Title: **Design and rationale of the INSYTE study: A randomised, placebo controlled study to test the efficacy of a synbiotic on liver fat, disease biomarkers and intestinal microbiota in non-alcoholic fatty liver disease**

Authors: Eleonora Scorletti[1,2], Paul R. Afolabi[2], Elizabeth A. Miles[1], Debbie E. Smith[1,2], Amal Almehmadi[1], Albandri Alshathry[1], Helen E. Moyses[2], Geraldine F. Clough[1], Mark Wright[3], Janisha Patel[3], Laure Bindels[4], Nathalie M. Delzenne[4], Philip C. Calder[1,2], Christopher D. Byrne[1,2]

[1] Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton, UK;
[2] National Institute for Health Research Southampton Biomedical Research Centre University of Southampton and University Hospital Southampton National Health Service (NHS) Foundation Trust, Southampton, UK
[3] Hepatology, University Hospital Southampton NHS Foundation Trust, Southampton, UK
[4] Metabolism and Nutrition Research Group, Louvain Drug Research Institute, Université Catholique de Louvain, Brussels, Belgium

Keywords:

NAFLD: Non-alcoholic fatty liver disease

Gut microbiota

Nutrition

Synbiotic

Probiotic

Bifidobacterium animalis

Prebiotic

Fructo-oligosacharide

Cardiovascular disease

Type 2 diabetes

List of abbreviations:

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic fatty liver disease

HA: Hyaluronic acid

PNPLA3: Patatin-like phospholipase domain-containing protein 3

TIMP1: Metallopeptidase inhibitor-1

Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) represents a spectrum of fat-related conditions ranging from simple fatty liver, to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. There is growing evidence that NAFLD is a multisystem disease, affecting several extra-hepatic organs and regulatory pathways. Furthermore, since the gut and liver are linked anatomically via the portal vein, disturbances of the gut microbiota (dysbiosis) can affect the liver.

Objectives: In patients with NAFLD, we are testing the effects of a synbiotic which is the combination of a prebiotic (fructooligosaccharides; 4 g/day) and a probiotic (*Bifidobacterium animalis* subsp. lactis BB-12 at a minimum of 10 billion CFU/day) on a) liver fat percentage, b) NAFLD fibrosis algorithm scores, c) gut microbiota composition. Additionally, there will be several hypothesis-generating secondary outcomes to understand the metaorganismal pathways that influence the development and progression of NAFLD, type 2 diabetes, and cardiovascular risk.

Design: In a randomised double-blind placebo-controlled trial, 104 participants were randomised to 10-14 months intervention with either synbiotic (n=55) or placebo (n=49). Recruitment was completed in April 2017 and the last study visit will be completed by April 2018.

Methods: Change in gut microbiota composition will be assessed using 16S ribosomal RNA gene sequencing. Change in mean liver fat percentage will be quantified by magnetic resonance spectroscopy (MRS). In addition, change in liver fat severity will be measured using two NAFLD fibrosis algorithm scores. The INSYTE study was approved by the local ethics committee (REC: 12/SC/0614) and is registered at www.clinicaltrials.gov as NCT01680640.

1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) comprises a wide spectrum of liver diseases, ranging from simple steatosis to hepatocyte inflammation, necrosis, and ultimately cirrhosis and hepatocellular carcinoma (1). The liver is exposed to endotoxin and other bacterial products derived from the gut microbiota. There is evidence that the adverse alteration of the intestinal ecosystem (termed dysbiosis) contributes to the development of metabolic diseases and NAFLD (2-4). The metaorganismal pathways by which dysbiosis may influence the development and progression of NAFLD are: i) alteration of gut hormone production affecting glucose control (5, 6); ii) alteration of short chain fatty acid production, influencing glucose and lipid metabolism (7-9); iii) translocation of bacterial lipopolysaccharide (LPS) influencing gut permeability, vitamin absorption and hepatic mitochondrial function (contributing to liver inflammation) (10, 11); and iv) perturbation of bile acid (12) and trimethyl-amine (TMA) metabolism, increasing liver toxicity and cardiovascular risk (13-15). Previous studies showed that in patients with non-alcoholic fatty liver disease (NASH), gut microbiota is altered with a prevalence of Gram-negative bacteria such as *Proteobacteria*, *Enterobacteriaceae*, and *Escherichia* (16, 17). In a recent study, Boursier et al. (4) showed that the severity of NAFLD (i.e., NASH and significant fibrosis) was associated with gut dysbiosis. Specifically, the increased abundance of *Bacteroides* genus was independently associated with NASH, and the increased abundance in *Ruminococcus* was independently associated with liver fibrosis. Recently, Loomba et al. described the metagenomic signature for non-invasive detection of advanced fibrosis in patients with NAFLD (18). They studied the stool microbiome and serum metabolome of a well-characterized cohort of patients with biopsy-proven NAFLD and they found that the gut microbiomes in NAFLD were dominated

by members of Firmicutes and Bacteroidetes, followed by *Proteobacteria* and *Actinobacteria*. The *Proteobacteria phylum* was significantly increased in abundance while the Firmicutes phylum decreased as the disease progresses from mild/moderate NAFLD to advanced fibrosis. Specifically, at the species level, *Eubacterium rectale* and *Bacteroides vulgatus* were the most abundant organisms in mild/moderate NAFLD while *Escherichia coli* (1%) was the most abundant in advanced fibrosis (18).

These studies suggest that interventions that modify the gut microbiota to correct the dysbiosis might be useful to reduce the severity of NAFLD and even to reverse some of its features. Such interventions might include the use of specific microbes (i.e. probiotics) or of substrates to promote the growth beneficial microbes (i.e. prebiotics), or the combination of both (i.e. synbiotic) (19). To assess whether a synbiotic ~~(fructo-oligosacharide with a degree of polymerization < 10 at 4 g/twice a day plus *Bifidobacterium animalis* subsp. lactis BB-12 at a minimum of 10 billion CFU/day)~~ will affect the gut microbiota composition, liver fat accumulation and biomarkers for liver fibrosis in people with NAFLD, we designed a randomised, double blind placebo controlled trial: the INSYTE study (**In**vestigation of **sy**nbiotic **t**reatm**e**nt in NAFLD).

2 STUDY DESIGN

The INSYTE study (**In**vestigation of **sy**nbiotic **t**reatm**e**nt in NAFLD; www.clinicaltrials.gov registration number NCT01680640).) is a double-blind, randomised, placebo-controlled trial testing the effects of a synbiotic intervention over 10 to 14 months in participants with NAFLD recruited from six hospitals in the South of England. The primary end points of the study are to test whether treatment with synbiotic decreases mean liver fat percentage from three discrete liver zones, measured by a magnetic resonance spectroscopy (MRS) scan; improves two validated algorithmically-derived liver fibrosis scores (20, 21); and changes the gut microbiota composition determined by 16S rRNA gene sequencing. The primary end points of the study will be tested using intention-to-treat (ITT) analysis and per protocol analysis. The INSYTE study was approved by the local ethics committee (REC: 12/SC/0614).

2.1 Patient selection

266 potential participants with fatty liver disease were identified from secondary care clinics, diagnosed by either radiological or biopsy criteria for NAFLD. 104 participants who met the inclusion criteria were randomised to the intervention or placebo. Participants were block randomised (according to age and sex) to either synbiotic ~~(fructo-oligosaccharide plus *Bifidobacterium animalis* subsp. lactis BB-12)~~ or placebo ~~(maltodextrin)~~.

2.2 Recruitment

Recruitment into the INSYTE study was completed in April 2017 and the final study visits will be completed by April 2018. Recruitment was conducted at University Hospital Southampton NHS Foundation Trust, where potential participants with a diagnosis of non-alcoholic fatty liver disease (NAFLD) established as part of their attendance at Hospital Clinics, were contacted by the research team **(Table 2)**. Contact occurred at their Hospital Clinic attendance or by letter of invitation from the research team.

Outside Southampton, at Poole Hospital NHS Foundation Trust, Portsmouth Hospitals NHS Trust, Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust, Hampshire Hospitals NHS Foundation Trust, and the Isle of Wight NHS Trust, our collaborators (medical doctors responsible for the care of people with NAFLD) identified and informed potential participants about the study, providing people with a patient information sheet and study team contact details.

2.3 Randomisation

All participants gave written, informed consent. After completion of baseline study tests, participants were randomised to synbiotic or placebo according to a list generated by the BRC Statistics and Data Management group. Participants were stratified by age (<50 and ≥50 years) and sex. Blocks were used to ensure a balance between the treatment groups within strata. Investigators are blinded to treatment allocation until cessation of the trial.

Table 2. Participant recruitment

• There was no payment of participants, but travel costs were reimbursed
• After discussion of their diagnostic liver biopsy test, or CT scan, or ultrasound, or MRI results, patients were invited to participate in the research study, by direct contact from a member of the research team, or by letter of invitation.
• Each patient had approximately 2 weeks, or as much time as they needed, to decide upon their participation after initial discussion with the research team doctor or nurse. Consent was obtained by a medical doctor or nurse within the research team.
• Translators were provided by the NHS Trust for non-English speaking participants. Only adults able to understand the nature of adipose tissue biopsy were recruited.
• We used the UHS clinical code database containing patients with a diagnosis of NAFLD. This information was sought from the UHS Information Team in accordance with UHS NHS policy.

