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Full Length Research Paper

Proteomic analysis of differentially expressed proteins in intestinal epithelial cell in response to Enteroinvasive *Escherichia coli* infection and *Lactobacillus plantarun* treatment

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A proteomic approach was taken to compare the proteomes of Enteroinvasive *Escherichia coli* (EIEC) infection alone, *Lactobacillus plantarun* pre-treatment and control group. Two-dimensional gel electrophoresis (2-DE), coupled with mass spectroscopy and protein database searching, 8 differentially expressed proteins was identified. Of them, Glutathione Transferase (down-regulated), Peroxisomal enoyl-coenzyme (up-regulated) and Peroxiredoxin (up-regulated) in EIEC infection group were compared with control group which are all associated with antioxidant-related proteins. Glyceraldehyde-3-phosphate dehydrogenase (up-regulated) and Triosephosphate Isomerase (up-regulated) were identified related with carbohydrate metabolism in EIEC infection group compared with control group. Keratin 8 (up-regulated) and hnRNP C1/C2 (down-regulated) were linked to antagonize cytoskeleton reorganization and apoptosis in *L. plantarun* pre-treatment group. Identification of these proteins provides insights that may lead to a better understanding of the molecular basis for EIEC infection process and *L. plantarun* protection function.

Key words: *Lactobacillus plantarun*, Enteroinvasive *Escherichia coli*, tight junction.

INTRODUCTION

Enteroinvasive *Escherichia coli* (EIEC) is a human intestinal pathogen responsible for the majority of cases of endemic bacillary dysentery prevalent in developing country (Song et al., 2005). The underlying pathogenesis is proposed as follows (Parsot, 2005; Croxen and Finlay, 2009). In the colonic mucosa, bacteria are supposed to cross the epithelial layer by invading M cells overlaying lymphoid follicles. Entry into epithelial cells involves rearrangements of the cell cytoskeleton (Cossart and Sansonetti, 2004). Bacteria released from M cells or epithelial cells interact with macrophages, escape from the phagocytic vacuole and induce apoptosis of infected cells. Apoptotic macrophages release pro-inflammatory cytokines facilitates further invasion by luminal bacteria (Steiner et al., 2000; Lahouassa et al., 2007). Recently, there are also evident that probiotic bacteria protect and enhance human intestinal epithelial barrier function. For example, enteropathogenic *E. coli* (EPEC)-induced neutrophil migration and EPEC binding to monolayers were inhibited by viable *Lactobacillus plantarum* but only when added to the monolayers before EPEC (Michail and Abernathy, 2003).

Identically, live *Streptococcus thermophilus* (ST)/*Lactobacillus acidophilus* (LA) interact with intestinal epithelial cells to protect them from the deleterious effect of EIEC via mechanisms that include interference with pathogen adhesion and invasion (Resta-Lenert and Barrett, 2003; Resta-Lenert and Barrett, 2006). In

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Abbreviations: EIEC, enteroinvasive *Escherichia coli*; **EPEC,** enteropathogenic *Escherichia coli*.

addition, *L. plantarum* have also been demonstrated reduce EIEC adhesion to Caco-2 by reducing intestinal permeability and increasing tight junction proteins (such as ZO-1, occludin and claudin-1 protein) in our previous reports (Qin et al., 2009). However, the detail mechanism was not very clear. Therefore, it is valuable to explore the regulation mechanism between *L. plantarun*, EIEC and intestinal epidermal-barrier function. In this study, we have, for the first time, taken a proteomic approach to identify differential proteins in EIEC infection alone, *L. plantarun* pre-treatment and control group. We anticipate these proteins may provide initial insights into the role of EIEC infection process and *L. plantarun* protection function.

MATERIALS AND METHODS

Preparation of bacteria

L. plantarum strain CGMCC No.1258 collected from Institute of Science Life of Onlly, Shanghai Jiao Tong University, Shanghai, China, a gift from Dr. Hang Xiaomin (Institute of Science Life of Onlly, Shanghai Jiao Tong University, Shanghai, China) was maintained on MRS agar (Difco Laboratories, Detroit, MI, U.S.A.). Enteroinvasive *Escherichia coli* EIEC strain 0124:NM (ATCC 43893, serotype O124:NM) was obtained from the Center of Diseases Prevention and Control of Shanghai, China and maintained in LB medium (Difco Laboratories, Detroit, MI, U.S.A). They were cultivated at 37°C for 16 h to reach stationary phase. The *L. plantarun* and EIEC suspensions were centrifuged for 5 min at 1500 × g. After removing the supernatant, the pellet was re-suspended in sterile PBS buffer to determine the bacterial concentration. Quantification of bacterial suspension was determined using a standard curve for visible absorbance (600 nm; Beckman DU-50 spectrophotometer) and adjusted the final concentration to 1×10^8/ml.

