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1 **Impact of maternal diet during pregnancy and breastfeeding on infant metabolic**  
2 **programming: a prospective randomized controlled study**

3  
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12  
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14  
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16  
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19  
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24 *GG* by Valio Ltd (Helsinki, Finland) and *B. lactis* by Chr. Hansen (Hoersholm, Denmark).

## 1 **Abstract**

2 **Objectives:** To evaluate the impact of maternal diet and intensive dietary counselling during  
3 pregnancy and breastfeeding on the infant's metabolic status.

4 **Subjects/Methods:** At the first trimester of pregnancy 256 women were randomized into a  
5 control/placebo group and two dietary counselling groups (diet/probiotics and diet/placebo). The  
6 counselling, with double-blind randomization to probiotics (*Lactobacillus rhamnosus GG* and  
7 *Bifidobacterium lactis*) or placebo, was targeting excessive saturated fat and low fibre  
8 consumption. The maternal diet was evaluated repeatedly during pregnancy and postpartum by  
9 means of three days' food diaries. Metabolic markers, serum 32-33 split and intact proinsulin,  
10 leptin/adiponectin ratio, skinfold thickness and waist circumference, were measured from 194  
11 healthy infants at six months of age, and the high levels were taken to mirror adverse metabolic  
12 status.

13 **Results:** The proportion of infants with high 32-33 split proinsulin was significantly lower in  
14 dietary counselling with probiotics(n=6/62, 9.7%) or placebo(n=7/69, 10.1%) compared to the  
15 control/placebo group(n=17/63, 27.0%). The high split proinsulin associated with larger skinfold  
16 thickness, waist circumference and higher leptin/adiponectin ratio in the infants(p<0.05). In  
17 respect of maternal diet during pregnancy, the highest and lowest tertiles of fat intake increased  
18 the infant's risk of high split proinsulin while those of butter associated correspondingly with the  
19 infants' waist circumference. Further, breastfed infants showed a reduced risk of high split  
20 proinsulin and leptin/adiponectin ratio compared to formula-fed infants.

21 **Conclusions:** Maternal diet during pregnancy and breastfeeding may benefit infant metabolic  
22 health. High split proinsulin reflects adverse metabolic status in infancy, which can be improved  
23 by early dietary counselling.

24 **Keywords:** Infant; Adiposity; Metabolism; Pregnancy; Counselling

## 1 **Introduction**

2 Industrialized countries worldwide have been faced with a progressive increase in metabolic  
3 conditions such as obesity, and the velocity of propagation is particularly outstanding in the  
4 paediatric population. Evidence that overweight or obesity, heightened blood pressure and  
5 impaired glucose metabolism are programmed (Barker 2004) by early nutrition points to foetal  
6 and early postnatal life as critical periods and intervention targets.

7

8 The data implicating early nutritional influences on the cardiometabolic risks in humans derive  
9 mainly from epidemiological studies of extreme prenatal circumstances such as exposure to  
10 famine (Painter *et al.* 2006; Ravelli *et al.* 1998). In these demonstrations low birth weight is the  
11 main risk factor, taken to reflect a poor intrauterine nutritional environment. In well-nourished  
12 women, again, child birth weight, low or high, and a suboptimal intrauterine environment have  
13 been linked to maternal intake of single dietary factors (Godfrey *et al.* 1996; Mikkelsen *et al.*  
14 2006; Moses *et al.* 2006; Zhang *et al.* 2006). However, a fact not previously properly addressed  
15 but not to be ignored is that the diet is always a mixture of several nutrients with possible  
16 complex interactions.

17

18 Previous demonstrations suggest that the gut microbiota composition is linked to the  
19 immunological and metabolic development of the child (Guarner and Malagelada 2003; Rinne *et al.*  
20 2005). The current intervention study targeted at dietary elements associated with these  
21 developmental components, specifically at the dietary low fibre and high saturated fat  
22 composition together with aberrant gut microbiota development by probiotics. We have shown  
23 that the joint actions of diet and probiotics benefited mothers' glucose metabolism (Laitinen *et al.*  
24 2009) and weight management (Ilmonen *et al.* Submitted).

1  
2 Here, we studied, whether these benefits achieved in mothers' diet can be extended to their  
3 children's metabolic health at the age of six months. The high 32-33 split proinsulin, a well  
4 characterized predictor of insulin resistance in adults and older children (Mykkanen *et al.* 1997;  
5 Singhal 2006; Temple *et al.* 1989), was taken as a novel marker of adverse metabolic status in  
6 infancy. To further investigate the infants' metabolic status and the usefulness of high split  
7 proinsulin in its assessment, waist circumference, skinfold thickness and adipocyte-derived  
8 cytokines – leptin and adiponectin – (Corvalan *et al.* 2010; Darendeliler *et al.* 2009; Shea *et al.*  
9 2003; Valle *et al.* 2002) were taken as secondary outcomes.

