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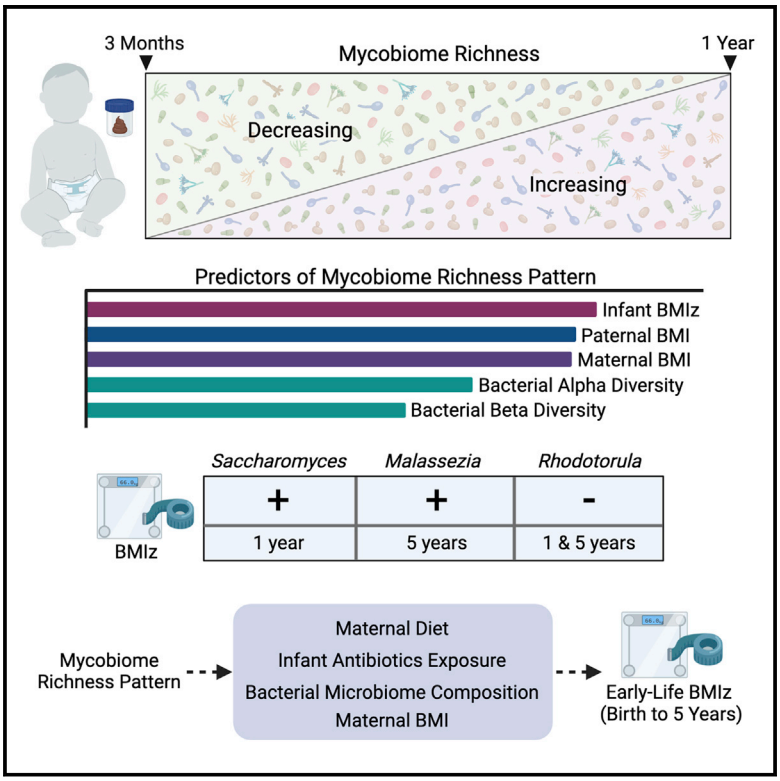
Maturational patterns of the infant gut mycobiome are associated with early-life body mass index

Graphical abstract



Highlights

- Divergent gut mycobiome maturation patterns are observed in the first year of life
- Mycobiome maturation patterns are associated with metabolism-related factors
- Specific fungal taxa are differentially associated with BMIz in early childhood
- Mycobiome influences on early childhood BMIz are likely mediated by gut bacteria

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In brief

Gutierrez et al. show that contrasting gut mycobiome maturation patterns in early life are linked to obesity-related factors, including associations between specific fungal taxa and BMI in early childhood. The influence of the gut mycobiome on BMI appears to be dependent on the bacterial gut microbiome.

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Report

Maturational patterns of the infant gut mycobiome are associated with early-life body mass index

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SUMMARY

Unlike the bacterial microbiome, the role of early-life gut fungi in host metabolism and childhood obesity development remains poorly characterized. To address this, we investigate the relationship between the gut mycobiome of 100 infants from the Canadian Healthy Infant Longitudinal Development (CHILD) Cohort Study and body mass index *Z* scores (BMIz) in the first 5 years of life. An increase in fungal richness during the first year of life is linked to parental and infant BMI. The relationship between richness pattern and early-life BMIz is modified by maternal BMI, maternal diet, infant antibiotic exposure, and bacterial beta diversity. Further, the abundances of *Saccharomyces*, *Rhodotorula*, and *Malassezia* are differentially associated with early-life BMIz. Using structural equation modeling, we determine that the mycobiome's contribution to BMIz is likely mediated by the bacterial microbiome. This demonstrates that mycobiome maturation and infant growth trajectories are distinctly linked, advocating for inclusion of fungi in larger pediatric microbiome studies.

INTRODUCTION

The developmental origins of health and disease paradigm hypothesizes that early-life events have long-lasting health impacts, including on the development of gut microbial communities, metabolic programming, and eating behavior,[1–3] all of which are implicated in the pathogenesis of obesity in children.[4] Childhood obesity rates are concerning, with 15% and 22% of Canadian and American children and adolescents classified as obese, respectively.[5,6] The bacterial gut microbiome has been well established to play an important role in host metabolism,[7] with studies finding strong associations between infant gut microbiome composition, infant growth trajectories,[8,9] and the risk of becoming overweight or obese in prospective birth cohorts,[10–15] as well as causal links confirmed in experimental models.[16–20] However, little is known about the role of gut fungal colonizers in these events as inherent members of this complex ecosystem.

The infant gut microbiome develops through a dynamic process of stepwise successions beginning at birth,[21,22] with strong links to host metabolism.[23,24] While descriptions of these early-life patterns are limited for gut fungi (collectively termed the gut mycobiome), there is evidence that fungi are also integrated in defined maturation patterns.[25–31] Infants are initially colonized by *Candida*, *Malassezia*, *Cladosporium*, and *Saccharomyces* and then exhibit communities abundant in *Debaryomyces*, *Candida*, *Malassezia*, and *Cladosporium* around 3 months of age.[25,27,28,30–34] From 1 year of age onward, the mycobiome shifts toward *Saccharomyces* dominance, alongside an increased presence of *Rhodotorula*.[25,27–29,31] These maturational patterns likely arise from an expanding variety of

Table 1. Participant characteristics of mother-infant dyads from the CHILD Cohort Study included in this analysis (n = 100)

Characteristics	n (%) or mean ± SD
Infant sex (female)[a]	46 (46%)
Mode of birth (vaginal)	64 (64%)
Prenatal antibiotics	12 (12%)
Intrapartum antibiotics	50 (52%)
Infant antibiotic exposure (6–12 months)	16 (16%)
Breastfeeding status at 3 months	
None	36 (36%)
Partial	30 (30%)
Exclusive	34 (34%)
Breastfeeding duration (months)	7.81 ± 7.29
Introduction of solid foods by 6 months	70 (70%)
Birth BMIz[b]	
Normal[c]	60 (60%)
Risk of becoming overweight/overweight	5 (5%)
Unknown	35 (35%)
3-month BMIz	
Normal[d]	87 (87%)
Risk of becoming overweight/overweight	10 (10%)
Unknown	3 (3%)
1-year BMIz	
Normal	64 (64%)
Risk of becoming overweight/overweight[e]	34 (34%)
Unknown	2 (2%)
3-year BMIz	
Normal[f]	54 (54%)
Risk of becoming overweight/overweight	31 (31%)
Unknown	15 (15%)
5-year BMIz	
Normal	59 (59%)
Risk of becoming overweight/overweight[g]	29 (29%)
Unknown	12 (12%)
Study site	
Vancouver	40 (40%)
Edmonton	22 (22%)
Manitoba[h]	29 (29%)
Toronto	9 (9%)
Maternal HEI	72.06 ± 8.14
Maternal AS beverage consumption during pregnancy	50 (50%)
Maternal BMI	26.52 ± 6.10
Paternal BMI	28.52 ± 4.54

AS, artificially sweetened; BMI, body mass index; BMIz, BMI *Z* score; HEI, healthy eating index; SD, standard deviation. See Tables S1 and S2 for additional information.

[a]Infant sex is based on sex assigned at birth.

[b]Normal is defined as a BMIz of 1.0 or less. Risk of becoming overweight/overweight is defined as a BMIz greater than 1.0.

[c]Includes 4 participants classified as underweight (BMIz < −2.0).

[d]Includes 5 participants classified as underweight (BMIz < −2.0).

[e]Includes 2 participants classified as obese (BMIz >3.0).

[f]Includes 1 participants classified as underweight (BMIz < −2.0).

[g]Includes 2 participants classified as obese (BMIz >3.0).

[h]Includes Winnipeg and two rural sites, Morden and Winkler.

exposures over the course of early life, with a 3-month-old infant having greater exposure to skin microbes, such as *Malassezia*, through breastfeeding and frequent skin-to-skin contact with caregivers.[30,34] In contrast, with the introduction of solid foods and development of crawling behavior, infants are exposed to a greater number of fungi with food or environmental origins, such as *Saccharomyces* and *Fomitopsis*.[27,30] From a diversity perspective, the patterns of mycobiome maturation are inconsistent across studies, with reports of increasing,[25,29,30] decreasing,[28,35] and unchanged or fluctuating[27,31,33,34] alpha diversity metrics over the course of early life. Similarly, differential associations between fungal beta diversity and infant age have also been reported.[27–31,33] Beyond these patterns of infant gut mycobiome maturation, it is not well understood how host and environmental factors influence gut mycobiome establishment and how this may subsequently impact infant growth trajectories.

