Bacteria-carried Iron Oxide Nanoparticles for Treatment of Anemia

Víctor Garcés,[†,#] *Alba Rodríguez-Nogales,*[‡,#] *Ana González,*[†] *Natividad Gálvez,*[†] *M. Elena Rodríguez-Cabezas,*[‡] *María L. García-Martin,*[§] *Lucia Gutiérrez,*[‖] *Deyanira Rondón,*[¦] *Mónica Olivares,*[¦] *Julio Gálvez*,*[‡] *and Jose M. Dominguez-Vera*,*[†]

[†] Department of Inorganic Chemistry and Biotechnology Institute, Universidad de Granada, 18071 Granada, Spain

[‡] Department of Pharmacology, CIBER-EHD, ibs. Granada, CIBM, Universidad de Granada, 18100 Granada, Spain

[§] BIONAND, Andalusian Centre for Nanomedicine and Biotechnology, Junta de Andalucía, Universidad de Málaga, 29590 Málaga, Spain

[‖] Departament of Analytical Chemistry, Institute of Nanoscience of Aragon, Universidad de Zaragoza y CIBER-BBN, 50018 Zaragoza, Spain

[¦] Biosearch S. A. Camino de Purchil, 66, 18004 Granada, Spain

[#]These authors contributed equally to this work

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ABSTRACT. The efficiency of maghemite nanoparticles for the treatment of anemia was sensibly higher when nanoparticles were incorporated onto the probiotic bacterium *Lactobacillus fermentum* (MNP-bacteria) than when administrated as uncoated nanoparticles (MNP). Plasma iron and hemoglobin, intestine expression of divalent metal transporter 1 (DMT1) and duodenal cytochrome B (DcytB), as well as hepatic expression of the hormone hepcidin were fully restored to healthy levels after administration of MNP-bacteria but not of MNP. A magnetic study on biodistribution and biodegradation showed accumulation of

maghemite nanoparticles in intestine lumen when MNP-bacteria were administrated. In contrast, MNP barely reached intestine.

In vivo MRI studies suggested the internalization of MNP-bacteria into enterocytes, which did not occur with MNP. Transmission electronic microscopy confirmed this internalization.

The collective analysis of results point out that *L. fermentum* is an excellent carrier to overcome the stomach medium and drive maghemite nanoparticles to intestine, where iron absorption occurs. Due the probiotic ability to adhere to the gut wall, MNP-bacteria internalize into the enterocyte, where maghemite nanoparticles are delivered, providing an adequate iron level into enterocyte. This paper advances a new route for effective iron absorption in the treatment of anemia.

INTRODUCTION

Iron deficiency, the most common cause of anemia, is the nutritional disorder of greatest impact in the world.[1] As well as affecting a large number of children and women in developing countries, it is the only prevailing nutrient deficiency in industrialized countries.[2-6] Iron has vital functions such as to carry O_2 from the lungs to tissues, to transfer electrons for a great variety of redox reactions or to carry out enzymatic oxidative reactions.[7] Iron deficiency provokes therefore interferences with these vital functions and leads to morbidity and death.[8] Although the importance of iron absorption in health has been recognized for decades, the mechanism by which the body regulates this process remains unclear. What is clear is that if loss of iron is not sufficiently compensated by adequate iron intake from the diet or supplements, an iron deficiency anemia occurs.[9]

To increase daily iron intake, numerous supplements based on different iron chemicals have been developed and some of them are commercially available.[10-12] It is well known the main side effect of these iron supplements: gastrointestinal discomfort.[13] Ferrous sulfate for instance, the most commonly used iron supplement due to its low cost, may cause stomach pain and nausea.[14,15] Likewise, supplements based on iron salts and complexes can suffer from hydrolysis going through stomach, and the delivered iron has low bioavailability and in addition, is inhibited by a variety of components of the diet.[10-12,17]

Iron oxide nanoparticles have emerged as promising iron supplements since they can be more bioactive than classical chemical forms and have better access to enter tissues.[18-22] However, some works showed that large uncoated iron oxide nanoparticle aggregate under physiological conditions and produce serious toxicological effects.[23-25] Therefore, it is necessary to consider the toxicological hazards of iron nanoparticles and establish the parameters (surface properties, charge, size, shape, and the engineered surface functionalization of the nanoparticles) that optimize their efficiency as iron supplements without side effects.

