

Baby-to-baby strain transmission shapes the developing gut microbiome

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors present a metagenome analysis of infants, family members and nursery staff (educators) to examine infant gut microbiome transmission processes in nursery setting. 643 samples from 133 individuals are analysed from 3 nurseries in Trento in Italy over 5 months. The authors report extensive transmission of gut microbiome strains occurring between infants in nurseries that is at higher levels compared to other family members and educators during this time period. The study highlights the importance of nursery settings in promoting infant to infant microbiome transmission. These findings are novel as they extend on previous research, much of it led by the same authors, highlighting the importance of transmission in seeding the infant gut microbiome and enabling microbiome assembly. While previous research has focused on the importance of maternal transmission and to a lesser degree, transmission from other family members, this study reveals childcare settings also contribute.

Overall, the authors demonstrate gut microbiome transmission is occurring in nursery settings between infants, however while well written, the results are quite descriptive and the impact of the findings are not entirely convincing.

One reason is the relatively small number of samples compared to other studies examining early-life gut microbiome transmission, e.g. Stewart et al. Nature and Shao et al. Nature analysed 11,000 and 1700 metagenomes from 900 and 600 babies respectively. In this study, 636 metagenomes from 133 individuals were analysed. These are still very impressive numbers and the authors are to be commended for designing and executing the study, but these numbers are not comparable to other similar studies.

Building on this, I was unsure how these findings fit into the entire sequence of early-life gut microbiome transmission initiated by mothers and continued here. The authors previously reported (Valles-Colomer et al. 2023) the number of strains shared between mothers and offspring (upto 1 year old) as 17. In this study, examining maternal transmission with infants of similar age the authors report number of strains shared as 5-6, which is much lower. Overall, the authors' conclusions (line 442) that the microbial transmission occurring in nurseries as a contributor to infant health is comparable to vaginal vs C-section or breastfeeding vs formula is not conclusive. Other studies from the same authors reported maternal transmission at birth or shortly afterwards contributes more strains (Ferretti et al 2018) compared to transmission reported here between infants in nurseries. Contextualisation of these results (e.g. maternal, paternal, sibling transmission) is needed to show that the findings here are representative of universal early-life microbiome ecological processes and are not isolated or stochastic.

As an example of how the results reported here could be given greater significance, can this study explain the transmission of strains that are prevalent and health associated in the early life intestinal microbiota such as *Bifidobacterium longum* subsp. *infantis* (less abundant in European children but still present) but which are not detected in mothers? Or to put it another way, are nursery settings the reservoir and infant-to-infant the transmission route for specific important early-life microbiome species?

The study focuses almost entirely on transmission processes, i.e. the number of species transmitted, the taxonomy of these species and the most transmissible species. As such, a lot of the results are descriptive as they do not address underlying mechanism. Figure 6 does touch on antibiotic usage and its impact on transmission but given the study focuses on early-life gut microbiomes I felt integration of infant dietary practices was required. For example, what was the role of breastfeeding in promoting or blocking transmission of specific species? I expect the infants in this study would be mixed feeding with breastfeeding and solids while others will be weaned. The impact of different diets on transmission in this setting should have been addressed.

Major comments

1. Better contextualisation of the importance of transmission within nurseries compared to maternal transmission or other family members should be provided. My specific comments related to this are above.
2. The role of diet in transmission in this study should be addressed. My specific comments related to this are above.
3. Greater clarity should be provided with results in terms of rate of strain sharing and numbers of strains shared. Fig 1A number of strains shared between babies at end of study is 7.2. SGB richness in babies is ~90 (Figure 1D) = 8% strains shared. How does this relate to the strain sharing rate of 20.2% reported in Line 234?
4. There is no information provided (as far as I can tell) on the extent of metagenome reads that are taxonomically profiled. At a minimum the authors should present the percentage of reads mapped per metagenome.
5. Results identifying SGBs with prevalence of transmission in Figure 3D and those identified as most transmissible in Figure 5 appear redundant. If they start with low prevalence but become highly prevalent (Figure 3D), they are by definition highly transmissible. I suggest these results be combined or else Fig. 3D be removed.

Other comments:

1. Line 153- The data on transmission between pets and humans is not very convincing given only 4 animals are profiled. I suggest removing from paper or moving to supplemental material.
2. Line 173- given the small sample numbers (4 pets) and the fact that *P. vulgatus* and *R. gnavus* also have health-associated properties, this statement is very speculative. I suggest removing it.
3. Line 147- It is not clear what triangles with numbers in Figure 2A represent and how they relate to numbers within family or nursery box. Please clarify further.
4. Line 195- The authors convincingly present examples of transmission event such as *A. muciniphila* in Figure 2D and validate StrainPhlAn approach by comparing CRISPR sequences. However, the extent of *A. muciniphila* transmission is unclear. Is it transmitted in all 3 nurseries? Does the phylogeny represent transmission events in different nurseries? It is not clear why this species was chosen apart from to validate the technical feasibility of detecting transmission events.
5. Line 200- typo , replace with 'in contrast'
6. Line 219- what does 'retained' mean in this context?
7. Line 428- please re-word. This sentence can be interpreted as saying nursery settings are most important factor in early-life microbiome assembly and not mother.
8. Line-442- To justify this statement, the authors would need to demonstrate some health benefit accrued from transmission and colonisation of most transmissible species. There is limited data on many of the species reported as highly transmissible in Figure 3d and Figure 5 in comparison to many early-life *Bifidobacteria* species in terms of reported health benefits. Also, its hard to reconcile this statement with the reported transmission of pathogens such as *Clostridiodes difficile*.

Referee #2

(Remarks to the Author)

Ricci et al describe the changes that occur in the infant gut microbiome following daycare admission, comparing three nurseries in Italy, collecting longitudinal samples from the infants, their parents, and also nursery staff. The cohort is very unique and has great value, and it is also sequenced at extreme depth, allowing for many strain-analysis methods to be applied. The main message is that quickly after entering the nursery, multiple strains are transferred across infants, and sometimes from the staff as well, highly impacting the infant gut microbiome.

The field of the infant gut microbiome has grown rapidly in the past decade, with many cohorts and manuscripts describing early life events and how they shape the infant gut composition. One missing aspect has long been the entrance to childcare, especially when comparing the microbiome pre- and post- childcare admission. This manuscript bridges this gap

beautifully, and will have a great interest in the field. Having said that, there are some important points the authors should take into consideration before this manuscript should be published.

Major comments:

- There is something wrong with the flow of the manuscript, it reads as if we go back and forth between some topics, and makes it challenging to follow the story. For example, here are the topics of the various sections:

- 1- A metagenomic monitoring of the dynamic nursery community microbiomes
- 2- An intricate network of inter-individual strain-sharing and longitudinal strain retention
- 3- Strain-sharing between babies and pets is detectable but not extensive
- 4- Tracking intricate strain transmission between babies, families, and educators
- 5- Infant microbiome is built on infant-to-infant transmission in the nursery
- 6- Strains spreading in the nursery
- 7- Strains from the nursery contribute more than those from family to the infant microbiome
- 8- Strain acquisition and donation rates of babies in nursery is variable and linked with transmission from siblings
- 9- The most transmissible species are environmentally persistent or prevalent gut anaerobes
- 10- Antibiotic treatment is associated with reduced strain retention and, in infants, with enhanced strain influx

First, there are way too many sections here, the whole manuscript can be more concise. Second, sections 1, 2, 4, 5, 6, 7, 8 all discuss strains sharing and compare the various groups of: within a child, across children, within a family, etc. It is very very confusing. The main message is washed out.

Another example is in Figure 2, where panel D jumps forward to one example, but most of the text to follow is general. I would add examples after the description of the general phenomenon.

- During this age of 4-15 mo, many factors are impacting the infant gut microbiome, inducing diet changes and weaning. Childcare classes have a range of ages in each class, and it is unclear from the text whether the samples collected at the same time are from children from the same age. Namely, what is the age distribution of each of the time points? Even if the authors can't do much about this difference now, they should add this point to the discussion.

- One of the more important distinctions in this work is between strain acquisition and strain replacement. The text describes mostly replacement, yet the figures support that the family contribution remains stable, while the nursery contribution to strain sharing grows over time (Figure 4). I think the text needs to be more subtle in making statements such as "Strains from the nursery contribute more than those from family to the infant microbiome" (header at line 269). It could very well be that these early sharing of strain has more impact than the later (larger) number of strain shared.

- The color choices in the figures are extremely confusing. Overall, the same colors represent different things across figures, and even in different panels of the same figure. In addition, the same feature is represented in different colors across figures (like nurseries, and groups within nurseries). I suggest the authors pick consistent colors across the entire manuscript: three main colors for the nurseries, with a shade for the groups, that will be used only for this purpose.

- It is accepted that there are many strains per species in each person, yet most computational tools assume a single dominant strain to enable these comparisons. The authors should at least acknowledge that there are likely multiple strains per species, and how it impacts their calculations (for example, do they expect to have chimeras in their strain profiling?)

- Statistical analysis -

The Mann-Whitney and Kruskal-wallis tests assumes the samples are independent between the two groups, and here the authors use it in cases when they are not independent:

*Comparing two samples of the same individual in different time points (Figure 1D boxplots, supp Figure 9, maybe more)

* There are data points of the form $f(x,y)$ in one group and $f(x,z)$ in the other (f is some strain sharing calculation for example), which makes the data points not independent (Figure 2B, 4A, 4B, Supplemental Fig. 5, maybe more - comparing strain sharing within the families to strain sharing between families).

Minor comments:

- The title should include the word childcare or nursery

- The introduction is lacking more information of what is known and moves directly to the hypothesis of the paper. Is anything known about infants in daycare vs. those that aren't? At least mention the NEJM paper describing decrease in Asthma and wheezing in kids who attended daycare (DOI: 10.1056/NEJM200008243430803). Additional comparison to known strain transability to infants from family, pets etc. will also be useful.

- line 97: what is "baseline alpha diversity"? At time point T0?

- There are multiple terms that are used interchangeably, like % strains shared and rate of strain sharing (for example, throughout Fig 3 & 4). It is confusing to read, and would be better if simplified when possible. Is strain replacement defined as (1-strain sharing rate)?

- Why were SGB coming from food discarded? Appears in line 609. This could have been an interesting point.
- The paragraph starting in line 618 was completely unclear to me. What does "Among the possible pairs we chose the one to maximize the lowest estimated coverage among the two samples" mean?
- Discussion: There is no evidence in the paper that higher strain sharing rate is good for infants' health, however it is mentioned in the discussion (line 443).

Figure comments:

Many many tiny and complex figures with multiple (sometimes confusing) variables compared that make it difficult to follow and understand. It would be better to cut some and leave the highlights clear and well explained. The font is extremely small, and can't be read on the printed version.

- Figure 1B - what dominates the PCoA plot? Diversity? The authors do not explain the rationale of using Jaccard as a distance measure, I believe it is to capture the qualitative similarities rather than most quantitative ones, but the rationale should be mentioned in the text.
- Figure 1C, is the average here for each person over all time points? So each dot is a person and the y-axis is average SGB richness across the three or more time points? It was unclear from the text. If so, I would also add the teachers and the siblings. Note, the teachers appear once as teachers and once as educators.
- Figure 1C: There are very few families that have siblings and even fewer that have pets, so this comparison could be biased. How many samples are in each group of the box plot?
- Figure 1C: The richness of babies with siblings is higher - are these babies age-matched?
- Figure 1C: Perhaps consider showing that while pets and siblings impact the baby's richness, also show that antibiotics and mode of delivery do not.
- Figure 1D right, this is confusing. Is this the beta diversity across all pairwise comparisons within each group and time point? It wasn't clear from the text. Is the beta diversity calculated within individuals from the same nursery? Also, the difference in diversity in babies is as significant as that of the fathers, how can you explain this? I am not confident this analysis is helpful for the general flow of the manuscript.
- Figure 2B bottom, the small dots should be organized in space according to their nurseries, so we can see cliques in the graph, and also inter-nursery connections.
- Figure 2D, the numbering should be per family, so M14 and F14 should be in the same family, and not as it is now.
- Figure 2D: this figure and example seems out of place and dives into specific strain sharing events longitudinally while we are still at a "snapshot" level.
- Figure 2D: In cases where SGB is not found (for example in B16) can this be as a result of very low relative abundance? Can the authors also explain why sometimes CRISPR spacers aren't found?
- Figure 3B, it would be useful to add a panel of shared SGB across time, so the readers can understand the upper bound of shared strains.
- Figure 4AB, what does baseline mean in the title?
- Figure 4F, ratio of 1 should correspond to 0 number of strains (fall on the x-axis line)
- Figure 5: What is the difference between transmissibility and strain sharing rate? Again, multiple terms that seem to describe the same thing makes it really hard to follow the story.
- Figure 6 can easily move to supplementary material

Referee #3

(Remarks to the Author)

Summary

Ricci et al., employs strain-resolved metagenomics of DNA from fecal samples obtained from infants attending different nursery homes, as well as their teachers, family members and pets, in order to investigate gut microbial transmission between these. The study focus on infant-to-infant transmission of gut microbes within nurseries and compares the extent of transmission to that coming from family members. The study is novel and very elegantly designed to detect microbial transmission in nurseries, involving multiple different nurseries and different nursery-groups within each nursery, as well as the longitudinal sampling regime. Likewise, the comparison to family-infant transmission enables assessment of the relative contributions of transmission from family vs nursery to infant gut microbiome development. The study concludes that extensive infant-to-infant transmission of gut microbes is occurring in nurseries and that these transmissions with time

outweigh family-to-infant transmissions.

Comments

Overall, this paper is of very high quality and the presented data on the extend of infant-to-infant transmission of gut microbes in nurseries using a dense longitudinal sampling regime is compelling and novel. I do, however, have some comments and questions for the authors to consider.

Major comments

1) Data on Pets: Any conclusion regarding transmission from pets seems hard to trust given the very low N of 4 (probably also even different pets?). And statistical testing here almost doesn't make sense. In my opinion this is the weakest part of the manuscript and it takes up too much space and speculation, given that is not of importance to the manuscript's conclusions. I would suggest that the authors remove or limit the statistical analysis and, if at all including them, transfer fig.2c to the supplementary materials. I do acknowledge, though, that pets are important to consider and that it is important to mention in the manuscript.

2) Metagenomic seq: I highly appreciate the strain level analyses and the longitudinal design of the study – this is highly needed in the field. However, it also seems to me that you do have a significant background signal. E.g. how is it possible that infants – before attending nursery (and thus with no or very limited contact to other babies?) - on average share 2.5 strains with unknown babies they will later come to share nursery with? Can you somehow normalize to this background? Or do you believe that it does reflect actual strain sharing?

Furthermore, a big limitation of metagenomic seq is the relatively high limit of detection, meaning that you risk missing strain sharing events if the microbes colonize at lower abundances. If I understand correctly, you disregard taxa at rel abundance <0.1%. Assuming 10^{11} bacteria/g feces, this means anything below 10^8 bacteria/g feces is not seen. Therefore, I find it important that these type of analyses are backed up by much more sensitive methods, such as actual culturing (and strain isolation) and/or qPCR. I do understand that this is extremely cumbersome, but validating your method in a few cases really adds to the credibility (e.g. qPCR targeting the Akkermansia strain in Fig. 2d).

3) Conclusions regarding nursery transmission outweighing family transmission: I'm not convinced about the author's conclusion regarding this. The data presented in Fig. 2A and Fig. 4C do not provide strong support for this conclusion, as detailed in my specific comments below.

4) Missing analysis of important metadata: It is mentioned in the methods and in Fig.1 that there is available data on the infant's diet and I assume that you also have data on infants exact age. As far as I can see, these data are not analysed together with the microbiome data. We might expect that the establishment of transmitted strains could depend on infant feeding and age. So, why where these not included?

5) Sudden jump in shared strains: The authors observe a sudden jump in # and %shared strains within infants in the nurseries between T07-T08 (Fig. 4c,d and Extended data Fig. 11). This is quite interesting, but not really discussed much in the manuscript. It seems to be 6-7 weeks after start in the nursery - what happens at that point?

Specific comments:

L. 24-26. Please consider modifying this sentence. The evidence of nursery transmission actually surpassing the family contribution is not overwhelming, e.g. Fig. 2A (showing 8.6 vs 8.0 for “family” vs “nursery”) and Fig. 4C (only showing slight significance in %strain shared at T15, but no difference in Avg. shared strains?). It would be fair to state that transmission in nurseries reaches similar levels as observed for baby-family.

L. 28-30: “microbiome transmission between babies interacting in the nursery outweighs parent-to-infant transmission after birth”. Please modify this sentence. What data in this manuscript demonstrate that transmission in nursery outweigh parent-to-infant transmission? Fig. 2A indicates higher cumulative strain sharing parental(mother+father)-to-baby, than nursery-to-baby? And Fig. 4C does not show significantly higher avg. #shared strains from nursery-to-baby than family-to-baby. Also “after birth” is not specific as you see large differences according to time-in-nursery.

L. 95: please explain (to the unfamiliar reader) what “expected trends” are.

L. 154-168: I don't find this part about pet-baby/parents strain particular strong. It is based on only 4 pet-infant pairs AND only 2 mother/father-pet pair? How can you draw conclusions on such low sample sizes?

The statement is very speculative given the actual data presented: “This may be evidence of the fact that the lower colonization resistance of the infant gut microbiome may facilitate microbial acquisition or may be indicative of a more intimate interaction between pets and babies with respect to pets and adults.”

L. 180-214: I find these case-observations very interesting, but also anecdotal. First, directionality is hard to infer if the strain is detected for the first time in two individuals at the same sampling point. This seems to be the case in both examples (Fig. 2D and Extended data Fig. 6), but is not highlighted in the figure.

One thing that I am missing here is an effort to validate these transmissions either by qPCR or cultivation and isolation of the strains from the fecal samples. The “problem” with relying solely on metagenomic data is the high detection limit, meaning that you risk missing detection of the strains at some timepoints (and individuals).

L. 225: What is meant by “fast-mutating ecological niches”?

L. 360: rho-values of 0.35-0.4 are not “strong” correlations. Please re-phrase.

L. 442-445: I find this overstated. You do not present any data linking these transmissions to infant health. In fact, these social interactions also result in spread of pathogens. Please rephrase.

L.448-450: How can you exclude that other factors associated with having a sibling is influencing these patterns. E.g. infants with older siblings might attend nursery less often? Or might interact less with other babies?

Figure 1

It would be helpful to indicate somewhere in this figure or elsewhere in the manuscript what T01-T15 translates to in terms of 1)weeks of nursery attendance and 2) infant age.

Figure 2

Panel A. These analyses only show the absolute average number of strains shared, but it does not tell us the average proportion of strains shared. E.g. how large a proportion of all strains detected in an individual are actually shared with the baby (from family and from nursery?). These numbers would be very informative, especially when assessing if the baby-to-baby strain sharing is actually “extensive”.

Panel B. Why is the average number of baby-to-baby shared strains from panel A “final” (=7.2) 2-to-4-fold higher than those numbers displayed in panel B for intra-nursery strain sharing (1.85, 2.56 and 3.44)?

I'm not sure I understand how the statistics are applied here – are the Mann Whitney test comparing all inter-nursery values (AvsB, AvsC, BvsC), to one intra-nursery value? Or only AvsB and AvsC to within A? Please specify. Also here it would be informative to show the average proportion of strains shared within and between nurseries.

Figure 3

Panel D. It seems like most of these strains were not detected at baseline? How do you know that they actually originate from one of the babies in the study? Are these strains also detected in teachers or family members?

Figure 4

Panel C. I assume that these comparisons are all to “baseline samples”, as for panel A and “B”? What happens if you split “with family” into “father”, “mother” and “sibling”. It seems like the contribution from siblings is quite a bit higher than maternal and paternal. It seems like sibling-baby sharing is quite extensive at baseline and at T15 (Fig. 2A and Fig. 4b), so I'm wondering whether siblings are actually contributing more than “nursery” during the entire sampling period?

Panel C and D. You see a quite interesting jump in % and #of shared strains occurring between T07 and T08. Why is this increase in strain sharing occurring so suddenly? Wouldn't we expect it to be more gradual?

Figure 6

I'm missing some non-Ab exposed control infants in these analysis. How can you exclude a “time-effect” here?

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have addressed my comments very comprehensively. Its a significant piece of work that adds to our understanding of microbiome transmission in early-life.

Remaining comments are very minor:

1. Line 25- use of word clone in abstract. This is only mention of 'clone' so either define it in main text or use a different word here e.g. strain
2. Figure 1 c- Pet panel is not mentioned in main text
3. Figure 1b- symbols are very small in printed version and difficult to interpret. Please make this panel bigger.
4. Line 310- please re-phrase this section title. It does not make sense.
5. Line 407- B. breve is generally considered anaerobic. Please re-phrase or add reference to demonstrate aerotolerance

Referee #2

(Remarks to the Author)

The authors have dramatically changed the manuscript, improving the flow of the story and adding much needed information. The story is still somewhat too complex, and will probably benefit from further pruning and adjustments (either by the editors or by the authors themselves).

The underlying question of the role of family vs. daycare in microbial transmission in early life is of great importance, and this work is the first to tackle it heads-on with a strong cohort that enables this comparison.

General comments:

- There are many terms that are used in the manuscript, and it is hard to keep track of all definitions (oftentimes having rates AND absolute numbers, on top of a customized new metric). Perhaps it would be best to keep one type, and have the rest in the supplementary (which is already huge...). For example in figure 6 - can the authors present 3 out of the 6 statistics?

- There is incoherence between figures and sections in the text. Multiple sections refer to the same figure (which is not that bad), but then within the same section the authors refer to multiple figures. This makes following the story to be more complicated. Another challenge in following the story is the somewhat confusing section titles (see below in the minor comments).

- The text in the figures is still too small, and some figures require much zooming in (example - figure 4 with tiny boxplots).

- The story with pets and siblings is very interesting (not that I am comparing the two entities...). From the figure, it seems like having pets is associated with *decreased* microbial diversity, which is a very interesting point. The authors do not describe this in the text at all (the entire Figure 3C-right panel is not mentioned in the text). The siblings provide a very interesting point of view, yet in the details, it seems that some of the siblings are teenagers and even older (21)? I understand the numbers are small, but I would imagine there is more transmission in younger siblings, is that right? If the cohort is too small

to examine this properly, I would expect the authors to discuss both these points in the discussion (pets and siblings)

- The ages of the participants at sampling are important, and are downplayed in the text. At the minimum, they should be added to supplementary table 2, so readers can re-examine some of these analyses (for example regarding the delivery mode).

- The clustering of the transmission network in Figure 3B is very nice, yet I have to make sure which SGBs were used for this evaluation. In the Methods, it reads "When the SGB was not present among at least 3 pairs within a category, transmissibility of the SGB within the category was set as undefined." Does this mean that only SGBs that were very common in babies were used? If so, that could explain why nurseries are the strongest factor. For example, if I need to paraphrase Anna Karenina *Nurseries are all microbially-alike; yet each family is microbially-distinct in its own way*, then this plot is very biased towards the nursery end of analysis.

In the paragraph starting in line 152 - the authors describe the 9.47M strain sharing events, and then start describing the % of events associated with each comparison (same individual, within family, between families etc). I think a better way to examine this topic is to "what are the chances that two individuals with relationship X will share strains?", and "how many strains do they share?" This also makes the statistical angle easier, without the need to compare across groups of comparisons of different sizes, etc.

Figure comments:

- Figure 1D was very hard to parse. Perhaps better y-axis titles can help. In general, I would separate the two panels in 1C and 1D to have their own letter, thus their own separated caption.

- The arrows in Figure2 and ExtFig12 that go between subjects are speculative. Especially when timepoints are not shared, the transmission probably did not occur on the last time point, but rather earlier. In Figure2, the assumption that the baby transmitted to the mother who then transmitted to the father, might be wrong. The father may have received it earlier. The figure stipulates that the father and mother are sharing, but the baby shares only with the mother. Same for transmission from B06 to B03 and B09 in ExtFig12. I imagine this is not what the authors meant, but the figure should be altered to make it clearer.

- Figure 2, if it is possible to change the color of the 05 or 06 families, it will be clearer to see in the tree that there is a shared strain between the families.

- Figure 3D, bottom, the double y-axis is misleading. From the figure, it seems like most of the common strains are shared by the end of the term, yet one scale is up to 20 and the other up to 4...

- Figure 4C bottom, is this the relative abundance of the SGB? If so, the caption should be clearer

- Figure 4CDE - There seems to be something interesting between timepoints T07 and T08, any idea what causes this increase?

Figure 6 - I find it confusing to follow and understand the 6 variables they describe. As mentioned above, I think it would be better to show less. But if you choose to leave them all, at least order them in a more meaningful way (facet like - rows are rates vs counts, cols are acquired SGBs/retained strains/replaced strains).

- Figure 6B - The axis of the strain retention rate goes up to 1.2 which is confusing as the ratio should be in [0,1]. I would remove this tick so it won't look as if the values are greater than 1.

- Colors of extended Figure 2 and 3 do not match on sample type and with the rest of the manuscript

- Extended Data Figure 23-24 - The shapes should have no color filling, it is currently very confusing.

Minor Comments:

Line 68 - the title is "Microbiome transmission in nurseries" but it is more of a "study design" section.

Line 103 - the subtitle does not reflect the text. The title is "Baby microbiome development in nurseries", but this seems to be the only section where the authors focus on species-level results, and many of them are not related to the nursery at all: babies vs adults, Mother GBS treatment, delivery mode, siblings vs no siblings.

Line 127 - the authors write that the pool of species did not change noticeably but they only mention there were 310 at the first time point and 305 at the last time point, not how many are shared between the timepoints.

Line 188 what is the difference between a baby and an "infant volunteer"? Is the infant volunteer a sibling? This is very confusing.

Line 268 - "Over the study period, babies' gut 268 microbiomes showed by far the lowest rate of SGB retention compared to

adults...". This is unclear. What is SGB retention? How many time points are needed to be considered retained? "by far the lowest" is not a very scientific description, can the authors compare these to other groups (parents, educators, etc) to have some statistics?

Line 288 - The authors report the average SSR, yet the rest of the paragraph describes the median SSR and statistical tests performed on the median. So better to start with the median from the beginning.

Line 354 - It is unclear to me how the strain acquisition "stabilized" and then "gradually increased". Perhaps better phrasing will be helpful.

Line 541 - I think it is better to describe vaginal delivery as "vaginal delivery" rather than "natural"

Line 637 - There are results that are described in the Method's section of Dietary information collection and analysis. I think it would be best to keep the Methods section more neutral, and to highlight all results in a more visible section of the text.

Lines 646, 650 - when writing reversed, did the authors mean negative?

Lines 364, 662 - replace "till" with "until".

Line 798 "microbiome assembly" - I would change to composition, not to confuse it with metagenomic assembly.

Referee #3

(Remarks to the Author)

Please find my few remaining comments (green text) to the revision in the attached pdf.

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RESPONSE TO EDITOR AND REVIEWERS' COMMENTS for the paper "Baby-to-baby strain transmission shapes the developing gut microbiome"

Comments from the Editor

The referees find the dataset and analysis of interest in principle, but there is a general sense that the manuscript is lacking in sufficient context of the findings, and also lacking in compelling insights into the factors influencing transmission. In addition, important variables (which may have an important bearing on the results, such as exact age and feeding regime) are not considered, and the referees feel that they must be included to ensure that the findings are robust.

