Current Microbiology (2019) 76:896–903
https://doi.org/10.1007/s00284-019-01706-8

Adhesion and Colonization of the Probiotic *Lactobacillus rhamnosus* Labeled by Dsred2 in Mouse Gut

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Received: 19 January 2019 / Accepted: 9 May 2019 / Published online: 21 May 2019

Abstract

The health-promoting effects of the probiotic strain *Lactobacillus rhamnosus* are based on its adherence and colonization ability. However, little is known about its adhesion and colonization rates. *Lactobacillus rhamnosus* in mouse intestinal mucosa a mutant of the red fluorescence protein (RFP) DSred2 was used to tag *L. rhamnosus* to observe the adhesion and distribution of *L. rhamnosus* in mouse intestinal mucosa. A mutant of the red fluorescence protein (RFP) *Dsred2* was used to tag *L. rhamnosus* to allow us to observe and distinguish it in the mouse intestine. Seven-week-old female BALB/c mice were fed once (at day 0) with an oral administration of the labeled *L. rhamnosus*, and the number of labeled bacteria was detected in different regions of the intestinal tract at 3 h and at day 1, 2, 3, 4, 5, 6, 7, and 15 after administration. The labeling process changed the morphology of *L. rhamnosus*, as it appeared after observation under the microscope, but did not change its basic probiotic properties in vitro. In vivo, labeled *L. rhamnosus* reached the colonization peak at the fourth day after gavage. From the distribution point of view, the number of colonization strains increased from the proximal to the distal small intestine (duodenum < jejunum < ileum) and the number of strains in the colon was less than the distal small intestine (ileum). The labeling protocol actually allowed the detection of the distribution and adhesion of this bacterium to the intestine, thus demonstrating that the health-promoting effects of this probiotic are satisfied. This study provides a scientific basis in the use of probiotics such as *L. rhamnosus* in functional foods.

Introduction

Lactobacillus rhamnosus is a probiotic strain that has been safely used in a variety of functional foods for nearly 30 years [16]. *L. rhamnosus* possesses many effects on health such as prevention and relieve of certain types of diarrhea [8], modulation of the inflammatory response [7] and lipid metabolism [5], and occasional beneficial effects for other disorders [4, 6, 15]. One postulated feature that is indispensable for the action of some probiotic lactobacilli is the adherence and colonization ability, which is considered the basis of these health-promoting effects [17].

In a previous study, *L. rhamnosus* showed resistance to bile and stomach acids and ability of adhering to epithelial cells in vitro [18]. In vivo, *L. rhamnosus* can adhere to the adult intestinal mucosa for more than 1 week after oral in take [1], and it can also adhere to the infant intestinal mucosa [12]. Actually, it is also able to colonize the digestive tract of mice [9]. However, these studies regarding the colonization ability of this bacterium in human or mice are mainly based on its detection in fecal samples and colony hybridization assays or other experimental technique of molecular biology. We previously discovered that the SpaC protein is involved in its adherence and colonization [11, 19]. However, little is known about its adhesion and colonization rates. Novel research methods should be developed to successfully observe and identify it. The fluorescent protein tagging system may be a good genetic tool to further determine its distribution and amount [3].

We now present a study performed to observe *L. rhamnosus* adhesion to the mice intestinal mucosa and determine its distribution in different parts of the intestine. *Dsred2* is a mutant of the red fluorescent protein (RFP), which is widely used to detect yeast or other eukaryotes [10, 21], although rarely used in bacteria. Thus, in this work, *L. rhamnosus* was tagged with *Dsred2*, which did not change its probiotic

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properties in vitro. The labeled *L. rhamnosus* could colonize mice digestive tract mainly attaching to the intestinal mucosa. In the small intestine, the number of colonized *L. rhamnosus* increased from the proximal to the distal intestine. In the colon, the amount of *L. rhamnosus* was not as high as the amount in the ileum at the end of the small intestine. According to our results, the use of RFP and other methods of probiotic labeling might be useful to evaluate probiotic adherence and colonization. Further efforts in research methods might improve the understanding of the probiotic health-promoting effects.

