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Centro de Investigación en Ciencias de la Salud y Biomedicina (CICSaB)

EFECTO DEL CONSUMO DE SINBIÓTICOS EN LA COMPOSICIÓN CORPORAL, NIVELES DE AGCC Y EXPRESIÓN DE FFAR2/3 EN ESCOLARES CON SOBREPESO Y OBESIDAD

TESIS QUE PRESENTA

MC. MARIELA VEGA CÁRDENAS

PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOMÉDICAS BÁSICAS

DIRECTOR DE TESIS
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Probiotics and synbiotics reduce metabolic obesity-associated markers in childhood by regulation G protein coupled receptors and gut microbiota: A Randomized Pilot Study

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ABSTRACT

Gut microbiota (GM) dysbiosis has been implicated in obesity-related metabolic disorders. The consumption of probiotics, prebiotcs and synbiotics have some beneficial effects on body weight and lipid metabolism. We investigated the effects of modulation of gut microbiota by probiotic and synbiotics on obesity related markers in children with overweight or obesity (n=37). Children were randomly assigned to a control group (probiotic L. casei), a group who receiving L. casei+3 g per day of inulin (Orafti®, Beneo, Belgium) or a group receiving L. casei+3 g per day of fructans from *A. salmiana* for a period of 6 weeks. The principal component analysis (PCA) showed the relations among the microbial abundance, GM metabolites and other obesity-related markers. Supplementation with probiotics and synbiotics improved HDL-cholesterol levels of children with overweight or obesity, although no changes in body composition were detected. Although, we found an increase in butyrate concentrations among both synbiotic groups, when we compared the studied groups at the end of the intervention ($p<0.03$). Among obesity-related markers we observed a diminished level of angtpl4 within the synbiotic with fructans group ($p=0.04$) but no differences were observed for Lipopolysaccharide binding protein (LBP). Supplementation with probiotic or synbiotic did not significantly change the cell frequency of FFAR2+ or FFAR3+ from total peripheral mononuclear cells (PBMC) in the three groups neither when it was compared the groups at the end of intervention. But when we analyzed the FFAR2 cell frequency between baseline and at the end of intervention it was found a decrease of FFAR2+ cells after 6-week intervention in the groups with synbiotics with inulin ($p=0.02$), and synbiotics with fructans ($p=0.04$), which was not observed for the group with probiotics. The percentage of FFAR3+ from total PBMC was no affected by the treatments, but we observed that CD14+FFAR3+ frequency increased in synbiotics with inulin ($p=0.02$) and synbiotic with fructans ($p=0.04$) groups when it was compared between baseline and at the end of intervention. In conclusion, the use of probiotic and synbiotics modulates the GM, which leads to an improvement in the lipid profile, although it was not effective in reducing body weight. However, changes were identified at the molecular level, such as the expression of FFAR3 and FFAR2, in the levels of angtpl4, propionate, and butyrate after probiotics, prebiotcs and synbiotics interventions. Our results suggest that FFAR2/3 could be evaluated as therapeutic targets in the regulation of the inflammatory state during children obesity**.**

BACKROUND &AIMS

Childhood obesity and overweight represent a major world health problem, being considered a global epidemic. It is estimated that the population with childhood obesity reaches 107.7 million without presenting differences in terms of gender[1]. Children with obesity are in high risk to present glucose intolerance, hyperinsulinemia[2], dyslipidemia[3,4], asthma[5], obstructive sleep apnea[6] and steatohepatitis[7]. The appearance of this co-morbidities associated with obesity in childhood will be maintained in adulthood[8]. Therefore, it is crucial to elucidate the factors associated with this physiopathology.

Obesity has been linked to dysbiosis defined as imbalance of intestinal microbiota in the composition, function, and diversity of gut-microbiota (GM)[9,10]. A decreased gut microbial diversity[9] may favor the onset of obesity associated to low-grade inflammation and insulin resistance[11]. It was identified different composition of intestinal microbiota between children with normal-weight and those with obesity[14]. The intestinal microbiota in obesity is characterized by an elevated *Firmicutes/Bateriodetes* ratio, enriched of *Ruminococaceae* at family level and depletion of *Bacteroides* at the genus and OTU levels[12,13], which characterize the shift from symbiotic relationship towards a dysbiotic state between microbiota and the host and impair their metabolism.

The effects of GM on the host metabolism and energy regulating homoeostasis are partially mediated by short chain fatty acids (SCFA)[14], which are produced –by some species of bacteria and consequently released to the circulation- during the anaerobic fermentation of the dietary fiber and non-digestible carbohydrates[15]. SCFA can directly modulate host health through a range of tissue-specific mechanisms related to gut barrier function, glucose homeostasis, immunomodulation, appetite regulation and obesity[16–18]. A greater abundance of SCFA has been associated in subjects with obesity compared to normoweight. However, the SCFA role in the development and maintenance of children obesity is still undefined. The SCFA have action through the signaling of receptors coupled to G proteins; FFAR2 (GPR43) and FFAR3 (GPR41), designated free fatty acid receptors (FFAR). The expression of FFAR3 and FFAR2 at the mRNA level has been detected in adipose tissue, pancreas, spleen, lymph nodes, bone marrow and immune cells[19,20]. However, their expression at protein levels in blood lymphocytes from children with obesity is unknown.

FFAR2 and FFAR3 could be the link between microbiota dysbiosis and chronic inflammatory disorders -as obesity- due to their ligand specificity for the major microbiota metabolites and also are expressed on various immune cells. The binding of acetate (C_2), propionate (C_3) and butyrate (C_4) to their receptors induce intracellular pathways including the mitogen-

activated protein kinase (MAPK) pathway, PKC and transcriptional factors such as ATF-2[21]. Butyrate is able to inhibits the production of TNF-alpha, NO and IL-6 but also the increase of IL-10 in monocytes/macrophages[22]. The SCFAs mechanisms include the inhibition of histone-deacetylase activity[23,24], an increase of the acetylation of proteins such as NF-κB, p53 and NF-AT as well as the regulation of gene expression associated with the inflammatory process in vitro[25].

Synbiotic -a combination of probiotics and prebiotics- are described as modifiers of the composition of microbiota, promoting the restoration and maintenance of intestinal dysbiosis and also modulating some immune parameters[26,27]. Our research group has previously studied the prebiotics effects of *Agave Salmiana* fructans in decrease energy intake and less weight gain in an animal model[28] with an increment in the growth of the *Lactobacillus* and *Bifidobacterium* genera[29]. However, little is known about the capability of probiotics and synbiotics to modify FFARs expression and their implications in children with overweight or obesity. Therefore, our objective was to assess the effect of synbiotic supplementation on metabolic outcomes (body composition, lipid profile and fasting glucose), excretion levels of SCFAs and the mRNA and protein expression of FFARs in monocytes and CD4+ T lymphocytes from overweight or obesity children.

MATERIALS AND METHODS

Study participants and protocol

The study was a randomized, simple-blind controlled trial with nutritional intervention. After providing detailed information, it was obtained the written informed consent from parents and written and oral assent from participants prior to the initial test day. This trial comprised 37 male and female children aged 6-11 years with overweight or obesity (≥ 85th body mass index (BMI)-percentile), were randomly assigned to a control group (probiotic *L. casei*), a group who receiving *L. casei*+3 g per day of inulin (Orafti®, Beneo, Belgium) or a group receiving *L. casei*+3 g per day of fructans from *A. salmiana* during a period of 6 weeks. The randomization was performed by an investigator that did not interact with the subjects and the research staff were responsible for all the product distribution. The participants were blinded to the treatments which were provided in identical presentation. Our objective was to examine the effects of the synbiotic supplementation independent of any other lifestyle changes, therefore participants were instructed to eat as usual does and maintain their level of physical activity. To address the dietary assessment, we used a 24 h dietary recall at the beginning and at the end of the study. Participants were asked to write down the type and amount of food eaten, using scales or household measures to gauge portion sizes where possible. Energy and macronutrient intakes were analyzed by the NutriKcal®VO software. The pilot study was conducted in accordance with the guidelines of the Committee of Health Education and Research of the Health Secretary of San Luis Potosi (SLP/012-2017).

Body composition and sample collection

At baseline and at the end of 6 weeks of intervention period, body weight, body mass index (BMI) and composition were determined. Segmental body composition was measured using the Tanita-BC418 bioelectrical impedance analyzer (Tanita Corp., Tokyo, Japan). Height was measured without shoes and with minimum of clothing with portable stadiometer (Seca 213, Hamburg, Germany). The waist, hip and neck circumferences were measured with an anthropometric tape (Lufkin W606ME, Sparks, Maryland). Fasting blood and stool samples were collected from each participant at baseline and at week 6 follow-up visit. With one of the parents accompanying his/her child, blood samples were taken from the antecubital vein between 7:00 and 8:30 am. After collecting blood samples, the participants received a healthy snack provided by the project team. Fasting plasma glucose, and serum lipids (TG,

TC, LDL-C and HDL-C) and hematic biometry were analyzed in the Laboratory of Clinical Analysis, Faculty of Chemical Sciences, Autonomous University of San Luis Potosí.

Flow cytometry analysis

After blood collection, peripheral blood mononuclear cells (PBMC) were isolated by the ficoll-hypaque method. The cells were fixed with 4% PFA and stored at -80°C in a freezing medium with 10% DMSO and 90% FBS. For the cell surface antigens, $5x10^5$ cells were incubated with fluorescent labeled monoclonal antibody anti CD4-PE (eBiosciences) or anti CD14-PE (BD Biosciences) for 30 minutes. For the FFAR2 and FFAR3 intracellular staining, 0.01% saponin was added to the cells and incubated for 10 minutes at 4°C in the dark, then were centrifuged at 10000 rpm for 15 minutes. After, the cells were incubated in different tubes with rabbit anti-human FFAR2 (BD Biosciences) or rabbit anti-human FFAR3 (Invitrogen) primary antibodies for 60 minutes at 4°C. The, 0.01% saponin step was repeated and a goat anti-rabbit FITC secondary antibody (Millipore) was added to each tube for 30 min at 4°C in the dark. After the wash step, the cells were fixed with 1% paraformaldehyde (PFA). Fluorescence positive cells were quantified by FACS Canto II cytofluorometer (BD Bioscience, San Diego, CA) and the FACS Diva software was used to analyze the data.

