Proteomic response and molecular regulatory mechanisms of *Bacillus cereus* spores under ultrasound treatment

Ruiling Lv\(^a\),\(^b\),\(^c\), Donghong Liu\(^b\),\(^c\), Wenjun Wang\(^b\),\(^c\), Enbo Xu\(^b\),\(^c\), Tian Ding\(^b\),\(^c\), Xingqian Ye\(^b\),\(^c\), Jianwei Zhou\(^a\),\(^b\),\(^c\)

\(^a\) NingboTech University, Ningbo 315100, China
\(^b\) Ningbo Research Institute, Zhejiang University, Ningbo 315100, China
\(^c\) College of Biosystems Engineering and Food Science, National-Local Joint Engineering Laboratory of Intelligent Food Technology and Equipment, Zhejiang University, Hangzhou 310058, China

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**ABSTRACT**

This study was aimed at providing new insights on the proteomic response of bacterial spores to ultrasound. Data-independent-acquisition method was used to quantify the proteome change of *Bacillus cereus* spores after ultrasound treatment (200 W). This study revealed that 2485 proteins were extracted from *Bacillus cereus* spores, most of them were related to metabolism. After ultrasound treatment, the expression of 340 proteins were significantly changed (the fold change $\geq 2$ and $p < 0.05$), of which 207 proteins were significantly down-regulated. KEGG pathway analysis showed that differentially expressed proteins mainly distributed in metabolism pathway, cell process pathway and genetic information processing pathway after ultrasound treatment. Furthermore, this study analyzed the differentially expressed proteins in significant enrichment pathways. In particular, the expression of key proteins in the phosphorylation reaction of spores was significantly decreased after ultrasound treatment. Thus, ATP synthesis rate decreased and the phosphorylation reaction inhibited. Also, the decrease of the expression of key proteins related to the tricarboxylic acid cycle led to the decrease of nutrients metabolism of spores. Ultrasound treatment induced the down-regulation of fatty acid synthetase expression and promoted fatty acid metabolism at the same time. The content of fatty acids decreased in spores consequently.

**1. Introduction**

Spores are metabolically dormant bacterial cells and can survive even under extreme conditions for a long time [1]. They have multilayered structure which promotes their highly resistance to both chemical and physical (thermal or non-thermal) processes [2]. Besides, they can germinate and continue to grow once under appropriate conditions [3]. Unfortunately, spore-forming bacteria are ubiquitous and can be commonly found in foods, water, environment even in the soil. *Bacillus cereus* (*B. cereus*) are Gram-positive, rod-shaped, aerobic spore-forming bacteria and responsible for food spoilage of various foods [4]. *B. cereus* greatly impact on food safety and quality due to their spoilage-causing capabilities and disease-causing potential [5]. The production of heat labile enter toxins begins once the *B. cereus* grow which is associated with food-borne illnesses: nausea and vomiting; abdominal cramps and diarrhea [4]. *B. cereus* can survive in a wide range of temperatures, pH values, water activities, even in industrial pasteurization. What’s more, *B. cereus* can form biofilm and spore, *B. cereus* frequently exist in nutrition-rich food products, such as meat, dairy products [6]. Thus, *B. cereus* vegetative cells and spores are a major concern in food industry.

Spores are focal point of food industry because of the highly resistance, lots of studies focused on the spore inactivation using thermal and non-thermal technologies. Thermal treatment at 88°C for up to 90 min inactivated 99.9% *B. cereus* spores [7]. During thermal treatment of spores, the release of dipicolinic acid (DPA) appeared to be an all or nothing phenomenon, since there is no spore with partial DPA release [8]. Thermal treatment inactivated *B. subtilis* spores by destroying one or several essential proteins [9].