Materials and Methods

Strains and Vectors Construction

Lactobacillus rhamnosus CGMCC 1.2466^T was purchased from China General Microbiological Culture Collection Center and was used for the experiments presented within this study. *E. coli* DH5α was purchased from Beijing Tiangen Technology Co., Ltd. (Beijing, China). Plasmid pMG36e, pPIC9 k-*Dsred2* was donated by Prof. Guo Xinghua, Institute of Microbiology, Chinese Academy of Sciences. The restriction enzymes *Hin*dIII, *Xba*I and T4 DNA ligase were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China).

In order to construct the vector pMD19-*Dsred2*, the red fluorescent gene *Dsred2* upstream primer containing an *Xba*I restriction site (*Dsred2*F: 5′-TCTAGACATGGCCTCCTC CGAGA-3′), and the downstream primer containing a *Hin*dIII restriction site (*Dsred2*R: 5′-CCCAAGCTTCTACAG GAACAGGTGG-3′), were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The *Dsred2* gene amplified from the plasmid pPIC9 k-*Dsred2* was cloned into the vector pMD19-T to construct the recombinant vector pMD19-*Dsred2*, and then the plasmids pMD19-*Dsred2* and pMG36e were digested using *Xba*I and *Hin*dIII, and connected by T4 ligase. The ligation product was transferred into *E. coli* DH5α to construct the vector pMG36e-*Dsred2*.

RFP-Labeled *L. rhamnosus* and Detection In Vitro

The resulting plasmid (pMG36e-*Dsred2*) was introduced into *L. rhamnosus* by electroporation as previously described [3]. Bacteria were cultured on MRS plates containing 0.5 μg/mL erythromycin, incubated at 37 °C for 2–3 days, and positive clones were collected and verified by plasmid extraction and sequencing.

Lactobacillus rhamnosus and the RFP-labeled *L. rhamnosus* in the liquid MRS medium were separately collected by centrifugation, washed three times with PBS, and then 10 μL of bacterial fluid was coated on a glass slide, naturally dried, and observed using a fluorescent microscope.

Tolerance Test and Adhesion Assay In Vitro

The in vitro tolerance test described by Carteris et al. [2] was used with some modifications. Briefly, *L. rhamnosus* and *Dsred2*-labeled *L. rhamnosus* were cultured on MRS, incubated overnight at 37 °C, and bacteria were collected by centrifugation. Bacteria concentration was adjusted to 10^9 CFU/mL using PBS at pH 3.0, and 1 mL of each bacterium type was inoculated into 9 mL of simulated gastric juice, and incubated at 37 °C. Next, 1 mL was collected at 0 h and 2 h for serial dilution, and the number of viable bacteria was measured. In addition, 1 mL of the culture digested in the simulated gastric juice for 2 h was inoculated in 9 mL simulated intestinal juice, and incubated at 37 °C for 4 h, and the number of viable bacteria was measured. The survival rate of *Dsred2*-labeled *L. rhamnosus* after simulated intestinal and gastric juice treatment was calculated.

The in vitro adhesion assay described by Ouwehand et al. [14] was performed as follows. The healthy mouse intestine was collected, rinsed with sterile PBS (0.01% gelatin) three times, and its inner surface was scraped. The mucus protein mixture was mixed with Hepes-Hanks buffer (10 mmol/L, pH 7.4), and the supernatant was collected after centrifugation at 12,000 r/min for 15 min at 4 °C. The protein concentration was determined by Bradford method using 1 mg/mL bovine serum albumin standard curve and adjusting the protein concentration to 1.0 mg/mL. The obtained intestinal mucus protein was stored at − 80 °C; an amount of 100 μL of the obtained intestinal mucus protein was added to each well of a 96-well cell culture plate and incubated overnight at 4 °C. The 96-well plate was washed twice with 200–250 μL Hepes-hanks buffer; then 100 μL of *Dsred2*-labeled *L. rhamnosus* suspension was added to each well, incubated at 37 °C for 1 h, and washed 2–3 times with Hepes-hanks buffer. Finally, 200–250 μL of lysis buffer containing 1% SDS and 0.1 mol/L NaOH was added and lysis was allowed at 37 °C for 0.5 h. The number of viable bacteria was determined by serial dilution and the adhesion rate was calculated.

