Investigation of synbiotic treatment in non-alcoholic fatty liver disease (INSYTE study): a double-blind, randomised, placebo-controlled, phase 2 trial

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Abstract

Background: Recent evidence suggests that dysbiosis may play a role in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and treatment with a synbiotic (a combined probiotic and prebiotic) may be beneficial. Our aim was to investigate the effect of synbiotic treatment on: a) liver fat content; b) liver fibrosis-biomarker scores and c) gut microbiota composition in NAFLD patients.

Methods: 104 patients with NAFLD were randomised in a double-blind, placebo-controlled phase 2 trial, to a ~12-month intervention with either synbiotic (fructo-oligosaccharides (4 g/twice day) + *Bifidobacterium animalis* subsp. *lactis* BB-12) or placebo. Liver fat content was measured by magnetic resonance spectroscopy (MRS), liver fibrosis by validated biomarker scores and vibration-controlled transient elastography, and faecal microbiota by faecal 16S rDNA sequencing.

Results: Mean(SD) age was 50.8(12.6) years and 65% were men. 37% had diabetes**.** Mean(SD) baseline and end-of-study MRS liver fat percentage was 32.3%(24.8) and 28.5%(20.1) (synbiotic-group) and 31.3%(22) and 25.2%(17.2) (placebo-group). In the unadjusted intention to treat (ITT) analysis, there was no significant difference in liver fat reduction between groups (β=2.8; 95%CI: -2.2, 7.8; p=0.3). In a fully adjusted regression model (adjusted for baseline measurement of the outcome *plus* age, sex, weight difference, baseline weight), only weight loss was associated with a significant decrease in liver fat (β=2; 95%CI: 1.5, 2.6; p=0.03). Synbiotic treatment fostered growth of *Bifidobacterium* and *Faecalibacterium* at the expense of *Oscillibacter* and *Alistipes*. However, change in gut microbiota composition was not associated with any primary outcome.

Conclusions: Synbiotic treatment was effective in changing gut microbiota but this pro- and prebiotic combination was ineffective in improving liver fat content or liver fibrosis markers in NAFLD.

The INSYTE is registered at www.clinicaltrials.gov as NCT01680640.

Keywords: NAFLD: Non-alcoholic fatty liver disease; Gut microbiota; Nutrition; Synbiotic; Cardiovascular disease; Type 2 diabetes.

1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a pathologic condition defined by the deposition of triglyceride (TG) in the liver greater than 5% of the total liver weight [1-3]. The term NAFLD encompasses a spectrum of pathologic conditions where the first stage is characterized by simple steatosis with liver fat accumulation in the hepatocytes [3, 4]. The second stage is non-alcoholic steatohepatitis (NASH) characterized by hepatocyte injury due to inflammation, ballooning and possible collagen deposition. NASH is a progressive form of fatty liver that can worsen over time and may lead to cirrhosis and liver failure [2].

NAFLD has become one of the most common causes of chronic liver disease and liver related mortality worldwide, and is now becoming a major reason for liver transplantation [5]. Approximately 10-20% of people with NAFLD progress to NASH [6]. NAFLD is also an independent risk factor for extra-hepatic diseases, such as type 2 diabetes and cardiovascular disease [7, 8]. There is recent growing interest in the role of gut microbiota in NAFLD pathogenesis, and there are several metaorganismal pathways linking altered gut microbiota (termed dysbiosis) and NAFLD [9-12]. Recent literature shows that the faecal microbial composition of patients with NAFLD differs from that of healthy individuals. Some studies showed a preponderance of Gram-*negative* bacteria, such as *Proteobacteria*, *Enterobacteriaceae* and *Escherichia* [13, 14]. Other studies have described a gut metagenomic signature in NAFLD patients with fibrosis of an increased abundance in *Ruminococcus* associated with the worsening of liver fibrosis and/or a more complex signature, involving 37 bacterial species in a model that distinguished mild/moderate NAFLD from advanced fibrosis [15, 16]. However, there is currently a lack of consistency in these findings due to the marked variance in the population studied, with differing ages, diets and geographical locations [17]. Nonetheless, despite these inconsistencies, there is the possibility that manipulation of the gut

microbiota to a more favourable profile, could provide a beneficial effect on liver disease in patients with NAFLD.

Treatments that can change the gut microbiota include, but are not limited to, probiotics, prebiotics and synbiotics. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [18-21]. A synbiotic is a mixture of one or more probiotics and one or more prebiotics that beneficially affect the host by promoting the survival and colonisation of the live microbes in the gastrointestinal tract [22]. Small randomized controlled trials (RCT), mostly of short duration (up to 6 months) and using ultrasonography, or surrogate markers of NAFLD, have suggested that administration of prebiotics and/or probiotics may reduce hepatic steatosis, hepatic inflammation, and insulin resistance in patients with NAFLD [9-12, 23]. However, these studies did not test the effect of the pro-, pre- or synbiotic treatments on gut microbiota to show that modification of gut microbiota benefits the liver in patients with NAFLD. In addition, no studies have reported the effect of a synbiotic treatment on both microbiota and liver fat accumulation in NAFLD.

In a proof of concept, phase-2 RCT, we tested the hypothesis that ~12 months of synbiotic treatment was effective in: 1) ameliorating liver fat content; 2) improving two histologically validated liver fibrosis scores and 3) changing the gut microbiota in patients with NAFLD. In addition, we tested the effect of the synbiotic on liver stiffness measurement assessed by vibration-controlled transient elastography (VCTE).

