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20 May 2022

An exoproteome approach to monitor safety of a cheese-isolated *Lactococcus lactis*

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Abstract

The safety of the cheese-isolated and potential starter *Lactococcus lactis* 11D was explored by means of an extracellular proteomic study. A preliminary analysis showed good caseification/proteolytic behavior of the strain, absence of production of biogenic amines and good survival at acidic pH. The extracellular proteome map was analyzed to investigate the presence of potential virulence factors. Five moonlighting proteins with adhesive properties (ornithine carbamoyltransferase, fructose bisphosphate aldolase, trigger factor, EF-Tu and GroEL) were identified in the exoproteome, as well as the potential plasminogen binding proteins enolase, GAPDH and phosphoglycerate mutase. Adhesive properties are fundamental features for good starters and commensal strains, although some controversial aspects arise from plasminogen binding proteins as we shall discuss. Noticeably, GroEL, chitinase, and triose phosphate isomerase were abundant in the *L. lactis* 11D exoproteome. These proteins play a role in bacterial aggregation and in bacteria–fungi interactions, therefore their presence may indicate a good competition potential of the strain against other microorganisms in both food and the gastrointestinal habitat. A DIGE comparative exoproteomic analysis was performed on the *L. lactis* 11D strain grown on glucose and the disaccharide trehalose, examined here due to its common use as lyophilization stabilizer, respectively. The experiment showed that chitinase biosynthesis was enhanced in presence of trehalose. This is to our knowledge the first extracellular proteomic mapping of *L. lactis* with relevance for bacterial strain-typing in food safety.

Highlights

► Bacterial strain-typing is an important issue in food safety. ► The potential starter *L. lactis* 11D was analyzed by an exoproteome approach. ► The exoproteome showed high presence of moonlighting proteins involved in adhesion. ► A DIGE exoproteome comparing growth on glucose and on trehalose was performed. ► Chitinase biosynthesis was enhanced in presence of trehalose.

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1. Introduction

Lactic acid bacteria (LAB) are widely exploited in food industry as spontaneous or starter cultures for several fermentation processes (Hugas, Garriga, & Aymerich, 2003). They are also used as bio-control agents for food safety improvement, due to their ability to counteract spoilage and pathogenic bacteria by means of nutritional competition, acidification, available enzymes and bacteriocin production (De Vuyst & Leroy, 2007).

The search for new starter microorganisms to be employed in food manufacturing for improving texture and for adding particular aromas is also of growing interest to satisfy consumers' demand of novel tastes. New starter microorganisms can be searched in the spontaneous microbiota of PDO (Protected Designation of Origin) products. Strain safety is an essential pre-requisite for their use in industrial food production, thus lack of excessive biogenic amine production and of secreted toxic molecules must be verified.

In the past few years production of biogenic amines by several LAB involved in food fermentation processes was highlighted (wine, beer, and cheese). Thus high contents of histamine and tyramine can cause allergies, hypertension and headache, and may have severe consequences such as brain hemorrhage and death (Millichap & Yee, 2003). The ability to biosynthesize amines is related to a specific strain rather than to a species, therefore even bacteria generally regarded as safe (GRAS) may present risks (Chander, Batish, Babu, & Bhatia, 1988).

Another important feature to be excluded is the secretion by the strain of known toxic molecules including proteins that may be involved in the activation of proteolytic cascades. This process is well known in pathogenic bacteria, nevertheless also non-virulent bacterial species can display this feature (Candela et al., 2007 and Hurmalainen et al., 2007). Most of these proteins are intracellular metabolic enzymes or stress proteins that act as plasminogen (Pg) receptors

triggering localized proteolysis when secreted (Carneiro et al., 2004, Kinnby et al., 2008, Pancholi and Fischetti, 1992 and Sibbald et al., 2006) and thus facilitating pathogenic bacteria invasion (Gohar et al., 2005 and Hurmalainen et al., 2007). It is therefore important to confirm the absence of potential proteolytic activators in strains employed in food technology in particular considering that some food bacteria display good resistance to acidic pH allowing them to reach the gut ecological niche. Exoproteome analysis is a valid method to exclude the presence of secreted virulence factors in bacteria of both biotechnological importance (Hansmeier, Chao, Puhler, Tauch, & Kalinowski, 2006) and food application interests (Gagnaire et al., 2004).

The aim of the present investigation was to identify possible starter candidates among the microbiota of Piedmont PDO artisanal-made cheeses, to be employed in cheese manufacturing. Towards this goal a preliminary screen focused on technological properties and biogenic amine production was performed. Subsequently the potential safety for human health was evaluated by exoproteome analysis of the chosen strain *Lactococcus lactis* 11D. Furthermore, a differential exoproteomic analysis of the strain grown on glucose and on trehalose, respectively, was performed by DIGE to exclude occurrence of trehalose induction of virulence factors. This is an important consideration since trehalose is a common starter cultures stabilizer used in the lyophilization process as cryoprotectant (Tymczyszyn, Gomez-Zavaglia, & Disalvo, 2007).

2. Materials and methods

2.1. Bacterial strains selection and their technological evaluation

Three *L. lactis* subsp. *lactis* strains were selected directly from an artisanal made cheese isolated and used to produce miniature cheeses as previously described (Bertolino, Zeppa, Gerbi, & McSweeney, 2008). Proteolysis parameters (total nitrogen and soluble nitrogen) were determined after 60 days of ripening using Official Methods of Analysis (Helrich, 1990). The ripening index was calculated as percentage of water soluble nitrogen of the total nitrogen.

