Ameliorative Function of a Probiotic Bacterium, *Lactobacillus rhamnosus* MR1 on Acute Iron Toxicity in Rats

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Abstract: Overuse of iron supplements can lead to an acute inflammation of the gastrointestinal tract. This study investigates the ameliorative and prophylactic effects of a probiotic bacterium, *L. rhamnosus* MR1, on acute iron poisoning in rats. In this study, a probiotic strain was isolated from yogurt and characterized for its probiotic properties, including antibiotic-resistant, bile salt (BS) and acid resistance, iron tolerance, cell hydrophobicity of the bacterial cells. The anti-inflammatory effect of strain MR1 was studied on the iron exposed-Caco-2 cell line. *In vivo* experiments were conducted for the assessment of survival in rats overdosed with treatment. These findings indicate high bacterial tolerance in acidic conditions, high concentrations of bile salts, and iron. The anti-inflammatory effects of strain MR1 were confirmed by decreasing the concentration of pro-inflammatory cytokine IL-8 and increasing anti-inflammatory cytokine IL-4 in treated groups. Prophylactic and acute effects of strain MR1 in rats caused a significant reduction in intestinal iron poisoning by 50 % during 6 h. Prophylactic regimen by *L. rhamnosus* MR1 increased the viability of about 33% in acutely poisoned rats. Since no report is found in the current literature about the effect of probiotic supplements on iron's acute toxicity, these interesting results can provide a useful background for further studies on dietary supplements.

Keywords: *Lactobacillus rhamnosus* MR1; acute iron toxicity; bacterial probiotics; oral administration.

1. Introduction

Iron is one of the most essential elements in the body that plays a fundamental role in many biological processes. Iron contained in heme, which includes hemoglobin and myoglobin, is ferrous iron (Fe^{2+}); however, more than 25% of the iron content of the body is stored in the complex with hemosiderin, ferritin, and transferrin in tissues such as the liver, spleen, and bone marrow [1]. Nowadays, iron supplements have attracted particular attention in society due to increasing iron deficiency [2]. On the other hand, excessive iron consumption

can lead to severe complications, including corrosive injury of the gastrointestinal tract and hemorrhagic necrosis of the gastric mucosal membrane. Since excessive iron in the body cannot bind to iron carriers, it converts to toxic free radicals causing acute intoxication [3,4]. Acute iron poisoning often occurs when an individual orally consumes a large number of iron-containing supplements. Complications of acute poisoning due to increased iron intake can cause various symptoms such as nausea, vomiting, abdominal pain, and diarrhea [5]. Iron in the form of ferrous sulfate is a common form of pharmaceutical preparations available in multiple formulations for oral and injectable applications. Oral iron supplements such as drops, syrups, elixirs, capsules, and tablets often contain 325 mg of ferrous sulfate. Only about 20% of them are absorbable by the intestine. In iron overdose cases, an increase in serum iron up to 500 µg/dl appears severe clinical complications that may lead to death [6].

As aforementioned, the first useful step to prevent acute oral poisoning is the inhibition of iron intake in the gastrointestinal system. Therefore, some compounds and drugs that can bind to iron in the gastrointestinal tract could be applied for preventing iron intoxication. One of the most essential gastrointestinal hemostatic regulators is gut microbial flora that acts as a pivotal mediator in the absorption of substances and the neutralization of harmful compounds [4,7]. Probiotics constitute a live part of fermented dairy products that provide many health benefits to the host. Many studies have documented the role of probiotic bacteria like *Lactobacillus* and *Bifidobacterium* species in the gastrointestinal tract's metabolic and physiological balance [8,9]. Studies have shown that probiotics are healthy, safe, and easily available because they have originated from food sources. Several reports have claimed the protective role of probiotics in heavy metals, chemical compounds, and toxins [10]. This study aimed to investigate the effects of a probiotic bacterium isolated from yogurt from iron sulfate's acute toxicity in rats.

2. Materials and Methods

2.1. Bacterium isolation and identification.

Several probiotic bacteria were isolated from yogurt fermented from goat milk. Before the isolation experiment, the yogurt sample was maintained at room temperature for 72 h. Then, 1 ml of yogurt sample was taken in a glass tube and diluted 10-fold series by sterile distilled water. A 10-µl volume of each dilution was spread on MRS (Man, Rogosa, and Sharpe) agar plates. The plates were then incubated at 37 °C for 72 h until different bacterial colonies appeared over the agar media. The single purified colonies were characterized based on their morphology, biochemical characterization, and 16S rRNA-based phylogenetic study. These isolates were frozen at -70 °C in MRS broth containing 20 % (v/v) glycerol and maintained until further investigations. Among the isolated strains, *L. rhamnosus* MR1 was selected based on the most resistant bacterium to ferrous sulfate into MRS broth compositions.

