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Maternal age is correlated with decreased infant gut microbial diversity and changes in eating behaviour

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SUMMARY Delay in motherhood to later stages of life has been increasing in many developed countries and has been associated with several negative health outcomes such as a decrease in fertility, increase in cesarian section operations and delay in onset of lactogenesis. These health outcomes associated with increased maternal age have been identified as contributors to microbial diversity in the infant's gut; however, the effect of maternal age on the infant gut microbiome has not been well understood. Using the dataset generated from 325 infant-mother dyads by Dr. Kyung Rhee and her research group from the University of California, we wanted to investigate the effects mothers aged 30 and above have on the diversity of the infant gut microbiome and other infant health factors compared to their younger counterparts under 30. We identified maternal age as a significant contributor to microbial diversity in the mothers' gut microbiome. Furthermore, we identified that maternal age does not result in a significant difference in the infant gut microbiome at 2 weeks but has a more significant impact on diversity at 2 months when analyzing diversity metrics that take phylogenetic relatedness into account. Lastly, we found significant correlations between maternal age and infant eating behaviour such as infant food responsiveness, slowness to eat, and number of sucks per feeding session. Our findings demonstrate the important role maternal age plays in microbial diversity in both mothers and infants as well as its role in infant eating behaviour.

INTRODUCTION

In the last three decades, there has been a significant increase in maternal age at childbirth in many high income and developed countries such as the United States, where they have seen an increase in the birth rate of women aged 40 to 44, and a decrease in women below the age of 40 (1). Similarly, on the report of Statistics Canada, there has been an increase in the average age of mothers at first birth and all births in Canada since 1966 and 1976, respectively (2). The cause of delayed motherhood is not linked to specifically one factor, but a multitude of factors that have impacted the landscape of having children early on in life. Some identified factors include effective contraception, changes in societal gender roles, prioritization of post-secondary education and careers, and financial security (3). Although recent studies have shown that a delay in childbearing age has been associated with several negative health outcomes for both the mother and child, we wanted to investigate whether maternal age affects the infant gut microbiome (1, 4-8).

A study investigating the effects of maternal age on the success rate of pregnancies found that women below the age of 30 had a stable pregnancy success rate of 400 out of 1000 women compared to women above the age of 30 who saw a substantial decline in fertility success (9). Furthermore, it has been shown that fertility declines in women at the age of 30, having a success rate of only 75% in terms of conceiving a child within a year compared to 92% in

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those aged 19-26 (10, 11). A decrease in fertility success due to maternal age has been associated with pregnancy complications such as preterm birth which has been shown to affect the infant gut microbiome (9, 12). Infants who are born pre-term are exposed to a different set of exposures including antibiotics in the first few days of life, feeding tubes, and neonatal intensive care units (12). The use of antibiotics in an infant's early life appears to have short-term and long-term influences on the development of the infant gut microbiome that increases the percentage of potentially pathogenic bacteria while decreasing microbes associated with a healthy microbiota, such as Bifidobacteriaceae and Bacilli (13, 14). Additionally, infants who are born preterm also have a microbiome similar to colonizing bacteria found on hospital surfaces and feeding tubes enriched in Staphylococcus epidermis and *Escherichia coli* (14, 15).

Given that an increase in maternal age has also been associated with an increase in caesarean section operations, we believe maternal age may also influence the infant gut microbiome. A previous study has shown that increased maternal age from 20 years to 30 years, sees an increase in caesarean section operations from 7% to 15% alongside a 10% increase in labour duration (16). Furthermore, the risk of caesarean section was found 3.6 times higher in women aged 40 years and above and doubled in multiparous women compared to women below the age of 30 (17). Infants born from caesarean sections have shown a decrease in intestinal bacterial richness and phylogenetic relatedness compared to infants who were born vaginally throughout the first 2 years of life and a significant decline in evenness during the first year of life (18). This may be due to the lack of major microbial exposure that occurs in the birth canal in vaginally delivered infants (19). Additionally, it was found that infants who were born vaginally had a microbiome that resembled the mother's vaginal microbiome while infants born to caesarean sections harbour bacterial communities similar to those found on the skin (19).

Another important factor that may affect the infant gut microbiome is delayed breast milk production due to an increase in maternal age. A study showed that 58.5% of mothers aged 30 and above see a delay in breast milk production while only 39% of mothers aged below 30 see similar effects (20). This delay in breastmilk production can increase formula feeding during the first 3 days of life, which has been associated with an increase in the risk of excess weight loss in infants (20). Furthermore, formula feeding has been shown to increase the diversity of microbial colonization while breastfeeding reduces diversity and induces a gut microbiome that is rich in Bifidobacteria (21). This strongly connects with the idea that the type of feeding is instrumental in establishing the gut microbiota of infants during their first few years in life.

