ABSTRACT

Probiotic microorganisms have many health-beneficial effects on gastrointestinal system and their therapeutic usage is becoming increasingly common in human and veterinary medicine. Many different species and strains of bacteria, yeast and even fungi have been extensively used as potential probiotics. Among the probiotic strains, Lactobacillus rhamnosus is one of the most commonly used strain for probiotic treatment and health promoting functions of this strain are well documented. To enhance therapeutic effects of probiotics, prebiotics have been extensively used. Prebiotics stimulate the proliferation of probiotics and this may have positive effects on the maintenance of the balance between pathogenic and nonpathogenic bacteria. In this study, we aimed to evaluate the effect of inulin and Auricularia polytricha aqueous extract on the proliferation of L. rhamnosus. For this purpose, L. rhamnosus was inoculated in three different MRS broth supplemented with inulin 5%, A. polytricha extract 5% and with the mixture of inulin 5% plus A. polytricha extract 5%. Our results indicated that L. rhamnosus was able to use inulin and fungus extract as a carbon source. Moreover, combined use of inulin and A. polytricha improved prebiotic efficacy.

Keywords: Lactobacillus rhamnosus; Probiotic; Prebiotic; Inulin; Auricularia polytricha.
activity. And also, mushrooms with the rich β-glucan and oligo-β-glucan ingredients may be the potential sources for prebiotics. Auricularia polytrichia is known for its nutritional ingredients and it has many beneficial effects on human health. Its benefits include increasing blood circulation, lowering cholesterol and blood sugar, natural anti-viral properties, and thought to prevent dryness due to its moistening properties [11-13]. But properly use of prebiotics is important for stimulation of the probiotic proliferation. Therefore, in the present study, the effects of inulin and A. polytricha extract on the proliferation of L. rhamnosus which is a probiotic bacteria were evaluated.

MATERIALS AND METHODS

Bacterial strains and culture media
For the preparation of active cultures, L. rhamnosus strains were grown in Man Rogosa Sharpe (MRS) broth (Oxoid) for 18-20 h at 37 ± 1 °C. Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 25923), Enterococcus faecalis (ATCC 29212), Pseudomonas aeruginosa (ATCC 27853), Candida albicans (ATCC 10231) were used as test microorganisms. Test microorganisms were obtained from culture collection at Hitit University, Faculty of Science and Arts, Department of Molecular Biology and Genetic, Microbiology Research Laboratory. Lactobacillus rhamnosus strain was obtained from Gazi University, Faculty of Dentistry, Division of Medical Microbiology. The bacterial strains were stored at -80 °C in Nutrient broth containing 10% glycerol as cryoprotective agent.

Antimicrobial activity of L. rhamnosus
The antifungal and antibacterial activities of L. rhamnosus were evaluated by well diffusion method on Mueller-Hinton Agar (MHA). Briefly, MHA plates were inoculated with bacterial strains (100 μl) that were activated two times and optically standardized (OD600=0.6Å). Then wells were composed 6mm diameter on MHA and wells were filled with 10 or 15 μl of L. rhamnosus suspension in MRS broth and incubated at 37°C for 16-18 hours. After the incubation period, all petri dishes were investigated by zone inhibition for their antimicrobial activity.

Preparation of fungi extract
A commercially cultivated strain of Auricularia polytrichia was purchased from Agroma Food (Turkey). To prepare the extract of A. polytrichia, distilled water was used and 20 g sample weighted for extraction. Extraction was made with soxhlet machine during 8-12 hours. After extraction, solvent was concentrated in rotary vacuum evaporator machine (Stuart Rotary Evaporator, RE300P). The extract was then membrane filtered (0.45-μm pore size) to avoid contamination in works. Extracts were protected from light and kept in +4⁰C until executing the experiments.

