Oral administration of *Bifidobacterium longum* CECT 7347 ameliorates gliadin-induced alterations in liver iron mobilisation

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Abstract

Coeliac disease is an autoimmune disorder triggered by gluten intake, causing intestinal inflammation and mucosal damage commonly associated with the malabsorption of nutrients and ferropenic anaemia. The present study evaluates the effects of the oral administration of *Bifidobacterium longum* CECT 7347 on gliadin-mediated alterations in hepatic Fe deposition and Hb concentration, liver transferrin receptor (TfR)-2, IL-6, TNF-α and hepcidin (*Hamp*) expression (mRNA), and active hepcidin peptide production by liquid chromatography–MS/MS. Weanling rats, sensitised or not with interferon (IFN)-γ, were fed with gliadins and/or the bifidobacterial strain. Gliadin feeding increased hepatic Fe deposition; however, only gliadin-fed sensitised animals showed lower Hb concentrations than the controls. *TfR2* expression decreased after gliadins were fed to both sensitised and non-sensitised animals, and restored by the administration of *B. longum*. These observations were accompanied by increases in *IL-6* expression levels in all the treatment groups; however, *TNF-α* expression only increased significantly in animals fed gliadins alone or together with *B. longum* if they had previously been sensitised with IFN-γ. Liver expression levels of *Hamp* diminished in all cases to the lowest values in animals sensitised with IFN-γ after being fed with gliadins and/or bifidobacteria. In these animals, plasma *Hamp* active peptide concentrations significantly increased when compared with the controls. Significant correlations were calculated between *Hamp* expression and liver Fe contents (liver Fe $= 1/0.0032 + 0.032 \times Hamp_{\mathrm{exp}}$), and Hb concentrations (Hb $= 11.49 + 10.13 \times (Hamp_{\mathrm{exp}})^{1/2}$). These data indicate that oral administration of *B. longum* ameliorates gliadin-mediated perturbations in liver Fe deposition and mobilisation.

Key words: Coeliac disease: Bifidobacteria: Gliadin: Iron metabolism: Enteropathy

Coeliac disease (CD) is an autoimmune enteropathy caused by permanent intolerance to cereal gluten proteins, in particular gliadins[1]. The main CD-related lesions occur in the intestinal epithelium, consisting of villous flattening, crypt cell hyperplasia and infiltration of the lamina propria by lymphocytes and macrophages. CD patients display many symptoms and signs, but is classically characterised by the defective absorption of nutrients and the failure to gain weight. Gliadin ingestion triggers inflammatory responses in the intestinal mucosa with the production of cytokines and related mediators such as IL-6[2,3], TNF-α and NF-κB[4]. TNF-α and IL-6 play important roles in up-regulating T-cell functions and mediating tissue toxicity[5,6]. Currently, the mainstay of treatment is a strict lifelong adherence to a gluten-free diet; however, compliance with this dietary treatment is difficult because wheat flour is present in a great percentage of processed foods. Thus, in this context, the development of alternative or adjuvant nutritional strategies to improve the health status of coeliac sufferers acquires special relevance.

Recent epidemiological data indicate that environmental factors, other than gluten, can contribute to the pathogenesis and/or presentation of CD[7,8]. In particular, the disease has been associated with reduced numbers of bifidobacteria and the increased prevalence of Enterobacteriaceae in the gut microbiota[9,10]. Data from *in vitro* and *in vivo* animal studies have revealed that some bifidobacterial strains can counteract the toxicity and inflammatory potential of gliadin on enterocytes[11], which are the cells responsible for Fe absorption in the upper intestine, and contribute to the gut immune function. A previous animal study has shown marked differences in the effects of gliadins in the jejunum of weanling animals, sensitised or not with interferon (IFN)-γ[12]. Moreover, oral administration of *Bifidobacterium longum* CECT 7347 to a gliadin-induced enteropathy model ameliorated the production of inflammatory cytokines in jejunal samples and decreased

the number of both $CD4^+$ and $CD4^+Foxp3^+$ T-regulatory cells. The positive effects of *B. longum* CECT 7347 feeding on alleviating inflammation were parallel to increased expression of proteins involved in energy and lipid metabolism, cell structure, protein folding and immune responses[13].

In CD patients, ferropenic anaemia is one of the most common extra-intestinal manifestations[14,15], probably secondary to this is inadequate intestinal absorption due to gliadin-mediated alterations in the architecture of the intestinal epithelium. Fe is an essential micronutrient for nearly all living organisms, which is poorly absorbed from the diet, whose status is tightly controlled by signals sensing dietary composition, body Fe stores and metabolic requirements[16]. Inflammatory cytokines such as TNF-α[17] and IL-6[18] have been suggested to influence dietary Fe assimilation and systemic Fe homeostasis[19]. These cytokines mediate a decrease in serum Fe concentrations accompanied by increased Fe storage in both enterocytes and hepatocytes. In addition, systemic Fe homeostasis is regulated by hepcidin (*Hamp*), a β-defensin-like peptide mainly synthesised by hepatocytes, which acts as a key regulator of Fe metabolism reducing its intestinal absorption[20,21]. Ferropenic anaemia in CD patients develops as a refractory to dietary Fe supplementation[22]. This particular characteristic implies the need to develop additional nutritional strategies to prevent or improve the health status of CD patients.

