PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Effects of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in children with newly diagnosed type 1 diabetes: protocol of a randomised controlled trial.
AUTHORS	Groele, Lidia; SZAJEWSKA, Hania; Szypowska, Agnieszka

VERSION 1 - REVIEW

REVIEWER	Przemyslawa Jarosz-Chobot Medical University of Silesia, Katowice, Poland
REVIEW RETURNED	23-Apr-2017

GENERAL COMMENTS	Authors have written a randomized trial protocol to examine the effects of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in children with newly diagnosed T1D. The protocol is written in accordance with the Consort statement. The study is innovative, the concept of the proposed work and methodology are clearly defined, the statistical methods are appropriate. Minor comments: 1. Why did the authors choose for testing Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 instead of other strains of bacteria? 2. Please describe in detail the change of bacterial flora in people with T1D taking into account strains of bacteria 3. Inclusion criteria: why did the authors choose as inclusion criteria age group of 8-17 years 4. Anthropometric parameters – add “height” 5. Side effects (abdominal pain, diarrhoea, constipation, vomiting, flatulence) - Were only these side effects analyzed? 6. Occurrence of other autoimmune disease (autoimmune thyroid disease, coeliac disease)-Were only these autoimmune diseases analyzed? 7. Introduction: please write “(e.g. lack of breastfeeding..” instead of “(lack of breastfeeding..)

REVIEWER	Jannet Svensson Herlev and Gentofte University hospital We are also studying remission phase as in this study. We do though not have any intervention with probiotics or similar intervention
REVIEW RETURNED	25-May-2017

GENERAL COMMENTS	Review of the protocol:

	Effects of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in children with newly diagnosed type 1 diabetes: protocol of a randomised controlled trial. The overall study aim is to compare a group of children/adolescents treated with or without probiotics. The background for the study and scientific basis is sound and valid. The intervention seems feasible and appropriate to see an effect if this intervention modulates the gut permeability or the immune system. The outcome measures are appropriate. The design is a randomized controlled trial, which is the optimal design. The biggest drawback is the lack of support of a beneficial effect, when applying this in all ready diagnosed cases. As stated in one of the references the best timing is just after weaning – there is no comments on this issue or discussion of the likelihood of a positive effect. Limitations/lack of information: Regarding the MMTT: • Blood Glucose: How are they going to proceed with the MMTT, if BG is high when arriving for the test? If BG is > 10 mmol/l there is already a stimulation of C-peptide leading to less residual insulin response to the test • Insulin: When are they going to stop the insulin treatment. The level of exogenous insulin in the blood at test start may influence the results. There are markedly differences between Degludec, other types of basal insulin and pump treated in regards to insulin on board at test start. • There is no test after 6 months – even if this is the timing for stopping the intervention, since use of probiotics not necessary have long-term effect on microbiota this could potentially leads to lack of any knowledge about a temporary positive effect during intervention period Regarding other possible outcomes/secondary: • They state that fasting c-peptide is a secondary outcome, but it is unclear if this is measured during the MMTT – or the first C-peptide is after 30 min? • Gut permeability is not measured – even if this is one of the possible effects of probiotics – this could be used to prove if the effect on gut is there – but the intervention too late to affect the ongoing autoimmune response. Information regarding confounders/influential factors: • Diet and antibiotics during the trial may impact the intervention, and some information regarding this could be included. • Pump treatment leads to lower insulin needs, therefore information regarding treatment modality should be included if the insulin need per kg is an outcome. Regarding statistics: • The statistics is appropriate, though they could think of more complex statistics trying to differentiate between confounders and mediators. Further in line with the prospective design and repeated measures of clinical parameters and hbA1c, they could consider a model with repeated measures. Despite these drawbacks or comments the protocol seems sound and the study well prepared

REVIEWER	Heli Siljander University of Helsinki, Finland
REVIEW RETURNED	27-May-2017

