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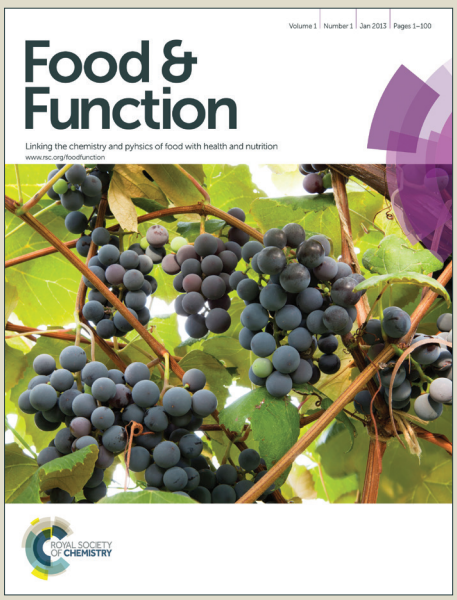

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Structural differences of prebiotic oligosaccharides influence their capability to enhance iron absorption in deficient rats

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Abstract

This study evaluates the influence of novel galacto-oligosaccharides derived from lactulose (GOS-Lu), kojibiose or 4'-galactosyl-kojibiose in hematological parameters of Fe homeostasis using Fe-deficient animals. Liver TfR-2, IL-6, NFκB and PPAR-γ expression (mRNA) were also determined by RT-qPCR analyses, and active hepcidin peptide production and short chain fatty acids by LC coupled to MS/MS or UV detection. Feeding animals with GOS-Lu or kojibiose together with $FeCl_3$ increased hemoglobin (Hb) production (by 17%) and mean Hb concentration into erythrocytes relative to animals administered with $FeCl_3$ alone (14.1% and 19.7%, respectively). Animals administered with prebiotics showed decreased plasmatic hepcidin levels, contributing to a higher intestinal absorption of the micronutrient. These data indicate that concurrent administration of these potentially prebiotic oligosaccharides together with a supplement of Fe ameliorates inflammation-mediated perturbations in the liver, according to the particular structure of the prebiotic compound, and result an attractive strategy to improve Fe absorption.

Keywords: Prebiotics, oligosaccharides, iron homeostasis, hepcidin, inflammation.

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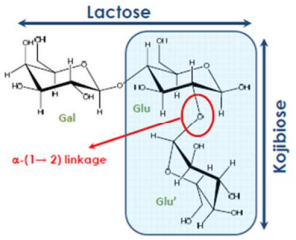

Structural differences of prebiotics improve Fe homeostasis in a Fe-deficient animals decreasing the liver secretion of inflammatory hepcidin peptide

1. INTRODUCTION

Iron (Fe) deficiency is the most prevalent nutrient deficiency worldwide, affecting nearly 2 billion people, particularly populations at risk such as women and children.[1] This nutritional deficiency is associated, among others, to aggravated severity of diseases based in defective function of immune responses. [2] In this sense, it is widely accepted the dynamic mutualism between the host and the commensal microbiota which has deep implications for health, and contributes to the maintenance of intestinal immune homeostasis. The intestinal tract harbors a massive and diverse microbiota, including both anaerobes and aerobes, containing at least 100 times as many genes as within our own genome with an enormous impact in the digestion of dietary compounds, salvage of energy, supply of (micro)nutrients and transformation of xenobiotics. [3] The composition of this bacterial ecosystem is dynamic and potentially modifiable in response to dietary factors.

Prebiotics are selectively fermentable ingredients that induce specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring potential benefit(s) on host health. [4] This concept assumes that they exert major effects in the colon, where most of gastrointestinal microorganisms live. However, accumulating evidences demonstrated that prebiotics also influence mineral absorption, which takes place mainly in the upper part of the intestine. The most compelling data have demonstrated the prebiotic-promoted positive effects increasing calcium and magnesium absorption and also that zinc balance can be improved by their consumption.[5] However, data from *in vivo* studies have showed conflicting data about iron absorption. [6,7]

Fermentation of prebiotics leads to the production of short chain fatty acids (SCFA) able to modulate cytokines secretion [7] and stimulate mucin production. [9] Prebiotics such as sialyl-lactose and Raftilose P95 (oligofructose) promoted anti-inflammatory effects via activation of peroxisome proliferator activator receptor (PPAR)-γ. [10] Other inflammatory mediators such interleukin (IL)-6 and the hepatic hepcidin have important roles in iron homeostasis. [11] Anemia of chronic diseases usually occurs as secondary to infections and it is characterized by an immune activation with an increase in inflammatory cytokines and hepcidin levels. [12]

In humans, recent research about the influence of prebiotics on iron absorption studied the influence in absorption processes and nutritional status concerning the micronutrient. [7] These studies mainly investigated the effects of fructooligosaccharides

(FOS) such as inulin showing positive trend in the fractional iron absorption in women with low iron status, although this influence did not result statistically significant. The effects of inulin on iron absorption appear of much higher magnitude in pig [6] and rat models. [13] Similarly to inulin, feeding galactooligosaccharides (GOS) with a degree of polymerization of 2-6 to young healthy men did not improve nutritional biomarkers of iron status. [14] A common conclusion of these studies is that inulin does not interfere with the molecular mechanisms of iron absorption. To the best of our knowledge there are no experimental data with in vivo animal models about the influence of either GOS derived from lactulose (GOS-Lu) and kojibiose (2-*O*-α-D-glucopyranosyl-α-D-glucopyranose) as well as 4'-galactosyl-kojibiose on iron absorption.

Likewise, scarce data are available associating micronutrient intake with markers of inflammation. [11,15] Data from human studies provided evidences about the positive effects of inulin in inflammatory processes [16] preventing impaired iron homeostasis. This is concordant with the results from a long-term feeding study indicating the negligible impact of consumption of prebiotic and *Bifidobacterium lactis* HN019 fortified milk on nutritional Fe indicators, although, the proportion of children with Fe deficiency was reduced by 39%. [17] These positive benefits could also be favored by bifidobacteria-mediated influence in liver Fe homeostasis. [18]

In the present study an *in vivo* iron-deficient rat model was used to evaluate the influence of a mixture of novel GOS-Lu, kojibiose or 4'galactosyl-kojibiose on restoration of hemoglobin (Hb) levels and liver expression of inflammatory biomarkers and hepcidin production. SCFA profile in the colon contents of the different experimental groups was measured to monitor the effect of prebiotic compounds on gut microbiota metabolic activity.

