Proteomics-Based Mechanistic Investigation of Escherichia coli Inactivation by Pulsed Electric Field

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The pulsed electric field (PEF) technology has been widely applied to inactivate pathogenic bacteria in food products. Though irreversible pore formation and membrane disruption is considered to be the main contributing factor to PEF’s sterilizing effects, the exact molecular mechanisms remain poorly understood. In this study, by using mass spectrometry (MS)-based label-free quantitative proteomic analysis, we compared the protein profiles of PEF-treated and untreated Escherichia coli. We identified a total of 175 differentially expressed proteins, including 52 candidates that were only detected in at least two of the three samples in one experiment group but not in the other group. Functional analysis revealed that the differential proteins were primarily involved in the regulation of cell membrane composition and integrity, stress response, as well as various metabolic processes. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis was conducted on the genes of selected differential proteins at varying PEF intensities, which were known to result in different cell killing levels. The qRT-PCR data confirmed that the proteomic results could be reliably used for further data interpretation, and that the changes in the expression levels of the differential candidates were, to a large extent, caused directly by the PEF treatment. The findings of the current study offered valuable insight into PEF-induced cell inactivation.

Keywords: pulsed electric field, E. coli, proteomics, cell inactivation, molecular mechanisms

INTRODUCTION

The pulsed electric field (PEF) technology is a mild, non-thermal method that processes various biological materials with intermittent high-intensity electric energy (Clark, 2006). Compared to other thermal processing techniques, PEF generates substantially less heat, which ensures better preservation of flavor, color and nutrition in food products. PEF also offers obvious advantages over both enzyme- and chemical-based cold processing methods in that it does not introduce any potentially quality-degrading additives. One of the main applications of PEF in food and beverage industry is the inactivation of microbial pathogens. For example, liquid whole egg processed by combination of PEF and mild thermal treatment at 55°C exhibited significantly longer shelf-life at 4°C than those treated with heat alone (Hermawan et al., 2004). In another study, Listeria...
monocytogenes populations in different liquid milk products were reduced by a factor of $10^4$ with a 0.6-ms PEF treatment at 50°C (Reina et al., 1998). Recently, PEF has also been evaluated for the preservation of solid food products, such as fruits (Sotelo et al., 2015; Yu et al., 2017), and for facilitating the extraction of valuable chemicals from plants and fungi (Goettel et al., 2013; Xue and Farid, 2015).

Currently, studies that aim to investigate the mechanism of PEF-induced cell inactivation have focused predominantly on its deleterious effects on membrane integrity (Unal et al., 2002; Wu, 2008). It is generally accepted that cell membranes exposed to PEF are locally distorted by electromechanical compression, culminating in pore formation and even membrane rupture (Weaver and Chizmadzhev, 1996). In comparison, there have been very few studies on whether PEF also affects other cellular components or processes, particularly proteins (Li et al., 2011; Rivas et al., 2013). In this regard, it has been demonstrated that PEF could induce significant structural changes in solid-state egg white proteins (Qian et al., 2016). Meanwhile, PEF-processed malting barley seeds showed reduced α-amylase expression compared to the untreated controls (Dymek et al., 2012). In recent years, the rapid development of proteomic technologies has allowed researchers to systematically analyze proteome-wide changes in response to external stimuli. For example, Rivas et al. (2013) examined the proteomic changes in PEF-damaged *Escherichia coli* DH5α cells with or without a 1-h recovery in Luria-Bertani (LB) medium after the treatment. The recovery and non-recovery groups showed different sets of differentially expressed proteins, which were mainly associated with cell metabolism, membrane structure, aerobic respiration, and protein folding (Rivas et al., 2013). There have also been a number of other studies regarding the effects of PEF treatment or electroporation on the mRNA and/or protein expression in various prokaryotic and eukaryotic cells (Mlakar et al., 2009; Heller et al., 2010; Zhao et al., 2018). However, further research is needed in order to provide a more comprehensive and detailed elucidation of how PEF disrupts various aspects of cellular structures and functions to achieve its sterilizing effects.

**MATERIALS AND METHODS**

**Cell Culture and PEF Treatment**

The *E. coli* strain CGMCC44102 was obtained from the China General Microbiological Culture Collection Center (CGMCCC), and cultured in LB medium at 150 rpm and 37°C to a final optical density (OD) of 1.0 at 600 nm. Subsequently, 1 mL of the culture broth was collected and centrifuged at 8,000 rpm, 4°C for 2 min. The cell pellet was re-suspended in 2 mL of ice-cold 0.1% (w/v) bacteriological peptone solution (Solarbio Life Science, China) and divided into 70 µL aliquots. For PEF treatment, each aliquot was pipetted into a new, sterile 1.0-mm electroporation cuvette and pulsed using a BTX ECM830 Square Wave Electroporation System (BTX, United States) based on a protocol described previously (Liu Z.Y. et al., 2016). The cell density in the aliquot was approximately $10^8$ CFU/mL and electric conductivity was 0.19 mS/cm. The general setup of the device and the wave shape of the pulse were illustrated in Figure 1. After treatment, the cells were serially diluted and counted based on a previously described protocol (Aronsson et al., 2005) to calculate the inhibition rate. To achieve different cell killing levels, we employed different sets of pulse conditions as previously described. For a cell killing extent of 95 ± 2.0%, pulse intensity, number and duration were set to 14.5 kV·cm⁻¹, 26 and 67 µs, respectively. The killing extent was 51 ± 1.3% with the treatment parameters of 6.10 kV·cm⁻¹, pulse number of 54, pulse duration of 77 µs. And when the pulse intensity was 2.88 kV·cm⁻¹, pulse number was 62, pulse duration was 82 µs, the killing extent was 29 ± 0.7%. Based on measurement on an RC05 Infrared Thermal Imaging Camera (Rinich Industrial, China), the culture temperature showed an average increase of less than 3°C following the PEF treatment, which would not significantly reduce the viability of the cells.

The electroporated aliquots were pooled together to a final volume of 3 mL (~43 aliquots) and divided in a clean 1.5 mL microcentrifuge tube placed on ice. This was used as an experimental replicate. The pooled *E. coli* suspension was then centrifuged at 4°C, 5,000 × g for 5 min, washed twice with and then re-suspended in ice-cold phosphate-buffered saline (PBS), and then quickly flash-frozen in liquid nitrogen for storage at ~80°C. For controls, a mock treatment without the application of PEF was performed, with identical downstream procedures. The whole experiment described above was conducted in triplicate.