Evaluation of prebiotic effect of inulin and A. polytricha extract
Inulin, A. polytricha aqueous extract and a mixture of inulin and the extract were used as prebiotic to improve the proliferation of L. rhamnosus. For this purpose, L. rhamnosus was activated two times in MRS broth and incubated 16-24 hours at 37°C. The bacterial suspension was adjusted to an optical density at 600 nm (OD600) of 0.6 to standardize the cell density of the samples. Activated and optically standardized microorganisms were inoculated (2% v/v) in 5 ml of MRS and culture medium containing different types of prebiotics. Table 1 shows the composition of these mediums. Prebiotics were added to MRS broth instead of glucose. Fermentations were carried out at 37°C independently, in duplicate, without any agitation. At the end of the incubation time, bacterial cell concentration in modified MRS was determined spectrophotometrically at 600 nm.

Counts of viable bacteria
Cell counts were made by plating in duplicate after fermentation. Samples (1.0 mL) were added to 4.0 mL of MRS broth and serial dilutions were made. After from

<table>
<thead>
<tr>
<th>Components (g/L)</th>
<th>MRS broth</th>
<th>MRS + inulin</th>
<th>MRS+ Fungus extract</th>
<th>MRS+ inulin+ Fungus extract</th>
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<tbody>
<tr>
<td>Pepton (g/L)</td>
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<tr>
<td>Yeast extract (g/L)</td>
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<tr>
<td>Glucose (g/L)</td>
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<tr>
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<tr>
<td>Sodium acetate.5H2O (g/L)</td>
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<tr>
<td>Triammonium citrate (g/L)</td>
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<tr>
<td>Manganese sulfate. H2O (g/L)</td>
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<tr>
<td>Magnesium sulfate.7H2O (g/L)</td>
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<tr>
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<td>1</td>
<td>-</td>
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<tr>
<td>Tween80 (mL/L)</td>
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<tr>
<td>A. polytricha</td>
<td>-</td>
<td>-</td>
<td>5%</td>
<td>5%</td>
</tr>
</tbody>
</table>

Table 1. Composition of MRS broth and medium containing prebiotics.
dilutions, L. rhamnosus was inoculated on MRS Agar, with pH adjusted to 6.8, and incubated at 37 °C for 16-18 h. Then, colony forming units (CFU) were enumerated in plates containing colonies, and cell concentration was expressed as CFU/ml.

**Statistical analysis**
The all experiments were done in duplicate. The results were expressed as means ± standard deviations (SD). Statistical analysis was performed on the data by SPSS 20.0 bivariate Correlation Analysis (SPSS Inc., Chicago) with statistical significance determined at 0.05.

**RESULTS AND DISCUSSION**

**Antimicrobial activity of L. rhamnosus**
Previous studies provided evidence that Lactobacillus species had inhibitory effect on pathogenic microorganisms [14]. In this study, L. rhamnosus was examined for antimicrobial activity against pathogenic microorganisms Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Enterococcus faecalis and Candida albicans. In our study, no antimicrobial effect of L. rhamnosus was detected on tested pathogens. Davoodabadi et al. [15] tested Lactobacillus strains with human origin for their antimicrobial activity against diarrheagenic Escherichia coli. A total of 20 Lactobacillus isolates were identified from stool samples. Lactobacillus fermentum was the most frequently isolated strain, followed by L. plantarum and L. rhamnosus. The findings showed that Lactobacillus strains with human origin had a mild inhibitory effect against the diarrheagenic E. coli. It was mentioned that the mechanism of antimicrobial activity of Lactobacillus strains appeared to be due to the production of organic acids or hydrogen peroxide. In another study, antimicrobial activity and antibiotic susceptibility were tested for 23 Lactobacillus and three Bifidobacterium strains. Agar-well diffusion method was used to test the antagonistic effect against Staphylococcus aureus, E. coli, Bacillus cereus and C. albicans of acid and neutralized lyophilized concentrated supernatants. Inhibition of two pathogens with neutralized L. bulgaricus, L. helveticus, L. plantarum, L. fermentum was detected. Some strains maintained activity after pH neutralization, indicating presence of active substances [16].