Response to the Editor and summary of changes in the revision

We thank the Editor and Reviewers for their constructive feedback on our manuscript. We have undertaken extensive revisions to address the concerns raised, and we believe the manuscript is now significantly strengthened. The six major improvements are summarized in topics below:

1. Largely expanded dataset and analysis set:

We expanded our dataset by integration with 367 new samples from additional timepoints, to reach a new total $n = 1013$ samples from 137 volunteers.

The newly integrated samples were collected in subsequent timepoints with respect to the first term of the first nursery school year ("autumn term", from September till December 2022) that was presented in the first version of the manuscript, and encompass:

- The second term ("spring term" of the school year, from January till July 2023): dense longitudinal sample collection was extended till the end of the first school year, as previously performed during the first term, only for babies in group 1 of nursery A and for their close social network (i.e. parents, siblings, educators, pets, with total $n = 32$ participants, including 2 newly enrolled twin babies that started the nursery in the second term, their mother and their pet, and with total $n = 221$ samples).
- Two follow-up samples were collected for all participants across the three nurseries, with the first sample collected at the end of the first year (July 2023, identified as "TA") and the second before the start of the second school year, after the summer break (September 2023, "TB"), with $n = 146$ follow-up samples.

Our newly expanded cohort and dataset are reported in the updated **Supplementary Table 1** ("The microTOUCH-baby cohort overview") and **2** ("Metadata for the 1013 metagenomes analyzed"), respectively. We also edited the study overview in **Figure 1A** (below) and the first result section (as well as the **Methods**), to showcase this data integration, as follows:

"For all participants in group 1 of nursery A (babies, parents, educators, and pets), sample collection continued through the second term, maintaining sampling frequencies. Two additional follow-up samples were collected for all participants: one

at the nursery year's conclusion (TA) and another at the end of the summer break (TB) (**Fig. 1A**)”.

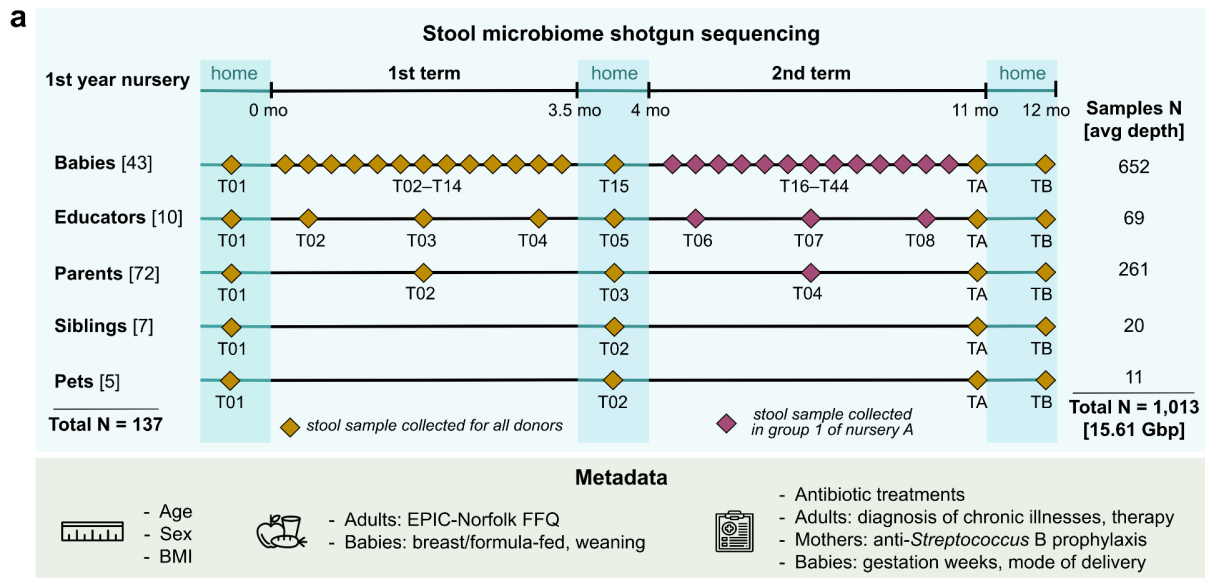
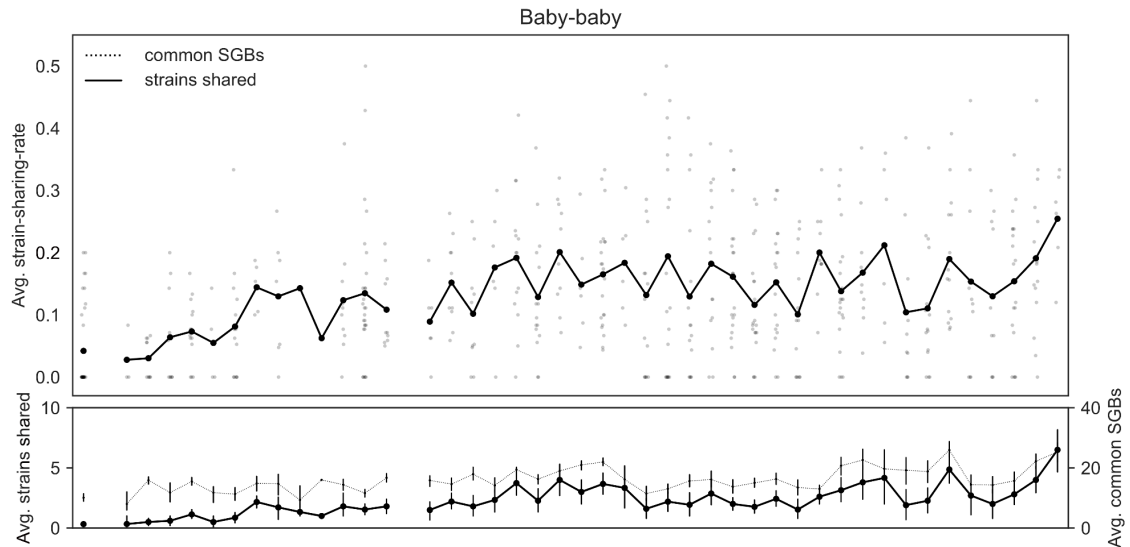


Figure 1A. microTOUCH-baby study design and overview.

Leveraging this expanded dataset, five newly performed analyses were integrated in different figures and supplementals, listed below and numbered 1 to 5 in order of appearance in the main text:

1.1. **Extended Data Figure 16.** We commented on this result in L308:

“By extending the investigation of baby-to-baby transmission to the second term of nursery (i.e. to the end of the scholastic year) for one of the classes in our cohort (group 1 of nursery A, which showed the lowest intra-nursery strain sharing among the three nurseries, **Fig. 3B**), we observed that SSR between babies in the same group continued to progressively increase over time, reaching an average of 25% at the end of the term (**Extended Data Fig. 16**)”.



Extended Data Fig. 16. Longitudinal baby-baby strain-sharing rate and average number of strains shared during the entire first year of nursery, for group 1 of nursery A. Only pairs with at least 5 SGBs in common were included. In the avg. strains shared subplot, the vertical lines report the standard deviation.

1.2. new **Figure 3E**. Integrated in the text as follows:

“When considering all babies enrolled across the three nurseries and the entire study period (**Fig. 3E**), baby-baby SSR within the same nursery (regardless of group) substantially grew during the second term, reaching a median 33.3% at the end of school year (TA) (vs median 17.9% at T15; Wilcoxon signed-rank test, $n = 58$, $T = 86$, $P = 6.2e-9$). Although baby-baby SSR decreased during the summer break (TB), in line with nursery closure and ceased regular contact among babies, it remained significantly higher compared to post-Christmas break levels (T15) (median 23.7% at TB vs 17.9% at T15; Wilcoxon signed-rank test, $n = 31$, $T = 68$, $P = 2.0e-4$).”

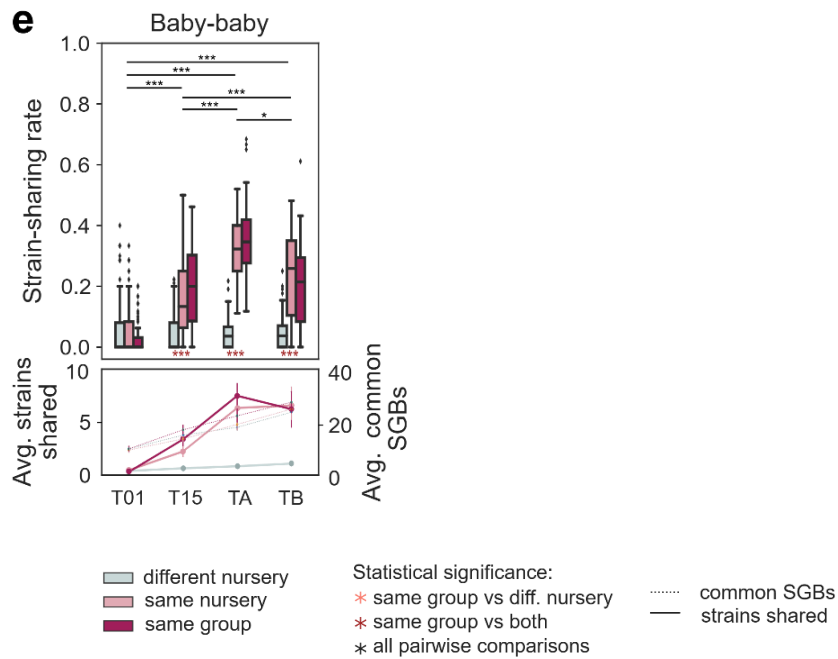
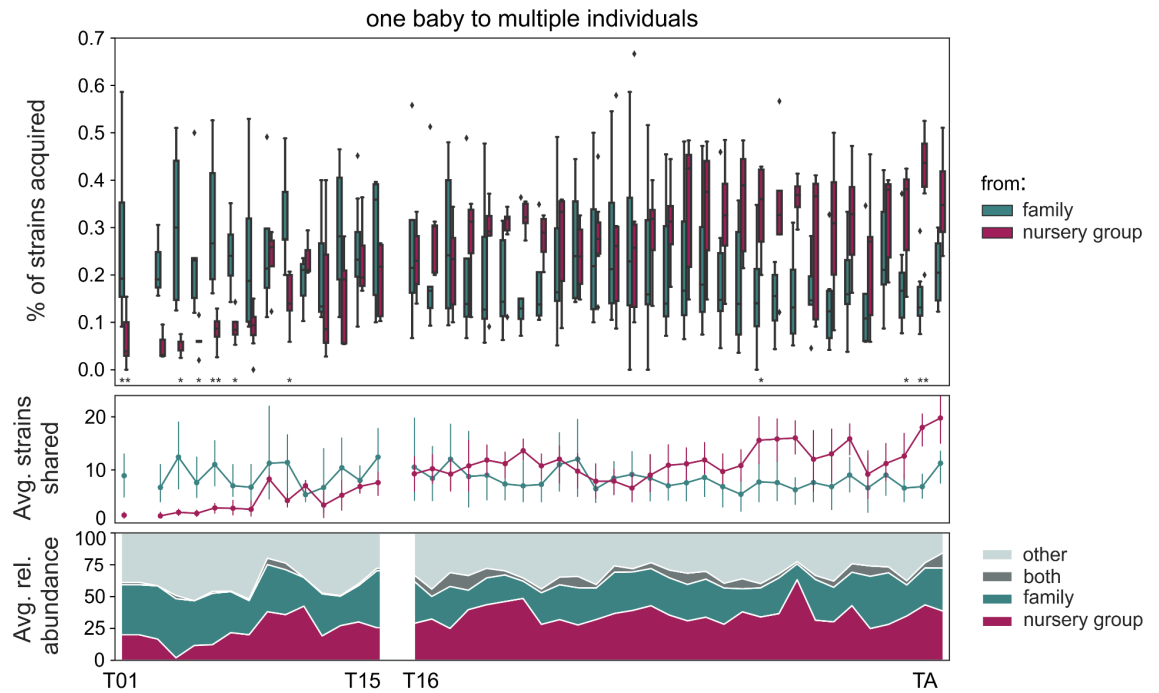


Figure 3E. Baby-baby strain-sharing rate and average number of strains shared at T01, at the beginning and the end of the second term (T15 and TA, respectively), and at the end of summer break (TB), across all babies enrolled in the three nurseries. Only pairs with at least 5 SGBs in common were included. In box plots, box edges indicate the lower and upper quartiles, the center line represents the median, and whiskers expand the IQR. Statistical significance asterisks refer to the highest P -value ($P < 0.001$, ***; permutation test for medians; **Methods**) with red asterisks indicating a significant result when comparing same nursery (different group) vs different nursery, and same group vs different nursery. Black asterisks at the top of the figure refer to the P -value ($P < 0.05$, *; $P < 0.001$, ***) of a Wilcoxon signed-rank test evaluating longitudinal baby-baby SSR trends for paired baby-baby pairs attending the same nursery (with “same nursery” and “same group” categories combined).

1.3. Extended Data Figure 18. We commented on this in the main text as follows:

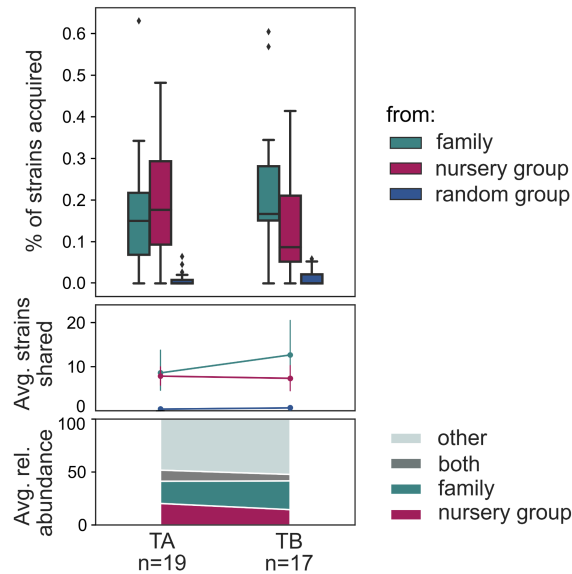
“The extended longitudinal analysis of group 1 of nursery A revealed that the proportion of strains acquired from the nursery peers stabilized after three months of attendance (T15) to gradually increase during the second term (**Extended Data Fig. 18**).”



Extended Data Figure 18. Proportion of strains acquired from group vs family, corresponding average number of strains acquired and total relative abundance, during the entire first year of nursery, for group 1 of nursery A only. For each baby timepoint, comparisons were performed against past/contemporaneous samples of the family and the nursery group (**Methods**). Only babies with at least one family member enrolled in the study were included. Statistical significance asterisks refer to a Mann-Whitney U test for the proportion of strains acquired from the same group vs the family. In the avg. strains shared subplot, the vertical lines report the standard deviation.

1.4. **Extended Data Figure 19**, that was referred to in the main text, namely:

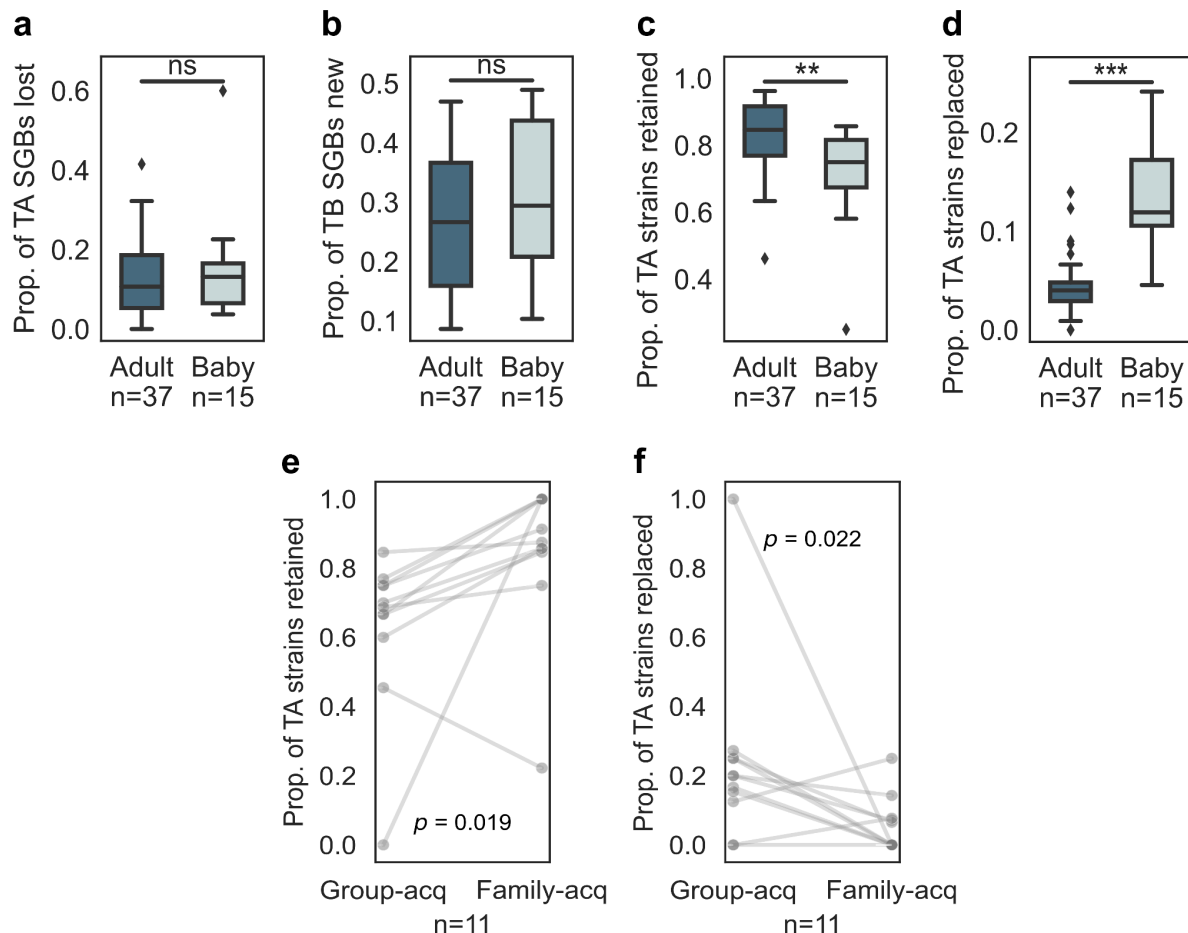
“However, comparable contributions to infant microbiome of family and nursery became evident when collectively analyzing samples from all babies across three nurseries at year-end (TA) (17.6% median proportion of strains acquired from nursery vs 15% from family; Mann-Whitney U test, $n = 19$, $U = 218$, $P = 0.29$; **Extended Data Fig. 19**), to then shift again (although not significantly) toward a greater family contribution after summer nursery closure, when contact among nursery peers ceased (TB) (8.7% median proportion of strains acquired from nursery vs 16.7% from family; Mann-Whitney U test, $n = 17$, $U = 122$, $P = 0.43$; **Extended Data Fig. 19**).”



Extended Data Figure 19. Proportion of strains acquired by babies from the nursery group vs family, corresponding average acquired number of strains and total relative abundance, at the end of the second trimester (TA) and at the end of summer break (TB), for all participants in the study. For each baby timepoint, comparisons were performed against past/contemporaneous samples of the family and the nursery group (**Methods**). Only babies with at least one family member enrolled in the study were included. In the avg. strains shared subplot, the vertical lines report the standard deviation.

1.5. Extended Data Figures 20. We commented on this evidence as follows:

“Moreover, babies exhibited lower retention of strains from the end of the nursery school year (TA) till the end of summer (TB), as well as higher strain replacement, compared to adults (i.e. parents and educators), despite no differences were observed between groups in the overall proportion SGBs typed at the strain level that were lost or newly acquired during this period (**Extended Data Fig. 20A-D**). In babies, in the same period of time, we found family-acquired strains to be significantly more retained (Wilcoxon signed-rank test, $n = 11$, $T = 5$, $P = 0.019$; **Extended Data Fig. 20E**) and less replaced (Wilcoxon signed-rank test, $n = 11$, $T = 5$, $P = 0.022$; **Extended Data Fig. 20F**) than nursery-acquired strains, suggesting that continuous seeding linked to continued contact is a factor behind long-term colonization, as well as potential priority effects making early-acquired familial strains outcompete in the long-term nursery-acquired ones”.



Extended Data Figure 20. (A) Proportion of SGBs typed at strain-level that were lost after the summer break (TB) over those present at year-end (TA), in adult and infant participants. In A–D, significance asterisks refer to a Mann-Whitney U test, with $P \geq 0.05$, ns; $P < 0.01$, **; $P < 0.001$, ***. **(B)** Proportion of SGBs typed at strain-level that were newly acquired during the summer break (TB) over the total present at TB, in adult and infant participants. **(C)** Proportion of strains retained after summer break (TB) over the total found at year-end (TA), in adult and infant participants. **(D)** Proportion of strains replaced at TB over the total strains present at TA, in adult and infant participants. **(E–F)** **E**, Proportion of baby strains acquired from either family or nursery that were retained or **F**, replaced by different conspecific strains after summer break (TB) over the total strains found at year-end (TA), in infant participants. In E–F, significance asterisks refer to Wilcoxon signed-rank tests.

2. Study of the effect of diet on the microbiome and its transmission.

We performed a thorough evaluation of the effect of diet on interindividual microbial transmission, doing so for both adult and infant participants. Infant dietary data was gathered by analyzing dietary information collected prior to the start of the study using *ad hoc* questionnaires for infants (to record weaning status and milk type in case of mix feeding during weaning), while adults provided EPIC Food Frequency Questionnaires (FFQ). FFQs were used to derive the healthy Plant-based Dietary Index (hPDI) to assess quality of long-term dietary habits. We newly described the calculation of PDI from FFQs in the **Methods**, as follows, and reported the indexes for each adult participant in **Suppl. Table 3**:

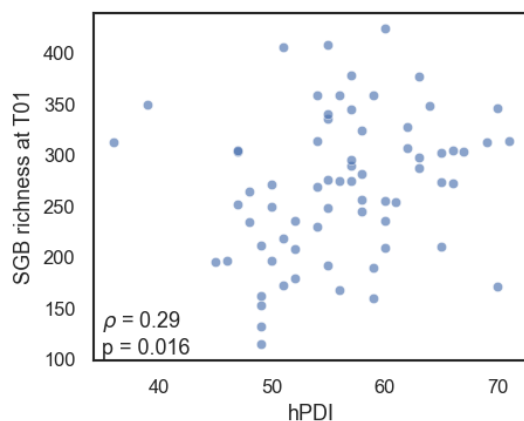
“FFQs were used to calculate the healthy Plant-based Dietary Index (hPDI) ¹. Quality and quantity of plant-based foods were considered and derived from FFQs for a total of eighteen food groups, and divided into quintiles and assigned positive or reverse scores. Participants whose intake exceeded the highest quintile received a score of 5, while those below the lowest quintile received a score of 1. Healthy plant-based foods received positive scores, whereas less healthy or unhealthy plant-based and animal-based foods received a reverse score. A final score was derived by summarizing the scores of each participant”.

Baby feeding practices were summarized in “Dietary information collection and analysis” in the **Methods** as follows:

“Briefly, most babies had begun weaning at T01 (n weaned=38, n non-weaned=2, NA=1) and received identical solid meals while in the nursery. The majority followed a mixed feeding approach during weaning, combining solid foods with any type of milk (n mixed diet=24, n exclusively solid food=14, NA=5). Among those babies receiving milk supplementation, feeding types were relatively balanced (n breastfed=9, n formula-fed=10, n receiving both=5).”

We then moved on to investigate the impact of diet on infant and adult microbiome composition (2.1, below) and transmission (2.2):

2.1. In order to test the effect of infant feeding practices on microbiome maturation, we first assessed the association between feeding practices and microbiome richness but did not identify any significant association (statistical details below). In contrast, we found a significant link between diet quality in adult volunteers (as indicated by higher healthy PDI) and microbiome richness, which was illustrated in the new **Extended Data Fig. 6**:



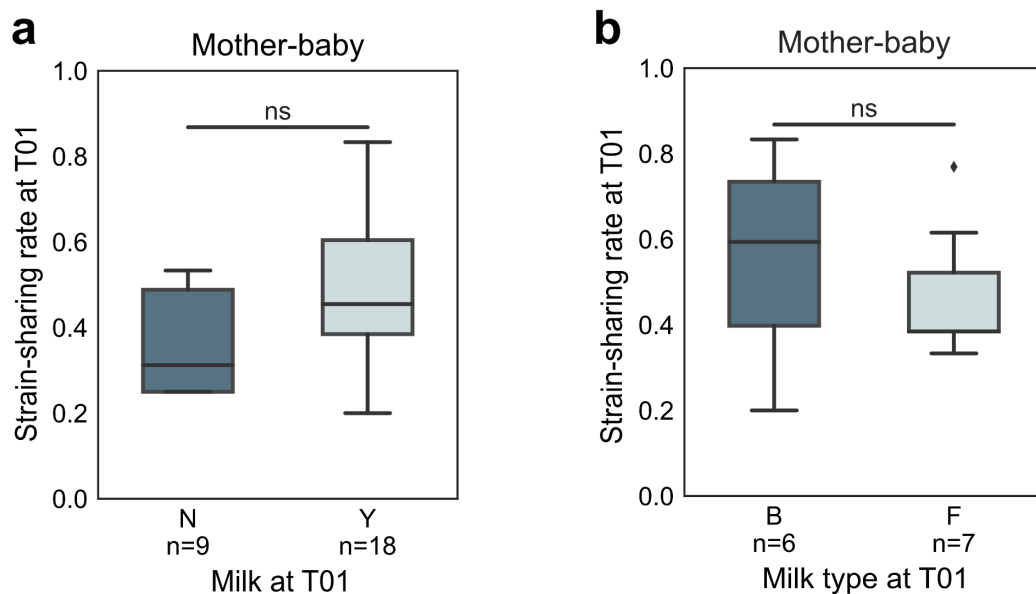
Extended Data Figure 6. Association between SGB richness before nursery start (T01) and healthy Plant Dietary Index (hPDI), as an indicator of diet quality, in adult volunteers. Statistical significance refers to the Spearman’s test.

