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# The impact of probiotic supplementation during pregnancy on DNA methylation of obesity-related genes in mothers and their children

Sanna Vähämiko[1], Asta Laiho[3], Riikka Lund[3], Erika Isolauri[2, 4], Seppo Salminen[1], Kirsi Laitinen[5]

1) Functional Foods Forum, Faculty of Medicine, 20014 University of Turku, Finland

2) Department of Clinical Medicine, Faculty of Medicine, 20014 University of Turku, Finland

3) Turku Centre for Biotechnology, 20520 University of Turku, Finland

4) Department of Pediatrics and Adolescent Medicine, Turku University Hospital, PL 52, 20521, Finland

5) Institute of Biomedicine, Faculty of Medicine, 20014 University of Turku, Finland

To whom correspondence should be addressed:

Sanna Vähämiko, Functional Foods Forum, 20014 University of Turku, Finland

Tel. +358 2 333 6821, Fax +358 2 333 6862, sanvah@utu.fi

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**Abstract**

*Purpose* Dietary supplementation with probiotics during pregnancy has been suggested to decrease the risk for obesity in women after delivery and to minimize excessive weight gain in their children. Epigenetic DNA methylation has been proposed to impact on gene activity thereby providing a plausible molecular mechanism for a broad range of biological processes and diseases. This pilot study aimed to evaluate whether probiotic supplementation during pregnancy could modify the DNA methylation status of the promoters of obesity and weight gain-related genes in mothers and their children.

*Methods* A sample of 15 pregnant women was taken from a prospective, randomized mother and infant nutrition and probiotic study. Seven women received the probiotic supplementation and eight served as controls. The women's and their children's DNA methylation status of obesity (623 genes) and weight gain-related (433) gene promoters was analyzed from blood samples at the mean of 9.8 months (range 6.1-12.7 months) postpartum.

*Results* Probiotic supplementation led to significantly decreased levels of DNA methylation in 37 gene promoters and increased levels of DNA methylation in one gene promoter in women. In their children, 68 gene promoters were significantly affected consistently with a lower level of DNA methylation in the probiotic-group.

*Conclusions* On the basis of our pilot study we suggest that probiotic supplementation during pregnancy may affect the DNA methylation status of certain promoters of obesity and weight gain-related genes both in mothers and their children thereby providing a potential mechanism for long-lasting health effects.

Keywords: probiotic, pregnancy, diet, obesity, methylation

## Introduction

Obesity has become a global epidemic and is now a major threat to human health [1, 2]. The main cause of obesity is thought to be an imbalance between energy intake and expenditure. However, there is a growing body of evidence highlighting the contribution of gut microbiota to the development of obesity, extending the relationship between the composition of the gut microbiota and host nutritional status, immune system and disease susceptibility [3, 4]. The formation of human intestinal microbiota begins prior to birth, within the intrauterine environment [5, 6]. Thereafter, it is modified by the maternal microbiota composition, mode of delivery, and type of infant feeding [4, 7]. In this light, pregnancy represents a unique time period to modify the gut microbiota composition of both the mother and newborn. Indeed, supplementation with probiotics during pregnancy has been shown to decrease the risk for central adiposity after pregnancy [8] and to modify the growth pattern of the child by minimizing excessive weight gain during the first years of life and thereby potentially decreasing the child's later risk for obesity [9].

Epigenetic DNA methylation is one possible molecular mechanism which modulates a broad range of biological processes and diseases [10]. In short, DNA methylation refers to the binding of a methyl-group to DNA primarily to CG dinucleotides which can regulate the accessibility of DNA to regulatory factors; when DNA methylation occurs in gene promoters, it can convert chromatin into a transcriptionally silent state which may decrease the transcription activity of the gene. The extent of demethylation of the gene promoter, in turn, may correlate with transcriptional activation or readiness. Interestingly, environmental factors, such as dietary components, have been reported to modify DNA methylation. In particular, when occurring in utero or during the early neonatal stages, these changes in DNA methylation have been postulated to induce long-term changes in gene expression and further to act as causative agents for lifelong effects on health [10, 11].

In this pilot study we aimed at analyzing the impact of specific probiotic supplementation during pregnancy on the modifications of DNA methylation status, especially of the promoters of obesity and weight gain-related genes in mothers and their children. The target was to reveal whether DNA methylation could be modified by probiotics, for example whether it could be used as a potential tool for future weight management and obesity risk modification.

## Experimental methods

### *Subjects, study design and ethics*

The study population comprised pregnant women participating in a prospective, randomized mother and infant nutrition and probiotic study. The recruitment, randomization, and study design have been described elsewhere [12, 13]. In brief, recruitment took place during the women's first visit to maternal welfare clinics in South-West Finland. The criteria for inclusion in original study were early pregnancy (≤18 weeks) and an allergy in the family (mother, father or sibling of the unborn child). The criteria for exclusion were any chronic diseases, such as diabetes or celiac disease.

From the original study, a cohort of 15 pregnant women was enrolled into the present pilot study. One criterion for inclusion was the willingness to provide a blood sample for DNA methylation analysis from both the mother and infant at same time point after the delivery. All women received dietary counselling during the study visits to follow the recommendations for pregnancy and breast feeding. In addition, seven of the women received in a double blind manner, probiotic capsules (one capsule /day) containing *Lactobacillus rhamnosus* GG (American type culture collection 53103, Valio Ltd, Helsinki, Finland) and *Bifidobacterium lactis* Bb12 (C.Hansen, Hoersholm, Denmark), $10^9$ cfu/day each, and eight received placebo capsules. Dosing with standard content capsules commenced on the first study visit and lasted until the end of exclusive breast-feeding, maximum 6 months. All capsules were stored at + 5 C and the viability of the probiotic capsules was confirmed by regular analysis in the laboratory. Compliance about consumption of study capsules was assessed by interview. The participants visited the study clinic in the first and third trimester of pregnancy, and with their infants when they were 1, 6, and 12 months of age.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of the Hospital District of South-West Finland (355/11/2000). Written informed consent was obtained from all of the subjects involved. The study is registered at clinical trials (NCT00167700, section 3; http://www.clinicaltrials.gov).

### *Dietary counseling and food records*

All of the women in the present study received dietary counseling. In short, the counseling aimed at modifying the mother's diet as recommended for pregnant and breastfeeding women and particularly with information on how to affect the type of fat used as well as increasing the amount of fiber in the diet [12].

