

Original Article

The Effects of Inulin on Characteristics of *Lactobacillus paracasei* TD3 (IBRC-M 10784) as Probiotic Bacteria *in vitro*

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Abstract

Introduction: Probiotics, as live microbial food ingredients or functional foods, are often related to health promotion and diseases prevention. The growth of these beneficial probiotics was improved by non-digestible food ingredients that are defined as prebiotics. Inulin is one of the known prebiotics that improves the gastrointestinal health.

Aim: The aim of this study was to evaluate the functional properties of *Lactobacillus paracasei* TD3 (IBRC-M 10784) and the effects of inulin as prebiotic on these properties *in vitro*.

Methods: The tolerance of *L. paracasei* TD3 for low pH and pepsin (stomach condition), bile salt and trypsin conditions (small intestine conditions), antibiotics and pathogenic bacteria were evaluated by different methods.

Results: *Lactobacillus paracasei* TD3 was obtained from TakGene Company. *L. paracasei* TD3 was resistant to acidic pH higher than 3.5 and was tolerant to pepsin condition lower than 0.723 µg/mL and to bile salts ≤ 0.4% w/v and trypsin condition equal to 72.32 u/g and lower. This strain was resistant to vancomycin, nalidixic acid, colistin and gentamicin while was sensitive to penicillin, streptomycin and tetracycline. *L. paracasei* TD3 showed antimicrobial activity against *S. typhimurium*, *Sh. dysenteriae*, *Sh. flexneri* and *E. coli*, respectively. Inulin promoted the growth of *L. paracasei* TD3 (log CFU) in different conditions and increased the antibacterial activity of *L. paracasei* TD3 against pathogenic bacteria without changing the tolerance of bacteria to pH and other conditions ($P > 0.05$).

Conclusion: *L. paracasei* TD3 (IBRC-M 10784) in combination with inulin can be considered as valuable strain for further investigations in human clinical studies.

Keywords: Inulin, *Lactobacillus paracasei* TD3, probiotic, prebiotic, small intestine, stomach

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Introduction

Live microbial feed supplements or probiotics are a group of microorganisms which improve the health of human and animals. *Lactobacillus* and *Bifidobacteria* are the major genera of probiotics.¹ Several health benefits of probiotic bacteria include inhibition of pathogenic bacteria in intestine, reduction of the risks associated with mutagenicity and carcinogenicity, enhancing the immune system and improving the Irritable Bowel Syndrome.² The criteria for selecting a strain as probiotic include bile stability, persistence to human intestinal tract's conditions, production of antimicrobial substances, antagonism against pathogenic bacteria and resistance to antibiotics.²

Many investigations have confirmed that non-digestible food ingredients are selectively fermented by probiotic bacteria and are defined as prebiotics. They improve the growth of probiotics in the intestinal tract.³ The outstanding prebiotic compounds include inulin-type fructans, lactulose, lactosucrose, xylo-oligosaccharides, gluco-oligosaccharides and fructo-oligosaccharides.^{4,5}

Inulin, a mixture of oligo-polymers with different degrees of polymerization, has been identified as a prebiotic compound.⁶ In

this study, we evaluated the functional properties of *Lactobacillus paracasei* TD3 (IBRC-M 10784) and the prebiotic potential of inulin on these properties *in vitro*.

Materials and Methods

Strain and its activation, measuring the Colony Forming Unit (CFU) of live bacteria

Lactobacillus paracasei TD3 (IBRC-M 10784) was obtained in lyophilized powder from TakGene Company (Tehran, Iran). *L. paracasei* TD3 was suspended in liquid MRS broth (Himedia, India), and then transferred into the MRS Agar (Merck, Germany) and incubated at 37°C under anaerobic condition for 48 hr. The purified colony was stored in skim milk containing 15% glycerol at -20°C for further examination.

For enumerating the CFU of *L. paracasei* TD3, 1 g of lyophilized bacteria was inoculated into 10 mL of MRS broth (10⁻¹ dilution). The solution was diluted (10⁻²–10⁻¹⁰) sequentially in phosphate buffer saline (PBS). Then, 100 µL of diluents was transferred in petri dishes and pour plated with MRS-Agar. Then, they were cultured at 37°C for 48 hr. The CFU per g of probiotic powder was enumerated and recorded. The experiments were performed in triplicate.