2 MATERIALS AND METHODS

2.1 Enzymatic synthesis of the studied potentially prebiotic oligosaccharides

Enzymatic synthesis of GOS derived from lactulose (GOS-Lu) was carried out via the hydrolysis and transgalactosylation of the prebiotic carbohydrate lactulose (Duphalac, Solvay Pharmaceuticals) by using a β-galactosidase from *Aspergillus oryzae* and following the procedure described elsewhere [19] with slight modifications.

Oligosaccharide mixture with high proportion (i.e., 44%) of 4'-galactosyl-kojibiose (*O*-β-D-galactopyranosyl-(1→4)-*O*-(α-D-glucopyranosyl-(1→2))-α-D-glucopyranose) was obtained through a biotechnological process based on the dextransucrase-catalysed

synthesis followed by a purification step with β-galactosidase hydrolysis and yeast treatment. Enzymatic synthesis was conducted with a dextransucrase from *L. mesenteroides* B-512F by the transfer of a glucosyl unit from the hydrolysis of sucrose to lactose acceptor through the formation of an α-(1→2)-glucosyl bond. [20] The oligosaccharide mixture obtained (38.5% lactose, 31.2% 4'-galactosyl-kojibiose, 21.3% fructose, 5.2% leucrose, 2.8% lactosucrose, 0.9% glucose and 0.1% sucrose) was purified by *Kluyveromyces lactis* β-galactosidase hydrolysis and *Saccharomyces cerevisiae* yeast treatment in order to reduce the large amount of lactose as well as eliminating residual monosaccharides and sucrose.

Oligosaccharide mixture with high proportion of kojibiose (66%) was obtained from the complete hydrolytic action of a *Kluyveromyces lactis* β-galactosidase on 4'-galactosyl-kojibiose, after removal of residual monosaccharides by using a *Saccharomyces cerevisiae* yeast treatment as previously shown. [21]

2.2 Animals

Forty-two female Wistar albino rats, aged 3 weeks with an average weight of 61.4 ± 5.6 g were obtained from the University of Valencia Animal Service. Animal experiments were carried out in strict accordance with the recommendations include in the Guide for the Care and Use of Laboratory Animals of University of Valencia (SCSIE, University of Valencia, Spain) and the protocol was approved by its Ethic Committee (A1351244049254).

2.3 Experimental design

Animals were randomly distributed into six different groups (n=7 per group), 1) a control group receiving a standard AIN-93G diet, and five iron-deficient groups receiving a AIN-76A diet (Harlan) for 15 days that were subjected to different treatments: 2) administered without $FeCl_3$; 3) administered with $FeCl_3$ (2.5 μg); 4) administered with $FeCl_3$ together with GOS-Lu; 5) administered with $FeCl_3$ together with kojibiose; and 6) administered with $FeCl_3$ together with 4'-galactosyl-kojibiose. Potentially prebiotic oligosaccharides were administered at 0.5% (w/w daily consumption of diet) during two consecutive days. The rats were maintained in an environment of controlled temperature (21–23ºC), humidity (55 %) and light (12 h) – dark (12 h) cycle, with *ad libitum* food and mineral-free water available. Records of weight and food intake were collected daily.

After treatment, rats were anaesthetised (isofluran) and sacrificed by exsanguination. Whole blood samples were preserved in EDTA-treated tubes to prevent coagulation (at room temperature) for haematological analyses and the rest of the blood was used for hepcidin peptide quantification. Sections (±100 mg) of the liver were immersed in RNA *later* buffer (Qiagen, CA, USA) and snap-frozen in liquid nitrogen for gene expression analyses. Colon content samples were kept in 0.5 mL H_2SO_4 (2N) and immediately analysed for SCFA content.

2.4 Hemoglobin (Hb) measurement

Hb concentrations were measured photometrically using cyanmethemoglobin standard solution according to the manufacturer's instructions (Sigma-Aldrich). This method is based on the oxidation of Hb and its derivatives (except sulfhemoglobin) to methemoglobin in the presence of potassium ferricyanide to form cyanmethemoglobin. The absorbance, measured at 540 nm, is proportional to the total Hb concentration.

2.5 Hematological parameters

The number of erythrocytes was calculated by using a Neubauer improved cell counting chamber and hematocrit was estimated by centrifugation of whole blood in microcapillar tubes. Mean corpuscular volume (MCV) was calculated using the following equation: (hematocrit x 10)/nº erythrocytes (10^6/mm^3 blood), and mean corpuscular Hb (MCH) (%) as: (hemoglobin (g/dL)x100)/hematocrit. The globular sedimentation speed (VSG) was determined according to the Westergren's method as proposed by the International Council for Standardization in Hematology (ICSH).

2.6 Real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from liver tissue samples using an RNeasy mini kit (Qiagen) following the protocol provided by the manufacturer. One microgram of total RNA was converted to double-stranded cDNA using AMV Reverse Transcriptase (Promega, WI, USA). PCR was performed with primers designed for the following *Rattus norvegicus* genes: Hamp (forward: 5'- AGC GGT GCC TAT CTC CGG CA-3'; reverse: 5'- CGG AGG GGA GGC AGT GTG TTG-3'); TfR2 (forward: 5'- GGC AGA GTG GTC GCT GGG TG -3'; reverse: 5'- GGC CAG AGC TCG GCA GTG TG -3'); IL-6 (forward: 5´-TCT CGA GCC CAC CAG GAA C -3´; reverse: 5´-AGG GAA GGC AGT GGC TGT CA -3´); NFκB (forward 5'- CTT CTC GGA GTC CCT CAC TG-3',

reverse 5'- CCA ATA GCA GCT GGA AAA GC-3') ; PPARγ (forward 5'- TGA TCC TAC GGC CAG ACA GA-3', reverse 5'-GGG AGG TTG TCC CTG GAA TG-3') and β-actin (forward 5'- CTC TTC CAG CCT TCC TTC CT-3'; reverse 5'- TAG AGC CAC CAA TCC ACA CA-3'), the latter used as a housekeeping gene. The PCR mix (20 μL reaction volume) consisted of 7.5 μL SYBR Green I master mix, 1.3 μmol/L primers, and 2.5 μL cDNA. PCR reactions were performed in triplicate in a LightCycler 480 (Roche) with the following program: 1 cycle at 95 ºC for 5 min, 35 cycles at 60 ºC for 20 s and 72 ºC for 45 s. Samples of each animal tissue were measured in duplicate and gene expression was expressed as fold-change. The relative mRNA expression of the tested gene compared to β-actin expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7 Quantification of hepcidin