Recent evidence in adults highlights the potential role of the gut mycobiome in obesity. For example, obesity has been associated with a reduction in mycobiome diversity; the relative abundance of *Rhodotorula mucilaginosa* has been negatively correlated with body mass index and visceral adiposity; and selective expansion of *Mucor* has been observed in adults undergoing diet-induced weight loss.[24,36,37] Mouse models have demonstrated causal contributions of the gut mycobiome to host energy balance, with one study highlighting the potential for *Candida parapsilosis*-derived lipases in enhancing fatty acid harvest from a high-fat diet.[38] While these findings have provided some insights into the role of certain mycobiome features in nutrient metabolism,[39] our understanding of infant gut mycobiome development and its role in childhood obesity is limited. To address this knowledge gap, we defined mycobiome maturation patterns in the first year of life and evaluated their association with body mass index *Z* score (BMIz) during the first 5 years of life in a sub-cohort of 100 of the 3,264 infants followed in the Canadian Healthy Infant Longitudinal Development (CHILD) Cohort Study.[40] By evaluating ecological metrics at the individual level and accounting for the effects of covariates relevant to microbiome maturation and metabolism, we identified a distinct relationship between infant mycobiome maturational patterns and early childhood growth.

RESULTS

Participant characteristics

We carried out this work as a secondary analysis of a previously reported case-control study on the relationship between maternal consumption of non-nutritional sweetener, early-childhood BMIz, and the bacterial microbiome.[13] The sub-cohort consisted of 100 infants with a rich dataset that allowed us to study other relevant associations, including between the gut mycobiome and BMIz. In this sub-cohort, 46% of the infants were female based on sex assigned at birth, with 64% of infants delivered vaginally. Prenatal and postnatal antibiotic exposure was limited to 12% and 16%, respectively, but intrapartum antibiotic exposure occurred in half of the cohort. At 3 months, breastfeeding status was classified as none (36%), partial (30%), or exclusive (34%), and the mean overall breastfeeding duration was

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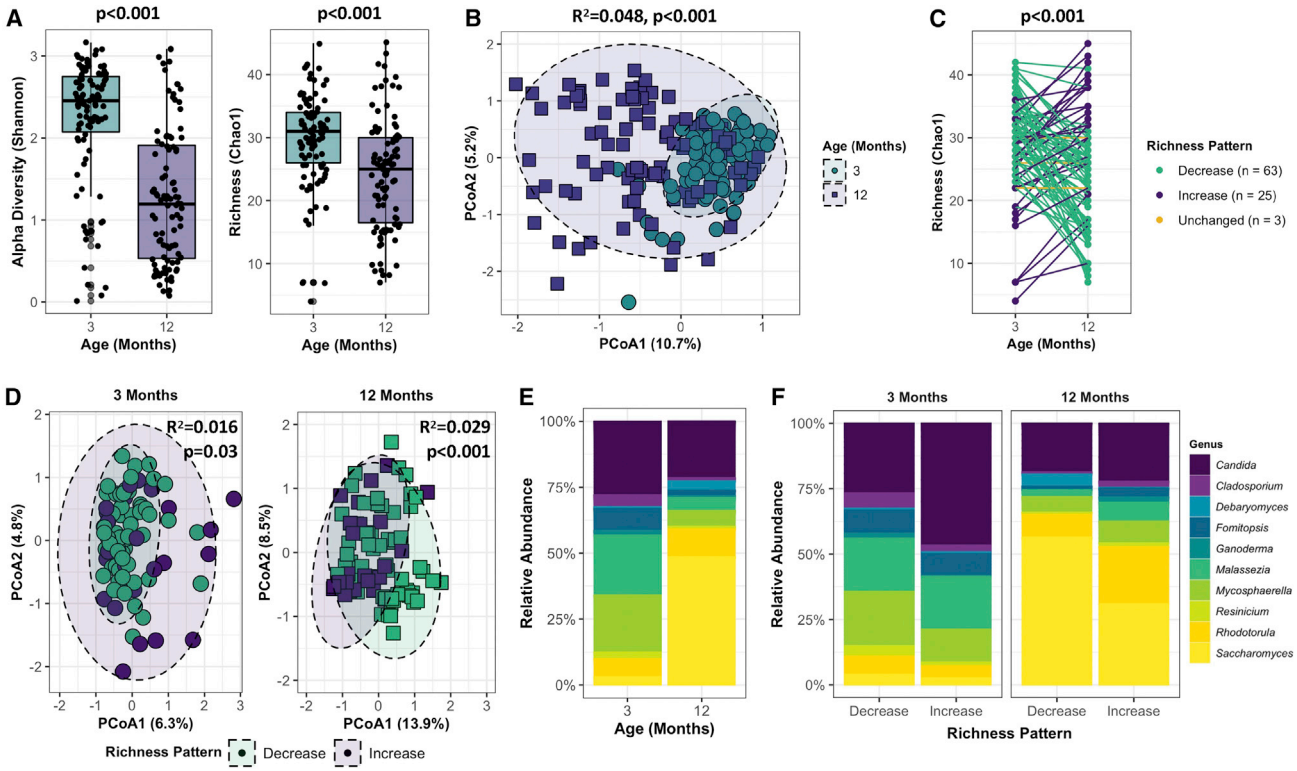

Figure 1. Divergent fungal richness patterns are observed in the first year of life that exhibit compositional and structural differences at 3 and 12 months of age

(A) Fungal Shannon and Chao1 alpha diversity indices at 3 and 12 months, assessed by Mann-Whitney U test (3 months: n = 95, 12 months: n = 95). Boxplots show median and interquartile range.

(B) Comparison of mycobiome beta diversity at 3 and 12 months based on Bray-Curtis dissimilarity indices, assessed by permutational multivariate analysis of variance (PERMANOVA; 3 months: n = 95, 12 months: n = 95). Ellipses represent 95% CI.

(C) Changes in fungal richness (Chao1) per individual from 3 to 12 months, assessed by paired t test (n = 91; see Figure S1 for alpha diversity).

(D) Comparison of mycobiome beta diversity by fungal richness pattern at 3 and 12 months, assessed by PERMANOVA (decrease: n = 63, increase: n = 25; see Figure S1 for alpha diversity). Ellipses represent 95% CI.

(E) Relative abundance of the 10 most abundant fungal genera at 3 and 12 months (3 months: n = 95, 12 months: n = 95).

(F) Relative abundance of the 10 most abundant fungal genera by fungal richness pattern at 3 and 12 months (decrease: n = 63, increase: n = 25). See Figures S1 and S2 for alpha diversity and differential abundance.

7.81 ± 7.29 months. At 6 months, 70% of infants had solids introduced into their diets (Table 1). Infants were grouped into BMI categories based on BMIz cutoffs established by the World Health Organization (WHO);[41] infants with a BMIz of 1.0 or less were classified as normal, while infants with a BMIz greater than 1.0 were classified as at risk of becoming overweight/overweight. BMIz values below −2.0 were classified as wasted under WHO guidelines[41] and were excluded from further analysis. BMIz values greater than 4.0 were also excluded. No significant sex differences in BMI *Z* scores were observed in the first 5 years of life in our sub-cohort (Table S1). Further, when comparing the evaluated covariates between BMI categories (normal vs. risk of becoming overweight/overweight), no significant differences were observed in the first 5 years of life, except for increased maternal and paternal BMI in infants at risk of becoming overweight or overweight at 3 and 5 years (data not shown).

Compared to the rest of the CHILD cohort (n = 3,164), our sub-cohort had a greater proportion of infants born by Cesarean section (C-section) ($p = 0.018$), reduced breastfeeding duration ($p < 0.001$), greater maternal BMI ($p < 0.001$) and paternal BMI ($p = 0.031$), and early childhood BMIz scores. This included a higher BMIz at 3 months in females ($p = 0.049$), at 1 year in both sexes (females: $p = 0.004$, males: $p = 0.005$), and at 5 years in males ($p = 0.008$; females trended toward significance at $p = 0.052$). As anticipated, given the original selection criteria, greater maternal consumption of artificially sweetened beverages during pregnancy was observed in our sub-cohort at 50% compared to 29% in the rest of the CHILD cohort ($p < 0.001$; Table S2). This indicates that our sub-cohort represents a population with higher BMIz, which allowed us to investigate the risk of elevated BMIz in relation to the gut mycobiome in a pan-Canadian cohort.