The use of an appropriate coating is essential to overcome the stomach medium and carry the iron nanoparticles to intestine, where iron absorption takes place.[9,26] We have recently reported that probiotic bacteria, *Lactobacillus fermentum* and *Bifidobacteria breve*, serve as platforms to densely arrange small iron oxide nanoparticles on their external surfaces.[27-29] These are promising materials for iron supplements since, first we have found that the bacteria remain alive after grafting nanoparticles, and secondly because probiotic bacteria constitute part of natural microbiota and are able to survive the stomach conditions and nest in different sites of the gastrointestinal tract.

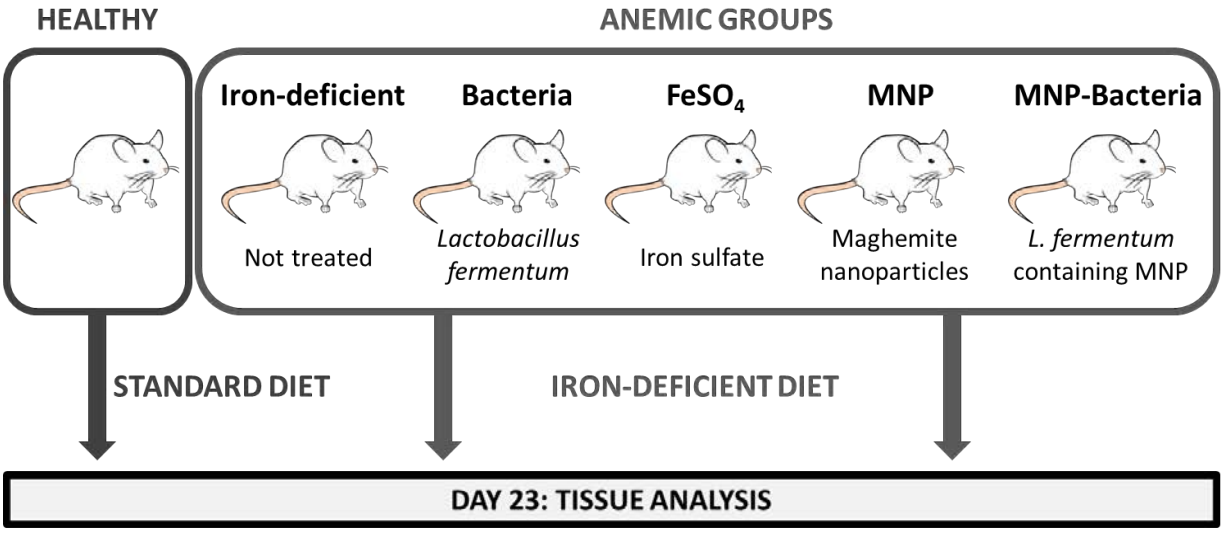
The aim of the present study was to investigate small maghemite nanoparticles (average size 10 nm), uncoated (MNP) or incorporated on *L. fermentum* (MNP-bacteria) as new iron supplements for iron deficiency in anemic rats. No adverse symptoms or mortality of anemic rats were observed after 23 days of oral administration of MNP or MNP-bacteria. However, the efficiency of these materials in alleviating anemia was sensibly higher when incorporated onto the probiotics (MNP-bacteria) than when isolated (MNP).

A full set of experiments, including magnetic analysis of the different rat tissues after administration of MNP and MBP-bacteria, *in vitro* MRI and electronic microscopy studies on HT-29 human cell line after treatment with MNP and MNP-bacteria pointed out that bacteria with nanoparticles (MNP-bacteria) are internalized by enterocytes, which advances a new route to ensure iron absorption. Restoration of healthy levels of blood parameters and iron-related protein expressions after administration of MNP-bacteria to anemic rats confirmed the efficiency of this material as a new drug for anemia.

RESULTS AND DISCUSSION

Thirty rats were rendered anemic by feeding them with an iron-deficient diet. Then, they were randomly divided into five groups: an untreated anemic group, and anemic groups treated with *L. fermentum*, iron sulfate, MNP or MNP-bacteria at the same iron dose. A control group

of six healthy (non-anemic) rats was also included in the study (Scheme 1). The different types of iron supplements were given to rats once a day by intragastric administration for 23 days. Observation of food intake and body weight revealed no adverse signs, symptoms, or mortality after the 23 days of treatment.



Scheme 1. Animals Treatment. Rats were randomly divided into six groups: a control group (healthy, non-anemic), an untreated anemic group, and four anemic groups treated with: *L. fermentum*, iron sulfate, MNP or MNP-bacteria. The last three groups at the same iron dose.