All sample preparation steps were performed at room temperature as previously described. [18] Briefly, aliquots (50 μL) of plasma were mixed with 100 μL aliquot of acetonitrile (Burdick and Jackson, Muskegon, MI, USA) by pipetting. The samples were then centrifuged at 3,000 x *g* for 10 minutes at 4°C (Jouan, Winchester, VA, USA) and the supernatant (100 μL) was mixed with 0.02% *(v/v)* aqueous acetic acid. The analysis was performed on an Agilent HPLC system connected on line to quadrupole ion trap mass spectrometer (Bruker Daltonics, Billerica, MA) via an electrospray interface. The HPLC system was equipped with a quaternary pump, an in-line degasser, an automatic injector, and a variable wavelength absorbance detector set at 214 nm (1200 Series, Agilent Technologies, Waldbronn, Germany). The column used in these analyses was a BioBasic C_{18} (250 × 4.6 mm, 5 μm particle size) (Thermo, Waltham, MA, USA). The mobile phases consisted of, trifluoroacetic acid/isopropanol/water (0.125/1/500, *v/v/v*, A) and trifluoroacetic acid/isopropanol/water/methanol/acetonitrile (0.125/1/50/350/100, *v/v/v/v/v*, B). Aliquots (50 μL) of the precipitation supernatants were injected in each cycle and the analysis was performed using the following gradient: 0 min, 5 % B; 30 min, 90 % B; 33 min, 100 % B; 35 min, 100 % B; 45 min, 5 % B. Two independent samples from each animal were analyzed.

2.8 Analysis of short chain fatty acids (SCFA)

Aliquots (0.17 ± 0.04 g) of colon samples were kept in 0.2 mL of 2N H_2SO_4. The samples were homogenized (1 min) using a TissueRuptor (Qiagen) and vortexed for 30

s. Afterwards, the mixtures were centrifuged (10,000 x *g*, 10 min) and the supernatant was collected and diluted (1:20) in deionized water prior filtration (0.45 µm, MillexGN, Millipore).

The quantification of organic acids was carried out on 1200 Agilent HPLC system equipped with a multisolvent pump and a wavelength absorbance detector set at 214 nm (1200 Series, Agilent Technologies, Waldbronn, Germany). The separation was performed on a BioBasis C_{18} column (250 x 4.6 mm, 5 µm particle size) (Thermo, Waltham, MA, USA). The elution was performed using 1% acetronitrile in 20 mM phosphate buffer adjusted to pH 2.20 with phosphoric acid (A), water/acetronitrile (80/20, *v/v*, B) according to the following gradient: 0 min, 0 % B; 5 min, 0 % B; 12 min, 10 % B; 19 min, 10 % B. The following organic acids were analyzed: formic acid, acetic acid, propionic acid, *D*-/*L*-Lactic acid, *i*-butyric acid, *i*-valeric acid.

2.9 Statistical analysis

Statistical analyses were performed using SPSS v.15 software (SPSS Inc., Chicago, IL, USA). Variance analysis by one-way method was used to compare the influence of feeding different prebiotic compounds in the iron-deficient groups of animals. Individual means were tested using pair-wise comparison with Tukey's multiple comparison test when effects were significant. Statistical significance was established at $P<0.05$ for all comparisons.

3. RESULTS

3.1 Effects in hematological parameters and hepatic expression of transferrin receptor (TfR2)

Animals fed with the Fe-deficient diet alone showed a significant decrease in hemoglobin (Hb) concentrations compared to animals fed with the standard diet (**Table 1**). In this period of treatment (15 days) there were not provoked changes in the hematocrit, which have been reported to occur after 20 days in animals under a Fe-deficient diet. [22] Nevertheless, the decrease in mean corpuscular hemoglobin concentration (MCH) became significantly ($P<0.05$) reduced in animals receiving the Fe-deficient diet. Animals administered with the supplement of Fe alone showed normalized Hb concentrations, but lower ($P<0.001$) than values quantified in controls. Notably, there were not quantified significant ($P>0.05$) alterations between the Hb concentration of animals administered with the supplement of Fe together with the

prebiotic compounds GOS-Lu or kojibiose. This prebiotic-promoted positive effect in Hb concentration was not observed in animals administered with 4'-galactosyl-kojibiose. A similar trend with higher increases in MCH was calculated in animals administered with GOS-Lu and kojibiose in comparison to 4'-galactosyl-kojibiose or the supplement of Fe alone. There were not significant (P>0.05) changes in body weight gain in animals fed with the Fe-deficient diet compared to the controls at the end of period of study (*data not shown*).

Changes in TfR2 expression (mRNA) levels in animals administered with the supplement of Fe alone or together with the oligosaccharides assayed are shown in **Figure 1**. Animals administered with the supplement of Fe alone showed a trend increasing (P>0.05) TfR2 expression levels in comparison to controls with normal Hb concentration. Feeding the supplement of Fe together with GOS-Lu or kojibiose did not affect TfR2 expression values. Notably, feeding animals with 4'-galactosyl-kojibiose down-regulated (P=0.001) TfR2 expression levels compared to controls, but to similar (P=0.28) levels found in animals fed with the Fe-deficient diet.