Traditionally, rats have been the animal model of choice when performing nutritional studies. There are a number of differences concerning Fe absorption in rats compared with humans, limiting their predictive value in relation to human responses[23,24]. However, rats can develop Fe deficiency when fed deficient diets[25] and exhibit comparable responses of regulatory factors of systemic Fe homeostasis that involve liver transferrin receptor-2 (TfR2)[26] and *Hamp*[26,27]. A recent study in rats has associated Fe depletion and metabolic activity with imbalances in the microbiota, showing higher abundances of lactobacilli and Enterobacteriaceae and a significant decrease in the *Roseburia* spp./*Eubacterium rectale* group[28]. Many members of the Enterobacteriaceae have developed mechanisms, including siderophores, to acquire Fe in competition with other bacteria and the host[29], and favour pro-inflammatory milieu[30].

The objective of the present study was to evaluate the effects of the oral administration of *B. longum* CECT 7347 on Fe homeostasis in weanling animals, sensitised or not with IFN-γ to induce an experimentally gliadin-sensitive enteropathy.

Materials and methods

Gliadin preparation

A commercially available extract of gliadin (G3375; Sigma-Aldrich) was subjected to *in vitro* digestion in order to obtain partially digested gliadin-derived peptides[11]. The dialysate (15 kDa, molecular weight cut-off) was diluted in a commercially available hypoallergenic milk-based formula and administered orally by means of a micropipette.

Bacterial strain and culture conditions

B. longum CECT 7347 was isolated from the faeces of healthy infants as described elsewhere[7]. Bacterial cultures were grown in Man–Rogosa–Sharpe agar and broth (Scharlau) supplemented with 0·05 % (w/v) cysteine (MRS-C; Sigma), and kept at 37°C in anaerobic conditions (AnaeroGen; Oxoid) for 24 h. For animal studies, a pure culture of the strain was grown overnight and used to inoculate fresh MRS-C broth for 22 h. Cells were harvested by centrifugation (6000 g for 15 min) at a stationary growth phase, washed twice in PBS (130 mM-NaCl and 10 mM-sodium phosphate, pH 7·4) and resuspended in 10 % (w/v) hypoallergenic milk-based formula (Nutramigen©; Mead Johnson B.V.). Aliquots of these suspensions (10^8 colony-forming units/ml) were frozen in liquid N_2 and stored at -80°C until used. More than 90 % of cells were alive upon thawing and no significant differences were found related to storage time (1 month). For every new experiment, one fresh aliquot was thawed to avoid variability in bacterial cell viability between the experiments.

Animals

All animal experiments were performed according to the University of Valencia Ethics Committee Guidelines for Animal Experiments (Central Support Service for Experimental Research). The experimental animals were female weanling Wistar rats. Adult females were date-mated, and fed *ad libitum* with a normal diet (Harlan Bioproducts). Shortly after spontaneous birth, offspring were randomly distributed in different groups (n 6)[12]: (1) artificially reared (AR); (2) AR + *B. longum* CECT 7347; (3) AR + gliadin-derived peptides (0·5 mg/ml hypoallergenic milk-based formula); (4) AR + gliadin-derived peptides + *B. longum* CECT 7347 ($6·0 \times 10^7$ and $8·2 \times 10^8$ colony-forming units/ml); (5) AR + gliadin-derived peptides + IFN-γ (sensitised with 1000 U ($1·6 \times 10^3$ pg) IFN-γ intraperitoneally immediately after birth); (6) AR + gliadin-derived peptides + IFN-γ + *B. longum* CECT 7347 suspension.

Artificially fed animals

Newborn animals were hand-fed (100 μl) using a micropipette. They were hand-fed by using a micropipette with a hypoallergenic milk-based formula for newborns, every 4 h (four to five times per d) until 10 d old, with composition per 100 ml (13·6 %, w/v) being as follows: 285 kJ (68 kcal); 1·9 g protein; 3·4 g fat; 7·5 g carbohydrates; 260 mOsm/l. Changes in body weight were monitored every 2 d. Weanling rats were fed (0·1 ml) with a gliadin (0·5 mg/ml) solution in a single dose per d for 10 d, and received a provocative dose of gliadin (1 mg/ml) approximately 2 h before killing.

Liver tissue and blood collection

Rats were anaesthetised (Isofluran) and killed by exsanguination by intracardiac puncture. Whole-blood samples were preserved in EDTA-treated tubes to prevent coagulation. For total

RNA isolation, one lobe from each liver section was quickly dissected and stored in RNAlater (Qiagen). Another liver lobe was used to quantify tissue Fe content. All tissue samples were stored at −80°C until further analysis.