Preparation of monolayer

DMEM supplemented with 10% fetal bovine serum, 1×10^5 U/L of penicillin and 100 mg/L streptomycin was used as a standard medium to cultivate Caco-2 cells (human colonic epithelial-like cancer cell line obtained from the Cell Institute Affiliated China Science Research Institute, Shanghai, China). When cell growing to 80 to 90% fusion cells under 5% CO_2 saturated humidity and 37°C conditions, 0.25% pancreatic enzyme with 0.03% EDTA was performed to digest cells to subculture (1:3). Then, the cells were inoculated to glass slide in six-well culture plate (gelatin treatment) and cell concentration was 5 × 104 cells/cm^2. After 7 to 10 days, monolayer cells were collected and used in later experiment.

Infection of intestinal epithelial monolayer

Caco-2 cells were washed three times in Hank's balanced salt solution (Life Technologies) to remove the antibiotic media. For rapid infection of the monolayer, 100 µl EIEC at 1.0×10^8 /ml was added to the apical side of the cell culture insert, and the insert was placed in a 50 ml tube and centrifuged at 200 g for 4 min. *L. plantarum* (100 µl of 1.0×10^8/ml) was added to the monolayers in different groups for 24 h. Caco-2 cells monolayers were cultured and served as the control group, Caco-2 cells were infected EIEC as the EIEC group, Caco-2 cells infected EIEC were co-incultured with *L. plantarum* as the *L. plantarum* group. The average number of Caco-2 cells in each monolayer was approximately 1×10^6. The inoculation ratio of EIEC to Caco-2 cells was 100:1. The ratio of lactobacillus to EIEC was 10:1.

Protein sample preparation

The Caco-2 cells monolayer cells in each group were harvested by centrifugation, rinsed in phosphate-buffered saline and re-suspended in 300 µl lysis buffer (9.5 M urea, 4% CHAPS, 65 mM DTT, 2% carrier ampholyte and protease inhibitor cocktail). Then, the Caco-2 cells were removed from the surface with a cell scraper and collected to a 1.5 Eppendorf tube. The crude extract solution was obtained by ultrasonic disruption (80 W, 2 min, with a 15 s interval every 10 s) and centrifugation (14000 rpm, 60 min). The resulting supernatant was concentrated on Biomax-5 K ultra-filtration membrane. The protein concentration was determined by a standard Bradford protein assay and stored at -80°C until use for 2-DE analysis.

Gel electrophoresis and analysis

First-dimensional electrophoresis was carried out using an IPGphor II (Amersham Biosciences) isoelectric focusing system. 100 g of total extract were loaded. IPG dry strips (pH 3-10, linear) were rehydrated at 30 V for 12 h. After rehydration, isoelectric focusing was performed under the following conditions: 500 V for 1 h, 1000 V for 1 h, 8000 V for 6 h and 500 V for 4 h. After equilibration of the isoelectric focusing strips, SDS electrophoresis was performed on 12.5% gels. SDS-PAGE was performed using a Hofer SE 600 System (Amersham Biosciences): 15 mA for 30 min and 30 mA until the Bromophenol Blue front reached 0.5 cm of the gel. After two-dimensional gel electrophoresis, proteins were stained with silver for subsequent mass spectrometry. To ensure data reliability, sample preparation and 2-DE were performed in triplicate. Silver-stained gels were scanned with Bio-Rad GS710 scanner. Images were analyzed using the specialized software program Image Master 2D Elite software (Amersham Biosciences).

MALDI-TOF mass spectrometry and protein identification

Proteins of interest were excised and digested in gel using trypsin for 20 h (sequencing grade, Promega, Charbonnie`res, France). Digest products were completely dehydrated in a vacuum centrifuge and resuspended in 10 ul of formic acid (2%), desalted using Zip Tips C18 (Millipore, Bedford, MA), eluted with acidα-cyano-4-hydroxy-trans-cinnamic acid (Sigma, 5 mg/ml in 0.1% TFA) and loaded on the target of a Bruker-Daltonics AutoFlex TOF−TOF LIFT mass spectrometer (Bruker Daltonics, Bremen, Germany). Analysis was performed in reflectron mode with an accelerating voltage of 20 kV. Identification of proteins was performed using both Mascot and PeptIdent software (available at www.matrixscience.com and www.expasy.org/tools/peptident.html, respectively). Search parameters were as follows: database: NCBInr (release date: 20070326); taxonomy: homo sapiens (human); type of search: peptide mass fingerprint; enzyme: trypsin; fixed modifications: carbamidomethyl (C); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 100 ppm; peptide charge state: 1+; and max missed cleavages: 1.

Statistics analysis

All data were analyzed by SPSS13.0 and the results were

Table 1. Differentially expressed proteins between EIEC and normal group.

