Proteomic analysis of an engineered isolate of *Lactobacillus plantarum* with enhanced raffinose metabolic capacity

Jicheng Wang, Wenyan Hui, Chenxia Cao, Rulin Jin, Caixia Ren, Heping Zhang & Wenyi Zhang

Lactic acid bacteria that can produce alpha-galactosidase are a promising solution for improving the nutritional value of soy-derived products. For their commercial use in the manufacturing process, it is essential to understand the catabolic mechanisms that facilitate their growth and performance. In this study, we used comparative proteomic analysis to compare catabolism in an engineered isolate of *Lactobacillus plantarum* P-8 with enhanced raffinose metabolic capacity, with the parent (or wild-type) isolate from which it was derived. When growing on semi-defined medium with raffinose, a total of one hundred and twenty-five proteins were significantly up-regulated (>1.5 fold, *P* < 0.05) in the engineered isolate, whilst and one hundred and six proteins were significantly down-regulated (<−1.5 fold, *P* < 0.05). During the late stages of growth, the engineered isolate was able to utilise alternative carbohydrates such as sorbitol instead of raffinose to sustain cell division. To avoid acid damage the cell layer of the engineered isolate altered through a combination of *de novo* fatty acid biosynthesis and modification of existing lipid membrane phospholipid acyl chains. Interestingly, aspartate and glutamate metabolism was associated with this acid response. Higher intracellular aspartate and glutamate levels in the engineered isolate compared with the parent isolate were confirmed by further chemical analysis. Our study will underpin the future use of this engineered isolate in the manufacture of soymilk products.

Soy-derived products contain the alpha-galactooligosaccharide sugar, raffinose. Due to a lack of pancreatic alpha-galactosidase (α-Gal) that could catalyze its hydrolysis, humans are unable to digest this sugar. However, this sugar is used by gas-producing bacteria in the large intestine, resulting in documented intestinal disorders such as nausea, diarrhoea and abdominal pain. To overcome this drawback which reduces the nutritional value of soy products, much attention has been paid to the use of α-Gal-producing lactic acid bacteria (LAB) in the production of soy products. Isolates from several LAB species including *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Leuconostoc mesenteroides* have the potential to reduce raffinose levels in soy products. Fermentation with these isolates can eliminate undesirable physiological effects associated with the consumption of soy products.

In the last few years, modeling of the transport and catabolic pathways for raffinose have made significant progress alongside advances in the understanding of LAB genomics. There have also been some studies that attempt to use genetic engineering techniques for the constructing isolates with enhanced α-Gal activity. Within the genus *Lactobacillus*, the first study on the molecular mechanism behind raffinose utilization was done using microarrays, which revealed that genes involved in the metabolism of raffinose by *L. plantarum* WCSF were differentially expressed. Recently, the genetic loci coding for the catabolic pathway of raffinose were accurately assigned in *L. acidophilus* NCFM, also using microarray techniques. Both of these studies demonstrated the importance of understanding the catabolic pathway in selected strains if they are to be better exploited in industrialized production, but also the high efficiency of new genomic research tools.

*Lactobacillus plantarum* P-8 is a probiotic isolate from traditionally fermented dairy products. It grows well in soymilk and is able to metabolize α-galactosides (stachyose and raffinose). In this study, comparative
Proteomic analysis was performed on an isolate of *L. plantarum* P-8 that had been engineered for enhanced raffinose metabolic capacity and its original parent strain. Our aim was to compare the behaviour of both isolates in the presence of raffinose. The information provided here will underpin the future use of probiotics with enhanced raffinose metabolism in the manufacturing of soymilk products.

Results

Growth of engineered *L. plantarum* P-8 and its parent isolate on media supplemented with raffinose. Growth curves based on viable counts, pH values and OD values were produced for both isolates growing in media supplemented with raffinose (Fig. 1a–c). Although the initial inoculum densities of the two strains in the media were the same, the growth rates were completely different. The number of viable counts increased more rapidly for the engineered isolate than the parent isolate although they ultimately achieved a similar density (above $9.24 \times 10^7$ cfu/mL); thereafter the viable cell count fell off more rapidly in the engineered isolate than the parent isolate (Fig. 1a). Compared with its original strain, the pH of the semi-defined medium (SDM) inoculated with the engineered isolate dropped much faster than for the parent isolate indicating it more rapid fermentation rate (Fig. 1b).

Organic acids after fermentation. After fermentation, lactic acid and acetate were the main end products present in the medium with raffinose (Fig. 2). A slightly higher concentration of these organic acids was detected in the medium fermented by the engineered isolate compared with the parent isolate (Fig. 2), which was in accordance with the pH values determined.

Intracellular amino acid profile. A total of 17 intracellular amino acids were quantified in the engineered and parent isolates (Table 1). There were higher intracellular levels of aspartate and glutamate in the engineered isolate than in the parent isolate (Table 1). No serine or phenylalanine was detected within the growing cells.

Up-regulation of proteins during late growth in media containing raffinose. A total of 125 proteins were significantly up-regulated (>1.5 fold, $P < 0.05$) in the engineered isolate compared with the parent isolate (Table 2). Most of these proteins could be assigned to the category from the clusters of orthologous groups of proteins (COGs) (Fig. 3), with 12.6% proteins involved in posttranslational modification, protein turnover, chaperones and 11.5% of proteins involved in carbohydrate transport and metabolism.