Hb measurement

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

Total iron quantification

Liver samples were washed twice with saline solution (140 mM-NaCl and 5 mM-KCl) and total Fe content was determined by atomic absorption spectrometry (Perkin-Elmer) in a solution of the ashes.

RT and real-time PCR analyses

Total RNA was extracted from liver tissue samples using an RNeasy mini kit (Qiagen) following the protocol provided by the manufacturer. Briefly, 1 μg of total RNA was converted to double-stranded complementary DNA using avian myeloblastosis virus RT (Promega). 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′); *TNF-α* (forward: 5′-AGG GTA CCA CAG AAA GAT GC-3′; reverse: 5′-GGA GAT GAG ACC CTT AGG TT-3′); *IL-6* (forward: 5′-TCT CGA GCC CAC CAG GAA C-3′; reverse: 5′-AGG GAA GGC AGT GGC TGT CA-3′); *NF-κB* (forward: 5′-CTT CTC GGA GTC CCT CAC TG-3′; reverse: 5′-CCA ATA GCA GCT GGA AAA GC-3′); *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 complementary DNA. PCR were performed in triplicate in a LightCycler® 480 system (Roche) with the following conditions: one cycle at 95°C for 5 min, thirty-five cycles at 60°C for 20 s and 72°C for 45 s. The relative mRNA expression of the tested gene compared with β-actin expression was calculated using the $2^{-\Delta\Delta C_p}$ method[31]. Samples of each animal tissue were measured in duplicate and gene expression was expressed as fold change.

Quantification of hepcidin in plasma (liquid chromatography–MS/MS)

All sample preparation steps were performed at room temperature as described previously[32] with slight modifications.

Aliquots (50 μl) of plasma were pipetted into 200 μl cone tubes, and a 100 μl aliquot of acetonitrile (Burdick and Jackson) was added to each tube and mixed by pipetting. The samples were then centrifuged at 3000 *g* for 10 min at 4°C (Jouan). After centrifugation, the protein precipitation supernatant (100 μl) was mixed with 0·02 % (v/v) aqueous acetic acid and injected onto an Agilent HPLC system connected on line to an Esquire-liquid chromatography electrospray system equipped with a quadrupole ion trap mass spectrometer (Bruker Daltonics). 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 (1100 Series; Agilent Technologies). The column used in these analyses was a BioBasic C18 5 μm 4·6 × 250 mm (Thermo). The elution phases consisted of mobile phase A (0·125:1:500, trifluoroacetic acid–isopropanol–water) and mobile phase B (0·125:1:50:350:100, trifluoroacetic acid–isopropanol–water–methanol–acetonitrile). Aliquots (50 μl) of the precipitation supernatants were injected in each cycle and the analysis was performed with the following gradient (min, %B): 0, 5; 30, 90; 33, 100; 35, 0; 40, 90; 45, 5.

N_2 was used as the nebulising and drying gas, and He collision gas pressure was approximately 5×10^{-3} bar. The capillary was held at 4 kV. Mass spectra were recorded over the mass/charge (m/z) range of 100–3500. About fifteen spectra were averaged in the MS analyses and about five spectra in the MS/MS analyses. The signal threshold to perform auto-MS/MS analyses was 5000, and precursor ions were isolated within a range of 4·0 m/z and fragmented with a voltage ramp from 0·39 to 2·6 V. The m/z spectral data were processed and transformed to spectra representing mass values using the program Data Analysis version 3·0 (Bruker Daltonics). BioTools version 2·1 (Bruker Daltonics) software was used to process the MS/MS spectra and to perform peptide sequencing. From each animal, two independent samples were analysed.

Statistical analysis

Data are expressed as means and standard deviations. Statistical analyses were performed using SPSS version 15 software (SPSS, Inc.). One-way ANOVA and Tukey's *post hoc* test were applied. In animal studies, comparison procedures were performed using a pairwise comparison to detect statistically significant differences. Simple linear regression analysis was used to determine the relationships between hepcidin expression, liver Fe contents and Hb concentrations. Statistical significance was established at $P < 0.05$ for all comparisons.

Results

Effect of gliadins on Hb and liver iron deposition

The animals fed with gliadins, alone ($P = 0.275$) or together with *B. longum* CECT 7347 ($P = 0.168$), did not show significant alterations in Hb concentrations compared with the controls (Fig. 1). This trend was also observed in the animals administered the bifidobacterial strain alone ($P = 0.529$). The animals sensitised with IFN-γ, but not fed gliadins, did

not exhibit significant changes in Hb concentrations (data not shown). However, the IFN-γ-sensitised gliadin-fed animals showed significant lower Hb concentrations than the controls ($P=0.001$) and non-sensitised gliadin-fed animals ($P=0.004$). The administration of *B. longum* CECT 7347 to the IFN-γ-sensitised gliadin-fed animals partially restored ($P=0.043$) Hb concentrations, although these animals still exhibited lower ($P=0.005$) concentrations than the controls.

