Organic Acid Profiling Analysis in Culture Media of Lactic Acid Bacteria by Gas Chromatography-Mass Spectrometry

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Abstract: Organic acid (OA) profiling analysis was performed in culture media from Lactobacillus pentosus K34 (L. pentosus K34) and Pediococcus lolli PL24 (P. lolli PL24) by gas chromatography-mass spectrometry (GC-MS) following methoxime/tert-butyldimethylsilyl derivatives. 12 OAs were positively identified in culture media. Most of OA levels from L. pentosus K34 of hetero lactic fermentation were found to be higher when compared with those from P. lolli PL24 of homo lactic fermentation, which may explain different OA metabolism in each strain. In addition, the distorted dodecagonal star patterns were readily distinguishable, and the characteristics of each strain were well represented. The present study demonstrates that the OA metabolic profiling method by GC-MS combined with star pattern recognition is useful for the monitoring study of characteristic OA metabolism in various microorganisms.

Key words: Organic acid, Profiling analysis, Lactobacillus pentosus K34, Pediococcus lolli PL24, GC-MS

Introduction

Organic acids (OAs) were well known as the final products in the metabolic process, which therefore supply information for altered biochemical metabolism from various biological samples including microorganisms. In particular, lactic acid in industrial chemistry fields was reported as important metabolite in fermentation step. In our previous report, phenyllactic acid was produced in high abundance with lactic acid and acetic acid. Phenyllactic acid is a novel antimicrobial compound active against Gram-positive and Gram-negative bacteria. Phenyllactic acid can be converted from phenylpyruvic acid by NADH dependent lactic acid dehydrogenase (LDH, EC 1.1.1.27). Therefore, the analysis of OAs in culture media and cells of microorganisms is important in the monitoring and screening of microbial availability. In our previous reports, OA profiling analyses by gas chromatography (GC) and GC-mass spectrometry (GC-MS) were found to be useful for the comparative analysis between control and experimental groups. Gram positive Lactobacillus pentosus K34 (L. pentosus K34) is facultative anaerobic and hetero-fermentative as rod-shaped lactic acid bacterium with rounded ends, which may occur singly, in pairs or short chains. It is nonmotile and grows at 10°C and 40°C but not at 45°C. In contrast, Pediococcus lolli PL24 (P. lolli PL24) is homo-fermentative Gram-positive lactic acid bacterium. It is coccus-shaped, nonspore-forming, nonmotile, and occurs in pairs or tetrads. L. pentosus K34 and P. lolli PL24 have two different characteristics. First, P. lolli PL24 has a thermo-tolerance and grows at high temperature of 47°C, while L. pentosus K34 cannot grow at high temperature of 47°C. Thermotolerant lactic acid bacteria has a beneficial probiotic advantage, for example, maintaining cell viability during manufacturing processes such as heat drying. Second, P. lolli PL24 has been known to be obligately homo-fermentative, while L. pentosus has been known to be facultatively hetero-fermentative. Normally, the metabolic pathway of homo lactic fermentation uses the Embden-Meyerhoff-Parnas (EMP) pathway and the metabolic pathway of hetero lactic fermentation uses the pentose phosphate pathway. Thus, in this study, our previous OA
profiling method\textsuperscript{9,14,15} using methoxime/tert-butylidimethylsilyl (MO/TBDMS) derivatives in combination with GC-MS was applied to culture media of \textit{L. pentosus} K34 and \textit{P. lolli} PL24 for the monitoring of altered OA metabolism.

**Experimental Section**

**Strain and culture condition**

\textit{L. pentosus} K34 with the strong inhibitory activity against gastro-intestinal pathogenic bacteria was isolated from the small intestine of Korean native chicken\textsuperscript{10,11} and \textit{P. lolli} PL24 with the inhibitory activity against gastro-intestinal pathogenic bacteria as well as thermo-tolerance at 47 °C was newly isolated from the waste of milk processing (n = 3). Each strain was activated at 37 °C for 2 days on de Man, Rogosa and Sharpe (MRS) (BD, USA) agar plate (n = 3). Then one loopful of each grown single colony was suspended into 1 mL 0.85% NaCl solution. Using syringe, 300 µL of each cell suspension was inoculated into 30 mL glass vial sealed by rubber and aluminum cap containing 20 mL MRS broth and incubated at 37 °C for 3 days without shaking. After cultivation, the culture broth was centrifuged at 10,000 g for 10 min. The supernatant was taken to the fresh new conical tube (SPL, Korea) and stored at −70 °C deep freezer (Ilsin, Korea).