Group ID	Normal group	EIEC infection group	Group ID	Normal group	EIEC infection group
3780	-6.46544	6.46544	4399	1.94149	-1.94149
4075	-3.90939	3.90939	3676	-1.93011	1.93011
3959	-3.35281	3.35281	4388	1.87136	-1.87136
3969	-3.03349	3.03349	4118	-1.75635	1.75635
4364	3.00393	-3.00393	4448	1.74678	-1.74678
3863	-2.66937	2.66937	4106	-1.74489	1.74489
3792	-2.48479	2.48479	3096	-1.6833	1.6833
4435	2.46231	-2.46231	3410	-1.6324	1.6324
4241	2.37954	-2.37954	3368	1.6012	-1.6012
4445	2.30779	-2.30779	3392	1.56976	-1.56976
4250	-2.30228	2.30228	3777	-1.56414	1.56414
4110	-2.27937	2.27937	3597	-1.56405	1.56405
4070	2.19601	-2.19601	3380	-1.51271	1.51271
3299	-2.05878	2.05878	4050	-1.51244	1.51244
4337	1.96883	-1.96883	2930	-1.50543	1.50543

measured by average ± standard deviation. One-way ANOVA was performed on all experiments with Tukey Kramer post-hoc comparison. $P < 0.05$ was considered as statistically significant.

RESULTS

Differentially expressed proteins analysis

The differentially expressed proteins between different groups were analyzed based on image master software (class report ratio > = 1.5). The results showed that there were 30 spots identified as differentially expressed between normal group and EIEC group. Of this, 19 genes were up-regulated and 11 genes were down-regulated expression compared with normal group (Table 1, Figures 1 and 2). When comparison betweennormal group and *L. plantarum* group, the results indicated 25 differentially expressed proteins (14 genes up-regulated and 11 genes down-regulated) (Table 2, Figures 3 and 4). 15 differentially expressed proteins were identified between *L. plantarum* group and EIEC group. Among them, 5 genes were down-regulated expression, and 10 genes were up-regulated expression compared with EIEC group (Table 3, Figures 5 and 6).

MALDI-TOF mass spectrometry and protein identification

Total of 16 differentially expressed proteins were excised from 2-DE gels, in-gel digested by trypsin and subjected to MALDI-TOF-TOF/MS analysis. Of them, 8 differentially expressed proteins have been identified by NCBInr database searching (Table 4).

DISCUSSION

In this study, we have used 2-DE and MS to establish the proteomic profiles of intestinal epithelial cell in response to EIEC infection and *L. plantarun* pre-treatment. 2-DE was employed as it permits the identification of the alteration of protein isoforms and determination of protein expression levels and post translational modifications. The 2-DE gel in each group was analyzed to screen differentially expressed proteins by Image Master 2D Elite software. Based on this analysis, 16 significantly differential proteins was chosen to identify by MALDI-TOF-TOF/MS, of them, 8 differentially expressed proteins have been identified by NCBInr database searching. Literature searches were conducted for all the proteins identified in the up- and down-expressed lists to find possible links of the change in expression and their implications in intestinal epithelial cell in general. Most proteins identified show homology with antioxidant-related proteins, such as glutathione transferase, peroxisomal enoyl-coenzyme A hydratase-like protein and peroxiredoxin. Glutathione-S-transferases (GSTs) are a family of Phase II detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds (Townsend and Tew, 2003; Wu et al., 2004). GST was down-regulated expression in EIEC group, indicating decrease in detoxification function. This result led to EIEC invasion and damage intestinal epithelial cell. Peroxiredoxin 1 (PRDX1) is a ubiquitously expressed antioxidant with vital roles in basal metabolic functions. In addition, PRDX1 is involved in cell differentiation and proliferation, apoptosis and innate immunity (Daly et al., 2008). In addition, glyceraldehyde-3-phosphate