The administration of *B. longum* CECT 7347 alone to weanling animals slightly, but significantly ($P=0.040$), increased liver Fe contents compared with the controls. The non-sensitised animals fed gliadins alone ($P<0.001$) or together with *B. longum* CECT 7347 ($P=0.002$) exhibited significantly ($P<0.05$) increased liver Fe contents compared with the controls. In the IFN-γ-sensitised gliadin-fed animals, liver Fe contents were also increased ($P<0.001$), but were similar to those quantified in the non-sensitised gliadin-fed animals ($P=0.198$). However, a significantly lower ($P=0.038$) liver Fe content was quantified in the sensitised animals fed gliadins and the bifidobacterial strain compared with those sensitised animals fed gliadins alone, although also markedly higher ($P<0.001$) than that in the controls. There were no significant ($P>0.05$) changes in liver Fe contents in the IFN-γ-sensitised animals not fed gliadins compared with the controls at the end of the treatment (data not shown).

Changes in *TfR2* expression levels in animals, sensitised or not with IFN-γ, fed gliadins and the bifidobacterial strain are shown in Fig. 2. Feeding *B. longum* CECT 7347 alone to weanling animals significantly ($P=0.003$) decreased *TfR2* expression levels compared with the controls. However, *TfR2* expression levels were more markedly down-regulated

($P=0.006$) in the animals fed gliadins alone. The co-administration of *B. longum* CECT 7347 slightly restored ($P=0.019$) *TfR2* expression levels, although it was still lower ($P=0.013$) than those in the controls. In the sensitised animals, gliadin feeding down-regulated *TfR2* expression levels, which were partially restored ($P=0.041$) by the concurrent administration of the bifidobacterial strain. Feeding *B. longum* CECT 7347 alone also down-regulated ($P=0.003$) *TfR2* expression levels compared with the controls, but similar levels found in the IFN-γ-sensitised gliadin-fed animals.

Effect of gliadins on plasma active hepcidin peptide concentrations and hepatic hepcidin expression (mRNA)

Liquid chromatography−MS/MS methods are able to quantify human urinary[33] or human and animal plasma[32,33] hepcidin (*Hamp*) from 0·25 to 500 ng/ml. However, protocol standardisation is still needed for the results to be comparable between laboratories. In the present study, to avoid misinterpretation of the reported results, we followed a previously optimised method for analysis in mouse plasma and serum samples[32]. The active *Hamp* peptide was identified by sequencing its amino acid backbone (Table 1). To better understand the role of *Hamp* in the alterations described in Fe homeostasis, circulating concentrations of the active *Hamp* peptide were quantified in animals from the different treatment groups (Table 2). The median plasma *Hamp* concentration in the control animals was 24·5 ng/ml. This value is of a similar order to those previously reported in mice[32].

