Increased Enterocyte Production in Gnotobiotic Rats Mono-Associated with *Lactobacillus rhamnosus* GG

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There is increasing scientific and commercial interest in using beneficial microorganisms (i.e., probiotics) to enhance intestinal health. Of the numerous microbial strains examined, *Lactobacillus rhamnosus* GG has been most extensively studied. Daily intake of *L. rhamnosus* GG shortens the course of rotavirus infection by mechanisms that have not been fully elucidated. Comparative studies with germfree and conventional rats have shown that the microbial status of an animal influences the intestinal cell kinetics and morphology. The present study was undertaken to study whether establishment of *L. rhamnosus* GG as a mono-associate in germfree rats influences intestinal cell kinetics and morphology. *L. rhamnosus* GG was easily established in germfree rats. After 3 days of mono-association, the rate of mitoses in the upper part of the small intestine (jejunum) increased as much as 14 and 22% compared to the rates in germfree and conventional counterparts, respectively. The most striking alteration in morphology was an increase in the number of cells in the villi. We hypothesize that the compartmentalized effects of *L. rhamnosus* GG may represent a reparative event for the mucosa.

![Table 1. Effect of *L. rhamnosus* GG on the mitotic index in the intestines of young male rats](https://example.com/table1.png)

<table>
<thead>
<tr>
<th>Rats</th>
<th>Duodenum (15 cm)</th>
<th>Jejunum (40 cm)</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon (2–3 cm)</th>
<th>Colon (3–5 cm)</th>
<th>Colon (8–9 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control GF</td>
<td>27.3 ± 2.4</td>
<td>31.4 ± 1.7</td>
<td>33.0 ± 2.2</td>
<td>32.7 ± 3.7</td>
<td>15.9 ± 2.6</td>
<td>12.7 ± 3.9</td>
<td>11.5 ± 2.7</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 3 days</td>
<td>29.3 ± 2.7</td>
<td>35.8 ± 2.3⁶</td>
<td>36.1 ± 2.1⁶</td>
<td>32.6 ± 2.6⁵</td>
<td>19.5 ± 3.9⁵</td>
<td>13.7 ± 2.7⁵</td>
<td>14.2 ± 2.9⁵</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 21 days</td>
<td>28.0 ± 2.9</td>
<td>34.4 ± 2.2⁶</td>
<td>31.9 ± 4.2</td>
<td>29.4 ± 2.8</td>
<td>19.8 ± 1.6⁶</td>
<td>11.9 ± 1.0</td>
<td>11.2 ± 2.1</td>
</tr>
<tr>
<td>Control Conv</td>
<td>27.5 ± 2.4</td>
<td>29.3 ± 2.0</td>
<td>28.9 ± 4.4</td>
<td>28.1 ± 0.8</td>
<td>15.9 ± 1.2</td>
<td>11.8 ± 0.9</td>
<td>11.2 ± 2.7</td>
</tr>
</tbody>
</table>

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* Value significantly different from the value for GF rats.

* Value significantly different from the value for Conv rats.
with a significant reduction in fecal viral excretion (8, 9). These findings might have some relevance in light of the major role of rotavirus in diarrhea in day care centers (11). Another possible mechanism, not previously suggested, is that oral ingestion of _L. rhamnosus_ GG could have a stimulatory effect on production of enterocyttes, thereby causing wash-out of virus.

Recently, by performing comparative studies with GF and Conv rats and mice, we found that age, gender, and microbial status influence intestinal cell kinetics and morphology in a compartmentalized manner (1, 2). Reviewing previous investigations, we realized that little attention had been paid to standardization of these variables. Additionally, we are not aware of any previous publication describing intestinal cell kinetics in rats or mice mono-associated with probiotic microbes. The aim of the present study was to investigate the influence of _L. rhamnosus_ GG on mucosal cell kinetics and morphology in different regions of the intestine of young male rats mono-associated with bacteria.

