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Proteome analysis of *Lactobacillus plantarum* strain under cheese-like conditions

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ABSTRACT

As a food grade fermentation starter, *Lactobacillus plantarum* (*L. plantarum*) also named as the secondary starters during cheese ripening. In this study, the concentration of NaCl was screened as the main factor in the cheese-like conditions (15 °C, pH 5.2, 6% NaCl) to assess the potential properties of *L. plantarum*. A comprehensive proteome profile of *L. plantarum* strain was analyzed with iTRAQ proteomics methods fractionated by SCX chromatography. Proteins involved in carbohydrate transport and metabolism, cell envelope, peptide-glycan biosynthesis and lipid transport and metabolism were found significant changes. Meanwhile, the same trends were found in mRNA expression levels analyzed by RT-PCR. Some general transportation proteins related to ion transporters were detected as more abundant, which may reveal a rescue mechanism of the microbe in sodium-dependent glucose transfer. The understanding of *L. plantarum* proteome in salt tolerance could be useful to get strain adapted for specific applications.

Biological significance: The bacterial biota has a primary role in affecting cheese quality. Under cheese-like conditions, *L. plantarum* mainly increased the levels of enzymes that responsible for the flavour development during cheese ripening. However, the mechanisms of proteomic adaptation remain largely unclear in unraveling details of the salt tolerance of *L. plantarum*. This study revealed a dramatic change involved in carbohydrate transport and metabolism, cell envelope, peptide-glycan biosynthesis, lipid transport and metabolism, and glycolysis. Meanwhile, these pathways provide a comprehensive proteome profile of *L. plantarum* survived under cheese-like conditions. Furthermore, this study shows that iTRAQ proteomics provide more reliable information in describing the molecular rescue strategy of *L. plantarum* in sodium-dependent glucose transfer.

1. Introduction

As a food grade fermentation starter, lactic acid bacteria have been evaluated for their potential use as probiotics. It was found that LAB have various special therapeutic and prophylactic properties by the investigation of bacteria's behavior in the gastrointestinal tract [1].

Fermentation is one of the oldest ways to preserve milk, traditional fermented milk products have been developed independently worldwide, especially yoghurt and cheese [2]. Milk for cheese is coagulated by acidification, or enzymatic action, and the resulting gel is processed to encourage moisture loss. Cheeses are made by adding dry salts frequently to its surface for 2–3 weeks and then ripened up to two years, such as Grana Trentino, an Italian hard cooked cheese [3]. During this long ripening process, extensive biochemical reactions occur in the curd metabolize milk components such as lactose, protein and fat. Meanwhile, cheese texture and flavour are also obtained through this series of chemical changes [4,5].

The bacterial biota has a primary role in affecting cheese quality. Among these, non-starter lactic acid bacteria (NSLAB) from the environmental microbial communities could have a significant effect on most ripened cheeses [6]. These NSLAB also been named as the secondary starters during cheese ripening, for they do not make a difference on acid production during cheese manufacture as the starter culture does, but do contribute to flavour development in the ripening cheese. *Lactococci*, *Pediococci*, *Enterococci*, *Leuconostoc* spp. and *Thermophilic* lactic acid bacteria are part of this population. Among mesophilic and facultative heterofermentative lactobacilli, *Lactobacillus paracasei*, *Lactobacillus casei*, *Lactobacillus plantarum* (*L. plantarum*), and *Lactobacillus rhamnosus* are identified [7]. NSLAB make a contribution to cheese ripening by increased proteolysis and peptidolysis and production of aroma compounds. In addition, NSLAB have frequently been screened with some degree of success for health-promoting properties [8].

Under cheese-like conditions, intended as the sum of various types of environmental stress, NSLAB mainly increased the levels of enzymes that are responsible for nitrogen metabolism and free amino acid

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catabolism. And indeed, the maximum aminopeptidase and peptidolytic enzymes activity was detected in cheese-ripening conditions [9]. During early cheese ripening, contaminating NSLAB account for a small proportion, which markedly increases to lager numbers during late maturation [6]. Proteolysis is a very complex biochemical process during cheese ripening, and specificity of proteolytic enzymes depends on environmental conditions, such as pH value and salt concentration. Meanwhile, some research also found the enzyme activities of aminopeptidase, aminotransferase and esterase activities were reduced in *Lactococci*, *Lactobacilli*, and *Brevibacteria* in cheese-ripening conditions [10].

Throughout the ripening, facultatively heterofermentative group of *lactobacilli* prevailed in cheese samples. *Lactobacillus casei*/*paracasei* and *L. plantarum* take the majority of NSLAB in most ripened cheese varieties, especially in Cheddar or Gouda cheese with pH 5.25 and 5.0% (w/w) NaCl [11]. Previous research also revealed *L. plantarum* strain isolated from Italian and Argentinean cheeses are highly resistant to lysozyme, well adapted to simulated gastric juice, and a moderation to low bile tolerance [12]. Some kinds of *L. plantarum* strain have the bacteriocidal activity against *Listeria innocua* and *Listeria monocytogenes* with the production of a potent class IIa bacteriocin [13].

Proteomics plays a decisive role in linking genome and transcriptome to potential biological functions [14]. Although various studies have been conducted in recent years about characterization of cheeses, and the adaptation of NSLAB to the hostile environmental conditions of the cheese during ripening. Only a few studies considered a proteomic approach to investigate the metabolic versatility and diversity of NSLAB [15]. And the mechanisms of proteomic adaptation remain largely unclear in unraveling details of the salt tolerance of *L. plantarum* during cheese ripening.

