**Lactobacillus acidophilus La5 and Bifidobacterium lactis Bb12 Induce Different Age-Related Metabolic Profiles Revealed by \(^1\text{H}-\text{NMR} \) Spectroscopy in Urine and Feces of Mice\(^ 1\text{-}^3\)**

Elisa Brasili,\(^ 4\) Elena Mengheri,\(^ 4\)* Alberto Tomassini,\(^ 5\) Giorgio Capuani,\(^ 5\) Marianna Roselli,\(^ 4\) Alberto Finamore,\(^ 4\) Fabio Sciubba,\(^ 5\) Federico Marini,\(^ 5\) and Alfredo Miccheli\(^ 5,^6\)

\(^4\)National Research Institute on Food and Nutrition (INRAN), Department of Nutritional Sciences, Rome, Italy; \(^5\)Department of Chemistry, La Sapienza University, Rome, Italy; and \(^6\)Institute of Crystallography, National Research Council (CNR), Bari, Italy

**Abstract**

Age-related dysbioses of intestinal microbiota and decline in the overall metabolic homeostasis are frequently found in the elderly. Probiotic supplementation may represent a way to prevent or reduce the senescence-associated metabolic disorders. The present study evaluated the metabolic impact of *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 supplementation in relation to age by analyzing urine and feces metabolic profiles using \(^1\text{H}-\text{nuclear magnetic resonance} \) spectroscopy and multivariate analysis. Adult (3 mo old) and aged (16 mo old) mice received an oral supplementation of the 2 probiotics (1 \(\times\) \(10^9\) colony-forming units/d each) or phosphate buffered saline (control) daily for 30 d. Urine and feces were collected for 48 h before the end of the study. Partial least squares–discriminant analysis showed that the urinary discriminant metabolites for the probiotic treatment included higher dimethylglycine in adult and aged mice, lower sarcosine and nicotinate in adult mice, higher N-methyllnicotinamide in adult mice and lower N-methyllnicotinamide in aged mice compared with their controls. These results indicate a probiotic-induced modulation of homocysteine and NAD metabolism pathways, which have important implications because these pathways are involved in essential cellular processes that can be altered in senescence. The probiotic supplementation also modified the fecal metabolic profiles, inducing in both adult and aged mice higher 4-hydroxyphenylacetate and lower xylose in treated mice compared with their control mice, whereas valerate was greater in treated adult mice and lower in treated aged mice compared with their controls. The ANOVA simultaneous component analysis on urinary and fecal metabolic profiling showed an age \(\times\) treatment interaction (\(P < 0.05\)), confirming the age-related modulation of the metabolic response to probiotic supplementation. The results suggest that *L. acidophilus* and *B. lactis* may prevent or reduce age-related metabolic dysfunction. J. Nutr. 143: 1549–1557, 2013.

**Introduction**

An important role in the maintenance of health and well-being is ascribed to the functionality of the complex ecosystem constituted by the many microorganisms living in the gut and interacting with the host system. In these mutual exchanges, the microbiota prevents pathogen infection, maintains membrane barrier, improves immune response, and produces essential metabolites, whereas the host provides a nutrient-rich environment for microbial growth. The host-microbiota interaction is not confined to the gut but includes a series of metabolic axes connecting the microbiota with the gut, brain, liver, and muscle (1). In this context, probiotic bacteria, such as lactobacilli and bifidobacteria, may exert substantial health-promoting effects to the host. Yet, the mechanisms through which probiotics induce benefits are not completely understood (2–5).

The gut progressively acquires a stable microbiota composition from birth through adult life that may transiently change in response to several factors, including diet and environmental stress (6,7). An altered intestinal microbiota has been associated...
with gut diseases, including inflammatory bowel diseases and irritable bowel syndrome (8,9), as well as systemic diseases, such as obesity and type 2 diabetes (10,11). Perturbations in the microbial ecosystem occur during aging with an increase in pathogenic bacteria and a decrease in health-promoting bacteria, especially bifidobacteria (12–14). These changes are not surprising because the characteristic age-related alterations in gut functions, modification in lifestyle and nutritional behavior, metabolic dysfunctions, and immunosenescence inevitably affect the microbiota composition (15–19). However, the complex networks connecting the unbalanced microbiota with the pathological diseases of aging are not fully elucidated.

Dietary manipulation of the gut microbiota through probiotic supplementation has been suggested to improve or restore a healthy microbial community and in this way to induce several benefits (20–23). Recent studies have shown that lactobacilli can modulate several compounds of the host metabolism. For instance, altered hepatic lipid metabolism associated with lowered plasma lipoprotein concentrations was found in germ-free mice instance, altered hepatic lipid metabolism associated with lowered plasma lipoprotein concentrations was found in germ-free mice (24). Administration of Lactobacillus paracasei or Lactobacillus rhamnosus to germ-free mice induced beneficial changes in lipid profiles, gluconeogenesis, and amino acid metabolism in different host compartments (25,26). L. paracasei normalized the energy metabolism altered by Trichinella spiralis infection in mice (27). Beyond these studies, there are no data, to our knowledge, on other strains or mixtures of probiotics and whether they can modulate the metabolic profile during aging and ameliorate metabolic disorders occurring in the elderly.