GENERAL COMMENTS	Outline of the proposed study Colleagues Groele et al. present a protocol for investigating the effects of the combination of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in 8 to 17 years old children (targeted N=96) with newly diagnosed type 1 diabetes (T1D) in a placebo-controlled double-blinded randomized trial. Investigators hypothesise that modulating the gut microbiota might prevent further islet cell destruction via immunomodulatory pathways. They also speculate that children receiving the active combination may have more preserved beta-cell function than children receiving the placebo. Study subjects are expected to be free of gastrointestinal complaints, recent bacterial infections, and use of probiotics, and they should have retained some of their endogenous insulin production (fasting C-peptide ≥0.4ng/ml at diagnosis). The studied treatment (daily p.o. dose of 109 colony-forming units or placebo) has been expected to last for 6 months, after which an active follow-up continues for another 6 months. After enrolment, study visits with clinical and anthropometric assessments as well as laboratory measurements are scheduled to take place at 3, 6, and 12 months. The major strengths The major strengths of the proposed study are that 1. The intended immunomodulatory means can be regarded as safe therapies for minors 2. The study protocol does not require extra visits from the families and appears feasible 3. The planned laboratory assessments support the clinical care 4. The recruitment in a tertiary hospital setting with 2 x 200 cases of newly diagnosed T1D/year may provide the number of study subjects needed. Open questions and weaknesses There are indications in the recent literature that 1. Compared to healthy controls, subjects with T1D exhibit a less diverse and less stable gut microbiota that have proinflammatory characteristics 2. The main changes in the gut microbiota precede T1D-associated seroconversion 3. The low abundance of lactate- and butyrate-producing species in children with T1D may alter the intestinal barrier function, thus allowing increased leaking in the gut epithelium and altered presentation of foreign antigens 4. Early postnatal administration of p.o. probiotics (mainly Lactobacillus and Bifidobacterium) may be associated with a reduced risk of islet autoimmunity 5. In diabetes-prone rodents, modulation of gut microbiota leads to decreased insulitis and protection of the beta cell function. All these findings (except # 5) promote the idea that early modulation of the gut microbiota of the T1D-prone individuals might prevent seroconversion or even postpone the overt disease, even in human. However, lessons from earlier experimental immunomodulatory therapies introduced at T1D diagnosis have taught us at least that at this advanced disease stage 1. Means of therapy required to achieve significant results should be potent and relatively heavily immunomodulatory 2. Retention effect on beta-cell function is mainly temporary and

	often clinically speaking quite modest 3. Reliable assessment of the beta-cell mass and the level of the insulitis are challenging in human Thus, it does not seem too plausible that the intended therapy presented in this proposal could provide the expected effects. If launching a study of even this magnitude, I would suggest that investigators might invest on studying the potential mechanisms introduced by the intended therapy. This would not require too much extra blood volume; for example, enough serum for proper cytokine profiling, cells for immune stimulation assays, samples for epigenetic/RNA assessments in case some interesting effects are otherwise observed. Consecutive stool sampling throughout the study and assessment of the gut permeability might be advisable procedures. The clinical aspect of studying both prepubertal, pubertal, and late pubertal minors should also be considered, as their hormonal background may have interesting effects on the outcomes. Data analysis has been presented here in quite a restricted way. That should be improved significantly with proper effect estimates and power calculations, pre-planned stratification of the study subjects (to be able to see subgroup effects, if there are any), analysing policy ("intent-to-treat" or else), etc. To conclude, I would suggest that investigators downgrade their expectations and aim at providing solid data on the mechanistic effects of the proposed immunomodulatory therapy. They should take some time to clarify especially the setting for the data analyses, to ensure optimal recruitment and study procedures. Otherwise, I'm looking forward to hearing from this study group and their results.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Przemyslawa Jarosz-Chobot

COMMENT: Authors have written a randomized trial protocol to examine the effects of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in children with newly diagnosed T1D. The protocol is written in accordance with the Consort statement. The study is innovative, the concept of the proposed work and methodology are clearly defined, the statistical methods are appropriate.
RESPONSE: We thank the Reviewer for these kind words.

COMMENT: Minor comments:
1. Why did the authors choose for testing Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 instead of other strains of bacteria?
RESPONSE: As stated in the Introduction, Lactobacillus rhamnosus GG (LGG) and Bifidobacterium lactis Bb12 (Bb12) were chosen due to their impact on glucose control. Previous studies found that the supplementation with LGG and Bb12 improved blood glucose control in normoglycaemic pregnant women and reduced the frequency of gestational diabetes mellitus.