The animals administered *B. longum* CECT 7347 alone exhibited slightly increased median values (1·25-fold), but
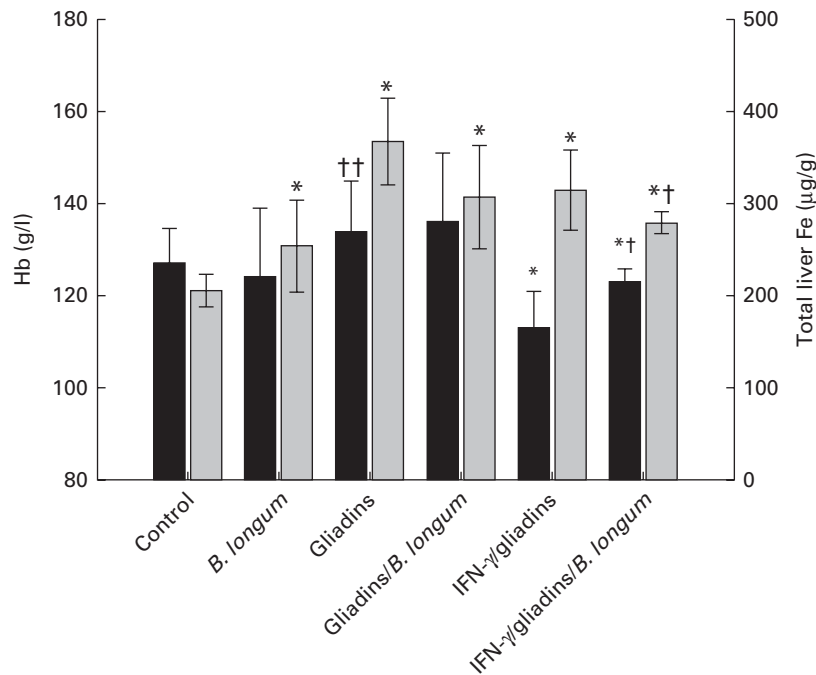
Fig. 1. Hb (■) and liver iron contents (□) in weanling animals, sensitised or not with interferon (IFN)-γ, fed with gliadins with or without *Bifidobacterium longum* CECT 7347. Values are means (*n* 6), with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control animals ($P<0.05$). Mean value was significantly different from that of the IFN-γ/gliadins-treated animals: † $P=0.04$, †† $P<0.01$.
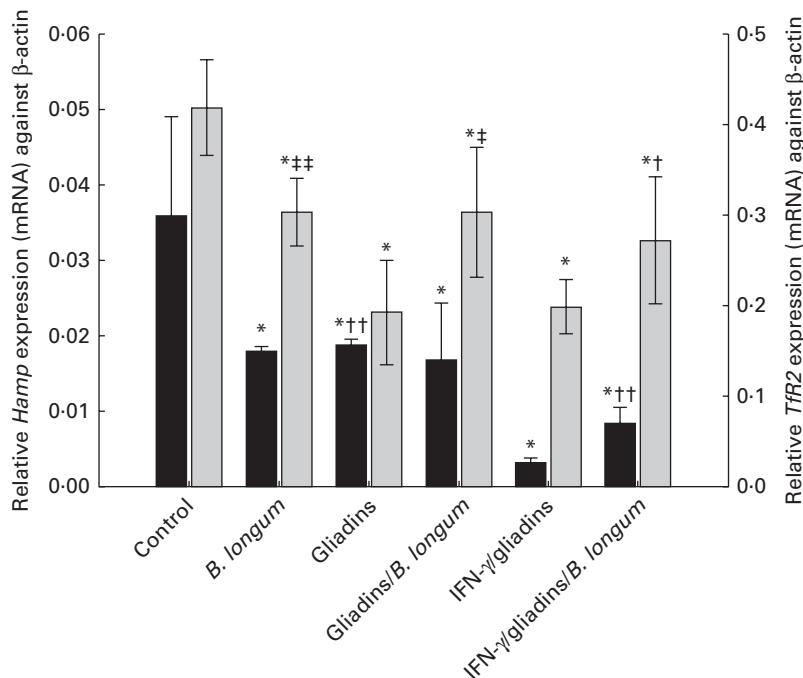
Fig. 2. Hepatic hepcidin (*Hamp*; ■) and transferrin receptor-2 (*TfR2*; ▢) expression (mRNA) levels in weanling animals, sensitised or not with interferon (IFN)-γ, fed with gliadins with or without *Bifidobacterium longum* CECT 7347. Values are means (*n* 6), with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control animals (*P* < 0·05). Mean value was significantly different from that of the IFN-γ/gliadins-treated animals: † *P* < 0·02, †† *P* < 0·01. Mean value was significantly different from that of the gliadins-treated animals: ‡ *P* < 0·02, ‡‡ *P* < 0·01.

not significantly different (*P* = 0·116) from the controls. Feeding gliadins produced *Hamp* concentrations up to 1·45-fold that of the controls, bordering statistical significance (*P* = 0·072). However, when the bifidobacterial strain was administered with gliadins, differences in plasma *Hamp* concentrations quantified were reduced to values close to those of the control animals (0·96-fold). Sensitisation of the animals with IFN-γ gave median plasma *Hamp* concentrations 0·88-fold (*P* = 0·965) of the controls, but after gliadin feeding, the values were quantified at 1·26-fold of the control values, albeit not statistically significant (*P* = 0·113). The only significant increase in plasma *Hamp* concentrations was found in the IFN-γ-sensitised gliadin-fed animals administered *B. longum* CECT 7347.

Initially, *Hamp* is synthesised as a pre-pro-peptide (84 amino acids; aa), which is finally processed to a 24 aa bioactive peptide[34]. Previous studies have reported differences in hepatic *Hamp* expression (mRNA) of anaemic rats[26], prompting us to investigate potential changes in the different animal groups in the present study. Animals from all the treatment groups showed a decreased expression (mRNA) of hepcidin (*Hamp*) compared with the controls (Fig. 2). The administration of gliadins significantly (*P* = 0·021) down-regulated *Hamp* expression, as well as when administered together with *B. longum* CECT 7347 (*P* = 0·021); however, statistical (*P* = 0·535) differences were not detected between either treatment. The administration of bifidobacteria alone also caused the down-regulation of the hepatic expression of *Hamp*. The animals sensitised with IFN-γ exhibited a higher down-regulated expression of *Hamp* after being fed

with gliadins (*P* < 0·001), which was partially restored (*P* = 0·001) by the bifidobacterial strain. In the present study, a significant reciprocal correlation was observed between *Hamp* expression (mRNA) and total hepatic Fe contents (μg/g) (*R²* 0·504; *P* < 0·005). In addition, *Hamp* expression was significantly (*R²* 0·453; *P* < 0·012) correlated with Hb concentrations through a square root model (Fig. 3). The correlation calculated for *Hamp* expression was able to explain 25·43 % (liver Fe = 1/0·0032 + 0·032 × *Hamp* expression) and 20·52 % (Hb = 11·49 + 10·13 × (*Hamp* expression)$^{1/2}$) of the variations in total hepatic Fe content and Hb concentrations, respectively.