2 METHODS

2.1 *Study design*

The INSYTE study (**In**vestigation of **sy**nbiotic **t**reatm**e**nt in NAFLD; www.clinicaltrials.gov registration number NCT01680640) was approved by the Southampton and South West Hampshire Local Research Ethics Committee (REC: 12/SC/0614). The protocol, study design and methods for the trial have been reported previously [24]. 104 participants with proven NAFLD were randomised to the intervention or placebo **(Figure 1)** for a minimum of 10 months and a maximum of 14 months [24]. This period of intervention was chosen to allow for the potential of a maximum of two prescribed courses of antibiotics. There was a further requirement that any antibiotic therapy had ceased a minimum of one month prior to end of study tests, including collection of stool sample.

Fifty-five participants were randomised to receive synbiotic treatment consisting of fructo-oligosaccharides 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) and forty-nine participants were randomised to receive placebo consisting of 4 g/twice a day of maltodextrin (1 capsule a day plus two sachets a day, to stir into a cold drink). Participants were stratified by age (<50 and ≥50 years) and sex. Block randomisation in groups of four was used to ensure a balance between the treatment groups. Synbiotic and placebo were provided free of charge by Christian Hansen A/S, Boege Alle 10-12, 2970 Hoersholm, Denmark. The prebiotic component of the synbiotic was Actilight®950P. The conduct of the trial, data analyses and writing of the manuscript were all undertaken by the authors and were completely independent from Chr. Hansen.

2.2 *Liver fat measurement*

Measurement of liver fat content was undertaken at baseline and at the end of the study using proton magnetic resonance spectroscopy (MRS). Three 20×20×20 mm^3 spectroscopic voxels were 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 voxel positions were copied from the first scan, to ensure measurements at the end of the study were taken in the same regions of the liver, as at baseline. Values for the lipid and water peak integrals were produced for each voxel and recorded for each participant [24]. For each participant to test the effect of the intervention on liver fat percentage we calculated the mean of the three spectroscopic voxels for liver fat percentage at baseline and end of study.

2.3 *Faecal microbiota analysis*

Faecal genomic DNA was extracted using the QIAamp DNA Stool Mini Kit, including a bead-beating step. Total bacteria, *Bifidobacterium* spp. and *Bifidobacterium animalis* levels were quantified using qPCR as previously described [25]. The samples were PCR-enriched for the V5-V6 region of the 16S rRNA gene and then underwent a library tailing PCR as described in Pötgens et al. [26]. The amplicons were purified, quantified and sequenced using an Illumina MiSeq to produce 2x300 bp sequencing products. Initial quality-filtering of the reads was conducted with the Illumina Software, yielding an average of 169,858 pass-filter reads per sample. Quality scores were visualized and reads were trimmed to 220 bp (R1) and 200 bp (R2). The reads were merged with the merge-Illumina-pairs application [27]. For all samples but two, a subset of 26,000 reads was randomly selected using Mothur 1.32.1 [28]. The UPARSE pipeline implemented in USEARCH v10.0.240 [29] was used to further process the sequences.

Putative chimaeras were removed using the *cluster_otus* command. Non-chimeric sequences were subjected to taxonomic classification using the RDP MultiClassifier 1.1 from the Ribosomal Database Project [30]. The phylotypes were computed as percent proportions based on the total number of sequences in each sample. Raw sequences can be found in the SRA database (project ID: PRJNA559052).

2.4 *Statistical analysis*

The sample size for the INSYTE was based on testing the effect of the synbiotic treatment on changes in MRS liver fat content as a one of three key primary outcomes for the trial [See INSYTE Protocol paper [24]]. Statistical analyses were performed using SPSS for Windows, Stata v14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP.) and R [31]. For each primary outcome (MRS liver fat percentage, ELF score and NAFLD fibrosis score), a change variable was calculated as the difference between end-of-study and baseline measurements. For continuous variables, multiple regression analysis was used to assess the effect of synbiotic treatment on each of the change variables of interest. Multivariable (adjusted) linear regression model testing the effects of synbiotic treatment (primary analyses) and changes in *Bifidobacterium* levels between baseline and end of study were undertaken. Models were adjusted for baseline measurement of the outcome variable only or adjusted for baseline measurement of the outcome *plus* age, sex, weight difference and baseline weight.

The ITT analysis included all patients randomized who had baseline and end-of-study measurements, regardless of whether they were later found to be ineligible, a protocol violator, given the wrong treatment allocation, or never treated. Multiple imputation (MI) was used to handle any missing data. As a secondary analysis, the above was repeated with change levels

of *Bifidobacterium* genus at the end of the study. A *P*-value of <0.05 was considered statistically significant [24].

In addition, for quantifying the changes in gut microbiota following intervention, we compared taxonomic and operational taxonomic unit (OTU) profiles between treatment and placebo arms. Statistical tests for changes in abundance over time and differences in changes of abundance over time were performed for each taxonomic unit, OTU and alpha-diversity indexes, by using the Wilcoxon paired rank-sum test and the Mann-Whitney U test, with a Benjamini-Hochberg correction for multiple testing (with a false discovery rate [FDR] cut-off of 0.1). Beta-diversity indexes were first visualized through a Principal Coordinates Analysis (PCoA) using Emperor [32] to explore the dissimilarity between microbial communities. Beta-diversity values for each pair of samples were compared between treatment and placebo arms using Mann-Whitney U tests. Partial Spearman rank-based correlations were computed using the *pcor.test* function (http://www.yilab.gatech.edu/pcor.html).

