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# The impact of probiotics and n-3 long-chain polyunsaturated fatty acids on intestinal permeability in pregnancy: a randomised clinical trial

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**RESEARCH ARTICLE**

## Abstract

A disruption in intestinal barrier integrity may predispose individuals to metabolic aberrations, particularly during the vulnerable period of pregnancy. We investigated whether intestinal permeability, as measured by serum zonulin concentration, changes over the duration of pregnancy and whether this change is reflected in lipopolysaccharide (LPS) activity. Second, we tested in a randomised double-blind placebo controlled clinical trial the impact of consuming dietary probiotics and/or long chain polyunsaturated fatty acid (LC-PUFA) supplements in lowering serum zonulin concentration and LPS activity. The probiotic supplement was a combination of two bacteria, *Bifidobacterium animalis* ssp. *lactis* 420 and *Lactobacillus rhamnosus* HN001. This study included 200 overweight pregnant women participating in an on-going study; participants were randomised to consume either (1) probiotics, (2) LC-PUFA, (3) probiotics and LC-PUFA, or (4) placebo for each supplement. Blood samples were obtained at early, the baseline, and late pregnancy (mean 14 and 35 weeks of gestation, respectively). Serum zonulin concentration increased from early (mean (standard deviation): 62.7 (12.9) ng/ml) to late pregnancy by 5.3 (95%CI 3.7-6.9) ng/ml, and LPS activity increased from (0.16 (0.04) EU/ml) by 0.04 (95%CI 0.03-0.05) EU/ml. No differences among the intervention groups were detected in the change from early to late pregnancy in serum zonulin concentration ($P$=0.8) or LPS activity ($P$=0.2). The change in serum zonulin concentration during the pregnancy was associated with the weeks of follow up (r=0.25, $P$<0.001). Serum LPS activity was correlated with higher maternal weight gain (r=0.19, $P$=0.008). As a conclusion, intestinal permeability increased with the progression of pregnancy in overweight and obese women and was reflected in LPS activity. No efficacy of supplementation with probiotics and/or LC-PUFA was demonstrated in pregnancy-induced changes in serum zonulin concentration or LPS activity.

**Keywords:** serum zonulin, overweight and obese pregnant women, lipopolysaccharide

## 1. Introduction

The changes that occur in maternal physiology and metabolism during pregnancy have multiple, and even long-term effects on the health of both the mother and the child. Recent research highlights the importance of the gut, particularly the role of the intestinal epithelium, as a regulator of human health (König *et al.*, 2016). The intestinal epithelium has many functions, an essential one being the absorption of nutrients which, during pregnancy, is enhanced to support foetal development (Astbury *et al.*, 2015). How the intestinal epithelium adapts to pregnancy and the consequences of the adaptation on maternal health is poorly known. In non-pregnant individuals, increased intestinal permeability has been associated with an increased risk of low grade inflammation related metabolic disorders, including adverse alterations in glucose metabolism (Teixeira *et al.*, 2012), type 2 diabetes (Cox *et al.*, 2017; Jayashree *et al.*, 2014; Moreno-Navarrete *et al.*, 2012) and metabolic syndrome (Leber *et al.*, 2012). This may be explained by the increased passage of gut components, such as lipopolysaccharide (LPS), into host circulation, which

induces the production of proinflammatory mediators and low grade inflammation (Boroni Moreira and De Cássia Gonçalves Alfenas, 2012; Cani *et al.*, 2008).

A range of factors, including the inadequate intake of dietary nutrients, malnutrition, alcohol consumption, infections, and gut pathogens, may disrupt intestinal epithelium integrity (Bischoff *et al.*, 2014; De Santis *et al.*, 2015). Dietary factors shown to benefit the intestinal epithelium include vitamin D (Kong *et al.,* 2007; Zhao *et al.,* 2012), n-3 long-chain polyunsaturated fatty acids (LC-PUFA) (Mokkala *et al.*, 2016a) and the amino acid glutamine (Li *et al.*, 2003). Initial evidence indicates that intestinal permeability may be increased in pregnant compared to non-pregnant women (Kerr *et al.*, 2015; Reyes *et al.,* 2006). However, longitudinal studies investigating the changes in intestinal permeability throughout pregnancy, and the means for regulation through dietary components, are lacking. A search for novel dietary means to reinforce the intestinal epithelium could provide public health significance by improving the health of the mother and the child during a vulnerable period of life.

