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Adaptive response of *Lactobacillus sakei* 23K during growth in the presence of meat extracts: A proteomic approach

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ABSTRACT

Lactobacillus sakei is a lactic acid bacterium mainly found in meat and meat products. In order to understand the factors favoring its adaptation to meat matrix, growth parameters and survival of the strain *L. sakei* 23K in the presence of sarcoplasmic or myofibrillar extracts were assessed. Cytosolic proteins putatively involved in the response of this strain to meat proteins were determined using 2D electrophoresis and the significantly regulated proteins were identified by Maldi Tof-MS analyses. From the 31 differentially expressed spots, 16 occurred in the presence of myofibrillar extract while 6 proteins were modulated by the sarcoplasmic extract. Two dipeptidases were overexpressed in the presence of sarcoplasmic proteins, in correlation to the protein degradation patterns obtained by SDS-PAGE. In the presence of the myofibrillar extract, *L. sakei* 23K overexpressed proteins related to energy and pyrimidine metabolism as well as ala- and tyr-tRNA synthetases, involved in translation, while others corresponding to general stress response, pyrimidine, vitamin and cofactor biosynthesis were down-regulated. The supplementary nutrients furnished by meat extracts modulated the overexpression of proteins related to translation, peptide/amino acid metabolism and energy production while the stress proteins were under regulated. The results obtained here suggest that meat proteins would not represent a stress environment *per se* for *L. sakei* 23K in contrast to the harsh conditions during meat processing. This study has extended the understanding of the molecular responses and growth mechanisms of *L. sakei* 23K in the presence of meat proteins. The transference of genomic information into useful biological insight is an important step for the selection of well-adapted strains for the achievement of high-quality fermented products.

1. Introduction

Lactobacillus sakei is a ubiquitous lactic acid bacterium commonly associated with the food environment. Although this organism was isolated from various fermented vegetables such as sauerkraut and kimchi (Choi et al., 2003; Vogel et al., 1993) and fish products (Najjari et al., 2008), it was mostly isolated from the meat environment (Chenoll et al., 2007; Fontana et al., 2005, 2006). *L. sakei* represents the major population of many fermented meat products and vacuum-packaged raw meats and is recognized as an important component of the starter cultures used for fermented sausage production in Western Europe (Vignolo et al., 2010). In addition, *L. sakei* has been found to produce bacteriocins and consequently it has been used as bioprotective culture to preserve fresh and processed meat and fish (Castellano et al., 2008; Katla et al., 2001). More recently, it was shown that this species is also a transient member of the human gastrointestinal microbiota (Chiaramonte et al., 2009; Dal Bello et al., 2003).

Due to the ubiquitous occurrence, *L. sakei* displays notable differences in physiological and biochemical properties compared to other lactobacilli (Chaillou et al., 2005). The prevalence of *L. sakei* in a variety of habitats indicates its potential to adapt and/or to compete in such different ecosystems. Ecologically, meat represents a diverse and changing environment that influences the growth potential of different bacterial species during storage (Labadie, 1999). As bacterial substrate, meat represents a relatively poor source of carbohydrates but an important source of proteins. During ageing, endogenous proteolytic enzymes still act releasing amino acids from meat proteins. Consistently, *L. sakei* adaptation to meat has led to a lack of bacterial biosynthetic pathways for amino acid synthesis; this species being auxotrophic for all amino acids except for aspartic and glutamic acids (Champomier-Vergès et al., 2002a,b). Moreover, this species has psychrotrophic and osmotolerant properties and is able to grow at low temperatures as well as in 10% NaCl concentrations; these

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features being associated with the presence of genes coding for stress response proteins in *L. sakei* genome (Marceau et al., 2004). The analysis of *L. sakei* 23K genome revealed potential survival strategies as well as metabolic properties enabling it to effectively compete in the raw-meat environment (Chaillou et al., 2005). It has been argued that niche-specific genes showing greater expression in a particular ecosystem are more likely to contribute to better ecological performance than those equally expressed in a range of environments. A transcriptomic approach dealing with the detection of *L. sakei* 23K genes specifically induced during meat fermentation has been reported by Hüfner et al. (2007). As consequence, intraspecies genomic diversity may be required for successful adaptation in this complex habitat; the extent of *L. sakei* intraspecies variation has been recently reported (Chaillou et al., 2009).

Despite of the huge amount of DNA information provided by the completed genome sequencing projects, the biological function of the proteins encoded by the detected genes still remain to be revealed. Indeed as the next step in the post genomic era, proteomics focuses on the functionality of these genes contributing to establish the connection between genome sequences and their biological role. The introduction of bioinformatics to extract information from these genetic data constitutes a key tool of the post genomic era. In combination with transcriptional profiling expression, proteomics provides access to interesting candidate genes and proteins that can be further characterized. For lactic acid bacteria (LAB), cold adaptation and salt stress challenges have been illuminated by this approach (Cotter and Hill, 2003; van de Guchte et al., 2002). Moreover, a proteomic approach has been undertaken to elucidate adaptation mechanisms of *L. sakei* 23K to the fermented sausage environment by studying various stress factors such as high NaCl concentrations or cold temperatures (Champomier-Vergès et al., 2002a).