Clustered genes (LBP_cg2912- LBP_cg2914, LBP_cg2917) could be distinguished that were significantly up-regulated (>3.8 fold). The set of sorbitol-related proteins consisted of a sorbitol PTS EIIA, a sorbitol PTS
EIIBC, a sorbitol PTS EIIIC and a sorbitol-6-phosphate 2-dehydrogenase. This was similar to the genetic organization of the sorbitol operon identified in L. casei ATCC BL2315. The operon coded in the parent isolate also included an activator.

Proteins associated with the cell membrane and cell wall metabolism, namely an alanine racemase (LBP_cg0413), a D-alanine-poly (phosphoribitol) ligase subunit (LBP_cg1580), a large-conductance mechanosensitive channel (LBP_cg1017), a glutamate racemase (LBP_cg1859) and a cyclopropane-fatty-acyl-phospholipid synthase (LBP_cg0412) were all up-regulated in the engineered isolate compared with the parent isolate. This may be because the rapid growth of the engineered isolate compared with the parent isolate means that it is likely to be challenged by a more acidic environment in the medium. Other characteristics of the acid response in the engineered isolate include activation of classic stress response proteins. These include a small heat shock protein (LBP_cg0109), a 60 kDa chaperon and a cold shock protein (LBP_cg0785).

**Down-regulation of proteins during late growth on media containing raffinose.** A total of 106 proteins were significantly down-regulated (< -1.5 fold, P < 0.05) in the engineered isolate compared with the parent isolate (Table 3). As can be seen from the COG category (Fig. 3), these proteins were mainly involved in amino acid transport and metabolism (18.2%), and carbohydrate transport and metabolism (20.2%).

Amidst them, proteins coding for an alpha-galactosidase (LBP_cg2832) and a beta-galactosidase (LBP_p2g004) have been predicted to be involved in the raffinose metabolism of LAB species16. Other repressed proteins associated with carbohydrate metabolism were involved in galactitol utilization (LBP_cg2877-LBP_cg2878) and the pentose phosphate pathway (LBP_cg2869 and LBP_cg2868). Two proteins coding for an aspartate aminotransferase (LBP_p3g040) and an AAE family aspartate:alanine exchanger (LBP_p3g041) were found to be flanked by transposases in the plasmid. Unexpectedly, some transporters of oligopeptides (LBP_cg0966 and LBP_cg0963) and amino acids (LBP_cg0653, LBP_cg0601 and LBP_cg0602) were detected, suggesting a low requirement for these materials during the late growth of the engineered isolate.

**Discussion**

LAB isolates that can produce α-Gal are a promising way to improve the nutritional value of soy-derived products. For their further exploitation in the manufacturing process, it is important to understand the catabolism mechanisms involved in growth. In the present study we used comparative proteomic analysis to compare the metabolic capacity of an engineered isolate of L. plantarum with the parent isolate when growing in media supplemented with raffinose.

Isolates of L. plantarum are included on the list of α-Gal-producing LAB17. Genes coding for α-Gal hydrolysis have been characterized at the biochemical and molecular levels in L. plantarum18. In the isolate L. plantarum ATCC 8014, genes involved in galactose catabolism were clustered in a galactose operon14. The protein coding for α-Gal that often initiates the first degradation step in α-Gal hydrolysis of α-1,6-galactosides links in raffinose were flanked by a raffinose transporter and two subunits of the heterodimeric β-galactosidase16. Inspection of the genome of the isolate used in this study (L. plantarum P-8) revealed a cluster of genes with the same organization on the chromosome12. In addition, a copy of the cluster, except for the raffinose transporter, was found on a plasmid12. Redundant coding genes associated with α-Gal in L. plantarum P-8 seem to endow this isolate with a

| Amino acid | Content (mg/L) | L. plantarum P-8 | The engineered strain |
|------------|----------------|------------------|----------------------|
| Aspartic acid | 0.151 ± 0.011a | 0.422 ± 0.054a |
| Threonine | 0.152 ± 0.007b | 0.141 ± 0.037b |
| Serine | 0.049 ± 0.005a | 0.031 ± 0.006b |
| Glutamine | 1.061 ± 0.073a | 1.585 ± 0.225a |
| Glycine | 0.092 ± 0.004a | 0.114 ± 0.012a |
| Alanine | 7.489 ± 0.393b | 4.952 ± 0.285b |
| Cysteine | 0.492 ± 0.010a | 0.470 ± 0.006a |
| Valine | 0.395 ± 0.016a | 0.594 ± 0.020a |
| Methionine | 0.429 ± 0.040a | 0.498 ± 0.061a |
| Isoleucine | 0.110 ± 0.005a | 0.084 ± 0.010a |
| Leucine | 0.154 ± 0.014a | 0.173 ± 0.025a |
| Tyrosine | 0.979 ± 0.019a | 0.819 ± 0.336a |
| Phenylalanine | 0.291 ± 0.011 | — |
| Lysine | 0.731 ± 0.042a | 0.682 ± 0.059a |
| Histidine | 0.167 ± 0.009a | 0.133 ± 0.016a |
| Arginine | 0.301 ± 0.031a | 0.264 ± 0.024a |
| Proline | 0.246 ± 0.023a | 0.114 ± 0.009a |

