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

Jae Yeon Lee[a,†], Duc-Toan Nguyen[b,†], Young Shik Park[a], Kyo Yeol Hwang[a], Yong Seok Cho[a], Kyung-Don Kang[a], Jaehwan Yoon[b], Jundong Yu[c], Sung-Tae Yee[d], Young Hwan Ahn[e], Gwang Lee[b,f], Su-Il Seong[a,g,#] and Man-Jeong Paik[d,*]

[a]*R&D Center for Life Science, Biotopia Co., Ltd., Chuncheon, Gangwon-do 200-883, Korea*
[b]*Department of Molecular Science and Technology, Ajou University, Suwon 443-721, Korea*
[c]*Racing Laboratory, Korea Racing Authority, Gwacheon 427-711, Korea*
[d]*College of Pharmacy, Sunchon National University, Suncheon, 540-950, Korea*
[e]*Department of Neurosurgery, Ajou University School of Medicine, Suwon 443-721, Korea*
[f]*Institute for Medical Science, School of Medicine, Ajou University, Suwon 443-721,Korea*
[g]*Department of Life Science, The University of Suwon, Hwaseong, Gyeonggi-do 445-743, Korea*

Received May 30, 2012; Revised June 29, 2012; Accepted August 20, 2012
First published on the web September 20, 2012; DOI: 10.5478/MSL.2012.3.3.74

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.[1–3] In particular, lactic acid in industrial chemistry fields was reported as important metabolite in fermentation step.[4–9] In our previous report,[10] 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.[11,12] Phenyllactic acid can be converted from phenylpyruvic acid by NADH dependent lactic acid dehydrogenase (LDH, EC 1.1.1.2.7).[14] 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.[9,10,14,15]

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.[16] In contrast, *Pediococcus lolli* PL24 (*P. lolli* PL24) is homo-fermentative Gram-positive lactic acid bacterium. It is coccus-shaped, non-spore-forming, nonmotile, and occurs in pairs or tetrads.[17] *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. Thermo-tolerant lactic acid bacteria has a beneficial probiotic advantage, for example, maintaining cell viability during manufacturing processes such as heat drying.[18] Second, *P. lolli* PL24 has been known to be obligately homo-fermentative, while *L. pentosus* has been known to be facultatively hetero-fermenative. 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

*Reprint requests to Dr. Man-Jeong Paik
E-mail: paik815@sunchon.ac.kr
#Co-corresponding author
†These authors contributed equally to this study

profiling method[9,14,15] using methoxime/*tert*-butyldimethylsilyl (MO/TBDMS) derivatives in combination with GC-MS was applied to culture media of *L. pentosus* K34 and *P. lolli* PL24 for the monitoring of altered OA metabolism.

Experimental Section

Strain and culture condition

L. pentosus K34 with the strong inhibitory activity against gastro-intestinal pathogenic bacteria was isolated from the small intestine of Korean native chicken[10,11] and *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) methoxyamine hydrochloride were purchased from Sigma-Aldrich. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamid (MTBSTFA) + 1% *tert*-butyldimethylchlorosilane 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 *L. pentosus* K34 and *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 *L. pentosus* K34 and *P. lolli* PL24 were normalized to the corresponding mean of those in the *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.[19]

Results and Discussion

GC-MS profiles from the control media (A), *L. pentosus* K34 (B), and *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 *L. pentosus* K34 (B) and *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 *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 *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 *L. pentosus* K34 were higher when compared with those of the *P. lolli* PL24. This finding may suggest that OAs produced by *P. lolli* PL24 was smaller than those of *L. pentosus* K34. In particular, *P. loll* 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