In the present study, our aim was to verify whether Lactobacillus acidophilus La5 and Bifidobacterium lactis Bb12 modulate host and gut microbiota metabolism in relation to age. We performed metabolic profiling of urine and feces of adult and aged mice by 1H-NMR spectroscopy and multivariate analysis. 1H-NMR spectroscopy is a powerful technique to detect diverse microbial metabolites in feces and metabolic changes at the systemic level induced by probiotic intervention (28–31). In particular, metabolic profiles in urine result from the host-gut microbiota interaction (32), whereas fecal metabolomics provide information directly related to gut microbiota metabolism (33), thus allowing a comprehensive view of the host-microbiota metabolism.

Materials and Methods

Bacterial growth. L. acidophilus La5 and B. lactis Bb12 were provided in frozen milk aliquots (courtesy of Parmalat, Parma, Italy). Each bacterial strain was grown in De Man Rogosa Sharpe medium (DMRS) at 37°C in anaerobic conditions. After overnight incubation, bacteria were diluted 1:15 in fresh De Man Rogosa Sharpe medium and grown to mid-log phase. Bacterial concentrations were first determined by densitometry and viability was tested by CFU counts after agar plating. Bacteria were harvested by centrifugation at 3000 × g for 10 min and resuspended in PBS.

Animals and treatment. All mice were from Charles River Laboratories. Two groups of adult and aged male BALB/c mice were used. The old mice were initially maintained in the animal facility at Italian National Research Centres of Aging (INRCA, Ancona, Italy) until they were 14 mo old, then transferred to our animal rooms (INRAN) and maintained an additional 2-mo acclimation period to minimize stress-related impact on the metabolic profiles (aged group: 16 mo old). One-month-old adult mice underwent the same 2 mo of acclimation simultaneously with the aged mice (adult group: 3 mo old). All mice were fed a standard complete diet (4RF21 standard diet, Mucedola) with free access to food before and throughout the experiments. The mice were housed in an air-conditioned room at 23°C with a 12-h light-dark cycle. Body weight and food intake were recorded every other day. The groups of adult and aged mice were randomly divided into 2 subgroups (n = 10 each) receiving an oral supplementation of a mixture of L. acidophilus La5 and B. lactis Bb12 (1 × 10⁵ CFU/d each, freshly prepared; treated) or PBS (control) daily for 30 d. The bacterial suspensions or PBS (both 140 μL) were added to small amounts of diet in plastic petri dishes to ensure complete bacteria intake and to avoid any stress related to manipulation. The total ingestion of bacteria was controlled daily. The 2 probiotics were chosen because they normally reside in the human small intestine, exert beneficial effects, and are a component of commercialized yogurt (34). In addition, mixtures of lactobacilli and bifidobacteria have been suggested to be more efficient than single probiotic strains, and the probiotic mixture used in this study has been shown to induce several benefits (35–37). Feces and urine were collected by using metabolic cages for the last 48 h of the experimental period. Urine samples were collected in a solution of 0.05% sodium azide to avoid contamination and frozen at −80°C until 1H-NMR spectroscopy analysis. Overnight feces were collected between 0800 and 1000 and immediately stored at −80°C until analysis. Urine and fecal samples were not obtained from every animal because of the death of 2 aged mice (1 control, 1 treated), urine contamination by feces, or an insufficient feces amount for 1H-NMR analysis. All experimental procedures were approved by the ethics committee at the National Health Ministry, Department of Food, Nutrition, and Animal Health.

