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Mass-Based Metabolomic Analysis of *Lactobacillus sakei* and Its Growth Media at Different Growth Phases

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Changes in the metabolite profiles of *Lactobacillus sakei* and its growth media, based on different culture times (0, 6, 12, and 24 h), were investigated using gas chromatography-mass spectrometry (MS) and liquid chromatography-MS with partial least squares discriminant analysis, in order to understand the growth characteristics of this organism. Cell and media samples of *L. sakei* were significantly separated on PLS-DA score plots. Cell and media metabolites, including sugars, amino acids, and organic acids, were identified as major metabolites contributing to the difference among samples. The alteration of cell and media metabolites during cell growth was strongly associated with energy production. Glucose, fructose, carnitine, tryptophan, and malic acid in the growth media were used as primary energy sources during the initial growth stage, but after the exhaustion of these energy sources, *L. sakei* could utilize other sources such as trehalose, citric acid, and lysine in the cell. The change in the levels of these energy sources was inversely similar to the energy production, especially ATP. Based on these identified metabolites, the metabolomic pathway associated with energy production through lactic acid fermentation was proposed. Although further studies are required, these results suggest that MS-based metabolomic analysis might be a useful tool for understanding the growth characteristics of *L. sakei*, the most important bacterium associated with meat and vegetable fermentation, during growth.

Keywords: *Lactobacillus sakei*, GC-MS, LC-MS, metabolomics

Introduction

Lactobacillus is a gram-positive facultative anaerobic bacterium found widely on fresh meat, fish, and fermented food products. It is one of the most important species of lactic acid bacteria (LAB), having many functional properties, including improvement of the immune system, prevention of fungal infection, and balance of gut microflora [1]. Moreover, previous studies suggest that *Lactobacillus* species led to enhanced shelf life and improved nutritional quality and microbial safety of fermented foods [2, 3]. Among *Lactobacillus* species, *L. sakei* is mainly involved in meat preservation and salt-fermentation of meat [4, 5] and vegetables such as doenjang (a fermented soybean paste) and kimchi (a traditional Korean fermented cabbage). In contrast to other strains, *L. sakei* has distinctive biochemical and physiological features, such as producing the lactic acid isomer and ammonia from arginine, and having a unique sugar fermentation pattern [6]. Although many studies on its taxonomic status, physiology, nutrient requirement, end-product properties, morphological characteristics, and energy metabolism have been conducted using biochemistry and molecular genetics [7, 8], knowledge of its microbial, genetic, growth, and physiological characteristics is still obscure.

Recently, changes in the intercellular metabolome of

various microorganisms, including *Bacillus subtilis*, *Escherichia coli* [9], *Halomonas* sp. [10], and the marine diatom *Skeletonema marinoi* [11], during different growth phases have been studied using mass spectrometry (MS)-based metabolomic analysis, in order to better understand the growth characteristics of these bacterial species. The metabolomic profiles of *Lactobacillus* species, including *L. bulgaricus* [12], *L. paracasei* [13] and *L. plantarum* [14], have also been analyzed by gas chromatography (GC)-MS-based metabolomic analysis, thus promoting the development of protocols for metabolite analysis of *L. delbrueckii* subsp., *L. bulgaricus*, and *L. paracasei*, and the relationship between *Saccharomyces cerevisiae* and *L. plantarum*. However, the change in the metabolome of the *Lactobacillus* species, especially *L. sakei*, during different growth phases was not investigated.

Therefore, in this study, cell metabolite profiles of *L. sakei* and its culture media at different growth phases were analyzed by GC-MS and liquid chromatography (LC)-MS in order to better understand its growth characteristics. Moreover, the metabolomic pathway of *L. sakei* was proposed, based on the identified metabolites.