2.4 Inclusion and exclusion criteria

Briefly, the inclusion criteria for both men and women for participation in the study were age > 18 years and: 1) a recent (< 3 years) diagnosis of liver fat on normal clinical grounds including in most cases liver assessed by Kleiner scoring system (22), with no known aetiological factors for underlying liver disease (e.g. exclusion of hepatitis A, B and C, primary biliary cirrhosis, autoimmune hepatitis, haemochromatosis); or 2) liver fat diagnosed by ultrasound, CT or magnetic resonance imaging (MRI) within 3 years in patients who also have either diabetes and/or features of the metabolic syndrome, and 3) alcohol consumption ≤ 14 units /week for women ≤ 21 units / week for men (23). Exclusion criteria included: Previous bariatric or other abdominal surgery, three or more courses of broad spectrum of antibiotics in a year that may change gut microbiota, or consumption of probiotic supplement, within the 2 months preceding enrolment, and body weight above 155 kg, as this is the maximum weight capacity for the magnetic resonance imaging table.

2.5 Intervention and placebo group

Fifty-five participants were randomised to receive synbiotic treatment consisting of fructo-oligosacharide with a degree of polymerization < 10 at 4 g/twice a day (two sachets a day, to stir into a cold drink) plus *Bifidobacterium animalis* subsp. lactis BB-12 at a minimum of 10 billion CFU/day (1 capsule a day) for a minimum of 10 months and a maximum of 14 months (See 3.6 Milestones and plan for explanation). The quantity of live bacteria in the capsules and the quality of the fructo-oligosacharide have been monitored through the study.

The synbiotic composition selected for the INSYTE study was chosen using the "synergistic approach" to maximize beneficial effects on specific microbiotic species. The probiotic (*Bifidobacterium animalis* subsp. lactis BB-12) was chosen based on specific beneficial effects on the host, and the prebiotic (fructo-oligosacharide with a degree of polymerization < 10) was chosen to specifically stimulate growth and activity of the selected probiotic. The prebiotic was selected to have a higher affinity for the probiotic and was chosen to improve its survival and growth in the host (19, 24).

Synbiotic supplements are generally well tolerated and the side effects include: increased bloating, increased flatulence and increased regularity of bowel movements (25).

Forty-nine participants were randomised to receive placebo treatment consisting of 4 g/twice a day of maltodextrin (1 capsule a day plus two sachets a day, to stir into a cold drink).

The synbiotic and the placebo were supplied in identical packaging. Participants were asked to consume the synbiotic and placebo sachets soon after stirring the powder into cold drink. The synbiotic and placebo were provided gratis by Chr. Hansen Holding A/S, Boege Alle 10-12, 2970 Hoersholm, Denmark.

2.6 Outcome measures

The primary outcomes are to assess: a) change in liver fat percentage (measured by MRS); b) change in the liver fibrosis score which is a non-invasive fibrosis biomarker score calculated using the following algorithm: score = -7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494) (20), and change in the NAFLD fibrosis score which is calculated using the following algorithm: -1.675 +0.037 ×age (years) +0.094 ×BMI (kg/m^2) +1.13 ×IFG/diabetes (yes=1, no=0) +0.99 ×AST/ALT ratio -0.013 ×platelet ($\times 10^9/l$) -0.66 ×albumin (g/dl) (21); c) change in gut microbiota composition determined by 16S rRNA gene sequencing.

Additionally, there will be several hypothesis-generating secondary outcomes to understand the metaorganismal pathways that potentially influence the development and progression of NAFLD, type 2 diabetes, and cardiovascular risk. These are to test the effects of the synbiotic on change in: a) liver stiffness (determined by transient elastography); b) microvascular function; c) plasma cardiovascular risk markers; d) markers of lipid metabolism and inflammation (bile acid metabolism, TMAO and LPS); e) satiety and satiety factors; f) liver inflammation and fibrosis score (LIF score); g) hepatic mitochondrial dysfunction and h) intestinal permeability. Additionally, relevant magnetic resonance sequences will be collected to analyse degree of liver inflammation and liver fibrosis, and to generate a liver inflammation and fibrosis score (LIF score) (26) and subcutaneous adipose tissue biopsies have been taken at baseline and end of study, for future studies which will aim to investigate effects of synbiotic on adipose tissue.

2.7 Sample size and power calculations

The sample size calculation for INSYTE was based on testing the effect of the synbiotic on change in liver fat percentage as a key primary outcome for the trial. There was (and still is) little published literature upon which to base a sample size calculation to test the effects of the synbiotic treatment on the primary end point of change in liver fat. That said, a total sample size of 100 participants, with a 14% drop out during the study and 43 participants in each group completing the study provides 85% power to detect a difference of 40% in liver fat (in the treatment arm compared with the placebo), assuming that the common standard deviation is 62% of baseline mean liver fat percentage, using a power calculation test with a 0.05 two-sided significance level. In our recent randomised placebo-controlled trial over a longer period of intervention (the WELCOME Study), 5% of the randomised cohort withdrew between randomisation and end of study measurements (27). In the WELCOME study (completed in 2013) testing the effects of omega-3 fatty acids on the primary outcome (change in liver fat percentage) (27, 28), we reported that the mean percentage fat content was 28.5% with a similar standard deviation to that presented above for the INSYTE study sample size calculation. MRS spectroscopy can reproducibly detect liver fat levels from zero to 100%. The CV of the MRS methodology for detecting liver fat is ~8%. One recent publication of a small study in people with NAFLD testing the effect of a synbiotic over 24 weeks, showed a 69% decrease in liver fat (29). Another more recent study with only 10 patients in the treatment arm and 10 in the placebo arm, showed a significant 34% decrease in liver fat after only 6 months probiotic treatment (25).

2.8 Statistical analysis

Statistical analyses will be performed using SPSS for Windows, Stata v14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.) and R (30). There

will be three primary endpoints: 1) change in liver fat, 2) change in fibrosis biomarker scores, and 3) change in gut microbiota composition. The normal distribution of the data will be tested by the Shapiro–Wilk and Kolmogorov–Smirnov tests (See below for gut microbiota statistical analysis). For each outcome of interest, a change variable will be calculated where possible, as the difference between end of study measurement and baseline measurement. For continuous variables, multiple regression analysis will be used to assess the effect of the synbiotic on each of the change variables of interest.

Liver fat percentage statistical analysis. For change in liver fat percentage as the outcome, the baseline measurement and randomisation group will be included in the model as predictors, as we undertook recently for the analysis of an omega-3 fatty acid intervention in the similarly designed WELCOME trial (27, 28). This approach allows for adjustment of potential confounders, such as change in body weight, change in diet, age, sex, medications, change in physical activity assessed by a physical activity monitoring device (Sensewear Pro 3) worn for at least 4 days at baseline and 4 days at the end of the study. For change in liver fat percentage (and other secondary outcomes), ANCOVA will also be undertaken to assess effect sizes in the intervention group and placebo as we reported previously for the WELCOME trial (31).

Gut microbiota statistical analysis. At the end of the study, taxonomic and operational taxonomic unit (OTU) profiles between treatment and placebo arms will be compared to quantify significant changes in gut microbiota following intervention. Predictive functional profiling will be performed using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (32). Statistical tests for differences in abundances for each taxonomic unit, OTU or predicted function, as well as difference in alpha-diversity indexes, will be established using Wilcoxon rank-sum tests with false discovery rate correction for multiple tests. PCA will be performed at the microbial feature level (absolute

values and difference associated to treatment) for a first unsupervised exploration of the data, whereas beta-diversity indexes will be visualized through a Principal Coordinates Analysis (PCoA) to explore the dissimilarity between microbial communities.

For all outcomes, the treatment effect with 95% confidence interval will be reported (regardless of whether statistical significance is met). This will allow discussion of whether the trial result is compatible with a clinically important effect. When reporting results, it will be acknowledged that care should be taken in interpreting results from several outcome measures, as some statistically significant findings are likely to result from chance alone; consequently, we will allow for multiplicity of testing when interpreting our results.

2.8.1 *Analyses by intention to treat (ITT) and Per Protocol*

Analyses will be undertaken by an intent-to-treat (ITT) analysis and per-protocol. The ITT analysis will include all patients in the groups to which they were randomised (regardless of whether they were later found to be ineligible, a protocol violator, given the wrong treatment allocation or never treated). Multiple imputation (MI) will be used to handle any missing data. This will involve using a regression model to generate a number of datasets with values imputed for participants who have missing outcomes. The model will take into account the participant's observed values at baseline, and then the parameters estimated will be averaged to give a single estimate. In order to assess the impact of MI on the results, the analysis will be repeated without MI (i.e. analysis of participants with complete data (i.e. having baseline and end of study measurements). This analysis assumes that any missing data is missing at random (MAR), i.e. that there is no difference between missing and observed values, once adjusted for any baseline variables which predict for missingness. If appropriate, in order to explore the effect of departures from the MAR assumption, a sensitivity analyses may be performed (33). This assumes that d= the mean of the missing data minus the mean of the

observed data. Under MAR, d=0. The value of d will then be varied in order to model different scenarios (i.e. that the patients who are lost to follow up have systematically worse outcomes), and we will report whether the significance of the main analysis is maintained in the sensitivity analysis. All randomised participants will be accounted for in these analyses. Per protocol analysis will exclude participants consuming <50% of their allotted medication or participants who were later found to be ineligible or who did not complete the study.