COMMENT: 2. Please describe in detail the change of bacterial flora in people with T1D taking into account strains of bacteria.
RESPONSE: Due to the word limit, a detailed description of the changes in gut microbiota in people with T1D is beyond the scope of this paper. However, the reader is directed to a reference for additional information (i.e., "For a detailed review of studies evaluating the role of the gut microbiota in these patients, see the review by Gulden et al.20").

COMMENT: 3. Inclusion criteria: why did the authors choose as inclusion criteria age group of 8-17 years
RESPONSE: Children younger than 8 years were not included, as the autoimmune process is usually very dynamic at a very young age. This may reduce the chance of prolonged remission.

COMMENT: 4. Anthropometric parameters – add “height”
RESPONSE: Done.

COMMENT: 5. Side effects (abdominal pain, diarrhoea, constipation, vomiting, flatulence) - Were only these side effects analyzed?
RESPONSE: All adverse effects reported by the participants will be considered. In the revised manuscript, we clarified that these are just some examples.

COMMENT: 6. Occurrence of other autoimmune disease (autoimmune thyroid disease, coeliac disease)-Were only these autoimmune diseases analyzed?
RESPONSE: All autoimmune diseases will be considered. In the revised manuscript, we clarified that these are just some examples.

COMMENT: 7. Introduction: please write “(e.g. lack of breastfeeding..” instead of “(lack of breastfeeding..)
RESPONSE: Done.

Reviewer: 2
Reviewer Name: Jannet Svensson

COMMENT: The overall study aim is to compare a group of children/adolescents treated with or without probiotics. The background for the study and scientific basis is sound and valid. The intervention seems feasible and appropriate to see an effect if this intervention modulates the gut permeability or the immune system. The outcome measures are appropriate. The design is a randomized controlled trial, which is the optimal design.
RESPONSE: No response is needed.

COMMENT: The biggest drawback is the lack of support of a beneficial effect, when applying this in all ready diagnosed cases. As stated in one of the references the best timing is just after weaning – there is no comments on this issue or discussion of the likelihood of a positive effect.
RESPONSE: The Reviewer is likely correct that a preventive effect rather than a therapeutic effect is more likely. However, this assumption needs to be confirmed in a clinical trial.

COMMENT: Limitations/lack of information:
Regarding the MMTT:
Blood Glucose: How are they going to proceed with the MMTT, if BG is high when arriving for the test? If BG is > 10 mmol/l there is already a stimulation of C-peptide leading to less residual insulin response to the test
RESPONSE: In the revised manuscript, we clarified that the MMTT will be rescheduled if a child has a capillary glucose value >180 mg/dl or <70 mg/dl (>10 mmol/l or <3.9 mmol/l).

COMMENT:
Insulin: When are they going to stop the insulin treatment. The level of exogenous insulin in the blood at test start may influence the results. There are markedly differences between Degludec, other types of basal insulin and pump treated in regards to insulin on board at test start.
RESPONSE: In the revised manuscript, we clarified the MMTT will be initiated before 10 AM with

children in the fasting state. Children treated with an insulin pump will continue use of this pump at the usual basal rate. A long-acting insulin analogue (glargine or detemir) will be given in the evening of the previous day. In Poland, Degludec insulin is not reimbursed. Thus, children are not treated with this insulin.

COMMENT: There is no test after 6 months – even if this is the timing for stopping the intervention, since use of probiotics not necessary have long-term effect on microbiota this could potentially leads to lack of any knowledge about a temporary positive effect during intervention period
RESPONSE: As stated in study procedure, the MMTT will be performed at months 6 and 12.

COMMENT: Regarding other possible outcomes/secondary: They state that fasting c-peptide is a secondary outcome, but it is unclear if this is measured during the MMTT – or the first C-peptide is after 30 min?
RESPONSE: C-peptide will be measured with children in the fasting state.

COMMENT: Gut permeability is not measured – even if this is one of the possible effects of probiotics – this could be used to prove if the effect on gut is there – but the intervention too late to affect the ongoing autoimmune response.
RESPONSE: We agree with the Reviewer and we decided to add gut permeability to the secondary endpoints.

COMMENT: Information regarding confounders/influential factors:
• Diet and antibiotics during the trial may impact the intervention, and some information regarding this could be included.
RESPONSE: We agree with the Reviewer that not only antibiotics, but also diet, may have an impact on gut microbial community profiling. However, the latter will not be assessed in our study. Still, we believe the comment with regard to antibiotics is relevant. Thus, we decided to add antibiotic use <2 months prior to enrolment as one of our exclusion criteria.