While the aforementioned negative health outcomes associated with an increase in maternal age have been shown to affect the infant gut microbiome, there is still uncertainty on the effect of maternal age as a direct contributor to infant microbiome diversity. Given past associations with poor health outcomes due to increased maternal age, we hypothesize that an increase in maternal age affects the infant gut microbiome. Using the unpublished dataset generated by Dr. Kyung Rhee and her research group from the University of California-San Diego (22), we separated mothers aged 30 and above (M-O) from mothers below the age of 30 (M-Y) all with their respective infants. We performed 16S rRNA gene amplicon analysis using the QIIME2 (23) pipeline to determine the effects of maternal age on the microbial diversity of the mother and infant gut microbiome. We also performed correlational analyses in R (24) to determine what other infant health factors are correlated with maternal age that may provide reasons as to why we may or may not see differences in the microbiome of both mothers and infants. We found that maternal age had a significant effect on the microbial diversity in the mother's gut microbiome and the infant's gut microbiome at 2 months. We also identified significant correlations between maternal age and infant health factors such as infant food responsiveness, slowness to eat, and number of sucks per feeding session as possible contributors to differences seen in the infant gut microbiome.

METHODS AND MATERIALS

Dataset. For this paper, the unpublished dataset generated by a research group directed by Dr. Kyung Rhee from the University of California-San Diego (UCSD) was used to examine

the gut microbiomes of mothers and their infants across maternal age categories (22). The dataset included rich metadata spanning 171 fields from each subject's health, diet, medical history, and feeding behaviours which were looked at through the lens of maternal age. The dataset is especially useful in that it contained 325 infant-mother dyads with infants sampled at up to 4 different time points during the first 12 months of life, while mothers were at most sampled twice with the first occurring 2 weeks after giving birth. Infant-mother dyads were recruited from the state of Michigan through the University of Michigan in Ann Arbor. Only infants born without neonatal and perinatal complications between 37-42 weeks gestation time with average weight were included. Furthermore, foster children, those born to mothers below 18 years of age, and those born to parents with non-fluency in English were all excluded. In terms of microbiome analysis, stool samples were collected from each subject and underwent 16S rRNA amplicon sequencing using the V4 region which was then further analyzed for microbial composition. This dataset is publicly available on the European Nucleotide Archive (ENA) website (PRJEB39437).

Preliminary Metadata Processing. Processing of metadata in preparation for microbiome analysis was done through R (Version 4.1.0) (24) in RStudio (Version 1.4.1717) (25) using the packages tidyverse and dplyr (26, 27). Metadata from mothers and infants were separated from one another for downstream analysis. Within the infant samples, all-time points were filtered into separate metadata files. Since infants had samples carried out in multiple time points, duplicate mothers were filtered out in the mother data frame. Mothers were then categorized into two categories older mothers (M-O), for those aged 30 and above, and younger mothers (M-Y), for those below the age of 30. These same maternal age categories were applied to the infant samples by merging them based on their shared anonymized names that connect mother-infant dyads. The processed metadata for mothers and infants of 2 weeks, 2 months, 4 months, 6 months, and 9 months were exported from R and uploaded to an external server where microbiome analysis was performed. These filtration steps brought the number of unique mothers down to 74, the number of infants at 2 weeks to 54, and the number of infants at 2 months to 60. Furthermore, the same filtering above was performed on the manifest file for sequence importation which together with the metadata form the foundation for the microbiome analysis through QIIME2 (23).

QIIME2 Sequence Preparation. Sequence data was imported into each respective directory using the necessary filtered manifest files using QIIME2 (23). Sequence files were then denoised and quality control was applied across them using DADA2 (28). No truncating of reads was necessary given the high quality of reads as indicated by a median quality of above 30 for all sequence categories. Once all reads were processed, each respective timepoints' sequence data had its sampling depth determined based on alpha-rarefaction plots that showed a plateauing of observed features at that depth. The rarefaction depth selected for mothers, infants at 2 weeks, and infants at 2 months were 20000, 24960, and 30000, respectively. This was then applied to all downstream analyses.

Diversity Metric Generation. QIIME2's core diversity metrics command was used to generate the full suite of alpha and beta diversity metrics for all respective categories. This was done based on the representative sequences from DADA2 (28) earlier along with a reference database SILVA (29) to construct a phylogeny tree which is then combined with the features table output of DADA2 (28) to obtain a wide range of both alpha and beta diversity metrics. The significance of these metrics was determined by statistical tests intrinsic to each form of diversity metric within the QIIME2 visualized files with the Kruskal-Wallis test used for alpha-diversity and the PERMANOVA test for beta-diversity. Those that were found to be significant were visualized as plots using QIIME2's visualization feature through the qzv files obtained from the diversity metrics command.