Table 1. Concentration of intracellular amino acids in an engineered isolate of L. plantarum P-8 and its parent isolate after fermentation in SDM for 12 h. a,bWithin the same row followed by different superscript letters differ significantly (P < 0.05).
| Function group and ORF | Description | Fold change |
|------------------------|-------------|-------------|
| **Energy production and conversion** | | |
| LBP_cg2666 | Glutathione reductase | 1.56 |
| LBP_cg1616 | D-lactate dehydrogenase | 1.79 |
| **Cell cycle control, cell division, chromosome partitioning** | | |
| LBP_cg1932 | Bacterial cell division membrane protein FtsW | 1.86 |
| **Amino acid transport and metabolism** | | |
| LBP_cg0323 | Glycine betaine/carnitine/choline ABC transporter, substrate binding and permease protein | 1.65 |
| LBP_cg1441 | putative 5-methyltetrahydropteroyltriglutamate–homocysteine S-methyltransferase | 1.5 |
| LBP_cg1849 | Xaa-Pro dipeptidase | 1.54 |
| LBP_cg2294 | Amino acid transport protein | 1.69 |
| **Carbohydrate transport and metabolism** | | |
| LBP_cg1118 | Cell-cycle regulation histidine triad protein | 1.52 |
| LBP_cg0026 | Malto phosphorylase | 1.81 |
| LBP_cg0226 | Alpha, alpha-phosphotrehalase | 1.54 |
| LBP_cg0227 | Phosphoenolpyruvate-dependent sugar PTS family porter EIIABC, trhalose specific | 1.85 |
| LBP_cg0470 | Mannose PTS, EIIA | 1.51 |
| LBP_cg2634 | Phosphotransferase system fructose-specific component IIA | 1.59 |
| LBP_cg2912 | Sorbitol PTS, EIIA | 5.8 |
| LBP_cg2913 | Sorbitol PTS, EIIBC | 5.72 |
| LBP_cg2914 | Sorbitol PTS, EIIC | 3.81 |
| **Lipid transport and metabolism** | | |
| LBP_cg0412 | Holo-[acyl-carrier-protein] synthase (Putative) | 2.82 |
| LBP_cg0469 | Acyltransferase (Putative) | 1.54 |
| LBP_cg2725 | Phosphatidylglycerophosphatase | 2.01 |
| LBP_cg1580 | D-alanine–poly(phosphoribitol) ligase subunit 2-1 | 2.09 |
| LBP_cg2490 | Short-chain dehydrogenase/oxidoreductase | 1.95 |
| LBP_cg2917 | Sorbitol-6-phosphate 2-dehydrogenase | 10.6 |
| **Translation, ribosomal structure and biogenesis** | | |
| LBP_cg0495 | 50S ribosomal protein L33 | 3.28 |
| LBP_cg0799 | Seryl-tRNA synthetase | 1.56 |
| LBP_cg1733 | 30S ribosomal protein S20 | 1.51 |
| LBP_cg1826 | Polynucleotide nucleotidyltransferase (Putative) | 1.58 |
| **Transcription** | | |
| LBP_cg0225 | GntR family transcriptional regulator | 2.17 |
| LBP_cg0785 | Cold shock protein CspC | 1.72 |
| LBP_cg1077 | Arginine regulator | 1.69 |
| LBP_cg1588 | Heat-inducible transcription repressor hrcA | 2.06 |
| LBP_cg2038 | Hypothetical protein | 1.59 |
| LBP_cg2611 | Transcription regulator | 6.92 |
| LBP_cg1703 | Transcription regulator of fructose operon | 1.65 |
| LBP_cg2916 | Sorbitol operon transcription regulator | 1.78 |
| **Replication, recombination and repair** | | |
| LBP_cg1218 | Esx012c (Esx12c) | 1.96 |
| LBP_cg1718 | UvrABC system protein C | 1.5 |
| **Cell wall/membrane/envelope biogenesis** | | |
| LBP_cg0413 | Alanine racemase | 2.73 |
| LBP_cg0619 | Glucosamine–fructose-6-phosphate aminotransferase | 2.15 |
| LBP_cg1017 | Large-conductance mechanosensitive channel | 1.67 |
| LBP_cg1636 | Prophage Lp1 protein S8, lysin | 2.21 |
| LBP_cg1859 | Glutamate racemase | 1.9 |
| LBP_cg2578 | Cyclopropane-fatty-acyl phospholipid synthase | 1.64 |