Food and nutrient intakes were evaluated using a 3-day food record, including one weekend day, at the first and third trimester of pregnancy and one month postpartum. Daily energy and nutrient intakes were calculated using the Micro-Nutrica® computerized program version 2.5 (Research Centre of the Social Insurance Institution, Turku, Finland).

*Sampling and DNA methylation profiling*

Blood samples were taken from the mothers and their children at the same time point, according to mother's request, either 6 or 12 months after the delivery (mean 9.8 months, range 6.1-12.7 months, placebo group mean 10.8 months, range 6.1-12.7 and probiotic group 8.8 (6.1- 12.7) months). Whole blood samples were stored in EDTA at -70ºC until the analysis.

The DNA methylation profiling was carried out in the Finnish Microarray and Sequencing Center (FMSC, Turku Centre for Biotechnology, University of Turku and Åbo Akademi University). In the DNA methylation profiling, the genomic DNA was extracted from the EDTA blood sample with a QIAamp DNA Blood Maxi kit (Qiagen). From each sample, 5 µg of genomic DNA was sheared with a Covaris S2 sonicator for 10 min (Duty cycle 10; Intensity 5; Cycles/burst 100) into an average fragment size of 150 bp, as determined with an Agilent 2100 Bioanalyzer and Bioanalyzer High Sensitivity DNA kit. The methylated DNA was enriched with a MethylMiner™ Methylated DNA Enrichment kit (Invitrogen) by following the high-salt (2M NaCl) single elution workflow as described in the kit manual. For the next-generation sequencing, 500 ng of enriched methylated DNA was processed with a SOLiD Fragment Library Construction kit (Life Technologies) according to the kit manual. Briefly, the double stranded DNA fragments were subjected to end-repair, which was followed by adaptor ligation, nick-translation and PCR amplification. The SOLiD™ Fragment Library Barcoding Kit Module 1–16 (Life Technologies) was used for multiplexing the samples. The libraries were purified with AMPure XP beads (Agencourt) and size selected from 1% agarose gel to collect 150 - 300 bp fragments. A qiaquick gel extraction kit (Qiagen) was used to purify the size-selected libraries. The size distribution of the libraries was determined with a Bioanalyzer DNA 1000 kit. The quantity of the libraries was measured with both a Qubit and SOLiD Library TaqMan Quantitation Kit (Life Technologies). Equal amounts of barcoded libraries were pooled for multiplexed sequencing. The bead preparation was carried out according to the SOLiD4 System Templated Bead Preparation Guide. A SOLiD™ EZ Bead™ System was used for automated templated bead preparation. The libraries were run with a SOLiD4 or SOLiD 5500XL Sequencer (Life Technologies) with 50 bp chemistry.

*Analysis of the DNA methylome data*

The raw sequence data was mapped to hg19 reference genome sequence with Life Technologies Bioscope (version 2.0) software using the default parameters, yielding on average 41.8M mapped reads per sample (stdev 8.84M reads). The read counts for proximal promoters (region between 1000 bp upstream and 500 bp downstream TSS, coordinates derived from Refseq gene annotations) were calculated using bedtools (version 2.17.0).

Functional enrichment analysis toward the GO and KEGG databases was carried out using the topGO and GOstats packages in R/Bioconductor. Alterations in the DNA methylation status of 623 obesity and 433 weight gain associated genes were visualized and functional associations were examined with the Ingenuity Pathway Analysis Tool (Ingenuity Systems).

To evaluate the role of maternal pre-pregnancy BMI in more detail, the level of DNA methylation was illustrated in individual study subjects according to the mother's BMI. Figures show DNA methylation of FTO, MC4R and MSRA in women.

*Statistical analysis*

The subject characteristics and the women's dietary intake at the first and third trimester of pregnancy as well as one month postpartum are shown as means with 95% confidence interval (CI). Univariate analysis ANOVA and multivariate analysis of variance MANOVA were used to compare the groups.

Statistical analysis for comparing differentially methylated promoters between sample groups was carried out using R/Bioconductor limma package on TMM normalised and voom transformed count values as suggested in the limma manual. The promoters with an absolute fold-change above 2 and moderated t-test p-value below 0.05 were considered as being significantly differentially methylated.

**Results**

*Subject characteristics and dietary intake*

The subject characteristics are shown in Table 1. The women were Caucasian, in good health, and well educated; 6 of the women in the placebo group (n=8) and 6 of the women in the probiotic group (n=7) had completed college or

university education. None of the women smoked during the pregnancy. In the placebo group the mean pre-pregnancy BMI was 24.5 including two mothers with BMI over 30 (range 18.7 – 32.7). In the probiotic group the mean pre-pregnancy BMI was 21.7 (range 19.4 – 24.0). The mean weight gain during pregnancy was 13.2 (range 6.9- 22.0) kg in probiotic group and 15.7 (13.0-20.4) kg in placebo group. The differences between the groups in the pre-pregnancy BMI or weight gain were not statistically significant. The intakes of energy and energy yielding nutrients are shown as means (and 95% CI) at the first and third trimester of pregnancy and one month postpartum in Table 2. The intakes of folate, riboflavin, B6, and B12 are also shown in Table 2 since they have previously been shown to be able to act as methyl-donors [14]. No significant differences between the groups were found in the women's characteristics or in their dietary intake during pregnancy in univariate ANOVA. At one month postpartum, fiber intake was significantly higher in the placebo group compared to the probiotic group. When analyzing the dietary intakes with multivariate MANOVA, no statistically significant differences were found at the first trimester ($p=0.29$), third trimester ($p=0.47$), or one month postpartum ($p=0.41$) between the groups. All infants were born at term.

*Probiotics intake alters the DNA methylation status of obesity risk genes*

Genome-wide association studies have recently revealed several genetic variants and risk factors associated with obesity [15]. Therefore, we first examined whether the intake of probiotics affects the DNA methylation status of any of these gene promoters previously linked with obesity in genome-wide association studies. Interestingly, three of the known risk genes were affected specifically in the mothers and five in the children (Table 3). Importantly, the DNA methylation of the promoter of the fat mass and obesity associated (FTO) gene, the strongest known genetic risk factor for obesity, was decreased in the women in response to the intake of the probiotics. The gene promoter of the methionine sulfoxide reductase A (MSRA) gene was affected in both the women and their infants (Table 3) with decreased DNA methylation in the probiotic group. To evaluate the role of maternal pre-pregnancy BMI on DNA methylation of these genes, the scatter-plots describe the level of methylation in the individual study subjects (figure 1). These figures reveal that maternal pre-pregnancy obesity does not explain the differential methylation of obesity related genes here.