Tolerance of *L. paracasei* TD3 for low pH and pepsin (stomach condition)

The pH of MRS broth was adjusted to pH values of 1.5, 2.5, 3.5,

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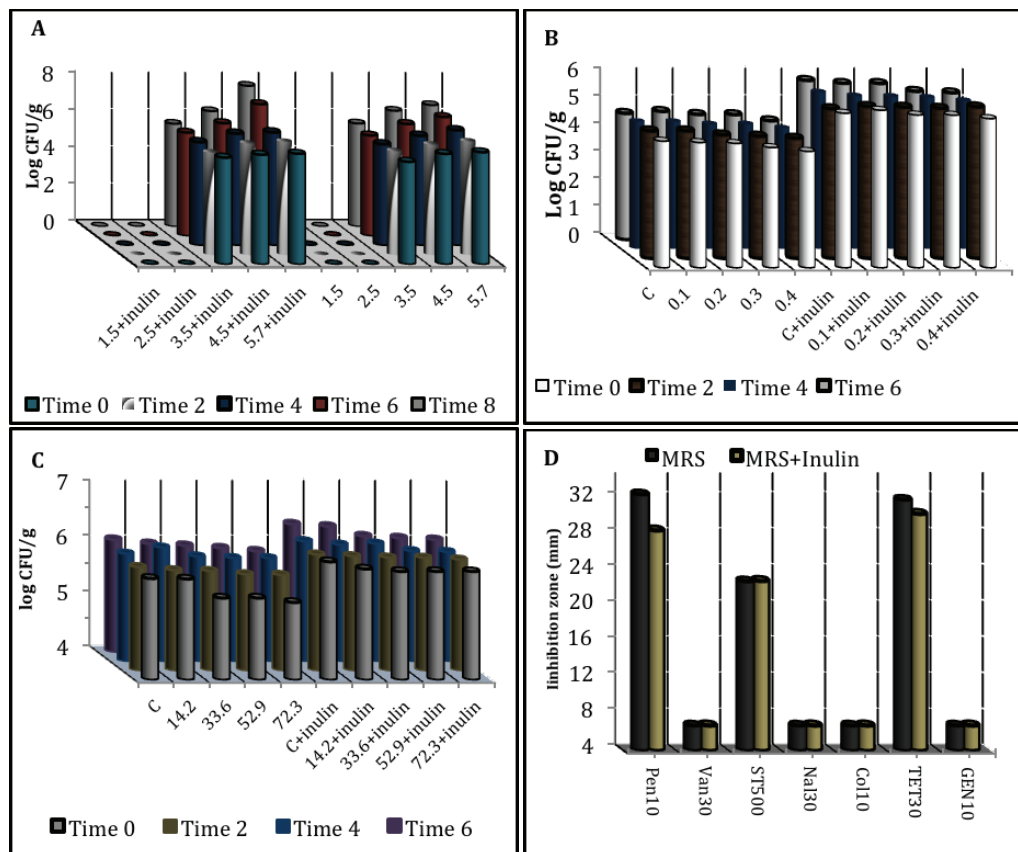


Figure 1. The effects of different conditions A) pH, B) Bile salts; C) 0.2% bile salts and trypsin D) antibiotics on the surveillance of *L. paracasei* TD3 in presence and absence of inulin 5%.

and 4.5 and then they were sterilized. The original *L. paracasei* TD3 liquid (10^{-1} dilution) was inoculated in MRS broth at different pH values and was incubated at 37°C for 8 hr, then the CFU/mL of *L. paracasei* TD3 was enumerated at different points of times from 0 to 8 hours.