Magnetic study of luminal contents from tissues. Since iron absorption takes place at specific sites of the gastrointestinal tract, mainly in intestine,[9,26] we focused to analyze the presence of maghemite nanoparticles in the lumen of different sections of the gastrointestinal tract after administration of MNP or MNP-bacteria. Magnetic measurements of lyophilized lumen from small intestine, cecum and colon were performed. In particular, we studied the temperature dependence of the magnetic ac susceptibilities, both the in-phase (χ') and out-phase (χ'') components of these three samples for every treatment with MNP or MNP-bacteria (Figure 1). The study of the temperature dependence of the ac susceptibility is a highly

sensitive technique for the detection of superparamagnetic iron oxide nanoparticles in biological tissues as well as for monitoring their partial or full degradation processes.[30-32]

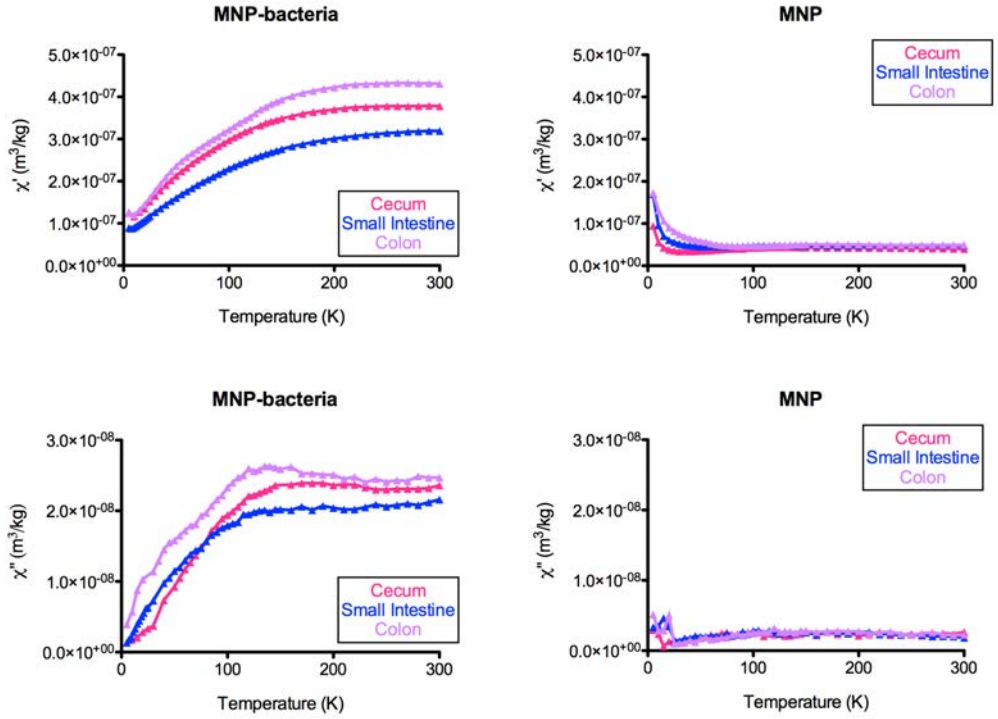


Figure 1. Temperature dependence of the ac magnetic susceptibility, both the in-phase (χ') and out-phase (χ″) components of lumen samples from different sections of the gastrointestinal tract: cecum (pink), colon (violet) and small intestine (blue).

The in- and out-phase susceptibilities of samples from the treatment with MNP-bacteria exhibited maxima in all the tissues, which is indicative of the presence of superparamagnetic particles. In contrast, the magnetic behavior corresponding to the luminal contents of the different tissues after administration of MNP did not exhibit any maxima, which informs of the absence of maghemite nanoparticles. In addition, a paramagnetic low temperature contribution in the in-phase susceptibility was clearly observed. This contribution corresponds to the presence of iron cations resulting from the degradation of maghemite nanoparticles.

From these data it can be concluded that the incorporation of the maghemite nanoparticles onto the probiotic (MNP-bacteria) allow nanoparticles overcoming the stomach medium and

reaching the intestine. In contrast, MNP undergoes biodegradation, probably at the stomach, which makes negligible the amount of maghemite nanoparticles accumulated in the intestinal lumen.

Plasma iron and hemoglobin levels. Anemia is characterized by a decrease of iron and hemoglobin (Hb) levels in blood. As it can be seen in Figure 2, due to the iron-deficient diet, plasma iron and Hb levels of anemic rats were lower ($p < 0.05$) than those of healthy rats (controls). The group that was supplemented with MNP showed a slight increase in plasma iron and practically no significant difference in Hb level when compared with the anemic group ($p > 0.05$). In contrast, the administration of MNP-bacteria restored the physiological plasma iron and Hb levels as occurred with the administration of iron sulfate, the most common iron supplement.

