The Gastrointestinal Transit Tolerance of *Lactobacillus plantarum* Strain No. 14 Depended on the Carbon Source

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This study aimed to assess variations in the human gastrointestinal transit tolerance of *Lactobacillus plantarum* strain No. 14 after culture with glucose and with fructose. Transit tolerance was determined at 37 °C against simulated gastric juices at pH values of 2.5, 3.0, and 3.5, and against simulated small intestinal juices containing 0%, 0.2%, or 0.4% oxgall. The simulated gastrointestinal transit tolerance of *Lactobacillus plantarum* strain No. 14 varied with the carbon source. Hence we compared the amounts of exopolysaccharide from *Lactobacillus plantarum* strain No. 14 cultured in various carbon sources. The exopolysaccharide levels were 146.5 ± 8.1 mg/l (culture) with glucose, and 20.1 ± 17.0 mg/l (culture) with fructose. Exopolysaccharide was removed by centrifugation, the simulated gastric tolerance of *Lactobacillus plantarum* strain No. 14 cultured with glucose decreased markedly, but that with fructose did not decrease. These results suggest that the gastrointestinal transit tolerance of *Lactobacillus plantarum* strain No. 14 is related to exopolysaccharide contents.

**Key words:** plant origin lactic acid bacteria; gastric tolerance; intestinal tolerance; carbon source; exopolysaccharide (EPS)

Certain lactic acid bacterial strains are known for their probiotic (beneficial to human health) effects during gastrointestinal tract passage.1,2 Roy Fuller’s definition of probiotic (1989),2 “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance,” is widely used. However, the definition of probiotic has since become broader, to the extent that almost anything, living or dead, can be included. According to Salminen et al. (1999),3 “probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the host system and well-being of the host.” It has been found that heat-killed lactic acid bacteria cells affect the host systemic immune system. For example, the symptoms of Japanese cedar-pollen allergy improved significantly in a group of volunteers who consumed fermented milk containing *Lactobacillus (L.) acidophilus* strain L-92 cells (heat-treated).4 When microorganisms are consumed orally, it is important to consider whether the microorganisms are dead or alive in the intestine, as this should provide insight into the mechanisms of the effects on the host.

Probiotic bacteria delivered through food systems must first survive transit through the upper gastrointestinal tract and then persist in the gut in order to provide the beneficial effects for the host.5 The low pH of the stomach and the antimicrobial action of pepsin are known to provide an effective barrier against the entry of many bacteria into the intestinal tract.5 The pH of the stomach can be as low as pH 1.5,7 or as high as pH 6 after food intake,8 but generally ranges from pH 2.5 to 3.5.9 The nature of the food also affects the transit time through the stomach. Food typically stays in the stomach for between 2 and 4 h.9 Another barrier against probiotic bacterial transit is the intestine. Adverse conditions in the intestine include the presence of bile salts.10 The transit time of food through the intestine is generally between 18 and 36 h.9 A bile salt concentration of 0.15–0.3% has been recommended as a suitable concentration for selecting probiotic bacteria for human use.11

*Lactobacillus plantarum* strain No. 14 (No. 14) was isolated from pickled shallots, a traditional Japanese food. It is used in starter cultures in the food industry. We conducted a placebo-controlled, double-blind study in order to evaluate the effects of No. 14 on Japanese cedar pollen allergy in 2005.12 Daily oral intake of No. 14 suppressed Japanese cedar pollen-specific Immunoglobulin E (IgE) levels, eosinophils, and subjective symptoms, and decreased the body fat percentage. No. 14 is a plant-origin lactic acid bacterium that is generally considered to be capable of surviving in severe environments.13 It has been reported that the gastrointestinal transit tolerance of some strains of *Lactobacillus plantarum* isolated from rice is high.14

In this study, we evaluated the gastrointestinal tolerance of No. 14 in vitro. In cultures of lactic acid bacteria, glucose is typically used as a carbon and energy source. No. 14 produced much viscous material when cultured with glucose, but produced smaller amounts with fructose. There are several reports on lactic acid bacteria that make different amounts of viscous material, or exopolysaccharide (EPS), depending on the carbon source. For instance, there are *Lactobacillus amylovorus*
DU-21\textsuperscript{15} and Lactobacillus casei CRL 87.\textsuperscript{16} We also evaluated the transit tolerance and yield of EPS when No. 14 was cultured with glucose or fructose as carbon source.