The knowledge of bacterial response to a specific environment is of paramount importance in the selection of the most appropriate functional starter cultures. The aim of this work was to evaluate the response of *L. sakei* 23K during growth on meat sarcoplasmic and myofibrillar proteins to better understand their impact on bacterial adaptation to the meat environment by using a proteomic approach.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Lactobacillus sakei 23K, originally isolated from a French sausage, and plasmid cured is used as model strain (Berthier et al., 1996). A single colony was grown in MRS broth at 30 °C for 24 h, transferred to a chemical defined medium (CDM) (Lauret et al., 1996) and then grown at 30 °C for 16 h. This subculture was used to inoculate 250 mL of CDM containing sarcoplasmic (S-CDM) or myofibrillar (M-CDM) extracts at an initial $OD_{600} = 0.1$. A culture grown in CDM without meat extracts was used as control (C).

2.2. Meat protein preparation

Ten grams of meat (*Longissimus dorsi*) was homogenized with sterile phosphate buffer 20 mM (pH 6.0), 1:10 (w/vol) in a laboratory blender (Stomacher 400 London, UK) for 2 cycles of 4 min each. The supernatant obtained after centrifugation (10,000 *g*, for 20 min at 4 °C) containing the soluble fraction was filter sterilized (Steritop GP, BIOPORE, Buenos Aires, Argentina) and constituted the sarcoplasmic extract. Myofibrillar proteins were extracted from the pellet using a high-ion strength phosphate buffer (0.1 N Na_2HPO_4–NaH_2PO_4; 0.7 M KI; pH 6.0 containing 0.2 g/L sodium azyde) as reported previously (Sanz et al., 1999). Separately, both meat extracts were incubated at 30 °C during 5 days to allow endogen proteolysis to occur and then lyophilized and stored at 4 °C until used. The mixture of peptides and proteins obtained was independently used for CDM supplementation. Sarcoplasmic and myofibrillar lyophilized extracts were added to CDM medium at 0.19 g/L and 0.075 g/L of protein final concentration, respectively.

2.3. Bacterial growth and survival

Growth of *L. sakei* 23K cultured microaerobically in CDM at 30 °C during 72 h was followed by measuring OD_{600} and cell viability. Growth rates (μ) were estimated from the growth curve by fitting the data to the Eq. (1):

$$\mu = \mathrm{Ln}N_t - \mathrm{Ln}\ N_0 / \Delta t \quad (1)$$

μ: growth rate.

N_t and N_0 are the cell densities at time t_n and time zero on exponential phase, respectively.

For bacterial enumeration, decimal dilutions were prepared, plated on MRS agar (Merck, Darmstadt, Germany) and incubated at 30 °C for 48 h. Sample pH was determined by using a Metrohn 692 pH/Ion Meter (Metrohn Ltd, Herisau, Switzerland). Three independent cultures were used for each condition.

2.4. Sample preparation and SDS-PAGE

Cultures of 0 and 72 h were centrifuged (10,000 *g* for 15 min at 4 °C) and supernatants were subjected to protein precipitation with cold acetone (MERCK, Darmstadt, Germany) (1:3 v/v) and stored overnight at −20 °C. Protein pellet was separated by centrifugation (10,000 *g*, for 10 min at 4 °C), air-dried and solubilized with sample buffer containing glycerol, SDS, β-mercaptoethanol (SIGMA Chemical CO, St. Louis, MO, USA), 0.5 M Tris–HCl pH 6.8 and brome phenol blue (BIORAD, Richmond, UK). These protein samples were subjected to SDS-PAGE analysis using a Mini Protean 3 gel Unit (BIORAD; Richmond, CA) on 12% (w/v) polyacrylamide gels (Laemmli, 1970). Wide range protein markers (from 212 to 6.5 kDa) were used as molecular weight standards (BioLabs Inc, Hitchin Herts, UK). Proteins were visualized by Biosafe colloidal Coomassie blue (BIORAD; Richmond, CA) according to the manufacturer's instructions.

2.5. Cell-free proteins extraction

Two hundred and fifty milliliter-cultures grown to mid-exponential phase ($OD_{600} \cong 0.8$, 4.5 h for C and S-CDM, and 7 h for M-CDM) were centrifuged (4500 *g* for 10 min at 22 °C) and washed twice in 0.1 M Tris–HCl buffer, pH 7.5, for 15 min, centrifuged and frozen at −80 °C until cell lysis. Bacterial pellets were resuspended in 5 mL of 1 M Tris–HCl buffer, pH 7.5, and cells were broken by a single pass through a cell disrupter (Basic Z; Constant Systems Ltd., Daventry, UK) at 2.5×10^5 Pa. Unbroken cells and cell debris were removed by centrifugation at 4500 *g* for 15 min at 4 °C. Membrane vesicles were discarded from the solution by ultracentrifugation at 50,000 *g* for 30 min at 4 °C. Protein concentration was estimated using the Bradford method (Bradford, 1976) according to the manufacturer's instructions (Coomassie Protein Assay Reagent; Pierce Biotechnology, Rockford, IL). Aliquots of 350 µg of proteins were stored at −80 °C until isoelectrofocusing assay.

2.6. 2D electrophoresis

Sample preparation and 2D electrophoresis gels were carried out according to Sánchez et al. (2005). Isoelectrofocusing strips of pH 4.0 to 7.0 (BIORAD), were rehydrated for 12 h at 50 V using the Protean IsoElectric Focusing Cell II (BIORAD) and then focused at 60,000 V/h. The second dimension was performed by SDS-PAGE on gels containing 12.5% polyacrylamide and carried out with a BIORAD Protean II xi cell. Proteins were resolved overnight at a constant current of 10 mA/gel at 4 °C. Gels were stained with Biosafe colloidal Coomassie blue (BIORAD) and scanned with an Image Scanner (Amersham Biosciences, Piscataway, NJ,

USA). Spot detection and volume quantification were carried out with ImageMaster 2D Elite (version 3.10; Amersham Biosciences). At least three independent experiments for each growth condition were performed.

