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Global metabolomic analysis of blood from mice infected with *Brucella abortus*

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ABSTRACT. To better understanding *Brucella abortus* infection, serum metabolites of *B. abortus*-infected and -uninfected mice were analyzed and twenty-one metabolites were tentatively identified at 3 and 14 days post-infection (d.p.i.). Level of most lysophosphatidylcholines (LPCs) was found to increase in infected mice at 3 d.p.i., while it was decreased at 14 d.p.i. as compared to uninfected mice. In contrast, acylcarnitines were initially reduced at 3 d.p.i then elevated after two-weeks of infection, while hydroxysanthine was increased at 14 d.p.i. in infected mice. Our findings suggest that the significant changes in LPCs and other identified metabolites may serve as potential biomarkers in acute phase of *B. abortus* infection.

KEY WORDS: *Brucella abortus* infection, lysophosphatidylcholine, metabolomics profiling, serum, ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry

Brucella spp. are facultative, non-spore producing intracellular bacteria residing in animal host and causing brucellosis. *Brucella* proliferates inside professional phagocytes and hampers their functional activation [13]. In order to successfully replicate within dendritic cells, *Brucella* relies on its VirB type IV secretion system that allows them to escape from lysosomal killing. As differences in factors required for implementing chronic infection *in vivo* and *in vitro* were identified, distinct cell populations and metabolic state may account for the persistent infection *in vivo* [4].

Diagnosis of human brucellosis using serum has been suggested as a reliable approach with the maximum specificity [15]. Classical approaches such as the Rose Bengal test, agglutination test and enzyme-linked immunosorbent assay have been used for serological diagnosis and remained valuable in clinical practice in many parts of the world [1]. Cross reactivity of *Brucella* positive samples, however, with other bacterial infection [3] implies the need of developing new diagnostic methods with high specificity, affordability and accessibility. Earlier-developed fields capable of systemic identification and quantification of biological molecules such as transcriptomics and proteomics have greatly contributed to improving human and animal health. Immunoproteomics analysis has paved the way for identifying immunodominant antigens in *B. abortus*-challenged animals contributing to generation of potential effective vaccines [10]. On the other hand, transcriptional profiling by microarray analysis of both murine macrophages and *Brucella* has suggested a significant number of genes involved in *B. abortus* intracellular growth that may provide insights to better understand host-*Brucella* interaction [7]. However, metabolomics profiling regarding *Brucella*-host interaction remains limited, although metabolomics has effectively served as a reliable platform for numerous fields of study [2].

Therefore, in the study, metabolomics profiles of serum collected from *B. abortus*-infected mice were analyzed to better understand *Brucella*-host interaction.

The *B. abortus* 544 strain (ATCC 23448) was cultured in Brucella broth (BD Biosciences, San Jose, CA, USA) at 37°C for 48 hr to reach stationary phase. Female ICR mice (Samtako Bio Co., Ltd., Osan, Korea), arrived as 7-week-old, were randomly picked up to make two groups (8 mice each). The animals were acclimatized with the surrounding environment for one week before either intraperitoneally infection with 2×10^4 colony-forming units (CFUs) of *B. abortus* or phosphate buffered saline (PBS). Food and water were given *ad libitum*. Blood collection from tail vein was performed at 3 and 14 days post-infection (d.p.i.) which belong to acute phase of *Brucella* infection [6]. Serum samples were obtained after centrifugation at 2,000 × *g* for 5 min at 4°C then stored at −70°C until needed. The animal study was authorized by Animal Ethical Committee of Chonbuk National University (Authorization Number CBNU-2018-101).

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Blood metabolites from *Brucella*-infected or uninfected mice were analyzed as previously described [8] with some modifications. Briefly, blood proteins were precipitated with cold methanol. The supernatants were dried using a Centrivap Speedvac concentrator (Labconco Co., Kansas City, MO, USA). The residues were resolved by 20% methanol with terfenadine and abscisic acid as internal standards. After centrifugation, sample supernatants were analyzed by ultra-high performance liquid chromatography-quadrupole-time-of-flight (UPLC-Q-TOF) mass spectrometry (MS) (Xevo, Waters, Milford, MA, USA). An Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters) filled with blood metabolites extracts was equilibrated with 0.1% formic acid in water. The metabolites were then eluted with a gradient from 1 to 100% acetonitrile containing 0.1% formic acid. The eluted metabolites were analyzed using Q-TOF MS with a positive electrospray ionization mode. The capillary and sampling cone voltages were set at 2.5 kV and 20 V, respectively. The cone gas and desolvation flow rate were 30 l/hr and 900

