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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.

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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 *Lactobacillus rhamnosus* GG and *Bifidobacterium 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-h 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: The study protocol is under registration at ClinicalTrials.gov. NCT03032354

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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 a mode of birth, early infant diet (lack of breastfeeding, early introduction of cow's milk, gluten), viral infections, and antibiotic use.[5,6,7] Recent evidence has demonstrated that dysbiosis, defined as imbalances in the composition and function of the gut microbiota, also may be relevant.

Gut microbiota & type 1 diabetes

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 T1D patients 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 to healthy controls, subjects with T1D exhibit a less diverse and less stable gut microbiota.[9 ,10, 11, 12, 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.[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 T1D patients 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, or insulinoma antigen 2 on at least two consecutive visits) in children genetically at increased risk for T1D. This study found that early (i.e., 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 [hazard ratio (HR) 0.66, 95% confidence interval (CI) 0.46–0.94], especially in children with the highest-risk HLA genotype of DR3/4 (HR 0.4, 95% CI 0.21–0.74). Of note, no reduction

was seen in children with moderately higher-risk genotypes (HR 0.97, 95% CI 0.62–1.54).[26] Further studies to confirm this association are needed.

Lactobacillus rhamnosus GG and Bifidobacterium lactis B12

These are among the world's best-studied probiotics. In the US, both have received a Generally Recognised As Safe (GRAS) status by the Food and Drug Administration.[27] In Europe, both have been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) - a status granted on a species level.[28] Previous studies found that supplementation with *Lactobacillus rhamnosus* GG (*L rhamnosus* GG) and *Bifidobacterium lactis* Bb12 (*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 April 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 ISPAD criteria [31], diagnosed within 60 days;
- Presence of at least one positive autoantibody [autoantibodies to glutamic acid decarboxylase (GAD), the tyrosine phosphatase-related insulinoma-associated 2 molecule (anti-IA-2), islet-cell antibodies (ICA)];
- Age 8-17 years
- Single fasting C-peptide level ≥0.4ng/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 4 weeks prior to enrolment
- Use of probiotics within 2 weeks prior to enrolment
- Gastrointestinal infection within 2 weeks prior to enrolment
- Chronic gastrointestinal diseases (e.g., 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 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 (CFU) 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 month 3, 6, and 12 will be coordinated 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 HbA1c levels (performed by high-performance liquid chromatography). As T1D is frequently associated with autoimmune thyroid disease, anti-thyroid peroxidase (anti-TPO), anti-thyroglobulin (anti-Tg), serum thyroid-stimulating hormone (TSH), and and free thyroxine (fT4) 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 (anti-TG2) antibodies (analyzed by Elisa test) and/or endomysial antibodies (EMA) (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 month 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 were plotted on WHO 2007 growth curves.[34] 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 type 1

diabetes.[35] The MMTT will be performed 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 minutes for 2 hours after the mixed meal consumption.

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/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 of the C-peptide level (AUC CP) during 2-h responses to a mixed meal.

Secondary

- Fasting C-peptide concentration
- Insulin requirement (U/kg body mass)
- HbA1c,
- Interleukins: IL-1, IL-2, IL-10, TNF-α, IFN-γ
- Anthropometric parameters (weight, BMI z-score)
- Side effects (abdominal pain, diarrhoea, constipation, vomiting, flatulence)
- Occurrence of other autoimmune diseases (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 T1D patients by Lachin. [36] A normalizing 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 T1D patients, the study plans to detect a 50% increase in the (untransformed) 2-hour AUC of C-peptide values in MMTT at 12th month 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 randomization and assuming a drop-out rate of 10%, a sample size of 96 subjects is needed (calculation based on mean and 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, which is separate for each centre, 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: 2 in the intervention group and 2 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.

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.

