Metabolomic analysis of significant changes in *Lactobacillus casei* Zhang during culturing to generation 4,000 under conditions of glucose restriction

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ABSTRACT

Lactic acid bacteria are being consumed more frequently as awareness of their health benefits has increased. The industrial production of lactic acid bacteria requires a comprehensive understanding of their survival stress, especially regarding changes in metabolic substances in a glucose-limited environment. In the present study, a metabolomic approach was applied to investigate *Lactobacillus casei* Zhang using cultures from a common ancestor that were permitted to evolve under conditions with normal or glucose-restricted media for up to 4,000 generations. Metabolomic analyses of intracellular and extracellular differential metabolites under De Man, Rogosa and Sharpe broth (2% vol/vol glucose; Oxoid Ltd., Basingstoke, UK) and glucose-restricted (0.02% vol/vol glucose in De Man, Rogosa and Sharpe broth) conditions were performed at generations 0, 2,000, and 4,000 and revealed 23 different metabolites. Myristic acid, ergothioneine, Lys-Thr, and palmitamide contents exhibited significant reductions between 0 and 4,000 generations, whereas nicotinate, histidine, palmitic acid, L-lysine, urocanate, thymine, and other substances increased. The dynamics of the pathways involved in AA metabolism, including glycine, serine, and threonine metabolism, histidine metabolism, lysine degradation, and arginine and proline metabolism, were also a focus of the present study. There were also changes in several other metabolic pathways, including vitamin B₆, thiamine, nicotinate, and nicotinamide, according to generation time. Additionally, in the present study we screened for key metabolites involved in the glucose-restricted response and provided a theoretical basis for comprehensively revealing the regulatory mechanisms associated with *L. casei* Zhang glucose restriction at the metabolic level. These findings also provide novel ideas and methods for

analyzing the glucose-restricted stress response at the metabolic level.

Key words: metabolomics, *Lactobacillus casei* Zhang, glucose restriction

INTRODUCTION

Lactic acid bacteria (LAB) can ferment lactose to produce lactic acid and are widely distributed in nature. Lactic acid bacteria in the form of probiotics play a vital role in human health and as an important industrial microorganism. Lactic acid bacteria and their metabolites have been widely used in food, medicine, feed, and so on (Burgess et al., 2004; De Angelis and Gobbetti, 2004; Parvez et al., 2006).

Lactic acid bacteria inevitably face various environmental stresses during their production and application, such as osmotic pressure, acid environments, oxidative stress, cold, heat, pH, and starvation. These stresses can affect the physiological state and characteristics of cells and may limit the growth of LAB in other manufacturing processes (De Angelis and Gobbetti, 2004). Among these stresses in particular, glucose restriction has been reported as one of the most important limitations to growth. During the process of reproduction, if LAB cannot replenish glucose in time, the bacteria will enter a state of starvation and exhibit survival stress; it is worth noting that LAB can also indirectly enter a nutrient-deficient state through other stresses; LAB regulate genetic regulators of different responses associated with other stresses when faced with carbohydrate restriction (Hussain et al., 2009). Al-Naseri et al. (2013) demonstrated that *Lactobacillus casei* GCRL163 inhibits phosphotransferase abundance, lactose metabolism, and galactose metabolism under starvation conditions. The same process occurs during synthesis of nucleotides and proteins (Al-Naseri et al., 2013). Thus, for an experimenter, it is important to understand the conditions that are beneficial or harmful to LAB and the mechanisms that allow them to survive under stress and carry out metabolic activities.

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Lactobacillus casei Zhang is a probiotic bacteria strain isolated from the traditional homemade milk products of herdsman in Zhenglan Banner, Xilinhot, in the Inner Mongolia region of China. *Lactobacillus casei* Zhang is highly tolerant to acid and bile salt stress and has probiotic functions that lower blood lipids, improve immune regulation, enhance antioxidation, and antagonize the growth of intestinal pathogens (Ya et al., 2008; Wu et al., 2009; Zhang et al., 2010). To assess the effects of *L. casei* Zhang on upper respiratory tract infections and intestinal diseases, researchers divided 137 volunteers into adult and elderly groups to perform randomized double-blind controlled trials using a daily oral dose of *L. casei* Zhang (9 log cfu). The administration of *L. casei* Zhang reduced plasma proinflammatory cytokines (IL-1), increased anti-inflammatory cytokines (IL-4 and IL-10), and upregulated the whole-blood expression levels of cluster of differentiation (CD) 4, CD8, CD44, CD27, and C-X-C motif chemokine receptor 5 (CXCR5). Additionally, the elderly group had an increased number of bowel movements per week, which alleviated red blood cell abnormalities, ameliorated the symptoms of upper respiratory tract infection in adults, and significantly shortened the duration of symptoms. These findings demonstrated the potential of *L. casei* Zhang for reducing the symptoms of upper respiratory tract infections and maintaining gastrointestinal health (Hor et al., 2018).

Metabolomics can be used to qualitatively and quantitatively analyze low molecular weight metabolites in cells that are produced by microbial metabolism, including peptides, AA, carbohydrates, nucleic metabolites, vitamins, organic acids, and minerals (Jewett et al., 2006; Wishart, 2008). Foschi et al. (2017) combined 16S rRNA gene sequencing with proton nuclear magnetic resonance to determine the classification and metabolic properties of LAB and found that metabolomics can be used to correlate specific biological activities with taxonomy as well as to understand the mechanisms underlying bacteriostasis in some LAB species. Under glucose-restricted conditions, *Lactobacillus plantarum* H4 produces acetate in addition to acetic acid in the pH range of 3.8 to 6, and L-lactate accumulation was greater than that of D-lactate (Bobillo and Marshall, 1992). These findings demonstrate that *L. plantarum* H4 adjusts to changes in environmental conditions by altering its metabolic pathways. There is also evidence showing that heterofermentative *L. casei* can degrade arginine during culture and lead to the formation of ammonia and citrulline (Toh et al., 2013). Under stress, *Lactobacillus* will become deficient in essential nutrients, which limits the exponential phase of microbial growth, allowing it to enter the stationary phase and engage in carbohydrate starvation, resulting in the consumption

of cellular energy (De Angelis and Gobbetti, 2004). At present, LAB, as an important fermenting microorganism, are faced with widespread carbon source stress in production applications, which will seriously affect their physiological functions and restrict the development of related products.

Ultraperformance liquid chromatography quadrupole time-of-flight MS in the MS^E mode (UPLC-Q-TOF MS^E) was used to analyze changes in metabolites during the different evolutionary processes between the original probiotic *L. casei* Zhang and other bacteria that were subcultured to generations 2,000 and 4,000 under glucose-restricted conditions. Understanding metabolic changes in *L. casei* Zhang under nutrient stress will provide practical guidance for the industrial production of probiotics.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

The original strain of *L. casei* Zhang was deposited in the Key Laboratory of Dairy Biotechnology and Engineering, Education Ministry of China. The obtained ampoule strain was activated in De Man, Rogosa and Sharpe (MRS) broth medium (CM0359; Oxoid Ltd., Basingstoke, UK) and then subcultured twice at 37°C in an incubator (MIR-1620; Sanyo, Osaka, Japan).