2.2. Biogenic amine determination

Biogenic amine extraction was performed on cheese (20 g) in 95 mL pure HPLC water (Milli-Q, Millipore). Each sample was homogenized (2 min), and thereafter added trichloroacetic acid (5 mL; TCA, Fluka, 100% w/v). After centrifugation at $2500 \times g$ (15 min), the supernatants (50 mL) were extracted three times with ether (each 15 mL). The volume of the aqueous solution was adjusted to 50 mL with HPLC water and filtered (0.22 μm syringe filter; Millipore, Bedford, MA, USA) before HPLC analyses (Arlorio, Coïsson, & Martelli, 1998). Four biogenic amines (tyramine, histamine, 2-phenylethylamine and tryptamine) and their precursor amino acids (tyrosine, histidine, phenylalanine and tryptophan) were quantified using an optimized ion-pair HPLC method (Coïsson, Cerutti, Travaglia, & Arlorio, 2004) and 10 μL of sample injected.

2.3. Strain maintenance

The selected strain was maintained in 50% glycerol aliquots of frozen culture, and transferred to fresh M17 broth before performing proteomic analysis.

2.4. Acid tolerance of *L. lactis* 11D

Simulated gastric fluid (SGF) was formulated according to the guidelines of the U.S. Pharmacopeia. Briefly, SGF was composed of 3.2 g/L pepsin and 2.0 g/L NaCl, and the pH was adjusted to 1.5, 2.0, 2.5, and 3.0 by the addition of 5 M HCl. Middle exponential phase cultures

(1 mL) of *L. lactis* subsp. *lactis* 11D in M17 broth were added to SGF (19 mL) at 37 °C under mild agitation (200 rpm). After 30 min 1 mL was collected, mixed in sterile M17 broth and plated onto M17 agar after serial 10-fold dilutions. Plates were incubated at 37 °C for 24 h. The average number of CFU from triplicate analyses of every dilution was determined.

2.5. Proteomic experiments

2.5.1. Bacterial growth

For the extracellular and cytosolic maps, *L. lactis* 11D was grown on M17 broth. For the comparative exoproteome analysis the strain was grown on M17 broth deprived of lactose supplemented with 1% (w/v) glucose and 1% (w/v) trehalose for the two different conditions.
L. lactis 11D was pre-cultured in M17 (10 mL) at 37 °C overnight (to late exponential phase) and inoculated into 45 mL of M17, glucose-M17 or trehalose-M17, respectively. These cultures were incubated at 37 °C until middle exponential phase. Four biological and four technical replicates were prepared at each condition.

2.5.2. Sample preparation for extracellular maps

The middle-exponential phase cultures were centrifuged (9000 $\times g$; 20 min) and the cell free supernatants were filtered (0.2 μm syringe filter, Millipore Millex filter unit); after a cooling step at -80 °C (3 h) cultures were freeze-dried overnight (Scanvac CoolSafe™ instrument, Labo-Gene). The freeze-dried supernatants were dialyzed (MWCO 3500 Da molecular porous membrane, SpectraPor) against 3×5 L 5 mM Tris HCl pH 7.5 (overnight at 4 °C) and precipitated by three volumes 10:90 (v/v) TCA:acetone. After incubation at -80 °C overnight, the samples were centrifuged (25,000 $\times g$ for 30 min, at 4 °C) and protein pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 3% CHAPS, 10 mM Tris HCl pH 8.5). Protein concentration was assessed by 2D-Quant kit (GE Healthcare).

2.5.3. Sample preparation for the cytosolic proteome

The cell pellet obtained after centrifugation of the middle-exponential phase culture of *L. lactis* 11D in M17 was washed twice with 0.95% NaCl and transferred to a Fast-Prep tube. One part of glass beads (< 106 μm; Sigma) was added to one part of cells. The cells were added 300 μL saturated phenol (phenol with 10 mM Tris HCl pH 8 kept at 4 °C for 6 h), 300 μL 10 mM Tris HCl pH 7.5 and placed on ice. The cell disruption was carried out (Bio 101 Savant FastPrep FP 120 bead beater, Savant, Farmingdale, USA) for four cycles (45 s, 6 m s^{-1}) with 60 s intermittent cooling on ice between cycles. After centrifugation (15,000 $\times g$; 15 min, at 4 °C) three phases were formed. Three volumes of 96% ethanol (v/v) were added to the isolated middle phase and stored overnight at -20 °C. After centrifugation as above, the protein pellet was resuspended in rehydration buffer and added 1 μL benzonase nuclease (25 U/μL, Novagen) to avoid DNA streaking. Protein concentrations were assessed by 2D-Quant kit (GE Healthcare).

2.5.4. Isoelectric focusing (IEF)

An equal amount of proteins resuspended in rehydration buffer were added 100 mM DTT, 10% isopropanol, 5% glycerol and 1% IPG buffer pH 4–7 (GE Healthcare) and loaded by anodic cup-loading on 11 cm Immobiline DryStrip (GE Healthcare) pH 4–7, which had been previously rehydrated overnight with 200 μL of rehydration buffer. IEF was carried out at 20 °C,

50 µA/strip, following the steps: 30 V 70 min, 100 V (gradient) 60 min, 100 V 8 h, 2000 V (gradient) 6 h, 8000 V (gradient) 2 h, 8000 V until 33,000 V h.

2.5.5. IEF for DIGE (Difference Gel Electrophoresis)

Prior to IEF, proteins were labeled with Cy2, Cy3 and Cy5 CyDye DIGE Fluors for Ettan DIGE (GE Healthcare) according to the manufacturer's instructions. Each dye (100 pmol) was added to sample or internal standard (each containing 15 µg protein) and incubated for 30 min on ice. The reaction was stopped by addition of 1.5 µL L-lysine (100 mg/mL) on ice for 10 min. Samples labeled with the different dyes were mixed together and added to the rehydration buffer. IPG strips, previously rehydrated overnight with 200 µL of rehydration buffer, were loaded by anodic cup-loading with the mixture of labeled proteins (45 µg). The run was performed using an IPG-phor (GE Healthcare) covered from direct light, following the program described in Section 2.5.4.