This study highlighted the proteome features of these molecular mechanisms of *L. plantarum* stress adaptation in cheese-like conditions. Comparison of differentially protein expression levels in different cellular signaling pathways, ion transport pathways related to sodium and carbohydrate metabolic pathways were affected to varying degrees. The understanding of *L. plantarum* proteome in salt tolerance could be useful to get strain adapted for specific applications.

2. Materials and methods

2.1. Bacterial strain and growth conditions

L. plantarum ATCC 14917 was purchased from the China General Microbiological Culture Collection Center and cultured at 37 °C in modified de Man-Rogosa-Sharpe (MRS) medium, in which the glucose was replaced by lactose. For the salt tolerance screening, *L. plantarum* was grown in MRS broth at 37 °C for 8 h and then transferred into MRS broth with different sodium concentrations (2, 4, 6, 8, 10, 12, 14, or 16%). To assess the potential properties of *L. plantarum* during cheese ripening, the cells were incubated in cheese-like conditions (15 °C, pH 5.2, 6% NaCl) [16]. Cells suspension were centrifuged at 8000 ×*g* for 15 min at 4 °C, and pellets were harvested for electron-microscopic observation and total proteins isolation.

2.2. Scanning electron microscope (SEM) and transmission electron microscope (TEM) analysis

Pellets (from 100 ml culture medium) isolated from stationary phase (18 h) cells were washed with 0.1 M phosphate buffer saline (PBS, pH 7.0) for three times. Then, the samples were dehydrated for 20 min in ethanol at concentrations of 30%, 50%, 70%, 80%, 90% and 100%, and then dehydrated with tertiary-butanol. After that, samples were freeze-dried with a freezer dryer (Christ, Alpha 1-4 LD plus, Germany). The sample specimens were viewed under SEM by Hitachi S-570 (Japan) at 15 kV in high-vacuum mode. TEM were carried out at follows: isolated pellets of *L. plantarum* were washed with 0.1 M PBS (pH 7.0) for three times, followed by draining of the excess liquid. The bacteria were observed with a Hitachi model H-800 transmission electron microscope, using an accelerating voltage of 200 kV.

2.3. Proteomic sample preparation

To assess the potential properties of *L. plantarum* during cheese making, the cells were incubated in (15 °C, pH 5.2, 6% NaCl) for 18 h before the total proteins extraction. *L. plantarum* cells in the control (MRS medium) and experimental groups (cheese-like conditions) were harvested by centrifuging at 8000 ×*g* for 15 min at 4 °C, and the sample pellets were washed three times with PBS (0.1 M, pH 7.0). After that, the pellets were lysed in lysis buffer containing 8 M urea, 1% dithiothreitol (DTT), with a protease inhibitor cocktail (Roche, USA). The total protein was extracted by using an ultrasonic processor (400 W, 3 × 50 times) at 4 °C. After that, the suspensions were centrifuged at 12,000 ×*g* for 30 min. The protein concentration in supernatant was measured with a Non-Interference Protein Assay Kit (Sangon Biotech, Shanghai, China). Protein concentration of 5–10 mg/mL is necessary for iTRAQ analysis. All the processes were operated on ice to prevent protein degradation.

2.4. iTRAQ labeling and fractionation

Protein samples were digested with trypsin (Promega, Madison, WI) overnight (20 μg/mL) at 37 °C in a 1:50 trypsin-to-protein mass ratio. After trypsin digestion, peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ kit (Applied Biosystems). The pooled mixtures of iTRAQ-labeled peptides were fractionated by SCX chromatography using the Shimadzu LC-20AB HPLC Pump system. The eluted peptides were pooled as 10 fractions, desalted by Strata X reverse column (Phenomenex) and vacuum-dried.

2.5. Liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) analysis and protein identification

LC-ESI-MS/MS analysis was performed on a Waters nanoAcquity UPLC by the autosampler onto a C18 trap column and 8 μL peptides supernatant (0.5 mg/mL) were eluted onto an analytical C18 column. The flow rate is 8 μL/min at the first 4 min with buffer A (5% ACN, 0.1% FA), then the 65 min gradient was run at 300 nL/min starting from 8 to 25% B (ACN, 0.1% FA), followed by 10 min linear gradient to 80%, and maintenance at 80% B for 5 min. Data acquisition was performed with a TripleTOF 5600 System. A rolling collision energy coupled with iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation.

The original tandem mass spectrometry (MS/MS) file data (*.wiff) were analyzed with ProteinPilot 4.2 (Applied Biosystems), with 1% FDR for protein and peptides. After that, the raw peptide identification results were classified according to the SWISS-PROT online database (http://www.uniprot.org/). Functional analysis of proteins identified were mapped to Gene Ontology (GO) Terms and classified by the GO enrichment analysis approach (http://beta.geneontology.org/). An online reference system Kyoto Encyclopedia of Genes and Genomes (KEGG) was used for the systematic interpretation of differentially expressed proteins (http://www.kegg.jp/kegg/pathway) [17].

2.6. mRNA detection related to gene expressions

Cells were centrifuged at 12,000 ×*g* for 10 min at 4 °C, and then washed with PBS three times. Total RNA was extracted with an EasyPure RNA Kit (TransGen Biotech, China) and cDNA was synthesized using an All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit from TransGen Biotech. TransStart Tip Green qPCR SuperMix kit were used for real-time PCR (RT-PCR) and performed with a LightCycler 96