| LBP_cg2781 | Extracellular protein, gamma-D-glutamate-meso-diaminopimelate murenopeptidase (Putative) | 2.83 |
| **Posttranslational modification, protein turnover, chaperones** | | |
| Continued | | |
| Function group and ORF | Description | Fold change |
|------------------------|-------------|-------------|
| **Small heat shock protein** | LBP_cg0109 | 3 |
| | LBP_cg0196 | 2.04 |
| | LBP_cg0537 | 2.24 |
| | LBP_cg0538 | 1.81 |
| **Glutathione peroxidase** | LBP_cg1585 | 1.52 |
| **10 kDa chaperonin** | LBP_cg1586 | 2.11 |
| **60 kDa chaperonin** | LBP_cg1587 | 1.73 |
| **Small heat shock protein** | LBP_cg2734 | 1.87 |
| **ATP-dependent Clp protease, ATP-binding subunit ClpE** | LBP_cg2905 | 1.85 |
| **Thioredoxin H-type** | LBP_cg2160 | 1.68 |
| **Phosphate ABC transporter, substrate binding protein** | LBP_cg0542 | 1.94 |
| **Metal uptake regulator** | LBP_cg0659 | 1.75 |
| **Ferric uptake regulator** | LBP_cg2648 | 2.09 |
| **Catalase** | LBP_cg2900 | 1.71 |
| **Stress induced DNA binding protein** | LBP_p6g011 | 1.92 |
| **Beta-phosphoglucomutase** | LBP_cg0025 | 1.54 |
| **Oxidoreductase** | LBP_cg0197 | 1.51 |
| **Cyanide hydratase** | LBP_cg0344 | 2.18 |
| **Prophage Lp3 protein 8, helicase** | LBP_cg2033 | 2.22 |
| **NADP oxidoreductase coenzyme F420-dependent** | LBP_cg2366 | 1.63 |
| **Putative universal stress protein** | LBP_cg1346 | 1.5 |
| **PemI-like protein** | LBP_p7g009 | 1.66 |
| **ABC transporter, ATP-binding protein** | LBP_cg2242 | 1.77 |
| **ABC transporter, permease protein** | LBP_cg2243 | 1.71 |
| **ABC transporter, permease protein (Putative)** | LBP_cg2310 | 1.53 |
| **ABC transporter, ATP-binding protein** | LBP_cg2311 | 1.83 |
| **Maltose/maltodextrin ABC transporter subunit (Putative)** | LBP_cg0151 | 1.65 |
| **Alkaline shock protein** | LBP_cg0721 | 2.43 |
| **Alkaline shock protein** | LBP_cg0722 | 2.92 |
| **Hypothetical protein** | LBP_cg1404 | 1.64 |
| **Hypothetical protein** | LBP_cg1866 | 1.54 |
| **Hypothetical protein** | LBP_cg1913 | 1.64 |
| **Integral membrane protein** | LBP_cg2375 | 2.6 |
| **Integral membrane protein** | LBP_cg2385 | 1.65 |
| **Hypothetical protein** | LBP_cg0392 | 1.79 |
| **Extracellular zinc metalloproteinase** | LBP_cg0481 | 6.94 |
| **Cell surface protein** | LBP_cg0600 | 2.45 |
| **Hypothetical protein** | LBP_cg0625 | 1.53 |
| **Hypothetical protein** | LBP_cg0719 | 1.92 |
| **Hypothetical protein** | LBP_cg0720 | 3.17 |
| **Hypothetical protein** | LBP_cg0889 | 2.66 |
| **Extracellular protein, membrane-anchored (Putative)** | LBP_cg1033 | 2.44 |
| **Hypothetical protein** | LBP_cg1196 | 10.39 |
| **Cell surface protein** | LBP_cg1257 | 1.85 |
| **Hypothetical protein** | LBP_cg1314 | 4.2 |
| **Lysin** | LBP_cg1367 | 1.69 |
| **Prophage Lp2 protein 54** | LBP_cg1637 | 1.77 |
| **Prophage Lp2 protein 53** | LBP_cg1638 | 3.22 |
| **Hypothetical protein** | LBP_cg1639 | 1.51 |
| **Hypothetical protein** | LBP_cg1640 | 2.61 |
good performance in the presence of the soy-derived products\textsuperscript{14}. In the present study, some of these proteins were down-regulated in the engineered isolate, consistent with the fact that most of the raffinose was depleted from the medium by the late stage of its growth. In contrast the parent isolate, which had a slower growth rate still required raffinose as the sole carbohydrate source to support its growth at the same stage.