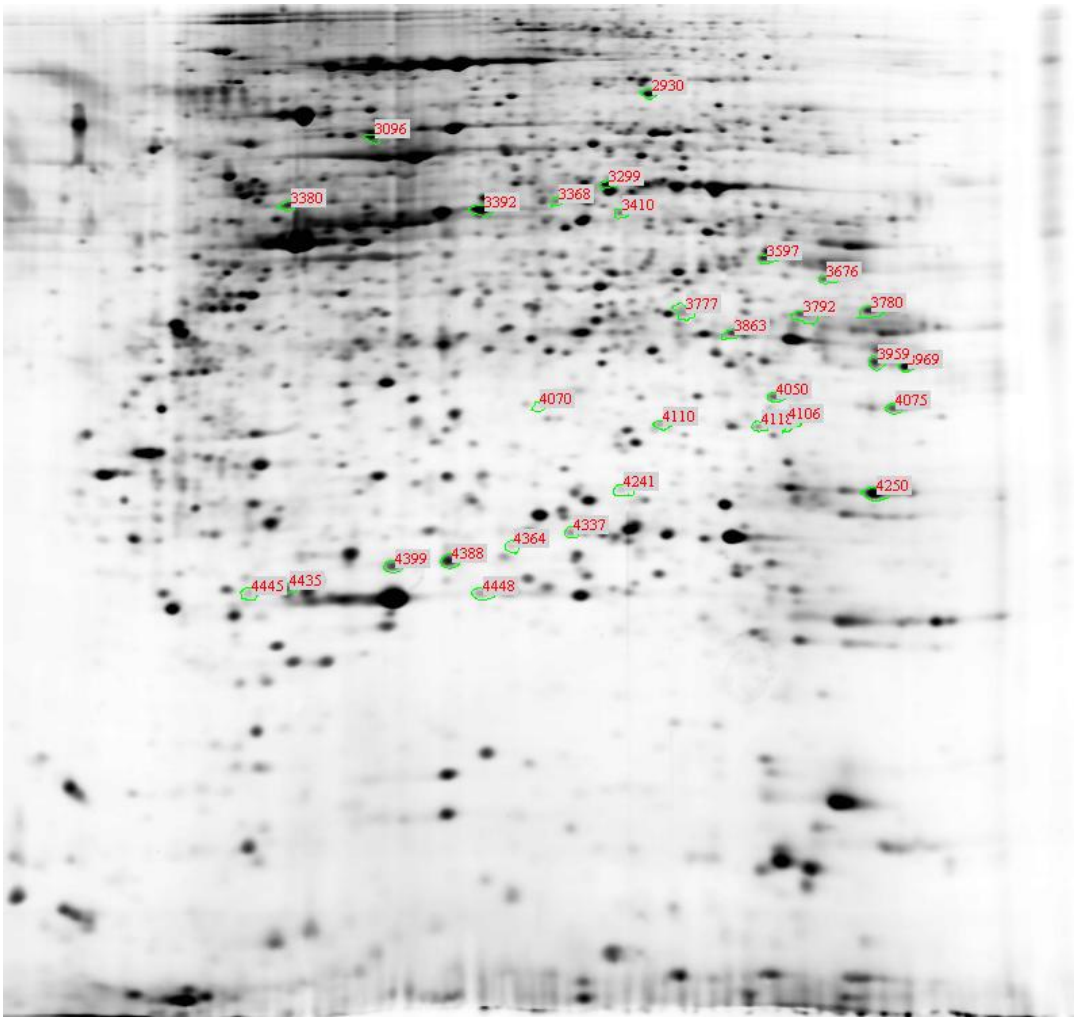

Figure 1. Differently expression spots in EIEC and normal group.

dehydrogenase (GAPDH) and triosephosphate isomerase (Tim) were also identified, which all play an important role in carbohydrate metabolism.

Of them, GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway. As part of the conversion, GAPDH converts NAD^+ to the high-energy electron carrier NADH. GAPDH has been referred to as a "housekeeping" protein based on the view that GAPDH gene expression remains constant under changing cellular conditions. Triosephosphate isomerase (TIM) is also an enzyme with a role in glycolysis and gluco-neogenesis by catalyzing the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (Moraes et al., 2011). Carbohydrate metabolism has been implicated in pathogenesis of enteroinva-sive *E. coli* (EIEC). In a cell culture model, an EIEC mutant defective in both glucose and mannose transport was significantly impaired in adherence and invasion (Gore and Payne, 2010). And Egea et al. (2007) found that GAPDH could be present in the surface of enteropathogenic (EHEC) and enterohaemorrhagic (EPEC) *E. coli* (all as Gram-negative bacteria) and GAPDH could adhere to the cell surface after co-cultivation with Caco-2 cells. This may partially explain the pathogenesis of EHEC and EPEC infection. Therefore, in our study, we found GAPDH and TIM were up-regulated expression in EIEC group contrast to normal group, suggesting EIEC also express the GAPDH and increase its ability of adherence and invasion to Caco-2 cells.

Recently, GAPDH has also been known to contribute to a number of diverse cellular functions unrelated to glycolysis such as cytoskeletal organization, apoptosis and viral pathogenesis (Tatton et al., 2000). These may also be associated with Caco-2 cells damage and apoptosis upon EIEC infection. The clinical study found probiotics can restrain the damage effect of intestinal pathogenic bacteria, and can be used to treat acute diarrhea (Sazawal et al., 2006) and intestinal flora