Divergent maturational patterns are observed in the gut mycobiome in the first year of life

Previous studies examining mycobiome maturation during early life have demonstrated inconsistent findings regarding shifts in

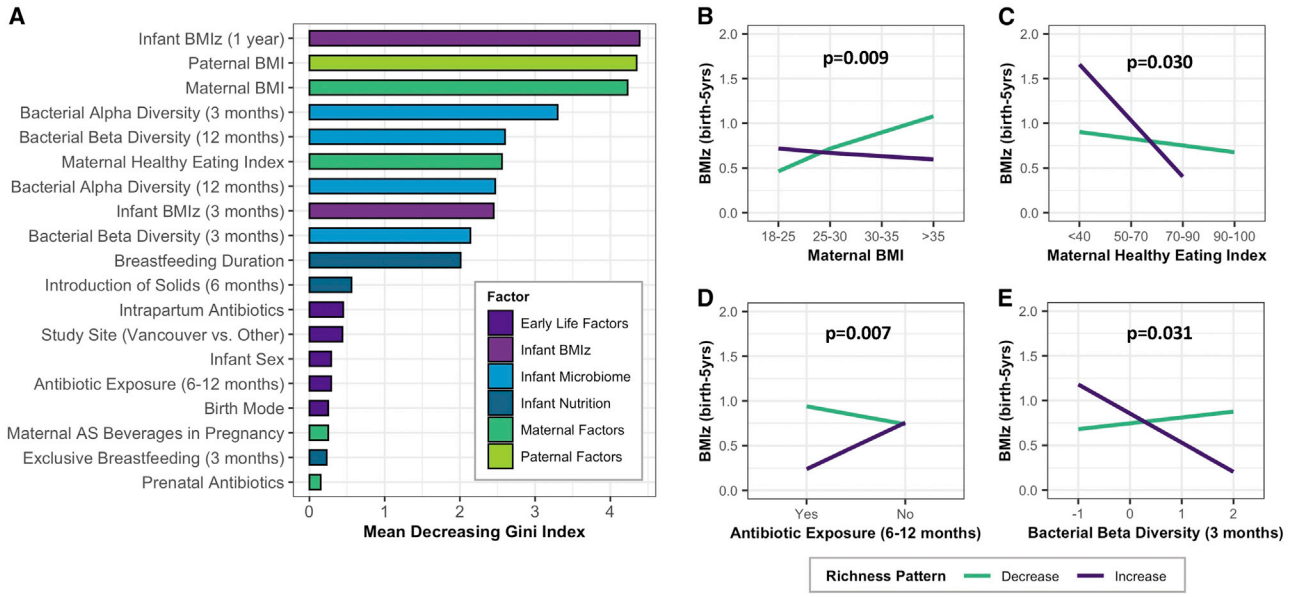

Figure 2. Maternal factors, antibiotic exposure, and bacterial community composition are associated with fungal richness pattern and modify its effect on BMIz in early life

(A) Predictors of fungal richness pattern identified by random forest analysis using 10-fold cross-validation, 500 trees, and 1,000 permutations (decrease: n = 60, increase: n = 23). The decreasing richness pattern was set as the reference level.

(B) Effect modification assessment of maternal BMI on the association of fungal richness pattern with early-life BMIz from birth to 5 years (decrease: n = 63, increase: n = 25).

(C) Effect modification assessment of maternal HEI on the association of fungal richness pattern with early-life BMIz from birth to 5 years (decrease: n = 63, increase: n = 25).

(D) Effect modification assessment of infant antibiotic exposure from 6–12 months on the association of fungal richness pattern with early-life BMIz from birth to 5 years (decrease: n = 63, increase: n = 25).

(E) Effect modification assessment of bacterial beta diversity at 3 months on the association of fungal richness pattern with early-life BMIz from birth to 5 years (decrease: n = 62, increase: n = 25). Regression interaction p values calculated with Stargazer v.5.2.2.[42]

See also Figures S3 and S4 and Table S3. AS, artificially sweetened; BMI, body mass index; BMIz, BMI *Z* score.

fungal alpha diversity over time.[25,27,28,30–33] In the mycobiome of Canadian infants assessed in this cohort, we observed an overall decrease in fungal alpha diversity (Shannon) and richness (Chao1) from 3–12 months of age (Shannon: 2.22 ± 0.76 vs. 1.28 ± 0.83; Chao1: 29.44 ± 7.61 vs. 24.18 ± 8.87; $p < 0.001$; Figure 1A). Significant differences in mycobiome composition were observed by age, accounting for 4.8% of the variance, with mycobiome composition across infants displaying less heterogeneity at 3 months compared to 12 months ($p < 0.001$; Figure 1B). However, upon assessing shifts in the mycobiome at the individual level, we observed differences in fungal richness and alpha diversity patterns from 3 to 12 months (Figures 1C and S1A). Of the 91 infants with available mycobiome data at both time points after bioinformatic quality control (STAR Methods), 63 (69%) displayed decreased, 25 (28%) displayed increased, and 3 (3%) displayed no change in mycobiome richness (Chao1 index) in the first year of life ($p < 0.001$; Figure 1C), with a similar pattern observed for alpha diversity (Shannon index; Figure S1A). These alpha diversity patterns were reflected in the significant compositional differences evident at 3 and 12 months (3 months: $R^2 = 0.016$, $p = 0.03$; 12 months: $R^2 = 0.029$, $p < 0.001$; Figures 1D and S1B). This highlights that the infant gut mycobiome follows varied maturational patterns from a diversity perspective, suggesting that these patterns may be differentially influenced by early-life exposures specific to an individual or characteristic of certain lifestyles.

Taxonomic community structure differs by mycobiome richness pattern

To identify the specific fungal taxa responsible for the compositional differences between the decreasing and increasing fungal richness patterns, we determined the relative abundance of the 10 most abundant genera at 3 and 12 months of age, which represented 82.87% ± 14.09% and 91.01% ± 12.97% of the total communities, respectively. Overall, the mycobiome at 3 months was dominated by *Candida*, *Malassezia*, and *Mycosphaerella* and then shifted toward *Saccharomyces* dominance at 12 months (Figure 1E), in agreement with previous reports.[25–31] Compositional differences by richness pattern only reached significance at 12 months, with the increasing richness pattern displaying increased relative abundance of *Fomitopsis* ($p = 0.02$), *Mycosphaerella* ($p = 0.006$), and *Malassezia* ($p = 0.006$) and reduced abundance of *Saccharomyces* ($p = 0.009$) relative to the decreasing pattern (Figures 1F and S2). The greater maintenance of taxa abundant at 3 months in the mycobiome of infants with an increasing richness pattern at 12 months is suggestive of reduced mycobiome maturity in these individuals.

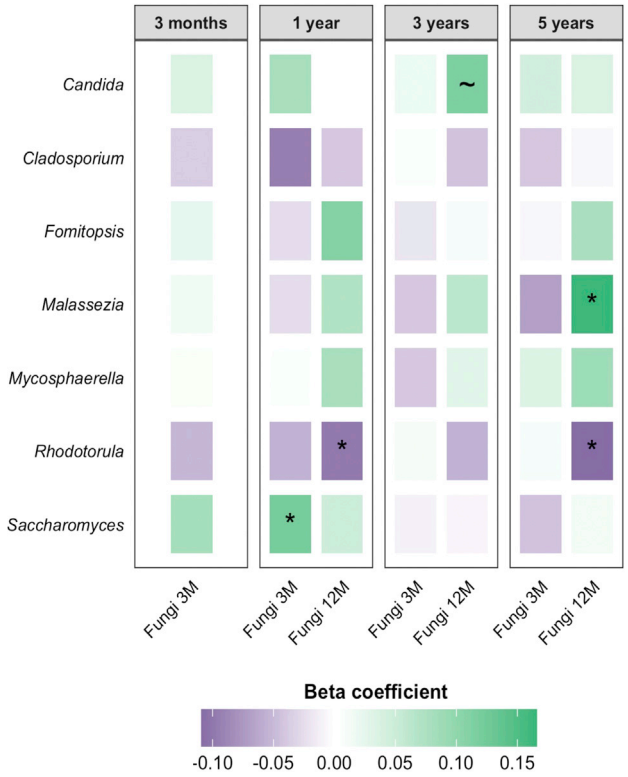

Figure 3. The core gut mycobiome members *Saccharomyces*, *Rhodotorula*, and *Malassezia* are differentially associated with early-life BMIz

Shown is the association of centered log ratio-transformed fungal genera with a minimum 2% mean relative abundance with BMIz from 3 months to 5 years of age using adjusted linear regression permutation tests (n = 95). Covariates known to influence the infant gut microbiota or infant growth (maternal BMI, maternal HEI, breastfeeding status at 3 months, antibiotic exposure from 6–12 months) were included in the models. ~p(false discovery rate [FDR]) < 0.10, *p(FDR) < 0.05. See Figure S6 for species-level associations with BMIz.