2.5.6. SDS-PAGE

IPG strips were soaked (15 min) in equilibration buffer (5 mL 6 M urea, 30% glycerol, 50 mM Tris HCl pH 8.8, 0.01% bromophenol blue and 2% SDS) with DTT (10 mg/mL) followed by 15 min in the same buffer (5 mL) containing iodoacetamide (25 mg/mL). The second dimension SDS-PAGE was performed on PROTEAN Plus Precast Gels for 2-D (BioRad) at constant voltage of 200 V until the bromophenol blue reached the bottom of the gel. Molecular weight marker MARK 12™ unstained standard (Invitrogen) was used.

For the comparative exoproteome analysis by DIGE, SDS-PAGE was performed in 24 cm gels using ETTAN™ DALT SIX Electrophoresis unit (GE Healthcare) with the following program: 2 W per gel, 600 V, 400 mA for 30 min, then 12 W per gel until the bromophenol blue reached the bottom of the gel. The electrophoresis unit was covered from direct light during the run. All gels were fixed and stained according to the Blue silver method for Colloidal Coomassie Blue (CCB) staining (Candiano et al., 2004) and destained in distilled water overnight.

2.5.7. Image acquisition

Gels were scanned with the ScanMaker 9800 XL scanner (Microtek), both in reflective and in transparent mode. DIGE gels were scanned with the Typhoon 9410 Variable Mode Imager (GE Healthcare). Imaging of DIGE gels was done immediately after the SDS-PAGE at excitation/emission wavelengths of 488/520 nm (Cy2), 532/580 nm (Cy3) and 633/670 nm (Cy5), respectively.

2.5.8. Image analysis

Progenesis SameSpots (version 3.3, nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) was used for spot detection in all experiments. DIGE gel images were also aligned by automated calculation of ten manually assigned alignment landmark vectors. Scanned gels were analyzed by intra-gel (difference in-gel) and inter-gel (biological variance) analysis. A 1.5-fold threshold (spot volume ratio change) and ANOVA $p \leq 0.05$ was chosen as criterion in the identification of differentially expressed protein candidates.

2.5.9. Mass spectrometry analysis

All visible spots in the exoproteome map and the selected spots in the DIGE maps were manually excised from the freshly CCB-stained gel with a scalpel and transferred to Eppendorf™ tubes. Trypsin digestion was carried out as previously described (Hellman, Wernstedt, Gonez, & Heldin, 1995) and the tryptic digests (2 μL) were loaded to the AnchorChip target plate (Brucker-Daltonics) with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Beavis, Chaudhary, & Chait, 1992).

MS and MS/MS spectra were obtained by Ultraflex II MALDI-TOF MS mass spectrometer (Bruker-Daltonics) in auto-mode using Flex Control v3.0 (Bruker-Daltonics) and processed by Flex Analysis v3.0 (Bruker-Daltonics). Peptide mass maps were acquired in reflectron mode with 500 laser shots per spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/μL); MS/MS data were acquired with stop conditions so that 1000–1600 laser shots were accumulated for each spectrum. The combined peptide mass fingerprint and MS/MS search was performed on NCBInr database for bacteria (NCBInr20100323; 10606545 sequences; 3615943919 residues) using the MASCOT 2.0 software (http://www.matrixscience.com) integrated together with BioTools v3.1 (Bruker-Daltonics). The parameters used for the search were: monoisotopic peptide mass accuracy of 80 ppm, fragment mass accuracy to $\pm\,0.7$ Da; maximum of one missed cleavage; carbamidomethylation of cysteine as fixed modification and partial oxidation of methionine as variable modification. There were no restrictions with respect to protein Mw and pI. Filtering of peaks was done for known autocatalytic trypsin peaks and keratin peaks; the signal to noise threshold ratio was set to 1:6. The significance threshold was set at $p < 0.05$, and identification required that each protein contained at least one peptide or an e-value < 0.05.

3. Results and discussion

3.1. Proteolytic potential, caseification properties and biogenic amines production

To select possible candidates to be employed as new starters in cheese manufuring, the microbiota of an Italian artisanal-made cheese was screened and three selected *L. lactis* strains were used to produce miniature-cheeses. To evaluate the technological value of these strains and their impact on human health, the proteolytic potential, the free amino acid and the most common biogenic amine concentrations were measured.

All three strains displayed good technological features as starter for cheese production in terms of pH and ripening index (Table 1). As regards biogenic amines, one strain (*L. lactis* 11D) did not produce any, another strain (*L. lactis* 150) gave < 20 mg kg^{-1} for both tyramine and histamine, and the third strain (*L. lactis* 370) showed a tyramine level reaching 73 mg kg^{-1} (Table 1). The findings demonstrate that *L. lactis* 11D is biogenic amine-free and this strain was therefore chosen for further analyses regarding its safety features.

Table 1.

Technological features (proteins, pH, and ripening index), free amino acids and biogenic amines concentration in cheese samples inoculated with *Lactococcus lactis* 11D, 150 and 370.