Adhesion and Colonization of Dsred2 Labeled *L. rhamnosus* in Mouse Gut

Sixty female BALB/c mice seven-week-old were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and housed in a controlled environment with a 12:12-h light–dark cycle at the Animal Holding Unit of College of Food Science and Technology, Agricultural University of Hebei. They had free access to a standard mouse diet and water ad libitum. Approximately 10^9 *Dsred2*-labeled *L. rhamnosus* dissolved in 1 mL of PBS was administered

once by orogastric intubation to 27 mice at day 0. Another group of 27 mice received oral PBS once (at day 0) and used as control.

Three mice per each group and each time point were randomly selected and sacrificed by N_2 asphyxiation at 3 h, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, and 15 days after intragastric administration. Mice body surface was disinfected and duodenum, jejunum, ileum, and colon were aseptically collected, embedded in paraffin and paraffin sections were cut and observed under a fluorescence microscope. The remaining portion of the intestines was washed with PBS to remove the intestinal contents and approximately 2 cm of each intestine were collected. The intestines were repeatedly washed with sterile PBS at a dose of 100 µL/cm, and this PBS used for washing was collected, uniformly mixed, and 100 µL was further collected. Then, it was diluted with sterile PBS, mixed, and 100 µL of the diluted solution was seeded into a MRS solid plate containing erythromycin 5 µg/mL, incubated at 37 °C for 24 h, the number of colonies was detected and 24 colonies were randomly collected. Colony PCR was performed and the proportion of positive strains was calculated. The number of colonized bacteria in the intestine was calculated according to the following formula: number of colonies counted on the plate × (number of bacteria that resulted positive by PCR/24).

Results

Construction of the Probiotic Labeling Vector using RFP

The *Dsred2* gene from plasmid pPIC9 k-*Dsred2* was amplified using primers *Dsred2*F and *Dsred2*R. The amplified *Dsred2* gene was cloned into the vector pMD 19-T to generate the plasmid pMD-*Dsred2*. Next, the plasmids pMD-*Dsred2* and pMG36e were isolated from *E. coli* DH5α and digested using *Xba*I and *Hin*dIII, resulting in a small DNA fragment containing the *Dsred2* gene and a large fragment. The small DNA fragment containing the *Dsred2* gene was inserted into multiple cloning sites (MCSs) of the pMG36e to obtain the probiotic labeling plasmid pMG36e-*Dsred2*. The overall strategy is outlined in Fig. 1. The plasmid pMG36e-*Dsred2* was designed for probiotics, although it can be used to obtain a labeled *E. coli* DH5α.