**RESULTS**

The animals remained healthy throughout the study. In all rats mono-associated with bacteria, _L. rhamnosus_ GG was present in the luminal contents at a concentration of at least $10^7$ CFU/g of contents (data not shown).

The microscopic evaluations created a vast amount of data; therefore, only significant results are commented upon below.

**Mitoic index.** The total numbers of mitotic cells and cell nuclei were counted in the left columns of 30 well-oriented crypts in all sections, and the mitotic index was calculated. The mitotic index (MI) is the percentage of cells in the metaphase or the mitotic phase before the metaphase (27), and it is calculated with the following formula: $MI = N_m/N_c \times 100$, where _N_m_ is the number of mitotic cells and _N_c_ is the total number of cells in the left column of the crypt.

**Number of epithelial cells.** The numbers of cells in the left column of 30 well-oriented small intestine and colonic crypts and in the villi of the small intestine were counted.

**Depth of crypts and height of villi.** In the small intestine the depths of 20 well-oriented crypts were measured from the base of the crypt to the crypt-villus junction, and the heights of 20 villi were measured from the crypt-villus junction to the tip of the villus by using a micrometer in the ocular eyepiece (magnification, $\times 200$; Leica DM LS, Wetzlar, Germany).

**Preparation of specimens.** Specimens (2 cm) were taken from the duodenum, from the jejunal 15 and 40 cm distal to the pyloric region (Jej-land Jej-2, respectively), and from ileum 5 cm proximal to the ileo-cecal junction. Samples (1 cm) were also taken from the cecum at its base and from the colon 2 to 3, 5 to 6, and 8 to 9 cm from the cecum (C-1, C-2, and C-3, respectively). Each specimen was placed on a microprobe filter (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany) and cut open along its longitudinal axis to obtain access to crypts and villi. The specimens were fixed for 3 h in Carnoy’s solution (60% methanol, 30% chloroform, 10% acetic acid) and for 20 h in 70% ethanol and embedded in paraffin. A number of six 3-μm-thick sections were cut 100 μm from each other and stained with hematoxylin and eosin. All specimens were coded and examined in a blind fashion with a light microscope (magnification, $\times 200$; Leica DM LS, Wetzlar, Germany).

**Mitoic count.** The numbers of mitotic cells and nuclei were counted in the left columns of 30 well-oriented crypts in all sections, and the mitotic index was calculated. The mitotic index (MI) is the percentage of cells in the metaphase phase before the metaphase (27), and it is calculated with the following formula: $MI = N_m/N_c \times 100$, where _N_m_ is the number of mitotic cells and _N_c_ is the total number of cells in the left column of the crypt.

**Depth of crypts and height of villi.** In the small intestine the depths of 20 well-oriented crypts were measured from the base of the crypt to the crypt-villus junction, and the heights of 20 villi were measured from the crypt-villus junction to the tip of the villus by using a micrometer in the ocular eyepiece (magnification, $\times 100$). In a similar way, the depths of colonic crypts were measured from the base of the crypt to the flat margin of the colonic mucosa.

**Statistical analysis.** Results are expressed below as means ± standard deviations. The one-way analysis of variance test was used to evaluate the differences between groups. The significance level used was _P_ < 0.05.

**TABLE 2. Effect of _L. rhamnosus_ GG on the total number of crypt cells and the depth of the crypt in young male rats**