Rao et al. inactivated spores by high pressure CO\(_2\) with high temperature, over 7 log CFU/mL reductions were achieved depending on temperature and pressure. About 90% DPA released after treatments...
UV-LED was proven to be synergistic to chlorine on spore inactivation, the reactive radicals formed by UV-Cl₂ treatment was significant to the synergism [11]. Blue light also inactivated spores efficiently by inducing DNA damage [12]. Wang et al. investigated the effect and mechanism of cold atmospheric plasma on bacterial spores and found that cold atmospheric plasma treatment for 3 min reached over 5 log CFU/mL reductions damaging inner membrane and germination proteins [13]. Previous study showed that ultrasound did minor inactivation effect to spores (less than 1 log CFU/mL). However, ultrasound treatment reduced the resistance of spores significantly, when it applied with thermal and pressure treatment, synergistic effect was observed [14]. However, there are few studies focused on the protein expression changes of spores after ultrasound treatment.

Recently, with the continuous improvement of proteomics methods by liquid chromatography (LC) and tandem mass spectrometry (MS / MS), we have a deeper understanding of human biology and diseases [15]. Wang et al. investigate the mechanism of high pressure processing and slightly acidic electrolyzed water on B. cereus spore inactivation by label-free quantitative proteomics approach. The results indicated that the metabolic, degradation, signaling, and biosynthesis pathways were involved in HPP-SAEW mediated spore inactivation [16]. The protein expression level of Bacillus subtilis spores mainly associated with metabolism under thermosonication treatment [17]. Since the first description by Venable etc., data independent acquisition (DIA) mass spectrometry has developed for over two decades [18]. Now it is a powerful technique which improving the reproducibility and throughput of proteomics studies [19,20]. Furthermore, this research determined the molecular regulatory mechanisms of the stress responses observed in our previous study [21]. This research aimed to evaluate the proteomic response of Bacillus cereus spores to ultrasound using DIA mass spectrometry. These results will likely provide valuable insights into the molecular mechanism of ultrasound treatment against spores.

2. Material and methods

2.1. Bacterial strain and sporulation

Bacillus cereus ATCC 14,579 was obtained from Hope Bio-Technology Co., Ltd (Qingdao, China). To prepare fresh suspension, Bacillus cereus was inoculated to nutrient broth (NB; 10% peptone, 3% beef extract, 5% NaCl and 15% agar; Hope Bio-Technology Co., Ltd, Qingdao, China), and incubated at 37 °C with constant shaking at 150 rpm overnight. The sporulation was as previously described [14]. The concentration of the spore suspension was 8.08 log CFU/mL for each experiment determined by plating on the nutrient agar with 0.1% soluble starch.

2.2. Ultrasound treatment

Ultrasound treatment was carried out with a 950-W, 20 kHz probe-style ultrasonic processor (Scientz-II D; Ningbo Scientz Biotechnology Co., LTD., Ningbo, China) with a 10-mm-diameter titanium solid probe. An 85-ml cylindrical glass tube was filled with 25 ml spore suspension, and the ultrasonic emitter was immersed 1.5 cm into the liquid. Spore suspension was ultrasonic treated with power at 200 W for 30 min at 20 °C maintained by a thermostatic water bath.

2.3. Protein extraction and quantitation

Treated and control samples were grounded into powder in liquid nitrogen, and 200 mg of each sample was mixed with 5-mm magnetic beads and Lysis buffer 3 (8 M Urea and 40 mM Tris-HCl containing 1 mM PMSF, 2 mM EDTA and 10 mM DTT, pH 8.5). Then, samples were grinded in a tissue grinder at 50 Hz for 2 min. After centrifugation with 25,000 × g at 4 °C for 20 min, the supernatant was treated by adding 10 mM DTT at 56 °C for 1 h. Later on, sample was incubated with 55 mM IAM in dark after cooling down to room temperature (alkylation). By adding 4 × volume of acetone, proteins were precipitated at –20°C for 2 h. Repeat the precipitation procedure until the supernatant was clear. The pellet after centrifugation with 25,000 × g at 4 °C for 20 min was mixed with lysis buffer 3 and grinded for 2 min to dissolve the protein. The supernatant after centrifugation was collected as protein sample and then quantified by Bradford and SDS-PAGE.

2.4. Protein digestion

The protein samples (100 µg) were mixed with Trypsin Gold (Promega, Madison, WI, USA) to digest the proteins (the ratio of protein : trypsin = 40 : 1) at 37 °C for 4 h. The same ratio of trypsin was added one more time and kept digesting at 37 °C for 8 h. Then, proteins were digested to peptides which were desalted with a Strata X C18 column (Phenomenex, Torrance, CA, USA) and vacuum-dried for further detection.