3 STUDY DESIGN SCOPE: VISIT AND ASSESMENT SCHEDULES

3.1 Study visit overview and follow-up

During the research trial, participants have been asked to attend the clinical research facility (NIHR Wellcome Trust Clinical Research Facility at University Hospital Southampton NHS Foundation Trust) for two days (visit 1 and visit 2) at the beginning of the study, after six months for simple haematological and biochemical blood tests that were undertaken as a safety check, and again at the end of the study (visit 5 and visit 6) to repeat the same tests that were undertaken at baseline in visit 1 and visit 2. Synbiotic and placebo have been stored at a controlled room temperature and were dispensed after the baseline tests and at 6 months follow-up visit. Participants were asked to return all used and unused sachets at their next study clinic visit and their final clinic visit. Compliance will be assessed at the end of the study as percentage of sachets and capsules used. Adverse effects possibly associated with trial medication have been recorded and reported according to University Hospital Southampton research governance policy. If a participant reported any untoward medical occurrences, these were recorded on an adverse event or serious adverse event form in accordance with the most current University Hospital Southampton Research Related Adverse Event Reporting Policy.

3.2 Milestones and plan

A key primary end point of the trial is to test the effect of the intervention on change in liver fat percentage. From another study that tested the effect of a synbiotic on liver fat in a small number (n=34) of people with NASH that showed a 69% decrease in liver fat on liver biopsy score over 24 weeks (25), we reasoned that a six month minimum period of intervention would be required to change liver fat percentage. To err on the side of caution, we determined that the invention period should be a minimum of 10 months and because of the necessary

staggering of patients at randomisation to allow us the flexibility to undertake all the complex end of study measurements with the limited numbers of trial staff that were available in our single centre study, we aimed for a 10-12 month intervention period. Additionally, we specified that a maximum of two courses of broad spectrum antibiotic would be allowed during the trial period, with a further one month of synbiotic or placebo added for each course of antibiotic. Thus, the maximum period of time within the trial was potentially 14 months (although very few participants, required 14 months of treatment with synbiotc or placebo).

The progress of the trial was divided into 4 steps:

Months 0-36: Recruitment of cohort, baseline measurements and randomisation to active compound or to placebo (10-14 months intervention)

Months 10-12: Follow up measurements began

Months 10-32: Follow up measurements completed

Months 32-40: Measurements and analyses of results

4 METHODS

4.1 Biochemical and body composition measurements

At baseline and end of study, participants have been characterized according to: i) well validated panels of serum markers and simple anthropometric measurements associated with severity of liver fibrosis; ii) measures of insulin sensitivity, body composition and other features of the metabolic syndrome that help provide an estimate of future risk of type 2 diabetes; iii) presence of diabetes mellitus; iv) assessment of prevalent cardiovascular disease; v) diet and appetite; vi) alcohol consumption; vii) quality of life indicators **(Table 3).**

Glucose, insulin, HbA1c measurement, total cholesterol, HDL-cholesterol, triglycerides, platelets, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase (GGT) have been measured in fasting serum at the Pathology Laboratory at the University Hospital Southampton. Serum was collected for the analysis of lipopolysaccharide (LPS). Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA) have been analysed using ELISA Kits (R&D) read on Luminex 200 (Bio-plex 200 - Bio-Rad, Watford, UK). The Procollagen-III N-terminal Propeptide (PIIINP) assay has been performed with a UniQ radioimmunoassay kit supplied by Orion Diagnostica (Product no.68570). We have estimated the homeostatic model assessment to quantify insulin resistance (HOMA-IR) using fasting insulin and glucose concentrations. We have generated two different histologically-validated liver fibrosis scores (20, 21). The first of these scores has been calculated using measurement of HA, PIIINP and TIMP-1 (20) and the second validated algorithmically-derived score (NAFLD fibrosis score) has been calculated using age (years), body mass index (BMI), impaired fasting glucose/ diabetes (yes/no), ALT/AST ratio, platelet count and albumin concentration (21). Blood pressure has been measured using a Marquette Dash 3000 monitor (GE Healthcare, Little Chalfont, Bucks, UK) on the non-dominant arm

after subjects had become acclimatised and had rested; the mean of three measurements has been calculated.

4.1.1 *Urine tests*

Urine samples have been collected to test: urinary albumin/creatinine ratio (as a measure of albuminuria and increased vascular risk) (34, 35).

4.1.2 *Body fat measurements*

Whole-body dual-energy X-ray absorptiometry (DEXA), and magnetic resonance imaging (MRI) and liver fat magnetic resonance spectroscopy (MRS) have been performed at the beginning and at the end of the study. DEXA and horizontal five-slice cross-sectional MRI have been used to evaluate in detail the absolute amount and relative percentages of body fat, truncal fat, sub cutaneous abdominal fat, and visceral fat. DEXA scanning is undertaken with a Discovery 17 instrument (Hologic, Bedford, MA, USA) using a standard visual method to divide images into trunk, limb and head. MRI images have been acquired from five non-contiguous slices of the abdomen, extending from 5 cm below to 15 cm above L4–L5, to obtain an estimation of sub cutaneous abdominal and visceral fat. Participants have been scanned in the supine position with a 1.5 T MR scanner (Siemens Avanto, Syngo software release B17; Siemens AG, Munich, Germany) using a 32-channel body coil. A gradient echo 2D FLASH (fast low angle shot) sequence (TR = 111 ms, TE = 4.18 ms, flip angle = 70°, slice width = 10 mm, slice spacing = 50 mm) has been used to obtain T1-weighted images. In order to accommodate the circumference of the individual being scanned within the image, the field of view has been varied. The MR images have been analysed using a proprietary software package (Mimics 14.0; Materialise NV, Leuven, Belgium) to identify regions of subcutaneous and visceral fat within the cross-sectional abdominal MR images. This package enabled identification of subcutaneous and visceral fat. By examining the histogram of pixel

values present in the image, threshold levels can be set. Since fat pixels have been the highest value pixels in the image, fat tissue can be identified from other tissue in the images. Some manual intervention could be required when using this technique, as there has been some variation in signal intensity across the image, which is often the case in large field-of-view MR images. Three different masks have been created; one comprising the whole cross-section of the body, one containing the visceral fat region and one containing the subcutaneous fat region. The number of pixels contained within each of these masks could be determined, and hence calculate the areas of subcutaneous fat and visceral fat and compare them with the total cross-sectional area. Adipose tissue volume has been converted to mass in kg using a density of 0.92 kg/l for adipose tissue.

4.1.3 *Anthropometric measurements*

Total body fatness has been measured using bioimpedance and regional body fat by skin fold thickness. Waist circumference has been measured over bare skin midway between the costal margin and the iliac crest (36). Hip circumference has been measured at the widest part between the greater trochanter and lower buttock level. BMI has been calculated as body weight (kg) divided by the square of body height (m^2) (37). Bioelectrical impedance (Bodystat 1500; Bodystat, Isle of Man, UK) has been used to determine body composition. Metabolic syndrome has been defined using the International Diabetes Federation criteria (38)

4.1.1 *Energy expenditure and indirect calorimetry*

VO_2 max has been measured. Basal metabolic rate has been assessed by indirect calorimetry (GEM Nutrition, UK). This measurement of indirect calorimetry involves participants lying on a bed with a transparent Perspex hood over the head and neck for approximately 30 minutes. Inspired oxygen consumption and expired carbon dioxide measured by indirect

calorimetry at rest has been used to calculate respiratory quotient at the beginning and at the end of the study.

4.2 Primary outcomes

4.2.1 *Liver fat measurement with magnetic resonance imaging*

Liver fat estimation by MR has been undertaken at baseline and at the end of the study by both MR imaging and MR spectroscopy where possible, as the techniques provide an estimation of liver fat percentage using different methodologies. A 'change' variable will be calculated as the arithmetic difference between end of study and baseline measurements. The scans have been undertaken and read by radiographers independent from the study. The total scanning time for both techniques combined has been around ~40 minutes for each participant. The MR spectroscopy (MRS) of the liver is a measurement of the quantity of liver fat accumulated in three discrete liver zones, at baseline and follow-up. Three 20 × 20 × 20 mm^3 spectroscopic volumes of interest (VOI) are positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions have been copied from the first scan, to ensure consistency. The pulse sequence used a TR = 1500 ms, TE = 3 ms, flip angle = 90°, bandwidth = 1000 Hz, 8 averages and acquisition duration of 1024 data points, with no water suppression. The acquisition has been obtained in a breath hold examination of 18 s. Spectra have been post-processed using Siemens scanner software. This has been a fully automated process and involved several steps, starting with filtering the data using a Hanning filter, zero-filling the data, baseline correction, phase correction and finally curve fitting has been performed (with

4 iterations) to identify the water and lipid peaks. Values for the lipid and water peak integrals have been produced for each VOI and recorded for each subject. Moreover, MR scan acquisitions for the assessment of pancreatic fat quantity have been undertaken at baseline and end of study, as there is evidence that pancreatic fat is associated with type 2 diabetes and NAFLD (39).