10

## 11 **Subjects and methods**

### 12 *Study design*

13 A total of 256 pregnant women were recruited from the maternal welfare clinics in the area of  
14 Turku, Southwest Finland, in the first trimester of pregnancy between April 2002 and November  
15 2004 to participate in a prospective, randomized mother-infant nutrition and probiotics study  
16 (NCT00167000; section 3, <http://www.clinicaltrials.gov>) (Laitinen *et al.* 2009). Exclusion criteria  
17 for pregnant women were any chronic diseases except for allergy. The women visited the study  
18 clinic three times during pregnancy, at a median of 14 (range, 7 to 18), 24 (20 to 27) and 34  
19 weeks of gestation (30 to 37), respectively. At the first study visit, representing the baseline,  
20 mothers were randomized into three study groups (Fig 1), two dietary intervention and one  
21 control group, according to computer-generated block randomization of six women. The study  
22 statistician, who was not involved in the study visits, generated the randomization list. The  
23 mother-infant pairs visited the study clinic at a median infant age of six months (range, 5 to 8).  
24 The study complies with the Declaration of Helsinki, as revised in 2000. Written informed

1 consent was obtained from the women and the Ethics Committee of the Hospital District of  
2 Southwest Finland approved the study.

3

#### 4 *Maternal dietary and probiotic intervention*

5 The pregnant women in the control group received standard dietary counselling in well-women  
6 clinics. At each study visit, women in the dietary intervention groups (diet/probiotics and  
7 diet/placebo) received intensive dietary counselling in accordance with that currently  
8 recommended (1996), and this being additional to standard counselling given to all pregnant  
9 women in well-women clinics in Finland. The counselling was given in layman terms by a  
10 nutritionist who encouraged the participants to pay attention to the amount and type of fat and the  
11 amount of fibre in the diet (Piiirainen *et al.* 2006). Further, to strengthen the dietary course and to  
12 demonstrate sources of favourable fat and fibre content, various food products, available on the  
13 market, were provided for use at home. The efficacy and safety of this dietary counselling during  
14 pregnancy have been reported elsewhere (Piiirainen *et al.* 2006). The dietary intervention groups  
15 (diet/probiotics and diet/placebo) received capsules of probiotics (*Lactobacillus rhamnosus GG*,  
16 American type culture collection 53103, Valio Ltd., Helsinki, Finland and *Bifidobacterium lactis*,  
17 Chr. Hansen, Hoersholm, Denmark,  $10^{10}$  colony-forming units each/day) or placebo  
18 (microcrystalline cellulose and dextrose anhydrate; Chr. Hansen, Hoersholm, Denmark) in  
19 double-blind manner, while the control group (control/placebo) received placebo in single-blind  
20 manner. The maternal probiotic intervention continued from early pregnancy until the end of  
21 exclusive breastfeeding, a maximum six months postpartum.

22

23

24

1 *Evaluation of maternal food and nutrient intake*

2 The maternal dietary intake was evaluated in the context of all study visits by means of three  
3 days' food diaries, including one weekend day, and using household measures. The daily dietary  
4 intakes of energy, foods and nutrients were calculated by the computerized program Micro-  
5 Nutrica, version 2.5 (Research Centre of the Social Insurance Institution, Turku, Finland).  
6 Maternal energy, fibre and energy-yielding nutrient intake was analyzed. The foods consumed  
7 were combined as groups (grain, meat, fish and dairy products, fruits and berries, soft margarine  
8 and vegetable oil, sugar and sweets) in the analyses, but milk, cheese, sour milk products,  
9 vegetables and butter consumptions were also analyzed separately.

10

11 *Evaluation of clinical characteristics*

12 Women's weight, height, blood pressure and fasting plasma glucose concentration were  
13 measured at every study visit (glucose not in the second visit) (Aaltonen *et al.* 2008; Laitinen *et*  
14 *al.* 2009). Total gestational weight gain was calculated and the appropriate gestational weight  
15 gain evaluated according to the pre-pregnancy body mass index (1990). We recorded maternal  
16 diagnoses, including gestational diabetes mellitus, from the well-women records, and asked their  
17 smoking habits. The duration of pregnancy was calculated from the last menstruation.

18

19 Infants' weight, length and head circumference at birth were measured in the hospital maternity  
20 ward with measurements comparable to those used in the study visits. At the six months of age  
21 they underwent a physical examination, breastfeeding status was recorded, and their  
22 anthropometrics were measured; weight with Data baby scale 930 (Oriola, Espoo, Finland),  
23 length with Infantometer (Pedihealth, Oulu, Finland), waist and head circumference with a

1 measuring tape, and the supra-iliac skinfold with a Holtain Tanner/Whitehouse Skinfold caliper  
2 (Marsden Weighing Group, Henley-on-Thames, Oxfordshire, UK).

3

#### 4 *Sampling*

5 To evaluate infants' metabolic status, serum 32-33 split proinsulin, intact proinsulin and  
6 adiposity-derived hormones, leptin and adiponectin were chosen as metabolic markers, as these  
7 are not sensitive to non-fasting state (Gil-Campos *et al.* 2010; Glauber *et al.* 1986; Karlsson *et al.*  
8 2004) unlike blood glucose and insulin concentrations. Indeed, for obvious ethical reasons  
9 overnight fasting was not possible at the age of six months. Venous blood samples were collected  
10 before noon and were successfully obtained from 194 (76%) infants (Fig 1).