Total RNA extraction and quantitative real-time reverse transcription

Total RNA was extracted from PBMC using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA concentration and purity were determined with a spectrophotometer (Synergy HT, BioTek). A total amount of 100 ng of RNA was used to synthetize cDNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A Total of 250ng of cDNA was used as a template to perform qPCR using TaqMan® Universal Master Mix (Applied Biosystems™). To evaluate the mRNA expression of the FFARs, iTaq™Universal SYBR® Green Supermix and specific primers for FFAR2 [sense: 5'- gtagctaacacaagtccagtcct- 3', antisense: 5'- ctaggtgttgctttgaagcttgt- 3'] and FFAR3 [sense: 5'- caccatctatctcaccgccc- 3', antisense: 5'- cagtgagcagaggccaacag- 3'] were used. The analysis was performed on the CFX96 Touch™ Real-Time System (BioRad). The data were analyzed with the $2^{-\Delta\Delta Cq}$ method against the level of the transcripts GADPH for mRNAs.

SCFA quantification

Fecal samples were processed at two years of storage and analyzed using a method previously reported[30]. The fecal homogenates were analyzed by High Performance Liquid Chromatography -Agilent 1100 series HPLC system (Germany)- equipped with a quaternary bomb, thermal compartment and a the refractive index detector was used in reverse. An Hi-Plex H for organic acids ion exchange column at a flow rate of 0.6 ml/min with dilute acid at 5 mM H_2SO_4 as eluent. The temperature of the column and the detector was 55 °C with an operation time per sample of 26 min. The samples were quantified in relation to standards measured in parallel.

Multiplex assay procedure

Serum levels of ANGTPL4 (angiopoietin-like 4) and LBP (lipopolysaccharide-binding protein) were evaluated using a Luminex Human Magnetic Assay 2-plex (R&D SYSTEMS). The assays were carried out following the manufacturer´s instructions. Data was analyzed using the software Bio-Plex Manager™ Software (BioRad).

Statistical analysis

The distribution of each one of the variables was assessed by the Kolmogorov-Smirnov test. Relative abundances of OTUS were used for principal component analysis and to visualize variance distribution using R studio software 1.1.419, R packages ggplot2, ade4, factoextra, FactoMineR and NMF. Differences between groups at baseline were determined by using independent T tests. Changes in anthropometric, clinic, dietetic variables were analyzed by using repeated-measures two-way ANOVA test for variations among groups and Bonferroni post-hoc test to compare the three groups. The assessment of differences in the expression of FFAR2 and FFAR3 was determined using both parametric and non-parametric tests. The differences in the expression of FFARs between the groups were determined by 2-way ANOVA. The differences were considered significant at $p < 0.05$. The statistical analysis was performed using InStat GraphPad software (InStat GraphPad Inc., San Diego, CA, USA), version 5.0.

RESULTS

Characteristics of the participants in the study groups

A total of 37 subjects consented to participate in the study of which 10 were randomized to the probiotic group (*L. casei*), 13 to synbiotic group with inulin, and 14 to the synbiotic group with fructans from *A. salmiana.* Participants demonstrated good compliance with the supplement consumption and no adverse effects or symptoms were reported. The age of the participants ranged from 6 to 11 years. There were no significant differences in baseline characteristics (Table 1), either on the intake of energy and nutrient in the three groups throughout the study (Table 2).

Probiotics and synbiotics consumption lead to the improvement of HDL levels with no effect on body composition

Distributions of weight, weight z-score, weight gain, BMI, waist circumference, neck circumference, fat mass and lean mass of the participants at baseline and after the 6-week intervention were not significantly different between groups (Table 3). Intervention with synbiotic containing fructans from *Agave Salmiana* and *Lactobacillus casei*, showed the weight gain ($p=0.04$) compared to synbiotic group with inulin ($p=0.08$) and probiotic ($p=0.93$). Although absolute body weight increased in the three groups over the 6 weeks. The age and sex-specific analysis of body weight showed no significant change in body weight z-score within synbiotic containing fructans from *Agave Salmiana* group compared with the increased of weight z-score in probiotic and synbiotic with inulin groups. However, the effect of time was significant for weight, weight z-score, BMI, and fat mass (Table 3). Given the growing evidence for a critical role of the gut microbiome in metabolism, we aimed to evaluate the lipid profile and glucose levels of the participants (Table 4). All groups showed a significant increase in fast glucose throughout the study. However, the mean and SD are within the normal ranges and that increase not showed differences between the intervention groups. Regarding the lipid profile, we observed an increase in serum triglycerides and total cholesterol within the synbiotic with inulin group through the study which was not observed for the other groups. The serum levels of LDL-cholesterol showed no differences through the study between groups. Nevertheless, the intervention with probiotic and both synbiotics promotes a significant increase in HDL-cholesterol levels without differences between groups (Table 4).

Principal component analysis (PCA)

Diet interventions seem to influence microbiome composition and in consequence the immune response. To explore the relationship among all the variables studied, the first step was a descriptive principal component analysis (PCA) (Figure 1). The first axis accounted for more than half of the variance (>50%), showed that individuals randomized assignment within the three groups of intervention tended to cluster together. The factorial map for all the study variables did no showed a clear grouping (Fig 1a), for this reason different factorial maps were performed by classifying the variables. For instance, clinical and anthropometric variables (Fig 1b) and dietary variables (Fig 1c) are showed but no a clear grouping was detected. PCA based on molecular features (Fig 2a) showing the grouping pattern of the individuals by intervention group and the relations between them (Fig 2b), which includes the GM metabolites and other obesity-related markers. The absolute abundance of OTUs (Fig 2c) revealed a separation of the three groups of treatment based on the first principal component PC scores, confirmed the above findings, showing a separation of individuals within the treatment group. Our PCA shows the relations among the microbial abundance (Fig 2d).

Probiotics and synbiotics consumption promote fecal short chain fatty acid (SCFA) production in overweight and obese children.

Representative chromatogram of a fecal homogenate extracts from a study participant are shown in Fig 3a and the arrows indicate the retention times of acetic acid (15.9 min), propionic acid (19.0 min) and butyric acid (23.8 min). Fecal SCFA concentrations did not differ between the three groups neither when it was compared the studied groups at the end of the intervention (Fig 3b-d). However, synbiotics with fructans intake resulted in higher fecal propionate (Fig 3c, $p=0.03$) and butyrate (Fig 3d, $p<0.0001$) concentrations when we compared baseline and at the end of intervention levels. There was no significant difference in fecal acetate when compared with probiotic or synbiotics with inulin treatment, however, resulted in a trend towards higher fecal acetate concentrations when compared with synbiotics over the entire test period (Fig 3b).

Angptl4 and LBP serum levels in study subjects

Propionate and butyrate activated Angptl4 production in the human intestinal tract and it has been proposed its role in lipid metabolism in fasting/feed states by inhibiting LPL activity. In the total study population, the angptl4 levels ranged from 46660 to 209496 pg/ml. Plasma

glucose levels has been negatively associated with Angptl4 serum levels suggesting that an increase of Angptl4 could play a role in glucose homeostasis. We observed a diminished level of angtpl4 within the synbiotic with fructans group (Fig 4a, p=0.04) when we compared baseline and at the end of the study. To assess if the consumption of probiotics and synbiotics influenced intestinal permeability through LBP measurement, we compared the concentrations in the three groups after 6-week intervention but there was no significant difference in serum levels LBP between groups (Fig 4b).

Increase in levels of CD14+FFAR3+ cells and decrease of CD4+FFAR2+ in PBMC from individuals with overweight or obesity after the intervention with synbiotics

Proinflammatory Th cell subsets and monocytes contribute to inflammation and metabolic alterations associated with obesity. However, there is a lack of agreement on the cell type responsible for the effect of FFAR2 and FFAR3. Representative histograms for the identification of FFAR2+ and FFAR3+ in CD4+ or CD14+ cells from PBMC after defining the leucocyte population, based on forward and side scatter parameters, are shown in Figure 5 a-b. Flow cytometry analysis showed significantly lower FFAR2+ than FFAR3+ cell frequencies in PBMC from the participants through the study (p= 0.002) (Fig 5c). In addition, the diminished levels of FFAR2+ from total PBMC after 6-week intervention was detected by synbiotic with inulin (p=0.03) and synbiotic with fructans (p<0.001) when it was compared baseline and at the end of treatment (Fig 6a). In contrast, the FFAR3+ from total PBMC was not modified by intervention (Fig 6d).

To elucidated which leukocyte subsets are more involved in the SCFAs receptor expression, we analyzed the frequency of CD4+ or CD14+ cells expressing either FFAR2+ or FFAR3+. After the intervention, the percentage of CD4+FFAR2+ cells decrease significantly only in the synbiotic with fructans group (p=0.01) (Fig 6b). However, the interaction time and treatment were statistically significant for the percentage CD14+FFAR2+ (Fig 6c, p=0.042), where the frequency of CD14+FFAR2+ cells decrease significantly after the synbiotics with fructans intervention and in comparison, with the decrease in the synbiotic with inulin and probiotic groups. In the case of FFAR3+CD14+, we observed a increased levels after the intervention with both synbiotics (Fig 6f, p<0.05) without differences between groups or in CD4+ cells (Fig 6e).

Regulation of FFAR2 and FFAR3 mRNA levels after the intervention with probiotics and synbiotics

The mRNA levels of FFAR2 and FFAR3 in the PBMC of the participant were very variables and not presented significant differences at the begining of the study. However, after 6-week intervention we observed a diminished level of FFAR2 mRNA transcripts compared to baseline but no differences for FFAR3 levels (Fig 7a). When it was compared the synbiotic effect, there was a diminished relative expression of FFAR2 within the synbiotic with fructans group (p=0.02) but no between-groups. Also, no differences were detected for FFAR3 mRNA transcripts through the study (Fig. 7 b-c).