*Epigenetic alterations in obesity and weight gain signaling pathways*

In order to examine more extensively the epigenetic status of obesity and weight gain genes, we used Ingenuity Pathway tool (Qiagen) to extract all of the genes functionally associated with obesity (n= 623) and weight gain (n= 433) in the literature. Subsequently, we examined the DNA methylation status of the promoter of these genes in our data with Ingenuity Pathway analysis tool. This analysis revealed epigenetic changes in a large set of additional genes that are functionally associated with obesity or weight gain based on the literature. Tables 4 and 5 show the gene promoters that were significantly affected by probiotics in the women and their children. In the women, 37 gene promoters showed decreased levels of DNA methylation in the probiotic group. In addition, one gene promoter HTR3D (5-hydroxytryptamine (serotonin) receptor was more methylated in the probiotic group (Table 4). In the children, 68 gene promoters were found to be significantly affected; all of these were less methylated in the probiotic group (Table 5). In the pathway analysis, five genes were identified as being influenced in both the mothers and infants, IGFBP1 (insulin-like growth factor binding protein 1), C3 (complement component 3), IL5 (interleukin 5), SLC6A5 (solute carrier family 6 (neurotransmitter transporter), member 5) and MYH11 (myosin, heavy chain 11, smooth muscle); all of them were less methylated in the probiotic group in both the mothers and their children.

**Discussion**

We postulate here that supplementation with specific probiotics during pregnancy may affect the DNA methylation status of the promoters of obesity- and weight gain related genes in both mothers and their children. We measured DNA methylation from peripheral blood samples and while these do not necessarily describe the methylation status in primary tissues, and although we had no RNA samples available to evaluate whether the changes in DNA methylation were actually translated into the levels of gene expression, the results were encouraging. Altogether, the affected genes included cytokines or other growth factors, enzymes, receptor-molecules, ion channels, kinases, transmembrane proteins, and transporters, providing one explanation for the probiotics' clinical effects in obesity prevention [8] and treatment [16] but also potentially affecting other metabolic [13, 17] and inflammatory conditions [18-20].

There are previous studies that the use of specific probiotics decreases the risk for central adiposity [8], abdominal visceral fat areas, BMI, as well as waist and hip circumferences and body fat mass [16]. Here, our results revealed that probiotic supplementation during pregnancy may be able to decrease the DNA methylation status in the promoter of the women's FTO (fat mass and obesity associated gene) gene which may potentially increase its transcription. FTO is the strongest risk gene associated with obesity and it has been linked with body mass index, obesity risk, and type II diabetes in numerous studies [21, 22]. The exact molecular mechanisms through which FTO participates in modulating

the obesity risk remain unclear, but it seems obvious that the altered levels of FTO have multiple and diverse consequences in obesity risk modification [23, 24]. In support of our result, a recent study concluded that the FTO methylation level may be involved in one of several mechanisms of the underlying the obesity risk of FTO polymorphism [25] whereas another study with rat white adipose tissues indicated that diet did not affected DNA methylation although diet was important factor modulating the transcription of FTO [26]. In our study another well-known obesity associated gene promoter, MC4R (melanocortin 4 receptor), was also less methylated by the probiotic combination. MC4R is known to be an important regulator of food intake by participating in appetite and energy control regulation in the brain [27]. MC4R defects have been shown to lead to a clinical phenotype characterized by lack of satiety and early-onset obesity [28]. Taken together, our present findings suggest that specific probiotics may affect the DNA methylation status of obesity and weight gain related genes, such as FTO and MC4R, and this finding may provide one explanation for the clinical effects of specific probiotics in the prevention and treatment of obesity.

Here we also detected alterations in the epigenetic regulation of several components of the insulin signaling pathways in response to probiotic intervention, which may partly explain the beneficial effects of probiotics on glucose metabolism. Interestingly, the promoter of the insulin-like growth factor binding protein 1 (IGFBP1) was less methylated in both the mothers and their children in the probiotics group. IGFBP1 encodes a protein that binds both insulin-like growth factors I and II, and a low concentration of this protein has previously been associated with insulin resistance and diabetes. Furthermore, animal experiments have indicated that increased IGFBP1 concentrations may be an effective approach to prevent insulin resistance and diabetes [29] . On the other hand, the decreased placental expression of IGFBP1 has been reported in pregnancies complicated by fetal growth restriction [30]. The MSRA (methionine sulfoxide reductase A) gene promoter was also less methylated in the probiotic group both in the mothers and children. MSRA has been shown to reduce oxidized methionine residues and thereby participate in the repairing and protection of proteins from oxidation. Mice experiments have revealed that animals lacking the MSRA gene are prone to the development of high-fat-diet induced insulin resistance and display a reduced physiological insulin response when compared to wild-type mice [31]. In the light of these findings, we speculate that the decreased methylation of IGFBP1 and MSRA may provide a mechanism that confers health benefits in both women and their children by decreasing the risk of aberrant glucose metabolism.

Our present results suggest that probiotic supplementation during pregnancy may influence the DNA methylation of obesity and weight gain related genes also in children. This highlights the question of whether probiotic

supplementation during pregnancy and the resulting changes in DNA methylation and gene activity may evoke long-term health consequences in children. For example, the promoters of STAT 3 (signal transducer and activator of transcription 3), TLR5 (Toll-like receptor 5) and IL6R (Interleukin 6 receptor) were less methylated in the probiotic group. All of those genes participate in essential metabolic and immunological processes [32-35] and changes in their activity may explain the clinical benefits of the probiotics, for instance in the prevention and treatment of allergies [18-20] and infections [36, 37] or in the treatment of necrotizing enterocolitis [38]. Nevertheless, specific trials will be needed to clarify the effect of probiotics on the developmental programming of fetus and further on lifelong health-effects in children [39-41].