2.2. Bile salt and pH resistance.

The survival test was conducted according to a method described by Sharma *et al.* (2019) with a little modification [10]. All experiments were performed in different conditions such as pH (2-8) and sodium taurocholate (0.1-3 %) in sterile glass tubes containing 10 ml of MRS broth medium. Overnight grown bacterial inoculum in the volume of 100 µl containing 1.5×10^8 cells/ml was inoculated in glass tubes, incubated at 37 °C for 1 h. After that, 100 µl of

each sample was taken, 10-fold diluted and spread on MRS agar plate. Finally, the number of colonies was determined using a colony counter (Gallenkamp, England).

2.3. Antibiotic resistance of L. rhamnosus MR1.

The antibiotic resistance of *L. rhamnosus* MR1 was characterized based on the Clinical and Laboratory Standards Institute (CLSI) guideline using the disc diffusion method. For this, the bacterial inoculum (1.5×10^8 CFU/ml) was spread on MRS agar using a sterile swap. For this, ten antibiotics discs including cephalexin (30 μg), ampicillin (10 μg), penicillin G (2 IU), tetracycline (10 μg), erythromycin (15 μg), chloramphenicol (30 μg), streptomycin (10 μg), kanamycin (30 μg), azithromycin (15 μg) and ciprofloxacin (5 μg) were used. Different antibiotic paper discs were placed on the plates and incubated at 37 °C for 24 h. A ruler measured the diameter of the growth inhibition zone.

2.4. In vitro cell adhesion ability of L. rhamnosus MR1.

The adhesion ability of *L. rhamnosus* MR1 was investigated on Caco-2 cells in the presence of iron sulfate. For this, Caco-2 cells were seeded in 24-well culture plates in high glucose DMEM supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) at 37 °C in a humidified atmosphere with 5% CO_2 until 80% confluent monolayer cells were formed. After that, the culture medium was removed from the wells, washed with PBS, and replaced with a fresh medium containing different numbers of the bacterial cells (10^6, 10^7, and 10^8 CFU/ml) supplemented with 30 mg/l of ferrous sulfate. After 4 h the incubation, the culture medium was removed and washed twice with PBS. Adherent bacterial cells were detached using 1 % Triton X-100. The number of viable bacterial cells was counted by the colony counting method on MRS agar plates.

2.5. Ferrous ion tolerance assay.

The iron resistance pattern was determined using MIC and MBC methods against different concentrations (0-500 mg/ml) of ferrous sulfate as previously [11]. To assay MIC for Iron treatment, ferrous sulfate stock (500 mg/ml) was prepared, and then 2-fold dilutions were performed until it reached the lowest concentration. The overnight bacterial cells were grown in MRS broth containing one of the Iron diluted solutions. After 24 h incubation, the bacterial growth rate was examined by measuring the culture media's optical density at 600 nm. MIC was defined as the lowest iron concentration at which there was no visible growth in optical density (OD_{600}). MBC has defined as the lowest concentration of the iron that no colony was growing on the MRS agar plates.

2.5. Cell hydrophobicity.

Hydrophobic of the bacterial cells was evaluated using n-hexane and olive oil as a non-polar phase according to a method described by Heravi *et al*. (2011) [12]. A bacterial suspension with an initial optical density of 0.3 (absorbance wavelength of 600 nm) was prepared from overnight bacterial culture. The effect of Ferrous sulfate, pH, and bile salt on the cell hydrophobicity were examined at the pH range of 2-8 and the different concentrations of ferrous sulfate (0-300 mg/l) and bile salt (0.5-3.0 %). Two-milliliter volumes of bacterial suspension were mixed with 500 μl of n-hexane and olive oil, then vortexed for 2 min and kept at room temperature for 1 h. Subsequently, the aqueous phase was taken for estimating bacterial

cell density using determining the optical density (OD$_{600}$ nm). The hydrophobicity index was calculated as the following formula:

$$\text{Hydrophobicity index} = \frac{\text{OD initial} - \text{OD final}}{\text{OD initial}} \times 100$$

2.6. Anti-inflammatory activity of L. rhamnosus MR1.

The anti-inflammatory function of *L. rhamnosus* MR1 was examined on the Caco-2 cell line in 24-well plates. The inflammatory response was stimulated by treating the cells with 10 or 30 mg/ml of ferrous sulfate. Additionally, one group was also stimulated with 250 ng/µl of lipopolysaccharide (LPS). Each well was treated with 200 µl of 10^8 CFU/ml of *L. rhamnosus* MR1 in MRS broth and incubated in the conditions described earlier for 3 h. Three untreated groups, including Fe-stimulated, LPS-stimulated, and un-stimulated groups, were considered for comparative controls. After that, the culture media were taken to measure the levels of immunomodulatory cytokines, including TNF-α and IL-4, using ELISA kits (ZellBio, GmbH, Germany).