3 RESULTS

Eighty-nine participants out of 104 (86%) completed the RCT (58 men and 31 women) **(**see **Table S1** for the reasons for participant withdrawal**)**. **Table 1** shows the baseline clinical characteristics for the whole cohort and a comparison between the two treatment groups. At baseline, there was no significant difference in the main clinical and biochemical parameters, liver profile and *Bifidobacterium* abundance between the synbiotic and placebo groups. Abundance of *Bifidobacterium* (a component of the synbiotic) was reported as a marker of good compliance during the study. **Table 2** shows the changes between baseline and end-of-trial measurements, stratified by randomization group, for the main anthropometric and biochemical variables. Baseline intestinal permeability measurement (lactulose/mannitol ratio) in the INSYTE cohort was: median = 0.11, interquartile range (IQR)=0.25; mean ± SD 0.27 ± 0.4 (Table 2). Since the INSYTE study was not designed with a healthy control group, we compared the INSYTE baseline lactulose/mannitol ratio with that for healthy subjects described in the literature. Notably, the mean and median values of lactulose/mannitol ratio were ~10-fold greater than those reported for healthy subjects in the literature [33-36], supporting the presence of altered intestinal permeability. That said, there were no significant changes in intestinal permeability between baseline and end-of-study measurements for the lactulose mannitol ratio in either the synbiotic or placebo groups. Interestingly, there was an overall reduction in MRS-measured liver fat content in 65% of the total 89 patients who completed the trial. With regard to LPS, there was no difference between baseline and end of study in either placebo (p=0.80) or treatment group (p=0.40) suggesting that synbiotic treatment did not affect circulating levels of LPS. At the end of the study, there was no significant association between changes in MRS-measured liver fat and changes in LPS levels (p=0.80). We tested the effect of synbiotic treatment on faecal SCFAs using a regression model with SCFAs difference, acetic acid difference, propionic acid difference or butyric acid difference as separate outcomes, and

adjusting for sex, age, treatment group and the baseline SCFA measure as exposures in the model. These data showed that there was no association between synbiotic treatment and change in SCFA concentrations (total SCFAs p=0.21; acetic acid p=0.23; propionic acid p=0.24; butyric acid p=0.40). With regard to MRS liver fat percentage, we undertook regression modelling using MRS difference as the outcome, adjusting the model for sex, age, weight difference, weight at baseline, liver fat content at baseline and SCFAs. There was no association between change in SCFAs and change in MRS liver fat percentage: SCFAs p=0.63; acetic acid p=0.62; propionic acid p=0.78; butyric acid p=0.73.

The results from the FFQs showed that there was an overall decrease in total calories, carbohydrates, protein and fat from visit 1 to visit 2 (after 6 months in the study). However, there was no evidence of a significant treatment effect as there was no difference between the two randomisation groups **(Table S2 and S3)**. With regard to physical activity, there was also no significant difference at the end of the study in either placebo or treatment group **(Table 2).**

3.1 Intention to Treat (ITT) Analysis

Table 3 shows baseline and end-of-study data for each of the primary outcomes and the results of multivariable linear regression models for the ITT analyses. In the synbiotic group, there was only a 4% MRS liver fat reduction (p=0.08) and a significant increase in *Bifidobacterium* abundance (p<0.001). Therefore, there was no significant effect of the synbiotic treatment on MRS liver fat content. In the placebo group, although there was no change in *Bifidobacterium* abundance at the end of the study (p=0.5), there was a significant 6% reduction of MRS liver fat (p=0.01). In the unadjusted ITT analyses, there was no difference between the two groups in changes in MRS liver fat (β=2.8; 95%CI: -2.2, 7.8; p=0.3. In the fully adjusted regression model (adjusted for baseline measurement of the outcome plus

age, sex, weight difference and baseline weight), there was a borderline significant difference in MRS-measured liver fat (β=4.1; 95%CI: -0.02, 8.3; p=0.05) between placebo and synbiotic groups **(Table 3)** (N.B. However, this analysis showed a borderline significantly greater reduction in liver fat in the placebo group than in the synbiotic group). In this multivariable regression model, weight loss was the only factor that was associated with a significant reduction in MRS liver fat at the end of the study with a 2% reduction of liver fat associated with every kg of weight loss (β=2.0; 95%CI: 1.5, 2.6; p=0.03) **(Table 3)**. We then performed regression modelling with MRS liver fat difference as the outcome and weight difference as an explanatory variable. These data showed that weight loss was strongly associated with a significant reduction liver fat (β coefficient=1.9; 95%CI 1.4, 2.5; p<0.001).