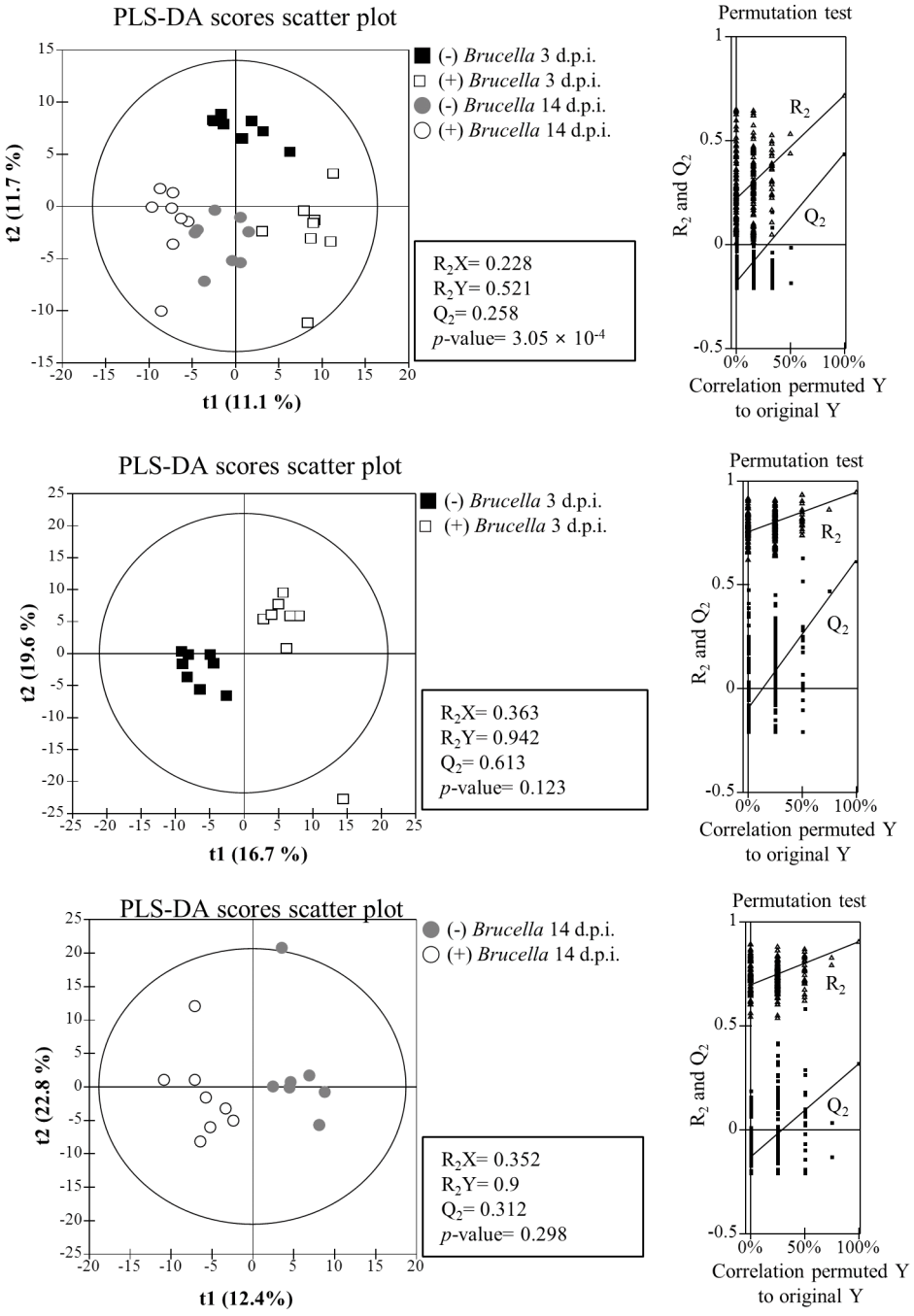

Fig. 1. Partial least-squares discriminant analysis (PLS-DA) score plots of blood metabolites for ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF MS) data at different collection times. The quality of the PLS-DA models was evaluated by R_2X, R_2Y, Q_2, and *P*-values and validated by 200 permutation tests.