Experimental Evolution

After 2 generations of cultivation (24 h/generation), *L. casei* Zhang was streaked on MRS agar medium (CM0361; Oxoid Ltd.). After incubation at 37°C for 72 h, 3 colonies were randomly selected and cultured in a normal 2% (vol/vol) glucose MRS broth medium and a 0.02% (vol/vol) glucose-restricted medium. Every 24 h, *L. casei* Zhang strains cultured in MRS broth or a glucose-restricted environment were inoculated into MRS broth and MRS containing 0.02% glucose sugar at an inoculation amount of 1% (vol/vol, 50 µL); 100-fold bacterial growth per day represents almost 6.6 generations per subculture (Wang et al., 2017). The 4,000-generation evolution in the laboratory took approximately 603 d to complete. Strains of different generations were obtained for vacuum freeze-drying preservation during the long-term cultivation (−50°C, 15.0 Pa for 48–72 h).

Sample Preparation for UPLC-Q-TOF MS^E Analysis

Cultivation of *L. casei* Zhang. The original strain of *L. casei* Zhang, and the *L. casei* Zhang grown in the

glucose-restricted medium deposited in the ampoule tube, were cultured in the MRS. The strains growing in normal or glucose-restricted environments were subcultured in MRS broth twice and the bacterial solution was transferred to fresh medium at 1% volume of inoculation every 24 h.

Sample Quenching and Metabolite Extraction.

Samples were collected at the mid-exponential phase (~8 h). The activated samples were dispensed into 2-mL centrifuge tubes and centrifuged at $7,000 \times g$ for 5 min at 4°C; supernatant retention was performed to collect extracellular metabolic samples. Briefly, 200 μ L of extracellular sample was obtained, and 800 μ L of methanol/acetonitrile at the ratio of 1:1 (vol/vol) was added to each extracellular sample. Then, the sample was vortexed for 30 s and subjected to ultrasound for 10 min (4°C cold bath). Following the ultrasound, samples were allowed to stand at -20°C for 60 min and were then centrifuged at $13,000 \times g$ for 15 min at 4°C. Next, 500 μ L of the supernatant was collected, vacuum-dried at 35°C, reconstituted with 200 μ L of 50% acetonitrile, vortexed for 30 s, and subjected to ultrasound for 10 min (4°C cold bath). Next, the sample was centrifuged at $13,000 \times g$ for 15 min at 4°C and kept on the machine for testing.

The bacteria sludge cells were quickly added to a precooled 0.9% NaCl, vortexed, centrifuged at 0°C for 5 min at $5,000 \times g$, and washed. This process was repeated twice, and the supernatant was discarded; then, the remaining bacterial sludge was collected and -40°C precooled 60% methanol/0.9% NaCl was quickly added to the centrifuge tube for quenching using 4 times the volume of the sludge (de Koning and Vandam, 1992; Jensen et al., 1999; Oldiges and Takors, 2005; Mashego et al., 2007; Canelas et al., 2008). The cells were then quenched for 5 min and centrifuged at $10,000 \times g$ for 5 min at 0°C; the supernatant was then discarded. The quenched cells were subjected to liquid nitrogen milling for 20 min (a mortar was precooled at -80°C) and ground to a fine powder; 50 mg of powder was placed in a 1.5-mL centrifuge tube. Then, 1 mL of -40°C 70% methanol/water mixture was added to the tube, vortexed, fully mixed, and extracted to suspend the cells. Liquid nitrogen was used to repeatedly freeze and thaw the cells (4 times; liquid nitrogen freezing for 3 min, -20°C refrigerator melting), followed by centrifugation at $10,000 \times g$ for 5 min at -20°C; the supernatant was then collected. The cell pellet was continuously suspended in 1 mL of -40°C 70% methanol/water, vortexed for 30 s, and centrifuged at $8,000 \times g$ for 5 min at 0°C; the supernatants obtained by the 2 extractions were then combined (Chen et al., 2014). The supernatant was stored at -80°C until analysis; this fraction was labeled as the intracellular sample. Each sample (2

replicates per sample) was processed in parallel to yield 6 replicates.

UPLC-Q-TOF MS^E Analysis

An Acquity UPLC/Xevo G2 Q TOF-MS^E instrument (Waters Corp., Milford, MA) was used to extract the MS spectral peaks of the samples. Samples were filtered and placed in a sample vial for testing. When the samples in this experiment were analyzed by metabolomics, the quadrupole time-of-flight mass spectrometer carried out data scanning using positive electrospray ionization (ESI⁺) and negative electrospray ionization (ESI⁻) ion modes. The cone voltage was 40 V, the ion energy was 1, the collision energy was 6, the sample infusion flow rate was 10 μ L/min, the sample fill volume was 250 μ L, the LockSpray capillary was at 3 kV, and the acquisition scan range was 100 to 1,000 mass-to-charge ratio (m/z) at a scan frequency of 10.00 s.

To ensure the accuracy of data acquisition, leucine-cephalin was used as the lock mass for both positive ion mode (556.2771 [M + H]⁺) and negative ion mode (554.2615 [M - H]⁻). Pretreated samples were chromatographed using a C18-UPLC system. The column was an HSS T3 (2.1 mm $\times$ 100 mm, 1.8 μ m; Waters Corp.) with a sample infusion flow rate of 0.3 μ L/min; the column was maintained at 35°C. The injection volume was 4 μ L.

For ESI⁺ ion mode, mobile phase A was an aqueous phase containing 0.1% formic acid (Sigma-Aldrich, St. Louis, MO), and mobile phase B was an organic phase containing 0.1% formic acid in acetonitrile (Optima LC/MS-grade; Fisher Scientific, Grand Island, NY). The elution gradient is shown in Table 1. For scanning in ESI⁻ ion mode, the mobile phase consisted of water (Millipore, Bedford, MA) containing 10% acetonitrile (solution A) and 50% acetonitrile (solution B). The elution gradient was as shown in Table 2.

UPLC-Q-TOF MS^E Data Processing and Multivariate Statistical Analyses

The data processing method has been described in detail previously (Pan et al., 2018). Briefly, the data were preprocessed in MassLynx version 4.1 (Waters Corp.) and then imported into the EZinfo software (Waters Corp.) for centralization and Pareto homogenization to reduce deviation due to large-scale peaks.

Simultaneous multivariate statistical analysis was performed using SIMCA-P + 11 (Umetrics AB, Umeå, Sweden) and MetaboAnalyst (www.metaboanalyst.ca) software. For each treatment, 3 comparisons were made: 0 versus 2,000 generations, 0 versus 4,000 generations, and 2,000 versus 4,000 generations. To determine

Table 1. Ultraperformance liquid chromatography quadrupole time-of-flight MS in the MS^E mode mobile phase gradient program for the analysis of samples in positive ion mode

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Curve
Initial	0.3	95	5	Initial
2	0.3	95	5	6
7	0.3	80	20	6
12	0.3	35	65	6
14	0.3	20	80	6
17	0.3	20	80	6
18	0.3	95	5	6

changes in metabolites between the different generations of *L. casei* Zhang, variables with variable importance in the projection values greater than 1 (fold change ≥ 2 and $P \leq 0.5$) were selected as potential metabolic discriminant variables (Chong and Jun, 2005; Xi et al., 2017). Based on these variables, the different metabolic substances were further analyzed and identified.

Identification of Differential Metabolites

The significantly different substances obtained by screening were automatically searched for in several databases, including METLIN (<http://www.metlin.scripps.edu/>), KEGG (www.genome.jp/kegg/), ChemSpider (www.chemspider.com), and the Human Metabolome Database (www.hmdb.ca) based on their mass-to-charge ratio and retention time. Finally, correlation and metabolic pathway analyses were performed on the various retrieved metabolites.