**Chemicals and reagents**

OA standards including 3,4-dimethoxybenzoic acid as internal standard (IS), triethylamine (TEA) methoxamine hydrochloride were purchased from Sigma-Aldrich. N-Methyl-N-(tert-butylidimethylsilyl) trifluoroacetamid (MTBSTFA) + 1% tert-butylidimethylchlorosilane was provided from Thermo Scientific (Bellefonte, PA, USA). Toluene, diethyl ether, ethyl acetate, and sodium chloride (pesticide grade) were obtained from Kanto Chemical (Chuo-ku, Tokyo, Japan). Sulfuric acid was purchased from Samchun pure chemical Co. Ltd. (Pyeongtaek, Gyeonggi-do, South Korea). All other chemicals were of analytical reagent grade.

**Gas chromatography-mass spectrometry**

Derivatized samples were analyzed using an Agilent 6890N gas chromatograph interfaced to an Agilent 5975B mass-selective detector (70 eV, electron ionization source). The mass spectra were scanned in the mass range of 50-650 u at a rate of 0.99 scans/s. The temperatures of the injector, interface, and ion source were 260, 300, and 230 °C, respectively. An Ultra-2, cross-linked capillary column coated with 5% phenyl-95% methylpolysiloxane bonded phase (25 m × 0.20 mm I.D., 0.11 mm film thickness, Agilent Technologies, Santa Clara, CA, USA) was used for all analyses. Helium was used as the carrier gas at a flow rate of 0.5 mL/min in the constant flow mode. Samples (1 µL) were introduced in split-injection mode (10:1), and the oven temperature was set initially at 100 °C (2 min), then increased to 250 °C at rate of 5 °C/min and finally programmed to 300 °C at rate of 20 °C/min (5 min).

**Sample preparation for measurements of organic acids in cell culture media**

Control media (MRS), and culture media from \textit{L. pentosus} K34 and \textit{P. lolli} PL24 were used for experiments (n = 3). Aliquots of culture media (20 µL) containing IS (5 µg) were spiked to distilled water (1 mL) and reacted with methylhydroxylamine hydrochloride (1 mg) in alkaline condition at 60 °C for 30 min for conversion into MO derivative. The reaction mixture was then acidified to pH < 2 with 10% sulfuric acid solution, saturated with sodium chloride, and extracted with diethyl ether (4 mL) followed by ethyl acetate (2 mL). After addition of TEA (5 µL), the combined extracts were evaporated under a gentle stream of nitrogen (40 °C) to dryness. Toluene (20 µL) as the solvent and MTBSTFA (20 µL) as the silylation reagent were added to the residue, and the mixture was heated at 60 °C for 30 min to form MO/TBDMS derivatives prior to analysis by GC-MS.

**Star symbol plotting**

The mean peak area ratios to IS of OAs confirmed in the control media and culture media from \textit{L. pentosus} K34 and \textit{P. lolli} PL24 were normalized to the corresponding mean of those in the \textit{L. pentosus} K34. Then normalized levels of 12 OAs were plotted with lines radiating for star symbol plotting using Microsoft Excel (Microsoft, Redmond, WA), as described in previous report.\textsuperscript{19}