Table 1. Identification of major metabolites contributing to the separation among sample groups on the partial least-squares discriminant analysis (PLS-DA) scores plot of the data set analyzed by ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF MS)

No.	RT (min)[a]	Compound	Extact mass (M+H)	MS fragments	VIP[b]	*P*-value[c]
1	1.09	Hydroxyxanthine	169.04	141, 124	1.20	1.58E-04
2	3.26	Unknown	362.20	344	0.96	3.06E-02
3	6.3	Linoleoylcarnitine	424.34	85	1.89	5.13E-06
4	6.38	Lpc (c14:0)	468.31	450, 184, 104	1.41	5.91E-03
5	6.41	Lpc (c20:5)	542.32	524, 184, 104	2.00	1.38E-06
6	6.49	Lpc (c18:3)	518.32	184, 104	1.96	2.04E-06
7	6.51	Palmitorylcarnitine	400.34	85	1.92	6.31E-06
8	6.57	Lpc (c16:1)	494.32	476, 184, 104	1.72	2.63E-04
9	6.58	Vaccenylcarnitine	426.36	85	1.91	1.76E-05
10	6.67/6.81	LPC (C18:2) 2M	1,039.67	520, 184, 104	1.55	4.55E-03
11	6.72	LPC (C20:3)	526.29	184, 104	1.59	1.33E-03
12	6.73	LPC (C22:6) 2M	1,135.67	568, 184, 104	1.73	2.96E-04
13	6.76	LPC (C15:0)	482.32	184, 104	1.89	9.80E-06
14	6.78	LPC (C20:4) 2M	1,087.67	544, 184, 104	1.84	7.61E-06
15	6.93	LPC (C22:5)	570.36	184, 104	1.87	1.69E-05
16	6.94	LPC (C17:1)	508.34	184, 104	1.66	5.11E-04
17	7.07	LPC (C20:3) 2M	1,091.70	546, 184, 104	1.88	2.40E-06
18	7.3	LPC (C18:1) 2M	1,043.70	522 184, 104	1.79	4.02E-05
19	7.33	LPC (C22:4)	572.37	184, 104	1.82	8.14E-05
20	7.47	LPC (C20:2)	548.37	184, 104	1.30	1.56E-03
21	7.52	LPC (C17:0)	510.36	184, 104	1.59	1.49E-03

[a] RT, retention time. [b] Variable importance in the projection (VIP) values were determined by PLS-DA. [c] *P*-values were processed by ANOVA with Duncan's test.

l/hr, respectively, and desolvation and source temperatures were set to 400°C and 100°C, respectively. The TOF MS data were collected in the range of m/z 50 to 1,500 with a scan time of 0.2 sec. To ensure accuracy and reproducibility of the analyzing system, leucine–enkephalin ([M+H]=556.2771) was used as a reference compound. A quality control composed of the mixture of all samples was analyzed after every ten samples.

The datasets obtained by UPLC-Q-TOF MS were collected, aligned, and normalized by UNIFI® v1.8.1 software (Waters). The peaks were collected using peak-to-peak baseline noise of 1, noise elimination of 6, a peak-width at 5% height of 1 sec and an intensity threshold of 10,000. The collected and aligned data were normalized by each internal standard. Metabolites were identified by online databases, including ChemSpider (www.chemspider.com), human metabolome databases (www.hmdb.ca), and the METLIN database (metlin.scripps.edu).

Processed data sets of blood metabolites were analyzed with multivariate statistics using SIMCA-P+ version 12.0.1 (Umetrics, Umea, Sweden). Partial least-squares discriminant analysis (PLS-DA) was used to visualize the differences among sample groups. The quality of PLS-DA models was evaluated using three parameters (R_2X, R_2Y, and Q_2Y) and validated by permutation tests (n=200). Normalized chromatogram intensities of metabolites were statistically analyzed using one-way analysis of variance (ANOVA) with Duncan's test ($P<0.05$) or *t*-test ($P<0.05$) using SPSS 17.0.0.0 (SPSS Inc., Chicago, IL, USA). Identified metabolites were also visualized in a heat map by R-project. The heat map was drawn with ggplot2 representing the z-score transformed data of identified metabolites in a blue-red color scale, with red indicating a decrease and blue indicating an increase in metabolite levels.