Infant BMIz, parental BMI, and bacterial diversity are associated with mycobiome richness pattern in the first year of life

We identified factors associated with mycobiome richness pattern using random forest and multivariable logistic regression analyses. Random forest analysis identified infant BMIz at 1 year, paternal BMI, maternal BMI, bacterial alpha diversity at 3 months, and bacterial beta diversity at 12 months as the top five factors associated with an increasing fungal richness pattern (Figure 2A). Maternal healthy eating index (HEI), bacterial alpha diversity at 12 months, infant BMIz at 3 months, bacterial beta diversity at 3 months, and breastfeeding duration were also strongly associated with fungal richness pattern (Figure 2A). Intriguingly, BMI- and bacterial microbiome-related factors had greater predictive power of mycobiome richness pattern than those commonly associated with bacterial microbiome development, such as delivery mode and exclusive breastfeeding (Figure 2A). Next, we performed logistic regression adjusted for the confounding effects of infant, early-life, maternal, and paternal factors known to influence the microbiome and/or infant growth to try to generate an improved understanding of the probability that a given factor was related to fungal richness pattern. However, we did not detect significant associations between infant BMIz, maternal BMI, or paternal BMI and fungal richness pattern, with only a weak positive association between introduction of solid foods by 6 months and an increasing fungal richness pattern emerging in the model (odds ratio [OR]: 4.363, 95% confidence interval [CI]: 1.050–23.369, p = 0.058; Figure S3). Given logistic regression is best used to identify simple, linear relationships, whereas random forests can handle non-linearity and more robustly reduce noise in the data, this suggests that the associations between an increase in mycobiome richness in the first year of life and metabolism-related factors identified by random forest may be driven by more complex or non-linear relationships.

Maternal factors, antibiotic exposure, and the bacterial microbiome modify associations between mycobiome richness pattern and early-life BMIz

To further explore the relationship between metabolism-related factors and mycobiome richness, we first examined the distribution of early-life BMIz scores (Figures S4A–S4E), maternal BMI (Figure S4F), maternal HEI (Figure S4G), paternal BMI (Figure S4H), and the proportion of infants classified as normal or at risk of becoming overweight/overweight (Figure S4I) between the increasing and decreasing fungal richness patterns. However, we did not observe any significant differences. Given this conflicted with the random forest findings, we carried out effect modification analysis to determine whether more complex relationships were underlying our results. Indeed, factors linked to fungal richness pattern were found to be effect modifiers of the association between richness pattern and BMIz by defining interaction terms in linear regression models (Figures 2B–2E; Table S3). Specifically, an increasing fungal richness pattern was associated with higher early-life BMIz when maternal BMI or HEI were low, while it was associated with lower early-life BMIz when maternal BMI or HEI were high (BMI: $\beta = -0.043$, p = 0.009; HEI: $\beta = -0.028$, p = 0.030; Figures 2B and 2C). In addition, an increasing richness pattern was associated with lower early-life BMIz when an infant was exposed to antibiotics from 6–12 months ($\beta = -0.719$, p = 0.007; Figure 2D). Bacterial community composition (first principal coordinate axis [PCoA1]) at 3 months also strongly interacted with the effect of fungal richness pattern on early-life BMIz ($\beta = -0.391$, p = 0.031; Figure 2E). The remaining factors shown in Figure 2A displayed non-significant interactions with fungal richness pattern (Table S3). It is important to note that these interactions should be interpreted cautiously, given the limited sample size of this sub-cohort. Together, these results highlight the complexity of the relationship between early-life BMIz and mycobiome development, suggesting that this relationship likely involves maternal factors that are associated with metabolic health, such as BMI and diet, antibiotic exposure, and multi-kingdom interactions.

Relationship between increasing richness pattern and BMIz in early life is not sex-dependent

Infant sex is known to be associated with infant growth patterns and microbiome development.[43–46] We next explored whether

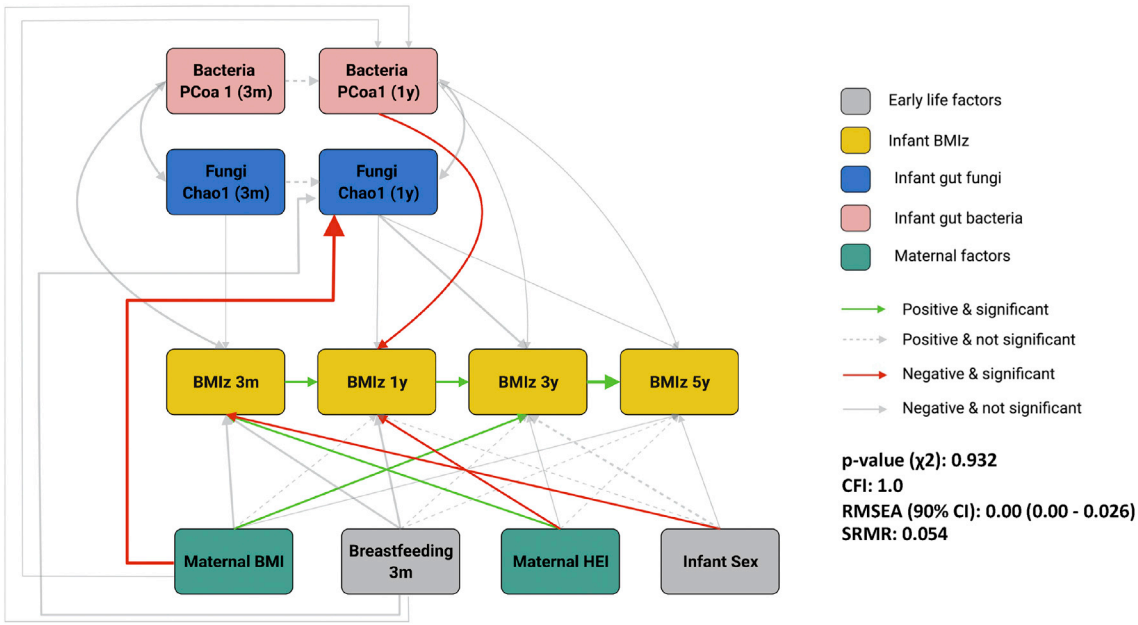

Figure 4. Association between fungal richness (Chao1) and BMIz in early life is dependent on the infant bacterial microbiome

Structural equation modeling was used to differentiate the influence of fungal richness on BMIz while considering the structure of the associations between other early-life factors and bacterial microbiome composition (n = 95). Model fit was assessed by Pearson's Chi-squared ($\chi 2$) test, the comparative fix index (CFI), root-mean-square error of approximation (RSMEA) and its 90% confidence interval (CI), and the standardized root mean residuals (SRMR). Non-significant $\chi 2$ test, CFI $\geq$ 0.9, RMSEA < 0.05, and SRMR < 0.08 were considered as indications of good model fit. The edge width is proportional to standardized β coefficients. HEI, healthy eating index; PCoA1, first principal coordinate axis; green, positive and significant; red, negative and significant; gray, statistically not significant. See also Table S4 for β coefficients and significance by BMIz time point and related factors.

sex-dependent associations existed between fungal richness pattern and BMIz in early life based on infant sex assigned at birth, but we did not detect any significant differences (Table S1). Further, the proportion of infants of either sex that were classified as normal or at risk of becoming overweight/overweight between birth and 5 years of age did not differ (Figure S5A). Infant sex assigned at birth was not associated with mycobiome richness pattern (Figures 2A and S3), and we did not observe a significant interaction effect between richness pattern and infant sex for early-life BMIz outcomes (Figure S5B). This suggests that infant sex was not involved in mediating or moderating the observed association between an increasing fungal richness pattern and BMIz in this sub-cohort.