Microbial correlations with clinical biomarkers at baseline

We conducted a Spearman's correlation analysis to evaluate the relationship between gut microbial abundance and body composition, biological parameters, and GM metabolites at the baseline. The Pearson matrix correlation in the Fig 8, showed similar relations to the PCA analysis, there was a correlation among the 26 OUTs significantly associated with overweight or obesity. Among body composition parameters we found a several OTUs which were correlated, for example BMI-for-age with *Holdemanella* (r^2-0.3), neck circumference (NC) and lean body mass (LBM) with *Catenibacterium* ($r^2$0.52), and also it was found that LBM correlated with *Bifidobacterium* ($r^2$0.34), and fat mass (FM) with *Veillonella* ($r^2$0.43). Within the metabolic biomarkers fasting glucose were correlated with *Christen_R7* (r^2 0.49), *Akkermansia* ($r^2$0.19) and *Clostr_innoc* ($r^2$0.44), total cholesterol were correlated with *Agathobacter* ($r^2$0.44) and triglycerides levels were significantly and positively correlated with *Eubac_hallii (*$r^2$0.28), *Alistipes (*$r^2$0.4), *Blautia* ($r^2$0.29), *Dorea (*r^2*0.44),* and *Erysipelatoclostridium* ($r^2$0.33). Among the SCFA such acetate correlated with *Romboutsia (*r^2*-0.39)* and *Clostridiumsensu (*r^2*-0.19),* meanwhile butyrate with *Blautia* (r^2-0.4*)* and *Veillonella* (r^2-0.16). Nevertheless, no correlation between propionate concentrations and OTUs were detected.

DISCUSSION

Diet can play a beneficial role on modulating GM[31] and influence its composition then, it is used as a therapeutic tool. The consumption of prebiotics and synbiotics could reduce body weight[32] and the percent of body fat therefore present an effect on inflammatory markers such as CRP[32], IL-6 [33] and serum lipids levels[27]. Several reports have shown that changes in GM composition by synbiotic consumption regulate changes in lipid levels in blood[34]. In our study we identified an improve in HDL cholesterol after the synbiotic intervention, demonstrating properties of *Lactobacillus casei* strain by induction of *Bifidobacterium* within the intestine[35] which leads in improvement of metabolic profile in children with obesity[36]. Also, it is important to notice that at baseline, HDL cholesterol levels were correlated with *Bacteroides* ($r^2$0.25), *Romboutsia* ($r^2$0.25), *Agathobacter* ($r^2$0.43), *Blautia* (r^2-0.31), *Catenibacterium* (r^2-0.59) and Veillonella ($r^2$0.49), which have been determined as markers of intestinal microbiota obesity-related metabolic abnormalities[37].

Weight loss is the expected outcome for obesity interventions. However, childhood obesity protocols should consider the confounding effect of normal growth, since children aged 6 to 11 years old should gain weight approximately 3kg per year [38]. In this regard, it would be expected at least 0.3kg of weight gain over a period of 6 weeks. However, it has been reported in prospective studies weight gains between 5 to 6 kg per year, on those study cohort the participants did not make lifestyle modifications and correlated the weight gain with hypersinulinemia[39] and loss of control eating experience[40]. In our study the participants gained a mean of 0.76kg over 6-week follow-up period and only 19 children increase more than 0.3 kg (Supplementary Figure 1). It is important to note that interventions for childhood weight loss are determined by the child's age and severity of obesity and related comorbidities. Experts in nutrition indicate that a weight loss of 0.45kg per month is considered safe for children aged 2 to 11 years old[41]. There is a lack of consensus about which are the best and most structured strategies for pediatric weight control. However, our findings indicate that despite that not all the analyzed parameters were improved, nutritional intervention exclusively with the complementation of the habitual diet with a synbiotic product constitutes a useful tool that should be complementary with behavioral strategies focused on the reduction of total caloric intake, increase of physical activity and involvement of the children family and school environment for pediatric weight control.

SCFA are the principal physiologically active metabolites that contribute to the maintenance of mucosal homeostasis, constitute energy source for host colonocytes and exert potent

anti-inflammatory activitIes[42] by modulating cell signaling pathways in immune cells[43]. Fecal acetate and butyrate were significantly associated with *Rombutsia*, *Blautia* and *Veinollena* members of butyrate producer group. SCFAs have a key role in the maintenance of the normal structure, integrity and function of the intestines[44]. Several clinical trials have demonstrated the key role of dietary intervention on modulate GM composition and in consequence the production of their metabolites. In this study, we analyzed the fecal excretion of the three principal SCFA; acetate, propionate and butyrate and we observed a change in the amount of propionate and butyrate only in the synbiotic with fructans as prebiotic group.

Disruption of the intestinal barrier function is associated with local and systemic inflammation and play an important role in the onset of diseases associated with obesity. Barrier defects enhanced epithelial permeability and in consequence excessive translocation of inflammatory molecules such as LPS to the circulation. LPS is bound in the bloodstream to LBP and transferred to TLR4 activating immune downstream signaling pathways to the production of proinflammatory cytokines [45,46]. Therefore, LBP constitute a potential marker of metabolic endotoxemia and permeability. Obesity-associated GM dysbiosis have also been associated with the absorption of LPS and increased intestinal permeability, due the capacity of probiotics and prebiotics as regulators of GM composition, we analyzed changes in LBP levels before and after the intervention. We found that serum concentrations of this protein were correlated with *Bifidobacterium* ($r^2$0.38) at baseline. However, no differences to LBP levels were observed between groups.

Another mechanism by which SCFAs have effects on metabolism is by regulating the production of adipose factor induced by fasting Fiaf (Fasting-induced Adipose Factor) or also known as Angptl4 (angiopoietin-like 4), an inhibitor of the lipoprotein lipase (LPL)[47]. Angptl4 is an adipokine expressed in various tissues such as adipose tissue, liver, intestine and heart; promotes lipolysis resulting in an increase in serum triglyceride levels and a decrease in free fatty acids, thereby reducing fat storage[48]. One study demostrated that supplementation with *Lactobacillus* strains such as *L. rhamnosus GGL* and paracasei F19 increase serum concentrations of Angptl4 and it was associated with lower adiposity in murine models[49]. Therefore, we expected to find changes in the amount of circulating levels Angptl4 after intervention, due to Angptl4 has been considered a circulating mediator of the microbiota and fat storage, it has also been reported that circulating concentrations are associated with an inflammatory phenotype and higher adiposity in murine models. Our

study is the first report to evaluate this adipokine in metabolically healthy children with overweight or with obesity.

Adipocytes from individuals with obesity showed higher levels of FFAR2 compared with lean counterparts and a correlation with TNFα mRNA levels. Also, it has been demonstrated that FFAR2 regulates inflammatory signals in Gpr43TG model. FFAR2 and FFAR3 have been detected in a variety of tissues, among peripheral blood mononuclear cells (PBMC)[50] and neutrophils[19]. FFAR2 showed a higher amount of transcript in immune cells[51] which seems to be trigger by inflammation challenges such as LPS[52] likewise IL-1β and TNFα which shown raise both receptors expression by dose dependent[53]. Therefore, is interesting the role of FFAR3 and FFAR2 as therapeutic targets. We analyzed the expression levels of both receptors in the PBMC from study participants and at baseline the transcripts of FFAR3 and FFAR2 (GPR41 and GPR43) negatively correlate with *Agathobacter* and *Veillonella,* respectively which it is consistent with other report in inflammatory syndroms[54]

Probiotics, prebiotics and synbiotics have been used in dietary interventions as modifiers of GM, promoting the restoration of dysbiosis by the maintenance of the intestinal barrier and modulating some immune parameters[26]. It has been reported that the consumption of this products in children and adolescents with obesity affect body weight and metabolic parameters, suggesting that GM has an impact on energy balance[27,55]. Nevertheless, this study shown for the first time the regulation of FFAR expression by the intervention of a probiotic and synbiotic products in humans. This is consistent with various studies in murine models that showed the sinergestic effects of synbiotics in order to regulate FFAR expression[56]. It is important to highlight the fact that these findings are based on mRNA measurements, which may not correlate with the expression levels of the functional protein. However, we also showed for the first time the expression of this FFAR as a cell frequency, which is a measurement of indirect protein levels. We found that the intervention with synbiotics can modulate the expression of FFAR2 and FFAR3 in CD14+ monocytes but no in CD4+ T cells.

We have demonstrated that targeting gut microbiota with commercial fermented milk containing prebiotics components constitute a relevant and promising intervention in the nutritional approach to reduce biomarkers of obesity in human subjects. The results obtained support that probiotic and synbiotic supplements used in this pilot study modulates gut microbiota by shaping the abundance of beneficial microbial species, the role of these changes in relation to metabolic parameters requires further studies. Understanding the

mechanisms by which SCFA and their receptors affect metabolic homeostasis could contribute to the development of strategies aimed in reducing the prevalence of obesity.

Our study has some limitations such as is not a double-blind placebo versus control study. The period of synbiotic interventions was only six weeks then a longer follow-up is needed. It was included a combined commercial fermented milk as a probiotic enriched with prebiotics components and thus it cannot be evaluated the effects of probiotics and prebiotics separately.

CONCLUSION

The use of synbiotics by modulating the intestinal microbiota, leading to an improvement of the lipid profile, and changes at the molecular level, such as the expression of SCFA receptor FFAR3 and FFAR2 after the intervention. Also, we identify changes in the amount of excreted SCFA specially propionate and butyrate. Our results suggest that these SCFA receptors could be evaluated as therapeutic targets in the regulation of the inflammatory state during obesity. The findings of this trial suggest beneficial effects of a synbiotic, however more studies with longer follow-up should be conducted.

