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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

Lidia Groele, Hania Szajewska, Agnieszka Szypowska

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Department of Paediatrics, The Medical University of Warsaw, Warsaw, Poland

Correspondence to
Dr Agnieszka Szypowska; agnieszka.szypowska@gmail.com

ABSTRACT

Introduction Recent evidence has demonstrated that, among other factors, dysbiosis (imbalances in the composition and function of the gut microbiota) may be relevant in the development of type 1 diabetes (T1D). Thus, gut microbiota may be a target for improving outcomes in subjects with T1D. The aim of the study is to examine the effects of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 on beta-cell function in children with newly diagnosed T1D.

Methods and analysis A total of 96 children aged 8 to 17 years with newly diagnosed T1D, confirmed by clinical history and the presence of at least one positive autoantibody, will be enrolled in a double-blind, randomised, placebo-controlled trial in which they will receive *L. rhamnosus* GG and *B. lactis* Bb12 at a dose of 10^9 colony-forming units or an identically appearing placebo, orally, once daily, for 6 months. The follow-up will be for 12 months. The primary outcome measures will be the area under the curve of the C-peptide level during 2-hour responses to a mixed meal.

Ethics and dissemination The Bioethics Committee approved the study protocol. The findings of this trial will be submitted to a peer-reviewed paediatric journal. Abstracts will be submitted to relevant national and international conferences.

Trial registration number NCT03032354; Pre-results.

Strengths and limitations of this study

- The study design (randomised controlled trial, RCT) is the most robust methodology to assess the effectiveness of therapeutic interventions.
- The findings of this RCT, whether positive or negative, will contribute to the formulation of further recommendations on the use of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 for improving beta-cell function in children with newly diagnosed type 1 diabetes (T1D).
- It remains unclear which probiotics, alone or in combination, and at which doses, are potentially the most useful for management of T1D.

INTRODUCTION

Type 1 diabetes

Type 1 diabetes (T1D) involves the autoimmune destruction of the insulin-secreting pancreatic islets of Langerhans, leading to insulin deficiency. [1 2] The prevalence of T1D varies considerably geographically,[3] but in many regions it is rising.[4] The pathophysiology is multifactorial. In addition to genetic background, a number of environmental factors have been claimed to influence the T1D epidemiology, including the mode of birth, early infant diet (eg, lack of breast feeding, early introduction of cow's milk, gluten), viral infections and antibiotic use.[5–7] Recent evidence has demonstrated that dysbiosis, defined as imbalances in the composition and function of the gut microbiota, may also be relevant.

Gut microbiota and TID

Current studies suggest that the interaction between gut microbiota and the immune system may be a major factor influencing T1D development. Alterations in gut microbiota composition observed in patients with T1D would increase gut permeability. Microbes, microbial metabolites, bacterial products and the immune responses to them may promote inflammation and induce an alteration in intestinal barrier function. If so, that facilitates greater exposure to the immune system of dietary antigens and microbiota-derived products, which may cause a proinflammatory response and stimulate beta-cell autoimmunity in genetically predisposed subjects.[8]

Compared with healthy controls, subjects with T1D exhibit a less diverse and less stable gut microbiota.[9–13] A low abundance of lactate and butyrate-producing species

has been noted in children with T1D.[14] Butyrate is the main energy source for colonic epithelial cells. It induces mucin synthesis and increases the barrier mechanisms of tight junctions. It also decreases bacterial transport across the gut epithelium.[15 16] Adequate butyrate production is essential for gut integrity and may have a protective effect on the development of anti-islet cell autoantibodies (ICA).[17]

It seems that the diabetic gut is underequipped with bacteria that promote protective immune mechanisms.[18] In children with beta-cell autoimmunity, a significant decrease in the numbers of *Lactobacillus* and *Bifidobacterium* was observed.[11] These are the major genera of bacteria that make up the colon flora in humans, constitute intestinal microbial homeostasis, inhibit growth of pathogens, improve the gut mucosal barrier and modulate local and systemic immune responses. Dysbiosis causes changes in the local immune systems of patients with T1D demonstrated, for example, by the low expression of FOXP3 and impaired induction of FOXP3-positive regulatory T cells by small intestinal dendritic cells. [19]

For a detailed review of studies evaluating the role of the gut microbiota in these patients, see the review by *Gulden et al*.[20]

Microbiota modulation strategies

With the growing recognition of the role of gut microbiota in health and disease, it has become clear that gut microbiota may be a target for improving outcomes in subjects affected by or at risk for certain diseases, including T1D. To date, modification of the gut microbiota via the provision of probiotics (defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host) [21] is the most extensively studied strategy. In humans, by far, the most commonly used probiotics are bacteria from the genus *Lactobacillus* or *Bifidobacterium*.