<table>
<thead>
<tr>
<th>Rats</th>
<th>Duodenum</th>
<th>Jejunum (15 cm)</th>
<th>Jejunum (40 cm)</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon (2-3 cm)</th>
<th>Colon (5-6 cm)</th>
<th>Colon (8-9 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control GF</td>
<td>25.6 ± 2.5</td>
<td>21.0 ± 0.8</td>
<td>20.9 ± 1.6</td>
<td>20.1 ± 0.9</td>
<td>24.2 ± 3.5</td>
<td>22.2 ± 3.6</td>
<td>25.0 ± 2.6</td>
<td>23.3 ± 2.3</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 3 days</td>
<td>28.9 ± 1.9</td>
<td>22.6 ± 2.1</td>
<td>23.7 ± 3.1</td>
<td>22.3 ± 1.6</td>
<td>33.5 ± 3.1</td>
<td>26.9 ± 2.2</td>
<td>29.4 ± 2.4</td>
<td>29.3 ± 3.6</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> GG, 21 days</td>
<td>30.3 ± 2.2</td>
<td>23.3 ± 1.7</td>
<td>23.0 ± 0.8</td>
<td>22.1 ± 1.0</td>
<td>36.7 ± 2.0</td>
<td>25.3 ± 1.3</td>
<td>30.5 ± 3.8</td>
<td>29.3 ± 2.1</td>
</tr>
<tr>
<td>Control Conv</td>
<td>26.5 ± 1.0</td>
<td>21.7 ± 1.3</td>
<td>22.0 ± 1.8</td>
<td>20.3 ± 1.1</td>
<td>28.7 ± 2.4</td>
<td>24.5 ± 2.6</td>
<td>27.4 ± 3.3</td>
<td>28.5 ± 1.5</td>
</tr>
</tbody>
</table>

| a Value significantly different from the value for GF rats. |
| b Value significantly different from the value for Conv rats. |

**Preparation of specimens.** Specimens (2 cm) were taken from the duodenum, from the jejunal 15 and 40 cm distal to the pyloric region (Jej-land Jej-2, respectively), and from ileum 5 cm proximal to the ileo-cecal junction. Samples (1 cm) were also taken from the cecum at its base and from the colon 2 to 3, 5 to 6, and 8 to 9 cm from the cecum (C-1, C-2, and C-3, respectively). Each specimen was placed on a microprobe filter (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany) and cut open along its longitudinal axis to obtain access to crypts and villi. The specimens were fixed for 3 h in Carnoy’s solution (60% methanol, 30% chloroform, 10% acetic acid) and for 20 h in 70% ethanol and embedded in paraffin. A number of six 3-μm-thick sections were cut 100 μm from each other and stained with hematoxylin and eosin. All specimens were coded and examined in a blind fashion with a light microscope (magnification, $\times 200$; Leica DM LS, Wetzlar, Germany).

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**Number of epithelial cells.** The numbers of cells in the left columns of 30 well-oriented small intestine and colonic crypts and in the villi of the small intestine were counted.

**Depth of crypts and height of villi.** In the small intestine the depths of 20 well-oriented crypts were measured from the base of the crypt to the crypt-villus junction, and the heights of 20 villi were measured from the crypt-villus junction to the tip of the villus by using a micrometer in the ocular eyepiece (magnification, $\times 100$). In a similar way, the depths of colonic crypts were measured from the base of the crypt to the flat margin of the colonic mucosa.
pared with the number in GF rats, and after 21 days, the numbers of crypt cells in all intestinal compartments (except C-1) were increased compared with the numbers in GF rats (Table 2).

After 3 days of mono-association, the number of crypt cells in the cecum was increased compared with the number in Conv rats, and after 21 days, the numbers of crypt cells in the duodenum, ileum, and cecum were increased compared with the number in Conv rats.

**Depth of the crypts in rats mono-associated with L. rhamnosus GG compared with the depths of the crypts in GF and Conv rats.** After 3 days of mono-association, the crypts in the small intestine and cecum were shorter in the rats mono-associated with L. rhamnosus than in GF rats, and after 21 days, the crypts in the jejenum were shorter in the rats mono-associated with L. rhamnosus than in GF rats. However, after 21 days, the crypts in the cecum and C-2 were deeper in the rats mono-associated with L. rhamnosus GG than in the GF rats (Table 2).

After 3 days of mono-association, the crypts in the duodenum, ileum, cecum, C-1, and C-2 were shorter in the rats mono-associated with L. rhamnosus than in Conv rats, and after 21 days, the crypts in the duodenum, Jej-1, and C-1 were shorter in the rats mono-associated with L. rhamnosus than in Conv rats.