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FIGURE LEGENDS

FIGURE 1. The PCA analysis shows the grouping pattern of the individuals by study group. a) All the variables studied without a categorization b) Biochemical and anthropometric variables c) Macro and micronutrient intake. Percentages are the variance explained for the first two dimensions produced by the PCA**.**

FIGURE 2. The PCA showing the grouping pattern of the individuals by study group. Molecular features (a) and their relations among these variables (b). The abundance of microbes (c) and PCA showing the relations among the OTUs (d). In both figures the numbers are our internal subject identification number, the percentages showed are the variance explained for the first two dimensions produced by the PCA

FIGURE 3. **Fecal SCFAs excretion in children with overweight and obesity.** a) Chromatograms of SCFAs obtained from feces. b) Concentration of fecal acetate c) propionate and d) butyrate did not vary within groups throughout the study.
p=Value resulted from independent t-test for difference between probiotic and synbiotics groups after intervention ($p<0.05$). circles indicate control group *L. casei* (probiotic), triangle represent a group who receiving L. casei+3 g per day of inulin and diamonds a group receiving L. casei+3 g per day of fructans from *A. salmiana.*

FIGURE 4. Serum angtPL4 and LBP concentrations of individuals in the probiotic and synbiotics group over the study period. Concentrations were measured by multiplex immunoassays and analyzed by 2-way ANOVA for test the time and treatment effect.
p=Value resulted from independent t-test for difference between probiotic and synbiotics groups after intervention ($p<0.05$).
circles indicate control group *L. casei* (probiotic), triangle represent a group who receiving L. casei+3 g per day of inulin and diamonds a group receiving L. casei+3 g per day of fructans from *A. salmiana.*

FIGURE 5. SCFA receptors expression in T CD4+ cells and CD14+ monocytes after the intervention with probiotics and synbiotics. The frequency of FFAR2+ or FFAR3+ cells in PBMC from the individuals within *L.casei, L.casei* +inulin and *L.casei* +fructans groups was analyzed. a) Representative FACS profile showing the cell frequencies b)

Percentage of FFAR2+ or FFAR3+ cells from individuals within the three intervention groups. circles indicate control group L. casei (probiotic), triangle represent a group who receiving *L. casei*+3 g per day of inulin and diamonds a group receiving *L. casei*+3 g per day of fructans from *A. salmiana.*

FIGURE 6. Levels of CD4+FFAR2+ or CD4+FFAR3+ and CD14+FFAR2+ or CD14+FFAR3 cells in PBMC from study participants. The frequency of FFAR2+ on a) PBMC, b) CD4+ and c) CD14+ cells from the individuals within *L.casei*, *L.casei* +inulin and *L.casei* +fructans groups was analyzed. The frequency of FFAR3+ on d) PBMC, e) CD4+ and f) CD14+ cells from the individuals within L.casei, L.casei +inulin and L.casei +fructans groups was analyzed. # p=Value resulted from independent t-test for difference between probiotic and synbiotics groups after intervention ($p<0.05$). circles indicate control group L. casei (probiotic), triangle represent a group who receiving *L. casei*+3 g per day of inulin and diamonds a group receiving *L. casei*+3 g per day of fructans from *A. salmiana.*

FIGURE 7. Relative expression of FFAR2 and FFAR3 through intervention with probiotic and synbiotics. Analysis of FFAR2 and FFAR3 expression in the PBMC from individuals within *L.casei*, *L.casei* +inulin and *L.casei* +fructans groups was performed. a) Relative expression of FFAR2 compared with FFAR3 from individuals within the three intervention groups. b) Relative expression of FFAR2 through the study c) Relative expression of FFAR3 through the study. Graphs show medians with a range.
Statistical significance is shown as $*p < 0.05$.
p Value resulted from independent t-test for difference between probiotic and synbiotics groups after intervention ($p<0.05$).
circles indicate control group L. casei (probiotic), triangle represent a group who receiving *L. casei*+3 g per day of inulin and diamonds a group receiving *L. casei*+3 g per day of fructans from *A. salmiana.*

FIGURE 8. The Pearson matrix correlation analysis for the whole studied variables. The circles within cells are the significant correlations. The horizontal blue to red-scale color bar at the bottom indicates the direction (negative or positive) of correlation values.

Suplementary figure 1. Effects of probiotic and synbiotics consumption on weight gain. We aimed at determining the effect of the interventions within changes in body composition. We analyzed total body weight gain among three groups.

circles indicate control group L. casei (probiotic), triangle represent a group who receiving *L. casei*+3 g per day of inulin and diamonds a group receiving *L. casei*+3 g per day of fructans from *A. salmiana.*

Table 1. Baseline characteristics of the participants in the study groups.

Variables	**Probiotic**	**Synbiotic (inulin)**	**Synbiotic (fructans)**	**p Value**
N	10 (3M/7F)	13 (8M/5F)	14 (6M/8F)	
Age	8.6±1.4	9.0±1.6	8.5±1.7	0.59
Weight (kg)	39.7±11.2	42.6±12.4	36.6±9.4	0.30
Weight z-score	1.56±0.56	1.60±0.82	1.42±0.73	0.79
BMI (kg/m2)	20.8(17.4-27.4)	22.3(18.4-28.9)	20.5(19.08-23.6)	0.77
Weist circunference (cm)	70.4 ±11.0	73.6±9.5	73.9±10.3	0.53
Neck circunference (cm)	29.5±2.2	31.8±3.3	30.6±3.6	0.17
Fat mass (%)	30.4(20.9-41.5)	30.6(20.3-48.6)	30.1(26.3-38.8)	0.92
Trunk fat mass (kg)	5.5±2.3	6.0±2.5	5.2±1.5	0.62
Lean mass (kg)	16.3±3.1	17.3±2.8	16.0±3.0	0.50

*p Value resulted from ONE-way ANOVA for difference within groups at baseline.
M males and F females

Table 2. Dietary intake of participants throughout the study.

Variables	Probiotic	Synbiotic (inulin)	Synbiotic (fructans)	P Value
Calories				
Before	1323±246.9	1670 ±352.9	1675 ±483.6	0.33[#]
After	1509 ±411	1601 ±387.3	1502 ±344.5	0.18
p	0.153	0.61	0.26	
Protein (g)				
Before	48.8±10.6	57.4±7.2	57.8±18.8	0.24"
After	54.0±14.9	58.6±14.9	54.7±1.0	0.86
p	0.28	0.77	0.63	
Fat (g)				
Before	50.6±12.6	65.5±21.6	65.6±21.7	0.08[#]
After	57.4±18.4	59.7±20.9	58.9±17.5	0.24
p	0.16	0.48	0.26	
Saturated fatty acids				
(g)	15.2(8-26.10)	17.8(8.4-30.2)	17.1±7.1	0.46[#]
Before	14.7(6.2-27)	16.3 (8.4-35.5)	15.9±6.6	0.90
After	0.75	0.62	0.49	
p				
Monounsaturated				
fatty acids (g)	16.5(11.4-30.3)	18.9±7.7	18.3±7.4	0.58[#]
Before	16.2 (10.1-34)	16.2±6.6	17.2±6.7	0.65
After	0.99	0.30	0.66	
p				
Polyunsaturated fatty				
acids (g)	11.7±2.9	11.0(5.0-20.6)	12.1±5.3	0.83[#]
Before	13±3.7	10.6(2.4-23)	11.5±4.4	0.61
After	0.23	0.23	0.61	
p				
Carbohydrates (g)				
Before	171.9±34.3	217.3±61.2	209.5±75.5	0.12[#]
After	198±86.3	216.8±63.1	192.6±57.3	0.40
p	0.34	0.97	0.44	
Fiber (g)				
Before	15.7(3.9-23.8)	17.1±8.6	12.4(3.3-27.3)	0.09[#]
After	17.7(6-57.3)	26.4±16.8	15.1(5.6-25.7)	0.25
p	0.89	**0.05**	0.29	

p Value resulted from independent t-test for difference between probiotic and synbiotic groups after intervention

[#]p Value resulted from ONE-way ANOVA for difference within groups at baseline.

P Value resulted from two-way ANOVA for difference within groups through the study

Table 3. Effects of probiotic and synbiotics consumption on anthropometric parameters of participants.

Variables	**Probiotic**	**Synbiotic (inulin)**	**Synbiotic (fructans)**	**p[1] Value**
Weight (kg)				
Before	39.7±11.2	42.6±12.4	36.6±9.4	0.10
After	40.8±11.4	43.6±13.0	37.1±9.3	
p	**0.0002**	**0.0005**	**0.0011**	
Weight z-score	1.56±0.5	1.60±0.8	1.42±0.73	0.46
before	1.64±0.5	1.65±0.8	1.46±0.71	
after	**0.01**	**0.03**	0.20	
p				
weight gain (kg)	1.11±0.7	1.08±1.0	0.56±0.5	0.11* **0.04[a]** **0.08[b]** 0.93[c]
BMI (kg/m²)				
Before	20.8(17.4-27.4)	22.3(18.4-28.9)	20.5(19-23.6)	0.65
After	21.4(16.1-28.8)	23.9(18.2-34)	21.4(17.7-27.3)	
p	0.58	0.50	0.64	
Weist circunference (cm)	70.4 ±11.0	73.6±9.5	73.9±10.3	0.13
Before	70.8 ±10.0	73.0±10.4	73.0±10.5	
After	0.15	0.90	0.81	
p				
Neck circunference (cm)	29.5±2.2	31.8±3.3	30.6±3.6	0.43
Before	29.8±2.0	31.5±3.3	30.7±3.8	
After	0.70	0.84	0.95	
p				
Fat mass (%)				
Before	30.4(20.9-41.5)	30.6(20.3-48.6)	30.1(26.3-38.8)	0.57
After	33.1(21.3-42.6)	32(20.7-50.3)	31.7(27-42.7)	
p	0.28	0.56	0.30	
Trunk fat mass (kg)				
Before	5.5±2.3	6.0±2.5	5.2±1.5	0.37
After	6.0±2.1	6.0±2.4	5.6±1.6	
p	0.57	0.98	0.59	
Lean mass (kg)				
Before	16.3±3.1	17.3±2.8	16.0±3.0	0.90
After	16.3±3.2	17.2(±3.3)	15.9±2.8	
p	0.99	0.93	0.88	

p[1] Value resulted from TWO-way ANOVA for difference within groups throughout the study

p Value resulted from independent t-test for difference between probiotic and synbiotic groups after intervention

* Value resulted from ONE-way ANOVA for difference within groups at the end of the study

[a] p Value resulted from independent t-test for difference between probiotic and synbiotic (fructans) groups after intervention.

