[1]H NMR-BASED METABOLIC PROFILE OF *LACTOBACILLUS SALIVARIUS* FDB89 UNDER OSMOTIC STRESS

QI, N. L.[1,2†] – GONG, X.[1,3†] – YANG, C. L.[1,2*] – CHENG, Z. H.[1,4] – ZHOU, W.[1,3] – LI, J. H.[1,3*]

[1]*Agricultural Product Processing Research Institute, Chinese Academy of Tropical Agricultural Sciences, Zhanjiang 524001, China*

[2]*Chinese Academy of Tropical Agricultural Sciences Center for Food Quality Supervision and Testing Ministry of Agriculture (Zhanjiang), Zhanjiang 524001, China*

[3]*Key Laboratory of Tropical Crop Products Processing of Ministry of Agriculture, Zhanjiang 524001, China*

[4]*College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430000, China*

**Corresponding author*
e-mail: zjyangcl@126.com (C. L. Yang), foodpaper@126.com (J. H. Li)

[†]*These authors should be considered co-first authors.*

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Abstract. In their natural habitats and application environments, lactic acid bacteria are often exposed to osmotic changes. At metabolic level, bacteria are able to *de novo* synthesize or uptake specific solutes from environment to maintain constant turgor pressure. In the work, the [1]H nuclear magnetic resonance (NMR) spectroscopy based on intracellular metabolites profiles of *Lactobacillus salivarius* under hyper-osmotic growth conditions was characterized. New characteristic peaks of compatible compounds, such as betaine, carnitine, choline and/or their derivatives were detected. Especially, the cytoplasmic concentrations of proline increased dramatically when the osmolarity was raised from about 0.40 to 1.84 osmol/kg by adding 0.8 mol/l NaCl to the medium. Comparative analysis of the metabolites of *L. salivarius* FDB89 grown under aerobic and anaerobic conditions with NaCl stress revealed an overlap of up-regulated compounds of more than 50%. 2-aminobutyrate, pyruvate, glycine, trimethylamine-*N*-oxide, and acetylcholine were detected only under anaerobic condition, and 2-oxoglutarate, tyrosine, glutamine and glutamate were detected only under aerobic condition, which suggested oxygen also has a non-negligible effect on osmotic response in *L. salivarius* FDB89.
Keywords: *Lactobacillus salivarius*, metabolomics, hyper-osmotic stress, quantitative [1]H NMR spectroscopy, principal component analysis

Introduction

Lactobacillus is a genus of gram-positive facultative anaerobic or micro-aerophilic bacteria that produce lactic acid as the predominant end-product during the sugar fermentation. *Lactobacillus* is normally isolated from oral cavity, intestinal tract, vagina and fermented products, and generally recognized as safe (GRAS). As a major part of lactic acid bacteria (LAB) group, they especially have an important role in the development of the organoleptic, nutritional and/or hygienic quality of fermented foods, and are extensively exploited for the health-promoting properties (Machado et al., 2004; Shellhammer et al., 2017). *Lactobacillus salivarius* FDB89 used in this study, was isolated from the healthy centenarian's fecal samples from the volunteers inhabited in Bama longevity villages in China (the fifth longevity area in the world), and was

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confirmed to be able to convert the carcinogen 4-nitroquinoline-*N*-oxide to a less toxic compound and its geno-toxicity is significantly inhibited in previous study (Zhao et al., 2013), which highlights its potential probiotic use.