11

#### 12 *Analytical methods*

13 Serum was separated immediately and the samples were initially stored at  $-20^{\circ}\text{C}$  and then at  
14  $-70^{\circ}\text{C}$ . The NIHR Cambridge Biomedical Research Centre, Core Biochemical Assay Laboratory  
15 (Cambridge, UK) analyzed the infants' samples. Serum leptin, adiponectin, 32-33 split proinsulin  
16 and intact proinsulin concentrations were assayed on a 1235 AutoDELFI A immunoassay system  
17 (Perkin Elmer Life Sciences, Boston, MA). All assays were in-house, two-step time-resolved  
18 fluorometric assays as previously described (Hales *et al.* 1991; Semple *et al.* 2006; Temple *et al.*  
19 1989), and samples were analyzed in duplicate. Samples in which the coefficient of variation of  
20 the duplicates was greater than 10% were repeated. Quality Control samples with concentrations  
21 spanning the working range of the assay were run each day. The between-batch imprecision for  
22 the Quality Control samples was less than 8% for all assays and analyte concentrations. Maternal  
23 plasma glucose concentrations were analyzed on the day of sampling as previously reported  
24 (Laitinen *et al.* 2009).



1

2 *Statistical analyses*

3 The serum 32-33 split and intact proinsulin concentrations were primary outcome variables,  
4 leptin and adiponectin concentrations, waist circumference and supra iliac skinfold were  
5 secondary outcome variables. Any 32-33 split and intact proinsulin values above the 85<sup>th</sup>  
6 percentile of the concentrations (7.9pmol/l and 6.64pmol/l, respectively) were considered high  
7 values. These were dichotomized, as the clinically important difference in mean levels is not  
8 known and we considered differences in proportions of higher values to be more relevant than  
9 those in overall mean levels. As the agreement between high concentrations was good (kappa-  
10 coefficient  $\kappa= 0.80$ ), only the dichotomized 32-33 split proinsulin concentration was analyzed as  
11 a final outcome variable. To evaluate high split proinsulin as a metabolic marker logistic  
12 regression analysis was used for dichotomized split proinsulin which was explained by  
13 continuous or dichotomized (the 85<sup>th</sup> percentile as a cut-off point) adipocytokine ratio and  
14 anthropometrics measured at age of six months.

15

16 The group comparisons in categorized and continuous outcome variables were assessed by  
17 univariate logistic regression analysis or by analysis of variance (ANOVA) when appropriate. As  
18 most of the associations between the clinical characteristics (Table 1) and high split proinsulin  
19 were nonlinear, the characteristics of mother and child were categorized according to median,  
20 tertiles or quartiles. The effects of other possible explaining factors (Table 1) on infants' high 32-  
21 33 split proinsulin were analyzed using univariate logistic regression analyses. In the final  
22 multivariate models the intervention was forced and the explaining factors, if  $p < 0.10$  in  
23 univariate analysis, were introduced to the forward stepwise logistic model (criterion for entry  $p <$

1 0.10). The group comparisons are given as unadjusted and adjusted odds ratios (OR) with 95%  
2 confidence intervals using the control/placebo group as a reference group.  
3  
4 Maternal dietary intakes during and after pregnancy were divided into tertiles (T1=lowest,  
5 T2=middle and T3=highest). The Chi-squared test was used to study associations between dietary  
6 intakes and outcome variables. The effect of probiotic intervention on the association between  
7 dietary components and infant's high split proinsulin was analyzed by the method of Mantel-  
8 Haenszel and the Breslow-Day test,  $p < 0.20$  was used to indicate interaction. P-values  $< 0.05$  were  
9 considered statistically significant. Statistical analyses were performed with SPSS version 15.0  
10 (SPSS Inc. Chicago, IL, USA).

11

## 12 **Results**

13 Clinical characteristics of mothers and infants are shown in Table 1. All pregnant women were  
14 Caucasian, and the majority had college or university education (74%). The infants were healthy  
15 and their anthropometrics were comparable in the diet/probiotics, diet/placebo and  
16 control/placebo groups. The infants' median 32-33 split proinsulin concentration was 3.3 pmol/l  
17 (range: 1.2 to 21.9), intact proinsulin 3.7 pmol/l (1.2 to 20.0), leptin 3.7 ng/ml (0.7 to 14.8) and  
18 adiponectin 13.5 ug/ml (3.6 to 37.3) at age of six months.

19

### 20 *The cluster of metabolic markers in infancy*

21 In evaluating the usefulness of high split proinsulin as a marker of adverse metabolic status in  
22 infancy we found that higher skinfold thickness (OR=1.24,  $p=0.045$ ), waist circumference  
23 (OR=1.14,  $p=0.035$ ) and leptin/adiponectin ratio (OR=1.90,  $p=0.029$ ), each of them included as  
24 continuous variables, were associated with increased likelihood of high 32-33 split proinsulin.

1 Lower (<3320g; Q1 vs Q2-Q4) birth weight infants had an increased risk for subsequent high  
2 split proinsulin (OR=2.59, p=0.027), while the other infants' anthropometrics at birth or at the  
3 age of six months were not statistically significantly related to the high split proinsulin.