1H-NMR spectroscopy. Urine samples were centrifuged at 11,000 × g for 15 min at 4°C. Samples (400 μL) were added to 3-trimethylsilyl-propionic-2,2,3,3-d4 acid (TSP)7 in cold PBS-D2O (200 μL, 2 mmol/L final concentration) as internal standard. Fecal samples were prepared as previously described (31,33). Briefly, 320 mg of fecal samples were homogenized in 1.4 mL cold PBS-D2O and centrifuged at 11,000 × g for 15 min at 4°C. Supernatants were filtered through a cell strainer (100-μm pore size), centrifuged, and refiltered through a sterile syringe filter (0.2-μm pore size). Supernatants (600 μL) were added to TSP in PBS-D2O (60 μL, 2 mmol/L final concentration). 1H-NMR spectra of both urine and feces were acquired at 298 K by using a Bruker AVANCE 400 spectrometer (Bruker BioSpin GmbH) equipped with a magnet operating at 9.4 T and at 400.13 MHz for 1H frequency. The pulse sequence adopted for spectrum acquisition was as follows: presaturation pulse, single 90° detection pulse, acquisition, and relaxation delay. The relaxation delay was 7.5 s, whereas the presaturation pulse was applied for 2 s. The acquisition time needed to collect the 32-K points was approximately 40 s. The quantification of metabolites was made by comparing the specific signal integrals to the internal standard. The acquired NMR spectra were processed and quantified by using ACD Lab 1D-NMR Manager version 12.0 software (Advanced Chemistry Development), whereas two-dimensional NMR spectra were processed by using Bruker Top Spin version 3.1 (Bruker BioSpin GmbH). The acquired NMR spectra were manually phased, baseline corrected, and referenced to the chemical shift of the TSP methyl resonance at 1.0 ppm. The quantification of metabolites was made by comparing the specific signal integrals to the...
internal standard TSP integral. Metabolite concentrations are expressed as μmol/L for fecal samples and as μmol/mmol creatinine for urine samples, normalized to the integral of creatinine methyl group signal at 3.05 ppm.

Data and statistical analyses. The effect of the treatment on each age group was performed by partial least squares–discriminant analysis (PLS-DA), as previously described (41). Multivariate analysis was carried out by using in-house written routines operating in a Matlab R2011a environment (MathWorks). Data were mean-centered and scaled to unit variance before analysis. The PLS-DA method was applied to build models for the discrimination between groups on the basis of their different metabolic responses. Cross-model validation using 9 cancellation groups in the external and 8 in the internal loops together with permutation tests with 10,000 randomizations were used for testing the significance of the model and the relevance of supposedly important metabolites (42,43), which were identified on the basis of the values of PLS-DA regression coefficients and variable importance in projection (VIP) scores (44). VIP expresses the contribution of the individual variable in the definition of the model. Because the mean of squared VIP scores is equal to 1, values >1 are considered to be significant variables.

The comparison between treated and control groups of each age as well as between adult and aged control mice was performed by univariate unpaired Student’s t test. P values <0.05 were considered significant.

Because PLS-DA identified different metabolic profiles in adult and aged mice in relation to the probiotic treatment, to further investigate the age-dependent response to probiotic supplementation the whole data field (control and probiotic-treated adult and aged mice) was explored by ANOVA simultaneous component analysis (ASCA) on urinary and fecal 1H-NMR–based metabolic profiling according to Smilde et al. (45). Details of ASCA methods are provided in the Supplemental Data Analysis.

Results

Body weight and food consumption. After probiotic treatment, the body weight of adult and aged mice did not differ from that of their respective control mice (Supplemental Table 1). There was no difference in food intake between control and probiotic-treated adult or aged mice.

Influence of probiotic treatment on urine metabolome. Fifty-two metabolites were assigned and integrated (Supplemental Fig. 1A and Supplemental Table 2). The application of PLS-DA to the adult mouse urine data set provided complete discrimination among control and treated mice at each level of validation, the optimal complexity being 2 latent variables (LVs; \(R^2_Y = 0.75\)) explaining 22% and 21% of X-variance, respectively (Fig. 1A). Similarly, when the classification algorithm was applied to the aged mouse data set, a perfect separation between control and treated mice was achieved with three LVs (\(R^2_Y = 0.93\)), explaining 34%, 19%, and 7% of X-variance, respectively (Fig. 1B). In both cases, the significance of the multivariate classification model was assessed by comparing the validated results on the true data set with those obtained after random permutation of the class labels (permutation test). A P value of 0 for each of the figures of merit accounting for model performance (46) (i.e., number of misclassifications), area under the receiver operating characteristic curve, and discriminant Q² were obtained, indicating that the differences in metabolite concentrations induced by the treatment were significant (Supplemental Figs. 2 and 3).

The inspection of the bootstrapped VIP scores, together with cross-model validation, indicated 9 urine metabolites relevant for the discrimination between control and treated adult mice, namely as follows: N-methylnicotinamide (MNam), dimethylglycine (DMG), and choline, which were higher in probiotic-treated mice than in control mice; and sarcosine, phenylacetylglycine, nicotinate (NA), α-ketosocaprate, 2-oxo-4-methylvalerate, and 2 unassigned metabolites (U5+U6, overlapping signals), which were lower in treated mice than in control mice (Table 1). Among the discriminant metabolites, U5+U6 (P < 0.01), MNam, DMG, sarcosine, and phenylacetylglycine (P < 0.05) also showed significant variation by univariate analysis. The same approach identified 12 urine metabolites relevant to discriminate the probiotic supplementation in aged mice: DMG, butyrate, 3-hydroxyisovalerate, 2-oxo-4-methylvalerate, threonine, and 1 unassigned metabolite were greater in probiotic-supplemented mice than in control mice, whereas succinate, MNam, citrate, and 3 unassigned metabolites were lower in treated mice than in control mice (Table 2). Among the discriminant metabolites, U17 (P < 0.01), U20, and MNam (P < 0.05) also showed significant variation by univariate analysis.