2.7. Antioxidant activity of L. rhamnosus MR1.

The antioxidant property of *L. rhamnosus* MR1 metabolites was quantitatively determined using DPPH assay method. The metabolites of an overnight bacterial culture (OD=1.0 at 600 nm) were extracted via filtration using 0.22 µm Whatman filter paper. The filtrate was diluted two-fold, and 1 ml of serial dilutions was mixed with 1 ml of DPPH solution (0.05 mM). After that, the samples were incubated at 37 °C in darkness for 30 min. Their absorbance was determined at 517 nm using a UV–Visible spectrophotometer (Jenway UV-6420, UK). Ascorbic acid (AA) was used as a standard positive control, and Deionized water was a blank sample. The following equation was used for calculating the scavenged DPPH radicals by bacterial metabolites.

$$\text{Scavenging capacity (\%)} = \frac{1 - \text{OD Sample}}{\text{OD Blank}} \times 100$$

2.8. Animal experiments.

A total of 20 male Wistar rats (aged 9 weeks; weighing 200–250 g) were obtained from the Pasteur Institute Animal center, Tehran, Iran. To acclimate the animals to laboratory conditions, they were subjected to regular periods of 12 h-light and 12 h-dark. Relative humidity and room temperature were adjusted at 4% and 24 ±1°C, respectively. Two groups were served to study the prophylactic effect of probiotics. One group was fed a standard diet without probiotics (control), and another was fed a probiotic-supplemented diet (3×10^8 cells/g dried substance). These groups were kept for 3 weeks under the twice-a-day feeding regimen. After this period, control and treatment groups were gavaged by 1 ml of the diet without probiotics and 1 ml of probiotic-supplemented diet, respectively. Another experiment was conducted to examine the effect of probiotic administration on acute toxicity induced by ferrous sulfate. Therefore, two acute experiment groups (control and treatment) were fed the same regimen for prior groups. One hour later, all groups were gavaged with 500 mg/l ferrous sulfates. Finally, according to a time-schedule, all rats were anesthetized by intraperitoneal injection of ketamine/xylazine (90 mg ketamine plus 10 mg xylazine per kg animal weight). Blood samples were obtained from the retro-orbital venous plexus by sterilized glass capillary

tubes. Firstly, basal blood iron was determined before overdose iron administration. Then, blood samples were obtained after 1, 4, and 6 h of iron administration. Blood samples were collected in 2 ml microvials, maintained at laboratory temperature for 30 min, and then centrifuged at 3500 rpm for 15 min to separate the serum. Serum iron was measured by flame atomic absorption spectrophotometry. All animal experiments were conducted according to the human and animal ethical committee's protocol, Mashhad University of Medical Sciences.

2.9. Statistical analysis.

The results obtained from the experiments were presented as mean±SD. The data values were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post hoc test. All analyses were performed using GraphPad Prism Version 5.0 (GraphPad, Softwares Inc., San Diego, CA, USA). A confident level of 95% was considered for significance at P-value <0.05.

3. Results and Discussion

3.1. Bacterium isolation and identification.

Amongst the 9 isolates, the most resistant *Lactobacillus* strain to ferrous sulfate with the highest tolerance of about 100 mg/l ferrous sulfates was primarily selected, identified, and deposited as *L. rhamnosus* MR1 (accession number: KT215644.1) in the Genebank, NCBI according to 16S rRNA sequencing and global alignment in the BLAST online software. The bacterium was kept in 20 % glycerol for the subsequent studies. Figure 1 represents the closest strains and relation between different *Lactobacillus* genus spices that their 16S rRNAs were retrieved from NCBI Genebank, and the corresponding phylogenetic tree was constructed by Mega X software.
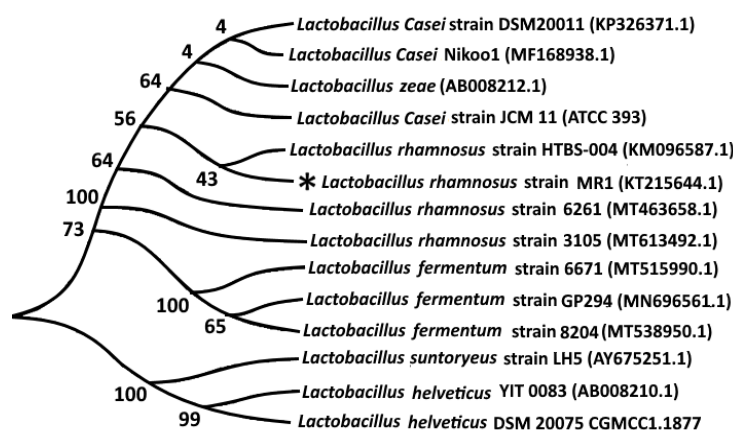
Figure 1. Phylogenetic tree of Lactobacillus strains and *L. rhamnosus* MR1 based on 16S rRNA gene sequences constructed by a neighbor-joining algorithm.

