is valid only on an individual, sample-to-sample basis.”

Their study argues that mode of delivery impacts infant microbiome structure and performed several analysis comparing two or more groups. All studies that compare variables of interest are subjected to statistics. Thus, all of these studies must be statistics-driven. I have learned this not as a biostatistician but after analyzing many human microbiome studies, where controlling for possibly confounding co-variates is the only valid method to link a microbiome feature with a variable of interest. There are enough studies available on the effect of antibiotics on infant microbiome, for example, to help direct future studies in terms of study design and number of samples necessary.

Manuscript reference number: NCOMMS-17-19613B

Responses to the reviewers' comments

N.B.: The original remarks by the reviewers are provided below in boxes whereas the authors' responses are listed in black type and cited passages from the manuscript are listed in blue.

Reviewer 1

1.1. The authors performed a substantial amount of work and additional experiments for this revised manuscript. The new data is strong and all the crucial issues pointed out on the original submission seem to be fixed. One of the strongest points of the work is the increased vertical transmission of microbes in vaginally delivered infants compared to delivery via C-section.

We thank the reviewer for his/her positive evaluation of the efforts that were put into improving the manuscript.

1.2. The paper is convincing on the fact that vaginal deliver impacts the infant microbiome. The authors state that this was quite controversial, but I partially disagree with this. What was controversial is whether the effect on the microbiome of vaginal versus C-section delivery is long-lasting and whether it is stronger than other conditions including breast-feeding versus formula-feeding. Because in this study the infants are not followed for months and breast feeding is not discussed in depth, these questions cannot be answered. I think the authors should be a bit more careful when discussing the effect of delivery mode on the microbiome, because there are not really many controls for other conditions (again feeding regime).

We thank the reviewer for this comment. Indeed, we agree with the reviewer that the current discussions in the field of the neonatal microbiome do not only focus on the question of potentially long-lasting effects of delivery mode but also on the diverse set of factors which may affect the neonatal gut microbiome. In the introduction of the revised manuscript, we now more explicitly mention one specific publication (Chu et al. 2017), which claimed that delivery mode had no effect on the microbiome structure and which thereby sparked the

aforementioned controversy regarding the impact of delivery mode on the neonatal microbiome. Although our multivariate analysis showed that delivery mode was the strongest driver in our study, we do agree that our timeframe is limited. In this sense, our manuscript will most likely serve as a model for future metagenomic studies that will need to include more mother-neonate pairs. Additionally, these future studies will also be able to evaluate the specific effects of certain conditions such as feeding regime after the timeframe of the first 5 days after delivery. We have revised the manuscript to be more careful with our phrasing and have additionally adapted the title.

1.3. The introduction now is very comprehensive. However, it is also redundant and repeated in several passages. I feel the introduction should be shortened a bit and repeated concepts minimized. Also the discussion is very verbose.

We thank the reviewer for pointing this out. We have revised the introduction accordingly.

1.4. Is multiple hypothesis testing correction applied on the identification of 1,697 differential KEGG KOs at line 226?

We thank the reviewer for his/her comment. Yes, these tests were performed with multiple testing correction by controlling false discovery rate according to Benjamin and Hochberg (1995).

1.5. Figure 3A. It is difficult to see the vertical transmission events because of the use of a color gradient (shade of orange). More distinct colors should be adopted here, and in general the figure is not very intuitive. In the legend, it is not clear what "taxon without link" means

We thank the reviewer for pointing this out. We aimed to make the figures as clear and intuitive as possible to support our findings. Therefore, we tested many different options that allowed us to include all the current information into one graph. As several different methods for shared taxa between mother and neonate and strain identification were applied, the current version was the clearest in conferring all necessary information to the reader. We agree that gradient colours might be sometimes difficult to assess, however in this case it reflects best the fact that the darker the colour is, the more evidence was found to prove that strain transfer

between mother and neonate gut occurred. Additionally, as the taxa are already highlighted by distinct colours, we wanted to refrain from another panel of distinct colours and opted for an orange colour gradient instead.

A ‘taxon without link’ in this context described a taxon that was found in the maternal samples, but not found to be shared between mother and neonate based on all of the applied levels of analyses (e.g. neither the same taxon was identified, nor was the same strain detected in both mother and neonate gut microbiomes). We have revised the legend within Figure 3A to clarify this point.

Manuscript, figure legends, lines 1681 to 1684: The level of evidence of transmission is indicated by the shading colour, with darker shading for stronger evidence. A taxon without link describes a taxon that was found in the maternal samples, but not shared between mother and neonate.