**Number of villus cells in rats mono-associated with L. rhamnosus GG compared with the numbers of villus cells in GF and Conv rats.** After 3 days and 21 days of mono-association, the number of villus cells in the small intestine was increased compared with the number of villus cells in GF rats (Table 3).

After 3 and 21 days of mono-association, the number of villus cells in the small intestine was also increased compared with the number of villus cells in Conv rats.

**Height of villus in rats mono-associated with L. rhamnosus GG compared with height of villus in GF and Conv rats.** After 3 days of mono-association, the villi were taller in the duodenum and jejunum but shorter in the ileum (Table 3). After 21 days of mono-association, the villi in the duodenum and jejunum were taller in rats mono-associated with L. rhamnosus GG than in Conv rats.

**DISCUSSION**

Our results demonstrate that L. rhamnosus GG is easily established as a mono-associate in GF rats. Establishment did not cause any discomfort in the animals. The results indicate that L. rhamnosus GG had a compartmentalized effect upon rat intestinal mucosa. After 3 days of mono-association, the mitotic index for the upper part of the jejunum increased 14 and 22% compared to data obtained for the GF and Conv rats, respectively. A similar increase in cell production has been observed in GF rats exposed to a Conv flora for 3 days (26). As is shown in Table 1, young male GF rats express higher jejunal mitotic activity than Conv rats in that region of the intestine, for reasons thought to be due to increased dietary intake (1). It is tempting to speculate that differences in the mitotic indices between GF and Conv animals may partially account for the differences in the intestinal persistence of rotavirus in such animals (12). The increased number of crypt cells was not followed by deeper crypts or taller villi, indicating that there was a reduction in individual cell size. In fact, both the villi and the crypts tended to be shorter, especially after 3 days of mono-association. Most probably, the reduction in cell size is a cophenomenon of increased cell production.

Several mechanisms may contribute to these effects. Utilizing a human colonic adenocarcinoma cell line (IEC-6), Olaya et al. showed that components of the cell wall of Escherichia coli and the cytols of Lactobacillus acidophilus are sources of epithelial cell mitogens (19). Members of the same group presented data linking this mitogenic effect(s) to the presence of lipopolysaccharides in gram-negative bacteria and lipotheoid acid/peptidoglycan in a gram-positive organism (20). In a recent study, Kitazawa

**TABLE 2—Continued**

<table>
<thead>
<tr>
<th>Crypt depth (µm)</th>
<th>Duodenum</th>
<th>Jejunum (15 cm)</th>
<th>Jejunum (40 cm)</th>
<th>Ileum</th>
<th>Cecum</th>
<th>Colon (2-3 cm)</th>
<th>Colon (5-6 cm)</th>
<th>Colon (8-9 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>126.9 ± 8.2</td>
<td>95.4 ± 9.6</td>
<td>96.0 ± 11.6</td>
<td>85.7 ± 9.7</td>
<td>135.3 ± 4.1</td>
<td>124.8 ± 12.4</td>
<td>134.8 ± 10.5</td>
<td>134.9 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>105.0 ± 13.3ab</td>
<td>79.3 ± 10.7a</td>
<td>76.7 ± 11.0a</td>
<td>71.1 ± 11.0ab</td>
<td>121.3 ± 10.7ab</td>
<td>115.4 ± 12.3a</td>
<td>132.7 ± 18.6a</td>
<td>131.0 ± 28.4a</td>
<td></td>
</tr>
<tr>
<td>123.0 ± 16.1</td>
<td>81.0 ± 4.8ab</td>
<td>80.6 ± 8.6ab</td>
<td>76.5 ± 9.4</td>
<td>145.8 ± 7.7b</td>
<td>110.2 ± 19.5b</td>
<td>163.1 ± 22.4b</td>
<td>134.8 ± 24.1b</td>
<td></td>
</tr>
<tr>
<td>143.2 ± 8.1</td>
<td>89.9 ± 5.7</td>
<td>84.4 ± 7.4</td>
<td>84.6 ± 4.7</td>
<td>151.6 ± 21.8</td>
<td>148.8 ± 19.0</td>
<td>162.7 ± 20.6</td>
<td>157.4 ± 24.9</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 3. Effect of L. rhamnosus GG on the number of cells and the height of the villus in young male rats**