2.5. Peptide fractionation

The peptide samples were mixed and separated on a Shimadzu LC-20AB HPLC Pump system coupled with a high pH RP column. The peptides power was resuspended in mobile phase A (5% ACN, 95% H₂O, pH 9.8) and loaded onto a 4.6 × 250 mm Gemini C18 column containing 5-µm particles (Phenomenex, Torrance, CA, USA). The peptide samples were gradient separated at a flow rate of 1 mL/min with a gradient of 5% mobile phase B (5% H₂O, 95% ACN, pH 9.8) for 10 min, 5–35% mobile phase B for 40 min, 35–95% mobile phase B for 1 min. Finally, the system was maintained in mobile phase B for 3 min and equilibrated with 5% mobile phase B for 10 min. Elution peaks were monitored by absorbance at 214 nm, and fractions were collected in each 1 min. The eluted peptides were pooled as 10 fractions according to the elution peak diagram and vacuum-dried at last.

2.6. HPLC

Each fraction was reconstituted in mobile phase A (2% ACN and 0.1% FA in water) and centrifuged at 20,000 × g for 10 min. First of all, the supernatant was loaded onto a C18 trap column to enrich and desalinate. Then, the peptides were eluted from trap column and separated by an analytical C18 column with inner diameter 150 µm and containing 1.8 µm particles which was self-assembly. The gradient ran at 500 nL/min starting 5% of mobile phase B (2% H₂O and 0.1% FA in ACN) for 5 min, then from 5 to 35% linearly in 155 min, after going up to 80% in 10 min mobile phase B maintained at 80% for 5 min, and finally returned to 5% and equilibrated for 5 min.

2.7. Date analysis by mass spectrometry

Data dependent acquisition (DDA) and data independent acquisition (DIA) analysis were both performed on Q Exactive HF mass spectrometer (Thermo Fisher Scientific, San Jose, USA). For DDA analysis, the mass spectrometry parameters were as follow: ion source voltage 1.6 kV, MS scan range from 350 to 1500 m/z, MS resolution 60,000; MS/MS HCD scans with resolution 15,000, m/z 100, dynamic exclusion duration 30 s; intensity threshold 10,000, charge exclusion, exclude greater than 7 + or less than 2 +. The DIA MS parameters were as follow: full scan range 350–1500 m/z at resolution 120,000; DIA isolation window was set to 17 m/z with loop count 40 and automatic MIT, scanned at resolution 30000; MS/MS HCD detected ion fragment in Orbitrap.

2.8. Data analysis

Each group had triple biological replicates including control and ultrasound treated (US), and each biological replicate had three technical replicates. The protein database was an important in the protein
identification strategy, NCBI was used for identification of proteins in this research. DIA data were analyzed by Spectronaut, and iRT was used to accomplish the correction of the retention time. In addition, Spectronaut integrated mProphet scoring algorithm which could accurately indicate the matching degree of ion pairs. Finally, based on the Targetdecoy model of the SWATH-MS, the false positive control was completed with FDR 1%, and significant quantitative results could be obtained. MSstats is an R package which used for statistical analysis of significant differences between different samples, it supports both label-free and label-based experimental workflows, also data-dependent and data-independent spectral acquisition [22]. Proteins with differential abundance with a \( p \)-value of less than 0.05 and fold change greater than 2.0 were defined as differentially expressed proteins (DEPs).

3. Results

3.1. The identification and quantification of proteins

The mass spectrometric data were acquired using data dependent acquisition method at first. This shotgun proteomics was the first and most widely used strategy [23]. MS/MS spectral database including fragment ion intensity and retention time of the peptides spectral peak characteristics was established with MaxQuant for the further data independent acquisition quantification. Totally, 14,326 peptides and 2485 proteins were detected from Bacillus cereus spores in this research. In NCBI database, there were 5231 proteins totally in Bacillus cereus vegetative cells, previous references detected about 2800 proteins from Bacillus cereus spores using proteomics [16]. The molecular masses of these proteins were mainly in the range of 10–50 kDa (Fig. S1), the number of unique peptide of majority proteins were 1–5 (Fig. S2), and the protein coverage mainly spread in 0–40% (Fig. S3).