3.2 Effects in hepcidin production and liver biomarkers

The consumption of Fe-deficient diet did not provoke alterations in bioactive hepcidin peptide production relative to animals fed with the Fe-adequate diet (**Figure 2**). Animals administered with the supplement of Fe alone exhibited a significantly (P=0.029) increased circulating hepcidin concentration. These animals showed hepcidin concentrations up to 1.64-fold that of control animals demonstrating the physiological inflammatory response at liver level. However, the concurrent administration of the supplement of Fe and all prebiotic compounds studied tended to decrease to similar values the circulating concentration of hepcidin that not differed from the control group.

Changes in hepatic NFκB, IL-6 and PPARγ expression levels in the different treatment groups are shown in **Figure 1**. Nutritional deficiency of iron induced NFκB (Nuclear Factor Kappa-B) expression (mRNA), which was not normalized neither by the administration of the Fe supplement alone or together with none of the prebiotic compounds. Additionally, Fe-deficient animals showed increased IL-6 expression (mRNA) compared to control animals as well as those groups administered with the supplement of Fe alone and together with kojibiose. However, there were not significant differences in IL-6 mRNA levels in animals administered with GOS-Lu or 4'-galactosyl-kojibiose relative to controls. All animals from the different treatment groups

showed an increased PPARγ expression (mRNA) compared to controls. The administration of the supplement of Fe alone decreased PPARγ expression values relative to Fe-deficient animals. Besides, animals administered with the Fe supplement together with the different prebiotic structures revealed significant differences affecting PPARγ expression in the different treatment groups.

3.3 Effects in short chain fatty acids (SCFA) production

The concentration of several different SCFA quantified in colon contents of animals from the different groups of treatment are shown in **Table 2.** Significant differences in the concentration of formic acid and *i*-valeric acid in colon content of Fe-deficient animals were found as compared to controls. On the other hand, the level of acetic, propionic, *i*-butyric and *i*-valeric acid was higher in animals administered with the supplement of Fe alone than those fed with the standard diet. Neither the concentration of propionic acid nor butyric acid in both Fe-adequate or deficient groups presented significant (P>0.05) correlation with Hb levels. Animals fed the concurrent administration of the supplement of Fe together with GOS-Lu showed decreased concentration of *i*-valeric acid relative to animals administered with the supplement of Fe alone. Feeding animals with the supplement of Fe and kojibiose changed, but not significantly (P=0.065), the mean value for formic acid and normalized the levels of *i*-valeric acid to values similar to concentrations found in controls. Animals administered with 4'-galactosyl-kojibiose exhibited lower concentration of formic, acetic, propionic, *i*-butyric and *i*-valeric acids in comparison to animals fed with the supplement of Fe alone.

4. DISCUSSION

This study demonstrated the rapid restoration of normal Hb levels in Fe-deficient animals fed with potential prebiotics such as GOS-Lu and kojibiose together with a supplement of $FeCl_3$, to even higher (by 14%) mean values than those quantified in animals fed only with the supplement of Fe. This positive effect in Fe absorption is clearly evident in the increased MCH calculated in animals fed kojibiose. In contrast, 4'-galactosyl-kojibiose showed a much less influence on the studied hematological parameters. These results highlight the role played by the oligosaccharide structure in mineral absorption.

The assayed oligosaccharides have been comprehensively characterized prior to this study. GOS-Lu is a complex mixture predominantly dominated by the presence of di- and trisaccharides (31% and 42%, respectively) followed by tetrasaccharides (24.6%) and pentasaccharides in trace amounts. [23] The disaccharide fraction was mainly composed of galactosyl-fructoses with 1→1, 1→4 (i.e., lactulose), 1→5, and 1→6 glycosidic linkages, in addition to galactobioses linked by 1→1, 1→2, 1→3, 1→4, and 1→6 glycosidic linkages, whereas the trisaccharide fraction was mainly composed by the trisaccharide 6′-galactosyl-lactulose. [24]

In the case of the mixture obtained with high proportion of kojibiose (2-*O*-α-D-glucopyranosyl-α-D-glucopyranose), carbohydrate composition determined by GC-FID was as follows: 66% kojibiose, 20% leucrose, 8% yeast metabolites and 6% trisaccharides. Kojibiose was purified by LC-RID and identified by GC-MS. [21]

Carbohydrate composition of the mixture with high proportion of 4'-galactosyl-kojibiose was determined by gas chromatography with flame ionization detector showing that it was composed of 44% 4'-galactosyl-kojibiose, 30% galactosylated derivatives, 13% kojibiose, 8% leucrose, 3% lactose and 2% yeast metabolites (minor amounts of polyalcohols and organic acids). 4'-galactosyl-kojibiose was isolated and chromatographically purified by LC-RID from the reaction mixture obtained and then fully characterized by 1D and 2D (^{1}H, ^{1}H) and (^{1}H-^{13}C) nuclear magnetic resonance studies (gCOSY, TOCSY, ROESY, multiplicity-edited gHSQC, and gHMBC). [20]

These results evidence the avidity of Fe-deficient organisms for the micronutrient and taken together with previous studies with humans [7,14] indicate that prebiotic-mediated positive effects on Fe homeostasis are mostly relevant when suffering nutritional deficiency of the micronutrient. These human studies also stressed that the magnitude of the prebiotic-mediated effects seemed to be conditioned by the nutritional status on the micronutrient of the subjects. For example, feeding GOS to young healthy men did not improved nutritional biomarkers of Fe status. [14] Further studies in women with low iron status also pointed out the inulin-promoted improved (P<0.05) fractional iron absorption. [7]

Previous research associated the dietary micronutrient intake with markers of inflammation, which effects that can be aggravated after long-term consumption. [15] Although, Fe absorption is tightly regulated and controlled at intestinal level these processes are influenced by the hepatic production of inflammatory mediators such as hepcidin [18, 25] and IL-6. [18] Serum hepcidin displays an inverse relationship with Fe

intestinal absorption either from foods or dietary supplements. [11, 18] Thus, prebiotic-mediated decrease in the production of hepcidin (Fig. 2) is reflected in improved hematological parameters such as Hb and MCH (Table 1). These anti-inflammatory effects appear to be dependent on the prebiotic structure considered and can explain, at least in part, their differential capacity to influence Fe absorption. Human and experimental animal models indicate that control of liver inflammatory processes in response to free Fe can result in an improved iron homeostasis and nutritional status.

