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Original Article

1H NMR-based metabolomics approach to understanding the temperature-dependent pathogenicity of *Lactococcus garvieae*

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Abstract:
Lactococcus garvieae is known as main agent of the bacterial diseases, Lactococcosis in trout farms. The present study was aimed to study the metabolic bases of the temperature-dependent pathogenicity of the *L. garvieae* using ^{1}H NMR spectroscopy. The bacteria were grown at different temperatures, including 10, 14, 18 and 22˚C and then the metabolites extracted, identified and quantified. The results of PLS-DA analysis clearly separated the experimental treatments. The main metabolites responsible for this separation were acetate, acetoacetate, creatine phosphate, succinylacetone and trehalose. Furthermore, the result of the analysis of variance indicated also significant differences in metabolome content between temperature treatments. The bacteria exposed to higher temperatures showed more concentration of acetate and acetoacetate compared to those grown at 10°C. The concentrations of trehalose were higher in the bacteria grown at 14 and 18°C compared to other temperature treatments. The higher levels of succinylacetone were found in the bacteria exposed to the temperature less than 14°C compared to those grown at 18 and 22°C. The creatine phosphate concentrations increased with temperature, however, a significant decline occurred at 22°C. The levels of isoeugenol, methionine and betaine significantly declined with increase of temperature from 10 to 22°C. Also, the concentration of N-Acetylglutamine significantly raised as the temperature increased from 10 to 22°C. In conclusion, the temperature altered the metabolome of *L. garvie*, which this may be linked to the pathogenicity. The temperature probably affects fermentation, homeostasis, energetic condition and metabolism of amino acids in *L. garvieae*.

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Introduction

The bacteria of genus *Lactococcus* are well-known as agent of a wide spectrum of clinical lesions in aquatic animals, particularly in fish and shellfish (Vendrell et al., 2006). In marine and freshwater aquaculture, the *L. garvieae* (a non-motile, anaerobic and Gram-positive species) has been well-recognized as the most important species of this genus, causing considerable economic losses (exceed approximately 50–80%) in fish farms throughout the world, especially in salmonid farms. In Turkey, *L. garvieae* caused approximately 80% mortality in rainbow trout farms (Diler et al., 2002). Furthermore, this pathogen was identified as responsible for outbreaks (>50%) in rainbow trout production in Australia, South Africa, Japan, Portugal, France, Taiwan, England and countries of the Mediterranean area (Baeck et al., 2006; Eyngor et al., 2004; Pereira et al., 2004). *Lactococcus garvieae* is the etiological agent of Lactococcosis in fish with clinical signs, including anorexia, melanosis, lethargy, loss of orientation, erratic swimming, exophthalmia, accumulation of ascitic fluid in peritoneal cavity, surface (in the base of fins, opercula-bucal region) and internal hemorrhages (in the swim bladder, intestine, liver, spleen and kidney) and also necrosis in the liver and spleen (Vendrell et al., 2006). Based on epidemiological studies, the clinical signs of *L. garvieae* are temperature-dependent and usually emerged in fish when water temperature increases over 16°C in summer months (Eldar and Ghittino, 1999; Soltani et al., 2008; Vendrell et al., 2006).

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However, to our knowledge, there is no comprehensive information regarding the mechanisms underlying the temperature-dependent pathogenicity of this pathogen.

