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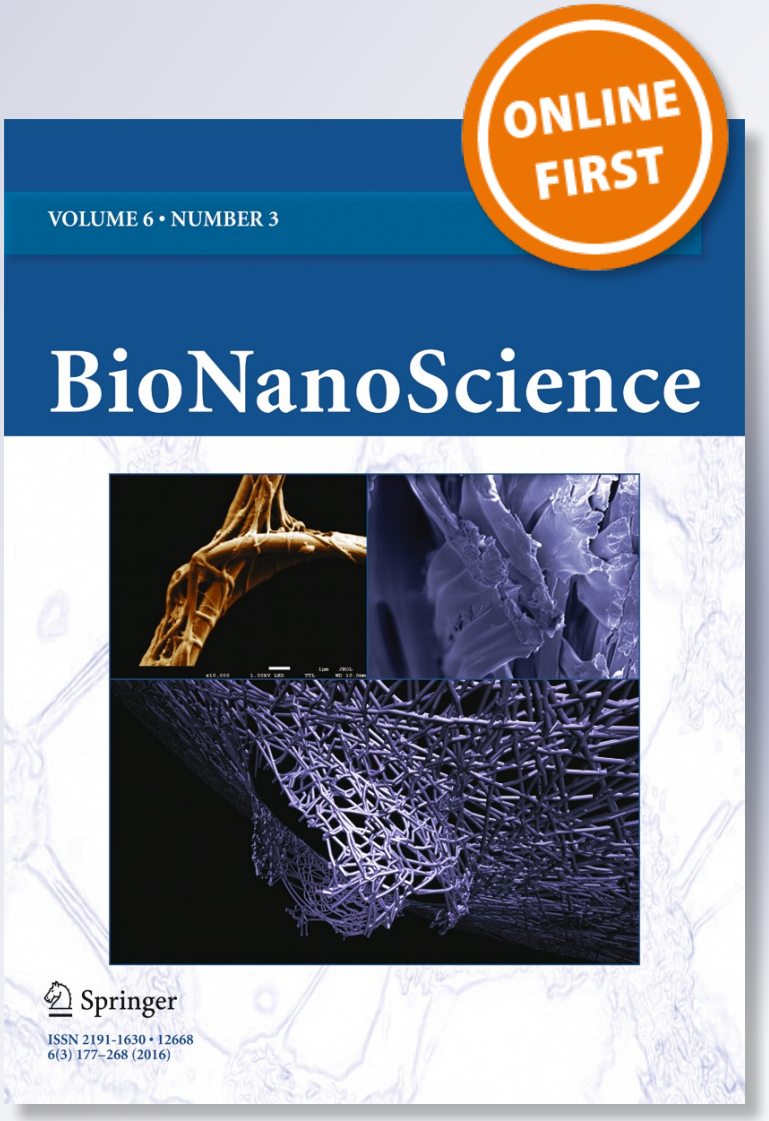
BioNanoScience

ISSN 2191-1630

BioNanoSci.
DOI 10.1007/s12668-016-0290-1



Springer

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Involvement of Iron in Biofilm Formation by *Lactobacillus rhamnosus*

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Abstract We investigated the effect of high iron availability on growth and biofilm formation of *Lactobacillus rhamnosus* strain BB. Biofilm development by *L. rhamnosus* BB was significantly increased by adding iron into the MRS medium, while bacterial growth was not affected. Iron chelator EDTA had no effect on *L. rhamnosus* BB growth and biofilm formation, but prevented the stimulation of biofilm development by iron. Our results are the first evidence of iron involvement in biofilm formation by lactobacilli—bacteria that were considered to be iron independent with poor biofilm formation capacity.