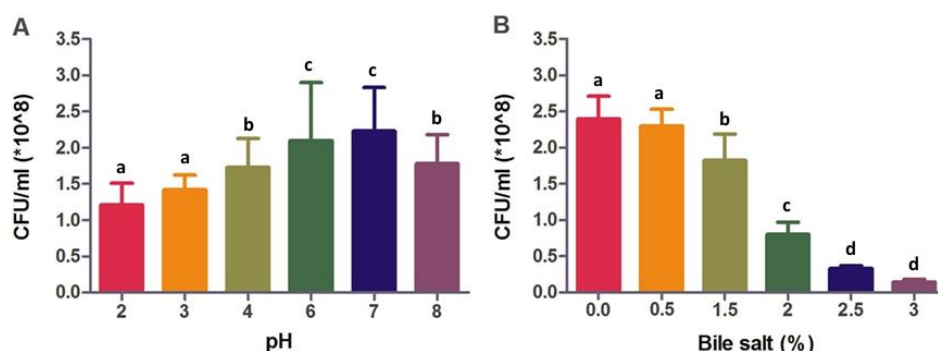### 3.2. The effects of pH and Bile salt on bacterium survival.

Although *L. rhamnosus* MR1 showed remarkable tolerance to pH value in the neural condition, a significant level of living bacterial cells was obtained under the acidic conditions (Fig. 2A). One critical point in oral administration of probiotics is to survive their normal activities when they pass through the stomach in contact with a high acidic environment [13]. Gastric acid is an essential barrier against living probiotics in the gastrointestinal tract that can cause metabolism inhibition and enzyme inactivation in microorganisms [14,15]. On the other hand, cell viability declined gradually in high alkali conditions, as observed in acidic

conditions. Therefore, one of the essential criteria for assessing the probiotic efficacy of bacteria is acid tolerance capacity that has been defined as the stability of their biological activities in acidic environments [13]. The strain MR1 exhibited a relatively acceptable tolerance in acidic conditions corresponding to the acid tolerance standard established based on several studies that most efficient probiotic strains could tolerate at least acidity level about pH 3.0.

Bile salt (BS) tolerance of the strain MR1 was determined at a satisfactory level by exposure to 1.5 % of BS for 1 h. However, the highest viability was observed in control, which no bile salt presented in bacterial cells' contact (Fig. 2B). BS is mainly produced from cholesterol, in which the small intestine facilitates the uptake of fatty acids and cholesterol. Since the bile salts act as detergents, they facilitate the uptake of lipophilic nutrients from the gastrointestinal tract. Therefore, they can affect the bacterial membrane, both microbiota and pathogens [16].

Meanwhile, overexposure of probiotic microorganisms to BS causes lost cellular integrity, increasing membrane permeability, and ultimately cell death. The selection of those probiotic strains that would be capable of passing safely through the gastrointestinal tract is considered a serious challenge [13]. Therefore, to obtain effective probiotics, their ability to tolerate stomach acid, intestinal osmolarity, and high concentrations of bile acids must be considered [17]. The results obtained from the pH and BS tolerance tests implied that strain MR1 had promising administration characteristics as a nutraceutical compound that remained its bioactivity under gastrointestinal conditions.

Figure 2. The effects of different pHs and bile salt concentrations on bacterial growth. The different letters indicate the significant differences between groups (p-value<0.05).

3.3. Antibiotic resistance of L. rhamnosus MR1.

Antibiotic susceptibility is the main criterion for evaluating the safety of probiotics. To assess the resistance to common antibiotics, the disc diffusion method is preferably used. As shown in Table 1, *L. rhamnosus* MR1 was susceptible to penicillin G and tetracycline. Strain MR1 showed intermediate resistance to erythromycin and azithromycin. Antibiotic resistance in probiotics is controversial in two respects, as resistant strains can benefit gastrointestinal microbiota during the treatment of bacterial infections.

On the other hand, antibiotic-resistant probiotics can donate genes to pathogenic species through conjugation [18,19]. Two antibiotic resistance types are found in bacterial strains, including innate (natural) and acquired resistance. The intrinsic type that originates the chromosome's resistance genes cannot be transferred horizontally to other bacterial strains. Numerous studies have reported vancomycin resistance in *L. rhamnosus* and *L. reuteri* is non-transferable due to its chromosomal origin. Some reports have attributed the development of

multiple resistance in probiotics to spontaneous mutations [20]. Therefore, the transfer of antibiotic resistance traits to probiotics tends to be more beneficial than a threat [21]. In this research, *L. rhamnosus* MR1, with high resistance to widely used antibiotics, has demonstrated that it can have a strong protective function against injuries caused by long-term antibiotic use in patients.

Table 1. Antibiotic resistance profile of *L. rhamnosus* MR1.