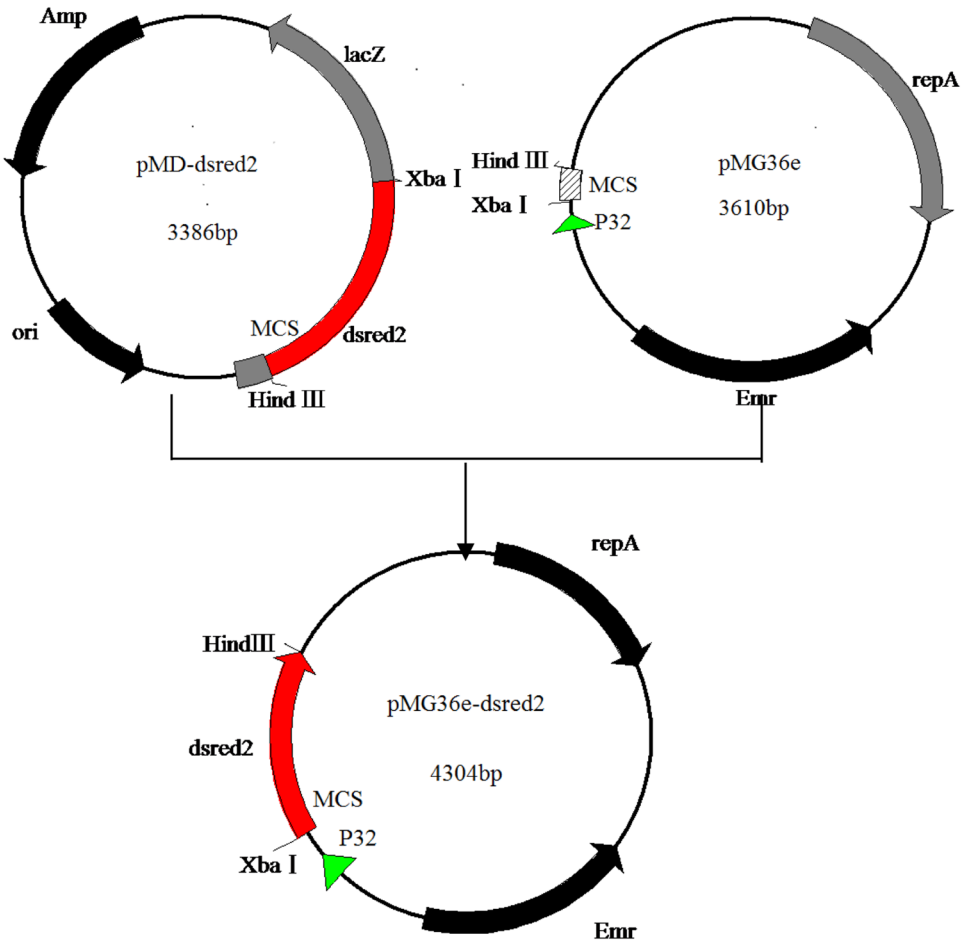

Fig. 1 Schematic representation of the strategy employed to construct the probiotic labeling vector pMG36e-dsred2

L. rhamnosus Detection

The presence of pMG36e-*Dsred2* into *L. rhamnosus* transferred by electroporation conferred to the bacteria the ability to emit red fluorescence. This phenotype was easily observable under a fluorescent microscopy, while the wild-type stain of *L. rhamnosus* cannot be detected by the same instrument because of the lack of fluorescence (Fig. 2). Interestingly, the transformation protocol changed the shape of bacteria. However, the purpose of *L. rhamnosus* labeled by the RFP was for its easy detection in vivo. Thus, the RFP cannot change the adherence and colonization ability of *L. rhamnosus*. For this reason, we analyzed the probiotic properties of this bacterium in vitro before the in vivo experiments.

The In Vitro Characteristics of *L. rhamnosus* Labeled with Dsred2

The tolerance test in vitro showed that the survival rate of *L. rhamnosus* and *Dsred2* labeled strain in simulated gastric juice was 83.08% and 83.55%, respectively, with no significant difference. Surviving strains in simulated gastric fluid were left in simulated intestinal fluid for 4 h, and the survival rates were reduced to 25.67% and 25.04%, respectively, and the difference between the two strains was not significant (Fig. 3a). In vitro adhesion assay showed that the adhesion rate of *L. rhamnosus* and the *Dsred2* labeled strain to the intestinal mice mucus was 26.95% and 28.12%, respectively, while the adhesion rate was 4.53% and 6.29% when bovine serum albumin was used as an adhesion matrix. The relative adhesion rate of the two strains was 22.42% and 21.83%, respectively, and the variance analysis was not significant (Fig. 3b).

These results indicated that although the electroporation transformation changed the morphology of *L. rhamnosus*, the probiotic properties such as tolerance to simulated gastric juice, simulated intestinal juice and relative adhesion rate in vitro did not change.

Adhesion and Colonization Properties of *L. rhamnosus* Labeled with Dsred2 in Mouse Gut

The paraffin sections of the ileum, jejunum, duodenum, and colon of mice after gavage demonstrated that the number of labeled strains was the highest in the ileum and they were of easy identification. In addition, the fluorescence was mainly concentrated on the intestinal mucosa. According to the chronological order of the time points after gavage, the fluorescence intensity of the ileum was the highest in the mice 3 h after gavage, and it gradually decreased with time, indicating that the amount of the labeled strain was gradually reduced (Fig. 4).