4

#### 5 *Impact of maternal dietary intervention on the infants' metabolic status*

6 The infants' risk of high 32-33 split proinsulin concentration was lower in dietary counselling  
7 with probiotics or placebo groups compared to the control group (Fig 2); diet/probiotics  
8 unadjusted OR=0.29 (95% CI 0.11 to 0.80, p=0.016) and diet/placebo OR=0.31 (0.12 to 0.80,  
9 p=0.015). To find the difference between diet/probiotics (9.7%) and diet/placebo group (10.1%)  
10 with a 0.05 two-sided significance level and 90% power, the required sample size were more than  
11 100 000 infants per group. The independent effect of dietary intervention on the infants' high  
12 split proinsulin concentration remained statistically significant in multivariate analysis with  
13 breastfeeding at six months, mother's glucose concentration at six months and weight gain during  
14 pregnancy. These adjusted and unadjusted logistic regression analyses are shown in Table 2.  
15 According to univariate analyses the intervention did not affect other metabolic markers  
16 measured in the infants (data not shown), but the breastfed infants tended to have a reduced risk  
17 of high leptin/adiponectin ratio compared to formula-fed infants (OR 0.54; p=0.054).

18

#### 19 *Impact of diet on the infants' metabolic status*

20 To evaluate the impact of maternal nutrition during pregnancy and at one and six months  
21 postpartum on the infants' risk of adverse metabolic programming, by means of high 32-33 split  
22 proinsulin, skinfold thickness, waist circumference and leptin/diponectin ratio, maternal dietary  
23 consumption was investigated in tertiles, as depicted in Table 3. Infants whose mothers' intake of  
24 fat, cheese or soft margarines and vegetable oil was in the highest or in the lowest tertile were

1 more prone to the high split proinsulin than those whose mothers' intake was in the middle  
2 tertile. A corresponding non-linear association was found between maternal butter intake and  
3 infants' waist circumference and a tendency between maternal fat intake and infants' waist  
4 circumference. Extreme consumption of fruits and berries, butter and milk in the maternal diet  
5 resulted in contrast in lower infant risk of high split proinsulin compared to the middle tertile.  
6 The highest tertile of grain products intake in the maternal diet was associated with the greatest  
7 risk of high split proinsulin and high skinfold thickness in the infants.

8

#### 9 *Interaction between probiotics and dietary components – exploratory analysis*

10 To take into account the complexity of the diet, the interaction between probiotics and dietary  
11 components were studied. The effect of probiotic versus placebo administration on the percentage  
12 of infants with high 32-33 split proinsulin in tertiles of intake of dietary components are shown in  
13 Table 4. The probiotic intake contributed to the pattern of association between dietary  
14 components and infants' high split proinsulin according to the Breslow-Day test. The interaction  
15 was suggested if  $p < 0.200$ . Interestingly, even when Bonferroni-adjusted, the maternal high milk,  
16 fruits and berries consumption combined with probiotics culminated in more prevalent high split  
17 proinsulin concentration in this group of infants compared to children whose mothers were not  
18 receiving probiotics. Further, the probiotics tended to reduced the detrimental effect of maternal  
19 low cheese, middle milk and low or middle fruit and berries consumption as well as high fat  
20 intake on the infant's risk of high split proinsulin concentration.

21

## 22 **Discussion**

23 Our results indicate that dietary counselling and a balanced nutritional environment early in life  
24 support a beneficial metabolic development of the infant. Together with our previous

1 demonstration that maternal nutrition during pregnancy contributes to the infant's blood pressure  
2 (Aaltonen *et al.* 2008), the present findings would further support the conception that several risk  
3 factors in the metabolic syndrome may be modifiable by diet during critical and sensitive periods  
4 of life, as previously shown in adults (Shiell *et al.* 2000).

5  
6 From our prospective study we have thus far learned that by modifying particularly the dietary  
7 intake of fat and fibre, by detailed dietary counselling and the provision of appropriate food  
8 products combined with probiotics, we are not only improving the quality of the maternal diet  
9 (Piirainen *et al.* 2006) but also the maternal glucose metabolism up to one year after pregnancy  
10 (Laitinen *et al.* 2009). In the present study the risk reduction in infants' high 32-33 split  
11 proinsulin concentration was already detected at six months of age, although, the intervention did  
12 not influence the infants' adiposity measurements or leptin/adiponectin ratio. Further, maternal  
13 dietary counselling *per se*, with or without probiotics, was independently related to a lowered risk  
14 of high split proinsulin concentration in infants irrespective of possible confounding variables  
15 such as maternal gestational diabetes mellitus, pregnancy weight gain or child birth size,  
16 previously linked to adverse metabolic outcomes in the child (Boney *et al.* 2005; Wrotniak *et al.*  
17 2008).