The pepsin condition in stomach was related to the pH of gastric juice. The corresponding pepsin condition related to the pH of 5, 3–4, and 1.5–2.5 were 0.592, 1.482, and 0.723 $\mu\text{g/mL}$, respectively. Therefore, pepsin related CFU for *L. paracasei* TD3 were estimated and reported from 0 to 4 hr. The experiments were performed in triplicate.⁷

Tolerance of *L. paracasei* TD3 to bile salt and trypsin condition (small intestine condition)

L. paracasei TD3 liquid (10^{-1} dilution) was inoculated in sterilized MRS broth containing 0.1, 0.2, 0.3, and 0.4% (w/v) bile salts and was incubated at 37°C. The CFU of *L. paracasei* TD3 was estimated at different points of time (0–4 hr).

Tolerance to trypsin changes in the small intestine was evaluated at concentrations of 72.32, 52.96, 33.60, and 14.24 u/g, and 0.2% (w/v) bile salt simultaneously. Then, *L. paracasei* TD3 was inoculated and cultured at 37°C. CFU was estimated from 0 to 4 hr.⁷

Tolerance of *L. paracasei* TD3 to antibiotics

The resistance of *L. paracasei* TD3 to antibiotics discs was evaluated by the disc diffusion method. The antibiotics included (μg); penicillin-G (Pen 10), vancomycin (Van 30), nalidixic acid (Nal 30), streptomycin (ST 500), tetracycline (Tet 30), gentamicin

(Gen10) and colistin (Co 10). The disc antibiotics were purchased from Rosco (Diagnostica A/S, Taastrupgaardsvej DK-2630 Taastrup).

One or two colonies of cultured *L. paracasei* TD3 (IBRC-M 10784) were inoculated in PBS and its turbidity was adjusted to 0.5 McFarland (1×10^7 – 1×10^8 CFU/mL). Using a sterile cotton swab, the microbial suspensions were cultured on MRS-Agar. Subsequently, the above antibiotic discs were put on the cultured media. The plates were incubated at 37°C for 24 hr. The inhibition zone (IZ) diameters were measured in millimeters (mm) and averages of IZ were recorded as means \pm SD (Standard Deviation).⁸

Antimicrobial activity of *L. paracasei* TD3 against pathogenic bacteria using dual agar overlay method

L. paracasei TD3 was inoculated in spot on the center of MRS agar and was incubated in anaerobic condition at 37°C for 24 hr. The indicator bacteria were cultured on nutrient agar and were incubated at 37°C for 24 h in aerobic conditions. The plates were overlaid with 15 mL of nutrient agar containing 10^6 CFU/mL of indicator bacteria (*Escherichia coli* ATCC 6538, *Salmonella typhimorium* ATCC 14028, clinical isolates of *Shigella dysenteriae* and *Shigella flexneri*). After 24 hr of aerobic incubation at 37°C, the diameter of inhibition zone was measured. The tests were performed in triplicate and the mean of values were reported.⁹

Antibacterial activity of *L. paracasei* TD3 supernatant using micro titer plates