2.7. Protein identification by peptide mass fingerprinting

Stained proteins displaying significant and reproducible intensities that differed among the tested growth conditions were excised from the gels and submitted to tryptic digestion and then to mass spectrometry analyses as described previously (Guillot et al., 2003). MS-Fit (University of California, San Francisco, Mass Spectrometry Facility; http://prospector.ucsf.edu) and Mascot (Matrix Science Inc., Boston, MA; http://www.matrixscience.com/search_form_select.html), installed locally, were used to identify proteins from peptide mass fingerprints. All searches were performed against the database for *L. sakei* 23K from the annotated genome (http://migale.jouy.inra.fr/sakei/genome-server).

3. Results

3.1. Growth of L. sakei 23K in CDM supplemented with sarcoplasmic and myofibrillar proteins

L. sakei 23K showed similar growth rates either in non-supplemented CDM (C) or in myofibrillar- (M-CDM) and sarcoplasmic- (S-CDM) supplemented CDM; the observed μ values being in a range of 0.24 to 0.28 h^{-1}. However, after 24 h incubation *L sakei* exhibited higher OD_{600} values in C and S-CDM than in M-CDM (Table 1). At this time, cell viability was 7.81, 6.20 and 7.63 log CFU/mL for C, S-CDM and M-CDM respectively; a dramatic decrease in cell count in C and no survival in sarcoplasmic and myofibrillar protein-containing media were observed after this period. The absence of viable cells together with the deep OD_{600} decrease registered after 24 h in M-CDM were probably due to cell lysis. A decrease in pH values from 6.2 to approximately 4.5, indicating an active metabolism during the first 24 h, was obtained in the three assayed conditions.

3.2. Metabolism of meat proteins during L. sakei 23K growth

The proteolytic activity of *L. sakei* 23K as well as that of the muscle enzymes was evaluated on S- and M-CDM at 30 °C for 72 h and analyzed by SDS-PAGE. No bands were obtained in non-supplemented and non-inoculated CDM (C) from 0 to 72 h (Fig. 1, lanes 1–2), while in the presence of *L. sakei* 23K, the appearance of faint bands were detected (Fig. 1, lane 3). As expected, when CDM was supplemented with S and M extracts, protein profiles revealed the presence of additional bands corresponding to added meat proteins (Fig. 1, lanes 4–6 and 8–10). Even when no major changes occurred on sarcoplasmic proteins in non-inoculated media (Fig. 1, lane 5), new bands of approximately 67 and 22 kDa were generated at 72 h as a result of *L. sakei* 23K hydrolytic activity (Fig. 1, lane 6). Conversely, new bands between 134 and 42 kDa arisen from myofibrillar proteins were observed in absence of bacterial inoculum (Fig. 1, lanes 8–9), whereas a richer band pattern was registered in the presence of *L. sakei* (Fig. 1, lane 10). These data suggest not only the occurrence of endogenous proteolysis but also bacterial protein hydrolysis during myofibrillar degradation; additional bands may be likely attributed to bacterial lysis.

Table 1
Growth and pH measurements of *L. sakei* 23K on CDM containing meat extracts at 30 °C during 72 h.

Cultures	Time (h)	CD_{600}	Log CFU/mL	pH
C[a]	0	0.10 ± 0.05	7.39 ± 0.07	6.23 ± 0.12
	6	1.27 ± 0.09	8.32 ± 0.05	4.60 ± 0.10
	24	1.80 ± 0.08	7.81 ± 0.04	4.47 ± 0.06
	48	1.74 ± 0.09	3.00 ± 0.04	4.47 ± 0.02
	72	2.03 ± 0.09	1.48 ± 0.02	4.48 ± 0.05
S[b]	0	0.10 ± 0.07	8.04 ± 0.07	6.21 ± 0.07
	6	1.60 ± 0.09	8.60 ± 0.05	4.58 ± 0.09
	24	1.85 ± 0.07	6.20 ± 0.07	4.43 ± 0.12
	48	1.79 ± 0.09	0.00	4.43 ± 0.10
	72	2.09 ± 0.08	0.00	4.43 ± 0.02
M[c]	0	0.12 ± 0.09	7.49 ± 0.04	6.11 ± 0.08
	6	0.65 ± 0.09	9.13 ± 0.06	5.82 ± 0.07
	24	1.00 ± 0.08	7.63 ± 0.05	4.89 ± 0.03
	48	0,69 ± 0.08	0.00	4.87 ± 0.02
	72	0.59 ± 0.09	0.00	4.88 ± 0.10

[a] C; CDM without meat proteins.
[b] S: CDM supplemented with sarcoplasmic.
[c] M: CDM supplemented with rnyofibrillar proteins.

3.3. Proteomic patterns of L. sakei 23K during growth on meat extracts

Proteins putatively involved in the response of *L. sakei* 23K to the presence of meat proteins were determined by comparison of protein cytosolic patterns of mid-log phase cells grown at 30 °C, in the absence or presence of meat extracts. The genome data of *L. sakei* 23K 1.8-Mb chromosome (Chaillou et al., 2005) were used to assign genes encoding proteins experimentally obtained by comparison of their peptide mass fingerprinting.