Gene Ontology (GO, Fig. 1A), Cluster of Orthologous Groups of proteins (COGs, Fig. 1B) and KEGG pathway analysis (Fig. 1C) were performed to classify the total identified proteins functionally from Bacillus cereus spores. GO included cellular component, biological process and molecular function. Proteins were classified into 12 classes according to the cellular component GO database. Majority of proteins located in cell (31.23%), cell part (30.66%) and membrane (25.19%). In the biological process database, there were 18 classes, in which metabolic process and cellular process predominated, accounting for 33.53% and 32.83%, respectively. Molecular function database identified proteins sorted in 11 classes. Most of the proteins played roles in catalytic activity (51.56%) and binding (35.26%). Total proteins classified into 24 classes in COG database. The top 5 categories were general function prediction only (9.55%); amino acid transport and metabolism (9.51%); translation, ribosomal structure and biogenesis (9.31%); transcription (8.96%) and energy production and conversion (6.40%). KEGG pathway analysis was used to classified the protein functions. KEGG database includes 7 branches: cellular processes, environmental information processing, genetic information processing, human diseases,
metabolism, organismal systems and drug development. The proteins of *Bacillus cereus* spores distributed in five branches except the latter two (Fig. 1C). Obviously, metabolism-related proteins had an absolute advantage in quantity, including amino acid, carbohydrate, cofactors and vitamins, nucleotide, lipid and energy.

### 3.2. The differentially expressed proteins

In order to increased signal-to-noise ratio, sensitivity, selectivity and dynamic range, the mass spectrometric data acquired using the data-independent acquisition method based on the date dependent collected MS/MS spectral database [18]. As described before, proteins with differential abundance with a p-value of less than 0.05 and fold change greater than 2.0 were defined as differentially expressed proteins (DEPs). DEPs were identified between control and ultrasound treatment groups. The volcano plot (Fig. 2A) plotted p value against fold change of proteins for all DEPs. It represented the expression level difference and statistically significant degree between two groups overall. Each point represented a protein, the gray dots represented a protein that did not have significant difference. The green spots represented down-regulated proteins while the red represented up-regulated. Fig. 2B was the cluster analysis of protein expression patterns difference between control and ultrasound group. The blue lines represented the down-regulated proteins, red lines represented the up-regulated proteins while the blank part meant no significant expression change. The results showed that there were 340 DEPs totally after 30 min ultrasound treatment. Among them, 207 proteins were down-regulated while 133 proteins were up-regulated. The number of DEPs of *Bacillus cereus* spore after ultrasound treatment was higher than that treated by slightly acidic electrolyzed water [16]. Though previous study showed that ultrasound had little inactivation effect on *Bacillus cereus* spore, a large number of proteins regulated to respond the ultrasound treatment. This was the possible reason for the resistance decrease after ultrasound treatment [13].

### 3.3. GO analysis of DEPs after ultrasound treatment

GO analysis were executed to classify the 340 DEPs after ultrasound treatment (Fig. 3). From the biological process database, DEPs after ultrasound treatment belonged to 12 categories, in which metabolic process, cellular process and localization were in the majority. Particularly, 68 metabolic process related proteins were down-regulated after ultrasound treatment (64.15%), indicating that ultrasound decreased the metabolic activity which might increase the survival rate of spores under ultrasound stress. The number of DEPs related to localization was also in the majority, including localization of cell, cellular localization and macromolecule localization (like proteins). DEPs after ultrasound treatment mainly located in membrane (23.67%), cell (22.86%), cell part (22.65%) and membrane part (22.04%). Using the molecular function database, the DEPs classified into 8 classes after ultrasound treatment. The majorities were catalytic activity, binding and transporter activity taking 50.00%, 27.81% and 17.70 %, respectively.