We summarized infant dietary practices and reported the newly generated results, in the second subsection of results:

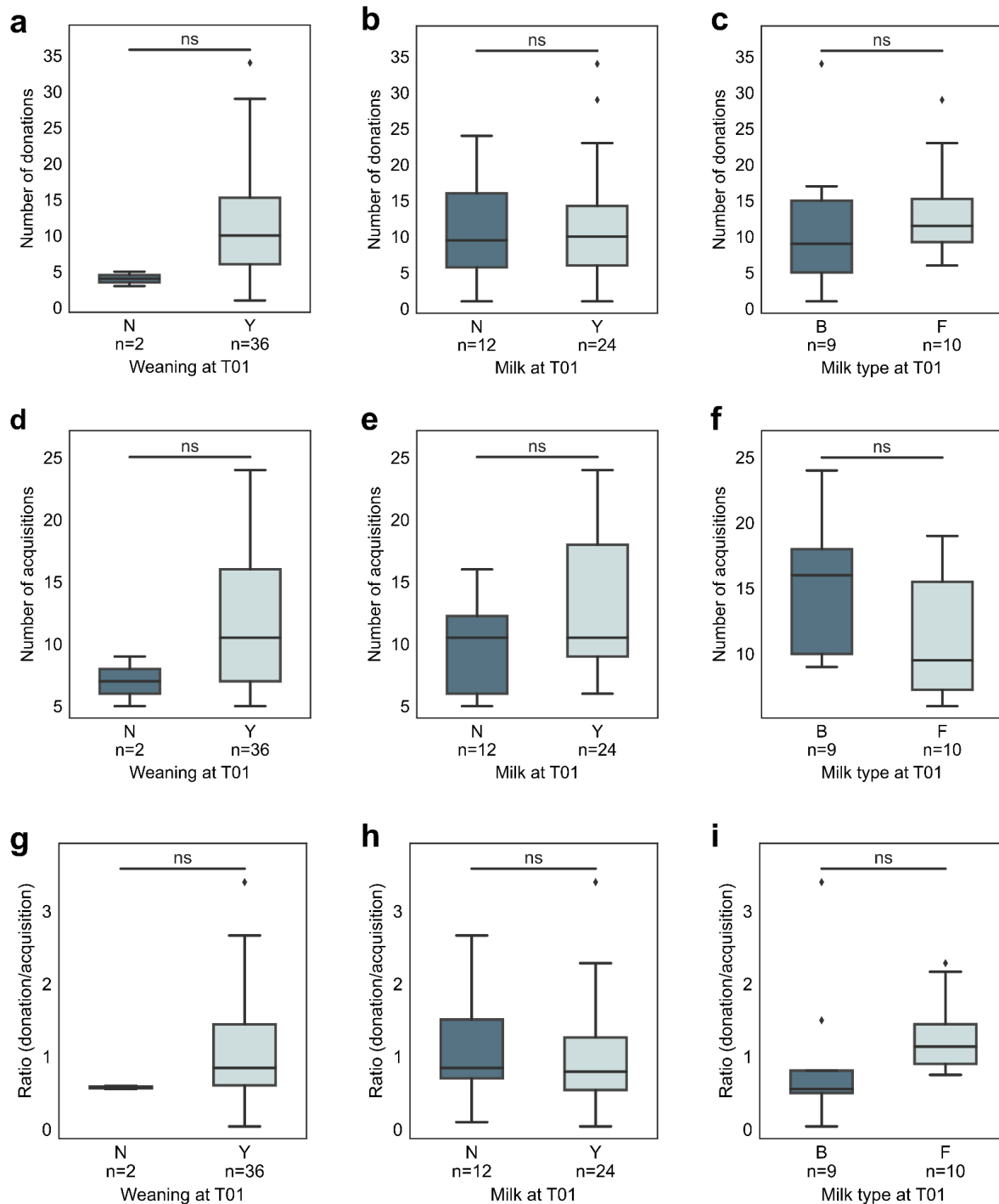
“Microbial richness was associated with dietary habits in adults i.e. higher healthy plant-diversity index (hPDI; Spearman’s test, $n = 69$, $\rho = 0.29$, $P = 0.016$; **Extended**

Data Fig. 6), but not in babies after accounting for age ($\beta = 23.16$, $SE = 22.26$, $P = 0.298$, 95% CI [-20.46, 66.79], intercept = -9.83 ($SE = 20.37$, $P = 0.630$)).”.

2.2. Secondly, we analysed the effect of infant diet on strain-sharing rates and found that babies consuming milk at T01, and among them, those consuming maternal milk, showed higher strain-sharing rate with the mother, however to a non-significant extent (new **Extended Data Figure 27**, below). We also explored the putative impact of infant diet on rates of acquisition and donation of new strains but did not identify any significant effects (new **Extended Data Figure 28**).



Extended Data Figure 27. (A–B) A, Mother-baby strain-sharing rate in babies drinking vs not drinking any type of milk and **B**, in babies receiving maternal milk vs formula milk at T01. In box plots, box edges represent the lower and upper quartiles, the center line indicates the median, and whiskers expand the IQR. Statistical significance refers to Mann-Whitney U tests, with $P \geq 0.05$, ns.



Extended Data Figure 28. (A–C) Association between number of strain donations and **A**, weaning status **B**, supplementation of milk and **C**, milk type at T01. **(D–F)** Association between number of strain acquisitions and **D**, weaning status **E**, supplementation of milk and **F**, milk type at T01. **(G–I)** **G**, Association between ratio of strain donations over acquisitions and weaning status, **H**, supplementation of milk and **I**, milk type at T01. In box plots, box edges represent the lower and upper quartiles, the center line indicates the median, and whiskers expand the IQR. Statistical significance refers to Mann-Whitney U tests, with $P \geq 0.05$, ns.

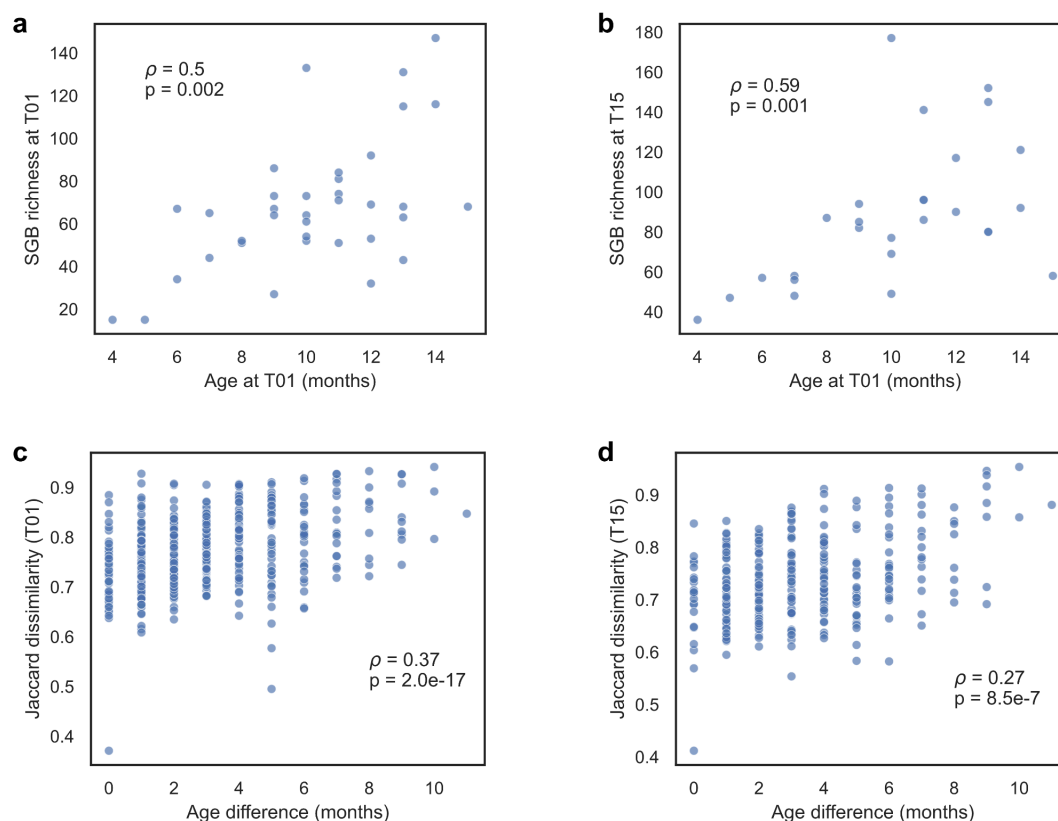
Overall, we found that diet appears to exert a minimal impact on interpersonal microbial transmission. We mentioned this set of newly produced evidence in the new dedicated

paragraph of the results section, titled “Host factors and microbiome transmission”, as follows:

“We then investigated the effect of diet on interpersonal microbial transmission dynamics in infant and adult participants. Analysis of influence of infant diet on strain-sharing revealed that infants consuming milk at T01, particularly those receiving maternal milk rather than formula, exhibited elevated strain-sharing rates with their mothers at T01, though this trend did not reach statistical significance (**Extended Data Fig. 27**). Further exploration of dietary impacts on strain acquisition and donation patterns failed to identify significant associations (**Extended Data Fig. 28**), indicating an overall negligible impact of diet on interpersonal microbiome transmission.”

3. Study of the effect of exact age on infant microbiome development (3.1) and transmission patterns (3.2);

3.1. First, we observed that age correlated, as expected, with infant microbiome alpha diversity both at T01 and T15 (new **Extended Data Figure 5A–B**, below). In addition, we observed that babies with more similar age had more similar microbiome compositions, also both at T01 and T15 (new **Extended Data Figure 5C–D**).



Extended Data Figure 5. (A–B) A, Association between baby microbiome diversity at T01 and **B**, T15 and baby age before nursery start (T01). **(C–D) C**, Association between Jaccard dissimilarity at T01 and **D**, T15 and age difference (months), in babies. Statistical significance refers to the Spearman’s test.

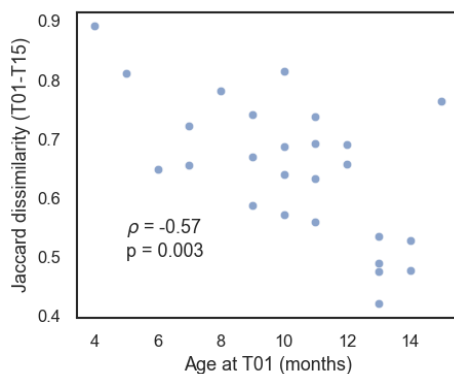
We incorporated this new evidence briefly as follows:

“In our data we observed the expected microbiome structures ²⁻⁴, with large compositional divergence between adults and babies (**Fig. 1B, Extended Data Fig. 2-4, Supplementary Table 5**), and age-dependent differences in babies (**Extended Data Fig. 5**)”.

In light of this significant effect of age in baby microbiome composition, we also adjusted the analyses in Figure 1C for age, and found that the positive association between having a sibling and SGB richness was still significant after accounting for age (new Supplementary Table 6), which we reported as follows:

“Babies having a sibling had, for example, an overall higher SGB richness compared to babies without brothers and sisters (avg. SGB richness across all timepoints with vs without sibling, $n = 40$, $U = 271$, $P = 0.012$; **Fig. 1C**), with this association remaining significant after adjusting for age (**Supplementary Table 6**), further supporting previous observations”.

Additionally, **Extended Data Fig. 8** revealed that the microbiomes of younger infants undergo more extensive compositional changes during nursery attendance than their older peers.



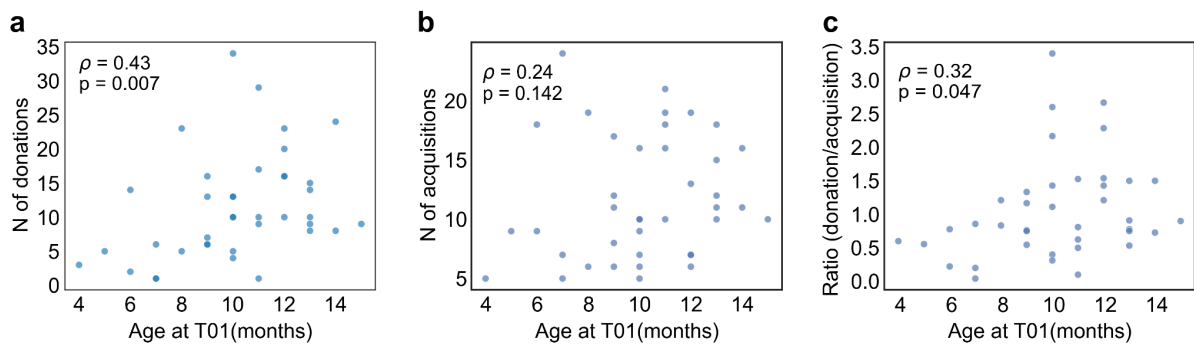
Extended Data Figure 8. Association between Jaccard dissimilarity during the first term of nursery attendance (T01-T15) and baby age (months) at T01. Statistical significance refers to the Spearman’s test.

We described this effect in the same subsection, as follows:

“Also, babies’ alpha diversity substantially increased during the 3 months of nursery attendance (57% avg. increase; T01 vs T15 SGB richness, Wilcoxon signed-rank test, $n = 27$, $T = 69$, $P = 0.003$; **Fig. 1D**), with a more pronounced microbiome change among younger ones (**Extended Data Fig. 8**)”.

3.2. As part of this newly added set of analyses, we explored the effect of age on strain acquisition and donation in babies attending the nursery using our strain inference approach

(Methods). We found that older infants had significantly higher rates of strain donation, compared to younger ones, whereas strain acquisition frequencies showed no significant correlation with age.



Extended Data Fig. 25. (A–C) A, Association between number of strain donations **B**, acquisitions and **C**, ratio of donations over acquisitions and baby age (months) at T01. Statistical significance refers to the Spearman’s test.

This evidence was newly integrated in the main text, referring to new **Extended Data Fig. 25**:

“Among these, age was uncovered to exert a significant effect on strain donation, with this occurring at higher frequency in older infants compared to younger ones (Spearman’s test, $n = 39$, $\rho = 0.43$, $P = 0.007$); in contrast, strain acquisitions were not associated with age (Spearman’s test, $n = 39$, $\rho = 0.24$, $P = 0.14$; **Extended Data Fig. 25**)”.

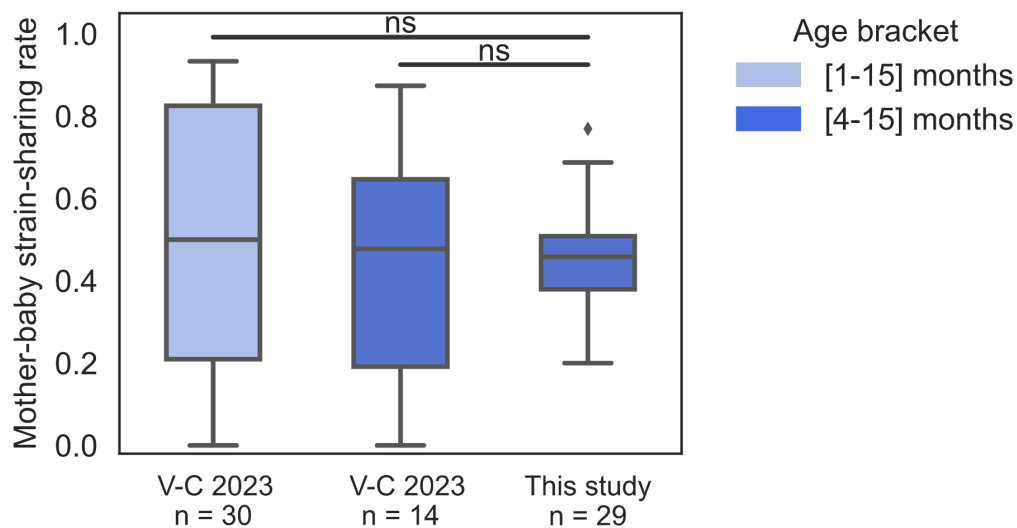
In conclusion, while infant age predictably correlates with microbiome diversity and longitudinal changes, and was revealed to affect baby interindividual transmission, our core findings remain consistently robust.

4. contextualization of results by comparison of mother-to-infant microbial transmission in this and the study Valles-Colomer *et al.*, 2023⁵, where seven different datasets are incorporated;

Because the previous paper (Valles-Colomer *et al.* 2023) considered younger children than the ones analyzed in our new study, we performed a comparison of the strain-sharing rates in mother-offspring pairs stratified by comparable age of the offspring in Valles-Colomer *et al.* (V-C) 2023 and in this study. To do so, we considered only one sample per mother-baby pair from this study (the T01 sample, since Valles Colomer *et al.* 2023 did not include multiple longitudinal samples for infants) and only samples in V-C 2023 of babies in a comparable age range (either 4-15 months, as in our study, or 1-15 months, to reach a comparable sample size). Notably, V-C 2023 encompasses multiple datasets from different populations, while our cohort was recruited within one municipality in northern Italy. Still, we found very comparable mother-baby strain-sharing rates (SSRs) across the two studies without any significant differences when tested with the Mann-Whitney U test (median SSR: 0.48, 0.45 and 0.50 in V-C [1-15] months, V-C [4-15] months and in this study, respectively, with median

number of shared strains: 7.5 and 5 in V-C [4-15] months and this study, respectively). This evidence displays that the extent of mother-infant transmission is consistent across different metagenomic studies and geography. We report this new analysis in **Extended Data Figure 10** below and referred to it as follows:

“We assessed the consistency of the improved strain-sharing inference method by comparing the mother-baby strain-sharing rate detected in this study before nursery entry with that quantified in Valles-Colomer *et al.*, 2023 across seven independent datasets ⁸, and found that mother-infant microbial transmission was highly comparable across different metagenomic studies and geographic regions (median SSR = 0.50% in this study vs 0.48% and 0.45% in Valles-Colomer *et al.*, 2023, for babies in the age ranges of 1-15 and 4-15 months, respectively, **Extended Data Fig. 10**)”.

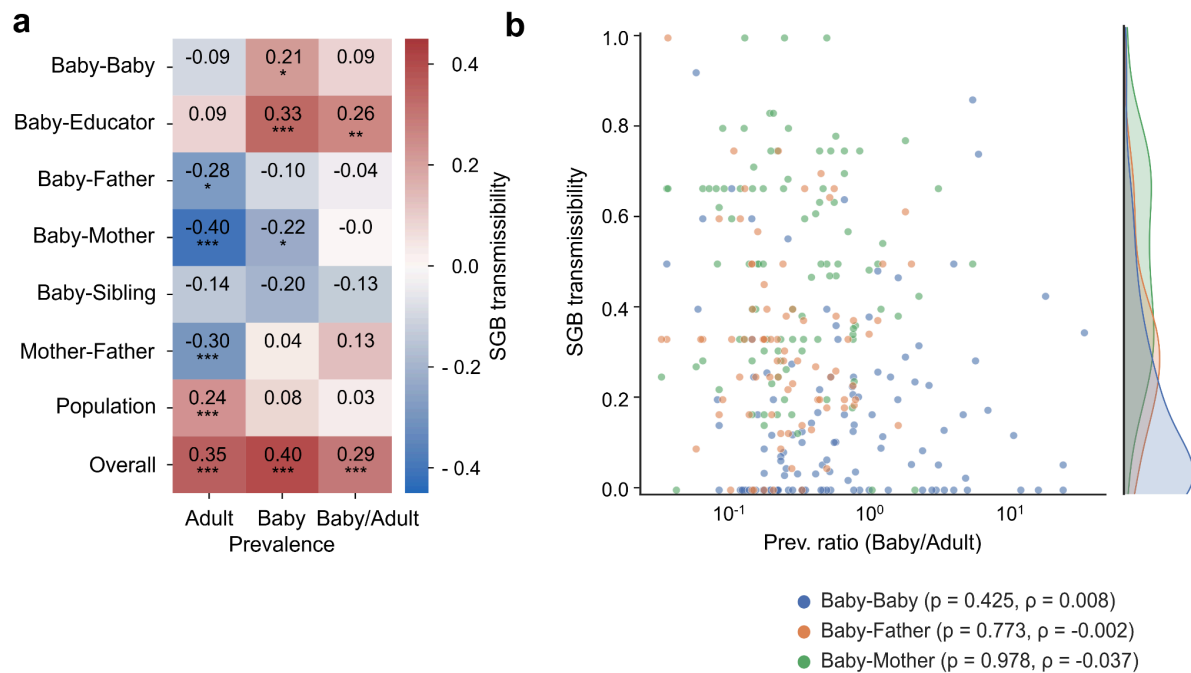


Extended Data Figure 10. Mother-baby strain-sharing rates in Valles-Colomer *et al.* (V-C), 2023, stratified in the [1-15] and [4-15] months age ranges, and in this study. Only pairs of samples with at least 10 SGBs in common were included. In box plots, box edges indicate the lower and upper quartiles, the center line represents the median, and whiskers expand the IQR. Statistical significance refers to Mann-Whitney U, V-C 2023 [1-15] months vs this study ($U = 474.0$), and V-C 2023 [4-15] months vs this study ($U = 198.0$), with $P \geq 0.05$, ns, for both comparisons.

5. Identification of microbial species, being differentially transmitted baby-to-baby in the nursery setting (over parent to baby), in 4 main analyses, described below;

5.1. In the first version of our manuscript we had already explored differential patterns of transmissibility of SGBs between adult and baby participants and presented the results of the analysis in Extended Data Figure 12 (now **Extended Data Fig. 22A**). Upon Reviewers' suggestion we investigated the relation between the ratio of prevalence in babies over prevalence in adults of the same SGBs, as an indicator of “age-specific” microbial distribution, and SGB transmissibility. We found this association to be not significant in any of

the participant dyads tested. This result was newly integrated in **Extended Data Fig. 22B**, below.



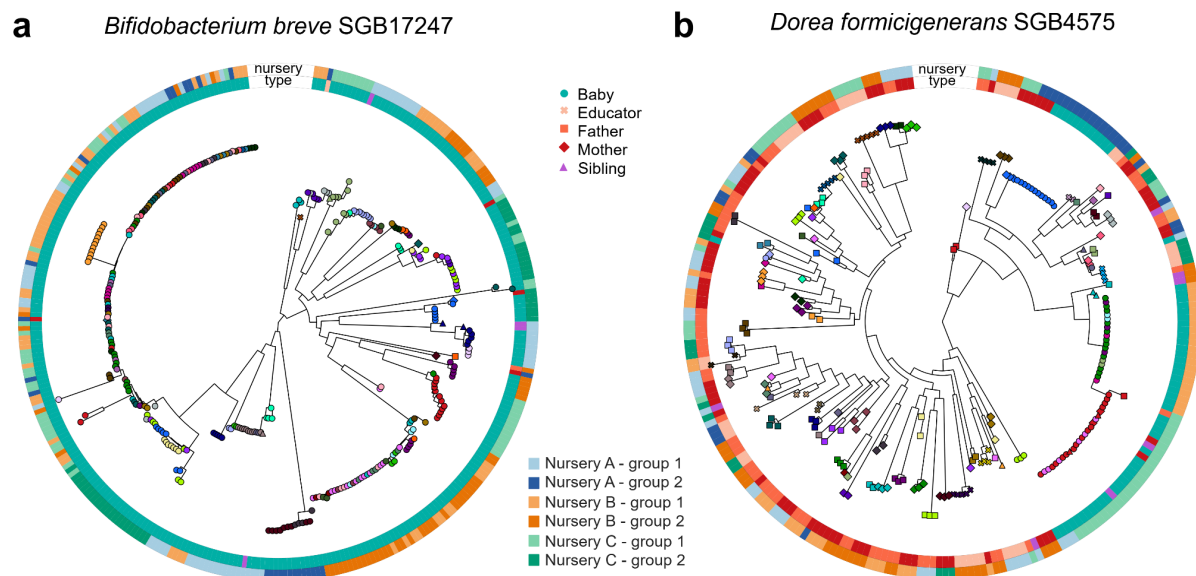
Extended Data Figure 22. (A) Association between SGB transmissibility between pairs of different volunteer types and SGB prevalence (in adults, babies, or the ratio of prevalence baby/adults). “Population” includes all unrelated adults in the microTOUCH-baby study cohort, while “Overall” reports the average of all category pairs in which the SGB transmissibility could be computed, including unrelated volunteers. Higher transmissibility of prevalent SGBs was captured among unrelated volunteers, such as adults (Spearman’s test, $n = 427$, $\rho = 0.24$, $P_{\text{adj}} = 4.6\text{e-}6$) and babies in the nursery (Spearman’s test, $n = 129$, $\rho = 0.21$, $P_{\text{adj}} = 0.031$). Statistical significance asterisks refer to Spearman’s tests, with $P_{\text{adj}} < 0.05$, *; $P < 0.01$, **; $P_{\text{adj}} < 0.001$, ***. **(B)** Association between SGB transmissibility and SGB Prevalence ratio (Baby/Adult).

5.2. Moreover, we looked further into our dataset to identify species exclusively or significantly more transmitted baby-to-baby in the nursery setting (compared to baby-mother and baby-father pairs). We summarize these results in the new **Supplementary Table 10**, generated as described in the **Methods** section:

“Differential transmissibility between baby-baby and baby-mother or baby-father pairs was assessed for all SGBs having at least 10 baby-baby pairs sharing it, with application of a Fisher test (including FDR control) in case there were at least 10 baby-mother or 10 baby-father pairs sharing the SGB, otherwise the number of SGB- and strain-sharing events were reported for each group without application of the test.”

Specific species of particular interest and relevance were further investigated. *Bifidobacterium breve* (SGB17247), a species known for its high prevalence in (breast-fed) babies^{6,7} and established favorable effects on early-life health^{8,9}, was highlighted by our analysis to be considerably more transmissible among baby-baby pairs (as already shown in **Figure 5** and in the new **Extended Data Figure 23A**, below): this SGB was present in 124 baby-baby pairs, 107 of which shared the same strain ($T = 0.86$), which is especially

noteworthy in face of it being rarely ever typed in mothers (present in four mother-baby pairs, two of which shared the same strain) and fathers (present in one father-baby pair, not sharing the same strain). *Dorea formicigenerans*, consistently reported as predictive of age in healthy infant microbiomes^{10,11}, was also significantly more transmissible among baby-baby pairs ($T = 0.92$) compared to baby-mother ($T = 0.27$; Fisher's exact test, $P_{adj} = 0.017$) and baby-father ($T = 0.09$; Fisher's exact test, $P_{adj} = 0.001$) (**Figure 5, Extended Data Figure 23B**).