The investigation by the ASCA model confirmed an age-related response to probiotic supplementation, highlighting a treatment-induced metabolic variation occurring in the same or opposite directions in adult and aged mice (Supplemental Data Analysis and Supplemental Table 3). The results of the ASCA model were in agreement with those of the PLS-DA models, both identifying DMG as the metabolite involved in the interaction of age × treatment, and showed higher values in probiotic-treated adult and aged mice compared with their controls. In addition, MNam and U5+U6 were the metabolites affected by the age ×

![Figure 1](image-url)
Analogously, when the classification algorithm was applied to explaining 22% and 39% of the X-variance, respectively (Brasili et al. 2013). From those in adult control mice (MNam and U5+U6 in aged control mice differed significantly treated aged mice compared with their controls. The amounts of treatment interaction: MNam was greater in treated adult mice and lower in treated aged mice compared with their controls, whereas U5+U6 was lower in treated adult mice and higher in treated aged mice compared with their controls. The amounts of MNam and U5+U6 in aged control mice differed significantly from those in adult control mice (P < 0.05).

**Influence of probiotic treatment on fecal metabolome.** Forty-six metabolites were assigned and integrated (Supplemental Fig. 1B and Supplemental Table 2). The application of PLS-DA to the adult mice feces data set provided complete discrimination among control and treated mice at each level of validation, the optimal complexity being 2 LVs (R^2_Y = 0.92) explaining 22% and 39% of the X-variance, respectively (Fig. 2A). Analogously, when the classification algorithm was applied to the aged mouse data set, a perfect separation between control and treated aged mice was achieved with 3 significant LVs explaining 15%, 16%, and 50% of the X-variance, respectively (Fig. 2B).

Inspection of the bootstrapped VIP scores, together with cross-model validation, indicated 9 fecal metabolites relevant for the discrimination between control and treated adult mice, namely the following: 3-hydroxybutyrate, uridine, 4-hydroxyphenylacetate (4-HPA), and valerate, which were greater in probiotic-treated mice than in control mice; and xylose, lactate, methionine, and 2 unknown metabolites, which were lower in treated mice than in control mice (Table 3). Among the discriminating metabolites, 3-hydroxybutyrate, xylose, uridine, 4-HPA, Uy (P < 0.01), and U4 (0.05 < P < 0.01) were also significant by univariate analysis. In the case of aged mice, the inspection of the bootstrapped VIP scores and cross-model