4.2.2 *NAFLD fibrosis biomarkers*

NAFLD fibrosis biomarkers have been measured at baseline and end of study. A 'change' variable will be calculated as the arithmetic difference between end of study and baseline measurements. Two algorithmically-derived fibrosis scores have been calculated: 1) Liver Fibrosis Score, which is a non-invasive which is a non-invasive fibrosis biomarker score using the following algorithm: score = -7.412 + (ln(HA)*0.681) + (ln(P3NP)*0.775) + (ln(TIMP1)*0.494) (20); 2) NAFLD Fibrosis Score which is calculated using the following algorithm: score = -1.675 +0.037 ×age (years) +0.094 ×BMI (kg/m^2) +1.13 ×IFG/diabetes (yes=1, no=0) +0.99 ×AST/ALT ratio -0.013 ×platelet (×10^9/l) -0.66 ×albumin (g/dl) (21).

4.2.3 *Gut microbiota analysis*

Gut microbiota composition will be assessed at baseline and end of study through 16S rRNA gene sequencing. 16S sequencing is well suited for analysis of large number of samples, i.e., multiple patients and longitudinal studies (40). Gut microbiota analyses will be performed at the Louvain Drug Research Institute (Université Catholique de Louvain, Belgium) as previously described (41). Genomic DNA will be extracted from fecal material using a QIAamp DNA Stool Mini Kit (Qiagen, Germany), including a bead-beating step. The V5-V6 region of the 16S rRNA gene will be amplified by PCR with modified primers. The amplicons will be purified, quantified and sequenced using an Illumina Miseq. After an initial quality-filtering of the reads with the Illumina Software, quality scores will be visualized, and

reads will be trimmed. The reads will be merged with the merge-Illumina-pairs application (42). Reads will be randomly subsampled using Mothur to avoid large disparities in the number of sequences (43). Subsequently, the UPARSE pipeline implemented in USEARCH will be used to further process the sequences. Clustering will be performed using the cluster_otus function which includes a chimera filtration, with 97% similarity cut-off to designate Operational Taxonomic Units (OTUs) (44). Statistical analysis will be performed using multivariate techniques including principal component analysis and partial least squares discriminant analysis.

4.2.4 *Cardiovascular risk assessment*

4.2.4.1 Cardiorespiratory fitness

Cardiorespiratory fitness has been measured in terms of maximal oxygen uptake (VO_2 peak) and determined from breath-by-breath analysis of oxygen consumption and CO_2 production using a Cortex metalyser 3B instrument (Cortex Biophysik, Germany) during maximal treadmill exercise (Woodway P55 treadmill) with 12-lead ECG monitoring throughout the test. Participants have been advised to avoid strenuous exercise for 24 hours and alcohol on the day prior to testing. They have been fitted with an air-tight facemask, which allows analysis of expired air, and ECG leads. To allow participants to become acclimated to the facemask and also to determine resting energy expenditure, resting measurements have been taken for 3 minutes prior to commencement of activity induced measurements. Participants have been encouraged to continue until the respiratory exchange ratio was >1.1 and they reached at least 90% of their predicted maximum heart rate (as determined by 220 − age), unless they experienced chest pain or felt unwell. Cardiorespiratory fitness has been measured by peak VO_2 which has been corrected for total body weight. We undertake an ECG before and after the treadmill test.

4.2.5 *Dietary assessment*

A validated food frequency questionnaire (FFQ) has been used to explore food preferences covering a list of regularly consumed food and drink via measured at baseline, 6 months and end of study (45). Participants have been asked to complete a standard questionnaire for food and alcoholic drink recall.

4.2.6 *Physical activity and energy expenditure assessment*

Physical activity has been assessed using a monitor (Sensewear Pro2) (46) worn by each participant for approximately 4 days, as well as by means of a modified Baecke questionnaire

(47). Baecke's questionnaire includes 16 questions, focusing on a composite score for 3 components of physical activity in the last 12 months: (1) occupational physical activity (eight questions); (2) physical activity in leisure (four questions); and (3) leisure and locomotion physical activities (four questions). A simple formula is used that takes account of the intensity of the activity, the amount of time spent undertaking the activity and the proportion of time annually spent undertaking the activity. The SenseWear Pro2 armband is a compact and lightweight (82 g) device worn around the upper arm that is well tolerated and contains sensors for 2 plane accelerometery, near body temperature, skin temperature and the galvanic skin response. The SenseWear Pro armband allows reliable measurement of physical activity levels and calculation of total energy expenditure recordings.

4.3 Additional measurements and analysis that we intend to undertake in the future

A major strength of the INSYTE trial is the extensive and detailed phenotypic characterisation of participants at baseline and at the end of the study. The following secondary outcomes are being assessed by undertaking measurements at baseline and at the end of study. A 'change' variable will be calculated as the arithmetic difference between end of study and baseline measurements.

4.3.1 *Liver stiffness, and liver inflammation and fibrosis (LIF) score*

Transient elastography has been undertaken at the beginning and at the end of the study to measure liver stiffness. This is a new non-invasive, painless method allowing the evaluation of liver "stiffness". The probe for the elastography is an ultrasound transducer mounted at the end of a vibrating cylinder. The cylinder produces a vibration that is transmitted towards the tissue. The ultrasound detects the propagation of the vibration by measuring its velocity. The

velocity of the wave towards the tissue is directly related to the tissue stiffness. High velocity is related to higher tissue stiffness that corresponds to an increased severity of liver disease. The performance of the transient elastography has been assessed in a meta-analysis including fifty studies: the mean area under receiver operating characteristics curve (AUROC) was 0.84 (95% CI, 0.82-0.86) for diagnosis of significant fibrosis, 0.89 (95% CI, 0.88-0.91) for severe fibrosis and 0.94 (95% CI, 0.93-0.95) for diagnosis of cirrhosis (48).

In addition, the liver inflammation and fibrosis score (LIF score) (26) will be estimated. The LIF score developed by Dr Banerjee and colleagues from Perspectum Diagnostics (Oxford) is a composite score derived from estimations of liver fat, inflammation and fibrosis. A LIF score <1, 1-1.99, 2-2.99, and ⩾3 represent validated scores for normal, mild, moderate, and severe liver disease (26, 49).

4.3.1.1 Hepatic mitochondrial function

^{13}C-Ketoisocaproic (^{13}C-KICA) breath test

Each participant has been studied using a standard ^{13}C-KICA breath test procedure. Participants have been asked to rest for 10 minutes before the oral administration of the ^{13}C-label and throughout the breath test study period. After resting for 10 minutes, breath samples have been collected after an overnight fast by blowing into two non-evacuated breath tubes using a straw. This has been followed by the oral ingestion of 1 mg/kg body weight of 2-keto-[1-^{13}C]-isocaproic acid (99%; ^{13}C) along with 20 mg/kg body weight of L-leucine dissolved in 200 ml of water. After 5 minutes the breath sample has been collected into 2 non-evacuated tubes every 10 min for 1 hour using a straw. The ratio of $^{13}CO_2$ to $^{12}CO_2$ in each breath sample collected has been analysed using an Isotope Ratio Mass Spectrometer (SERCON ABCA). The results of the ^{13}C-KICA breath test has been expressed as the

percentage of the administered dose of ^{13}C recovered per min (% dose/min) and as a cumulative percentage ^{13}C-dose recovered over time (cPDR). From calculating the % dose/min and cPDR for the ^{13}C-KICA breath test, we will determine the extent to which there is concordance between these pharmacokinetic variables and measurements of liver fat percentage and other measurements of NAFLD disease severity and liver function.

4.3.2 *Intestinal permeability*

4.3.2.1 Lactulose-Mannitol test

The lactulose and mannitol test has been used to measure the intestinal permeability before and after the intervention. After an overnight fast, participants have been asked to drink a solution containing 10 g of lactulose and 5 g of mannitol in 35 ml of water (1300 mOsml/L). Urine has been collected (over the 6 hours after the drink) into plastic containers with 1 ml of chlorexidine 2% as preservative, to prevent bacterial degradation of sugars. A 10 ml urine sample has been store at -20°C until assayed. Lactulose and mannitol concentrations in the urine samples have been measured using enzymatic colorimetric techniques as described previously on an autoanalyser (Konelab 20; Thermo Scientific, Swedesboro, NJ, USA)(50, 51). All reagents, unless stated otherwise, have been purchased from Sigma Aldrich (Poole, UK) and Biocataysts (Nantgarw, Wales). The results of the lactulose mannitol test have been expressed as a ratio of percentage of urinary excretion of lactulose to the percentage of urinary excretion of mannitol in relation to the amount of each probe consumed (L/M ratio).

4.3.2.2 Serum zonulin

Serum zonulin was measured by an ELISA technique used for the quantitative determination of human zonulin. Serum zonulin concentrations were measured by ELISA (Immundiagnostik AG, Bensheim, Germany). The Elisa kit used for zonulin measurement only detects the active (uncleaved) form of zonulin. The lower limit of detection was

0.23ng/mL. Intra- and interassay coefficients of variation were 3%-7% and 5%-12%, respectively.