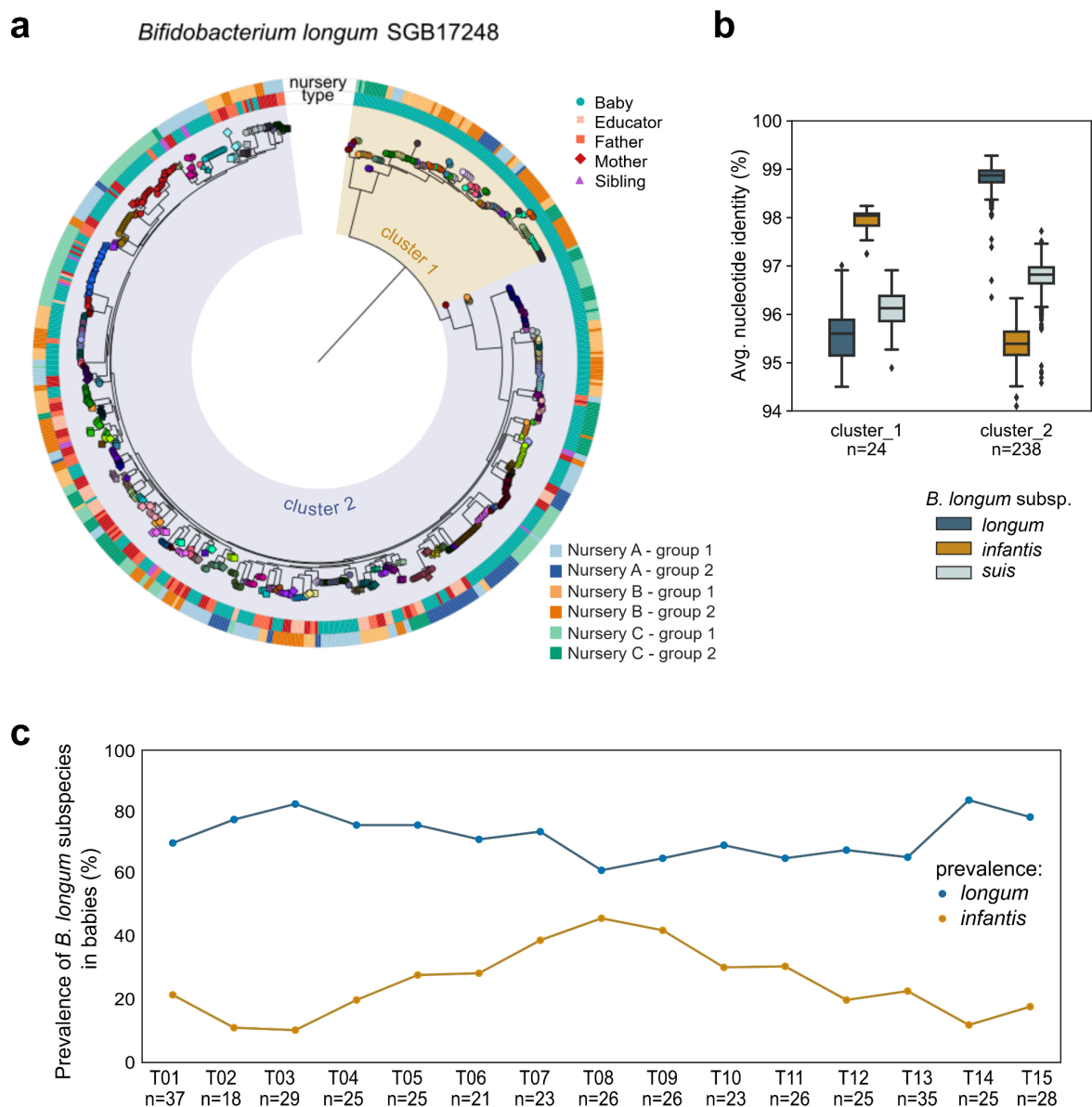


Extended Data Figure 23 (A–B). **A**, Phylogenetic tree of *Bifidobacterium breve* SGB17247 and **B**, *Dorea formicigenerans* SGB4575 that were found to be differentially more transmitted baby-to-baby in the nursery setting, compared to parent-baby pairs. Volunteers categories are identified by shape and colour in the inner ring, while nursery group affiliation by colour in the outer ring, as indicated in the legend.

5.3. We also followed the specific suggestion of Rev #1 to assess the dynamics and transmissibility of the distinct *Bifidobacterium longum* subspecies in the nursery setting. As *B. longum* subspecies fall within the same SGB (SGB17248), we had first to assign each *B. longum* strain profiled in our study to one of the *B. longum* subspecies (subsp. *longum*, subsp. *infantis*, or subsp. *suis*). Inspecting our *B. longum* phylogeny (**Extended Data Figure 24A**), we noticed two clusters of strains (provisionally named cluster_1 and cluster_2), with cluster_1 composed exclusively of baby strains (and as such hypothesized to be subsp. *infantis*) and cluster_2 having both baby and adult strains (hypothesized to be subsp. *longum*). To evaluate these hypotheses, we built metagenome-assembled genomes (MAGs) of those strains, managing to do so for 262 out of the 591 strains present in the tree of **Extended Data Figure 24A**, and calculated the average nucleotide identity between the *B. longum* MAGs of our study and five *B. longum* reference genomes of each of the three known *B. longum* subspecies ($n = 15$ reference genomes in total). As represented in the new **Extended Data Figure 24B** (shown below), cluster_1 MAGs ($n = 24$) were all genomically closer to *B. longum* subsp. *infantis* (median ANI = 98.04%) than the other *B. longum* subspecies (median ANI to subsp. *longum* = 95.60%; median ANI to subsp. *suis* = 96.13%), while cluster_2 MAGs ($n = 238$) were considerably closer to *B. longum* subsp. *longum*

(median ANI to subsp. *longum* = 98.87%; median ANI to subsp. *suis* = 96.82%; median ANI to subsp. *infantis* = 95.39%), validating our hypotheses.

After using this genome-based subspecies assignment to assign accordingly all 591 *B. longum* strains profiled with StrainPhlAn 4, we confirmed *B. longum* subsp. *infantis* was never detected in microbiomes of adult participants in our cohort, either parents or educators, while its prevalence increased in babies attending the nursery to reach a maximum of ~50% (at T08) to then decrease at the end of the first term (T15), as shown in the plot below (**Extended Data Figure 24C**).



Extended Data Figure 24. (A) Phylogenetic tree of *Bifidobacterium longum* SGB17248, with inner and outer rings identifying participant type and nursery group, respectively, as per legend. **(B)** Intra-subspecies average nucleotide identity (%) used to define subspecies-level clusters within *B. longum*. **(C)** Prevalence of *B. longum* subsp. *longum* and subsp. *infantis* in babies during the first term of nursery.

This indeed suggests this species acquisition may specifically occur via interpersonal transmission among infants in the nursery, possibly facilitated by similar intestinal ecology and lifestyle characteristics of the infant hosts, in addition to close physical contact during attendance. Hence, this evidence points to the nursery as a potential epidemiological reservoir of this species. Moreover, we found that transmission of *B. longum* subsp. *infantis* was significantly higher than *B. longum* subsp. *longum* among baby-baby pairs (TS = 85.3% vs 19.4%, respectively; Fisher's exact test, $n = 142$, $P = 5e-12$).

All in all, this analysis provides supporting evidence that various species that are considered relevant for infant health are exclusively or significantly more transmitted among babies in the nursery. We collectively report these examples in **Figure 5** and **Extended Data Figure 23** and **24** commenting the evidence in the subsection "The most transmissible species" (*slightly shortening the previous text to accommodate this addition*), as follows:

"We looked further into our dataset to identify species differentially more transmitted baby-to-baby in the nursery setting (compared to baby-mother and baby-father pairs; **Supplementary Table 11**). *B. breve*, a highly prevalent species in (breast-fed) babies^{6,51} with known favorable effects on early-life health^{52,53}, was differentially more transmissible among baby pairs, compared to mother-baby pairs in our study (**Fig. 5**, **Extended Data Fig. 23A**). *Dorea formicigenerans*, a known biomarker of age progression in healthy infant microbiomes^{54,55}, was also more transmissible among babies than between babies and either parent (**Extended Data Fig. 23B**). Notably, *Bifidobacterium longum* subsp. *infantis*, a specialized gut colonizer of breast-fed infants⁵⁶ (particularly in non-urbanized locations^{57,58}) with anti-inflammatory effects⁵⁹, was detected exclusively in babies in our cohort (**Methods**, **Extended Data Fig. 24A-B**), with prevalence peaking at ~50% mid-term (T08), before declining (**Extended Data Figure 24C**). Moreover, transmission of *B. longum* subsp. *infantis* was significantly higher than *B. longum* subsp. *longum* among baby-baby pairs ($T = 85.3\%$ vs 19.4% , respectively; Fisher's exact test, $n = 142$, $P = 5e-12$), showing that the acquisition of subsp. *infantis* may specifically occur via interpersonal transmission among infants in the nursery. This evidence substantiates that multiple species considered relevant for infant health are exclusively or significantly more transmitted among babies in the nursery."

We also mentioned their main health implications in early life in the conclusions ("particularly of key early-life gut colonizers such as *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium breve* (Henrick et al. 2020; Alcon-Giner et al. 2020)").

We also described the *B. longum* analysis in a newly added section of **Methods**, as follows:

"*Bifidobacterium longum* strains assignment to subspecies

To evaluate the transmissibility of distinct subspecies of *Bifidobacterium longum* SGB17248 in our dataset, we constructed a StrainPhlAn 4 phylogenetic tree of all 591 *B. longum* strains, which revealed two distinct clusters (cluster_1 and cluster_2, **Extended Data Figure 24**). Cluster_1 consisted exclusively of strains present in infants and hence was hypothesized to belong to *B. longum* subsp. *infantis*, while cluster_2 contained strains from both infants and adults, possibly representing other subspecies of *B. longum*. To definitively assign these strains to subspecies, we

succeeded in generating MAGs (as described above) for 262 of the 591 strains and then calculated the ANI between these MAGs and 15 reference genomes representing the three known *B. longum* subspecies¹¹¹ (five reference genomes each for subsp. *longum*, subsp. *infantis*, and subsp. *suis*). The 24 MAGs from cluster_1 showed highest similarity to *B. longum* subsp. *infantis* reference genomes (median ANI = 98.04%), compared to lower ANI values with subsp. *longum* (95.60%) and subsp. *suis* (96.13%). Conversely, the 238 MAGs from cluster_2 displayed the highest genomic similarity to *B. longum* subsp. *longum* reference genomes (median ANI = 98.87%), with substantially lower similarity to subsp. *suis* (96.82%) and subsp. *infantis* (95.39%). The 591 *B. longum* strains as profiled with StrainPhlAn 4 were then assigned according to their genome-level assignment.”

5.4. Additionally, we integrated our analysis on interindividual bacterial transmission in the nursery environment, exploring these dynamics for the understudied microeukaryote *Blastocystis*. We report the presence of *Blastocystis* and the distribution of different subtypes in our dataset in the new **Supplementary Table 12**, and discussed the new results as follows:

“Finally, we investigated transmission patterns of *Blastocystis*, a common human gut microeukaryote recently linked to cardiometabolic health⁶⁰. We identified *Blastocystis* in 26 subjects and 9.18% of the samples, exclusively as single-subtype (ST) carriage of its most common subtypes in the gut (ST1-ST4^{60,61}) and with longitudinal subtype retention (**Supplementary Table 12**). Despite detecting *Blastocystis* in at least one parent across 15 families in our cohort, no infants harbored this microeukaryote (**Supplementary Table 12**), consistently with recent findings⁶⁰.”

6. Improved readability, done by targeted-shortening of specific sections and thorough restructuring of the manuscript. More specifically, this included:

6.1 Removing subsections and incorporating their results into other ones (e.g., integration of the previous independent subsection on pet-human strain-sharing into “Tracking multi-host strain transmission”)

6.2 Reordering of subsections in the results section to obtain the following updated structure:

- Microbiome transmission in nurseries
- Baby microbiome development in nurseries
- Mapping intricate nursery strain-sharing
- Tracking multi-host strain transmission
- Strains spreading in the nursery
- Relations in nursery shape microbiomes
- Baby microbiomes built via transmission
- Nursery strains impact as family ones
- Long-term effect on transmission
- Siblings affect baby strain acquisition
- The most transmissible species
- Host factors and microbiome transmission
- Antibiotics may alter strain acquisition

6.3 Splitting large subsections into shorter ones (e.g. the first two subsections above obtained from the previous “A metagenomic monitoring of the dynamic nursery community microbiomes”);

6.4 Addition of two new subsections with novel analyses (namely, “Long-term effect on transmission” and “Host factors and microbiome transmission”).

Response to the specific comments and request of Reviewer #1

The authors present a metagenome analysis of infants, family members and nursery staff (educators) to examine infant gut microbiome transmission processes in nursery setting. 643 samples from 133 individuals are analysed from 3 nurseries in Trento in Italy over 5 months. The authors report extensive transmission of gut microbiome strains occurring between infants in nurseries that is at higher levels compared to other family members and educators during this time period. The study highlights the importance of nursery settings in promoting infant to infant microbiome transmission. These findings are novel as they extend on previous research, much of it led by the same authors, highlighting the importance of transmission in seeding the infant gut microbiome and enabling microbiome assembly. While previous research has focused on the importance of maternal transmission and to a lesser degree, transmission from other family members, this study reveals childcare settings also contribute.

Overall, the authors demonstrate gut microbiome transmission is occurring in nursery settings between infants, however while well written, the results are quite descriptive and the impact of the findings are not entirely convincing.

One reason is the relatively small number of samples compared to other studies examining early-life gut microbiome transmission, e.g. Stewart *et al.* Nature and Shao *et al.* Nature analysed 11,000 and 1700 metagenomes from 900 and 600 babies respectively. In this study, 636 metagenomes from 133 individuals were analysed. These are still very impressive numbers and the authors are to be commended for designing and executing the study, but these numbers are not comparable to other similar studies.

We appreciate Rev#1' comment on the sample size, and we have always been convinced that properly sized datasets are key in microbiome study design. Specifically, the sample size should be large enough to convincingly support the findings. In this work, our core hypothesis was that the daycare environment had a major effect on strain transmission dynamics and in the building of diverse and personal microbiomes in infants. We think that our study design was able to prove that, and thus sample size was sufficient for the scope of these specific findings.

Notice that when the hypotheses needed larger sample sizes, we were able to expand the sample size to over 30,000 individuals and metagenomes as recently reported in Piperni *et al.* in Cell, 2024, Manghi *et al.*, 2024 and Fackelmann *et al.*, 2025 in Nature Microbiology. Regarding the mother-infant transmission studies mentioned by the Reviewer (Stewart *et al.*

and by Shao et al.), these indeed represent notable efforts focused on addressing specific advanced hypotheses within an already established context of mother-infant transmission, which was previously demonstrated in studies of more modest scale but sufficient power (e.g. our paper by Ferretti et al. in *Cell Host & Microbe*, 2018 with 25 mother-infant pairs, now cited over 1.2k times). As such, we think the sample size should be discussed for the extent to which it is sufficient to prove the hypothesis.

Nonetheless, as we introduced and detailed at the beginning of this document, we have now expanded the study to a total of over 1013 samples from 137 volunteers, strengthening our conclusions. Crucially, we maintained a very high sequencing depth (avg. sequencing depth = 15.61 Gbp). This high depth is a critical aspect for accurate strain-level transmission modeling, distinguishing our approach from other large-scale transmission surveys employing significantly lower depths (e.g., Shao et al. with ~4.82 Gbp and Stewart et al. with ~20 Mbp), and as such, the depth of sequencing is another dimension of a properly sized study design to consider and directly impacting strain-level investigations.

Referee#1 Comment#1 (henceforward, R#1 C#1)

Building on this, I was unsure how these findings fit into the entire sequence of early-life gut microbiome transmission initiated by mothers and continued here. The authors previously reported (Valles-Colomer et al. 2023) the number of strains shared between mothers and offspring (up to 1 year old) as 17. In this study, examining maternal transmission with infants of similar age the authors report number of strains shared as 5-6, which is much lower.

We thank Rev #1 for this comment as we agree on the importance of contextualizing the results shown in the study with what has been reported by us and others in previous studies. Firstly, we'd like to clarify that the number "17" mentioned by Rev #1 describes in Valles-Colomer et al. 2023 the median number of species (not strains) shared between mothers and offspring during the 1st year of life (from birth to 1 year of age). The number of strains was reported in the study to be a median of 9 shared strains during the same period.

Nonetheless, we agree on the importance of comparing our results with previous studies, especially given the insights offered by recent work on strain-level transmission. To this end, and considering the comprehensive nature of the Valles-Colomer et al. 2023 study, we performed a comparative analysis of strain-sharing rates (SSRs) in mother-baby pairs between this study and Valles-Colomer et al. 2023.

Briefly, for this comparison, we focused on comparable age ranges for the offspring: specifically, we utilized only the T01 sample from each mother-baby pair in our study (as Valles-Colomer et al. 2023 did not include multiple longitudinal infant samples), and we selected samples from Valles-Colomer et al. (V-C) 2023 where babies were within 4-15 months of age (matching our study's age range at T01) or 1-15 months (to ensure a comparable sample size).

It is important to acknowledge that V-C 2023 included 7 different datasets across different populations, whereas our cohort was exclusively recruited within a single municipality in northern Italy. Despite these contextual differences, and as detailed in the the response to the Editor (point #4), we observed highly comparable mother-baby SSRs across both studies, with no statistically significant differences. Specifically, the median SSRs were 0.48,

0.45, and 0.50 in V-C 2023 (1-15 months), V-C 2023 (4-15 months), and our study, respectively, and the median number of shared strains was 7.5 in V-C 2023 (4-15 months) and 5 in our study. This evidence displays that mother-infant transmission is consistent across different metagenomic studies and geographic locations. This was integrated as **Extended Data Figure 10** (see point #4 in response to the Editor, above, for how this was discussed in the main text).

Finally, we'd like to note that the direct comparison of absolute count of shared strains across different studies should be interpreted cautiously, because the result of this quantification is contingent on sequencing depth and microbiome compositional diversity that are to some extent specific to the study and cohort analysed (and Valles-Colomer et al. 2023 combined multiple cohorts from multiple independent studies). This is evident when comparing our results in **Extended Data Figure 10**: while strain-sharing rates across studies remain robustly consistent, even for the age-matched comparison (i.e. 4-15 months bracket), we observed a varying number of shared strains, although of comparable magnitude (lower-upper quartiles: 4.25–11.5 for V-C 2023 and 5–8.5 for this study; median: 7.5 vs 5 shared strains in V-C 2023 and this study, respectively). This is because the absolute number of typed SGBs in common differed substantially across studies (lower-upper quartiles: 11.25–33 for V-C 2023 and 13–16 for this study; median: 18 vs 13.5 SGBs in V-C 2023 and in this study, respectively). These differences in absolute counts that are dependent on technical aspects and may vary in different studies, despite similar transmission rates, reinforce why we prefer strain-sharing rates for comparing interpersonal transmission across studies.

R#1 C#2

Overall, the authors' conclusions (line 442) that the microbial transmission occurring in nurseries as a contributor to infant health is comparable to vaginal vs C-section or breastfeeding vs formula is not conclusive. Other studies from the same authors reported maternal transmission at birth or shortly afterwards contributes more strains (Ferretti et al 2018) compared to transmission reported here between infants in nurseries. Contextualisation of these results (e.g. maternal, paternal, sibling transmission) is needed to show that the findings here are representative of universal early-life microbiome ecological processes and are not isolated or stochastic.

To better support our conclusions, as detailed in R#1 C#1, we contextualized strain transmission between the baby and the mother as quantified in this study and in Valles-Colomer et al 2023, which incorporates data from Ferretti et al 2018 and applies comparable strain-sharing inference methods (**Extended Data Figure 10**) and showed that our approach is robust in quantifying person-to-person microbial exchange, across different metagenomic studies and geographical locations. While, generally, the conclusive statement Rev #1 refers to was intended to provide a qualitative assessment of microbial transmission in the nursery relative to other significant perinatal variables, rather than to establish a quantitative comparison between these contributory factors, it was mainly drawn on the evidence shown in **Figure 4C**. This illustrates that the proportion of strains of the infant gut microbiome acquired from the community of peers in the nursery group is significantly higher than those acquired from the family members at the end of the first term and contributes to a combined larger relative abundance. We based this conclusion specifically on this result

because we consider the “proportion of strains acquired” (previously “% strains shared”) to be the best metric available to compare family versus nursery contributions because it is calculated over all the strains present in the baby, considering the overall baby gut microbiome composition. For instance, a baby may show 100% strain-sharing rate (SSR) with their mother, yet simultaneously acquire a significantly higher percentage of strains from the nursery than from family members. This apparent contradiction exists because the SSR calculation only considers species that are in common to the baby and mother pair, while the “proportion of strains acquired” examines strain acquisition across all strains present in the baby's microbiome, providing a more comprehensive picture of the “sources” of microbial transmission. Nonetheless, we acknowledge that the use of distinct, yet similarly named metrics throughout the manuscript was confusing to the reader and therefore renamed “% of strains shared” to “proportion of strains acquired” and added the label “one baby to multiple individuals” over **Figure 4C**. We discussed in more detail the significance of different metrics in the response to minor comment #4 of Rev #2.

Finally, we would also like to acknowledge that the previous formulation of the statement had additional sources of confusion in that we were comparing the importance of the role played in infant health by baby-baby microbial transmission occurring during the first year of nursery (hence involving babies in the age range of 4-15 mo at nursery admission), to established factors exerting key roles in earlier stages of life (from birth to the first months of age). To avoid any misinterpretation, we clarified this by re-phrasing the statement as follows:

“Our data indeed suggest that social and physical interaction between infants should be regarded as a key contributor to microbiome maturation during infancy comparably to previously outlined perinatal factors that influence the earliest stages of life, such as delivery mode (natural versus C-section) and feeding practices (breastfeeding versus formula feeding).”

And accordingly, in previous lines:

“Our study uncovered that after a few months of nursery attendance, the microbial strains acquired from peers in the same nursery group accounted for a larger proportion of the infant microbiome than those from the mother and – more generally – family members (**Fig. 4C**), who are known to exert the greatest influence on infants’ microbiome in the first months of life”.

Finally, we are aware that defining numerically the contribution of the daycare environment vs maternal and family environment is subject to a number of variables that differ in different studies and context, so we are avoiding any definitive or too strong statements on this in the revised version of the paper.

R#1 C#3

As an example of how the results reported here could be given greater significance, can this study explain the transmission of strains that are prevalent and health associated in the early life intestinal microbiota such as *Bifidobacterium longum* subsp. *infantis* (less abundant in European children but still present) but which are not detected in mothers? Or to put it

another way, are nursery settings the reservoir and infant-to-infant the transmission route for specific important early-life microbiome species?

We thank Rev #1 for this valuable note that we addressed extensively and detailed in the response to the Editor (point #5 of the response). Briefly, in the previous version of the manuscript, we had already explored SGBs transmissibility patterns in babies and adults (shown in Extended Data Figure 22A). To address Rev #1 suggestion, we delved deeper into the transmission patterns of SGBs, focusing on the ratio of SGB prevalence in babies over adults, as an indicator of "age-specific" microbial distribution, and found no significant association between this ratio and SGB transmissibility across the participant pairs we examined (new **Extended Data Figure 22B**).

As part of this new set of analyses, we also investigated the species that are exclusively or significantly more transmitted from baby-to-baby within the nursery setting, over parent-to-baby. These results are now summarized in the newly generated **Supplementary Table 10**. We found two particularly interesting species; *Bifidobacterium breve* SGB17247 and *Dorea formicigenerans* SGB4575, both differentially more transmissible among baby-baby pairs compared to baby-mother and baby-father pairs, as highlighted in **Figure 5** and new **Extended Data Figure 23**.

Following the Rev #1 specific suggestion, we investigated the transmissibility of distinct *Bifidobacterium longum* subspecies. Since *B. longum* subspecies fall within the same SGB (SGB17248), firstly we assigned strains to its specific subspecies (i.e. *longum*, *infantis*, or *suis*) (**Methods**, new section "*Bifidobacterium longum* strains assignment to subspecies"). Our phylogenetic analysis (**Extended Data Figure 24A**) showed two distinct strain clusters, with cluster 1 representing subsp. *infantis* (**Extended Data Figure 24B**) that was later confirmed to be exclusively detected in babies in our cohort. Next, we analyzed transmission dynamics of this subspecies and observed that *B. longum* subsp. *infantis* prevalence peaked in babies mid-term in the nursery (**Extended Data Fig. 24C**), and its transmission was considerably higher among baby-baby pairs compared to *B. longum* subsp. *longum*. This suggests the nursery as a reservoir for this species.

Finally, we explored the transmission of *Blastocystis*, a common gut microeukaryote. While we detected its presence in parents, no infants in our cohort harbored *Blastocystis* (**Supplementary Table 12**), consistently with recent research¹².

Overall, these new findings provide evidence that several species crucial for infant health are primarily or exclusively transmitted among babies within the nursery. We address Rev #1 to the response to the Editor for details on how these were integrated into the manuscript.

R#1 C#4

The study focuses almost entirely on transmission processes, i.e. the number of species transmitted, the taxonomy of these species and the most transmissible species. As such, a lot of the results are descriptive as they do not address underlying mechanism. Figure 6 does touch on antibiotic usage and its impact on transmission but given the study focuses on early-life gut microbiomes I felt integration of infant dietary practices was required. For

example, what was the role of breastfeeding in promoting or blocking transmission of specific species? I expect the infants in this study would be mixed feeding with breastfeeding and solids while others will be weaned. The impact of different diets on transmission in this setting should have been addressed.

We strongly agree with the observation of Rev #1 and integrated the manuscript with multiple analyses to address the investigation of the effect of diet of both baby and adult participants, on microbiome composition, as well as interpersonal microbial transmission. As done previously for other suggestions shared by the Reviewers and the Editor, we detailed how we address this point collectively in the response to the Editor, and we refer Rev #1 specifically to point #2 of the initial response to the Editor.

In summary, prior to nursery admission, we collected as much information as possible regarding infant dietary practices using an *ad hoc* questionnaire to record weaning status and milk type in case of mix feeding during weaning, while adult volunteers were asked to fill the EPIC Food Frequency Questionnaire (FFQ) that were used to derive the healthy Plant-based Dietary Index (hPDI) to assess quality of long-term dietary habits.

At nursery admission, the vast majority of babies were already weaned (38 weaned at T01, with only 2 unweaned), and received a mixed diet during weaning, combining solid food with some form of milk (24 mixed diet, 14 only solid food). Among those on a mixed diet, about half received maternal milk (9 breast-fed, 10 formula-fed, 5 receiving either). When investigating the impact of these feeding practices on infant microbiome richness, we found no significant association between the two, as newly mentioned in the results section. In contrast, we observed a significant relation between higher hPDI (an indicator of diet quality) in adult volunteers and their microbiome richness, as shown in the new **Extended Data Figure 6**.

Next, we explored the putative impact of infant diet on strain transmission patterns. Our analysis revealed that babies consuming milk at T01, particularly those receiving maternal milk, displayed a non-significant trend toward higher strain-sharing rates with their mothers (depicted in the new **Extended Data Figure 27**). Furthermore, we tested whether diet had any discernible impact on the acquisition or donation of new microbial strains in babies, but our investigations yielded no significant effects (new **Extended Data Figure 28**), overall indicating a negligible impact of diet on interindividual strain transmission. For more detail on how we incorporated and discussed these new evidence, we address Rev #1 to the above mentioned response to the Editor.

Other metadata were already included the manuscript in analyses investigating the relation between host-associated and lifestyle factors on microbial strain transmission, as various figures or supplementaries (e.g. **Figure 6** on antibiotic treatment and old Extended Data Figure 3, now **Extended Data Figure 7**, on intrapartum anti-*Streptococcus* B prophylaxis and mode of delivery) and their discussion was now collected in the “Host factors and microbiome transmission” subsection together with diet.

Major comments

1. Better contextualisation of the importance of transmission within nurseries compared to maternal transmission or other family members should be provided. My specific comments related to this are above.

We agree with Rev #1 that contextualization is necessary to demonstrate robustness of our results, and accordingly we added the text regarding **Extended Data Figure 10** in the third subsection of the results section titled “Mapping intricate nursery strain-sharing” (as explained in more detail in **R#1 C#1** and **C#2**, and response to the Editor point #4). The comparative analysis on mother-to-baby strain-sharing rates demonstrated consistency between our findings and existing literature, but was also instrumental in providing support for our methodological approach and strengthening the conclusions regarding strain transmission within the nursery setting. More details on how we addressed these comments are in the response to the general comments R#1 C#1 and C#2 above, and response to the Editor point #4.