In the present study, we used for the first time a ^{1}H-NMR based metabolic method to investigate the molecular bases of the temperature-dependent pathogenicity of the *L. garvieae*. Metabolomics is known today as a new and growing approach to study low molecular mass metabolites (MTs) (<500 Da) within a cell, tissue, or biofluid of an organism (Bundy et al., 2009; Lankadurai et al., 2013; Nicholson et al., 1999). The structure and function of almost all MTs have been well-recognized, which this makes them easier to interpret compared to genomic and proteomic information. Furthermore, MTs are more stable during sample preparation, enhancing the accuracy of metabolic data in comparison with proteomic ones (Ankley et al., 2006; Lankadurai et al., 2013; Van Ravenzwaay et al., 2012). NMR based metabolic is one of the most important methods to analyse metabolome. Nuclear magnetic resonance (NMR) is nondestructive, nonselective and laboratory reproducible (Pan and Raftery, 2007; Robertson, 2005; Viant et al., 2008). These advantages have made NMR as a good tool to assay metabolites within a biological sample (Åslund et al., 2011; Ritota et al., 2012; Simpson and McKelvie, 2009; Verpoorte et al., 2008). The results of the present study provide comprehensive data on temperature-dependent changes of metabolites in *L. garvieae*. The study also may help us to understand the molecular bases of the bacterial pathogenicity and subsequently to find solutions for reducing the pathogenicity using metabolic information.

Materials and Methods

Bacterial strain and preparation: A previously proven strain of *L. garvieae* was provided from faculty of veterinary sciences, Shahrekord University, Iran. The bacteria were initially inoculated into Trypticase Soy Agar (TSA) (Merck, Germany) and incubated at temperatures of 10, 14, 18 and 22°C for 24 h. After incubation, the bacteria were separately dissolved in distilled water to assay optical density (1-1.5) by spectrophotometer. 1.5 ml of the suspension was grown in 15 ml Tryptic Soy Broth (TSB) for 24 h. The culture was regenerated using 4 ml of the suspension in TSB (96 ml) for 14 h. After that, the suspension was centrifuged (8000 rpm) at 8°C for 1.5 min and then 0.5 ml of deposit was separately inoculated in 45 ml TSB at 10, 14, 18 and 22°C for 24 h. After incubation, 15 ml of liquid nitrogen was added to each medium to reduce the metabolism in bacteria. The suspensions were then centrifuged (12000 rpm at 4°C) for two times, followed with washing by 10 ml and 1 ml NaCl solution respectively. Eventually, the bacterial was centrifuged (16100 rpm at 4°C for 1 min) and lyophilized overnight at -41°C and stored in liquid nitrogen until metabolite extraction (Boroujerdi et al., 2009).

Metabolite extraction: The metabolites of bacteria were extracted using 2:1 (v/v) MeOH:H_2O at a constant cell mass/solvent ratio of 9 mg (dry weight) of lyophilized cells and 0.5 mL of MeOH:H_2O according to Boroujerdi et al. (2009). The solutions were vortexed gently and then centrifuged at 11000 g for 6 min at 8°C to eliminate cell debris. Finally, the supernatants containing metabolites were dried by a vacuum centrifuge drier (Eppendorf Vacufuge, Westbury, NY) for 150 min at 30°C to obtain concentrated extracts. The extracts were then stored in liquid nitrogen (-196°C) until nuclear magnetic resonance (NMR) assay.

^{1}H NMR assay: ^{1}H NMR assay was carried out based on the method described by Boroujerdi et al (2009) with some modifications. In brief, 500 MHz NMR spectrometer were used (BrukerBiospin. 165 Corporation, Billerica, MA, USA). The bacterial extracts were resuspended in 600 µl NMR buffer [60 µL internal standard solution (5 mM sodium 2,2 dimethyl-2-silapentane-5-sulfonate (DSS) (as internal reference) in 99% deuterium water (D_2O) +540 µL of 0.2 M sodium phosphate buffer (pH 7.0) containing 0.018% sodium azide (NaN3)] and then transferred into a 5-mm NMR tube (Wilmad, Buena, NJ). The Carr-Purcell-Meiboom-Gill (CPMG) technique was used to block signals related to proteins and other