	L. lactis 11D	*L. lactis* 150	*L. lactis* 370

	L. lactis 11D	*L. lactis* 150	*L. lactis* 370
Proteins (%)	18.35 ± 0.24	21.24 ± 1.19	20.79 ± 0.08
pH	5.53 ± 0.01	4.93 ± 0.02	5.47 ± 0.03
Ripening index	48.53 ± 0.01	29.74 ± 0.01	68.99 ± 0.03
Tyrosine	40.15 ± 0.15	42.27 ± 0.15	14.13 ± 0.31
Histidine	13.12 ± 1.07	15.23 ± 0.31	5.15 ± 0.31
Phenylalanine	170.85 ± 0.16	116.20 ± 0.75	161.92 ± 0.59
Tryptophan	4.53 ± 0.04	18.60 ± 0.09	1.51 ± 0.08
Tyramine	< LOD	10.90 ± 0.65	73.35 ± 0.94
Histamine	< LOD	18.36 ± 0.20	10.66 ± 1.89
2-Phenylethylamine	< LOD	< LOD	< LOD
Tryptamine	< LOD	< LOD	< LOD

Ripening index = $N_{sol} / N_{tot} * 100$, biogenic amine and amino acid concentrations are measured in mg kg^{-1}, LOD = limit of detection.

3.2. Acid tolerance

As shown in Table 2, *L. lactis* 11D is able to survive under acidic conditions for 30 min. No significant differences between the initial population at 0 min (cell count of $6.77 \times 10^9 \pm 4.9 \times 10^8$) and the population after 30 min at pH 2.5 (cell count of $6.47 \times 10^9 \pm 1.05 \times 10^9$) and pH 3 (cell count of $6.08 \times 10^9 \pm 4.4 \times 10^8$) were observed; by contrast *L. lactis* 11D is not able to grow after 30 min at pH ≤ 2.0. It is thus demonstrated that the strain may be able to overcome the gastric acidic barrier and reach the gut. This promising property motivated the analysis of proteins released to the extracellular environment that may be important both for understanding adhesion/invasion mechanisms and for assessing the strain safety for human consumption.

Table 2.

Identified extracellular proteins of *Lactococcus lactis* 11D.

Protein	Spot #	Gene ID	Score	E-value	Mw☐	pI☐	Accession no.
Cell wall processing, chitinase and surface antigen							

Protein	Spot #	Gene ID	Score	E-value	Mw☐	pI☐	Accession no.
N-acetylmuramidase	5	*acmD*	242	3.10E-18	37,517	4.5	15672523
N-acetylmuramoyl-L-alanine amidase	11	*acmB*	165	1.60E-10	49,556	4.82	15673889
Chitinase	58	*chiA*	160	4.90E-10	54,487	5.32	15673932
Surface antigen	70–80		186	1.20E-12	46,212	7.68	116513168
Adhesion proteins							
Fructose-bisphosphate aldolase	9	*fbaA*	146	1.20E-08	32,197	5.04	15673891
Ornithine carbamoyltransferase	30	*arcB*	148	7.80E-09	39,488	5.44	15674018
Trigger factor	87A	*tig*	107	9.80E-05	46,901	4.43	15672533
Triosephosphate isomerase	63	*tpiA*	263	2.50E-20	27,060	4.63	15673116
Chaperonin GroEL	2	*groEL*	363	2.50E-30	57,166	4.75	15672376
Elongation factor Tu	64–65	*tuf*	410	4.90E-35	43,185	4.89	15673843
Plasminogen-binding proteins							
Glyceraldehyde 3-phosphate dehydrogenase	85	*gapB*	268	7.80E-21	35,911	5.57	15674228
Phosphoglycerate mutase	69	*gpmA*	173	2.50E-11	26,297	5.3	116511195
α-Enolase	55	*eno*	557	9.80E-50	46,940	4.68	15672626
Molecular chaperone DnaK	23	*dnaK*	420	4.90E-36	64,947	4.62	15672936

Protein	Spot #	Gene ID	Score	E-value	Mw	pI	Accession no.
Putative moonlighting proteins and carbohydrate metabolism							
Phosphoglucose isomerase	28	*pgi*	147	9.80E-09	49,564	4.89	15674150
Elongation factor Ts	40	*tsf*	118	7.80E-06	36,647	4.92	15674134
Elongation factor G	66	*fusA*	157	9.80E-10	77,907	4.75	15674242
Thioredoxin reductase	8	*trxB1*	82	0.031	33,987	4.76	15672922
30S Ribosomal protein S1	17	*rpsA*	350	4.90E-29	44,683	4.99	15672820
Phosphofructokinase	32	*pfk*	124	2.00E-06	35,638	5.56	399697
Phosphotransferase system mannose/fructose-specific component IIA	1	*ptsIIB*	129	6.20E-07	35,085	5.23	116512556
Phosphoenolpyruvate-protein phosphotransferase	15	*ptsI*	97	0.00092	62,635	4.67	15672100
Tagatose 1.6-diphosphate aldolase	83	*tbpa*	423	2.50E-36	36,641	4.82	116326612
UDP-Glucose 4-epimerase	60	*galE*	102	0.00031	36,321	5.18	15673961
Galactose-6-phosphate isomerase subunit LacB	36–45	*lacB*	93	0.0027	19,085	5.13	116326614
L-Lactate dehydrogenase	24	*ldh*	121	3.90E-06	35,086	4.97	15673313
Proteins with other function							
50S Ribosomal protein L10	54	*rplJ*	101	0.00039	18,076	5.08	15673251
DNA polymerase III subunit beta	46	*dnaN*	81	0.039	42,238	5.17	15671984
Ubiquitin	16	*ub*	214	2.00E-15	3592	4.99	207094432

Protein	Spot #	Gene ID	Score	E-value	Mw□	pI□	Accession no.
Dipeptidase PepV	34	*pepV*	97	0.0011	52,025	4.75	15672824
Cell division protein FtsZ	87E	*ftsZ*	115	1.60E-05	43,961	4.54	15673851
Hypothetical protein L22960	10	*ymcF*	278	7.80E-22	38,305	4.95	15673185
Hypothetical protein L107870	57	*ytcC*	107	2.10E-05	39,963	6.92	15673838
Hypothetical protein L0370	43	*typA*	103	0.00025	68,258	4.91	15673997
Hypothetical protein L96658	21–29–42	*usp45*	173	2.50E-11	47,036	8.33	15674211
Hypothetical protein L22496	19	*yacG*	412	3.10E-35	50,338	8.47	15672000

□

The values indicated for Mw and pI are the theoretical ones.