2. The role of diet in transmission in this study should be addressed. My specific comments related to this are above.

We agree with Rev #1’ perspective and have incorporated the suggested analyses in **Extended Data Figures 27** and **28**, and discussed them in the newly dedicated subsection in the results section, as reported in the response to the Editor, point #2. We also described the observed effect of diet on microbiome richness in baby and adult volunteers (**Extended Data Figure 6**), as specified in the mentioned comment above.

3. Greater clarity should be provided with results in terms of rate of strain sharing and numbers of strains shared. Fig 1A number of strains shared between babies at end of study is 7.2. SGB richness in babies is ~90 (Figure 1D) = 8% strains shared. How does this relate to the strain sharing rate of 20.2% reported in Line 234?

We understand the source of confusion when comparing the extent of strain-sharing between babies at T15 as displayed in **Figure 2A** (now **Fig. 3A**, showing the average number of strains shared between a baby and other babies in the same nursery at T15=7.2) and the SSR in old L234. As done in previous work from our lab ⁵, we calculated samples-level descriptive diversity metrics such as SGB richness (shown **Figure 1D**) using MetaPhlAn 4 taxonomic profiles (**Methods**), while strain-sharing rates are defined between a pair of microbiomes and are calculated with respect to the number of shared SGBs typed at strain level (i.e. those profilable at strain-level and present across a pair of microbiome samples) according to StrainPhlAn 4. For a given metagenome, the number of SGBs typed at strain level with StrainPhlAn will necessarily be less or equal the number of SGBs detected in MetaPhlAn 4 profiles. This is the case because having reads mapping against the SGB-specific marker genes in the MetaPhlAn database is enough for a species to be detected/quantified, while for genetic/phylogenetic typing SGBs in StrainPhlAn these markers must be sufficiently covered so that the consensus alleles for each marker gene can be robustly identified. The mismatch between the number of SGBs present in a sample and the ones that can be typed is precisely the reason why we focus our analysis on strain-sharing rates instead of on the absolute number of shared strains.

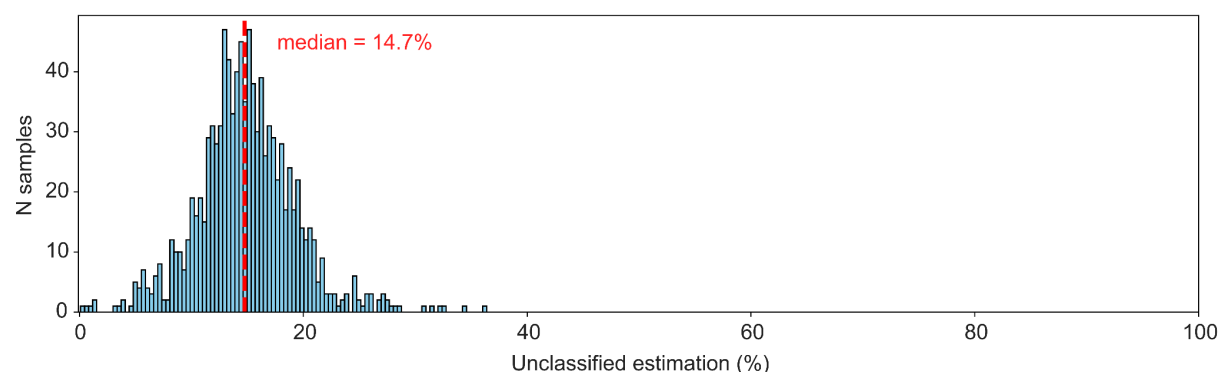
In addition, referring to the specific example indicated by Rev #1, as SSR is calculated as the number of shared strains over the number of shared species (typed at the strain-level) between a pair of microbiome samples, the avg. number of species commonly typed across same-nursery babies at T15=15.1, while the avg. number of shared strains among baby-baby pairs having at least 5 SGBs typed at strain level in common at T15=3.1, such that we obtain an avg. SSR of 18.3%. We note this avg. SSR of 18.3% among same nursery babies is different from the 20.2% indicated by Rev #1 because 20.2% refers to the avg. SSR among babies in the same nursery *group* (**Fig. 3D**).

Finally, in **Fig. 3A**, the avg. number of shared strains among babies in the same nursery at T15=3.1 reported above is different from the one of 7.2 indicated by Rev #1 because 7.2 refers to the avg. number of strains that a baby will share with at least one of the other babies in the same nursery at T15 and not the avg. number of strains shared at T15 across baby-baby pairs (the latter is the one used as the numerator in the calculation of SSRs).

To avoid misunderstandings, we specified throughout the main text when SGBs were typed at the strain-level (i.e. where StrainPhlAn 4 is implemented), e.g. for calculation of strain-sharing rates.

4. There is no information provided (as far as I can tell) on the extent of metagenome reads that are taxonomically profiled. At a minimum the authors should present the percentage of reads mapped per metagenome.

We agree and, in order to provide that information, we have rerun the metagenomic profiling of all metagenomes using the `–unclassified_estimation` mode of MetaPhlAn v4.1, recently shown to be very precise even for environments less characterized than the human gut¹³. Our newly generated **Supplementary Table 4** integrates the total number of metagenomic reads with the percentage of these that were unclassified. We also report a visual representation of this alongside the summary statistics (mean = 14.88%; range 0.08–36.39%) that was integrated as **Extended Data Figure 1** (below). We note that the large proportion of classified reads highlights a comprehensive mapping of the infant microbiome, representing a substantial improvement over metagenomic surveys in the past decade and reflecting the expanded coverage provided by having unknown SGBs (uSGBs) in current databases.



Extended Data Figure 1. Distribution of unclassified reads percentage across all samples analyzed in the study, as estimated using MetaPhlAn v4.1.

We refer to these new analyses as follows:

“Following shotgun metagenomic sequencing we used MetaPhlAn 4 ¹⁶ to generate taxonomic profiles at species-level genome bin (SGB) ¹⁷ resolution that successfully accounted for large fractions of metagenomes (mean unclassified reads = 14.88%, fraction of unknown SGBs i.e. uSGBs = 46.37% accounting for an average of 9.45% abundance, **Extended Data Fig. 1, Supplementary Table 4**)”.

5. Results identifying SGBs with prevalence of transmission in Figure 3D and those identified as most transmissible in Figure 5 appear redundant. If they start with low prevalence but become highly prevalent (Figure 3D), they are by definition highly transmissible. I suggest these results be combined or else Fig. 3D be removed.

We think the two analyses report different things as SGB transmissibility is computed at the whole SGB-level, showcasing efficiency of transmission as a generalized “feature” of a certain species, as it is computed by quantifying the number of strain-sharing events for each SGB over the total potential number of strain-sharing events, while Figure 3D (now **Fig. 2C**) is pinpointing single strains spreading effectively from 1 individual into many in the same group of participants and their longitudinal kinetics of spread. While the two analyses occasionally yield overlapping insights (for instance, the identification of specific *Tyzzarella nexilis* and *Streptococcus gallolyticus* strains with both high nursery transmission and broader species-level transmissibility) they reveal different aspects. One highlights species that may possess conserved phenotypes that promote efficient spread and colonization, the other describes a strain’s efficient transmission patterns, a phenomenon previously mainly seen in infectious microorganisms, in health-associated microbiome members.

To better convey this, we rephrased the description of the transmissibility metric, namely:

“We next assessed species-level transmissibility by counting the number of strain-sharing events for each SGB in our cohort over the total potential number of strain-sharing events”.

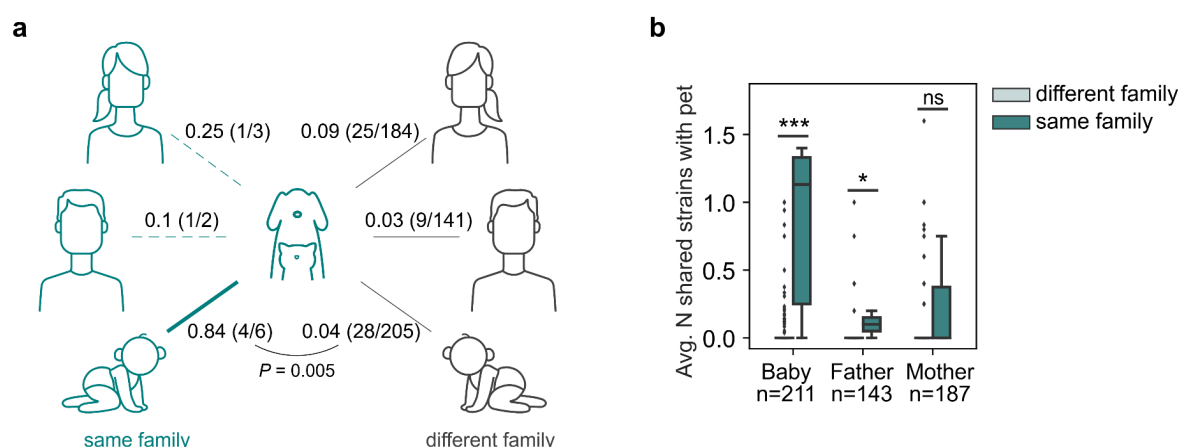
Other comments:

1. Line 153- The data on transmission between pets and humans is not very convincing given only 4 animals are profiled. I suggest removing from paper or moving to supplemental material.

We agree and we moved the figure on pets (old Figure 2C) to supplementary material (current **Extended Data Figure 13**, below). We note, however, that by incorporating extension and follow-up samples to the analysis of strain-sharing between pets and humans we managed to include an additional animal (from a family enrolled only during the extension) for a total of 5 pets included. To further strengthen the sample size of our analysis we now opted to include all pet samples available ($n = 11$) as opposed to only the T01 sample of each pet ($n = 4$). This means that our new **Extended Data Figure 13** reports the avg. number of strains shared for human-pet pairs across all the contemporaneous samples available for each human and pet, as well as the fraction of pairs for each comparison category (same family vs. different family) in which human-pet strain-sharing was detected. As previously, we could detect a signal of increased strain-sharing within the family only for

baby-pet pairs (Fisher's exact test, $P = 0.005$). New results were briefly discussed in the new subsection "Tracking multi-host strain transmission" (removing the independent subsection dedicated to pets present in the previous version of the manuscript), as follows:

"Our unique dataset also enabled us to explore strain transmission between different animal hosts by examining gut microbiomes of household pets and their baby or adult owners. Even though the findings are rather anecdotal given the reduced number of pets considered ($n = 5$), we overall identified a low total number of within family pet-human strain-sharing events. Pet-human sharing occurred with significantly higher frequency among pet-baby pairs compared with unrelated pairs (Fisher's exact test, $n = 211$, $P = 0.005$, **Extended Data Fig. 13**), while no increased human-pet strain-sharing within families was noticed among pets and parents. Strains found to be transmitted between babies and pets belonged to human-associated species that had also been previously detected in pet gut microbiomes (*Faecalimonas umbilicata*, *Ruminococcus gnavus*, *Clostridium* sp AT4 and *Phocaeicola vulgatus*^{27–30}), indicating they may be more ecologically fit to overcome host species boundaries and to adapt into new host populations".



Extended Data Figure 13. (A) Strain-sharing between pets ($n=5$) and human hosts within the same family (turquoise) and across different ones (gray). The average total number of strains shared between participant pairs across contemporaneous samples are reported above the connecting lines; in brackets are indicated the number of pet-human pairs with at least one strain shared over the total number of pet-human pairs. Statistical significance according to a Fisher's exact test is depicted for the same family vs different family baby-pet comparison. All other comparisons did not reach statistical significance. **(B)** Average number shared strains between pet and human across contemporaneous samples, in different vs same family. Statistical significance asterisks refer to Mann-Whitney U tests, with $P \geq 0.05$, ns; $P < 0.05$, *; $P < 0.001$, ***.

2. Line 173- given the small sample numbers (4 pets) and the fact that *P. vulgatus* and *R. gnavus* also have health-associated properties, this statement is very speculative. I suggest removing it.

As described in the previous answer, we generally edited the section on pets and removed the statement.

3. Line 147- It is not clear what triangles with numbers in Figure 2A represent and how they relate to numbers within family or nursery box. Please clarify further.

We thank Rev #1 for pointing this out. The figure caption was rephrased to improve clarity as follows: “Under each colored box dedicated to “family” or “nursery”, a triangle displays the average number of strains shared between the baby and any participant in “family” (mother, father, sibling) or “nursery” (other baby, educator). “

4. Line 195- The authors convincingly present examples of transmission event such as *A. muciniphila* in Figure 2D and validate StrainPhlAn approach by comparing CRISPR sequences. However, the extent of *A. muciniphila* transmission is unclear. Is it transmitted in all 3 nurseries? Does the phylogeny represent transmission events in different nurseries? It is not clear why this species was chosen apart from to validate the technical feasibility of detecting transmission events.

Rev #1 is right in suggesting the need for a contextualization of this analysis. While the tree represents all the strains of *Akkermansia muciniphila* SGB9226 traced in our study, including several transmission events occurring in different nurseries, in the panel on the right we focus on a particular nursery group where one specific strain of *A. muciniphila* transmission was more extensive and where we managed to strengthen our strain tracking approach by specific CRISPR spacers identification. To make this clear and immediate, we modified old **Figure 2D** (now **Figure 2C**, below) adding a coloured frame in the tree that highlights the strain of *A. muciniphila* tracked across some volunteers (group 1, nursery B) in the right side of the figure, and edited the figure caption accordingly:

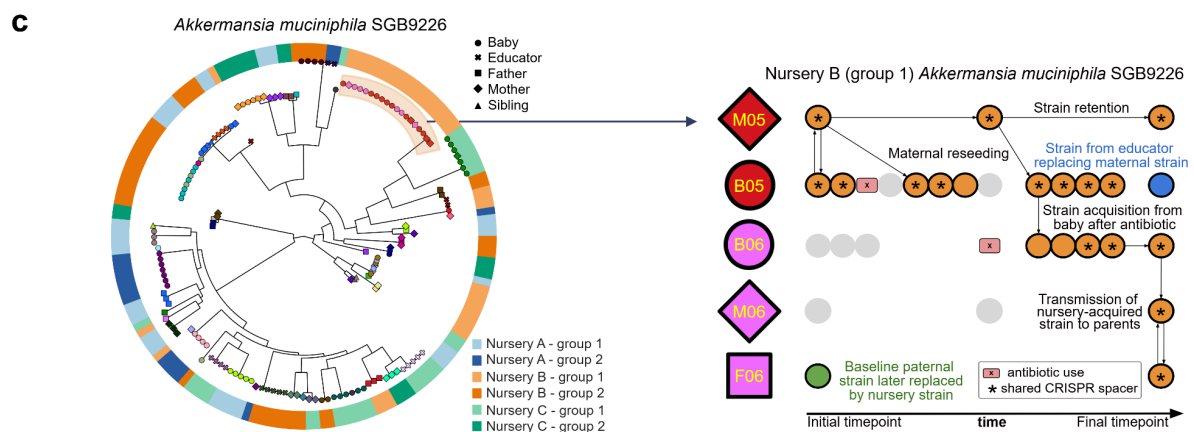


Figure 2C. Strain-level profiling for *Akkermansia muciniphila* SGB9226 (left) uncovers the chain of transmission events of one strain of this species in group 1 of nursery B (right). Volunteer types are identified by shape (mother, diamond; baby, circle; father, square) containing participant identifiers, composed of the first letter indicating volunteer type (M, mother; B, baby; F, father) and the family number; familiar relations are also highlighted by same-color filling. On the right, each circle represents a sample collected from the participants depicted, with color filling indicating the identity of the strain of *A. muciniphila* SGB9226 detected in the sample (except gray, used to indicate the SGB was not detected/typable at the strain level) and arrows indicating the most likely transmission event. The identification of shared CRISPR spacers of the target strain of *A. muciniphila* SGB9226 (orange circles) across different samples is indicated by an asterisk.

We implemented the same edits in a similar analysis, presented in **Extended Data Fig. 12**.

5. Line 200- typo , replace with 'in contrast'

We replaced "in opposition" with "As expected".

6. Line 219- what does 'retained' mean in this context?

In this context, as generally throughout the manuscript, we define as "retained" a taxa (either a strain or SGB) that is detected across a pair of longitudinal samples of the same individual. When discussing strain replacement rate, as this can be calculated only for SGBs typed at strain level across a pair of longitudinal samples, we specified the definition of retained SGBs as "fraction of SGBs with strains typed in initial and final timepoints for the same individual".

7. Line 428- please re-word. This sentence can be interpreted as saying nursery settings are most important factor in early-life microbiome assembly and not mother.

We agree with the Rev #1' suggestion and rephrased the sentence to avoid misinterpretation, as follows:

"Our study uncovered that after a few months of nursery attendance, the microbial strains acquired from peers in the same nursery group accounted for a larger proportion of the infant microbiome than those from the mother and – more generally – family members (**Fig. 4C**), who are known to exert the greatest influence on infants' microbiome in the first months of life".

8. Line-442- To justify this statement, the authors would need to demonstrate some health benefit accrued from transmission and colonisation of most transmissible species. There is limited data on many of the species reported as highly transmissible in Figure 3d and Figure 5 in comparison to many early-life Bifidobacteria species in terms of reported health benefits. Also, its hard to reconcile this statement with the reported transmission of pathogens such as Clostridiodes difficile.

We agree with Rev #1 for the reasons previously detailed **R#1 C#2** and rephrased the statement mentioned that now reads:

"Our data indeed suggest that social and physical interaction between infants should be regarded as a key contributor to microbiome maturation during infancy comparably to previously outlined perinatal factors that influence the earliest stages of life, such as delivery mode (natural versus C-section) and feeding practices (breastfeeding versus formula feeding)".

While the analyses mentioned by the Rev #1 in the original version of the manuscript were focusing on transmissibility in our cohort (both in adult and infant volunteers, and both in family and nursery settings), our newly added findings (described in detail in the response to the Editor, point #5, and integrated in **Figure 5, Supplementary Table 10 and Extended Data Fig. 24**) put the attention specifically on the baby-baby transmission route and indicate

that the species that are differentially more transmitted between babies, in comparison to baby-mother and baby-father, include key health-promoting species. In addition, while pathogenic spread in the nursery is well-established and we agree it is not to be overlooked, the result showing that *Clostridioides difficile* is exclusively transmitted baby-to-baby (**ST10**), is in line with the state of the art and is not per se indicative of *C. difficile* long-term colonisation, nor linked to detrimental effects on infant health. Indeed, *C. difficile* carriage is reportedly widespread in asymptomatic infants, with prevalence peaking at ~40% in the perinatal period, progressively decreasing to ~3% by age 3, matching adult rates^{14–18}. Moreover, while *C. difficile* causes colonic inflammation in adults, infants show resistance to *C. difficile* toxins with rare clinical infections^{19,20}.

All in all, we think these results, alongside the observed increase in microbiome richness associated with nursery attendance, suggest that the microbial transmission occurring in nurseries may potentially contribute positively to infant microbiome development. However, further research is needed to fully establish the long-term health implications of these transmission patterns.

Response to the specific comments and request of Reviewer #2

Ricci et al describe the changes that occur in the infant gut microbiome following daycare admission, comparing three nurseries in Italy, collecting longitudinal samples from the infants, their parents, and also nursery staff. The cohort is very unique and has great value, and it is also sequenced at extreme depth, allowing for many strain-analysis methods to be applied. The main message is that quickly after entering the nursery, multiple strains are transferred across infants, and sometimes from the staff as well, highly impacting the infant gut microbiome.

The field of the infant gut microbiome has grown rapidly in the past decade, with many cohorts and manuscripts describing early life events and how they shape the infant gut composition. One missing aspect has long been the entrance to childcare, especially when comparing the microbiome pre- and post- childcare admission. This manuscript bridges this gap beautifully, and will have a great interest in the field. Having said that, there are some important points the authors should take into consideration before this manuscript should be published.

Major comments:

R#2 C#1

- There is something wrong with the flow of the manuscript, it reads as if we go back and forth between some topics, and makes it challenging to follow the story. For example, here are the topics of the various sections:

- 1- A metagenomic monitoring of the dynamic nursery community microbiomes
- 2- An intricate network of inter-individual strain-sharing and longitudinal strain retention
- 3- Strain-sharing between babies and pets is detectable but not extensive
- 4- Tracking intricate strain transmission between babies, families, and educators
- 5- Infant microbiome is built on infant-to-infant transmission in the nursery
- 6- Strains spreading in the nursery
- 7- Strains from the nursery contribute more than those from family to the infant microbiome
- 8- Strain acquisition and donation rates of babies in nursery is variable and linked with transmission from siblings
- 9- The most transmissible species are environmentally persistent or prevalent gut anaerobes
- 10- Antibiotic treatment is associated with reduced strain retention and, in infants, with enhanced strain influx

We thank Rev #2 for suggesting formal improvements to the overall readability, we followed this suggestion and re-organized the manuscript, while maintaining its comprehensive scope regarding transmission, by: i) heavily reordering the original subsections to improve the flow; ii) splitting existing large subsections in shorter ones to streamline the sections' main messages (new "Relations in nursery shape microbiomes" and "Siblings affect baby strain acquisition", below) and adding two subsections to integrate newly performed analyses (e.g. "Long-term effect on transmission" and "Host factors and microbiome transmission"); iii) removing the previously independent subsection dedicated to pets (integrating its key

message in the subsection “Tracking multi-host strain transmission”). We note that in this phase we also edited subheadings titles to meet Nature formatting guidelines (40 character limit) and recapitulate the subsection's updated content; while subsections' content are well defined, subheadings shortening contributed to making them less descriptive of subsections' content, specifically in the case of the first two subheadings. Following we report the updated structure of the manuscript:

- Microbiome transmission in nurseries
- Baby microbiome development in nurseries
- Mapping intricate nursery strain-sharing
- Tracking multi-host strain transmission
- Strains spreading in the nursery
- Relations in nursery shape microbiomes
- Baby microbiomes built via transmission
- Nursery strains impact as family ones
- Long-term effect on transmission
- Siblings affect baby strain acquisition
- The most transmissible species
- Host factors and microbiome transmission
- Antibiotics may alter strain acquisition

R#2 C#2

First, there are way too many sections here, the whole manuscript can be more concise. Second, sections 1, 2, 4, 5, 6, 7, 8 all discuss strains sharing and compare the various groups of: within a child, across children, within a family, etc. It is very very confusing. The main message is washed out.

Another example is in Figure 2, where panel D jumps forward to one example, but most of the text to follow is general. I would add examples after the description of the general phenomenon.

We hope we refined the manuscript's overall structure, aiming to address Rev #2's suggestions for simplification. As outlined in our previous response, these modifications included reformatting the main text into simpler and more focused subsections to highlight our main findings more effectively, although also resulting in an increased total number of subsections. Regarding **Figure 2D** (now **Fig. 2A**), we were probably not clear about that; this is not a way to anticipate much real results, but rather to introduce the reader to the resolution of our methodological approach and effectiveness in tracking microbial interpersonal transmission before delving into the more descriptive part of the study. We do think that for the broad readership of Nature, it is not straightforward to grasp that microbiome members can be tracked at such resolution and precision that is comparable only to what has been done for cultured pathogens or well characterized pathogens with targeted PCR protocols. Nonetheless, to accommodate the suggestion of Rev #2, we tried to fit **Figure 2D** elsewhere but found this placement most effective for introducing readers to our approach before presenting the broader and longitudinal trends of interpersonal transmission. Moving this illustrative example would interrupt the logical flow from methodology demonstration to general findings, potentially making the manuscript less coherent for readers new to strain-level microbiome analysis. Therefore, we edited the main text to clarify the significance of Fig. 2D:

“In another example highlighting the resolution of our strain-resolved approach, we tracked the interpersonal transfer of a nursery-acquired strain of *Akkermansia muciniphila* (specifically SGB9226, one of the 4 SGBs labeled as *A. muciniphila* and the one to which the type strain of the species belongs) in group 1 of nursery B”.

R#2 C#3

- During this age of 4-15 mo, many factors are impacting the infant gut microbiome, inducing diet changes and weaning. Childcare classes have a range of ages in each class, and it is unclear from the text whether the samples collected at the same time are from children from the same age. Namely, what is the age distribution of each of the time points? Even if the authors can't do much about this difference now, they should add this point to the discussion.

We agree with Rev #2 that age is a key factor when investigating infant microbiome development, and we addressed this feedback on multiple levels. Age, and more specifically, time, were carefully taken into consideration in the study design. In detail, we recruited 43 babies attending their first year of nursery, aged 4-15 months at admission (T01) that represents the typical age breadth in first-year nursery groups. This age breadth was maintained (by definition) throughout the study length (from before nursery start-T01, throughout the first term, with weekly sample collection from babies, to after 2 weeks after the term concluded-T15, with follow-up sampling at the end of the nursery year-TA and summer-TB, with the latter two timepoints incorporated as part of this revision), for a total study timeframe of 12 months. We refined **Figure 1A** to better illustrate the study timeline and the intervals between timepoints. We also specified participants' age at nursery entry in the **Methods** and in the **Introduction**.

In addition to these formal edits, to address Rev #2' comment, we incorporated an additional set of analyses investigating the links between age, infant microbiome composition, and strain transmission. These were collectively described in detail in the response #3 to the Editor, at the beginning of this document.

In summary, first, we observed a correlation between age and infant microbiome diversity at both T01 and T15, as shown in the new **Extended Data Figure 5**. Recognizing this significant effect, we adjusted our analyses in Figure 1C for age. Notably, the positive association between having a sibling and SGB richness remained significant even after accounting for age, further supporting previous observations (new **Supplementary Table 6**). Our newly added analysis in **Extended Data Figure 8** revealed that younger infants' microbiomes undergo more extensive compositional changes during nursery attendance than those of older infants.

Furthermore, we explored how age influences strain acquisition and donation in nursery-attending babies. We found that older infants had significantly higher rates of strain donation compared to their younger peers, whereas strain acquisition frequencies showed no significant correlation with age (new **Extended Data Figure 25**).

This new set of evidence shows that while age predictably correlates with infant microbiome diversity and with the extent of infant longitudinal compositional changes (both expected), our key findings remain robust independently of age (e.g. the sibling effect on microbiome richness persists after age adjustment, infants act as more frequent strain donors while acquisition patterns remain age-independent across all infants). Overall, this suggests that the nursery environment hosts microbial transmission patterns that are influenced but are not determined by age.

R#2 C#4

- One of the more important distinctions in this work is between strain acquisition and strain replacement. The text describes mostly replacement, yet the figures support that the family contribution remains stable, while the nursery contribution to strain sharing grows over time (Figure 4). I think the text needs to be more subtle in making statements such as “Strains from the nursery contribute more than those from family to the infant microbiome” (header at line 269). It could very well be that these early sharing of strain has more impact than the later (larger) number of strain shared.