The effects and influence in liver physiology of the prebiotic compounds assayed could be explained by differences in the monomer and linkage type of the different prebiotic compounds tested that also could influence their prebiotic selectivity. Data from animal studies have demonstrated the prebiotic effect of GOS-Lu (1% *w/w* diet for 14 days) increasing the numbers of beneficial bifidobacteria and lactobacilli together with the number of *Eubacterium rectale*/*Clostridium coccoides* group and bacteroidetes. [26] However, from *in vitro* studies it has been calculated a high prebiotic index for kojibiose promoting increases in the numbers of bifidobacteria, but not for lactobacilli, *Eubacterium rectale*/*Clostridium coccoides* and bacteroidetes group. [27] In this context, it has been reported the inhibition of NFκB signaling by anaerobic commensal bacteria, particularly *Bacteroides thetaiotaomicron* exerted potential anti-inflammatory effects by promoting nuclear export of NFκB subunit relA in complex with PPAR-γ.[28] However, the GOS-Lu mediated increase in *Bacteroides* spp. it is not reflected in a significant down-regulation of NFκB expression (mRNA) in this group of treatment (Fig. 1).

PPAR-γ expression is also associated to insulin signaling and inhibition of monocyte and macrophage inflammatory responses by preventing the activation of nuclear transcription factors, such as NFκB, activating Protein-1 and STAT1 (Signal Transducer and Activator of Transcription-1. [29] Inulin is a heterogeneous collection of fructose polymers (glucosyl and fructosyl moieties), which are linked by β(2→1) bonds and a degree of polymerization ranged from 2 to 60. Besides, the prebiotic structures used in the present study are galactooligosaccharides, which are linked by α(1→2)-glucosyl bonds and predominantly dominated by the presence of di- and trisaccharides. These structural differences could explain the different expression patterns in relation to PPAR and NFB (Fig. 1). Positive prebiotic-mediated effects on gene expression have been evidenced in newborn animals because of the different regulation of circulating satiety hormones and genes involved in glucose transport and energy metabolism in offspring. [30] Findings supporting the influence of dietary prebiotics in modulation of gut

388 microbiota or their direct influence in PPAR-γ expression suggest a potential use for
389 prebiotics in type-2 diabetes, hypertension in the absence of obesity and, a number of
390 components of the metabolic syndrome. [31] Notably, the data presented in this study
391 revealed significant differences of the prebiotic compounds assayed in PPAR expression
392 evidencing the importance of the prebiotic structure on their potential physiological
393 effects and utilization as adjuvants in therapeutic strategies.

394 Fermentation of prebiotics with the subsequent production of SCFA plays a pivotal
395 role in some beneficial activities in the gut. [32] Deficiencies in Fe absorption processes
396 have been associated to acetic-induced inhibition of glucose metabolism in diabetic
397 animal models. [33] In good accordance with these data, the decreased colon acetic acid
398 concentration in the groups of animals fed with GOS-Lu and kojibiose likely associated
399 inversely with MCH (Table 1), but not in animals fed with the 4'-galactosyl-kojibiose.
400 Otherwise, the higher production of both propionic and butyric acid in the groups
401 administered with the prebiotics GOS-Lu and kojibiose compounds could be
402 hypothesized to favor low oxidative stress, due to Fe incorporation into cells, reflected
403 in improved MCH values. This hypothesis is supported by the interaction of propionates
404 with heme oxygenase leading to the production of precursors to the powerful
405 antioxidant bilirubin. [34] Also, ketone body D-β-hydroxybutyrate (βOHB) has been also
406 reported as an endogenous and specific inhibitor of class I histone deacetylases reducing
407 oxidative stress. [35] It cannot be ruled out that butyrate and propionate constitute the main
408 source of energy for host colonocytes and are also important for gastrointestinal health,
409 immunity, and host metabolism contributing to maintain angiopoietin-like protein 4
410 (ANGPTL4) levels stimulating additional routes to gut microbiota. [36]

411 Overall, the data presented indicate that administration of certain prebiotic structures
412 could help preventing cellular alterations as consequence of the dietary micronutrient
413 intake.

414

415 **5. CONCLUSIONS**

416 The data reported herein on the influence of different prebiotic compounds, reveal
417 novel findings on how structural differences of prebiotics can affect Fe homeostasis in a
418 Fe-deficient animal model. Physiological response(s) in Fe homeostasis can be
419 modulated by the concurrent administration of supplements of Fe together with
420 prebiotics. The data reported point to prebiotic-mediated beneficial effects to liver
421 function that are reflected in higher Hb concentration (up to 17%) improving nutritional

status of the micronutrient. Accordingly, animals administered with GOS-Lu and kojibiose showed significantly higher MHC than those administered with the supplement of Fe alone. Moreover, feeding animals with potential prebiotic compounds decreased the Fe supplement-induced liver secretion of inflammatory bioactive hepcidin peptide, thus contributing to an improved intestinal absorption of the micronutrient. These effects were accompanied of different expression patterns of liver iron sensing biomarkers indicating their different influence in the cross-talk within the gut-liver axis. The fact that infants constitute a population at a high risk to suffer iron deficiency, points out the attractive potential use prebiotics in the formulation of infant foods for improving bowel function due to its prebiotic function and prevent the risk of nutritional deficiency in iron. However, further human trials are needed to support the clinical relevance of this potential nutritional intervention.

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References

1 E. McLean, M. Cogswell, I. Egli, D. Wojdyla and B. de Benoist, Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. Public Health Nutr 2009, **12**, 444-454.

2 H. Abu Daya, B. Lebwohl, S.K. Lewis and P.H. Green, Celiac disease patients presenting with anemia have more severe disease than those presenting with diarrhea. Clin Gastroenterol Hepatol, 2013, **11**, 1472-1477.

3 R.I. Mackie, A. Sghir and H.R. Gaskins, Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 1999, **69**, 1035S-45S.

4 ISAPP (www.isapp.net). 6th Meeting of the International Scientific Association of Probiotics and Prebiotics. 2008, London, Ontario, Canada.