Sorbitol, also referred to as D-glucitol, is unlikely to have been present in the medium used in our study, but could be produced at a low level as the by-product of \textit{L. plantarum} fermentation\textsuperscript{19}. Within the sorbitol-related protein set, sorbitol-6-phosphate 2-dehydrogenase (\textit{SrlD}) that usually catalyzes the conversion of sorbitol-6-phosphate to fructose-6-phosphate\textsuperscript{20}, was the most highly expressed. Similarly, Laakso \textit{et al.}\textsuperscript{21} found that the expression of \textit{SrlD} and glucitol/sorbitol-PTS increased over time in \textit{L. rhamnosus} GG during growth in industrial-type whey medium, especially when the culture shifted from the exponential growth phase to the stationary phase. They therefore proposed that \textit{L. rhamnosus} GG began to use alternative energy sources, namely sorbitol, at the beginning of the stationary phase. This also seems to be a reasonable interpretation of the up-regulation of proteins for sorbitol utilization observed in our study, because the engineered isolate was entering the stationary phase at the time of sampling (Fig. 1).

Acid stress in LAB often invokes a variety of protection mechanisms\textsuperscript{22}. Amongst them, the structure of cell layers is considered to be a significant factor in sensing the acidic environment\textsuperscript{23}. Alteration of the cell layer by changing the saturated and cyclopropane fatty acids (FA) of the membrane in response to acidification has been observed in \textit{L. casei} ATCC 334\textsuperscript{24}. The authors suggested that increasing the rigidity and compactness of the cytoplasmic membrane decreased the permeability for protons\textsuperscript{24}. For the engineered isolate we showed that the alteration of the cell layer occurred through a combination of \textit{de novo} FA biosynthesis and modification of existing lipid membrane phospholipid acyl chains. In addition to the proteins related to cell wall metabolism, a cyclopropane-fatty-acyl-phospholipid synthase was up-regulated, as well as a protein that catalyzes the reactions for modifying the lipid membrane. Interestingly, glutamate racemase, which is involved in constructing cell walls\textsuperscript{25}, was also induced, suggesting high levels of intracellular glutamate. Consistently, higher intracellular glutamate levels were found in the medium in which the engineered isolate had been growing compared with the parent isolate.

| Function group and ORF | Description | Fold change |
|------------------------|-------------|-------------|
| LBP_cg1641             | Tail fiber  | 4.79        |
| LBP_cg1642             | Hypothetical protein | 2.02        |
| LBP_cg1643             | Hypothetical protein | 1.52        |
| LBP_cg1646             | Hypothetical protein | 2.08        |
| LBP_cg1647             | Phage major tail protein | 2.12        |
| LBP_cg1651             | Phage protein DNA packaging protein | 2.25        |
| LBP_cg1661             | tRNAs family phage transcriptional regulator | 2.15        |
| LBP_cg1663             | Hypothetical protein | 3           |
| LBP_cg1664             | Hypothetical protein | 4.27        |
| LBP_cg1673             | Hypothetical protein | 2.16        |
| LBP_cg1674             | Hel protein | 1.62        |
| LBP_cg1675             | Hypothetical protein | 2.72        |
| LBP_cg1677             | Hypothetical protein | 2.02        |
| LBP_cg1682             | Hypothetical protein | 3.01        |
| LBP_cg1721             | Hypothetical protein | 7.13        |
| LBP_cg1851             | Hypothetical protein | 1.55        |
| LBP_cg1933             | Hypothetical protein | 1.54        |
| LBP_cg1962             | Hypothetical protein | 1.6         |
| LBP_cg1970             | Hypothetical protein | 1.82        |
| LBP_cg2009             | Hypothetical protein | 1.6         |
| LBP_cg2032             | Prophage Lp4 protein 12 | 1.64        |
| LBP_cg2034             | Prophage Lp3 protein 7 | 1.56        |
| LBP_cg2185             | Hypothetical protein | 8.34        |
| LBP_cg2289             | Cell surface protein | 2.84        |
| LBP_cg2450             | Hypothetical protein | 15.41       |
| LBP_cg2519             | Extracellular protein (Putative) | 3.74        |
| LBP_cg2530             | Muramidase (Putative) | 1.84        |
| LBP_cg2736             | Hypothetical protein | 30.12       |
| LBP_cg2777             | Transcription regulator | 1.5         |
| LBP_cg2779             | Hypothetical protein | 3.75        |
| LBP_cg2898             | Hypothetical protein | 2.43        |

Table 2. Up-regulated proteins in the engineered isolate of \textit{L. plantarum} P-8 compared with its parent isolate.
Another interesting finding was the regulation of aspartate metabolism in the engineered isolate. Two proteins involved in aspartate metabolism were significantly depressed. According to Wu et al., the twist of aspartate flux may help L. casei to perform better under acid stress. Similarly, we observed that the engineered isolate had a greater capacity to manipulate aspartate metabolism by enriching higher amounts of the intracellular aspartate.

In this study, comparative proteomic analysis was used to compare the metabolic capacity of an engineered isolate of L. plantarum with its parent isolate. During the late stages of growth, the engineered isolate used alternative carbohydrate such as sorbitol instead of raffinose to sustain its cell division. To avoid acid damage the engineered isolate altered the cell layer through a combination of de novo FA biosynthesis and modification of existing lipid membrane phospholipid acyl chains. Interestingly, aspartate and glutamate metabolism was associated with this acid response. Our study contributes to underpinning the future use of these isolates in manufacturing soy-milk products.

Methods
Bacterial isolates and culture conditions. An engineered isolate of L. plantarum P-8 with enhanced raffinose metabolic capacity and its original parent isolate were cultured in SDM supplemented with 1.0% (w/w) raffinose. The engineered isolate was obtained from a laboratory evolution experiment that lasted for 150 days. During the experimental process, L. plantarum P-8 was continuously subcultured in de Man-Rogosa-Sharpe broth for lactobacilli with 0.2 g/L glucose (unpublished data). The composition of the SDM was as described by Kimmel et al. A growth curve was constructed in relation to optical density (OD), pH and the number of viable counts determined after 0, 2, 4, 6, 8, 10, 12 14, 16, 18, 20, 22, 24, 26, 28, 30 and 32 h of fermentation. All analyses were performed in triplicate.