As we know, microorganisms are often exposed to hyper-osmotic environments generally produced by high concentrations of common salt or sugar used in food processing, which has been accepted as a desired and effective preservation method to suppress spoilage of microbes (Tsakalidou et al., 2011). However, high-osmolarity might initially result in plasmolysis, loss of turgor, shrinkage of the cell, and further elevate reactive oxygen species (ROS), cause cytoskeleton rearrangement, which increases DNA strand breaks and oxidation of proteins and DNA bases. It is interesting that cells could accommodate by accumulating osmoprotectants or increasing the expression of relative shock proteins, and the failure to adapt usually results in cell apoptosis (Burg et al., 2007). Osmotic stress response of some microorganisms (e.g., *Escherichia coli*, *Salmonella typhimurium*, *Lactococcus lactis*, and *Lactobacillus plantarum*) have been studied intensively by genetic, proteinic, physiological, and biochemical methods (Glaasker et al., 1996; Hoffmann et al., 2013; Meadows and Wargo, 2015; Métris et al., 2016; Rep et al., 1999; Teo et al., 2009). But the osmotic response studies at metabolic level are limited. And it was found that different microorganisms may have different response mechanisms. Bacteria reduced the deleterious osmotic effects by uptake or synthesis of some compatible solutes including amino acids (glutamine, glutamate and proline) and their derivatives (peptides, glycine-betaines and *N*-acetylated amino acids), polyols (glycerol) and/or sugars (trehalose and sucrose), which are not inhibitory to most cellular processes, but rather stabilize the biological structure and function of macromolecules (Hoffmann et al., 2013; León et al., 2018).

Nuclear magnetic resonance (NMR), as an important non-targeted analytical techniques, enables to rapidly produce reliable, reproducible, and unbiased metabolite profiles of each sample, and has been widely applied in analyzing metabolic networks and distinguishing biopolymers (Pinto et al., 2014; Tomita et al., 2018).

In this work, we aimed at employing ^{1}H NMR technique combined with principal component analysis (PCA) approach to evaluate the metabolic response of *L. salivarius* to osmotic stress (under aerobic and anaerobic condition) and indicate potential biomarkers that may provide comprehensive information of osmo-adaptation on the microbial fermentative behavior.

Materils and methods

Bacterial strain and growth conditions

L. salivarius FDB89 (*L. salivarius* subsp. *salivarius*) was provided by culture collection of MOE Key Laboratory of Functional Dairy (China Agriculture University, China). Two kinds of culture mediums incluing control (MRS medium) and hyper-osmotic medium obtained by adding NaCl (final concentration 0.8 mol/l) to the basal MRS were used. The changes of physiochemical characteristics in aerobic and anaerobic condition for hyper-osmotic medium was compared with control (only aerobic condition). The strain was sub-cultured twice in deMan-Rogosa-Sharpe (MRS) medium for 12 h at 37°C prior to experimental use, 20 ml of cell suspensions in 100 ml flasks were incubated at 120 rpm under aerobic conditions as control. For typical anaerobic growth condition in hyper-osmotic medium, cells were cultivated in Hungate

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tube previously excluding oxygen with high purity nitrogen at 37°C without shaking. For each treatment, the assay was repeated at least three times.

Growth and pH assays

Samples were taken at two-hourly intervals for 24 h. The absorbance at 600 nm (OD_{600}) was determined for growth curve of *L. salivarius* FDB89 (Michael et al., 2010). And the pH was measured after calibration of the pH meter (Sartorius, Goettingen, Germany) with standardized pH buffer solutions 4.01 and 7.00 (Hamilton, Switzerland) prior to the analysis.

Preparation of intracellular extract

Stationary phase cells were carefully micro-filtrated to avoid lysis and washed for twice with physiological saline solution to remove any remaining nutrient solution. The sediment was quickly transferred to a clean Eppendorf tube and quenched immediately into liquid nitrogen. The whole process should be accomplished within 2 min (De Angelis and Gobbetti, 2011).

A biphasic system was performed to extract the intracellular metabolites with some modifications (Aliferis and Jabaji, 2010; Tomita et al., 2017). Briefly, 2.0 ml of cold methanol/chloroform (1:1, v/v) was added to the tube containing the cells and fully vortexed for 60 s, then 1.0 ml of Milli-Q water (0°C) was added, sonicated for 2 min and centrifuged at 8944 × *g* for 5 min at -20ºC. And 1.5 ml of the aqueous layer was carefully pipetted into a new tube and immediately frozen. The extraction was lyophilized and stored at -80ºC for analysis.

^{1}H NMR data acquisition and processing

Lyophilized extraction were dissolved in 650 µl of 0.1 mol/l phosphate buffer (pH 7.0), in deuterium oxide (D_2O, Norell Inc., USA), containing 0.5 mmol sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP, Sigma-Aldrich, UK)(chemical shift and reference indicator). Additionally, sodium azide (0.2% w/v) was added to preserve the samples for longer storage. Insoluble material was removed by centrifugation at 8944 × *g* for 2 min at 4°C, 600 µl of the supernatant was transferred into a 5-mm diameter NMR tube for determination (Galafassi et al., 2013; Graham et al., 2010).