We acknowledge that the probiotics' clinical effects are known to be dependent on which specific species and strains of probiotic are being used. Furthermore, we propose that each probiotic strain may have an independent effect on DNA methylation. Moreover, the DNA methylation in blood cells may vary from that occurring in primary tissues and in addition, exposure to other environmental or lifestyle factors may impact on DNA methylation. Furthermore, the relatively small number of study subjects examined in this study decreases its statistical power and therefore the results will need to be verified in a larger setting with the samples from specific tissues. However, as far as we are aware, this is the first report describing the effects of specific probiotics on DNA methylation in human subjects; moreover the existence of parallel data from mothers and their children adds significantly value to the results.

In summary, we conclude that probiotic supplementation during pregnancy may modify the DNA methylation status of obesity and/ or weight gain related genes both in mothers and their children. The current findings are certainly encouraging; we hope they will stimulate future investigations to verify these observations in primary tissues, in other populations and with other probiotic strains.

**Conflict of Interest**

None of the authors have any conflict of interest to declare.

**Authorship**

The authors' responsibilities were as follows: SV, KL, EI and SS designed the research and SV, KL, EI, SS, RL and AL conducted the research. All of the authors participated in the preparation of the manuscript and are responsible for the final content.

**Table 1.** Characteristics of the women and their children in the study groups. Probiotics refers to the groups of mothers who received probiotics and placebo indicates the mothers who received placebo. Children did not receive probiotics in their diet.

| | **Placebo (n=8)** | | **Probiotics (n=7)** | |
|---|---|---|---|---|
| | Mean (95% CI) | n | Mean (95% CI) | n |
| **Women** | | | | |
| Age | 28.6 (25.5 - 31.7) | | 29.5 (26.2 - 32.7) | |
| BMI pre-pregnancy (kg/m2) | 24.5 (21.6 - 27.4) | | 21.7 (18.6 - 24.8) | |
| Weight pre-pregnancy (kg) | 69.4 (59.7 - 79.2) | | 58.8 (48.4 - 69.3) | |
| **Children** | | | | |
| Male | | 5 | | 3 |
| Birth weight (g) | 3973 (3685 – 4260) | | 3703 (3395 - 4011) | |
| Birth height (cm) | 52.4 (51.3 - 53.5) | | 51.0 (49.8 - 52.2) | |
| Weight at one month of age (g) | 4846 (4372 - 5319) | | 4803 (4297 – 5310) | |
| Weight at six months of age (g) | 8733 (7785 - 9680) | | 7973 (6960 – 8986) | |

**Table 2.** Women's dietary intake of energy, energy yielding nutrients and methyl donors in the first and third trimester of pregnancy and at one month postpartum. Probiotics refers to the groups of mothers who received probiotics and placebo to mothers who received placebo. Children did not receive probiotics in their diet.

| | | | **Placebo (n= 8)** | **Probiotics (n=7)** |
|---|---|---|---|---|
| **Energy yielding nutrients** | | | | |
| | | | Mean (95% CI) | Mean (95%CI) |
| Energy | MJ | 1st tri | 7.58 (6.40 – 8.76) | 8.12 (6.87 – 9.38) |
| | | 3rd tri | 7.60 (6.58 - 8.63) | 8.96 (7.86 –10.06) |
| | | 1 month pp | 8.87 (6.79 –10.94) | 9.16 (6.94 – 11.38) |
| Fat total | g | 1st tri | 56.9 (42.8 – 71.0) | 66.9 (51.8 – 82.1) |
| | | 3rd tri | 61.1 (47.5 – 74.7) | 73.9 (59.4 – 88.4) |
| | | 1 month pp | 73.1 (54.6 – 91.7) | 78.5 (58.7 – 98.4) |
| SAFA | g | 1st tri | 23.0 (16.7 – 29.2) | 27.5 (20.9 – 34.2) |
| | | 3rd tri | 20.8 (15.2 – 26.5) | 26.3 (20.2 – 32.4) |
| | | 1 month pp | 27.7 (19.5 – 35.9) | 32.7 (23.9 – 41.5) |
| MUFA | g | 1st tri | 17.7 (12.5 – 22.8) | 22.7 (17.2 – 28.2) |
| | | 3rd tri | 22.7 (16.8 – 28.5) | 27.6 (21.3 – 33.8) |
| | | 1 month pp | 26.9 (19.9 – 33.9) | 27.2 (19.7 – 34.7) |
| PUFA | g | 1st tri | 9.6 (5.6 – 13.6) | 10.2 (5.9 – 14.6) |
| | | 3rd tri | 12.2 (9.5 – 14.8) | 13.5 (10.6 – 16.3) |

| | | | | |
|---|---|---|---|---|
| | | 1 month pp | 12.4 (9.5 - 15.3) | 12.1 (9.0 – 15.1) |
| Protein | g | 1st tri | 74.4 (60.5 – 88.3) | 82.8 (68.0 – 97.7) |
| | | 3rd tri | 77.5 (66.5 – 88.6) | 81.3 (69.5 – 93.0) |
| | | 1 month pp | 86.1 (67.7 – 104.5) | 88.1 (68.4 – 107.7) |
| Carbohydrates | g | 1st tri | 244.0 (205.8 – 282.2) | 243.1 (202.2 – 283.9) |
| | | 3rd tri | 231.7 (196.7 – 266.6) | 277.9 (240.5 – 315.2) |
| | | 1 month pp | 265.6 (199.3 – 331.8) | 269.7 (198.9 – 340.6) |
| Fiber | g | 1st tri | 20.9 (15.9 – 25.8) | 17.3 (12.0 – 22.5) |
| | | 3rd tri | 21.6 (15.8 – 27.3) | 20.6 (14.5 – 26.8) |
| | | 1 month pp | 21.5 (16.0 – 27.1) | 13.2 (7.3 – 19.1) |
| **Methyl-donors** | | | | |
| | | | Mean (range) | Mean (range) |
| Folate | µg | 1st tri | 296.2 (231.6 – 360.9) | 317.6 (248.5 – 386.7) |
| | | 3rd tri | 287.7 (251.0 – 324.5) | 299.1 (259.8 – 338.4) |
| | | 1 month pp | 284.3 (214.0 – 354.6) | 301.7 (226.6 – 376.9) |
| Riboflavin | mg | 1st tri | 2.2 (1.7 – 2.6) | 1.9 (1.3 – 2.5) |
| | | 3rd tri | 2.0 (1.6 – 2.4) | 2.1 (1.7 – 2.5) |
| | | 1 month pp | 2.3 (1.8 – 2.7) | 2.1 (1.6 – 2.6) |
| B6 | mg | 1st tri | 2.2 (1.7 – 2.6) | 2.3 (1.8 – 2.8) |
| | | 3rd tri | 2.0 (1.7 – 2.3) | 2.3 (2.0 - 2.7) |

| | | | | |
|---|---|---|---|---|
| | | 1 month pp | 2.6 (1.4 – 3.7) | 2.4 (1.2 – 3.7 |
| B12 | µg | 1st tri | 4.9 (3.7 – 6.2) | 6.0 (4.7 – 7.3) |
| | | 3rd tri | 5.7 (4.8 – 6.7) | 6.2 (5.2 – 7.3) |
| | | 1 month pp | 6.9 (3.6 – 10.2) | 7.4 (3.9 – 10.9) |