**Protein Extraction**

The cell pellet of strain was suspended in 200 µL SDT lysis buffer consisting of 4% (w/v) sodium dodecyl sulfate (SDS), 0.15 M Tris–HCl at pH 7.6 and 100 mM dithiothreitol (DTT), and transferred to a 2-mL tube pre-filled with 1/3 vol of quartz sand and ten steel grinding beads. The resultant mixture was subsequently homogenized twice on a Fastprep-24 homogenizer (MP Biochemicals, United States) at 6.0 m/s, followed by ten cycles of 10-s sonication at 80 W with 15-s intervals. The homogenate was boiled for 15 min and centrifuged at 14,000 × g for 40 min, after which the supernatant was sterilized with a 0.22-µm filter. Total protein in the filtrate was quantified with a BCA Protein Assay Kit (Bio–Rad, United States). All filtered protein extracts were stored at ~80°C until use.

**Trypsin Digestion**

Based on the BCA assay results, 200 µg of the extracted protein were denatured with 30 µL of SDT buffer and incubated at 56°C for 30 min. Low-molecular-weight components, including SDS and DTT, were removed by repeated ultrafiltration through a Microcon 10-kDa Centrifugal Filter Unit (Millipore, United States) with UA buffer, which consisted of 8 M urea in 0.1 M Tris–HCl at pH 8.0, as the wash buffer. The sample was mixed with 100 µL of 100 mM iodoacetamide in UA, followed by a 30-min incubation in darkness. The filter unit was subsequently washed with 100 µL of UA twice and 100 µL of 25 mM NH₄HCO₃ twice. The resultant protein sample was digested by adding 4 µg of trypsin (Promega, United States) in 40 µL of 25 mM NH₄HCO₃ to the filter unit and then incubating at 37°C for 18 h. The filter unit was centrifuged at 13,400 rpm for 30 min and the peptide products were collected...
as a flow-through. The filter unit was next washed with 40 µL of 25 mM NH₄HCO₃ and centrifuged as above. The two flow-through were combined, desalted on an Empore C-18 Standard Density Solid-Phase Extraction Cartridge (bed I.D. 7 mm, volume 3 mL; Sigma-Aldrich, United States) and concentrated by vacuum centrifugation, followed by reconstituting the peptides in 40 µL of 0.1% (v/v) formic acid. Peptide quantification was performed by measuring the absorbance at 280 nm on an ultraviolet-visible spectrophotometer.

**Liquid Chromatography (LC)-MS/MS Analysis**

Proteomic analysis was performed on an Easy-nLC 1,000 System (Thermo Fisher Scientific, United States) coupled to a Q Exactive HF Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, United States). Each peptide sample was loaded onto an Acclaim PepMap100 C18 reverse-phase trap column (100 µm × 2 cm, nanoViper fitting; Thermo Fisher Scientific, United States) connected to an Easy C18 reverse-phase analytical column (75 µm × 10 cm, 3 µm resin; Thermo Fisher Scientific, United States) pre-equilibrated in buffer A [0.1% (v/v) formic acid]. The peptides were separated with a linear gradient of buffer B [84% (v/v) acetonitrile and 0.1% (v/v) formic acid] at a constant flow rate of 300 nL/min. The gradient of B was first increased from 0 to 55% over 110 min, then to 100% over 5 min, before being maintained at 100% for another 5 min.

Following the separation, the eluted peptides were immediately analyzed under positive-ion mode with peptide recognition enabled. The ions were first subjected to one survey scan in the m/z range of 300–1,800 at a mass resolution of 70,000 at m/z 200. Automatic gain control target, maximum inject time and dynamic exclusion were set to 3 × 10⁶, 10 ms and 40.0 s, respectively. MS2 spectra were obtained by using a data-dependent top 10 method to dynamically select the most abundant precursor ions from the survey scans for high-energy collisional dissociation (HCD) fragmentation. The resolution of the MS2 scans was set to 17,500 at m/z 200. Isolation width, normalized collision energy and underfill ratio were set to 2 m/z, 30 eV and 0.1%, respectively.

**Protein Identification and Quantification**

The raw mass spectrometry (MS) data for each sample were combined and imported into MaxQuant (version 1.5.3.17, Max Planck Institute of Biochemistry, Germany) (Cox and Mann, 2008). For protein identification, the MS data were searched against the Uniprot E. coli protein database represented by the file uniprot_Escherichia_coli_1124415_20180910.fasta. Software settings were adjusted as follows: enzyme-trypsin; max missed cleavage-2; fixed modification-carbamidomethyl (C); variable modification-oxidation (M), acetyl (Protein N-term); main search ppm-6; MS/MS tolerance ppm-20; database pattern-reverse; include contaminants-true; false discovery rate (FDR)
Hochberg method was used to calculate the adjusted molecular function and cellular component. The Benjamini-Hochberg method is a step-down procedure that controls the false discovery rate (FDR). The GO terms were then transferred and combined with those of the corresponding query E. coli proteins. To reduce the workload, only the top 10 blast hits of each query sequence with an E-value below $1 \times 10^{-3}$ were selected. These candidates were annotated by Blast2GO (version 3.3.5; Stefan et al., 2008) with an E-value filter of $1 \times 10^{-6}$, GO weight of 5, annotation cutoff of 75, and default gradual EC weights. Sequences that could not be annotated under these settings were then subjected a new round of annotation using more permissive parameters. Subsequently, the sequences that could not be annotated and those without BLAST hits were then searched against database by using InterProScan (Sun et al., 2016) to retrieve functional annotations of their conserved motifs. The combined GO annotation results were plotted by R scripts. For Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, the sequences of differentially expressed proteins were searched via BLAST against the KEGG Database at https://www.genome.jp/kegg/kaas. InterProScan (Sun et al., 2016) to retrieve functional annotations of their conserved motifs. The combined GO annotation results were plotted by R scripts. For Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation, the sequences of differentially expressed proteins were searched via BLAST against the KEGG Database at https://www.genome.jp/kegg/kaas (Moriya et al., 2007).

Next, enrichment analyses were conducted using Fisher’s exact test in order to unearth the cellular and physiological roles of the differentially expressed proteins. The GO terms were categorized into three subcategories, including biological process, molecular function and cellular component. The Benjamini-Hochberg method was used to calculate the adjusted $p$-values. $p < 0.05$ was considered statistically significant.

Hierarchical Clustering
Hierarchical clustering was performed to reveal global differences in protein expression profiles between the PEF treatment group and the control group. To this end, the data from protein relative expression were analyzed by Cluster 3.0 (Lu et al., 2012) using the average-linkage method for clustering, with similarity between genes expressed in Euclidean distance. The obtained dendrogram was visualized in Java Treeview (version 3.0) (Zhu et al., 2014). In addition, a heat map was also generated as a visual aid.

