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Response of *Listeria monocytogenes* to liquid smoke

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Abstract:

Aims: To investigate the effect of liquid smoke on growth, survival, proteomic pattern and haemolytic potential of Listeria monocytogenes.

Methods and Results: Growth and survival curves were recorded in brain–heart infusion broth supplemented with three concentrations of liquid smoke. L. monocytogenes growth was inhibited in the presence of 15 μg ml−1 phenol while a rapid decrease in cell viability occurred in the presence of 30 μg ml−1 phenol. The proteome of L. monocytogenes cytosoluble proteins was slightly modified after 2-h incubation with 30 μg ml−1 phenol but no protein already characterized in response to other known stresses was induced, except the protease ClpP. Liquid smoke inhibited the haemolytic potential without affecting hly gene expression, showing a potential inhibition of protein activity or stability.

Conclusions: The presence of liquid smoke in a rich medium strongly affected growth and survival of L. monocytogenes. Brief smoke stress affected the metabolic pathways and inhibited the haemolytic activity of L. monocytogenes.

Significance and Impact of Study: This study is a first step in the investigation of the influence of a smoked product on L. monocytogenes strains.

Keywords: haemolysis, liquid smoke, Listeria monocytogenes, proteome analysis, stress, survival

1. Introduction

Listeria monocytogenes can grow and multiply under harsh conditions which explains its ubiquity. It has been isolated from a wide variety of produce (Farber and Peterkin 1991),especially ready-to-eat (RTE) products. Raw products contain generally low concentrations of *L. monocytogenes* (Autio *et al.* 1999) and contamination occurs principally during processing (Vogel *et al.* 2001). The prevalence of *L. monocytogenes* in the RTE seafood products ranges from 6 to 36% and sometimes reaches 78% (Ben Embarek 1994; Eklund *et al.* 1995; Jorgensen and Huss 1998). This high prevalence could be explained by a combination of worker handling, extended shelf-life and processing, which sometimes does not include a listericidal step. Some listeriosis cases have been linked to seafood products like smoked mussels (Brett *et al.* 1998), shrimps (Riedo *et al.* 1994), imitation crab meat (Farber *et al.* 2000), gravad rainbow trout (Ericsson *et al.* 1997) and smoked rainbow trout (Miettinen *et al.* 1999). There is a paradoxical contrast between the high prevalence of *L. monocytogenes* in the seafood products and the few related cases of listeriosis, especially for lightly preserved products like cold-smoked fish. This could be partly explained by the low contamination levels of *L. monocytogenes* generally detected (<100 CFU g^{-1}). Moreover, some strains isolated in the smoked salmon industry may have an attenuated infectious potential (Norton *et al.* 2001; Gudmundsdóttir *et al.* 2006).
L. monocytogenes contaminating seafood products are submitted to technological stress conditions. Growth of *L. monocytogenes* has been largely studied in seafood (Jorgensen and Huss 1998) and modeled using media (Membré *et al.* 1997) or directly in cold-smoked fish (Gimenez and Dalgaard 2004; Cornu *et al.* 2006). Cold-smoking of fish includes salting and smoking and the fish are then stored at low temperatures*. L. monocytogenes* can grow at temperatures as low as -0·1°C (Walker *et al.* 1990) and in more than 10% NaCl (Farber and Peterkin 1991). The minimal water activity for growth of *L monocytogenes* is 0.90 (Nolan *et al.* 1992). Inhibition of *L. monocytogenes* growth in a synthetic medium has been observed with a phenol concentration in the smoke as high as 20 µg ml^{-1} (Membré *et al.* 1997). Proteomic responses of *L. monocytogenes* to cold and salt stresses have been studied (Bayles *et al.* 1996; Hébraud and Guzzo 2000; Duché *et al.* 2002) but the mechanisms by which these bacteria survive and overcome smoking have not yet been investigated. Traditional smokes and liquid smokes are known for their antimicrobial properties and some studies have described the inhibition of *L. monocytogenes* growth in the presence of smoke (Faith *et al.* 1992; Sunen 1998). Liquid smoke is obtained by hard wood combustion; smoke is then filtered and condensed. It contains acid, phenyl and carbonyl compounds having an effect upon food colour, flavour and preservation. Predictive models of growth on cold-smoked salmon now include phenol concentration, to which is commonly attributed the bacteriostatic activity of the smoke (Gimenez and Dalgaard 2004; Cornu *et al.* 2006). Proteome analysis of *Pseudomonas putida* exposed to sudden sub-lethal inhibitory concentrations of phenol (Santos *et al.* 2004) showed that the level of expression of 81 proteins was consequently modified including the over-expression of general and oxidative stress proteins. To date, no study has described the response of *L. monocytogenes* or other Gram-positive bacteria exposed to smoke. Our study is a first investigation of the effect of liquid smoke on the physiology of *L. monocytogenes*. We studied bacterial growth and survival of exposure to different phenol concentrations as well as the effect of a 2-h treatment on proteome variation and hemolytic activity.