NMR spectra were acquired at 300 K using a DRX-600 NMR spectrometer (Bruker, Germany, proton frequency 600.13 MHz, 14.1 T) equipped with a 5-mm PATXI probe. One-dimensional solution ^{1}H NMR experiments were done with the following parameters (Galafassi et al., 2013; Li et al., 2018; Nieva-Echevarría et al., 2017): pulse program for water suppression; relaxation delay, 4 s; mixing time (for noesy), 1 s; acquisition time, 2.28 s; number of steady states transients (dummy scans) is 4; number of transients, 256; solvent suppression, pre-saturation with spoil gradient; spectral width is 12 ppm; time domain size of 32 k. Peak-width at half height of TSP was less than 1.50 ± 0.15 Hz (mean ± SD).

All NMR spectra were zero filled to 65, 536 points and applied with an exponential line broadening factor of 0.3 before Fourier transformation, manually phased, baseline corrected and calibrated by the TSP signal at 0 ppm using TOPSPIN 2.1 (Bruker Topspin GMBH, Rheinstetten, Germany). Selected peaks were assigned and quantified using the 600 MHz library from Chenomx NMR suite 6.0 (Chenomx Inc., Edmonton, Canada).

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Principal component analysis

The spectral region between 0.20 and 10.00 ppm of each spectrum was divided into equally sized buckets with a width of 0.04 ppm. The regions of water (between 4.75 and 5.00 ppm), methanol (between 3.35 and 3.37 ppm) and chloroform (between 7.67 and 7.70 ppm) were excluded. Scaled to the total spectrum area of the residual regions, each bucket area was integrated and each spectrum was transferred into a 1 × 238 vector. Principal component analysis (PCA) was performed on processed ^{1}H NMR data with AMIX 3.7.10 (Bruker BioSpin, Rheinstetten, Germany).

Results

Growth of L. salivarius FDB89 under different conditions

The growth rate and final pH were similar for *L. salivarius* FDB89 at NaCl stress with or without oxygen (*Figs. 1* and *2*). The absence of NaCl stress in the growth media resulted in about 1.4-fold increased maximum absorbance of *L. salivarius* FDB89. The result was in agreement with previous study (data not shown) and supported by other reports (Arsenijevic et al., 2013; Varelas et al., 2017). Based on the results, the cell culture in stationary phase was sampled at 12 h for NMR measurement.