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Urine metabolites discriminating Lactobacillus acidophilus- and Bifidobacterium lactis-treated and control adult mice</th>
<th>Metabolite</th>
<th>Regression coefficient sign^2</th>
<th>VIP^3 value</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>µmol/mmol creatinine</td>
<td></td>
</tr>
<tr>
<td>U5 + U6^4</td>
<td>-</td>
<td>4.49</td>
<td>2750 ± 208</td>
<td>1970 ± 70.0^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methylnicotinamide</td>
<td>+</td>
<td>3.87</td>
<td>20 ± 2.0</td>
<td>26 ± 1.0^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>+</td>
<td>3.76</td>
<td>469 ± 27.0</td>
<td>644 ± 53.2^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcosine</td>
<td>-</td>
<td>2.87</td>
<td>716 ± 55.1</td>
<td>559 ± 30.2^7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylacetylethylamine</td>
<td>-</td>
<td>2.96</td>
<td>325 ± 28.2</td>
<td>252 ± 9.3^8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinate</td>
<td>-</td>
<td>1.89</td>
<td>30 ± 3.0</td>
<td>23 ± 2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Ketoisocaproate</td>
<td>-</td>
<td>1.61</td>
<td>3540 ± 181</td>
<td>3180 ± 110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline</td>
<td>+</td>
<td>1.34</td>
<td>3470 ± 241</td>
<td>3590 ± 134</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxo-4-methylvalerate</td>
<td>-</td>
<td>1.16</td>
<td>1310 ± 106</td>
<td>1140 ± 46.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Unless otherwise indicated, values are means ± SEMs, n = 7 (control) or 10 (treated). Letters indicate different from control as assessed by univariate unpaired Student's t test: a P < 0.01, b P < 0.05.
2 Regression coefficient signs of discriminant metabolites vs. the partial least squares-discriminant analysis model: positive regression (+) or negative regression (−) in treated mice compared with control mice.
3 Variable importance in projection.
4 Unassigned.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Urine metabolites discriminating Lactobacillus acidophilus- and Bifidobacterium lactis-treated and control aged mice</th>
<th>Metabolite</th>
<th>Regression coefficient sign^2</th>
<th>VIP^3 value</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>µmol/mmol creatinine</td>
<td></td>
</tr>
<tr>
<td>U17^1</td>
<td>-</td>
<td>4.29</td>
<td>30 ± 6.0</td>
<td>9 ± 2.0^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>+</td>
<td>3.17</td>
<td>376 ± 61.0</td>
<td>612 ± 95.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate</td>
<td>+</td>
<td>2.50</td>
<td>415 ± 49.4</td>
<td>563 ± 58.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>-</td>
<td>2.37</td>
<td>1270 ± 146</td>
<td>970 ± 92.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U30^3</td>
<td>-</td>
<td>1.87</td>
<td>24 ± 3.0</td>
<td>13 ± 3.0^5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methylnicotinamide</td>
<td>-</td>
<td>1.84</td>
<td>44 ± 6.0</td>
<td>30 ± 2.0^6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Hydroxyisovalerate</td>
<td>+</td>
<td>1.44</td>
<td>380 ± 31.1</td>
<td>444 ± 52.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxo-4-methylvalerate</td>
<td>+</td>
<td>1.35</td>
<td>1140 ± 87.0</td>
<td>1310 ± 119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U16^3</td>
<td>-</td>
<td>1.23</td>
<td>58 ± 5.0</td>
<td>54 ± 5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U5 + U6^4</td>
<td>+</td>
<td>1.13</td>
<td>1830 ± 209</td>
<td>2090 ± 219</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>+</td>
<td>1.02</td>
<td>608 ± 23.5</td>
<td>675 ± 56.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td>-</td>
<td>1.01</td>
<td>4310 ± 376</td>
<td>3780 ± 360</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Unless otherwise indicated, values are means ± SEMs, n = 7 (control) or 8 (treated). Letters indicate different from control as assessed by univariate unpaired Student's t test: a P < 0.01, b P < 0.05.
2 Regression coefficient signs of discriminant metabolites vs. the partial least squares-discriminant analysis model: positive regression (+) or negative regression (−) in treated mice compared with control mice.
3 Variable importance in projection.
4 Unassigned.
validation indicated 12 fecal metabolites relevant for the discrimination between control and treated mice. In particular, urocanate, methanol, succinate, 4-HPA, and 1 unknown metabolite were higher in probiotic-treated mice than in control mice, whereas dimethylamine, acetone, valerate, butyrate, methionine, acetate, and xylose were lower in probiotic-treated mice than in control mice (Table 4). Among the discriminant metabolites, urocanate, methanol, and acetone ($P < 0.001$) and dimethylamine, valerate, butyrate, 4-HPA, and U6 ($0.01 < P < 0.05$) were also significant by univariate analysis.

The ASCA model built from feces data confirmed the PLS-DA results, providing the evidence of an age-related modulation of the metabolic response to probiotic supplementation. The results showed treatment-induced metabolic variations occurring in the same or opposite directions in adult and aged mice (Supplemental Data Analysis and Supplemental Table 4). Both PLS-DA and ASCA models identified in both adult and aged mice higher 4-HPA and lower xylose in treated mice than in control mice, whereas valerate was greater in treated adult mice and lower in treated aged mice compared with their controls.

### Discussion

The aim of the present study was to evaluate the metabolic response to *L. acidophilus* La5 and *B. lactis* Bb12 in relation to age through $^1$H-NMR–based metabolic profiling. We showed that the 2 probiotics influence several urinary and fecal biochemical pathways differently in adult and aged mice. To our knowledge, this is the first study indicating different metabolic profiles induced by probiotic treatment in relation to age.

Interestingly, the PLS-DA models between treated and control mice revealed either common or diverse metabolic signatures induced by the 2 probiotics in the biofluids of aged and adult mice, mainly involving the methyl group metabolism (transmethylation process), as indicated by changes in the related metabolites, namely DMG, MNam, sarcosine, and choline in the urine and methionine, dimethylamine, and methanol in the feces. In particular, the PLS-DA models assigned the enhanced DMG level in treated adult and aged mice among the main discriminant metabolic changes. These results indicate that *L. acidophilus* and *B. lactis* may stimulate betaine-homocysteine methyltransferase (BHMT) activity producing DMG, and consequently may control the homocysteine concentration through the transmethylation and transsulfuration pathways. In fact, BHMT catalyzes methyl transfer from betaine, a product of choline oxidation, to homocysteine.