Sample preparation. To ensure the reliability of the proteomic analysis, samples were obtained from 4 biological replicates after 12 h cultivation. Cells of the two isolates were harvested by centrifugation and washed with phosphate buffered saline (PBS) four times. One milliliter of lysis buffer (7 M urea, 4% sodium dodecyl sulfate, 30 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, 1 mM phenylmethylsulfonyl fluoride, 2 mM ethylenediamine tetraacetic acid, 10 mM DL-dithiothreitol, 1× protease inhibitor cocktail) was added to each sample, followed by sonication on ice and centrifugation at 13,000 rpm for 10 min at 4°C. The supernatants from each sample were transferred to fresh tubes.

Protein digestion and isobaric tags for relative or absolute quantitation (iTRAQ) labeling. We determined the protein concentration of the supernatants using the bicinchoninic acid protein assay, and then transferred 100 µg protein per condition into new tubes and adjusted each to a final volume of 10 µL with 100 mM triethylammonium bicarbonate (TEAB). To this 5 µL of 200 mM DL-dithiothreitol were added and incubated at 55°C for 1 h, then 5 µL of the 375 mM iodoacetamide was added to the sample and incubated for 30 min protected from light at room temperature.
| Function group and ORF | Description | Fold change |
|------------------------|-------------|-------------|
| **Energy production and conversion** | | |
| LBP_p2g050 | Pyridine nucleotide-disulfide oxidoreductase family protein | −10.15 |
| LBP_cg2288 | Flavodoxin | −4.62 |
| LBP_cg2434 | Nitroreductase | −3.34 |
| LBP_cg0327 | Glycerol-3-phosphate dehydrogenase | −3.15 |
| LBP_cg0326 | Glycerol kinase 1 | −2.67 |
| LBP_cg2703 | Formate C-acetyltransferase | −2.19 |
| LBP_cg0631 | Glycerol kinase 2 | −2.09 |
| LBP_cg2927 | Bifunctional acetaldehyde-CoA/alcohol dehydrogenase | −2.03 |
| LBP_cg0052 | Nitroreductase | −1.93 |
| LBP_cg0871 | L-lactate dehydrogenase 2 | −1.71 |
| LBP_cg0992 | Oxidoreductase | −1.76 |
| **Cell cycle control, cell division, chromosome partitioning** | | |
| LBP_p1g033 | Copy number control protein | −18.2 |
| **Amino acid transport and metabolism** | | |
| LBP_cg0219 | Cystathionine beta-lyase | −36.76 |
| LBP_p3g040 | Aspartate aminotransferase | −18.9 |
| LBP_cg0220 | Cysteine synthase | −6.96 |
| LBP_cg0585 | Lipoprotein, peptide binding protein OppA-like protein | −4.87 |
| LBP_cg0963 | Oligopeptide ABC superfamily ATP binding cassette transporter, substrate binding protein | −3.86 |
| LBP_cg0017 | Lipoprotein, peptide binding protein OppA-like protein | −3.55 |
| LBP_p7g004 | ABC-type polar amino acid transport system, ATPase component | −2.78 |
| LBP_cg0602 | Glutamine ABC transporter, ATP-binding protein | −2.45 |
| LBP_cg0601 | Glutamine ABC transporter, substrate binding and permease protein | −2.32 |
| LBP_cg2273 | Putative D-serine dehydratase | −1.96 |
| LBP_cg2253 | Bifunctional cystathionine gamma-lyase/maltose regulon repressor | −1.55 |
| LBP_cg0653 | Amino acid transport protein (Putative) | −1.5 |
| LBP_cg2156 | Pyruvate oxidase | −3.17 |
| LBP_cg911 | Pyruvate oxidase | −1.85 |
| LBP_cg0966 | Oligopeptide ABC transporter, ATP-binding protein | −2.01 |
| LBP_cg2875 | L-iditol 2-dehydrogenase | −1.75 |
| LBP_p7g005 | ABC-type amino acid transport/signal transduction system, periplasmic component/domain protein | −3.67 |
| LBP_cg0650 | X-prolyl-dipeptidyl aminopeptidase | −1.59 |
| **Nucleotide transport and metabolism** | | |
| LBP_cg2224 | Phosphoribosylamine-glycine ligase | −2.34 |
| LBP_cg0658 | Guanylate kinase | −1.59 |
| **Carbohydrate transport and metabolism** | | |
| LBP_p1g007 | Cupin 2 conserved barrel domain protein | −20.36 |
| LBP_p2g004 | Beta-galactosidase | −19.36 |
| LBP_p2g010 | GPH family glycoside-pentoside-hexuronide:cation symporter | −18.77 |
| LBP_p2g005 | Beta-galactosidase large subunit | −15.86 |
| LBP_cg2869 | Putative transaldolase | −2.8 |
| LBP_cg2868 | Transketolase | −2.2 |
| LBP_cg1489 | Pyruvate,water dikinase | −2.07 |
| LBP_cg1967 | Prophage Lp2 protein 59; xylanase/chitin deacetylase (Putative) | −1.98 |
| LBP_cg1383 | Phosphoglycerate mutase (Putative) | −1.87 |
| LBP_cg0206 | Protein-N(P)-phosphohistidine–sugar phosphotransferase | −1.81 |
| LBP_cg2857 | Beta-glucosides PTS, EIIBC | −1.73 |
| LBP_cg2862 | Alpha-glucosidase | −1.66 |
| LBP_cg0954 | 6-phosphogluconate dehydrogenase, decarboxylating | −1.62 |
| LBP_cg0148 | Maltose/maltodextrin ABC transporter, substrate binding protein | −1.6 |
| LBP_cg2856 | 6-phospho-beta-glucosidase | −1.58 |
| LBP_cg2832 | Alpha-galactosidase | −1.57 |
| LBP_cg0146 | Alpha-glucosidase | −1.56 |
| LBP_cg2877 | Galactitol PTS, ElIB | −1.55 |