Antibiotic	Susceptibility status	MIC (µg/ml)	Inhibition zone (mm)
Cephalexin	R	300	8.5±0.72
Ampicillin	R	250	12±0.13
Penicillin G	S	10	24.6±1.3
Tetracycline	S	15	21.3±3.2
Erythromycin	MR	30	20.3±0.30
Chloramphenicol	R	150	8.04±0.38
Streptomycin	R	250	10.1±2.12
Kanamycin	R	350	5.6±0.61
Azithromycin	MR	25	13.7±3.2
Ciprofloxacin	R	400	0.0±00

3.4. In vitro cell adhesion ability of L. rhamnosus MR1.

One of the features of probiotics is the ability to bind to and colonize gastrointestinal cells. As a result, the gastrointestinal tract's protection against infections and destructive factors is supported by increasing probiotics' compatibility. In this respect, various studies have demonstrated the regulating role of gastrointestinal microbiota in immune response and inflammatory reactions. [34]. Furthermore, the attachment of probiotics and their metabolites to the intestinal mucosa can function as a defensive shield. In this study, the *L. rhamnosus* MR1 showed that its ability to bind to Caco-2 cells was more than 50%. As seen in Figure 3, the binding potential of bacteria is diminished by interactions with iron ions. According to other studies, bacterial cells may compete with chemicals and biology to bind to intestinal cells.

Zhai *et al*. (2016) reported reduced cadmium toxicity to intestinal epithelial cells, HT-29 exposed to *Lactobacillus plantarum* [22]. Several reports have shown that *L. rhamnosus* strains are highly capable of detoxifying heavy metals such as cadmium, lead, and arsenic [23,24].
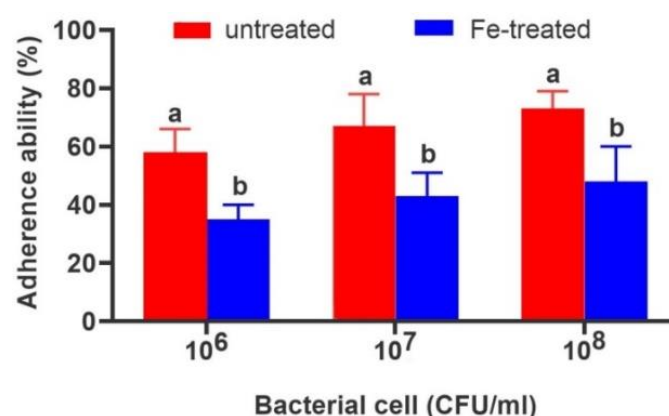

Figure 3. Adherence of *L. rhamnosus* MR1 to Caco-2 cells. The different letters indicate the significant differences between groups (p-value<0.05).

L. rhamnosus supplementation has been shown to reduce heavy metals, especially iron, in pregnant women and children [4]. Some of the secretory peptides of probiotics neutralize food toxins such as aflatoxins that cause food poisoning [25]. This study's findings indicate

that iron ions decrease the adherence of bacteria to Caco-2 cells. However, it can be proposed that probiotics may play a protective role against acute iron toxicity.

3.5. Ferrous ion tolerance assay.

The iron tolerance experiment showed that the strain MR1 could grow in the presence of a high level of ferrous sulfate *in vitro*. As seen in Figure 4, MIC for iron tolerance of the strain MR1 was determined 125 mg/ml, and MBC value was estimated at 250 mg/ml. Several mechanisms have occurred among various bacteria and metal ions, especially iron ions, including a ferric reduction to ferrous iron, siderophore production, and surface absorption by iron-binding proteins [26,27]. Since extracellular proteins are usually affected by environmental stress, such as electrochemical, osmolarity, and other physicochemical factors, a high level of the metal ions within the bacterial cell could influence the cellular metabolism and lead stress response [28]. A significant factor influencing bacteria's iron tolerance is the amount of soluble iron accessible for the bacterial cells to bacterial cells, increasing its absorption capacity [29].
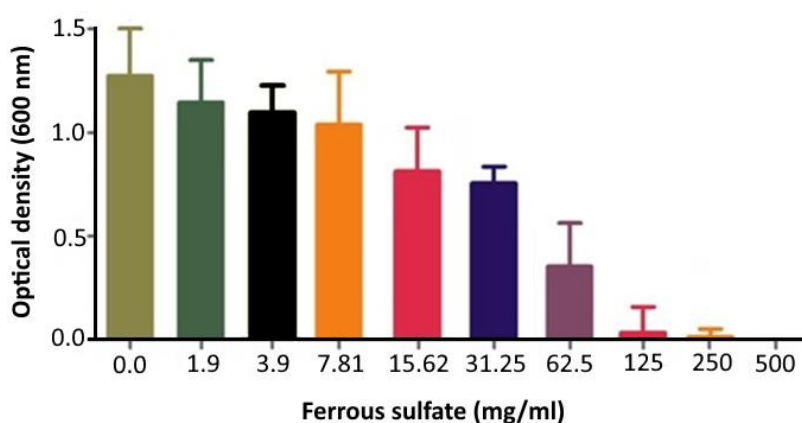

Figure 4. Iron tolerance by bacterial cells. The MIC value was determined based on the inhibition of iron growth in the MRS broth medium. MBC was calculated for those concentrations considered in the MIC assay.