4.3.2.3 Satiety assessment

Satiety has been measured at the beginning and at the end of the study. Participants have been instructed to abstain from alcohol and strenuous physical activity for 2 days prior to the day of the test. Participants have been offered a free-choice buffet breakfast (comprising yogurt, bread, butter, cheese, jam, fruit, orange juice, and water; approximately 470 kcal (52)) and have been instructed to complete breakfast within 15 minutes. Food and drink have been weighed before and after the meal and energy intake has been calculated. Before and after breakfast (at -5, 0, 15, 30, 60, 120, 180 minutes) appetite rating has been assessed, using a 100-mm visual analogue scale. Participants have been asked to draw a vertical mark across the line corresponding to their feelings from 0 (not hungry at all) to 100 (very hungry). The level of satiety has been quantified by measuring the distance from the left end of the line to the mark (53). Serum levels of: glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), pancreatic polypeptide (PP), ghrelin, insulin and glucose have been measured in response to breakfast (9, 54). Venous blood samples were collected at six time points, pre, immediately after breakfast, 15 min, 30 min, 60 min, and 120 min. The blood was collected into a tube containing EDTA and aprotinin as anticoagulant and protease inhibitor, respectively. One millilitre of blood is mixed immediately with an inhibitor cocktail (a mix of 2 ml of Pefabloc SC and 1 ml of DPPIV). Then, the blood sample was then centrifuged at 3000 rpm at 4° C for 10 minutes and plasma stored at -80°C until analysed. Gut hormones have been analysed using the HMHEMAG-34K-06 MILLIPLEX® $_{MAP}$ Human Metabolic Panels kit. The Luminex kit contains color-coded microparticles with two fluorescent dyes. Each of these dyes has been coated with a specific capture antibody and distinctly colored bead. After an

analyte from a test sample has been captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is incubated with the reporter molecule (Streptavidin-PE) to complete the reaction on the surface of each microsphere. Reagents, standards and samples dilutions have been prepared, as recommended by the manufacturer.

4.3.3 *Quality of life indicators*

Quality of life has been studied by assessing an array of key indicators measured via the following:

- EuroQol (EQ-5D): this questionnaire provides a descriptive profile and a single index value for health status (55). It consists of two parts: a) descriptive system and b) visual analogue scale. The descriptive system comprises 5 items: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. Each item has 3 levels: no problems, some problems, extreme problems. The 5 items do not have an arithmetic sum; they describe the respondent's health state. The visual analogue scale is a quantitative measure of health as perceived by the respondents. It is designed for self-completion by respondents**.**
- Hospital Anxiety and Depression scale (HADS): this questionnaire assesses psychological status such as depression and anxiety (56) as obesity is associated with psychological morbidity. The questionnaire is composed using a 14 item scale, half relating to depression and half relating to anxiety, each item is scored from 0-3, giving a total score range from 0 to 21. The cut-off point for anxiety or depression is 8. It is designed for self-completion by respondents.

4.3.3.1 Fatigue assessment

Fatigue is a common systemic feature of NAFLD though mechanisms remain poorly understood (57, 58). The severity of fatigue (Fatigue Severity Scale) and sleepiness (Epworth Sleepiness Scale) has been assessed using validated scores at baseline and after 6 and 12 months intervention. Associations between measures of NAFLD disease severity, daytime sleepiness, autonomic dysfunction, muscle function via grip test, inflammatory markers and mitochondrial dysfunction via ^{13}C-ketoisocaproate breath testing will also be explored at the end of the study. This will provide a comprehensive assessment of this symptom which can cause functional impairment in people with NAFLD.

4.3.3.2 Handgrip strength assessment

Muscle function has been tested using a simple and non-invasive technique (hand grip strength). This will allow us to better understand the influence of fatigue, disease severity, micronutrient status and mitochondrial function on muscle function in NAFLD. Handgrip strength has been measured at baseline and after intervention using a Jamar dynamometer with participants seated and their arms rested on the chair arms, alternating between each arm when taking measurements. The best score out of a total of three measurements from each hand will be used. The Jamar dynamometer is simple to use, accurate, reproducible in its measurements and the most widely used device used for assessing muscle strength. There is evidence in literature that there is association between impaired physical function, weaker muscle strength and hyperglycaemia in people with diabetes (58, 59). At the end of the study, we will assess whether there is an association between hand grip strength and other features of metabolic syndrome including NAFLD and muscle strength.

4.3.4 *Cardiovascular and peripheral vascular function assessment*

4.3.4.1 Carotid Artery Ultrasound

Carotid intima-media thickness (CIMT) and carotid plaque have been measured in all participants at the beginning and end of the study by a trained Clinical Research Fellow (ES). CIMT is a well-known marker of subclinical atherosclerosis and is a surrogate marker for coronary disease (60). It also has independent prognostic value in cardiovascular disease (61). The carotid arteries have been studied with a duplex scanner using a 7.5 MHz linear array transducer (Philips IE33 4–8 MHz) with ECG monitoring. Ultrasound parameters (dynamic range, depth range, power output and greyscale) for B-Mode carotid imaging have been adjusted during image acquisition to optimize image quality. All scans have been carried out according to a standardised protocol (62). Briefly, subjects have been asked to lie supine with the neck slightly rotated and a transverse scan is first performed as a screening measure and also to identify the carotid bifurcation. Longitudinal images of the near and far walls of the common, proximal portion of the internal and external carotid arteries and the carotid bifurcation have been examined and multiple images of 4 cine-loop cycles of the carotid artery have been recorded and stored digitally for subsequent off-line analysis using Philips Q-Lab version 8 software. For each subject, a 10 mm plaque-free segment of IMT at the far wall of the common carotid artery immediately proximal to the carotid bulb has been measured using QLAB automated software. The presence of carotid plaque at the distal common carotid, carotid bulb and proximal internal carotid arteries has also been recorded (62).

4.3.4.2 Ankle / Brachial Pressure Index (ABPI)

ABPI has been measured at baseline and at the end of the study. ABPI is a simple measurement for assessing peripheral macrovascular function. Low ABPI (< 0.9) is an

independent predictor of increased cardiovascular disease risk (63). We want to assess correlation of ABPI with other measures of macro- and microvascular function. With the participant rested and lying supine blood pressure cuffs are placed bilaterally on the upper arm (brachial pressure) and ankle, and inflated to 20 to 30 mmHg above systolic pressure. An ultrasound Doppler probe is placed over the brachial, dorsalis pedis and posterior tibialis arteries and used to detect return of the arterial signal at the highest systolic pressure. The ABPI has been calculated by dividing the ankle pressure by the highest brachial systolic pressure.

4.3.4.3 Central aortic pressure and haemodynamic indices wave analysis (PWA)

This has been undertaken using applanation tonometry and SphygmoCor software to derive non-invasively central aortic pressure and haemodynamic indices.

4.3.4.4 Microvascular function (Laser Doppler flowmetry)

Laser Doppler flowmetry has been undertaken to assess microvascular function. Two small Laser Doppler probes are placed on the volar surface of the forearm to detect blood flow in the superficial dermal vasculature. A blood pressure cuff is placed around the upper arm and blood flow measured before, during and after inflation of the cuff to supra-systolic pressure maintained for up to 3 minutes. At the end of the study, the reactive hyperaemic response will be used to assess the capacity of the vasculature to dilate under rested conditions (64).

4.3.5 *Blood DNA, urine and tissue analyses*

Blood has been collected and will be used for DNA extraction and analysis. All samples have been anonymised and stored in -80^{o}C freezers behind locked doors in the researchers' laboratory on the Southampton General Hospital site.

4.3.5.1 Adipose tissue

Lower abdominal adipose tissue biopsy has been undertaken at baseline and at the end of the study. Prior to the biopsy the participant received an injection of local anaesthetic. A scalpel has been used to make a small (≤ 1 cm) transverse incision to expose the subcutaneous fat. Lobules of fat pulled up into the wound (~1 g of fat lobules) have been excised using a scalpel or scissors. The adipose tissue sample has been collected in a sterile container and stored immediately, one sample is stored in formalin, one with RNA later at -80° and three in liquid nitrogen.

4.3.5.2 Assessment of lipids and proteins in plasma and urine

At the end of the study, we intend to undertake a detailed assessment of serum, plasma and urine lipids and proteins. We intend to analyse these samples using novel LC-MS/MS metabolomics and proteomics methodologies. Statistical analysis will be performed using multivariate techniques including principal component analysis and partial least squares discriminant analysis.

4.3.5.3 Micronutrient status

Patients with NAFLD are more likely to be obese than the general population. Obesity is associated with increased risk of micronutrient deficiency which may adversely influence insulin sensitivity, energy expenditure, mitochondrial function and muscle function. Therefore, at the end of the study, we intend to measure plasma and serum values of: vitamin C and vitamin E, calcium, phosphorus, magnesium, iron, copper, zinc, manganese, iodine, chromium, selenium, vitamin A (65, 66), vitamin B12, vitamin E, vitamin K, vitamin D (67), and folate.

