1H NMR-based Metabonomic Assessment of Probiotic Effects in a Colitis Mouse Model

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Metabolic profiling of the fecal extracts of male mice was carried out to assess the effects of probiotics on colonic inflammation using 1H NMR spectroscopy coupled with multivariate data analysis. The control group (n = 5) was administered phosphate buffered saline for 14 days. Acute colitis was induced with dextran sulfate sodium (DSS) for 7 days following administration of phosphate buffered saline for 7 days (DSS-treated group, n = 5). LAB + DSS-treated group (n = 5) was administered lactic acid bacteria (LAB) daily for 7 days followed by treatment with DSS for 7 days to investigate protective effect of LAB against DSS-inducible colitis. Histological damage, myeloperoxidase activity, and malondialdehyde content of colon tissue were reduced, whereas colon length increased in LAB + DSS-treated mice compared to those in DSS-treated mice. DSS treatment was associated with fecal excretion of amino acids, short chain fatty acids, and nucleotides, revealing significant decreases of threonine, alanine, glutamate, glutamine, aspartate, lysine, glycine, butyrate, uracil, and hypoxanthine together with increases of monosaccharides, glucose, and trimethylamine in the feces of mice with DSS-induced colitis. Increased levels of acetate, butyrate, and glucose and decreased levels of trimethylamine were found in the feces of LAB + DSS-treated mice compared to DSS-treated mice alone. The increased short chain fatty acids levels in the feces of mice fed with LAB indicate that the probiotics have protective effects against DSS-induced colitis via modulation of the gut microbiota. This work highlights the possibility for alternative approach of metabonomics in feces for assessing the probiotic effect in an animal model of inflammatory bowel disease.

Key words: Probiotics, Colitis, Dextran sulfate sodium, Lactic acid bacteria, NMR, Metabonomics

INTRODUCTION

Crohn’s disease (CD) and ulcerative colitis (UC) are the major types of inflammatory bowel disease (IBD) in the gastrointestinal tract. In particular, UC is restricted to the colon and affects only the mucosa of the colon (Stenson, 1995). Dextran sulfate sodium (DSS) is often used to induce colitis models because DSS treatment produces symptoms similar to those found in human ulcerative colitis (Gaudio et al., 1999). Dietary supplements, including probiotics and prebiotics, have been reported to ameliorate chronic intestinal inflammation in DSS-induced colitis (Herias et al., 2005; Osman et al., 2006; Larrosa et al., 2009; Lee et al., 2009). To date, the dietary effects of probiotic intake have typically been assessed based on histology, lipid peroxidation and myeloperoxidase activity in the colonic mucosa or tissues obtained from animal colitis models. Perturbations in intestinal bacterial flora, or
dysbiosis, are also indicative of the effects of dietary treatment on colitis. Dysbiosis is also observed in IBD patients and the intestinal bacteria are now believed to be involved in the initiation and perpetuation of IBD (Swidsinski et al., 2002; Seksik et al., 2003).

Recently, metabonomic characterization of IBD through metabolic profiling of fecal extracts revealed the potential to generate novel noninvasive diagnostics for IBD and to understand the disease mechanism (Marchesi et al., 2007). Bertini et al. (2009) characterized the metabolic signature in the sera of celiac disease patients and investigated the effect of gluten-free diet on this disease. These authors also showed alterations in urinary metabolites, indicating changes in the gut microbiota. Metabonomics broadly aims to measure the global, dynamic metabolic response of living systems to biological stimuli or genetic manipulation and thus simply represents the recognition and measurement of the entire metabolic reaction of an organism in response to an internal or external influence (Nicholson et al., 1999; Nicholson and Lindon, 2008). Metabonomics, especially based on nuclear magnetic resonance (NMR) spectroscopy, has the potential to identify biomarkers and prognostic factors and to enhance clinical diagnosis. It has been successfully applied to the study of human diseases, toxicology, microbes, nutrition and plant biology in the past few decades (Bjerrum et al., 2008). In particular, top-down multivariate analysis of metabolic profiles revealed a significant association of specific metabolotypes with the resident microbiome in a germ-free mouse model colonized by flora from a human infant (Martin et al., 2007) and housed in a well-ventilated room at temperatures of 25 ± 2°C and humidity of 50 ± 10% with a 12 h light/12 h dark cycle. During acclimatization for 7 days, animals were fed standard laboratory diet (Samyang) in individual standard stainless steel cages. Water and food were supplied ad libitum. All procedures relating to animals and their care conformed to the international guidelines outlined in ‘Principles of Laboratory Animal Care’ (NIH publication no. 85-23 revised 1985 and Kyung Hee University 2006).