Comparison of 2D protein patterns revealed a total of 31 spots clearly displaying differential expression (Fig. 2). In the presence of myofibrillar extract, 16 proteins were differentially expressed from which, 5 were overexpressed (spots 23F, 17, 24, 13, 20) and 11 were synthesized in lower amounts (spots 16, 19 = 28, 18, 26, 27, 1 = 15 = 22 = 25, 23, 14, 9, 21, 29). When *L. sakei* was grown on sarcoplasmic proteins, 6 spots showed intensity variation; 3 proteins were up-regulated and 3 were under-shifted. Comparative data for identified spots is given in Table 2. The identified spots were assigned to different functional categories, namely energy, lipid, cofactor/vitamin, pyrimidine/purine and amino sugar metabolisms as well as cellular processing and signaling, translation, genetic information, general stress and other cell processes. Different spots were identified as the same protein (1, 15, 22, 25 corresponding to cytidine triphosphate synthase and spots 19, 28 as 3-glycerol phosphate dehydrogenase) proving the existence of protein isoforms derived either as artifacts due to sample manipulation and separation, or stemming from post translational modifications.

Protein overexpression during *L. sakei* 23 K growth on myofibrillar extract involved 2 proteins related to energy metabolism (6-phosphofructokinase, spot 23F and 5-phosphate epimerase, spot 17) which catalyze carbohydrate (glucose, fructose, and mannose) hydrolysis and the conversion of ribose 5P into ribulose 5P in the pentose phosphate pathway, respectively. Moreover, spot 24 identified as aspartate carbamoyltransferase catalytic subunit was also up-regulated on M-CDM; this enzyme catalyzes the conversion of N-carbamoyl-L-aspartate to carbamoyl phosphate in pyrimidine pathway and it is also involved in the alanine–aspartate–glutamate metabolism (Fig. 3). However, other related enzymes from pyrimidine metabolism such as dihydroorotase and carbamoyl-phosphate synthase (*pyr*AA and *pyr*AB) codified by genes of *pyr* operon were not differentially expressed or were under regulated, as cytidine triphosphate synthase (spot 1) in M-CDM and S-CDM. Myofibrillar extract also promoted the overexpression of alanyl- and tyrosyl-tRNA synthetases (spots 13 and 20) assigned to translation processes. In addition, 30S ribosomal protein S1, a translation factor and two different dipeptidases from U34 family, (spots 2 and 3) involved in signaling and synthesis of structural molecules and peptide hydrolysis, were synthesized in higher amounts during growth of *L. sakei* 23K on sarcoplasmic proteins.

On the other hand, many other proteins were underexpressed in the presence of meat extracts (Table 2). When *L. sakei* was grown on M

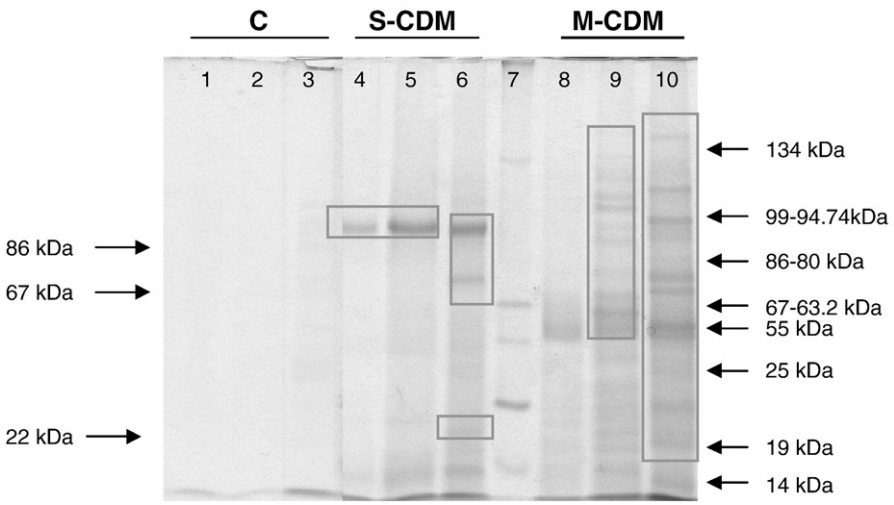

Fig. 1. SDS-PAGE of CDM supernatants (C, S-CDM and M-CDM) inoculated or not with *L. sakei* 23K and incubated during 72 h at 30 °C. Lane 1: C (T0); lane 2: C (T72); lane 3: C + *L. sakei* (T72); lane 4: S-CDM (T0); lane 5: S-CDM (T72); lane 6: S-CDM + *L. sakei* (T72); lane 7: MWm; lane 8: M-CDM (T0); lane 9: M-CDM (T72) and lane 10: M-CDM + *L. sakei* (T72).