Effect of gliadin on the hepatic expression of cytokines

Changes in hepatic IL-6 and TNF-α expression levels in the different treatment groups are shown in Fig. 4. All animals

Table 1. Amino acid sequences used to identify hepcidin (*Hamp*) in animal plasma

Ion (*m/z*) for MS/MS	Charge	Observed *m/z*	Calculated *m/z*	Fragment of amino acid sequence*: TNFPICLFCCK CCKNSSCGLCCI
964·3	1	964·3	965·3	ICLFCCKCC
798·3	1	798·3	799·1	PICLFCC
859·3	1	859·3	860·1	SSCGLCCI
988·3	1	988·3	989·3	CLFCCKCCK
921·4	1	921·4	922·1	CKNSSCGL

* Q99MH3, HEP_RAT (SwissProt).

Table 2. Plasma hepcidin (*Hamp*) peptide concentrations in animals administered with *Bifidobacterium longum* CECT 7347 or gliadins alone, gliadins together with the bifidobacterium strain, animals sensitised with interferon (IFN)-γ and fed gliadins and those co-administered with the bifidobacterial strain

(Medians and ranges; six animals per group)

Treatment	*Hamp* (ng/ml)		
	Median	Range	Fold change‡
Control	24·51	20·61–26·33	
B. longum	30·68	27·94–34·95	1·25
Gliadins	35·54	30·46–40·62	1·45
Gliadins/*B. longum*	23·59	21·18–31·73	0·96
IFN-γ	21·69	16·91–30·63	0·88
IFN-γ/gliadins	30·97†	27·69–31·38	1·26
IFN-γ/gliadins/*B. longum*	40·35*	36·56–44·09	1·64

* Median value was significantly different from that of the control animals (*P*=0·007; Student's *t* test).
† Median value was significantly different from that of the IFN-γ/gliadins-treated animals (*P*=0·033; Student's *t* test).
‡ Fold change *v.* median plasma *Hamp* concentration quantified in the control animals.

from the different treatment groups showed an increased expression (mRNA) of *IL-6* compared with the controls. There were no significant differences in *IL-6* mRNA levels in animals fed gliadins with or without the bifidobacterial strain. A significant (*P*=0·001) increase in *IL-6* expression levels was also observed after feeding *B. longum* CECT 7347 alone. Feeding gliadins to the sensitised animals with IFN-γ increased (*P*=0·002) hepatic *IL-6* expression levels, although to a lesser extent (*P*<0·001) compared with the non-sensitised animals. The administration of gliadins with *B. longum* CECT 7347 to the IFN-γ sensitised animals did not differ from the effect observed in the non-sensitised animals.

The administration of *B. longum* CECT 7347 alone to weanling animals did not cause significant (*P*=0·381) alterations in the basal expression of this inflammatory marker. Gliadin feeding significantly (*P*=0·008) increased *TNF-α* mRNA expression, while the simultaneous administration of *B. longum* CECT 7347 decreased (*P*=0·004) its levels to the values similar (*P*=0·162) to those of the controls (Fig. 4). In the IFN-γ-sensitised gliadin-fed animals, *TNF-α* expression increased (*P*=0·002) compared with the expression level found in the controls, but did not show a significant (*P*=0·071) variation compared with the non-sensitised gliadin-fed animals. However, the IFN-γ-sensitised animals fed both gliadins and the bifidobacterial strain exhibited higher *TNF-α* expression levels than those animals fed gliadins without the bacterium.

Discussion

Gliadins cause different symptoms in CD patients, and nutritional Fe deficiency-related manifestations are common[15,35]. Epidemiological studies have reported a higher prevalence of ferropenic anaemia in individuals with undiagnosed CD (50%)[35] and CD patients with normal mucosa (32%)[15] in comparison with healthy controls. According to the present data, deficient Fe status seems to be caused by the impairment

of micronutrient homeostasis processes and liver physiology. The present study shows that gliadin feeding causes alterations in liver Fe deposition and mobilisation, and induces the expression of hepatic inflammatory mediators, even in weanling animals lacking perceptible mucosal damage[12]. In the IFN-γ-sensitised animals, changes in Fe homeostasis were greater, resulting in a more severe decrease in circulating Hb concentration. This effect on weanling animals with high physiological requirements of Fe during early growth stages can have a profound negative impact on the development of adequate physiological and immune responses. *B. longum* CECT 7347 administration to gliadin-fed animals, sensitised or not with IFN-γ, partially reduced the severity of the gliadin-mediated effects observed in these animals, and could theoretically contribute to ameliorating the severity of anaemia in CD patients.