**Results and Discussion**

GC-MS profiles from the control media (A), \textit{L. pentosus} K34 (B), and \textit{P. lolli} PL24 (C) are showed in Figure 1. Seven OAs were screened in the control media (A), while 12 and 10 OAs were identified in \textit{L. pentosus} K34 (B) and \textit{P. lolli} PL24 (C), respectively (see also Table 1). In control media, lactic acid (No.2) was most abundant, followed by oleic acid (No.9), pyruvic acid (No.1), glycolic acid (No.3), succinic acid (No.7), and citric acid (No.12). Lactic acid (No.2) in \textit{L. pentosus} K34 was most abundant, followed by oleic acid (No.9), succinic acid (No.7), pyruvic acid (No.1) and phenyllactic acid (No.8). In contrast, in \textit{P. lolli} PL24, lactic acid (No.2) was most abundant, followed by oleic acid (No.9), pyruvic acid (No.1), phenyllactic acid (No.8), and succinic acid (No.7). The overall OA levels of \textit{L. pentosus} K34 were higher when compared with those of \textit{P. lolli} PL24. This finding may suggest that OAs produced by \textit{P. lolli} PL24 was smaller than those of \textit{L. pentosus} K34. In particular, \textit{P. lolli} PL24 mainly produced lactic acid (No.2) and phenyllactic acid (No.8) which are assumed to be produced from pyruvic acid and phenylpyruvic acid by LDH via EMP pathway. The difference in the overall OA levels is due to the different metabolic pathway of each.
strain. *P. lolli* PL24 with homo lactic fermentation mainly produced lactic acid from pyruvic acid by LDH, while *L. pentosus* K34 with hetero lactic fermentation produced the various OAs including lactic acid throughout the different metabolic pathway. Typically, hydroxyl acids such as No.4, No.5, and No.6 produced by *L. pentosus* K34 might be produced by central metabolism and/or amino acid metabolism. Specifically, α-keto acids were produced by biosynthetic enzymes involved in the central metabolism and/or also by aminotransferases from amino acids. Subsequently, hydroxyl acids were produced by hydroxyl acid dehydrogenases from α-keto acids.20

The OA levels in the media of control and *P. lolli* PL24 were normalized to the corresponding mean OA levels in the media of *L. pentosus* K34 (Table 1), which proved very informative by expressing the alteration (ranging from 0.00 to 8.20) of the OA values. In control media, the levels of glycolic acid (No.3) and citric acid (No.12) were highly elevated compared to those of *L. pentosus* K34 and *P. lolli* PL24. This is because these two OAs are used for growth of *L. pentosus* K34 and *P. lolli* PL24. In *P. lolli* PL24, the

### Table 1. Values of organic acids in control media, and cultured media with *L. pentosus* K34 and *P. lolli* PL24.

<table>
<thead>
<tr>
<th>No.</th>
<th>Organic acid</th>
<th>Control media (mean ± SD, n = 3)</th>
<th><em>L. pentosus</em> K34</th>
<th><em>P. lolli</em> PL24</th>
<th>Culture media (mean ± SD, n = 3)</th>
<th><em>L. pentosus</em> K34</th>
<th><em>P. lolli</em> PL24</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pyruvic acid</td>
<td>0.420 ± 0.066</td>
<td>0.355 ± 0.013</td>
<td>0.397 ± 0.015</td>
<td>1.18</td>
<td>1.00</td>
<td>1.12</td>
</tr>
<tr>
<td>2</td>
<td>Lactic acid</td>
<td>1.081 ± 0.181</td>
<td>23.840 ± 1.008</td>
<td>21.363 ± 0.752</td>
<td>0.05</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>3</td>
<td>Glycolic acid</td>
<td>0.404 ± 0.012</td>
<td>0.102 ± 0.007</td>
<td>0.116 ± 0.004</td>
<td>3.96</td>
<td>1.00</td>
<td>1.14</td>
</tr>
<tr>
<td>4</td>
<td>2-Hydroxyisovaleric acid</td>
<td>N.D.</td>
<td>0.038 ± 0.002</td>
<td>N.D.</td>
<td>0.00</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>2-Hydroxyisocaproic acid</td>
<td>N.D.</td>
<td>0.159 ± 0.009</td>
<td>0.012 ± &lt;0.001</td>
<td>0.00</td>
<td>1.00</td>
<td>0.08</td>
</tr>
<tr>
<td>6</td>
<td>2-Hydroxy-3-methylvaleric acid</td>
<td>N.D.</td>
<td>0.031 ± 0.002</td>
<td>N.D.</td>
<td>0.00</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>Succinic acid</td>
<td>0.213 ± 0.013</td>
<td>0.430 ± 0.025</td>
<td>0.247 ± 0.005</td>
<td>0.49</td>
<td>1.00</td>
<td>0.57</td>
</tr>
<tr>
<td>8</td>
<td>Phenyllactic acid</td>
<td>0.001 ± &lt;0.0010.164 ± 0.009</td>
<td>0.353 ± 0.011</td>
<td>0.00</td>
<td>1.00</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Oleic acid</td>
<td>0.881 ± 0.076</td>
<td>0.856 ± 0.058</td>
<td>0.808 ± 0.039</td>
<td>1.03</td>
<td>1.00</td>
<td>0.94</td>
</tr>
<tr>
<td>10</td>
<td>4-Hydroxyphenyllactic acid</td>
<td>N.D.</td>
<td>0.108 ± 0.007</td>
<td>0.162 ± 0.006</td>
<td>0.00</td>
<td>1.00</td>
<td>1.49</td>
</tr>
<tr>
<td>11</td>
<td>Indole-3-lactic acid</td>
<td>N.D.</td>
<td>0.005 ± 0.001</td>
<td>0.008 ± &lt;0.001</td>
<td>0.00</td>
<td>1.00</td>
<td>1.51</td>
</tr>
<tr>
<td>12</td>
<td>Citric acid</td>
<td>0.155 ± 0.015</td>
<td>0.019 ± 0.002</td>
<td>0.053 ± 0.003</td>
<td>8.20</td>
<td>1.00</td>
<td>2.82</td>
</tr>
</tbody>
</table>