With regard to the two algorithmically derived fibrosis scores (i.e. the ELF and NAFLD fibrosis scores), we undertook the same regression models we used for the MRS liver fat adjusting for baseline measurement of the outcome *plus* age, sex, weight difference, and baseline weight. We did not find any significant changes in either liver fibrosis scores with synbiotic treatment (**Table 3**). We also tested the effect of the synbiotic intervention of liver stiffness measurement (kPa) (assessed by VCTE). In a regression model that included change in liver stiffness measurement between baseline and end of study as the outcome, and that adjusted for baseline measurement of the outcome *plus* age, sex, weight difference, and baseline weight in the model, synbiotic was not associated with change in liver stiffness measurement (B coefficient = -0.78 (95%CI -1.93, 0.37), p=0.18). Therefore, taken together, these data show that it is unlikely that the synbiotic influenced liver fat or liver fibrosis as components of NASH pathology. Weight loss was associated with significant improvements in ELF (p=0.039) and NAFLD fibrosis score (p=0.027) (**Table 3**), and also liver stiffness measurement (p=0.025). Interestingly, within the synbiotic group, a negative association was found between the changes in *Bifidobacterium* spp. abundance and the changes in MRS fat

liver content (Spearman's *rho* = -0.36, p= 0.017). However, this association was no longer significant after controlling for body weight change, age and sex (Spearman's *rho* = -0.10, p=0.51). We also tested the association between changes in *Bifidobacterium* spp. abundance at the end of the study and each of the primary outcomes (adjustments as per the ITT analysis). Notably, we did not find any significant associations between changes in *Bifidobacterium* spp. abundance at the end of the study and MRS liver fat or the two algorithmically derived fibrosis scores. Moreover, there was no association between weight loss and synbiotic treatment or *Bifidobacterium* spp. abundance at the end of the study. In a multivariable regression model using *Bifidobacterium* spp. abundance difference (end of study minus baseline) as the outcome and adjusting the model for treatment allocation, age, sex and weight difference at the end of the study, interestingly, weight difference, age and sex were not significantly associated with *Bifidobacterium* spp. abundance.

3.2 Faecal microbiota analysis

At baseline, faecal microbiome was not significantly different between the two groups of participants **(Table S4, Table S5, Figure S1)** and was characterised by a positive association between levels of *Sphingomonas* and *Sutterella* and liver fibrosis scores, whereas there was a negative association between *Olsenella* and MRS-measured liver fat and between *Clostridia* and *Firmicutes* and liver fibrosis scores **(Table 4)**. Total bacteria levels were not significantly affected by the intervention (**Figure S2**). The synbiotic treatment was associated with a reduced within-sample microbial diversity (α-diversity) at the richness level, but not at the evenness or evenness and richness levels (**Table S4**), likely signalling the disappearance of taxa of low abundance. Dissimilarities between microbial communities were visualised through a Principal Coordinates Analysis (PCoA) of the β-diversity index Morisita-Horn (**Figure 2A**). The observed distribution of β-diversity was mainly due to inter-personal variability (explaining 86% of the total variation, ANOSIM, 1000 permutations, p=0.001). To evaluate whether the

synbiotic treatment had a greater effect upon the microbiota structure over time compared to the placebo treatment, we compared the distance between each pair of samples (baseline and end of study, computed through a β-diversity index) between the two treatment arms (**Figure 2B**). The synbiotic group displayed higher β-diversity values than the placebo group, indicating that the synbiotic treatment had a greater effect on gut microbiota structure than placebo. Similar results were obtained when looking at other β-diversity indexes, such as Binary-Jaccard and Bray-Curtis (**Figure 2B**). In accordance with these observations, compared to the placebo, the synbiotic treatment fostered the abundance of *Bifidobacterium* and *Faecalibacterium* at the expense of *Oscillibacter* and *Alistipes* (**Figure 3**, **Table S6**), with parent taxa showing the same trends (**Table S6**). No other significant changes were found either at the phylum level (**Figure S3**) or at any other taxonomic level (**Table S7**, **Figure S4**). In particular, bacteria classically linked to inflammation, obesity and NAFLD were not affected (**Figure S5**). Surprisingly, only *Oscillibacter* was negatively associated with MRS-measured liver fat after adjusting the model as per the ITT analysis (β= -2.2; 95%CI: -4.3, -0.1; p=0.04). The 2.2-fold increase in *Bifidobacterium* spp. was also confirmed by qPCR (**Figure S2**).

4 DISCUSSION

Our study is the first RCT to test the efficacy of a synbiotic treatment for ~12 months in NAFLD on a quantitative measurement of liver fat content, two algorithmically derived fibrosis scores in NAFLD (the ELF and NAFLD fibrosis scores), liver stiffness measurement, and changes in gut microbiota. At the end of the trial, we did not find any significant difference between synbiotic and placebo groups for MRS liver fat content, the two algorithmically derived fibrosis scores and the liver stiffness measurement. We found an overall reduction in MRS liver fat in 65% of participants independently associated only with weight loss. Although our RCT was not specifically designed to test the effect of weight loss on our primary outcomes, all participants were given healthy lifestyle advice as part of their usual care. There was only a mean weight loss of 1 kg in and yet this very small amount of weight loss was independently associated with a decrease in MRS-measured liver fat. Interestingly, from the unstandardized β coefficients, 1 kg of weight loss was associated with a 2% decrease in percentage of liver fat and weight loss was also associated with an improvement in the ELF and NAFLD fibrosis scores and liver stiffness measurement. In our proof of concept RCT, the fact that most of the patients had an improvement in MRS-measured liver fat, regardless of treatment allocation, is consistent with the so-called "clinical trial effect" [37], whereby participants benefit from participating in clinical trials.