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. The Student t-test will be used to compare mean values of continuous variables approximating a normal distribution. For non-normally distributed variables, the Mann-Whitney U test will be used. The mixed-effects ANCOVA model, using the baseline value as a covariate, will assess the effect of probiotic treatment on the AUC of the C-peptide level, HbA1c levels, and insulin dosage. The chi-square test or Fisher exact test will be used, as appropriate, to compare percentages. Moreover, relative risk (RR), 95% CI, and number needed to treat (NNT) will be calculated using the same computer software. The differences between study groups will be considered significant when the P-value is <0.05 or when the 95% CI for RR does not include 1 (equivalent to $P<0.05$) All statistical tests performed will be two-tailed tests. All analyses will be performed on the intention-to-treat basis (i.e., 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 (i.e., 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

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 (CONSORT) will be followed for this RCT.

Contributorship statement: 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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Competing interests statement: The authors declare that they have no competing interests.

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Table 1 Timetable of activities planned during the study.

	Study period						
	T1D onset	Enrolment	Allocation	Post-allocation			Close-out
Time point	- 60 days	0	0	Day 1st	Month 3rd	Month 6th	Month 12th
Enrolment:							
Eligibility screen		+					
Informed consent		+					
Allocation			+				
Interventions:							
Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12				←	—	→	
Placebo				←	—	→	
Assessments:							
Anthropometric measurement (body weight and height; BMI)			+		+	+	+
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			+				+
Side effects (abdominal pain, diarrhea, constipation, vomiting, flatulence)					+	+	+
Severe hypoglycemia, ketoacidosis					+	+	+
Return of non-used study products						+	

BMI: body mass index; HbA1c: glycated hemoglobin; IL-1: interleukin-1; IL-2: interleukin-2; IL-10: interleukin-10; TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma; anti-Tg: antithyroglobulin antibody; anti-TPO: anti-thyroid peroxidase antibodies; TSH: thyroid - stimulating hormone; fT4: free thyroxine; TTGA: tissue transglutaminase antibody; IgA: immunoglobulin A; ICA: islet cell cytoplasmic autoantibodies; GADA: glutamic acid decarboxylase autoantibodies; IA2A: tyrosine phosphatase autoantibodies

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	1
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4-6
	2b	Specific objectives or hypotheses	6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	
Participants	4a	Eligibility criteria for participants	7
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	
Sample size	7a	How sample size was determined	10
	7b	When applicable, explanation of any interim analyses and stopping guidelines	
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	10
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	10
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	10
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	10
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	10

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	11
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	
	13b	For each group, losses and exclusions after randomisation, together with reasons	
Recruitment	14a	Dates defining the periods of recruitment and follow-up	
	14b	Why the trial ended or was stopped	
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	11
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	
Other information			
Registration	23	Registration number and name of trial registry	2
Protocol	24	Where the full trial protocol can be accessed, if available	2
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	7,12

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

BMJ Open

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.

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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.

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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 *Lactobacillus rhamnosus* GG and *Bifidobacterium 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-h 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: The study protocol is under registration at ClinicalTrials.gov. NCT03032354

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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 (e.g., lack of breastfeeding, early introduction of cow's milk, gluten), viral infections, and antibiotic use.[5,6,7] Recent evidence has demonstrated that dysbiosis, defined as imbalances in the composition and function of the gut microbiota, also may be relevant.

Gut microbiota & type 1 diabetes

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 T1D patients 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 to healthy controls, subjects with T1D exhibit a less diverse and less stable gut microbiota.[9 ,10, 11, 12, 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.[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 T1D patients 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, or insulinoma antigen 2 on at least two consecutive visits) in children genetically at increased risk for T1D. This study found that early (i.e., 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 [hazard ratio (HR) 0.66, 95% confidence interval (CI) 0.46–0.94], especially in children with the highest-risk HLA genotype of DR3/4 (HR 0.4, 95% CI 0.21–0.74). Of note, no reduction

was seen in children with moderately higher-risk genotypes (HR 0.97, 95% CI 0.62–1.54).[26] Further studies to confirm this association are needed.