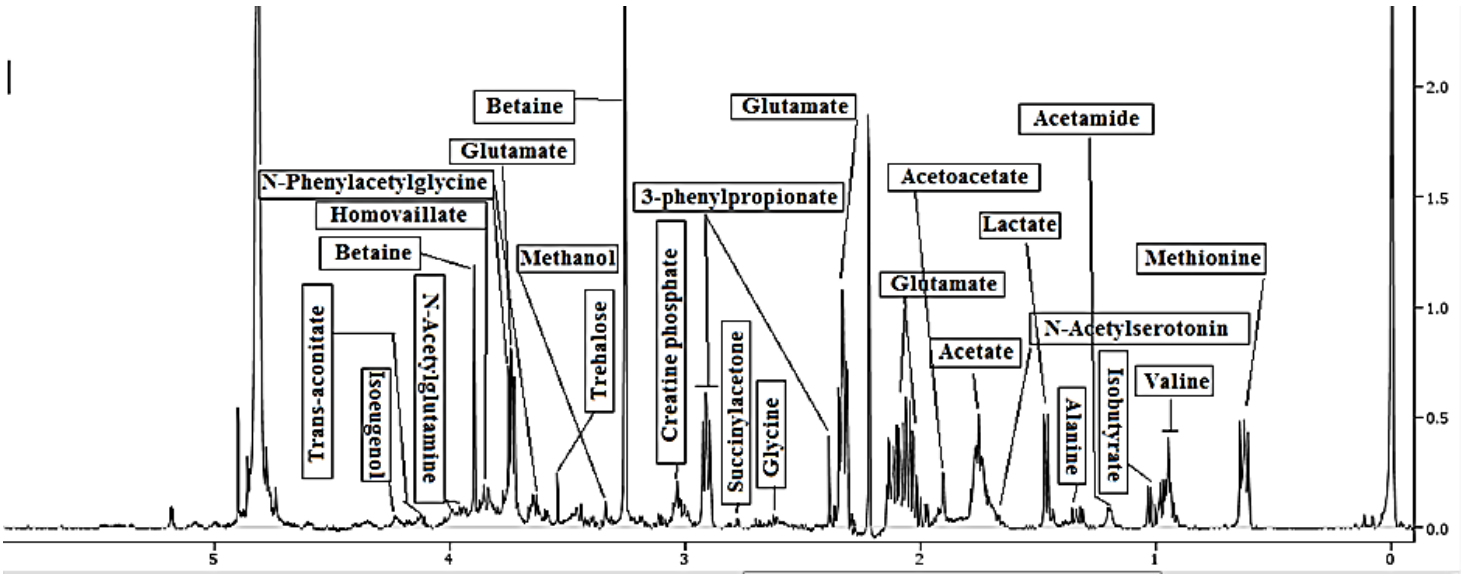

Figure 1. Representative 500 MHz 1H NMR spectrum of the bacterial metabolome (*Lactococcus garvieae*).

macromolecules (Tian et al., 2013). Free Induction Decays (FIDs) were obtained with 150 scans into 32K data points, using a spectral width of 8389.262 Hz with a relaxation time of 1.5s, an acquisition time of 4s, and a mixing time of 400 ms. NMR spectra (NSPs) were analyzed by Chenomx NMR suite (CHNS) ver. 7.6 software (Chenomx Inc., 172 Edmonton, AB, Canada). The NSPs were firstly phased and baselined by the Processor program and then metabolites identified and quantified using Profiler program.

Data analysis: Principal Component Analysis (PCA), followed by Partial Least Squares Discriminant Analysis (PLS-DA), which converts multidimensional data into smaller model, was used for analysis of metabolome data (Keun et al., 2003; Xu and Shao, 2004). Before PCA and PLS-DA analysis, NMR datasets were normalized using log transformation and autoscaling options. All above analyses were conducted using MetaboAnalyst, a comprehensive Web-based tool designed for processing, analyzing, and interpreting metabolomic data (Xia and Wishart, 2011).

The analysis of variance (ANOVA) and comparison of means between temperature treatments was performed by SPSS software (SPSS 19.0, IBM software, Inc., Chicago, IL, USA). Before analysis, the normality of data was evaluated by Kolmogorove-Smirnov test. After calculating significant F-ratios by ANOVA, the Tukey test was used to identify which groups were different (P<0.05). All data were presented as mean and standard deviation (SD). The P<0.01 was used as significance level for data evaluation.