2. Material and methods

2.1. Strain and culture conditions

Listeria monocytogenes EGDe (CIP107776), whose genome was sequenced by (Glaser et al. 2001) was used throughout this study. The strain was stored at -80°C in brain heart infusion (BHI) broth containing 40% glycerol. The liquid smoke L1114 was kindly provided by Lutetia (Arnouville-les-Gonesse, France) and its phenol concentration (1440 µg ml-1) was determined by IFREMER, Nantes (Cardinal et al. 2004). All media including those containing liquid smoke were filtered with 0·22 µm filters.
L. monocytogenes was precultured overnight at 37°C in BHI broth pH 6.2 with shaking (150 rpm). Then, 0.1% of the preculture was used to inoculate BHI broth pH 6.2 supplemented or not with different volumes of liquid smoke in order to obtain a final phenol concentration of 7 µg ml-1, 15 µg ml-1 or 30 µg ml-1. Cultures were incubated at 37°C with shaking (150 rpm) for 22 h. Cells were recovered by centrifugation and washed in saline water (8.5 g l-1 NaCl) to eliminate the liquid smoke

and to prevent the inhibition of the bacterial growth by phenols. Viable cell counts were determined by plating serial dilutions of cultures. Plates were incubated for 48 h at 37°C. The experiments were repeated twice.
For the survival study, proteome analysis and qRT PCR experiments, a preculture was grown as described above and used to inoculate BHI broth pH 6.2 for 5 h at 37°C with shaking (150 rpm). This culture was transferred at 50% (v/v) to BHI broth supplemented or not with liquid smoke. The final concentrations of phenol in the cultures corresponded to 15 µg ml-1, 22 µg ml-1, 30 µg ml-1 for the survival experiments and 30 µg ml-1 for the proteomic and qRT PCR experiments. Cell survival was assessed for 72-h incubation at 37°C with shaking by serial dilution of cells with a previous washing step and plating on BHI agar. Plates were incubated for 48 h at 37°C. For the proteomic study and the qRT PCR experiments, the control culture and the culture with liquid smoke were incubated for 2 h at 37°C with shaking (150 rpm). Bacteria were harvested by centrifugation (7500 g, 10 min, 4°C) and washed twice in saline water. For the proteomic study, the bacterial pellets were resuspended in TE Buffer pH 9 (20 mM Tris, 5 mM EDTA, 5 mM MgCl2) and stored at -20°C. Viable cells were enumerated before freezing.

2.2. Intracellular protein extraction

The intracellular protein fraction was obtained as previously described (Folio *et al.* 2004). Briefly, the bacterial suspensions stored in TE Buffer pH 9 were sonicated five times for 2 min (Vibra cell, Bioblock, Illkirch, France) using a microtip setting at power level 5 and 50% pulse duration. After a 30-min treatment with Dnase I/Rnase A, a solution with 20 mM Tris HCl, 5 mM EDTA, 5 mM $MgCl_2$, 4% CHAPS, 8 M urea, 4 M thiourea and 4 mM Tributyl phosphine (TBP) was added to the suspension (100% v/v). After a 30-min incubation on ice with intermittent agitation, the soluble protein sample was separated from cell debris by centrifugation (13 000 *g*, 20 min, 4°C). The supernatant was collected and proteins were quantified according to the method of Bradford (Bradford 1976) using the Protein Assay Kit (Bio-Rad, Marnes-la-Coquette, France) with bovine serum albumin as standard. The protein sample was then precipitated with three volumes of cold acetone at -20°C overnight and pelleted by centrifugation (13 000 *g*, 40 min, 4°C). The protein pellet was resuspended in isoelectric focusing buffer (7 M urea, 2 M thiourea, 4% CHAPS, and trace of bromophenol blue) at a final concentration of 5 µg proteins μl^{-1} and stored at -20 °C.