Abundance of specific fungal taxa in the first year of life is associated with early-life BMIz

Next, we evaluated taxon-specific associations with BMIz in early childhood while controlling for relevant confounding factors. Infant breastfeeding status at 3 months, antibiotic exposure from 6–12 months, maternal HEI, and maternal BMI were included as covariates based on our initial findings (Figure 2) and *a priori* knowledge.[47] We found several fungal taxa at the genus and species levels to be significantly associated with BMIz between 1 and 5 years of age (Figures 3 and S6). The relative abundances of *Saccharomyces* at 3 months and *Malassezia* at 12 months were positively associated with BMIz at 1 and 5 years, respectively (β = 0.122, p = 0.043 and β = 0.166, p = 0.033, respectively; Figure 3), while *Rhodotorula* abundance at 12 months was negatively associated with BMIz at 1 and 5 years (β = −0.099, p = 0.014 and β = −0.109, p = 0.028, respectively; Figure 3). *Candida* abundance at 12 months also trended toward being positively associated with BMIz at 3 years (β = 0.115, p = 0.055; Figure 3). Species-level analysis identified parallel negative associations for the relative abundance of *R. mucilaginosa* at 12 months with BMIz at 1 and 5 years (β = −0.096, p = 0.017 and β = −0.108, p = 0.032, respectively; Figure S6). This suggests that specific fungal genera present in the mycobiome in the first year of life are differentially associated with BMIz outcomes in early childhood.

Association between the gut mycobiome and childhood BMIz is dependent on bacterial gut microbiome composition

A strong association exists between infant gut bacterial community composition and risk of becoming overweight or obese later in life.[10–15] We also identified an association between bacterial composition with the fungal richness pattern in the first year of life (Figures 2A and 2B) and the potential of gut bacteria to modify the association between fungal richness pattern and early-life BMIz (Figure 2E). While we observed associations between infant gut fungi and BMIz in the first 5 years of life (Figure 3), the direct versus indirect association between gut bacteria and fungi in relation to BMIz is unclear. To address this question, we performed structural equation modeling based on the theoretical structure of the associations between the factors included. Our model was based on the assumption of co-variation of gut

bacterial and fungal compositions at each available time point (Figure 4). The mycobiome, as the main focus of this study, was modeled as fungal richness (Chao1), community composition (beta diversity) using the first principal coordinate axis (PCoA1) based on Bray-Curtis dissimilarity or richness pattern in the first year of life (Table S4). The bacterial microbiome was not the focus of this study and, thus, was only modeled as PCoA1 based on Bray-Curtis dissimilarity to represent the overall bacterial microbiome composition. We modeled the direct association of bacteria and fungi on longitudinal BMIz measurements while considering the structure of associations among other important factors known to affect infant BMIz, the gut bacterial microbiome, and/or the gut mycobiome. This included incorporation of maternal HEI, maternal BMI, breastfeeding status at 3 months, and infant sex based on sex assigned at birth into our theoretical framework. We confirmed that BMIz at each time point was positively associated with subsequent BMIz measurements in early life (Figure 4; Table S4). We did not observe any associations between fungal richness, community composition, or richness pattern and BMIz in early life (Table S4). However, bacterial community composition at 1 year was negatively associated with infant BMIz at 1 year ($\beta = -0.241$, $p = 0.010$; Figure 4; Table S4). Thus, our results suggest that the mycobiome's contribution to early-life BMIz is likely influenced by the bacterial microbiome.

DISCUSSION

The infant gut mycobiome exhibits defined maturation patterns over the course of early life from the perspective of taxonomic community structure.[25–33] However, our understanding of how fungal diversity metrics change over this period remains limited, largely due to inconsistent findings in the literature, small sample sizes used in mycobiome studies, and the substantial inter-individual differences observed in mycobiome composition and maturational patterns.[25,27,28,30–34] While it has been established that the early-life bacterial microbiome plays an important role in metabolic development and obesity risk,[48–51] very few studies have explored this from the fungal perspective. Using a variety of modeling techniques to examine the relationship between gut mycobiome development and infant growth, we found associations between the maturational patterns of the mycobiome in the first year of life and BMIz up to 5 years of age. Specifically, we observed an overall trend toward a reduction in fungal alpha diversity metrics from 3–12 months, with a subset of infants deviating from this maturational pattern, exhibiting an increase in fungal richness over this period. The mycobiome of these infants exhibited compositional and structural differences relative to those who displayed reduced fungal richness, suggestive of reduced mycobiome maturity at 12 months of age. The increasing fungal richness pattern was associated with early childhood BMIz through its interactions with maternal diet, maternal BMI, infant antibiotic exposure from 6–12 months of age, and bacterial community composition. Common members of the gut mycobiome were also found to be differentially associated with BMIz at various time points, including *Malassezia* (positive), *Saccharomyces* (positive), and *Rhodotorula* (negative). Together, these findings provide insights into the maturation patterns of the infant gut mycobiome and highlight the important role of differences in maturational patterns on growth outcomes in early life.

Characterizations of the infant gut mycobiome and the factors influencing its development to date have been limited in scope, with emphasis being placed on generating group-based average descriptions of maturation patterns in early life. While successional shifts in the taxonomic structure of the mycobiome have been comparable across studies, descriptions of alpha diversity changes over the course of early life are inconsistent.[25,27–31,33–35] This is likely in part due to differences in geographical, cultural, and socioeconomic influences on early-life exposures, which are already known to influence the bacterial microbiome.[52–54] In this study, we observed taxonomic shifts comparable with previous reports,[25–31] with a strong presence of *Candida*, *Malassezia*, and *Mycosphaerella* at 3 months and a shift toward *Saccharomyces* dominance at 12 months, alongside an overall decrease in fungal alpha diversity. However, upon assessing individual-level alpha diversity patterns, we identified a considerable subset of infants (>25%) who displayed an increase in fungal richness between 3 and 12 months of age. We observed differences in mycobiome composition and taxonomic structure at 3 months in infants with an increasing or atypical fungal richness pattern, which became more pronounced at 12 months. This included significant differences in the relative abundance of *Fomitopsis* (increased), *Mycosphaerella* (increased), *Malassezia* (increased), and *Saccharomyces* (decreased) in participants with an increasing richness pattern relative to a decreasing pattern at 12 months. *Saccharomyces* has been previously identified as a strong predictor of microbial community age,[30] likely in part because of its ability to grow rapidly under anaerobic conditions[55,56] and broad presence in solid foods,[57] resulting in diet-induced shifts in mycobiome composition.[27,58] Given the plasticity of infant gut microbial communities in the first 2–3 years of life,[59] it is plausible that the lower levels of *Saccharomyces* observed in the increasing richness pattern at 12 months are indicative of an unstable fungal community that remains in a transitional or immature state, lacking the selective pressures of a primarily anaerobic environment or typical dietary transitions. This is further supported by the maintenance of microbes observed in high abundance at 3 months in participants with an increasing richness pattern at 12 months compared with individuals with a decreasing richness pattern. Together, this highlights the role of early fungal colonizers in influencing the ecological patterns of mycobiome development and the importance of individual-level analyses in generating a more nuanced understanding of the ecological dynamics exhibited by the gut mycobiome in early life.

We discovered factors relating to metabolism and multikingdom dynamics to be most strongly associated with fungal richness patterns when assessed by random forest. These included infant BMIz at 3 months and 1 year, maternal and paternal BMI, maternal diet, breastfeeding duration, and bacterial alpha and beta diversity in the first year of life. Although previous studies have linked these factors with the infant gut bacterial community and obesity or BMI,[47,60–67] it is unclear whether their association with the gut fungal community is direct or indirect via modulation of bacteria and/or host-intrinsic obesogenic

pathways. Other factors known to influence the bacterial microbiome, such as birth mode and exclusive breastfeeding, were less strongly associated with fungal richness pattern, suggesting that our knowledge of factors influencing early-life bacterial microbiome maturation may not equally apply to infant gut mycobiome establishment. Alternatively, it is plausible that these factors weakly influence the infant mycobiome via their impact on the bacterial microbiome. Our finding of bacterial community composition at 3 months modifying the association between early-life BMIz and fungal richness pattern is supportive of this hypothesis. While we cannot establish causality in our study, the association of infant BMIz with fungal richness patterns highlights the potential and underexplored role of gut fungi in childhood obesity.