Protein-Protein Interaction (PPI) Network
The differentially expressed proteins were imported into STRING to analyze their potential interactions (version 10.5) (Zhou et al., 2012). The results were downloaded in XGMML format and visualized in the form of an interaction network by importing into Cytoscape (version 3.2.1) (Zhu et al., 2014). The degree value of each protein node was calculated to evaluate its importance in the PPI network.

**Parallel Reaction Monitoring (PRM) Analysis**
Parallel reaction monitoring analysis of selected proteins were conducted as previously described (Qu et al., 2018). Briefly, digested peptides were prepared as elucidated above. Then, 2 μg of the peptides were spiked with 20 fmol standard (PTRC: GISNEGQNASIK) and separated on an Easy nLC 1,200 System (Thermo Fisher Scientific, United States) with the same buffer A, B and flow rate as above. The gradient of B was set as follows: 5–10% over 2 min, 10–30% over 43 min, 30–100% over 10 min and then 100% for another 5 min. The eluted peptides were immediately analyzed under positive-ion mode on a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, United States). The ions were first subjected to one survey scan in the m/z range of 300 to 1,800 at a mass resolution of 60,000 at m/z 200. Automatic gain control target and maximum injection time were set to $3 \times 10^6$ and 200 ms, respectively. The full MS1 scan were followed by 20 MS2 scans at a resolution of 17,500 at m/z 200 (Liu et al., 2019). Precursor ions were fragmented via the HCD method at normalized collision energy of 27 eV. Isolation window and maximum injection time were set to 1.6 Th and 120 ms, respectively. The obtained raw PRM data were imported into Skyline (version 3.5.0) (MacLean et al., 2010) for analysis.

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis
Following the PEF treatment, total RNA was immediately extracted using anRNAsiso Plus Kit (TaKaRa, Japan), followed by reverse transcription with a PrimeScript Reverse Transcriptase Kit (TaKaRa, Japan). All gene-specific primers were designed using Primer Premier 5.0 software (Premier Biosoft International, United States) and synthesized by Sangon Biotech, China. Gene expression levels were analyzed by qRT-PCR using SYBR Green qPCR Mix (TaKaRa, United States) on a CFX96 Real-Time PCR System (Bio-Rad, United States) according to the manufacturers’ instructions. Glucan biosynthesis protein G (mdoG) was used as a control (Heng et al., 2011). All reactions were performed with at least three replicates. Fold changes were calculated based on the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001).

**Statistical Analysis**
All statistical analyses were performed using the SPSS software (version 13.0; IBM, United States). Data were expressed as mean ± standard error of mean (SEM). Differences between two groups were analyzed using Student’s $t$-test. $p < 0.05$ was considered statistically significant.

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1[^1]: http://geneontology.org
RESULTS

Protein Identification and Differential Expression Analysis

We used an untargeted LC-MS/MS approach to systematically investigate the effects of PEF on E. coli proteome. To this end, we applied PEF to freshly grown E. coli cells under abovementioned conditions that resulted in a 95 ± 2.0% reduction of bacterial population. In the meantime, we set up a control group in which cell aliquots from the same culture were similarly processed, but without the application of PEF. In total, we detected 16,829 peptides from our MS data and identified 2,252 proteins by sequence comparison with the Uniprot E. coli protein database. We then calculated the relative abundances of all identified proteins in the two experimental groups. Comparison of the expression data revealed 123 differentially expressed proteins based on the criteria of fold-change > 1.5 or < 0.67 and p < 0.05. Among them, 99 proteins exhibited significantly increased expression levels in PEF-processed E. coli, whereas 24 were found to be down-regulated (Table 1). The annotations, accession numbers and fold-change values of all differential proteins were summarized in Table 1. Notably, 33 proteins were confidently detected in at least two PEF-treated E. coli cultures but not in any of the controls, whereas another 19 were identified in at least two control samples but showed no apparent expression in the PEF treatment group. These candidates were therefore also considered as differentially expressed between the two groups and subsequently merged with the 123 proteins mentioned above. It is worth emphasizing that the reason that some proteins were only detected in two of the three samples in an experimental group could be caused by a combination of biochemical, analytical and statistical factors, such as miscleavage, ionization competition, ion suppression, peptide misidentification, ambiguous matching, etc. (Lazar et al., 2016).

To better compare the global proteomic profiles of the PEF-treated E. coli samples and the controls, we performed hierarchical clustering analysis of the 123 differentially expressed proteins and illustrated the results in an expression heat map with dendrogram (Figure 2). As depicted, the plot indicated clear differences in the number of detected proteins between the two experimental groups.

Functional Analysis of the Differentially Expressed Proteins

We next performed functional analysis of all 175 differentially expressed proteins (including the ones that could not be confidently detected in all samples as explained above) to shed light on their potential molecular and cellular roles. GO annotation by Blast2GO and InterProScan indicated that the differential proteins were predominantly involved in catalytic activity (47.43%) and binding (38.86%) under the subcategory of molecular function (Figure 3A). For biological process, the overwhelming majority of the candidates could be assigned to GO terms of cellular process (36%) and metabolic process (38.29%) (Figure 3A). Subsequent GO enrichment analysis demonstrated that PEF-treated E. coli underwent a wide range of metabolic alterations, such as those associated with sulfate, sulfide, propionate, 2-methylcitrate and short-chain fatty acids. In addition, we also identified significant changes in the transmembrane transport of inorganic anions, particularly phosphate ions. Other notably enriched GO terms included enzyme binding and enzyme inhibitor activity, including ribonucleoside inhibitor activity, endonucleoside inhibitor activity and methyl isocitrate lyase activity (Figure 3B).

On the other hand, the differentially expressed proteins could be mapped to 81 KEGG pathways. The top 5 KEGG pathways with the greatest numbers of protein candidates included those associated with ABC transporters, two-component system, propanoate metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, as well as sulfur metabolism (Figure 4A). Further examination suggested that sulfur metabolism was significantly enriched (Figure 4B), which comprised four differentially expressed proteins W8ZVZ2 (sulfate adenylytransferase subunit 1, EC:2.7.7.4, CysND), W8ZV88 (Sulfite reductase [NADPH] hemoprotein beta-component, EC:1.8.1.2, CysJ1), I0VX12 (Sulfate-binding protein, CysPUWA) and A0A1V2T430 [Adenyl-sulfate kinase (Fragment), EC:2.7.7.2, CysC]. Among them, W8ZVZ2 and W8ZV88 were up-regulated in PEF-treated E. coli cells by 2.0-fold and 1.8-fold, respectively, compared to the untreated cells, whereas I0VX12 and A0A1V2T430 were only detected in PEF-treated cells. The metabolic roles of these proteins were illustrated in Figure 4C.