RESULTS

Sample Principal Component Analysis Results

To compare the differences between *L. casei* Zhang generation 0 and glucose-restricted generation 2,000, multivariate analyses were used to segment the effective spectral area, and principal component analysis (PCA) was performed. In the PCA model, the abscissa is the first principal component and the ordinate is the second principal component. In each score graph, the

addition of the 2 principal components explained more than 95% of the variance, as shown in Figure 1.

In Figure 1, it can be seen that all PCA score plots have clear separation between different generations, indicating that the chemical compositions of samples differed among *L. casei* Zhang under stress conditions. Taking Figure 1A as an example, the 2 sets of samples are distributed in different regions, without intersection or overlap, and there is an obvious discrete phenomenon. Six parallel samples of each type are closely aggregated.

Samples between generation 0 and glucose-restricted generation 2,000 had values of 78.90 and 19.90% in the principal component 1 and principal component 2 directions, respectively, and the 2 principal components accounted for most of the variance among the different treatment samples. Principal component 1 had the largest contribution rate; this indicates that the metabolites of generations 0 and 2,000 are clearly dissociable and confirms the effectiveness of the sampling method and the reproducibility of the test. The extracellular analysis method is the same as the intracellular method; the PCA results are shown in Figure 2.

Figures 2A and D and Figures 2B and E are well separated by PCA analysis; only Figures 2C and F overlap, which indicates that the PCA model cannot adequately distinguish between the 2 groups. In addition, the overall dispersion of the 2 types of sample is large, indicating that the individual differences among the samples are also relatively large. However, as can be seen from Figures 1 and 2, the PCA plots of both

Table 2. Ultraperformance liquid chromatography quadrupole time-of-flight MS in the MS^E mode mobile phase gradient program for the analysis of samples in negative ion mode

Time (min)	Flow rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)	Curve
Initial	0.3	90	10	Initial
2	0.3	90	10	6
7	0.3	80	20	6
12	0.3	30	70	6
14	0.3	30	70	6
15	0.3	90	10	6

Figure 1. Principal component (PC) analysis score plots of intracellular (IC) samples. Data were obtained in positive electrospray ionization (ESI⁺: A, B, C) and negative electrospray ionization (ESI⁻: D, E, F) ion modes. A and D: generation 0 versus generation 2,000; B and E: generation 0 versus generation 4,000; C and F: generation 2,000 versus generation 4,000.

intracellular and extracellular metabolites are well separated, indicating that the metabolites of different generations will vary significantly during the laboratory evolution of *L. casei* Zhang in a glucose-restricted environment.

Sample Orthogonal Partial Least Squares Discriminant Analysis Results

The differences between the extracellular and intracellular metabolite samples were further examined by orthogonal partial least squares discriminant analysis (OPLS-DA). In OPLS-DA, The R^2Y parameter describes the percentage of variation explained by the model and Q^2 describes the predictive ability of the model. In Figure 3A, obtained through OPLS-DA in positive ion mode, the R^2Y and Q^2 values of the generation 0 versus generation 2,000 intracellular metabolite

samples are equal to 0.996 and 0.936, respectively. This indicates that the 2 models are reliable and that the metabolic samples had significant differences. The R^2Y and Q^2 values of all models were relatively high, indicating that the models were reliable and that both sets of samples in each OPLS-DA score plot had metabolic differences.

In Figure 3D, each point represents a variable, and the greater the distance of a variable from the origin point, the higher the confidence level regarding its contribution to the clustering observed in the OPLS-DA score plots. The variables in each S-plot map were screened for final identification of the substance based on variable importance in the projection and fold-change values. The OPLS-DA score plots and S-plots from generation 0 to 4,000 in both positive and negative ion modes are shown in Supplemental Figures S1 through S3 (<https://doi.org/10.3168/jds.2018-15702>).

Figure 2. Principal component (PC) analysis score plots of extracellular (EC) samples. Data were obtained in positive electrospray ionization (ESI⁺; A, B, C) and negative electrospray ionization (ESI⁻; D, E, F) ion modes. A and D: generation 0 versus generation 2,000; B and E: generation 0 versus generation 4,000; C and F: generation 2,000 versus generation 4,000.

Identification of Different Metabolites

According to the data on retention time and mass-to-charge ratio, a total of 8,376 differentially expressed metabolites were found between generations 0 and 2,000, including intracellular and extracellular metabolites; among these, 5,298 were in ESI⁺ ion mode. A total of 10,234 substances differed between generations 0 and 4,000, including 7,374 in ESI⁺ ion mode, whereas 3,952 differentially expressed substances were found between generations 2,000 and 4,000 (of which 2,205 were in ESI⁺ ion mode). The differential substances ultimately conforming to the screening principle described above are discussed below, and the differential metabolites of intracellular generations 0 and 2,000 are summarized in Table 3.

In Table 3 we can see that, via metabolomic analysis, 53 significantly different substances between generations

0 and 2,000 were identified based on the screening principle, containing AA, organic acids, vitamins, pyrimidines, and short peptides. In total, 39 were upregulated and are involved in histidine metabolism; tryptophan metabolism; biotin metabolism; pyruvate metabolism; alanine, aspartate, and glutamate metabolism; pyrimidine metabolism; purine metabolism; valine, leucine, and isoleucine biosynthesis; and vitamin digestion and absorption. There were 14 downregulated substances involved in arginine and proline metabolism; glycine, serine, and threonine metabolism; histidine metabolism; arginine biosynthesis; and biosynthesis of AA.

In the extracellular metabolomic analysis of generations 0 and 2,000, 81 significantly different metabolites were identified under ESI⁺ and ESI⁻ modes (Table 4). These 81 substances are involved in metabolic pathways including carbohydrate, energy, lipid, nucleotide, and AA metabolism. The number of significantly different

metabolites was larger than that of intracellular substances, and more metabolic pathways were involved.

In addition, *L. casei* Zhang was subcultured to generation 4,000. The differential metabolites of generations 0 and 4,000 are shown in Supplemental Tables S1 and S2 (<https://doi.org/10.3168/jds.2018-15702>). Forty-seven different substances, including the following were found in generation 4,000 but not in generation 2,000: the AA L-ornithine, L-leucine, and L-homoserine; the organic acids salicylic acid, succinic acid, and linolenic acid; the short peptides valine-alanine, isoleucine-proline, leucine-serine, histidine-cysteine, and tartrate; and pyridoxamine, 2-phospho-glycerate, *N*-acetylcytidine, guanosine, and indole. Meanwhile, 49 metabolites in generation 2,000 were not detected in generation 4,000 and hence were not screened.

Finally, we conducted a comparative analysis of the metabolites that differed between *L. casei* Zhang

generations 2,000 and 4,000. The results are shown in Supplemental Tables S3 and S4 (<https://doi.org/10.3168/jds.2018-15702>). There were 66 metabolomic differences between generations 2,000 and 4,000, including both intracellular and extracellular metabolites, of which 37 were upregulated and 29 were downregulated. Figure 4 shows that 23 metabolites were shared by all 3 groups; generation 0 versus generation 2,000, generation 0 versus generation 4,000 and generation 2,000 versus generation 4,000.

The content changes of the 23 different substances in the 3 groups from generation 0 to 4,000 are shown in Figure 5. In generation 0, the contents of myristic acid, ergothioneine, lysine-threonine, and palmitamide were higher than the contents of other metabolites. As the experiment progressed and they were subcultured to generation 4,000, the levels of the substances with a high content in generation 0 decreased, which may

Figure 3. Orthogonal partial least squares discriminant analysis score plots and S-plots of the data for intracellular (IC) samples obtained in positive electrospray ionization (ESI⁺) ion mode. A and D: generation 0 versus 2,000; B and E: generation 0 versus 4,000; C and F: generation 2,000 versus 4,000.