3.3. Exoproteome analysis

Proteins related to adhesive/invasive properties are generally distributed in the pH 4–7 range (www.jvirgel.de). The present study therefore focused on the acidic exoproteome analyzed for cells collected during the mid-logarithmic phase, to reduce the number of dead and lysed cells that increase in the late phases of the bacterial growth, when lack of nutrients occurs. Even though a non-specific release of cytoplasmatic components by cell autolysis cannot be excluded, a 2-DE map of the intracellular proteins was performed under the same conditions and displayed a different protein pattern (Fig. 1).
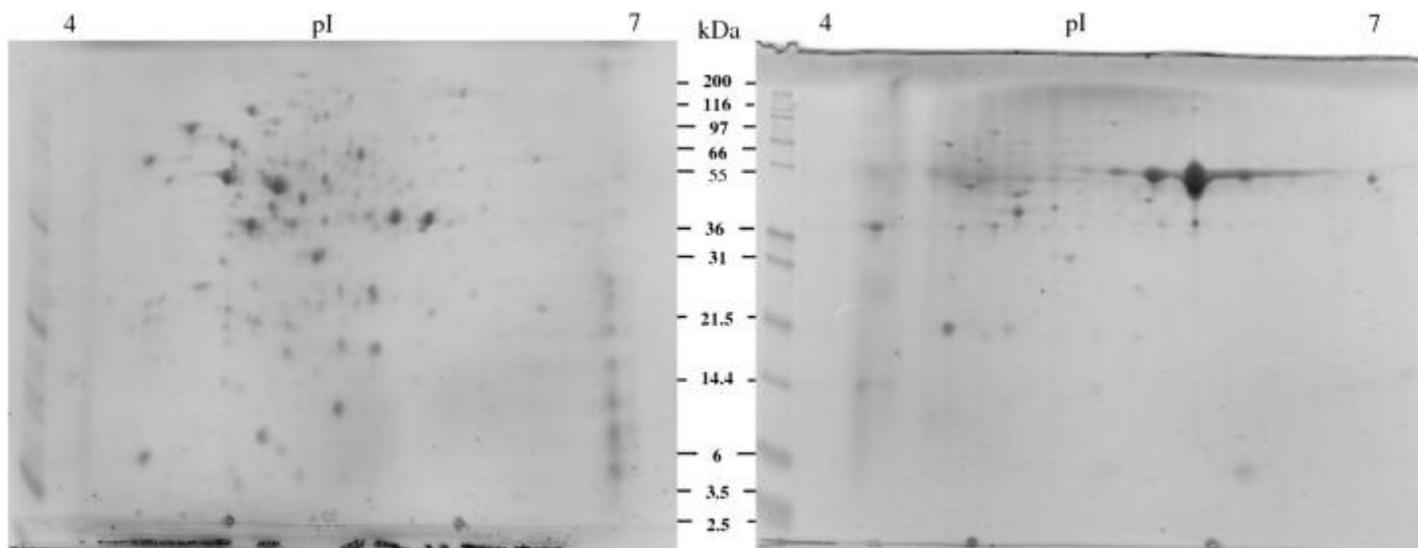
Fig. 1.

Comparison between the cytosolic (left) and the extracellular (right) map of *Lactococcus lactis* 11D. The maps exhibit different patterns, demonstrating no predominant cytosolic contamination in the extracellular sample.

Progenesis SameSpots analysis revealed 91 protein spots in the exoproteome (Fig. 2) representing 37 identified unique proteins (Appendix A Table 1), while the remaining spots represented multiple forms of the same proteins with a different pI or were too faint to be adequately identified by mass spectrometry analysis. It is worth noticing that no known virulence protein was identified in the acidic exoproteome of *L. lactis* 11D. The identified proteins were divided into five main functional groups (Table 2) according to the KEGG pathways (http://www.genome.jp/kegg/): (i) cell wall processing, chitinase and surface antigen; (ii) adhesion proteins; (iii) plasminogen binding proteins; (iv) putative moonlighting and carbohydrate metabolism; (v) proteins with other functions.
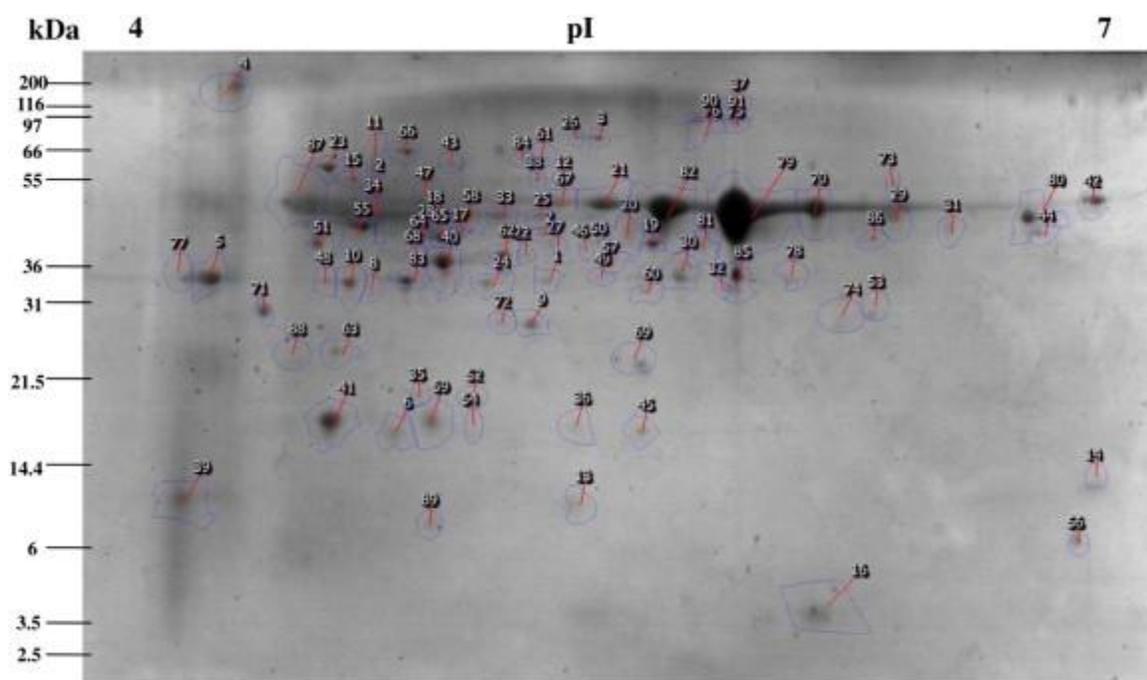
Fig. 2.

