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IDENTIFICATION OF BACTERIAL PROTEINS INVOLVED IN INULIN METABOLISM FROM COLON-DERIVED *LACTOBACILLUS CASEI* STRAINS

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ABSTRACT
The *Lactobacillus casei* strains AP and AG are lactic acid bacteria originating in the gastrointestinal tract. The strains were grown in inulin as the only carbon source to identify inulin-degrading proteins and their metabolism. To examine their inulin metabolism, sugar analysis was performed using high performance liquid chromatography (HPLC) for intracellular- and supernatant-derived samples. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify inulin-degrading proteins; this was followed by electrospray ionisation - liquid chromatography mass spectrometry (ESI-LC-MS/MS) analysis of specific protein bands. The cell wall protein profiles of bacterial cells grown in inulin showed different band sizes at 30 and 50 kDa from those grown in glucose. The 30-kDa proteins belonged to the phosphotransferase system mannose/fructose/sorbose-specific IID component, ATP-binding cassette transporter and ATP-binding cassette transporter substrate-binding protein. These proteins may be involved in the intracellular inulin transport in the *L. casei* strains AP and AG.

Keywords: inulin, inulin metabolism, *Lactobacillus casei*, ESI-LC-MS/MS

INTRODUCTION
Prebiotics are non-digestible components on which digestive enzymes, such as α-glucosidase, maltase, isomaltase and sucrose, have no effect, and which selectively stimulate the growth and/or activity of colonic microflora and provide health benefits. Inulin is a carbohydrate complex consisting of glucose and fructose with a degree of polymerisation (DP) between 10 and 65 (Kaur and Gupta, 2002). Inulin is categorised as a prebiotic because it is beneficial for maintaining the gastrointestinal tract. For example, inulin can increase calcium absorption, reduce triglycerides in individuals with hyperlipidaemia and imp-rove gut function (Roberfroid, 2005). Consumption of inulin suppresses the growth of pathogens such as *Clostridium perfringens*, *Escherichia coli*, *Campylobacter jejuni*, *Enterobacter* spp., *Salmonella enteri-tidis* and *S. typhimurium* (Florowska et al., 2016).

The colonic microflora comprises bacteria that play a role in maintaining the gastrointestinal tract, including lactobacilli and bifidobacteria, which combat pathogenic bacteria by increasing the concentration of short-chain fatty acids, thereby altering colonic pH (Florowska et al., 2016). Lactobacilli and bifidobacteria are probiotic, which means that they can degrade inulin by releasing specific enzymes that hydrolyse β-(2→1) fructosyl–fructose linkages, producing oligofructose with a low DP. *Lactobacillus paracasei* 1195 (Kaplan and Hutkins, 2003) and *L. acidophilus* NCFM (Barrangou et al., 2003) intracellularly metabolise fructo-oligosaccharides (FOS) through the ATP-binding cassette (ABC) transport system and β-fructofuranosidase. FOS metabolism by *L. paracasei* 1195 also involves the fructose/mannose phosphotransferase system (PTS) and β-fructosidase (Goh et al., 2006). Moreover, *L. plantarum* ST-III metabolises FOS through PTS1 and PTS 26 (Chen et al., 2015). *L. delbrueckii* also degrades inulin intracellularly (Tsujikawa et al., 2013). By contrast, some lactic acid bacteria degrade inulin extracellularly, such as *L. paracasei* JCM 8130T, DSM 20020 (Gänzle and Follandor, 2012), DSM 23505 (Petrova et al., 2015) and *L. casei* IAM 1045 (Kuzuwa et al., 2012).

L. casei strains AP and AG are two strains of lactic acid bacteria isolated from the faeces of Indonesian infants who were fed breast milk (Widodo et al., 2012). Previously, both strains showed potential as probiotics based on their growth in a medium with inulin as the sole carbon source (Widodo et al., 2014), confirming that they can degrade inulin through metabolic pathways that involve certain proteins. However, detailed information on how inulin is metabolised by these strains is limited. Therefore, we attempted to identify the proteins used by the *L. casei* AP and AG in degrading inulin to elucidate their role in inulin metabolism.