[b] p Value resulted from independent t-test for difference between synbiotic (fructans) and synbiotic (inulin) groups after intervention.

[C] p Value resulted from independent t-test for difference between probiotic and synbiotic (inulin) groups after intervention

Table 4. Effects of probiotic and synbiotics consumption on serum biochemical factors.

Variables	**Probiotic**	**Synbiotic (inulin)**	**Synbiotic (fructans)**	**p[1] Value**
Glucose (mg/dl)	83.4±5.9	86.4±5.6	86.1±8.6	0.49
before	93.3±5.5	95.2±8.9	92.±8.9	
after	**< 0.0001**	**0.002**	**0.02**	
p				
Triglyceride (mg/dl)	126.2±61.7	155.1±81.3	138.1±41.8	0.20
before	134.8±48.6	191.9±94.3	149.5±68	
after	0.57	**0.04**	0.50	
p				
Colesterol total(mg/dl)	167.5±33.1	165.1±22.1	161.4±16.1	0.07
before	167.7±24.4	178.4±25.1	171.8±28.4	
after	0.93	**0.0006**	0.14	
p				
HDL (mg/dl)	44.5±10.3	45.5±9.3	43.65±9.3	0.77
before	54.8±7.3	48.2±8.9	56.0±12.6	
after	**0.001**	**0.024**	**0.0002**	
p				
LDL (mg/dl)	106(39-134)	90.5(62.3-114)	94(41-107)	0.18
before	89.9(40.3-115.1)	91.5(73.7-116.7)	82.3(62.7-125.5)	
after	0.11	0.98	0.26	
p				

p1 Value resulted from two-way ANOVA for difference within groups throughout the study
p Value resulted from independent t-test for difference between probiotic and synbiotic groups after intervention

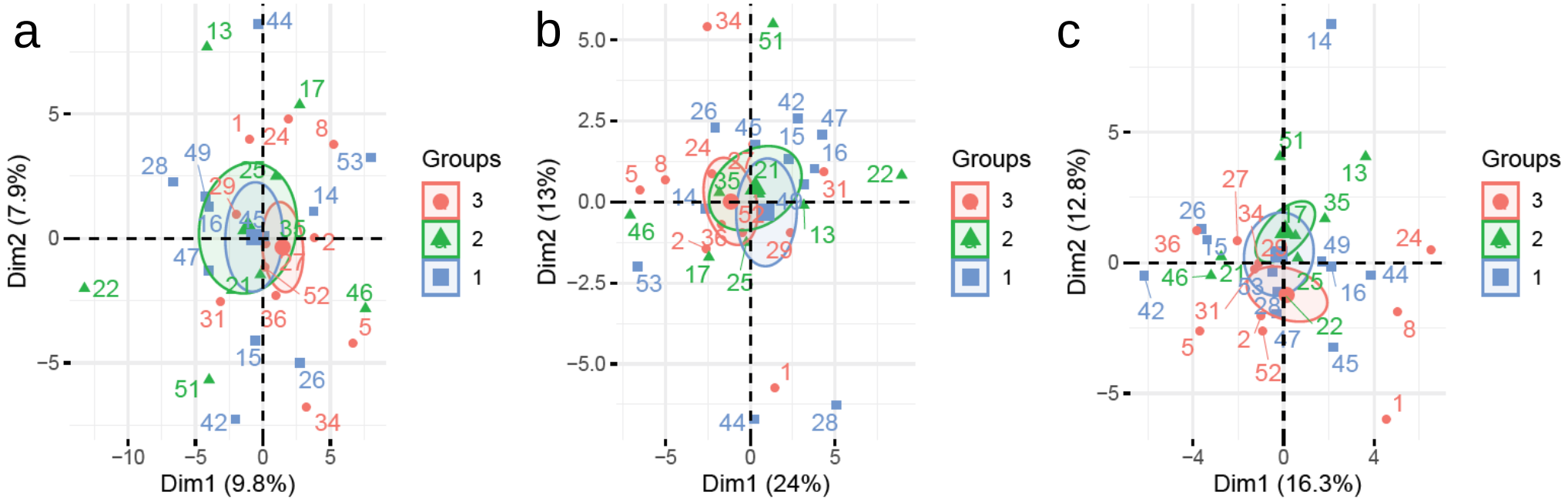

a
b
c
Dim2 (7.9%)
Dim1 (9.8%)
Dim2 (13%)
Dim1 (24%)
Dim2 (12.8%)
Dim1 (16.3%)
Groups
3
2
1

Fig 1

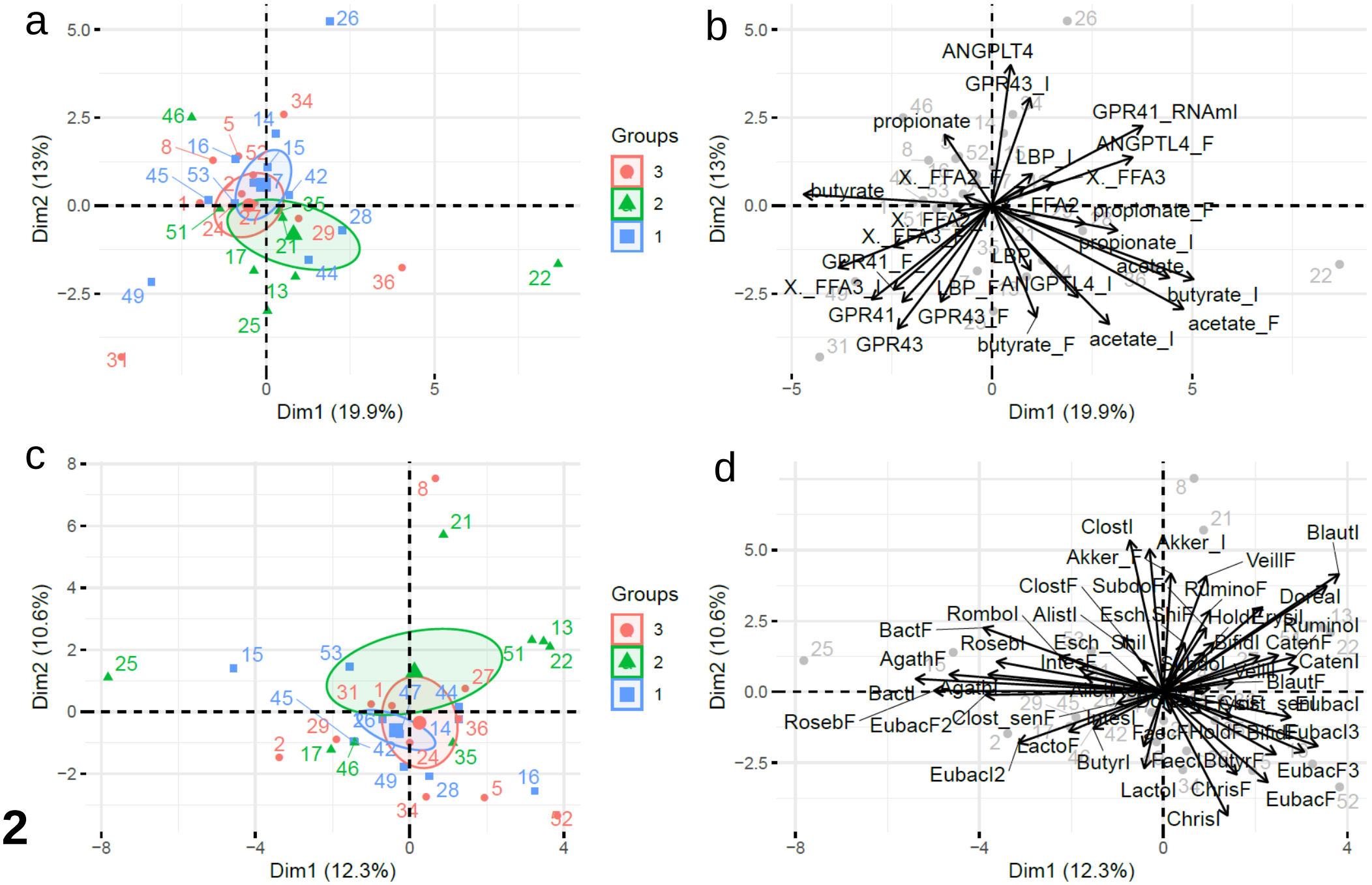
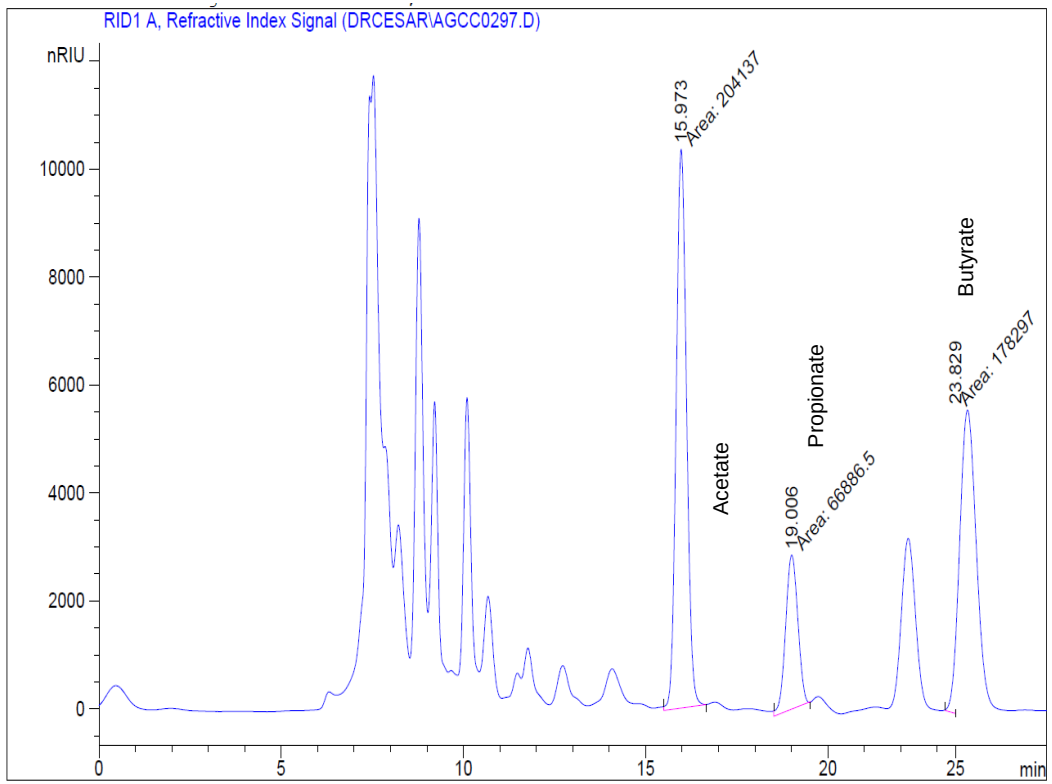
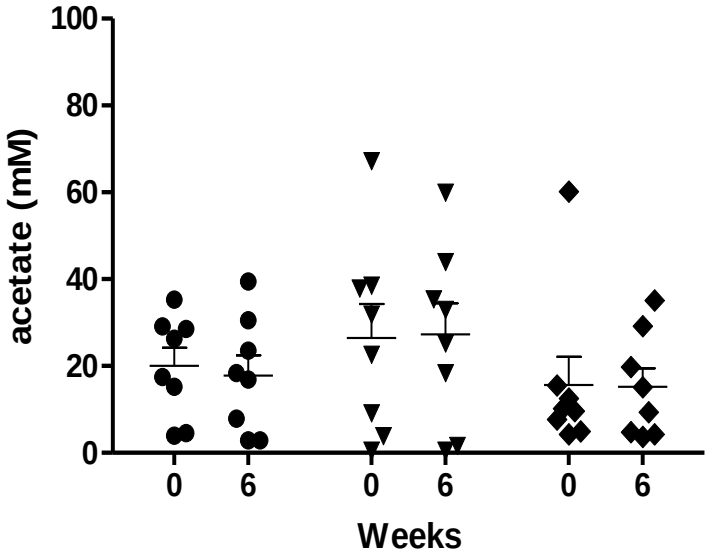