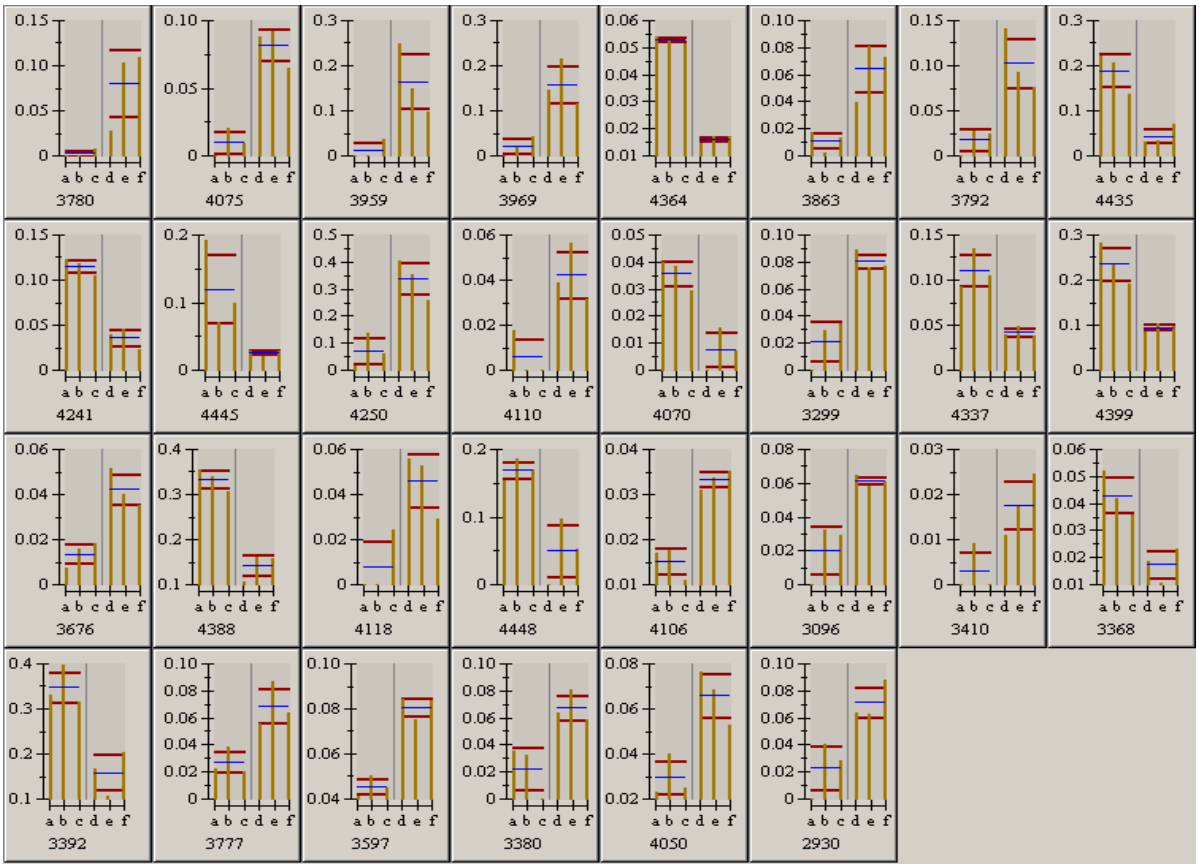

Figure 2. Relative expression profile of differently expression spots in EIEC and normal group.

Table 2. Differentially expressed proteins between *L. plantarum* and normal group.

Group ID	Normal group	*L. plantarum* group	Group ID	Normal group	*L. plantarum* group
1122	-3.01255	3.01255	1118	-1.85844	1.85844
1615	-2.79918	2.79918	1804	-1.78829	1.78829
2007	-2.67766	2.67766	1955	1.75013	-1.75013
900	-2.58965	2.58965	1698	1.72375	-1.72375
1746	-2.56488	2.56488	639	-1.59668	1.59668
1656	2.51169	-2.51169	925	-1.59597	1.59597
1558	-2.49958	2.49958	1842	-1.5682	1.5682
1944	2.46618	-2.46618	641	-1.55929	1.55929
390	-2.04106	2.04106	1684	1.54977	-1.54977
1917	1.99593	-1.99593	1754	1.53794	-1.53794
1914	1.9928	-1.9928	638	-1.52214	1.52214
1883	1.90046	-1.90046	2159	1.51294	-1.51294
1886	1.89765	-1.89765			

(Kuehbacher et al., 2006). Keratin 8 was induced up-regulated expression upon *L. plantarum* treatment. Keratins 8 and 18 belong to the keratin family of intermediate filament proteins and they can be covalently conjugated to constitute a hallmark for all simple epithelia (Gilbert et al., 2001; Magin et al., 2007). Phosphorylation in some sites may affect the structure and function of this protein and even cells signal transduction (Feng et al., 1999; Ridge et al., 2005). Phosphorylation facilitates formation of Keratin 8/18 aggregates, but is not crucial.