Materials and Methods

L. sakei Cultivation and Sample Collection

L. sakei K040706 used in this study was provided by the Korea Food Research Institute and was cultured at 37°C for 6, 12, and 24 h in 100% Difco Lactobacilli MRS broth (Becton Dickinson and Company, USA). *L. sakei* cultures were centrifuged at 3,515 ×*g* for 15 min at 4°C. Supernatant media were filtered using a syringe filter (0.22 μm) and the collected cells were washed three times with 0.9% cold saline for quenching. After immersing in 5 ml of cold methanol, the quenched cells were collected. The collected cells and media were lyophilized and stored at −80°C until analysis.

Sample Extraction and Preparation

For LC/MS analysis, the media were pre-separated on a solid-phase extraction (SPE) C_{18} column (Waters, USA). The medium was loaded on a SPE column activated by 100% methanol and equilibrated with water. After washing with water, samples were eluted by 100% methanol and dried using a CentriVap Centrifugal Vacuum Concentrator (Labconco, USA). Dried samples were resolved by 50% methanol containing terfenadine as an internal standard. Cell metabolites were extracted from lyophilized cells with 50% methanol containing terfenadine, using a bullet blender (Next Advance, USA). After centrifugation, the supernatant was collected.

For GC-MS analysis, cell metabolites were extracted with a mixture solvent of 50% methanol:chloroform (5:1) using the bullet blender. After centrifugation, the supernatant was completely dried using the CentriVap Centrifugal Vacuum Concentrator. Dried cell extracts and media were derivatized. For derivatization, both dried samples were mixed with 50 μl of 2% methoxyamine hydrochloride in pyridine containing dicyclohexyl phthalate as an internal standard. The reaction mixtures were sonicated for 15 min and incubated at 37°C for 90 min. After the methoxylation, samples were derivatized with 100 μl of *N,O-bis*(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane at 70°C for 30 min. The derivatized samples were analyzed by GC-MS.

Ultra-Performance Liquid Chromatography–Quadrupole–Time-of-Flight (UPLC-Q-TOF) MS Analysis

Metabolites extracted from cells and their growth media were analyzed on a UPLC system (Waters). The samples were injected into an Acquity UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 μm; Waters) equilibrated with water containing 0.1% formic acid, and eluted in a gradient with acetonitrile containing 0.1% formic acid at a flow rate of 0.4 ml/min for 9 min. The eluted metabolites were analyzed using electrospray ionization-Q-TOF MS (Waters) with positive-ion mode. The capillary voltages and sampling cones were set at 3 kV and 40 V, respectively. The source temperature was set to 120°C. The desolvation flow rate and temperature were 400 l/h and 120°C, respectively. TOF MS data were collected in the *m/z* 100–1,200 range with a scan time of 0.1 sec. Leucine-enkephalin (*m/z* 556.2771 for positive-ion mode) was infused as the lack mass at a flow rate of 5 μl/min and a frequency of 10 sec to ensure the accuracy and reproducibility for all analyses. For quality control, a mixture of all samples was injected after every 10 samples. The MS/MS spectra of the metabolites were collected in the *m/z* 70–1,200 range using a collision energy ramp at 20–45 eV. All MS data were obtained using MassLynx software (Waters), including retention time, *m/z*, and ion intensity.

GC-MS Analysis

Derivatized cell and medium metabolites were analyzed using a Shimadzu GC-2010 plus (Japan). The derivatized sample (1 μl) was injected into a DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, Agilent J&W, USA) with a split ratio of 1:100. Helium was used as the carrier gas at a flow rate of 1 ml/min and the injection temperature was set at 200°C. The oven temperature was maintained at 80°C for 2 min, increased to 320°C at 10°C/min, and then held at 320°C for 6 min. The GC column effluent was analyzed using a Shimadzu GCMS–TQ 8030 operated in the electron ionization mode (70 eV). The temperatures of the ion source and interface were set to 230°C and 280°C, respectively. The MS spectra were monitored in the full-scan mode from *m/z* 45 to 550 with a scan event time of 0.3 sec and a scan speed of 2,000 u/sec. The detector voltage was 0.1 kV and the threshold was 100.