a
b
c
d
Dim1 (19.9%)
Dim2 (13%)
Dim1 (12.3%)
Dim2 (10.6%)
Groups
3
2
1
ANGPLT4
GPR43_I
GPR41_RNAmI
propionate
ANGPTL4_F
LBP_I
X._FFA3
butyrate
FFA2
propionate_F
propionate_I
acetate
butyrate_I
acetate_F
GPR41_F
LBP
X._FFA3_I
LBP_F
ANGPTL4_I
GPR41
GPR43_F
GPR43
butyrate_F
acetate_I
ClostI
Akker_I
BlautI
Akker_F
VeillF
ClostF
SubdoF
RuminoF
DoreaI
RomboI
AlistI
EschShiF
HoldEryIF
RuminoI
BactF
RosebI
Esch_ShiI
BifidI
CatenF
AgathF
IntesF
CatenI
BactI
AgathI
BlautF
RosebF
EubacF2
Clost_senF
EubacI
LactoF
FaecFHoldF
BifidF
EubacI3
EubacI2
ButyrI
FaecI
ButyrF
EubacF3
LactoI
ChrisF
EubacF
ChrisI

Fig 2

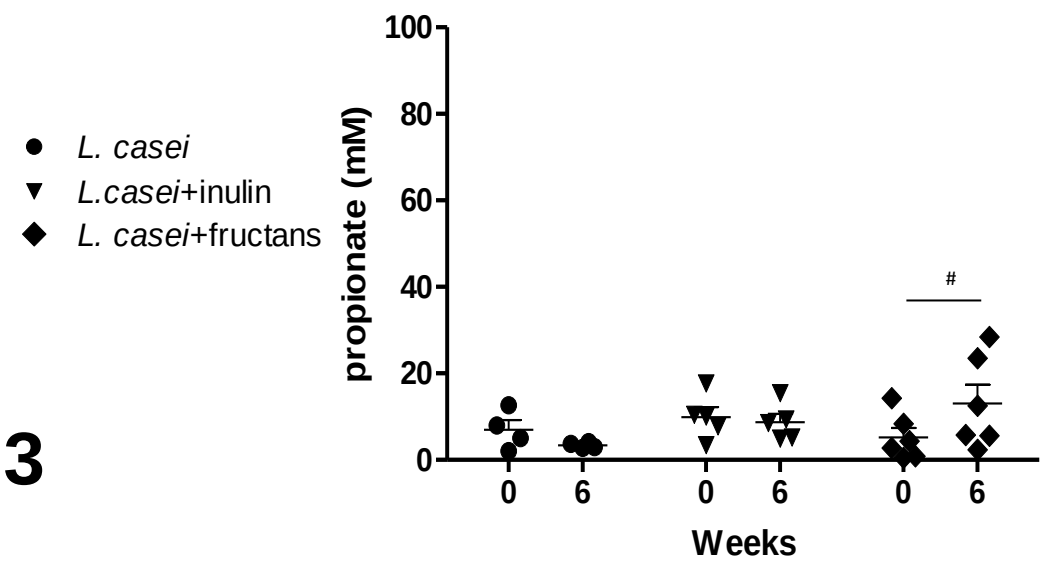
a


RID1 A, Refractive Index Signal (DRCESAR\AGCC0297.D)
nRIU
10000
8000
6000
4000
2000
0
15.973
Area: 204137
Acetate
19.006
Area: 66886.5
Propionate
23.829
Area: 178297
Butyrate
0
5
10
15
20
25
min

b


acetate (mM)
100
80
60
40
20
0
0
6
0
6
0
6
Weeks

c


L. casei
L.casei+inulin
L. casei+fructans
propionate (mM)
100
80
60
40
20
0
#
0
6
0
6
0
6
Weeks

d

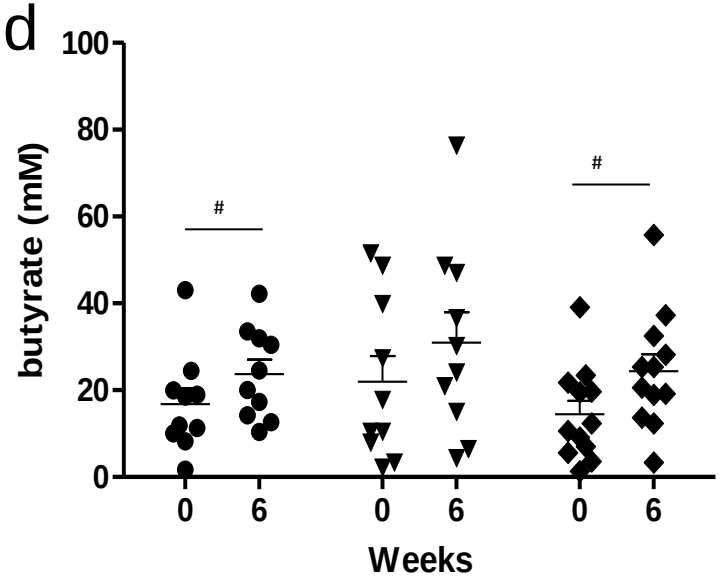

butyrate (mM)
100
80
60
40
20
0
#
#
0
6
0
6
0
6
Weeks

Fig 3

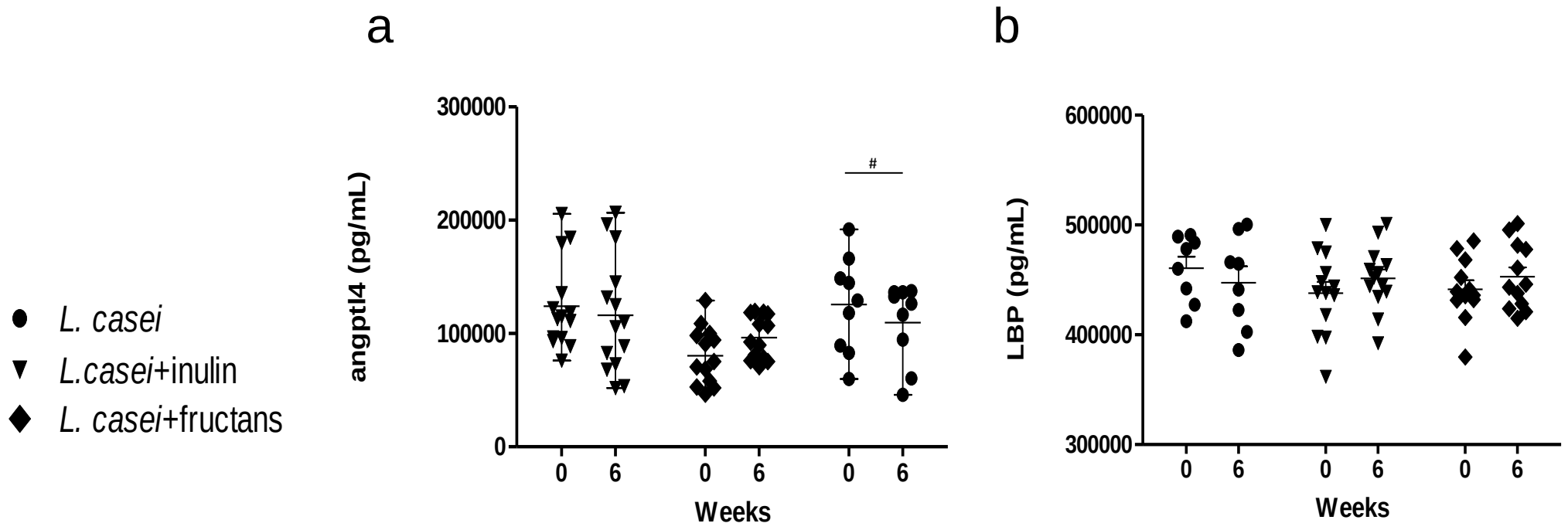

a
b
L. casei
L.casei+inulin
L. casei+fructans
angptl4 (pg/mL)
300000
200000
100000
0
#
0
6
0
6
0
6
Weeks
LBP (pg/mL)
600000
500000
400000
300000
0
6
0
6
0
6
Weeks