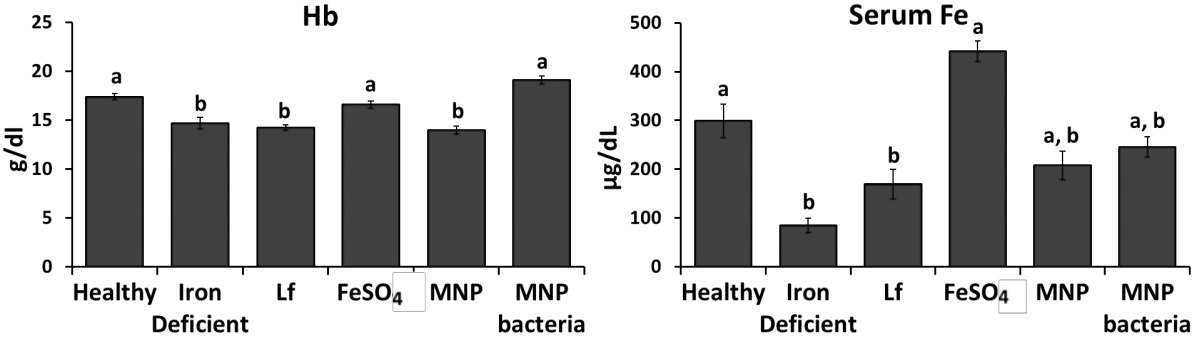


Figure 2. Plasma iron and Hb values in the six rat groups: healthy, iron-deficient and treated with (from left to right) L. fermentum, ferrous sulfate, MNP and MPN-bacteria. Error bars, ±sem (n=6). Different letters show statistically significant differences ($p < 0.05$).

Expressions of biomolecules of iron homeostasis. Figure 3 shows the results of DMT1 and DcytB protein gene expression analysis in the small intestine from the different groups of rats. A proper functioning of this tandem of proteins is essential for iron absorption. Iron enters the small intestine mainly as Fe(III) as the result of Fe(II) oxidation by gastric juice components. The ferrireductase protein DcytB is highly expressed at duodenal enterocytes and reduces

Fe(III) to Fe(II). Upon reduction, Fe(II) is transferred across the apical membrane of enterocytes by divalent metal transporters, mainly DMT1. Accordingly to these functions, the expressions of DcytB and DMT1 dramatically increase in periods of high body iron demand, as occurs during anemia. Figure 3 shows that in fact, the expression of DcytB and DMT1 increased in anemic rats with respect to those of the control (healthy rats). The administration of MNP-bacteria leads to a restoration of healthy levels of both proteins, pointing out that the supplementation of iron is effective. However, the administration of iron sulfate or MNP does not move the DcytB and DMT1 levels until those of healthy rats. This suggests that the administration of iron sulfate or MNP at this dose in these conditions is not efficient to decrease the iron demand caused by anemia. Interestingly, the effect of the administration of MNP-bacteria on DcytB is similar to that of bacteria without iron. As recently reported, some compounds excreted by *L. fermentum* have ferrireductase activity,[33] making functions of DcytB and therefore, the expression of DcytB does not increase in rats administrated with *L. fermentum*.

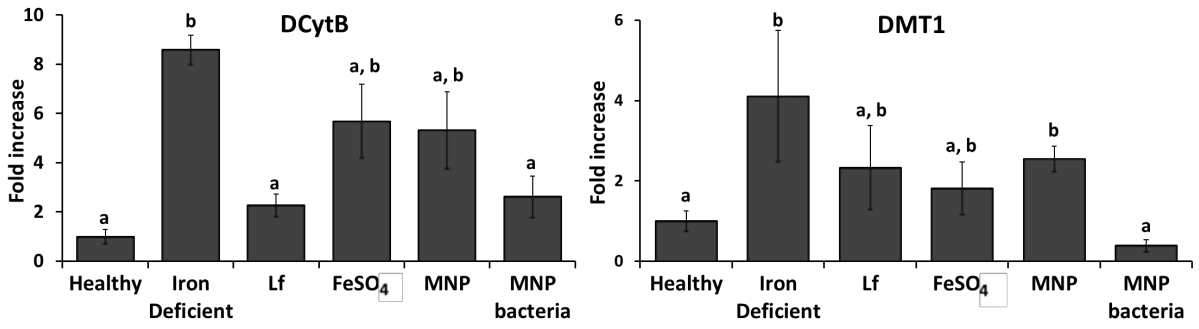


Figure 3. DMT1 and DcytB mRNA expression in small intestine tissue of: healthy, iron-deficient and treated rats with (from left to right): *L. fermentum*, ferrous sulfate, MNP and MPN-bacteria. Error bars, ±sem (n=6). Different letters show statistically significant differences ($p < 0.05$).