2.3. Two-dimensional gel electrophoresis (2-DE)

2-DE was performed as previously described (Folio *et al.* 2004). ReadyStrip IPG strips with linear gradient from pH 4 to 7 (Bio-Rad, Marnes-la-Coquette, France) were rehydrated overnight with 60 µg of proteins for analytic gels or 600 µg of proteins for semi preparative gels in the isoelectric focusing buffer containing 1.5% ampholytes pH 4-7 and 2 mM TBP in a 400 µl final volume. The first dimension was performed with the Protean IEF cell (BioRad) for 24 h at 19°C at a maximum voltage of 8000 V in a total of 73 kVh. IPG strips were then stored at -20°C. Strips were equilibrated twice for 15 min in an equilibration solution (6 M urea, 2% w/v SDS, 50 mM Tris-HCl pH 8.8, 30% v/v glycerol) containing 5 mM TBP for the first step and 2.5% w/v iodoacetamide and traces of bromophenol blue for the second step. The second dimension was carried out with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% T, 3.3% C polyacrylamide gels in a Multicell Protean II XL system (Bio-Rad, Hercules, USA). For the separation of the proteins, 15 mA were applied per gel until the marker dye reached the bottom of the gel.

2.4. Staining of 2-DE gels and image analysis

Analytic gels were silver stained according to a method previously described by (Rabilloud 1992). The protein spots were identified from semi-preparative gels stained with colloidal Coomassie blue (Neuhoff *et al.* 1988).
Stained gels were scanned using GS-800 imaging densitometer (Bio-Rad) and images were analyzed using Image Master 2D Platinum 5.0 (Amersham Biosciences, Saclay, France). At least seven gels were done for the control and the stressed conditions from three independent cultures. The reproducibility of gels was checked by using the scatter plot analysis tool (experimental replications *per* condition n_{epx}=7). Statistical analyses were performed with Image Master 2D Platinum 5.0 software

to evaluate the relative quantity variation of the spots from gels of a condition to assess reproducibility of the experiments. Only spots showing significant relative quantity differences between the control and the stress conditions were removed from the gels.

2.5. Identification of proteins by mass spectrometry

To identify proteins separated by 2-DE, spots were removed from the gels and washed in a first destaining solution for 30 min (25 mM NH_4HCO_3, 5% acetonitrile). Then, spots were washed twice for 30 min in a second destaining solution (25 mM NH_4HCO_3, 50% acetonitrile). Spots were dehydrated with 100% acetonitrile for 10 min. Proteins were then digested at 37°C using trypsin (Promega, Madison, USA) overnight and peptides were extracted using 100% acetonitrile and finally placed on the plate. The samples were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) (Voyager DE-Pro, PerSeptive BioSystems, Farmingham, USA) as previously described (Folio *et al.* 2004). The peptide masses were used for the NCBI databases searches with the "Mascot" software (http://www.matrixscience.com). Several parameters were considered for the protein identification: a fragment ion mass tolerance of ± 25 ppm, cysteine modifications with carbamidomethylation, methionine oxidation and one only possible missed enzymatic cleavage.

2.6. Hemolysis assay

A semi quantitative hemolysis assay (Roberts *et al.* 2005) was used to assess the hemolytic potential of the *L. monocytogenes* EGDe incubated in smoke stress conditions. An overnight culture in Luria Bertani broth (LB) at 37°C was used to inoculate LB supplemented with 2·5 mM $CaCl_2$, 20 mM $MgCl_2$ and 20 mM $MgSO_4$. The suspension was incubated at 37 °C for 5 h with shaking. Then this culture was transferred at 50% (v/v) to LB pH 6.2 (control condition) and LB supplemented with liquid smoke pH 6.2 (3·5 µg ml^{-1}, 7 µg ml^{-1}, 15 µg ml^{-1}). Cultures were incubated for 2 h at 37°C with shaking.
Two solutions of 1% erythrocytes were prepared from sheep blood and horse blood (Bio-Rad). Two blood types were used according to previously published data showing that hemolyse efficiency could be different between bloods (Van der Kelen and Lindsay 1990; Roche *et al.* 2005). Ten milliliters of blood were centrifuged at 1000 *g* for 30 min. The pellet was washed once with sterile phosphate buffer saline (PBS) and resuspended in 50 ml of PBS. After centrifugation of 1 ml of each bacterial culture, pellets were washed once in saline water and resuspended in 0.5 ml of sterile PBS containing 6 mM cysteine at pH 5·8. Serial 1:2 dilutions were done in the PBS cysteine solution and incubated at 37°C for 30 min without shaking. 0.5 ml of the 1% erythrocyte solution was added to each dilution and suspensions were finally incubated for 1 h at 37°C without shaking. The number of hemolytic units was defined as the inverse of the last dilution to show complete hemolysis. Results were expressed as a percentage of the hemolysis of *L. monocytogenes* incubated under control conditions.