PPI Analysis

It is a well-established concept that cellular processes are often the results of specific interactions between two or more proteins rather than the actions of a single protein. Based on these considerations, we constructed a PPI network of all the differentially expressed proteins that we identified in order to gain a deeper understanding of how the various PEF-altered biological functions in E. coli cells were interconnected with each other. As shown in Figure 5, the network map comprised 123 nodes and 208 interactions. W8ZUR8 (uncharacterized protein elB), predicted by GO annotation to participate in ribosome binding, represented the largest node with ten putative associations. Other differential proteins predicted to interact with seven or more partners included W9ADR5 (uncharacterized protein yccJ), W9ADK0 (uncharacterized protein cbJ), A0A1 × 3L920 (beta-lactamase induction protein AmpE), W8SS44 (hydrolase ycaC), W8ZR12 (tryptophan synthase alpha chain, trpA), and V6FUM8 (universal stress protein B UspB). Notably, all these proteins were up-regulated in the PEF treatment group, with V6FUM8 detected only in PEF-processed samples.

Data Verification by PRM Analysis

The proteomic data were verified by selecting five differentially expressed proteins for PRM analysis, including M9GK20 (Citrate synthase), W8ZVZ2 (Sulfate adenylytransferase subunit 1), V6FYL6 (Bifunctional polymyxin resistance protein ArnA), M9FZM4 (2-methylcitrate dehydratase) and W9AM8 (Phosphate-binding protein PstS). We quantified the levels of 1–3 unique peptides for each selected protein in the PEF-processed E. coli and untreated controls. As shown in (Table 2),
### TABLE 1

The significantly upregulated and downregulated proteins in *E. coli* after PEF-treated.

| Accession | Description | Coverage | Unique peptides | Folds | Exe/Con | p-value  |
|-----------|-------------|----------|----------------|-------|---------|----------|
| M9GK20    | Citrate synthase, GN=ECMP0215612_0833 | 60.9 | 20 | 181.5173 | 0.0277 |
| A0A0H3JNL0| Putative glucurate dehydratase, GN=ECs3648 | 5 | 1 | 28.3712 | 0.0232 |
| V0JH66    | Octanoyltransferase, GN=IbpB | 35.1 | 3 | 18.3945 | 0.0002 |
| W8TL6     | 2-methylisocitrate lyase, GN=prpB | 24 | 1 | 15.7546 | 0.0040 |
| A0A2B7LLZ2| Aconitate hydratase B, GN=BM230_09860 | 73.8 | 0 | 12.0020 | 0.0275 |
| M9FZM4    | 2-methylcitrate dehydratase, GN=prpD | 48.4 | 16 | 7.4411 | 0.02615 |
| A0A039K113| Putative protein PutA, GN=putA | 49.8 | 1 | 5.9676 | 0.0224 |
| W1G3P2    | Aldehyde-alcohol dehydrogenase | 53.6 | 1 | 5.8303 | 0.0452 |
| W8ZMF2    | Putative resistance protein, GN=yygT | 10.1 | 2 | 4.0865 | 0.0211 |
| A0A0K5XJ9 | Ribosomal RNA small subunit methyltransferase F, GN=rsmF | 10.1 | 2 | 3.6706 | 0.0003 |
| A0A234Q1M9| GTP-binding protein, GN=AL530_011080 | 18.3 | 1 | 3.4667 | 0.0275 |
| A0A234Y2B9| Bifunctional protein PutA, GN=putA | 73.8 | 0 | 2.9581 | 0.0116 |
| W1F3      | FeS assembly scaffold SufA, GN=sufA | 61.4 | 1 | 2.8832 | 0.0261 |
| W9AMD8    | Phosphate-binding protein PstS, GN=pstS | 50.3 | 5 | 2.8444 | 0.0022 |
| W1FRP3    | ATP-dependent Clp protease ATP-binding subunit ClpA | 34.4 | 0 | 2.7513 | 0.0314 |
| W1FTY7    | Galactose/methyl galactoside ABC transport system, D-galactose-binding periplasmic protein MglB (TC 3.A.1.2.3) | 49.4 | 1 | 2.6992 | 0.0013 |
| W1F3 × 3 | Phosphate transport ATP-binding protein PstB (TC 3.A.1.7.1) | 24.7 | 5 | 2.6913 | 0.0155 |
| A0A234Q1M9| L-cystinetransporter, GN=CG691_13490 | 8.7 | 2 | 2.5515 | 0.0080 |
| A0A193RPM2| Ornithine decarboxylase, GN=WM48_15995 | 11.3 | 6 | 2.4748 | 0.0009 |
| A0A075XPP6| Molydbdenopterin biosynthesis protein MoeA, GN=AOX65_16030 | 3.2 | 1 | 2.4707 | 0.0008 |
| A0A27Y3666| Bifunctional glutamine amidotransferase/anthranilate phosphoribosyltransferase, GN=APX76_17630 | 35.3 | 11 | 2.4593 | 0.0006 |
| W8SPU9    | Nicotinate phosphoribosyltransferase, GN=ncpB | 21.2 | 6 | 2.3065 | 0.0056 |
| A0A1 × 3L920| Protein AmpE, GN=EAXG_02432 | 14.8 | 1 | 2.2675 | 0.0084 |
| D7 × 9F1 | Uncharacterized protein (Fragment), GN=HMPREF9552_03278 | 50 | 2 | 2.2191 | 0.0032 |
| W9ADK0    | Uncharacterized protein, GN=ycbJ | 20.2 | 4 | 2.1980 | 0.0033 |
| A0A0T5XIP1| Regulator of ribonuclease activity A, GN=rraA | 27.3 | 1 | 2.1974 | 0.0017 |
| W9ADRS    | Uncharacterized protein, GN=yccJ | 60 | 3 | 2.1729 | 0.0246 |
| W1WSH0    | Protein mocC, GN=Q609_ECAC01910G0002 | 44.9 | 3 | 2.1698 | 0.0108 |
| A0A1 × 3KHH3| Alchohol dehydrogenase YghD, GN=EATG_01859 | 39 | 1 | 2.1604 | 0.0003 |
| W8ZNL1    | UFP0250 protein YbeD, GN=ybeD | 55.2 | 4 | 2.1440 | 0.0009 |
| A0A096ZJW9| Sigma-38 (Fragment), GN=rpOS | 38.7 | 9 | 2.1201 | 0.0050 |
| V8FH58    | Spermidine/putrescine ABC transporter substrate-binding protein, GN=Q458_15770 | 56.2 | 15 | 2.0609 | 0.0268 |
| A0A2B7MPJ0| Dihydroxyacetone kinase subunit DhaM, GN=BM230_01885 | 17.4 | 1 | 2.0210 | 0.0283 |
| W1 × 9K1 | Galactonate operon transcriptional repressor (Fragment), GN=Q609_ECAC00550G0001 | 35 | 4 | 2.0176 | 0.0017 |
| W8ZNL8    | Nucleic Acid binding protein subunit C, GN=ssbC | 5.8 | 4 | 2.0107 | 0.0127 |
| W8ZJY8    | Uncharacterized protein, GN=yecA | 35.7 | 5 | 2.0036 | 0.0011 |
| W8ZVZ2    | Sulfate adenylytransferase subunit 1, GN=cysN | 32.2 | 12 | 2.0004 | 0.0294 |
| W9AE4D    | Fatty acid metabolism regulator protein, GN=fadR | 56.9 | 10 | 1.9864 | 0.0041 |
| W8STE3    | BdA DNA-binding transcriptional dual regulator, GN=bola | 42.3 | 3 | 1.9619 | 0.0064 |
| V6FLY6    | Bifunctional polymyxin resistance protein ArnA, GN=arnA | 25.9 | 13 | 1.9311 | 0.0075 |
| A0A0K9TED1| Smg protein, GN=ERYG_01892 | 45.3 | 3 | 1.8818 | 0.0119 |
| W8ZV88    | Sulfite reductase [NADPH] hemoprotein beta-component, GN=cysl | 30.5 | 6 | 1.8737 | 0.0141 |
| W1WPC4    | Cell division protein ZapB, GN=zapB | 88.6 | 7 | 1.8712 | 0.0026 |
| W8T248    | Protein YciE, GN=yciE | 51.8 | 6 | 1.8429 | 0.0174 |