18  
19 Since the maternal and foetal nutritional environments are closely related, an explanation for the  
20 beneficial effect of dietary counselling could be extrapolated from the current knowledge that a  
21 higher intake of unsaturated fatty acids can improve insulin sensitivity (Riserus *et al.* 2009),  
22 whereas a high saturated fat content in the diet promotes the secretion of pro-inflammatory  
23 cytokines (Cani *et al.* 2007), causally linked to insulin resistance (Shoelson *et al.* 2006). Further,  
24 higher fibre consumption, by reducing the risk of gestational diabetes mellitus (Zhang *et al.*

1 2006) or by facilitating maternal weight control (Howarth *et al.* 2001), was presupposed to  
2 benefit the infant's metabolic programming. These previous data are in agreement with our  
3 findings that maternal intake of fat and specific fat-containing food products affected metabolic  
4 markers in infancy. The impacts of grain products, fruits and berries on the infants' risk may be  
5 mediated by the effect of dietary fibre. Interestingly, in contrast to what has previously been  
6 shown (Godfrey *et al.* 1996), maternal energy, protein or carbohydrate intakes were not related to  
7 infants' high split proinsulin concentration in our analyses. Furthermore, a longer duration of  
8 breastfeeding reduced the infants' risk of high split proinsulin and leptin/adiponectin ratio. The  
9 advantage of breastfeeding may be related to protection against obesity (Armstrong and Reilly  
10 2002) or to the collective composition of the gut microbiota. Indeed, bifidobacteria, which typify  
11 the gut microbiota of the healthy breast-fed infant (Fanaro *et al.* 2003), may dampen the systemic  
12 endotoxemia induced by bacterial lipopolysaccharides (Griffiths *et al.* 2004), and thereby improve  
13 metabolic status (Cani *et al.* 2007; Shoelson *et al.* 2006). The independent effect of maternal  
14 probiotic intervention on the infant's risk of high split proinsulin was not possible to study in this  
15 study population as the estimated sample size for such evaluation were more than 100 000  
16 mother-infant pairs per group. However, our exploratory results suggest that probiotics may  
17 interact with dietary components and enhance the association between maternal dietary  
18 components and infants' high split proinsulin concentration. In the light of previous studies this is  
19 not surprising. For example, dietary fatty acids and gut microbiota share similar signalling  
20 pathways in immune responses possibly controlling the low-grade inflammation (Laitinen *et al.*  
21 2006) frequently detected in metabolic disorders (Fantuzzi 2005). Thus our results, together with  
22 those of recent human and animal studies, point to the relevance of late gestation and early  
23 postnatal life (Jones and Ozanne 2009), and the importance of dietary quality (Cani *et al.* 2007)  
24 in the metabolic development.

1  
2 A large body of evidence supports the role of 32-33 split proinsulin as a marker of insulin  
3 resistance. Raised concentrations of split proinsulin in adults have been interpreted as evidence of  
4 beta-cell dysfunction (Temple *et al.* 1989) and a risk of impaired glucose tolerance (Hales *et al.*  
5 1991), but its role as a metabolic marker in infancy has remained poorly understood (Hawdon *et*  
6 *al.* 1993; Singhal 2006). We found a positive association between the high split proinsulin  
7 concentration and the ratio of adipocyte-derived cytokines - leptin and adiponectin - which have  
8 been found to correlate with adiposity (Mantzoros *et al.* 2009; Schubring *et al.* 1999), and  
9 metabolic disorders (Darendeliler *et al.* 2009; Valle *et al.* 2002) in newborns and children, and,  
10 further, to reflect efficaciously cardio-metabolic risks (Norata *et al.* 2007; Steinberger *et al.*  
11 2003). On the other hand, weight status at the age of six months predicts obesity in childhood  
12 (Taveras *et al.* 2009), and here abdominal obesity, known to impair beta-cell function (Hanley *et*  
13 *al.* 2002), was linked to the high split proinsulin concentration. A limitation to acknowledge is  
14 that the infant were not fasted before blood sampling. As this being unethical we selected  
15 metabolic markers not very sensitive to non-fasting state (Gil-Campos *et al.* 2010; Glauber *et al.*  
16 1986; Karlsson *et al.* 2004). Taken together, a high level of split proinsulin at the age of six  
17 months likely indicates susceptibility to adverse metabolic programming in these infants.

18  
19 In conclusion, we have shown for the first time in humans that favourable metabolic  
20 programming, measured especially by a lower incidence of high 32-33 split proinsulin, can be  
21 achieved by balancing the diet of the mother during pregnancy and by breastfeeding the infant.  
22 Thus, the intrauterine and immediate postnatal period comprises a window of opportunity for  
23 interventions aiming to reduce the risk of metabolic disorders in both mother and infant and  
24 implies a prospect of achieving health benefits for two generations.

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1 **Figure legends**

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3 **Figure 1** Subject flow. The mothers were randomized into three study groups at the first  
4 study visit. The study was open respect to the dietary intervention, double-blinded (black arrow  
5 head) to intervention with probiotics or placebo, and single-blinded (white arrow head) to  
6 controls with placebo.

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8 **Figure 2** The impact of dietary intervention on the infants' high (above the 85<sup>th</sup> percentile  
9 cut-off point) 32-33 split proinsulin concentration. The number of infants with high 32-33 split  
10 proinsulin was 6 (9.7%) in diet/probiotics, 7 (10.1%) in diet/placebo and 17 (27.0%) in  
11 control/placebo group. The group comparison, diet/probiotics and diet/placebo groups combined  
12 versus control/placebo group, is given as unadjusted odds ratio (OR) with 95% confidence  
13 interval.