Exoproteome map of *Lactococcus lactis* 11D shows 91 spots by analysis with Progenesis SameSpots. Unique proteins (37) were identified with statistical significance, these included several moonlighting proteins

Proteins of the first group are chitinase, N-acetylmuramidase, and N-acetylmuramoyl-L-alanine amidase. Chitinase (spot 58) is of a special interest as it hydrolyzes chitin, the major cell-wall component of fungi and a prominent component of yeast cell walls. Since often bacteria and fungi share the same ecological niche, this enzyme may play a role in competition for nutrients. Carranza et al. (2009) reported evidence of an extracellular chitinase in *Chronobacter turicensis* displaying a fungi-inhibiting action. N-acetylmuramidase (spot 5) and N-acetylmuramoyl-L-alanine amidase (spot 11) are proteins involved in peptidoglycan renewal (maturation and degradation), but also in bacterial interspecies interactions since they act as general cell-wall lysis factors (Salazar & Asenjo, 2007). The presence of these proteins is consistent with the harvesting in mid-exponential phase, when the highest bacterial cells duplication occurs. The most abundant protein (spots 70–80) was identified as a generic surface antigen. Considering its high abundance it is of future interest to elucidate the function of this protein.

Among the proteins identified in the supernatant from *L. lactis* 11D more than 20% are anchorless multifunctional proteins (Jeffery, 2009) involved in adhesion and plasminogen binding mechanisms. These proteins are usually expressed in the intracellular compartment, where they exert metabolic roles, but when released to the extracellular environment (Bergonzelli et al., 2006 and Granato et al., 2004) or expressed on the cell surface (Alam et al., 2009, Hussain et al., 1999 and Tunio et al., 2010) they display additional functions. Such proteins are generally referred to as moonlighting (Jeffery, 2009).

The finding in the extracellular proteome of several adhesion proteins that should exert their function when surface-bound is a common phenomenon reflecting that a dynamic equilibrium between these two cell districts establishes, especially during the logarithmic growth phase.

Several factors such as pH variations and cell wall renewal may favor the cell wall detachment of several proteins (Antikainen, Kuparinen, Lahteenmaki, & Korhonen, 2007).

Fructose bisphosphate aldolase (FBA) (spot 9) and ornithine carbamoyltransferase (OTC) (spot 30), exert their adhesion activity when surface-bound. FBA is involved in the binding to a large trans-membrane receptor of the cadherin superfamily in the pathogenic Gram-negative *Neisseria meningitidis* and Gram-positive *Streptococcus pneumoniae* (Tunio et al., 2010). OTC, a nitrogen metabolism enzyme, has been found on the surface of several streptococci, *Clostridium perfringens* (Alam et al., 2009), and the opportunistic *Staphylococcus epidermidis* (Hussain et al., 1999), where it acts as adhesin. Trigger factor (spot 87A) and DnaK (spot 23) are also connected with adhesion, since it has been reported that surface adhesin P1 from *Streptococcus mutans* cannot efficiently work in the absence of DnaK and trigger factor (Crowley et al., 2008). Furthermore, it has been demonstrated in *Lactobacillus reuteri* NCIB 11951 that a collagen I binding protein, not belonging to the S-layer, shares high sequence homology with *Escherichia coli*trigger factor (Aleljung et al., 1994). Triose phosphate isomerase (TPI) (spot 63) was also proven as an adhesion factor for fungal mannans in *Staphylococcus aureus* when surface exposed (Furuya & Ikeda, 2009). Besides, both GroEL (spot 2) and EF-Tu (spots 64 and 65) in *Lactobacillus johnsonii* display adhesive capabilities towards human tissues (Bergonzelli et al., 2006). In *L. johnsonii*, GroEL induces a strong aggregation of the pathogen *Helicobacter pylori*, thus decreasing the bacterial load and facilitating clearance of the aggregated pathogens by the mucus (Bergonzelli et al., 2006). Its presence has been detected in the spent culture medium throughout the whole logarithmic phase, which is in agreement with the results of the present investigation, where high abundance of both GroEL and EF-Tu is evident in the extracellular environment. GroEL and EF-Tu have also been detected in the extracellular proteome of *Bifidobacterium animalis* subsp. *lactis* as putative moonlighting proteins with adhesion roles that contribute to the probiotic features of the strain (Gilad, Svensson, Viborg, Stuer-Lauridsen, & Jacobsen, 2011).