In order to analyze the colonization of *L. rhamnosus* in the mice intestine and to study its distribution, we counted

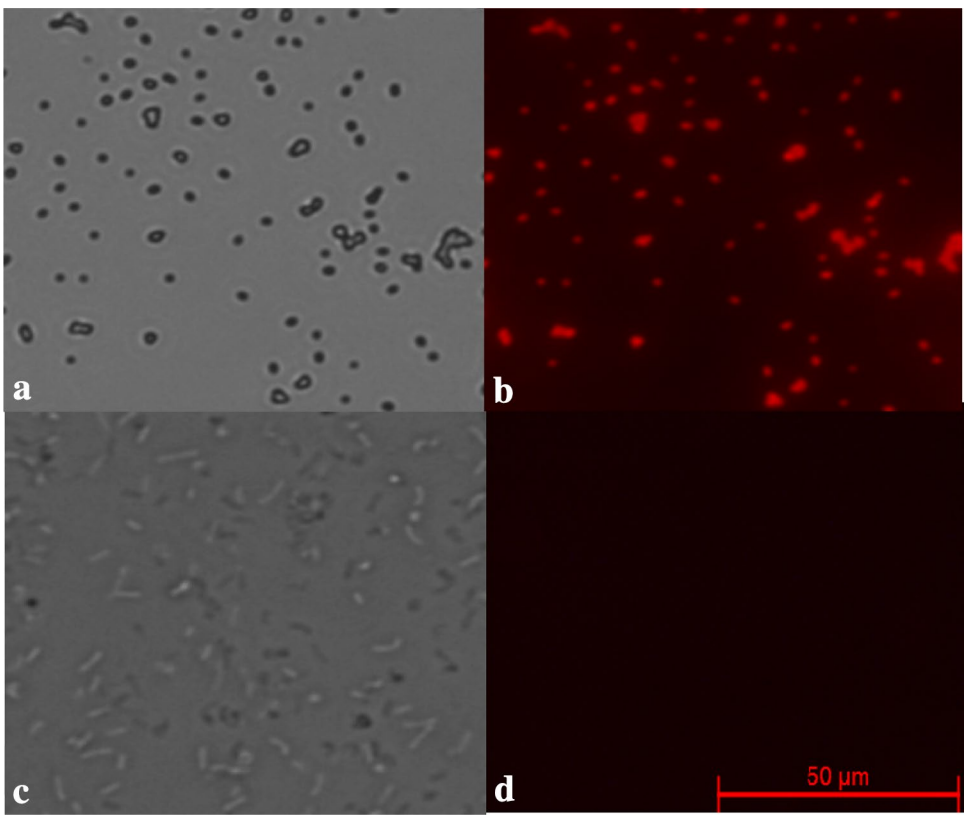

Fig. 2 Strain fluorescent detection: bright-field images are shown on the left (**a** and **c**); fluorescence images are shown on the right (**b** and **d**). **a**, **b** dsred2-labeled *L. rhamnosus*; **c**, **d** *L. rhamnosus*; (scale bar = 50 μM)