We consider here two diet components of particular interest; probiotics, 'live microorganisms that when administered in adequate amounts confer a health benefit to the host' (Hill *et al.,* 2014), and LC-PUFA, particularly the n-3 series LC-PUFAs. Previous studies that investigated the effects of probiotics on intestinal permeability in non-pregnant humans showed a beneficial impact in patients with diarrhoea-predominant irritable bowel syndrome (Zeng *et al.*, 2008) and no impact in patients with cirrhosis, metabolic syndrome, acute pancreatitis, chronic liver disease, or in critically ill patients (Horvath *et al.*, 2016; Kwak *et al.*, 2014; Leber *et al.*, 2012; McNaught *et al.*, 2005; Sharma *et al.*, 2011). Studies with LC-PUFA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have thus far demonstrated beneficial effects on intestinal epithelium integrity in *in vitro* settings (Li *et al.*, 2008, Mokkala *et al.*, 2016b). It is of significance that both probiotics and LC-PUFAs, when consumed during pregnancy, have been found to benefit maternal and child health (Mokkala *et al.*, 2017a). It is possible that one underlying mechanism relates to the reinforcement of intestinal integrity.

The aim of this study was to determine whether intestinal permeability measured by serum zonulin concentration changes over the duration of the pregnancy and whether this change is reflected in LPS activity, an indicator of potential adverse immune response. Second, we aimed to study the impact of consuming dietary probiotics and/or LC-PUFA supplements in lowering serum zonulin concentration and LPS activity. Third, we investigated the possible factors associated with changes in serum zonulin concentration and LPS activity. Our hypothesis is that intestinal permeability in overweight pregnant women increases during pregnancy and that the increase may be lowered by dietary supplementation with probiotics and LC-PUFA, separately and/or combined, resulting in decreased serum LPS activity.

## 2. Subjects and methods

### Participants and study design

A total of 200 healthy overweight women who provided blood samples during both early (mean: 14 weeks of gestation) and late (mean: 35 weeks of gestation) stages of pregnancy were taken for the analysis of serum zonulin concentration and LPS activity (Supplementary Figure S1). This study is part of a larger mother-infant dietary intervention trial (ClinicalTrials.gov, NCT01922791) that is being conducted in Southwest Finland and was started in October 2013. The inclusion criteria for the study were overweight (self-reported pre-pregnancy body mass index (BMI) ≥25) and early pregnancy (<17 weeks of gestation). The exclusion criteria were gestational diabetes diagnosed during the current pregnancy, multifoetal pregnancy, and the presence of metabolic or inflammatory diseases, including type 1 and type 2 diabetes, celiac disease and inflammatory bowel disease, whilst presence of allergy was allowed. Leaflets with the study information and invitations to participate in the study were distributed in welfare women clinics. In addition, media and social media were used to inform about the study. The women interested in the study contacted the project coordinator for further information and to schedule the study visit. The participants were assigned to the intervention groups according to the randomisation list by the project coordinator.

At the early pregnancy visit, which was the baseline of the intervention trial, the women were randomised in a double-blind manner into four intervention groups; one group received probiotics and placebo, the second received n-3 LC-PUFA and placebo, the third group received both probiotics and n-3 LC-PUFA, and the fourth acted as a control group and received placebo for both test compounds. A stratified allocation list was drawn by a statistician, leaving the research personnel blinded, by using block randomisation within each stratum (3) according to previous deliveries/pregnancies (primipara, multipara without or with previous gestational diabetes mellitus) with block sizes of n=4. Women consumed the capsules from the first study visit onwards. The mothers were guided not to consume any probiotics or n-3 LC-PUFA containing products during the intervention. Three women reported that they consumed probiotics within one month before the late pregnant study visit: one woman due to gastroenteritis (also reported to consume probiotics containing sour milk daily) and two due to the consumption of antibiotics.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures that involved human subjects were approved by the Ethics Committee of the Hospital District of Southwest Finland (permission number 115/180/2012). Written informed consent was obtained from all subjects.