Baseline measurements Visits 1 and 2 (+/- visit 3)	Visits 4 at 5-6 months	Follow-up measurements Visits 5 and 6 (+/- visit 7)

Table 3. A summary table of participants' visits and tests during the study

Questionnaires: quality of life, physical activity, fatigue, Epworth sleepiness scale, anxiety and depression scale, food frequency, and satiety.	Food frequency questionnaire, Epworth sleepiness scale, anxiety.	Questionnaires: quality of life, physical activity, fatigue, Epworth sleepiness scale, anxiety and depression scale, food frequency, and satiety.
Body composition: Anthropometric measurements, body fat distribution (DEXA scan, bioimpedance, magnetic resonance imaging), indirect calorimetry.	Anthropometric measurements Blood pressure	Body composition: Anthropometric measurements, body fat distribution (DEXA scan, bioimpedance, magnetic resonance imaging), indirect calorimetry.
Fasting blood tests: Glucose, insulin, HbA1C, lipid profile, liver function test, gut hormones, inflammatory markers, microparticles	Fasting blood tests: Glucose, insulin, HbA1C, lipid profile, liver function test	Fasting blood tests: Glucose, insulin, HbA1C, lipid profile, liver function test, gut hormones, inflammatory markers, microparticles
Cardiovascular measurements: carotid intima media thickness (carotid artery ultrasound), blood pressure, pulse wave velocity/analysis, ankle-brachial index, cardiorespiratory fitness (Treadmill), physical activity energy expenditure (SenseWear Pro), hand grip strength, laser doppler flowmetry		Cardiovascular measurements: carotid intima media thickness (carotid artery ultrasound), blood pressure, pulse wave velocity/analysis, ankle-brachial index, cardiorespiratory fitness (Treadmill), physical activity energy expenditure (SenseWear Pro), hand grip strength, laser doppler flowmetry
Gut microbiota analyses (stool sample collection) Intestinal permeability (Lactulose-mannitol test)	Gut microbiota analyses (stool sample collection)	Gut microbiota analyses (stool sample collection) Intestinal permeability (Lactulose-mannitol test)
Liver fat (magnetic resonance spectroscopy) Liver stiffness (Fibroscan) Hepatic mitochondria function (13C-Ketoisocaproic) Metabolic inflammation (fat tissue biopsy) Fibrosis biomarker scores		Liver fat (magnetic resonance spectroscopy) Liver stiffness (Fibroscan) Hepatic mitochondria function (13C-Ketoisocaproic) Metabolic inflammation (fat tissue biopsy) Fibrosis biomarker scores

5 Discussion

The INSYTE trial is a unique proof-of-concept double blind, randomised, placebo controlled clinical trial with a 10-14 month intervention, testing the effects of synbiotic treatment in patients with NAFLD. There is recent growing interest in the role of the gut microbiota in the pathogenesis of NAFLD and there are several metaorganismal pathways linking dysbiosis and liver disease. Previous studies have tested the effect of synbiotic on NAFLD (25, 68-70); however, these studies differ from our clinical trial in several aspects. Firstly, the duration of the treatment in our study was between 10-14 months, depending on whether patients had one or two courses of antibiotics. In previous studies the duration of the synbiotic treatment was between 8 to 28 weeks without accounting for possible antibiotic treatment during the trial. Secondly, in the INSYTE study we have tested the effect of the treatment on liver fat percentage using MRS. We acknowledge that a limitation of the trial is that we did not include liver biopsy, although it should also be recognised that NAFLD is known to be a patchy disease and liver biopsy is invasive, expensive, and is subject to sampling variability. Consequently, we did not include the requirement for liver biopsy in the protocol and decided to measure any change in liver fat percentage precisely, using MRS. MRS is currently considered the non-invasive gold-standard technique for assessing liver fat percentage and has excellent reproducibility and sensitivity, with a coefficient of variance of only 8%, and liver fat signals of only 0.2% are clearly evident above the noise level (71). Thirdly, we are evaluating the effect of the synbiotic supplement on change in gut microbiota. This is a novel and timely outcome and hopefully the results will help to understand the effect of synbiotic on dysbiosis. Fourthly, we are undertaking several hypothesis-generating secondary outcomes that will help to better understand the not only the role of intestinal and systemic

inflammation in the pathogenesis of NAFLD but also the effect of synbiotic on intestinal and systemic inflammation.

The liver is a key organ exposed to high levels of intracolonic fermentation products and almost three quarters of its blood supply is from the intestine via the portal vein. Consequently, changes in specific microbial products, secondary to altered gut microbial composition and changes in intestinal permeability and function, can affect structure and function of the liver (72). Alterations in hepatic structure and function can include altered lipid accumulation, inflammation, fibrosis, insulin sensitivity, and mitochondrial energetic status, which in turn may not only adversely affect the liver but also increase risk of type 2 diabetes and cardiovascular disease. For example, it has been shown that in patients with NAFLD, *Bacteroides* species are independently associated with NASH and *Ruminococcus* species are associated with significant liver fibrosis (18). There is evidence that in the presence of NAFLD there is often "functional dysbiosis", with changes in certain microbial species affecting metabolic and inflammatory pathways. For example, certain species affect gut oxidative stress and butyrate production (e.g. *Akkermantia muciniphila*, and *Faecalibacterium prausnitzii*) (73-75). Increased amounts of *Akkermantia muciniphila* are also associated with higher L-cell activity and the consequent increased production of glucagon-like peptide -1 (GLP-1) that is responsible for better glucose tolerance and higher levels of satiety (76). Moreover, in the intestine, microbial fermentation of dietary fibre by anaerobic bacteria (inter alia *Roseburia*, *Escherichia rectale* and *Blautia*) produces short chain fatty acids (SCFAs) (8, 77, 78). SCFAs are mainly acetate, propionate and butyrate, and these molecules can influence host lipogenesis and gluconeogenesis. A meta-analysis of predominantly short-term probiotic treatments in patients with type 2 diabetes has suggested a beneficial effect on plasma glucose and insulin sensitivity by increasing butyrate production (79). With dysbiosis, there is also increased production of endotoxins from Gram-negative

bacteria that can damage the intestinal barrier, affect vitamin absorption, and increase gut permeability with the potential for LPS to enter the portal and systemic circulation (80, 81). LPS causes disruption of the gut intracellular tight junctions, favouring release of cytokines and gut microbiota components (i.e. gut microbiota DNA) into the circulation and consequently into the liver (80-83). LPS not only promotes inflammation in the liver and adipose tissue but also systemic inflammation, which is now recognised as being associated with increased risk of cardio-metabolic diseases, such as type 2 diabetes and cardiovascular disease. Since LPS production provides a direct inflammatory stimulus to the liver via the portal vein, LPS might increase risk of steatohepatitis. It is therefore plausible that the inflammatory burden affects hepatic mitochondrial function since we and others have shown abnormal hepatic mitochondrial function occurs with steatohepatitis (84, 85). The INSYTE study addresses an important research question given the increased prevalence of NAFLD; namely does synbiotic treatment improve liver fat content in patients with NAFLD.

The FAO/WHO definition of a probiotic: “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (86-88). Prebiotics were first described by Gibson and Roberfroid as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” (86, 89). Administration of prebiotics and/or probiotics reduces hepatic inflammation (90), insulin resistance and diabetes (25, 91) **(Table 4).** A synbiotic is defined as “a mixture of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare” (19).

Synbiotic treatment is safe, well tolerated and inexpensive; therefore, if it can be shown that these agents have clinical efficacy to ameliorate liver fat, synbiotics could be used in primary

care settings to treat patients with NAFLD who are in the early stages of liver disease. Additionally, by richly phenotyping participants with NAFLD at baseline and at the end of the study, we are testing the effect of the synbiotic treatment on many hypothesis-generating secondary outcomes. The secondary outcomes will hopefully inform understanding of the relationships between NAFLD; dysbiosis, and extra-hepatic complications of NAFLD such as type 2 diabetes and cardiovascular disease (92-94).

Table 4. Potential beneficial effects of pre- and probiotics.

Potential mechanism of action of probiotics
Competitive exclusion of intestinal bacteria
Lower pH gradient in the intestine, thus promoting butyrate production and decreasing acetate concentration. Inhibition of the growth of Gram-negative pathogenic bacteria **(79)**.
Improvement of epithelial barrier function
Potential mechanism of action of prebiotics
Promotion of growth of specific health-promoting intestinal bacteria
Increase of enteroendocrine L cells, resulting in increased serum levels of GLP-1, GLP-2 and PYY **(9, 95)**
Reduced plasma triglyceride levels, muscle lipid infiltration, adipose tissue mass and oxidative stress **(95, 96)**
Reduced inflammation and improved immune function **(29)**.

Disclosures/Conflicts of interest

The synbiotic and placebo were provided at no cost by Chr. Hansen Holding A/S, Boege Alle 10-12, 2970 Hoersholm, Denmark. Chr. Hansen had no input into any aspect of study design or conduct of the trial. Furthermore, Chr. Hansen will have no input into data analysis or subsequent reporting of the trial results. PCC has received consulting fees from Chr. Hansen, but not in relation to this trial. None of the other authors has any disclosures.

Acknowledgements

The authors would like to thank the INSYTE Trial Investigators who helped with the recruitment. We would also like to thank the INSYTE Trial participants and also Chr. Hansen Holding A/S, Boege Alle 10-12, 2970 Hoersholm, Denmark, who provided the synbiotics and placebo at no cost. This work was supported by the National Institute of Health Research through the NIHR Southampton Biomedical Research Centre and by the Parnell Diabetes Trust. The authors thank Sanchia Triggs, Gemma Rood, Andria Staniford, Norma Diaper and Jennifer Hedges who were the research nurses, Lucinda England for research governance administration; Cheng Yueqing who helped with the data management and data cleaning, Colin Newell, and Karen Long who helped with data entry.