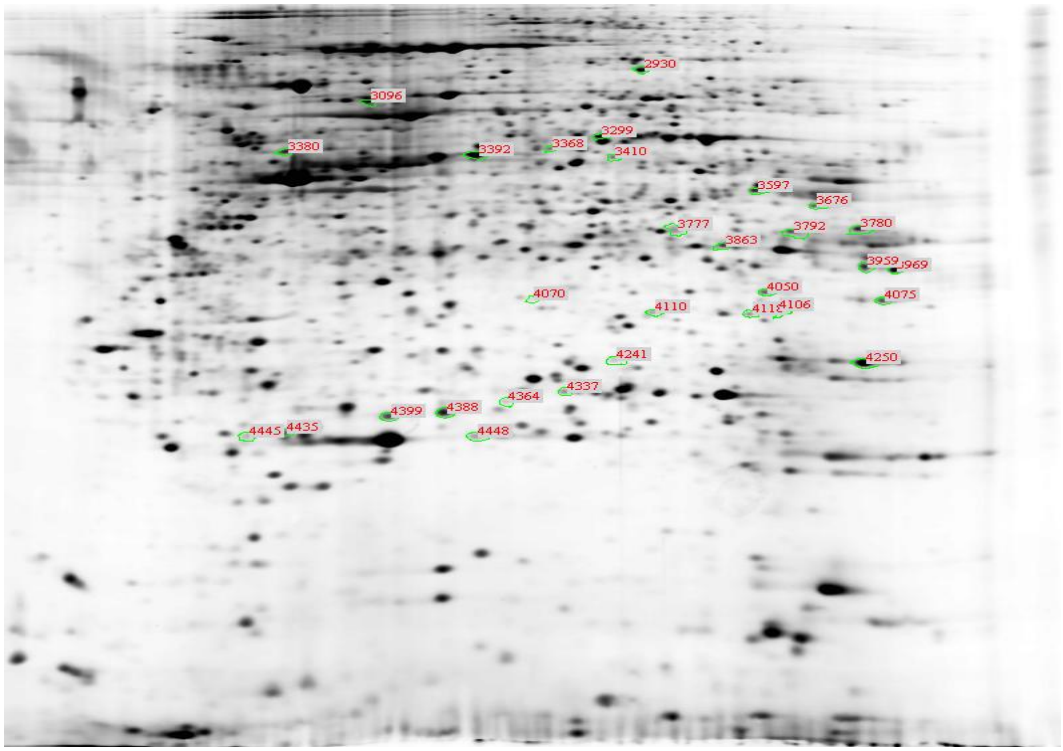

Figure 3. Differently expression spots in *L. plantarum* and normal group.

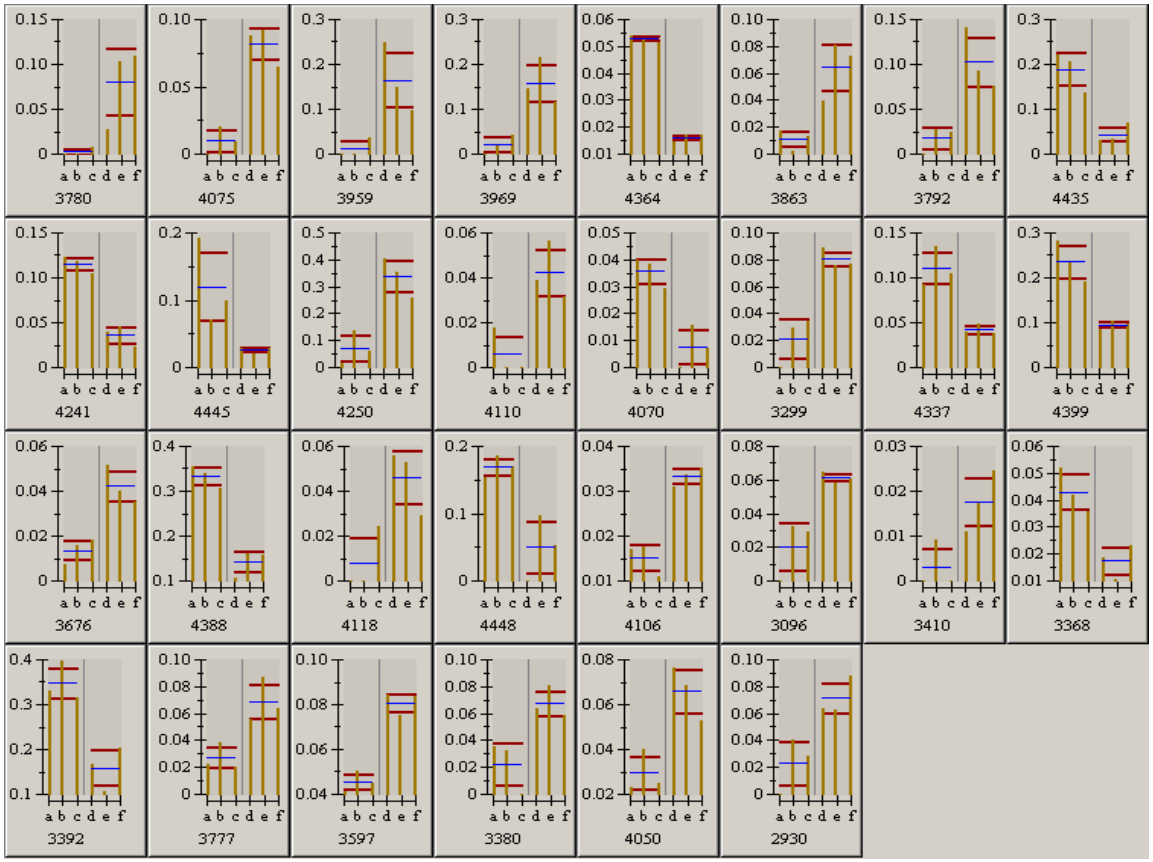

Figure 4. Relative expression profile of differently expression spots in *L. plantarum* and normal group.