Three glycolytic enzymes, enolase (spot 55), GAPDH (spot 85) and phosphoglycerate mutase (PGM) (spot 69) as well as the stress protein DnaK (spot 23) behave as plasminogen (Pg) receptors in pathogenic bacteria, like *S. aureus* and streptococci (Kinnby et al., 2008). Once bound on the bacterial cell-wall, Pg is converted by specific virulence factors produced by both staphylococci (staphylokinases) and streptococci (streptokinases) into plasmin (Lahteenmaki, Kuusela, & Korhonen, 2001) able to activate proteolytic cascades, resulting in the degradation of the extracellular matrix and subsequent migration of bacteria through host tissues (Gladysheva, Turner, Sazonova, Liu, & Reed, 2003).α-enolase, in particular, is able to directly bind to extracellular matrix proteins like laminin (Carneiro et al., 2004), collagen and fibronectin (Candela et al., 2007), thus favoring damage via the proteolytic cascade. Nevertheless GAPDH and α-enolase have both been detected on the surface of certain LAB (*Lactobacillus acidophilus*, *L. amylovorus*, *L. gallinarum*, *L. gasseri*, *L. crispatus* and *L. johnsonii*) (Hurmalainen et al., 2007) and bifidobacteria (*Bifidobacterium lactis*, *B. longum*, *B. bifidum* and *B. animalis*) (Candela et al., 2007 and Gilad et al., 2011). Both of these enzymes allow Pg-binding on the cell-wall, but no homologue genes coding for any of the known Pg-activators (strepto- and staphylokinases) have been found in the genome of the sequenced lactobacilli species. This may indicate that LAB lacks an endogenous potential for Pg activation by conversion to plasmin. Some authors, however, suggest that Pg attachment to the cell-wall receptor can cause a conformational change of the latter, enhancing Pg susceptibility to host Pg-activating factors, namely uPA (urokinase-type Pg activator) and tPA (tissue Pg activator) (Candela et al., 2007 and Crowley et al., 2008). These controversial reports concerning bacteria–Pg interactions and the possibility to activate proteolytic cascades, suggest the need of an accurate

typing of both surface and extracellular proteome of starter strains before use, especially for those able to bypass the gastric barrier.

Many ribosomal proteins have been predicted to be extracellular by in silico analysis (CELLO: sub cellular localization predictive system (Yu, Chen, Lu, & Hwang, 2006)) and several identified proteins, such as EF-G (spot 66), EF-Ts (spot 40), thioredoxin reductase (spot 8) and 30S ribosomal protein S1 (spot 17) were previously classified as signal peptide-lacking exoproteins in *S. aureus* (Sibbald et al., 2006) and *Bacillus anthracis* (Gohar et al., 2005) and as surface protein in *L. lactis* NZ9000 (Berlec, Zadravec, Jevnikar, & Strukelj, 2011). All these considerations suggest that some of the detected proteins might play an extracellular role that remains to be identified.

3.4. Comparative exoproteome map by DIGE (Difference Gel Electrophoresis)

In order to ensure the viability and to control safety of the selected strain during and after the freeze-dry process, a culture was set up on the cryoprotectant sugar trehalose. Growth curves kinetic analysis of *L. lactis* 11D highlighted a lower growth rate ($\mu_{trehalose-M17} = 0.58\ h^{-1}$, $\mu_{glucose-M17} = 0.78\ h^{-1}$) but a higher final biomass concentration (0.97 g/L in trehalose-M17, 0.69 g/L in glucose-M17) for bacteria grown in presence of trehalose, as compared to the control (Fig. 3). This suggests that trehalose is a suitable carbon source for *L. lactis* 11D, which does not damage physiological functions. Furthermore, the lower growth rate is a good index that the strain cannot consume all the cryoprotectant/antioxidant present.
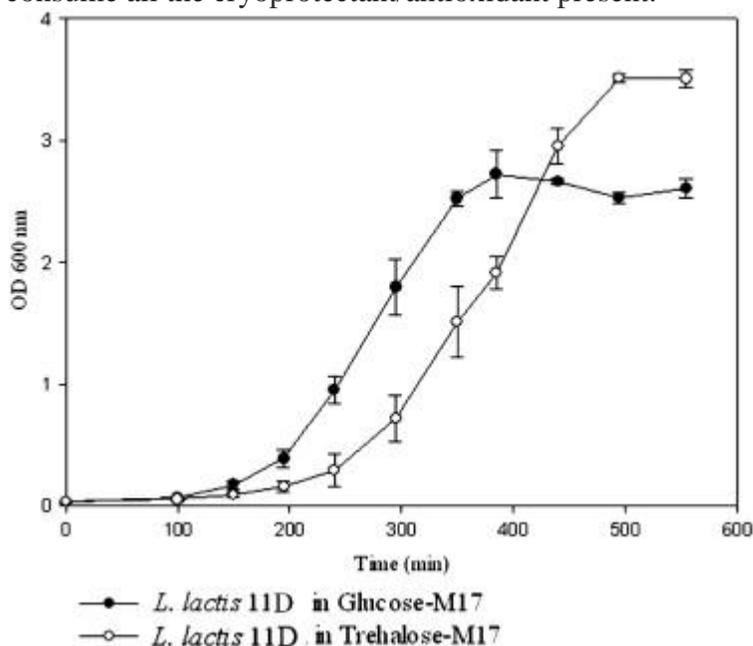

Fig. 3.