Data on the effects of probiotics in subjects with T1D are very limited. However, preliminary data are promising. In animals, studies using non-obese diabetic (NOD) mice or a rat model showed that the development of T1D can be prevented or delayed through modulation of the intestinal microbiota.[22 23] Administration of *Lactobacillus johnsoni* N6.2, isolated from BioBreeding diabetes-resistant rats, delays or inhibits the onset of T1D in BioBreeding diabetes-prone rats. Transmission of segmented filamentous bacteria to the NOD mouse correlates with disease prevention and the upregulation of Th17 cells in the intestine.[24 25]

In humans, one recent, prospective, cohort study, which was carried out as part of the TEDDY (The Environmental Determinants of Diabetes in the Young) study, aimed to examine the association between early probiotic exposure and islet autoimmunity (positive antibodies to insulin, glutamic acid decarboxylase (GAD) or insulinoma antigen 2 on at least two consecutive visits) in children genetically at increased risk for T1D. This study found that early (ie, during the first 27 days of life) administration of probiotics (mainly *Lactobacillus* and *Bifidobacterium*, given either as a supplement or in infant formula supplemented with probiotics) may be associated with a reduced risk of islet autoimmunity (HR 0.66, 95% CI 0.46 to 0.94), especially in children with the highest-risk human leukocyte antigen (HLA) genotype of DR3/4 (HR 0.4, 95% CI 0.21 to 0.74). Of note, no reduction was seen in children with moderately higher-risk genotypes (HR 0.97, 95% CI 0.62 to 1.54).[26] Further studies to confirm this association are needed.

L rhamnosus GG and *Bifidobacterium lactis* Bb12

These are among the world's best-studied probiotics. In the USA, both have received a generally recognised as safe status by the Food and Drug Administration.[27] In Europe, both have been granted Qualified Presumption of Safety status by the European Food Safety Authority—a status granted on a species level.[28] Previous studies found that supplementation with *L. rhamnosus* GG and *B. lactis* Bb12 improved blood glucose control in normoglycaemic pregnant women and reduced the frequency of gestational diabetes mellitus,[29 30] thus suggesting a role for these probiotics in glucose control.

TRIAL OBJECTIVES AND HYPOTHESIS

The aim of the study is to examine the effects of *L. rhamnosus* GG and *B. lactis* Bb12 on beta-cell function in children with newly diagnosed T1D. We hypothesise that gut microbiota modulation with the combination of these two probiotics may be used as a tool to modulate the immune system for preventing islet cell destruction. We also speculate that children who receive *L. rhamnosus* GG and *B. lactis* Bb12 at the recognition of T1D will have more preserved beta-cell function than children who receive placebo.

METHODS

Trial design

This study is designed as a randomised, double-blind, placebo-controlled trial with allocation of 1:1. The trial was registered at the ClinicaTrials.gov (NCT03032354) prior to the inclusion of the first patient. Any important changes in the protocol will be implemented there.

Settings and participants

Recruitment will be through the paediatric diabetes outpatient clinics at two participating centres in Warsaw, Poland (Department of Paediatrics, the Medical University of Warsaw and Department of Endocrinology and Diabetology, Children's Memorial Health Institute). Both are tertiary care hospitals that provide annually diabetes care to more than 200 children with newly recognised T1D. The personnel are adequately trained and competent in conducting clinical trials. The start of the recruitment is planned in July 2017 and should be completed within the following 1 year.

Eligibility criteria

Eligible children must fulfil all of the following inclusion criteria:

- T1D as defined by International Society for Pediatric and Adolescent Diabetes (ISPAD) criteria [31], diagnosed within 60 days;
- presence of at least one positive autoantibody (autoantibodies to GAD, the tyrosine phosphatase-related insulinoma-associated 2 molecule (anti-IA-2), ICA;
- age 8–17 years;
- single fasting C-peptide level ≥0.4 ng/mL;
- written informed consent signed by parents (and patients if older than 16 years).

