We compared the colonization of lactobacilli in the stomachs of mice fed nonpurified and purified diets and examined to determine whether the expression of Toll-like receptor 2, which is involved in the recognition of lactobacilli, is influenced by diet. Female BALB/c mice were fed a nonpurified or a purified diet for 2 weeks. Conventional cultivation and cultivation-independent molecular biological analysis based on the 16S rRNA gene sequence revealed that the number of lactobacilli associated with the gastric tissue was significantly higher in the mice fed the nonpurified diet than in those fed the purified diet. Sequencing analysis indicated that L. gasseri and L. johnsonii were predominant Lactobacillus species associated with the gastric tissue of the mice fed the nonpurified diet. The mRNA levels of Toll-like receptor 2, but not of 9, in the gastric tissue were significantly higher in the mice fed the purified diet than in those fed the nonpurified diet. We propose that nonpurified and purified diets have different impacts on gastric microbiota, which can in turn influence the expression of Toll-like receptor 2 in the stomach.

Key words: purified diet; nonpurified diet; lactobacilli; stomach; mice

There are two outstanding types of diet for experimental animals: nonpurified and purified. The primary ingredients in nonpurified diet are derived from natural sources, such as corn, wheat bran, defatted soybean, defatted rice bran, fishmeal, defatted milk, soybean oil, and brewer’s yeast. In contrast, purified diet is made of refined ingredients. Not surprisingly, purified and nonpurified diets have different impacts on the physiology and pathophysiology of certain diseases in animals. For instance, our previous study found that in the mice fed purified diet, fructo-oligosaccharide supplementation exacerbated the symptoms of DSS-induced colitis, whereas in those fed nonpurified diet, fructo-oligosaccharide supplementation reduced the symptoms. In addition, we have reported that Candida albicans, an opportunistic fungal pathogen, successfully colonized the gut of the mice fed purified diet but not those of the mice fed nonpurified diet. Purified diet is widely used in nutritional studies, but purified diet is quite different from the human diet, which contains a wide variety of ingredients. Therefore, studies using purified diet may not properly reflect physiological and pathophysiological phenomena under certain circumstances. Nevertheless, the different impacts of feeding nonpurified and purified diets on the physiology and pathophysiology of animals have not been studied vigorously.

The gastrointestinal tract harbors various strains of microbes, predominantly anaerobic bacteria, which have symbiotic relationships with the development and health of host animals. Lactobacilli have the ability to grow and colonize in the acidic conditions of the stomach, and are the predominant bacterial species in the stomachs of laboratory rodents throughout the life span. The stomachs of mice are compartmentalized into the forestomach, lined with keratinized stratified squamous epithelium, and the glandular stomach, with a columnar secreting epithelium. Lactobacilli colonize the forestomach. Previous studies based on histological examination and conventional cultivation have found that the number of lactobacilli colonized in the forestomach is much lower in mice fed purified diet than in those fed nonpurified commercial rodent diet, but the mechanism by which these diets influence lactobacillus colonization of the stomach remains to be elucidated. In addition, it is also unclear whether different levels of lactobacilli colonization in the stomachs of mice fed nonpurified and purified diets influence the host immune system. In order to discriminate further the differences in the gastric microbiota of mice fed nonpurified and purified diets, the present study employed cultivation-independent molecular biological methods based on the 16S rRNA gene sequence. In addition, we compared the gene expression of Toll-like receptors (TLRs) in the stomachs of mice fed nonpurified and purified diets. Because TLRs play a crucial role in the innate immune recognition of microbes, it is possible that the expression of TLR genes in the stomach is influenced by lactobacilli colonized in the stomach.

Materials and Methods

Animals and diets. All study protocols were approved by the Animal Use Committee of Hokkaido University (approval no. 08-0139), and all the mice were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hokkaido University.

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Abbreviations: CFU, colony-forming unit; DGGE, denaturing gradient gel electrophoresis; MRS, de Man Rogosa Sharpe; RT-qPCR, real-time quantitative PCR; TLR, Toll-like receptors
Female specific pathogen-free BALB/cCrSlc mice (age, 5 weeks) were purchased from Japan SLC (Hamamatsu, Japan) and housed in standard plastic cages in a temperature-controlled (23 ± 2 °C) room under a 12-h light:dark cycle. They were allowed free access to food and water. After acclimatization with a nonpurified commercial rodent diet (MR Stock; Nason Corporation, Yokohama, Japan) for 1 week, the mice were fed either the nonpurified diet or a purified diet prepared according to the composition of AIN-93G.1)