### 3.4. KEGG pathway analysis of DEPs after ultrasound treatment

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of pathway maps in order to have a better understanding of molecular interaction, reaction and relation network of proteins [24]. Generally, DEPs after treatment belonged to cellular processes, environmental information processing, genetic information processing, human diseases and metabolism (Fig. 4). Metabolic pathways were in an absolute majority, including carbohydrate metabolism, energy metabolism, lipid metabolism, metabolism of cofactors and vitamins, metabolism of other amino acids, nucleotide metabolism, and so on. Besides, the number of down-regulated proteins were more than two times of the up-regulated which was coincident with the GO analysis results. Similarly, Schultz et.
investigated that low-intensity pulsed ultrasound affected the metabolism of pyrimidine and five amino acids of *Saccharomyces cerevisiae* [25].

3.5. Subcellular distribution of DEPs after ultrasound treatment

After transcription and translation, proteins are synthesized on ribosome and transported to specific sites. Proteins play its existing function and cells complete their normal life activities once in the right position. Therefore, subcellular localization of proteins was a very important part of protein function annotation. The subcellular distribution was predicted using PSORTb after ultrasound treatment [26]. As shown in Fig. 5, DEPs mainly located in the cytoplasm (154 DEPs, accounting for 54.80%) after ultrasound treatment. Cytoplasm was the place where cell life activities carried out, including protein synthesis, respiration and so on. It was consistent with the previous study that ultrasound treatment destroyed the inner membrane permeability [14]. In addition, 113 differential proteins were distributed on the cytoplasmic membrane, accounting for 40.21% of all DEPs. Plasma membrane played an important role in maintaining the relative stability of intracellular microenvironment and external material and energy exchange. The results of subcellular distribution indicated that the proteins in cytoplasm also had corresponding stress response after ultrasound treatment. In addition, a small portion of DEPs were distributed in the cell wall and extracellular.

4. Discussions

The DIA proteomics results showed that 340 proteins expressed differentially after ultrasound treatment. The DEPs were mainly distributed in metabolism, cell process, and genetic information processing pathways. The key pathways involved in DEPs were further discussed.

4.1. Changes associated with oxidative phosphorylation

The main source of ATP in organisms is oxidative phosphorylation reaction [27]. NADH dehydrogenase was the key protein in the electron transport chain, which catalyzed the oxidation of NADH and released two electrons [28]. The expression of NADH dehydrogenase subunit M/L/H/A decreased significantly after ultrasound treatment. Thus, the activity of the dehydrogenation reaction and electron chain reduced significantly. The expression of several cytochrome c oxidase...
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polypeptides also decreased significantly (Table 1), which causing the significant decrease in the ability of transmembrane proton pumping and the rate of electron consumption. ATP synthase was the terminal enzyme in oxidative phosphorylation, which was known as the smallest rotating engine [41]. Interestingly, the expression of flagellum-specific ATP synthase in oxidative phosphorylation pathway, glucose 6-phosphate dehydrogenase was certainly of central metabolism. As the first enzymatic step of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase was certainly of central metabolism. As the first enzymatic step of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase was certainly of central metabolism. As the first enzymatic step of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase was certainly of central metabolism. 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Cereus spores through pentose phosphate pathway was significantly improved after ultrasound treatment. Tricarboxylic acid (Kreb's, TCA) cycle was the key pathway of nutrient metabolism and the main channel of obtaining energy. Thus, the expression of proteins related to TCA cycle could reflect the basic energy production and metabolism level of cell [37]. TCA cycle converted NAD$^+$ to NADH and FAD to FADH$_2$. Meanwhile, it produced a GTP at the same time. The DEPs involved in TCA cycle were down-regulated after ultrasound treatment of spores, including the key enzyme in the first step: cysteine synthase. Thus, the ability of carbohydrate catabolism of spores decreased, and the capacity of energy production decreased after ultrasound treatment.