Subjects will be excluded for the following reasons:

- antibiotic treatment within 2 months prior to enrolment
- use of probiotics within 2 weeks prior to enrolment
- gastrointestinal infection within 2 weeks prior to enrolment
- chronic gastrointestinal diseases (eg, inflammatory bowel disease, coeliac disease, food allergy)
- immunodeficiency.

Interventions

The intervention will be administration of a combination of two probiotics: *L. rhamnosus* GG (strain deposit number ATCC 53103) and *B. lactis* Bb12 (strain deposit number DSM15954). A placebo comparator was chosen as the gold standard for testing the efficacy of a new treatment.[32] The placebo will contain maltodextrin, and its taste and appearance will be identical to those of the active product. The study products will be manufactured in capsules and supplied free of charge by Chr. Hansen Holding A/S, Denmark. The manufacturer will have no role in the conception, protocol development, design or conduct of the study, or in the analysis or interpretation of the data.

Study procedure

The study procedures are described in table 1. Patients and parents/caregivers will receive oral and written information regarding the study during their regular diabetes outpatient clinic visits within 60 days after T1D recognition. Written informed consent, signed by the legal caregivers and/or the patients, will be obtained by a physician involved in the study.

Participants will be randomly assigned to two groups, receiving either *L. rhamnosus* GG and *B. lactis* Bb12 at a dose of 10^9 colony-forming units or placebo, orally, once daily, for 6 months. All study participants will be followed up for up to 12 months after the start of the intervention. Study visits at months 3, 6 and 12 will be co-ordinated with diabetes outpatient clinic visits.

During the hospitalisation at diabetes recognition, a blood sample will be obtained for the measurement of fasting C-peptide, anti-GAD, anti-IA-2, ICA (analysed by radiobinding assays) and glycated haemoglobin (HbA1c) levels (performed by *high-performance liquid chromatography*). As T1D is frequently associated with autoimmune thyroid disease, anti-thyroid peroxidase, anti-thyroglobulin, serum thyroid-stimulating hormone and free thyroxine will be assessed (by chemiluminescence method) at diabetes onset and at month 12. Similarly, as T1D is associated with coeliac disease, all subjects will be tested for anti-tissue transglutaminase type 2 antibodies (analysed by ELISA test) and/or endomysial antibodies (performed by indirect immunofluorescence method) at diabetes recognition and at month 12. In addition, at diabetes onset, total serum IgA will be measured (by nephelometric analysis) to exclude IgA deficiency. In the case of IgA deficiency, the same type of antibodies in the IgG class will be analysed. In the case of positive serology, a small intestine biopsy will be considered to confirm the diagnosis of coeliac disease in line with current European guidelines.[33] Interleukins as inflammatory markers will be compared (by ELISA tests) between groups at diabetes onset and months 6 and 12.

At study entry and at all study visits, all eligible children will undergo a physical examination, including evaluation of anthropometric measurements (weight, height and body mass index (BMI)), which will be plotted on WHO 2007 growth curves.[34] The participants will also be stratified accordingly to Tanner developmental stage ≤3 or >3, as assessed by physical examination. All information regarding treatment modality (eg, pump, infusion) and antibiotic use will be collected at these visits. Children will be treated with continuous subcutaneous insulin infusion or multiple daily injections. The total daily insulin dose and basal insulin will be downloaded from insulin pumps or will be collected from patients' diaries.

In our study, we chose to use the mixed-meal tolerance test (MMTT), as it is widely regarded as the gold standard for measuring endogenous insulin production among patients with T1D.[35] The MMTT will be performed three times: at allocation and at months 6 and 12. For the MMTT, all eligible participants will consume a standard, mixed meal (liquid-meal BOOST test (6 mL/kg max 360 mL, Nestle S.A., Vevey, Switzerland; 237 mL contains 41 g carbohydrates, 10 g protein, 4 g fat, energy value 240 kcal). The C-peptide levels will be measured in blood samples drawn every 30 min for 2 hours after the mixed meal consumption. The MMTT will be initiated before 10:00 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. The MMTT will be rescheduled if a child has a capillary glucose value >180 or <70 mg/dL (>10 or <3.9 mmol/L).