### Intervention supplements

The intervention supplements, both the probiotics and LC-PUFA, and their doses, were selected based on actual scientific knowledge to be optimal for the purpose of the larger on-going trial (ClinicalTrials.gov, NCT01922791). The probiotic supplement was a combination of two bacteria, *Bifidobacterium animalis* ssp. *lactis* 420 (DSM 22089; DuPont, Niebuell, Germany) and *Lactobacillus rhamnosus* HN001 (ATCC SD5675; DuPont, Niebuell, Germany); it contained $10^{10}$ cfu of each in a capsule, and 1 capsule was consumed per day. *L. rhamnosus* HN001 is a well characterised probiotic (Dekker *et al.,* 2007) and *B. lactis* 420 is a novel probiotic with demonstrated health benefits related to inflammation and body fat mass in humans (Klein *et al.,* 2008; Roessler *et al.,* 2008; Stenman *et al.,* 2016). The previous evidence of the impact of the selected probiotics on intestinal permeability is scarce. In our *in vitro* study (Mokkala *et al.,* 2016b), we detected enhancement of intestinal barrier function with *B. lactis* 420, which is in line with previous *in vitro* study (Putaala *et al.,* 2008). In a recent study, serum zonulin concentration tended to be lower in the *B. lactis* 420 than in the placebo group after six months of supplementation in overweight and obese non-pregnant adult men and women (Stenman *et al.,* 2016). The n-3 LC-PUFA capsule (Croda Europe Ltd., Leek, England) consisted of 1.2 g of n-3 LC-PUFA (79.6% DHA and 9.7% EPA), two capsules consumed per day to give a total daily dose of 2.4 g. Placebo for the probiotics consisted of microcrystalline cellulose and for the n-3 LC-PUFA, medium chain fatty acids (capric acid C8 54.6% and caprylic acid C10 40.3%). Both probiotics and n-3 LC-PUFA have been used in clinical studies during early critical periods. The probiotic capsules were stored at -20 °C until they were provided to the subjects, who were instructed to store the capsules in the refrigerator. The viability of the probiotic capsules was confirmed by regular analysis by the manufacturer. The n-3 LC-PUFA capsules were stored at room temperature, and the quality was controlled by regular analysis by the manufacturer. The capsule containers were numbered according to the randomisation list by someone not involved in the study. Compliance in the consumption of study capsules was assessed via interview. Overall, 97.5% (194 out of 200) of participants reported that they regularly consumed the capsules daily. The women who did not consume the capsules either consumed them in fewer than 5 days a week (one woman), discontinued the consumption or had a 12-day break before the late pregnancy visit (five women).

### Primary and secondary outcomes

The primary outcomes reported here were the change in serum zonulin concentration (intestinal permeability) from early to late pregnancy and the impact of the consumption of dietary probiotics and/or n-3 LC-PUFA supplements on the changes in serum zonulin concentration. The secondary outcomes were the change in serum LPS activity from early to late pregnancy and the impact of the consumption of dietary probiotics and/or n-3 LC-PUFA supplements on the changes in serum LPS activity.

### Clinical procedures and dietary intake

Pre-pregnancy BMI ($kg/m^2$) was calculated by dividing self-reported weight in kilograms, obtained from welfare women clinic records, by height measured with a wall stadiometer to the nearest 0.1 cm during early pregnancy. The weight was measured with an electronic scale (the Bod Pod system, COSMED, Inc., Concord, CA, USA). Weight gain was calculated as the difference between the weight measured at the late and early pregnancy visits.

Three-day food diaries were recorded by the women within a week prior to the study visit. The women were given written and oral instructions on how to record food intake and diaries were checked for completeness and accuracy with the help of a portion picture booklet during the study visits. Mean daily intakes of energy and nutrients, not including the supplements, were calculated with computerised software (Aivo diet 2.0.2.3, Aivo, Turku, Finland). Five women at early pregnancy visit and six women at late pregnancy visit did not return the food diaries.

### Sampling and analytical methods

Fasting (10 h minimum) blood samples were drawn from the antecubital vein of mothers, and the serum was separated and frozen in aliquots at -80 °C until it was analysed for zonulin concentration and LPS activity. Serum zonulin, a protein responsible for regulating paracellular transport in the intestine (Sapone *et al.*, 2006) was measured with the Zonulin ELISA kit (Immundiagnostik AG, Bernsheim, Germany). The inter-assay variation for the zonulin assay was <8%. One subject (in the control group) was excluded from the statistical analysis due to very high serum zonulin concentration (>4× standard deviation), which was considered an outlier. LPS activity was analysed with a Limulus amebocyte lysate assay coupled with a chromogenic substrate (HyCult Biochemistry B.V., Uden, the Netherlands). The interassay coefficient of variation was 5.1%.