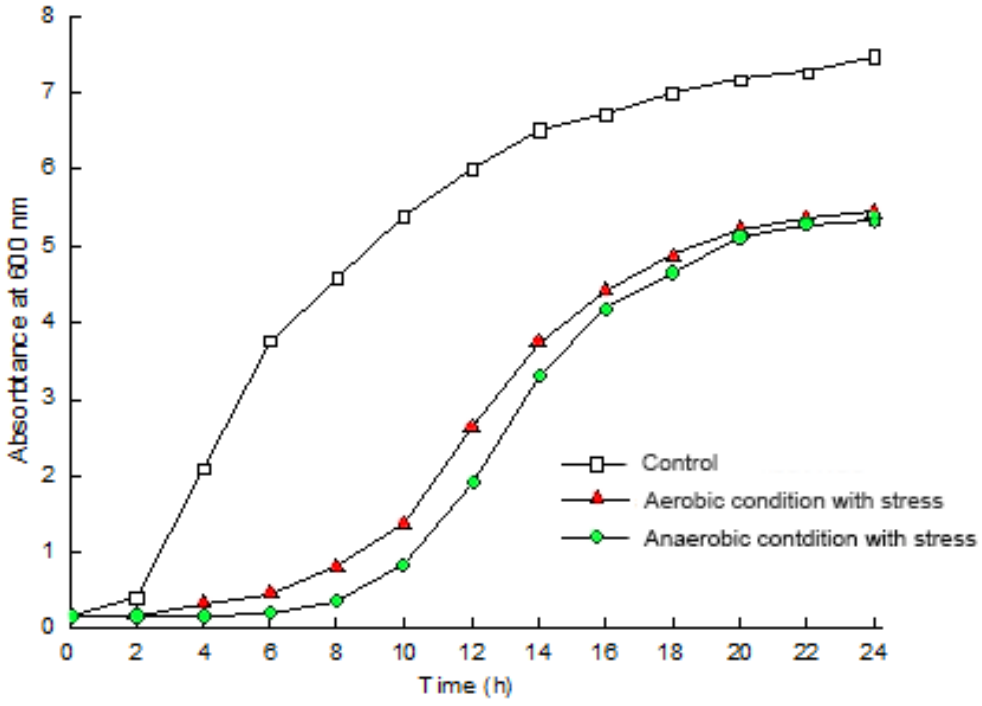

Figure 1. *Growth curves of L. salivarius FDB89 cultivated under different growth conditions*

Visual inspection of ^{1}H NMR spectra and assignment of compounds

Representative ^{1}H NMR spectra are presented in *Fig. 3*, which shows the metabolic profiles of *L. salivarius* FDB89 stationary phase cultivated under normal and NaCl (aerobic and anaerobic) stress conditions. About 44 metabolites were presumptively identified by Chenomx software (*Table 1* in Supplemental Data).

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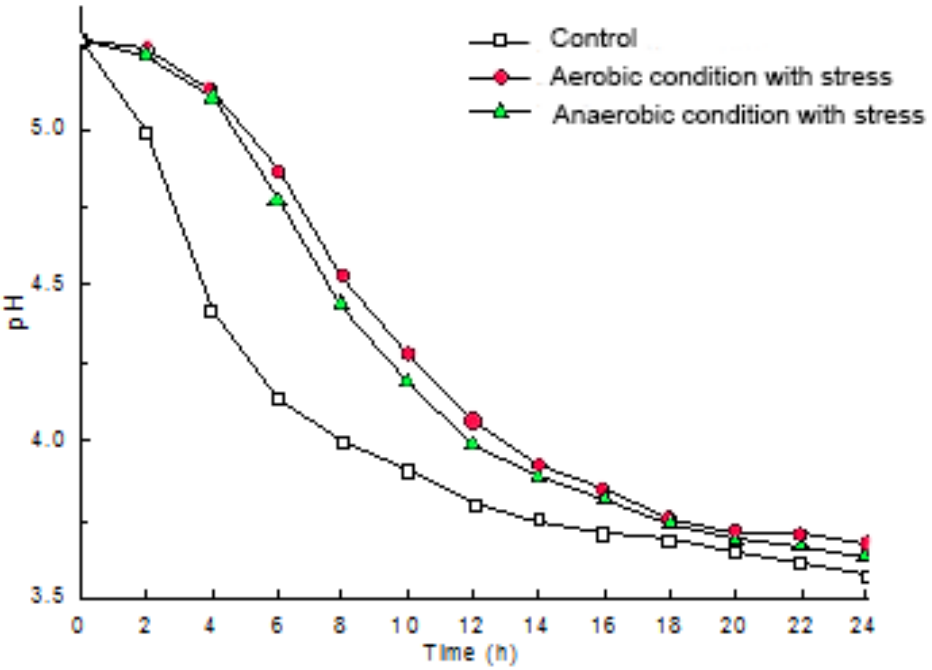

Figure 2. *Changes in pH when L. salivarius FDB89 grown under different culture conditions*