Given that infant, maternal, and paternal BMI emerged as factors strongly associated with mycobiome richness pattern, we sought to better understand the relationship between these measures and the infant mycobiome. While we did not observe direct associations between BMIz and fungal richness pattern, potentially because of the size of the cohort studied, we discovered that maternal BMI and maternal diet modified the association between fungal richness pattern and early-life BMIz. Maternal BMI and diet are known to play an influential role in the infant microbiome and obesity.[60–64] Intriguingly, we observed that an increasing fungal richness pattern was associated with lower early-life BMIz when maternal BMI was high or when the maternal diet was deemed less healthy. While it is unclear whether these factors directly impact the composition of the infant mycobiome, their effect on the bacterial microbiome and independent effect on obesity-inducing pathways may modify the association between gut fungi and early-life BMIz. Further, infant dietary practices during complementary feeding and after weaning are influenced by maternal and paternal diets, which likely also impacts gut mycobiome development. The increase in *Saccharomyces* abundance from 3 to 12 months likely reflects the transition to solid food, especially considering the evidence that the presence of this genus is strongly influenced by diet.[58] The reduced relative abundance of *Saccharomyces* in infants with an increasing fungal richness pattern at 12 months suggests that this could be indicative of dietary differences, which, in turn, may have consequences for obesity development. However, further research is needed to confirm this association. Interestingly, the increasing fungal richness pattern also displayed significantly higher relative abundance of *Fomitopsis* at 12 months, a fungal genus that has been linked to improved insulin sensitivity in rodent models.[68,69] While speculative, it is possible that the greater presence of *Fomitopsis* in the mycobiome of infants with an increasing richness pattern has a postnatal buffering effect against the potential influences of maternal BMI and diet on fetal programming of obesogenic pathways that may lead to higher BMIz in infancy. Together, our results suggest that the association between gut fungi and childhood obesity varies depending on other contributing factors; in this case, maternal BMI and diet.

Given that infant growth and the microbiome are affected by infant sex,[43–46] we examined whether the association of the gut mycobiome with early-life BMIz was modified by infant sex assigned at birth. While there is some evidence that the gut mycobiome may exhibit sex-dependent features,[35] we did not identify infant sex to be associated with BMIz in our cohort. This may be in part due to the subset of infants selected for this study and the early BMIz measurements examined, with greater potential for sex differences to arise later in childhood and adolescence, as evidenced by our observation of sex differences in BMIz beginning to emerge at 5 years of age. Additionally, the characteristics of our sub-cohort, including elevated BMIz, increased maternal consumption of artificially sweetened beverages, and elevated parental BMI, may be contributing to these outcomes. It is also plausible that infant sex modifies the association of fungal richness pattern with early-life BMIz, but our study was underpowered to statistically demonstrate this interaction. Further research is required to examine the dynamic relationship between the gut mycobiome, maternal factors, and potential sex-dependent variations in relation to infant growth.

Very few studies have explored the relationship between the mycobiome and infant growth in early life. However, a recent publication found absolute fungal abundance at 1 year to be negatively correlated with infant BMIz from birth to 1 year and absolute fungal abundance at 2 years to be positively associated with height at 2–9 years[25]; although, the reliability of these results is limited by the small number of samples with detectable fungi in this study. We found that the relative abundance of common fungal colonizers may be differentially associated with early-life BMIz outcomes, providing better-defined implications for mycobiome community dynamics on BMIz in early life. We identified associations between core members of the infant mycobiome, *Saccharomyces* (positive), *Rhodotorula* (negative), and *Malassezia* (positive)[28,34,35] and BMIz between 1–5 years of age in our cohort. While causal associations between obesity development and *Rhodotorula* and *Malassezia* have yet to be reported, *Saccharomyces boulardii* has displayed anti-obesogenic properties in mice,[70] and studies in human adults have also pointed to beneficial outcomes for obesity management.[71] Given *Saccharomyces* is found in several food products and certain strains are employed as probiotics,[72] further explorations clarifying its role in early-life weight gain are needed. Based on our initial findings, translational studies aimed at understanding the causal contributions of common mycobiome members to childhood obesity development are strongly warranted.

While we have provided evidence of the role of the early-life gut mycobiome in infant growth, it remains to be demonstrated how this relationship is influenced by the infant gut bacterial community. We have shown previously that the gut microbiome of the infants included in this study was linked to infant BMI downstream of maternal consumption of artificially sweetened beverages during pregnancy.[13] In the current study, we found that bacterial beta diversity at 3 months modified the effect of fungal richness pattern on early-life BMIz, where an increasing fungal richness pattern was associated with lower BMIz when bacterial community dissimilarity was increased. We also found that an increasing fungal richness pattern was associated with lower BMIz when an infant was exposed to antibiotics between 6 and 12 months. These findings are consistent with the well-known ability of antibiotics to disrupt the gut microbiome and provide an opportunity for fungal community alterations,[73] which may be involved in the relationship between early-life antibiotic

exposure and obesity risk later in life.[74] This suggests a role of inter-kingdom interactions in mediating infant growth trajectories, such as cross-kingdom competitive exclusion and selective pressures. We further modeled the complex inter-relation of bacteria and fungi at 3 and 12 months with BMIz to investigate this relationship. Using several microbiome features, including bacterial microbiome composition (PCoA1), fungal richness, and fungal richness pattern, we found that bacterial community composition and infant BMIz were associated with each other at 12 months, whereas fungal diversity metrics were not associated with BMIz. Together, this suggests an inter-dependent role of the gut mycobiome and bacterial microbiome in early-life BMIz outcomes alongside a role of cross-kingdom microbiome features in mediating infant growth trajectories. Changes in the metabolic activity of gut bacteria during childhood obesity can result in altered nutrient bioavailability in the gut,[75] potentially impacting the physiological and ecological fitness of fungi. This highlights the need to consider inter-kingdom interactions between bacteria and fungi when evaluating the role of the infant gut mycobiome in childhood obesity.

The main strength of our study was our multi-method analysis incorporating longitudinal infant, maternal, paternal, and environmental factors to assess individual maturation patterns of the mycobiome and its association with BMIz in the first 5 years of life. Many explorations of the infant gut mycobiome and infant growth trajectories lack maternal and paternal data, but by leveraging the CHILD Cohort Study, we were able to perform analyses involving relevant factors. Further, the assessment of factors extending into gestation, such as maternal HEI, provides valuable insights into the importance of pre-partum exposures that may be modified to influence infant health outcomes.

Overall, our findings suggest that early-life and maternal factors may collectively influence growth dynamics in the first 5 years of life, in association with mycobiome maturational patterns and specific fungal taxa. Further characterization of mycobiome maturational patterns in large infant cohorts is warranted because right now, our understanding of mycobiome establishment and its relationship with infant development is very limited. Future evaluations of the role of differences in the directionality of fungal richness patterns in early life on BMIz and metabolism will be crucial to understanding this intriguing relationship. In particular, it will be important to validate these findings at the general population level to determine whether specific patterns of mycobiome maturation may predispose infants to metabolism-related health outcomes. In parallel, mechanistic studies are needed to draw conclusions about the exact role of the mycobiome in early-life determinants of growth and metabolism.

Limitations of the study

We recognize that this study was constrained by the lack of repeated mycobiome measures into early childhood and the small sample size of our cohort, which was reflected in the limited number of infants exhibiting an increasing fungal richness pattern and/or at risk of becoming overweight or obese. This hindered the strength of our analysis, making it difficult to generalize our results to the pediatric population, particularly given that the cohort used was specifically selected for a previous nested case-control study assessing gestational artificially sweetened beverage consumption.[13] It is also possible that mycobiome measures at later time points may provide alternative insights into the relationship between the gut mycobiome and BMIz trajectories in early childhood. However, this analysis was performed with the intention of exploring the relationship between early-life mycobiome maturation patterns and infant growth, given the recognized role of the early-life microbiome in the developmental origins of health and disease.[1–3] Despite our limited sample size and lack of mycobiome measures beyond the first year of life, we were still able to find significant associations between the mycobiome and BMIz in the first 5 years of life, highlighting the need for further evaluations in large cohort studies that include gut mycobiome sampling extending into childhood.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, M.-C.A.; methodology, M.W.G., E.M.M., S.M., and I.L.-L.; investigation, M.W.G., E.M.M., S.M., and M.E.R.; writing – original draft, M.W.G., E.M.M., S.M., and M.-C.A.; writing – review & editing, I.L.-L., M.E.R., A.B.B., E.S., P.J.M., S.E.T., T.J.M., M.R.S., P.S., and M.B.A.; funding acquisition, M.-C.A. and M.B.A.; resources, A.B.B., E.S., P.J.M., S.E.T., T.J.M., M.R.S., P.S., M.B.A., and M.-C.A.; supervision, M.-C.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We worked to ensure that the study questionnaires were prepared in an inclusive way. One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper self-identifies as living with a disability.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Infant stool samples (3 & 12 months)	Subbarao et al., 2015[40]	CHILD Cohort Study http://childstudy.ca
Critical commercial assays		
DNeasy PowerSoil Pro Kit	Qiagen	Cat # 47016
Deposited data		
ITS2 rRNA gene sequencing Fastq. files	This paper	BioProject Accession Number: PRJNA814728
16S rRNA gene sequencing Fastq. files	Laforest-Lapointe et al., 2021[13]	BioProject Accession Number: PRJNA624780
R Scripts	This paper	https://github.com/ArrietaLab/CHILD_Mycobiome_BMI_CellReportsMedicine_Files
Oligonucleotides		
ITS2 rRNA Forward Primer CCTCCGCTTATTGATATGC	Op De Beeck et al., 2014[42]	ITS1F
ITS2 rRNA Reverse Primer CCGTGARTCATCGAATCTTTG	Op De Beeck et al., 2014[42]	ITS4
16S rRNA Forward Primer GTGCCAGCMGCCGCGGTAA	Caporaso et al., 2012[76]	515F
16S rRNA Reverse Primer GGACTACHVGGGTWTCTAAT	Caporaso et al., 2012[76]	806R
Software and algorithms		
R v.4.1.1	R Core Team[77]	https://www.r-project.org
UNITE database v.8.0	Nilsson et al., 2019[78]	https://unite.ut.ee/index.php
SILVA database v.132	Quast et al., 2013[79]	https://www.arb-silva.de/documentation/release-132/
DADA2 R package v.1.20.0	Callahan et al., 2016[80]	https://www.bioconductor.org/packages/release/bioc/html/dada2.html
phyloseq R package v.1.36.0	McMurdie and Holmes, 2013[81]	https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html
mice R package v.3.13.0	Van Buuren et al., 2011[82]	https://cran.r-project.org/web/packages/mice/index.html
vegan R package v.2.5.7	Oksanen et al., 2017[83]	https://cran.r-project.org/web/packages/vegan/index.html
zCompositions R package v.1.3.4	Palarea-Albaladejo et al., 2015[84]	https://cran.r-project.org/web/packages/zCompositions/index.html
CoDaSeq R package v.0.99.6	Gloor and Reid, 2016[85]	https://github.com/ggloor/CoDaSeq
randomForest R package v.4.6.14	Liaw and Wiener et al., 2002[86]	https://cran.r-project.org/web/packages/randomForest/index.html
caret R package v.6.0.90	Kuhn, 2020[87]	https://cran.r-project.org/web/packages/caret/index.html
stats R package v.4.1.1	R Core Team[77]	https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html
performance R package v.0.8.0	Lüdecke et al., 2021[88]	https://cran.r-project.org/web/packages/performance/index.html
car R package v.3.0.10	Fox and Weisberg, 2019[89]	https://cran.r-project.org/web/packages/car/index.html
effects R package v.4.2.0	Fox et al., 2022[90]	https://cran.r-project.org/web/packages/effects/index.html
stargazer R package v.5.2.2	Hlavac, 2018[91]	https://cran.r-project.org/web/packages/stargazer/index.html
lmPerm R package v.2.1.0	Wheeler and Torchiano, 2016[92]	https://cran.r-project.org/web/packages/lmPerm/index.html
lavaan R package v.0.6.6	Rosseel, 2012[93]	https://cran.r-project.org/web/packages/lavaan/index.html
MVN R package v.5.9	Korkmaz et al., 2014[94]	https://cran.r-project.org/web/packages/MVN/index.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Marie-Claire Arrieta (marie.arrieta@ucalgary.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Demultiplexed ITS2 and 16S rRNA gene sequencing data were deposited into the Sequence Read Archive (SRA) of NCBI and can be accessed via BioProject accession numbers PRJNA814728 and PRJNA624780. This information can also be found in the key resources table.
- All code (R scripts) has been deposited at: https://github.com/ArrietaLab/CHILD_Mycobiome_BMI_CellReportsMedicine_Files. The software and packages used for this analysis are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Inclusion and exclusion criteria of study participants

We studied 100 mother-infant dyads which represents a subset of the population-based prospective CHILD Cohort Study.[40] Women with singleton pregnancies were enrolled between 2008 and 2012 across four Canadian provinces into the CHILD Cohort Study and remained eligible if they delivered a healthy infant at >35 weeks' gestation (n = 3,264).[40] Recruitment occurred at four sites: Vancouver (British Columbia), Edmonton (Alberta), Toronto (Ontario), and Manitoba (Winnipeg and two adjacent rural towns, Morden and Winkler). The mother-infant dyads included in this study were previously selected for a nested case-control study, equally divided between mothers who reported little to no artificially sweetened beverage consumption (less than one per month) or high artificially sweetened beverage consumption (one or more per day) during pregnancy.[13] Each artificially sweetened beverage exposure group was balanced for six factors known to influence the gut microbiome: infant sex (assigned at birth), mode of birth, breastfeeding status at 3 and 12 months, infant antibiotic exposure before 12 months (those exposed prior to 3 months were excluded), and maternal BMI.

Ethics approval

The study was approved by the University of Calgary Conjoint Health Research Ethics Board and ethics committees at the Hospital for Sick Children, and the Universities of Manitoba, Alberta, and British Columbia. Written informed consent was obtained from mothers during study enrollment.

Anthropometric measurements

Weight and length/height were measured at birth, 3 months, 1, 3, and 5 years by trained study staff to the nearest 0.1 unit according to a standardized protocol. Age- and sex-specific BMI *Z* scores (BMIz) were calculated according to the 2011 World Health Organization (WHO) standards.[95] Infants were grouped into BMI categories modified from BMIz cutoffs established by the WHO.[41] Infants with BMIz $\leq$1.0 were classified as normal, while infants with BMIz >1.0 were classified as at risk of overweight/overweight (also referred to as high BMI). As BMI *Z* score is a more accurate predictor of childhood obesity compared to weight-for-length *Z* score,[96] the former was used as the main outcome in this work.

Infant, early life, maternal, and paternal factors

Infant sex (assigned at birth), birthweight, gestational age, mode of birth, parity, and intrapartum antibiotic use were documented from hospital records. We focused on early-life factors known to influence the gut microbiome, including mode of birth, intrapartum antibiotics, antibiotic exposure from 6 to 12 months of age, and infant feeding. Infant feeding was reported using a standardized questionnaire at 3, 6, and 12 months. Infant feeding included breastfeeding status at 3 months, total breastfeeding duration, and introduction of solid foods into the infant diet by 6 months. Breastfeeding status was classified as "exclusive" (human milk only) or "zero/partial" (no human milk or human milk supplemented with formula milk or solid foods). Prenatal, intrapartum, and infant antibiotic exposure was obtained from maternal and infant medical records. Maternal BMI was calculated from measured height and self-reported pre-pregnancy weight. Paternal BMI was calculated from weight and height measurements collected during clinic visits. Maternal Healthy Eating Index (HEI) was used as an indication of maternal diet quality. Maternal diet was assessed using a validated semi-quantitative food frequency questionnaire during the second or third trimester of pregnancy, as previously described,[40] and maternal HEI was derived based on the 2010 guidelines.[97] Consumption of artificially sweetened beverages during pregnancy was assessed based on consumption of diet sodas (serving = 355mL/one can) or artificial sweetener added to tea or coffee (serving = 1 packet), as previously described.[13]

METHOD DETAILS

Sample collection and processing

Stool samples (n = 200) were collected from soiled diapers by CHILD Cohort Study staff at the 3-month home visit and 12-month clinic visit using standardized methods and were stored at −80°C until further processing.[98]

DNA extraction of infant stool samples

Genomic DNA was extracted from fecal samples using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) following the manufacturer's instructions. Following extraction, DNA concentration and quality were quantified using a NanoDrop spectrophotometer (Thermo Scientfic). DNA was stored at −20°C until downstream analysis.

ITS2 and 16S rRNA gene sequencing

In-house extracted DNA was sent to Microbiome Insights (Vancouver, Canada), where PCR was used to amplify the V4 region of 16S rRNA gene with 515F/806R primers[76] and the internal transcribed spacer region 2 (ITS2) rRNA gene with ITS1F/ITS4 primers[42] for bacteria and fungi, respectively, using Phusion Hot Start II DNA Polymerase (Thermo Scientific). This generated ready-to-pool, dual-indexed amplicon libraries, as described previously.[99] Controls without template DNA and mock communities with known amounts of selected bacteria or fungi were included in the PCR and downstream sequencing steps to control for microbial contamination. The pooled and indexed libraries were denatured, diluted, and sequenced in paired-end modus on an Illumina MiSeq (Illumina Inc., San Diego, USA). For additional details, bacterial sequencing methods have been previously reported.[13]

QUANTIFICATION AND STATISTICAL ANALYSIS

Sequencing processing

Sequence processing was conducted in R v.4.1.1.[77] Sequences were checked for quality, trimmed, merged, and checked for chimeras using the *DADA2* v1.20.0 pipeline for ITS2 or 16S data.[80] Taxonomy was assigned as amplicon sequence variants (ASVs) using the UNITE v.8.0 (fungi)[78] and SILVA v.132 (bacteria)[79] databases at 99% sequence similarity. Preprocessing of the ASV table was performed using the *phyloseq* package v.1.36.0.[81] Overall, 3,328 unique fungal ASVs were detected. ASVs belonging to kingdom Plantae (n = 673) were removed, leaving 2,655 unique fungal ASVs. Samples with less than 2,000 sequencing reads were excluded (n = 10) and ASVs appearing in only one sample were removed (n = 59). The remaining dataset was filtered for ASVs appearing at least two times in the dataset, leaving 604 unique fungal ASVs. This dataset was used for all subsequent analyses. Bacterial sequence processing methods have been previously reported[13] and only alpha- and beta-diversity metrics derived from published results are used in this analysis.

Handling missing data

Missing paternal BMI (n = 23) and infant BMIz data at 3 months (n = 3) and 1 year (n = 2) was imputed for random forest and multivariable logistic regression using multivariate imputation by chained equations (*MICE*) package v.3.13.0.[82] Samples with missing data for other covariates were excluded from multivariable analyses. These included bacterial alpha-diversity at 3 months (n = 1) and 12 months (n = 1), bacterial beta-diversity (PCoA1) at 3 months (n = 1) and 12 months (n = 1), and prenatal (n = 2) and intrapartum (n = 2) antibiotics exposure, resulting in the exclusion of 5 infants from analyses. Missing data for BMIz measurements in the first 5 years of life (birth: n = 28; 3 months: n = 3; 1 year: n = 2; 3 years: n = 14; 5 years: n = 11) was maintained in regression interactions and multivariable linear regression permutation analyses, as the models employed were capable of handling missing data.

Exclusion of data

Infants lacking samples at one or both time points (n = 9) or having an unchanging fungal richness pattern (n = 3) were excluded from all downstream analysis involving fungal richness pattern and/or associations with early-life BMIz. Additionally, samples were excluded from multivariable analyses if they were missing essential covariate data (n = 5). BMIz values <−2.0 and >4.0 were considered outliers and excluded from all analyses (birth: n = 4; 3 months: n = 4; 1 year: n = 1; 3 years: n = 1).

Statistical analysis

Fungal alpha-diversity was assessed by Chao1 (richness) and Shannon (diversity) and reported as mean and SD. Association of alpha-diversity with infant age was assessed by the Mann-Whitney U test, after determining data was non-normally distributed using the Shapiro-Wilk test. Chao1 and Shannon metrics were categorized according to their pattern over the first year of life into increase, decrease, and unchanged categories and assessed by paired t-test. Bray-Curtis dissimilarity (beta-diversity) with variance-stabilizing transformation of the infant gut mycobiome by age and alpha-diversity pattern was assessed by permutational ANOVA (PERMANOVA) using the *vegan* package v.2.5.7.[83] The relative abundance of the ten most abundant fungal genera were compared by age and alpha-diversity pattern. Relative abundances were center log-ratio (CLR) transformed following zero-replacement using *zCompositions* v.1.3.4 and *CoDaSeq* v.0.99.6[84,85] to control for compositionality before statistically assessing differential abundance. Differences in CLR-transformed abundance between 3 and 12 months of age were assessed by Mann-Whitney U test for non-normally distributed data, as determined by the Shapiro-Wilk test.

Random forest was performed to determine relevant clinical and early-life factors predictive of fungal richness (Chao1) pattern using 10-fold cross-validation, 500 trees, and 1,000 permutations with the *randomForest* v.4.6.14 and *caret* v.6.0.90 packages.[86,87] Factors known to be associated with bacterial microbiome maturation in early-life and/or infant growth were included,[100–106] alongside bacterial microbiome diversity measures to examine for inter-kingdom influences. The factors most strongly associated with fungal richness pattern were identified by mean decreasing Gini index. Multivariable logistic regression examining factors related

to an increasing fungal richness pattern was performed using the package *stats* v.4.1.1,[77] and the absence of multi-collinearity was confirmed using the package *performance* v0.8.0.[88] Bacterial microbiome diversity measures were excluded from this analysis to prevent model overfitting and enable explorations of factors influencing mycobiome maturation in isolation, given limited understandings exist in this realm. Results are reported for each factors as log-transformed odds ratio and 95% confidence interval. The decreasing fungal richness pattern was set as the reference level for both random forest and logistic regression analyses.

To assess differences in early-life BMIz from birth to 5 years between increasing and decreasing fungal richness patterns, normality was first assessed by Shapiro-Wilk's test, homogeneity of variance was assessed by F-test, and differences were determined by two-sample t-test or Mann-Whitney U test, accordingly. Associations between mycobiome richness pattern or sex (assigned at birth) and early-life BMI categories were assessed using Pearson's Chi-squared ($\chi 2$) test with Yates' continuity correction. Regression interactions between infant, early life, paternal, and maternal factors, and mycobiome diversity pattern for early-life BMIz was performed with *car* v.3.0.10,[89] *effects* v.4.2.0,[90] and *stargazer* v.5.2.2[91] packages. BMIz was modeled as the outcome, and BMIz time point, fungal richness (Chao1) pattern, and the variable of interest were specified as explanatory variables. The former two variables were modeled as individual or crossing variables. These models were inputted into *stargazer*[91] and plotted with *effects*.[90]

Multivariable linear regression permutation tests were performed using *lmPerm* v.2.1.0[92] for genera or species with greater than 2% mean relative abundance. As described above, relative abundances were CLR-transformed following zero-replacement using *zCompositions* v.1.3.4 and *CoDaSeq* v.0.99.6[84,85] to control for the compositionality. The model included maternal BMI, breastfeeding status at 3 months, infant antibiotic exposure from 6 to 12 months, and maternal HEI as covariates. The Benjamini-Hochberg procedure was used to correct p values for multiple comparisons.

Structural equation modeling (SEM) was performed using the *lavaan* package v.0.6.6.[93] Multivariate normality was verified using *MVN* package v.5.9.[94] The model was estimated using maximum likelihood (ML) parameter estimation and NLMINB optimization method with bootstrapping (N = 1,000).[107] Model fit was assessed by $\chi 2$ test, the comparative fix index (CFI), root-mean-square error of approximation (RSMEA) and its 90% confidence interval (CI), and the standardized root mean residuals (SRMR). Non-significant $\chi 2$ test, CFI $\geq$ 0.9, RMSEA<0.05, and SRMR<0.08 were considered as indications of good model fit.[107]