Taxonomic Analysis. Sklearn machine learning (30, 31) was used to train a classifier based on the SILVA database (29) to allow for taxonomic assignment of all sequence samples up to the level of species. From this taxonomic organization, a taxonomic barplot was generated to visualize the taxonomic diversity within sequence samples. The taxonomic barplot also

had a corresponding csv file which listed the abundance of different taxonomic groups across categories. This was then processed in R to collapse all samples in each maternal age category for the abundance of the listed taxonomic groups. This was transformed into a mean taxonomic abundance value for all taxonomic groups across the 2 maternal age categories. Taxonomic groups with a mean abundance value of 2 or lower were filtered out if they were unique to one age category. Those taxonomic groups that were shared were reassigned to the unique group if they had a mean abundance value of 2 or lower and the other age group had 10 times the abundance value. The results of this revealed taxonomic groups that were unique to one maternal group based on a mean abundance value of 0 in the other maternal age category. Those taxonomic groups with abundance values above 0 for both categories were considered shared taxonomic groups. The resulting analysis was used to generate a Venn diagram using the R package VennDiagram (32) that showed taxonomic groups that are shared or unique to each category of interest. Lastly, the most abundant taxonomic groups in each of the two groups were determined.

Correlation Analysis. The infant dataset had rich metadata that included many quantitative health metrics which could be correlated with absolute maternal age. To investigate potential health associations to maternal age, 64 quantitative health variables were examined with the top 7 health variables being of particular interest due to their relationship with eating behaviour (**Table S1**). Using R and linear regression analysis through Spearman's Correlation Test, given the distribution of maternal was uneven, all quantitative health variables within the 2 months infant categories were looked at. Data wrangling of the many quantitative variables was done using the R package tidyverse (26). The 7 p-values were then adjusted using a Benjamini-Hochberg adjustment. Those with an adjusted p-value < 0.05 were visualized and plotted using the R package ggplot2 (33) to show the statistically significant correlations with maternal age within the 2-month infant categories.

RESULTS

Maternal age is a significant contributor to microbial diversity in the maternal gut microbiome. To determine if maternal age has a significant effect on the maternal gut microbiome, we performed alpha and beta diversity analyses across the maternal age categories.

A statistically significant difference was found across all alpha and beta diversity metrics for the two maternal age categories that were examined including Shannon Diversity, Observed Features, Pielou's Evenness, Jaccard Distance, Bray-Curtis Distance, and the Unweighted Unifrac Distance (Figure S1). Faith's Phylogenetic diversity and Weighted UniFrac distance were then chosen as representative plots to highlight the microbial diversity differences that exist across the age categories. When performing Faith's Phylogenetic diversity, we found a significant difference in gut microbial diversity of the M-O group compared to the M-Y group (Figure 1A). When performing Weighted UniFrac distance, we were also able to determine that the M-O had a significantly different microbiome compared to the M-Y group (Figure 1B). Older mothers were thus found to have greater gut microbial diversity.

Maternal age is not a significant contributor to microbial diversity in the infant gut microbiome at two weeks. To determine if maternal age has a significant effect on the infant gut microbiome at the two-week period, we performed alpha and beta diversity analysis using Faith's Phylogenetic diversity and Weighted UniFrac distance.

We found a small decline in the microbial diversity of infants belonging to the M-O group compared to infants belonging to the M-Y group. Although there was a slight decline, we found no significant differences in the gut microbiome of infants when calculating Faith's Phylogenetic diversity or Weighted UniFrac distance (Figure 2A and 2B). In fact, there were no statistically significant differences in microbial diversity across any alpha or beta diversity metrics generated from the analysis (Figure S2).

Maternal age is a significant contributor to microbial diversity in the infant gut microbiome at two months when comparing phylogenetic relatedness. To determine if maternal age has a significant effect on the microbial diversity of the infant gut microbiome