Fe homeostasis is mediated through the regulation of duodenal Fe absorption and Fe release from macrophages, and other cell types, according to signals from different compartments reflecting dietary sources or availability, status
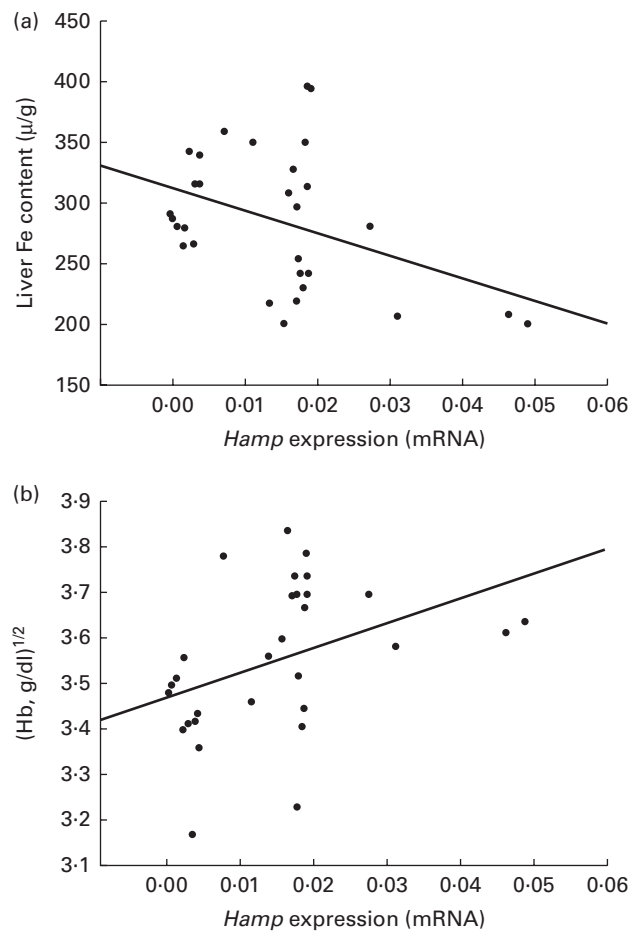
Fig. 3. Correlation of hepatic expression of hepcidin (*Hamp*) with liver iron contents (a) and Hb concentrations (b) in weanling animals, sensitised or not with interferon (IFN)-γ, fed with gliadins and/or *Bifidobacterium longum* CECT 7347. *Hamp* expression was significantly related to liver iron contents (*P*<0·005, liver Fe = 1/0·0032 + 0·032 × *Hamp* expression) and Hb concentrations (*P*<0·012, Hb = 11·49 + 10·13 × (*Hamp* expression)$^{1/2}$).
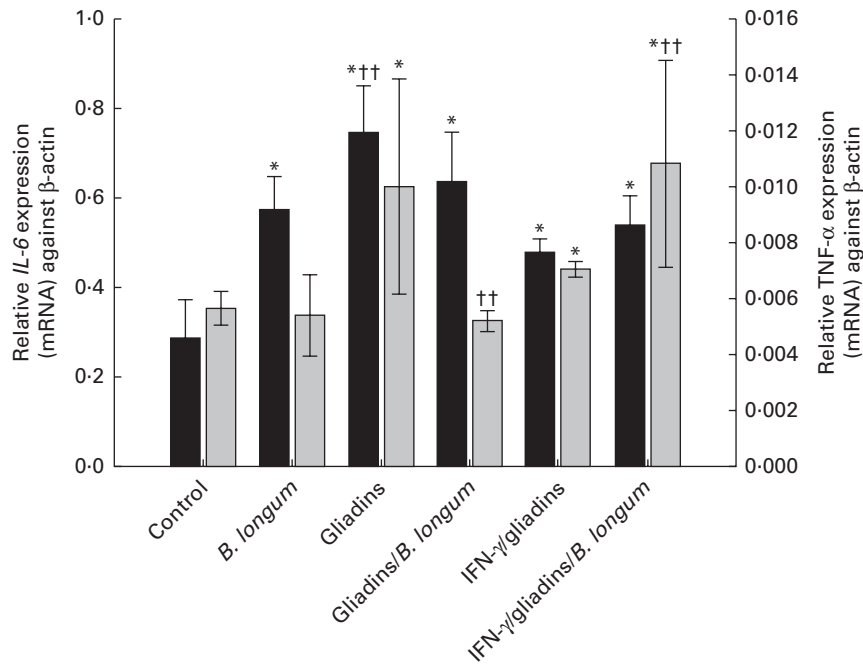
Fig. 4. Hepatic *IL-6* (■) and *TNF-α* (□) expression (mRNA) levels in weanling animals, sensitised or not with interferon (IFN)-γ, fed with gliadins with or without *Bifidobacterium longum* CECT 7347. Values are means (*n* 6), with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control animals (*P* < 0·05). †† Mean value was significantly different from that of the IFN-γ/gliadins-treated animals *P* < 0·01.

of the body Fe stores and metabolic requirements. In this context, the liver plays a central role in the regulation of Fe metabolism, producing key regulators and mediators such as *Hamp*[20,36]. *Hamp* is predominantly expressed in the liver, although various tissues, such as the heart, brain and monocytes, can express *Hamp* at low levels in response to adhesion and pro-inflammatory cytokines[37], regulating not only Fe metabolism but also intestinal Fe absorption.