SAFA= saturated fatty acids, MUFA=monounsaturated fatty acids PUFA= polyunsaturated fatty acids

**Table 3**. DNA methylation changes in the promoters of obesity and weight gain associated risk genes in response to the intake of either a placebo or the probiotics. Positive fold change = less methylated in the probiotics group, negative fold change = more methylated in the probiotics group.

| | **Mothers** | | **Children** | | |
|---|---|---|---|---|---|
| **Gene Symbol** | **Fold change** | **p-value** | **Fold change** | **p-value** | **Genomic location** |
| FTO | 3.13 | 0.021 | 1.06 | 0.872 | chr16:53,736,875-53,738,375 |
| MC4R | 3.47 | 0.007 | 1.89 | 0.107 | chr18:58,039,501-58,041,001 |
| MSRA | 2.59 | 0.042 | 2.57 | 0.016 | chr8:9,910,830-9,912,330 |
| MTMR9 | 2.36 | 0.093 | 2.31 | 0.024 | chr8:11,141,000-11,142,500 |
| TNKS | 1.90 | 0.180 | 2.76 | 0.012 | chr8:9,412,445-9,413,945 |
| CTNNBL1 | 1.63 | 0.221 | 2.19 | 0.044 | chr20:36,321,434-36,322,934 |
| BDNF | -1.08 | 0.873 | 2.02 | 0.047 | chr11:27,743,105-27,744,605 |

**Table 4.** Obesity- and weight gain- related genes with significantly (absolute fold-change >2 and moderated t-test value <0.05) altered methylation in the mothers. Positive fold change = less methylated in the probiotics group, negative fold change = more methylated in the probiotics group.

| Symbol | Entrez Gene Name | Fold Change | p-value |
|---|---|---|---|
| ABCC9 | ATP-binding cassette, sub-family C (CFTR/MRP), member 9 | 2.01 | 0.016 |
| ADCYAP1 | adenylate cyclase activating polypeptide 1 (pituitary) | 5.38 | <0.001 |
| ADRB1 | adrenoceptor beta 1 | 3.13 | 0.008 |
| ADRB2 | adrenoceptor beta 2, surface | 2.76 | 0.029 |
| BBS2 | Bardet-Biedl syndrome 2 | 3.06 | 0.014 |
| C3 | complement component 3 | 3.51 | 0.002 |
| CA3 | carbonic anhydrase III, muscle specific | 2.34 | 0.036 |
| CAV1 | caveolin 1, caveolae protein, 22kDa | 3.03 | 0.013 |
| CXCL11 | chemokine (C-X-C motif) ligand 11 | 2.95 | 0.020 |
| ESR1 | estrogen receptor 1 | 2.15 | 0.026 |
| FOXA2 | forkhead box A2 | 3.17 | 0.013 |
| FTO | fat mass and obesity associated | 3.13 | 0.021 |
| GABRA1 | gamma-aminobutyric acid (GABA) A receptor, alpha 1 | 2.35 | 0.039 |
| GABRB1 | gamma-aminobutyric acid (GABA) A receptor, beta 1 | 3.14 | 0.018 |
| GABRB3 | gamma-aminobutyric acid (GABA) A receptor, beta 3 | 2.62 | 0.031 |
| GRIN1 | glutamate receptor, ionotropic, N-methyl D-aspartate 1 | 3.67 | 0.013 |
| GYS1 | glycogen synthase 1 (muscle) | 2.41 | 0.012 |
| HTR1F | 5-hydroxytryptamine (serotonin) receptor 1F, G protein-coupled | 3.75 | 0.007 |
| HTR3D | 5-hydroxytryptamine (serotonin) receptor 3D, ionotropic | -2.00 | 0.024 |
| IGF2R | insulin-like growth factor 2 receptor | 3.24 | 0.019 |
| IGFBP1 | insulin-like growth factor binding protein 1 | 4.71 | <0.001 |
| IL18 | interleukin 18 | 2.87 | 0.025 |
| IL1B | interleukin 1, beta | 2.48 | 0.033 |

| | | | |
|---|---|---|---|
| IL2 | interleukin 2 | 2.53 | 0.042 |
| IL5 | interleukin 5 | 3.28 | 0.015 |
| IRS1 | insulin receptor substrate 1 | 3.00 | 0.021 |
| LDLR | low density lipoprotein receptor | 3.17 | 0.023 |
| MC4R | melanocortin 4 receptor | 3.47 | 0.007 |
| MYH11 | myosin, heavy chain 11, smooth muscle | 2.40 | 0.048 |
| OMA1 | OMA1 zinc metallopeptidase | 2.46 | 0.038 |
| PANK1 | pantothenate kinase 1 | 2.74 | 0.015 |
| POU3F4 | POU class 3 homeobox 4 | 2.12 | 0.036 |
| PTEN | phosphatase and tensin homolog | 2.27 | 0.014 |
| RGS7 | regulator of G-protein signaling 7 | 3.87 | 0.001 |
| SLC6A5 | solute carrier family 6 (neurotransmitter transporter), member 5 | 3.28 | 0.010 |
| SP4 | Sp4 transcription factor | 2.02 | 0.021 |
| SPTLC1 | serine palmitoyltransferase, long chain base subunit 1 | 2.68 | 0.013 |
| SST | somatostatin | 3.16 | 0.002 |
| TIMP2 | TIMP metallopeptidase inhibitor 2 | 2.75 | 0.021 |
| TNFRSF1B | tumor necrosis factor receptor superfamily, member 1B | 2.23 | 0.033 |