Gut permeability will be measured using zonulin, a biomarker of impaired gut barrier function, via ELISA, at months 6 and 12 of the study.[36]

Compliance will be assessed by collecting empty packages and the remainder of the product that was not used as well as by direct interview with the patient and/

Table 1 Timetable of activities planned during the study

	Study period						
	T1D onset	Enrolment	Allocation	Postallocation			Close-out
Time point	–60 days	0	0	Day 1	Month 3	Month 6	Month 12
Enrolment:							
Eligibility screen		+					
Informed consent		+					
Allocation			+				
Interventions:							
Lactobacillus rhamnosus GG and *Bifidobacterium lactis* Bb12					←	→	
Placebo					←	→	
Assessments:							
Anthropometric measurement (body weight and height; BMI; Tanner stage)			+		+	+	+
Fasting C-peptide	+						
GADA, IA2A, ICA	+					+	+
Total IgA	+						
TTGA	+						+
Anti-Tg, anti-TPO, TSH, fT4	+						+
HbA1c	+		+		+	+	+
Interleukins: IL-1, IL-2, IL-10, TNF-α, IFN-γ	+					+	+
C-peptide during mixed-meal test			+			+	+
Gut permeability						+	+
Side effects (eg, abdominal pain, diarrhoea, constipation, vomiting, flatulence)					+	+	+
Severe hypoglycaemia, ketoacidosis					+	+	+
Return of non-used study products						+	

anti-Tg, antithyroglobulin antibody; anti-TPO, anti-thyroid peroxidase antibodies; BMI, body mass index; fT4, free thyroxine; GADA, glutamic acid decarboxylase autoantibodies; HbA1c, glycated haemoglobin; IA2A, tyrosine phosphatase autoantibodies; ICA, islet cell cytoplasmic autoantibodies; IFN-γ, interferon gamma; IL, interleukin ; TNF-α, tumour necrosis factor alpha; TSH, thyroid-stimulating hormone; TTGA, tissue transglutaminase antibody.

or caregiver. Participants receiving <75% of the recommended doses will be considered as non-compliant.

At any point of time, caregivers will have the right to withdraw the participating child from the study without giving the reason for discontinuation. There will be no effect of this discontinuation on subsequent physician and/or institutional medical care.

End points

Primary

- Area under the curve (AUC) of the C-peptide level during 2-hour responses to a mixed meal.

Secondary

- Fasting C-peptide concentration
- Insulin requirement (U/kg body mass)
- HbA1c
- Interleukins: IL-1, IL-2, IL-10, tumour necrosis factor alpha, interferon gamma
- Gut permeability
- Anthropometric parameters (weight, height, BMI z-score)
- Side effects (eg, abdominal pain, diarrhoea, constipation, vomiting, flatulence)
- Occurrence of other autoimmune diseases (eg, autoimmune thyroid disease, coeliac disease)
- Acute complications of T1D such as severe hypoglycaemia or ketoacidosis

Participant timeline

The time schedule for enrolment, interventions, assessment and visits for the participants is described in table 1.

Sample size

The sample size was calculated based on recommendations on sample size calculation to be used in studies on the effects of new agents on the 2-hour AUC of the C-peptide in MMTT in newly diagnosed patients with T1D by Lachin *et al*. [37] A normalising transformation ln(x+1) for C-peptide AUC is planned to be used. Since there are no studies that have evaluated the use of probiotics in patients with T1D, the study plans to detect a 50% increase in the (untransformed) 2-hour AUC of C-peptide values in MMTT at month 12 in the treated group relative to the placebo group. It is assumed that fractions of children in age groups 8–12 and 13–17 years of age are equal (50%). To provide 85% power using a one-sided test at the confidence level of 0.05, with 1:1 randomisation and assuming a drop-out rate of 10%, a sample size of 96 subjects is needed (calculation based on mean and root mean square error (RMSE) estimates 0.25 and 0.142, and 0.30 and 0.204 for age groups 8–12 years and 13–17 years, respectively).

Randomisation

The randomisation list will be generated using the statistical program StatsDirect by an independent person and will be kept by a staff member not involved in the trial. In order to obtain comparable groups, block randomisation will be performed (each block will contain four patients: two in the intervention group and two in the control group).