<table>
<thead>
<tr>
<th>Rats</th>
<th>No. of villus cells</th>
<th>Height of villus (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Duodenum</td>
<td>Jejunum (15 cm)</td>
</tr>
<tr>
<td>Control GF</td>
<td>75.4 ± 0.6</td>
<td>63.0 ± 3.0</td>
</tr>
<tr>
<td>L. rhamnosus GG, 3 days</td>
<td>100.1 ± 7.0ab</td>
<td>79.7 ± 4.7ab</td>
</tr>
<tr>
<td>L. rhamnosus GG, 21 days</td>
<td>101.4 ± 6.7ab</td>
<td>84.2 ± 6.8ab</td>
</tr>
<tr>
<td>Control Conv</td>
<td>68.0 ± 4.8</td>
<td>56.7 ± 4.4</td>
</tr>
</tbody>
</table>

a Value significantly different from the value for GF rats.
b Value significantly different from the value for Conv rats.
et al. (16) showed that chromosomal DNA, purified from 12 of 16 strains of L. acidophilus, induced proliferation of splenic lymphocytes. The nucleotide sequences, consisting of only A and T nucleotides, were characterized as B-lymphocyte-specific mitogen (16). However, no experiments were performed on enterocytes. It seems reasonable to assume that the enterocytes in our rats mono-associated with bacteria were exposed to a large amount of lipoteichoic acid/peptidoglycan and probably also to oligonucleotides similar to those present in L. acidophilus. Whether and to what extent these three substances may act alone or in concert as a driving force(s) for increased mitogenic activity in enterocytes are still not known.

Whatever the mechanism is, the mitogenic effect of L. rhamnosus GG found in the present study may have a beneficial effect(s) in some clinical situations. Increased cell production contributes to enhanced mucosal regeneration. In addition to this reparative effect, the increased cell production could act as a wash-out mechanism for pathogenic microbial agents. Thus, use of L. rhamnosus GG in the treatment of a rotavirus diarrhea may increase cell production in the jejunum, leading to wash-out of infected cells, thereby shortening the period of fecal excretion of the virus. Previous investigations have also shown that L. rhamnosus GG reduces the severity of rotavirus infections (8, 9). The wash-out function related to the presence of L. rhamnosus GG may also be involved in this reduction of diarrhea symptoms.

In the present study, the observation period was limited to 3 weeks. Consequently, extrapolation of our results to more long-lasting or chronic inflammatory bowel conditions has to be made with the greatest care. If the mitogenic effect(s) of L. rhamnosus GG lasts for extended periods of time, it seems reasonable to assume that there is a reparative effect on more chronic conditions as well. The chronic inflammatory factors might be microbial pathogens or chemical agents. Experimentally, ex-GF rats challenged with acute or chronic proinflammatory agents provide excellent models for testing possible prophylactic and/or therapeutic effects of probiotics.

This study represents a first attempt to study the mitogenic effect(s) of a probiotic strain in ex-GF animals. It would be reasonable to assume that a similar mitogenic mechanism(s) may be found when other probiotics are used. It can be hypothesized that various probiotics may act on different compartments within the gastrointestinal tract. A strategic goal could be to orchestrate a group of probiotics that act on specific compartments, which could be used in specific pathophysiologic conditions in patients.

ACKNOWLEDGMENTS

We are most grateful to Anna-Karin Persson, Sandra Stenberg, and Ewa Osterlund, Laboratory of Medical Microbial Ecology, Karolinska Institutet, and Margareta Rodensjö, Department of Histopathology and Research Laboratory, Karolinska Hospital, for their excellent technical assistance.

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REFERENCES