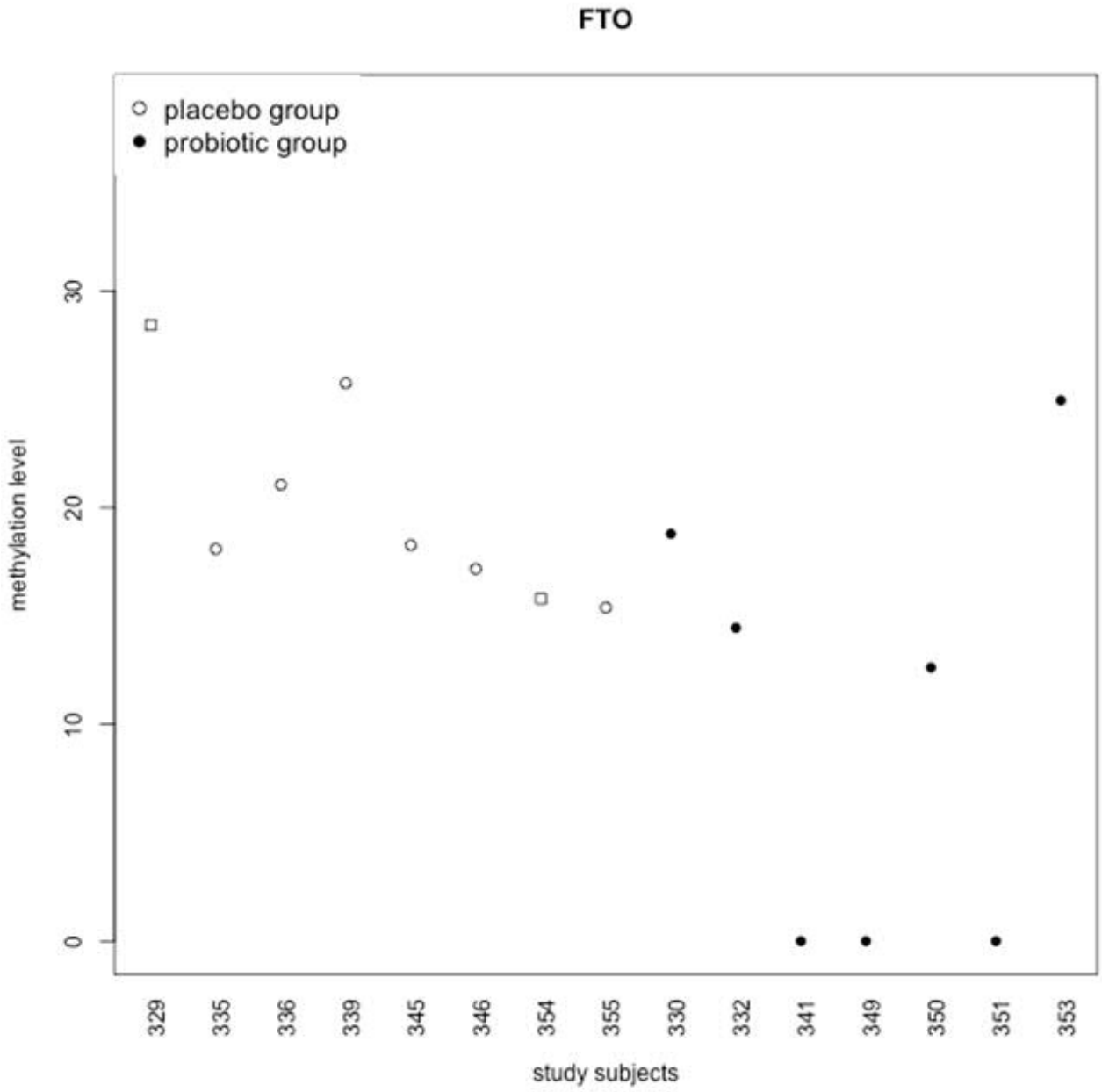
**Table 5.** Obesity- and weight gain- related genes with significantly (absolute fold-change >2 and moderated t-test value <0.05) altered methylation in the children. Positive fold change = less methylated in the probiotic group, negative fold change = more methylated in the probiotics group.

| Symbol | Entrez Gene Name | Fold Change | p-value |
|---|---|---|---|
| ABCC8 | ATP-binding cassette, sub-family C (CFTR/MRP), member 8 | 2.20 | 0.043 |
| ADORA2A | adenosine A2a receptor | 2.40 | 0.023 |
| ADRA1D | adrenoceptor alpha 1D | 2.09 | 0.035 |
| APP | amyloid beta (A4) precursor protein | 2.39 | 0.029 |
| ARNT | aryl hydrocarbon receptor nuclear translocator | 2.50 | 0.019 |
| ARRB1 | arrestin, beta 1 | 2.11 | 0.031 |
| BDNF | brain-derived neurotrophic factor | 2.02 | 0.047 |
| C3 | complement component 3 | 2.51 | 0.034 |
| CCND3 | cyclin D3 | 2.08 | 0.026 |
| CCRN4L | CCR4 carbon catabolite repression 4-like (S. cerevisiae) | 2.37 | 0.024 |
| CD38 | CD38 molecule | 2.32 | 0.030 |
| CGB | chorionic gonadotropin, beta polypeptide | 2.23 | 0.015 |
| CRHR1 | corticotropin releasing hormone receptor 1 | 2.17 | 0.025 |
| CXCR4 | chemokine (C-X-C motif) receptor 4 | 2.09 | 0.012 |
| DGAT1 | diacylglycerol O-acyltransferase 1 | 2.14 | 0.037 |
| DPP4 | dipeptidyl-peptidase 4 | 2.18 | 0.027 |
| DRD2 | dopamine receptor D2 | 2.06 | 0.022 |
| FABP2 | fatty acid binding protein 2, intestinal | 2.65 | 0.017 |
| GABRA5 | gamma-aminobutyric acid (GABA) A receptor, alpha 5 | 2.65 | 0.005 |
| GABRG2 | gamma-aminobutyric acid (GABA) A receptor, gamma 2 | 2.24 | 0.041 |
| GAL | galanin/GMAP prepropeptide | 3.29 | 0.002 |
| GAS6 | growth arrest-specific 6 | 3.34 | 0.003 |