On the other hand, once inside the enterocytes, Fe(II) is transported to plasma by the membrane protein ferroportin, the activity of which is regulated by the hormone hepcidin.[34] This hormone is expressed in the liver and is up-regulated when hepatic iron stores are high and down-regulated when iron stores are decreased. Because liver iron levels are representative of the store regulator for iron absorption, hepcidin expression inversely correlates with the amount of iron absorbed by the intestine. Likewise, the decrease of hepcidin correlates with an increase in the duodenal expression of DcytB and DMT1.

Figure 4 shows how in fact, the expression of hepcidin decreases in anemic rats. However, after administration of MNP-bacteria, iron sulfate and, to a lesser extend MPN, the hepcidin expression levels increases with respect to that of anemic rats, reaching values corresponding to those of healthy rats.

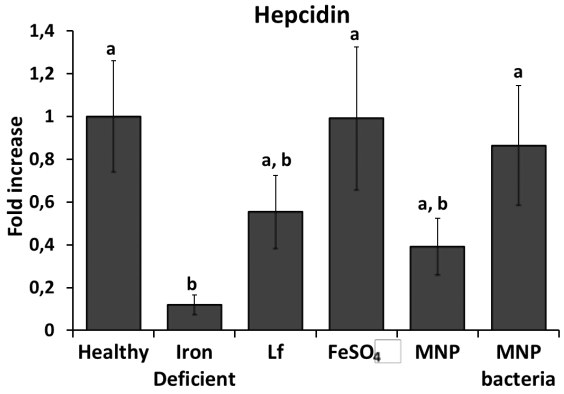


Figure 4. Hepcidin mRNA expression in liver of healthy, iron-deficient and treated rats with (from left to right): *L. fermentum*, ferrous sulfate, MNP and MPN-bacteria. Error bars, ±sem (n=6). Different letters show statistically significant differences ($p < 0.05$).

After magnetic study on biodistribution it is clear that MNP-bacteria allow accumulating maghemite nanoparticles at sites where iron absorption takes place. After the collective analysis of blood parameters and mRNA expressions of DcytB, DMT1

and hepcidin reveals that MNP-bacteria is more effective for treatment of anemia since it restores the healthy blood parameters. This means that the accumulation of maghemite nanoparticles at intestine seems an ideal scenario to ensure appropriate iron absorption. However, no information from these data can be infered about the mechanism by which the presence of MNP-bacteria at intestine does ensure the enter of iron into blood to remediate anemia. With the aim to explore this mechanism we performed an *in vitro* MRI experiment and a transmission electron microscopy (TEM) study to evaluate the internalization of maghemite nanoparticles into HT-29 human cells after incubation with MNP or MNP-bacteria.

Internalization of maghemite nanoparticles into enterocyte cells. We compared the internalization level of maghemite nanoparticles into enterocytes by measuring the transversal relaxation times (T2) of water protons when incubated the cells with MNP or MNP-bacteria. After incubation for 30 min, the wells were exhaustively washed with water to remove any material not internalized in cells.

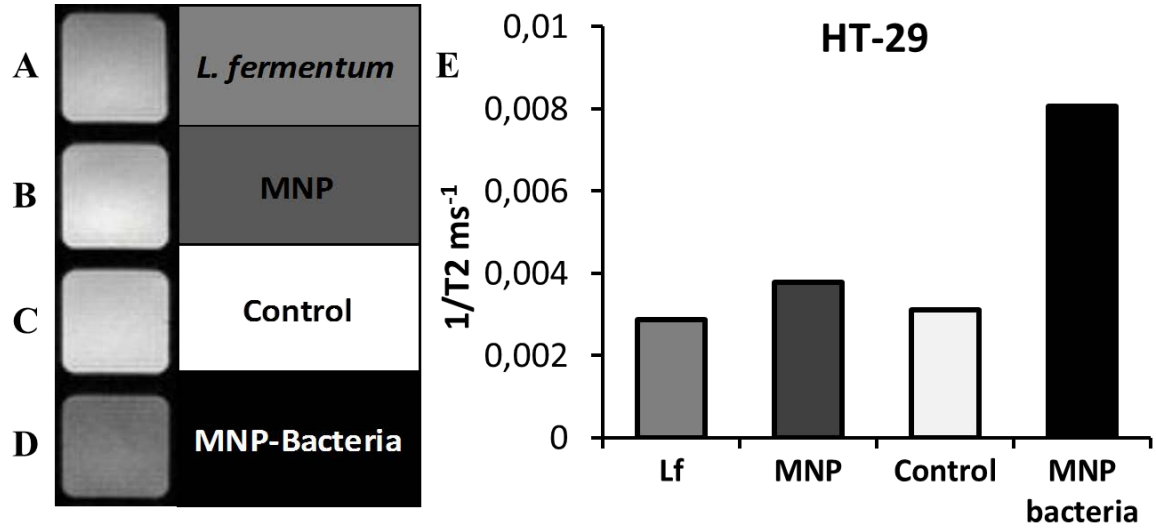


Figure 5. T2-weighted MR images after incubation of enterocyte cells (line HT-29) with a) *L. fermentum*; b) MNP; c) untreated well and d) MNP-bacteria. The negative