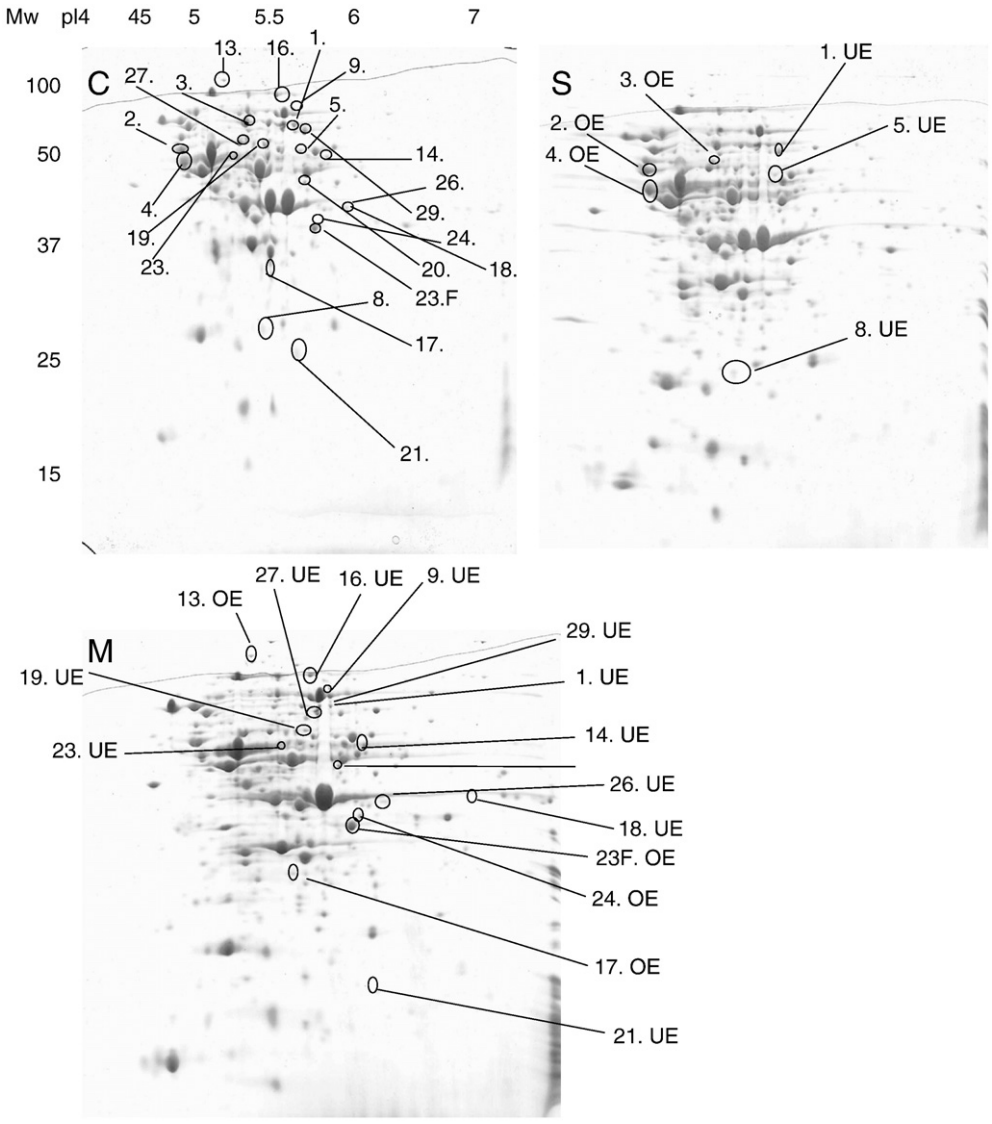

Fig. 2. Proteomes of *L. sakei* 23K during growth in the presence or absence of meat proteins. 2D gels showing intracellular protein expression of mid-log phase *L. sakei* 23K cells on CDM (C), supplemented with sarcoplasmic (S) and myofibrillar (M) proteins. OE overexpressed; UE underexpressed.

Table 2
Identified *L. sakei* 23K proteins significantly modified during growth on CDM supplemented with sarcoplasmic (S-CDM) or myofibrillar (M-CDM) protein extracts.

Functional category	Spot-putative function[b]	Gene	Accession no.	Mass (kDa)	p1	MOWSE[c]	Protein expression levels relative to C (mean spot ± SEM)[a]			Comments
							C	S-CDM	M-CDM	
Energy metabolism: Glycolysis,	23F. 6-phosphofructokinase	*pfk*	Lsa1033	34.35	5.36	3.40 E + 12	1.00 ± 0.09		1.35 ± 0.06	QE[d] in M
Energy metabolism: Methane fixation	16. Putative phosphoketolase	*xpk*	Lsa0289	88.70	5.14	7.60 E + 18	1.00 ± 0.09		0.40 ± 0.08	UE[e] in M
Pentose phosphate pathway, Carbon fixation	17. Ribose 5-phosphate epimerase	*rpiA*	Lsa1685	24.97	5.02	1.21 E + 06	1.00 ± 0.08		1.38 ± 0.10	OE in M
Lipid metabolism: Glycerophospholipid metabolism	19 = 28. Glycerol-3-phosphate dehydrogenase	*glpD*	Lsa0650	66.78	5.13	4.80E + 18	1.00 ± 0.10		0.57 ± 0.08	UE in M
Cofactors and Vitamins: pantothenate and CoA biosynthesis	18. 2-dehydropantoate 2-reductase	*panE*	Lsa0041	33.33	5.62	4.15 E + 02	1.00 ± 0.10		0.16 ± 0.05	UE in M
Other cell processes: Zinc ion binding	26. Putative zinc-containing alcohol dehydrogenase (oxidoreductase)	*LSA1395*	Lsa1395	33.64	5.51	5.32 E + 03	1.00 ± 0.10		0.26 ± 0.08	UE in M
Cell wall formation: Glycan biosynthesis; peptidoglycan biosynthesis.	27. UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	*murD*	Lsa0747	49.54	5.07	1.40 E + 11	1.00 ± 0.09		0.031 ± 0.05	UE in M
Pyrimidine metabolism	1 = 15 = 22 = 25. Cytidine triphosphate synthase	*pyrG*	Lsa1629	58.99	5.28	1.67 E + 13	1.00 ± 0.10	0.61 ± 0.05	0.59 ± 0.08	UE in M/S
	24. Aspartate carbamoyltransferase catalytic subunit	*pyrB*	Lsa0952	34.33	5.38	4.32 E + 08	1.00 ± 0.10		2.41 ± 0.11	OE in M
	5. Inosine-5-monophosphate dehydrogenase	*guaB*	Lsa0276	52.30	5.39	2.61 E + 11	1.00 ± 0.10	0.025 ± 0.08		UE in S
Cellular processing and signaling: membrane and intracellular structural molecules	3. Dipeptidase D-type (U34 family)	*pepD5*	Lsa0897	53.26	5.03	4.58 E + 04	1.00 ± 0.10	2.66 ± 0.08		OE in S
	2. Dipeptidase D-type (U34 family)	*pepD2*	Lsa0196	54.05	4.97	1.86 E + 06	1.00 ± 0.09	1.73 ± 0.1		OE in S
Translation	4. 30 S Ribosomal protein S1	*rpsA*	Lsa1017	43.91	4.61	1.10 E + 08	1.00 ± 0.10	2.67 ± 0.07		OE in S
	23. Elongation factor Tu	*tuf*	Lsa1063	43.28	4.72	7.39 E + 09	1.00 ± 0.09		0.35 ± 0.07	UE in M
	13. Alanyl-tRNA synthetase	*alaS*	Lsa0387	97.15	4.81	1.70 E + 08	1.00 ± 0.10		1.67 ± 0.1	OE in M
	20. Tyrosyl-tRNA synthetase	*tyrS*	Lsa769	47.42	5.32	7.10 E + 12	1.00 ± 0.10		2.16 ± 0.08	OE in M
Genetic Information Processing: chaperones and folding catalysts; Sorting and Degradation	14. ATP-dependent Hsl protease, ATP-binding subunit HslU	*hslU*	Lsa0984	52.99	5.35	6.3 E + 05	1.00 ± 0.07		0.05 ± 0.02	UE in M
	8. Methionine sulfoxide reductase	*msrA*	Lsa0886	19.56	5.12	4.66 E + 02	1.00 ± 0.05	0.45 ± 0.07		UE in S
General Stress	9. ATPase/chaperone ClpE, putative specificity factor for ClpP protease	*clpE*	Lsa1465	79.69	5.25	4.63 E + 11	1.00 ± 0.1		0.123 ± 0.08	UE in M
	21. Similar to universal stress protein, UspA family	*Usp1*	Lsa0042	17.19	5.54	2.37 E + 05	1.00 ± 0.10		0.220 ± 0.10	UE in M
Amino sugar metabolism: Glutamate, Aminosugars	29. Glucosamine-fructose-6-phosphate aminotransferase	*glmS*	Lsa1355	65.97	5.26	3.73 E + 13	1.00 ± 0.10		0.14 ± 0.08	UE in M