| | | | |
|---|---|---|---|
| GNB5 | guanine nucleotide binding protein (G protein), beta 5 | 2.40 | 0.038 |
| GPT2 | glutamic pyruvate transaminase (alanine aminotransferase) 2 | 2.73 | 0.010 |
| GRIN2C | glutamate receptor, ionotropic, N-methyl D-aspartate 2C | 2.29 | 0.017 |
| HDAC9 | histone deacetylase 9 | 2.77 | 0.004 |
| HIF1A | hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) | 2.97 | 0.002 |
| HMGCR | 3-hydroxy-3-methylglutaryl-CoA reductase | 2.11 | 0.027 |
| HTR1A | 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled | 2.81 | 0.006 |
| HTR2A | 5-hydroxytryptamine (serotonin) receptor 2A, G protein-coupled | 2.43 | 0.008 |
| IAPP | islet amyloid polypeptide | 2.19 | 0.009 |
| IGF1R | insulin-like growth factor 1 receptor | 2.02 | 0.023 |
| IGFBP1 | insulin-like growth factor binding protein 1 | 3.31 | 0.001 |
| IL5 | interleukin 5 | 2.41 | 0.017 |
| IL6R | interleukin 6 receptor | 2.33 | 0.019 |
| INSR | insulin receptor | 2.15 | 0.033 |
| ITGAM | integrin, alpha M (complement component 3 receptor 3 subunit) | 3.66 | 0.003 |
| KDM3A | lysine (K)-specific demethylase 3A | 3.19 | 0.003 |
| LCLAT1 | lysocardiolipin acyltransferase 1 | 2.86 | 0.009 |
| LOX | lysyl oxidase | 2.19 | 0.044 |
| MFSD2A | major facilitator superfamily domain containing 2A | 2.98 | 0.003 |
| mir-103 | microRNA 107 | 2.07 | 0.029 |
| MMP11 | matrix metallopeptidase 11 (stromelysin 3) | 2.19 | 0.042 |
| MYH11 | myosin, heavy chain 11, smooth muscle | 2.37 | 0.008 |
| NHLH2 | nescient helix loop helix 2 | 2.29 | 0.047 |
| NR4A2 | nuclear receptor subfamily 4, group A, member 2 | 2.06 | 0.017 |
| PNRC2 | proline-rich nuclear receptor coactivator 2 | 2.22 | 0.047 |
| PPARGC1A | peroxisome proliferator-activated receptor gamma, coactivator 1 alpha | 2.37 | 0.044 |
| PRL | prolactin | 3.17 | 0.003 |

| | | | |
|---|---|---|---|
| RETSAT | retinol saturase (all-trans-retinol 13,14-reductase) | 2.77 | 0.008 |
| SCN3B | sodium channel, voltage-gated, type III, beta subunit | 2.72 | 0.020 |
| SCN9A | sodium channel, voltage-gated, type IX, alpha subunit | 2.69 | 0.006 |
| SERPINE1 | serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 | 3.10 | 0.006 |
| SIRT2 | sirtuin 2 | 2.11 | 0.033 |
| SLC17A6 | solute carrier family 17 (vesicular glutamate transporter), member 6 | 2.55 | 0.011 |
| SLC4A10 | solute carrier family 4, sodium bicarbonate transporter, member 10 | 2.68 | 0.006 |
| SLC6A4 | solute carrier family 6 (neurotransmitter transporter), member 4 | 2.31 | 0.022 |
| SLC6A5 | solute carrier family 6 (neurotransmitter transporter), member 5 | 2.20 | 0.033 |
| SPTLC2 | serine palmitoyltransferase, long chain base subunit 2 | 2.77 | 0.005 |
| STAT3 | signal transducer and activator of transcription 3 (acute-phase response factor) | 2.07 | 0.009 |
| STC1 | stanniocalcin 1 | 2.34 | 0.029 |
| STC2 | stanniocalcin 2 | 2.13 | 0.021 |
| TACR1 | tachykinin receptor 1 | 2.83 | 0.003 |
| TLR5 | toll-like receptor 5 | 2.45 | 0.036 |
| TP53INP1 | tumor protein p53 inducible nuclear protein 1 | 2.91 | 0.004 |
| TRPC1 | transient receptor potential cation channel, subfamily C, member 1 | 2.76 | 0.010 |
| UCP2 | uncoupling protein 2 (mitochondrial, proton carrier) | 2.17 | 0.022 |
| UGCG | UDP-glucose ceramide glucosyltransferase | 2.29 | 0.009 |
| VEGFA | vascular endothelial growth factor A | 2.75 | 0.004 |