Table 3. Generation 0 versus generation 2,000: differential intracellular metabolites at positive and negative ions

RT_Exact MS ¹	Metabolites	Molecular formula	VIP ²	Fold change	P-value
Increased in positive ion mode					
1.13_119.0473	L-Threonine	C ₄ H ₉ NO ₃	1.04172	0.13642	8.49E-05
2.13_120.0790	L-Erythrose	C ₄ H ₈ O ₄	3.85927	0.11167	2.49E-09
1.12_123.0424	Nicotinate	C ₆ H ₄ NO ₂	1.36029	0.090243	5.56E-08
4.89_126.0533	Thymine	C ₅ H ₆ N ₂ O ₂	1.64726	0.27725	1.05E-05
8.52_135.0793	Adenine	C ₅ H ₅ N ₅	1.89482	0.28561	0.010734
4.66_136.0599	Hypoxanthine	C ₅ H ₄ N ₄ O	1.47924	0.094283	5.87E-06
3.32_138.0532	Urocanate	C ₆ H ₆ N ₆ O ₂	1.36621	0.039506	0.00082435
4.89_144.0645	Octanoic acid	C ₈ H ₁₆ O ₂	1.08949	0.072831	0.0016261
4.20_146.0588	L-Lys	C ₆ H ₁₄ N ₂ O ₂	1.61573	0.026049	4.72E-07
4.90_150.0542	D-Ribulose	C ₅ H ₁₀ O ₅	1.27149	0.18516	3.58E-06
1.13_165.0556	L-Phe	C ₉ H ₁₁ NO ₂	1.03563	0.066446	9.08E-06
2.13_166.0857	D-Arabinonic acid	C ₅ H ₁₀ O ₆	1.14851	0.08138	5.05E-08
4.58_168.0648	Homogentisate	C ₈ H ₇ O ₄	1.27214	0.27723	0.0067467
6.77_169.0758	Pyridoxine	C ₈ H ₁₁ NO ₃	1.22469	0.22546	0.0003405
4.88_186.0762	Ala-Pro	C ₈ H ₁₄ N ₂ O ₃	2.29469	0.29218	1.05E-07
4.19_188.0713	Homo-cis-aconitate	C ₇ H ₈ O ₆	1.71221	0.046498	2.83E-08
1.99_189.0875	N-Acetyl-glutamate	C ₇ H ₁₁ NO ₅	2.30644	0.2841	2.35E-06
4.07_204.0869	Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	2.66964	0.18814	4.20E-07
4.07_210.0767	D-Glucaric acid	C ₆ H ₁₀ O ₈	1.70103	0.13964	1.78E-07
11.45_228.2328	Myristic acid	C ₁₄ H ₂₈ O ₂	1.03995	0.25929	0.00015464
8.62_243.0886	Cytidine	C ₉ H ₁₃ N ₃ O ₅	2.34393	0.25464	1.18E-06
9.31_255.0661	Dihydroneopterin	C ₉ H ₁₃ N ₅ O ₄	2.62242	0.22783	1.92E-05
13.32_256.2646	Palmitic acid	C ₁₆ H ₃₂ O ₂	1.26038	0.43152	0.010902
9.49_285.0758	Leu-Gly-Pro	C ₁₃ H ₂₃ N ₃ O ₄	1.87168	0.15803	1.83E-06
4.89_294.0978	Ile-Tyr	C ₁₅ H ₂₂ N ₂ O ₄	1.22201	0.30706	5.19E-05
13.39_300.2901	Retinoic acid	C ₂₀ H ₂₈ O ₂	1.14088	0.15403	0.00024178
9.64_309.0878	Tyr-Gln	C ₁₄ H ₁₉ N ₃ O ₅	1.55137	0.20551	3.47E-05
4.89_372.1299	Biocytin	C ₁₆ H ₂₈ N ₄ O ₄ S	1.08822	0.061536	1.61E-05
Reduced in positive ion mode					
7.65_131.0685	Creatine	C ₄ H ₉ N ₃ O ₂	1.05928	0.0091171	0.0091171
8.25_133.0845	Aspartic acid	C ₄ H ₇ NO ₄	2.66956	0.0020091	0.0020091
10.07_139.1100	Histidinal	C ₆ H ₉ N ₃ O	1.48395	0.0018336	0.0018336
7.65_175.0966	Citrulline	C ₆ H ₁₃ N ₃ O ₃	1.10533	0.0036725	0.0036725
7.65_177.1121	N-Formyl-L-methionine	C ₆ H ₁₁ NO ₃ S	2.67214	0.0042931	0.0042931
7.32_221.1387	N-Acetyl-D-glucosamine	C ₈ H ₁₅ NO ₆	1.52476	0.002552	0.002552
1.06_230.1872	Ergothioneine	C ₉ H ₁₅ N ₃ O ₂ S	1.67643	0.00050511	0.00050511
7.64_283.1750	Gln-His	C ₁₁ H ₁₇ N ₅ O ₄	1.24846	0.00043446	0.00043446
7.32_309.1909	Tyr-Lys	C ₁₅ H ₂₃ N ₃ O ₄	1.40144	0.0086228	0.0086228
Increased in negative ion mode					
1.05_100.9841	1-Aminocyclopropane 1-carboxylate	C ₄ H ₇ NO ₂	1.59147	0.0021828	2.93E-08
1.47_112.9843	Creatinine	C ₄ H ₇ N ₃ O	2.38491	0.090431	0.00021513
2.16_122.9663	Nicotinate	C ₆ H ₅ NO ₂	1.51372	0.1068	6.97E-05
1.84_144.9737	2-Oxoglutarate	C ₅ H ₇ NO ₄	1.42925	0.16986	0.004854
1.04_147.0443	O-Acetyl-L-serine	C ₅ H ₉ NO ₄	1.58474	0.0087079	3.48E-05
1.20_150.9617	Guanine	C ₅ H ₅ N ₅	1.03773	0.0051694	5.16E-09
1.99_162.9586	N-Acetyl-L-cysteine	C ₅ H ₉ NO ₃ S	1.8844	0.13853	0.0001293
9.67_243.1951	Lys-Pro	C ₁₁ H ₂₁ N ₃ O ₃	1.77331	0.0014116	0.0018486
12.62_246.9924	Pyridoxal-5-phosphate	C ₈ H ₁₀ NO ₆ P	1.02409	0.12221	0.0057913
11.54_283.2632	Stearamide	C ₁₈ H ₃₇ NO	4.0032	0.00029692	0.0004265
1.81_116.9579	L-Aspartate-4-semialdehyde	C ₄ H ₇ NO ₃	1.07003	0.0046003	7.43E-06
Reduced in negative ion mode					
9.09_199.1696	Lauramide	C ₁₂ H ₂₅ NO	1.35532	536.06	0.0060526
9.46_243.1958	Gln-Pro	C ₁₀ H ₁₇ N ₃ O ₄	1.76496	525.26	3.21E-05
10.01_265.1458	L-Aspartic acid L-ornithine	C ₉ H ₁₉ N ₃ O ₆	2.30661	1,107.9	2.84E-05
10.67_293.1779	Phe-Gln	C ₁₄ H ₁₉ N ₃ O ₄	2.14739	503.54	0.0021298
10.37_309.1712	Tyr-Gln	C ₁₄ H ₁₉ N ₃ O ₅	1.2100	339.55	0.00059251