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

No.	Organic acid	Peak area ratio (mean ± SD, n = 3)			Normalized values[b]		
		Control media	*L. pentosus* K34	*P. lolli* PL24	Culture media	*L. pentosus* K34	*P. lolli* PL24
1	Pyruvic acid	0.420 ± 0.066	0.355 ± 0.013	0.397 ± 0.015	1.18	1.00	1.12
2	Lactic acid	1.081 ± 0.181	23.840 ± 1.008	21.363 ± 0.752	0.05	1.00	0.90
3	Glycolic acid	0.404 ± 0.012	0.102 ± 0.007	0.116 ± 0.004	3.96	1.00	1.14
4	2-Hydroxyisovaleric acid	N.D.[a]	0.038 ± 0.002	N.D.	0.00	1.00	0.00
5	2-Hydroxyisocaproic acid	N.D.	0.159 ± 0.009	0.012 ± < 0.001	0.00	1.00	0.08
6	2-Hydroxy-3-methylvaleric acid	N.D.	0.031 ± 0.002	N.D.	0.00	1.00	0.00
7	Succinic acid	0.213 ± 0.013	0.430 ± 0.025	0.247 ± 0.005	0.49	1.00	0.57
8	Phenyllactic acid	0.001 ± < 0.001	0.164 ± 0.009	0.353 ± 0.011	0.00	1.00	2.16
9	Oleic acid	0.881 ± 0.076	0.856 ± 0.058	0.808 ± 0.039	1.03	1.00	0.94
10	4-Hydroxyphenyllactic acid	N.D.	0.108 ± 0.007	0.162 ± 0.006	0.00	1.00	1.49
11	Indole-3-lactic acid	N.D.	0.005 ± 0.001	0.008 ± < 0.001	0.00	1.00	1.51
12	Citric acid	0.155 ± 0.015	0.019 ± 0.002	0.053 ± 0.003	8.20	1.00	2.82

[a]Not determined

[b]Values normalized to corresponding mean values of organic acids in cultured media with *L. pentosus* K34. IS = 3,4-Dimethoxybenzoic acid

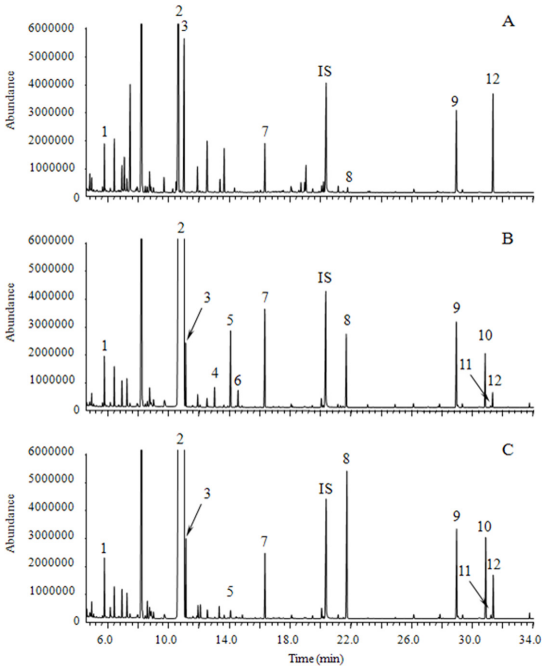

Figure 1. Organic acid profiles as methoxime/*tert*-butyldimethylsilyl derivatives of control media (A), culture media from *L. pentosus* K34 (B) and *P. lolli* PL24 (C). 1 = Pyruvic acid, 2 = Lactic acid, 3 = Glycolic acid, 4 = 2-Hydroxyisovaleric acid, 5 = 2-Hydroxyisocaproic acid, 6 = 2-Hydroxy-3-methylvaleric 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.

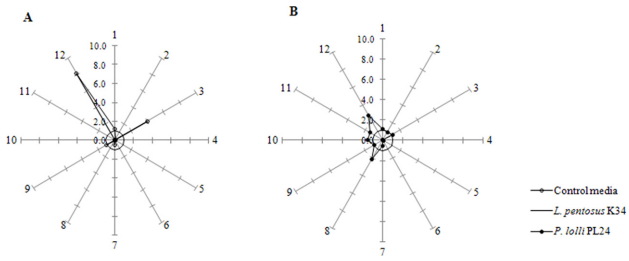

Figure 2. Star symbol plots of control media, *L. pentosus* K34, and *P. lolli* PL24 based on the mean levels of the 12 OAs obtained through normalization to the corresponding mean values of *L. pentosus* K34. (A) *L. pentosus* K34 vs. control and (B) *L. pentosus* K34 vs. *P. lolli* PL24. The numbers on the rays correspond to those in Table 1.