Colitis was induced by administration of 2.5% dextran sulfate sodium (DSS, MW 40,000-50,000, United States Biochemical Corp.) in the drinking water for 7 days, following administration of phosphate buffered saline (PBS) for 7 days, according to the method of Fukata et al. (2006) and these mice were assigned to the DSS-treated group (n = 5). The control group (n = 5) was administered PBS for 14 days, while the LAB (lactic acid bacteria) + DSS-treated group (n = 5) was administered 2.5% DSS for 7 days following daily oral administration of LAB (3 × 10^9 cfu/mL, mixture of Lactobacillus brevis HY7401, Lactobacillus sp. HY7801 and Bifidobacterium longum HY8004 in PBS) for 7 days. All animals were sacrificed on day 22 by cervical dislocation. The mouse colons were removed, and lengths of the colons were determined. The colon tissues were frozen in liquid nitrogen and then stored at −80°C until needed. The colon tissues were fixed with 10% neutral buffered formalin and embedded in paraffin. Sections (3 µm thick) were stained with hematoxylin and eosin (H&E) to reveal the structural features and were then examined under a light microscope. Tissue inflammation was assessed by a pathologist using the method of crypt scoring in a double-blinded fashion, as described by Cho et al. (2007) and graded as follows: Grade 0, a few inflammatory cells; Grade 1, mild inflammation of the lamina propria and submucosa; Grade 2, severe inflammation of both the lamina propria and submucosa; Grade 3, severe inflammation of the entire wall of the colon; and Grade 4, more severe inflammation of the entire wall of the colon.

**Fecal sample collection**

Fecal samples were collected directly from the large intestine after sacrifice on day 22 and were immediately transferred into Eppendorf tubes and kept at −80°C. Feces (300 - 400 mg) were mashed in sterile phosphate buffer (5 mL, 100 mM, pH 7.4) and centrifuged at 10,000 g at 4°C for 30 min; 800 - 900 µL of the supernatant was then transferred into a new Eppendorf tube and stored at −80°C until NMR analysis.

**MATERIALS AND METHODS**

**Animals and Treatment**

Fifteen male ICR mice (age, 4 weeks; weight, 20-22 g) were obtained from Central Laboratory Animal Inc. and housed in a well-ventilated room at temperatures of 25 ± 2°C and humidity of 50 ± 10% with a 12 h light/12 h dark cycle. During acclimatization for 7 days, animals were fed standard laboratory diet (Samyang) in individual standard stainless steel cages. Water and food were supplied ad libitum. All procedures relating to animals and their care conformed to the international guidelines outlined in ‘Principles of Laboratory Animal Care’ (NIH publication no. 85-23 revised 1985 and Kyung Hee University 2006).
Myeloperoxidase activity
To determine the myeloperoxidase (MPO) content in colonic mucosa, colonic segments were homogenized in 1 mL phosphate buffer (10 mM, pH 7.0) containing 0.5% hexadecyl trimethyl ammonium bromide and then centrifuged at 14,000 g and 4°C for 10 min. The supernatant (50 µL) was added to a reaction mixture consisting of 1.6 mM tetramethyl benzidine and 0.1 mM H₂O₂, and incubated at 37 °C for 10 min. The MPO activity was calculated by spectrophotometrically time-scanned monitoring at 450 nm. MPO (Sigma Chemical Co.) was used as a standard and values are expressed as MPO units/mg tissue.