1.6. line 212-220: microbiomes from different body sites of the mother and infants are here compared using correlation. Giving the large differences in the overall structure of the microbiome across body sites and hosts, I don't think correlation is the correct statistical tool to use.

We thank the reviewer for pointing this out. In the revised manuscript, we have additionally assessed the functional potentials of the distinct microbiome samples (vaginal swab and neonatal stool samples) using a principal coordinate analysis (PCoA) representing the ordination of their respective Jensen–Shannon distances. Based on these results, a large fraction of the vaginal swab microbiomes cluster together and represent a common microbial community structure. However, as both vaginal swab and early neonatal stool samples were marked by generally low numbers of identified KOs, some of the maternal vaginal microbiomes clustered together with neonatal microbiomes, making a clear distinction impossible. Therefore, we decided that a subsequent statistical analysis using correlation was valid in this specific case. We have included the PCoA plot and the matching plots representing the Jensen-Shannon distances comparing neonatal samples to maternal gut and vaginal swab samples as Supplementary Figure 4 and discuss the figure in the main manuscript.

Supplementary information, legend of figure 4: Assessment of functional potential across sample types and between maternal and neonatal samples. **a,** Principal coordinate analysis of Jensen-Shannon divergence was generated for the functional data of all different sample types. Lines connect samples which originated from the same neonate according to the order of sampling. **b,** Jensen-Shannon divergences of the functional profiles of the neonatal gut microbiomes to those of the respective maternal gut microbiomes. **c,** Jensen-Shannon divergences of the functional profiles of the neonatal gut microbiomes to those of the respective maternal vaginal microbiomes. Samples are coloured according to birth mode and small for gestational age (SGA) status. VD, delivery; CSD, caesarean-section delivery; M, maternal faeces; MV, maternal vaginal swab: N, neonatal faeces collected at day1 / day 3 / day 5. Boxplots: centre line – median, bounds – first and third quartile, whisker <= 1.5 x interquartile range.

Manuscript, results lines 271 to 277: To assess whether the apparent taxonomic differences between the gut microbiomes of VD and CSD neonates are reflected at the level of functional potential, we used the metagenomic sequencing data to calculate Jensen-Shannon divergences for all samples (Supplementary Fig. 4a). Overall, comparison of the functional profiles of all neonates to the gut microbial potential of their respective mothers highlighted that the neonatal gut microbiota were more divergent from the maternal vaginal microbiota than the corresponding gut microbiota (Supplementary Fig. 4a, b & c).

Reviewer 2

2.1. In this manuscript, Wampach et al collected stool samples from mothers and matched neonates. They perform shotgun sequencing and analyze the gene-level differences between the stool microbiomes of vaginally born babies vs. C-section born babies. They find that genes encoding LPS synthesis are enriched in the vaginally born babies - and that the LPS extracted from these two stool types, when tested on primary human immune cells, elicits differences in cytokine release. The LPS extracted from the Vaginally born babies appears to be more immunostimulatory. Thus, the authors conclude that C-section disrupts transfer of specific microbial strains that are highly immunostimulatory, and that this may impact "neonatal immune system priming".

Overall, the manuscript is much improved from its prior version. A notable strength of this manuscript is the collection and evaluation of very early post-natal samples (day 0-5) and the strong effort to characterize the functional impact of LPS on the immune system. The manuscript, as it stands, is a bit lengthy (especially in the discussion section), but is well carried out and interesting. I offer the following suggestions for improvement.

We thank the reviewer for re-evaluating the manuscript and for kindly acknowledging the efforts that were put into the revision.

Major suggestions

2.2. The authors now allude to the existing controversy in evaluating the impact of birth-mode on microbial "transmission". I propose that it might be better for the authors to steer clear of the transmission question and to simply report the results in the context of vaginally born and CS born babies having different microbiomes (which may be the consequence of confounding factors other than just birth mode - e.g. antibiotic exposure).

We thank the reviewer for his/her comment. Mention of the controversy regarding transmission according to delivery mode was in fact suggested by reviewer 1 in the previous revision phase. We agreed that this question should not go unmentioned, since our approach not only addresses differential analyses of gut microbiome structure and function according to delivery mode, but through integration of strain-level information points to differences in vertical strain transmission (see also doi: 10.1016/j.chom.2018.06.007 and doi: 10.1016/j.chom.2018.06.005). Furthermore, we want to highlight that an extensive multivariate analysis had identified the delivery mode as the main driver of the neonatal gut microbiome structure during the earliest days after birth.