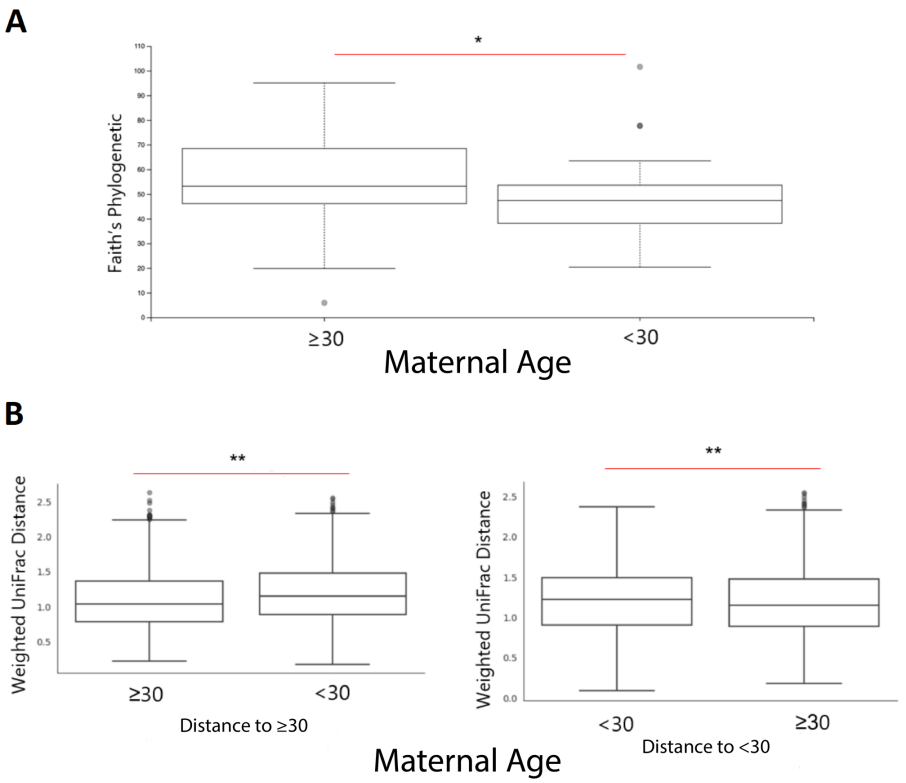

FIG. 1 Mothers aged 30 and above have a significantly different microbiome compared to mothers below 30. Comparing microbial diversity of mothers aged 30 and above to mother below the age of 30 (n = 74) using alpha and beta diversity metrics. Samples from the two groups show statistically significant results using **(A)** Faith's Phylogenetic diversity (p-value < 0.05) and **(B)** Weighted UniFrac distance (p-value < 0.05). Statistical significance was determined by a Kruskal-Wallis test for alpha-diversity, and a PERMANOVA test for betadiversity.

at two months, we performed alpha diversity metrics, Faith's Phylogenetic diversity and Pielou's Evenness, and beta diversity metrics, Unweighted UniFrac and Weighted UniFrac distance.

From our results, we found that infants at two months did not show any significant differences in their microbiome when comparing infants from M-O and those from M-Y when using metrics such as Pielou's Evenness and Weighted UniFrac distance (Figure 2C and 2D). However, there was a significant difference in the microbiome of infants from M-O compared to infants from M-Y when using Faith's Phylogenetic diversity (Figure 2E) and Unweighted UniFrac distance (Figure 2F). A major difference between these metrics is the consideration of phylogenetic relatedness and abundance. Faith's Phylogenetic diversity and Unweighted UniFrac take phylogenetic distance without abundance into account. Furthermore, Pielou's Evenness only considers abundance while Weighted UniFrac distance considers abundance and phylogenetic distance. Our results suggest that taxonomic groups unique to each category of infants at 2 months are the driving factor for significant differences.

Distinct bacterial species emerge based on maternal age. To determine if taxonomic groups drive the difference in significance among diversity metrics based on phylogenetic relatedness rather than abundance, we carried out an analysis in R using the taxonomic csv file from the taxa bar plot (24). From this, we were able to determine the unique and shared taxonomy groups with respect to maternal age following a filtration step of removing taxonomic groups of mean abundance values of less than 2. We noted 15 taxonomy groups specific to infants belonging to the M-O group, 33 taxonomy groups unique to infants from the M-Y group, and 61 shared by infants from both groups (Figure 2G). Lastly, the 5 most abundant taxonomic groups unique to each group were determined (Table S3). Functional analysis of these taxonomic groups only revealed one relevant group, the Lachnospiraceae, while the others were commensal or had no clear role.

Increased maternal age is correlated with decreased infant food responsiveness, increased slowness to eat, and decreased number of sucks per feeding session. To explore if maternal age affects other health outcomes, we evaluated the correlational strengths between maternal age and all other quantitative health outcomes relating to feeding behaviour within the dataset.