Materials and Methods

Preparation of bacteria. In simulated gastrointestinal transit assays, No. 14 was serially transferred twice into MRS medium (2% glucose or fructose, 1% peptone, 0.8%LAB-Lemco, 0.5% CH\textsubscript{2}COONa\textsubscript{2}, 0.4% yeast extract, 0.2% K\textsubscript{2}HPO\textsubscript{4}, 0.2% triammonium citrate, 0.1% Tween80, 0.02% MgSO\textsubscript{4}•7H\textsubscript{2}O, and 0.005% MnSO\textsubscript{4}•4H\textsubscript{2}O, pH 6.8), followed by aerobic incubation at 30 °C for 20h. For comparison, Leuconostoc sp. strain GLT 36 cultured with glucose was also treated. To determine the simulated gastric tolerance of No. 14 in the absence of EPS, cultures incubated with glucose or fructose were centrifuged for 10 min at 9,000 × g. The precipitates were suspended by vortexing in fresh MRS broth (volume equal to original culture volume), and then centrifuged again under the same conditions. Centrifugation and suspension were repeated 3 times.

Lactobacillus plantarum strain No. 14 has been deposited as FERM P-11550. It was isolated from pickled shallots. Leuconostoc sp. strain GLT 36 has been deposited as FERM P-13131. It was isolated from soil. Momoya Co., Ltd., has these two strains.

Preparation of simulated gastric and intestinal juices. Simulated gastric and intestinal juices were prepared fresh daily. Simulated gastric juices were prepared by suspending peptic (1:10,000, Sigma, St Louis, MS, USA) in sterilized MRS broth (glucose was used as carbon source, Oxoid, Basingstoke, UK) at a final concentration of 0.04%, and by adjusting the pH to 2.5, 3.0, or 3.5 with concentrated HCl or sterilized 0.1 mol/l NaOH. Simulated intestinal juices were prepared by suspending 0.2%, or 0.4% oxgall (Difco, Detroit, MI, USA) in sterilized MRS broth (glucose was used as carbon source, Oxoid).

Gastrointestinal transit tolerance assay. Simulated gastric and intestinal transit tolerance was determined by the methods of Azuma et al. (2001)\textsuperscript{17} and Kumagai et al. (2001).\textsuperscript{18} No. 14 was cultured with glucose and with fructose, as described above, and then mixed with simulated gastric pH (2.5, 3.0, or 3.5) or intestinal juices (0%, 0.2%, or 0.4% oxgall) at a final concentration of 1%. As a control, Leuconostoc sp. strain GLT 36 was cultured with glucose. The mixture was then vortexed at maximum speed for 10s and incubated at 37 °C. To determine the tolerance of combined gastric and intestinal transit, 3% of the simulated gastric juice mixture (pH 3.0, 3h) was added to the intestinal juices (0%, 0.2%, or 0.4% oxgall). The culture medium of No. 14 cultured with glucose or fructose and removed EPS was used only for the examination of simulated gastric transit tolerance at pH 2.5.

Gastric transit tolerance was evaluated based on the total viable count after incubation for 0, 1, 2, or 3 h. Total viable counts of bacteria were determined by the pour-plate method using MRS agar supplemented with 0.5% CaCO\textsubscript{3} after serial 10-fold dilution in maximum recovery diluents. MRS plates were incubated anaerobically at 30 °C for 2 d, and the colonies on the MRS plates were counted using a colony counter.