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**Table 1** Clinical characteristics of mothers and their children (n=194) in the dietary intervention groups (diet/probiotics and diet/placebo) and in the control group (control/placebo)

	Diet/probiotics n=62	Diet/placebo n=69	Control/placebo n=63	P-value
<b>Mother</b>				
Age, y	29.6 (4.3)	30.4 (5.2)	30.3 (4.9)	0.513
Smokers 1 y prior to pregnancy	20/61 (32.8%)	30/69 (43.5%)	19/62 (30.6%)	0.256 <sup>b</sup>
Prepregnancy BMI (kg/m <sup>2</sup> ) <sup>a</sup>	22.7 (3.0)	24.2 (4.0)	24.0 (3.3)	0.045
Gestational weight gain, kg	15.2 (4.1)	15.1 (5.2)	14.7 (4.9)	0.773
Gestational diabetes mellitus	0/56 (0%)	8/67 (11.9%)	6/62 (9.7%)	0.033 <sup>b</sup>
Plasma glucose (mmol/l)				
1 <sup>st</sup> trimester	4.66 (0.40)	4.68 (0.32)	4.70 (0.29)	
3 <sup>rd</sup> trimester	4.47 (0.30)	4.62 (0.48)	4.59 (0.35)	
6 months	4.88 (0.32)	4.97 (0.39)	5.01 (0.41)	0.041 <sup>c</sup>
<b>Child</b>				
Sex, male	32 (52 %)	33 (48%)	35 (56 %)	0.674 <sup>b</sup>
Gestation age at delivery, wk	40.3 (36.9-42.3)	40.4 (33.3-42.4)	40.1 (34.9-43.3)	0.985 <sup>d</sup>
5' Apgar	9 (6-10)	9 (3-10)	9 (4-10))	0.409 <sup>d</sup>
Weight, g				
At birth	3536 (379)	3666 (363)	3621 (525)	
At 6 mo	8174 (1043)	8182 (949)	8256 (973)	0.703 <sup>e</sup>
Length, cm				
At birth	50.9 (1.7)	51.4 (1.6)	51.0 (2.2)	

At 6 mo	68.4 (2.5)	69.0 (2.4)	69.0 (2.5)	0.310 <sup>e</sup>
Head circumference, cm				
At birth	34.8 (1.2)	35.1 (1.2)	35.3 (1.4)	
At 6 mo	44.1 (1.5)	44.0 (1.2)	44.2 (1.2)	0.455 <sup>e</sup>
Waist circumference, cm				
At 6 mo	44.2 (2.9)	43.3 (3.3)	44.4 (3.3)	0.102
Skinfold, mm				
At 6 mo	6.7 (1.9)	6.6 (1.8)	6.7 (1.8)	0.934
Breast-fed, exclusive or partial				
At 6 mo	66 (67%)	69 (75%)	44/62 (71%)	0.395 <sup>b</sup>

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Results are given as means (SD) or medians (range) or number of subjects (percentage). The statistically significant differences in mothers among the study groups have been published elsewhere (Laitinen et al. 2009, Luoto et al. 2010, Ilmonen et al. Submitted).

<sup>a</sup> BMI = body mass index

The groups were compared using ANOVA unless stated otherwise.

<sup>b</sup> Chi-squared test, <sup>c</sup> ANCOVA for repeated measurements with 1<sup>st</sup> trimester as a covariate, <sup>d</sup> Kruskal-Wallis test, <sup>e</sup> ANOVA for repeated measures.

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**Table 2.** The effect of interventions and clinical characteristics on infant's high 32-33 split proinsulin (above the 85<sup>th</sup> percentile cut-off point) in univariate and multivariate analysis.

Explaining factors	Univariate analysis		Multivariate analysis <sup>a</sup>	
	OR (95% CI)	P-value	OR (95% CI)	Adjusted P-value
<b>Intervention<sup>b</sup></b>				
Control/placebo (n=63)	1.00		1.00	
Diet/probiotics (n=62)	0.29 (0.11 to 0.80)	0.016	0.24 (0.07 to 0.79)	0.019
Diet/placebo (n=69)	0.31 (0.12 to 0.80)	0.015	0.27 (0.09 to 0.79)	0.017
<b>Breastfeeding at 6 months</b>				
Ended (n=57)	1.00		1.00	
Exclusive or partial (n=136)	0.21 (0.09 to 0.47)	<0.001	0.24 (0.09 to 0.60)	0.002
<b>Glucose 6 mo postpartum</b>				
< 4.90mmol/l (n=90)	1.00		1.00	
≥ 4.90mmol/l (n=104)	3.37 (1.37 to 8.28)	0.008	3.06 (1.10 to 8.50)	0.032
<b>Weight gain during pregnancy<sup>c</sup></b>				
As recommended (n=77)	1.00		1.00	
> recommended (n=80)	2.12 (0.81 to 5.58)	0.128	2.77 (0.92 to 8.30)	0.070
< recommended (n=32)	3.33 (1.09 to 10.17)	0.034	4.04 (1.13 to 14.46)	0.032

The results are given as non-adjusted odds ratios (95% CI) and as adjusted for clinical characteristics using multivariable logistic regression analysis. Only explaining factors included in the final multivariate model of infants' high 32-33 split proinsulin concentration are shown.