![Score plot derived from fecal $^1$H-NMR spectra of Lactobacillus acidophilus– and Bifidobacterium lactis–treated and control adult (A) and aged (B) mice. The partial least squares–discriminant analysis models, characterized by $R^2_y = 0.92$ (A) or $R^2_y = 0.89$ (B), were based on significant latent variables (LVs), namely the following: LV1 and LV2 in A, explaining 22% and 39% of the X-variance, respectively; and LV1, LV2, and LV3 in B, explaining 15%, 16%, and 50% of the X-variance, respectively.](https://example.com/image)

### Table 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Regression coefficient sign</th>
<th>VIP value</th>
<th>Control $\mu$mol/L</th>
<th>Treated $\mu$mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxybutyrate</td>
<td>+</td>
<td>5.85</td>
<td>392 ± 12.5</td>
<td>965 ± 43.1$^a$</td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td>220 ± 21.2</td>
<td>65 ± 5.0$^*$$^a$</td>
</tr>
<tr>
<td>Uracil</td>
<td>+</td>
<td>4.24</td>
<td>238 ± 19.4</td>
<td>484 ± 39.8$^a$</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetate</td>
<td>+</td>
<td>2.78</td>
<td>220 ± 13.0</td>
<td>312 ± 24.1$^b$</td>
</tr>
<tr>
<td>$U^4$</td>
<td></td>
<td></td>
<td>2.25</td>
<td>960 ± 87.4</td>
</tr>
<tr>
<td>$U^4$</td>
<td></td>
<td></td>
<td>2.17</td>
<td>379 ± 38.2</td>
</tr>
<tr>
<td>Valerate</td>
<td>+</td>
<td>1.41</td>
<td>884 ± 68.2</td>
<td>997 ± 54.0</td>
</tr>
<tr>
<td>Lactate</td>
<td></td>
<td></td>
<td>1.13</td>
<td>1439 ± 81.0</td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td></td>
<td>1.03</td>
<td>528 ± 48.1</td>
</tr>
</tbody>
</table>

$^a$ Unless otherwise indicated, values are means ± SEMs, $n = 7$ (control) or 10 (treated). Letters indicate different from control as assessed by univariate unpaired Student’s t test: *$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

$^b$ Regression coefficient signs of discriminant metabolites vs. the partial least squares-discriminant analysis model: positive regression (+) or negative regression (−) in treated mice compared with control mice.

$^2$ Variable importance in projection.

$^a$ Unassigned.
yielding DMG and methionine (Fig. 3). Methionine may be transformed into $S$-adenosylmethionine (SAM) by methionine adenosyltransferase, and SAM may lead to homocysteine through SAM-dependent transmethylation reactions, after the formation of $S$-adenosylhomocysteine (SAH). Finally, homocysteine can be either re-methylated back to methionine or irreversibly catabolized to cysteine in the transsulfuration pathway (47–50). The metabolism of methyl groups and homocysteine represents critical pathways for health improvement and disease prevention (47). Homocysteine metabolism is a highly regulated cellular process, but high concentrations of plasma homocysteine are frequently found in the elderly population (51). High plasma concentrations of homocysteine are positively correlated with increased risk of cardiovascular disease, Alzheimer disease, bone weakness, and renal dysfunction (51–53). In this context, although we have not measured the plasma homocysteine concentrations, our results suggest that changes in the gut microbiota induced by $L$. acidophilus and $B$. lactis supplementation may help in preventing the risk of the above-mentioned age-related diseases by regulating homocysteine metabolism. Notably in mice, the BHMT pathway appears to be the major route for homocysteine removal (54). Our results are consistent with the findings of previous studies showing an effect on betaine-homocysteine metabolism by probiotics. Martin et al. (55) observed an increase in dimethylglycine amounts in the liver and pancreas of mice after pre- and symbiotic microbial modulation, suggesting a stimulation of transmethylation in the methionine cycle.

We further show that other pathways involved in the regulation of homocysteine amounts are modulated by $L$. acidophilus and $B$. lactis, namely the NA and nicotinamide (Nam) methylation pathways influenced by $Lactobacillus acidophilus$ and $Bifidobacterium lactis$ treatment, based on the metabolite changes in urine of adult (Ad) and aged (Ag) mice. In bold type are the metabolites whose VIP are shown in Tables 1 and 2. BHMT, betaine homocysteine; CBS, cystathionine $\beta$-synthase; DMG, dimethylglycine; Gly, glycine; GNMT, glycine N-methyltransferase; GSH, glutathione; MNA, N-methylnicotinamide; MMN, nicotinamide acid mononucleotide; NA, nicotinate; Nam, nicotinamide; NAMN, nicotin acid mononucleotide; NamPRT, Nam phosphoribosyltransferase; NAPRT, nicotinate phosphoribosyltransferase; NNMT, nicotinamide N-methyltransferase; PARP, poly (ADP-ribose) polymerases; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, $S$-adenosylhomocysteine; SAM, $S$-adenosylmethionine; VIP, variable importance in projection; YNDase, nicotinamide deaminase.