Keywords *Lactobacillus rhamnosus* · Iron · Biofilm

1 Introduction

Iron is an essential cofactor in almost all biological systems. In many bacterial species, most notably pathogenic *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Escherichia coli*, iron is required for biofilm formation. Thus, successful establishment of the infection is mediated by the availability of iron in a host [1]. On the other hand, bacteria of the genus *Lactobacillus* have little or no requirement for iron. *Lactobacillus rhamnosus* strains are widely used as probiotics and in functional foods. Moreover, Gorbach-Goldin (GG) strain is one of the best-studied probiotic microorganisms [2]. Health-benefiting properties of *L. rhamnosus* GG are dependent in part on its prolonged residence in the gastrointestinal tract and its strong adhesive capacity. In the search for factors responsible for high colonization ability of *L. rhamnosus* GG, it has been shown that bacteria of this strain are able to form biofilms with an efficiency that exceeds that of related *Lactobacillus* species [3]. In this study, we investigated the impact of iron on *L. rhamnosus* biofilm formation. Iron-responsive lactobacilli have strong probiotic potential because they would compete with pathogens under conditions of high iron availability such as intestinal bleeding, trauma, or stress.

2 Material and Methods

L. rhamnosus strain was derived from the drink yogurt branded as "Bio Balance" (JUnimilk, Russia) and was termed strain BB according to the name of the product. The isolation of the strain was carried out by generating single colonies via serial dilution of the product in sterile phosphate-buffered saline (PBS), subsequent plating onto MRS agar (BD Difco), and incubation under microaerophilic conditions at 37 °C. The identification of bacterial isolates to the species level was performed by MALDI-TOF mass spectrometry (Bruker Biotyper system, Bruker Daltonics, Germany), as described elsewhere [4]. Overnight cultures of *L. rhamnosus* BB were diluted 1:100 with fresh MRS broth supplemented with $FeCl_3$ (Sigma-Aldrich) and loaded into sterile polystyrene 96-well microplates (flat bottom, Cellstar Grenier Bio-one). Growth curves were obtained using a Tecan Infinite F200 PRO (Switzerland) microplate reader. Measurement of biofilms was performed in microtiter plate biofilm formation assay. The broth was removed from the wells, and biofilms were washed and stained with 0.1 % (*w*/*v*) crystal violet (Sigma-Aldrich) for 1 h at room temperature. After vigorous washing

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Published online: 04 October 2016

with water, the stained biofilms were dissolved in 96 % ethanol and biofilm cell-associated dye was measured in a microplate reader (Bio-Rad, USA) as A_{570} of 100 μL aliquots [5]. Iron concentrations were measured using sulfosalicylic acid photometric method. The data were analyzed using the MS Excel and Statistica 6.0. Statistical significances were determined by Mann-Whitney *U* test and set at $P \leq 0.05$.

3 Results and Discussion

Addition of $FeCl_3$ had no effect on *L. rhamnosus* BB growth (Fig. 1a, b, d), but significantly increased its biofilm formation in response to iron concentration 1000 μM (Fig. 1c, d). Furthermore, *L. rhamnosus* BB formed robust biofilms regardless of the level of iron in the medium in contrast to other *Lactobacillus* species tested, which were not able to form biofilms (data not shown). This finding is consistent with earlier data [3] that also showed biofilm formation was a rare property among lactobacilli. The stimulation of biofilm formation in *L. rhamnosus* BB by iron was verified using an iron chelator EDTA. EDTA in all tested concentrations (500–2500 μM) did not affect the growth, but prevented the increase in biofilm formation by depleting iron from the culture medium, when added together with $FeCl_3$ (Fig. 1d).

Furthermore, on plating, this strain was characterized by the presence of rough (R) and smooth (S) morphotypes. However, both were identified by MALDI-TOF MS as *L. rhamnosus* species. While S and R forms may differ in exopolysaccharide synthesis [6], further studies are now needed to elucidate which morphotype responds to high iron availability with increased biofilm formation.

4 Conclusions

We propose that iron-responsive biofilm formation contributes to the probiotic potential of the strain. Stimulation of biofilm formation by iron makes *L. rhamnosus* BB able to compete with the pathogenic flora under high iron conditions of intestinal bleeding, trauma, or stress, when most probiotics are usually outcompeted. Further investigations should provide deeper understanding of iron involvement in probiotic activity of lactobacilli.