While the header was meant to point to a higher quantitative contribution (rather than a general impact) of the nursery strains to the infant microbiome at the end of the first term (i.e. nursery strains accounted for a larger proportion of the infant microbiome, compared to family strains, now referred to as “proportion of strains acquired” as shown in new **Figure 4C**), we acknowledge the wording was misleading and we thank Rev #2 for pointing this out. We addressed this by rephrasing the specific header, also considering the newly incorporated evidence indicating that the proportions of strains acquired from family and nursery peers become comparable at the end of the school year (**Extended Data Fig. 19**) as follows:

“Nursery strains impact as family ones”.

In light of other similar comments such as **R#1 C#2** and **minor comment #4** raised by **Rev #2**, we applied additional minor changes throughout the manuscript to avoid overstating our findings and also to better identify the results shown in **Figure 4C** and their significance:

- “The proportion of strains acquired from family members fluctuated from an average of 24.0% per infant at T01 to 20.0% at the end of the first term of nursery (Wilcoxon signed-rank test, $n = 25$, $T = 112$, $P = 0.18$; **Extended Data Fig. 17A**), while those shared with/acquired from the nursery group increased from an average of 6.5% to 28.4% at the end of the first term (Wilcoxon signed-rank test, $n = 25$, $T = 0$, $P = 6.0e-8$; **Extended Data Fig. 17A**), significantly surpassing the proportion of strains acquired from the family (Mann-Whitney U test, $n = 52$, $U = 463$, $P = 0.023$; **Fig. 4C**).”
- “A similar trend was observed in terms of cumulative relative abundance of strains acquired either from the family or the nursery group (**Fig. 4C**). Family contribution slightly diminished over time (from an avg. 33.2% at T01 to 20.6% at T15; Wilcoxon signed-rank test, $n = 25$, $T = 72$, $P = 0.014$; **Extended Data Fig. 17B**) and in contrast, the cumulative relative abundance of strains acquired from the nursery

group expanded during nursery attendance (reaching an avg. of 39.6% at T15 from a starting 10.2%; Wilcoxon signed-rank test, $n = 25$, $T = 18$, $P = 1.5e-5$; **Extended Data Fig. 17B**), suggesting that nursery collectively contributes to a larger extent to infant strain composition than the family by the end of the first term”

- Finally, in the conclusions: “Our study uncovered that after a few months of nursery attendance, the microbial strains acquired from peers in the same nursery group accounted for a larger proportion of the infant microbiome than those from the mother and – more generally – family members (**Fig. 4C**), who are known to exert the greatest influence on infants’ microbiome in the first months of life”.

R#2 C#5

- The color choices in the figures are extremely confusing. Overall, the same colors represent different things across figures, and even in different panels of the same figure. In addition, the same feature is represented in different colors across figures (like nurseries, and groups within nurseries). I suggest the authors pick consistent colors across the entire manuscript: three main colors for the nurseries, with a shade for the groups, that will be used only for this purpose.

We thank Rev #2 for this suggestion. We edited all figures applying a specific colorblind safe colour scheme to improve visualization and clarity of results:

- 3 colours to identify nursery A, B, and C (blue, orange, and green, respectively, as previously done in the tree of Figure 2D, now **Fig. 2A**) with shading for groups, as suggested;
- family (turquoise) vs nursery (magenta, with darker shading for same group) (i.e. new **Figures 3A, 3D and 3E, and 4C**)
- a specific colour code to identify participant types (e.g. family members and educators), was already exclusively used throughout the manuscript and matching across figures in the old version of the manuscript (e.g. old Fig. 1B, 3A, 4A, 4B); in this version we decided to simplify this in boxplots where colour schemes were not necessary (i.e. new **Fig. 3C**) but maintained it where distinction was necessary to identify volunteer types (**Fig. 1B**).
- dark grey (446578ff) vs light grey (cad8d8ff) were used for comparisons throughout the manuscript where specific colours were not needed to identify participant types or other categories (e.g. Y/N or before/after) (i.e. **Figures 1C and 1D, Fig. 6**); similarly, we used lighter green (a8bb37ff) as a colour filling without specification, when needed for visualization (e.g. **Figures 3C, 4F and Ext. Data Fig. 7**).

R#2 C#6

- It is accepted that there are many strains per species in each person, yet most computational tools assume a single dominant strain to enable these comparisons. The authors should at least acknowledge that there are likely multiple strains per species, and how it impacts their calculations (for example, do they expect to have chimeras in their strain profiling?)

We acknowledge the importance of reporting this methodological limitation and discussed it in the conclusion:

“Our strain-sharing pipeline models the genetics of single dominant strains present within species (SGBs) in individual gut ecosystems in any given sample ⁸⁵ (as per recent surveys ²⁵) to enable identification of strain transmission events; further advances in metagenomic strain-profiling tools could reveal the complexity of multiple coexisting conspecific strains and shed light on their role in influencing strain(s) transmission dynamics and long-term colonization in the gut microbiome.”

Nonetheless, considerable evidence supports single-strain predominance within single microbial species in the human gut microbiome. In particular, a recent study integrating bacterial culturing and whole-genome sequencing of isolates, coupled with metagenomic sequencing and strain tracking, demonstrated that most gut bacterial species exist as only 1-3 strains per person, with average strain richness per species of 1.2 ²¹. This low strain diversity appears unique to the human gut ecosystem compared to environmental microbiomes (like soil or lakes, considered in the same study), which is possibly explained by the unique features of the gut, such as its semi-closed ecosystem and fast microbial growth rates, leading to increased inter- and intra-taxa competition. The same study showed that single-strain predominance of the human gut is stable, since even when strain richness is artificially elevated through pooled fecal microbiota transplantation (FMT), strain richness was temporarily increased but eventually returned to baseline levels ²¹.

Moreover, although analyses focusing on dominant strains may not capture the full spectrum of coexisting strains per species within samples, we think this approach represents a necessary methodological compromise. Indeed, as argued elsewhere ²²⁻²⁴, current challenges in profiling non-dominant strains include strain-specific sequencing inaccuracies, due to insufficient metagenomic sequencing depth, and the inherent log-normal abundance distribution, as well as within-species strain dominance patterns. These factors collectively render non-dominant allele frequencies either indistinguishable from background sequencing noise or only sporadically detectable at reliable thresholds ^{22,23}.

All in all, in light of this evidence and the ecological constraints operating within the human gut microbiome, it is reasonable to think that single-strain predominance assumption in metagenomic studies may represent an acceptable proxy.

R#2 C#7

- Statistical analysis -

The Mann-Whitney and Kruskal-wallis tests assumes the samples are independent between the two groups, and here the authors use it in cases when they are not independent:

*Comparing two samples of the same individual in different time points (Figure 1D boxplots, supp Figure 9, maybe more)

We thank the Rev #2 for this comment, to which we agree. We now have opted to use the Wilcoxon signed-rank test in all analyses involving longitudinal comparisons with matched

individuals, which we now also described in the **Methods**: “Longitudinal comparisons between timepoints were performed using the Wilcoxon signed-rank test”. Following, the figures reporting the updated analyses using the Wilcoxon signed-rank test:

- **Figure 1D**

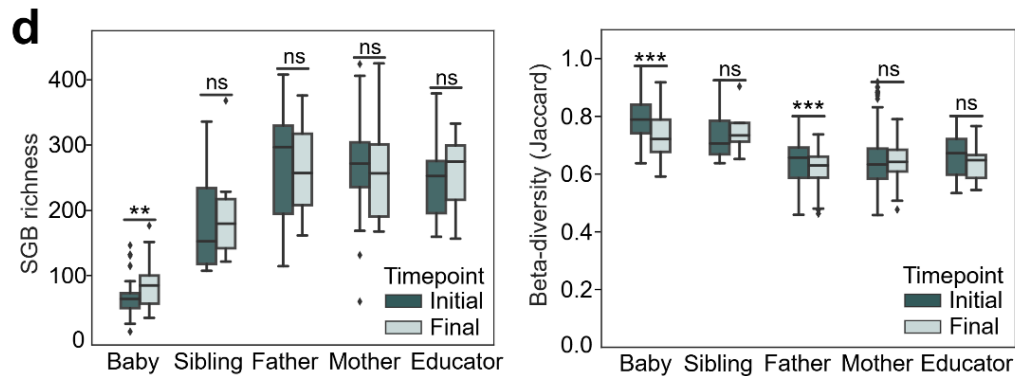
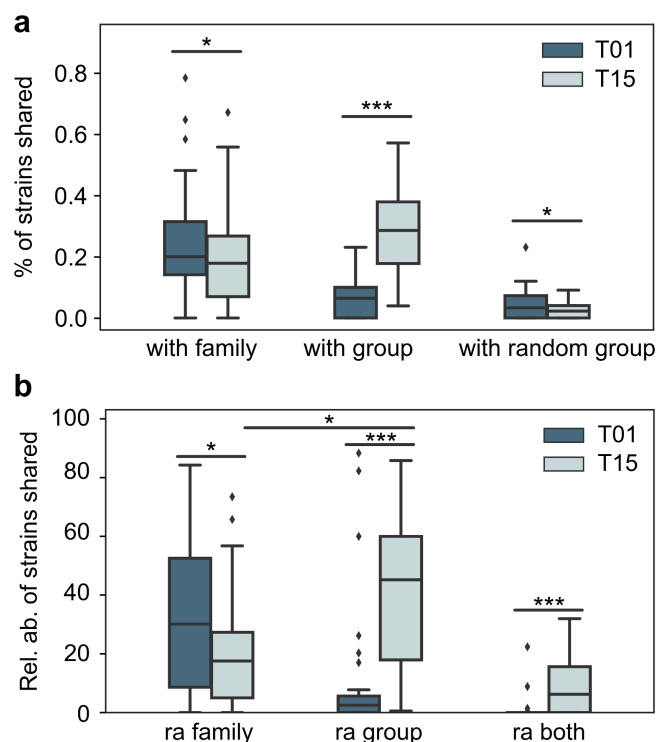


Figure 1D. Change in alpha (SGB richness) and beta diversity (Jaccard) across participant types between beginning and the end of the first term. Beta diversity refers to the all-vs-all within-nursery dissimilarities. In box plots, box edges show the lower and upper quartiles, the center line indicates the median, and whiskers expand the interquartile range (IQR). Statistical significance asterisks refer to Wilcoxon signed-rank tests, with $P \geq 0.05$, ns; $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***.

- **Extended Data Fig. 17:**



Extended Data Figure 17. (A) Percentage of strains shared between babies and either their family, nursery group and a random different group from another nursery (as a control) at T01 and T15. **(B)** Relative abundance (ra) of strains shared with either family, nursery group and both, at T01 and T15. In box plots, box edges indicate the lower and upper quartiles, the center line represents the median, and whiskers expand the IQR. Statistical significance asterisks refer to Mann-Whitney U tests, with $P < 0.05$, *; $P < 0.001$, ***.

R#2 C#8

* There are data points of the form $f(x,y)$ in one group and $f(x,z)$ in the other (f is some strain sharing calculation for example), which makes the data points not independent (Figure 2B, 4A, 4B, Supplemental Fig. 5, maybe more - comparing strain sharing within the families to strain sharing between families).

We agree with the concern raised by the reviewer. In the revised version of the manuscript, for all comparisons between groups where there are observations computed in relation to the same microbiome sample in both groups (making them not independent) we apply a permutation-based approach performed as follows:

1. Compute the difference in the median observed between groups in the original data ($D_{original}$)
2. Shuffle the labels between groups maintaining the same original sample sizes
3. Compute the difference in the median observed between groups with shuffled labels from the previous step ($D_{shuffled}$)
4. Repeat steps (2) and (3) a total of 1,000 times, storing $D_{shuffled_i}$ in each case ($D_{shuffled_1} \dots D_{shuffled_{1000}}$)
5. Compute the proportion of times that the difference in the median observed between groups with shuffled labels was equal or more extreme than the one observed in the original data (i.e. number of cases in which $D_{shuffled_i} \geq D_{original}$ for i in 1...1000 divided by the total number of permutations), which is then taken as the nominal P -value.

We refer to this statistical test as “permutation test for medians”, as now described in **Methods**:

“Cross-sectional dependent observations were compared with a permutation test for medians (or means, when comparing the number of shared strains), with P -value calculated as the proportion of times (out of a 1,000 permutations) that the observed difference in the median between groups with shuffled labels is equal or more extreme than the observed in the correctly labeled data.”

We applied this test in the data reported in **Figures 3D, 3E, 4A, 4B** and **Extended Data Fig. 11**. The previously reported conclusions still hold, although with expectedly higher p -values considering the lowest non-zero P -value that can be achieved is 0.001 with 1,000 permutations (and, given our sample size, having more permutations would frequently involve repeated comparisons). Still, often the P -value was nominally zero, cases in which we indicated it as P -value < 0.001. Overall, we believe that reaching similar conclusions with independent statistical approaches is a further testament to the soundness of our results.

Minor comments:

1. The title should include the word childcare or nursery

We thank Rev #2 for the valuable feedback regarding the title. Our original title excluded it because it focused on the core of the study, which is the modeling of interpersonal microbial transmission in early life, with the nursery as an ideal setting. Nonetheless, while we agree that emphasizing the nursery context is important, unfortunately, the journal's character limit for titles makes it challenging to incorporate "in nursery" while maintaining conciseness and content. Hence, we would propose to keep the original title:

"Baby-to-baby strain transmission shapes the developing gut microbiome".

However, if the Reviewers and Editor prefer its inclusion, we are flexible in accommodating this suggestion by proposing:

"Strain transmission in nurseries shapes the baby microbiome".

This option adheres to the character limit and highlights the nursery's context of the study.

2. The introduction is lacking more information of what is known and moves directly to the hypothesis of the paper. Is anything known about infants in daycare vs. those that aren't? At least mention the NEJM paper describing decrease in Asthma and wheezing in kids who attended daycare (DOI: 10.1056/NEJM200008243430803). Additional comparison to known strain transability to infants from family, pets etc. will also be useful.

The main goal of this study was to quantify interpersonal transmission in early life, utilizing the nursery as an ideal setting for the study, as babies first admitted to this are unrelated and can be assumed to be generally naive with regards to social contacts outside of the household or larger family. With this in mind, we tailored our introduction to showcase progress in studying interpersonal microbial transmission at the strain level. As suggested we integrated the introduction to the study with examples of evidence on the general long-term effects of nursery attendance on children's health (e.g. reduced risk of respiratory infections, and of developing allergy and asthma), as reported below. While these are numerous, limited studies are available investigating the effects of childcare on the gut microbiome (with the exception of Amir et al. 2022²⁵ already referenced in the discussion and that was newly included in the introduction, and Thompson et al. 2015²⁶, demonstrating increased richness associated with nursery attendance). Even more so, to our knowledge, there is a substantial gap in studies looking at interpersonal transmission in the nursery setting. State of the art on strain-level inference of microbiome transmission from family to infants was already included at the beginning of the introduction. Hence, we integrated the introduction in this manner:

"The role of nurseries as potential hotspots for pathogen spreading^{9,10} and corresponding long-term improved immune competence and allergy desensitization¹¹⁻¹³ is well established. On the other hand, nursery attendance was associated with increased species-level diversity of the infant gut microbiome¹⁴."

3. line 97: what is "baseline alpha diversity"? At time point T0?

We uniformed the use of “T01” throughout the text to indicate the sample collected before nursery start to avoid confusion (previously identified as “baseline” or “initial”). Similarly, we also modified **Figures 2A and 2B** (now **3A and 3B**) to convey that we are comparing specifically “T01” and “T15”, instead of “initial” and “final” timepoints, as previously indicated. The use of “initial” and “final” wording was maintained in **Figure 1D** to indicate the timepoints collected before and after the first term, across different participant types, where numbering of timepoints were non-matching.

4. There are multiple terms that are used interchangeably, like % strains shared and rate of strain sharing (for example, throughout Fig 3 & 4). It is confusing to read, and would be better if simplified when possible. Is strain replacement defined as (1-strain sharing rate)?

We thank the Reviewer for this constructive comment. While “% strains shared” and “rate of strain sharing” identify two different metrics that highlight different aspects of transmission dynamics, we acknowledge that terms utilized were confusing. As described in the **Methods**, strain-sharing rate summarizes the extent of the strain-level overlap between two microbiome samples, while the percentage of strains shared refers to the percentage of strains of a given microbiome that was exclusively shared with, and hence, putatively acquired from, a specific source (by incorporating comparison between one microbiome sample of focus and multiple other samples). Notice that only the % of strains shared enables comparison of the cumulative effects or contributions of strain-sharing to baby microbiome build-up from different sources (in our case, family or nursery). We in any case agree with the reviewer, and to enhance clarity, have replaced “% strains shared” with the more direct wording “proportion of strains acquired” throughout the text and figures. We want to emphasize, however, that this term continues to refer to strains putatively acquired from a given source, acknowledging the inferential nature of the quantification. We further explained its definition in the **Methods** as follows:

“We computed, for each baby, the proportion of strains in the infant microbiome that were exclusively shared with, and hence putatively acquired from, either family members or other babies in the nursery group (**Methods**) and we refer to it as “proportion of strains acquired”. ”

The definition of strain replacement (1-strain sharing rate) was previously found only in the figure caption; to improve clarity, we added it to the main text: “defined as one minus the SSR”.

5. Why were SGB coming from food discarded? Appears in line 609. This could have been an interesting point.

While we agree with Rev #2 that microbial transmission from food sources to humans is of great interest, particularly for its health implications, the goal of this study was to identify strain transfer events occurring specifically person-to-person, and to quantify them in the nursery setting. Even though interpersonal transmission of strains from food-associated SGBs (i.e. those also found in foods) may occur and be substantial, in some cases it is not possible to disentangle this from dietary co-acquisition; therefore, we excluded strains closely related to food-associated strains from our study to avoid misinterpretation of results

(Methods L661). Over the 23 food-associated SGBs StrainPhlAn 4 phylogenies with sufficient alignment length (≥ 1000 nt) evaluated in our dataset, we discarded strains from 13 due to being too close (ANI on marker genes $> 99.85\%$) to the food strains from Carlino et al., 2024²⁷. In only 7 cases the discarded strains composed more than 20% of the phylogenetic tree, such that we discarded the SGB altogether from the strain-sharing analysis. This included mostly relatively minor SGBs, specifically:

SGB discarded	Prev. in adults (%)	Prev. in babies (%)
<i>Lactococcus lactis</i> SGB7985	24.7	21.6
<i>Lacticaseibacillus paracasei</i> SGB7142	5.5	24.3
<i>Lacticaseibacillus rhamnosus</i> SGB7144	0	40.5
<i>Lactococcus cremoris</i> SGB7984	4.1	5.4
<i>Lactobacillus delbrueckii</i> SGB7020	13.7	18.9
<i>Hafnia paralvei</i> SGB10207	15.1	2.7
<i>Latilactobacillus curvatus</i> SGB7249	2.7	2.7

In contrast, the most important infant SGBs that were also food-associated (e.g., *Bifidobacterium longum* SGB17248, *Escherichia coli* SGB10068, and *Bifidobacterium breve* SGB17247, all with $>90\%$ prevalence in babies), despite in some cases having strains discarded, were still kept in the analysis. This means that strain-sharing inference was done for the large majority and for the most important food-associated SGBs present in our dataset, with the decision of discarding food-related strains having likely a minor and overall positive impact in our methodology. This filtering mirrors the methodology previously applied in Valles-Colomer et al, 2023, where, for example, most strains of *Bifidobacterium animalis* (Fig. 1d of the article) were excluded from the analysis because they were associated with dairy products (Extended Data Fig. 4a), while only a subset of strains of other spp (e.g. *Streptococcus thermophilus*) were associated with fermented food intake and hence filtered out (last section of “Profiling microbiome transmission” and in Methods, “Detection of strain-sharing events”). Finally, we note that we have preliminarily investigated microbial transmission from dietary sources in other work²⁷ and will continue to do so as part of the DOMINO consortium (<https://www.domino-euproject.eu/>).

6. The paragraph starting in line 618 was completely unclear to me. What does “Among the possible pairs we chose the one to maximize the lowest estimated coverage among the two samples” mean?

We agree the paragraph was unclear. We rewrote it as follows:

"Among the possible pairs, we chose the one maximizing the chances to type the strains of the SGB of interest in both samples, and we did so by choosing the pair

for which the sample with the lowest coverage for the SGB between the two samples was the highest among all pairs."

7. Discussion: There is no evidence in the paper that higher strain sharing rate is good for infants' health, however it is mentioned in the discussion (line 443).

We acknowledge Rev #2's valid concern that our initial conclusions may have overstated the health implications of strain transmission in the nursery environment, as they were based on broad assumptions about benefits of gut microbiome enrichment and maturation via strain transmission. However, our refined analyses (performed for this revision and detailed in the response to the **Editor**, point #5 **3**) demonstrate the circulation of health-associated microbiome species, such as *Bifidobacterium longum* subsp. *infantis* and *B. breve* (**Figure 5**), with notably higher frequency among infants than between parent-baby pairs. These new findings suggest that peer-to-peer strain sharing in the nursery may indeed confer beneficial effects on infant microbiome development and, by extension, infant health outcomes. In any case, we rephrased the sentence to avoid overstating the impact of our results as follows:

"In addition to their already established effects in emotional and cognitive development ^{66,67}, social relations among peers in the nursery are thus a hub for microbial enrichment during infancy, particularly of key early-life gut colonizers, such as *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium breve* ^{52,53}, with their known favorable effects on health including via immune development of the infant ^{68,69}. Our data indeed suggest that social and physical interaction between infants should be regarded as a key contributor to microbiome maturation during infancy, comparably to previously outlined perinatal factors that influence the earliest stages of life, such as delivery mode (natural versus C-section) and feeding practices (breastfeeding versus formula feeding) ⁶⁹⁻⁷²."

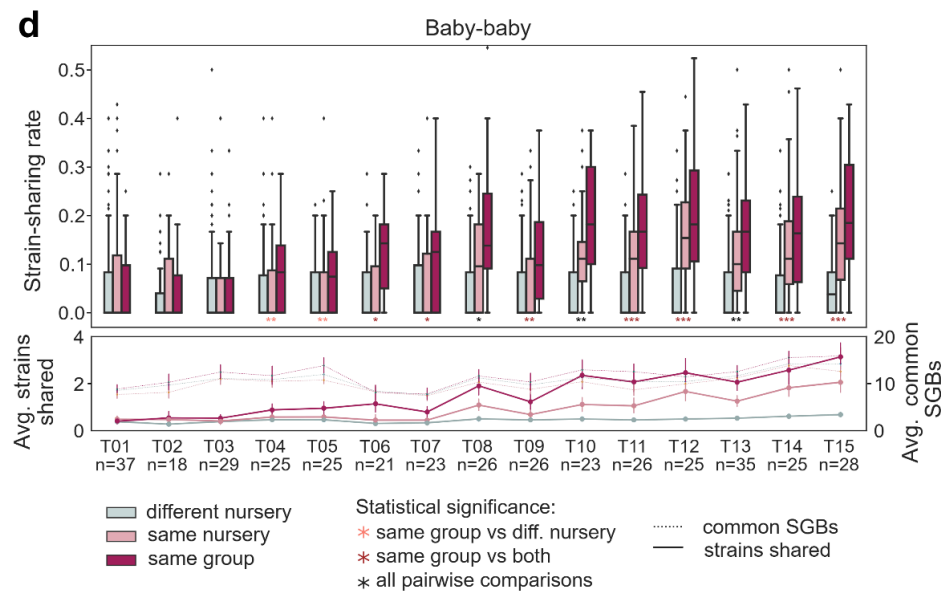
Figure comments:

1. Many many tiny and complex figures with multiple (sometimes confusing) variables compared that make it difficult to follow and understand. It would be better to cut some and leave the highlights clear and well explained. The font is extremely small, and can't be read on the printed version.

We thank Rev #2 for suggesting formal improvements that ameliorated visualization of our findings. We modified our figures to improve clarity, by applying multiple edits:

- we used clearer color schemes (as suggested by Rev #1) and bigger font sizes,

- we displayed both strain-sharing rate, average number of strains and species shared, when relevant (such as in **Figure 3D**, below, and **Fig. 3E**):

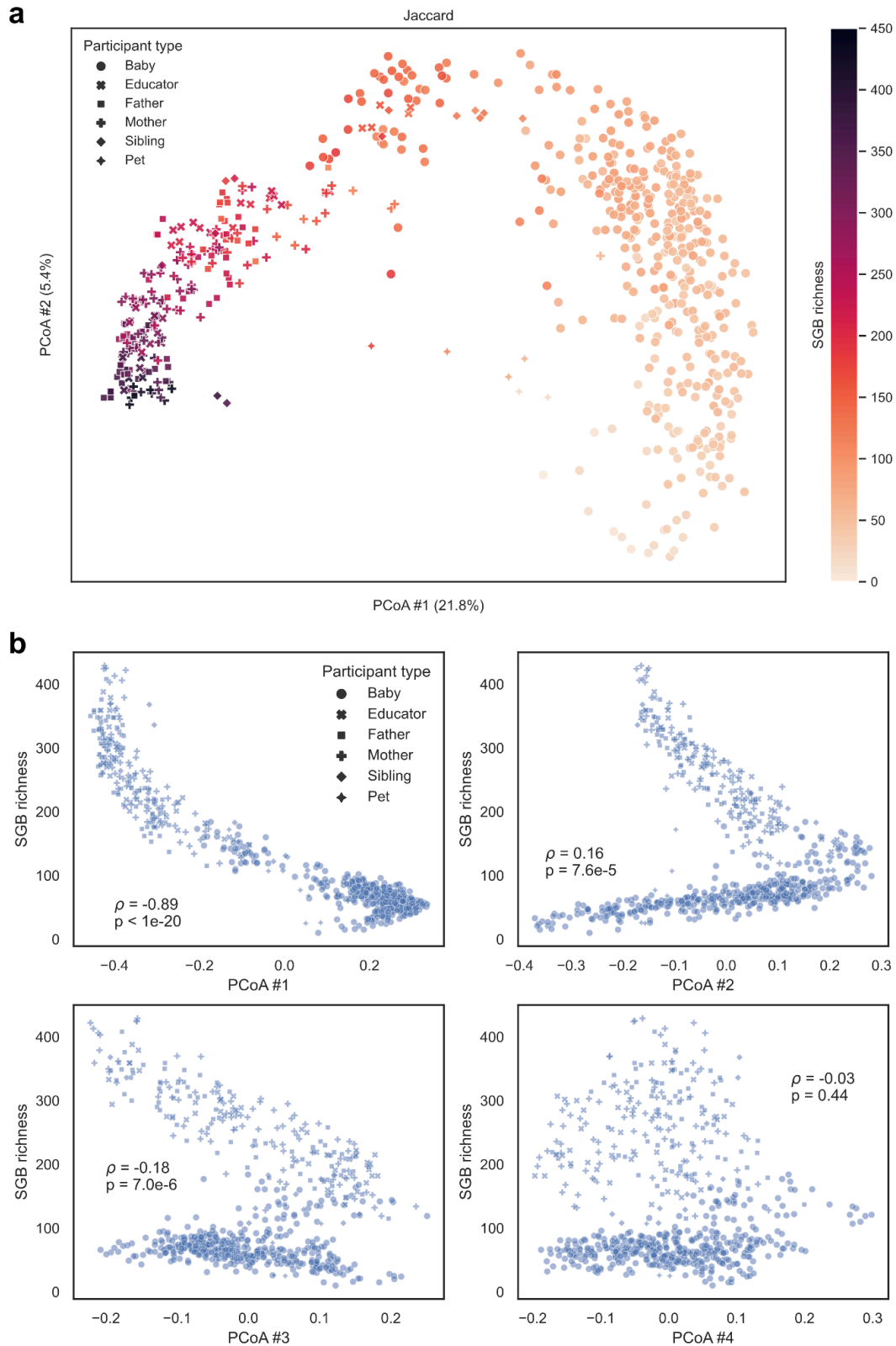


- we moved some of our analyses (as previously detailed in **R#2 C#2**) to supplementary material to simplify the overall structure of the manuscript.

