STUDY PROTOCOL

# Effect of probiotics on T2DM and pre-diabetes in China ---the role of gut microbiota composition

## Background

According to recent surveyed，diabetes prevalence rate in Chinese adults had gone up to 11.6%，the pre-diabetics diabetes prevalence rate was 50.1%. Effective intervention is urgently needed. Study of relationship between intestinal flora and diabetes is a hot topic at present.Gut microbiota disorders may be involved in the development of diabetes by means of increasing the number of inflammatory mediators, chronic inflammation, insulin resistance, increased energy intake, and obesity ect. In view of the correlation between gut microbiota and diabetes, gut microbiota can be taken as an important target for diabetes intervention. In this study, we aim to explore whether probiotics intervention affect blood glucose level and Risk factors for diabetes in T2DM and pre-diabetes via modification of gut microbiota composition and other post-interventional effects.

## Objectives :

1. To investigate the effects of probiotics intervention on blood glucose metabolism (blood glucose, insulin sensitivity) and diabetes risk factors (blood lipid, weight, body composition, inflammatory state) in diabetes and pre-diabetes.
2. To investigate the effect of probiotics intervention on gut microbiota composition and its correlated metabolite.
3. To investigate the mediating effect of gut microbiota on glucose control in diabetes and pre-diabetes.

## Methods/design

Trial design

This is a four-arm randomized controlled trial （Diabetes-intervention n=30，Diabetes-control n=30， prediabetes-intervention n=80， prediabetes-control n=80）. Participants are recruited from the outpatient registration pool of those who have participated in annual health checks for diabetes during 2017 to 2018 at Health care Center, Southwest Hospital, Chongqing, China. After an initial screening, potential subjects will be assessed for inclusion in the run-in trial. Those subjects who meet the inclusion criteria will be then invited to the intervention study for a total duration of 3 month. Those participants who are qualified for the intervention study will be randomized into intervention or control group after baseline assessments.

## Eligibility criteria

Inclusion criteria:
1. The patient was diagnosed with pre-diabetes or diabetes by OGTT
2. Age: 18-60y
3. BMI:18-35kg/m2

Exclusion criteria:

1. Patients who have diabetes， hyperlipemia , and need regular use of drugs.
2. Secondary obesity or diabetes.
3. Serious systematic diseases, such as digestive diseases, tumor, cardiac, pulmonary, renal, rheumatic and immune system diseases.
4. Pregnant women, women ready for pregnancy, and nursing mothers.
5. Take antibiotics or bacterial agents within 1 month
6. Diarrhea or abscess in 1 month or blood or other abnormal feces
7. Diabetics history more than 2 years
8. The OGTT experiment FG≥9mmol/L or 2h≥14mmol/L in diabetic patients

## Study settings

The interventions and laboratory tests and performed and managed at the Health Care Center, Southwest Hospital, Chongqing, and Health management institute, PLA General hospital, Beijing, China.

Trial design

## Interventions

1. Probiotics group: Diabetes/prediabetes participants take four packs of probiotics a day on an empty stomach with 200ml water every morning. The probiotics is called KAWAI, each packs contains 880 billion dead Lactobacillales（S. thermophilus）.
2. Placebo group:
   Diabetes/prediabetes participants take four packs of probiotics a day on an empty stomach with 200ml water every morning.

## Primary and secondary outcomes

## Primary outcomes:

1. Blood glucose levels(FBG and OGTT)，insulin sensitivity(Insulin，C-peptide)
2. Gut microbiota composition

## Secondary outcomes:

1. Blood inflammatory markers
2. Diabetes risk factors (blood pressure, blood lipids)
3. Weight, waist circumference，BMI, Body composition
4. Intestinal endocrine function（GPL-1）
5. Gut microbiota metabolites
   The primary and secondary outcome measures will be assessed at baseline, 1- and 3-month time points.

## Measurements procedures

## 1. Screening:

The potential subjects will be selected from the outpatient pool of those who have records of abnormal blood glucose level in 2017. Registered nurses from health care center will first contact the potential subjects and invite them to a

study information meeting. Detailed information of the objectives of the study, its nature and constraints, the anticipated risks and the expected benefits are given by the investigating physician of endocrinology and professor of sport medicine in the recruitment information meetings. When the screening consent form has been signed, patients will be asked to fill in a screening questionnaire to check their health and medication background as well as alcohol consumption, and to undergo a glucose tolerance test (blood draw after overnight fasting, followed by samples of 2 hours after the intake of 75 g of glucose).

## Follow-up visits

The purpose of the follow-up visits is to monitor the compliance, enhance retention of the subject in the study, and obtain information on factors that may affect outcomes of the study. A study coordinator will contact participants every month to check their oral probiotics records. The study coordinator will also give feedback. The participants will have follow-up visits to the laboratory of Health care Center, Southwest Hospital, Chongqing at months1, 3. Their height, weight, blood pressure, blood samples, fecal samples, and changes in health status and body composition will be checked at those time points.

## Methods of measurements

## 1.Questionnaire, anthropometry and physical examinations

Background information regarding lifestyle, behavioral and motivational characteristics as well as medical history will be collected by questionnaires. Data gathered from eligible subjects will then be used to describe the study populations, and individual results will be used to screen the changes and for monthly feedback.

  Height will be determined using a wall-fixed measuring device, and weight using a calibrated scale. Height and weight will also be used to determine body mass index (BMI, weight(kg)/(height(m))2. Blood pressure will be measured after 5 min rest. A physician will examine the physical conditions of subjects and ensure that subjects meet the inclusion criteria.