MATERIALS AND METHODS
Chemicals: Commercial inulin (Sigma-Aldrich) was used as a substrate. The media used in this study were as follows: (1) MRS broth purchased from

Merck and used as a complex growth medium; (2) modified MRS (mMRS) medium containing 1% (w/v) glucose, referred to as glucose mMRS and (3) mMRS medium containing 1% (w/v) inulin, referred to as inulin mMRS. All other chemicals were of analytical grade.

Bacterial strains and culture conditions: The *L. casei* AP and AG were used in this study. The bacterial cells were initially cultured in MRS broth (Merck) at 37°C for 24 h under micro-aerobic conditions. After 24 h incubation, the culture was centrifuged (Eppendorf 5804 R) at 3000 rpm for 20 min. The pellet was washed with 0.85% NaCl solution and was inoculated in the mMRS medium. For assessing bacterial growth and small-scale fermentation, the cells were grown in mMRS supplemented with 0.05 g/L L-cysteine HCl and different carbon sources, each at a final concentration of 10 g/L.

Growth of bacteria in inulin mMRS: The *L. casei* AP and AG were grown in MRS broth for 24 h at 37°C, followed by centrifugation at 3000 rpm at 4°C for 20 min to harvest the bacterial cells. The biomass was then washed with 0.85% (w/v) NaCl solution and was separately inoculated in inulin mMRS, foll-owed by incubation for 24 h at 37°C. The optical density (OD) was measured at 620 nm for each medium (Taleghani et al., 2014).

Sugar determination using high performance liquid chromatography (HPLC): Analysis of sugars was performed using HPLC to determine the inulin and simple sugar content as a product of inulin hydrolysis for elucidating the inulin degradation pattern. The *L. casei* AP and AG were incubated with inulin mMRS and were harvested after 6 h of incubation (exponential phase) and 20 h (stationary phase) of incubation. A sample from both incubation periods was centrifuged (Eppendorf 5804 R) at 3000 rpm for 20 min to separate the pellet from the supernatant. The supernatant was heated in a water bath for 5 min, followed by centrifugation at 13000 rpm for 15 min. The supernatant was then stored at −20°C as the supernatant sample. The pellet was washed twice with 1 mL PBS, followed by centrifugation at 3000rpm at 4°C for 15min. Then, the pellet was sonicated for 4 min, followed by centrifugation at 3000 rpm at 4°C for 20 min and extraction with chloroform and water (1 : 1). The water extract was stored at −20°C as the intracellular sample. Each sample was filtered with a 0.22-µm filter (Millipore) and diluted before injecting into the HPLC column. Inulin mMRS without the bacteria was used as a control. Sugar analysis was carried out using HPLC Instrument (Knauer) with Smartline Pump 1050 (Knauer), MetaCarb 87C column 300 x 7.8 mm (Varian) and Refractive Index Detector (Knauer). Standard and sample solution (20 µL) was injected into the column which is maintained at 85°C in column oven (Shimadzu). The analysis was performed by isocratic elution for 20 min at flow rate of 0.5 mL min^{-1} using purified water (Aqua Pro Injection) (PT. Kapharmindo Putramas) as the mobile phase.

For calibration standard, ten milligrams of each sugar (Inulin, glucose, fructose, and sucrose) was weighed and dissolved in purified water (Aqua Pro Injection) to produce the stock solution with concentration 10000 ppm. The stock solution was diluted to provide 250, 500, 1000, 1500 and 2000 ppm working solution for calibration standard curve. The working solutions were filtered through 0.22 um filter (Millipore) and placed in 2 mL vial screw tube (Axygen). The calibration of each sugar resulted in the equation which correlates the sugar concentration with peak area. The calibration equation from inulin, sucrose, fructose and glucose were presented in equation 1, 2, 3 and 4 respectively.

$$y = 71286x - 685863 \quad (1)$$
$$y = 25114{,}5x - 341024 \quad (2)$$
$$y = 78824x - 72756 \quad (3)$$
$$y = 69213x - 2.6E + 006 \quad (4)$$

These equations were used to calculate sugar concentration of samples (x) with peak area data (y) from each sample which, for example, is shaded in Figure 1.

Protein extraction and SDS-PAGE analysis: Three types of proteins were selected: cell wall, intracellular and extracellular. The pellet was used to isolate cell wall and intracellular proteins, whereas the supernatant was used to extract extracellular proteins. The proteins were then separated using SDS-PAGE to identify those belonging to the mMRS inulin bacteria to compare them with the bacteria grown in glucose.