Malondialdehyde content
Malondialdehyde (MDA) concentrations in colonic tissues were determined as an index of lipid peroxidation using Ohkawa’s method (Ohkawa et al., 1979). Colonic segments were homogenized in 1.5% KCl solution. The homogenate (100 µL) was added to a mixture of 8.1% SDS (200 µL), 20% acetic acid (1500 µL, pH 3.5), 0.8% thiobarbituric acid (1500 µL), and distilled water (700 µL), boiled for 1 h at 95°C and then centrifuged at 14,000 g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 532 nm. The MDA concentration was expressed as µmol/mg tissue.

¹H NMR spectroscopic analysis of fecal extracts
Before spectral acquisition by NMR spectrometry, the fecal supernatant was defrosted and 640 µL of the supernatant was mixed with 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (60 µL, DSS, 97%) dissolved in deuterium oxide (D₂O, 99.9%). The mixture was then centrifuged at 10,000 g for 10 min. The supernatants (600 µL) were transferred into 5-mm NMR tubes. D₂O and DSS provided a field frequency lock and chemical shift reference (¹H, δ 0.00), respectively. ¹H NMR spectra were acquired on a Varian Inova-600 MHz NMR spectrometer (Varian Inc.) operating at 599.84 MHz ¹H frequency and a temperature of 298 K, using a triple resonance 5-mm HCN salt tolerant cold probe. A NOESYPRESAT pulse sequence was applied to suppress the residual water signal. For each sample, a total of 32 transients were collected into 64 K data points using a spectral width of 9615.4 Hz with a relaxation delay of 2.0 s, an acquisition time of 4.00 s, and a mixing time of 400 ms. A 0.3 Hz line-broadening function was applied to all spectra prior to Fourier Transformation (FT). Signal assignment for representative samples was facilitated by acquisition of two-dimensional (2D) total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum correlation (HSQC), spiking experiments and comparisons to literature (Saric et al., 2008a, 2008b).

NMR data preprocessing and multivariate data analysis
All NMR spectra were phased manually and baseline corrected using Vnmrj software 2.1B (Varian Inc.) and then converted to ASCII format. The ASCII format files were imported into MATLAB (R2008a, Mathworks, Inc., 2008).Probabilistic quotient normalization of the spectra using the median spectrum to estimate the most probable quotient was carried out (Dieterle et al., 2006) and the spectra were aligned by the recursive segment-wise peak alignment (RSPA) method (Veselkov et al., 2009) to reduce variability in the peak positions. The residual water resonance signal (δ 4.70 - 4.90) and the spectra region (δ -1.00 - 0.50) were removed prior to the normalization and spectra alignment. In addition, the regions for residual ethanol (δ 1.15 - 1.20 and δ 3.62 - 3.68) were removed. The resulting datasets with full resolution including 40,725 variables were then imported into SIMCA-P version 12.0 (Umetrics) for multivariate statistical analysis. All imported data were mean centered for the multivariate analysis. Principal components analysis (PCA), a supervised pattern recognition method, was first employed to visualize the global variance of the data sets and find out outliers. To maximize the separation between samples, orthogonal projections to latent structures or orthogonal partial least-squares discriminant analysis (OPLS-DA) model was applied to maximize covariance between the measured data (X variable, peak intensities in NMR spectra) and the response variable (Y variable, predictive classifications), and simultaneously to remove non-correlated variation in X variables to Y variables or variability in X that is orthogonal to Y (Trygg and Wold, 2002; Trygg et al., 2007). Hotelling’s T² region (Hotelling, 1931), shown as an ellipse in the scores plot, defines the 95% confidence interval of the modeled variation. The quality of the models was described by R² and Q² values. R² is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and Q² is defined as the proportion of variance in the data predictable by the model, and indicates predictability (Mahadevan et al., 2008). In addition, to validate the model permutation tests where the Y variable was permuted randomly 20 and 200 times and PLS-DA models were created between the metabolites data and the permuted Y variables, highlighting metabolites having stronger correlation to the original Y variables compared to permuted Y variables.
Statistical analysis

All data, including the normalized relative intensities of 1H NMR spectra, are presented as mean ± S.D. Significance in differences of metabolites between groups were evaluated for individual values using the Aspin-Welch t-test in Excel.