Experimental design. The mice were randomly allocated to two groups (n = 6 for each group) and fed either the nonpurified or the purified diet. Two weeks after feeding the test diets, mice were anesthetized by inhalation of diethyl ether and killed by exsanguination from the carotid artery. Following laparotomy, the stomach was excised and cut open along the greater curvature. The contents were removed gently with a spatula and subjected to examination of microbiota, as described below. The gastric tissue was washed gently with cold sterile PBS to remove the remaining contents, and a portion of tissue was subjected to histological examination, as described below. The other portion of tissue was immediately frozen in liquid nitrogen and stored at −80 °C for isolation of RNA. The remaining portion of the tissue was subjected to examination of microbiota, as described below.

Histology. Cryostat sections (5 μm) of gastric tissue were prepared and stained with hematoxylin and eosin for observation of tissue structure. Gram staining was also performed to detect bacteria associated with the gastric tissue.

Conventional cultivation of gastric bacteria. Gastric contents were suspended in anaerobic phosphate buffer. Gastric tissue samples were added to 1 mL of anaerobic phosphate buffer and, after vigorous shaking for 1 min, the washings were collected. The washings were centrifuged at 700 × g for 1 min and the supernatant was collected. This washing procedure was repeated, and the supernatants were combined and analyzed for microbes associated with the gastric tissue by a conventional cultivation method. A portion of the supernatant was further centrifuged at 10,000 × g for 3 min, and the resulting pellet was subjected to extraction of bacterial DNA, as described below. Microbes in the gastric contents and the gastric tissue washings were enumerated by the method of Mitsuoka et al.12) In brief, the samples were diluted stepwise 10-fold with anaerobic phosphate buffer. A sample (0.05 mL) of each dilution was inoculated onto two nonselective media (glucose- and tryptophanase-soy agar for total anaerobic bacteria and total aerobic bacteria respectively) and one selective medium (lactobacillus selection agar). Anaerobic incubation was carried out at 37 °C for 48 h using the AnaeroPack system (Mitsubishi Gas, Tokyo), and aerobic incubation was done at 37 °C for 48 h. After incubation, the number of colonies was counted, and was represented in logarithm colony forming units (CFUs). In addition, a number of single colonies that appeared in the lactobacillus selection agar were selected and suspended in de Man Rogosa Sharpe (MRS) broth (Becton Dickinson, Franklin Lakes, NJ). After incubation at 37 °C for 72 h under anaerobic conditions, the medium was removed by centrifugation at 10,000 × g for 10 min, and the resulting pellet subjected to extraction of bacterial DNA for sequencing, as described below.

Molecular biological analysis of microbiota. DNA was isolated from the gastric contents and the pellet of gastric tissue washings using a fecal DNA isolation kit (MO Bio Laboratories, Carlsbad, CA), following the manufacturer’s instructions. Similarities and differences in microbiota composition were analyzed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. DNA samples (10 ng) were added to a 25-μL reaction mixture containing 0.5 μM each of U968-GC and L1401 primers,13) 1 × Green Go Taq Flexi Buffer (Promega, Madison, WI), 1 mM MgCl2, 0.2 mM dNTP, and 1.25 U of Go Taq Hot Start polymerase (Promega). The reaction conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 10 s, 56 °C for 20 s, and 68 °C for 40 s, and a final extension at 68 °C for 7 min. The amplicons were analyzed by DGGE, as described above, except that an 8% polyacrylamide gel containing a 32.5 to 40% gradient of urea-formamide was used. The bands of interest were excised from the DGGE gel with a sterile blade under a Safe Imager Blue-Light Transilluminator (Invitrogen, Carlsbad, CA). The excised gels were added to 20 μL of sterile double-distilled water and incubated at 4 °C overnight. A portion (2 μL) of the samples was subjected to PCR with Lac1 and Lac2 primers.15) The reaction conditions were 94 °C for 5 min, followed by 34 cycles at 94 °C for 30 s, 61 °C for 1 min, and 68 °C for 1 min, and a final extension at 68 °C for 5 min. The amplicons were analyzed by DGGE, as described above, except that an 8% polyacrylamide gel containing a 32.5 to 40% gradient of urea-formamide was used. The bands of interest were excised from the DGGE gel with a sterile blade under a Safe Imager Blue-Light Transilluminator (Invitrogen, Carlsbad, CA). The excised gels were added to 20 μL of sterile double-distilled water and incubated at 4 °C overnight. A portion (2 μL) of the samples was subjected to PCR with Lac1 and Lac2 primers.15) Amplification was assessed by 1% agarose gel electrophoresis. Amplicon bands were excised from the gel, and the amplicons were purified using a QIAquick Gel Extraction Kit (Qiagen, Germantown, MD). Purified samples were sequenced using a BigDye Terminator v3.0 cycle sequencing kit (Applied Biosystems Japan, Tokyo) following the manufacturer’s instructions, with the Lac1 and Lac2 primers.15) The sequences were compared to those in the GenBank database with the BLAST N algorithm. Similarly, DNA samples isolated from bacterial colonies appearing in the lactobacillus selection agar were also subjected to PCR with the U968 and L1401 primers,15) followed by sequencing as described above.