Lactococcus lactis 11D growth curves in glucose-M17 and trehalose-M17: trehalose stimulated growth of the strain (white dots), although the growth rate was lower as compared with the control (glucose, black dots).

Figure options

A comparative proteomic analysis was performed by DIGE to detect different extracellular protein expression between the growth of *L. lactis* 11D on trehalose and the control (glucose). The comparative image analysis of the DIGE gels revealed 72 differentially expressed spots: 35

spots were down-regulated and 37 spots were up-regulated in trehalose-M17 (Fig. 4). A significant number of spots were faint and very difficult to identify by mass spectrometry, successful hits were obtained for 8 spots. Thus five down-regulated and three up-regulated proteins were identified with statistical significance (Appendix A Tables 2–3). In spite of these limits, DIGE proved to be a sensitive method for spot detection, as expected (Marouga, David, & Hawkins, 2005).
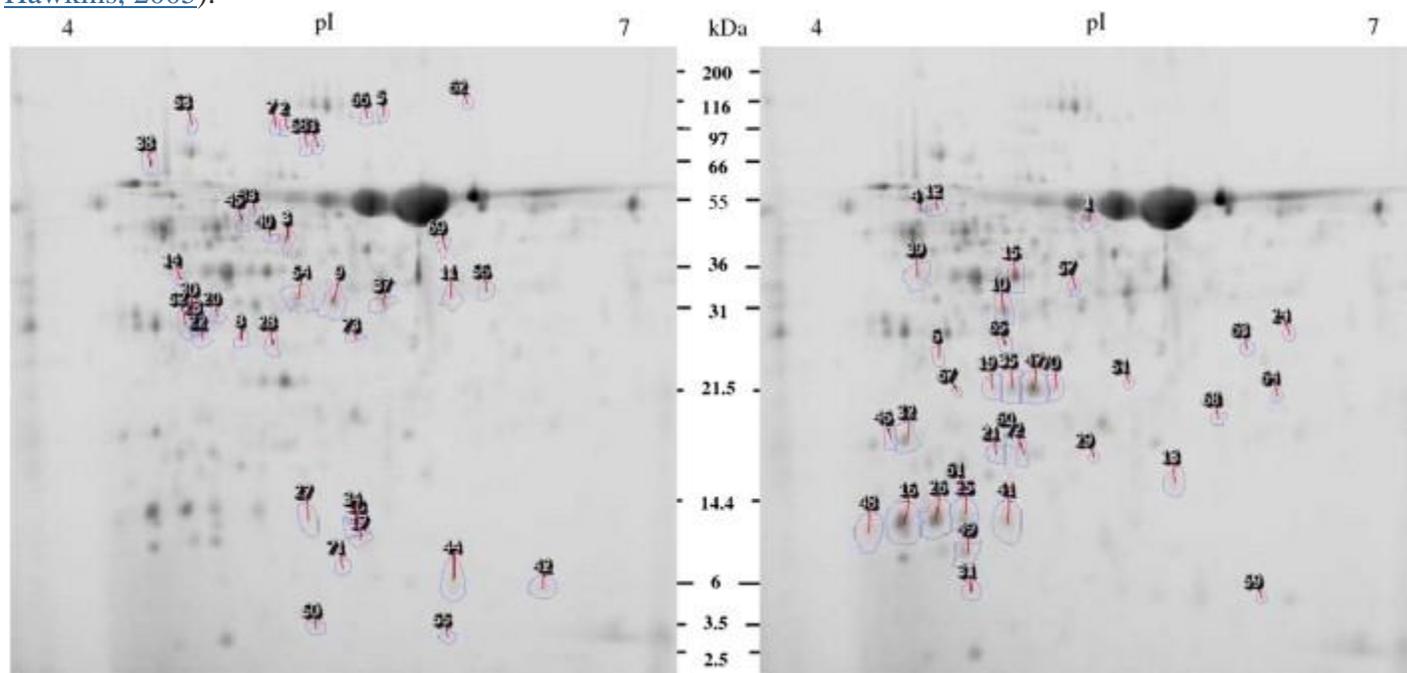

Fig. 4.

Down-regulated (left) and up-regulated (right) spots in trehalose-M17. Numbers indicate spots with a different expression ratio among the two tested conditions higher than 1.5 fold and with an ANOVA $p < 0.05$.

It is worth noticing that even in presence of trehalose no known virulence factor was up-regulated in the acidic exoproteome indicating that as regards the analyzed proteins, the use of this cryoprotectant seems not to enhance risks for human health.

Chitinase proved to be up-regulated in *L. lactis* 11D grown on trehalose suggesting that this carbon source, commonly used in freeze-dry processes, can enhance the antagonistic potential of the bacterial strain against fungi. Moreover, the growth in trehalose seems to attenuate adhesin expression, as demonstrated by GroEL and FBA down-regulation.

4. Conclusions

The selection of bacteria to be directly employed as starters in the artisanal cheese matrix opens new possibilities for improving organoleptic properties, texture and added value of food, provided that a reliable safety control is carried out. Several analyses to verify the safety of a strain for human health must be performed especially in the light of several pathogenic pathways being strain-specific and not species-specific. An example of this is the controversial interaction between bacteria and plasminogen, which can lead to proteolytic cascades activation. This suggests the need of an accurate typing of extracellular proteomes of starter strains, especially for

those able to bypass the gastric barrier that can reach and interact with the gut epithelium. In the present study the acidic exoproteome of *L. lactis* 11D was analyzed and shows potential to become a relevant tool for bacterial strain-typing to ensure bacterial safety.

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