RESULTS

Histological Features and Colonic Inflammation

Fig. 1 shows the changes in the histological features of mucosal cells, colonic inflammation, colon length, and lipid peroxidation in colonic tissues from control, DSS-treated, and LAB + DSS-treated groups. Compared to control mice, LAB + DSS administration resulted in less cellular damage than DSS-treatment alone as shown from histological measurements (Fig. 1A), reduced inflammatory scores (Fig. 1B), reduced myeloperoxidase (MPO) activity (Fig. 1C), and reduced malondialdehyde (MDA) content (Fig. 1D). Moreover, DSS-treatment alone resulted in significantly decreased colonic length compared to control and DSS + LAB-treated mice (Fig. 1E).

Fig. 2 shows representative one-dimensional (1D) 1H NMR spectra of fecal extracts obtained from control (A), DSS-treated (B), and LAB + DSS-treated (C) mice. Spectra of fecal extracts were dominated by a number of metabolites, such as butyrate, propionate, leucine, isoleucine, valine, lactate, threonine, alanine, lysine, acetate, glutamate, glutamine, succinate, trimethylamine, aspartate, ethanol, methanol, glycine, methionine, glucose, xylose, monosaccharides, galactose, dextran, uracil, tyrosine, hypoxanthine, phenylalanine...
and formate. In particular, ethanol peaks were observed in all fecal extracts due to inadequate removal of ethanol from surgical instruments sterilized with alcohol during direct feces removal from the large intestine after sacrifice. Large variations in residual ethanol were observed across the $^1$H NMR spectra of all samples and thus ethanol peaks were removed prior to multivariate statistical modeling. In general, $^1$H NMR ethanol peaks are never found in fecal extracts of mice, rats and human (Saric et al., 2008a).

OPLS-DA score plot revealed more clear discrimination among the group than PCA score plot (Fig. 3), demonstrating that spectral noise and systematic uncorrelated variation to classifiers or class identity, e.g., control, DSS and LAB + DSS, in PCA, an unsupervised pattern recognition technique, model led to less discrimination, whereas OPLS-DA, a supervised algorithm, removed effectively the noise and uncorrelated variation (Trygg and Wold, 2002). Two NMR spectra from each one fecal extract of control and DSS-treated groups were excluded for the multivariate data analysis due to large dilution from small amounts of feces.

**Variations in fecal metabolites of DSS-treated and LAB-treated mice**

The OPLS-DA score plot showed clear differentiation between the DSS-treated and control groups (Fig. 4A), demonstrating high goodness of fit but low predictability with a $R^2_Y$ value of 0.883 and a $Q^2$ of 0.358, respectively. To test the validity of the OPLS-DA model, we performed permutation test with 20 and 200 random permutations in a PLS-DA model with same number of components of the OPLS-DA

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**Fig. 2.** Representative 600 MHz $^1$H NMR spectra obtained from fecal extracts of control (A), DSS-induced colitis (B), and LAB + DSS-treated (C) mice. LAB administration was followed by DSS-induced colitis. Key: 1, butyrate; 2, propionate; 3, leucine; 4, isoleucine; 5, valine; 6, lactate; 7, alanine; 8, lysine; 9, acetate; 10, glutamate; 11, glutamine; 12, succinate; 13, trimethylamine; 14, aspartate; 15, ethanol; 16, methanol; 17, glycine; 18, β-glucose; 19, α-glucose; 20, xylose; 21, an unidentified monosaccharide; 22, galactose; 23, dextran; 24, uracil; 25, tyrosine; 26, hypoxanthine; 27, phenylalanine; 28, formate; 29, threonine; 30, methionine. DSS represents sodium 2,2-dimethyl-2-silapentane-5-sulfonate used as chemical shift reference. Number 21 was tentatively assigned to a monosaccharide. U1 and U2 are unknown compounds.
When the PLS components were 2, the model was valid which resulted in the extrapolated intercept values ($Q^2_{\text{inter}}$) of $-0.062$ (20 permutations) and $-0.023$ (200 permutations) (Fig. 4G and Table II). In general, statistical models are considered as valid significantly when the corresponding $Q^2_{\text{inter}}$ value is negative (Mahadevan et al., 2008), avoiding an overfitting of the models. The number of components could be determined in the model validation. In the present study, all models were therefore valid with 2 components in PLS-DA models and 1-predictive and 1-orthogonal components in OPLS-DA models.