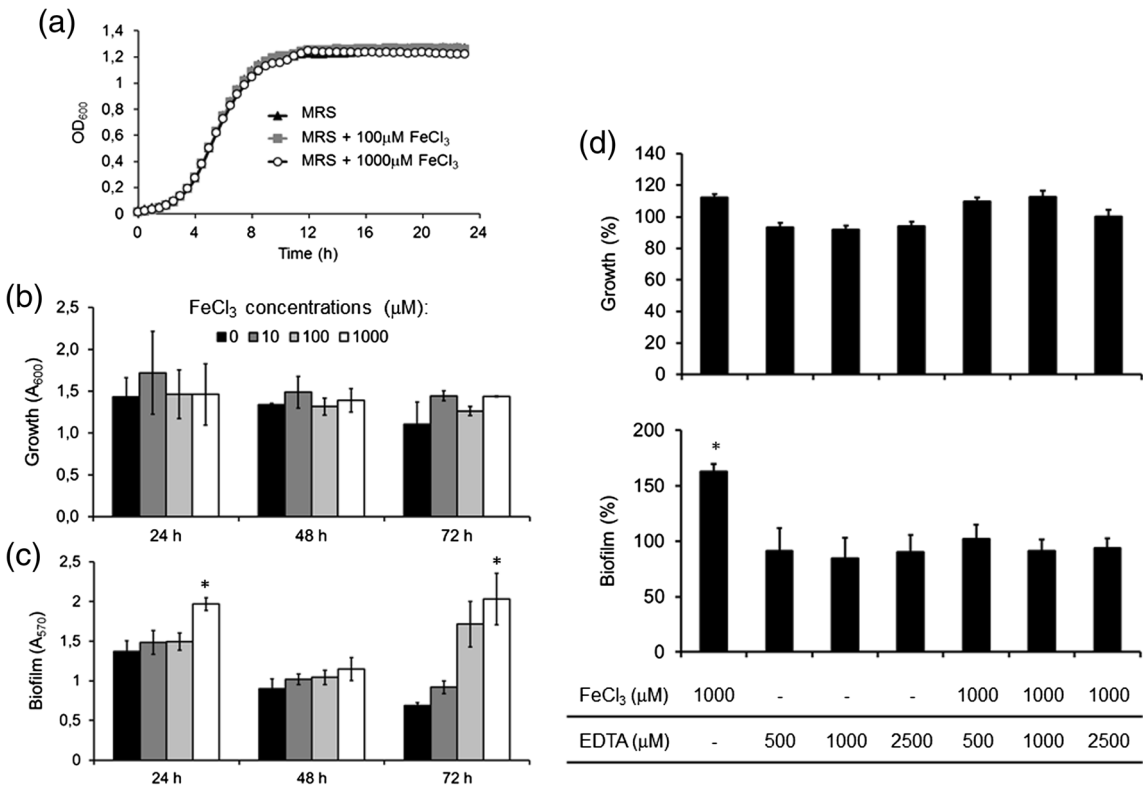

Fig. 1 Effect of iron on *L. rhamnosus* BB growth (**a**, **b**, **d**) and biofilm formation (**c**, **d**). Growth and biofilm formation assays were conducted in MRS broth supplemented with $FeCl_3$ and/or iron chelator DETC. Biofilm formation was measured in microtiter plate biofilm assay. Data shown represent the mean absorbance ± SD. Biofilm and growth levels were measured at 72 h (**d**). Biofilm formation and growth of *L. rhamnosus* BB in MRS broth was taken as 100 %. (**d**). *Asterisk* denotes significantly higher biofilm formation in the presence of 1000 μM $FeCl_3$ than without iron ($P < 0.05$)

Acknowledgments This work was supported by the program of competitive growth of Kazan Federal University and the Russian Science Foundation (RSF 15-14-00046). The research was performed using the equipment of the Interdisciplinary Centre for shared use of Kazan Federal University. The authors declare no conflict of interest.

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