Cultured *L. paracasei* TD3 in MRS broth was centrifuged

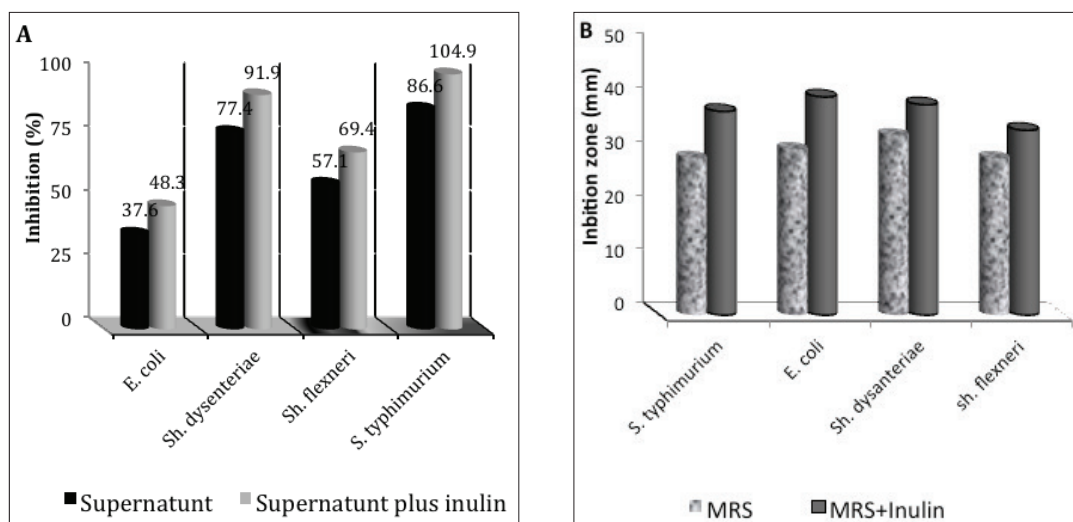


Figure 2. The antimicrobial potency of *L. paracasei* TD3 A) supernatant using micro titer plates and B) using dual agar overlay method against pathogenic bacteria.

(6000×g for 15 min) and supernatant of cultured MRS broth was poured in the wells of micro titer plates.

The above pathogenic bacteria were diluted in nutrient broth (10^4 CFU/mL). Then, 100 μ L of the supernatant and 100 μ L of the above diluted bacteria were pipetted into the wells of micro titer plates. In this case, 200 μ L of bacteria in nutrient broth was used as positive control. All micro titer plates were incubated at 37°C for 24 hr. The Optical densities (OD) of bacterial growth at 560 nm were evaluated by ELISA Reader. The analysis was carried out in triplicates and the means were estimated. The percentage growth of target bacteria was measured using the equation:

$$(\text{OD}_{560} [\text{A-B}] / \text{OD}_{560} \text{B}) * 100$$

Where A is the well containing bacteria in supernatant after 24 h incubation and B is the broth with the bacteria at time 0.⁹

All of the above experiments were performed with free cell and also probiotic with 5% (w/v) inulin (BNP company, batch number: BNPI131026) and the results were compared and reported.

Statistical analysis

Statistical analysis of the \log_{10} -transformed data was performed to ensure variance homogeneity and normality. The GraphPad prism 6 software was used for data and statistical analysis. Numerical data were analyzed using the one-way analysis of variance (ANOVA) in order to compare the mean values. A probability equal to or less than 5% was considered as statistically significant.

Results

Tolerance of *L. paracasei* TD3 (IBRC-M 10784) for low pH and pepsin in stomach condition

The CFU of live *L. paracasei* TD3 per gram of lyophilized powder was 10^7 CFU/g. The tolerance of *L. paracasei* TD3 for low pH condition in presence of inulin and without it was reported in Figure 1A. As the figure shows, in both conditions (in presence and without inulin), *L. paracasei* TD3 was sensitive to pH 1.5 and 2.5 while at pH 3.5, 4.5, and 5.7, the log CFU of *L. paracasei* TD3

increased with time. Values of pH 4.5 and 5.7 were the best pH for growing *L. paracasei* TD3. Adding the inulin to growth medium of probiotic bacteria increased the log CFU of *L. paracasei* TD3 from 6 to 8 at pH 5.7 but the differences were not significant ($P > 0.05$).

The tolerance of *L. paracasei* TD3 to pepsin was the concentration 0.723 μ g/mL and lower. The differences were not significant ($P > 0.05$).

Tolerance of *L. paracasei* TD3 to bile salt and trypsin condition

The tolerance of *L. paracasei* TD3 to different concentrations of bile salts and trypsin were reported at the time of 0 to 4 hr (Figures 1B and 1C), the results of tolerance to bile salts showed *L. paracasei* TD3 (IBRC-M 10784) was resistant to bile salts at concentrations of 0.1–0.4% (w/v) and there was no difference between the log CFU of probiotic bacteria in the presence of bile salts and control group (Figure 1B). Adding the inulin to the media containing bile salts increased the log CFU of *L. paracasei* TD3 with time in comparison to media without inulin ($P < 0.05$).

The tolerance of *L. paracasei* TD3 to tripsin enzyme showed this strain was resistant to tripsin, in conditions similar to the small intestine, although in high concentrations of tripsin, the log CFU was decreased. Inulin increased log CFU of *L. paracasei* TD3 in the presence of tripsin (Figure 1C), and these differences were significant ($P < 0.05$). The increase in tripsin enzyme decreased the log CFU of *L. paracasei* TD3 but the log CFU was higher than 5.