## 2.Blood samples

Glucose tolerance tests will be performed after overnight fasting and 2 hours

after the intake of 75 g glucose for the assessment of serum insulin, and glucose. Venous blood samples will be taken in standardized fasting conditions at 7–8 a.m. Total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, will be measured from serum sample by conventional methods. ELISA can be used to assess serum samples for adiponectin,tumor necrosis factor alpha (TNFα), C-reactive protein (CRP), interleukin (IL-6), interleukin 10 (IL-10), and lipopolysaccharides (LPS).Gut microbiota metabolites will be measured from serum sample by LC-MS/MS.

## 3.Fecal samples collection

The fecal samples (two samples per each time) will be collected in the health care center during the second day of the 3-day dietary records collection. The study will provide to subjects the materials for sample collection. The subjects are advised to sample approximately 2–3 grams of feces by spatula attached to the cover of the plastic specimen jar. Fecal samples are frozen after collection within 30 min , stored at −80°C. 16SrDNA sequencing will be performed. Different bacterial indices will be calculated to evaluate the interrelationship of different bacterial species and how they are associated with diabetes.

## 4.Body composition

Bioelectrical impedance analysis will be used to estimate lean tissue mass (LM), fat mass (FM) and bone mass (BM) of the whole body, legs, arms, and trunk as well as android and gynoid regions.

## Concomitant adherences and procedures

To ensure good compliance in probiotics and placebo group, we will keep in contact with them via phone call monthly questionnaire to check on their physical condition and lifestyle at 1 and 3months follow-up during the intervention.

## Sample size estimation

The sample size calculation with an estimate of how many participants will be needed for the primary outcome to be statistically, clinically and/or politically significant. For the specific primary outcome of blood glucose and microbiota, based on our plot study, 68 prediabetes subjects in each group would have 85% power for mean comparison between the randomized groups for the fast

blood glucose. We set the significance level at 0.05 and allowed for 10 mean comparisons with the Bonferroni correction between the groups. Taking into account the compliance and drop-out (15%), we increase our simple size to 80 in each prediabetes group. With the same approach, sample size for each diabetes group is 60 subjects.

## Randomization methods

### 1. Randomization Process:

SPSS will be used for generating the randomization assignment for Diabetes groups and prediabetes groups respectively. Randomization numbers will be generated and sealed in an envelope and kept by the study coordinator. On the basis of subjects' enrolment time when 20 qualified subjects are reached, the study coordinator will open one block to allocate subjects into each group.

### 2.Blinding:

Both subjects and investigators will be blinded with respect to the randomization assignment of each participant. The investigators will remain blinded until the completion of the study. The study coordinator will be blinded from which group the participants are signed in. The accuracy of the randomization log is the responsibility of the study coordinator.

## Statistical methods

All data collected by questionnaires will be entered into database management software (EpiData), double checked by independent researchers, and exported to SPSS statistics version. If data is not normally distributed, their natural logarithms will be used for further analysis. Descriptive statistics will be used to present the background and anthropometric data at the baseline and follow-up assessments as mean and 95% confidence interval (CI) unless otherwise stated.

An intention-to-treat (ITT) analysis will be performed to compare the Probiotics group to Placebo group. The effects of the interventions will be assessed using analysis of variance (ANCOVA) for repeated measures (treatment group × time) and baseline difference as a covariate. If the significance of the group by time interaction is $p < 0.05$, the effect will be localized utilizing Bonferroni for multiple comparisons. The level of statistical significance chosen for the contrasts will be $p < 0.05$. In addition to the ITT

analysis, efficacy or active treatment analysis will be done when the compliance of the participation of the intervention is ≥60% of the whole trial [ANCOVA for repeated measures (treatment group × time) and baseline difference as a covariate.] The percentage differences (0–3month) will be calculated from duration between baseline and end point measurements for each individual. The comparison of percentage changes in different groups will be performed using ANCOVA (two factor interactions: compliance/ noncompliance × treatment group) controlled for the baseline value using Bonferroni for multiple comparisons. If the significance of the overall group difference is $p < 0.05$, then the effect will be localized by contrast to the Con group. When the 95% CI does not include zero, the difference is regarded as statistically significant at $\alpha = 0.05$.

Linear Pearson's correlation, partial correlation, Kendall Tau's, and bivariate, logistic, and multivariate regression analysis will be used for analysing relationships. In addition, systems biology approaches will be used to develop models which integrate the different types of data and the high-throughput data measured in terms of gut microbiota composition, as well as LPSs and inflammation.

## Ethical issues, research permits or information permit applications

The current study will continue to adhere to all relevant guidelines for good scientific and clinical practice. All subjects in this study are volunteers. None of the measurements are known to have any significant health risk.

The benefits and associated risks of the study will be carefully explained to the subjects and voluntary participation will be stressed. Informed consent will be obtained from all subjects. The study physician will be available during the laboratory tests. The study has been approved by Ethics Committee of PLAGH, ( S2016-068-01).

All data will be handled and archived confidentially. All image scans and background information will be electronically transferred and stored in the specific database. The blood samples and fecal samples will be stored at -80°C. If the analyses will be performed outside the data collection site, the sample transportation will be handled according to the relevant safety standards. The duration of the sample storage is generally 10 years. If the samples storage time exceeds the permitted time, a new permit will be obtained from both the subjects and the Ethical committee. If the subjects are deceased, permission will be sought from the relatives.