After 24 h of incubation in inulin or glucose, mMRS bacterial cells were centrifuged at 3000 rpm at 4°C for 20 min to separate the pellet and supernatant. Cell wall proteins were extracted following a previously described method (Gatti et al., 1997). The bacterial pellet was extracted using 0.01 M Tris-HCl, 0.01 M EDTA, 0.01 M NaCl, 2% (w/v) SDS at pH 8 at 100°C for 5 min. The suspension was centrifuged (Sigma Sartorius 3K30) at 13000 rpm for 10 min. The supernatant was stored at −20°C until further analysis. The pellet was dissolved in 0.5 mL PBS, and 0.2 g glass beads were added. The solution was then mixed vigorously by vortexing for 7 min,

followed by centrifuging at 12000 rpm for 5 min. The resulting supernatant was referred to as the intracellular protein. The remaining pellet was dried and suspended in 50mM Tris-HCl at pH 7.3. The resulting solution was referred to as cell wall protein.

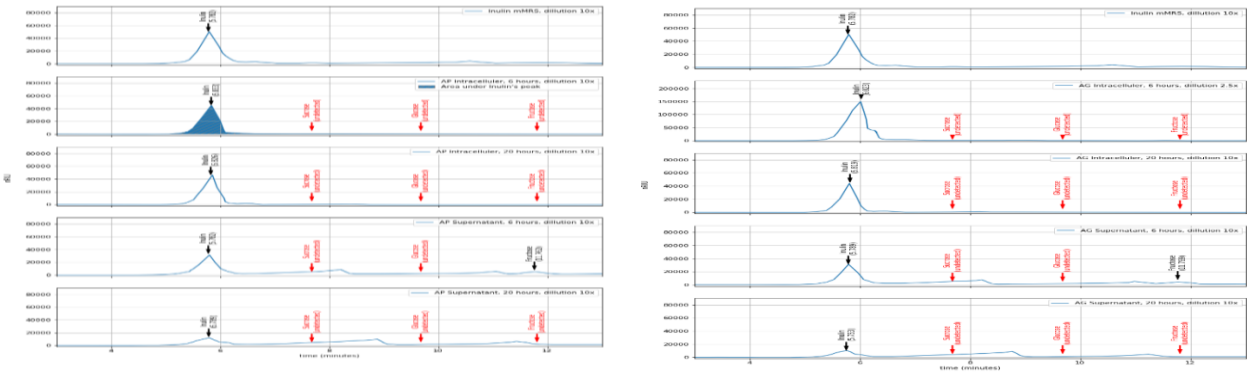

Figure 1: HPLC chromatogram of inulin mMRS medium, sample of *L. casei* AP and AG. Analysis was performed by MetaCarb 87C column 300 x 7.8 mm (Varian) with Refractive Index Detector (Knauer), flow rate of 0.5 mL min^{-1} for 20 min and purified water (aqua pro injection) as mobile phase.

Extracellular proteins were extracted using 7 mL of 16% trichloroacetic acid. The solution was shaken and incubated overnight at 4°C. The solution was then centrifuged at 16000 rpm for 20 min at 4°C in a high-speed centrifuge (Sigma Sartorius 3K30). The pellet was washed with absolute ethanol and centrifuged at 16000 rpm for 20 min at 4°C. The pellet was dried and suspended in 50 mM Tris-HCl at pH 7.3. Then, 1 mL cold acetone was added to the solution, and it was incubated overnight. The next day, solution was centrifuged at 13000 rpm for 20 min at room temperature. The pellet was dried and suspended in 50 mM Tris-HCl at pH 7.3.

SDS-PAGE analysis: The concentration of cell wall, intracellular and extracellular proteins was measured using the Bradford method with a microplate reader (Bio-Rad) at 595nm. Protein samples of equal concentration were mixed with a loading buffer containing 25% (w/v) 4× Tris-HCl at pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.001% (w/v) bromophenol blue and were boiled for 5 min before loading on to polyacrylamide gels. Electrophoresis was performed in a Mini Protean II cell unit (Bio-Rad) at 100 V. SDS-PAGE was performed using a 5% stacking gel and 12% resolving gel. The gel was stained by the silver staining method. The protein profile of inulin mMRS was compared with that of glucose mMRS.