[a] Statistical differences were significant at a P value of <0.05.
[b] The quantities of the spots in each medium are shown as the means ± standard error of the means (SEM). Quantities are scaled to set the average quantity in CDM without meat extracts (C) to 1.00.
[c] Molecular weight search score.
[d] OE: overexpressed.
[e] UE: underexpressed.

medium, putative phosphoketolase (spot 16), glycerol-3-phosphate dehydrogenase (spots 19 and 28) and 2-dehydropantoate 2-reductase (spot 18), enzymes from energy, lipid and cofactor/vitamin metabolisms, respectively, were underexpressed. Proteins from other cell processes (putative zinc-containing alcohol dehydrogenase, spot 26); cell wall formation (UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase, spot 27); translation (elongation factor Tu, spot 23); genetic information (ATP-dependent protease ATP-binding subunit HslU; spot 14); general stress (ATPase/Chaperone ClpE, putative specificity factor for ClpP protease, spot 9; similar to universal stress protein, UspA family, spot 21) and amino sugar metabolism (glucosamine-fructose-6-phosphate aminotransferase, spot 29) were also underexpressed in the presence of myofibrillar proteins. Cytidine triphosphate synthase (spot 1), from pyrimidine metabolism, was underexpressed in both S and M supplemented media. In addition, inosine-5-monophosphate dehydrogenase (spot 5) and methionine sulfoxide reductase (spot 8) involved in pyrimidine metabolism and genetic information respectively were under-produced by *L. sakei* grown on the sarcoplasmic protein-containing medium.

4. Discussion

L. sakei 23K strategies to grow and survive on a Chemically Defined Medium (CDM) containing meat proteins, regardless other stressful technological stimuli such as high salt content, low temperature or redox potential changes, were investigated here for the first time. This analysis was undertaken on CDM separately supplemented with lyophilized sarcoplasmic and myofibrillar protein extracts and compared with the unsupplemented CDM. The impaired survival experienced by *L. sakei* after 24 h in CDM cannot be explained due to depletion of limiting factors in the medium as previously reported by Champomier-Vergès et al. (1999). Contrary to what happens in rich environments such as CDM with meat extracts, stressful conditions shuts off most metabolic activities in *L. sakei* committing it to