The profiles of blood metabolites from *Brucella*-infected mice were analyzed using UPLC-Q-TOF MS and compared to those of uninfected mice. The differences in blood metabolite profiles of sample groups were visualized by PLS-DA score plots (Fig. 1). The sample groups were clearly separated along the first two-component PLS-DA score plots with statistically acceptable quality parameters ($R_2X=0.228$, $R_2Y=0.521$, and $Q_2=0.258$; *P*-value <0.0003) and cross-validation values (R_2 intercept <0.2 and Q_2 intercept < −0.2) analyzed by the permutation test (n=200). To find the metabolites contributing to these observed differences, the *P*-values of all normalized chromatogram intensities of blood metabolites were analyzed. Twenty-one metabolites [lysophosphatidylcholines (LPCs) (C14:0, C15:0, C16:1, C17:0, C17:1, C18:1, C18:2, C18:3, C20:2, C20:3, C20:4, C20:5, C22:4, C22:5, and C22:6), acylcarnitines (linoelaidylcarnitine, palmitoylcarnitine, and vaccenylcarnitine), and hydroxyxanthine] having significant difference were tentatively identified and VIP values of all identified metabolites were above 1.0, indicating as major metabolites contributing to the separation on the PLS-DA (Table 1).

The normalized chromatogram intensities of all the identified metabolites were relatively compared (Fig. 2). Interestingly, at 3 d.p.i., the levels of most blood LPCs were increased during *Brucella* infection, but at 14 d.p.i., their levels were lower except LPC (14:0 and 20:2) than those of uninfected mice suggesting a potential complicated role of the phospholipid in initiating immune