Protein identification by electrospray ionisation (ESI)-LC-mass spectrometry (MS)/MS: Protein samples from a specific band in the SDS-PAGE analysis were treated with trypsin. Peptides were extracted by a previously described method (Bringans et al., 2008). Peptides were analysed by ESI MS using a Shimadzu Prominence nano HPLC system (Shimadzu) connected to a 5600 Triple TOF mass spectrometer (Sciex). They were then injected into an Agilent Zorbax 300SB-C18 3.5µm column (Agilent Technologies) and were separated by linear gradi-ent from water/acetonitrile/0.1% (v/v) formic acid. Mass spectra were analyzed to identify proteins using the Mascot sequence matching software (Matrix Science) with the MSPnr100 database. Mass spectrometry proteomics data have been deposited in the Proteom exchange Consortium via the PRIDE partner repository (Vizcaíno et al., 2016) with the dataset identifiers PXD006082 and 10.6019/PXD00 6082.

RESULTS AND DISCUSSION

Growth of the bacteria in inulin-containing media: In the present study, we grew bacterial cells in MRS broth for 24 h. After being harvested, the bacterial culture was transferred to inulin and glucose mMRS. The *L. casei* AP and AG were able to degrade inulin as the sole carbon source based on their growth curves shown in Figure 2. During small-scale fermentation (30 mL), the growth patterns of the *L. casei* AP and AG grown in inulin were similar to those grown in glucose mMRS. This indicates that the bacterial strains could grow in and metabolize inulin as the sole carbon source. The strains cultured in both media reached the exponential phase after 2 h of incubation. However, the ODs measured at 620 nm of the strains grown in glucose were higher than those grown in inulin during the exponential phase. The exponential phase of both strains grown in inulin mMRS was sustained for 8 h, while that of both strains grown in glucose was sustained for 6 h. The exponential phase of both strains grown in

inulin lasted longer than that of the strains grown in glucose, indicating that the addition of inulin to the growth medium promotes a metabolism different from that of glucose to the medium. Carbon metabolism influences the growth of bacteria based on energy production and energy utilization. The lower OD of the strains grown in inulin than that of the strains grown in glucose is attributable to the energy utilized to transport inulin into the bacterial cells (Buntin et al., 2017).

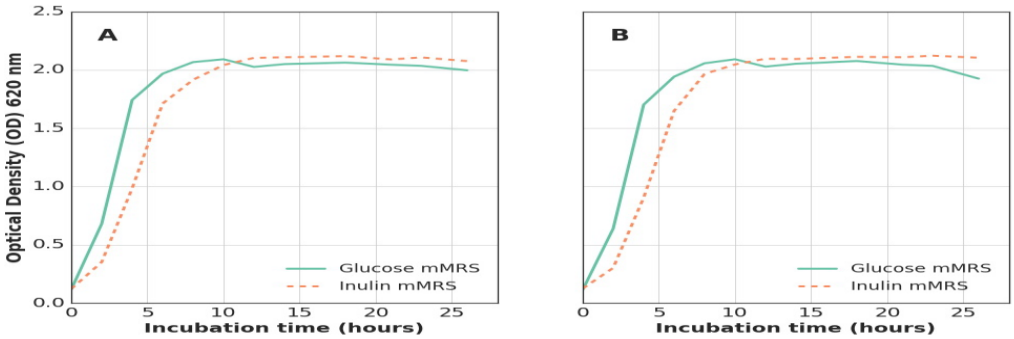

Figure 2: Growth curve of *L. casei* AP (A) and AG (B) in glucose and inulin mMRS during incubation for 26 h at 37 °C

The bacterial cells were harvested after 24 h incubation, by which time they had reached the stationary phase. The bacteria that reach the stationary phase usually develop a general stress response to adapt to environmental changes (Saarela et al., 2004). In this case, the change in environment was triggered by a carbon source alteration from glucose to inulin. Sugar is the main component for lactobacilli to generate energy and bio-mass. In food technology, sugar is also the most important compound to produce lactic acid as the main product in the fermentation process (Pessione et al., 2010). Changes in the carbon source in the growth media induce adaptation responses in bact-eria by protein and metabolite expression (Shimizu, 2014).