We were successful in showing a change in gut microbiota in the synbiotic group, and we significantly changed the diversity of the gut microbiota after synbiotic treatment. This increased diversity was reflected by an increased abundance of *Bifidobacterium* and *Faecalibacterium* and a reduction of *Oscillibacter* and *Alistipes. Bifidobacterium* spp. that are known to have some beneficial effects on lipid profile and gut barrier [38-40]. However, we did not find any association between *Bifidobacterium* spp. levels at the end of the study and change in MRS-measured liver fat (after adjusting for weight loss), or on intestinal permeability, after

adjusting for age, sex and baseline measurements for intestinal permeability (the lactulose/mannitol ratio). Levels of the anti-inflammatory bacterium *Faecalibacterium prausnitzii* have been reported to be decreased in NAFLD, independently of obesity and insulin resistance, and to be increased upon consumption of inulin-type fructan prebiotics [41-44]. *Faecalibacterium prausnitzii* has also been shown to be negatively associated with markers of lymphocytes and Kupffer cells in the portal tract [45]. In contrast, *Alistipes* spp. has been shown to be positively associated with hepatic inflammatory and oxidative stress markers in mice [46, 47]. Therefore, we may speculate that the increase in *Faecalibacterium prausnitzii* and the decrease in *Alistipes* associated with the synbiotic treatment might confer beneficial effects in terms of inflammation, an effect not investigated in this study. No other significant changes were found either at the phylum level (Figure S3) or at any other taxonomic level (Table S7, Figure S4). In particular, bacteria classically linked to inflammation, obesity and NAFLD were not affected (Figure S5).

A recent study by Raman et al. characterized the faecal microbiota of obese patients with NAFLD and compared the faecal microbiota with that of healthy controls [48]. In this study these authors showed that *Oscillibacter* was significantly lower in NAFLD patients than in healthy controls [48].

Because the INSYTE study was not designed with a healthy control cohort, we investigated the presence of gut microbiota dysbiosis in the INSYTE population by comparing the baseline microbiota composition at the phylum level with two previously published cohorts: Twins-UK and Food4Gut [49, 50]. As shown in Table S8, the INSYTE population presents a nearly 2-fold higher level of *Proteobacteria* compared to the two other cohorts (the first one with a similar geographical origin, the second one with identical analyses). Although we acknowledge that these two control cohorts were not assessed contemporaneously to the INSYTE study cohort, this comparison suggests that the baseline gut microbiota of the INSYTE cohort was different

from the gut microbiota found in a general UK population as *Proteobacteria* is one of the bacterial taxa presented in some studies as a signature of NAFLD [14].

The synbiotic composition selected for the INSYTE study was chosen to maximize the effects of the probiotic. The probiotic (*Bifidobacterium animalis* subsp. *lactis* BB-12) was chosen based on specific beneficial effects on the host and for its ability to colonize the human intestine [51] and based on its previously reported specific beneficial effects on impaired glucose tolerance, plasma lipid profile and gut barrier function [38, 40]. The prebiotic (fructo-oligosaccharide with a degree of polymerization <10) was chosen to specifically stimulate growth and activity of Bifidobacteria.

The prebiotic was selected to have a higher affinity compared to inulin for the probiotic and was chosen to improve its survival and growth in the host [22, 24, 52, 53]. Moreover, using a validated *in vitro* faecal bacterial culture system designed to mimic the conditions within the human colon, we have additionally investigated the effects of *Bifidobacterium lactis* BB-12 and fructo-oligosaccaride (contained within the INSYTE synbiotic) on human gut microbiota *ex vivo* [54]. These data showed that with either the synbiotic or the prebiotic alone, there was a significant increase in total bacteria and in total bifidobacteria number, compared to either the probiotic alone or the negative control. Furthermore, the synbiotic supplement was well tolerated. During the trial, only one patient experienced some minor gastro-intestinal distress symptoms (bloating and flatulence). We advised that participant to divide the dose of supplement during the day and the participant was able to complete the trial without further gastro-intestinal distress.

It is important to note that previous studies in NAFLD that have tested the effects of synbiotics have also included lifestyle interventions (or medication) with the synbiotic treatment [11, 12, 23, 55-58]. In contrast, our RCT was primarily designed to test the effect of synbiotic

alone, and not lifestyle intervention which is known to be effective in decreasing liver fat in NAFLD. Importantly, in previous studies, investigators have not adjusted their analyses for key potential confounding factors, such as changes in physical activity or weight loss. Moreover, in most of these studies, the power to detect differences with the study sample sizes has not been mentioned. With inadequate sample sizes there is high probability of type 1 statistical errors, with a high potential for different types of bias, or confounding, influencing the results [11, 12, 23, 55-59]. Previous studies that have tested the effects of synbiotic treatment in NAFLD [9-12] have also used a combination of multiple strains of probiotics as a component of the synbiotic treatment. Therefore, it might be possible that, because the intestine harbours trillions of bacteria, adding one single type of bacterium in a synbiotic may not be as effective as adding three or six different types of bacteria [10-12, 55, 60, 61] with the potential to influence many more bacterial species. There are some studies that have tested the effect of a single bacterium [*Bifidobacterium longum* [9] and *L. reuteri* [62]] in patients with NAFLD; however, these studies also included lifestyle modifications in addition to the synbiotic treatment. An additional parameter to be considered is the fact that patient responsiveness to microbiota-targeting intervention may be highly individualized [63, 64]. Our RCT was not designed to stratify patients based either on baseline gut microbiome or microbiome changes overtime, and it is therefore not possible to determine whether a subset of patients did respond more favorably.