REFERENCES

1. Byrne CD, Olufadi R, Bruce KD, Cagampang FR, Ahmed MH. Metabolic disturbances in non-alcoholic fatty liver disease. Clinical science (London, England : 1979). 2009;116(7):539-64.
2. Doulberis M, Kotronis G, Gialamprinou D, Kountouras J, Katsinelos P. Non-alcoholic fatty liver disease: An update with special focus on the role of gut microbiota. Metabolism: clinical and experimental. 2017;71:182-97.
3. Henao-Mejia J, Elinav E, Thaiss CA, Licona-Limon P, Flavell RA. Role of the intestinal microbiome in liver disease. Journal of autoimmunity. 2013;46:66-73.
4. Boursier J, Mueller O, Barret M, Machado M, Fizanne L, Araujo-Perez F, et al. The severity of nonalcoholic fatty liver disease is associated with gut dysbiosis and shift in the metabolic function of the gut microbiota. Hepatology (Baltimore, Md). 2016;63(3):764-75.
5. Abu-Shanab A, Quigley EM. The role of the gut microbiota in nonalcoholic fatty liver disease. Nature reviews Gastroenterology & hepatology. 2010;7(12):691-701.
6. Iacono A, Raso GM, Canani RB, Calignano A, Meli R. Probiotics as an emerging therapeutic strategy to treat NAFLD: focus on molecular and biochemical mechanisms. The Journal of nutritional biochemistry. 2011;22(8):699-711.
7. Delzenne NM, Cani PD. Interaction between obesity and the gut microbiota: relevance in nutrition. Annual review of nutrition. 2011;31:15-31.
8. Russell WR, Hoyles L, Flint HJ, Dumas ME. Colonic bacterial metabolites and human health. Current opinion in microbiology. 2013;16(3):246-54.
9. Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, et al. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. The American journal of clinical nutrition. 2009;90(5):1236-43.
10. Xue L, He J, Gao N, Lu X, Li M, Wu X, et al. Probiotics may delay the progression of nonalcoholic fatty liver disease by restoring the gut microbiota structure and improving intestinal endotoxemia. Scientific reports. 2017;7:45176.
11. Thuy S, Ladurner R, Volynets V, Wagner S, Strahl S, Konigsrainer A, et al. Nonalcoholic fatty liver disease in humans is associated with increased plasma endotoxin and plasminogen activator inhibitor 1 concentrations and with fructose intake. The Journal of nutrition. 2008;138(8):1452-5.
12. Janssen AWF, Houben T, Katiraei S, Dijk W, Boutens L, van der Bolt N, et al. Modulation of the gut microbiota impacts nonalcoholic fatty liver disease: a potential role for bile acids. Journal of lipid research. 2017;58(7):1399-416.
13. Chen YM, Liu Y, Zhou RF, Chen XL, Wang C, Tan XY, et al. Associations of gut-flora-dependent metabolite trimethylamine-N-oxide, betaine and choline with non-alcoholic fatty liver disease in adults. Scientific reports. 2016;6:19076.
14. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. The New England journal of medicine. 2013;368(17):1575-84.
15. Loscalzo J. Gut microbiota, the genome, and diet in atherogenesis. The New England journal of medicine. 2013;368(17):1647-9.
16. Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. Hepatology (Baltimore, Md). 2013;57(2):601-9.

17. Betrapally NS, Gillevet PM, Bajaj JS. Changes in the Intestinal Microbiome and Alcoholic and Nonalcoholic Liver Diseases: Causes or Effects? Gastroenterology. 2016;150(8):1745-55.e3.
18. Loomba R, Seguritan V, Li W, Long T, Klitgord N, Bhatt A, et al. Gut Microbiome-Based Metagenomic Signature for Non-invasive Detection of Advanced Fibrosis in Human Nonalcoholic Fatty Liver Disease. Cell metabolism. 2017;25(5):1054-62.e5.
19. Kolida S, Gibson GR. Synbiotics in health and disease. Annual review of food science and technology. 2011;2:373-93.
20. Guha IN, Parkes J, Roderick P, Chattopadhyay D, Cross R, Harris S, et al. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: Validating the European Liver Fibrosis Panel and exploring simple markers. Hepatology (Baltimore, Md). 2008;47(2):455-60.
21. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology (Baltimore, Md). 2007;45(4):846-54.
22. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology (Baltimore, Md). 2005;41(6):1313-21.
23. Armstrong MJ, Houlihan DD, Bentham L, Shaw JC, Cramb R, Olliff S, et al. Presence and severity of non-alcoholic fatty liver disease in a large prospective primary care cohort. Journal of hepatology. 2012;56(1):234-40.
24. Betrapally NS, Gillevet PM, Bajaj JS. Gut microbiome and liver disease. Translational research : the journal of laboratory and clinical medicine. 2017;179:49-59.
25. Malaguarnera M, Vacante M, Antic T, Giordano M, Chisari G, Acquaviva R, et al. Bifidobacterium longum with fructo-oligosaccharides in patients with non alcoholic steatohepatitis. Digestive diseases and sciences. 2012;57(2):545-53.
26. Banerjee R, Pavlides M, Tunnicliffe EM, Piechnik SK, Sarania N, Philips R, et al. Multiparametric magnetic resonance for the non-invasive diagnosis of liver disease. Journal of hepatology. 2014;60(1):69-77.
27. Scorletti E, Bhatia L, McCormick KG, Clough GF, Nash K, Calder PC, et al. Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chainomega-3 fatty acid treatment in non-alcoholic fatty liver disease [corrected]. Contemporary clinical trials. 2014;37(2):301-11.
28. Scorletti E, Bhatia L, McCormick KG, Clough GF, Nash K, Hodson L, et al. Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. Hepatology (Baltimore, Md). 2014;60(4):1211-21.
29. Lomax AR, Calder PC. Prebiotics, immune function, infection and inflammation: a review of the evidence. The British journal of nutrition. 2009;101(5):633-58.
30. Team RDC. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. 2012;. ISBN 3-900051-07-0, URL http://www.R-project.org/. In.
31. McCormick KG, Scorletti E, Bhatia L, Calder PC, Griffin MJ, Clough GF, et al. Impact of high dose n-3 polyunsaturated fatty acid treatment on measures of microvascular function and vibration perception in non-alcoholic fatty liver disease: results from the randomised WELCOME trial. Diabetologia. 2015;58(8):1916-25.
32. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature biotechnology. 2013;31(9):814-21.
33. White IR, Horton NJ, Carpenter J, Pocock SJ. Strategy for intention to treat analysis in randomised trials with missing outcome data. BMJ (Clinical research ed). 2011;342:d40.

34. Gerstein HC, Mann JF, Yi Q, Zinman B, Dinneen SF, Hoogwerf B, et al. Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals. Jama. 2001;286(4):421-6.
35. Wachtell K, Olsen MH, Dahlof B, Devereux RB, Kjeldsen SE, Nieminen MS, et al. Microalbuminuria in hypertensive patients with electrocardiographic left ventricular hypertrophy: the LIFE study. Journal of hypertension. 2002;20(3):405-12.
36. Han TS, Lean ME. Self-reported waist circumference compared with the 'Waist Watcher' tape-measure to identify individuals at increased health risk through intra-abdominal fat accumulation. The British journal of nutrition. 1998;80(1):81-8.
37. Lean ME, Han TS, Morrison CE. Waist circumference as a measure for indicating need for weight management. BMJ (Clinical research ed). 1995;311(6998):158-61.
38. Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009;120(16):1640-5.
39. Saisho Y. Pancreas Volume and Fat Deposition in Diabetes and Normal Physiology: Consideration of the Interplay Between Endocrine and Exocrine Pancreas. The review of diabetic studies : RDS. 2016;13(2-3):132-47.
40. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the Gut Microbiome Using 16S or Shotgun Metagenomics. Frontiers in microbiology. 2016;7:459.
41. Bindels LB, Neyrinck AM, Claus SP, Le Roy CI, Grangette C, Pot B, et al. Synbiotic approach restores intestinal homeostasis and prolongs survival in leukaemic mice with cachexia. The ISME journal. 2016;10(6):1456-70.
42. Eren AM, Vineis JH, Morrison HG, Sogin ML. A filtering method to generate high quality short reads using illumina paired-end technology. PloS one. 2013;8(6):e66643.
43. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology. 2009;75(23):7537-41.
44. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature methods. 2013;10(10):996-8.
45. Biro G, Hulshof KF, Ovesen L, Amorim Cruz JA. Selection of methodology to assess food intake. European journal of clinical nutrition. 2002;56 Suppl 2:S25-32.
46. Mignault D, St-Onge M, Karelis AD, Allison DB, Rabasa-Lhoret R. Evaluation of the Portable HealthWear Armband: a device to measure total daily energy expenditure in free-living type 2 diabetic individuals. Diabetes care. 2005;28(1):225-7.
47. Baecke JA, Burema J, Frijters JE. A short questionnaire for the measurement of habitual physical activity in epidemiological studies. The American journal of clinical nutrition. 1982;36(5):936-42.
48. Friedrich-Rust M, Ong MF, Martens S, Sarrazin C, Bojunga J, Zeuzem S, et al. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. Gastroenterology. 2008;134(4):960-74.
49. Pavlides M, Banerjee R, Sellwood J, Kelly CJ, Robson MD, Booth JC, et al. Multiparametric magnetic resonance imaging predicts clinical outcomes in patients with chronic liver disease. Journal of hepatology. 2016;64(2):308-15.