aNot determined
bValues normalized to corresponding mean values of organic acids in cultured media with *L. pentosus* K34. IS = 3,4-Dimethoxybenzoic acid

![Figure 1](image1.png)

**Figure 1.** Organic acid profiles as methoxime/tert-butylidimethylsilyl derivatives of control media (A), culture media from *L. pentosus* K34 (B) and *P. lolli* PL24 (C). 1 = Pyruvic acid, 2 = Lactic acid, 3 = Glycolic acid, 4 = 2-Hydroxyisovaleric acid, 5 = 2-Hydroxyisocaproic acid, 6 = 2-Hydroxy-3-methylvaleric acid, 7 = Succinic acid, 8 = Phenyllactic acid, 9 = Oleic acid, 10 = 4-Hydroxyphenyllactic acid, 11 = Indole-3-lactic acid, 12 = Citric acid, IS = 3,4-Dimethoxybenzoic acid.

![Figure 2](image2.png)

**Figure 2.** Star symbol plots of control media, *L. pentosus* K34, and *P. lolli* PL24 based on the mean levels of the 12 OAs obtained through normalization to the corresponding mean values of *L. pentosus* K34. (A) *L. pentosus* K34 vs. control and (B) *L. pentosus* K34 vs. *P. lolli* PL24. The numbers on the rays correspond to those in Table 1.
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abundances of phenyllactic acid (No.8), 4-hydroxyphenyllactic acid (No.10), indole-3-lactic acid (No. 11), and citric acid (No.12) in the P. loll PL24 were much larger, while the abundances of 2-hydroxyisovaleric acid (No.4), 2-hydroxyisocaproic acid (No.5), 2-hydroxy-3-methylvaleric acid (No.6), and succinic acid (No.7) were smaller than those of the L. pentosus K34. The star symbol plots drawn based on these values displays distorted patterns (Figure 2) for the control media (Figure 2A) and the P. loll PL24 (Figure 2B) when compared to the pattern of the L. pentosus K34. Thus, this result may explain different OA metabolism and fermentation process of L. pentosus K34 and P. loll PL24.

Conclusions

OA metabolic profiles in cell growth media from L. pentosus K34 as hetero fermentative and P. loll PL24 as homo fermentative showed different patterns. And the star patterns were readily distinguishable and characteristic of each strain. This may explain for usefulness of OA profiling method using GC-MS combined with star pattern recognition for monitoring of altered and characteristic OA metabolism in various microorganisms.

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