### Table 4

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Regression coefficient sign$^2$</th>
<th>VIP$^3$ value</th>
<th>Control (μmol/L)</th>
<th>Treated (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urocanate</td>
<td>+</td>
<td>4.09</td>
<td>68 ± 8.0</td>
<td>567 ± 110$^4$</td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>3.97</td>
<td>122 ± 10.0</td>
<td>213 ± 14.1$^4$</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>−</td>
<td>3.95</td>
<td>555 ± 78.1</td>
<td>334 ± 38.5$^5$</td>
</tr>
<tr>
<td>Acetone</td>
<td>−</td>
<td>3.70</td>
<td>77 ± 4.0</td>
<td>54 ± 4.3$^6$</td>
</tr>
<tr>
<td>Valerate</td>
<td>−</td>
<td>2.85</td>
<td>1010 ± 84.5</td>
<td>741 ± 47.5$^5$</td>
</tr>
<tr>
<td>Butyrate</td>
<td>−</td>
<td>2.45</td>
<td>4670 ± 30.0</td>
<td>3740 ± 194$^4$</td>
</tr>
<tr>
<td>Methionine</td>
<td>−</td>
<td>1.67</td>
<td>443 ± 43.0</td>
<td>352 ± 26.3</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>1.62</td>
<td>7360 ± 787</td>
<td>5550 ± 580</td>
</tr>
<tr>
<td>U6$^4$</td>
<td>+</td>
<td>1.55</td>
<td>600 ± 60.1</td>
<td>916 ± 117$^4$</td>
</tr>
<tr>
<td>Xylose</td>
<td>−</td>
<td>1.29</td>
<td>237 ± 23.4</td>
<td>196 ± 32.1</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
<td>1.18</td>
<td>222 ± 30.2</td>
<td>395 ± 104</td>
</tr>
<tr>
<td>4-Hydroxyphenylacetate</td>
<td>+</td>
<td>1.16</td>
<td>286 ± 21.2</td>
<td>364 ± 21.3$^4$</td>
</tr>
</tbody>
</table>

1 Unless otherwise indicated, values are means ± SEMs, $n=9$ (control) or 9 (treated). Letters indicate different from control as assessed by univariate unpaired Student’s t test: $^aP<0.001$, $^bP<0.01$, $^cP<0.05$.
2 Regression coefficient signs of discriminant metabolites vs. the partial least squares-discriminant analysis model: positive regression (+) or negative regression (−) in treated mice compared with control mice.
3 Variable importance in projection.
4 Unassigned.
reactions producing N-methylnicotinate and MNam, respectively, the last by the activity of nicotinamide N-methyltransferase, and yielding SAH by reacting with SAM (Fig. 3). Interestingly, the Nam methylation pathway was differently affected by L. acidophilus and B. lactis depending on age, as shown by the opposite effect in urinary MNam concentrations in treated adult and aged mice. Furthermore, the PLS-DA models assigned NA amounts as discriminant between treated and control adult mice (VIP value = 1.89) but not between treated and control aged mice (VIP value <1). However, the interaction between age and treatment analyzed by the ASCA model highlighted lower NA and N-methylnicotinate amounts in probiotic-treated mice of both age classes (Supplemental Table 3). All of these data support the involvement of the methylation pathway in response to probiotic treatment.

In addition to contributing to homocysteine regulation, Nam and NA, consumed with the diet or produced by the gut microbiota, are also involved in the pathway of NAD synthesis through the activity of nicotinamide phosphoribosyltransferase (NamPRT) or nicotinate phosphoribosyltransferase. Nam is also a product of a catabolism of NAD through the activity of poly (ADP-ribose) polymerases (PARP) and sirtuins (56,57). Because Nam concentration is strictly controlled by NamPRT activity toward NAD synthesis, and by the activity of nicotinamide N-methyltransferase producing MNam, the lower MNam concentration induced by L. acidophilus and B. lactis in aged mice suggests an enhanced Nam utilization toward NAD synthesis. Notably, the MNam urinary concentration in control mice was significantly higher in aged mice than in adult mice (Supplemental Table 3). These results are of particular relevance considering that a decline in systemic NAD biosynthesis has been found to be associated with aging, potentially causing a variety of age-associated diseases mediated by unbalanced sirtuin and PARP activity (58). In addition, a new systemic regulatory mechanism connecting NAD and aging, the so-called NAD World, has been hypothesized, whereby NamPRT and sirtuins work together to control NAD concentration (59). Further data are necessary to clarify the possible role of the 2 probiotics in regulating NAD metabolism through sirtuin and PARP activity. The lower NA concentration in probiotic-treated mice than in control mice suggests an increased utilization of NA to yield NAD through nicotinate phosphoribosyltransferase and a decreased conversion of Nam to NA by nicotinamide deaminase. In addition, in treated adult mice, Nam was preferentially methylated to MNam, as shown by the enhanced MNam concentration. Because the methylation reaction of Nam may yield SAH and then homocysteine, the 2 probiotics could lead to an overproduction of these metabolites. However, the greater production of SAH and homocysteine was likely balanced by either increased BHMT activity, as discussed above, and reduced glycine-N-methyltransferase activity, as suggested by the lower sarcosine concentration in treated mice than in control adult mice, thus leading to limited SAH formation.