There are strengths and limitations of our study that should be considered. The strengths of our study were: the rigorous design of the placebo-controlled trial; the long duration of the trial (~12 months); the detailed phenotyping of participants; the adjustment for potential confounders; and the fact that we have also evaluated the effect of the intervention on changes in gut microbiota using an *ex vivo* system designed to mimic the human colon. Our RCT also had some limitations. Firstly, we choose one strain of bacteria for inclusion in the synbiotic. The prebiotic was selected based on its effect on lipid metabolism [22, 52, 65, 66]. The probiotic

Bifidobacterium animalis subspecies lactis (BB12) was selected based on its ability to colonize the intestine [51] and its beneficial properties on the gut barrier [39, 40]. With dysbiosis, there is an increased production of endotoxins from Gram-*negative* bacteria that can damage the intestinal barrier with the potential for toxic metabolites to enter the portal circulation ultimately damaging the liver [67]. We reasoned that by improving the gut barrier using this *Bifidobacterium* as a probiotic, we could reduce the toxic effect of the gut microbiota and therefore improve liver fat. Secondly, the INSYTE study was designed as a phase 2 clinical trial to test the effect of the intervention on liver fat content using MRS and not liver biopsy. NAFLD is known to be a "patchy" disease and liver biopsy is invasive, expensive, and subject to sampling variability. Consequently, we did not include the requirement for liver biopsy in the design of this proof of concept RCT. MRS is currently considered the non-invasive, gold-standard technique for accurately measuring changes in liver fat percentage and has excellent reproducibility and sensitivity. Additionally, MRS is a very sensitive technique for measuring liver fat and liver fat signals of only 0.2% above the background noise level can be measured [68]. Furthermore, because synbiotics are safe, inexpensive nutritional supplements that are widely available, we also reasoned that if synbiotics have a place in the treatment of NAFLD, that place would be early in the course of the disease process, targeted at decreasing liver fat content. Therefore, our intention was to test the effect of the synbiotic intervention on liver fat content, rather than to assess the histological resolution of NASH, improvements in NAFLD activity score, or improvements in liver fibrosis, as required in phase 3 RCTs testing novel medications that would be potentially licensed as pharmaceutical agents for the treatment of NASH. Finally, we deliberately did not include a lifestyle intervention because *a priori*, our intention was to test the effect of the synbiotic intervention alone.

In conclusion, the results of our double-blinded, randomised, placebo-controlled phase 2 trial trial show that synbiotic treatment (consisting of fructo-oligosaccharides 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) for a period of ~12 months was effective in changing the gut microbiota in patients with NAFLD. However, this treatment was ineffective in decreasing MRS-measured liver fat content, or in improving two validated biomarker scores for liver fibrosis, or liver stiffness measurement, compared with placebo. Interestingly despite there only being a mean weight loss of 1 kg in both the synbiotic and placebo groups, weight loss of 1 kg was significantly associated with an improvement of 2% in MRS-measured liver fat content, and with improvement in the two algorithmically-derived fibrosis scores [69, 70] and liver stiffness measurement. Thus, our RCT suggests that changing the gut microbiota with this synbiotic may occur without clinically significant effects on the liver in NAFLD. The changes in specific microbes known to be involved with inflammation that we observed in the study, support a need to evaluate the effect of synbiotic treatment on inflammatory/immune-related parameters in NAFLD.

Post Script

Sadly, our friend and respected colleague, Professor Valerio Nobili died suddenly and unexpectedly after completion of this manuscript. We honour Valerio's contribution as an author of this work and as a highly respected clinician scientist in NAFLD research.

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Table 1. Baseline characteristics of study participants

	Total (n=89)	Placebo (n=44)	Synbiotic (n=45)
Age	50.8 (12.6)	51.6 (13.1)	50.2 (12.4)
Sex (M/F)%	65/35	61/39	69/31
Physical activity (METs)	1.2 (0.3)	1.26 (0.3)	1.16 (0.3)
Diabetes/No Diabetes (%)	37/63	36/64	38/62
HbA1c (mmol/mol)	40 (22.5)	39 (24.5)	41 (21.5)
Height (cm)	172.3 (0.2)	170.8 (10.3)	173.8 (10)
Weight (kg)	98.0 (16.5)	96.8 (17.1)	99.2 (16.1)
BMI (kg/m^2)	33.1 (5.2)	33.2 (4.9)	32.9 (5.5)
DEXA body fat %*	34.0 (11.2)	34.5 (11.0)	32.9 (12.1)
Systolic blood pressure (mmHg)	133.6 (16.1)	134.3 (18.9)	132.9 (12.9)
Diastolic blood pressure (mmHg)	74.1 (9.1)	72.5 (9.8)	75.7(8.2)
Fasting glucose (mmol/L)*	6.1 (2.3)	6.1 (2.2)	6.2 (2.5)
Fasting insulin (µU/mL)*	14.2 (9.0)	14.9 (9.5)	13.5 (7.9)
Total cholesterol (mmol/L)*	4.8 (1.4)	4.8 (1.2)	4.9 (1.2)
HDL-cholesterol (mmol/L)*	1.2 (0.34)	1.2 (0.4)	1.2 (0.2)
LDL-cholesterol (mmol/L)	2.7 (0.9)	2.2 (1.0)	2.8 (0.8)
Total cholesterol/HDL-cholesterol ratio	4.1 (0.8)	4.0 (0.8)	4.1 (0.8)
Triglycerides (mmol/L)*	1.8 (0.9)	1.7 (0.8)	1.8 (1.1)
ALT (U/L)*	58.0 (42.0)	61.7 (30.9)	59.0 (39.3)
AST (U/L)*	35.0 (23.5)	40.5 (29.3)	34.0 (18.0)
GGT (U/L)*	55.0 (53.5)	56.0 (56.0)	51.0 (48.0)
HA (ug/L)*	35.0 (40.0)	38 (28)	31 (47)
PIIINP (ug/L)*	8.3 (3.7)	8.2 (3.2)	8.3 (3.4)
TIMP-1 (ug/L)	158.6 (28.6)	160.2 (28.9)	155.5 (35.5)
MRS-measured liver fat (%)	25 (26.6)	22.9 (12.9, 44.7)	26.9 (9.7, 38.4)
†ELF score	6.8 (0.3)	6.9 (0.3)	6.9 (0.4)
NAFLD fibrosis score	-1.15 (1.3)	-1.2 (1.3)	-1.3 (1.3)
Fibroscan (kPa)	7.5 (3.2)	8.4 (3.5)	6.7 (2.7)
Fibroscan CAP score (dB/m)*	306 (60)	312 (84)	312 (65)
Lactulose (%)*	0.62 (1.4)	0.60 (1.2)	0.66 (1.7)
Mannitol (%)*	6.2 (3.3)	6.0 (4.1)	6.47 (2.7)
Lactulose-mannitol ratio	0.12 (0.3)	0.11 (0.2)	0.11 (0.3)
LPS (EU/mL)*	0.13 (0.1)	0.13 (0.1)	0.13 (0.1)
Bifidobacterium* spp. (%)	0.78 (1.85)	0.63 (1.89)	0.88 (1.75)
Acetic Acid (umol/g)*	146.2 (187.7)	155.68 (193.3)	145.99 (194.6)
Propionic Acid (umol/g)*	8.82 (6.1)	8.38 (5.1)	9.47 (6.4)
Butyric Acid (umol/g)*	6.53 (5.2)	6.22 (5.7)	6.97 (6.1)