¹RT_Exact MS = retention time and exact mass.²Variable importance in the projection.

have been due to adaptability during long-term generation. It was also obvious that the color of substances such as phosphocreatine, hypoxanthine, octanoic acid,

L-lysine, O-acetyl-L-serine, urocanate, L-phenylalanine, stearamide, lysine-serine, thymine, and D-ribulose changed from blue to green, which indicates that they

Table 4. Generation 0 versus generation 2,000: differential extracellular metabolites at positive and negative ions

RT_Exact MS ¹	Metabolites	Molecular formula	VIP ²	Fold change	P-value
Increased in positive ion mode					
13.04_106.0836	Glycerate	C ₃ H ₆ O ₄	1.37696	0.064754	0.00010451
9.04_111.0422	Histamine	C ₅ H ₉ N ₃	1.32371	0.060176	6.25E-06
9.05_113.0576	Creatinine	C ₄ H ₇ N ₃ O	1.1274	0.24478	9.76E-07
11.62_121.0276	L-Cysteine	C ₃ H ₇ NO ₂ S	1.33441	0.03647	0.00068041
3.07_126.0533	Thymine	C ₅ H ₆ N ₂ O ₂	1.38025	0.37788	2.22E-06
3.54_129.1007	Pipecolate	C ₆ H ₁₀ NO ₂	1.34534	0.10938	4.83E-07
9.01_131.0691	L-Isoleucine	C ₆ H ₁₃ NO ₂	1.36324	0.3464	1.11E-06
6.93_133.0848	Aspartic acid	C ₄ H ₇ NO ₄	2.62725	0.092483	1.01E-05
4.64_136.0608	Hypoxanthine	C ₅ H ₄ N ₄ O	1.12188	0.40962	2.02E-07
8.99_137.0588	Tyramine	C ₈ H ₁₁ NO	1.4478	0.25862	2.27E-08
5.45_138.054	Urocanate	C ₆ H ₆ N ₂ O ₂	1.192	0.10981	0.0033048
11.62_149.0229	L-Methionine	C ₅ H ₁₁ NO ₂ S	4.24085	0.22881	0.0011476
4.86_150.0543	D-Ribulose	C ₅ H ₁₀ O ₅	1.77383	0.20833	2.52E-09
4.51_150.0548	Guanine	C ₅ H ₅ N ₅ O ₅	1.15784	0.099719	0.0020137
8.84_155.0701	Histidine	C ₆ H ₉ N ₃ O ₂	1.56322	0.36495	1.90E-05
5.45_168.0654	Phosphoenolpyruvate	C ₃ H ₇ O ₆ P	1.37741	0.084776	0.0017744
6.71_169.0765	Pyridoxine	C ₈ H ₁₁ NO ₃	1.40996	0.31877	2.53E-06
9.00_175.0967	Citrulline	C ₆ H ₁₃ N ₃ O ₃	1.47653	0.36346	4.00E-06
7.95_177.1123	N-Formyl-L-methionine	C ₆ H ₁₁ NO ₃ S	3.51179	0.032643	1.56E-07
10.47_181.0767	Tyrosine	C ₉ H ₁₁ NO ₃	1.10225	0.12071	6.00E-07
1.78_189.0879	N-Acetyl-glutamate	C ₇ H ₁₁ NO ₅	2.38658	0.00053012	0.015579
4.16_195.1137	Dopaquinone	C ₉ H ₆ NO ₄	1.83436	0.24037	8.53E-08
9.03_199.0972	O-Phospho-L-homoserine	C ₄ H ₁₀ NO ₆ P	1.59952	0.098639	1.18E-05
9.82_200.2018	Lauric acid	C ₁₂ H ₂₄ O ₂	1.31753	0.18146	1.20E-05
5.41_204.0876	Tryptophan	C ₁₁ H ₁₂ N ₂ O ₂	1.85181	0.19821	0.0045673
5.56_210.0773	Phosphocreatine	C ₄ H ₁₀ N ₃ O ₅ P	1.35399	0.18712	0.0090698
4.48_218.1504	Thr-Val	C ₉ H ₁₈ N ₂ O ₄	1.37357	0.11998	3.61E-06
3.85_219.1164	Asn-Ser	C ₇ H ₁₃ N ₃ O ₅	1.2503	0.24715	3.86E-07
1.08_223.0049	N-Acetyl-L-tyrosine	C ₁₁ H ₁₃ NO ₄	1.14526	0.18651	2.46E-05
1.13_226.0459	Carnosine	C ₉ H ₁₄ N ₄ O ₃	1.07902	0.24006	1.05E-07
11.39_228.234	Myristic acid	C ₁₄ H ₂₈ O ₂	1.72647	0.1129	3.59E-07
11.78_240.2334	Palmitaldehyde	C ₁₆ H ₃₂ O	1.12981	0.31535	2.46E-06
9.03_243.1236	Cytidine	C ₉ H ₁₃ N ₃ O ₅	1.91826	0.13377	2.27E-06
2.44_252.0747	Ubiquinol	C ₁₉ H ₂₈ O ₄	1.02464	0.20019	0.00018987
13.15_256.265	Palmitic acid	C ₁₆ H ₃₂ O ₂	6.07164	0.094882	4.06E-11
4.46_258.1459	Cys-His	C ₉ H ₁₄ N ₄ O ₃ S	1.1113	0.089658	0.018617
9.03_261.1327	Gly-Trp	C ₁₃ H ₁₅ N ₃ O ₃	1.111	0.18045	0.0031832
9.61_263.0826	Asn-Met	C ₉ H ₁₇ N ₃ O ₄ S	1.44426	0.26998	6.02E-09
10.47_274.2172	Lys-Gln	C ₁₁ H ₂₂ N ₄ O ₄	1.44944	0.091774	3.14E-06
4.59_295.0962	Asn-Tyr	C ₁₃ H ₁₇ N ₃ O ₅	1.31443	0.0025136	9.77E-08
13.22_300.2906	9-Cis-retinoic acid	C ₂₀ H ₂₈ O ₂	2.59746	0.074304	2.66E-08
4.24_303.1382	Val-Trp	C ₁₆ H ₂₁ N ₃ O ₃	1.16046	0.45085	3.01E-09
9.04_305.1589	Trp-Thr	C ₁₅ H ₁₆ N ₃ O ₄	1.07658	0.27243	0.0062877
9.61_309.0875	Tyr-Gln	C ₁₄ H ₁₉ N ₃ O ₅	1.1821	4.7676	4.58E-05
4.85_332.1941	Gln-Trp	C ₁₆ H ₂₀ N ₄ O ₄	1.73175	0.097169	6.31E-08
Reduced in positive ion mode					
4.16_146.0595	Glutamine	C ₅ H ₁₀ N ₂ O ₃	1.60715	2.6254	4.15E-08
1.87_166.0473	D-Arabinonic acid	C ₅ H ₁₀ O ₆	1.12964	3.4992	6.09E-05
5.06_199.1808	Lauramide	C ₁₂ H ₂₅ NO	1.13257	32.168	1.43E-09
4.15_188.0715	Homo-cis-aconitate	C ₇ H ₈ O ₆	2.09792	990.71	7.71E-12
1.39_212.1041	Pro-Pro	C ₁₀ H ₁₆ N ₂ O ₃	1.08773	3.8416	0.001635
1.07_230.1873	Ergothioneine	C ₉ H ₁₅ N ₃ O ₂ S	2.88249	6.99	0.00089026
4.38_235.1089	Asn-Cys	C ₇ H ₁₃ N ₃ O ₄ S	1.317	3.3316	5.77E-05
2.80_247.1302	Lys-Thr	C ₁₀ H ₂₁ N ₃ O ₄	1.202	17.782	7.83E-09
1.60_254.1625	His-Val	C ₁₁ H ₁₈ N ₄ O ₃	1.07387	6.2752	7.48E-07
10.47_255.0778	N-Ribosyl-nicotinamide	C ₁₁ H ₁₆ N ₂ O ₅	1.33182	2.6137	1.13E-09
1.24_276.1448	Saccharopine	C ₁₁ H ₂₀ N ₂ O ₆	1.39699	11.101	1.49E-06
1.97_310.1292	Porphyrin	C ₂₀ H ₁₄ N ₄	1.12752	15.695	9.83E-07
3.87_343.15	His-Trp	C ₁₇ H ₁₉ N ₅ O ₃	1.12332	3.5089	1.95E-05
8.97_360.2057	Aldosterone	C ₂₁ H ₂₈ O ₅	1.23831	349.4	8.39E-09
Increased in negative ion mode					
1.14_100.0299	Isohexanal	C ₆ H ₁₂ O	1.92532	0.12398	0.014571
2.02_100.9743	1-Aminocyclopropane 1-carboxylate	C ₄ H ₇ NO ₂	2.11696	-1.8244	6.06E-05