Continued
| Function group and ORF | Description | Fold change |
|-----------------------|-------------|-------------|
| LBP_cg0767            | Alpha-ribazole-5′-phosphate phosphatase (Putative) | -1.51 |
| LBP_cg2878            | Galactol PTS, EIIA | -1.59 |
| Coenzyme transport and metabolism |          |            |
| LBP_cg2237            | Lipote-protein ligase | -2.32 |
| LBP_cg1190            | 5-formyltetrahydrofolate cyclo-ligase | -1.97 |
| LBP_cg0655            | Pyridoxal kinase | -1.81 |
| LBP_cg1219            | Geranyltranstranferase | -1.59 |
| Lipid transport and metabolism |          |            |
| LBP_cg1286            | (3R)-hydroxymyristoyl-(Acyl carrier protein) dehydratase | -2.05 |
| LBP_cg2422            | Putative acyltransferase | -1.64 |
| Transcription         |          |            |
| LBP_cg2245            | Transcription regulator | -1.71 |
| LBP_cg2858            | Transcription antiterminator | -1.66 |
| LBP_cg2353            | Transcription regulator | -1.58 |
| LBP_cg2921            | Ribose operon repressor | -1.51 |
| LBP_cg0477            | Transcription regulator | -1.51 |
| LBP_cg2612            | Putative aromatic-amino-acid transaminase | -6.1 |
| LBP_cg1416            | Transcription repressor | -2.06 |
| Replication, recombination and repair |          |            |
| LBP_p1g016            | Resolvase | -3.34 |
| LBP_cg2331            | Methylated-DNA-(Protein)-cyesteine S-methyltransferase | -2.41 |
| Cell wall/membrane/envelope biogenesis |          |            |
| LBP_cg1351            | Penicillin binding protein 1A | -3.18 |
| LBP_cg1937            | UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1 | -2.05 |
| LBP_cg1793            | Penicillin binding protein 2B | -1.78 |
| Posttranslational modification, protein turnover, chaperones |          |            |
| LBP_p2g013            | Cell envelope-associated proteinase, lactocepin PrtR | -5.22 |
| LBP_cg2704            | Formate acetyltransferase activating enzyme | -2.23 |
| LBP_cg1561            | Protein-methionine-S-oxide reductase | -1.78 |
| LBP_cg0698            | Hypothetical protein | -1.67 |
| LBP_cg1426            | Peptide methionine sulfoxide reductase msrB | -1.52 |
| LBP_p1g026            | Multicopper oxidase | -1.61 |
| General function prediction only |          |            |
| LBP_p3g041            | AAE family aspartate:alanine exchanger | -12.55 |
| LBP_cg2520            | HAD superfamily hydrolase | -2.77 |
| LBP_cg383             | ABC superfamily ATP binding cassette transporter, ABC protein | -2.36 |
| LBP_cg1029            | HAD superfamily hydrolase | -2.28 |
| LBP_cg2267            | Acetyltransferase | -1.69 |
| LBP_cg1295            | GTPase | -1.67 |
| LBP_cg2080            | ABC superfamily ATP binding cassette transporter, ABC protein | -1.63 |
| LBP_cg0694            | 2-nitropropane dioxygenase | -1.62 |
| LBP_cg2803            | NADH oxidase | -1.52 |
| Signal transduction mechanisms |          |            |
| LBP_p1g017            | Putative universal stress protein | -5.94 |
| Defense mechanisms    |          |            |
| LBP_cg1584            | Serine-type D-Ala-D-Ala carboxypeptidase | -1.57 |
| Function unknown      |          |            |
| LBP_p2g025            | Hypothetical protein | -7.13 |
| LBP_cg0505            | Lysyl-tRNA synthetase (Class II) | -2.2 |
| LBP_cg1165            | Lipoprotein | -2.13 |
| LBP_cg0676            | Hypothetical protein | -1.83 |
| LBP_p3g034            | Hypothetical protein | -10.71 |
| LBP_p3g025            | Hypothetical protein | -6.26 |
| LBP_p2g017            | Nisin resistance protein | -6.01 |
| LBP_cg1057            | Hypothetical protein | -3.6 |
| LBP_p3g035            | LtrC-like protein | -3.59 |
| Continued             |          |            |
Table 3. Down-regulated proteins in the engineered isolate of \textit{L. plantarum} P-8 compared with its parent isolate.