(Continued)
| Accession | Description | Coverage | Unique peptides | Folds | p-value |
|-----------|-------------|----------|----------------|-------|---------|
| W1HG9Y    | 6,7-dimethyl-8-ribityllumazine synthase, GN=ribH | 87.2     | 8              | 1.8411| 0.0024  |
| A0A2S7HHN4| Uncharacterized protein (Fragment), GN=C5P43_35025| 34.2     | 3              | 1.8367| 0.0432  |
| M9GR7     | Protein rcf, GN=rcf | 63.3     | 3              | 1.8317| 0.0361  |
| W1F1D7    | Anthranilate synthase component 1 | 32.5     | 14             | 1.8300| 0.0208  |
| V8K FY8   | Protein CsdD, GN=csdD | 64.6     | 17             | 1.8290| 0.0061  |
| W8ZY2     | Transcriptional regulator ModE, GN=modE | 51.9     | 8              | 1.8284| 0.0121  |
| D6I850    | YcfF protein, GN=ECDG_01154 | 45.2     | 6              | 1.8177| 0.0022  |
| V0AYA0    | Uncharacterized protein (Fragment), GN=HMPREF1608_01019| 24.2     | 1              | 1.8173| 0.0434  |
| A0A0K4H5N9| Transcriptional regulator, GN=ERS085411_00773 | 27.4     | 10             | 1.7957| 0.0022  |
| V0YHCA2   | DNA recombination protein RmuC, GN=HMPREF1608_02578| 20.6     | 8              | 1.7851| 0.0374  |
| A0A1x13NA3| Formate dehydrogenase-N subunit alpha, GN=fdhG | 41.6     | 30             | 1.7653| 0.0156  |
| V8FUE1    | Pimeloyl-[acyl-carrier protein] methyl ester esterase, GN=biocH | 30.1     | 4              | 1.7541| 0.0492  |
| W8I2J72   | Fused mannitol-specific PTS enzymes: IIA components/IIb components/III components/IlIA | 36.1     | 14             | 1.7486| 0.0103  |
| A0A1x3KKE8| Transketolase, GN=EATG_02582 | 37.5     | 1              | 1.7098| 0.0264  |
| W8Z4B2    | Regulator of ribonuclease activity A, GN=meng | 50.3     | 4              | 1.7003| 0.0006  |
| W8P2R2    | Protein TutB, GN=theL | 12.6     | 1              | 1.6993| 0.0332  |
| S0VE10    | PTS system trehalose-specific EIIbC component, GN=WE7_05383| 18.4     | 6              | 1.6949| 0.0410  |
| W8TSM7    | C-lysosome inhibitor, GN=lyv | 46.6     | 5              | 1.6947| 0.0206  |
| W8AP42    | Regulator of ribonuclease activity B, GN=yjdD | 39.1     | 3              | 1.6871| 0.0056  |
| W8Z4R1    | Tryptophan synthase alpha chain, GN=trpA | 59.7     | 1              | 1.6803| 0.0021  |
| W1EYP8    | L-proline glycine betaine ABC transport system permease protein ProW (TC 3.A.1.12.1) | 16.2     | 2              | 1.6793| 0.0206  |
| W8U2G6    | Carboxymethylenedebutenolodidase, GN=sga | 38.9     | 5              | 1.6667| 0.0317  |
| W8Z2G4    | Regulator of sigma D, GN=ylA | 27.8     | 3              | 1.6666| 0.0034  |
| A0A0T5X69 | Threonine synthase, GN=AOX85_13005 | 13.1     | 1              | 1.6576| 0.0007  |
| T9DJA4    | Glutaminase, GN=glkA | 48.4     | 3              | 1.6534| 0.0071  |
| W1x6R0    | Phosphate starvation-inducible protein psf, GN=QE09_ECAC01403002 | 39.6     | 4              | 1.6472| 0.0342  |
| W8ZFC1    | Uncharacterized protein, GN=EC9568_1708 | 73.0     | 12             | 1.6406| 0.0150  |
| A0A29HCSQ5| Deoxyuridine 5-triphosphate nucleotidohydrolase, GN=coaBC | 45.9     | 15             | 1.6347| 0.0003  |
| W1EQP2    | Tryptophan synthase beta chain, GN=trpB | 38.8     | 2              | 1.6319| 0.0017  |
| A0A0J2EWU6| Dihydroorotate dehydrogenase (quinone), GN=pyrD | 36.9     | 9              | 1.6316| 0.0007  |
| W1EWD5    | Transcriptional regulator YcjW, LacI family, possibly involved in maltodextrin utilization pathway | 15.9     | 1              | 1.6275| 0.0138  |
| W9A8B9    | Uridylate kinase, GN=pyrH | 45.2     | 7              | 1.6140| 0.0320  |
| W8Z8N9    | Regulator of nucleoside diphosphate kinase, GN=rmk | 26.5     | 2              | 1.6051| 0.0140  |
| S1JKX6    | Protein AroM, GN=AIWS_00874 | 16.1     | 1              | 1.6048| 0.0490  |
| W1XC14    | Iron-sulfur cluster insertion protein ErpA, GN=erpA | 22.8     | 2              | 1.5893| 0.0095  |
| U8YV3N3   | D-methionine-binding lipoprotein MetG, GN=HMPREF1599_05823 | 20.2     | 2              | 1.5838| 0.0117  |
| W1VWN8    | Replicative DNA helicase, GN=QE09_ECAC0209060002 | 25.3     | 8              | 1.5828| 0.0111  |
| A0A2A3VGN6| UDP-4-amino-4-deoxy-L-arabinose – oxoglutamate aminotransferase, GN=arnB | 20.3     | 2              | 1.5763| 0.0295  |
| H4UMP9    | Inner membrane protein yjrD, GN=yjrD | 8.2      | 1              | 1.5698| 0.0065  |
| W1F6U7    | Chromosome partition protein MukE, GN=mukE | 37.3     | 6              | 1.5425| 0.0203  |
| W8SO29    | GST-like protein with glutathione S-transferase domain protein YljU, GN=gstB | 52.4     | 8              | 1.5391| 0.0072  |
| A0A2HJDE7 | Arabino ABC transporter substrate-binding protein, GN=CRT55_12445 | 32.0     | 2              | 1.5267| 0.0465  |
| W8SS44    | Hydrolase, GN=ycAC | 48.6     | 3              | 1.5255| 0.0104  |
| W1EY44    | Zinc transport protein ZntB | 13.4     | 3              | 1.5160| 0.0148  |
| W8ZUF8    | Uncharacterized protein, GN=elaB | 44.6     | 4              | 1.5169| 0.0186  |
| H4LJ45    | Peptidyl-prolyl cis-trans isomerase, GN=slgD | 68.6     | 4              | 1.5151| 0.0356  |
| W1WTW0    | Arginine repressor, GN=argR | 42.9     | 4              | 1.5101| 0.0013  |