### 4.4. Changes associated with fatty acid synthesis and metabolism

Bacteria synthesized fatty acids through fatty acid synthesis cycles, starting with acetic acid, CoA and ATP [38]. The expression of acetyl-CoA acetyltransferase, enoyl-CoA hydratase and other key enzymes decreased significantly after ultrasound treatment (Table 4). These results indicated that ultrasound had an adverse effect on the fatty acid synthesis ability of Bacillus cereus spores.

| Protein ID   | Ratio | Change | Description                           |
|--------------|-------|--------|---------------------------------------|
| NP_830560.1  | 1.35  | up     | short chain dehydrogenase             |
| NP_833117.1  | 1.03  | up     | short chain dehydrogenase             |
| NP_833571.1  | 1.32  | up     | ACP S-malonyltransferase              |
| NP_834419.1  | 1.34  | up     | glucose-1-dehydrogenase               |
| NP_834943.1  | 1.09  | down   | (3R)-hydroxymyristoyl-ACP dehydratase |
| NP_833741.1  | 1.32  | down   | acetyl-CoA acetyltransferase          |
| NP_834232.1  | 1.65  | down   | enoyl-CoA hydratase                   |

Table 4: The DEPs in fatty acid synthesis and metabolism pathways.

The metabolism of fatty acids was also known as fatty acid oxidation or $\beta$-oxidation [39]. Firstly, fatty acid was activated, then converted into fatty acyl-CoA and into acetyl-CoA. The metabolism of fatty acid supplied acetyl-CoA for TCA and maintained the activity of the electron transport chain [40]. Table 4 showed that ultrasound up-regulated significantly the key enzymes of fatty acid metabolism such as glucose-1-dehydrogenase, short chain dehydrogenase. In general, ultrasound treatment decreased the ability of fatty acid synthesis, and promoted the metabolism of fatty acid at the same time. The reserve of fatty acid in Bacillus cereus spores decreased under ultrasonic stress.

### 4.5. Changes associated with ABC transporters

It is greatly important to complete the transport of organic and inorganic molecules on the cell membrane and maintain the intracellular balance for microorganisms [41]. For example, about 10% of the genome encodes transporters of E. coli that bind to the cell membrane [42]. Most ABC transporters pumped the transport substrate in a chemical gradient, and required ATP hydrolysis to provide the driving force [43]. ABC transporters were usually specific for substrate and in single direction, which meant one ABC transporter could only transport one or a class of compounds [44,45]. The expression of sugar transport ATP-binding protein was significantly down-regulated, which indicated that the sugar transport ability of Bacillus cereus spores was weakened after ultrasound treatment (Table 5). However, the expression of phosphate transporter ATP-binding protein was up-regulated, indicating the ability of phosphate transport was enhanced after ultrasound treatment. The main protein regulations of Bacillus cereus spores under ultrasonic stress were illustrated in Fig. 6. Red arrows indicated up-regulation of DEPs on related pathways, and green arrows indicated the down-regulated DEPs. Key proteins in TCA cycles, oxidative phosphorylation, amino acid synthesis and metabolism, fatty acid biosynthesis and ABC transporter pathways were down-regulated under ultrasound treatment.

| Protein ID          | Ratio | Change     | Description                        |
|---------------------|-------|------------|------------------------------------|
| NP_832710.1         | 1.60  | down       | sugar transport ATP-binding protein|
| NP_833980.1         | 1.63  | up         | phosphate transporter ATP-binding protein |
| NP_834244.1         | 1.08  | up         | bacitracin transport ATP-binding protein bcrA |
| NP_834561.1         | 1.72  | up         | lipase                             |

Table 5: The DEPs in ABC transporters pathways.

### 5. Conclusions

The present study illustrated that the actions of ultrasound treatment against B. cereus spores by proteomic response and molecular regulatory. Totally 340 proteins expressed differentially which mainly distributed in metabolic, cellular process and genetic information.

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Fig. 6. Schematic diagram of main protein regulations of Bacillus cereus spores under ultrasound treatment (arrows indicate up-regulation or down-regulation of DEPs on related pathways).
processing pathways. The analysis of DEPs on the significant enrichment pathway indicated that spores mainly respond to ultrasound treatment by regulating energy and carbohydrate metabolism pathways. However, the pentose phosphate pathway and fatty acid metabolism pathway were up-regulated.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultrasonch.2021.105732.

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