Blinding

All participants and investigators will be blinded to the assigned treatment throughout the study. The products for both groups will be similar in terms of smell and colour and will be packed in identical packages. Researchers, caregivers, outcome assessors and the person responsible for the statistical analysis will be blinded to the intervention until completion of the study.

Allocation concealment

The study products will be packaged and assigned consecutive numbers according to the randomisation list. Independent personnel not involved in the conduct of the trial will dispense the numbered study products.

Data collection and management

All study participants will be assigned a study identification number. Case report forms (CRFs) will be completed on paper forms. Data will then be entered and stored in a password-protected electronic database. The original paper copies of CRFs and all study data will be stored in a locker within the study site, accessible to the involved researchers only. Insulin requirements, HbA1c values and anthropometric parameters will be collected from all participants who discontinue or deviate from the intervention protocols.

Monitoring

An independent Data and Safety Monitoring Board (DSMB) will be set up prior to the start of the study. The DSMB will review data after recruitment of 25%, 50% and 75% participants to review the study progress and all adverse events.

Statistical analysis

All statistical analyses will be performed with the computer software StatsDirect.

In the case of descriptive statistics for categorical variables, the number and percentage of occurrences will be reported. The distribution of continuous variables will be first evaluated using the Shapiro-Wilk test, then, in the case of variables with a normal distribution, the mean and SD will be provided; if not, the median and the 25th and 75th percentile will be reported. For each variable, the number of missing data will be given. Categorical variables will be compared using the Fisher test or X^2 test, as appropriate. Normally distributed variables will be compared using the Student's t-test; the Mann-Whitney test will be used for variables that are not normally distributed. If applicable, paired tests will be used.

In the analysis of primary endpoint, a prespecified analysis of covariance (ANCOVA) model will be used; the change from baseline to the month 12 in the AUC of the C-peptide level will be used as the response variable, with study treatment and baseline AUC values as covariates. A normalising transformation ln(x+1) for the C-peptide AUC is planned to be used. In an additional exploratory analysis, the mixed-effect model for C-peptide AUC as a response variable will be built, with both random intercept and slope adjusting for treatment assignment, time (0, 6 and 12 months), baseline C-peptide AUC, age, gender and Tanner developmental stage (<=3 or >3).

Similarly, in the analysis of secondary endpoints, a prespecified ANCOVA model will be used; the change from baseline to the month 12 in HbA1c level, insulin dosage, fasting C-peptide concentration, interleukin levels and gut permeability will be used as the response variables, with study treatment and the baseline AUC value as covariates. In an additional exploratory analysis, mixed-effect models for each of the above variables as response variables will be built, with both random intercept and slope adjusting for treatment assignment, time (0, 6 and 12 months), baseline values, age, gender and Tanner developmental stage (<=3 or >3).

Moreover, for acute complications of T1D, the relative risk with 95% CI and number needed to treat will be calculated. For all models, coefficients with 95% CI and p values will be reported. The 95% CI will be provided in descriptive statistics for changes in time for continuous variables.

Subgroup analysis will be conducted, and the Tanner developmental stage (<=3 or >3) will be used to form the subgroups of the analyses.

Missing data will not be replaced. Two-sided tests will be used for all hypotheses. The significance level will be set at 0.05. All analyses will be performed on the intention-to-treat basis (ie, all participants are included in the arm to which they were allocated, whether or not they received

(or completed) the intervention given to that arm) and per-protocol analysis (ie, an analysis of the subset of participants who complied with the protocol).

Harms

Data on adverse events will be collected. All serious adverse events will be immediately reported to the project leader who will be responsible for notifying the ethics committee, all participating investigators and the manufacturer of the study products.

Auditing

The ethics committee did not require auditing for this study.

Ethics and dissemination

The study was approved by the Ethics Committee of the Medical University of Warsaw.

Verbal and written information regarding informed consent will be presented to the caregivers and/or patients. Any modifications to the protocol that may affect the conduct of the study will be presented to the committee. The full protocol will be available freely due to open access publication. The findings of this RCT will be submitted to a peer-reviewed journal. Abstracts will be submitted to relevant national and international conferences. The standards from the guidelines of the Consolidated Standards of Reporting Trials will be followed for this RCT. All investigators will have access to the final trial dataset.

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Contributors AS conceptualised the study. LG developed the first draft of the manuscript. HS contributed to the development of the study protocol and approved the final draft of the manuscript.

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