Tolerance of *L. paracasei* TD3 to antibiotics

Among the screened antibiotics, including penicillin-G, vancomycin, nalidixic acid, streptomycin, tetracycline, gentamicin and colistin (Figure 1D), *L. paracasei* TD3 exhibited sensitivity to penicillin-G, streptomycin and tetracycline. Other antibiotics showed no effect on *L. paracasei* TD3. Adding the inulin to the medium had no effect on the sensitivity of *L. paracasei* TD3 to these antibiotics.

Antimicrobial activity of *L. paracasei* TD3 against pathogenic bacteria using dual agar overlay method

The antimicrobial activity of *L. paracasei* TD3 against pathogenic bacteria was evaluated in the presence of inulin and without

it using the dual agar overlay method (Figure 1B). The results of our evaluation showed all of the pathogenic bacteria inhibited by *L. paracasei* TD3 growth with inhibition zone diameters higher than 25 mm and *Sh. dysenteriae* was the most sensitive bacteria to *L. paracasei* TD3 growth followed by *E. coli*, *S. typhimorium* and *Sh. flexneri*. In the presence of inulin, the sensitivity of all pathogenic bacteria increased to *L. paracasei* TD3. *E. coli* showed more sensitivity to *L. paracasei* TD3 followed by *Shigella dysenteriae*, *Salmonella typhimorium* and *Shigella flexneri*.

Antibacterial activity of *L. paracasei* TD3 supernatant using micro titer plates

Evaluating the antibacterial activity of *L. paracasei* TD3 supernatant in the presence of inulin and without it against pathogenic bacteria using microtiter plates (Figure 2A) showed that the growth of *S. typhimorium*, and *Sh. dysenteriae* was inhibited by the supernatant of *L. paracasei* TD3. In the broth medium, *E. coli* was inhibited by *L. paracasei* TD3 supernatant lower than the other pathogenic bacteria. Inulin enhanced the antibacterial activity of *L. paracasei* TD3 supernatant against pathogenic bacteria.

Discussion

Some strains of *L. paracasei* have been studied extensively due to their health benefits for humans and animals.¹⁰⁻¹²

The impact issues for selecting the new strain for probiotic studies are the tolerance of the strain to bile salts, tripsin in the intestinal tract, resistance to pepsin and acidic conditions of stomach, antagonism against pathogenic bacteria and resistance to antibiotics. In this study, we evaluated the functional properties of a new strain of *L. paracasei* TD3 for further studies. This strain tolerated acidic pH higher than 3.5.

The normal human stomach has a pH in the range of 1–3. After food consumption, the pH of stomach can rise to as high as 4–5. Therefore, *L. paracasei* TD3 from this study cannot tolerate the acidic conditions of stomach; therefore, it can be used as enterocoated tablets or used after a meal. This strain was tolerant to bile salts and tripsin, therefore it has the property of tolerance to intestinal conditions. The potency of inhibiting the growth of pathogenic bacteria helps with the treatment of certain infectious diseases caused by *S. typhimorium*, *Shigella* species and pathogenic *E. coli*. Indeed, *L. paracasei* TD3 strains adhere to human epithelial cell and human intestinal mucus and by producing the bacterial metabolites and occupying the attachment sites of pathogenic bacteria, they prevent the colonization of bacterial pathogens,¹³ thus stopping their pathogenesis.

Furthermore, *L. paracasei* prevents the amplification of pathogenic bacteria by producing bacteriocins.¹⁴

Inulin as a prebiotic compound increased the log CFU of *L. paracasei* TD3 in different conditions and enhanced the antibacterial potency of *L. paracasei* TD3. It has been shown that inulin can increase *bifidobacteria* populations.¹⁵

Our *in vitro* results show that inulin acts as growth enhancer and

increases the *L. paracasei* TD3 populations.

Therefore, inulin and *L. paracasei* TD3 (IBRC-M 10784) can be formulated in enterocoated form for further studies in human clinical trials.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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