Continued

Table 4 (Continued). Generation 0 versus generation 2,000: differential extracellular metabolites at positive and negative ions

RT_Exact MS ¹	Metabolites	Molecular formula	VIP ²	Fold change	P-value
1.12_101.0131	5-Aminopentanal	C ₅ H ₁₁ NO	2.11987	−1.7147	0.012176
1.20_112.9737	1-Pyrroline 2-carboxylate	C ₅ H ₇ NO ₂	1.50273	−1.1726	0.011679
1.70_126.9932	Piperidine-2-carboxylate	C ₆ H ₉ NO ₂	1.11773	−8.3604	0.0061875
2.21_144.9625	2-Oxoglutaramate	C ₅ H ₇ NO ₄	1.88478	−1.9446	0.014777
Reduced in negative ion mode					
1.15_116.0416	2-Oxoisovalerate	C ₅ H ₈ O ₃	1.19353	5.9119	0.0010377
1.12_130.0228	1-Pyrroline 4-hydroxy-2-carboxylate	C ₅ H ₈ O ₃ N	1.412	12.916	0.0017742
8.01_144.036	Octanoic acid	C ₈ H ₁₆ O ₂	1.04722	34.886	6.55E-05
9.14_199.161	Lauramide	C ₁₂ H ₂₅ NO	1.62047	630.16	0.0014492
8.85_201.1129	Heteropyrithiamine	C ₁₁ H ₁₃ N ₄	1.13337	279.32	9.77E-05
1.13_203.0665	Lys-Ala	C ₉ H ₁₉ N ₃ O ₃	1.28695	11.954	0.0031665
6.03_220.0714	Asp-Ser	C ₇ H ₁₂ N ₂ O ₆	1.23198	3.6768	0.016655
10.57_233.0657	Lys-Ser	C ₉ H ₁₉ N ₃ O ₄	1.12395	290.4	0.00056947
4.94_243.1724	Cytidine	C ₉ H ₁₃ N ₃ O ₅	1.33214	419.5	0.0010608
8.62_243.1911	Lys-Pro	C ₁₁ H ₂₁ N ₃ O ₃	2.24671	990.02	1.09E-09
1.12_251.0834	Deoxyadenosine	C ₁₀ H ₁₃ N ₅ O ₃	2.06978	39.629	0.00071983
5.16_255.0814	Dihydroneopterin	C ₉ H ₁₃ O ₄ N ₅	1.12851	4.2526	0.01321
10.60_255.2246	Palmitamide	C ₁₆ H ₃₃ NO	7.81627	1,963.7	1.01E-05
11.31_283.2548	Stearamide	C ₁₈ H ₃₇ NO	7.99357	13122	3.67E-06
9.35_299.1956	Dehydrosphinganine	C ₁₈ H ₃₇ NO ₂	1.10186	65.754	0.0013811
4.40_314.1949	Progesterone	C ₂₁ H ₃₀ O ₂	1.5214	591.05	0.0041312

¹RT_Exact MS = retention time and exact mass.

²Variable importance in the projection.

were slightly more abundant in generation 4,000 than generation 0.

Metabolite Enrichment Pathway Analysis

All 175 significantly different metabolites (including intracellular and extracellular metabolites from generation 0 vs. 2,000, generation 0 vs. 4,000, and generation 2,000 vs. 4,000) were analyzed by a pathway analysis (Figure 6). As shown in Figure 6, metabolites from the 0 versus 2,000, 0 versus 4,000, and 2,000 versus 4,000 generation comparisons are involved in various metabolic processes, such as nucleotide metabolism and AA metabolism.

Supplemental Figure S4 (<https://doi.org/10.3168/jds.2018-15702>) shows a metabolite enrichment analysis chart. In total, 32 metabolic subpathways were shared by all 3 generations, including histidine metabolism, fatty acid metabolism, arginine and proline metabolism, the urea cycle, β-alanine metabolism, glycine and serine metabolism, aspartate metabolism, purine metabolism, nicotinate and nicotinamide metabolism, glycerolipid metabolism, lysine degradation, tyrosine metabolism, and vitamin B₆ metabolism.

For the generation 0 versus 2,000 comparison, 3 metabolic pathways for metabolite enrichment, histidine metabolism, arginine and proline metabolism, and vitamin B₆ metabolism were observed, as shown in Figure 6. Similarly, arginine and proline metabolism as well as thiamine metabolism were observed in the

0 versus 4,000 generation comparison. For the 2,000 versus 4,000 generation comparison, lysine degradation, nicotinate and nicotinamide metabolism, and histidine

Figure 4. The distribution of significant metabolic differences among the 3 generations (generation 0 vs. 2,000; generation 0 vs. 4,000; generation 2,000 vs. 4,000).

Figure 5. Changes in the contents of 23 substances differentially expressed between generations 0 and 4,000.

metabolism were observed in both the enrichment and pathway analyses.

All of the differential substances identified in the present experiment were analyzed in terms of their metabolic pathways (Table 5). The metabolic pathway analysis revealed that the metabolites that significantly changed during subculturing of the original strain from generation 0 to 4,000 were mainly responsible for the changes in alanine, aspartate, and glutamate metabolism; glycine, serine, and threonine metabolism; histidine metabolism; cysteine and methionine metabolism; valine, leucine, and isoleucine biosynthesis; α -linolenic acid metabolism; and other metabolic pathways (Table 5).

The metabolites of generation 0 versus those of generation 4,000 were analyzed using iPath (<https://pathways.embl.de/ipath3.cgi>; Figure 7). The primary metabolic pathways of the laboratory evolution of *L. casei* Zhang affected by the differential metabolites were AA metabolism, carbohydrate metabolism, nucleotide metabolism and cofactors, and vitamin metabolism.

DISCUSSION

Effects of Glucose Restriction on Carbohydrate Metabolism

A glucose-restricted environment generally occurs during the stationary phase of microbial growth. Under normal culture conditions, metabolic diversity of probiotic *L. casei* Zhang will occur. A significant scarcity of carbon sources can cause lactobacillus to stop growing (to ensure survival), such that it enters a stable period. Because glucose is depleted during later growth stages, the cells begin to use other sugars (Wang et al., 2012).