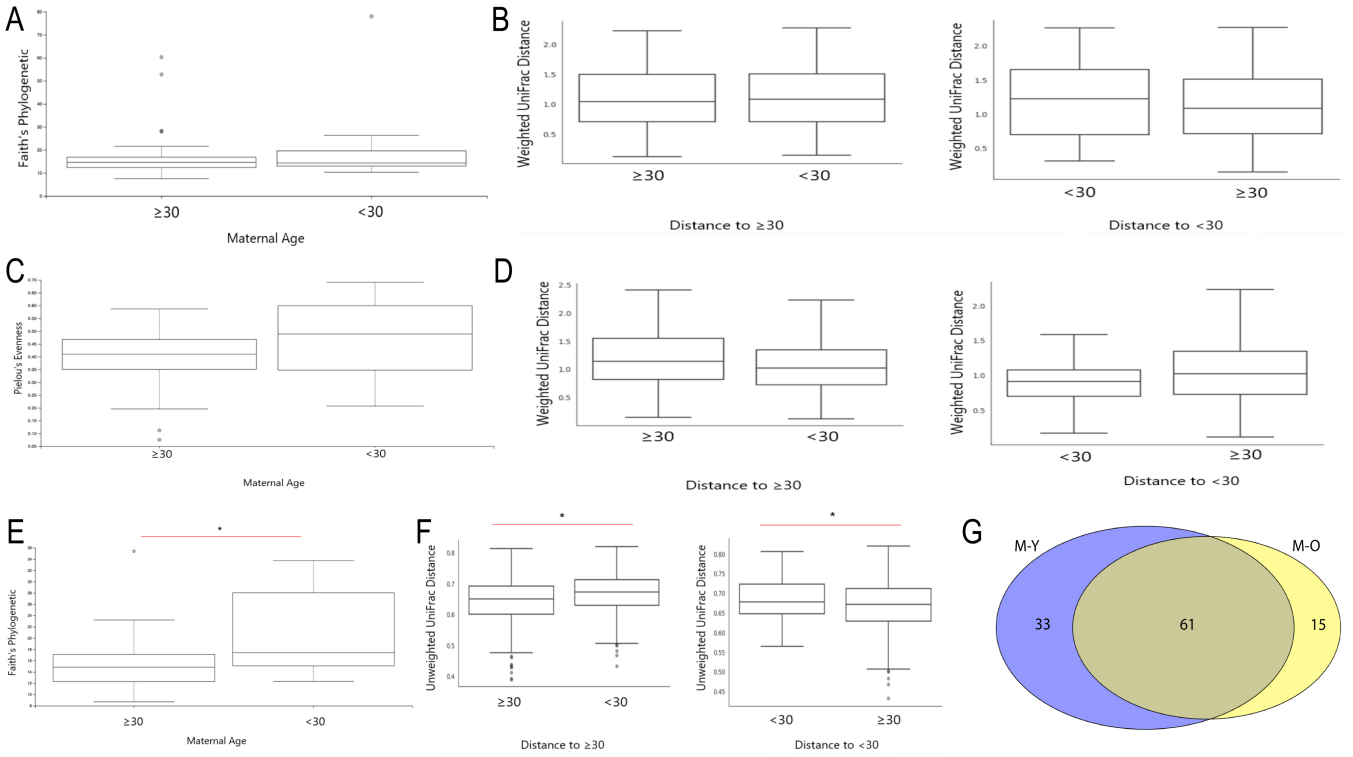

FIG. 2 Infants born to older mothers show statistically significant differences in their microbiome compared to infants born to younger mothers at 2 months but not at 2 weeks when comparing phylogenetic relatedness with unique and similar taxonomic groups being observed between them. Comparing microbial diversity of infants with mothers aged 30 and above to infants with mother below the age of 30 at 2 weeks (n = 54) using alpha and beta diversity metrics. Samples from the two groups do not show statistically significant results using **(A)** Faith's Phylogenetic diversity (p-value > 0.05) and **(B)** Weighted UniFrac distance (p-value > 0.05). For the same comparison at 2 months (n=60), we did not find significant results for **(C)** Pielou's Evenness (p-value > 0.05) and **(D)** Weighted UniFrac distance (p-value > 0.05). However, we did find significant results for **(E)** Faith's Phylogenetic diversity (p-value < 0.05) and **(F)** Unweighted UniFrac distance (p-value < 0.05). Further examination of the taxonomic groups yielded a venn diagram **(G)** generated from R using taxonomic abundance data shows the number of taxonomic groups that are unique to infants with mothers aged 30 and above (n = 90) compared to infants with mothers below the age of 30 (n = 84) as well as taxonomic groups that are similar to both groups (n = 127). Statistical significance was determined by a Kruskal-Wallis test for alpha-diversity, and a PERMANOVA test for beta-diversity.

The resulting correlational strengths were listed by increasing p-value (Table S1) obtained through a Spearman's correlational test which revealed 3 correlations that were significant with maternal age.

Increased maternal age was found to be correlated with decreased infant food responsiveness in a significant manner (Figure 3A). Infant food responsiveness is a qualitative score scored using a five-point Likert frequency scale (1 = never, 2 = rarely, 3 = sometimes, 4 = often and 5 = always) (34). The final score is computed through a 5-item questionnaire (Table S2) given to mothers who submit the score at the 4-month point. The 5 components that make up the score include consistent desire in infants to continue feeding even when full, infant desire to always be fed, infant desire to be fed 30 minutes after the last feed, infant fussiness when not fed, and overfeeding of breastmilk all of which are scored by the mothers themselves (35). The trend that is observed suggests that as maternal age increases, infants at 4 months see a decrease in their food responsiveness.