Lactobacillus rhamnosus GG and Bifidobacterium lactis B12

These are among the world's best-studied probiotics. In the US, both have received a Generally Recognised As Safe (GRAS) status by the Food and Drug Administration.[27] In Europe, both have been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) - a status granted on a species level.[28] Previous studies found that supplementation with *Lactobacillus rhamnosus* GG (*L rhamnosus* GG) and *Bifidobacterium lactis* Bb12 (*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 ISPAD criteria [31], diagnosed within 60 days;
- Presence of at least one positive autoantibody [autoantibodies to glutamic acid decarboxylase (GAD), the tyrosine phosphatase-related insulinoma-associated 2 molecule (anti-IA-2), islet-cell antibodies (ICA)];
- Age 8-17 years
- Single fasting C-peptide level ≥0.4ng/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 (e.g., 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 (CFU) 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 month 3, 6, and 12 will be coordinated 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-TPO), anti-thyroglobulin (anti-Tg), serum thyroid-stimulating hormone (TSH), and free thyroxine (fT4) 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 (anti-TG2) antibodies (analyzed by Elisa test) and/or endomysial antibodies (EMA) (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 month 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 also will be stratified accordingly to Tanner developmental stage ≤ 3 or >3, as assessed by physical examination. All information regarding treatment modality (e.g., 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 type 1 diabetes.[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 minutes for 2 hours after the mixed meal consumption. The MMTT will be initiated before 10 a.m. 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 mg/dl or <70 mg/dl (>10 mmol/l or <3.9 mmol/l).

Gut permeability will be measured using zonulin, a biomarker of impaired gut barrier function, via ELISA (enzyme-linked immunosorbent assay), 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/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 of the C-peptide level (AUC CP) during 2-h responses to a mixed meal.

Secondary

- Fasting C-peptide concentration

- Insulin requirement (U/kg body mass)
- HbA1c
- Interleukins: IL-1, IL-2, IL-10, TNF-α, IFN-γ
- Gut permeability
- Anthropometric parameters (weight, height, BMI z-score)
- Side effects (e.g., abdominal pain, diarrhoea, constipation, vomiting, flatulence)
- Occurrence of other autoimmune diseases (e.g., 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 T1D patients by Lachin. [37] A normalizing 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 T1D patients, the study plans to detect a 50% increase in the (untransformed) 2-hour AUC of C-peptide values in MMTT at 12th month 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 randomization and assuming a drop-out rate of 10%, a sample size of 96 subjects is needed (calculation based on mean and 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, which is separate for each centre, 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: 2 in the intervention group and 2 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 standard deviation 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 chi-squared 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 distribured. If applicable, paired tests will be used.

In the analysis of primary endpoint, a pre-specified analysis of covariance (ANCOVA) model will be used; the change from baseline to the 12th month 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 normalizing 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 pre-specified ANCOVA model will be used; the change from baseline to the 12th month 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 (RR) with 95% confidence interval (CI) and number needed to treat (NNT) 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 (i.e., 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 (i.e., 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. checklist 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 (CONSORT) will be followed for this RCT.

Contributorship statement: AS conceptualised the study. LG developed the first draft of the manuscript. HS, AS, LG contributed to the development of the study protocol and approved the final draft of the manuscript.

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Competing interests statement: The authors declare that they have no competing interests.

Table 1 Timetable of activities planned during the study.

	Study period						
	T1D onset	Enrolment	Allocation	Post-allocation			Close-out
Time point	- 60 days	0	0	Day 1st	Month 3rd	Month 6th	Month 12th
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 (e.g., abdominal pain, diarrhoea, constipation, vomiting, flatulence)					+	+	+
Severe hypoglycaemia, ketoacidosis					+	+	+
Return of non-used study products						+	

BMI: body mass index; HbA1c: glycated haemoglobin; IL-1: interleukin-1; IL-2: interleukin-2; IL-10: interleukin-10; TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma; anti-Tg: antithyroglobulin antibody; anti-TPO: anti-thyroid peroxidase antibodies; TSH: thyroid - stimulating hormone; fT4: free thyroxine; TTGA: tissue transglutaminase antibody; IgA:

immunoglobulin A; ICA: islet cell cytoplasmic autoantibodies; GADA: glutamic acid decarboxylase autoantibodies; IA2A: tyrosine phosphatase autoantibodies.