5 K.E. Scholz-Ahrens, G. Schaafsma, E.G. van den Heuvel and J. Schrezenmeir, Effects of prebiotics on mineral metabolism. Am J Clin Nutr 2001, **73**, 459S-464S.

6 K. Yasuda, K.R. Roneker, D.D. Miller, R.M. Welch and X.G. Lei, Supplemental dietary inulin affects the bioavailability of iron in corn and soybean meal to young pigs. J Nutr 2006, **136**, 3033-3038.

7 N. Petry, I. Egli, C. Chassard, C. Lacroix and R. Hurrell, Inulin modifies the bifidobacteria population, fecal lactate concentration, and fecal pH but does not influence iron absorption in women with low iron status. Am J Clin Nutr 2012, **96**, 325-331.

8 C.R. Cavaglieri, A. Nishiyama, L.C. Fernandes, R. Curi, E.A.Miles, and P.C. Calder, Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. Life Sci, 2003, **73**, 1683-1690.

9 M. Capuano, L. Iaffaldano, N. Tinto, D. Montanaro, V. Capobianco, V. Izzo, F. Tucci, G. Troncone, L. Greco and L. Sacchetti, MicroRNA-449a overexpression, reduced NOTCH1 signals and scarce goblet cells characterize the small intestine of celiac patients. PLoS One, 2011, **6**, e29094.

10 M. Zenhom, A. Hyder, M. de Vrese, K.J. Heller, T. Roeder and J. Schrezenmeir, Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinal Caco-2 cells via activation of PPARγ and peptidoglycan recognition protein 3. J Nutr 2011, **141**, 971-977.

11 M.F. Young, R.P. Glahn, M. Ariza-Nieto, J. Inglis and G. Olbina, Serum hepcidin is significantly associated with iron absorption from food and supplemental sources in healthy young women. Am J Clin Nutr 2009, **89**, 533-538.

12 N. Gangat and A.P. Wolanskyj, Anemia of chronic disease. Seminars in Hematology 2013, **50**, 232-238.

13 K. de Cássia Freitas, O.M.S. Amancio and M.B de Morais, High-performance inulin and oligofructose prebiotics increase the intestinal absorption of iron in rats with iron deficiency anaemia during the growth phase. Br J Nutr 2012, **108**, 1008-1016.

14 E.G. van den Heuvel, G. Schaafsma, T. Muys and W. van Dokkum, Nondigestible oligosaccharides do not interfere with calcium and nonheme-iron absorption in young, healthy men. Am J Clin Nutr 1998, **67**, 445-451.

15 E.K. Lund, S.J. Fairweather-Tait, S.G. Wharf and I.T. Johnson, Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. J Nutrition 2001, **131**, 2928-2931.

16 P. Dehghan, B.P. Gargari, M.A. Jafar-Abadi and A. Aliasgharzadeh, Inulin controls inflammation and metabolic endotoxemia in women with type 2 diabetes mellitus: a randomized-controlled clinical trial. International J Food Sci Nutr, 2014, **65**, 117-123.

17 S. Sazawal, U. Dhingra, G. Hiremath, A. Sarkar, P. Dhingra, A. Dutta, V.P. Menon and R.E. Black, Effects of Bifidobacterium lactis HN019 and prebiotic oligosaccharide added to milk on iron status, anemia, and growth among children 1 to 4 years old. J Pediatr Gastr Nutr 2010, **51**, 341-346.

18 J.M. Laparra, M. Olivares and Y. Sanz, Oral administration of Bifidobacterium longum CECT 7347 ameliorates gliadin-induced alterations in liver iron mobilisation. Br J Nutr 2013, **9**, 1-9.

19 A. Clemente, O. Hernández-Hernández, J.M. Laparra, A. Montilla, F.J. Moreno, A. Olano, L. Ruiz, M.L. Sanz M and Y. Sanz, Y. (2011) Multi-functional galactooligosaccharides derived from lactulose with immunomodulatory and prebiotic activities. Spanish patent, 2011, P201130784.

20 M. Díez-Municio, A. Montilla, M.L. Jimeno, N. Corzo, A. Olano and F.J. Moreno, Synthesis and characterization of a potential prebiotic trisaccharide from cheese whey permeate and sucrose by Leuconostoc mesenteroides dextransucrase. J Agricl Food Chem 2012, **60**, 1945-1953.

21 M. Díez-Municio, A. Montilla, F.J. Moreno and M. Herrero, M., A sustainable biotechnological process for the efficient synthesis of kojibiose. Green Chem 2014, **16**, 2219-2226.

22 M.J. Alférez, J. Díaz-Castro, I. López-Aliaga, M. Rodríguez-Ferrer, L.J. Pérez-Sánchez and M.S. Campos, Development of nutritional iron deficiency in growing male rats: haematological parameters, iron bioavailability and oxidative defence. Br J Nutrition 2011, **105**, 517-25.

23 M.C. Marín-Manzano, L. Abecia, O. Hernández-Hernández, M.L. Sanz, A. Montilla, A. Olano, L.A. Rubio, F.J. Moreno and A. Clemente, Galacto-oligosaccharides derived from lactulose exert a selective stimulation on the growth of Bifidobacterium animalis in the large intestine of growing rats. J Agric Food Chem 2013, **61**, 7560-7567.

24 O. Hernández-Hernández, F. Montañes, A. Clemente, F.J. Moreno and M.L. Sanz, Characterization of galactooligosaccharides derived from lactulose. 2011 J Chromatogr A 2011, **1218**, 7691-6.

25 S. Jenkitkasemwong, M. Broderius, H. Nam, J.R. Prohaska and M.D. Knutson, Anemic copper-deficient rats, but not mice, display low hepcidin expression and high ferroportin levels. J Nutrition, 2010, 140, 723-730.

26 O. Hernández-Hernández, M.C. Marín-Manzano, L.A. Rubio, F.J. Moreno, M.L. Sanz and A. Clemente, Monomer and linkage type of galacto-oligosaccharides affect their resistance to ileal digestion and prebiotic properties in rats. J Nutr 2012, **142**, 1232-1239.