Data Processing

MS data analyzed by GC-MS were processed using the analyzer Pro software (Spectralworks Ltd., UK). Metabolite peaks were

deconvoluted and collected using an area threshold of 7,000, height threshold of 1, signal-to-noise ratio of 3, width threshold of 0.01, scan windows of 5, and smoothing of 5. The data were aligned with a retention time window of 0.3 min. All mass data were normalized using the internal standard, dicyclohexyl phthalate. Metabolites were identified based on the comparison of their mass spectra and retention indices (RIs), which were calculated using a series of *n*-alkanes (C8–C40), with the Wiley and NIST mass spectral databases, the published RIs, and authentic standards.

The MarkerLynx software was used for collection, alignment, and normalization of the data sets analyzed using the UPLC-Q-TOF MS. Metabolite peaks were collected using a peak-width at 5%, height of 1 sec, peak-to-peak baseline noise of 1, noise elimination of 6, and an intensity threshold of 10,000. The data were aligned with a 0.05 Da mass window and a retention time window of 0.2 min. All mass spectra were normalized against an internal standard. The metabolites were identified using ChemSpider (http://www.chemspider.com), human metabolome databases (http://www.hmdb.ca), the METLIN database (http://metlin.scripps.edu), literature references, and authentic standards.

Statistical Analysis

Mass data sets processed by Analyzer Pro and MarkerLynx, respectively, were subjected to multivariate statistical analysis with SIMCA-P$^+$ ver. 12.0.1 (Umetrics, Sweden). Prior to analysis, data were scaled to unit variance. Differences among sample groups were visualized by principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). The quality of the PLS-DA models was evaluated by the goodness of fit measure (R2X and R2Y) and predictive ability (Q2Y), and validated by cross-validation with a permutation test (n = 200). Based on the variable importance in the projection (VIP) value > 1.0, calculated by PLS-DA and one-way analysis of variance (ANOVA) with Duncan's test ($p < 0.05$) using SPSS 17.0 (SPSS Inc., USA), metabolites contributing to the difference among groups were found and identified. Identified metabolites with significant differences ($p < 0.05$) were also visualized in a heat map drawn in R with ggplot2. The heat map was plotted in a green-red color scale, with red indicating an increase and green indicating a decrease in metabolite levels. Extraction of metabolites and mass analysis were processed in random order.

Results and Discussion

Growth Curve of *L. sakei*

After a 2 h lag phase, the cells were exponentially grown until 12 h, after which they entered a stationary phase (Fig. 1). This was similar to the growth curves of other *Lactobacillus* species [15]. Based on the growth curve, the cells and the culture media were collected at three representative time points of the growth curve (6 h, the middle of the exponential phase; 12 and 24 h, the entrance and the middle of the stationary phase, respectively) for metabolomic analysis. The fresh medium (0 h) was used as a control.

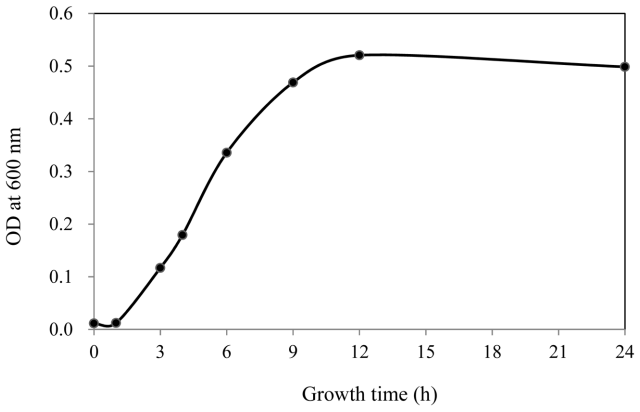

Fig. 1. Growth curve of *L. sakei* K040706 cultured in MRS broth medium at 37°C.