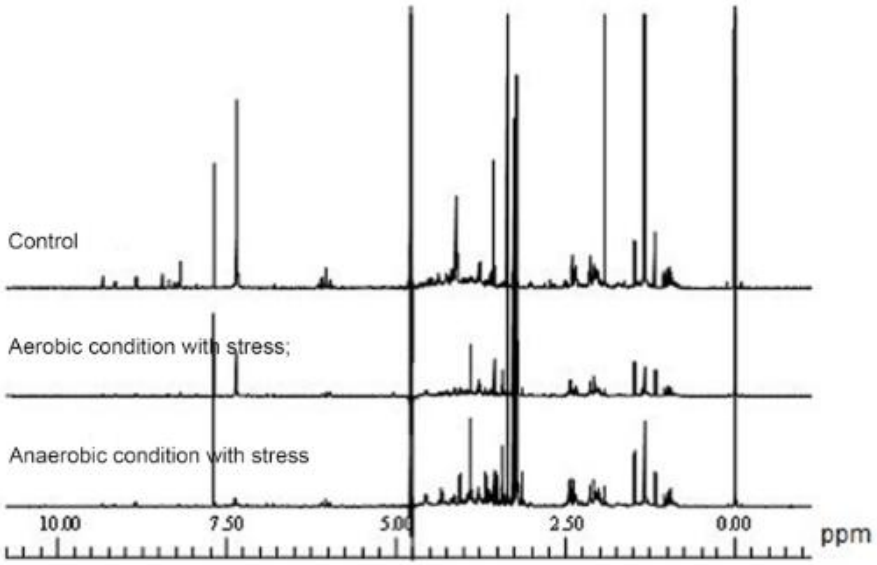

Figure 3. *Representative 600 MHz [1]H NMR spectra of intracellular extracts of L. salivarius FDB89 under different growth conditions*

It was difficult to discern the individual peaks with certainty in the [1]H NMR spectra between approximately δ 1.9-2.5 and δ 3.0-4.5 ppm due to overlapping amino acid and sugar resonances. However, conspicuous differences were observed among the three spectra patterns and peak intensities. In the osmotic spectrum, new peaks of betaine, carnitine, choline and its derivatives (e.g., sn-glycero-3-phosphocholine, phosphocholine, and acetylcholine), and isoleucine, phenylalanine, pyrimidine and

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cyclopentanecarboxylic acid were also observed. Higher peaks of methionine, proline, malonate, and aspartate have been discerned compared to control one. In the spectrum without NaCl stress, the characteristic peaks of isocitrate, arginine, kynurenine, propionate, UDP-glucose and succinate were identified, and the peak intensity of 2-hydroxybutyrate, alanine, glycine, valine, glutamine, acetate, lactate, pyroglutamate, threonine and NAD^{+} increased.

2-Aminobutyrate, pyruvate, acetylcholine and trimethylamine oxidexide were only observed in hyper-osmotic and anaerobic condition. In contrast, tyrosine, glutamine and glutamate were the particular compounds in aerobic condition regardless of NaCl stress. The peak intensity of methanol at δ 3.30 in anaerobic condition is 1.5-fold greater than those of aerobic ones. The results showed different metabolic pathways of *L. salivarius* FDB89 under anaerobic and aerobic conditions. Being a major part in one carbon metabolism, more methanol was observed in anaerobic condition, it might be used as energy and carbon skeleton in this condition.

As a rapid and non-targeted method, NMR technique provides information about a very wide range of different compounds, in spite of its deficiency in the database system. In this work, many unknown peaks in NMR spectra could not be assigned.

Principal component analysis

When visual inspections of ^{1}H NMR spectra of different growth conditions are compared, only marginal variations at metabolite levels are obvious. Both the peak overlapping and low intensities between the signals also affect the full interpretation, and it is difficult to accurately assess statistical differences between datasets and comprehend the individual variability in each sample. So PCA was performed to reduce and analyze the acquired NMR data for discrimination. The corresponding points from one sample group occur close together on score plot, but far from other ones (*Fig. 4*). The values of PC1 and PC2 cumulatively accounted for 99.59% of the total variability (*Fig. 4A*), so the preparation of intracellular extraction in this study was reproducible and reliable.

Score plots well define the notable separations between PCA comparisons with the PC1 of 99.50% (*Fig. 4B*) and 90.68% (*Fig. 4C*), respectively, illustrating significant differences in the metabolic profiles of *L. salivarius* FDB89 cultured under different growth conditions. The loading plots indicate the metabolites that are contributed significantly to the individual variables in terms of magnitude and direction to PCs, and permit the potential biomarkers to be identified.