2.3. The title suggests that birth mode is causal of strain-conferred gut microbiome functions and immunostimulatory potential - I think the title should be adjusted to reflect the fact that the authors identify an association, as opposed to a causal link between birth mode and these outcomes.

We thank the reviewer for the thoughtful suggestion and have changed the title in accordance with the editor's suggestion to 'Birth mode is associated with earliest strain-conferred gut microbiome functions and immunostimulatory potential'.

2.4. Are there any other notable differences between the VD and CSD babies? Or were these all elective CSD babies - which would be the best comparator.

We thank the reviewer for pointing this out. Indeed, the data is provided in the Supplementary Data 1: out of the CSD neonates for whom the high-resolution metagenomics approach was applied, only 1 was born by emergency C-section (C101), while all others were born by elective C-section.

2.5. I find the LPS aspect of the paper the most novel and exciting - but it is only briefly focused on in the manuscript. Also, is there a reason that only a subset of the total number of subjects was included in the LPS part of the study? Lastly, is there a reason the day 3 sample was selected (as opposed to the day 5 sample, which may have been higher biomass)?

We thank the reviewer for highlighting our work on the LPS extraction and stimulations assays. As highlighted in the manuscript (lines 1147 to 1150), only a subset of stool samples contained sufficient stool material (150 mg) for extracting measurable LPS concentrations. Additionally, some stool samples, although presenting the desired biomass (150 mg), did not yield any measurable LPS concentrations. The reason for this was either because the bacterial biomass in these samples was generally low or because some of these samples were just not rich enough in Gram-negative bacteria.

Furthermore, we chose to extract LPS from the neonatal stool samples of day 3 after delivery as both, the functional data (Figure 2a) and the cytokine data from plasma (Figure 4b), reflected the most significant differences according to delivery mode at this specific day of life. By selecting day 3, we were able integrate matching microbiome, LPS and plasma data. This rationale is now more clearly presented in the manuscript.

Manuscript, results, lines 502 to 517: Based on our data, the microbial composition differed most strongly in VD and CSD neonates on day 3 postpartum and, thereby, may critically

affect the developing immune system at this time[12]. We isolated LPS from faecal samples from day 3 with sufficient biomass from 16 neonates (7 VD, 7 CSD, 2 CSD+SGA; Supplementary Data 12; Methods).

Minor suggestions

2.6. Would remove the phrase "After data curation" in the abstract - it is vague.

We thank the reviewer for his/her suggestion and have adapted the abstract accordingly.

2.7. line 76 - I am not exactly sure what the authors are referring to when they state "Although CSD is not associated with improved health outcomes…".

We agree with this reviewer that this sentence was not clear and have removed the passage from the manuscript in line 139.

2.8. Line 86 - would call this 16S rRNA gene amplicon sequencing.

We agree with the reviewer and have changed this term accordingly.

2.9. Line 132 - for what proportion of samples could sufficient DNA be extracted for downstream metagenomic analysis?

While a total of 75 samples (including maternal vaginal swabs and stool and neonatal stool samples) were collected and extracted from the 16 mother-neonate pairs, a total of 65 samples yielded enough DNA to reliably proceed with metagenomic sequencing (87%). For only 2 out of these 65 samples a high proportion of the resulting data represented contaminant sequencing reads and were thus excluded from further analyses (samples MV_C100 and V1_C100). We have mentioned this in the revised manuscript, lines 973 to 975.

2.10. The majority of the analyses seem to focus on CSD +/- SGA compared to VD; I applaud the authors for including CSD -SGA controls - but am surprised that they chose not

to analyze the three groups (CSD -SGA, CSD + SGA and VD) separately, as this would be a much stronger analysis that lacked the likely confounding factors contributed by SGA.

We thank the reviewer for raising this comment. Indeed, the initial analyses were done by keeping the samples from CSD and CSD+SGA neonates as separate groups and comparing both independently to the microbiome characteristics of VD neonates. However, on each level of analysis, we observed that there were no pronounced differences between the CSD and CSD+SGA profiles, while both groups showed the same kind of differences compared to the VD microbiomes. Therefore, we report both groups together as CSD±SGA. We have clarified this aspect in the revised manuscript.

Manuscript, results lines 356 to 359: As comparisons between VD and CSD as well as VD and CSD+SGA were largely matching independent of SGA status, we combined both groups (CSD and CSD+SGA) to increase statistical power (CSD±SGA).

2.11. Line 422, 458 - calling the approach they employed "artefact-free" seems hyperbolic.

We agree with the reviewer and suggest to change 'artefact-free' to 'artefact-curated' throughout the entire manuscript.