Results

Representative ^{1}H NMR spectra of the bacterial metabolome are shown in Figure 1. Totally 23 metabolite were identified (Fig. 1). The scores plot obtained from PLS-DA clearly confirmed the effects of different temperature treatments on endometabolites of the *L. garvieae*, because the bacteria incubated at 18 and 22°C showed significant overall separation from those grown at 10 and 14°C (Fig. 2). The main metabolites responsible for this separation are indicated by corresponding loading plot (Fig. 3). 12 metabolites contributed more in the separation of temperature treatments according to VIP scores obtained from PLS-DA (Fig. 3). In this regard, the more significant contributions were related to acetate, acetoacetate, creatine phosphate, succinyl-acetone, trehalose (Fig. 3).

The result showed also significant differences in metabolome content between temperature treatments (Fig. 4A-E, P<0.05). The concentration of acetate and acetoacetate were lower in the bacteria exposed to 10°C compared to other temperature treatments (Fig.

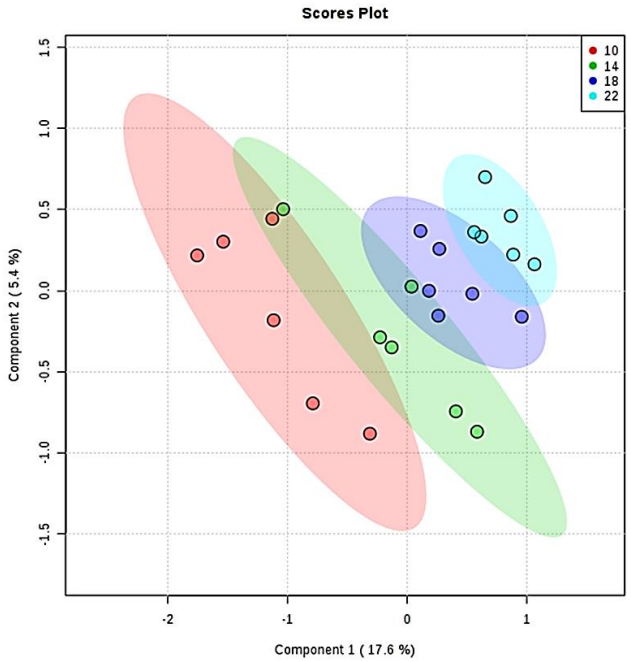

Figure 2. Partial Least-Squares Discriminant Analysis (PLS-DA) of the bacterial metabolome (*Lactococcus garvieae*) at different inoculation temperatures.

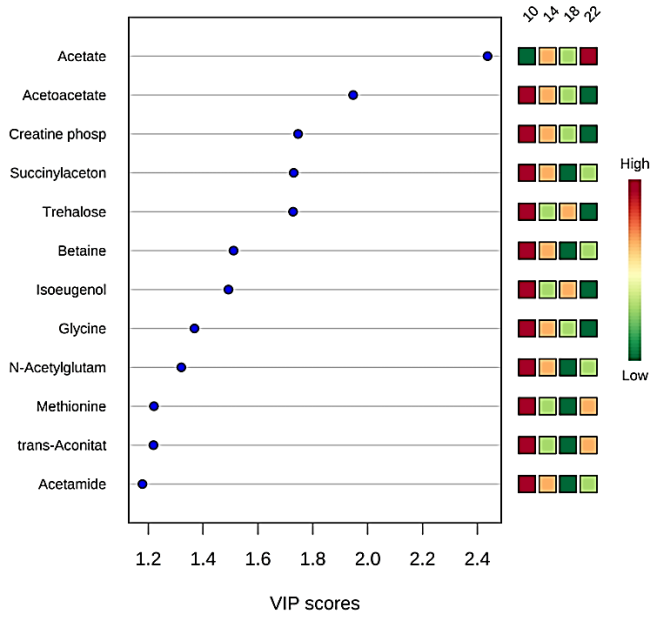

Figure 3. Main metabolites identified by PLS-DA. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