3.6. Cell hydrophobicity.

Hydrophobicity of the strain MR1 was measured in hydrophobic phases, namely olive oil and n-hexane, in which different concentrations of ferrous ion and BS and different pHs were investigated. The results showed that the bacterial cells' hydrophobic tendency to the n-hexane phase was more than the olive oil phase (Figure 5). On the other hand, cell hydrophobicity significantly decreased with the gradual increase of ferrous ion concentration. Similarly, hydrophobicity value in high concentrations of BS drastically declined to 1 % when the bacterial cells were tested for their affinity to the olive oil as the hydrophobic phase. As a general result, the highest hydrophobicity was found in those experiments related to control without ferrous ion and bile salt treatments and pH 2. Bacterial cells have different extracellular molecules that provide surface charge for attachment of a wide range of materials, including glass, metals, and various organic polymers [30]. Therefore, the bacterial cells' attachment capacity depends on some surface energy such as surface charge, hydrophobic interactions that mediate possible absorptions to extracellular structures [31]. Also, bacterial cells, especially probiotic bacteria, often secret many secondary metabolites like bacteriocins, siderophores, and some peptides that promote attachment of chemical compounds and nutrients [32-34].
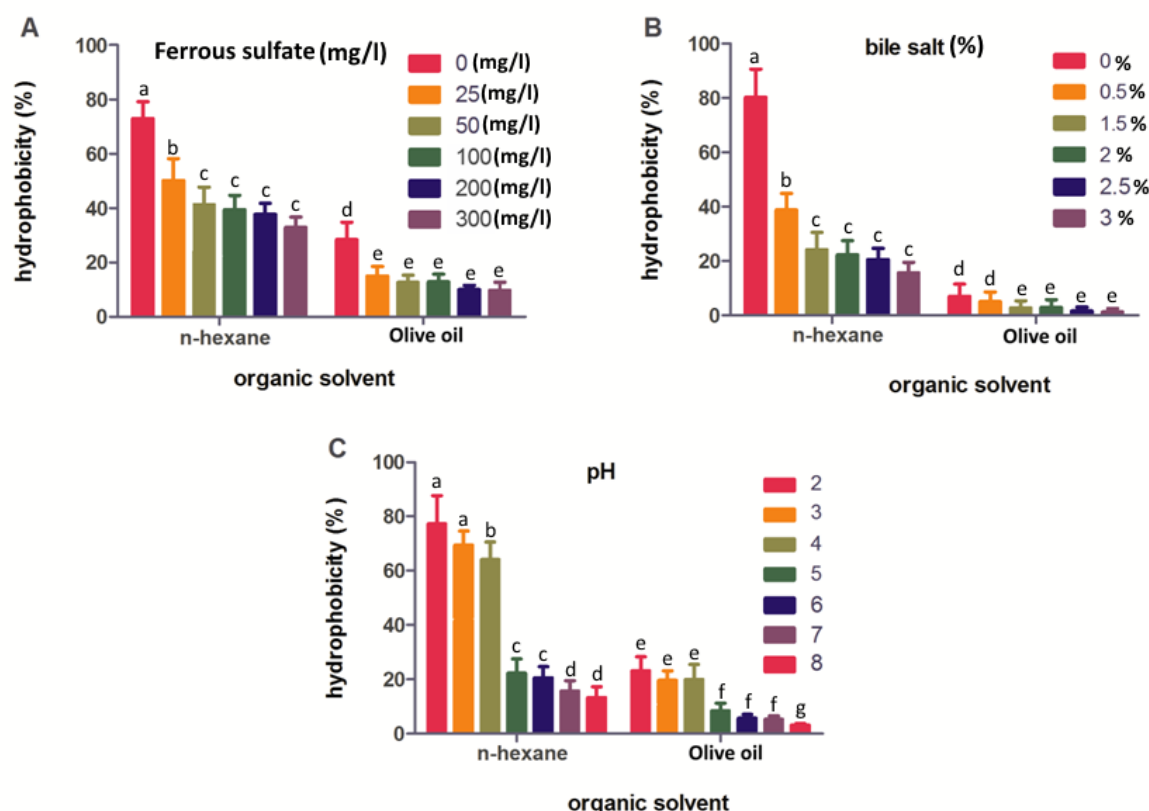
Figure 5. Hydrophobicity of the bacterial cells to two hydrophobic phases, n-hexan and olive oil, in different conditions. Hydrophobicity tendency of bacterial cells in the presence of (A) various iron concentrations. (B) different BS concentrations and (C) different pHs.