### Statistics

For the primary outcomes, no a priori data concerning how serum zonulin concentrations are altered or the effects of probiotics or n-3 LC-PUFA on serum zonulin concentration during pregnancy were found. The statistical power of the study was calculated using data from a previous study (Liu *et al.*, 2015) in which probiotics compared to placebo resulted in an ~70% decrease in serum zonulin levels in patients undergoing colorectal liver metastases surgery and data from our previous study that measured variability in serum zonulin concentrations in overweight women in early pregnancy (Mokkala *et al.,* 2016a). To detect a 15% difference in serum zonulin concentration from early to late pregnancy or between control and intervention groups at late pregnancy, the required sample size is 39/group (80% power, α-level of 0.05). For the power calculations regarding secondary outcomes, we used data from a study that compared lean and obese pregnant women (Basu *et al.*, 2011) in which a 50% difference in LPS levels between the groups was detected, and we used data from our previous study (Mokkala *et al.*, 2017b). To detect a 15% difference in serum LPS from early to late pregnancy or between control and intervention groups at late pregnancy, the required sample size is 25/group. As this is the first study in this population with the depicted dietary intervention, we decided to analyse samples from 50 subjects per group.

The normality was analysed via the visual inspection of histograms. The difference in baseline characteristics and dietary intake between the study groups was evaluated using one-way ANOVA for normally distributed variables, the Kruskal-Wallis Test for non-normally distributed variables and the Pearson Chi-Square for categorical variables. The differences in dietary intake between early and late pregnancy were evaluated with a paired sample t-test. When the changes in serum zonulin (primary outcome) and LPS (secondary outcome) concentrations from early to late pregnancy were evaluated, one-way ANOVA was conducted to compare differences between the study groups. Similarly, when the serum zonulin concentration and LPS activity was compared among the study groups at the late pregnancy stage, one-way ANOVA was performed. Adjustments for any baseline characteristics (Table 1) were not performed, since no differences were detected among the study groups. To further study the possible factors affecting serum zonulin concentration and LPS activity as well as the changes from early to late pregnancy, a Pearson correlation was conducted. Since the intervention had no impact on serum zonulin concentration and LPS activity, correlation was performed without adjustments for intervention groups. *P*-values of less than 0.05 were considered statistically significant. Statistical analyses were performed with SPSS for Windows, version 23.0 (IBM Corp., Armonk, NY, USA).

## 3. Results

### Clinical characteristics and dietary intake

Of all the women, 44% were obese, and 63% were highly educated with a college or university degree (Table 1). No statistically significant differences were detected in clinical characteristics among the four intervention groups (Table 1). Dietary intake, except for the intake of fibre, which was lower, and saturated fatty acids, SFA, which was higher, were within the recommended intake (Nordic Nutrition Recommendations, 2012) at early and late pregnancy stages (Table S1). When the dietary intake between the study visits was evaluated, the intake of carbohydrates