(Continued)
TABLE 1 | Continued

| Accession | Description | Coverage | Unique peptides | Folds | Exe/Con | p-value |
|-----------|-------------|----------|----------------|-------|---------|---------|
| V2T0T4    | ABC transporter periplasmic-binding protein, GN=G723_01103 | 17.6     | 5              | 1.5076 | 0.0307  |
| A0A2P9W5R2| Primosomal protein DnaT (Fragment), GN=C1157_22015 | 33.6     | 3              | 1.5058 | 0.0426  |
| A0A2P9W0   | L-arabinose isomerase (Fragment), GN=C1157_30235 | 10.6     | 3              | 1.5021 | 0.0302  |
| W8ZM76    | Uncharacterized protein, GN=yadG | 19.2     | 7              | 0.6651 | 0.0173  |
| W82VN7    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W9ACD0    | Proofreading thioregerase EntH, GN=ybdB | 18.2     | 2              | 0.6451 | 0.0007  |
| A0A0A0FBI3| Uncharacterized protein, GN=EL76_3316 | 10.6     | 3              | 1.5021 | 0.0302  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| M9FWG9    | Arginine transport ATP-binding protein ArtP, GN=artP | 23.1     | 4              | 0.6609 | 0.0215  |
| W8ZM76    | Uncharacterized protein, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |
| W8ZM76    | Putative YbH sigma 54 modulator, GN=EC958_2900 | 59.3     | 6              | 0.6611 | 0.0349  |

all five proteins were significantly up-regulated as a result of the PEF treatment, which was consistent with the results of the untargeted proteomic analysis. This suggested that the proteomic data that we obtained were sufficiently reliable for subsequent interpretation.

In the present study, we systematically analyzed PEF-induced changes in the protein expression profile of E. coli through MS-based label-free quantitative proteomics. Our experimental data echoed previous findings (Rivas et al., 2013; Yun et al., 2016; Zhao et al., 2018), that exposure to PEF exerted significant detrimental effects on cell membrane integrity, triggered a host of cellular stress response mechanisms, and altered important metabolic pathways. The results of our current study could increase our understanding of the mechanisms responsible for PEF-induced inactivation of bacterial cells in food products.

qRT-PCR Validation
We selected a list of significantly up-regulated proteins and measured their mRNA levels at different PEF intensities (which resulted in different cell killing levels). The detailed information and the selection rationale for each protein were summarized in (Table 3). As illustrated in Figure 6, all candidates demonstrated increased transcription at all PEF intensities that we tested, confirming the validity of our proteomic results. However, the genes showed different expression trends with increasing PEF intensities. In most cases, we did not observe a positive correlation between the mRNA level of the gene and the level of cell death. These results suggested that PEF treatment could directly stimulate the expression of the E. coli proteins, though indirect contribution via cell lysis could not be completely eliminated. In addition, at high intensities, PEF exerted a clear inhibitory effect on the transcription of most of the genes.

DISCUSSION
Notable among the most up-regulated proteins in the PEF treatment group are several enzymes related to the methylcitrate cycle and the TCA cycle, including 2-methylisocitrate lyase (PrpB; W8T6L6), the dual-functionalaconitate hydratase B (alsoaconitate, AconB; A0A2B7LUZ2), 2-methylcitrate dehydratase (PrpD; M9FZM4), and the rate-limiting citrate synthase (prpC; M9GK20). The methylcitrate cycle functions in a similar manner as the TCA cycle, and is responsible for converting propionyl-CoA to pyruvate for further catabolism (Luo et al., 2016; Serafini et al., 2019). As a result, both the TCA cycle and the methylcitrate cycle are functionally connected to and downstream of the β-oxidation of fatty acids. Since increased lipid oxidation is a well-established phenomenon...
under oxidative stress (Esterbauer et al., 1991) or PEF treatment (La et al., 2015; Yun et al., 2016), we speculate that the augmented expression of TCA or methylcitrate cycle-associated enzymes could reflect a pre-emptive metabolic defense mechanism of the stressed *E. coli* cells. This is supported by the results discussed in several studies with regard to the anti-oxidative roles of the TCA cycle (Tretter and Adam, 2000; Mailloux et al., 2010). It is worth mentioning that AcnB contains a critical Fe-S cluster that is particularly susceptible to oxidative stress. In fact, there is evidence that excessive accumulation of reactive oxygen species (ROS) could dramatically reduce the protein level of AcnB in superoxide dismutase-deficient *Salmonella enterica* (Thorgersen and Downs, 2009). Therefore, the effects of PEF treatment and oxidative stress on the expression of AcnB and other enzymes mentioned above could be both stimulatory and inhibitory. Another top-ranking up-regulated enzyme is bifunctional proline utilization A (PutA; A0A0G3K113), which catalyzes the conversion of proline to glutamate via two consecutive steps of dehydrogenation (Menzel and Roth, 1981). PutA is known to be involved in redox homeostasis and its deletion in *E. coli* has been shown to substantially increase cell susceptibility to oxidative injuries (Zhang et al., 2015). It has been postulated that the protective role of PutA against oxidative stress could be attributed to the generation of a low level of hydrogen peroxide by proline oxidative metabolism, which builds resistance in cells in a pre-adaptive manner (Zhang et al., 2015). Taken together, these results suggested that PEF treatment and the possibly associated oxidative stress could activate the stress response mechanism in the *E. coli* cells, leading to induced expression of various oxidation-combating enzymes.