<sup>a</sup>The intervention was forced to the model and the following categorical variables were given to the



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stepwise logistic regression model (criterion for entry  $p < 0.10$ ): breast-feeding at 6 months, mothers glucose at 6 mo (median = 4.90mmol/l), weight gain during pregnancy, gestational diabetes mellitus (yes vs. no), maternal smoking over one year prior pregnancy (yes vs. no) and birth weight (quartiles Q1 vs. Q2-Q4;  $< 3320$  g vs.  $\geq 3320$  g).

<sup>b</sup> Global effect  $p=0.012$  in univariate and  $p=0.014$  in multivariate model

<sup>c</sup> Global effect  $p=0.098$  in univariate and  $p=0.075$  in multivariate model

**Table 3** Tertiles for intake of foods and energy-yielding nutrients in maternal diet during pregnancy and breastfeeding, and the proportion of infants with high (above the 85<sup>th</sup> percentile cut-off point) 32-33 split proinsulin concentration, skinfold thickness, waist circumference and leptin/adiponectin ratio in each tertile of intake.

Dietary component	Study Visit	Tertile of intake	Tertile of intake		Infants with high split proinsulin		Infants with high skinfold thickness		Infants with high waist circumference		Infants with high leptin/adiponectin	
			Mean	Range	Number (%)	P <sup>a</sup>	Number (%)	P <sup>a</sup>	Number (%)	P <sup>a</sup>	Number (%)	P <sup>a</sup>
Grain products, g	1 <sup>st</sup> trim.	T1 (n=65)	134	43-176	7 (10.8)	0.034	11 (18.6)	0.011	7 (11.7)	0.065	10 (15.4)	0.491
		T2 (n=70)	206	176-239	8 (11.4)		3 (4.5)		5 (7.8)		8 (11.4)	
		T3 (n=58)	298	239-560	15 (25.9)		12 (23.1)		12 (22.2)		11 (19.0)	
Fruits and berries, g	2 <sup>nd</sup> trim.	T1 (n=69)	147	17-232	6 (8.7)	0.006	8 (12.3)	0.518	8 (12.5)	0.597	12 (17.4)	0.571
		T2 (n=62)	300	233-373	17 (27.4)		7 (12.7)		10 (16.9)		10 (16.1)	
		T3 (n=63)	543	373-1443	7 (11.1)		11 (19.0)		6 (10.7)		7 (11.1)	
Fat, g	3 <sup>rd</sup> trim.	T1 (n=59)	46	23-58	11 (18.6)	0.038	8 (14.8)	0.243	7 (12.3)	0.088	8 (13.6)	0.234
		T2 (n=66)	67	58-76	4 (6.1)		12 (20.3)		4 (7.0)		7 (10.6)	

		T3 (n=67)	93	76-175	14 (20.9)		6 (9.5)		13 (20.6)		14 (20.9)	
Cheese, g	3 <sup>rd</sup> trim.	T1 (n=64)	19	0-30	12 (18.8)	0.038	9 (15.0)	0.992	7 (12.1)	0.561	10 (15.6)	0.668
		T2 (n=66)	39	30-52	4 (6.1)		9 (15.0)		7 (11.3)		8 (12.1)	
		T3 (n=62)	77	52-177	13 (21.0)		8 (14.3)		10 (17.5)		11(17.7)	
Butter, g	3 <sup>rd</sup> trim.	T1 (n=62)	0.0	0.0-0.3	4 (6.5)	0.029	8 (13.6)	0.876	9 (15.3)	0.011	10 (16.1)	0.495
		T2 (n=64)	1.5	0.3-3.8	15 (23.4)		10 (16.7)		2 (3.4)		7 (10.9)	
		T3 (n=66)	11.5	3.8-53.3	10 (15.2)		8 (14.0)		13 (22.0)		12 (18.2)	
Soft margarine and vegetable oil, g	3 <sup>rd</sup> trim.	T1 (n=66)	15	1-22	15 (22.7)	0.045	8 (13.3)	0.554	7 (11.3)	0.808	10 (15.2)	0.905
		T2 (n=59)	28	22-34	4 (6.8)		10 (19.2)		8 (15.1)		8 (13.6)	
		T3 (n=67)	47	34-76	10 (14.9)		8 (12.5)		9 (14.5)		11 (16.4)	
Milk, g	6 mo	T1 (n=66)	72	0-151	9 (13.6)	0.035	12 (19.0)	0.450	7 (12.7)	0.891	10 (15.2)	0.591
		T2 (n=59)	246	151-350	15 (25.4)		6 (11.1)		7 (12.5)		11 (18.6)	
		T3 (n=59)	549	350-1016	5 (8.5)		7 (13.2)		9 (15.3)		7 (11.9)	

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Only dietary components significantly associated with infants' high 32-33 split proinsulin concentration are shown.