contrast informs of the accumulation of magnetic material into cells. 1/T2 values are represented in e.

As it is evident from Figure 5, the incubation with MNP-bacteria resulted in a strong effect on the signal intensity of the T2-weighted MR images. In contrast, MNP produced a negligible effect, with T2 values close to those of controls, without magnetic material (Figure 5). These results evidence the internalization of maghemite nanoparticles into enterocytes only in the case of MNP-bacteria, which points out some role of the probiotic to promote internalization.

TEM images of HT-29 cells treated with MNP-bacteria showed unequivocally the presence of MNP-bacteria and maghemite nanoparticles, localized intracellularly in endosomal/lysosomal compartments (Figure 6). No internalization of maghemite nanoparticles was observed when the cells were treated with MNP. These data make clear that the mechanism of the internalization of maghemite nanoparticles into enterocyte takes place by the internalization of nanoparticles-containing bacteria (MNP-bacteria), which are clearly observed in Figure 6. The presence of maghemite nanoparticles would be therefore consequence of the degradation of MNP-bacteria inside the endosome. Once delivered, these nanoparticles form the large aggregated found in Figure 6.

In this sense, it is interesting to note that some previous works reported by Juillerat-Jeanneret *et al.*[35] concluded that cationic magnetite nanoparticles, of the same size than those used in the present work, internalized in HT-29 cells. This apparent contradiction is explained in terms of the existence of a polyvinylamine coating around the magnetite nanoparticles whereas we used uncoated maghemite nanoparticles.

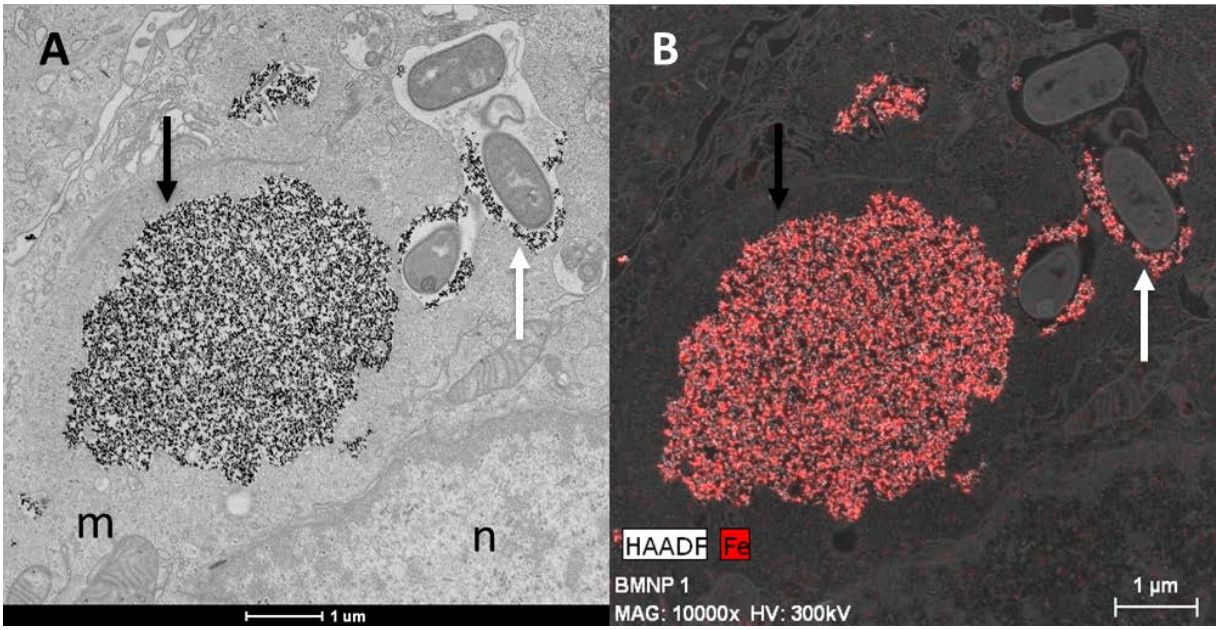


Figure 6. a) A typical TEM micrograph of thin epoxy resin sections of cell line HT-29 incubated with MNP-bacteria showing large accumulation of nanoparticles (black arrow) and MNP-bacteria (white arrow) at endosomal/lysosomal compartments of one HT-29 cell (m is the mitochondria and n the nucleus). b) EDX compositional maps of iron collected over the whole image in (a).