Multivariate Statistical Analysis of Cell and Medium Metabolites

The profiles of cell and medium metabolites detected by GC-MS and UPLC-Q-TOF MS were statistically analyzed by multivariate statistical analysis, including PCA and PLS-DA, to visualize the difference among samples and to find metabolites contributing to the difference (Fig. 2). Prior to PLS-DA analysis, significant outliers identified by the PCA-Hotelling's T2 test, at a 95% confidence interval of the modeled variation, were excluded from further analysis. Cell (6, 12, and 24 h) and medium samples (0, 6, 12, and 24 h) were clearly separated on the first two-component PLS-DA score plots. The PLS-DA model quality parameters were as follows: R2X > 0.80, R2Y > 0.96, and Q2 > 0.93 for cell metabolites; R2X > 0.77, R2Y > 0.65, and Q2 > 0.48 for medium metabolites. The values obtained from cross-validation (R_2 intercept < 0.4 and Q_2 intercept < -0.2 for cell and medium metabolites) indicated that the PLS-DA models for cell and medium metabolomic analyses were statistically acceptable, although the p-values of PLS-DA score plots for media were higher than 0.05.

Identification of Metabolites

Among metabolites detected by LC-MS and GC-MS, statistical analysis of the normalized metabolites showed that the levels of 66 cell metabolites and 136 medium metabolites changed significantly during cell growth. Of these metabolites, 10 cell metabolites (carnitine, triose phosphate, 5-deoxy methylthioadenosine, phosphoric acid,