Sugar analysis using HPLC: The results of sugar analysis of the samples of the *L. casei* AP and AG are presented in Tables 1 and 2. Sugar analysis of samples obtained from inulin mMRS as a control showed an inulin content of 16241 ppm and no presence of simple sugars (Figure 2). Inulin was detected in the intracellular sample of the *L. casei* AP and AG during the exponential and stationary phases. Fructose was detected only in the supernatant sample of the *L. casei* AP and AG during the exponential phase. Glucose and sucrose were not detected in the samples of the *L. casei* AP and AG during the exponential and stationary phases. The inulin concentration in the supernatant of both *L. casei* strains during the stationary phase was lower than that during the exponential phase, indicating that both bacterial strains consumed inulin as the sole carbon source.

Table 1 Sugar concentration of *L. casei* AP at different incubation time on different sample

Incubation time (h)	Sample	Sugar Concentration (ppm)*			
		Inulin	Glucose	Fructose	Sucrose
0	Inulin mMRS	16241.8	-	-	-
6	Intracellular	12976.0	-	-	-
	Supernatant	10332.6	-	945.5	-
20	Intracellular	13147.4	-	-	-
	Supernatant	4475.9	-	-	-

*Sugar concentration was calculated using the peak area based on a calibration equation (shown in equation (1), (2), (3) and (4)) from each sugar type in chromatogram data.

Table 2 Sugar concentration of *L. casei* AG at different incubation time on different sample

Incubation time (h)	Sample	Sugar Concentration (ppm)*			
		Inulin	Glucose	Fructose	Sucrose
0	Inulin mMRS	16241.8	-	-	-
6	Intracellular	13110.5	-	-	-
	Supernatant	10358.7	-	940.4	-
20	Intracellular	12400.2	-	-	-
	Supernatant	3851.7	-	-	-

*Sugar concentration was calculated using the peak area based on a calibration equation (shown in equation (1), (2), (3) and (4)) from chromatogram data.

Sugar analysis confirmed our assumption. HPLC analysis showed that inulin was detected inside the bacterial cells in the exponential and stationary phases of both strains, confirming that inulin is intracellularly transported through cell membrane transporters, and that inulin degradation occurs intracellularly. We previously found that fructans with a high DP tends to be intracellularly transported for degradation, which we confirmed by the absence of inulin in the supernatant similarly reported by Makras et al. (2005). This process occurred in *L. delbrueckii* JCM 1002^T after incubation for 12 h. Furthermore, fructose was detected in the supernatant of both *L. casei* strains during the exponential phase, indicating that the degradation of inulin in the strains can also occur extracellularly. However, no fructose was detected in the bacterial cells during the exponential phase or in the supernatant during the stationary phase.

Glucose, fructose and sucrose as inulin hydrolysis products were not detected in the bacterial cells in the exponential or stationary phases. This is likely attributable to the rapid utilization of simple sugars in the bacterial cells during glycolysis to produce energy. It was previously shown that the concentration of fructose, glucose and sucrose and inulin degradation products depletes quickly after 4 h fermentation with *L. paracasei* subsp. *paracasei* 8700:2 (Makras et al., 2005). The exponential phase is an active growth phase during which bacterial cells need considerable amount of primary energy for sustenance. Based on these data, the *L. casei* AP and AG showed similar sugar patterns, indicating a similar intracellular inulin metabolism.

Protein analysis using SDS-PAGE and ESI-LC-MS/MS: The proteins isolated from the *L. casei* AP and AG were identified to determine their profile when grown in inulin mMRS and glucose. SDS-PAGE analysis showed that protein bands from the *L. casei* AP and AG grown in glucose were different from those grown in inulin, as shown in Figures 3. Cell wall proteins with molecular weights ranging between 30 kDa and 50 kDa appeared for both bacterial strains during inulin treatment. Proteins of approximately 65 kDa appeared for the *L. casei* AP grown in inulin, whereas there was no differences on protein bands appeared in the intracellular protein sample of the *L. casei* AG. Considering the extracellular protein profile, both the *L. casei* strains showed a difference on 100 kDa protein band when grown in inulin but did not show the same pattern when grown in glucose (data not shown).