Cell membrane functions as the main barrier against harmful substances and regulates the exchange of essential materials between the cytoplasm and the environment. When exposed to a strong electric field, a cell becomes polarized due to the migration of ions in its cytoplasmic fluid. The realignment of opposite charges on both sides of the cell creates electrostatic attraction that squeezes the cell and its membrane (Zimmermann et al., 1974). Meanwhile, the various membrane constituents of the cell are also perturbed by the electromechanical forces. The polar lipids, including phospholipids and cholesterol, represent obvious targets. As the electric field intensifies, both electromechanical compression and polar lipid rearrangement will reach a breaking point that leads to pore formation (Zimmermann et al., 1974; Serpersu et al., 1985; Weaver and Chizmadzhev, 1996; Unal et al., 2002; Liu Z.W. et al., 2016). One of the direct consequences of this is increased membrane permeability, which not only allows entry of deleterious substances, but also generates an osmotic imbalance (Aronsson et al., 2005). Obviously, the electric induction of a few small pores is usually non-lethal, as the fluidity of the biolipid layer allows rapid restoration of a stable membrane structure (Aronsson et al., 2005). However, a sufficiently strong electric field is capable of creating an increased number of large pores on the membrane, leading to irreversible cell damage, disruption, and eventually death (Locke et al., 2006; Lin et al., 2012). Numerous studies have been performed to determine the boundary between PEF-induced temporary electroporation and permanent sterilization.
FIGURE 3 | Gene ontology (GO) annotation and enrichment analysis of all 175 differentially expressed proteins. (A) The primary Y axis denotes the number of annotated proteins categorized to each GO term. The secondary Y axis represents the percentage of annotated proteins belonging to each GO term in all differential proteins. GO terms are classified into three subcategories, including biological process (BP, red), molecular function (MF, purple) and cellular compartment (CC, orange). (B) The color gradient from orange to red represents the p-value; the closer the color to red, the lower the p-value and the higher the significance level corresponding to the enrichment. The small number above each column is the rich factor, which denotes the ratio of the number of differential proteins enriched to a given GO term to the number of all annotated proteins categorized to the same GO term.
Based on these results, it is generally believed that irreversible cell injury occurs when the field strength reaches 5–15 kV·cm$^{-1}$ (Guerrero and Welti, 2016). This is consistent with observations that PEF at an intensity above 15 kV·cm$^{-1}$ is necessary for membrane breakdown (Lampe, 1999; Lebovka et al., 2001). However, some researchers have noticed microbial resistance to field strengths as high as 19 kV·cm$^{-1}$ (Ulmer et al., 2002). As a result, it has been argued that PEF with a field strength of 25 kV·cm$^{-1}$ or above should be used to ensure pathogen inactivation in food processing (Toepfl et al., 2007).

Overall, 29 of the 175 differentially expressed proteins that we identified in our current proteomic study are localized on the cell membrane and/or potentially involved in membrane functions. Particularly, 16 of these proteins participate in the transmembrane transport of various small metabolites and macromolecules, including phosphate ions, zinc, glycine betaine, trehalose, as well as short peptides, carbohydrates and lipids in general. There is ample evidence that sugar molecules, especially trehalose, exert a protective effect on the physical structure and integrity of cell membrane (Crowe, 2002). It has been hypothesized that carbohydrates can increase the internal cohesion of cell membrane by forming stabilizing hydrogen bonds with its various components (Locke et al., 2006). Another theory argues that sucrose and trehalose can mitigate mechanical disruption of cell membrane by forming an amorphous glass structure (Crowe et al., 1998). In support of these roles, Pereira et al. demonstrated that trehalose could effectively reduce the physical stress that stretching induced on cell membrane by forming hydrogen bonds with the polar lipid components (Pereira and Hünenberger, 2008). Glycine betaine is another well-known cryoprotectant and osmoprotectant that is often accumulated in cells in response to external abiotic stress stimuli. Several studies have shown that transport of glycine betaine across bacterial cell membrane can be activated by osmotic shock and low temperature (Gerhardt et al., 2000; Guillot et al., 2000). Therefore, it seems more plausible that the altered expression of membrane transporters could be a cellular defense mechanism against PEF-induced structural disruptions, though it is also possible that the electromechanical forces might directly affect the expression or stability of these proteins.

It is worth mentioning that some of the differential membrane proteins were only detectable in the PEF treatment group. A careful survey of their functions implied that these proteins might have been activated by PEF for different reasons. The outer membrane protein F (ompF; W9ADK8) directly contributes to pore formation and has been shown to help E. coli cells maintain their structural stability (Nogami and Mizushima, 1983). However, the effects of ompF seem to be mixed, as Tan et al. (2017) reported that its deletion led to substantial enhancement of membrane integrity. Based on these findings, we speculated that the augmented expression of ompF could either be a sign of increased membrane damage or reflect the cellular response to PEF-induced perturbations of intracellular compounds. Universal stress protein B (uspB; V6FUM8) and other members of the USP family can be induced by a wide range of abiotic stress stimuli (Nachin et al., 2005) and participate in a variety of cytoprotective activities such as DNA protection (Gustavsson et al., 2002), arrest of cell growth (Neidhardt and Nyström, 2010) and re-adaptation of cell metabolism to nutrient shortage (Tkaczuk et al., 2013). The E. coli sensor histidine kinase (A0A2R9W207) belongs to the two-component signal-transducing system implicated in stimuli...
perception (Mascher et al., 2006). Thus, its stimulated expression could be the result of increased PEF-dependent stresses. The prenyltransferase family protein (ubiA; A0A080HYZ5) is responsible for lipimating a wide range of suitable acceptor compounds. Recent studies pointed out that many lipophilic products of prenyltransferase serve as important components of bacterial cell membrane (Li, 2016), raising the possibility that its up-regulation by PEF could be a cellular repair mechanism. We are especially intrigued to discover that a macrolide-specific efflux protein (MacA; H4L9U9) exhibited detectable expression only in PEF-treated cells. Mostly known for their antibiotic activities, macrolides are recently speculated to play an additional role in membrane organization. For example, Tyteca et al. (2003) examined the mechanism of azithromycin-based inhibition of endocytosis and found that the macrolide compound could diminish membrane fluidity by binding to the polar heads of phospholipids. This is further corroborated by findings that macrolide efflux pumps can be up-regulated by stress signals unrelated to drug resistance (Bolla, 2014).