Fig 4

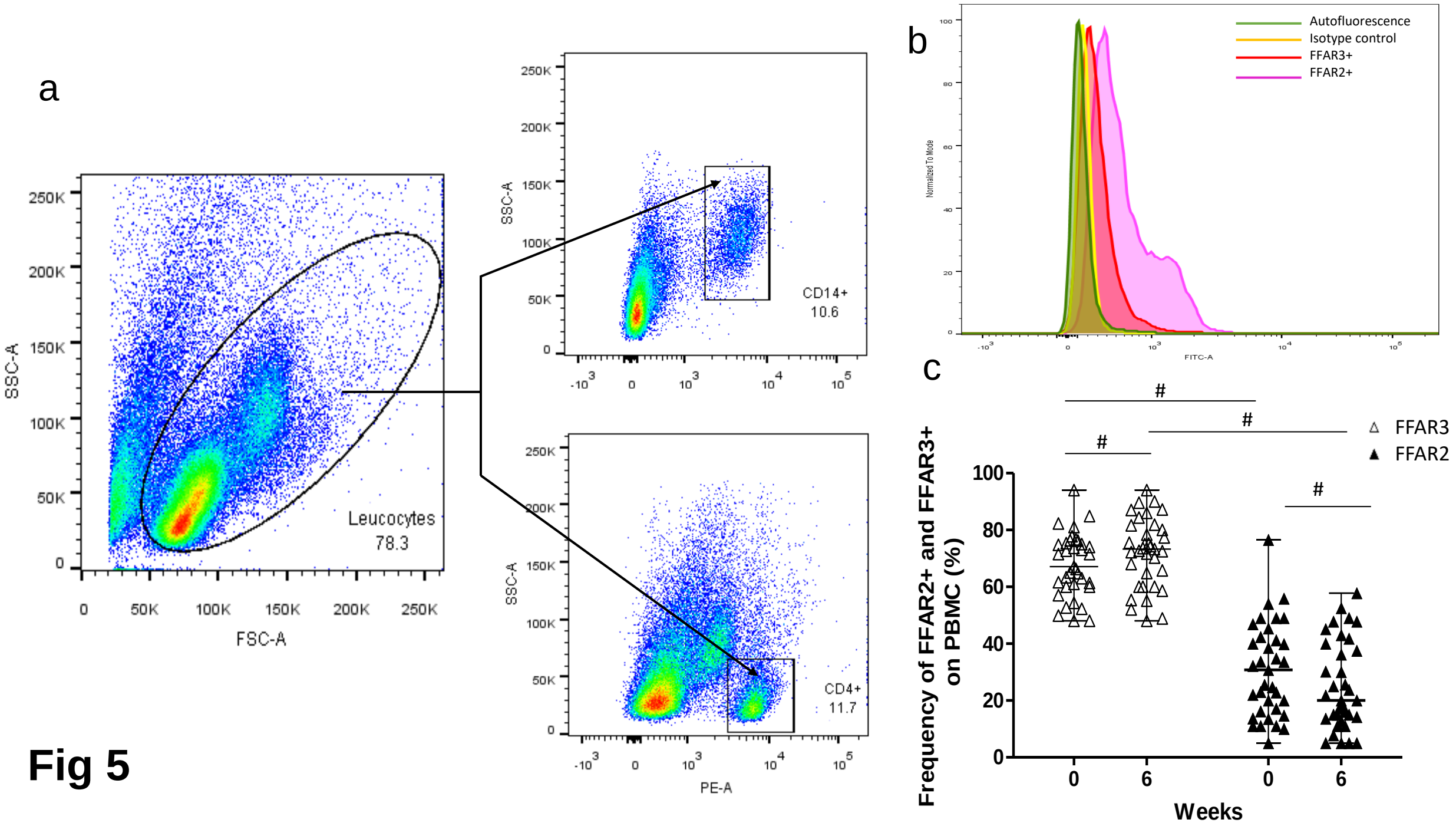

a
SSC-A
FSC-A
Leucocytes
78.3
CD14+
10.6
CD4+
11.7
PE-A
b
Autofluorescence
Isotype control
FFAR3+
FFAR2+
Normalized To Mode
FITC-A
c
FFAR3
FFAR2
Frequency of FFAR2+ and FFAR3+ on PBMC (%)
Weeks
#

Fig 5

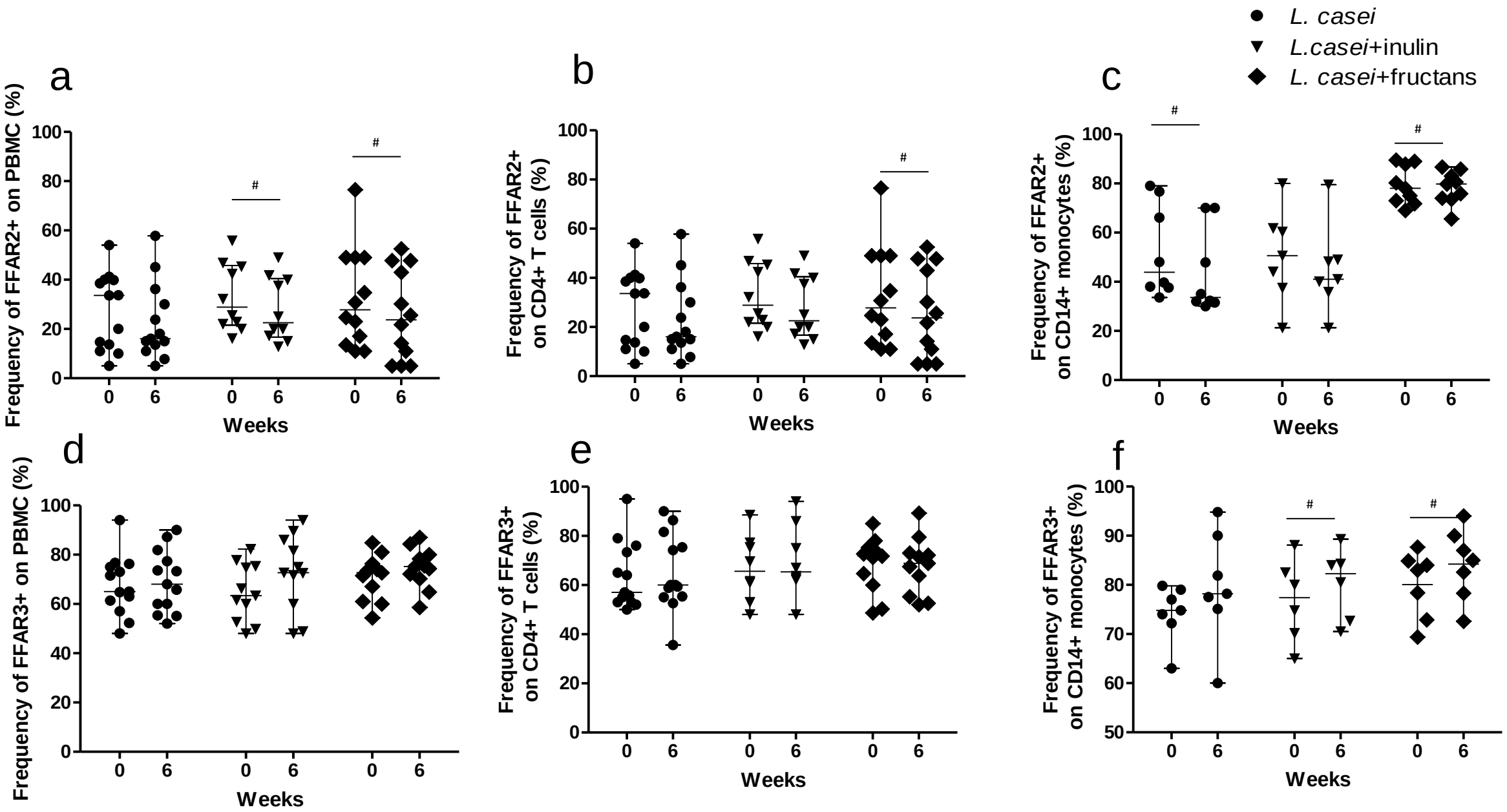

L. casei
L.casei+inulin
L. casei+fructans
a
b
c
d
e
f
Frequency of FFAR2+ on PBMC (%)
Frequency of FFAR2+ on CD4+ T cells (%)
Frequency of FFAR2+ on CD14+ monocytes (%)
Frequency of FFAR3+ on PBMC (%)
Frequency of FFAR3+ on CD4+ T cells (%)
Frequency of FFAR3+ on CD14+ monocytes (%)
Weeks
0
6
#