Intestinal transit tolerance was evaluated based on optical densities (OD\textsubscript{540}), which were determined after incubation for 0 or 18h. Growth rates were expressed as the percentage of turbidity in culture liquid containing 0.2% or 0.4% oxgall after incubation for 18h, as compared with those of broth containing no oxgall. Gastrointestinal transit tolerance was evaluated based on turbidity. The mixtures were observed under a microscope.

Determination of EPS from Lactobacillus plantarum strain No. 14. EPS was isolated and purified by the method of Kitazawa et al. (1998).\textsuperscript{19} No. 14 was serially transferred twice in MRS medium (glucose or fructose), and incubated aerobically at 30 °C for 20 h. The cultures were heated at 100 °C for 20 min, and bacterial cells were removed by centrifugation (13,000 × g, 20 min, 4 °C). The precipitates were then dissolved in PBS and centrifuged twice at 13,000 × g for 20 min at 4 °C. The supernatant was neutralized and concentrated 10-fold, and an equal volume of cold ethanol was added, followed by centrifugation at 13,000 × g for 20 min at 4 °C. Precipitated polysaccharide materials were collected and dissolved in distilled water, followed by the removal of insoluble material by centrifugation (13,000 × g, 20 min, 4 °C). Ethanol was then added to the resulting solution in order to precipitate the dissolved materials, as described above. The precipitates were treated for 6h at 37 °C with DNase and RNase (each at 7 μg/ml, Sigma) in 5 mmol/l Tris–HCl buffer (pH 8.0) containing 1 mmol/l MgCl\textsubscript{2}, and were subjected to digestion by protease K (200 μg/ml, Sigma) overnight at 37 °C. After inactivation of the enzyme by heating for 10 min at 100 °C, polysaccharide samples were precipitated with cold ethanol. The supernatants were centrifuged twice for 30 min at 13,000 × g at 4 °C. The precipitated polysaccharide materials were collected and dissolved in distilled water, and after removal of the insoluble material by centrifugation (13,000 × g, 20 min, 4 °C), the solution was dialyzed against distilled water for 48h at 4 °C. Polysaccharides were quantified by the phenol-sulfuric method.

Statistical analysis. The results were expressed as means and standard deviation. For gastric tolerance, two-way analysis of variance (ANOVA), one-way ANOVA, and Dunnett’s test were performed. For intestinal tolerance and gastrointestinal tolerance, two-way ANOVA and unpaired t-test were performed. For simulated gastric tolerance of No. 14 without EPS, two-way ANOVA, one-way ANOVA, and SNK (Student-Newman-Keuls) test were performed. To compare the amounts of EPS, an unpaired t-test was performed. p values of less than 0.05 were regarded as indicating a significant difference.

Results

Tolerance to simulated gastric juices at different pH levels

The effects of simulated gastric juices at different pH levels on viability are presented in Fig. 1. The average final pH of the simulated transit mixture did not change. All samples showed lower viability in simulated gastric juice at pH 2.5 as compared with pH 3.0 or pH 3.5.

In the simulated gastric juice at pH 2.5, the viability of No. 14 was reduced by 1-log unit after at was incubated in MRS medium (No. 14-Glc) for 3 h. No. 14 incubated in MRS medium with fructose (No. 14-Fru) showed a 4-log reduction in viability after 3 h. One-way ANOVA showed no differences among the time courses under glucose conditions, but in the case of No. 14-Fru, there was a significant difference (p < 0.01). Dunnett’s test indicated that the viability of No. 14-Fru decreased after 1 h. Leuconostoc sp. strain GLT 36 incubated in MRS medium (GLT36-Glc) lost viability after 1h of simulated gastric tract transit.

When the pH of the simulated gastric juice was raised to pH 3.0, No. 14-Glc retained a similar level of viability during simulated gastric tract transit for up to 3 h. In contrast, No. 14-Fru showed a 1-log reduction in viability after 3 h. One-way ANOVA indicated that there were no differences among the time courses under glucose conditions, but in the case of No. 14-Fru, there was a significant difference (p < 0.01). Dunnett’s test indicated that the viability of No. 14-Fru decreased after 2 h. GLT36-Glc showed a 2.5-log reduction in viability after 1 h of simulated gastric tract transit.