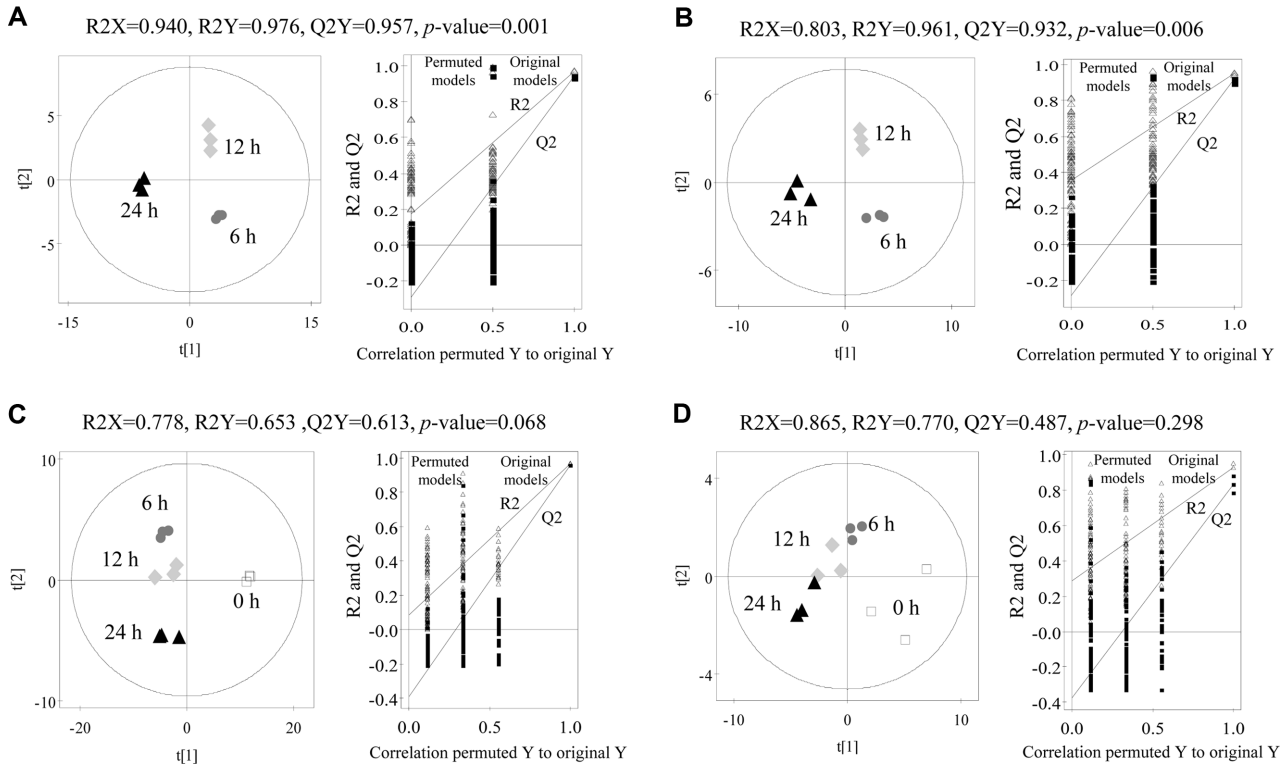

Fig. 2. Partial least squares discriminant analysis (PLS-DA) score plots of the datasets obtained by UPLC-Q-TOF MS (**A** and **C**) and GC-MS (**B** and **D**) analysis of *L. sakei* (**A** and **B**) and its growth media (**C** and **D**).
The quality of the PLS-DA models was evaluated by R2X, R2Y, Q2Y, and *p*-values, and validated by 200 permutation tests.

phenylalanine, citiric acid, talose, lysine, inositol, and trehalose) and 16 medium metabolites (arginine, carnitine, tyrosine, adenosine monophosphate, tryptophan, C16-sphinganine, palmitamide, oleamide, lactic acid, phosphoric acid, butanedioic acid, propanoic acid, malic acid, fructose, mannose, and glucose) were identified (Table 1). In particular, the energy metabolism-associated metabolites, including sugars (fructose, mannose, glucose, trehalose, talose, and triose phosphate), organic acids (lactic acid, citric acid, and phosphoric acid), and amino acids (carnitine and tyrosine), having VIP > 1.0 were identified as major metabolites along with two adenosine derivatives (5-doexy methlylthioadenosine and adenosine monophosphate (AMP)).

Heat Maps of Identified Metabolites

Changes in the levels of metabolites identified in the cell and the growth medium were visualized according to heat maps (Fig. 3). The levels of carnitine, triose phosphate, 5-deoxy methylthioadenosine, talose, inositol, and phenylalanine in the cell increased with an increase in growth time, whereas the levels of phosphoric acid decreased. Compared with 6 h cultured cells, the elevated levels of citric acid, lysine, and trehalose at 12 h decreased at 24 h.

From the medium, the levels of tryptophan, carnitine, fructose, glucose, phosphoric acid, butanedioic acid, propanoic acid, and malic acid were decreased with an increase in growth time, whereas the levels of other identified metabolites accumulated.

Metabolomic Pathway Associated with the Growth of *Lactobacillus sakei*

Based on the identified metabolites, a metabolic pathway associated with the growth of *L. sakei* was proposed (Fig. 4). The pathway suggested that the energy metabolism via substrate-level phosphorylation was significantly changed during cell growth. In general, carbohydrates and beef extracts used as energy sources in the growth medium for *L. sakei* are enzymatically broken down to small molecules, such as hexoses and amino acids. The small molecules were transported into the cell by the phosphotransferase system and other systems to produce energy [16], leading to decreased amounts in the medium with increasing growth time. Metabolomic analysis of the media demonstrated that

Table 1. Identification of *L. sakei* cell and medium metabolites contributing to the separation among sample groups on the PLS-DA score plots of the datasets analyzed using UPLC-Q-TOF MS and GC-MS.