Previous research has demonstrated the inverse relationship between serum *Hamp* concentration and Fe absorption from food and supplemental sources[36]. Besides, *Hamp* transcription is down-regulated when body Fe demand is high[26,27]. In the present study, gliadin feeding increased hepatic Fe deposition, impairing Fe supply to peripheral tissues. This was reflected by the significant inverse relationship between *Hamp* transcription levels and liver Fe concentrations in animals fed with gliadins, administered either with or without *B. longum* CECT 7347. In accordance with impaired Fe status, expression levels of *TfR2* were down-regulated in gliadin-fed animals. TfR2 is thought to serve as a body Fe sensor, relying on information from plasma Fe (holo-transferrin)[38], which is necessary for hepatic *Hamp* expression[26]. In human studies, serum protein concentration of *Hamp* has previously been associated with ferritin concentration, but not with Hb[39], as found in this study. *B. longum* CECT 7347 administration to the gliadin-fed animals partially restored *TfR2* expression to levels similar to those of the controls, although it was insufficient to reduce the marked gliadin-induced hepatic Fe deposition. Sensitisation with IFN-γ reduced serum Hb concentrations below the normal range in these animals (120 g/l). It is noteworthy that feeding

B. longum CECT 7347 significantly restored the Hb concentration and reduced the gliadin-mediated hepatic deposition of Fe, parallel with the small increase in *Hamp* expression levels. This trend is concordant with previous reports indicating that *Hamp* expression levels are lower in anaemic than in non anaemic individuals[40].

Inflammation and bacterial infection, in addition to body Fe status, are the main stimuli inducing *Hamp* synthesis[20,21]. In CD patients, gliadins induce the production of pro-inflammatory cytokines, among others IL-6, which has been suggested as a marker of both CD activity and response to treatment[2]. In the initial phase of the inflammatory process, IL-6 is a key inducer of hepatic acute-phase protein synthesis, but also plays diverse biological activities in immune regulation, inflammation and haematopoiesis[6,41]. The administration of gliadins to weanling animals, with or without *B. longum* CECT 7347, increased hepatic *IL-6* expression levels, which are hypothesised to be mediated by the increased hepatic deposition of Fe. IL-6 exerts a wide range of biological activities in immune regulation, favouring T-cell growth and the differentiation of cytotoxic T cells[42] and macrophages[43], and may be important in Th-17 commitment[3]. In this respect, the administration of *B. longum* CECT 7347 to weanling animals has proven effective to reduce both peripheral CD4+ and CD4+Foxp3+ T cells in gliadin-fed animals, revealing the positive immunomodulatory characteristics of this bacterial strain[12]. It should be noted that two types of IL-6-responsive elements have been identified, in genes encoding acute-phase proteins, acting through increased gene expression or its post-translational modification[6]. However, from the limited data available at present, one cannot speculate which of

these specific mechanisms underlies the bifidobacterial-induced activation of IL-6.

In vitro studies using the intestinal epithelial Caco-2 cell line have shown that TNF-α production reduces Fe intestinal absorption[17] and favours its hepatic deposition[19]. In animals exhibiting significantly increased TNF-α expression, compared with the controls, a similar relationship could be found between hepatic *TNF-α* expression and Fe deposition levels. Moreover, increased *TNF-α* expression levels in the non-sensitised gliadin-fed animals and the sensitised animals fed gliadins with or without the bifidobacterial strain could be associated with increased median values for plasma active hepcidin quantified in the different treatment groups. However, this association has not been reported for *in vitro* studies with human-derived hepatocytes[20], suggesting that Hamp response activation may require the interaction of liver and immune cells[44]. Gliadin-induced production of pro-inflammatory cytokines such as IL-1β and TNF-α favours lymphocyte infiltration and, thereby, further adaptive responses to counteract gliadin toxicity[45]. Hepcidin production, which greatly induces IL-6 and TNF-α production by macrophages[21], would suggest its potential contribution to antigen processing as well as to direct effects causing Fe sequestration.

Conclusions

An alteration in Fe homeostasis, leading to ferropenic anaemia, is one of the most common extra-intestinal manifestations of CD. The data reported herein, on the administration of *B. longum* CECT 7347 to weanling animals, reveal novel findings on how administration of this strain affects Fe homeostasis in a gliadin-induced enteropathy model. The sensitised gliadin-fed animals displayed low Hb concentrations, below the normal range in these animals, demonstrating enteropathy-associated anaemia; however, administration of *B. longum* CECT 7347 normalised the expression levels of *TfR2*, thus contributing to restoring normal Hb concentrations. Although both *TfR2* and *IL-6* are involved in *Hamp* expression regulation, their action seems to be dependent on Fe serum levels. To the best of our knowledge, this is the first study to provide evidence for the entero-hepatic link with the bifidobacterial-mediated regulation of essential micronutrient homeostasis, such as that of Fe. Further studies and human trials are needed to gain a better understanding of the clinical relevance of *B. longum* CECT 7347 in controlling CD-related ferropenic anaemia.

Acknowledgements

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