<sup>a</sup> The association between tertiled intake of dietary components and infants' high split proinsulin concentration, skinfold thickness, waist circumference and leptin/adiponectin ratio were analyzed by Chi-squared test in combined study groups (diet/probiotics, diet/placebo and control/placebo).

**Table 4.** The effect of probiotic intervention on the association between dietary components and infant's high 32-33 split proinsulin (above the 85<sup>th</sup> percentile cut-off point).

Dietary component	Study visit	Tertile of intake (total number)	Infants with high 32-33 split proinsulin		P <sup>a</sup>
			Probiotics Number (%)	Placebo Number (%)	
Fruits and berries, g	2 <sup>nd</sup> trim.	T1 (n=69)	0/23 (0.0)	6/46 (13.0)	0.003
		T2 (n=62)	2/21 (9.5)	15/41 (36.6)	
		T3 (n=63)	4/18 (22.2)	3/45 (6.7)	
Fat, g	3 <sup>rd</sup> trim.	T1 (n=59)	2/18 (11.1)	9/41 (22.0)	0.115
		T2 (n=66)	2/19 (10.5)	2/47 (4.3)	
		T3 (n=67)	2/25 (8.0)	12/42 (28.6)	
Cheese, g	3 <sup>rd</sup> trim.	T1 (n=64)	1/20 (5.0)	11/44 (25.0)	0.197
		T2 (n=66)	2/23 (8.7)	2/43 (4.7)	
		T3 (n=62)	3/19 (15.8)	10/43 (23.3)	
Milk, g	6 mo	T1 (n=66)	2/18 (11.1)	7/48 (14.6)	0.002
		T2 (n=59)	1/24 (4.2)	14/35 (40.0)	
		T3 (n=59)	3/16 (18.8)	2/43 (4.7)	

Only dietary components indicating interaction in Breslow-Day test ( $p < 0.200$ ) with infants' high 32-33 split proinsulin concentration are shown.

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<sup>a</sup> The results are given as number of children with high split proinsulin / as total number of children in the tertile group of intake (% of children with high 32-33 split proinsulin).

<sup>b</sup> The interaction was analyzed by the method of Mantel-Haenszel and the Beslow-Day test.

Figure 1

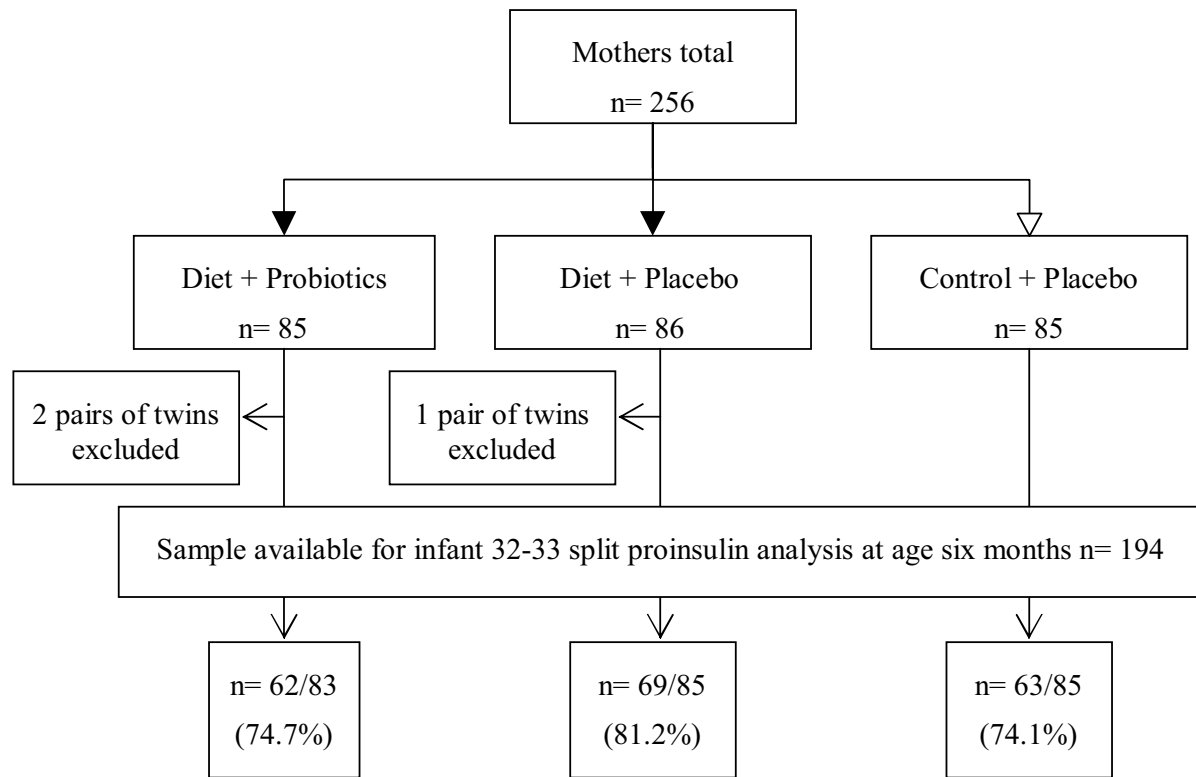


Figure 2