The collective analysis confirms the crucial role of the nanoparticle coating for internalization into enterocyte. In the present work we have used a living transporter, the probiotic bacterium *L. fermentum*. The present study demonstrates that maghemite nanoparticles grafted onto this probiotic bacterium (MNP-bacteria) has positive effects upon anemic rats sensibly better than uncoated nanoparticles (MNP) and the standard supplement iron sulfate. These results note the role of the probiotic as an efficient oral carrier of maghemite nanoparticles, since it allows overcoming the chemical medium of stomach, driving the nanoparticle to intestine, where maghemite nanoparticles internalize into enterocytes and ensure the restoration of blood parameters and alleviate anemia.

METHODS

Maghemite nanoparticles synthesis. MNP were synthesized according to Massart's method by co-precipitation of Fe(II) and Fe(III) salts in a stoichiometry of 0.5.[36] By adjusting the pH to 11 with 3M NaOH and ionic strength with 1M $NaNO_3$, the average size of the resulting magnetite nanoparticles is 10 nm. After oxidation of magnetite to maghemite with 1M $HClO_4$, a colloid of maghemite nanoparticles stable at pH 2 was obtained.

Grafting maghemite nanoparticles to bacteria. For the preparation of MNP–bacteria, a liquid culture of probiotic *Lactobacillus fermentum* CECT5716 was grown in a common bacterial growth medium such as MRS at 37 °C with orbital agitation for 24 h. Bacteria were centrifuged at 3000g for 5 min and washed with distilled water. Then, an acid solution (pH 2) of MNP (66.6 μL, 0.95 M) was added to the bacteria in an ice bath and mixed. The solution was diluted to 1 mL with distilled water. Bacteria labeled with maghemite nanoparticles (MNP–bacteria) were collected at 100g, 20 min.

MNP and MNP–bacteria characterization was carried out by Transmission Electron Microscopy (TEM) as previously reported.[27]

Animals. This study was carried out in accordance with the 'Guide for the Care and Use of Laboratory Animals' as promulgated by the National Institute of Health. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.[37,38]

Male Wistar rats (180–200 g) obtained from Janvier (St Berthevin Cedex, France) were housed individually in Makrolon cages, maintained in an air-conditioned atmosphere with a 12 h light–dark cycle, and provided with free access to tap water and food ad libitum. They were randomly assigned to two different experimental groups. One of them received only the standard diet, whereas the other was fed an iron deficient diet (TD.80396) provided by Harlan Laboratories (Madison, WI, USA). Blood from tail

veins were collected and tests for Fe content were carried out weekly until the fourth week. In this moment, IDA was established and the group fed iron deficient diet was subdivided into 5 groups. Three groups received a supplement of iron (1 mg per day per animal) by: iron sulfate solution, suspension of MNP or suspension of MNP–bacteria respectively. The two remaining groups were: a not treated anemic group; and an anemic group treated with *L. fermentum*. All the groups were treated for 23 days (**Scheme 1**). All the treatments were daily administered by oral gavage. Food and water intake was daily recorded for all groups (n=6). Rat body weight was measured twice a week. No significant differences were observed in animal weight among the groups throughout the experiment.

Biological samples. Blood samples were taken weekly from the hepatic portal vein in a heparinized tube to analyze the blood panel. After 23 days of treatment, the rats were sacrificed and the different parts of gastrointestinal tract and liver were collected and weighed. To obtain the luminal content of the different tissues of the gastrointestinal tract; small intestine, cecum and colon were emptied of their luminal material. Intestinal, colonic and cecal lumen were separately frozen in liquid nitrogen and freeze-dried and stored at −80 °C. Finally, lyophilized lumen of the tissue samples was pulverized and the corresponding powders were analyzed for their magnetic characterization.

Magnetic characterization. Lumen of the different tissues of the gastrointestinal tract from the groups treated with MNP–bacteria and MNP, separated by group and tissue, were freeze-dried and the corresponding powders were placed in gelatine capsules for magnetic characterization. Measurements were performed in a Quantum Design MPMS-XL SQUID magnetometer equipped with an AC (alternating current) magnetic susceptibility option. AC susceptibility measurements were performed with an AC

amplitude of 0.41 Oe, in the temperature range between 10 and 300 K and at a frequency of 11 Hz.