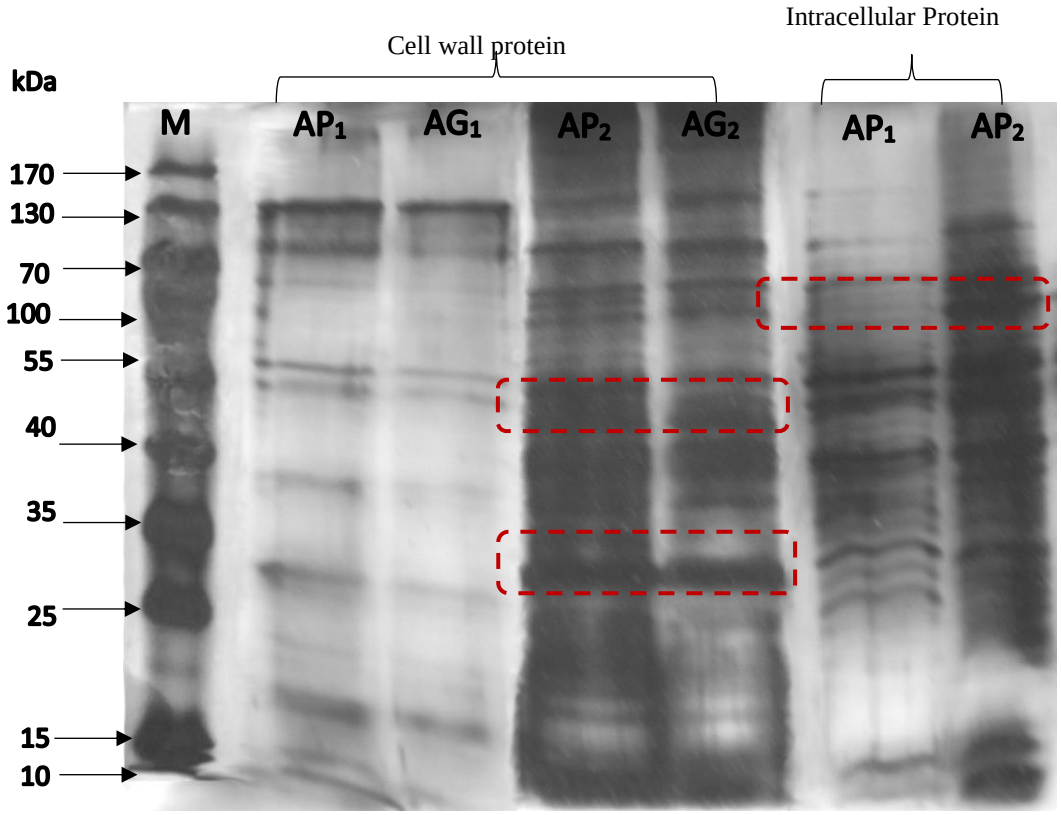

Figure 3: Profile of cell wall and intracellular proteins of *L. casei* strains in glucose (AP_1 and AG_1) and in inulin mMRS (AP_2 and AG_2)

Table 3 Proteins in the specific protein band of 30 kDa, from the cell wall of *L. casei* strain AP after 24 h incubation at 37°