To identify the metabolites responsible for the differentiation in the OPLS-DA score plot, an OPLS-DA loading plot was generated (Fig. 4D). The upper section in the loading plot represents metabolites elevated in the DSS group and the lower section represents metabolites decreased in the DSS group. Fecal extracts of the DSS-treated group were characterized by lower levels of butyrate.

### Table I. Metabolic effects of DSS-induced colitis and probiotic-administration on mouse feces

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift (multiplicity)</th>
<th>DSS-induced colitis vs control</th>
<th>LAB+DSS treatments vs DSS-induced colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>butyrate</td>
<td>0.89 (t)</td>
<td>↓ 0.24</td>
<td>↑ 0.09</td>
</tr>
<tr>
<td></td>
<td>1.55 (m)</td>
<td>↓ 0.20</td>
<td>↑ 0.08</td>
</tr>
<tr>
<td></td>
<td>2.16 (t)</td>
<td>↓ 0.21</td>
<td>↑ 0.09</td>
</tr>
<tr>
<td>isoleucine</td>
<td>0.94 (t)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>valine</td>
<td>0.98 (d)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>leucine</td>
<td>0.96 (t)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>propionate</td>
<td>1.05 (t)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2.19 (q)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>acetate</td>
<td>1.92 (s)</td>
<td>-</td>
<td>↑ 0.04</td>
</tr>
<tr>
<td>lactate</td>
<td>1.32 (d)</td>
<td>↓ 0.09</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4.11 (m)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>threonine</td>
<td>3.58 (d)</td>
<td>↓ 0.05</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4.21 (m)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>alanine</td>
<td>1.48 (d)</td>
<td>↓ 0.06</td>
<td>-</td>
</tr>
<tr>
<td>glutamate</td>
<td>2.35 (m)</td>
<td>↓ 0.03</td>
<td>-</td>
</tr>
<tr>
<td>glutamine</td>
<td>2.49 (m)</td>
<td>↓ 0.008</td>
<td>↑ 0.08</td>
</tr>
<tr>
<td>succinate</td>
<td>2.39 (s)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>aspartate</td>
<td>2.82 (d)</td>
<td>↓ 0.007</td>
<td>-</td>
</tr>
<tr>
<td>trimethylamine</td>
<td>2.88 (s)</td>
<td>↑ 0.006</td>
<td>↓ 0.03</td>
</tr>
<tr>
<td>lysine</td>
<td>3.05 (m)</td>
<td>↓ 0.02</td>
<td>-</td>
</tr>
<tr>
<td>glycine</td>
<td>3.56 (s)</td>
<td>↓ 0.06</td>
<td>↑ 0.10</td>
</tr>
<tr>
<td>monosaccharide</td>
<td>4.51 (d)</td>
<td>↑ 0.02</td>
<td>-</td>
</tr>
<tr>
<td>β-glucose</td>
<td>4.64 (d)</td>
<td>↑ 0.29</td>
<td>-</td>
</tr>
<tr>
<td>xlyose</td>
<td>5.19 (d)</td>
<td>↑ 0.11</td>
<td>-</td>
</tr>
<tr>
<td>α-glucose</td>
<td>5.24 (d)</td>
<td>↑ 0.06</td>
<td>-</td>
</tr>
<tr>
<td>dextran</td>
<td>4.40 (m)</td>
<td>↑ 0.01</td>
<td>↓ 0.004</td>
</tr>
<tr>
<td></td>
<td>5.30 (m)</td>
<td>↑ 0.0001</td>
<td>↓ 0.0005</td>
</tr>
<tr>
<td>uracil</td>
<td>5.80 (d)</td>
<td>↓ 0.02</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>7.53 (d)</td>
<td>↓ 0.01</td>
<td>-</td>
</tr>
<tr>
<td>hypoxanthine</td>
<td>8.19 (d)</td>
<td>↓ 0.07</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\)The arrows (↓ and ↑) represent a decrease or increase in metabolite levels in DSS-induced colitis compared to control and in LAB + DSS treatments compared to DSS-induced colitis. The levels were estimated from relative intensities of $^1$H NMR spectra of fecal extracts following spectral normalization. Metabolites above $p = 0.50$ are not indicated.