When the pH of the simulated gastric juice was further raised to 3.5, all the samples retained the same level of viability during the 3 h of simulated gastric tract transit, and there were no significant differences among the time courses.

Tolerance of simulated intestinal juices

The effects of different oxgall concentrations in the simulated intestinal juices on growth rate are presented
in Table 1. The pH of all samples was approximately 5.8. The growth rates of No. 14-Glc and No. 14-Fru in the presence of 0.2% oxgall were 37.7% and 37.5% respectively. The growth rates of No. 14-Glc and No. 14-Fru in the presence of 0.4% oxgall were 24.0% and 21.8% respectively. With 0.2% oxgall, there were no significant differences between No. 14-Glc and No. 14-Fru, but with 0.4% oxgall there was a significant difference between them. The growth rate of GLT36-Glc was 30.3% in 0.2% oxgall and 12.4% in 0.4% oxgall.

**Tolerance of simulated gastric and intestinal juices**

The pH values in all samples were approximately 5.3. The growth rates of No. 14-Glc and No. 14-Fru in gastric and intestinal juices (0.2% oxgall) were 31.8% and 29.5% respectively (Table 2). In gastrointestinal juices with 0.4% oxgall, the growth rates of No. 14-Glc and No. 14-Fru were 12.1% and 6.6% respectively. The growth rate in intestinal juices alone and in that in gastrointestinal juices containing oxgall at any concentration were significantly different (unpaired t-test, p < 0.01). Under gastrointestinal conditions with 0.2% oxgall, there was no significant difference between No. 14-Glc and No. 14-Fru, but with 0.4% oxgall, a significant difference was noted. The growth rate of GLT36-Glc was 0% in both 0.2% and 0.4% oxgall.

It has been reported that *L. casei* sp. Strain GLT 36 at pH 2.5, 3.0, and 3.5. There were no differences in time course at pH 3.5. One-way ANOVA and post-hoc Dunnett’s test were performed for No. 14-Glc and for No. 14-Fru at 0.2% and 0.4% oxgall. *p* < 0.05, **p** < 0.01.

**Table 1. Effects of Oxgall on the Growth of Lactobacillus plantarum Strain No. 14 and Leuconostoc sp. Strain GLT 36 in MRS Broth**

<table>
<thead>
<tr>
<th>%oxgall</th>
<th>0.2</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 14-Glc</td>
<td>37.7 ± 0.1</td>
<td>24.0 ± 0.6</td>
</tr>
<tr>
<td>No. 14-Fru</td>
<td>37.5 ± 0.6</td>
<td>21.8 ± 0.3 **</td>
</tr>
<tr>
<td>GLT36-Glc</td>
<td>30.3 ± 0.3</td>
<td>12.4 ± 1.3</td>
</tr>
</tbody>
</table>

Growth rates are expressed as percentage of turbidity in culture medium containing 0.2% or 0.4% oxgall after incubation for 18h, as compared to those in broth containing no oxgall. Each value represents the mean ± S.D. of triplicate determinations. Two-way ANOVA was performed for No. 14-Glc and No. 14-Fru in 0.2% and 0.4% oxgall. There were significant differences in oxgall concentrations and between carbon sources (p < 0.01). A post-hoc unpaired t-test was performed for No. 14-Glc and for No. 14-Fru at 0.2% and 0.4% oxgall. *p* < 0.05, **p** < 0.01.