2.12. line 490 - typo - "form" instead of "from".

Thank you for this comment. We have corrected this typo.

2.13. Only 16 mother/neonate pairs were collected; yet, additional neonates were studied - the methods section could be more clear as to how those additional neonates were recruited to the study.

We agree with the reviewer that this aspect could be better explained in the manuscript. We have now added another sentence to the methods section.

Manuscript, methods lines 899 to 903: From the 33 neonates that were recruited into the study, the gut microbiome of 15 (Supplementary Data 1) had previously been characterised

using a combination of 16S rRNA gene amplicon sequencing and quantitative real-time PCR[7]. For a subset of neonates, the mother was sampled additionally.

2.14. I presume the authors used standard V4 primers for 16S sequencing. Please state this, if so.

We thank the reviewer for pointing this out and have specified this in the revised manuscript.

Manuscript, methods lines 960 to 963: All DNA samples (along with 8 controls) underwent standard amplicon sequencing of the V4 region of 16S rRNA genes using primers 515F-GTGBCAGCMGCCGCGGTAA and 805R-GACTACHVGGGTATCTAATCC at the Center for Analytical Research and Technology–Groupe Interdisciplinaire de Génoprotéomique Appliquée (CART-GIGA; Liège, Belgium).

2.15. What is the "optimized low-quantity DNA library preparation kit" that was used?

We thank the reviewer for his/her comment. The library preparation was performed by the service provider GATC Biotech using validated standard procedures modified to optimize the method for automated library preparation. These protocols are proprietary to GATC Biotech and were not released to the authors. We referred to the fact that this sequencing was performed by GATC Biotech in the manuscript, lines 966 to 970.

2.16. Why were some of the metagenomic libraries (C105, 109, 110 and 119) prepared using another (presumably) kit?

During the revision of the manuscript, we additionally sequenced samples from 4 more mother-neonate pairs (C105, C109, C110, C119). At that time, our in-house sequencing platform was well established and experienced with low biomass samples so we chose to do the metagenomic sequencing in-house in order to have a shorter turn-around time. Although we could not use the exact same protocol or library preparation kit as the previous sequencing facility, the quality after in-house sequencing was at least as high as for the initial sequencing of the first samples of the 12 mother-neonate pairs.

2.17. The method for removing artifactual reads relies on contain assembly and alignment. What was the contig size cutoff used for this method? If the contig size cutoff is >read length, this might remove low abundance contaminants that are not present at a high enough abundance to assemble into contigs - thus compromising the effectiveness of this contamination removal effort.

We thank the reviewer for his/her comment. Indeed, we observed that the putative artefactual sequences were almost exclusively very small fragments that could not be assembled effectively or for which no genome reconstruction could be obtained. Therefore, as a defined contig size cutoff would have not taken into consideration the many low abundance contaminant reads, we opted to have no cut-off for any of the low biomass samples (neonatal samples and vaginal swab samples). More specifically, as the removal of putative artefactual sequences relies on the simultaneous binning of contigs from both study sample and contamination control samples, the cut-off for visualizing and considering contigs was set to >0 instead of the usual cut-off of >1000 nt (which was kept for the complex samples from maternal stool). Notably, the artefact removal was not only based on assembled contigs, but identified putative contaminant sequences were also removed from the original sample reads, which further highlights the need of properly identifying, assessing and removing putative artefactual contigs and reads from low biomass microbiome data.

Manuscript, methods lines 1002 to 1005: After removing the rRNA sequences from the contigs[56], we performed joint binning of control cell-culture contigs with each of the samples' contigs individually using VizBin[29] without any length cut-off.

2.18. line 854 - Please provide some additional detail in text regarding the "further purification" that was used to obtain the LPS.

We now added more detail in the text, specifying the nature of the "further purification".

Manuscript, methods lines 1150 to 1159: To maximise yields, LPS was purified from three aliquots of 50 mg of each neonatal faecal sample using the hot phenol–water method[73] and further purification was performed using a modified phenol re-extraction protocol[74].

2.19. Line 856 - why was LPS from the three aliquots of stool pooled - presumably because each extraction resulted in a very small amount of LPS. I would have very much liked to see this assay done in replicate (starting with the stool), as I am not familiar with the variability of LPS extraction efficiency.

LPS extraction was much more efficient if it was done on small quantities of stool samples (e.g. 50 mg) compared to LPS extraction done on bigger quantities of stool samples (e.g. 150 mg). Therefore, three small aliquots were used and pooled afterwards to have enough LPS to work with. The variability of LPS extraction efficiency using the method described within the manuscript was quite low as we could observe that for the control samples, where we extracted LPS from pure *E. coli* cultures (each culture with an OD600 of 0.5), we could obtain similar amounts of LPS (please refer to the table below).