**Table 1. The clinical characteristics of the women.**

| Characteristics | Probiotics + placebo | LC-PUFA + placebo | Probiotics + LC-PUFA | Placebo + placebo | All | *P*-value |
|---|---|---|---|---|---|---|
| Subjects | 51 | 49 | 49 | 51 | 200 | |
| Age (years) | 30.5 (4.9) | 30.7 (5.5) | 30.1 (5.3) | 30.2 (3.9) | 30.4 (4.9) | 0.9 |
| Prepregnancy BMI (kg/m$^2$) | 30.3 (5.1) | 30.3 (4.4) | 30.0 (4.1) | 29.8 (4.5) | 30.1 (4.5) | 0.9 |
| Highly educated[1] | 31/47 (66%) | 34/48 (71%) | 27/48 (56%) | 31/51 (61%) | 123/194 (63%) | 0.4 |
| Primipara[1] | 43% | 45% | 41% | 43% | 43% | 0.9 |
| Non-smoking/early pregnancy[1] | 44/46 (96%) | 47/48 (98%) | 46/48 (96%) | 48/51 (94%) | 185/193 (96%) | 0.9 |
| Non-smoking/late pregnancy[1] | 47/51 (92%) | 48/49 (98%) | 47/49 (96%) | 50/51 (98%) | 192/200 (96%) | 0.4 |
| Weight gain (kg) | 8.6 (3.8) | 8.9 (4.0) | 9.0 (4.2) | 8.8 (4.4) | 8.8 (4.0)[2] | 0.9 |
| Gestational weeks/early pregnancy | 16.5 (2.2) | 13.7 (2.4) | 14.0 (2.1) | 13.9 (2.1) | 13.8 (2.2) | 0.8 |
| Gestational weeks/late pregnancy | 35.2 (1) | 35.2 (1) | 35.2 (1) | 35.2 (1.2) | 35.2 (1) | 0.9 |
| Weeks of supplementation | 21.8 (2.6) | 21.5 (2.5) | 21.3 (2.5) | 21.3 (2.3) | 21.4 (2.5) | 0.7 |

[1] Pearson Chi-Square. Others: One-way ANOVA among groups, values are the mean (standard deviation).
[2] One measure is missing at late pregnancy due to hospitalisation.

as a proportion of energy intake (E%) (mean (standard deviation) difference -1.5 (8.2) E%, 95%CI: -2.6 to -0.3 E%, *P*=0.015) decreased, while the intake of fat (1.5 (7.6) E%, 95%CI 0.5-2.6 E%, *P*=0.006), SFA (0.6 (3.4) E%, 95%CI: 0.07-1.0 E%, *P*=0.025) and monounsaturated fatty acids, MUFAs, as absolute intake (1.7 (11.1)g 95%CI: 0.1-3.3 g; *P*=0.036) and as a proportion of energy intake (E%) (0.7 (3.5) E%, 95%CI: 0.18-1.20 E%, *P*=0.008) increased from early to late pregnancy. No differences in dietary intakes of energy, energy-yielding nutrients or fibre were found among the intervention groups at early or late pregnancy stages (Supplementary Table S1).

### Impact of dietary intervention on serum zonulin concentration and LPS activity

The consumption of probiotics and/or n-3 LC-PUFA supplements had no impact on either zonulin concentration or serum LPS activity, as no statistically significant differences were detected among the intervention groups from early to late pregnancy (*P*=0.8 and *P*=0.2, respectively) or at late pregnancy (*P*=0.7 and *P*=0.2, respectively).

### Change in serum zonulin concentration and LPS activity from early to late pregnancy

During the follow-up period, we observed a statistically significant increase in serum zonulin concentration of 5.3 (11.6) ng/ml when the differences between early and late pregnancy were compared (Table 2; Figure 1). The increase in serum zonulin concentration was detected in 67.8% (135/199) of the women. Similarly, a statistically significant increase (0.04 (0.07) EU/ml) was detected in LPS activity (Table 2, Figure 1). The increase in LPS activity was detected in 83.5% (167/200) of the women.

### Correlations between serum zonulin concentration, LPS activity and maternal characteristics

As both serum zonulin concentration and LPS activity increased during pregnancy, we investigated the interrelations between these factors. A statistically significant correlation was observed between serum zonulin concentration and LPS activity in early pregnancy (r=0.216, *P*=0.002) but not in late pregnancy (r=0.013; *P*=0.8). Additionally, the change in serum zonulin concentration did not correlate with the change in LPS activity from early to later pregnancy stages (r=0.02, *P*=0.7).

Further, the association of serum zonulin concentration and LPS activity with maternal pre-pregnancy BMI, weight gain and the duration of the follow-up were investigated. Maternal pre-pregnancy BMI was not associated with serum zonulin concentration or LPS activity either in early pregnancy (r=-0.06, *P*=0.4; r=0.043, *P*=0.5, respectively), or in late pregnancy (r=0.04, *P*=0.5;-0.079, *P*=0.2, respectively) or with changes in these levels between the visits (r=0.11, *P*=0.1; -0.103, *P*=0.1, respectively). Maternal weight gain was positively associated with the change in serum LPS activity (r=0.19, *P*=0.008), but not with the change in serum zonulin concentration (r=-0.09, *P*=0.2) over the follow up period. Maternal weight gain was negatively associated with serum zonulin concentration at the second study visit (r=-0.17, *P*=0.01) but not with serum LPS activity (r=0.12, *P*=0.09). The duration of the follow up (in weeks) correlated with the change in serum zonulin concentration (r=0.25,