Maternal age was also found to be correlated with increased slowness to eat in a significant manner (Figure 3B). Infant slowness to eat is a qualitative score scored using a five-point Likert frequency scale. The final score is computed through a similar 4-item questionnaire (Table S2) given to mothers to score. In the case of slowness to eat, the 4

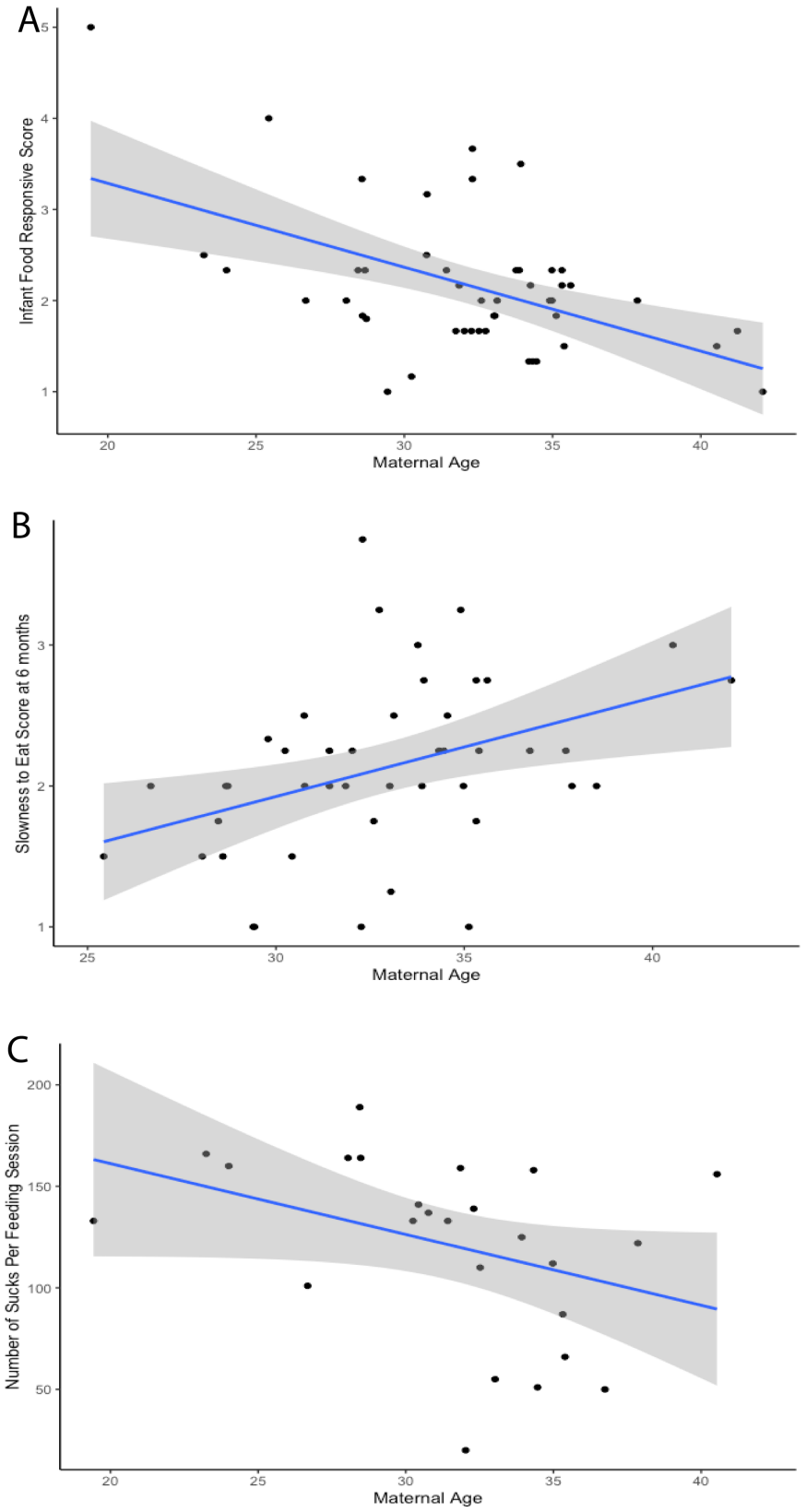

FIG. 3 Increased maternal age is correlated with changes in eating behaviour. Correlational analyses using Spearman correlation tests within the 2-month infant category (n = 60) revealed significant relationships between increased maternal age and decreased infant food responsiveness at 4 months **(A)**, increased slowness to eat at 6 months**(B)**, and decreased number of sucks per feeding session at 2 months **(C)**. Statistical significance for all relationships were p-value < 0.05 through a Spearman correlation test. Note that not all infants had data for all 3 eating behaviour factors leading to discrepancies in the x-axis limits.

components that make up the score include overall slowness to eat, feed durations that last more than 30 minutes, the rate of breast sucking during the feed, and how often feeds are completed quickly which are all scored by the mothers themselves (35). The trend observed

here suggests that infants born to older mothers are significantly slower at feeding compared to those born to younger mothers.