COMMENT: Pump treatment leads to lower insulin needs, therefore information regarding treatment modality should be included if the insulin need per kg is an outcome.
RESPONSE: The information regarding treatment modality will be included.

COMMENT: Regarding statistics:
• The statistics is appropriate, though they could think of more complex statistics trying to differentiate between confounders and mediators. Further in line with the prospective design and repeated measures of clinical parameters and hbA1c, they could consider a model with repeated measures.
RESPONSE: Done.

COMMENT: Despite these drawbacks or comments the protocol seems sound and the study well prepared.
RESPONSE: We think the reviewer for these kind words.

Reviewer #3.
COMMENT: Colleagues Groele et al. present a protocol for investigating the effects of the combination of Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 on beta-cell function in 8 to 17 years old children (targeted N=96) with newly diagnosed type 1 diabetes (T1D) in a placebo-controlled double blinded randomized trial. Investigators hypothesise that modulating the gut microbiota might prevent further islet cell destruction via immunomodulatory pathways. They also speculate that children receiving the active combination may have more preserved beta-cell function

than children receiving the placebo.
Study subjects are expected to be free of gastrointestinal complaints, recent bacterial infections, and use of probiotics, and they should have retained some of their endogenous insulin production (fasting Cpeptide≥0.4ng/ml at diagnosis). The studied treatment (daily p.o. dose of 109 colony-forming units or placebo) has been expected to last for 6 months, after which an active follow-up continues for another 6 months. After enrolment, study visits with clinical and anthropometric assessments as well as laboratory measurements are scheduled to take place at 3, 6, and 12 months.
RESPONSE: No response is needed.

COMMENT: The major strengths
The major strengths of the proposed study are that
1. The intended immunomodulatory means can be regarded as safe therapies for minors
2. The study protocol does not require extra visits from the families and appears feasible
3. The planned laboratory assessments support the clinical care
4. The recruitment in a tertiary hospital setting with 2 x 200 cases of newly diagnosed T1D/year may provide the number of study subjects needed.
RESPONSE: No response is needed.

COMMENT: Open questions and weaknesses
There are indications in the recent literature that
1. Compared to healthy controls, subjects with T1D exhibit a less diverse and less stable gut microbiota that have proinflammatory characteristics
2. The main changes in the gut microbiota precede T1D-associated seroconversion
3. The low abundance of lactate- and butyrate-producing species in children with T1D may alter the intestinal barrier function, thus allowing increased leaking in the gut epithelium and altered presentation of foreign antigens
4. Early postnatal administration of p.o. probiotics (mainly Lactobacillus and Bifidobacterium) may be associated with a reduced risk of islet autoimmunity
5. In diabetes-prone rodents, modulation of gut microbiota leads to decreased insulitis and protection of the beta cell function.

All these findings (except # 5) promote the idea that early modulation of the gut microbiota of the T1Dprone individuals might prevent seroconversion or even postpone the overt disease, even in human. However, lessons from earlier experimental immunomodulatory therapies introduced at T1D diagnosis have taught us at least that at this advanced disease stage
1. Means of therapy required to achieve significant results should be potent and relatively heavily immunomodulatory
2. Retention effect on beta-cell function is mainly temporary and often clinically speaking quite modest
3. Reliable assessment of the beta-cell mass and the level of the insulitis are challenging in human
RESPONSE: We agree with the Reviewer that these are the challenges of our study.

COMMENT: Thus, it does not seem too plausible that the intended therapy presented in this proposal could provide the expected effects. If launching a study of even this magnitude, I would suggest that investigators might invest on studying the potential mechanisms introduced by the intended therapy. This would not require too much extra blood volume; for example, enough serum for proper cytokine profiling, cells for immune stimulation assays, samples for epigenetic/RNA assessments in case some interesting effects are otherwise observed. Consecutive stool sampling throughout the study and assessment of the gut permeability might be advisable procedures.
RESPONSE: As presented in the original manuscript, blood samples will be collected and analysed

for cytokines. Stool sampling would require analysis by 16S rRNA sequencing and quantitative PCR. Current funding is limited, so this is beyond the resources of the current study. However, our team will make an effort to collect stool samples and store them at -80°C for future analysis (provided additional funding will be obtained).