50. Northrop CA, Lunn PG, Behrens RH. Automated enzymatic assays for the determination of intestinal permeability probes in urine. 1. Lactulose and lactose. Clinica chimica acta; international journal of clinical chemistry. 1990;187(2):79-87.
51. Lunn PG, Northrop-Clewes CA. Intestinal permeability: update on the enzymatic assay of mannitol. Clinica chimica acta; international journal of clinical chemistry. 1992;205(1-2):151-2.
52. Gregersen NT, Flint A, Bitz C, Blundell JE, Raben A, Astrup A. Reproducibility and power of ad libitum energy intake assessed by repeated single meals. The American journal of clinical nutrition. 2008;87(5):1277-81.
53. Flint A, Raben A, Blundell JE, Astrup A. Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2000;24(1):38-48.
54. Halford JC, Harrold JA. Satiety-enhancing products for appetite control: science and regulation of functional foods for weight management. The Proceedings of the Nutrition Society. 2012;71(2):350-62.
55. Brooks R. Quality of life measures. Critical care medicine. 1996;24(10):1769.
56. Marteau TM, Bekker H. The development of a six-item short-form of the state scale of the Spielberger State-Trait Anxiety Inventory (STAI). The British journal of clinical psychology. 1992;31 (Pt 3):301-6.
57. Newton JL. Systemic symptoms in non-alcoholic fatty liver disease. Digestive diseases (Basel, Switzerland). 2010;28(1):214-9.
58. Sayer AA, Syddall HE, Dennison EM, Martin HJ, Phillips DI, Cooper C, et al. Grip strength and the metabolic syndrome: findings from the Hertfordshire Cohort Study. QJM : monthly journal of the Association of Physicians. 2007;100(11):707-13.
59. Sayer AA, Dennison EM, Syddall HE, Gilbody HJ, Phillips DI, Cooper C. Type 2 diabetes, muscle strength, and impaired physical function: the tip of the iceberg? Diabetes care. 2005;28(10):2541-2.
60. Amato M, Montorsi P, Ravani A, Oldani E, Galli S, Ravagnani PM, et al. Carotid intima-media thickness by B-mode ultrasound as surrogate of coronary atherosclerosis: correlation with quantitative coronary angiography and coronary intravascular ultrasound findings. European heart journal. 2007;28(17):2094-101.
61. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. Circulation. 2007;115(4):459-67.
62. Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-Media Thickness Task Force. Endorsed by the Society for Vascular Medicine. Journal of the American Society of Echocardiography : official publication of the American Society of Echocardiography. 2008;21(2):93-111; quiz 89-90.
63. Wild SH, Byrne CD, Smith FB, Lee AJ, Fowkes FG. Low ankle-brachial pressure index predicts increased risk of cardiovascular disease independent of the metabolic syndrome and conventional cardiovascular risk factors in the Edinburgh Artery Study. Diabetes care. 2006;29(3):637-42.
64. Clough GF, L'Esperance V, Turzyniecka M, Walter L, Chipperfield AJ, Gamble J, et al. Functional dilator capacity is independently associated with insulin sensitivity and age in central obesity and is not improved by high dose statin treatment. Microcirculation (New York, NY : 1994). 2011;18(1):74-84.

65. Rhee EJ, Plutzky J. Retinoid metabolism and diabetes mellitus. Diabetes & metabolism journal. 2012;36(3):167-80.
66. Sauvant P, Cansell M, Atgie C. Vitamin A and lipid metabolism: relationship between hepatic stellate cells (HSCs) and adipocytes. Journal of physiology and biochemistry. 2011;67(3):487-96.
67. Zuniga S, Firrincieli D, Housset C, Chignard N. Vitamin D and the vitamin D receptor in liver pathophysiology. Clinics and research in hepatology and gastroenterology. 2011;35(4):295-302.
68. Hadi A, Mohammadi H, Miraghajani M, Ghaedi E. Efficacy of synbiotic supplementation in patients with nonalcoholic fatty liver disease: A systematic review and meta-analysis of clinical trials: Synbiotic supplementation and NAFLD. Critical reviews in food science and nutrition. 2018:1-12.
69. Mofidi F, Poustchi H, Yari Z, Nourinayyer B, Merat S, Sharafkhah M, et al. Synbiotic supplementation in lean patients with non-alcoholic fatty liver disease: a pilot, randomised, double-blind, placebo-controlled, clinical trial. The British journal of nutrition. 2017;117(5):662-8.
70. Eslamparast T, Poustchi H, Zamani F, Sharafkhah M, Malekzadeh R, Hekmatdoost A. Synbiotic supplementation in nonalcoholic fatty liver disease: a randomized, double-blind, placebo-controlled pilot study. The American journal of clinical nutrition. 2014;99(3):535-42.
71. Machann J, Thamer C, Schnoedt B, Stefan N, Haring HU, Claussen CD, et al. Hepatic lipid accumulation in healthy subjects: a comparative study using spectral fat-selective MRI and volume-localized 1H-MR spectroscopy. Magnetic resonance in medicine. 2006;55(4):913-7.
72. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science (New York, NY). 2005;308(5728):1635-8.
73. Horner-Devine MC, Carney KM, Bohannan BJ. An ecological perspective on bacterial biodiversity. Proceedings Biological sciences. 2004;271(1535):113-22.
74. Konturek PC, Haziri D, Brzozowski T, Hess T, Heyman S, Kwiecien S, et al. Emerging role of fecal microbiota therapy in the treatment of gastrointestinal and extra-gastrointestinal diseases. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 2015;66(4):483-91.
75. Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. Gut. 2016;65(2):330-9.
76. Simon MC, Strassburger K, Nowotny B, Kolb H, Nowotny P, Burkart V, et al. Intake of Lactobacillus reuteri improves incretin and insulin secretion in glucose-tolerant humans: a proof of concept. Diabetes care. 2015;38(10):1827-34.
77. Neuschwander-Tetri BA, Loomba R, Sanyal AJ, Lavine JE, Van Natta ML, Abdelmalek MF, et al. Farnesoid X nuclear receptor ligand obeticholic acid for non-cirrhotic, non-alcoholic steatohepatitis (FLINT): a multicentre, randomised, placebo-controlled trial. Lancet. 2015;385(9972):956-65.
78. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of lipid research. 2013;54(9):2325-40.
79. Walker AW, Duncan SH, McWilliam Leitch EC, Child MW, Flint HJ. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Applied and environmental microbiology. 2005;71(7):3692-700.
80. Ilan Y. Leaky gut and the liver: a role for bacterial translocation in nonalcoholic steatohepatitis. World journal of gastroenterology. 2012;18(21):2609-18.

81. Miele L, Valenza V, La Torre G, Montalto M, Cammarota G, Ricci R, et al. Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease. Hepatology (Baltimore, Md). 2009;49(6):1877-87.
82. Ley RE. Obesity and the human microbiome. Current opinion in gastroenterology. 2010;26(1):5-11.
83. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006;444(7122):1022-3.
84. Bruce KD, Cagampang FR, Argenton M, Zhang J, Ethirajan PL, Burdge GC, et al. Maternal high-fat feeding primes steatohepatitis in adult mice offspring, involving mitochondrial dysfunction and altered lipogenesis gene expression. Hepatology (Baltimore, Md). 2009;50(6):1796-808.
85. Szendroedi J, Chmelik M, Schmid AI, Nowotny P, Brehm A, Krssak M, et al. Abnormal hepatic energy homeostasis in type 2 diabetes. Hepatology (Baltimore, Md). 2009;50(4):1079-86.
86. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, et al. Prebiotic effects: metabolic and health benefits. The British journal of nutrition. 2010;104 Suppl 2:S1-63.
87. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature reviews Gastroenterology & hepatology. 2017;14(8):491-502.
88. FAO/WHO. Joint FAO/WHO working group report on drafting guidelines for the evaluation of probiotics in food. WHO. 2002;London, Ontario, Can.
89. Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a more comprehensive concept for prebiotics. Nature reviews Gastroenterology & hepatology. 2015;12(5):303-10.
90. Qing L, Wang T. Lactic acid bacteria prevent alcohol-induced steatohepatitis in rats by acting on the pathways of alcohol metabolism. Clinical and experimental medicine. 2008;8(4):187-91.
91. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50(11):2374-83.
92. Byrne CD, Targher G. NAFLD: a multisystem disease. Journal of hepatology. 2015;62(1 Suppl):S47-64.
93. Scorletti EB, C.D. Extrahepatic diseases and NAFLD: The triangular relationship between NAFLD, type 2-diabetes and dysbiosis Dig Dis. 2016;34 Suppl 1:11-8.
94. Targher G, Lonardo A, Byrne CD. Nonalcoholic fatty liver disease and chronic vascular complications of diabetes mellitus. Nature reviews Endocrinology. 2018;14(2):99-114.
95. Everard A, Lazarevic V, Derrien M, Girard M, Muccioli GG, Neyrinck AM, et al. Responses of gut microbiota and glucose and lipid metabolism to prebiotics in genetic obese and diet-induced leptin-resistant mice. Diabetes. 2011;60(11):2775-86.
96. Delzenne NM, Neyrinck AM, Cani PD. Modulation of the gut microbiota by nutrients with prebiotic properties: consequences for host health in the context of obesity and metabolic syndrome. Microbial cell factories. 2011;10 Suppl 1:S10.