No	Protein name	Accession no.	Mascot score	Mass (Da)	No. of matches	No. of sig. matches	No. of sequences	emPAI
	Carbohydrate and energy metabolism							
1	Triose phosphate isomerase	C2FGY5	236	27669	17	6	8	1.16
2	Acetoin reductase	A0A0C2S230	168	26866	6	2	6	0.30
3	Fructose-bisphosphate aldolase	S2TTT9	97	31469	13	7	6	0.76
4	Glyceraldehyde-3-phosphate dehydrogenase	S2PKG9	84	36712	7	4	6	0.34
5	Fructokinase	T0SEM5	67	31879	3	2	2	0.25
6	50S ribosomal protein	A0A0E2BNE3	225	22641	8	6	5	1.55
7	50S ribosomal protein L1	K0N7M7	182	25219	13	6	7	1.33
8	50S ribosomal protein L3	T0SLI2	66	23869	4	1	3	0.16
9	Large subunit ribosomal protein L1	770629518	115	24633	10	3	6	0.54
10	Cell envelope-associated transcriptional attenuator LytR-CpsA-Psr, subfamily L2	S2M782	137	32585	11	6	8	0.93
11	30S ribosomal protein S4	S2TYI6	61	23292	2	2	2	0.35
12	Ribosome biogenesis GTPase A	A0A0E2BSI0	70	32042	1	1	1	0.12
	Membrane transport							
13	PTS system mannose-specific IID component	K6QK79	118	24126	4	4	4	0.08
14	PTS system mannose/fructose/sorbose family IID component	D2EKT9	92	33270	3	2	2	0.24
15	PTS system mannose/fructose/sorbose family IID component	S6C2R4	99	33463	3	2	3	0.24
16	ATP-binding cassette transporter	A0A0C9P0J6	109	34032	3	1	3	0.23
17	ABC transporter substrate-binding protein	511466071	60	20581	2	2	2	0.41
18	Amino acid ABC transporter substrate-binding protein	A0A0C9QBN2	69	29605	2	2	2	0.27
19	Iron-sulphur cluster assembly ATPase protein SufC	A0A0E0Z9D4	60	28160	1	1	1	0.13
20	WxL domain surface protein	A0A0C9PD36	72	27142	7	3	6	0.48
21	Cell surface protein	A0A0C9QBN4	67	27519	5	2	5	0.29
	Lipid metabolism							
22	Lipoprotein	A0A0E2BT31	78	30443	6	3	4	0.42
23	Esterase C	410534861	54	9530	5	2	3	1.03
24	Esterase	A0A0B8TY18	44	29974	1	1	1	0.13
	Pyruvate metabolism							
25	L-lactate dehydrogenase	A0A0B8QK32	89	35020	1	1	1	0.11
26	1-deoxy-D-xylulose-5-phosphate synthase	A0A062XD99	45	35473	1	1	1	0.11
	Transcriptional regulators							
27	DeoR family transcriptional regulator	A0A0E2LZ02	74	26977	3	2	3	0.30
	Stress response							
28	60 kDa chaperonin	A0A011RPA1	64	57317	2	1	2	0.07
	Proteins with general function							
29	Haloacid dehalogenase (HAD) superfamily hydrolase	A0A0C9Q6R8	87	30314	3	2	3	0.27
30	HAD family hydrolase	A0A098R1Y1	73	28711	1	1	1	0.13
31	Acyltransferase	A0A0B8TMF1	89	33105	2	1	1	0.11
32	SAM-dependent methyltransferase	S2PWL2	48	28597	5	1	4	0.13
33	NLP/P60 family protein	A0A0C9PM75	198	41541	7	5	6	0.54
34	Surface antigen	A0A0C9P9Z1	157	42406	5	2	5	0.18
35	YkuN_2 protein	K0NA19	103	22906	3	2	3	0.36
36	Fimbriae subunit	A0A0C9P9T4	85	35833	1	1	1	0.10
37	Extracellular protein	A0A0E0ZF14	75	34693	2	2	2	0.23
38	Lysozyme M1 (1,4-beta-N-acetylmuramidase)	A0A0E2BQP1	55	74963	3	2	3	0.10
39	DNA-entry nuclease	A0A0C9PNY4	82	31083	2	2	2	0.26

40	Serine family D-Ala-D-Ala carboxypeptidase	A0A0C9PD74	71	46900	2	2	2	0.17
41	Methylpurine-DNA glycosylase	A0A0C9QU01	63	18932	1	1	1	0.20
42	Alpha/beta hydrolase fold-3 domain-containing protein	A0A0A8KSV2	50	32738	1	1	1	0.12
43	Purine biosynthesis operon repressor	A0A0A8KU54	41	30431	4	1	3	0.12
	Unknown function							
44	Uncharacterised protein	A0A0A8KV18	97	24920	3	2	3	0.33
45	Uncharacterised protein	S2N3J8	84	18692	4	2	4	0.46
46	Uncharacterised protein	A0A0E1PNM7	73	32985	2	1	2	0.11
47	Uncharacterised protein	A0A0A8KV30	70	30633	2	1	1	0.12
48	Uncharacterised protein	S2LY18	66	30262	1	1	1	0.12
49	Hypothetical protein	822506473	61	85557	1	1	1	0.04
50	Uncharacterised protein	A0A0E1PL73	53	37546	1	1	1	0.10
51	Uncharacterised protein	A0A0B8U064	45	19855	1	1	1	0.19
52	Uncharacterised protein	K0N8L5	34	32258	1	1	1	0.12
53	Uncharacterised protein	A0A0A3IYA3	48	18689	1	1	1	0.21

The cell wall-derived protein band of 30 kDa was further analyzed using ESI-LC-MS/MS to characterize its constituent proteins. The results of this analysis are presented in Table 3. We successfully identified 43 proteins, which were found to be involved in functions such as carbohydrate and energy metabolism, transport processes through the cell membrane, lipid metabolism, pyruvate metabolism, transcriptional regulation and stress response. However, the protein type and function could not be identified for some peptides.