COMMENT: The clinical aspect of studying both prepubertal, pubertal, and late pubertal minors should also be considered, as their hormonal background may have interesting effects on the outcomes.
RESPONSE: We agree with the Reviewer. We have added information about assessment of Tanner developmental stage to the manuscript. ("The participants also will be stratified according to Tanner developmental stage ≤ 3 or >3, as assessed by physical examination.").

COMMENT: Data analysis has been presented here in quite a restricted way. That should be improved significantly with proper effect estimates and power calculations, pre-planned stratification of the study subjects (to be able to see subgroup effects, if there are any), analysing policy ("intent-to-treat" or else), etc.
RESPONSE: As stated in the Methods, the sample size was calculated (see Sample size). All analyses will be performed on an intention-to-treat basis. Subgroup analyses based on a Tanner developmental stage will be performed. The Statistical analysis section has been substantially revised to address this comment.

COMMENT: To conclude, I would suggest that investigators downgrade their expectations and aim at providing solid data on the mechanistic effects of the proposed immunomodulatory therapy. They should take some time to clarify especially the setting for the data analyses, to ensure optimal recruitment and study procedures. Otherwise, I'm looking forward to hearing from this study group and their results.
RESPONSE: We appreciate this comment by the Reviewer. However, current funding allows us to focus mainly on clinical outcomes.

VERSION 2 – REVIEW

REVIEWER	Przemyslawa Jarosz-Chobot Medical University of Silesia, Katowice, Poland Dept. of Children's Diabetology
REVIEW RETURNED	31-Jul-2017

GENERAL COMMENTS	I'm deeply interested in the results of the study.

REVIEWER	Jannet Svensson Herlev University Hospital
REVIEW RETURNED	21-Jul-2017

GENERAL COMMENTS	I have no further comments to this protocol, all my queries have been answered

REVIEWER	Heli Siljander University of Helsinki, Finland, and The Children's Hospital, Helsinki, Finland
REVIEW RETURNED	18-Jul-2017

GENERAL COMMENTS	Thank you for your corrections and clarifications. They improved the paper significantly. Couple of additional thoughts about the protocol and data analyses: - Do you consider measuring zonulin alone to be adequate for assessing the intestinal inflammation and changes caused by the treatment? No calprotectin, beta-defencin or compositional assessments of the stool samples needed? -As the delay from the T1D diagnosis to the beginning of the therapy may be variable (ad 60 days), patients may be at different stages of their "honeymoon" both during the treatment and at the last MMTT; how do you adjust your results for the fact?

VERSION 2 – AUTHOR RESPONSE

Reviewer: 1
Reviewer Name: Przemyslawa Jarosz-Chobot
I'm deeply interested in the results of the study.
RESPONSE: No response is needed.

Reviewer: 2
Reviewer Name: Jannet Svensson
I have no further comments to this protocol, all my queries have been answered.
RESPONSE: No response is needed.

Reviewer: 3
Reviewer Name: Heli Siljander
Thank you for your corrections and clarifications. They improved the paper significantly. Couple of additional thoughts about the protocol and data analyses:
Do you consider measuring zonulin alone to be adequate for assessing the intestinal inflammation and changes caused by the treatment? No calprotectin, beta-defencin or compositional assessments of the stool samples needed?
RESPONSE: As suggested previously, we will measure zonulin as the marker of the gut permeability. As our aim is to focus on clinical outcomes; thus, the assessment of the intestinal inflammation is not planned by us. Moreover, our current grant will not allow us to cover the cost of any additional investigations.

COMMENT: As the delay from the T1D diagnosis to the beginning of the therapy may be variable (ad 60 days), patients may be at different stages of their "honeymoon" both during the treatment and at the last MMTT; how do you adjust your results for the fact?
RESPONSE: We thank the Reviewer for this valuable comment. As a matter of fact a number of immunotherapy studies in newly diagnosed diabetic patients have a delay of up to three months from the T1D diagnosis to the beginning of the immunomodulation treatment (e.g. Ludvigsson J et al. N Engl J Med. 2012;366:433-4)2. In our study, to evaluate the stage of the honeymoon phase, fasting C-peptide secretion and stimulation test will be performed in all children before the start of the study.