27 M.L. Sanz, G.R. Gibson and R.A. Rastall, Influence of disaccharide structure on prebiotic selectivity in vitro. J Agric Food Chem, 2005, **53**, 5192-5199.

28 D. Kelly, J.I. Campbell, T.P. King, G. Grant, E.A. Jansson, A.G. Coutts, S. Pettersson and S. Conway, Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-γ and RelA. Nature Immunology, 2004, **5**, 104-12.

29 M. Viladomiu, R. Hontecillas, L. Yuan, P. Lu and J. Bassaganya-Riera, J. (2013) Nutritional protective mechanisms against gut inflammation. J Nutr Biochem 2013, **24**, 929-939.

30 A.D. Maurer and R.A. Reimer, Maternal consumption of high-prebiotic fibre or -protein diets during pregnancy and lactation differentially influences satiety hormones and expression of genes involved in glucose and lipid metabolism in offspring in rats. Br J Nutr 2011, **105**, 329-338.

31 J.A. Parnell, M. Raman, K.P. Rioux and R.A. Reimer, The potential role of prebiotic fibre for treatment and management of non-alcoholic fatty liver disease and associated obesity and insulin resistance. Liver Int 2012, **32**, 701-711.

32 K. Venema, In vitro assessment of the bioactivity of food oligosaccharides. In Moreno, F.J. & Sanz, M.L., editors. Food Oligosaccharides: Production, Analysis and Bioactivity. Chichester, UK: John Wiley & Sons, Inc.; 2014.pp 219-237.

33 X. Jia, J. Kim, T. Veuthey, C.H. Lee, and M. Wessling-Resnick, Glucose metabolism in the Belgrade rat, a model of iron-loading anemia. Am J Physiol-Gastr L 2013, **304**, G1095-1102.

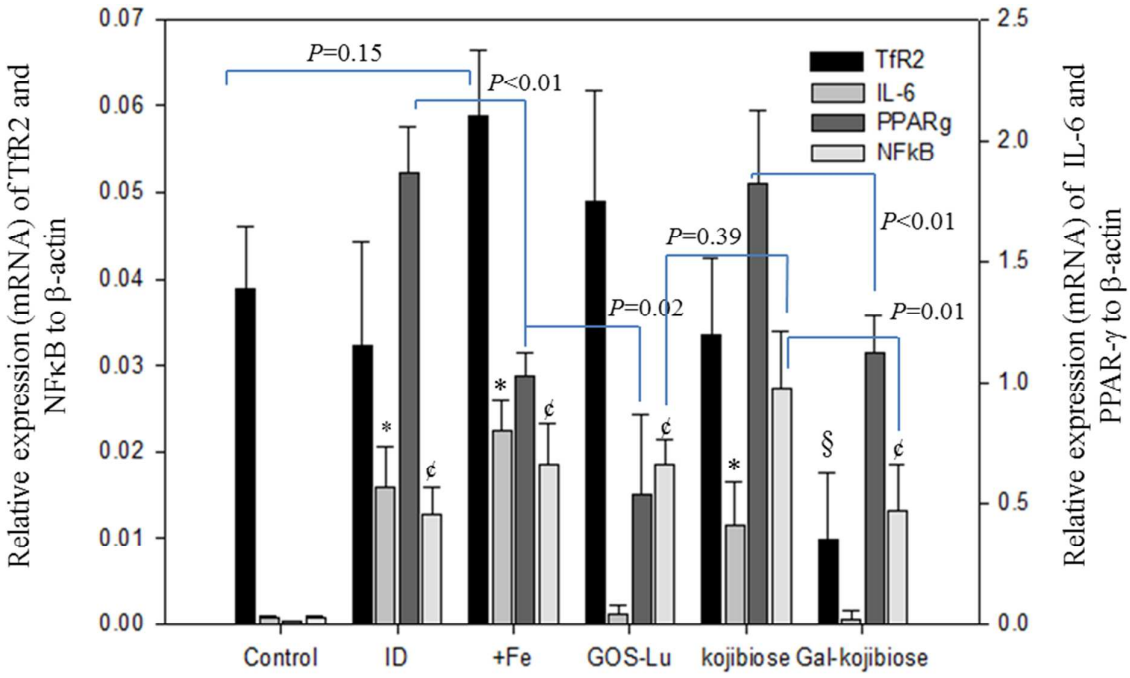
34 D. Peng, L.H. Ma, K.M. Smith, X. Zhang, M. Sato and G.N. La Mar, Role of propionates in substrate binding to heme oxygenase from Neisseria meningitidis: a nuclear magnetic resonance study. Biochemistry-US 2012, **51**, 7054-7063.

35 T. Shimazu, M.D. Hirschey, J. Newman, W. He, K. Shirakawa, N. Le Moan, C.A. Grueter, H. Lim, L.R. Saunders, R.D. Stevens, C.B. Newgard, R.V. Farese, R. de

Cabo, S. Ulrich, K. Akassoglou and E. Verdin, Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science 2013, **339**, 211-214.

36 A. Korecka, T. de Wouters, A. Cultrone, N. Lapaque, S. Pettersson, J. Doré, H.M. Blottière and V. Arulampalam, ANGPTL4 expression induced by butyrate and rosiglitazone in human intestinal epithelial cells utilizes independent pathways. Am J Physiol-Gastr L 2013, **1304**, G1025-37.

564 **Figure 1.** Hepatic expression of transferrin receptor (TfR2), interleukin (IL) -6, peroxisome
565 activator receptor (PPAR)-γ and nuclear factor kappa (NFκ)-B in control and iron
566 deficient (ID) animals and those administered with the supplement of Fe alone or together
567 with the different potential prebiotic compounds (GOS-Lu, kojibiose or galactosyl-
568 kojibiose). Results are expressed as median (lower-upper limits) (n=7). Superscript symbols
569 indicate statistically (P<0.05) significant differences. §, P=0.024; *,¢ P<0.05 relative to
570 controls.