Fig. 4B shows oxygen also has an important influence on the stress response of *L. salivarius* FDB89. For aerobic condition, higher levels of glycylproline, oxoglutarate, glutamine, tyrosine and lysine were characterized. Whereas trimethylamine-*N*-oxide, pyruvate, 2-aminobutyrate and glycine were the major metabolites accumulated in anaerobic condition. With betaine, carnitine, choline, alanine, leucine, proline, phenylalanine, valine, actate, lactate, malonate, pyroglutamate, serine, and isopropanol were all identified in the both salt stress conditions. But their contents in anaerobic condition are much lower than those in aerobic one except acetate and pyroglutamate. Those metabolites were regarded as the representative metabolites or biomarkers, respectively, for aerobic and anaerobic condition with osmotic stress.

Fig. 4C shows higher levels of betaine, carnitine, choline and its derivatives, sn-glycero-3-phosphocholine, proline, glycylproline, isoleucine, phyenylalanine, malonate, isopropanol, cyclopentanecarboxylic acid and taurine, which were identified as

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important metabolites for *L. salivarius* FDB89 under osmotic stress. And arginine, propionate, lactate, acetate, proglutamate, and succinate were shown to be important contributors in control condition.

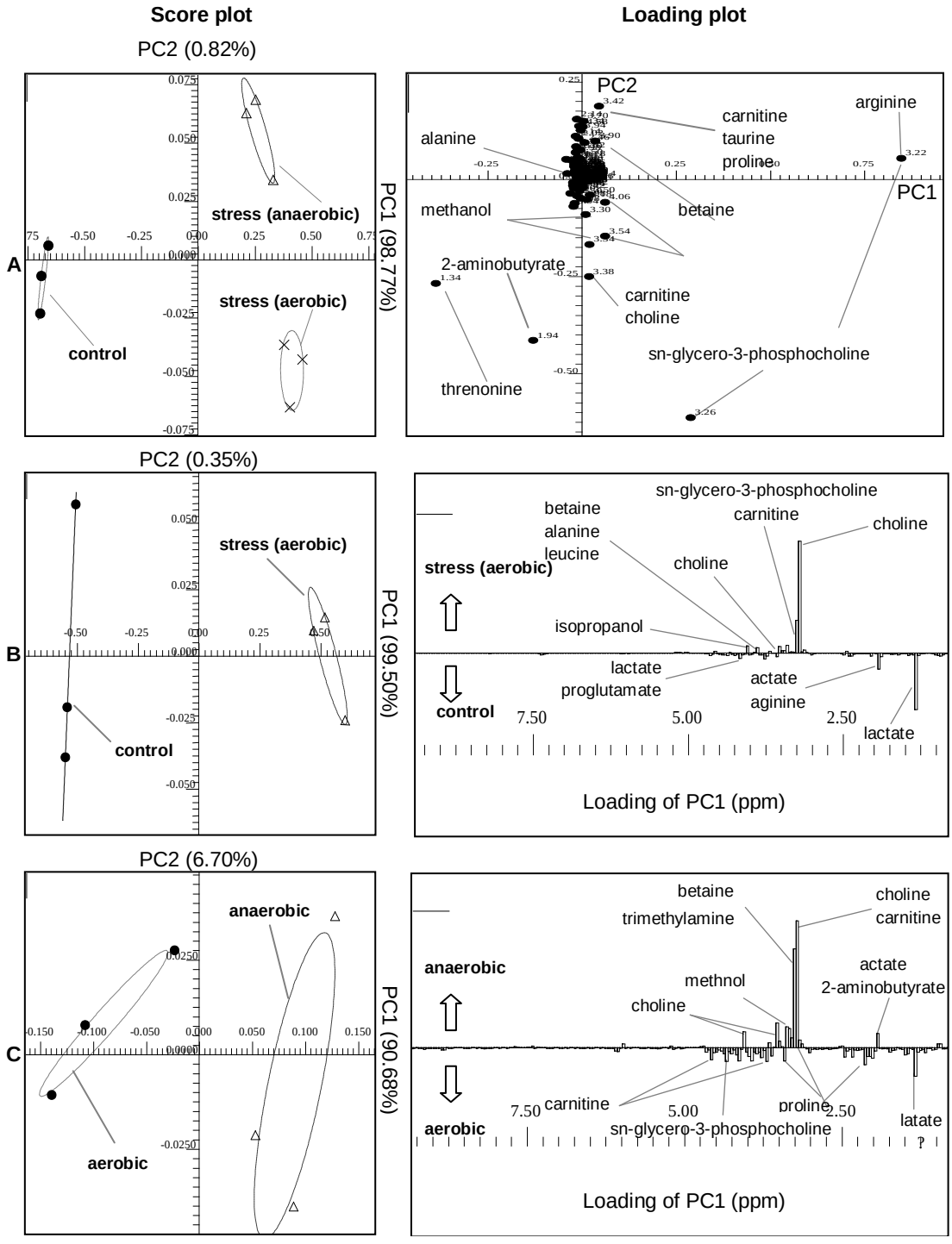

Figure 4. *PCA of intracellular extracts of L. salivarius FDB89 under different growth conditions: (A) PCA of control and aerobic and anaerobic conditions with stress; (B) control versus anaerobic condition with stress; (C) aerobic condition with stress versus anaerobic condition with stress*

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Biomarker identification by direct comparison of their peak intensities

The biomarkers identified by PCA could be identified directly by their increased metabolite resonances among the three types of growth models. In this study, PCA of ^{1}H NMR spectra indicated that betaine, carnitine, choline and its derivatives (mainly including sn-glycero-3-phosphocholine, phosphocholine, and acetylcholine), proline, malonate, aspartate, cyclopentanecarboxylic acid, and to a much lesser extent isoleucine, and phenylalanine were potential biomarkers of *L. salivarius* FDB89 when exposed to osmotic stress.

Discussion

Cellular responses induced by stress are essential for the survival of cells under adverse conditions. These responses, resulting in cell adaptation to the stress, are accomplished by a variety of processes at the molecular or metabolic level. Initial adaptive responses to high-osmolarity is a large increase in the uptake rate and the amount of cytosolic potassium, which normalizes cell turgor, and followed by a slower accumulation of different intracellular organic osmolytes at metabolic level, mainly including polyhydric alcohols, free amino acids and their derivatives. They are able