		RT (min)[a]	Compound	RI[b]	Exact mass (M+H)	Mass fragments	*p*-Value[c]	VIP[d]
LC-MS	Cells	0.59	Carnitine	-	162.11	103, 85, 60	2.79E-5	1.255
		0.61	Triose phosphate	-	585.14	243, 207	2.34E-4	1.158
		1.94	5-Deoxy methylthioadenosine	-	298.09	136	1.79E-3	1.099
	Media	0.53	Arginine	-	175.12	158, 130, 116, 70	2.78E-6	0.821
		0.57	Carnitine	-	162.11	103, 85, 60, 43	4.99E-8	1.078
		0.94	Adenosine monophosphate	-	348.07	136	6.81E-4	1.497
		1.52	Tyrosine	-	182.08	165, 136, 119	6.05E-4	2.159
		2..00	Tryptophan	-	188.07	170, 144, 118	6.07E-5	0.823
		3.70	C16-sphinganine	-	274.27	256, 106, 88	1.36E-6	0.714
		5.63	Palmitamide	-	256.26	116, 102, 88, 71	1.68E-3	0.734
		5.70	Oleamide	-	282.28	121, 111, 97, 83	9.44E-4	0.484
GC-MS	Cells	8.71	Phosphoric acid	1,261	-	299, 73	1.42E-3	4.304
		13.67	Phenylalanine	1,620	-	218, 192, 73	1.78E-3	0.334
		15.87	Citric acid	1,804	-	273, 147, 73	1.14E-2	2.978
		16.72	Talose	1,881	-	319, 205, 147, 73	1.50E-2	1.386
		17.04	Lysine	1,911	-	317, 174, 156, 73	9.91E-3	0.899
		18.76	Inositol	2,076	-	305, 217, 147, 73	1.41E-3	0.486
		24.52	Trehalose	2,741	-	361, 191, 73	3.17E-2	2.765
	Media	5.36	Lactic acid	1,050	-	148, 147, 117, 73	9.68E-7	4.310
		8.70	Phosphoric acid	1,261	-	299, 73	1.63E-2	0.826
		9.38	Butanedioic acid	1,307	-	147, 73	1.80E-3	0.134
		9.58	Propanoic acid	1,320	-	292, 189, 147	8.10E-4	0.134
		11.81	Malic acid	1,477	-	233, 147, 73, 45	5.05E-13	0.222
		16.43	Fructose	1,857	-	307, 217, 103, 73	7.31E-6	1.156
		16.59	Mannose	1,871	-	319, 205, 147, 73	4.45E-3	3.633
		16.93	Glucose	1,903	-	319, 205, 147, 73	1.07E-2	2.370

[a]RT, retention time. [b]RI, retention index. [c]*p*-Values were analyzed by one-way ANOVA. [d]VIP, variable importance in the projection.

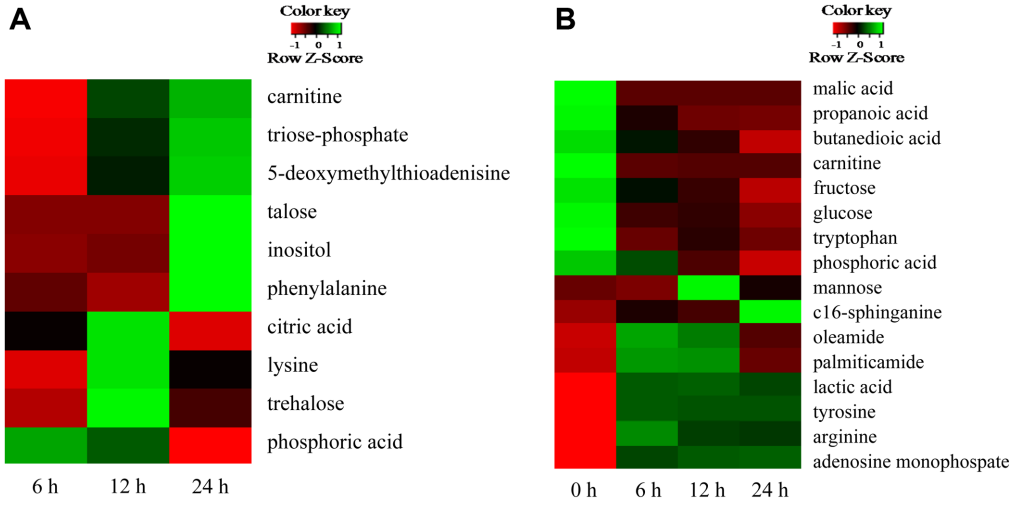

Fig. 3. Heat maps of identified *L. sakei* cell (**A**) and medium (**B**) metabolites analyzed using UPLC-Q-TOF MS and GC-MS.
The heat maps were drawn using R with gplots and the green-red color represents the z-score transformed raw data of cell and medium metabolites with significant differences among sample groups. Red and green colors indicate a decrease and an increase in metabolite levels, respectively.