a)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probiotic group
methylation level
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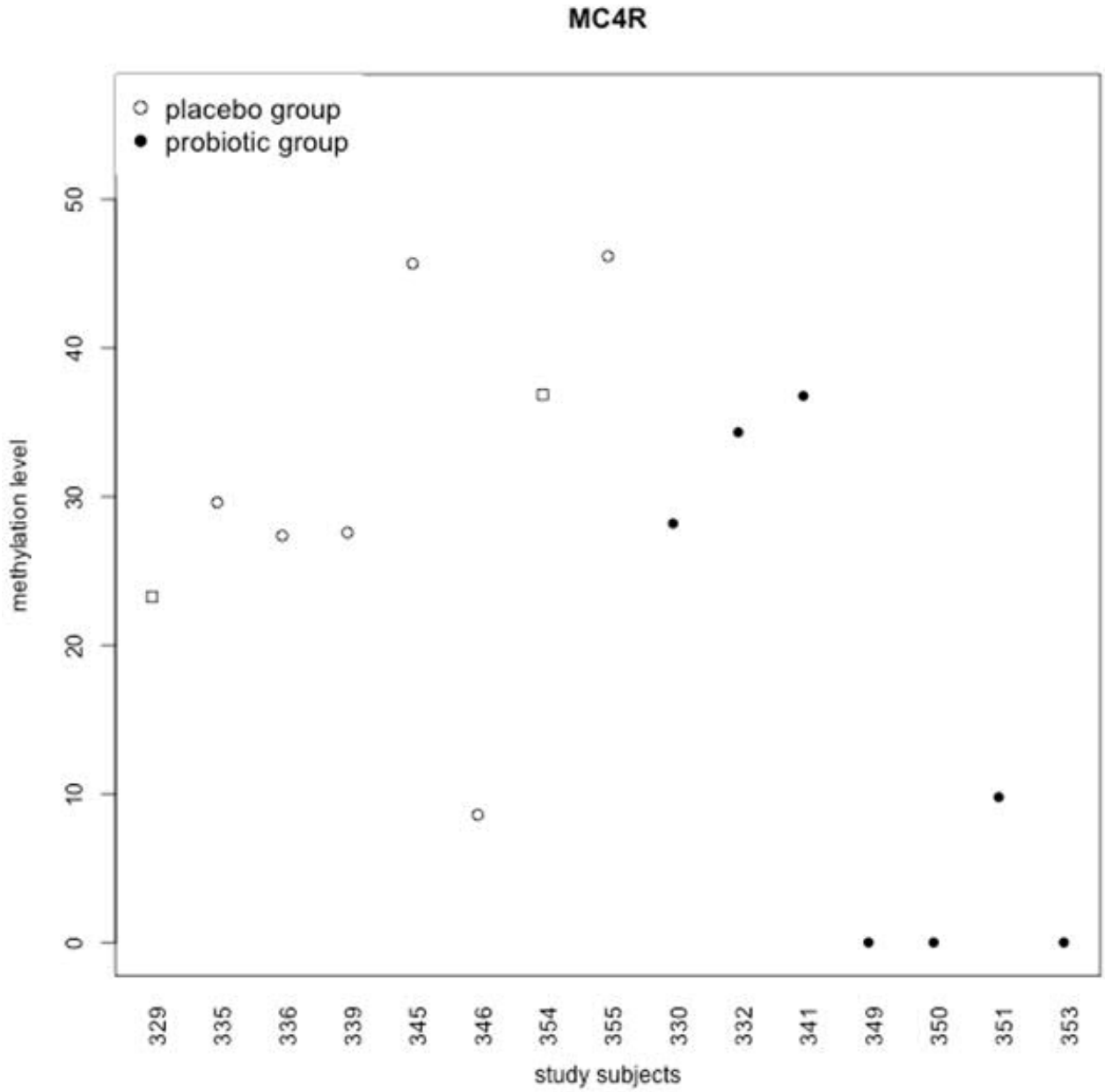
353
study subjects

b)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study subjects

c)

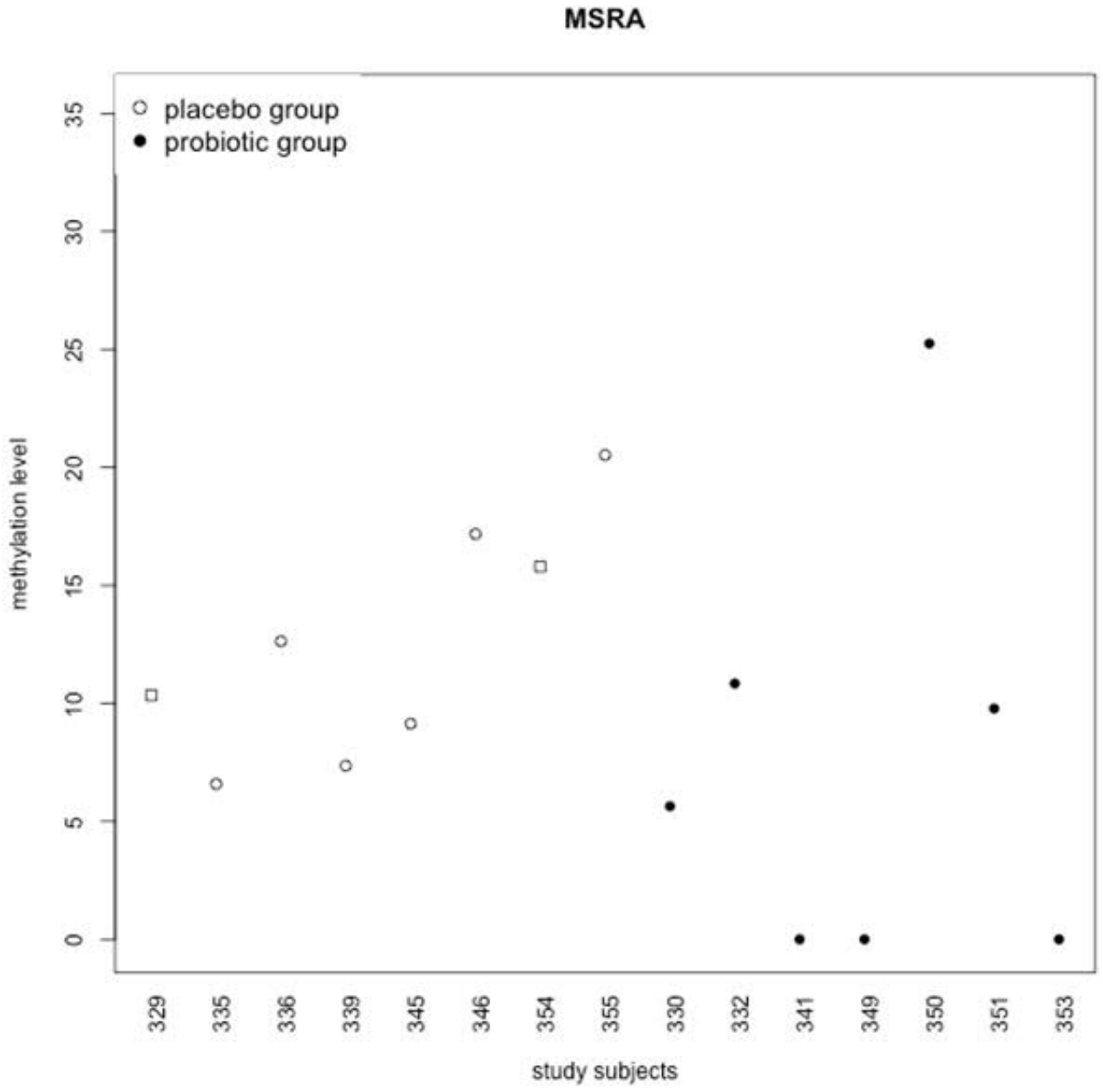


**Figure 1.** Methylation level of FTO (a), MC4R (b) and MSRA (c) genes in the individual study subjects. Open circles represent the placebo group whereas black circles are the probiotic group. Mothers with BMI over 30 are illustrated as squares.

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