**Table 2. Early and late pregnancy serum zonulin concentrations and LPS activities (mean (SD)) and 95% confidence intervals (95% CI) (n=199).**

| | Probiotics + placebo | LC-PUFA + placebo | Probiotics + LC-PUFA | Placebo + placebo | All | *P*-value[1] |
|---|---|---|---|---|---|---|
| Serum zonulin ng/ml | | | | | | |
| Subjects | 51 | 49 | 49 | 50 | 199 | |
| Early pregnancy | 61.9 (11.7) | 64.7 (16.4) | 61.7 (11.3) | 62.6 (11.8) | 62.7 (12.9) | |
| Late pregnancy | 68.4 (12.4) | 70.0 (18.5) | 67.2 (13.6) | 66.8 (13.5) | 68.1 (14.6) | 0.7 |
| Mean change (SD) | 6.5 (12.3) | 5.2 (11.2) | 5.5 (11.9) | 4.0 (11.2) | 5.3 (11.6) | 0.8 |
| 95%CI for change | 3.0-10.0 | 2.0-8.5 | 2.1-8.9 | 0.8-7.2 | 3.7-6.9 | |
| Serum LPS EU/ml | | | | | | |
| Subjects | 51 | 49 | 49 | 51 | 200 | |
| Early pregnancy | 0.15 (0.04) | 0.15 (0.04) | 0.16 (0.05) | 0.16 (0.04) | 0.16 (0.04) | |
| Late pregnancy | 0.18 (0.04) | 0.21 (0.11) | 0.19 (0.05) | 0.20 (0.05) | 0.19 (0.07) | 0.2 |
| Mean change (SD) | 0.03 (0.05) | 0.06 (0.11) | 0.03 (0.05) | 0.04 (0.05) | 0.04 (0.07) | 0.2 |
| 95%CI for change | 0.018-0.041 | 0.023-0.088 | 0.012-0.042 | 0.027-0.054 | 0.028-0.048 | |

[1] One-way ANOVA; tests among the intervention groups. 95% CI: 95% confidence interval.

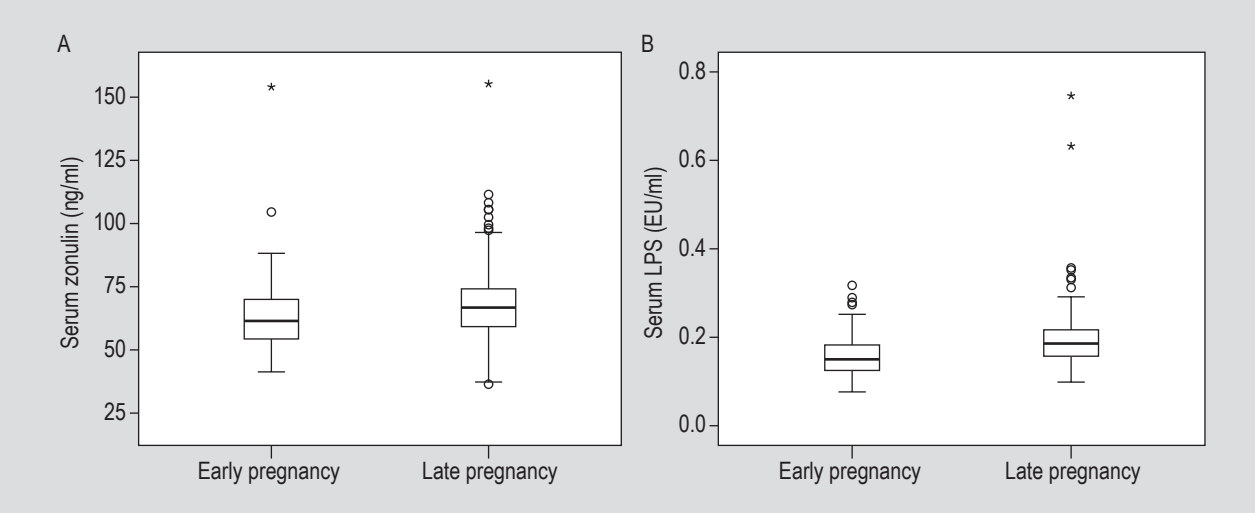


**Figure 1. Change in serum zonulin (A) and LPS (B) concentrations from early to late pregnancy. The whiskers represent 1.5 times the inter-quartile range (IQR). The circles are outliers and the asterisks are extreme outliers.**