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Reported on page No
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2,6
	2b	All items from the World Health Organization Trial Registration Data Set	YES
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	13
	5b	Name and contact information for the trial sponsor	13
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	7, 13
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-6
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	6
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and	7

		individuals who will perform the interventions (eg, surgeons, psychotherapists)	
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	9
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	9
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	8
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	9,10
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	10
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	10
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6
Methods: Assignment of interventions (for controlled trials)			
Allocation: Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	10,11
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	11
Implementation	16c	Who will generate the allocation sequence, who will enroll participants, and who will assign participants to interventions	10,11
Blinding (masking)	17a	Who will be blinded after assignment to	11

		interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	11
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	11
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	11
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	11
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11,12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11,12
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	11
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	11
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	13

Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	13
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	12
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	6
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	7,8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	11
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	N/A
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	6
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	12
	31b	Authorship eligibility guidelines and any intended use of professional writers	
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	yes
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons “Attribution-NonCommercial-NoDerivs 3.0 Unported” license.

BMJ Open

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.

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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.

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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 *Lactobacillus rhamnosus* GG and *Bifidobacterium 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-h 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: The study protocol is under registration at ClinicalTrials.gov. NCT03032354

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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 (e.g., lack of breastfeeding, early introduction of cow's milk, gluten), viral infections, and antibiotic use.[5,6,7] Recent evidence has demonstrated that dysbiosis, defined as imbalances in the composition and function of the gut microbiota, also may be relevant.

Gut microbiota & type 1 diabetes

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 T1D patients 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 to healthy controls, subjects with T1D exhibit a less diverse and less stable gut microbiota.[9 ,10, 11, 12, 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.[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 T1D patients 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, or insulinoma antigen 2 on at least two consecutive visits) in children genetically at increased risk for T1D. This study found that early (i.e., 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 [hazard ratio (HR) 0.66, 95% confidence interval (CI) 0.46–0.94], especially in children with the highest-risk HLA genotype of DR3/4 (HR 0.4, 95% CI 0.21–0.74). Of note, no reduction

was seen in children with moderately higher-risk genotypes (HR 0.97, 95% CI 0.62–1.54).[26] Further studies to confirm this association are needed.

Lactobacillus rhamnosus GG and Bifidobacterium lactis B12

These are among the world's best-studied probiotics. In the US, both have received a Generally Recognised As Safe (GRAS) status by the Food and Drug Administration.[27] In Europe, both have been granted Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) - a status granted on a species level.[28] Previous studies found that supplementation with *Lactobacillus rhamnosus* GG (*L rhamnosus* GG) and *Bifidobacterium lactis* Bb12 (*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 ISPAD criteria [31], diagnosed within 60 days;
- Presence of at least one positive autoantibody [autoantibodies to glutamic acid decarboxylase (GAD), the tyrosine phosphatase-related insulinoma-associated 2 molecule (anti-IA-2), islet-cell antibodies (ICA)];
- Age 8-17 years
- Single fasting C-peptide level ≥0.4ng/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 (e.g., 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 (CFU) 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 month 3, 6, and 12 will be coordinated 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-TPO), anti-thyroglobulin (anti-Tg), serum thyroid-stimulating hormone (TSH), and free thyroxine (fT4) 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 (anti-TG2) antibodies (analyzed by Elisa test) and/or endomysial antibodies (EMA) (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 month 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 also will be stratified accordingly to Tanner developmental stage ≤ 3 or >3, as assessed by physical examination. All information regarding treatment modality (e.g., 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 type 1 diabetes.[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 minutes for 2 hours after the mixed meal consumption. The MMTT will be initiated before 10 a.m. 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 mg/dl or <70 mg/dl (>10 mmol/l or <3.9 mmol/l).