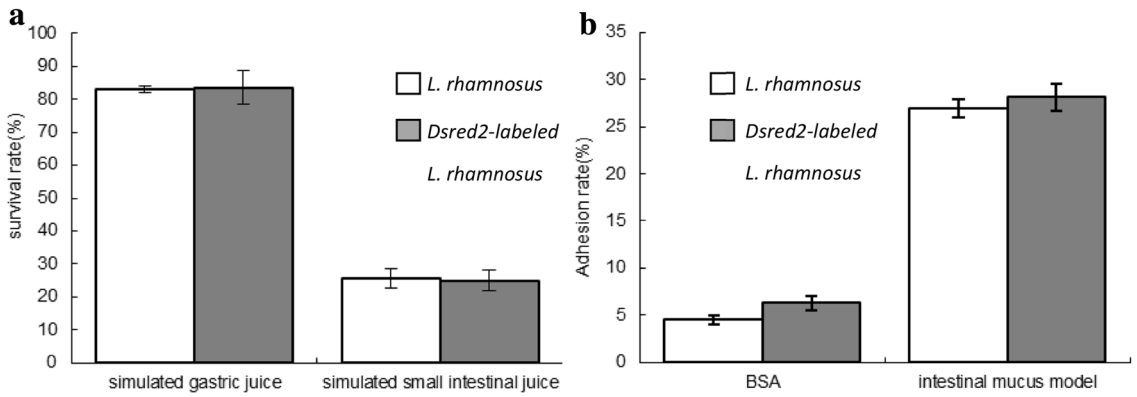

Fig. 3 Tolerance and adhesion rate of *L. rhamnosus* and dsred2-labeled *L. rhamnosus* in vitro: **a** Tolerance of *L. rhamnosus* and dsred2-labeled *L. rhamnosus* in simulated gastric juice and simulated small intestinal juice; **b** Adhesion rate of *L. rhamnosus* and dsred2-labeled *L. rhamnosus* in vitro

the viable bacteria on different parts of the mice intestine at different time points after intragastric administration. Since the labeled strain contained erythromycin resistance, the count performed on erythromycin-resistant plates can avoid the interference by other species and endogenous *L. rhamnosus*. Control group mice were intragastrically treated with PBS. The results indicated that erythromycin-resistant strains were naturally present in the mice intestine. In order to further eliminate the interference of other species and endogenous *L. rhamnosus*, we selected the unique *Dsred2* gene in the labeled strain for PCR identification. The proportion of the labeled strains was calculated from the number of PCR-positive colonies in 24 randomly selected colonies. The results showed that the *Dsred2* gene was not detected in the intestinal erythromycin-resistant strains of the mice treated with PBS.

According to the viable bacteria count, we excluded the part of the *Dsred2* gene that was not detected by PCR. We constructed the distribution map of the labeled strains in different parts of the mice intestine and at different time points after gavage (Fig. 5). The number of labeled strains in the intestinal tract was the highest at 3 h after gavage, and the distribution decreased from the proximal end of the small intestine to the distal end of the large intestine. This result reflected the effect of the intragastric administration, since after gavage the labeled strain moved from the proximal end of the small intestine to the distal end of the large intestine. The number of labeled strains decreased sharply 1 day after gavage, and each part was stable at approximately 10^5 CFU/mL. The number of strains continued to decrease until the third day. At day 3 the labeled strains began to proliferate in the intestine, reaching the peak of colonization at day 4, and began to decrease again at day 5. The labeled strain remaining on the mice intestinal mucosal surface after 7 days was 14.1% of the amount on the first day in the duodenum, 14.35% in the jejunum, 36.01% in the ileum, and 27.3% in the colon. From the distribution point of view, the number of colonization strains increased from the proximal to the distal small intestine (duodenum < jejunum < ileum); the number of strains in the colon was less than the number in the distal small intestine (ileum), and this distribution pattern appeared since the first day after gavage. At the 15th day, still colonies were present on the plate, but no positive strain was detected by PCR, and the number of colonies was basically the same as that of the blank control, indicating the absence of labeled *L. rhamnosus* in the mouse gut 15 days after gavage.

Discussion

As a representative probiotic that can regulate intestinal health, *L. rhamnosus* has been widely used for many years in functional foods, especially fermented dairy products, and its probiotic properties have always been the focus of many scientists. The ability to adhere and colonize the human intestine represents the basis of the health-promoting effects of this bacterium [17]. Most of the past studies used indirect methods to analyze their colonization ability and these methods are often based on molecular biology techniques, coupled with the interference of endogenous *L. rhamnosus*, which greatly reduced the reliability of the results. In this study, the vector expressing the RFP *Dsred2* was used to label *L. rhamnosus*, to count it by direct culture method and was verified by PCR of specific genes, which made the study of the colonization more intuitive and reliable. Such methods can be applied to in vivo studies of other probiotics.