4A, $P<0.01$). The levels of trehalose were higher in the bacteria treated with 14 and 18°C compared to other treatments (Fig. 4A, $P<0.01$). The higher concentrations of succinylacetone were observed in the bacteria exposed to the temperatures less than 14°C compared to those treated with the temperatures of 18 and 22°C (Fig. 4B, $P<0.01$). The creatine phosphate (Cr) levels increased, as the temperature reached 18°C (Fig. 4B, $P<0.01$). However, we found a considerable decline in Cr concentrations at 22°C (Fig. 4B, $P<0.01$). The concentration of isoeugenol (Fig. 4B), methionine (Fig. 4D) and betaine (Fig. 4E) significantly declined with increase of temperature from 10 to 22°C ($P<0.01$). The N-Acetylglutamine levels significantly elevated as the temperature increased from 10 to 22°C (Fig. 4C, $P<0.01$). Also, There were no significant differences between temperature treatments in terms of the levels of aconitate and N-Acetylglutamine (Fig. 4C, $P<0.01$).

Discussions

It is well-recognized that the pathogenicity of *L. garvieae* increases with raises in water temperature, especially when fish are exposed to the temperature higher than 16°C (Eldar and Ghittino, 1999; Soltani, et al., 2008; Vendrell et al., 2006). The present study was carried out to investigate the temperature-dependent pathogenicity of *L. garvieae* from a metabolic point of view.

The results of PLS-DA analysis showed significant separations between experimental groups, indicating the effects of temperature on the metabolome content of the bacteria. The metabolites, including acetate, acetoacetate, trehalose, succinylacetone, creatine phosphate, isoeugenol, betaine, glycine, N-Acetylglutamine, trans-aconitate, acetamide and methionine had more contribution in this separation based on VIP scores.

Acetate and acetoacetate are known as ketone bodies in biological systems and produced during the oxidation of the lipids for meeting energetic demands of cells (Newman and Verdin, 2014). In anaerobic bacteria, it is recognized that acetate is produced as a result of the fermentation of organic matters including fatty acids (Fujita et al., 2007; Ljungdhal, 1986; Mah et al., 1977). Furthermore, acetate along with formaldehyde, butyrate, lactate and succinate were found to be the final product of Lactic acid bacteria (Gottschalk 2012). However, we could not found any data regarding the metabolism of fatty acids and