TLR mRNA expression analysis. Total RNA was isolated from tissue homogenates using Trizol reagent (Invitrogen) following the manufacturer’s instructions. After digestion of genomic DNA with RNase-free Dnase (Promega), approximately 10 ng of total RNA was annealed with oligo (dT)18 primer (Invitrogen) at 70 °C for 10 min, and 1st-strand cDNA was then synthesized using M-MLV reverse transcriptase (Invitrogen), followed by RNA digestion with RNase-free RNase H (Invitrogen). RT-qPCR was performed using a Thermal Cycler Dice TP800 (Takara). The primer sequences for TLR2 and TLR9 were as in Gorczynski et al.16) The primer sequences for 18S rRNA were as in Johnson et al.19) Amplification was carried out in a 25-μL reaction volume containing 12.5 μL of 1 × SYBR Premix Ex Taq (Takara), 200 nM of each primer, and 1 μL of template cDNA. The reaction conditions were 95 °C for 10 s, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s, with a dissociation curve at 95 °C for 15 s, 60 °C (for TLR2), or 56 °C (for TLR9) for 30 s and 95 °C for 15 s. Relative gene expression levels for each sample were normalized to the levels for 18S rRNA.

Statistical analysis. Results are presented as means ± SEM. To compare mean values, Student’s t-test or two-way ANOVA with the Tukey-Kramer post hoc test was used. GraphPad Prism for Macintosh (version 5.0, GraphPad Software, San Diego, CA) was used for analysis. p values of <0.05 were considered statistically significant.

Results

The diversity of microbiota in the gastric contents and microbiota associated with the gastric tissue in the mice fed the nonpurified and purified diets was compared by

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Fig. 1. PCR–DGGE Analysis of Gastric Microbiota Based on 16S rRNA Gene Sequences in BALB/c Mice Fed Nonpurified and Purified Diets.

A DGGE gel image is shown in the lower part of chart A. Similarities among DGGE band profiles were calculated based on the positions and intensities of bands. The dendrogram of DGGE band profiles was constructed by the unweighted pair-group method using the arithmetic average clustering method (upper part of chart A). Each lane in the gel and each line in the dendrogram represents an individual mouse. Distances are measured in arbitrary units. N-C, gastric contents in the mice fed the nonpurified diet; P-C, samples associated with the gastric tissue in the mice fed the purified diet; N-A, sample associated with the gastric tissue in the mice fed the nonpurified diet; P-A, sample associated with the gastric tissue in the mice fed the purified diet. In chart B the number of DGGE bands is shown. Values are expressed as means with SEM, n = 6. A comparison of mean values among the groups of mice was done by two-way ANOVA with the Tukey-Kramer post hoc test. The p values for ANOVA were diet 0.0049, tissues <0.0001, and interaction, 0.0168.

PCR–DGGE analysis based on 16S rRNA gene sequences (Fig. 1). The intensity and position of the detected bands were subjected to cluster analysis. The dendrogram (Fig. 1A) shows two large clusters of mice fed the nonpurified and purified diets, but two of the mice fed the purified diet belonged to the cluster of mice fed the nonpurified diet. Additionally, each cluster was subdivided into two clusters: microbiota in the gastric contents and microbiota associated with the gastric tissue. The results indicate that gastric microbiota composition differed between the mice fed the nonpurified and the purified diet, as well as between the gastric contents and the gastric tissue. In addition, the number of bands was significantly higher in the gastric contents of the mice fed the nonpurified diet, and in the gastric contents and the samples associated with the gastric tissue of the mice fed the purified diet, than in the gastric tissue of the mice fed the purified diet, than in the gastric contents and the samples associated with the gastric tissue in the mice fed the purified diet, and in the gastric contents and the samples associated with the gastric tissue in the mice fed the nonpurified diet, and in the gastric contents and the samples associated with the gastric tissue in the mice fed the nonpurified diet, and in the gastric contents and the samples associated with the gastric tissue in the mice fed the nonpurified diet.