It is worth noting that the labeling with RFP *Dsred2* caused a change in the morphology of the strain from a typical rod shape to a spherical one, which might be due to the cell wall removal during transformation. Probiotics pass through the harsh conditions of the gastrointestinal

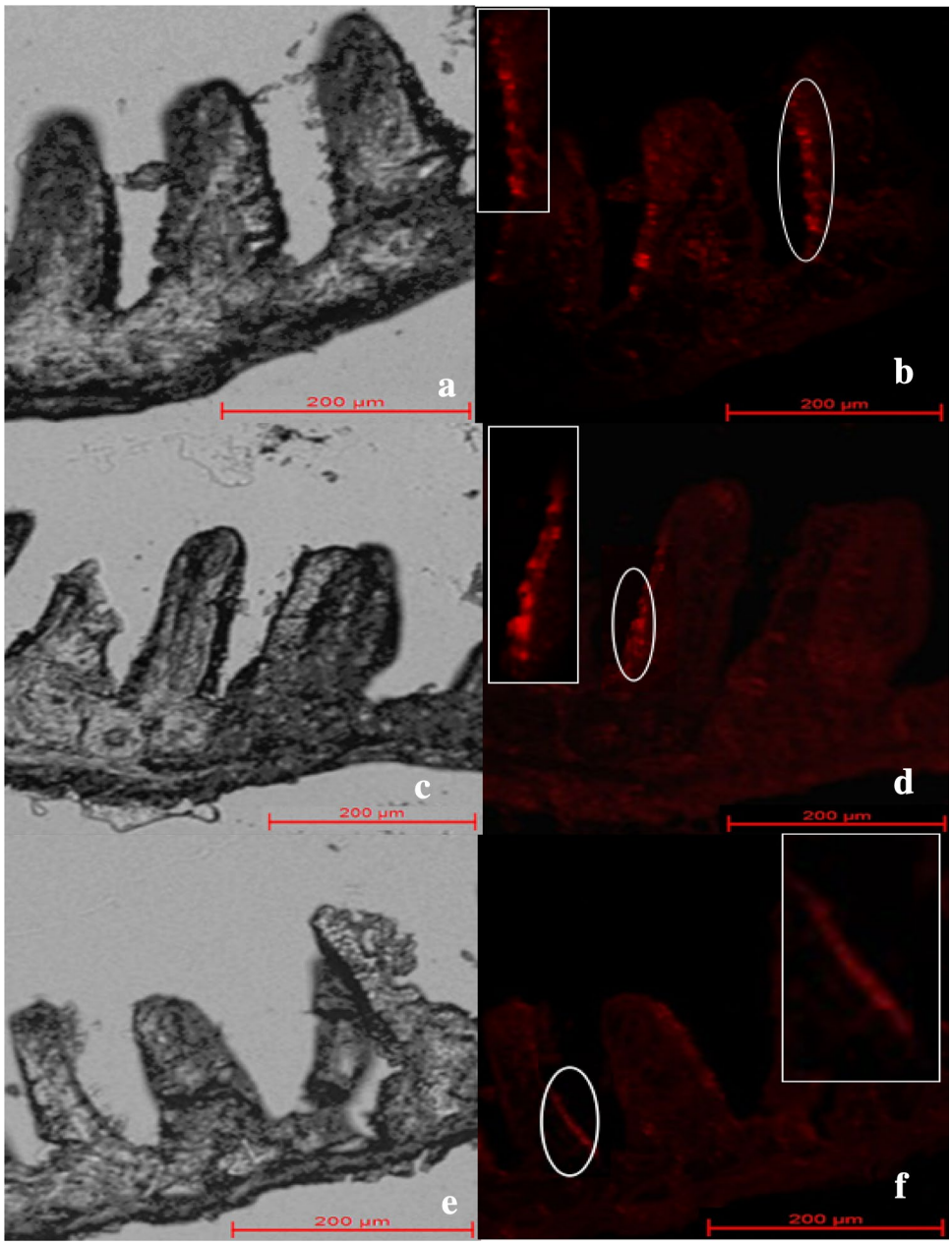

Fig. 4 Mice ileum after intragastric administration of Dsred2-labeled *L. rhamnosus*: bright-field images are shown on the left (**a**, **c** and **e**); fluorescence images are shown on the right (**b**, **d** and **f**). **a**, **b** 3 h after intragastric administration; **c**, **d** 1 day after intragastric administration; **e**, **f** 4 days after intragastric administration (scale bars = 200 μM)