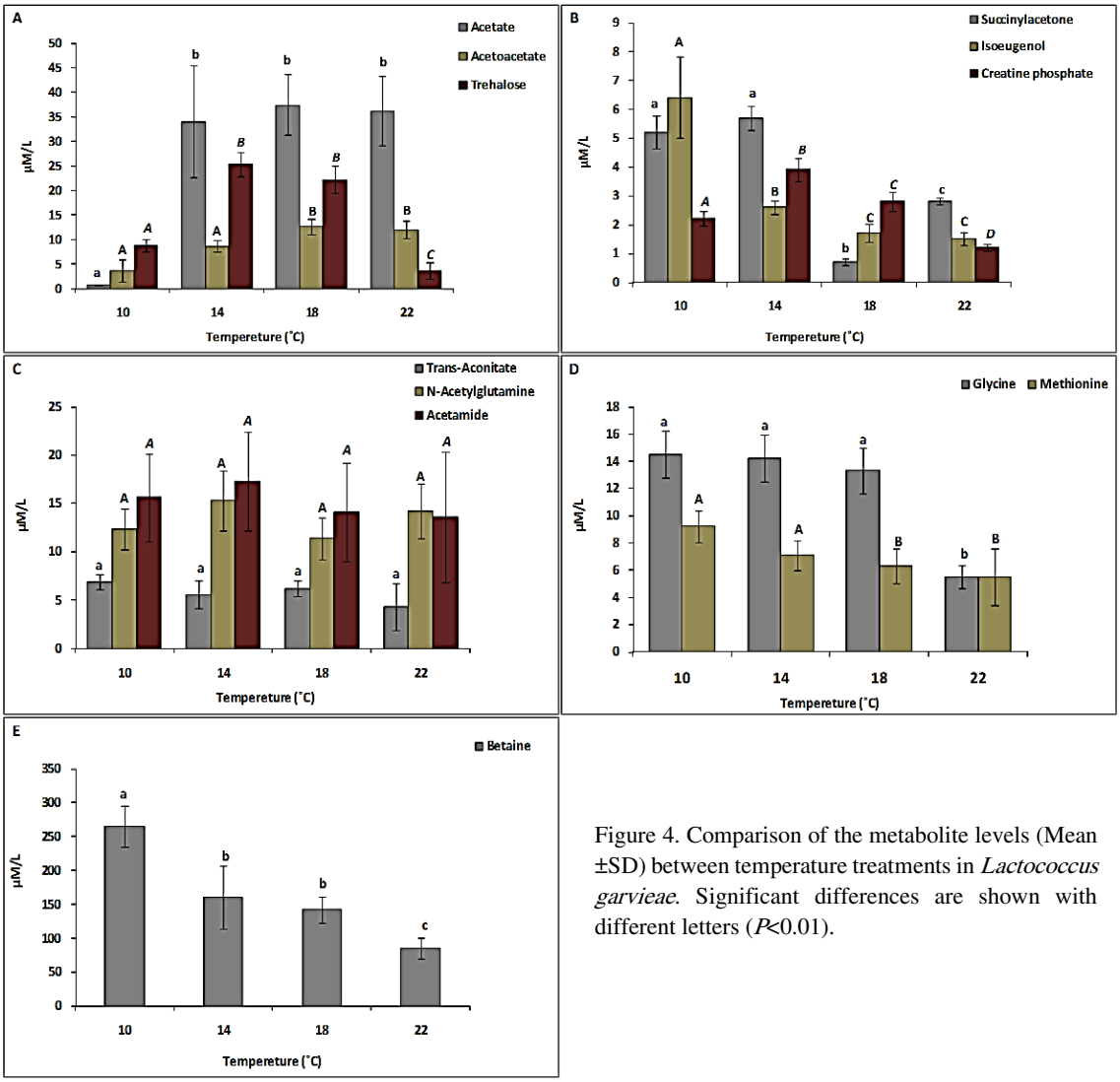

Figure 4. Comparison of the metabolite levels (Mean ±SD) between temperature treatments in *Lactococcus garvieae*. Significant differences are shown with different letters (P<0.01).

related metabolites such as acetate and acetoacetate in *L. garvieae*. In our study, lower levels of acetate and acetoacetate were observed in the bacteria treated with low temperature (i.e. 10°C) compared to other temperature treatments, probably indicating the retarding effects of low temperatures on the bacterial fermentation and subsequently its pathogenicity.

Trehalose, also known as mycose, is a 1-alpha disaccharide which plays important role in hydrobiosis, the ability of organism to withstand prolonged periods of dehydration. Trehalose is found to form a gel phase as cells dehydrate, which prevents the disruption of internal cell organelles by effectively splinting them in position (Filippov et al., 2015). In bacteria, trehalose is found to protect the bacteria against heat shocks and dehydration (Argüelles, 2000; Gomez Zavaglia et al., 2003; Leslie et al., 1995). In the *L. garvieae*, more accumulation of trehalose was observed in the bacteria exposed to temperatures of 14 and 18°C, which may be attributed to physiological response of the bacteria to high temperature. Nevertheless, the levels of trehalose decreased in the bacteria exposed to 22°C, indicating the possible disrupting effects of high temperatures on the accumulation of trehalose.

Succinylacetone is an abnormal tyrosine metabolite that produced as a result of defect in the fumarylacetoacetase, an enzyme responsible for hydrolysis of fumarylacetoacetate into fumarate and acetoacetate (Lerner, 2009). Fumarylacetoacetase is involved in metabolism of phenylalanine and tyrosine (Lerner, 2009). Succinylacetone can also be generated by the oxidation of glycine (Dindo et al., 2018). In the present study, the temperature less than 14°C induced

succinylacetone production, which this may be associated with the disrupting effects of low temperature on metabolism of some amino acids such as tyrosine, glycine and phenylalanine. However, we found no information on metabolism of succinylacetone in bacteria.