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

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. lolli* 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. lolli* 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. lolli* PL24.

Conclusions

OA metabolic profiles in cell growth media from *L. pentosus* K34 as hetero fermentative and *P. lolli* 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.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2010-0021443) to M. J. Paik and (2010-0023846). G. Lee. And the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0028294).

References

1. Matsumoto, I.; Kuhara, T. *Mass Spectrom. Rev.* **1987**, 6, 77.
2. Liebich, H. M.; Forst, C. *J. Chromatogr.* **1990**, 525, 1.
3. Duez, P.; Kumps, A.; Mardens, Y. *Clin. Chem*. **1996**, 42, 1609.
4. Caligiani, A.; Cirlini, M.; Palla, G.; Ravaglia, R.; Arlorio, M. *Chirality* **2007**, 19, 329.
5. Grabar, T. B.; Zhou, S.; Shanmugam, K. T.; Yomano, L. P.; Ingram, L. O. *Biotechnol. Lett.* **2006**, 28, 1527.
6. Joshi, D. S.; Singhvi, M. S.; Khire, J. M.; Gokhale, D. V. *Biotechnol. Lett*. **2010**, 32, 517.
7. John, R. P.; Nampoothiri, K. M.; Pandey, A. *Appl. Microbiol. Biotechnol*. **2007**, 74, 524.
8. Ewaschuk, J. B.; Zello, G. A.; Naylor, J. M.; Brocks, D. R. *J. Chromatogr. B* **2002**, 781, 39.
9. Paik, M. J.; Nguyen, D. T.; Yoon, J.; Chae, H. S.; Kim, K. R.; Lee, G.; Lee, P. C. *Bull. Korean Chem. Soc.* **2011**, 32, 2418.
10. Lee, J. Y.; Hwang, K. Y.; Kim, K.; Seong, S. I.; Park, Y. S.; Paik, M. J.; Kim, K.R. *Kor. J. Microbiol. Biotechnol.* **2002**, 30, 241.
11. Dieuleveux, V.; van der Pyl, D.; Chataud, J.; Gueguen, M. *Appl. Environ. Microbiol.* **1998**, 64, 800.
12. Dieuleveux, V.; Lemarinier, S. ; Gueguen, M. *Intl. J. Food Microbiol.* **1998**, 40, 177.
13. Li, X. F.; Jiang, B.; Pan, B. L.; Mu, W. M. ; Zhang, T. *J. Agric. Food Chem.* **2008**, 56, 2392.
14. Paik, M. J.; Kim, K. R. *J. Chromatogr. A* **2004**, 1034, 13.
15. Paik, M. J.; Cho, E. Y.; Kim, H.; Kim, K. R.; Choi, S.; Ahn, Y. H.; Lee, G. *Biomed. Chromatogr.* **2008**. 22, 450.
16. Zanoni, P.; Farrow, J. A. E.; Phillps, B. A.; Collins, M. D. *Intl. J. Systematic Bacteriol.* **1987**, 37, 339.
17. Doi, K.; Nishizaki, Y.; Fujino, Y.; Ohshima, T; Ohmomo, S.; Ogata, S. *Intl. J. Systematic and Evolutionary Microbiol.* **2009**, 59, 1007.
18. Ross, R. P.; Desmond, C.; Fitzgerald, G. F.; Stanton, C. *J. Appl. Microbiol*. **2005**, 98, 1410.
19. Paik, M. J.; Li, W. Y.; Ahn, Y. H.; Lee, P. H.; Choi, S.; Kim, K. R.; Kim, Y. M.; Bang, O. Y.; Lee, G. *Clin. Chim. Acta* **2009**, 402, 25.
20. van Kranenberg, R.; Leerebezem, M.; Vlieg, J. van H.; Ursing, B.M.; Boekhorst, J.; Smit, B. A.; Ayad, E. H. E.; Smit, G.; Siezen, R. J. *Intl. Dairy J.* **2002**, 12, 111.