Lastly, an interesting negative correlation was found between maternal age and the number of sucks an infant does per feeding session (Figure 3C), which could be a potential by-product of decreased food responsiveness that was correlated earlier. A decrease in the number of sucks per feeding session suggests decreased input of food which may have effects on other health indicators such as weight. While age-normalized weight at 12 months was shown to trend downwards with increased maternal age this was not found to be statistically significant (Table S1. These trends seem to suggest that older mothers tend to have infants that have a reduced number of sucks per feeding session while those born to younger mothers have slightly higher number of sucks per feeding session which may have implications for weight.

DISCUSSION

The principal goal of this investigation was to examine the impact of maternal age on the infant gut microbiome as poor health outcomes are associated with increased maternal age. This question was investigated by looking at the impact of maternal age on the maternal and infant gut microbiota, as well as infant health factors. We approached this question by performing 16S rRNA gene amplicon analysis using the QIIME2 (23) pipeline and R (24).

We found that there is an increase in maternal microbial diversity for the M-O group compared to the M-Y. This increase in diversity with maternal age has been shown in the literature with mothers aged 20-45 (37). Our finding prompted us to investigate the impact of maternal age on the infant gut microbiota because we hypothesized that there might be a difference carried over from what was observed within the mothers themselves since the maternal gut microbiota is known to impact the infant gut microbiota during breastfeeding and pregnancy (38).

We found a small decline in the microbial diversity of infants from M-O compared to those from M-Y at 2 weeks, however, this decline was not significant. These results suggest that although maternal age influences the microbial diversity of the mother's gut microbiome, this diversity does not translate to their infants at two weeks. This is contradictory to what we hypothesized would occur; we hypothesized that maternal age would result in a significant difference in the diversity of the infant gut microbiome since its establishment is partially linked to that of the mothers as indicated by the similar bacterial species found on the placenta, amniotic fluid, and infant meconium (39). In our case, it may be that two weeks is an insufficient period to see a difference or that the infant gut microbiota is more heavily impacted by an infant's diet rather than what is transferred from the mother until childbirth.

When we moved onto the infant gut microbiome at 2 months, there were significant differences in the infant gut microbiome between infants of the M-O and M-Y groups when it came to metrics that did not consider abundance (Faith's Phylogenetic diversity and Unweighted UniFrac distance). Unexpectedly, the infants from the M-Y group had higher levels of diversity as opposed to what was seen among the mothers where the M-O group had higher levels of diversity. This seems to indicate that while maternal age does affect gut microbial diversity it is not simply a recreation of the pattern seen in mothers. This would likely require further investigation to see if levels of diversity in mothers independently affect gut microbiota diversity.

Thus, our results suggest that the differences in the microbiome of infants from the M-O and M-Y groups are due to phylogenetic relatedness between taxonomic groups and not differential abundance. This prompted taxonomic investigation with the hypothesis that unique taxonomic groups amongst maternal groups may be driving the significance of specific metrics. Taxonomic analysis was used to identify taxonomic groups that were shared or unique to each of the two groups. Importantly we opted not to carry out differential abundance because of the concern that the taxonomic groups that were driving the microbial diversity difference were very rare and that they would likely be filtered out.

We were able to number and identify the unique and shared taxonomic groups with respect to maternal age. Delving deeper, we wanted to see if these taxonomy groups had any functional relevance with regard to their respective age category. After selecting the 5 most

abundant taxonomy groups that were unique to each maternal age category, we discovered only Lachnospiraceae had functional relevance, whereas the others were commensal or had an unclear role based on literature **(Table S3)**. It was uncovered that members of the Lachnospiraceae family may protect against colon cancer in humans by producing butyric acid (40). One study identified that those children born to mothers in older age groups had a 13-36% higher risk of pediatric cancer compared with children born to mothers aged 20-24 years (41). This opens the door for experiments that investigate whether Lachnospiraceae plays a role in the differences observed in pediatric cancer rates between children born to mothers in older age groups versus children born to mothers aged 20-24 years.

It was observed that as maternal age increases, infants at 4 months see a decrease in their food responsiveness and are significantly slower at feeding compared to those born to younger mothers. Additionally, it was found that older mothers tend to have infants that have reduced number of sucks per feeding session while those born to younger mothers have higher number of sucks per feeding session.

One plausible explanation of increased maternal age being correlated with decreased infant food responsiveness increased slowness to eat, and decreased number of sucks per feeding session is the possibility of differing maternal feeding practices. Maternal feeding practices including controlling or monitoring food intake and pressuring eating are known to influence young children's eating behaviour and weight (42). Although no literature could be found that investigated the link between maternal feeding style and age, it is plausible that the maternal age of mothers in the dataset is impacting maternal feeding style and subsequently having an impact on infant eating behaviour and potentially weight. As of now, it is difficult to say whether this is the case, as maternal feeding style is not a variable that was included in this dataset which is a factor that could be documented in future experiments. The potential impact of breastfeeding versus formula feeding was also not possible due to only 4 infants having formula only feeding. If more infants with formula feeding had been in the dataset, then a comparison of the two feeding groups based on maternal age could've been done. This would've allowed us to see whether the alteration of feeding behaviour seen correlated with maternal age could be explained by differing feeding styles across younger and older mothers. This is especially informative given the influence that breastmilk has on the gut microbiota and its development.