In biological systems, creatine phosphate (Cr) is known as an energetic buffer, which reduced to ATP and creatine to meet the immediate needs of cells to energy. Cr also affects the metabolism of arginine and proline (Pastoris et al., 1998). In our study, as the temperature reached the optimum temperature (18°C) of the bacteria, the intracellular concentration of Cr also increased, suggesting an efficient regeneration for Cr at temperatures in which the pathogenicity is high. Nevertheless, at 22°C, we observed a considerable decline in Cr concentrations, which may be related to the adverse effects of high temperature on the energetic homeostasis of the bacteria.

The isoeugenol is known as a natural antimicrobial component and identified in essential oils extracted from many terrestrial plants (Akhtar et al., 2012; Dal Pozzo et al., 2012; Janssens et al., 1990; Zemek et al., 1979; Zemek et al., 1987). The metabolic conversion of phenolic compounds such as isoeugenol has been the focus of many studies in bacteria. For example, the metabolic conversion of phenolic compounds to vanillin has been well-reviewed by Priefert et al. (2001). However, it is well-recognized that lactic acid bacteria are not able to produce vanillin from the components such as isoeugenol (Bloem et al., 2007). In the present study, the intracellular concentration of isoeugenol decreased with temperature, which may be due to the direct destructive effect of temperature on metabolite or metabolic conversion of the isoeugenol to other metabolites at temperatures in which the pathogenicity is high.

Betaine is usually recognized as an osmoprotectant metabolite in organisms including bacteria (Courtenay et al., 2000; Csonka, 1989; Csonka and Hanson, 1991; Roeßler and Müller, 2001). In various bacteria, including lactic acid bacteria (van de Guchte et al., 2002), the accumulation of betaine and other osmolytes have observed against high salinity shocks and other stressors, such as elevated temperature (Caldas et al., 1999). Most bacteria are not capable to synthesize betaine de novo and rely more on uptake from medium (Culham et al., 1993; Haardt et al., 1995; Molenaar et al., 1993; Racher et al., 1999). The results of present study were in contrast with above conclusions on betaine, since we observed declines in intracellular betaine with increases in temperature. These results may be attributed to the species-specific differences in the behavior of bacteria in response to the temperature shocks. In *L. garvieae*, the temperatures more than 16°C are recognized as optimum temperatures for bacterial growth and pathogenicity. Therefore, the concept of temperature shock and its relationship with betaine accumulation may be different in *L. garvieae* in comparison with other bacteria.

N-Acetylglutamine is a modified stable amino acid that used as a source of glutamine (López-Pedrosa et al., 2007; Snowden et al., 2002). The production of N-Acetylglutamine is also reported in bacteria during fermentation (Haran et al., 1983; Kinoshita and Tanaka, 1972; Nakanishi, 1978; Qu et al., 2002). However, we could not find evidences regarding the generation of this amino acid in *L. garvieae*. In our study, the N-Acetylglutamine concentrations showed significant elevations with temperature, indicating the possible effect of temperature on the metabolism of glutamine.

As an essential amino acid, methionine has a main role in the initiation of translation and following biosynthesis of the proteins. Almost all bacterial species possess biosynthetic pathways for methionine (Foster et al., 2005; McCutcheon and Moran, 2012). In the present study, the temperature affected the metabolism of the methionine, because the concentration of this amino acid decreased as the temperature elevated. The reduction in intracellular methionine may be attributed to the metabolic consumption of this amino acid for synthesis of proteins, as the temperature approached the optimum temperatures for growth and pathogenicity of the *L. garvieae*.

In conclusion, the temperature altered the

metabolome of *L. garvie*, which this may be linked to the pathogenicity. The temperature probably affects fermentation, homeostasis, energetic condition and metabolism of amino acids in *L. garvieae*.

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