2. Figure 1B - what dominates the PCoA plot? Diversity? The authors do not explain the rationale of using Jaccard as a distance measure, I believe it is to capture the qualitative similarities rather than most quantitative ones, but the rationale should be mentioned in the text.

Rev #2 is correct, the ordination of **Figure 1B** is driven by diversity (SGB richness), with the first three components significantly correlated with it (**Extended Data Figure 4**, below). Since the work focuses on microbial transmission, we used Jaccard to underline longitudinal changes in similarity across samples, in terms of presence/absence of taxonomic units possibly via interpersonal transmission (e.g. new SGB acquisition). We mentioned this in the main text when referring to **Figure 1B**, namely:

“In our data we observed the expected microbiome structures ²⁻⁴, with large compositional divergence between adults and babies (**Fig. 1B**, **Extended Data Fig. 2-4**, **Supplementary Table 5**), and age-dependent differences in babies (**Extended Data Fig. 5**).”



Extended Data Figure 4. (A) Ordination overview of the species-level microbiome composition of the microTOUCH-baby cohort (principal coordinate analysis on Jaccard dissimilarity, $n = 646$), with samples coloured by SGB richness and shapes indicating participant types. **(B)** Association between SGB richness and first four PCoA ordination components. Statistical significance refers to the Spearman's test.

3. Figure 1C, is the average here for each person over all time points? So each dot is a person and the y-axis is average SGB richness across the three or more time points? It was unclear from the text. If so, I would also add the teachers and the siblings. Note, the teachers appear once as teachers and once as educators.

We edited the figure caption to explain the average was performed across all individuals in each participant type. Unfortunately, it was not possible to include siblings in the second panel of **Figure 1C**, as no families with siblings were reported to have pets in our cohort. In addition, we did not collect metadata regarding family composition (including pets) of educators, so we could not apply this suggestion either.

While we uniformed the participant type “teacher” to “educators” throughout the text and in **Figure 1C** and we thank Rev #2 for pointing this out.

4. Figure 1C: There are very few families that have siblings and even fewer that have pets, so this comparison could be biased. How many samples are in each group of the box plot?

We collected data regarding the household composition (i.e. number of family members, presence of siblings and/or pets) for all families enrolled (with the exception of family G16 for which this data could not be retrieved, **Supplementary Table 2 and 3**), which evidently excludes educators. In this plot we are utilizing this information (i.e. if siblings or pets are present) regardless of siblings/pets participation (i.e. recruitment) in the study. Hence the numbers used in the analysis are larger than the number of participating siblings and pets (i.e., the total number of siblings of enrolled babies exceeds the number of enrolled siblings). In any case, we agree with Rev # 2’s note and specified the number of samples per group in the figure caption for clarity:

“**Figure 1C.** Average SGB richness across all timepoints and for all individuals in each family member category having a sibling (babies, n=14; mothers, n=12; fathers, n=8) vs not having a sibling (babies, n=26; mothers, n=25; fathers, n=21) (left panel). Average SGB richness in all family members having a pet (babies, n=9; mothers, n=8; fathers, n=7) vs not having a pet (babies, n=31; mothers, n=29; fathers, n=22) (right panel), across all timepoints and individuals in each category.”

5. Figure 1C: The richness of babies with siblings is higher - are these babies age-matched?

We further analysed the relation between age and gut microbiome diversity in babies with or without siblings, and with or without pets. First we found that the age of babies with siblings is close to significantly ($p = 0.10$) higher than babies with no siblings, and the age of babies with pets is significantly lower than babies without pets ($p = 0.04$), well justifying taking the age of babies into account in the association between gut microbiome diversity and presence of siblings/pets in the household, as hinted by Rev #2. After correcting for age, we observed that the association between average diversity of baby gut microbiome and presence of siblings is still significant, while relation between decreased average diversity and having a pet is non-significant. We incorporated this evidence in **Supplementary Table 6**, and mentioned it in the main text as follows:

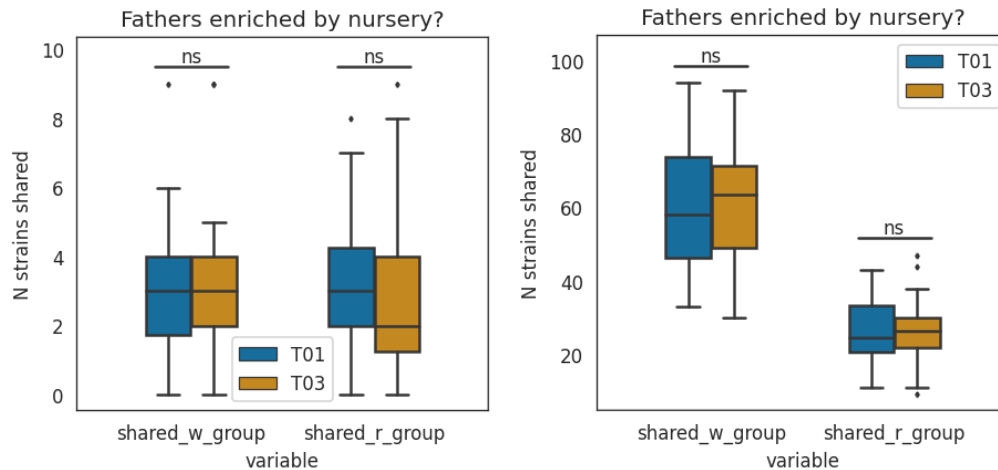
“Babies having a sibling had, for example, an overall higher SGB richness compared to babies without brothers and sisters (avg. SGB richness across all timepoints with vs without sibling, $n = 40$, $U = 271$, $P = 0.012$; **Fig. 1C**), with this association remaining significant after adjusting for age (**Supplementary Table 6**), further supporting previous observations^{21,22}”.

6. Figure 1C: Perhaps consider showing that while pets and siblings impact the baby's richness, also show that antibiotics and mode of delivery do not.

We thank Rev #2 for this observation. We discussed the option of including these results in **Figure 1C** but we reasoned that, in light of the limited space, it is preferable to leave them mentioned in the main text referring to **Extended Data Fig. 7**.

7. Figure 1D right, this is confusing. Is this the beta diversity across all pairwise comparisons within each group and time point? It wasn't clear from the text. Is the beta diversity calculated within individuals from the same nursery? Also, the difference in diversity in babies is as significant as that of the fathers, how can you explain this? I am not confident this analysis is helpful for the general flow of the manuscript.

Rev #2 is correct. In **Figure 1D** each box represents the all-vs-all Jaccard dissimilarities for a given participant type and timepoint (T01 or final), which is always calculated within individuals of the same nursery. We have clarified that by adding the following to **Figure 1D** caption: “Beta diversity refers to the all-vs-all within-nursery dissimilarities.” This means that a significant decrease in this metric is indicative of microbiomes becoming more similar to each other during the first term, potentially due to strain transmission in the nursery setting, which we set out to investigate in the following sections. We therefore disagree that this analysis is unhelpful for the general flow of the manuscript. Regarding the fact that not only babies show more similar microbiomes by the last timepoint, but also fathers, we do not have a simple answer for this. To investigate this trend, we checked whether fathers were enriched in nursery strains towards the end of the term (T03), compared to before nursery start (T01) but did not find a significant increase (figure below), and therefore excluded the hypothesis of indirect transmission routes of nursery strains as a driver of higher microbiome similarity among fathers of babies attending the same nursery. Hence, we speculate that behavioral and lifestyle convergence among fathers of nursery-attending babies, including similar dietary patterns, sleep schedules and childcare routines, may contribute to increased microbiome similarity independent of direct microbial transmission. However, we were unable to test this hypothesis and refrained from including the previous, inconclusive analysis in the manuscript.



Number strains shared between fathers and any baby sample from the nursery group (excluding the baby from the family) (left) and with any sample from the nursery group (excluding other participants from the family) (right) before (T01) and after nursery (T03), and for a random nursery group from another nursery for comparison purposes.

8. Figure 2B bottom, the small dots should be organized in space according to their nurseries, so we can see cliques in the graph, and also inter-nursery connections.

We understand the suggestion of Rev #2 and indeed there are two choices here: (1) perform an unsupervised strain-sharing network clustering analysis to try to let the structure of the graph in association with the nursery emerge, or (2) set in a supervised way the structure of the nursery to observe the level of connections between the two. While both options are perfectly fine and the reviewer is suggesting the second option, we preferred focusing on the first because we are worried the second option may be misunderstood. Specifically, while we can write in the caption that the position of the nodes is set with a supervised approach based on the belonging of the individuals to nursery, we are afraid many readers will more naively think that the transmission patterns are so powerful to be able to recapitulate *perfectly* the nursery structure. While we are still open to option 2, we would prefer to opt for the more conservative way of option 1. Specifically, as detailed in **Methods**, we built unsupervised strain-sharing networks using the strain-sharing matrices with the R packages ggraph (v2.2.1) and tidygraph (v1.3.1).

9. Figure 2D, the numbering should be per family, so M14 and F14 should be in the same family, and not as it is now.

We acknowledge the lack of clarity regarding the volunteer numbering in old Figure 2D (now **Figure 2A**). This was not indicative of familiar relations (that were instead identified by the colour filling, as specified in the figure caption), but simply reflected the listing order per volunteer category. To better clarify familiar relations, we revised **Fig. 2A** and **Extended Data Fig. 12** to display participants IDs (from **Supplementary Table 2** and **3**) showing participant type ("M", mother; "F", father; "S", sibling; "B", baby; "E", educator) and now also the family number, as detailed in the response to **Rev#1, other comment #4**.

10. Figure 2D: this figure and example seems out of place and dives into specific strain sharing events longitudinally while we are still at a “snapshot” level.

We understand Rev #2 concern as we experimented with figure order extensively and, finally, we reckon this is the ideal location for this example as it sets the scene to the resolution of our strain-tracking approach. As detailed in **R#2 C#2**, we hope we conveyed the significance of the old Figure 2D (new **Fig. 2A**) as a representative example of the resolution of our methods.

11. Figure 2D: In cases where SGB is not found (for example in B16) can this be as a result of very low relative abundance? Can the authors also explain why sometimes CRISPR spacers aren't found?

Yes, the lack of detection of a certain strain such as in some samples of B16 (now relabeled as B06), or also in B05, - where we expect to see the strain due to its presence in other longitudinal samples of the same individual - can indeed be attributed to the strain's relative abundance falling below the limit of detection; in any case this will impair recognition of the strain's signal from the background noise and not yield sufficient sequence reads for confident SGB identification and/or strain typing. However, this could also be attributed to true absence of the strain/SGB, for instance through its depletion following antibiotic usage, with potential re-acquisition of the strain in later timepoints. With regards to the CRISPR spacers identification in strains expected to have them this is more likely due to them falling below the limit of detection due to low coverage rather than true absence of the spacers, as the loss and re-acquisition of the same spacer by a given strain is unlikely. We commented on this methodological limit with the following:

“We note the lack of detection of a certain strain that is found in previous and subsequent timepoints from the same individual (such as in sample B05, **Fig. 2C**), may be due to its relative abundance falling below the sequencing limit of detection.”

12. Figure 3B, it would be useful to add a panel of shared SGB across time, so the readers can understand the upper bound of shared strains.

We modified **Figure 3D** to include longitudinal trends of shared SGBs as suggested by Rev #2, as follows:

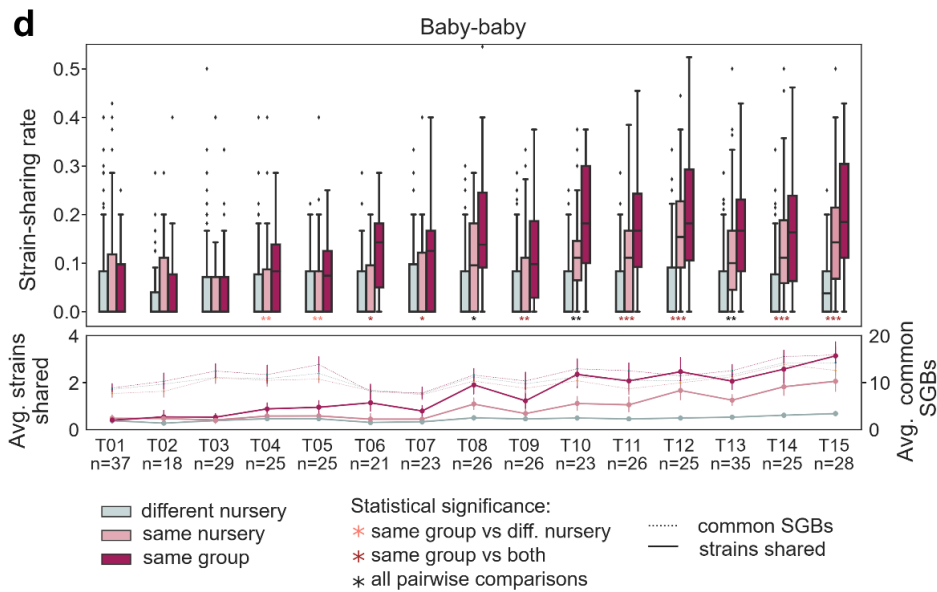


Figure 3D. Longitudinal baby-baby strain-sharing rate and average number of strains shared throughout the length of the first term.

13. Figure 4AB, what does baseline mean in the title?

In the previous version of the manuscript we used “baseline” interchangeably to indicate the timepoint before nursery start/admission (T01). For clarity, we uniformed the text and certain figures, when applicable (**Figures 4A** and **4B**), to “T01”.

14. Figure 4F, ratio of 1 should correspond to 0 number of strains (fall on the x-axis line)

We first would like to clarify that the left and right y axes are independent, with the left indicating the number of strains that are either donated (dark bars) or acquired (light green bars) and with the right y axis showing the ratio of donation/acquisition. Ratio of 1 means that the number of strains donated equals the number of strains acquired, which we agree should be the origin of the axis in the case of the right-side one. We have updated **Figure 4F**

accordingly:

f

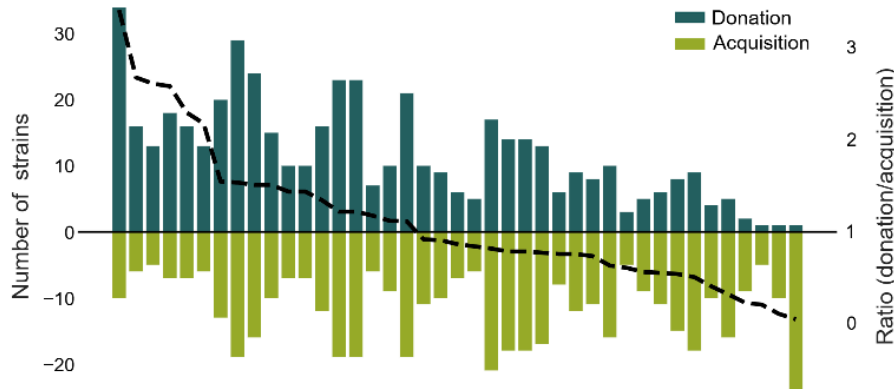


Figure 4F. Stacked barplot of the number of strains either donated or acquired by each baby over the first term.

15. Figure 5: What is the difference between transmissibility and strain sharing rate? Again, multiple terms that seem to describe the same thing makes it really hard to follow the story.

Transmissibility is a metric defined on SGBs (and not on individuals, on samples, or on a set of individuals or samples) and tries to quantify how prone to being transmitted the strains of a given SGB are. It is quantified as the ratio between the instances of strain-sharing between volunteer pairs for a given SGB over the number of volunteer pairs in which the strain of that SGB was typed in both individuals (regardless of the same strain being found). Strain-sharing rate is a metric defined on pairs of microbiome samples and estimates the strain-level overlap between them. It is quantified as the ratio between the number of strain-sharing events between two microbiome samples (all SGBs combined) divided by the number of SGBs for which strains were typed in both microbiome samples. We better explained SGB transmissibility in the main text to avoid misinterpretation of the two metrics:

“We next assessed species-level transmissibility by counting the number of strain-sharing events for each SGB in our cohort over the total potential number of strain-sharing events (i.e. the number of species-sharing pairs).”

16. Figure 6 can easily move to supplementary material

While we see how **Figure 6** represents a change in the tones and analyses shown in the manuscript, other reviewers suggested investigating more the link between strain sharing/acquisition/retention and removing this part from the main text would go in the opposite direction. We thus think that this figure that reports the significant impact of antibiotic treatment underlying the observed trends of microbial transmission and integrates the study with mechanistic insight should be kept. In contrast, negligible effects of other host-associated factors already present in the original version of the manuscript (intrapartum

antibiotics, delivery mode), or investigated during the revision phase, such as diet, and integrated as supplementary (details in the response to the Editor in point #2 and to **R#1 C#4**), were discussed in the new subsection titled "Host factors and microbiome transmission".

Response to the specific comments and request of Reviewer #3

Summary

Ricci et al., employs strain-resolved metagenomics of DNA from fecal samples obtained from infants attending different nursery homes, as well as their teachers, family members and pets, in order to investigate gut microbial transmission between these. The study focus on infant-to-infant transmission of gut microbes within nurseries and compares the extent of transmission to that coming from family members. The study is novel and very elegantly designed to detect microbial transmission in nurseries, involving multiple different nurseries and different nursery-groups within each nursery, as well as the longitudinal sampling regime. Likewise, the comparison to family-infant transmission enables assessment of the relative contributions of transmission from family vs nursery to infant gut microbiome development. The study concludes that extensive infant-to-infant transmission of gut microbes is occurring in nurseries and that these transmissions with time outweigh family-to-infant transmissions.

Comments

Overall, this paper is of very high quality and the presented data on the extend of infant-to-infant transmission of gut microbes in nurseries using a dense longitudinal sampling regime is compelling and novel. I do, however, have some comments and questions for the authors to consider.

We thank the reviewer for the very positive feedback and we are happy to further improve the manuscript as per our answers below.

Major comments

R#3 C#1

1) Data on Pets: Any conclusion regarding transmission from pets seems hard to trust given the very low N of 4 (probably also even different pets?). And statistical testing here almost doesn't make sense. In my opinion this is the weakest part of the manuscript and it takes up too much space and speculation, given that is not of importance to the manuscript's conclusions. I would suggest that the authors remove or limit the statistical analysis and, if at all including them, transfer fig.2c to the supplementary materials. I do acknowledge, though, that pets are important to consider and that it is important to mention in the manuscript.

We thank Rev #3 for this suggestion to which we largely agree. We still have tried to strengthen the sample size of our analysis by incorporating the newly sequenced extension and follow-up samples, collected during the second term of nursery (new **Fig. 1A**). This allowed us to include an additional animal (for a total of 5, 2 cats and 3 dogs) and 7 additional pet samples to the analysis of strain-sharing between pets and humans (for a total of 11). This means that our new figure on pets (**Extended Data Fig. 13**) reports the avg. number of strains shared for human-pet pairs across all the contemporaneous samples available for each human and pet (instead of considering only the T01 of each pet), as well as the fraction of pairs for each comparison category (same family vs. different family) in which human-pet strain-sharing was detected. As previously, we could detect a signal of increased strain-sharing within the family only for baby-pet pairs (Fisher's exact test, $P = 0.005$). We believe the application of a statistical test is reasonable because even with few

animals the number of human-pet pairs (the observations on which the tests were applied) can be relatively large, with contingency tables having 143–211 observations, depending on the participant type tested (with the number of observations concerning babies now specified in the text). Nevertheless, in light of this still limited sample size (affecting robustness of statistical testing) and the comments of the Reviewers, we updated and moved **Fig. 2C** to supplementary material (**Extended Data Fig. 13**) but discussed the main evidence in the results section, shortening the previously independent subsection and merging it with the previous one (“Tracking multi-host strain transmission”), as previously explained in detail in the response to **R#1 other comment #4**.

R#3 C#2

2) Metagenomic seq: I highly appreciate the strain level analyses and the longitudinal design of the study – this is highly needed in the field. However, it also seems to me that you do have a significant background signal. E.g. how is it possible that infants – before attending nursery (and thus with no or very limited contact to other babies?) - on average share 2.5 strains with unknown babies they will later come to share nursery with? Can you somehow normalize to this background? Or do you believe that it does reflect actual strain sharing?

We think that the observed background level of strain-sharing among unrelated individuals without direct contact may represent a biological signal rather than methodological noise. Indeed, this was reported in previous research⁵, where even unrelated individuals shared a few strains when living in the same geographical region. In particular, it was noticed that while the median SSR among unrelated individuals living in the same country (i.e. at population level) was 0%, the median SSR among unrelated individuals living in the same village was 8%. To evaluate where the unrelated individuals in our study fall in this SSR spectrum we computed their median SSR rate at T01, before nursery entry (focusing on adults, as in Valles-Colomer 2023). We found a median 4.5% baseline SSR (median 2 shared strains) for adults living in the city of Trento, with slight increased baseline strain-sharing among unrelated individuals with babies about to attend the same nursery (and therefore assumed to live in the same geographical area within Trento): 4.7% baseline SSR (median 3 shared strains). Considering the numbers reported above for country (0%) and villages (8%), we believe is reasonable that we found an in between estimate (4.5%) for a small urban area (as defined by The Organisation for Economic Co-operation and Development - OCDE, <https://www.oecd.org/en/data/indicators/urban-population-by-city-size.html>). Given the hypothesised authenticity of this background signal, normalization was deliberately omitted from the manuscript analysis, though upon Rev #3' suggestion we decided to deepen our analysis reported in Fig. 2A (now, after re-structuring of the manuscript, **Fig. 3A**) to calculate the avg. number of strains shared with babies at T15 after disregarding strains that were already shared at T01 (i.e. those present at baseline). As this analysis included only babies for which T01 and T15 samples were available we found an even higher avg. number of shared strains after taking into account this strain-sharing background (8.8 avg. strains shared with babies at T15 vs 7.2 originally reported). We kept both estimates in the manuscript, differentiating them by calling the former the *avg. number of strains shared with babies* and the latter the *avg. number of strains acquired from babies* (reflecting the fact that

if they were not shared before nursery start they were acquired from babies in the nursery). The new sentence reads as follows:

“We quantified the number of strains shared between babies attending the same nursery over time and found that they shared, on average, more strains at the end of the first term than before nursery admission (avg. number of strains shared with any other baby at T01 = 2.5 and at T15 = 7.2; **Fig. 3A**), which was even more evident after considering only babies with both T01 and T15 samples available and disregarding strains already present at T01 (avg. number of strains acquired per baby at T15 = 8.8) ”.

Finally, we would like to further clarify that the 2.5 shared strains at T01 reported in **Fig. 3A** indicate the average number of strains present in a given baby that are also present in *any of the other babies* (i.e. all other babies combined) in the same nursery before nursery start, and not the average number of strains that are shared between a baby and *each of the other babies* (i.e. each baby-baby pair considered) before nursery start. This is instead indicated in **Fig. 3B** and is much lower than the 2.5 reported in **Fig. 3A**, ranging between 0.2 and 0.55 depending on the nursery, with an avg. of 0.45 strains shared between baby-baby pairs about to start attending the same nursery. This was clarified in the main text and in the **Fig. 3** legend by using “any other baby” when referring to **Fig. 3A**.

R#3 C#3

3) Furthermore, a big limitation of metagenomic seq is the relatively high limit of detection, meaning that you risk missing strain sharing events if the microbes colonize at lower abundances. If I understand correctly, you disregard taxa at rel abundance <0.1%. Assuming 10^{11} bacteria/g feces, this means anything below 10^8 bacteria/g feces is not seen. Therefore, I find it important that these type of analyses are backed up by much more sensitive methods, such as actual culturing (and strain isolation) and/or qPCR. I do understand that this is extremely cumbersome, but validating your method in a few cases really adds to the credibility (e.g. qPCR targeting the *Akkermansia* strain in Fig. 2d).

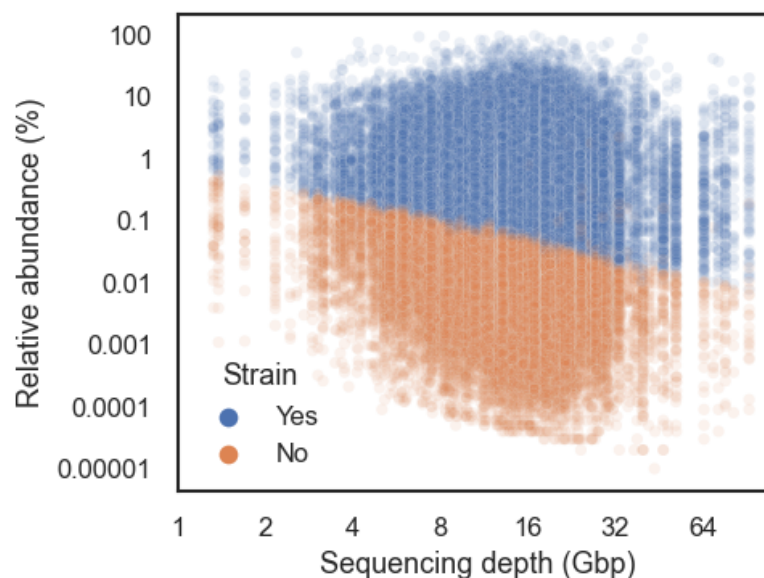
We agree in general that not all strains in microbiomes can be reconstructed via metagenomic sequencing and indeed to tackle this methodological limitation we sequenced samples at higher depth compared to standard metagenomic sequencing. We hope to convince the Reviewer that the current metagenomic approach has enough analytical sensitivity and qPCR would not add relevant information to the work.

1. Discussion on limit of detection for our metagenomic approach

First, we would like to clarify that when we wrote in the **Species- and strain-level profiling subsection of Methods** that “SGBs with <0.1% relative abundance in all stool samples were removed from taxonomic profiles” we were referring specifically to the analysis of SGB-level MetaPhlAn 4 taxonomic profiles for the calculation of diversity indices (i.e. not strain-sharing data), so this does not relate to our ability to reconstruct strains. We took this decision due to the fact that we performed diversity analyses with metrics that take into account only presence-absence of SGBs (i.e. SGB richness for alpha-diversity and Jaccard dissimilarity for beta-diversity). As such, we filtered out SGBs consistently found to have low abundance to avoid as much as possible presence of potential contaminants biasing our SGB-level diversity estimations, which might happen especially considering our very high sequencing

depth. We have clarified that by rewriting that sentence as: “SGBs with $<0.1\%$ relative abundance in all stool samples were removed from taxonomic profiles for calculation of diversity indices”. Furthermore, only SGBs with abundance this low ($<0.1\%$) in *all samples* were discarded (e.g., *Xanthomonas campestris* SGB12430), so if a given SGB had abundance $\geq 0.1\%$ in a single sample this would already justify keeping that SGB for the diversity analyses (which will include many examples in which the SGB has abundance $<0.1\%$ in the other samples).