In addition to Rivas et al.’s (2013) protein profiling study based on 2D gel electrophoresis, there have been several other proteomics or transcriptomics investigations of PEF-treated E. coli. Chueca and coworkers treated E. coli MG1655 cells, buffered at pH 4.0, with 50 exponential waveform electric pulses with a frequency of 0.08 Hz and pulse duration of 2 µs, followed by DNA microarray-based transcriptomic analysis (Chueca et al., 2015). Based on their data, a total of 47 genes showed differential expression as a result of the treatment. One of their major findings was the significantly elevated mRNA expression of several genes associated with the TCA cycle, including (sdhA, sdhB, sdhC, sdhD). Although these genes were different from those that encoded the differential proteins identified in this study, their finding of a link between PEF treatment and alterations in TCA-associated cell metabolism mirrored what

| Accession | Description                                              | PRM results | Label-free results |
|-----------|----------------------------------------------------------|-------------|--------------------|
| M9GK20    | Citrate synthase                                         | 6.96 (up)   | 181.5172 (up)      |
| W9AMD8    | Phosphate-binding protein                                 | 3.60 (up)   | 2.8444 (up)        |
| M9FZM4    | 2-methylcitrate dehydratase                              | 7.32 (up)   | 7.4411 (up)        |
| V6FYL6    | Bifunctional polymyxin resistance protein ArnA            | 1.55 (up)   | 1.9311 (up)        |
| W8ZVZ2    | Sulfate adenylyltransferase subunit 1                     | 1.87 (up)   | 2.0004 (up)        |
TABLE 3 | Differential protein use for fluorescence quantification PCR.

| Protein | Gene Name | Reason for verification |
|---------|-----------|-------------------------|
| M9GK20  | prpC      | Transferase activity, transferring acyl groups, acyl groups converted into alkyl on transfer, tricarboxylic acid cycle |
| W9AMD8  | pstS      | Part of the ABC transporter complex, involved in phosphate import |
| M9FZM4  | prpD      | Propionate catabolic process, 2-methylcitrate cycle |
| W8ZMF2  | yggT      | Resistance protein |
| ECs3648 | A0A0H3JNL0 | Amino acid metabolism |
| V0UHA6  | lipB      | Catalyzes the transfer of endogenously produced octanoic acid from octanoyl-acyl-carrier-protein onto the lipoyl domains of lipoate-dependent enzymes. |
| W8TBL6  | prpB      | Catalyzes the thermodynamically favored C-C bond cleavage of (2R,3S)-2-methylisocitrate to yield pyruvate and succinate. |
| W8ZVZ2  | cysND     | Sulfur metabolism |
| V6FYL6  | arnA      | The modified arabinose is attached to lipid A and is required for resistance to polymyxin and cationic antimicrobial peptides. |
| V6FUM8  | uspB      | Universal stress protein |
| W8ZUR8  | elB       | Ribosome binding |
| A0A1 × S9L20 | AmpE  | AmpE, PPI network important node |
| W8ADK8  | ompF      | Outer membrane protein |

In addition, Chueca et al.'s (2015) study also indicated the up-regulation of genes involved in cell respiration and membrane function, as well as the down-regulation of several mRNAs related to acid shock response, possibly as a result of the mild acidic environment of the cell suspension during the treatment. On the other hand, it has come to our attention that a recent PEF study by Guionet et al. (2014) did not result in as extensive changes in cells as what we have observed in our current study. Specifically, they reported that treating E. coli cells with 500 consecutive 60-ns pulses at 10^7 V m^-1, followed by a 1-h proteomic recovery in LB at room temperature, only led to the differential expression of one protein (Guionet et al., 2014). We speculated that the discrepancies could have stemmed from the fact that they used considerably shorter pulses and detected proteins by two-dimensional gel electrophoresis, which, in theory, tends to afford a lower diversity of protein candidates.

The development of resistance in pathogenic microbes against PEF treatment poses a significant problem to food preservation. In some cases, such resistance seems to be temperature- and/or pH-dependent (Somolinos et al., 2008), suggesting the involvement of enzymes and/or other bioactive molecules that confer damage mitigation. Liu and coworkers pointed out that the composition of membrane fatty acids plays a key role in the PEF resistance of E. coli by regulating membrane fluidity (Liu et al., 2017). A similar conclusion has also been obtained by Cebrián (Cebrián et al., 2016). On the other hand, microbial cells can activate an oxidative stress response upon PEF treatment, which has been shown to produce excessive ROS (Pakhomova et al., 2012). As evidence, Tanino et al. (2012) reported the up-regulation of several genes associated with oxidation stress, including those encoding glutathione synthase and superoxide dismutase, in PEF-stressed yeasts. Furthermore, the authors confirmed that the attenuation of such oxidative injuries strongly correlated with the PEF resistance of the cells (Tanino et al., 2012). García et al. (2010) suggested that enzymes involved in lipid biosynthesis and energy production are required for post-PEF membrane repair. In support of these findings, we demonstrated that PEF treatment significantly augmented the expression of several proteins involved in sulfate transport and reduction. This would in turn result in increased levels of sulfide and other reductive sulfur-containing species, presumably exerting a cytoprotective effect against PEF-induced oxidative stress (Shatalin et al., 2011; Ono et al., 2014;
Walmsley et al., 2017). Taken together, these studies offered clear evidence that microorganisms can adopt a number of strategies to combat the detrimental effects of PEF.

In summary, we reported the first systematic proteomic profiling study on PEF-treated *E. coli*. The results that we obtained suggested that the application of PEF could significantly impact the membrane integrity of the cells and could potentially stimulate a wide range of cellular defense mechanisms. These results can help researchers better understand PEF-induced microbial inactivation and develop more effective PEF-based food processing methods.

### DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the ProteomeXchange Consortium using accession number PXD014365 and also via the iProX partner repository (Ma et al., 2019).

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**Conflict of Interest:** YP was employed by company the Shanghai Applied Protein Technology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.