3.7. Anti-inflammatory activity of L. rhamnosus MR1.

Three inflammatory mediators (IL-4, IL-8, and TNF-α) were measured in the culture media from Caco-2 monolayers stimulated with iron sulfate or LPS and treated with *L. rhamnosus* MR1. As shown in Figure 6, a significant reduction was found in the IL-8 level in both LPS, and Fe stimulated groups. In contrast, the supplementation of probiotics had no significant amelioration in TNF-α level in all treatment groups. On the other hand, the level of anti-inflammatory cytokine IL-4 was drastically increased by *L. rhamnosus* MR1 supplementation in both LPS, and Fe stimulated groups. Considering the results, *L. rhamnosus* MR1 showed a significant role in the production of IL-4 by LPS or Fe-induced Caco-2 cells, which is thought to result from its immune modulation of anti-inflammatory cytokines. On the other hand, the levels of pro-inflammatory factors, TNF-α and IL-8, rose even after probiotic administration in both groups induced by LPS and iron. Similarly, Devi *et al*. (2018) Showed that *L. plantarum* and *L. rhamnosus* supplementation did not have a significant effect on the expression of pro-inflammatory genes, such as IL-6, IL-8, IL-1α, IL-1β, and TNF-α [35].

High doses of drugs and supplements, especially iron, can lead to acute inflammatory reactions in the gastrointestinal tract [5]. On the other hand, gut microbiota may modulate the inflammatory and oxidative stress response by affecting inflammatory cytokines. Although probiotics' influential role in stimulating the immune system, especially the gastrointestinal tract, has been investigated, probiotics' therapeutic role remains controversial [36]. Some scientists contend that probiotics simultaneously promote pro-inflammatory and anti-inflammatory influences. For instance, some probiotic strains can lead to increased levels of TNF-α and then trigger IL-8 expression in the gut [37]. In contrast, Bahrami *et al*. (2011)

concluded that HT-29 and Caco-2 cells treated with *L. plantarum* and *Bifidobacterium adolescentis* expressed high anti-inflammatory factors IL-4 and IL-10. On the other hand, they claimed probiotics such as *L. acidophilus*, *L. rhamnosus,* and *L. paracasei* significantly decreased IL-4 expression levels during the inflammatory process [38].
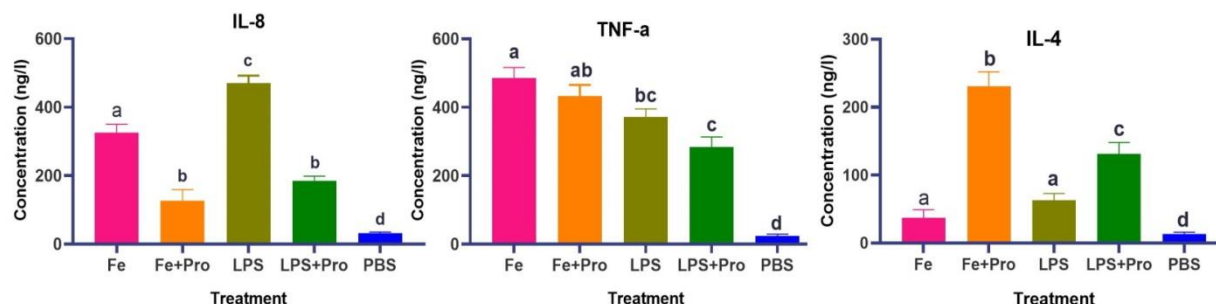

Figure 6. Level of pro- and anti-inflammatory cytokines A) IL-8, B) TNF-α, and C) IL-4 during LPS- and Fe-induced Caco-2 cells after treatment with *L. rhamnosus* MR1. The values represented are the gene expression fold change mean average ± SD (n = 3). The different alphabetical superscripts are defined as statistically significant (p < 0.05).

3.8. Antioxidant activity of L. rhamnosus MR1.

The DPPH scavenging assay evaluated antioxidant activity of L. rhamnosus MR1. The DPPH scavenging assay was recognized as a promising technique for the antioxidant potential of bioactive compounds. This method measures the proton donation capacity of antioxidants to DPPH radicals [36]. As seen in Figure 7, the antioxidant capacity of *L. rhamnosus* MR1 was found to be 47% at the highest concentration compared with ascorbic acid (100 %). As the concentration of probiotic metabolites in the CFS decreased, so did the antioxidant activity. In this experiment, the highest antioxidant activity was 47 % for a cell density of approximately 3×10^8 CFU/ml (OD_{600}=1.0).