*P*<0.001) but not with the change in LPS activity (r=0.12, *P*=0.098). We also detected a positive association between the number of gestational weeks in early pregnancy and LPS activity (r=0.21, *P*=0.003) but not with serum zonulin concentration (r=0.12, *P*=0.105).

The intake of fat as a proportion of energy intake did not correlate with serum zonulin concentration or LPS activity in early pregnancy (r=0.04, *P*=0.6; r=0.03, *P*=0.6). A trend in the correlation between the intake of fat as a proportion of energy intake and serum LPS activity was detected at late pregnancy stages (r=0.14, *P*=0.053) but not with serum zonulin concentration (r=-0.02, *P*=0.77).

## 4. Discussion

In this study, we demonstrated that the serum concentration of zonulin, a marker of intestinal permeability, increased from early to late pregnancy in overweight and obese pregnant women. At the same time, an increase in LPS activity was detected. Supplementation with probiotics and n-3 LC-PUFA, either separately or in combination, had no impact on these changes.

The detected elevation in serum zonulin concentration suggests that intestinal permeability is increased as pregnancy proceeds, and this was independent of maternal BMI or weight gain during pregnancy. To our knowledge, this is the first study to evaluate the longitudinal changes in intestinal permeability during pregnancy. In one previous study, intestinal permeability, measured via the lactulose-mannitol (L/M) – ratio urinary excretion – test at gestational week 25, was shown to be higher in normal weight pregnant women than in non-pregnant women (Kerr *et al.*, 2015). It is possible that the change in intestinal permeability is physiological and relates to an increase in the surface area of the intestine (Astbury *et al.,* 2015), which allows the enhancement of nutrient absorption as a response to the growing needs of the developing foetus. Further, an increase in intestinal permeability during pregnancy could allow the passage of maternal gut components enabling the education of foetal immune system towards immune tolerance (Khan *et al.*, 2015) and for encountering the pathogens (Gomez de Agüero *et al.,* 2016). It is also possible that the increase in intestinal permeability is harmful and may induce local or systemic inflammation. The local inflammatory response within the gut may arise from several causes, one being an aberrant gut microbiota, which may be plausible, as microbiota composition and richness is suggested to change as pregnancy proceeds (Koren *et al.*, 2012). Diet composition may also be a contributory factor (Bischoff *et al.*, 2014; De Santis *et al.*, 2015).

In our study, along with the increase in serum zonulin concentration, an increase in serum LPS activity was detected. However, as we found no association between the serum zonulin concentration and LPS activity, the increase in LPS activity may not reflect paracellular trafficking and, thus, intestinal permeability. Instead, the increase in LPS activity may result from receptor, such as scavenger receptor of the class B type I (SR-BI) and toll like receptor 4 (TLR4), mediated transport of LPS (Hersoug *et al.*, 2016). It may also be related to pregnancy induced alterations in lipoprotein profiles (Alvarez *et al.,* 1996) and hepatic LPS clearance, as LPS is mainly transported in lipoproteins (Hersoug *et al.,* 2016). A likely explanation for the elevated LPS activity may also relate to weight gain during pregnancy or to obesity status of the women (Basu *et al.*, 2011), also detected in non-pregnant populations (Gonzales-Quintela *et al.,* 2013; Lassenius *et al.*, 2011).

Probiotics and LC-PUFA intervention had no effect on the change in serum zonulin concentration or LPS activity. This is surprising because we hypothesised that based on previous, although not unanimous, experimental and human studies and plausible physiological mechanisms, supplementation with probiotics and/or n-3 LC-PUFA might enhance intestinal epithelium integrity (Horvath *et al.*, 2016; Leber *et al.*, 2012; Li *et al.*, 2008; McNaught *et al.*, 2005; Mokkala *et al.*, 2016a; Sharma *et al.*, 2011; Zeng *et al.*, 2008). In addition, decrease in serum LPS activity was expected, although studies in humans supplemented with LC-PUFA have been conducted only after LPS-induced inflammation (Ferguson *et al.,* 2014) and probiotics in disease conditions such as alcoholic hepatitis, where 7 days of supplementation with *Bacillus subtilis/Enterococcus faecium* (Han *et al.*, 2015) decreased serum LPS.