Among the discriminant urinary metabolites of the probiotic effect, the PLS-DA models displayed the intermediates of the tricarboxylic acid cycle, namely citrate and succinate, which were lower in treated aged mice than in control mice. These effects were also found in treated adult mice when the age × treatment interaction was analyzed by the ASCA model. Although the variations of citrate and succinate amounts suggest a probiotic-dependent effect on energy metabolism, different causes may contribute to these variations, including renal tubular functions or acid-base homeostasis, particularly in aged animals (60).

Of relevance, the body weight and food intake of probiotic-treated adult and aged mice did not differ from those of their controls; thus, the probiotic-induced variations in the metabolic profiles observed in the urine were not due to changes in food intake but rather to a direct interaction of L. acidophilus and B. lactis with the host or sensing of bacterial products by the cells.

We further investigated another aspect of the possible influence of probiotic supplementation on the gut-microbiota interaction by examining the microbiota metabolism in adult and aged mice. A common fecal metabolic signature was found in adult and aged mice after L. acidophilus and B. lactis treatment, mainly consisting of higher 4-HPA and lower xylose excretion, suggesting a change in the gut microbiota community or activity. The phenolic compounds may derive from dietary polyphenols or short peptides and amino acid fermentation by the activity of intestinal anaerobes, including lactobacilli and bifidobacteria. Because no change in food intake was found between control and treated mice, the probiotic-induced enhanced concentration of phenolic compounds is directly related to changes in the gut microbiota. The role of the microbiota in the formation of phenolic derivatives was highlighted by studies indicating that germ-free or antibiotic-treated rodents did not produce these compounds (61–63). The higher amounts of phenols may have important consequences, because 4-HPA has been shown to inhibit the growth of pathogenic bacteria and to stimulate the growth of Lactobacillus and Bifidobacterium species (64,65).

The decrease in fecal xylose concentrations observed in adult and aged mice is likely related to changes in the gut microbiota as well. Indeed, the xylose content may depend on the ability of the gut microbiota to degrade complex carbohydrates, such as xylose containing dietary hemicellulose (66). Because bifidobacterial strains can grow on xylose (67,68), our results suggest a probiotic-induced ecosystem favoring the growth of bifidobacteria both in adult and aged mice. On the other hand, the different increase and/or decrease in the fermentation products observed in the feces of adult and aged mice, namely valerate, butyrate, 3-hydroxybutyrate, lactate, acetone, acetate, methanol, succinate, and urocanate, indicates that L. acidophilus and B. lactis induced age-dependent modifications in the metabolic activities and/or composition of the microbiota. Interestingly, the main change in aged mice is the increase in urocanate concentration. Urocanate is an intermediate of histidine catabolism, which is produced mainly by the commensal Bacteroides thetaïotaomicron (69), a prominent bacterial species of mouse and human normal distal intestinal microbiota. Taking into account that several alterations occur in the microbiota of elderly people, with a decline in number and species diversity, including probiotics and commensal Bacteroides (16,19), our results suggest a beneficial effect of L. acidophilus and B. lactis in promoting a healthy microbiota community in aged mice. However, a metagenomic analysis is necessary to elucidate the relation of the metabolic changes found in this study with the modifications in the gut microbiota ecology in aged mice.

In conclusion, we show different metabolic responses to L. acidophilus and B. lactis in adult and aged mice. The 1H-NMR–based metabolic profiling of urine highlights a probiotic-mediated regulation of homocysteine and NAD metabolism in relation to age. Because these metabolic pathways have an important role in regulating fundamental cellular processes that can be altered in senescence, our findings suggest that supplementation with L. acidophilus and B. lactis may be a strategy to prevent or reduce age-related metabolic dysfunctions. In addition, the 2 probiotics induced several age-dependent modifications in the gut microbiota metabolism, which may favor a healthy microbiota and contribute to well-being.
Acknowledgments

We are grateful to Rita Rami for her excellent care of the animals and to E. Mocchegiani (Italian National Research Centres of Aging, INRCA, Ancona, Italy) for his collaboration in mice maintenance until 14 mo. E.B., A.E., and M.R. conducted the research; G.C. and F.M. performed the statistical analyses; G.C., A.T., and F.S. analyzed the 1H-NMR spectroscopy data; E.M., A.M., and E.B. designed the research; A.M. was responsible for 1H-NMR–based metabolic analysis; and E.M. and A.M. wrote the manuscript and had primary responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited