Summary

In Chapter 2, the association between the intestinal microbiota composition at 1 month of age and childhood weight development, measured repeatedly between the ages of 1 and 10 years, was investigated using data from the KOALA Birth Cohort Study. This cohort consists of two recruitment groups, a group with a conventional lifestyle was recruited from an ongoing prospective cohort study on pregnancy-related pelvic girdle pain (conventional recruitment group) and a second group was recruited through alternative channels, organic food shops, Steiner schools, magazines and anthroposophic doctors and midwives (alternative recruitment group). We found that gut colonization with Bacteroides fragilis group at one month postpartum in the conventional recruitment group was significantly associated with a higher BMI z-score, but only among infants with a low fibre intake. B. fragilis counts in children that were colonized were positively associated with BMI z-score in children from the conventional recruitment group with a high-fibre diet, but negatively among those with a low fibre intake and among children from the alternative recruitment group.

The early infant gut microbiota is susceptible to changes by a number of environmental factors such as mode of delivery, type of infant feeding and antibiotic use. At 2–3 years of age, an adult-like intestinal microbiota composition is established, although maturation of the microbiota may still continue for several years. In Chapter 3, using the HITChip, we comprehensively characterised the gut microbiota composition of children at age 6–7 years, when its composition is assumed to be more resilient. We then investigated the association between the gut microbiota composition at this age in relation to the anthropometric outcomes BMI z-score, weight z-score, and overweight. Using Redundancy Data Analysis (RDA), we found that part of the variation in the gut microbiota composition of these children was accounted for by the cumulative abundance of Prevotella melaninogena et rel., Prevotella oralis et rel., Dialister, and Uncultured clostridiales II (UCII). The anthropometric outcomes and potential confounders explained very little of the variation in the overall microbiota composition. However, the abundances of several specific bacterial groups were associated with the anthropometric outcomes BMI, weight, and overweight. Akkermania, and Bryantella formatexigens et rel. were found to be consistently associated with all three anthropometric outcomes while Sutterella wadsworthia et rel., was negatively associated with BMI and overweight. With regards to bacterial groups exhibiting a bimodal distribution, higher abundance of UCII was found to be inversely associated with all three outcomes under study while higher abundance of Prevotella (melaninogena et rel. and oralis et rel.) was inversely associated with overweight.

Taken together, Chapter 2 and 3, demonstrate that specific bacterial groups of the gut microbiota are associated with childhood overweight. In chapter 2 only a total of 5 bacterial genera and species were considered whereas in chapter 3 we used the HITChip to carry out an in-depth characterisation of the gut microbiota composition identifying 130 genus-like bacterial groups. Even though both studies considered B. fragilis (at the genus level) in association to childhood overweight, we only found associations for B. fragilis group in faecal samples collected at 1 month of age, and these associations were dependent on the level of fibre intake. Due to differences in age at faecal sampling, study design (longitudinal versus cross-sectional), and method of microbial identification, results are not directly comparable. Moreover, Bervoets et al. suggested that different species within the B. fragilis group might be responsible for the seemingly contradictory associations with weight development by showing that more B. fragilis was present in individuals suffering from obesity and more B. vulgatus in control subjects. Differences in the species composition of the B. fragilis group could explain both the opposite findings for the two recruitment groups at the age of 1 month, as well as the absence of an association at school-age.

Considering the fact that, next to bacteria, other microbial groups within the intestinal tract might influence host metabolism and energy balance, in Chapter 4 we examined the presence of archaea (M. smithii and M. stadtmanae) in the faecal samples of 472 children at age 6–7 years in association with childhood weight development. M. smithii, the dominant archaeal species in the human gut, was associated with higher weight z-scores and an increased likelihood of being overweight, with children harbouring the highest levels of this archaeon being at the highest risk. The biological interaction between archaea and bacteria was not investigated. A study by Samuel and Gordon, 2006, actually showed that M. smithii played a critical role in facilitating an increased capacity of B. thetaiotaomicron to digest polyfructose-containing glycans leading to increased production of short-chain fatty acids (SCFAs) and total liver triglycerides in mice. Mice colonized with B. thetaiotaomicron and Desulfovibrio piger instead of M. smithii did not show such an effect. These biological interactions have not yet been investigated in humans and therefore should be considered for future studies.

Antibiotics have been reported as one of the factors affecting the composition of the gut microbiota and have also been reported to be associated with childhood overweight. In Chapter 5, we investigated the timing, frequency and type of antibiotic exposure during the first 10 years of life in association with (over)weight across this period in 979 children participating in the KOALA Birth Cohort Study. We found that exposure to a single course of antibiotics in the first 6 months of life, as well as ≥2 courses during the first two years of life were associated with increased weight and height z-scores. These associations were stronger for exposure to -lactam antibiotics. We found no associations between antibiotic exposure beyond the second year of life and childhood weight development. This indicates that early childhood is a critical period where exposure to antibiotics may have most pronounced effects on
childhood overweight, possibly mediated by microbial perturbations. Our findings support existing evidence from previous studies that early exposure to antibiotics is associated with childhood (over)weight development. Our study is one of the first large-scale prospective studies that included information on the timing, frequency and type of antibiotic used during the first 10 years of life, in relation to anthropometric outcomes measured repeatedly over this entire follow-up period.

In the above-mentioned longitudinal studies, we investigated associations of microbial or antibiotic exposures with BMI over time while ignoring the fact that there may be distinct developmental patterns of growth between children. In Chapter 6, we aimed to identify such developmental patterns. Using Group-based trajectory modelling (GBTM), we identified 4 distinct latent BMI z-score trajectories in our study population. Latent trajectories were found to be associated with established risk factors of childhood overweight, including physical activity and total energy intake. Using statistical methods to reduce the high dimensional data of the gut microbiota in relation to these latent trajectories, we identified ten bacterial groups that were associated with the trajectories. This shows that GBTM can be used to summarize complex developmental patterns of children into a single multi-category variable, which facilitates the reduction of high dimensional data in relation to this.

Methodological considerations

Reliability of faecal samples
Due to the relative ease and non-invasiveness of stool collection, most of the studies on the intestinal microbiota, including ours, have been performed using this sample type. It can be debated whether stool samples are the best sample type to gain insight into the intestinal microbiota since the microbial profile of faeces is substantially different from that of the mucosa, which itself can vary along the length of the gastrointestinal tract. However, colonic biopsies are more difficult to obtain, which makes collection of biopsies not feasible within the context of large-scale epidemiological studies, especially when involving children. Moreover, patients are required to take laxatives before colonoscopy which may also significantly alter the gut microbiota composition. It can be assumed that despite all activities that go on along the passage of the intestinal tract, most viable as well as nonviable commensal intestinal bacteria will still be detectable in faeces with molecular methods. However, it is unlikely that analysing microbiota composition in faeces is representative of all important intestinal micro-organisms. Hence, using faeces represents a limitation of most studies on the gut microbiota even though at this point faeces remain the only reliable sample in large non-invasive studies.

Collection and processing of faecal samples
In order to minimise bias and transport time of faecal samples, detailed instructions were sent to parents directing them on how to collect their child’s faeces. Upon collection, faecal samples were sent to the laboratory by mail. Issues arise because anaerobic conditions are not maintained during faecal sample collection, transportation and laboratory analysis. This may have led to increased proliferation of certain types of microbes whereas others, especially strictly anaerobes, might die during the course of sample transportation and handling. However, it is likely that the impact of sample handling and transport on the composition of the samples is less pronounced when molecular methods are used instead of traditional culture techniques, as the non-viable bacteria will still be detectable. It can however not be ruled out that the potential overgrowth of some bacterial species and suppression of others, as well as degradation of bacterial DNA due to transportation could have influenced our results. In our study the transport time for faecal samples ranged from 1 to 3 days at ambient to room temperature. Several previous studies have shown that the microbial diversity and composition of faecal samples is much more affected by inter-individual differences and biases in molecular techniques rather than differences in short-term storage conditions, including storage for up to 2 weeks at room temperature. The method used for DNA extraction can also introduce bias as samples may contain both organisms that are more easily disrupted, such as gram-negative species, and those that are more difficult to lyse, such as gram-positive species, mycobacteria, and spores. We have minimised the potential effect of this bias by using a widely used and validated protocol that combines both mechanical and enzymatic disruption.

Temporal variations in microbiota composition
The studies presented in this thesis were based upon characterization of the faecal microbiota at the ages of respectively 1 month and 6-7 years of age. It is well-known that the microbiota in neonates is highly dynamic. By only examining the microbiota at a single time-point during infancy, some potential associations between the gut microbiota and weight development might have gone undetected. By the age of 2-3 years an adult-like microbiota has established. Hence, at age 6-7 years the microbiome becomes more adult-like with a more stable composition, although the composition might still be affected by bacterial infections, antibiotic treatment, as well as changes in lifestyle and dietary patterns over time.

Validity of outcome measures
We used sex- and age-standardised BMI as a measure of adiposity classification. BMI is not only determined by body fat mass but also by lean mass, and therefore may misclassify children into the overweight category when actually they are not overly fat. However, several studies have indicated a positive correlation between BMI and body
fatness. The World Health Organisation (WHO) also recommends BMI as the most useful population level measure of overweight and obesity. Together this justifies the use of BMI in our study as a measure to track childhood (over)weight development. The repeated weight and height measurements used in our longitudinal analysis were parent-reported. A validation study using data from the KOALA Birth Cohort Study, found an underestimation of overweight with parent-reported data compared to data collected during home visits. This so-called differential misclassification, where the rate of misclassification depends on BMI, with more misclassification for overweight and obese individuals, may have led to both under- and overestimation of the true associations in our studies. However, the fact that we had repeated measurements might have partially offset this drawback.

Study design
The current thesis contains both cross-sectional and longitudinal analyses. Longitudinal studies take into account associations between gut microbiota and weight development over a defined period of follow-up and have been considered to provide better evidence of causality than cross-sectional studies. The majority of previous studies on the role of the gut microbiota on (over)weight were of cross-sectional design or, if longitudinal, did not address the transitional evolution of childhood (over)weight over an extended period of time. The longitudinal study design therefore is one of the major strengths of our studies. In addition, this thesis contains the first study that has used GBTM to generate longitudinal childhood growth patterns (BMI z-score trajectories) while associating them to gut microbiota composition. However, longitudinal studies have their own limitations, such as loss-to-follow up that leads to a reduction in sample size and hence decreases the statistical power to detect associations. In our study, loss-to-follow up rates was lowest among children from parents with a higher economic status and with a healthier lifestyle, implying that most of the drop-outs were the unhealthier children, which might have resulted in a smaller chance of finding associations. Observational studies usually generate information on associations and not on causal relations. We therefore cannot rule out the fact that reverse causation might have occurred, particularly in the cross-sectional analyses on the gut microbiota and childhood body weight at the age of 6-7 years. Evidence for a causal role of the gut microbiota in overweight development comes mostly from animal studies, while a few human studies provide some evidence of causality by means of the effects of gut microbiota transplantation. With this in mind, evidence from previous studies and findings from this thesis further strengthens the hypothesis that the gut microbiota influences childhood over (weight) development.

Generalizability
Our study population consisted entirely of Dutch Caucasian children hence results might not be generalizable to children with another ethnic or racial background. Indeed, the gut microbiota composition has been shown to vary between populations from different geographic regions and racial backgrounds. It is however, unclear whether ethnicity or racial background has a direct impact on the gut microbiome since most of these variations between populations seem to be caused by differences in dietary habits. Nevertheless, there is a great need for similar studies in other regions of the world.

Future perspectives/recommendations
Several studies have reported associations between the gut microbiota and childhood overweight but till date, there is a lack of consensus in the findings between these studies. These inconsistent findings might, at least to some extent, be explained by differences in experimental techniques (DNA isolation, microbial identification) that affect the observed composition of the human microbiome. When multiple studies across the world are being carried out on large-scale cohorts while utilizing the same methods of DNA isolation and comprehensive microbial identification, this may likely clarify some of the present uncertainties.

In addition, most of the studies performed so far have been case-control studies. Such studies are important in actually providing the initial step in investigating if there is an association between the gut microbiota and overweight. However, causality cannot be established with such studies. Prospective studies or birth cohorts with multiple follow-up time-points from birth throughout infancy and childhood, and with faecal samples obtained at the same time of anthropometric outcomes will enable us to monitor the composition of the microbiome and its association to weight development. Some human intervention studies involving the transplantation of gut microbiota obtained from healthy donors to diseased recipients have already been carried out. Such studies could be used to investigate if effects of the microbiota are causal. However, such studies also have their drawbacks in that it carries the risk of transferring either unknown or undetectable pathogens. A recently reported type of faecal transplantation known as synthetic faecal transplantation seems to overcome the limitations of the direct or native faecal transplantation. Synthetic faecal transplantation utilises microbial strains from the faeces of a healthy donor that has been cultured and transplants these strains into an unhealthy recipient. Future studies, could therefore implement this method of faecal transplantation in place of the native faecal transplantation to investigate the causal effect of the gut microbiota on childhood over (weight) development. Such studies should be extended to the different continents of the world to enable us to have a deeper insight into whether effects are similar in different geographic and ethnic populations. In addition, the studies should be able to carry out a high interrogation of the gut microbiota composition up to the species level and hence advanced statistical
methodology for high dimensional data reduction and longitudinal data analysis are needed. The investigation of biological interactions between different microbial groups and the joint effect of these interactions on childhood weight development may further add meaning to the existing evidence.

Above all, it is important to mention that this is still a very new area of research which so far has revealed new leads as to how the gut microbial composition relates to different aspects of human health especially childhood obesity. *Akkermansia* has consistently been reported by previous studies and our study as being inversely associated with body weight in both mice and humans. Methanogenic archaea (specifically *M. smithii*) are also a group of gastrointestinal microorganisms that have been found to be positively associated with body weight. Further studies (e.g. trials) in which *Akkermansia* are being used as probiotics and animal studies which involve the administration of only archaea or archaea in combination with other microbes that compete with archaea for hydrogen are warranted. This will enable us to further confirm if the associations between *Akkermansia*, methanogenic archaea and weight development are causal. We also identified bacterial groups that have not been previously reported to be associated with childhood overweight such as *Sutterella wadsworthia* et rel. and *Bryantella formatexigens*. This will require the performance of mechanistic studies for such bacterial group to actually investigate their mechanisms involved in weight development. Future studies should aim at identifying what are beneficial (“good”) and non-beneficial (“bad”) microbes for an ideal gut microbial ecosystem for the human health.

As research in this field is ongoing, we hope that our findings and those of others will help in providing unique solutions to the growing epidemic of childhood obesity. To achieve this aim, the gut microbiota should be studied together and in interaction with established risk factors of overweight such as diet and physical activity.

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**References**


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