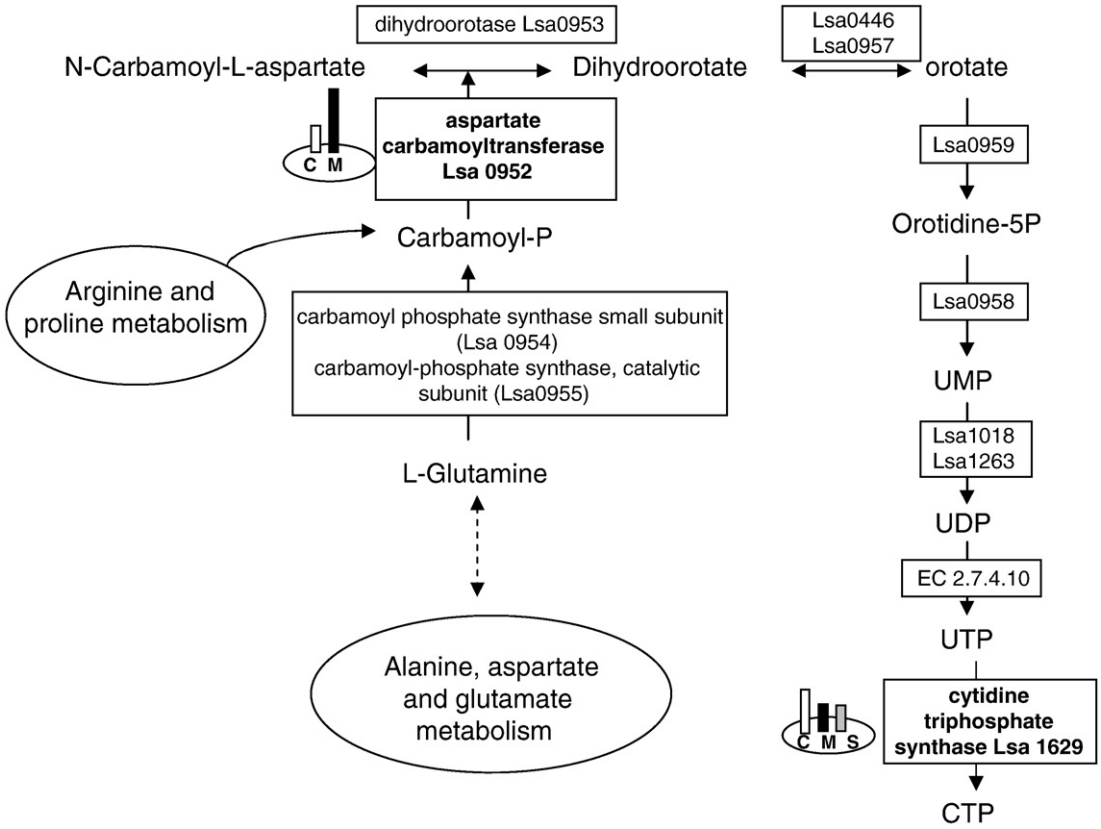

Fig. 3. Pyrimidine metabolism. Lsa0446: dihydroorotate dehydrogenase 1A, Lsa0957: dihydroorotate oxidase, catalytic subunit; Lsa0954: carbamoyl phosphate synthase small subunit; Lsa0955: carbamoyl-phosphate synthase, catalytic subunit; Lsa0958: orotidine 5′-phosphate decarboxylase; Lsa 0959: orotate phosphoribosyl transferase; Lsa1629: cytidine triphosphate synthase; EC 2.4.2.10: nucleoside triphosphate adenylate kinase; Lsa1018: cytidylate kinase; Lsa1263: uridylate kinase; UMP: uridine monophosphate; UDP: uridine diphosphate; UTP: uridine triphosphate; CTP: cytidine triphosphate.

particular adaptive strategies towards survival (Marceau et al., 2003). Indeed, CDM supplemented with meat extracts cannot be compared to meat as an optimal growth substrate for this meat-borne strain. In this sense, Fadda et al. (2008) reported previously that a 15 day-cell viability was attained by *L. sakei* 23K when incubating the strain on meat surfaces at refrigeration temperatures.

SDS-PAGE profiles showed *L. sakei* proteolytic activity on both, S- and M-CDM supernatants at 72 h. *L. sakei* proteolytic system played a major role on sarcoplasmic protein degradation, fact that was in agreement with previous proteolytic studies using this strain (Fadda et al., 2008). As regard as the myofibrillar proteolytic pattern, the endogenous activity observed was in accordance with previous findings by Sanz et al. (1999). In addition, these results correlate well with the highest amino acids release caused by muscle peptidase activity on M-CDM after 24 h (data not shown). However, the myofibrillar profile not only exhibited endogenous and *L. sakei* 23K hydrolytic activities but the presence of potential cell lysis products, this phenomenon being supported by the appearance of additional faint bands (Fig. 1, lane 10) and the decrease in OD_{600} values after 24 h on M-CDM (Table 1). Moreover, microbial proteins were identified by MS analyses from SDS-PAGE excised bands after incubation of *L. sakei* 23K on myofibrillar extract at 25 °C during 4 days (Fadda et al. unpublished data).

To unravel the functions involved in *L. sakei* 23K response to the presence of meat proteins, a proteomic approach was applied. Even weak, the simultaneous induction of 6-phosphofructokinase (Lsa1033) and ribose 5-phosphate epimerase (Lsa1685) in the presence of myofibrillar proteins may be related to the ability of *L. sakei* to ferment glucose and ribose, the two more common sugars in meat. Similarly, Koistinen et al. (2007) recently reported the simultaneous expression of proteins associated with different carbohydrate pathways in two *L. plantarum* strains and attributed them to adaptive purposes. Molecular analyses of the ribose gene cluster in *L. sakei* 23K revealed a strong correlation between these two sugar pathways due its chimeric conformation (Champomier-Vergès et al., 2002a). In previous studies, Marceau et al. (2004) observed that the enzyme 6-phosphofructokinase was negatively affected in *L. sakei* 23K under stressful conditions. Contrarily, this enzyme was induced under the mild conditions used in this study.