Table 1. Populations of Indigenous Bacteria Associated with Gastric Tissues in BALB/c Mice Fed the Nonpurified and the Purified Diet

<table>
<thead>
<tr>
<th></th>
<th>Nonpurified diet</th>
<th>Purified diet</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>log CFU/g tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total aerobes</td>
<td>7.46 ± 0.16</td>
<td>6.02 ± 0.07</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total anaerobes</td>
<td>8.59 ± 0.10</td>
<td>6.48 ± 0.07</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>8.61 ± 0.08</td>
<td>6.12 ± 0.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>log copies/g tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>6.02 ± 0.12</td>
<td>5.27 ± 0.22</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

1Values are expressed as means with SEM, n = 6. The comparison of mean values was done by Student’s t-test.

Fig. 2. PCR–DGGE Analysis of Gastric Microbiota in BALB/c Mice Fed the Nonpurified Diet.

PCR amplicons, generated using Lactobacillus genus-specific Lac1 and Lac2GC primers, were separated by DGGE, the bands indicated were amplified by Lactobacillus genus-specific Lac1 and Lac2 primers, and their sequences were analyzed. In the gastric contents, C1 and C3 correspond to L. gasseri or L. johnsonii, and C2 corresponds to L. reuteri. In the samples associated with the gastric tissue, T1 corresponds to L. gasseri or L. johnsonii. Bands without indication did not result in sequences on purification and sequencing.

To characterize the population profile of the lactobacilli in the stomach, PCR–DGGE with Lactobacillus genus-specific primers was performed. Although some bands were detected in the mice fed the nonpurified diet (Fig. 2), no detectable bands were observed in those fed the purified diet (data not shown). The major bands were cut from the gel and DNA sequences were analyzed. A BLAST search indicated that the sequences showed highest similarity to L. gasseri (99%), L. johnsonii (99%), and L. reuteri (98%). Although bands corresponding to these three species were detected in the gastric contents, no detectable bands corresponding to L. reuteri were observed in the samples associated with the gastric tissue. In addition, the sequences of the DNA samples isolated from the bacterial colonies appearing in the lactobacillus selection agar exhibited highest similarity to L. gasseri (99%) and L. johnsonii (99%).

The gastric tissue was examined histologically. In the mice fed the nonpurified diet, a number of Gram-positive rods were observed near the surface of the
keratinized stratified squamous epithelium of the forestomach (Fig. 3). These bacteria were considered lactobacilli, because lactobacilli were the most common bacterial species in the gastric contents (Table 1). In contrast, no Gram-positive bacteria were observed in the forestomach of the mice fed the purified diet. In the glandular stomach, no Gram-positive bacteria were observed in either dietary group (data not shown). Hematoxylin and eosin staining revealed no overt differences in the tissue structure of the forestomach as between the mice fed the nonpurified and purified diets (Fig. 3). Specifically, we observed no infiltration of inflammatory cells in the tissues.

TLR mRNA levels in the gastric tissues were measured by RT-qPCR. The TLR2 mRNA levels were significantly higher in the mice fed the purified diet than in those fed the nonpurified diet (Fig. 4). The TLR9 mRNA levels were the same between mice fed the nonpurified and purified diets.

**Discussion**

Lactobacilli are indigenous to the stomachs of experimental rodents.\(^5\)\(^-\)\(^9\) The ability of lactobacilli to grow in acidic conditions\(^5\) is thought to be responsible for its capacity to colonize the stomach. However, their number is drastically decreased by feeding a purified diet.\(^3\)\(^,\)\(^10\) The present study investigated gastric microbiota in mice fed nonpurified and purified diets using conventional cultivation and culture-independent molecular biological analyses. In line with our previous study,\(^3\) the number of culturable total anaerobes was 100-fold higher in the mice fed the nonpurified diet than in those fed the purified diet. In addition, nearly all the anaerobes were considered to be lactobacilli. PCR-DGGE based on the 16S-rRNA gene indicated differences in the compositions of gastric microbiota in the mice fed the nonpurified and purified diets. Histological examination revealed clumps of Gram-positive rods near the keratinized stratified squamous epithelium in the forestomachs of the mice fed the nonpurified diet, but not the purified diet. In sum, the present study confirms that mice fed a nonpurified diet have higher populations of microbiota, among which lactobacilli are predominant, colonizing the forestomach as compared to mice fed a purified diet.