Gene expression analysis. The expression of DMT1 and DcytB markers was determined in small intestine, the hepcidin expression was determined in liver. Total RNA from the small intestine and liver samples was isolated using TRI Reagent®, according to the manufacturer's instructions. Purity and RNA concentration were measured by the absorbance ratio at 260 and 280 in a Thermo Scientific Nano Drop TM 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA (2 µg) was reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Subsequently, 20 ng of complementary DNA was taken for real-time quantitative PCR amplification and detection, which was accomplished on optical-grade 48-well plates in an Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA) with the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) and specific primers for each gene (GAPDH: FW: CCATCACCATCTTCCAGGAG, RV: CCTGCTTCACCACCTTCTTG; hepcidin: FW:CACAGCAGAACAGAAGGCATG, RV: CTTCTGCTGTAAATGCTGT; DMT1: FW: GTGTTGGATCCTGAAGAAAAGATTCC, RV: GGGATTTTCTCATCAAAGTAGGTGGT; DCytB: FW: AGTGCAGCAAGTTCTTGATGAAAT, RV: CGTGGCAATCACTGTTCCAA).The expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured to normalize messenger RNA (mRNA) expression. The mRNA relative quantitation was calculated with the ΔΔCt method and expressed as fold-change compared with the non-colitic control.

Statistics. Statistical analysis was performed using SPSS version 20.0 software (SPSS, Inc.) and GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, United

States), and data were expressed as mean ± SEM. Differences between more than two groups were assessed using one-way ANOVA followed by Tukey's test. Significant differences were established at $P \leq 0.05$.

In vitro internalization in enterocyte cells. HT-29 epithelial human colorectal adenocarcinoma cells were provided by the Scientific Instrumentation Centre (University of Granada, Spain). Cell line was grown adherently and maintained in McCoy's 5a medium + 2 mM glutamine + 1 mM of sodium pyruvate + 10% of fetal bovine serum (FBS) at 37 °C in 5% CO_2. All experiments were performed in 12-well plates. Cells were seeded onto the plates at a density of 5×10^4 cells per well and incubated for 48 h prior to the experiments.

In vitro T2 measurement by MRI. For MRI experiments HT-29 cells were treated separately with: MNP-bacteria (0.94 mM of Fe), MNP (0.94 mM of Fe) and *L. fermentum*. Wells were filled up to a final volume of 2 ml with the grown medium. A blank sample containing only cells was also prepared. The plate was incubated at 37 °C for 30 min. After incubation wells were washed three times with PBS 6.8 solution. Finally cells were trypsinized and transferred to a suitable plate for MRI measurement. Transversal relaxation times, T2, were measured on a 9.4T Bruker Biospec system using a 128-echo CPMG (Carl-Purcell-Meiboom-Gill) imaging sequence and the following acquisition parameters: TE values from 7.5 ms to 960 ms, TR= 3000 ms, FOV = 6 cm, matrix size = 256x256, slice thickness = 1 mm. T2 values were calculated using automated scripts written in IDL (Interactive Data Language, Research Systems Inc., Boulder, USA).

In vitro internalization of MNP-bacteria study by Electronic Microscopy. After the same treatment as in MRI experiment, HT-29 cells were embedded in epoxy resine. Fixation was performed by adding glutaraldehyde 2.5% in 0.1 M sodium cacodilate

buffer at 4 °C for 4 h. The sample was washed 3 times in 0.1 M sodium cacodilate buffer for 15 min. 0.1% Osmium tretroxide in 0.1 M sodium cacodilate buffer was added and let stand for 1 h. Osmium tretroxide was washed three times with 0.1 M sodium cacodilate buffer for 15 min every time. Then, dehydration took place through a series of ethanol and propylene oxide. Finally, the sample was embedded in epoxy resin and left overnight at 4 °C. After ultramicro-cutting, samples were observed with a FEI TITAN G2 microscope.

AUTHOR INFORMATION

Corresponding Authors

*josema@ugr.es. [†]Department of Inorganic Chemistry and Biotechnology Institute, University of Granada, 18071 Granada, Spain.

*jgalvez@ugr.es. [‡]Department of Pharmacology, CIBER-EHD, ibs. Granada, CIBM, University of Granada, Spain.

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ABBREVIATION LIST

MNP, Maghemite nanoparticles; AC, Alternating current; Hb, Hemoglobin; DMT1, Divalent metal transporter 1; DcytB, Duodenal cytochrome B.

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Anemia

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