| Function group and ORF | Description                  | Fold change |
|------------------------|------------------------------|-------------|
| LBP_cg2528             | NmrA family protein          | −3.24       |
| LBP_cg0079             | Hypothetical protein         | −2.4        |
| LBP_cg1398             | Hypothetical protein         | −2.18       |
| LBP_cg1623             | Hypothetical protein         | −2.1        |
| LBP_cg1376             | Por-forming protein          | −2.09       |
| LBP_p3g036             | Hypothetical protein         | −1.83       |
| LBP_cg2330             | Extracellular protein        | −1.76       |
| LBP_cg0725             | Lipoprotein                  | −1.73       |
| LBP_cg2475             | Extracellular protein        | −1.6        |
| LBP_cg1507             | Hypothetical protein         | −1.56       |

For each sample, proteins were precipitated with ice-cold acetone, and then re-dissolved in 20μL TEAB. Proteins were then digested with sequence-grade modified trypsin (Promega, Madison, WI), and the resultant peptide mixture was labeled using chemicals from the iTRAQ reagents kit (Applied Biosystems, Foster City, CA). The labeled samples were combined, desalted using a C18 SPE column (Sep-Pak C18, Waters, Milford, MA) and dried under vacuum.

**High pH reverse phase separation.** Phase separation was performed as described by Gilar\(^2\) with some modifications. The peptide mixture was dissolved in buffer A (buffer A: 10 mM ammonium formate in water, pH10.0, adjusted with ammonium hydroxide), and then fractionated by high pH separation using an Aquity UPLC system (Waters Corporation, Milford, MA) connected to a reverse phase column (BEH C18 column, 2.1 mm × 150 mm, 1.7 μm, 300 Å, Waters Corporation, Milford, MA). High pH separation was done using a linear gradient. Starting from 0% B to 45% B in 35 min (B: 10 mM ammonium formate in 90% acetonitrile, pH 10.0, adjusted with ammonium hydroxide). The column flow rate was maintained at 250 μL/min and column temperature was maintained at 45°C. Sixteen fractions were collected and each fraction was dried in a vacuum concentrator prior to the next step.

**Low pH nanoscale high-performance liquid chromatography coupled to tandem mass spectrometry (nano-HPLC-MS/MS) analysis.** The fractions were re-suspended in a mixture of solvent C and solvent D (C: water with 0.1% formic acid; D: acetonitrile with 0.1% formic acid), separated by nano LC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on a Nano Acuity UPLC system (Waters Corporation, Milford, MA) connected to a quadrupole-orbitrap mass spectrometer (Q-Exactive) (Thermo Fisher Scientific, Bremen, Germany) equipped with an online nano-electrospray ion source. 8μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100μm × 2 cm), with a flow of 10 μl/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75μm × 25 cm) with a linear gradient, from 5% D to 30% D in 95 min. The column was re-equilibrated at initial conditions for 15 min. The column flow rate was maintained at 300 nL/min and column temperature was maintained at 45°C. An electrospray voltage of 2.0 kV was used against the inlet of the mass spectrometer.

The Q-Exactive mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350-1600) were acquired with a mass resolution of 70 K, followed by fifteen sequential high-energy-collisional-dissociation (HCD) MS/MS scans with a resolution of 17.5 K. In all cases, one micro-scan was recorded using dynamic exclusion of 30 seconds. MS/MS fixed first mass was set at 100.

**Database searching.** Tandem mass spectra were extracted by Proteome Discoverer software (Thermo Fisher Scientific, version 1.4.0.288). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3). Mascot was set up to search the NCBI database (Taxonomy: \textit{Lactobacillus plantarum} P-8, 3179 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethylation of cysteine and iTRAQ 8plex of lysine and the n-terminus were specified in Mascot as fixed modifications. Oxidation of methionine and iTRAQ 8plex of tyrosine were specified in Mascot as a variable modification.

**Quantitative data analysis.** We used the percolator algorithm lower than 1% to control peptide level false discovery rates (FDR). Only unique peptides were used for protein quantification and the method of normalization on protein median was used to correct experimental bias. The minimum number of proteins that must be observed was set to 1000. Statistical analysis was realized in the software package R; using students’ t tests, \( p < 0.05 \) was considered statistically significant. A 1.5-fold change was used as the threshold for selection of regulated proteins. All regulated proteins were distributed over COGs and were subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database\(^9\).
Measurement of organic acids. The content of lactate and acetate was determined by HPLC using the methods of Wang et al.26. Firstly, 1 mol/L HCl was used to denature protein at a volume of four times that of the samples. Then the samples were subjected to high speed centrifugation at 4, 200 rpm for 10 min. The supernatants were used for analysis after filter sterilization through a 0.45 μm. The mobile phase consisted of a phosphate buffered solution and methanol (97:3, v/v), with a flow rate of 0.5 mL/min. The UV detector was set at 210 nm and the ZORBAX SB-Aq column (5 μm, 4.6 × 150 mm, Agilent, USA) was operated at 35 °C.

Quantification of intracellular amino acids. Extraction of intracellular amino acids was achieved as described by Wu et al.26. The amino acids were quantified using a Hitachi L-8900 fully automatic amino acid analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan), which used ion-exchange chromatography to separate amino acids31.

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Author Contributions
W.Z. and H.Z. designed the study, and wrote the manuscript. J.W. and W.H. designed and performed experiments. C.C., R.J. and C.R. analyzed data. All authors reviewed the manuscript.
Additional Information

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