Fig 6

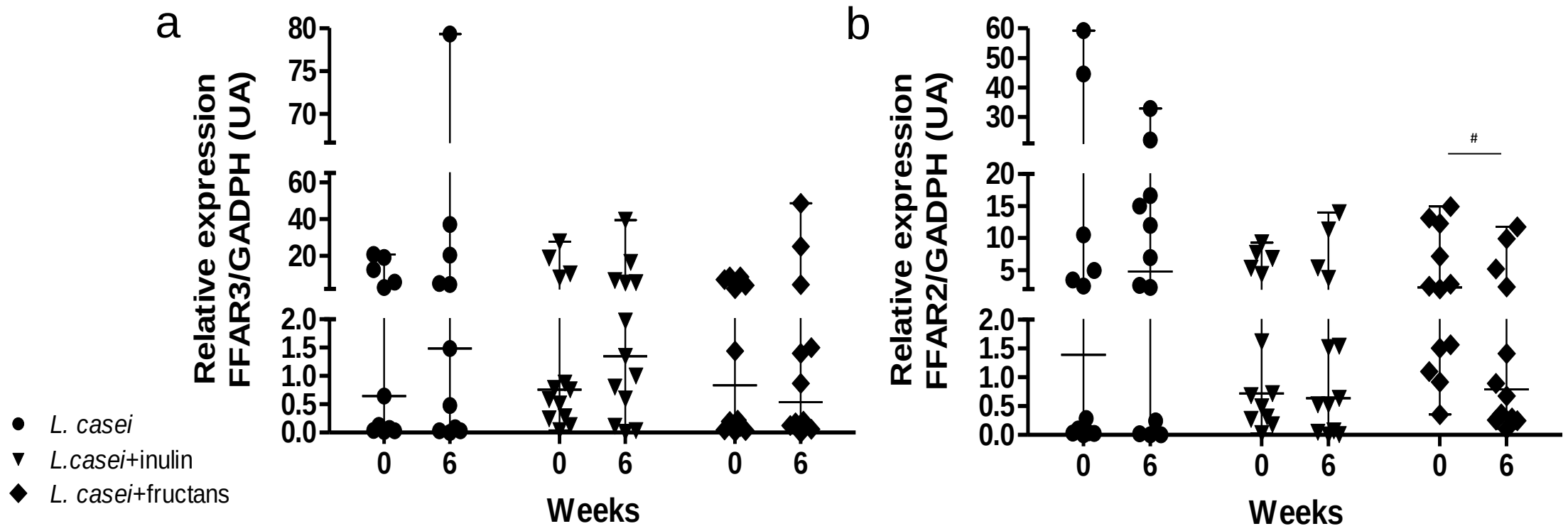

a
Relative expression FFAR3/GADPH (UA)
80
75
70
60
40
20
2.0
1.5
1.0
0.5
0.0
0
6
0
6
0
6
Weeks
L. casei
L.casei+inulin
L. casei+fructans
b
Relative expression FFAR2/GADPH (UA)
60
50
40
30
20
15
10
5
2.0
1.5
1.0
0.5
0.0
#
0
6
0
6
0
6
Weeks

Fig 7

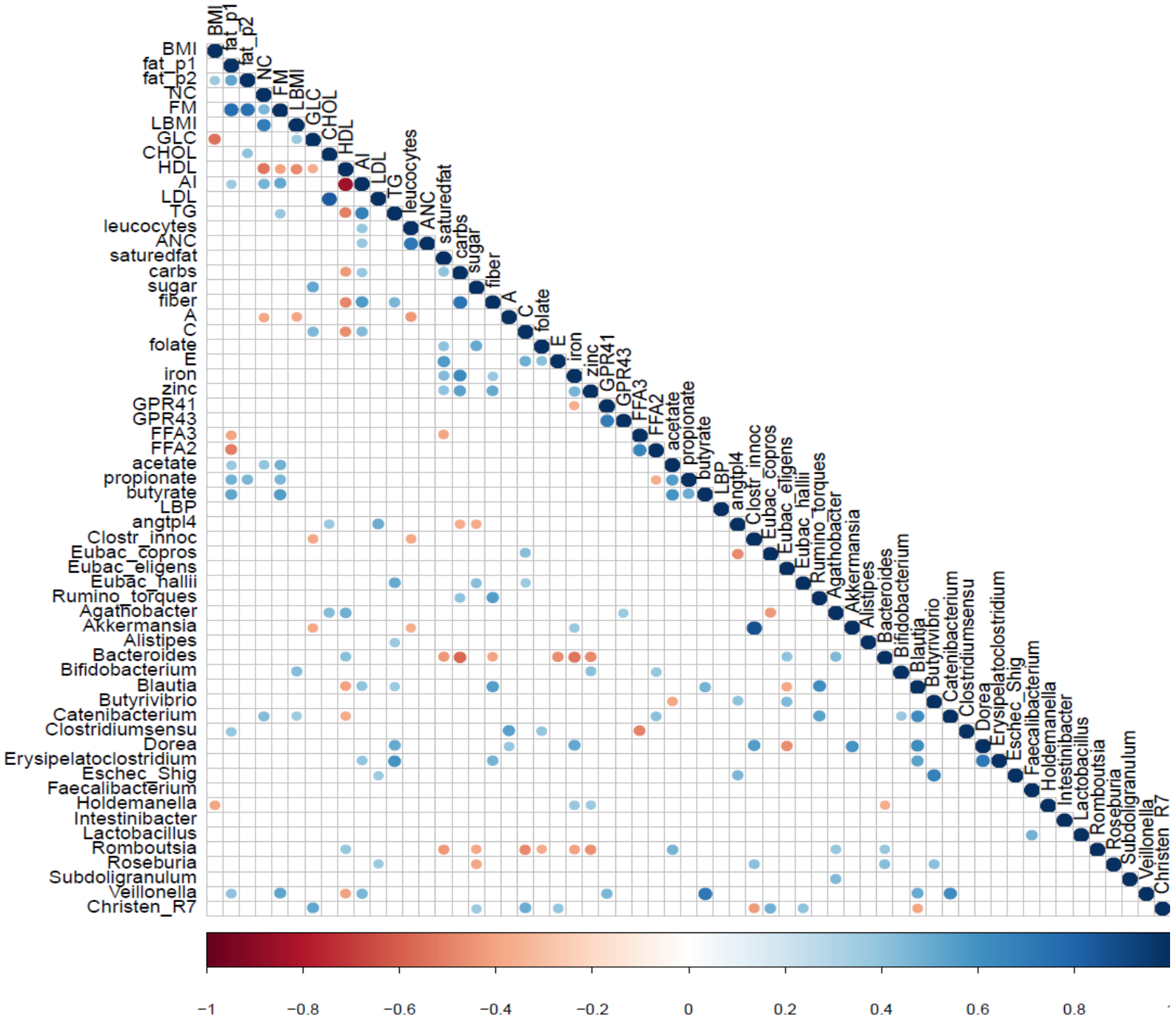
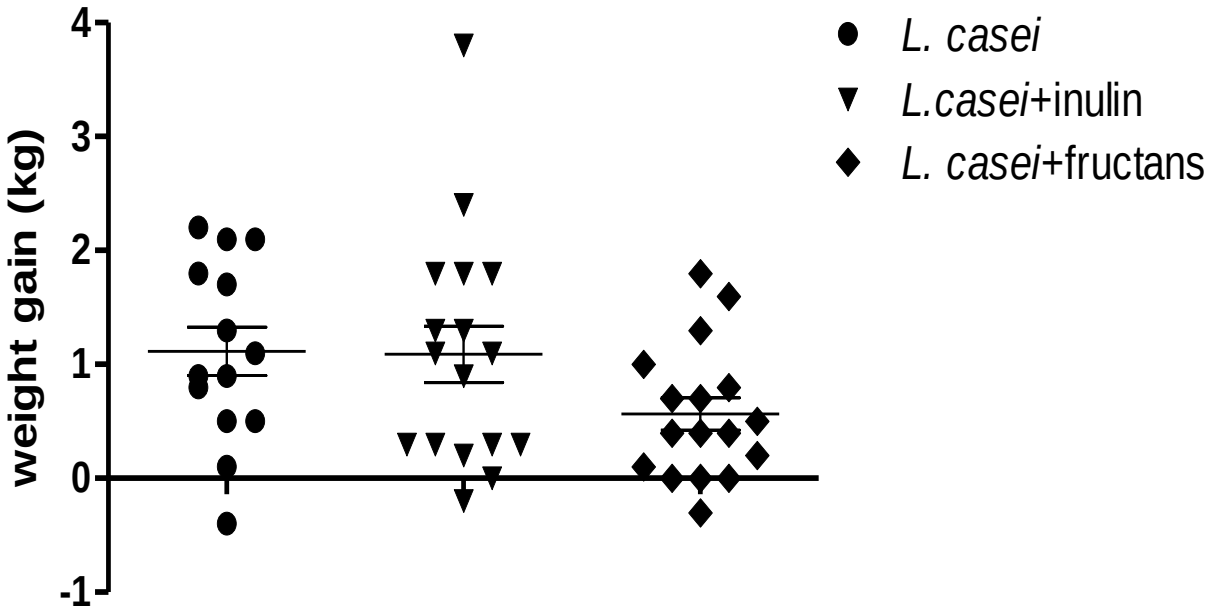

BMI
fat_p1
fat_p2
NC
FM
LBMI
GLC
CHOL
HDL
AI
LDL
TG
leucocytes
ANC
saturedfat
carbs
sugar
fiber
A
C
folate
E
iron
zinc
GPR41
GPR43
FFA3
FFA2
acetate
propionate
butyrate
LBP
angtpl4
Clostr_innoc
Eubac_copros
Eubac_eligens
Eubac_hallii
Rumino_torques
Agathobacter
Akkermansia
Alistipes
Bacteroides
Bifidobacterium
Blautia
Butyrivibrio
Catenibacterium
Clostridiumsensu
Dorea
Erysipelatoclostridium
Eschec_Shig
Faecalibacterium
Holdemanella
Intestinibacter
Lactobacillus
Romboutsia
Roseburia
Subdoligranulum
Veillonella
Christen_R7
−1
−0.8
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8

Fig 8



Supplementary figure1. Effects of probiotic and synbiotics consumption on weight gain.