Variables that are normally distributed are expressed as mean (SD).
*Variables that are non-normally distributed are expressed as median (IQR).
Acetic acid, propionic acid and butyric acid concentrations expression per gram of faeces.

Table 2. Anthropometric and biochemical characteristics at baseline and end of study according to randomization group

Parameters	Placebo		Synbiotic	
	Baseline	End of Study	Baseline	End of Study
Weight (kg)	96.8 (17.1)	96.8 (17.9)	99.2 (16.1)	98.4 (17.4)
Physical activity (METs)	1.26 (0.3)	1.19 (0.2)	1.16 (0.3)	1.2 (0.3)
BMI (kg/m^2)	33.2 (4.9)	33.1 (5.1)	32.9 (5.5)	32.6 (5.6)
DEXA body fat %*	34.5 (11.0)	33.9 (10.7)	32.9 (12.1)	31.9 (10.9)
Systolic blood pressure (mmHg)	134.3 (18.9)	132.6 (15.2)	132.9 (12.9)	131.4 (12.2)
Diastolic blood pressure (mmHg)	72.5 (9.8)	73 (9.2)	75.7 (8.2)	75.4 (7.7)
Fasting glucose (mmol/L)*	6.1 (2.2)	5.9 (2.8)	6.2 (2.5)	5.9 (2.6)
Fasting insulin (µU/mL)	14.9 (9.5)	13.6 (7.6)	13.5 (7.9)	13.1 (7.5)
HbA1c (mmol/mol)*	39 (24.5)	41.5 (17.2)	41.0 (21.5)	41.0 (19.5)
Total cholesterol (mmol/L)	4.8 (1.2)	4.7 (1.1)	4.9 (1.2)	4.96 (1.2)
HDL-cholesterol (mmol/L)	1.2 (0.4)	1.2 (0.3)	1.2 (0.2)	1.21 (0.2)
LDL-cholesterol (mmol/L)	2.2 (1.0)	2.2 (0.9)	2.8 (0.8)	2.6 (0.8)
Total cholesterol/HDL-cholesterol ratio	4.0 (0.8)	3.9 (0.89)	4.1 (0.8)	4.2 (1.1)
Triglycerides (mmol/L)*	1.7 (0.8)	1.7 (0.6)	1.8 (1.1)	1.6 (1.2)
ALT (U/L)	61.7 (30.9)	55.38 (30.72)	59.0 (39.3)	57.0 (35.0)
AST (U/L)	40.5 (29.3)	40.8 (24)	34.0 (18.0)	34.0 (16.8)
GGT (U/L)*	56.0 (56.0)	42 (61)	51.0 (48.0)	40 (36)
HA (ug/L)*	38 (28)	51 (42)**	31 (47)	39 (58)
PIIINP (ug/L)	8.2 (3.2)	9.53 (4.6)**	8.3 (3.4)	8.9 (3.3)
TIMP-1 (ug/L)	160.2 (28.9)	201.2 (91.1)	155.5 (35.5)	202.9 (77.5)
MRS-measured liver fat (%)	22.9 (12.9, 44.7)	21.4 (10.7, 35.9)**	26.9 (9.7, 38.4)	23.7 (13.0, 42.0)
ELF score	6.9 (0.3)	7.0 (0.4)	6.9 (0.4)	7.0 (0.3)
NAFLD fibrosis score	-1.2 (1.3)	-1.3 (1.3)	-1.3 (1.3)	-1.6 (1.4)**
Fibroscan (kPa)	8.4 (3.5)	8.0 (3.7)	6.7 (2.7)	7.1 (3.8)
Fibroscan CAP score (dB/m)*	312 (78)	332 (110)	312 (65)	327 (51)
Lactulose-mannitol ratio	0.11 (0.2)	0.10 (0.19)	0.11 (0.3)	0.11 (0.26)
LPS (EU/mL)*	0.13 (0.1)	0.15 (0.1)	0.13 (0.1)	0.11 (0.1)
Bifidobacterium spp. (%)*	0.63 (1.89)	0.45 (1.28)	0.88 (1.75)	2.03(3.70)
Acetic Acid (umol/g)*	155.68 (193.3)	152.38 (184.4)	145.99 (194.6)	127 (158.7)
Propionic Acid (umol/g)*	8.38 (5.1)	8.34 (7.7)	9.47 (6.4)	8.3 (5.4)
Butyric Acid (umol/g)*	6.22 (5.7)	5.7 (5.9)	6.97 (6.1)	5.69 (3.3)