Table 3. Differentially expressed proteins between *L. plantarum* and EIEC group.

Group ID	*L. plantarum* group	EIEC group	Group ID	*L. plantarum* group	EIEC group
1313	-3.59685	3.59685	1829	1.90584	-1.90584
1403	-3.56569	3.56569	1549	-1.79476	1.79476
1043	-3.33972	3.33972	613	1.61932	-1.61932
1217	-2.51966	2.51966	680	-1.57435	1.57435
1792	-2.32483	2.32483	1341	1.55353	-1.55353
1710	-2.29915	2.29915	927	-1.5412	1.5412
1664	1.95007	-1.95007	1851	1.50677	-1.50677
1413	-1.93483	1.93483			

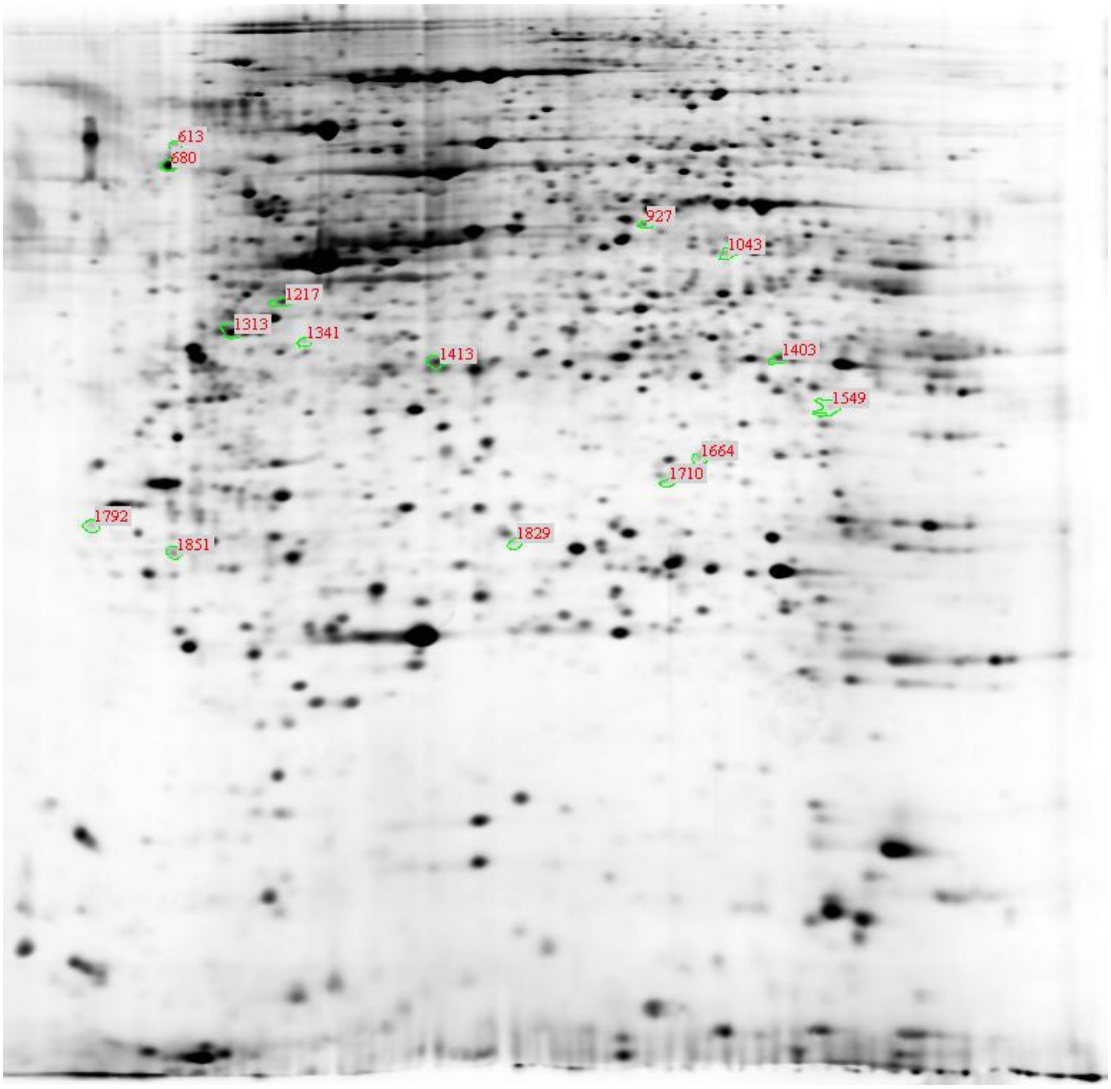

Figure 5. Differently expression spots in *L. plantarum* and normal group.