**Table 2. Effects of Oxgall on the Growth of Lactobacillus plantarum Strain No. 14 and Leuconostoc sp. Strain GLT 36 in MRS Broth after Treatment at pH 3.0 in MRS Broth Containing 0.04% Pepsin**

<table>
<thead>
<tr>
<th>%oxgall</th>
<th>0.2</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 14-Glc</td>
<td>31.8 ± 4.0</td>
<td>12.1 ± 1.3</td>
</tr>
<tr>
<td>No. 14-Fru</td>
<td>29.5 ± 1.3</td>
<td>6.6 ± 1.3 **</td>
</tr>
<tr>
<td>GLT36-Glc</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Growth rates are expressed as percentage of turbidity in culture liquid containing 0.2% or 0.4% oxgall after incubation for 18h, as compared to those in broth containing no oxgall. Each value represents the mean ± S.D. of triplicate determinations. Two-way ANOVA was performed for No. 14-Glc and No. 14-Fru at 0.2% and 0.4% oxgall. There were no significant differences in oxgall concentrations and between carbon sources (p > 0.01). A post-hoc unpaired t-test was performed for No. 14-Glc and for No. 14-Fru at 0.2% and 0.4% oxgall. *p* < 0.05, **p** < 0.01.

**Determination of EPS from Lactobacillus plantarum strain No. 14**

We compared the amounts of EPS from No. 14 after growth in glucose and in fructose. The concentrations of EPS obtained after culture in glucose and in fructose...
was significant. The concentration of EPS per viable count were 146.5 ± 8.1 mg/l and 20.1 ± 17.0 mg/l respectively (Table 3), and the difference was found to be significant. The concentration of EPS per viable cell count/ml was 4.6 x 10^{-11} mg/CFU in glucose and 2.4 x 10^{-11} mg/CFU in fructose.

Tolerance of simulated gastric juices of No. 14 when EPS was removed

In order to remove EPS from No. 14, cultures were incubated with glucose or fructose, centrifuged 3 times at 9,000 x g for 10 min, and then washed with fresh MRS broth. Viable counts of No. 14 before the gastric tolerance test did not change after centrifugation (data not shown). The tolerance of washed No. 14-Glc of simulated gastric juices at pH 2.5 decreased significantly, but that of washed No. 14-Fru did not. There was no significant difference between washed No. 14-Glc and untreated No. 14-Fru.

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach. Initially, this study evaluated the effects of the pH of simulated gastric juices on the viability of No. 14 during 3 h of simulated gastric transit. There was no loss of viability for No. 14 at pH 3.5 regardless of the carbon source. On the other hand, at pH 3.0, No. 14-Glc retained the same level of viability, while No. 14-Fru showed reduced viability after 2 h. At pH 2.5, all the samples showed reduced viability, and a difference between No. 14-Glc and No. 14-Fru was seen after 1 h. Kumagai et al. found that at pH 3.5, several strains of L. casei subsp. casei, L. plantarum, L. acidophilus, and L. gasseri retained the same level of viability. However, at pH 2.5, six strains of L. casei subsp. casei showed a 5-log reduction in viability, while the other species showed a 0- to 7-log reduction, depending on the strain. These results indicate that the gastric transit tolerance of No. 14-Glc is sufficiently high for it to transit successfully through the human stomach. However, the gastric transit tolerance of No. 14 varied with the carbon source, and when incubated with fructose, the viability of No. 14 decreased.

In evaluating the potential of lactic acid bacteria as probiotics, it is generally necessary to evaluate resistance to bile acids. We evaluated the growth rate of No. 14 in simulated intestinal juices containing various concentrations of oxgall during an 18-h simulated intestinal transit. The growth rates of No. 14-Glc and No. 14-Fru in the presence of 0.2% oxgall decreased to...
37.7% and 37.5% respectively. Other reports have indicated that the growth rates of some strains of *L. gasseri*, *L. casei*, *L. sakei*, and *L. acidophilus* decreased to 11–91% in simulated intestinal juices with 0.2% oxgall. Although the growth rate of No. 14 in simulated intestinal juice decreased, the present results indicate that No. 14 has the potential to grow in human intestinal juices. In 0.2% oxgall, there was no significant difference between No. 14-Glc and No. 14-Fru, but in 0.4% oxgall, a significant difference in growth rates was seen. However, the viable cell count of No. 14 after it was mixed with intestinal juice could not be measured because the colony count is difficult in a medium adding bile and the time course of tolerance is uncertain.