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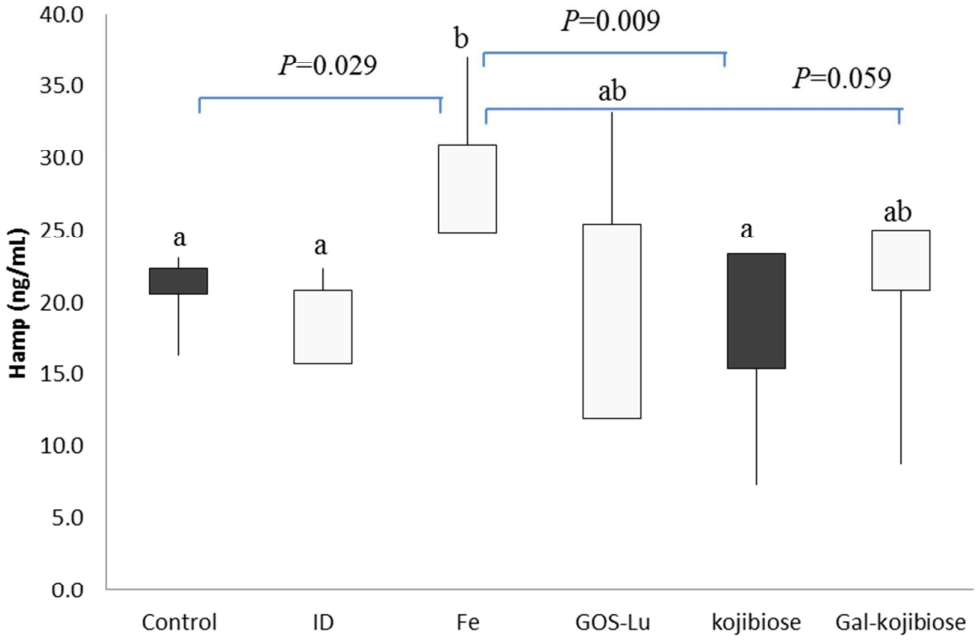
574 **Figure 2.** Plasma hepcidin peptide concentrations in control and iron deficient (ID) animals
575 and those administered with the supplement of Fe alone or together with the different
576 potential prebiotic compounds (GOS-Lu, kojibiose or galactosyl-kojibiose). Results are
577 expressed as median (lower-upper limits) (n=7). Superscript letters indicate statistically
578 (P<0.05) significant differences.



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581 **Table 1.** Hematological parameters of control and iron deficient (ID) animals and those administered with the supplement of Fe alone or together with
582 the different potential prebiotic compounds GOS-Lu, kojibiose, and galactosyl-kojibiose. Results are expressed as median (lower-upper limits) (n=7). [a-d] Different superscript letters indicate significant (P<0.05) statistical differences.
583

	Control	ID	$FeCl_3$	Oligosaccharide + $FeCl_3$		
				GOS-Lu	Kojibiose	Gal-kojibiose
Hemoglobin, Hb (g/dL)	19.8 ± 0.7 [a]	11.1 ± 2.7 [d]	16.2 ± 0.2 [bc]	18.1 ± 1.3 [ab]	18.5 ± 0.9 [ab]	15.2 ± 1.0 [c]
Hematocrit	55.3 ± 2.1 [a]	53.7 ± 1.4 [a]	56.0 ± 0.9 [ab]	54.0 ± 0.9 [a]	53.3 ± 1.3 [a]	58.4 ± 1.4 [b]
Erythrocytes ($x10^6/mm^3$)	3.25	3.26	3.30	3.50	3.35	3.14
MCV[1] ($x10^4$)	1.68	1.64	1.71	1.67	1.63	1.78
MCH[2] (pg)	35.7 ± 1.2 [a]	20.3 ± 5.5 [b]	28.9 ± 0.3 [cd]	33.0 ± 0.8 [cd]	34.6 ± 1.5 [a]	27.6 ± 2.4 [d]
VSG[3] (mm/h)	2.3	2.5	2.2	2.1	2.7	2.4

584 [1] MCV, mean corpuscular volume – mean standard deviation (SD) = ±4.09 $x10^{-6}$; [2] MCH, mean corpuscular hemoglobin concentration; [3]VSG,
585 corpuscular speed sedimentation

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587 **Table 2.** Short chain fatty acids (SCFA) concentration in colon contents (μg/g feces) of control and iron deficient (ID) animals and those administered
588 with the supplement of Fe alone or together with the different potential prebiotic compounds GOS-Lu, kojibiose, and galactosyl-kojibiose. Results are
589 expressed as median (lower-upper limits) (n=7). [a-d] Different superscript letters indicate significant (P<0.05) statistical differences.

SCFA	Control	ID	$FeCl_3$	Oligosaccharide + $FeCl_3$		
				GOS-Lu	Kojibiose	Gal-kojibiose
Formic	8.57 ± 1.06 [a]	14.49 ± 2.94 [b]	12.03 ± 2.32 [ab]	12.06 ± 0.26 [ab]	11.57 ± 2.54 [ab]	8.31 ± 2.50 [a]
Acetic	1.41 ± 0.25 [a]	1.92 ± 1.07 [ab]	3.04 ± 1.24 [b]	2.24 ± 0.13 [ab]	1.81 ± 0.35 [a]	0.98 ± 0.10 [a]
Propionic	0.54 ± 0.18 [ab]	0.50 ± 0.18 [ab]	0.96 ± 0.21 [c]	0.80 ± 0.07 [bc]	0.64 ± 0.20 [abc]	0.31 ± 0.02 [a]
D/L-Lactic	0.21 ± 0.02 [ab]	0.18 ± 0.09 [a]	0.32 ± 0.07 [ab]	0.30 ± 0.07 [ab]	0.28 ± 0.09 [ab]	0.36 ± 0.03 [b]
i-Butyric	0.24 ± 0.05 [a]	0.30 ± 0.11 [a]	0.62 ± 0.15 [b]	0.42 ± 0.13 [ab]	0.31 ± 0.09 [a]	0.21 ± 0.05 [a]
i-Valeric	8.10 ± 1.17 [a]	15.20 ± 3.02 [c]	15.26 ± 2.83 [c]	12.71 ± 0.51 [b]	8.59 ± 0.60 [a]	5.71 ± 0.19 [a]

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