PCR-DGGE analysis in the present study suggested that there are differences between the composition of microbiota in the gastric contents and that associated with the gastric tissue. In the mice fed the nonpurified diet, the number of DGGE bands was lower in the samples associated with the gastric tissue than in the gastric contents, suggesting that diversity is lower in microbiota associated with the gastric tissue than in those in the contents. These results are in line with previous findings for the intestine.\(^20\) For instance, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of Enterobacteriacea, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* have all been found in feces, whereas only *Clostridium*, *Lactobacillus*, and *Enterococcus* have been found in the mucus layer and epithelial crypts of the small intestine.\(^21\) Thus many bacterial species present in the gastric and intestinal lumen appear not to access the mucus layer or to attach to epithelial cells.

TLRs play a critical role in the innate immune response, and TLR2 recognizes peptidoglycan and lipoteichoic acid, components of the cell wall of Gram-positive bacteria, including *Lactobacillus* species.\(^11\) Although TLR2 has been reported to be expressed in the middle and distal colon, only sparse expression was observed in the stomach.\(^22\) In the present study, we postulated that different populations of lactobacilli in the stomach of mice fed the nonpurified and purified diets differentially influence TLR2 expression in the stomach. We expected that the mice fed the purified diet would show lower expression levels of TLR2 mRNA in the stomach, because administration of *Lactobacillus* strains has been reported to upregulate the expression of TLR2 in the mouse intestine.\(^23\)\(^,\)\(^24\) Contrary to our expectations, however, the TLR2 mRNA levels in the stomach were higher in the mice fed the purified diet than in those fed the nonpurified one. TLR9 mRNA expression, which recognizes bacterial DNA, was unaffected by diet. Based on these results, it is speculated that lactobacilli in the stomach influence the expression of the TLR2 gene differently from those in the intestine. Alternatively, the effect of exogenously

![Fig. 3. Histological Sections of Forestomach Tissue of BALB/c Mice Fed the Nonpurified and the Purified Diet.](image)

![Fig. 4. Relative mRNA Levels of TLR2 and TLR9 in the Gastric Tissue of BALB/c Mice Fed the Nonpurified and the Purified Diet.](image)
administered lactobacilli on the expression of the TLR2 gene might be different from that of indigenous lactobacilli. Therefore, whether administration of *Lactobacillus* strains influences expression of TLR2 mRNA in the stomach remains to be determined.

It remains to be elucidated how the purified diet reduces the number of lactobacilli colonizing the mouse stomach. *Lactobacillus* species generally demonstrate increased sensitivity at pH values <3; although differences exist between species and specific strains. However, we have found that the pH values of the gastric contents in mice fed nonpurified and purified diets are 4.3 and 4.0 respectively. Thus the possibility that gastric content pH is associated with bacterial growth should be excluded. Brockett and Tannock proposed that dietary fatty acid composition is associated with the number of tissue-associated lactobacilli in the stomachs of mice. Recently, we observed a negative correlation between gastric emptying and the number of tissue-associated lactobacilli in *Lactobacillus* strains influences expression of TLR2 mRNA.

However, we have found that the pH values of the stomachs of mice fed a purified diet allows viscous fiber. We are now investigating the possibility that lower gastric emptying allows lactobacilli to colonize the stomach.

Even though there were significant differences between colonization by lactobacilli and the expression of TLR2 mRNA in mice fed the unpurified and purified diets, we observed no overt differences in tissue structure or inflammatory cell infiltration of the forestomach between the two groups. Indigenous gut microbiota contribute to resistance against infectious agents, but the possibility exists that a reduced lactobacilli population in the stomachs of the mice fed the purified diet led to insufficient host resistance. Indeed, our previous study found that feeding a purified diet allows *C. albicans*, an opportunistic fungal pathogen, to colonize the guts of mice via reductions in the lactobacillus population. In addition, TLR2 is involved in sensing phospholipomannan, a cell-wall component of *C. albicans*. However, activation signals mediated by TLR2 are not always beneficial to the host, and TLR2 activation can be used by *C. albicans* to promote more severe infections. Hence we speculate that increased sensitivity at pH values <3, although differences exist between species and specific strains, is associated with the number of tissue-associated lactobacilli in the stomach.

The purified diet, which is made of refined ingredients, is widely used in nutritional studies and is quite different from the human diet which contains a wide variety of ingredients. The present study suggests caution in using a purified diet under certain conditions.

**References**