There are several possible explanations for why the dietary intervention induced no effects on intestinal permeability in this study. The women were well-nourished, healthy women, which suggests that the function and the integrity of their intestinal epithelium is normal and, thus, the impact of the intervention may be lower than in compromised cases. Further, the detected changes in serum zonulin concentrations and LPS activities at the group level were small, in this sense, the number of subjects in the study possibly was not sufficient to detect differences across the intervention groups. Also, the probiotic strains and LC-PUFA composition and concentration, as well as the duration of the intervention, may be influential factors. Although the dietary intervention products induced no effects on intestinal permeability, we consider this finding is important for designing future studies to investigate the possible mechanism of action of both probiotics and n-3 LC-PUFA. It may be that we were not measuring the best mechanism of action of the active dietary component. Other mechanisms whereby probiotics and LC-PUFA may induce beneficial effects on human metabolism and health are various, the one key impact relating to immunomodulation (Mokkala *et al.*, 2017a). Further, serum zonulin may not be an optimal marker of intestinal permeability. Serum zonulin is a commonly used as a marker for intestinal permeability (Moreno-Navarrete *et al.*, 2012; Pacificico *et al.*, 2014; Zak-Golab *et al.*, 2013; Zhang *et al.*, 2015), as a decreased concentration of serum zonulin has been associated with a decreased L/M-ratio (Liu *et al.*, 2013; Russo *et al.*, 2012), and a correlation (R=0.36, *P*=0.0004) with L/M-test results has been demonstrated (Sapone *et al.,* 2006). But, in addition to the intestine, zonulin is also produced in other tissues (Wang *et al.*, 2000), which may impact the validity of serum zonulin as marker of intestinal permeability. Faecal zonulin has also been used as an indicator for intestinal permeability, but no correlation with L/M-test results have been detected (Damms-Machado *et al.,* 2017). Since the promoter of the zonulin gene, haptoglobin 2, is under IL-6 control (Oliviero and Cortese, 1989), the inflammatory status may influence serum zonulin concentrations and thus hamper its use as a marker of intestinal permeability. Indeed, further studies are needed to evaluate the sources of zonulin in circulation and its role as a marker of intestinal permeability.

The limitations of the study include the population used. The study subjects were pregnant women, which limits the generalisability of the findings to other populations, such as non-pregnant overweight and obese women, who also have a high risk of metabolic complications. The further analysis of serum fatty acids and faecal microbiota composition would allow us to define the study population, the nutrient status and the possible impact of the baseline values on the intervention. Additional limitation was that we did not have pre-pregnancy samples with which to compare the individual changes in serum zonulin concentrations in non-pregnant women to verify the pregnancy effect, although considering the extensive resources that would have been needed, this was not feasible to accomplish. The strengths of this study are the well characterised study population, good compliance and long duration of the intervention, 21 weeks, over the course of the pregnancy with regard to the rapidly renewing intestinal epithelium.

To conclude, we found an increase in serum zonulin concentration and LPS activity during pregnancy in overweight pregnant women. Despite previous evidence, intervention with probiotics and n-3 LC-PUFA had no impact on the changes detected. It remains to be seen if an intervention can impact intestinal permeability in a larger study population or using other measurements, such as the L/M-test. Increased intestinal permeability and the resulting increase in LPS activity may be of significance regarding the pregnancy-related complications of maternal and child's health as both increased LPS activity (Creely *et al.*, 2007; Kallio *et al.,* 2015; Pussinen *et al.,* 2011) and increased intestinal permeability (Cox *et al.*, 2017; Jayashree *et al.,* 2014; Leber *et al.,* 2012; Moreno-Navarrete *et al.*, 2012; Teixeira *et al.*, 2012) have been related to metabolic risk markers.

## Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2017.0072.

**Figure S1.** Study participants and analysis.

**Table S1.** The dietary intake in the four intervention groups and all pregnant women.

## Conflict of interest

The authors have no conflicts of interest to disclose.

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