Similarly, Ghafari and Ansari (2018) showed that CFS of *L. casei* and *L. rhamnosus* had an antioxidant capacity of about 45% [39]. According to the studies, metabolites of probiotics contain different compounds with various bioactive properties such as antioxidant, anticancer, antimicrobial, etc. These compounds are often composed of low-weight peptides, exopolysaccharides, surfactants, antibiotics, and active short-chain fatty acids. Ji *et al*. (2015) noted that many *Lactobacillus* strains produce low molecular weight metabolites with remarkably high antioxidant capacity that can neutralize various toxins and mutagenic substances [40]. In this study, *L. rhamnosus* MR1 had a promising antioxidant potential that could reduce iron ions' acute toxicity in the intestine.
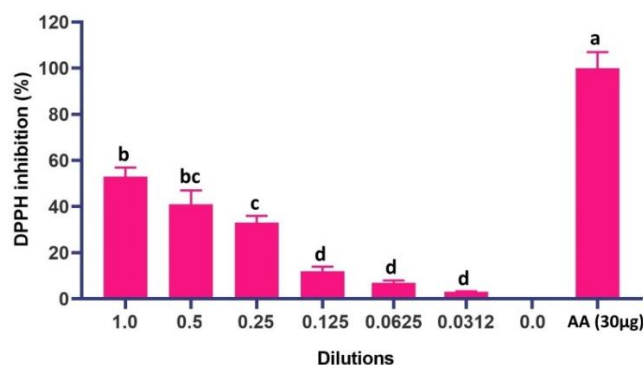
Figure 7. Antioxidant activity of *L. rhamnosus* MR1 in serial dilutions compared with ascorbic acid (AA). Different superscript alphabetic shows a significant difference between groups (P-value<0.05).

3.9. Animal experiments.

Prophylactic and ameliorative effects of *L. rhamnosus* MR1 in rats caused a significant reduction in intestinal iron uptake by 50 % during 6 h. However, the Prophylactic assay of *L. rhamnosus* MR1 on acute iron toxicity showed no significant difference to ameliorative effect when overdose iron feeding (Figure 8). Therefore, iron sequestration may be associated with the extracellular absorption capacity of the bacterial cells. In this regard, Skrypnik *et al.* (2018) reported that supplementation of multispecies probiotics, including 9 different bacterial strains, significantly reduced acute iron toxicity in rats [41]. According to studies, gut microbiota plays a vital role in the absorption, neutralization, and chemical changes in food compositions. As the gastrointestinal tract's pivotal living components, probiotics profoundly impact the processing of nutrients and toxic substances [4,8]. Since the bacterium MR1 has demonstrated a remarkable ability to bind to epithelial cells, it can compete with absorbable materials and even pathogens, mitigating its harmful effects.
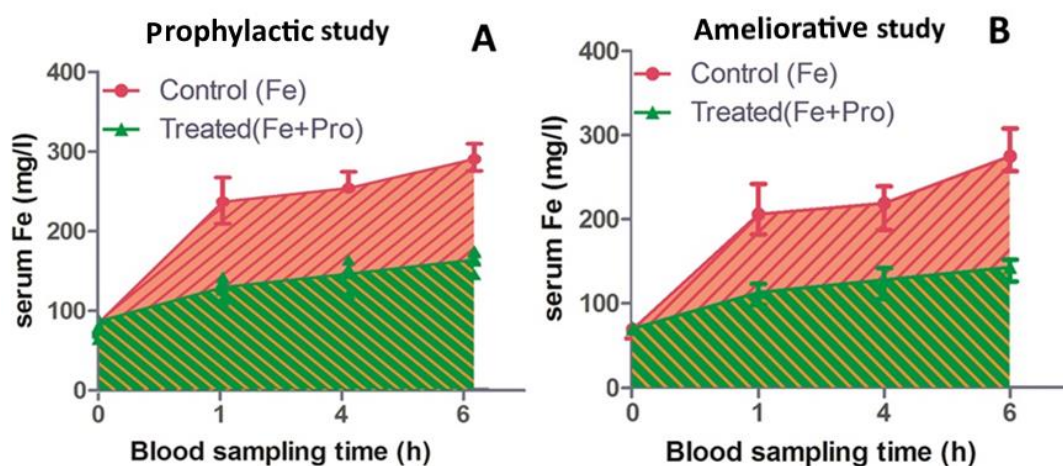
Figure 8. Iron binding abilities of probiotic bacteria. A. Prophylactic effect of *L. rhamnosus* for 2 weeks on overdose supplementation of iron sulfate and B. Ameliorative effects of *L. rhamnosus* on intestinal absorption of iron sulfate. The results are presented in three replicates with standard deviation. Total serum iron was measured in 6-h time intervals after treatment with probiotic and then ferrous sulfate.

4. Conclusions

This study concluded that *L. rhamnosus* MR1 effectively increases iron tolerance *in vitro* and mitigating iron toxicity *in vivo*. The results showed the potential of *L. rhamnosus* MR1 on decreasing lethality after overdose administration of ferrous sulfate and induction of acute toxicity in rats. Since no report is found in the current literature about the effect of probiotic supplements on iron's acute toxicity, these interesting results can provide a useful background for further studies on dietary supplements.

Conflicts of Interest

The authors declare no conflict of interest.

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