Considering the reviewer’s concern about the limit of detection of strains, we evaluated the range of SGB relative abundances for which we could reconstruct strains. As reconstruction of strains depend on certain number of reads mapping against SGB marker genes, this will also be a function of sequencing depth, for which we represent the cases in which we could or could not reconstruct strains as a function of SGB relative abundances and metagenome sequencing depth:



We calculated the median relative abundance as the lowest relative abundance SGB for which a strain was reconstructed (visually, the lowest blue dots) across all samples, which can be taken as a rough estimate of our strain-level detection limit. We found this to be 0.057%, which is indeed close to the threshold suggested by the reviewer. Still, as this is a function also of sequencing depth, for more deeply sequenced samples we managed to reconstruct the strains even for SGBs present at relative abundances as low as 0.007%. Please also notice that our strain-sharing analysis normalizes the number of identical strains over the number of strains that can be typed within shared species: as such, while higher depths are always good, the strain sharing rate is per se not dependent on the depth (on the contrary of the absolute number of shared strains).

2. Discussion on PCR to confirm "positive" strain sharing inference

To specifically answer the comment of Rev #3, while we understand the general suggestion regarding additional PCR confirmation, we would like to explain our perspective that this would not add to our specific study. The computational pipelines employed in strain-tracking analyses incorporate stringent quality controls and statistical thresholds that effectively

minimize technical artifacts and the risk of false positives (even for low relative abundance bacteria)²⁸. In our opinion, given this robust analytical framework and our high sequencing depth, additional PCR confirmation offers minimal gain for strains already detected above our limit of detection (LOD): if multiple marker genes of a given species are reconstructed at high coverage, qPCR defined on some of these markers would just confirm the DNA of the marker gene is present. In any case, a systematic qPCR evaluation of the finding would be theoretically feasible but extremely laborious undertaking. Indeed, MetaPhlAn 4 and StrainPhlAn 4 profiling is based on the identification and typing of multiple (up to 200) SGB-specific marker genes, for which the design of a targeted PCR-based validation with comparable resolution and accuracy would require the optimization of a substantial number of primers targeting strain-specific genes.

3. Discussion on PCR on "false negative" strain sharing inference

In contrast, we acknowledge that PCR confirmation could be valuable for detecting the presence of very low-abundance strains that fall below our already rather small LOD, or in the context of validating the implementation of a newly developed method, while instead metagenomics has been exclusively applied in the vast majority of published microbiome transmission research (we point Rev #3 to Table 1 in Heidrich et al., 2025²⁹ for a comprehensive summary). In light of these considerations, we think that performing PCR confirmation for some of the strains already detected by our metagenomic inference method would offer limited additional insight. In contrast, while PCR validation would be valuable for detecting and tracking low-abundance strains not identified by metagenomics, its systematic implementation for all strains in a large-scale transmission study would be practically unfeasible and, indeed, would diminish the utility (or replace altogether) the metagenomic analysis.

Hence, we believe our approach represents a robust and highly sensitive methodology, making additional PCR validation for already detected strains unnecessary for the scope and rigor of our findings and PCR for low-abundance microbes unfeasible if done systematically for all species (and not doing it systematically would not be correct), and conclude that our study aligns with the established practices of current and past microbiome transmission research that relies solely on metagenomics for detecting interpersonal microbial transfer.

R#3 C#4

Conclusions regarding nursery transmission outweighing family transmission: I'm not convinced about the author's conclusion regarding this. The data presented in Fig. 2A and Fig. 4C do not provide strong support for this conclusion, as detailed in my specific comments below.

Hopefully we adequately rephrased the statement to a satisfactory level, to avoid overstating our findings, and clarified our previous interpretations addressing this specific concern, in the response to **Rev#3 specific comment #1** below (as well as to **Rev#1 C#2** and **Rev#2 C#4**).

R#3 C#5

Missing analysis of important metadata: It is mentioned in the methods and in Fig.1 that there is available data on the infant's diet and I assume that you also have data on infants exact age. As far as I can see, these data are not analysed together with the microbiome

data. We might expect that the establishment of transmitted strains could depend on infant feeding and age. So, why were these not included?

We acknowledge that consideration of the exact age of babies is key for evaluation of infant microbiome development, and we integrated our set of results and the main text with extensive additional analyses. Briefly, in light of the strong association between age of babies at T01 and microbiome alpha diversity at T01 and T15 (now included in **Extended Data Figure 4**), we accounted for the effect of age in following analyses. First, we found that after correcting for age of babies, having a sibling was significantly associated with increased microbiome alpha diversity (SGB richness, **Figure 1C**, new **Supplementary Table 6**), while the effect of having a pet on microbiome richness was lost. Furthermore, as demonstrated by the Jaccard dissimilarity computed between samples collected before nursery start (T01) and after conclusion of the first term (T15), microbiome compositional changes occurring during nursery attendance were more pronounced in younger infants than in older infants (**Extended Data Fig. 8**). We also investigated the effect of age on strain acquisition and donation in babies attending the nursery, and uncovered that strain donation occurred at higher frequency in older infants compared to younger ones; in contrast, the number of strain acquisitions were not significantly correlated with age (**Extended Data Fig. 22**). We edited the manuscript to report the effect of age on microbiome diversity metrics and interindividual transmission as detailed in response to the Editor (point #3) and to **R#2 C#3**.

We agree with Rev #3 on the importance of investigating the effect of diet on patterns of microbiome development and transmission. As part of the revision, we thoroughly analysed dietary information of both infant and adult participants that was collected for the study prior to nursery admission via custom made questionnaires and EPIC FFQs, respectively. Briefly, while some weak correlations were detected (particularly regarding diet quality and microbiome richness in adults), none of the dietary factors examined showed strong or consistent effects on the actual transmission dynamics between participants, suggesting that diet plays an overall minimal impact on trends of microbial transfer in our cohort. We integrated this new set of results as supplementary data, and point Rev #3 to our response to the previous response to the Editor (point #2), specifically addressing this and reporting detailed new figures and discussion.

R#3 C#6

Sudden jump in shared strains: The authors observe a sudden jump in # and %shared strains within infants in the nurseries between T07-T08 (Fig. 4c,d and Extended data Fig. 11). This is quite interesting, but not really discussed much in the manuscript. It seems to be 6-7 weeks after start in the nursery - what happens at that point?

We think the increase in proportion of strains acquired (using the new adopted nomenclature, see response to **Rev #2 minor comment #4** and **R#1 C#2**) at T08 that Rev #3 refers to in **Fig. 4C** is largely due to the incorporation of new samples in the analysis at that point, as explained in the **Methods** (as well as experimental mishaps or missed collection of timepoints that may occur in long-term studies to a minor extent). In this analysis, we aim to identify the origin of the strain (i.e., nursery group or family) by evaluating strain-sharing between a focus baby at a given timepoint and past/contemporaneous samples of the nursery group or family. Because babies are more densely sampled than

adults, for a fairer comparison we consider only nursery group samples of the same timepoints that are available for parents. As parents samples were collected before nursery start (T01 for all participants), at midpoint of nursery attendance (equivalent of T08 for babies), and after the end of the semester (equivalent of T15 for babies), we include new baby samples in the analysis (in which we look for the strains present in the focus baby) at T08 and T15, both points in which we see a sudden increase in the proportion of strains acquired from the group. However, we believe that the sudden jumps are due to this analytical limitation and that a more linear increase in the proportion of strains acquired from the nursery group from T01 to T15 to be more likely. For clarity, we expanded on this in the **Methods** section:

“This likely explains the non-linear increases of proportion of strains acquired from the nursery group at baby T08 and T15 observed”.

Specific comments:

1. L. 24-26. Please consider modifying this sentence. The evidence of nursery transmission actually surpassing the family contribution is not overwhelming, e.g. Fig. 2A (showing 8.6 vs 8.0 for “family” vs “nursery”) and Fig. 4C (only showing slight significance in %strain shared at T15, but no difference in Avg. shared strains?). It would be fair to state that transmission in nurseries reaches similar levels as observed for baby-family.

There are multiple analyses incorporated in the paper that compare different strain transmission metrics between nursery and family, as Rev #3 rightfully points out, including: Figure 2A (now **Fig. 3A**, baby-centered quantification of average of the total number of strains shared between one baby and all the other babies or family members, as better detailed in **minor comment #14**, below) and **Figure 4C** displaying the community-level contribution in terms of the proportion of strains acquired by the baby from either the nursery or the family members. These figures show insight generated via different analyses but are nonetheless noncontradictory: after the first term of nursery, the absolute counts of strains shared between babies in the nursery is not higher than those shared with the family on average, but still these contribute to a higher proportion of the strain-build up of the infant microbiome, considering all the other species present. This is also evident when looking at the cumulative relative abundance of nursery-acquired strains (also in **Figure 4C**), that surpass that of strains shared with the family at end of the first term. We previously (response to **R#1 C#2**) explained why we consider the “proportion of strains acquired” as the optimal metric to evaluate community-based contributions to infant strain transmission; in particular, because it examines strain acquisition across all species in the baby's microbiome, providing a comprehensive view of transmission sources, unlike “strain-sharing rates” which only consider species common between pairs of microbiome samples and “absolute counts of strains shared” that does not consider the context of the infant microbiome. Hence, we think the evidence shown in **Figure 4C** is strong and indicative of a larger quantitative contribution to the infant microbiome of the nursery than the family at the end of the first term. Nonetheless, to avoid overstating our conclusions, and in light of the newly incorporated results illustrating that the proportion of strains acquired from the nursery becomes comparable to that from family at the second term of nursery (**Ext. Data Figures 18 and 19**), this statement (as well as others throughout the manuscript, as detailed in response to **R#2 C#4**) was toned down as follows:

“We detected extensive baby-to-baby microbiome transmission within nursery groups even just after only one month of nursery attendance, with nursery-acquired strains accounting for a proportion of the infant gut microbiome comparable with that from family by the end of the first term”.

2. L. 28-30: “microbiome transmission between babies interacting in the nursery outweighs parent-to-infant transmission after birth”. Please modify this sentence. What data in this manuscript demonstrate that transmission in nursery outweigh parent-to-infant transmission? Fig. 2A indicates higher cumulative strain sharing parental(mother+father)-to-baby, than nursery-to-baby? And Fig. 4C does not show significantly higher avg. #shared strains from nursery-to-baby than family-to-baby. Also “after birth” is not specific as you see large differences according to time-in-nursery.

Rev #3 is correct in suggesting that our previous statement was giving an inaccurate timeframe and summary of the main finding of our study. Hence, we modified it to specifically point to evidence shown in **Figure 4C** and referring to timepoints corresponding to the end of the first term:

“This study shows that microbiome transmission between babies interacting in the nursery is extensive during the first year of nursery, and points to social relationships in infancy as crucial drivers of infant microbiome development”.

3. L. 95: please explain (to the unfamiliar reader) what “expected trends” are.

We agree with this comment and expanded the sentence, by describing that different species-level compositions between the infant and the adult gut microbiomes, as well as higher alpha diversity in adults, are known features. We included supporting references for these, as well as evidence from our study in **Extended Data Figures 2, 3, 4, 5**. The expanded explanation reads as follows:

“In our data we observed the expected microbiome structures^{1,19,20}, with large compositional divergence between adults and babies (**Fig. 1B, Extended Data Fig. 2–4, Supplementary Table 5**), and age-dependent differences in babies (**Extended Data Fig. 5**)”.

4. L. 154-168: I don't find this part about pet-baby/parents strain particular strong. It is based on only 4 pet-infant pairs AND only 2 mother/father-pet pair? How can you draw conclusions on such low sample sizes?

We understand Rev #3 concerns that were also raised by other Reviewers and that we thoroughly addressed. Briefly, although the number of pets participating in the study is very limited, in this analysis we are also taking into consideration human-pet pairs from different families (including adults not having pets), making the number of observations considered in statistical tests range between 143 and 211, depending on the participant type tested. We also note that we have tried to strengthen our sample size by incorporating an additional animal (for a total of 5 pets included) and 7 additional pet samples (for a total of 11 pet samples included). This was possible because we opted to also include a pet from a family

only enrolled during the extension and because we chose to analyze all pet samples available (as opposed to only the T01 sample from each pet, as previously). This means that our new **Extended Data Figure 13** reports the avg. number of strains shared for human-pet pairs across all the contemporaneous samples available for each human and pet, as well as the fraction of pairs for each comparison category (same family vs. different family) in which human-pet strain-sharing was detected. As previously, we could detect a signal of increased strain-sharing within the family only for baby-pet pairs (Fisher's exact test, $n = 211$, $P = 0.005$). Nonetheless, we agree with this comment and, as previously detailed (**R#3 C#1**), we moved **Figure 2C** to Supplementary (**Extended Data Figure 13**) but decided to include the main messages of this analysis in the main text (after extensively shortening the previous subsection), as we think the investigation of microbial transmission between pets and human hosts (hence across different host species) is novel and important to consider. The rephrased text on pets is included in **other comments #1 of Rev#1**.

5. The statement is very speculative given the actual data presented: "This may be evidence of the fact that the lower colonization resistance of the infant gut microbiome may facilitate microbial acquisition or may be indicative of a more intimate interaction between pets and babies with respect to pets and adults."

We agree this was overspeculative, and we have removed the sentence from the main text.

6. L. 180-214: I find these case-observations very interesting, but also anecdotal. First, directionality is hard to infer if the strain is detected for the first time in two individuals at the same sampling point. This seems to be the case in both examples (Fig. 2D and Extended data Fig. 6), but is not highlighted in the figure.

We agree with the impossibility of determining directionality of transmission in those cases and thank Rev #3 for pointing out this inaccuracy in the visualization. We have added double-headed arrows when participants display the strain at the same time points, both in **Fig. 2D** and **Extended Data Fig. 12** (old Extended Data Fig. 6).

7. One thing that I am missing here is an effort to validate these transmissions either by qPCR or cultivation and isolation of the strains from the fecal samples. The "problem" with relying solely on metagenomic data is the high detection limit, meaning that you risk missing detection of the strains at some timepoints (and individuals).

We hopefully responded to the Rev #3 concerns in the response to **R#3 C#3**. In summary, we think that our computational metagenomics approach allows for the high-confidence detection of species with median relative abundance of 0.057% (previously indicated as our strain-level detection limit) thanks to identification based on hundreds of species-specific marker genes that renders additional PCR confirmation unnecessary for already detected strains, consistently with numerous published transmission studies relying exclusively on metagenomics.

8. L. 225: What is meant by "fast-mutating ecological niches"?

We rephrased it to avoid confusion, to: "This reflects the expected high plasticity of the infant gut microbiome with its rapidly evolving ecosystem and limited colonization resistance".

9. L. 360: rho-values of 0.35-0.4 are not “strong” correlations. Please re-phrase.

We agree with this comment and removed the “strongly” from the statement in current L424-425.

10. I. 442-445: I find this overstated. You do not present any data linking these transmissions to infant health. In fact, these social interactions also result in spread of pathogens. Please rephrase.

We thank Rev #3 for this constructive comment, as we acknowledge that our initial conclusions overemphasized the health benefits of nursery strain transmission, as they relied on general assumptions about the advantages of gut microbiome enrichment and maturation through transmission. We added to our previous analyses showing that several key infant gut bacteria are preferentially transmitted between babies in nursery settings rather than from parents to infants. Most notably, *Bifidobacterium longum* subsp. *infantis* - a specialized breast-fed infant colonizer with anti-inflammatory effects- was found exclusively in babies and showed dramatically higher transmission rates between baby pairs (85.3%) compared to other *B. longum* subspecies (19.4%), suggesting nurseries serve as important reservoirs for health-beneficial infant-specific microbes. *Bifidobacterium breve* was also found to be differentially more transmitted baby to baby, over parents to baby (details in response to the Editor, point #5). Nonetheless, we rephrased the statement in the conclusions to avoid overstating the potential impact on health:

“In addition to their already established effects in emotional and cognitive development^{66,67}, social relations among peers in the nursery are thus a hub for microbial enrichment during infancy, particularly of key early-life gut colonizers, such as *Bifidobacterium longum* subsp. *infantis* and *Bifidobacterium breve*^{52,53}, with their known favorable effects on health including via immune development of the infant^{68,69}. Our data indeed suggest that social and physical interaction between infants should be regarded as a key contributor to microbiome maturation during infancy, comparably to previously outlined perinatal factors that influence the earliest stages of life, such as delivery mode (natural versus C-section) and feeding practices (breastfeeding versus formula feeding)⁶⁹⁻⁷².”

11. I.448-450: How can you exclude that other factors associated with having a sibling is influencing these patterns. E.g. infants with older siblings might attend nursery less often? Or might interact less with other babies?

We agree we cannot exclude other factors associated with having a sibling as not playing an impact on infant microbiome, but we do not have additional metadata to support further speculations. Nonetheless, we have better rephrased the sentence to indicate there is an association between the two events, rather than infer a direct cause-effect relation namely:

“Our results show that overall baby-sibling strain-sharing surpasses transmission between parent-baby pairs (**Extended Data Fig. 11**) and correlates with a later decrease in infant microbial acquisition in the nursery (**Fig. 4D**)”.

12. Figure 1

It would be helpful to indicate somewhere in this figure or elsewhere in the manuscript what T01-T15 translates to in terms of 1) weeks of nursery attendance and 2) infant age.

We agree with this suggestion that we have implemented in the new **Figure 1A** (in the **dataset expansion** comment in response to Editor) that now provides the reader with an immediate visualization of the timeline of the study and intervals in months between different timepoints (already detailed in “sample collection” section, **Methods**). We also took this opportunity to integrate **Figure 1A** with the additional set of samples that we included during the revision (details in response to comments to Rev#1). Regarding the addition of infant age to **Figure 1A**, or per timepoint in other figures, we found this would have not been practical or informative as baby age ranged from 4 to 15 months before nursery admission (with this age distribution maintained in each consecutive timepoint during the study) and this range was actually larger than the change in age during the core of the study (encompassing the first term of school year). We have however addressed the detailing and discussion of the effect of age on infant microbiome diversity metrics and on strain donation in the response to the Editor (point #3) and to **R#2 C#3**, to show our main findings were not affected by age-adjustments.

13. Figure 2

Panel A. These analyses only show the absolute average number of strains shared, but it does not tell us the average proportion of strains shared. E.g. how large a proportion of all strains detected in an individual are actually shared with the baby (from family and from nursery?). These numbers would be very informative, especially when assessing if the baby-to-baby strain sharing is actually “extensive”.

We thank Rev #3 for the suggestion. Unfortunately, while we agree that as a concept this would be an informative addition to this set of results, we cannot in practice provide a fair proportional estimation of strains shared. This is because the average numbers of shared strains displayed in Figure 2A (now **Fig. 3A**) are computed only for specific subsets of participant pairs where samples from each participant type were available. For instance, strain-sharing with siblings is computed only for families having a sibling enrolled in the study as well as strain-sharing with fathers is computed only for cases in which father metagenomes are available. Because of this each percentage value would be calculated using a widely different subset of babies, which would create inconsistent percentages, as they would not sum to a meaningful 100% and could even exceed 100% when combined, making the results confusing and potentially misleading.

14. Panel B. Why is the average number of baby-to-baby shared strains from panel A “final” (=7.2) 2-to-4-fold higher than those numbers displayed in panel B for intra-nursery strain sharing (1.85, 2.56 and 3.44)?

The average number of shared strains baby-to-baby do not match across the two figures (now incorporated in **Figure 3**, as **3A** and **3B**, respectively) because:

- **Figure 3A** displays the number referring to one baby (i.e. average number of shared strains of each baby to all others). Mathematically, it is the average of the total number of strains shared between one baby and all the other babies. Visually, in a

network graph, is the average of the sum of the widths of the lines connecting one point (one baby) to any other point (baby). For clarity, we edited the main text and the **Fig. 3** legend by using “any other baby” when referring to **Fig. 3A**.

- **Figure 3B** is the average across baby-baby pairs (i.e. the average number of the sum of shared strains per each different baby pair), obtained by averaging the number of strains shared among all the couples of babies sharing a strain. Visually, it is the average width of all the lines in the network graph. We further note that during revision we realized the data used to compute the values present in the original **Fig. 3B** were the same used to generate the strain-sharing network depicted in the same panel, which excludes cases of no strains shared. After also considering those cases the values mentioned by the reviewer (1.85, 2.56, and 3.44) are 1.23, 2.11, and 3.23, respectively. **Fig. 3B** was updated accordingly:

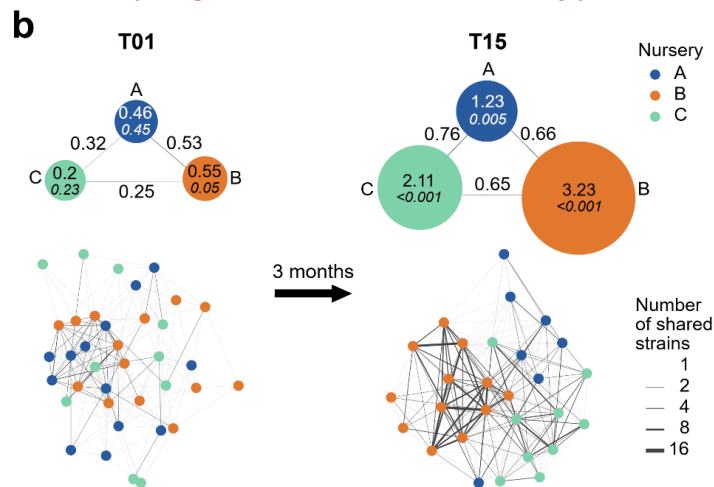


Figure 2B. Summary of the average number of shared strains between baby pairs in the same nursery (in each circle) vs inter-nursery (over connecting lines); *P*-values (permutation test for means; **Methods**) for intra- vs inter-nursery comparisons for each nursery are shown in italics in the circle. The average number of shared strains normalized by the total number of SGBs typed at strain-level in each baby is provided in **Supplementary Table 9**. The network analysis is built on strain-sharing matrices among all infants before nursery start and at the end of term. Thickness of connecting lines reflects the extent of strain-sharing between pairs of babies.

15. I'm not sure I understand how the statistics are applied here – are the Mann Whitney test comparing all inter-nursery values (AvsB, AvsC, BvsC), to one intra-nursery value? Or only AvsB and AvsC to within A? Please specify. Also here it would be informative to show the average proportion of strains shared within and between nurseries.

The Mann-Whitney test was computed as all inter-nursery observations vs all intra-nursery observations, with each observation of the absolute number of strains shared across a baby-baby pair. This is done separately for each nursery (e.g., intra-nursery A vs inter-nursery A-B and inter-nursery A-C) and timepoint (T01 or T15). We note that, as pointed out by Rev #2, this test is not appropriate for dependent observations (and there is dependence in the sense sharing is computed with relation to the same microbiome samples in both groups), for which we adopted a more suited permutation-based approach (as detailed in **Methods** and in the response to **R#2 C#8**), finding full concordance with previous results.

As suggested, by considering the number of SGBs typed at strain-level in each baby, the average proportion of strains shared within and between nurseries (computed over all baby-baby pairs) is now provided as **Supplementary Table 9**, which is referenced in the main text beside **Figure 3B** and in the **Figure 3B** legend as follows: “The average number of shared strains normalized by the total number of SGBs typed at strain-level in each baby is provided in **Supplementary Table 9**.”.

16. Figure 3

Panel D. It seems like most of these strains were not detected at baseline? How do you know that they actually originate from one of the babies in the study? Are these strains also detected in teachers or family members?

We understand the source of confusion. The condition for entering the analysis was for the strain not to be shared by babies in the group at T01, while it could be present in one baby and other participants (including parents and educators) at the same time point. We specified this in the text to improve clarity:

“We focused on strains initially detected in maximum one baby in the group (but potentially shared with other participants)”.

17. Figure 4

Panel C. I assume that these comparisons are all to “baseline samples”, as for panel A and “B”? What happens if you split “with family” into “father”, “mother” and “sibling”. It seems like the contribution from siblings is quite a bit higher than maternal and paternal. It seems like sibling-baby sharing is quite extensive at baseline and at T15 (Fig. 2A and Fig. 4b), so I’m wondering whether siblings are actually contributing more than “nursery” during the entire sampling period?

Comparisons in **Figure 4C** are not conducted to baseline. As detailed in the **Methods**, they are conducted in relation to all past or contemporaneous samples available from the family, and similarly for comparison with nursery group samples (with the distinction that not all samples from babies in the nursery are incorporated to keep the comparison balanced with the fact that gut microbiomes of family members were less densely sampled than those of babies, with them being added in the analysis in the same amount and at the same time that family samples are) comparisons among proportions of strains acquired from family or nursery group are performed between the baby samples and the most contemporaneous timepoint for parents. This was newly specified in the figure caption, like so: “For each baby timepoint, comparisons were performed against past/contemporaneous samples of the family and the nursery group, as detailed in **Methods**”.

Our data in old **Extended Data Fig. 10** (now **Extended Data Fig. 21**) show that strain-sharing between siblings and babies is extensive and indeed surpasses the one observed between parent-baby pairs. However, in **Figure 4** siblings are already included in the family proportion, so separating and comparing acquisition from the nursery vs acquisition from siblings cannot produce a greater contribution of strains from siblings than the nursery group.

18. Figure 4

Panel C and D. You see a quite interesting jump in % and #of shared strains occurring between T07 and T08. Why is this increase in strain sharing occurring so suddenly? Wouldn't we expect it to be more gradual?

As mentioned in a previous answer to the reviewer, the analysis is constrained by the uneven sampling of family members relative to infants. The T08 value is real, while the only marginal increase in the preceding trend is an artefact derived from the lack of samples from family to be included in the analysis in this interval (which hampered the inclusion of the available nursery group samples, to keep the analysis unbiased in terms of samples to which we compare against), with inclusion of new family samples (T02 of parents) - and therefore also of nursery group samples - only at baby T08. This is perceived as a sudden increase in the proportion of strains acquired from the nursery group at T08, but it is due to our inability to incorporate nursery samples with complementary family samples at each timepoint. We added a note about this limitation in the **Methods** section, as follows:

"This likely explains the non-linear increases of proportion of strains acquired from the nursery group at baby T08 and T15 observed".

19. Figure 6

I'm missing some non-Ab exposed control infants in these analysis. How can you exclude a "time-effect" here?

The analysis reported in **Figure 6** includes pairs of timepoints fully encompassing the antibiotic course (i.e. with treatment starting after the first timepoint and finishing before the second timepoint) for individuals who underwent antibiotic therapy ("pre-post"), and compares this to consecutive timepoints (or closer available) in individuals that did not undergo any antibiotic treatment ("pre-pre"). Therefore the analysis already accounts for any time effects of comparing longitudinal samples. However, we better identified our negative controls (untreated individuals) and exposed tests (treated individuals) by rephrasing from "pre-pre" and "pre-post" to "Ctrl pre-post" and "ATB pre-post", respectively.

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