tract before they adhere and colonize the intestines. The gastric juice in the stomach is strongly acidic, and its pH is usually around 3.0. The acidic environment created by the gastric juice activates pepsin. The lower pH or the activated pepsin has a bactericidal effect. In order to reach the intestinal tract in a viable state, bacteria should be resistant to low pH and pepsin [2]. The small intestine is the main place of probiotics exerting prebiotic properties, where the trypsin and bile salts can also affect the survival of the probiotics. Therefore, acid and bile salt-tolerant properties are important criteria for testing probiotic tolerance in vitro as we actually did, while in vitro tolerance after strain labeling did not change. Mucus is an elastic colloid secreted by the gastrointestinal tract and is mainly composed of mucin [13]. Since mucus blocks the receptors of epithelial cells, most of the microbes in the intestine are in direct contact with the mucosa, and the epithelial cells are not exposed. Mucus becomes the direct colonization environment of probiotics [20]. Therefore, the adhesion test was based on intestinal mucus in vitro, and the adherence ability in vitro after strain labeling did not changed, which mainly be due by the expression of adhesion-related proteins such as SpaC protein not affected by labeling [11, 19]. Therefore, the labeling process only changed the morphology of the strain, while its probiotic properties did not change.

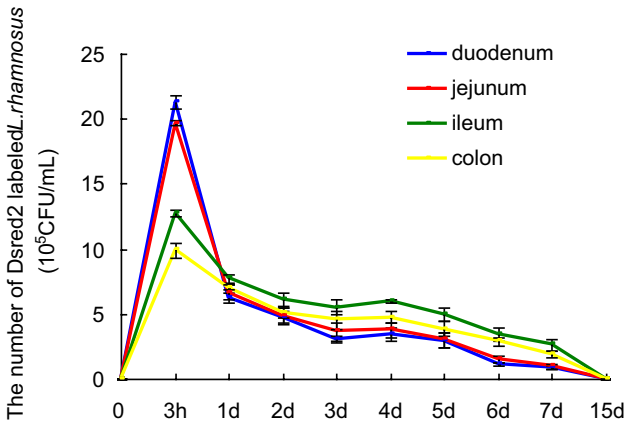

Fig. 5 The number of labeled *L. rhamnosus* in mice intestine after intragastric administration

The distribution of labeled strains orally administrated to mice was observed in the different tracts of the intestine. The number of labeled strains decreased from the proximal end of the small intestine to the distal end of the large intestine at 3 h after gavage, which reflected the effect of intragastric gavage. The labeled strain moved from the proximal end of the small intestine to the distal end of the large intestine after gavage. At 3 h, strains did not fully colonize the intestine, and the bacteria number decreased sharply from 1 to 3 days, while the number was stable and began to increase on the fourth day. This phenomenon represented an equilibrium of the colonizing strains during day 1–4, in which the colonization peak was also reached. After that, the colonizing strains gradually decreased and eventually disappeared completely. From the distribution point of view, the number of colonization strains increased from the proximal to the distal small intestine (duodenum < jejunum < ileum), which reflected the difference in affinity between different intestinal tracts and strains. The number of *L. rhamnosus* in the colon was less than in the distal small intestine (ileum), which was mainly due to the complex and diverse bacterial species in the colon.

In this study, the colonization and distribution of *L. rhamnosus* in the mice intestine were studied using RFP *Dsred2* labeling. The discovery of the colonization and distribution of *L. rhamnosus* provides a scientific basis for the application of probiotics, including *L. rhamnosus*, in functional foods.

Acknowledgements This work was supported by the National Natural Science Foundation of China (Grant No. 31301520) and Natural Science Foundation of Hebei Province (Grant Nos. C2016204129, C2017204094).

Compliance with Ethical Standards

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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