**Evaluation of prebiotic properties of inulin and A. polytricha aqueous extract**
Prebiotics such as oligosaccharides and inulin promote the development of probiotic microorganisms. Nowadays, some macrofungus are presumed to be prebiotic like inulin and various studies are being carried out [17]. In our study, we compare Lactobacillus rhamnosus growth in MRS broth and MRS supplemented with inulin and Auricularia polytricha aqueous extract. Growth activity of L. rhamnosus in MRS broth and in medium containing prebiotics resulted in different growth profile. Figure 1 shows the data for growth rate of L. rhamnosus in the medium with and without glucose. It was observed that the development of L. rhamnosus in glucose-containing MRS medium was fairly good (OD600nm = 1.972 A), while the removal of glucose resulted in a somewhat weaker development. The addition of inulin as prebiotic instead of glucose led to the continue development of L. rhamnosus (OD600nm=1.058 A). The prebiotic effect of A. polytricha, which is a macrofungus, was found to be weaker when compared to inulin (OD600nm=1.032 A). When the concurrent effect of inulin and fungus is examined, inulin was found that the effect was slightly higher than the fungus alone (OD600nm=1.046 A). However, no statistical difference was observed (p<0.05). Kaplan et al. [18] studied the effect of inulin on the growth profile of sixteen Lactobacillus strains. It was established that twelve of these strains were able to ferment inulin. In another study, a significant increase in lactobacilli levels in colon rats was observed when the culture medium was supplemented with inulin [19]. This is the first study, to our knowledge, to test the prebiotic effect of A. polytricha aqueous extract. Synytsya et al. tested the prebiotic effect of aqueous and alkali extracts of two cultivated mushrooms that belong to same class with A. polytricha. The difference between the values of maximum growth rate and maximum biomass concentration measured for the medium without and with the extract was compared for the extracts of Pleurotus ostreatus and Pleurotus eryngii. In most cases the extracts from P. ostreatus and P. eryngii support probiotic bacteria growth rate and biomass Lactobacillus strains. Extracts from P. eryngii proved better growth source than those from P. ostreatus. Lactobacillus strain Lac A grew with the same rate as control as with water extract of P. eryngii, but alkaline extract increased this rate twice [20].

![Figure 1. Proliferation of L. rhamnosus with effect of inulin and A. polytricha extract](image-url)
Counts of viable cells
Viability of L. rhamnosus was calculated as colony-forming units. As shown in Figure 2, L. rhamnosus viability determined 1.87 x 104 cfu/ml in control medium, 2.08 x 104 cfu/ml with inulin, 1.92 x 104 cfu/ml with A. polytricha and 2.22x104 with inulin plus A. polytricha. These results point out a generalized stimulation of the proliferation of L. rhamnosus induced by prebiotics. The influence of inulin on probiotic survival is consistent with the observations of several authors, who observed a clear beneficial action of this prebiotic on the viability of L. rhamnosus [21, 22]. Oliveira et al. [23] observed significant difference for viable counts of L. rhamnosus, which was strongly influenced by the inulin. The mean probiotic viable counts were 4.1% higher than in the control in the presence inulin. Although there is some information on the effect of A. polytricha on the intestinal flora in the literature, there is no definite study relation with the prebiotic effect of its.

**Figure 2. Viability of L. rhamnosus**

CONCLUSION
Probiotics are effective in the prevention and treatment of numerous diseases such as irritable bowel syndrome, cancer, urinary infections. Lactobacillus rhamnosus is one of the most used probiotic. To enhance their proliferation and therapeutic effects, substrate like inulin are widely used as a prebiotics. The prebiotic effects of inulin and several mushrooms have been confirmed in some clinical researches. In this research, we evaluated the effect of inulin and Auricularia polytricha extract on the growth profile of L. rhamnosus. Our results indicated that L. rhamnosus could use inulin and A. polytricha extract as a carbon source. Further studies on health benefit in animal models will be also conducted.

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REFERENCES