\(^b\)Indicates no change in the level.

\(^c\)Peaks corresponding to the methyl protons of lactate and threonine overlapped at 1.32 ppm; s, singlet; d, doublet; q, quartet; t, triplet, m, multiplets
Fig. 4. OPLS-DA scores (A-C) and loading (D-F) plots derived from the $^1$H NMR spectra of fecal extracts obtained from control, DSS-induced colitis (DSS), and LAB + DSS-treated groups. See Fig. 2 for the metabolite key. $R^2_Y = 90.8\%, Q^2 = 52.6\%$ between control and DSS groups; $R^2_Y = 87.4\%, Q^2 = 73.9\%$ between the control and LAB+DSS groups; $R^2_Y = 77.5\%, Q^2 = 36.1\%$ between the DSS and LAB + DSS groups. The permutation tests were carried out with 200 random permutations in PLS-DA models with same number of components of the OPLS-DA models (G, H, and I). Two components were used for permutation test of PLS model and 1 predictive and 1 orthogonal component were used for OPLS-DA model.
isoleucine, valine, lactate, threonine, alanine, glutamate, glutamine, aspartate, lysine, methanol, glycine, methionine, uracil, hypoxanthine, formate, and an unknown compound (U2), and higher levels of leucine, propionate, trimethylamine, α- and β-glucose, monosaccharides, dextran, xylose, phenylalanine, and an unknown compound (U1) compared to those of the control group.

Comparison of the control and LAB + DSS-treated groups using OPLS-DA also allowed clear differentiation of these two groups with high goodness of fit ($R^2_X = 0.821$) and low predictability ($Q^2 = 0.484$) (Fig. 4B). In the complementary loading plot (Fig. 4E), higher levels of propionate, acetate, trimethylamine, glucose, xylose, galactose, monosaccharides, dextran, an unknown compound (U1) and phenylalanine were observed in the LAB + DSS-treated group compared to the control group. In addition, levels of amino acids (leucine, isoleucine, valine, alanine, threonine, glutamate, lysine, methionine, tyrosine and glycine), organic acids (aspartate, formate and succinate), and nucleotides (uracil and hypoxanthine) were decreased in the LAB + DSS-treated group compared to the control group.

To investigate the effect of probiotics on DSS-induced colitis, we compared the metabolic differences in the fecal extracts of DSS- and LAB + DSS-treated mice and clear discrimination between the mice was observed in the OPLS-DA score plot (Fig. 4C). As shown in the OPLS-DA loading plot, the LAB + DSS-treated group was characterized by elevated levels of short-chain fatty acids (SCFAs, butyrate, acetate, and propionate), amino acids (isoleucine, valine, alanine, lysine, and tyrosine), nucleotides (uracil and hypoxanthine), carbohydrates (α- and β-glucose, xylose, and monosaccharides) and lactate, while levels of succinate, trimethylamine, and dextran were increased in the DSS-treated group (Fig. 4F).