LPS in EU/ml (Replicate A)	**LPS in EU/ml (Replicate B)**	**LPS in EU/ml (Replicate C)**	**LPS in EU/ml (Replicate D)**
10,610.07	8,018.01	7,923.36	9,363.41

LPS was extracted from a pure *E.coli* overnight culture grown in LB medium. Based on OD600 reading, cultures were diluted to an OD600 of 0.5 and 1.5 ml of each culture was taken for LPS extraction. Bacterial suspensions were centrifuged at 10,600 g for 10 minutes, the supernatants were discarded and pellets were used for LPS extraction. LPS concentration was quantified using an ELISA-based endotoxin detection assay (Endolisa; # 609033, Hyglos GmbH, Germany).

2.20. What proportion of all metagenomic reads were assigned a potential function? I ask because it seems surprising that 2% of all metagenomic reads are classified as part of a 2-component system, for example. That seems rather high.

We thank the reviewer for pointing this out. 77 % of the curated metagenomic reads mapped to genes that were assigned a potential function (standard deviation 13 %) in the neonate stool samples displayed in the figure. This is now mentioned in the manuscript, lines 1019 to 1022.

2.21. Figure 3 is very complex and does not communicate a strong, focused story. I wonder if the authors could simplify the figure (by, perhaps, moving some of the panels to the supplement)?

We thank the reviewer for his/her suggestion. Although we agree that Figure 3 contains much information, removing some of the panels would not seem as intuitive to us, since the order of the panels follows the main results text, while giving concrete visual examples for both a CSD and VD case. We would therefore prefer not to move any of the information that supports the main message of the manuscript to the supplement.

2.22. It is interesting (Fig 4) that one of the CSD+SGA neonates has a high proportion of Gram neg organisms and a very low amount of TNF-alpha release in the MoDC assay. What do the authors make of this? Perhaps this was in the discussion and I missed it?

We thank the reviewer for this observation. Indeed, we were also intrigued by this case and had already previously addressed this in the supplementary information, note 6. We think that this specific case could point to the importance of the composition of the LPS rather than the overall abundance being crucial for immune system stimulation.

Reviewer 3

3.1. After reviewing this manuscript again, I am quite impressed with the amount of attention and added work that the authors have given to the reviewers' comments and suggestions. While the previous manuscript was already strong, the authors acknowledged the rightful arguments made by all reviewers and submitted a revised paper that appropriately addressed all of the major points. The authors: added samples to the study (for microbiome analysis and immune assays), controlled for confounding variables known to influence microbiome structure, confirmed purity of LPS, performed the LPS assay under alternative conditions that normalized LPS concentrations across samples, provided added evidence that samples used for metagenomics were representative of all cohort samples, and limited the discussion to their findings, without associating to future health outcomes that were not assessed in their study. Truly, the authors should be commended for their effort. The result is an outstanding contribution to the field that in my view, confirms that mode of delivery impacts the infant

microbiome, both taxonomically, functionally and in relation to the immune consequence to the infant host. I would seriously question who would continue to debate on this issue after the findings presented by Wampach et al.

We are deeply thankful for the honest evaluation of our revised manuscript and for the overall positive feedback.

3.2. One comment I would like to leave with the authors, which does not impact this paper but may be helpful in future studies of a similar nature, is in reference to the answer in their rebuttal letter (Lines 1126-1228):
"We want to stress again that the main point of the manuscript is not statistics-driven, as the presence of strains, given their high degree of specificity, is valid only on an individual, sample-to-sample basis."

Their study argues that mode of delivery impacts infant microbiome structure and performed several analysis comparing two or more groups. All studies that compare variables of interest are subjected to statistics. Thus, all of these studies must be statistics-driven. I have learned this not as a biostatistician but after analyzing many human microbiome studies, where controlling for possibly confounding co-variates is the only valid method to link a microbiome feature with a variable of interest. There are enough studies available on the effect of antibiotics on infant microbiome, for example, to help direct future studies in terms of study design and number of samples necessary.

We thank the reviewer for this very thoughtful comment. As the domain of microbiome research and especially the analysis of shotgun metagenomic sequencing is still maturing, we see this study as a methodological model for further studies. We hope to be able to perform additional studies with larger sample sizes to determine the effects of different factors on the neonatal gut microbiome in the future. In this context, the presented data will prove helpful to perform the corresponding power analyses.