Gut permeability will be measured using zonulin, a biomarker of impaired gut barrier function, via ELISA (enzyme-linked immunosorbent assay), 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/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 of the C-peptide level (AUC CP) during 2-h responses to a mixed meal.

Secondary

- Fasting C-peptide concentration

- Insulin requirement (U/kg body mass)
- HbA1c
- Interleukins: IL-1, IL-2, IL-10, TNF-α, IFN-γ
- Gut permeability
- Anthropometric parameters (weight, height, BMI z-score)
- Side effects (e.g., abdominal pain, diarrhoea, constipation, vomiting, flatulence)
- Occurrence of other autoimmune diseases (e.g., 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 T1D patients by Lachin. [37] A normalizing 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 T1D patients, the study plans to detect a 50% increase in the (untransformed) 2-hour AUC of C-peptide values in MMTT at 12th month 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 randomization and assuming a drop-out rate of 10%, a sample size of 96 subjects is needed (calculation based on mean and 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, which is separate for each centre, 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: 2 in the intervention group and 2 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 standard deviation 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 chi-squared 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 distribured. If applicable, paired tests will be used.

In the analysis of primary endpoint, a pre-specified analysis of covariance (ANCOVA) model will be used; the change from baseline to the 12th month 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 normalizing 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 pre-specified ANCOVA model will be used; the change from baseline to the 12th month 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 (RR) with 95% confidence interval (CI) and number needed to treat (NNT) 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 (i.e., 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 (i.e., 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 (CONSORT) will be followed for this RCT. All investigators will have access to the final trial dataset.

Contributorship statement: AS conceptualised the study. LG developed the first draft of the manuscript. HS, AS, LG contributed to the development of the study protocol and approved the final draft of the manuscript.

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Competing interests statement: The authors declare that they have no competing interests.

Table 1 Timetable of activities planned during the study.

	Study period						
	T1D onset	Enrolment	Allocation	Post-allocation			Close-out
Time point	- 60 days	0	0	Day 1st	Month 3rd	Month 6th	Month 12th
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 (e.g., abdominal pain, diarrhoea, constipation, vomiting, flatulence)					+	+	+
Severe hypoglycaemia, ketoacidosis					+	+	+
Return of non-used study products						+	

BMI: body mass index; HbA1c: glycated haemoglobin; IL-1: interleukin-1; IL-2: interleukin-2; IL-10: interleukin-10; TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma; anti-Tg: antithyroglobulin antibody; anti-TPO: anti-thyroid peroxidase antibodies; TSH: thyroid - stimulating hormone; fT4: free thyroxine; TTGA: tissue transglutaminase antibody; IgA:

immunoglobulin A; ICA: islet cell cytoplasmic autoantibodies; GADA: glutamic acid decarboxylase autoantibodies; IA2A: tyrosine phosphatase autoantibodies.

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SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Reported on page No
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2,6
	2b	All items from the World Health Organization Trial Registration Data Set	YES
Protocol version	3	Date and version identifier	1
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	13
	5b	Name and contact information for the trial sponsor	13
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	7, 13
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-6
	6b	Explanation for choice of comparators	7
Objectives	7	Specific objectives or hypotheses	6
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	6
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	6
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and	7

		individuals who will perform the interventions (eg, surgeons, psychotherapists)	
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	7
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	9
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	9
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	8
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	9,10
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	10
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	10
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6
Methods: Assignment of interventions (for controlled trials)			
Allocation: Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	10,11
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	11
Implementation	16c	Who will generate the allocation sequence, who will enroll participants, and who will assign participants to interventions	10,11
Blinding (masking)	17a	Who will be blinded after assignment to	11

		interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	11
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	11
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	11
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	11
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	11,12
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	11,12
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	11
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	11
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	13

Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	13
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	12
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	6
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	7,8
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	11
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	13
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	6
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	12
	31b	Authorship eligibility guidelines and any intended use of professional writers	
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	yes
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons “Attribution-NonCommercial-NoDerivs 3.0 Unported” license.