Variables that are normally distributed are expressed as mean (SD). Variables that are non-normally distributed (*) are expressed as median (IQR). ** $p<0.05$. Acetic acid, propionic acid and butyric acid concentrations expression per gram of faeces.

Table 3. Baseline and end-of-study primary outcome results and regression models testing the effects of the synbiotic intervention and changes in *Bifidobacterium* spp. on the primary outcomes, adjusted for baseline measurement of primary outcome only* and fully adjusted**

	Placebo		Synbiotic		Difference in change from baseline to end of study* (95% CI) Synbiotic treatment Primary analysis	Adjusted difference in change from baseline to end of study** (95% CI) Synbiotic treatment Primary analysis	Adjusted difference in change from baseline to end of study** (95% CI) *Bifidobacterium* spp	Difference in change in weight from baseline to end of study*
Primary outcomes	**Baseline**	**End of study**	**Baseline**	**End of study**				
MRS-measured liver fat (%)	22.9 (12.9, 44.7)	21.4 (10.7, 35.9)	26.9 (9.7, 38.4)	23.7 (13.0, 42.0)	2.8 (-2.2, 7.8) p=0.30	4.1 (-0.02, 8.3) p=0.05	0.1 (-1.3, 1) p=0.80	1.9 (1.4, 2.5) p<0.001
ELF score	12.5 (0.7)	12.8 (0.8)	12.5 (0.9)	12.9 (0.8)	-0.0008 (-0.2, 0.2) *p*=1.0	0.02 (-0.2, 0.2) *p*=0.90	0.020 (-0.08, 0.04) p=0.50	0.015 (0.001, 0.03) p=0.039
NAFLD fibrosis score	-1.2 (1.3)	-1.3 (1.3)	-1.3 (1.3)	-1.6 (1.4)	-0.03 (-0.3, 0.2) *p*=0.80	-0.009 (-0.3, 0.3) *p*=0.90	0.005 (-0.02, 0.03) p=0.60	0.046 (0.008, 0.085) p=0.027

Results of multivariable (adjusted) linear regression models testing the effects of synbiotic treatment (primary analyses) and changes in *Bifidobacterium* levels between baseline and end of study.

For each pre-specified primary outcome (MRS liver fat content, ELF score and NAFLD fibrosis score; the difference in outcome represents the change in the outcome between baseline and end of study (* adjusted for baseline measurement of the outcome variable only, or ** adjusted for baseline measurement of the outcome *plus* age, sex, weight difference and baseline weight.

Table 4. Associations of baseline MRS-measured liver fat content, NAFLD fibrosis score and ELF score with gut microbiota at baseline

	MRS-measured Liver fat	**NAFLD Fibrosis Score**	**ELF score**	**MRS-measured Liver fat**	**NAFLD Fibrosis Score**	**ELF score**
pSRBC adjusted for age, sex, baseline weight	**Correlation coefficients (rho)**			**p-value**		
Olsenella genus	**-0.32**	-0.17	-0.10	**0.001**	0.108	0.354
Unclassified *Clostridia*	0.07	**-0.37**	-0.14	0.515	**0.000**	0.187
Unclassified *Clostridiales*	0.04	**-0.36**	-0.15	0.727	**0.000**	0.155
Unclassified *Firmicutes*	-0.03	**-0.35**	-0.11	0.803	**0.000**	0.284
Sphingomonadales order	-0.06	**0.32**	0.14	0.533	**0.001**	0.202
Sphingomonadaceae family	-0.06	**0.32**	0.14	0.533	**0.001**	0.202
Sphingomonas genus	-0.06	**0.32**	0.14	0.533	**0.001**	0.202
Sutterella genus	-0.02	0.10	**0.31**	0.833	0.320	**0.002**

pSRBC: partial Spearman rank-based correlation (adjusted for age, sex and body weight).

Figure 1. Consort diagram

Figure 2. Synbiotics increase microbial beta-diversity. (A) Principal coordinate analysis of the Morisita-Horn beta-diversity index computed based on the OTU table, for each group of patients, colored by time (baseline in red, end of study in blue) or patient ID. (B) Pairwise distance between baseline and end of study samples for each patient, computed using beta-diversity metrics. P-value of the Mann-Whitney test comparing placebo and synbiotics is presented below each graph.

Figure 3. Synbiotics boost *Bifidobacterium* and *Faecalibacterium* at the expense of *Oscilibacter* and *Alistipes*. Genera presenting a significant difference in change of abundance over time between arms (q-value < 0.1). The bold line represents the mean changes.

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