Borch et al. (1991) studied *Lactobacillus* sp. under limited glucose conditions in a continuous anaerobic culture with complete cell recycling and found that homofermentation transitioned into heterolactic fermentation when glucose was continuously depleted within the cell cycle such that the cells were starved. Under conditions of limited glucose, especially during cell cycle reculture, AA are widely produced and utilized, and

some sulfides are produced (Borch et al., 1991). This is consistent with the present experimental results.

Effects of Glucose Restriction on AA Metabolism

Amino acids are involved in many physiological functions of cells. Under stress conditions, AA can accumulate in significant amounts and function as cell-protective agents (Devantier et al., 2005; Jozefczuk et al., 2010; Kang et al., 2011; Li et al., 2012). Of the 23 substances that were detected at the intersection of the 3 experimental groups, the contents of substances involved in the AA metabolic pathway were elevated. For example, urocanic acid was detected in generation 0, 2,000, and 4,000, and the relative content increased with generation time. Urocanic acid is believed to be useful as a sun-screening agent in cosmetics and pharmaceuticals (Yamamoto et al., 1974), and Hogan et al. (1964) reported that elevated levels of urocanic acid may be due to reduced folate levels. Urocanic acid is the final product of histidine metabolism, which breaks histidine down into glutamic acid in the presence of formiminoglutamic acid (Chanarin, 1963). Humans metabolize histidine to uric acid via the activity of L-histidine ammonium lyase, which then enters the tricarboxylic acid cycle (Bentley et al., 1995). Several AA such as glycine, serine, threonine, and alanine in *Saccharomyces cerevisiae* cells increased significantly (Li et al., 2012). Similarly, changes in AA caused by starvation can also lead to changes in proteins. Ac-

cording to Kunji et al. (1993), arginine starvation in *Lactococcus lactis* ssp. *lactis* led to the induction of 15 proteins, whereas 14 proteins were induced after galactose starvation. Bacteria adapt to nutrition limitations by assuming a physiological state that is characterized by downregulation of nucleic acid and protein synthesis and simultaneous upregulation of protein degradation and AA synthesis (Chatterji and Ojha, 2001; Hussain et al., 2009). Some researchers also reported that the size and fatty acid composition of microbial cells will change under growth conditions adapted to nutrient deficiencies (Rice and Oliver, 1992). At the same time, the rate of cellular protein synthesis decreases, and the protein expression associated with long-term survival or environmental stress increases significantly (Schultz and Matin, 1991). In the present study, 23 unique metabolites were identified between generations 2,000 and 4,000. For example, O-phospho-L-serine was involved in glycine, serine, and threonine metabolism, glyceraldehyde-3-phosphate was involved in glycolysis and gluconeogenesis and vitamin B₆ metabolism, and 2-oxo-5-aminovalerate was involved in arginine and proline metabolism. The levels of these 3 substances were elevated when subcultured in a glucose-limited environment, which corresponds to the present finding that most of the significant metabolites participated in the AA metabolic pathway. In a study investigating the restricted carbon source culturing of *L. lactis* IL-1403, Redon et al. (2005) reported that citric acid is used preferentially when a carbon is scarce. To maintain the

Figure 6. Pathway analysis of significantly differentiated metabolites. (A) Generation 0 versus 2,000. 1 = alanine, aspartate, and glutamate metabolism; 2 = histidine metabolism; 3 = glycine, serine, and threonine metabolism; 4 = vitamin B₆ metabolism; 5 = cysteine and methionine metabolism; 6 = lysine biosynthesis; 7 = aminoacyl-tRNA biosynthesis; and 8 = arginine and proline metabolism. (B) Generation 0 versus 4,000. 1 = histidine metabolism; 2 = alanine, aspartate, and glutamate metabolism; 3 = glycine, serine, and threonine metabolism; 4 = arginine and proline metabolism; 5 = thiamine metabolism; 6 = phenylalanine metabolism; and 7 = aminoacyl-tRNA biosynthesis. (C) Generation 2,000 versus 4,000. 1 = histidine metabolism; 2 = nicotinate and nicotinamide metabolism; 3 = lysine degradation; 4 = arginine and proline metabolism; 5 = cysteine and methionine metabolism; 6 = alanine, aspartate, and glutamate metabolism; and 7 = aminoacyl-tRNA biosynthesis.

Table 5. Significant metabolic pathways of metabolites from generation 0 to 2,000 and 4,000

Pathway name	Raw <i>P</i> -value ¹	−log (<i>P</i> -value)	Holm <i>P</i> -value	FDR ²	Impact ³
Alanine, aspartate, and glutamate metabolism	0.0023171	6.0674	0.17146	0.023171	0.54606
Glycine, serine, and threonine metabolism	1.43E-05	11.158	0.0011265	5.70E-04	0.34468
Histidine metabolism	0.001661	6.4003	0.12457	0.022146	0.3032
Cysteine and methionine metabolism	0.0015956	6.4405	0.12127	0.022146	0.20644
Valine, leucine, and isoleucine biosynthesis	0.003994	5.523	0.28357	0.031952	0.20403
α-Linolenic acid metabolism	0.70259	0.35298	1	1	0.20335
Arginine and proline metabolism	0.0034295	5.6753	0.24693	0.030485	0.19684
Tryptophan metabolism	0.21683	1.5287	1	0.69182	0.17943
Retinol metabolism	0.22484	1.4924	1	0.69182	0.17514
Lysine degradation	0.29906	1.2071	1	0.85446	0.17297
Thiamine metabolism	0.0023171	6.0674	0.17146	0.023171	0.16019
Nicotinate and nicotinamide metabolism	0.26524	1.3271	1	0.78589	0.14392
Tyrosine metabolism	0.19482	1.6357	1	0.69182	0.14174
Lysine biosynthesis	0.0084794	4.7701	0.59356	0.061668	0.13361
Phenylalanine metabolism	0.033943	3.3831	1	0.21072	0.13206
Folate biosynthesis	0.51629	0.66109	1	1	0.13123
Biotin metabolism	0.071042	2.6445	1	0.37889	0.12195
Pyrimidine metabolism	0.094534	2.3588	1	0.4449	0.11351
Aminoacyl-tRNA biosynthesis	9.77E-07	13.839	7.82E-05	7.82E-05	0.11268
Steroid hormone biosynthesis	0.3772	0.97498	1	0.91442	0.11043
Sulfur metabolism	0.034242	3.3743	1	0.21072	0.10985
Vitamin B ₆ metabolism	0.37704	0.97541	1	0.91442	0.10376
Glycolysis or gluconeogenesis	0.7266	0.31937	1	1	0.1035
Ascorbate and aldarate metabolism	0.84865	0.16411	1	1	0.07571
Pantothenate and CoA biosynthesis	0.094541	2.3587	1	0.4449	0.07366
Ubiquinone and other terpenoid-quinone biosynthesis	0.43502	0.83237	1	1	0.0709
Sphingolipid metabolism	0.64813	0.43366	1	1	0.06438
Arachidonic acid metabolism	0.92654	0.076294	1	1	0.05672
Fatty acid metabolism	0.61213	0.4908	1	1	0.04482
Valine, leucine, and isoleucine degradation	0.22106	1.5093	1	0.69182	0.03889
Purine metabolism	0.16894	1.7782	1	0.67575	0.03469
Glyoxylate and dicarboxylate metabolism	0.33313	1.0992	1	0.88836	0.03291
D-Glutamine and D-glutamate metabolism	0.36758	1.0008	1	0.91442	0.02674
β-Alanine metabolism	0.10289	2.2741	1	0.45729	0.02328
Pentose phosphate pathway	0.73788	0.30397	1	1	0.02181
Glycerolipid metabolism	0.73788	0.30397	1	1	0.0206
Butanoate metabolism	0.81299	0.20704	1	1	0.01774
Amino sugar and nucleotide sugar metabolism	0.88254	0.12495	1	1	0.01487
Citrate cycle (tricarboxylic acid cycle)	0.19453	1.6371	1	0.69182	0.01446
Nitrogen metabolism	7.88E-04	7.146	0.06068	0.015761	0.0083
Phenylalanine, tyrosine, and tryptophan biosynthesis	5.77E-04	7.4572	0.045027	0.015394	0.008
Pentose and glucuronate interconversions	0.89222	0.11404	1	1	0.00638
Selenoamino acid metabolism	0.60091	0.50931	1	1	0.00482
Glutathione metabolism	0.19959	1.6115	1	0.69182	0.0019
Propanoate metabolism	0.76902	0.26264	1	1	0.00134
D-Arginine and D-ornithine metabolism	0.039144	3.2405	1	0.22368	0
Cyanoamino acid metabolism	0.1361	1.9943	1	0.57307	0
Fatty acid biosynthesis	0.32177	1.1339	1	0.88763	0
Taurine and hypotaurine metabolism	0.566	0.56917	1	1	0
Fatty acid elongation in mitochondria	0.67649	0.39083	1	1	0
Pyruvate metabolism	0.73788	0.30397	1	1	0
Porphyry and chlorophyll metabolism	0.98797	0.012104	1	1	0