Keratin 8/18 would breakdown and reorganize during apoptosis. And at later stages of the apoptotic process, that is, when the integrity of the cytoplasmic membrane becomes compromised, keratin aggregates are shed from the cells (Schutte et al., 2004). Our study discovered the keratin-dependent protection of Caco-2 cells from

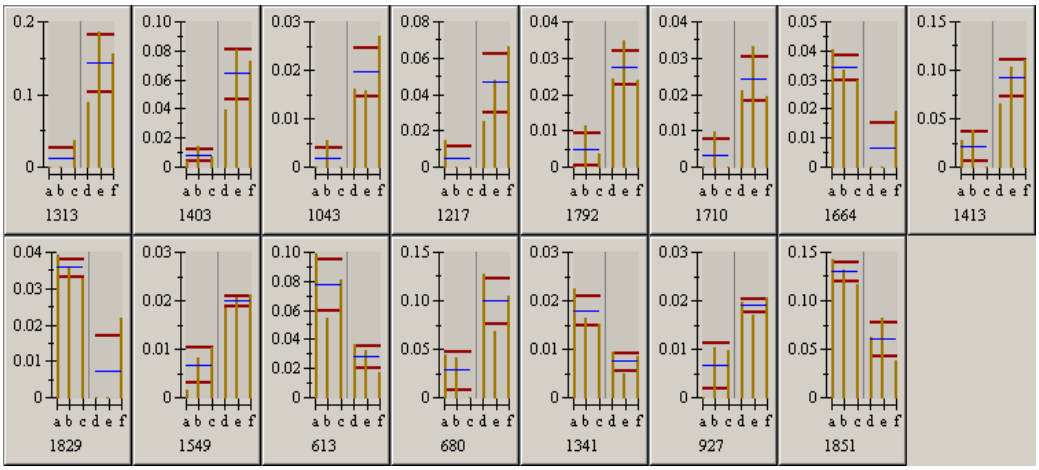

Figure 6. Relative expression profile of differently expression spots in *L. plantarum* and normal group.

Table 4. Identification of differentially expressed proteins by NCBInr database searching.

Spot No	Protein ID	Protein name
1403	Gi|31645	Glyceraldehyde-3-phosphate dehydrogenase.
1313	Gi|109082737	Predicted: heterogeneous nuclear ribonucleoprotein C (C1/C2) isoform 2 (Macaca mulatta).
1217	Gi|62913980	KRT8 protein.
1615	Gi|70995211	Peroxisomal enoyl-coenzyme A hydratase-like protein.
2007	Gi|55959887	Peroxiredoxin 1.
4445	Gi|20664358	Chain A, crystal structure of a recombinant glutathione transferase, created by replacing the last seven residues of each subunit of the human class Pi isoenzyme with the additional C-terminal helix of human class alpha isoenzyme.
4337	Gi|999892	Chain A, triosephosphate isomerase (Tim) (E.C.5.3.1.1) complexed with 2-phosphoglycolic acid.
4435	Gi|20664358	Chain A, crystal structure of a recombinant glutathione transferase, created by replacing the last seven residues of each subunit of the human class Pi isoenzyme with the additional C-terminal helix of human class alpha isoenzyme.

EIEC induced apoptotic challenge may be a key function of simple epithelial keratins (Jaquemar et al., 2003). And intermediate filament proteins might be induced over-expression by *L. plantarum* to antagonize the apoptosis and destructive effect on cytoskeleton by EIEC (Nishizawa et al., 2005).

The heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP-C1/C2) is one of the most abundant proteins in the nucleus, and shown to have roles in cellular differentiation and proliferation through post-transcriptional regulations of certain mRNA species (Williamson et al., 2000). Many hnRNP-C1/C2 have been found to be phosphorylated in response to extracellular stimulations. These changes have been proposed to regulate splice site selection in pre-mRNA alternative splicing, which is recognized as the cause or the consequence of numerous human diseases such as tumors and inflammatory injuries (Zhu et al., 2003). Translocation of hnRNP C1/C2 from nuclei to cytoplasm in PMA-induced pro-apoptotic cells have been identified dependent on ROCK-mediated cytoskeleton rearrangement (Lee et al., 2004). In our study, down-regulation of hnRNP-C1/C2

upon *L. plantarum* treatment might be as a novel mechanism to enhance the resistance of Caco-2 cells to apoptosis, inflammatory and indirectly decrease the hnRNP C1/C2 translocation.

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