As shown in Fig. 4, the differentiations between the groups were clear but predictabilities of the models were below 0.5, indicating poor predictabilities. Therefore, it is important to investigate the significances of variations in the metabolites responsible for the differentiations in the score plots because the metabolites could attribute to the differentiations even though their variations were not significant. Significant variations in metabolites between control and DSS-treated mice and between DSS-treated and LAB + DSS-treated mice are summarized in Table I. The significant metabolites were much more between control and DSS groups than between DSS and LAB+DSS groups, resulting in higher predictability of 0.484 than that of 0.096, respectively, in the OPLS-DA models. As a result, it was likely that significances of the metabolites are responsible for the predictability of the model. Although all OPLS-DA models had a low predictability being below 0.5 in the present study, we assessed the probiotic effect against colitis through the significant variations of individual metabolites.

**DISCUSSION**

Daily oral administration of LAB had a protective effect against acute colitis induced by DSS, as characterized by reductions in epithelial and submucosal cell damage, MPO activity, and MDA, as well as protection against changes in colon length. These results are consistent with many studies on probiotic effects in DSS-induced ulcerative colitis (UC) animal models (Sukumar et al., 1998; Osman et al., 2006, 2007, 2008; Yoon et al., 2008). To gain insight into the protective effect of LAB against inflammatory bowel disease, we investigated variations in fecal metabolites of LAB + DSS-treated mice through global profiling with high resolution $^1$H NMR spectroscopy and multivariate statistical analysis.

<table>
<thead>
<tr>
<th>PLS components</th>
<th>$R^2_X$</th>
<th>$R^2_Y$</th>
<th>$Q^2$</th>
<th>Extrapolated intercepts in permuted validate model$^a$</th>
<th>$R^2_{\text{inter}}$</th>
<th>$Q^2_{\text{inter}}$ $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.270</td>
<td>0.730</td>
<td>0.140</td>
<td>$0.509 (0.493)$</td>
<td>0.123 (0.063)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.562</td>
<td>0.883</td>
<td>0.178</td>
<td>$0.758 (0.718)$</td>
<td>-0.062 (-0.023)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.729</td>
<td>0.967</td>
<td>0.388</td>
<td>$0.918 (0.93)$</td>
<td>0.054 (0.030)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.897</td>
<td>0.979</td>
<td>0.460</td>
<td>$0.985 (0.981)$</td>
<td>0.382 (0.203)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.953</td>
<td>0.999</td>
<td>0.940</td>
<td>$0.997 (0.995)$</td>
<td>0.849 (0.618)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$The values with in baskets were calculated with 200 permutations while others with 20 permutations.

$^b$When the extrapolated intercepts of $Q^2$ in permuted validate model were negative, the models were recognized to be valid.
**Short chain fatty acids**

Short chain fatty acids (SCFAs) such as acetate, butyrate, and propionate are depleted in the feces of patients with inflammatory bowel diseases (IBD) including Crohn’s disease (CD) and ulcerative colitis (UC) (Marchesi et al., 2007). These SCFAs are produced from fermentation of carbohydrates, such as cellulose, fiber, and starches, by the gut bacteria and are an important energy source for the colonic epithelium and the host, providing an estimated 5-15% of energy requirements in humans and promoting cellular proliferation and differentiation (Bergman, 1990; Topping and Clifton, 2001; Hooper et al., 2002; Scheppach and Weiler, 2004). SCFAs also have immunomodulatory effects, suppressing inflammatory cytokine secretion in cultured epithelial cells and ameliorating colitis in mice model. In particular, luminal provision of butyrate has been considered an appropriate means to improve wound healing after intestinal surgery and to ameliorate IBD symptoms (Wachtershauser and Stein, 2000). In the present study, elevated levels of acetate and butyrate in the feces of mice fed LAB indicate that probiotics could attribute to protection against intestinal inflammatory disorders through modulation of gut microbiota, because butyrate is produced by intestinal bacteria such as *Clostridium*, *Eubacterium* and *Fusobacterium* during fermentation of nondigestable carbohydrates and responsible for the immunomodulatory effects in cultured epithelial cells and in colitis mice model (Salyers, 1995; Wachtershauser and Stein, 2000).