¹The original *P*-value calculated from the enrichment analysis.²False discovery rate.³The pathway impact value calculated from pathway topology analysis.

supply of cellular energy, the cells hydrolyze arginine to produce ornithine through the arginine deiminase pathway during the growth phase and then produce ATP to supply energy (Redon et al., 2005). The accumulation of these AA represents a stress response of the cells to carbon stress to preserve the integrity of the cells.

Effects of Glucose Restriction on Nucleotide Metabolism

Thymine was the base of deoxyribonucleic acid in the present experiment, and the thymine content increased during the subculturing of *L. casei* Zhang from

Figure 7. Analysis of significant metabolites using iPath (<https://pathways.embl.de/ipath3.cgi>) metabolic pathways between generation 0 and 4,000. Red node = metabolites; red line = interaction.

generation 0 to 4,000. Soska (1966) cultured *Lactobacillus acidophilus* R-26 in a medium without folic acid and found that the organism required thymine in addition to deoxynucleosides, purines, pyrimidines, and most AA. When thymine was present in the medium, the bacteria grew exponentially and without limitation. The presence of thymine can meet the demand for folic acid by *Lactobacillus leichmannii* (Thompson, 1952) and, in the absence of thymine, there is a 150% increase in DNA content that induces an extra cycle of chromosome replication (Elli et al., 1999; Soska, 1966). Uridine is a nucleoside that serves as a major component of ribonucleic acid. In the present study, uridine increased from generation 2,000 to 4,000, whereas guanosine decreased from generation 0 to 4,000. Guanosine is a proven taste enhancer when fermenting bread with *Lactobacillus plantarum* (McCabe and Rolls, 2007) and can provide significant growth stimulation for the *Lactobacillus johnsonii* NCC533 strain; however, nucleotide supplementation is required for *L. plantarum* growth in reconstituted skim milk (Elli et al., 1999). Furthermore, *Lactobacillus gasseri* PA-3 shows greater proliferation in the presence of guanosine during culture (Yamada et al., 2018). Metabolomic analyses were used to assess *Streptococcus intermedius* under aerobic and anaerobic conditions, and it was revealed that the intracellular guanosine concentration was higher during anaerobic growth and that the cells absorbed more under anaerobic conditions (Fan et al., 2016). Additionally, analyses of the presence of several typical molecules associated with the flavor and taste of foods during the fermentation of bread with *Lactobacillus plantarum* have detected uridine and guanosine (Valerio et al., 2017).

Effects of Glucose Restriction on the Metabolism of Cofactors and Vitamins

9-Cis-retinoic acid, which is associated with retinol metabolism, is the active substance in vitamin A (Priyamvada et al., 2015) and vitamin B₆ and is associated with the metabolism of pyridoxine and pyridoxamine during the metabolism of cofactors and vitamins. It has been reported that *L. casei* can be cultured on a medium containing riboflavin, pantothenic acid, nicotinic acid, and pyridine as AA sources. It has also been reported that *L. casei* can be cultured on a medium containing riboflavin, pantothenic acid, nicotinic acid, and pyridine as AA sources (Hutchings et al., 1941).

In the present study, there was a significant decrease in nicotinate, which is associated with nicotinate and nicotinamide metabolism, from generation 2,000 to 4,000. The essential growth factors needed by *Lactobacillus* include riboflavin, pantothenic acid, nicotinic acid, pyridoxine, biotin, and the eluate factor such as

folic acid (Hutchings et al., 1941). *Lactobacillus gasseri* can produce NAD using nicotinate and nicotinamide in the presence of nicotinamidase and nicotinate phosphoribosyltransferase (Foster and Moat, 1980), whereas *L. johnsonii* and *L. gasseri* lack the corresponding enzymes to synthesize thiamine, biotin, pyridoxine, and other substances involved in vitamin digestion and absorption (Azcarate-Peril et al., 2008). For *L. casei* LC2W, nicotinate is an essential nutrient under anaerobic and aerobic conditions and can increase the yield of *L. casei* LC2W exopolysaccharide (Xu et al., 2015).

Although nicotinate content was reduced by the influence of glucose restriction during the process of generation in the present study, *L. casei* Zhang still produced beneficial substances. Therefore, when *L. casei* Zhang is used as a starter, it may lead to a decrease in beneficial substances when long-term glucose sources are insufficient.

Due to the current practice of application of *L. casei* Zhang in food and feedstuffs, it is important to study the metabolic kinetics of *L. casei* Zhang under glucose-restricted conditions. Metabolomic analyses of the metabolites of *L. casei* Zhang during long-term passage under conditions of glucose restriction will provide indispensable supporting data for further understanding the properties of *L. casei* Zhang.

CONCLUSIONS

We studied metabolomics-related differences between an original strain of *L. casei* Zhang and strains cultured in a glucose-restricted medium for 2,000 and 4,000 generations using ultraperformance liquid chromatography quadrupole time-of-flight MS. Significant changes in metabolites were observed under the glucose-restricted conditions. Changes in the metabolites, including AA, nucleotides, and short peptides, resulted in changes in metabolic pathways; the changes were more pronounced when passaged to generation 4,000. The metabolically significant metabolites significantly affected several metabolic pathways, including histidine metabolism, arginine and proline metabolism, vitamin B₆ metabolism, thiamine metabolism, lysine degradation, and nicotinate and nicotinamide metabolism. Metabolomics was used to study changes in the metabolites associated with *L. casei* Zhang under conditions of glucose restriction and to screen for key metabolites involved in the glucose-restricted response. The present findings may provide a theoretical basis for a comprehensive understanding of the metabolic regulatory mechanisms of *L. casei* Zhang during glucose restriction. These findings may also lead to novel ideas and methods for assessing the glucose-restricted stress response at a metabolic level.

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