Potential probiotics are evaluated according to tolerance of gastric and intestinal transit respectively, but the intestinal tolerance of microorganisms after passage through the stomach is thought to decrease. Hence we evaluated intestinal tolerance after the microorganisms were treated under stomach conditions, by the method of Kumagai et al. The intestinal tolerance of No. 14 decreased after it was treated under stomach conditions, and the gastrointestinal transit tolerance of No. 14 was also found to depend on the carbon source.

No. 14 produced much viscous material when cultured with glucose, while it produced little when cultured with fructose. The physiological function of the EPS produced by *Lactococcus lactis* was studied by comparing the tolerance of the non-EPS-producing strain *Lactococcus lactis* ssp. cremoris MG1614 with an EPS-producing isogenic variant of this strain against several anti-microbial factors. There was no difference in the sensitivity of the strains to increased temperatures, freezing, or freeze-drying, or to antibiotics such as penicillin and vancomycin. A model system also showed that EPS production did not affect the survival of *Lactococcus lactis* during passage through the gastrointestinal tract. In contrast, *Escherichia coli* O157:H7 W6-13 and its colanic acid EPS-deficient mutant M4020 were examined for gastrointestinal tolerance. The results showed that colanic acid EPS might serve as a protective barrier for *Escherichia coli* O157:H7, improving its survival in the human gastrointestinal tract. Another study was carried out on whether the EPSs released by microorganisms have a protective effect on other members of similar and neighboring microbial communities. The results clearly demonstrated the importance of exogenous EPSs in desiccation tolerance, while mixed results were obtained in freezing trials.

We hypothesized that the gastrointestinal tolerance of No. 14 is related to EPS production and is affected by the EPS yield. The EPS yield of No. 14 cultured with glucose was greater than that during fructose culture. We then evaluated simulated gastric tolerance when EPS was removed by centrifugation. Viable counts of No. 14-Glc decreased significantly during treatment in gastric juice at pH 2.5 for 3 h after centrifugation. Viable counts of No. 14-Fru, which produced little EPS, did not significantly decrease after centrifugation. There was no significant difference between washed No. 14-Glc and untreated No. 14-Fru. We suggest that the gastrointestinal transit tolerance of No. 14 is related to EPS.

In this study, when No. 14 cultured with glucose or fructose was mixed with simulated gastric or intestinal juices based on MRS broth in which glucose was the carbon source, at a final concentration of 1%, there was a difference in tolerance with carbon source. Perhaps human gastrointestinal transit tolerance differs according to how the microorganism content food is made. Moreover it appears that the protective capacity is different according to whether the bacterial cell is wrapped in EPS when it faces gastrointestinal juice, because the gastric transit tolerance of No. 14 decreased with removal the EPS. But the physical and chemical relations between the bacterial cells and EPS of No. 14 is not yet clear.

Most studies of EPSs produced by lactic acid bacteria have focused on the influence of physiological growth conditions on EPS biosynthesis, the genetics of EPS biosynthesis, and elucidation of the composition and primary structures of these EPSs. Information on the physiological role of EPSs themselves is almost completely lacking. Some bacteria invest more than 70% of their energy in EPS production, presumably to obtain a selective advantage in the environment. Most proposed functions of EPS in general are of a protective nature, such as protection against dehydration, macrophages, bacteriophages, antibiotics, and toxic compounds. However, the protective mechanism of EPS against these anti-microbial factors has not been clarified. We want to clarify the relation between the chemical structure of EPS produced by No. 14 and gastrointestinal transit tolerance, and tolerance of other anti-microbial factors in the future.

In conclusion, this study indicates that the tolerance of gastric and intestinal transit of No. 14 is dependent on the carbon source, and that the gastrointestinal transit tolerance of No. 14 is related to EPS production.

References