**Amino acids**

In general, a disruption or dysbiosis of the normal gut bacterial ecology causes malabsorption due to inflammation, resulting in higher amounts of amino acids in the feces of patients with CD and UC compared to control patients (Marchesi et al., 2007). Perturbations in fecal amino acids have also been observed after vancomycin treatment, and the reduced bacterial population following vancomycin treatment is thought to cause the lower fecal concentrations of amino acids (Yap et al., 2008). On the other hand, derivation of amino acids from the bacteria themselves during fecal sample preparation such as sonication might contribute to the increased levels of amino acids before vancomycin treatment (Yap et al., 2008). Saric et al. (2008a) reported that during fecal extraction, sonication contributed to increases in amino acids, uracil and glucose, as well as decreases in SCFAs, demonstrating that sonication disrupts the bacterial cells and thus affects the levels of amino acids extracted from feces. However, we did not sonicate the feces and thus, the relative lower levels of several amino acids including alanine, threonine, glutamine, glutamate, lysine, glycine, and aspartate in the feces of DSS-treated mice compared to those of control mice are likely due to decreases in dietary protein degradation or amino acid biosynthesis caused by reduced bacterial populations in the feces of mice with DSS-treatment. These findings are in good agreement with observation of significant decreases in the population of fecal microflora in the *Lactobacillus acidophilus* group, *Bifidobacterium* species group and *Bacteroides-Prevotella-Porphyromonas* group in mice with DSS-induced colitis compared to control group (Nanda Kumar et al., 2008). However, in the present study, no significant differences in amino acids in mice fed LAB indicate that there was no evidence of changes in the fecal microbial population in mice fed LAB, may resulting from the insufficient LAB amount fed to these mice.

**Nucleotides in feces**

Uracil and hypoxanthine are pyrimidine and purine derivatives, respectively (Wasternack, 1980). The glycopeptide antibiotic, vancomycin, results in gut microbiota modification of mice and low levels of uracil are found in fecal extracts of vancomycin-treated mice (Yap et al., 2008). In particular, although there was no study on the relationship between bacterial population and hypoxanthine levels, it is likely that hypoxanthine levels can be served as an indicator of the freshness of food, because enzymatic conversion of nucleotides into inosine and hypoxanthine increase with increasing bacterial populations during food storage (Chang et al., 1998; Ozogul et al., 2006). In the present study, decreased levels of uracil and hypoxanthine in the feces of DSS-treated mice compared to control mice indicated the reduction in fecal microfloral population, consistent with the decreased fecal amino acid levels in DSS-treated mice. Relatively lower levels of glycine in feces of DSS-treated mice may also account for the effect of DSS-induced colitis on gut microbiota and could be associated with nucleotide metabolites such as uracil and hypoxanthine, because glycine is produced from purine metabolism by degradation of hypoxanthine (Abrams et al., 1948). Increased and decreased levels of trimethylamine in fecal extracts of mice with colitis and mice with administered LAB, respectively, also indicates disruption of the normal gut bacterial ecology in colitis.
because trimethylamine is produced by intestinal degradation of food components such as choline and carnitine by gut bacterial species (Smith et al., 1994; Seibel and Walsh, 2002).

By profiling fecal metabolites to assess IBD and to study the effects of probiotics in a chemicals-induced IBD animal model, we have highlighted potential of a metabonomics approach. Although pattern recognition models showed low the predictabilities accompanied by significant changes in few fecal metabolites, we found that probiotic treatment resulted in changes of SCFAs and trimethylamine metabolism through relative comparisons of individual intensities of the fecal metabolites, thereby demonstrating that probiotics administration provides a possibility to prevent IBD through modification of gut bacterial ecology. We suggest metabolic profiling as an alternative approach to assess the effects of probiotics in IBD models, especially when with more population of probiotic microorganism or longer treatment of the probiotics that gave more significant effect of probiotics than the present result, according to our unpublished data.

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