to additionally normalize intracellular ionic strength, and enhance their osmotic tolerance and survival. These responses have presumably occurred in cells chronically adapted to osmotic stress (Hoffmann et al., 2017). In this study, the intracellular metabolic profiles of *L. salivarius* FDB89 cells under NaCl stress has been investigated using ^{1}H NMR method, and the effect of oxygen on osmotic response was evaluated (*Fig. 4*). Our results indicated that *L. salivarius* FDB89 grown under osmotic stress, compared to control condition, consistently accumulated abundant betaine, carnitine, choline and its derivatives (e.g., sn-glycero-3-phosphocholine, acetylcholine and phosphocholine), regardless of the presence of oxygen, while none of them are observed in the cells without stress (*Table 1* in Supplemental Data). These compounds are presumed to play a dominating adaptive role in mediating osmotic adjustment and protecting macromolecular structures and function at high and variable concentrations in osmotic stressed cells, which was also in accordance with previous studies (Hoffmann et al., 2017; Meadows and Wargo, 2015; Sheehan et al., 2006; Li et al., 2013).

Proline, and to a lesser extent, isoleucine, phenylalanine and methionine as the most important amino acids increased in *L. salivarius* FDB89 when exposed to NaCl stress. However, it could not be considered that all these amino acids except proline are biomarkers due to an opposite result obtained using gas chromatography mass spectrometry (GC/MS) (data not shown). For example, aspartic acid was identified as an observably increased amino acid to defend against NaCl stress in GC/MS analysis. Moreover, cells grown in anaerobic condition with osmotic stress clearly face energy limitation, resulting in the differences of their osmotic response ways. In this work, more than 50% of the overlapping peaks down-regulated except methanol and pyroglutamate. 2-aminobutyrate, pyruvate, glycine, trimethylamine-*N*-oxide and acetylcholine were detected only under anaerobic high-osmolarity condition, which suggested oxygen also has a non-negligible effect on the selection of osmolytes for *L. salivarius* FDB89 under osmotic stress. In contrast to conventional methods, which most often focus on the analysis of few specific metabolites as markers, NMR technique combined with PCA approach enables to rapidly screen large quantities of metabolites and identify potential biomarkers easily using pattern recognition techniques in a single

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experiment (Li et al., 2013; Miller et al., 2007). But there are some unknown peaks with higher abundance and much lower abundance in the complex matrices have not distinguished, additional verification using other methods is often necessary to confirm that the biomarkers identified by NMR-based screening are reproducible and reliable (McKelvie et al., 2009).