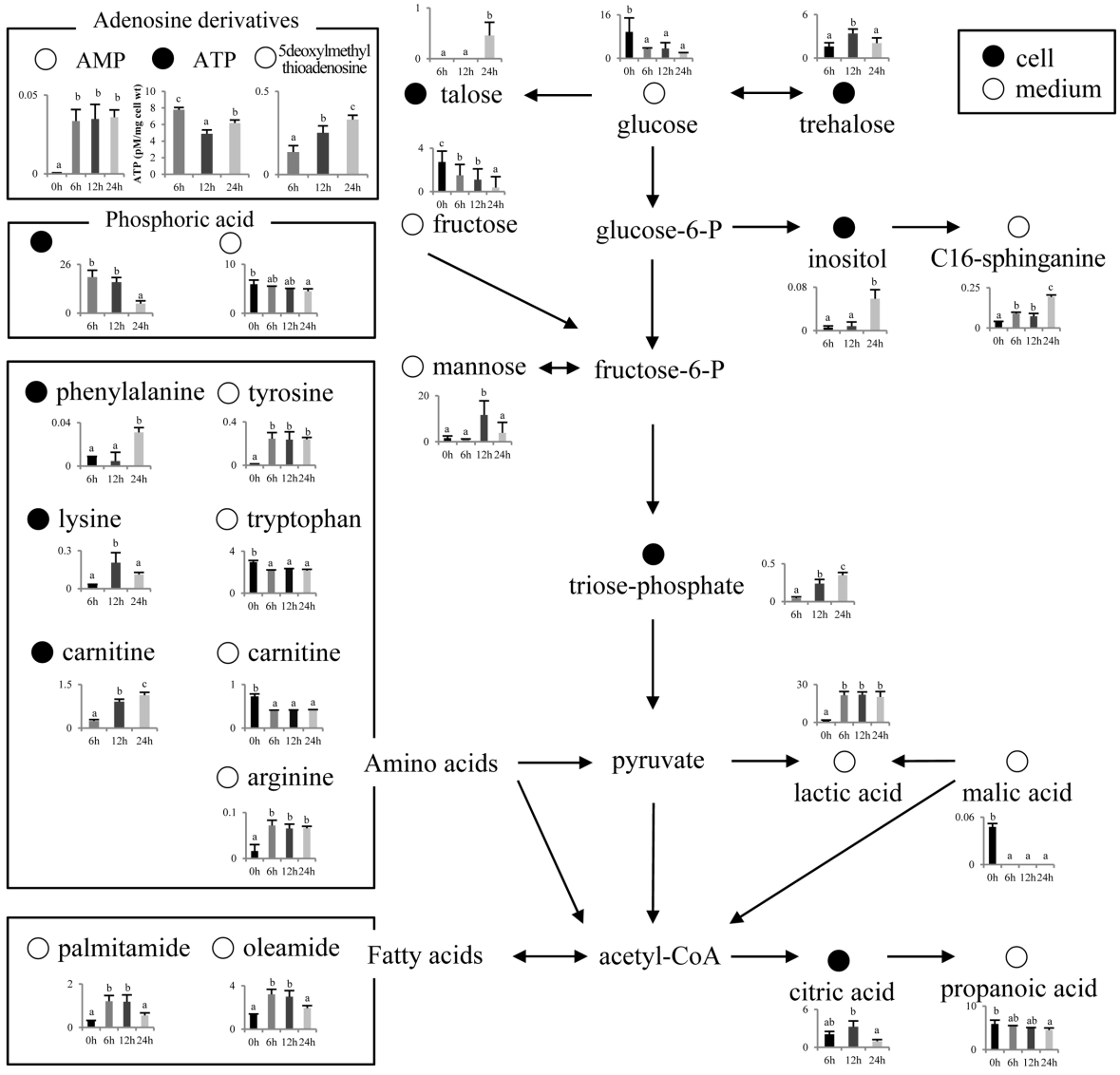

Fig. 4. Schematic of the proposed metabolic pathway associated with the energy production of *L. sakei* during growth, and the relative abundance of identified metabolites.