Proteins involved in translation such as Lsa0387; Lsa769 and Lsa1017 were also overexpressed in the presence of meat extracts; which reflects the accessibility of supplementary nutrients (peptides, amino acids, nucleotides and cofactors) that would stimulate protein synthesis. Coincidently, a lower expression of proteins involved in vitamin, cofactor and pyrimidine biosynthesis was registered on M-CDM and S-CDM, as observed for 2-dehydropantoate 2-reductase (Lsa0041) and cytidine 3-phosphate-synthase (Lsa1629) (Fig. 3). In this regard, it was reported that the induction of three polypeptides from pyrimidine pathway only occurred in absence of uracil (Marceau et al., 2001). Since this nucleotide may be provided by meat extracts, down regulation of pyrimidine biosynthesis may be expected. Nevertheless, aspartate carbamoyltransferase catalytic subunit (Lsa0952) involved in pyrimidine biosynthesis and in aspartate–alanine–glutamate metabolism was up-shifted, probably due to an active amino acid metabolism rather than *de novo* pyrimidine biosynthesis.

On the other hand, enzymes involved in peptide hydrolysis were synthesized in high amounts by *L. sakei* 23K grown on S-CDM. The overexpressed dipeptidases (Lsa0897 and Lsa0196) are cysteine-type, N-terminal nucleophile (Ntn)-hydrolases from U34 family peptidases, which are remote homologs of penicillin V acylases, sharing similar catalytic machinery but with a great sequence diversity to catalyze

reactions involving hydrolysis of amide bonds (Pei and Grishin, 2003). A similar peptidase, the AP4, has been characterized from the meat-borne *L. sakei* CECT 4808 strain (Sanz and Toldrá, 2002), as well as in other LAB from different origin (Goldstein et al., 2002; Seo et al., 2007). In our study, the maximum expression level of the two dipeptidases correlates well with the extra peptide source present in the S medium which could promote their metabolism; this being in accordance with the sarcoplasmic protein hydrolysis carried out by *L. sakei* 23K after 72 h (SDS-PAGE analyses, Fig. 1, lane 6). In fact, *in vitro* and *in situ* proteolytic activity for different *L. sakei* strains towards sarcoplasmic proteins have been reported in previous studies (Fadda et al., 1999, 2008).

In this study, proteins involved in stress response were under-expressed by *L. sakei* grown on myofibrillar proteins (Table 2). The activation of general stress response which allows the strain to withstand harsh conditions such as acid, oxidative, heat/cold, osmotic stress, and starvation have been reported (Hussain et al., 2009; Pieterse et al., 2005; Saarela et al., 2004). Even when stress proteins are normally expressed during the stationary phase, when carbon sources are exhausted and pH has considerably decreased, their induction was also reported during the lag-phase (Koistinen et al., 2007; Wu et al., 2009). Since the present study was carried out with log-phase cells that were not submitted to high salt concentration, extreme temperatures or starvation conditions, proteins involved in stress response, pyrimidine, vitamin and cofactor biosynthesis would be underexpressed. Among stress proteins, results showed that proteins from UspA family (Lsa0042) and methionine sulfoxide reductase (MsrA) (Lsa0886) related to general stress were repressed in M-CDM and S-CDM, respectively. It was reported that survival of *L. sakei* 23 K mutants for UspA and MsrA proteins, was reduced at low temperatures or in the presence of salt (Marceau et al., 2004). The lower induction of stress proteins in this study should have been responsible for *L. sakei* growth arrest after 24 h on S-CDM and M-CDM. It is noteworthy that stress proteins were already under regulated at mid-log phase when cell viability was still optimal. Consequently, stress protein regulation could be considered an early indicator of cell survival, demonstrating somehow a role of these proteins on *L. sakei* adaptive response.

A lower synthesis of glycerol-3-phosphate dehydrogenase (Lsa0650) involved in lipid metabolism and linked to membrane properties, was observed in M-CDM. An enhanced survival in a glycerol-3-phosphate dehydrogenase *L. sakei* mutant at 4 °C was attributed to a modified glycerol catabolic pathway (Marceau et al., 2004). In our study, this enzyme was synthesized in low amount on M-CDM and survival of *L. sakei* 23K was not positively affected in contrast to the findings reported by the former authors.

Summarizing, and by comparing results obtained on S- and M-CDM, a major role of myofibrillar extracts on *L. sakei* protein expression was observed as 16 differentially expressed spots were detected in contrast to only 6 proteins modulated by the sarcoplasmic extract. M-CDM exclusively regulated the expression of proteins related to energy, lipid and amino sugar metabolisms, cofactor and vitamin biosynthesis and cell wall formation, while cellular processing and signaling were modulated only by sarcoplasmic extracts. Nevertheless, most of cellular processes (pyrimidine metabolism, translation, genetic processing and stress) were affected by both meat extracts. When protein expression level was analyzed, the most strongly up- and down-regulated cellular processes were: cell wall formation, genetic information and pyrimidine metabolism by M-CDM; peptidases by S-CDM and translation by both media.

This study constitutes the first proteomic approach to understand *L. sakei* 23K growth response to the presence of meat proteins. The activity of muscle endogenous enzymes in cooperation with the up-regulated peptidases from *L. sakei* was demonstrated. Supplementary nutrients furnished by meat extracts were responsible for the overexpression of proteins involved in translation, peptide/amino acid metabolism and energy production. Conversely, the under-regulation of stress proteins in the presence of S- and M-CDM could play a role in cell death after 24 h.

As a whole, meat proteins would not represent a stress environment *per se* for *L. sakei* 23K, constituting a regulated state not subjected to a stress-induced response in contrast to the harsh conditions during meat processing (cold, salt, redox potential variations, etc.). Thus, technological conditions may be the main inducers for bacterial adaptive responses reported so far. A combination of proteomics and transcriptomics (focusing on the genes responsible for the affected enzymes), using a real meat system and some metabolic tests is currently ongoing in our laboratory to confirm or not the biological functions and mechanisms observed in the present work. The transference of genomic information into useful biological insight is an important step to achieve better product quality through the selection of well-adapted strains to particular niches.

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