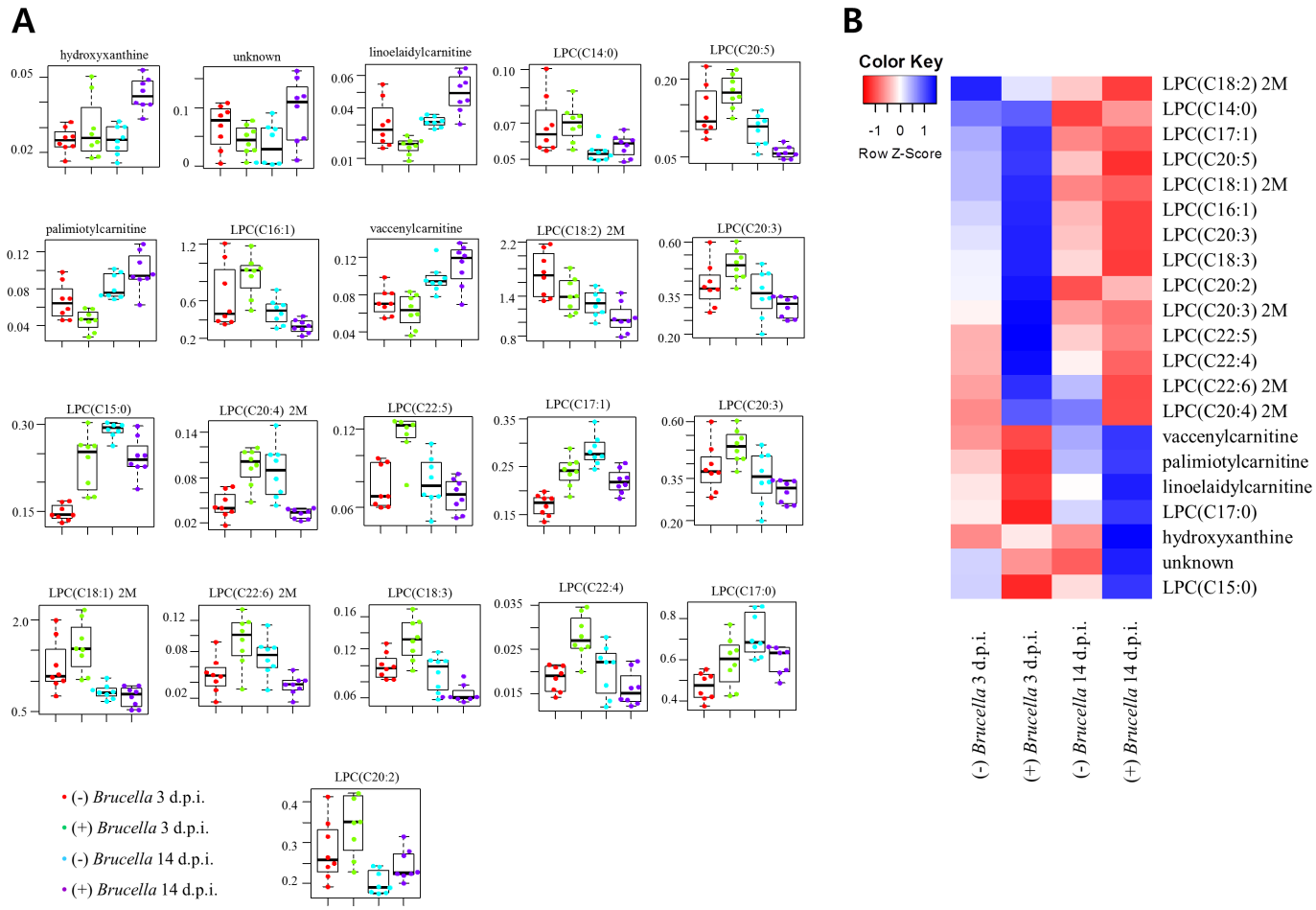

Fig. 2. Normalized chromatogram intensities and heat map of identified blood metabolites analyzed using ultra-high performance liquid chromatography-quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF MS). (A) Chromatogram intensity of detected metabolites was normalized with a proper internal standard. (B) The heat map was drawn by R with ggplot2 and the blue-red color shows the z-score transformed raw data of blood metabolites with significant differences among *Brucella*-infected and uninfected groups. Red and blue colors represent a decrease and an increase of metabolite level, respectively.

responses upon *B. abortus* infection. In contrast to LPCs, the levels of acylcarnitines were reduced during *Brucella* infection at 3 d.p.i. then showed to increase at 14 d.p.i., while an increased hydroxysanthine level was only observed at 14 d.p.i. in *Brucella*-infected group. Acylcarnitines are also suggested to possess immunomodulatory function [12] indicating pathology of murine brucellosis possibly associated with changes in LPCs and acylcarnitines release by host cells.

Metabolic shift is a prominent feature of *B. abortus* infection in human macrophage-like cells suggesting that *Brucella* invasion may disrupt or interfere with host metabolism, and likely enable metabolic modification in the bacteria itself [5]. Therefore, identification of metabolic differences between infected and uninfected host may suggest alternatives to upgrade diagnosis tools. Importantly, as products of body metabolism, the type and concentration of metabolites may infer the involvement of defined or undefined pathways associated with *Brucella* pathogenesis from which further examinations may provide insights into host-pathogen interactions for subsequent designing of efficient novel treatment, vaccine as well as diagnosis. In the present study, we observed that LPCs are among the groups of detected metabolites contributing to the separation between *Brucella*-infected and -uninfected animals contributing to the separation between *Brucella*-infected and uninfected animals. LPC, an endogenous phospholipid, is generated by the action of phospholipase A2 during low density protein oxidation. LPC was found to decrease during early *Acinetobacter baumannii* infection in mice, and its therapeutic efficacy against severe infections was associated with an elevation of the anti-inflammatory interleukin (IL)-10 that suppresses pro-inflammatory cytokines [14]. As other bacterial infection and viral infection may also exert significant changes in LPCs level, it is likely that the dynamics of detected LPCs in our study may not be specific to *B. abortus* infection. Study on LPCs and carnitines dynamics to determine whether these metabolites can also be found in other *Brucella* infection models such as human and cattle is encouraged to consolidate our findings. Additionally, LPC was suggested to promote phagosome maturation–a major killing mechanism of macrophage against intracellular pathogens [9] including *Brucella* spp. The metabolism of acylcarnitines stabilizes intracellular sugar and lipid metabolism by a series of reactions involving three components transporting system such as carnitine palmitoyltransferas I (CPTI),

the carnitine acylcarnitine translocase and CPTII [11]. Acylcarnitines are capable of inducing cyclooxygenase-2 expression and secretion of a number of pro-inflammatory cytokines in culture macrophages suggested to be dependent on TLR4/MyD88 signaling pathway [12].

Overall, findings in this study suggested a number of compounds which may potentially serve as biomarkers for murine brucellosis detection during the acute phase of infection. Nonetheless, further work remains significant. Specifically, animals' age, sex, genetics and infections may invoke wide range of heterogeneity, thus affecting metabolic status. Furthermore, how major detected metabolites contribute in the differences among *B. abortus*-infected and uninfected groups such as LPC and acylcarnitines affect host immune responses and dictate disease outcome requires further investigation.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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