The horizontal axis of the bars shows the normalized intensity, and letters on the bars represent significant differences at $p < 0.05$. Closed circles represent cell metabolites and open circles represent medium metabolites.

the levels of two sugars (glucose and fructose) and two amino acids (carnitine and tryptophan) significantly decreased in a time-dependent manner. Moreover, malic acid in the fresh medium was mostly exhausted at the initial growth stage (<6 h) because it was quickly converted into lactic acid for energy production via malolactic fermentation, a common process in winemaking [17]. Consequently, lactic acid, a main end-product of energy metabolism, was accumulated in the medium at the initial cell growth stage. Lactic acid levels at 6 h was 12 times higher than that in fresh medium, but further accumulation was not observed until 24 h. As observed with lactic acid production, the accumulated arginine and tyrosine in the medium at the initial cell growth stage were not decreased until 24 h, although it was reported that *L. sakei* can use arginine via the arginine deiminase pathway, leading to ATP synthesis regardless of cell growth [18, 19]. Furthermore, tyrosine can be converted to biogenic amine via the tyrosine decarboxylase activity of LAB [20]. Interestingly, mannose, which was almost undetectable before 12 h, accumulated at 12 h and then decreased at 24 h. Mannose might be produced as an intermediate compound of

mannitol biosynthesis via the hydrogenation of fructose during cell growth [17].

In contrast to their levels in media, the levels of some sugars (trehalose, talose, inositol, and triose phosphate), amino acids (phenylalanine, lysine, and carnitine), citric acid, and AMP accumulated in the cell during cell growth, but the accumulated levels of trehalose, citric acid, and lysine decreased at 24 h. Inversely, phosphoric acid was significantly decreased with increasing growth time. An elevated AMP concentration and a decreased phosphoric acid concentration could lead to decrease of energy production in the cell during the log and stationary phases because AMP concentration within the cell is inversely related to ATP concentration. In addition, phosphoric acid is directly related to substrate-level phosphorylation for energy production. To confirm this, the change in celluar ATP content during cell growth was determined. ATP (7 pM) at 6 h was significantly decreased to 4 pM at 12 h and then slightly increased to 6 pM at 24 h. Similar results reported in *L. rhamnosus* show that the amount of ATP per cell decreased dramatically during the exponential growth phase [18].

Low energy production caused by exhaustion of the main energy sources could lead *L. sakei* to utilize alternative energy sources such as citric acid to make energy. Citric acid is accumulated until the stationary phase, and is generally converted into pyruvate to assist with energy production. Sequentially, under aerobic or anaerobic conditions, pyruvate produced from citric acid in LAB can be further converted to different end-products such as lactic acid, formate, acetate, ethanol, and the C4 aroma compounds (diacetyl, acetoin, and 2,3-butanediol) known as the most important factors for evaluating the flavor of wine, beer, and sausage as well as dairy products [17, 21, 22]. However, these end-products, except lactic acid, were not observed in this study.

In conclusion, MS-based metabolomic analysis of *L. sakei* and its growth media showed that the profiles of monosaccharides, organic acids, amino acids, and adenosine derivatives associated with energy production were significantly changed during cell growth. Glucose, fructose, carnitine, tryptophan, and malic acid in the growth medium served as the primary energy sources during the initial growth stage, but after the exhaustion of these energy sources, *L. sakei* can use other sources such as trehalose, citric acid, and lysine in the cell. The change in the levels of these energy sources corresponded with energy production. The elevated ATP concentration at the initial stage decreased at the exponential growth stage via a decrease of primary energy sources. A slight increase at the stationary phase using secondary sources was also observed. Although further studies on the cellular functionalities of many metabolites and the validation of the proposed pathway using molecular and enzymatic tools are needed, our findings allow enhanced understanding of the growth characteristics, especially energy production, according to the growth phase of *L. sakei*, the most important bacterium associated with meat and vegetable fermentation.

Acknowledgments

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