A specific 30-kDa band isolated from the bacterial cell wall of the *L. casei* AP was identified to be a glycolysis and transporter protein by ESI-LC-MS/MS. Glycolysis proteins such as triose phosphate isomerase, fructose-bisphosphate aldolase, GAPDH and fructokinase are expressed in the bacteria grown in inulin. These proteins are involved in fructose glycolysis through the Feeder pathway (Nelson and Cox, 2013). Fructokinase catalyzes fructose to produce fructose-1-phosphate such that the glycolysis of fructose occurs through the feeder pathway. The high amount of fructose produced by inulin hydrolysis induces the expression of these proteins to catalyze enzymatic reactions during glycolysis.

Moreover, GAPDH was found in the cell wall of the *L. casei* AP. This protein is the most important housekeeping enzyme during glycolysis and is generally located intracellularly. However, in the present study, GAPDH was identified as a component of the bacterial cell wall. A previous study also identified GAPDH on the outer surface of *L. plantarum* 299v cells (Beck et al., 2009). It was thought to have an activity similar to *Streptococcus* GAPDH, which functions as an immunomodulatory protein related to virulence and binds to lipoteichoic acid in the cell wall of gram-positive bacteria (Madureira et al., 2007).

We also identified a cell surface protein belonging to the WxL superfamily group by Blastp analysis. This protein binds to the cell wall of Gram-positive bacteria and interacts with peptidoglycan. The presence of this protein in Gram-positive bacteria is expected to facilitate interbacterial interaction (Brinster et al., 2007). Moreover, this protein is predicted to mediate the adaptation of bacteria to specific biotope environments.

The transporter proteins detected in the *L. casei* AP grown in the inulin medium belonged to the PTS mannose/fructose/sorbose family IID component, ABC transporter and ABC transporter substrate-binding protein. This finding supports the HPLC data, which detected inulin inside the bacterial cells. In accordance with a previous study, we found that these proteins are involved in the transport of carbohydrates, particularly fructose and oligofructose, into the cell through the cell membrane (Gänzle and Follandor, 2012). PTS catalyzes the phosphorylation of sugars, which can then pass through the cell membrane. This protein consists of three peptides with a match value of 10%: AEEVHITK, FTPVVSDVPNQKs and ALELLQSQGLSLTK: The ABC transporter and ABC substrate-binding protein are thought to be responsible for the transport of inulin into cells of the *L. casei* AP and AG. The ABC transporter catalyzes the absorption of important nutrients and the excretion of toxic components. It was previously shown that the accumulation of FOS in *L. paracasei* 1195 occurs through the ABC transporter, whereas hydrolysis is catalyzed by β-fructosidase, a cytoplasmic protein (Kaplan and Hutkins, 2003). This mechanism is also present in *L. acidophilus* NCFM (Barrangou et al., 2003). Furthermore, inulin transport

through the ABC transporter requires energy that is produced via the hydrolysis of ATP to ADP. This explains why the growth curve of the bacteria grown in inulin was lower than that of the bacteria grown in glucose: it is caused by the utilization of energy to transport inulin into cells.

Inulin is also categorized as an FOS consisting of glucose and fructose polymers. The metabolism of FOS in lactobacilli can occur through three pathways: extracellular hydrolysis, transport and phosphorylation by PTS and transport by the ABC transporter (Gänzle and Follandor, 2012). Based on our data analysis, inulin metabolism in the *L. casei* AP occurs through two pathways involving inulin transport into bacterial cells: (1) PTS mannose/ fructose/ sorbose family IID component and (2) ABC transporter, followed by inulin degradation inside cells. This finding leads to further investigation in unravelling inulin transport-associated genes in *L. casei* AP.

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