Although the correlations within these results range from being weak to moderate in strength, they are significant and with the supporting literature, they signify plausible relationships that may lack a clear-cut signal due to data limitations. Such limitations could be confounding variables like socioeconomic status, education, type of interaction with mother, and lack of data at polar ends of maternal age.

Limitations The main limitations of our paper are the low sample sizes for infants due to discontinued data collection at later time points, low variation in maternal age and the inability to perform differential abundance on low abundant taxonomic groups. When calculating alpha and beta diversity metrics for infants at later time points, we were forced to accept results with low statistical power due to the discontinuation of data collection in metadata categories at later time points. The sample size of infants at six months and nine months was 21 and 5, respectively, which may have had an impact on the significance of microbial diversity in these infants. Another limitation that was true for our correlational analysis was that the bulk of our maternal age dataset was between 30-35 while those between 20-25 and above 40 had limited data points with only 3 mothers above 40 and only 7 below the age of 25. As a result of this limitation, we were not able to conclude with full confidence that infants have altered eating behaviour based on maternal age differences given the data was highly centered. Having additional data points at these extremes would provide a more complete picture of the relationship between eating behaviour and maternal age. Lastly, we could not perform differential abundance analysis on infants at 2 months to identify rare taxonomic groups within each infant category. We learned that maternal age at two months had a significant effect on microbial diversity of the infant gut microbiome due to rare taxa and not abundance which we further wanted to test with a differential abundance plot. However, as a concern with the regular differential abundance methods being insensitive to rare taxa, we were not able to perform this analysis. To troubleshoot this, we downloaded the

CSV file from our taxa bar plot and created a Venn diagram presenting taxonomic groups that were similar and unique to each category. A more robust approach to solve this for a future experiment is an indicator taxa analysis used to identify indicator taxa among levels of a categorical variable (43).

Conclusions This study investigated the effects of maternal age on the gut microbiome of mothers and their infants. Not only was a significant difference found in the gut microbiome of mothers based on maternal age, but this difference translated into that of infants sampled at 2 months. Furthermore, correlational analyses showed that maternal age is correlated with decreased food responsiveness, increased slowness to eat, and decreased number of sucks per feeding session in infants. This suggests that maternal age may be driving changes in feeding behaviour that affects the gut microbiota. The study lends support to the previously unexplored idea that maternal age can affect the gut microbiota of mothers and infants alike due to previously studied associations with health outcomes.

Future Directions To see why 2-month infant microbiomes are significantly different based on maternal age, we can longitudinally examine the taxonomic composition of infants across all time points to see changes in the microbiome. Such a longitudinal investigation could quantify the abundance of different taxonomic groups across all time points to see what is lost and gained over time and to explore the biological relevance of these changes. Furthermore, such an analysis can lend support to the idea that at 2 months the degree of contact with the mother and the resulting gut colonization is maximal while at 2 weeks gut microbial colonization is still in its beginning phase, so the effect of maternal age is minimal. The time points beyond 2 months sees the introduction of solid food, greater contact with other objects as greater motor skills are obtained (44), and increased contact with individuals besides the mother so the effect of maternal age is potentially lost.

The effect of maternal age can also be looked at using much smaller age brackets such as 5-year age brackets to examine whether the relationships that were found in this paper are still upheld. The difficult aspect of this however is the lack of samples within these age brackets. As seen in the correlational analyses, there is a general lack of samples from below the age of 25 and for those above the age of 40 which would make such an analysis more difficult to carry out. The dataset only had 7 Mothers under the age of 25 and only 3 mothers above the age of 40 and given that not all mothers had data for every metric the number of usable data further decreased. Nevertheless, it might reveal more granular differences across different maternal age brackets especially if a longitudinal approach is applied for gut microbial composition as maternal age increases.

Lastly, a grander study that integrates a more comprehensive background on mothers besides their maternal age may reveal other factors that play a role in the changing feeding behaviour in infants. It could consider aspects such as education, socioeconomic status, address, employment status, and more to understand if maternal age is the only factor that is correlated with changes in feeding behaviour in infants.

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CONTRIBUTIONS

Data analysis and manuscript writing were done collectively between Ali Anwari, Nemat Haroon and Jaskirat Malhi. Ali Anwari performed the alpha and beta diversity analysis on the mother and infant gut microbiome and wrote the abstract, introduction and limitation section of the manuscript. Nemat Haroon organized the metadata information, performed correlational analysis between maternal age and several other infant metadata categories and generated a Venn diagram to identify unique taxonomic groups. He also wrote the methods and materials, conclusion, and future directions section in the manuscript. Jaskirat Malhi performed taxonomic analysis and wrote the discussion and reference section of the manuscript. The results section was done together.

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