Conclusion

In their natural habitats and application environments, microorganism is usually exposed to osmotic changes. At metabolic level, bacteria are able to de *novo* synthesize or uptake specific solutes from environment to maintain constant turgor pressure. In the present study, the ^{1}H NMR and GC/MS metabolomics of *L. salivarius* FDB89 subjected to hyper-osmotic culture conditions was analyzed. Results indicate that nitrogen compounds (mainly amino acids and amines) including proline, isoleucine, phenylalanine and methionine may serve as potential biomarkers. However, not all organisms accumulate the same organic osmolytes, and the selection of osmolytes depends on the duration of the osmotic stress and the availability of substrates and osmolytes in the surroundings. The analysis of the metabolic profiles exposed to iso-osmotic concentrations of ionic and/or non-ionic (e.g., sucrose, lactose, trehalose, and sorbitol) compounds is also needed in furtuher metabolomics studies.

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SUPPLEMENTAL DATA

Table 1. *Presumptive metabolites and their contents in intracellular extracts of L. salivarius FDB89 under different growth conditions by [1]NMR method*

Metabolite	External concentration (mmol/l)	Intracellular concentration (millimoles per gram of dry weight)		
		Control	Aerobic growth (0.8 M NaCl)	Anaerobic growth (0.8 M NaCl)
2-hydroxybutyrate	0.16	1.42	0.82	0.84
2-aminobutyrate	nd[a]	nd	nd	1.36
Pyruvate	0.14	nd	nd	1.55
2-oxoglutarate	nd	nd	0.92	nd
Isocitrate	nd	1.10	nd	nd
Betaine	0.13	nd	8.66	6.91
Carnitine	nd	nd	4.46	2.05
Acetylcarnitine	nd	0.17	0.11	0.12
Choline	0.07	nd	13.95	13.74
Phosphocholine	nd	nd	0.59	0.93
Acetylcholine	nd	nd	nd	1.21
Sn-glycero-3-phosphocholine	nd	0.00	3.05	1.09
Alanine	1.42	6.32	4.10	3.11
Isoleucine	0.51	nd	1.61	0.97
Leucine	1.03	2.28	2.22	1.86
Lysine	0.59	nd	0.76	nd
Methionine	0.10	nd	0.27	0.08
Proline	nd	1.13	5.82	3.40
Glycyl proline	nd	nd	1.32	nd
Glycine	1.81	3.12	nd	0.65
Phenylalanine	nd	nd	0.77	0.93
Tyrosine	0.14	0.75	0.80	nd
Arginine	0.99	0.32	nd	nd
Threonine	0.74	8.16	6.85	5.29
Aspartic acid	0.15[b]	1.51[b]	3.36[b]	3.60[b]
Valine	0.61	2.50	2.09	1.28
Glutamine	nd	2.50	0.79	nd
Trimethylamine-*N*-oxide	nd	nd	nd	3.21
Kynurenine	nd	0.45	nd	nd
Acetate	1.44	16.54	1.51	2.77
Lactate	0.23	4.12	2.35	1.63
Malonate	nd	nd	4.33	3.37
Propionate	nd	1.01	nd	nd
Glutamate	0.83	3.40	3.62	nd
Pyroglutamate	0.77	8.87	3.15	4.78
Methanol	1.32	77.33	93.69	233.26
Isopropanol	nd	1.64	2.29	1.70
NAD^+	nd	0.31	0.09	0.05
Pyrimidine	nd	nd	0.50	0.16
UDP-glucose	nd	0.45	nd	nd
d TTP	nd	nd	0.37	nd
Cyclopentanecarboxylic acid	nd	nd	3.04	1.85
Succinate	0.04	0.38	nd	nd
Taurine	nd	0.79	3.10	0.71

[a] nd, not detected.
[b] based on GC/MS analysis (data not shown).

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