2.7. Quantitative Reverse transcription PCR (qRT PCR)

Growth conditions used for the qRT PCR experiments are described above. Total RNA was extracted as previously described (Milohanic *et al.* 2003) and stored at -80°C. A DNase treatment was performed with the RQ1 RNAse-free DNAse following the manufacturer's instructions (1 U per µg of RNA) (Promega, Charbonnières, France) at 37°C for 45 min. Then RNA precipitation was performed with sodium acetate and RNA was finally quantified with a UVIKON XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France) using absorbance at 260 nm. The purity and integrity of RNA was checked on agarose gels then RNA was stored at -80°C. The forward primer (5'-ACCGTAACCCAGAAGAACTTCC-3') and the reverse primer (5'- TGCTGGAGCGGAGATAACAAC-3') were used for qRT PCR of *gap* gene used as a control gene (Corvec *et al.* 2003; Kazmierczak *et al.* 2006). The *gap* gene primers amplified a 120 bp sequence from bp 230 to 349 of the *L. monocytogenes* EGDe strain genome (GenBank accession number AL591824). The forward primer (5'-GGGAAATCTGTCTCAGGTGATGT-3') and the reverse primer (5'- CGATGATTTGAACTTCATCTT TTGC-3') previously published (Hough *et al.* 2002) were used for qRT PCR of *hly* gene. The *hly* primers amplified a 106 bp sequence from bp 973 to 1078 of the *L. monocytogenes* EGDe strain genome. cDNA synthesis was performed for 60 min at 42°C using 2 µg of RNA, 1·2 mmol l^{-1} of dNTP, 2 µmol l^{-1} of specific reverse primer and 200 U of MMLV reverse transcriptase (Promega). Quantitative PCR was performed in the thermocycler (Chromo-4 System; Bio-Rad, Marnes-la-Coquette France) for

45 cycles at 95°C for 15 s and 62°C for 1 min with a first enzyme activation step for 3 min at 95 °C. The qRT PCR reaction mixture contained 4·5 µl of cDNA, 7.5 µl of IQ^{TM} SYBR Green Supermix (Bio-Rad, France) and 0·25 µmol l^{-1} of forward and reverse primer in a final volume of 15 µl. A standard curve generated from dilutions of *L. monocytogenes* EGDe DNA was used for both primer pairs to evaluate the cDNA quantity of corresponding genes (*hly* and *gap*). The amplified products were subjected to electrophoresis in 1.5 % (w/v) agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2). Gels were stained with ethidium bromide and visualised under UV light. The quantity of *hly* cDNA was normalized to the quantity of *gap* cDNA in each sample. Statistical analyses from three independent assays were performed on the *hly* expression between the stress and the control conditions using the Fisher Least Significant Difference with a confidence interval of 99% (LSD test).

3. Results

3.1. Growth and survival of *L. monocytogenes* in the presence of liquid smoke

The *L. monocytogenes* growth curves obtained with three liquid smoke concentrations are shown in Figure 1. When the BHI medium contained a phenol concentration of 7 µg ml^{-1}, the growth started more slowly compared to the control conditions but in both conditions there were 10^9 CFU ml^{-1} after 15 h of growth. With 15 µg ml^{-1} phenol, no growth was observed during the experiments. Moreover, a slight decrease appeared after 20 h.
The growth of *L. monocytogenes* was also inhibited by 30 µg ml^{-1} phenol. The number of cells was stable during the eight first hours and then a rapid decrease occurred.
The survival study was performed in BHI supplemented with liquid smoke (Figure 2). When *L. monocytogenes* was cultured in control condition, the viable cell population was stable for 72 h. The presence of 15 or 22 µg ml^{-1} phenol affected bacterial survival and no viable cell was detected after 48 h. With 30 µg ml^{-1} phenol in the medium, no viable cell was detected after 24 h.

3.2. Identification of proteins involved in the response to the liquid smoke stress

Results showed that the level of expression of more than 50 protein spots varied between proteome patterns of control and phenol-treated cells. Among these protein spots, we identified 23 from their peptide mass fingerprint obtained by MALDI-TOF mass spectrometry (Figure 3). These spots corresponded to 17 different proteins of which 8 were under-expressed (Table 1) and 8 over-expressed (Table 2) following exposure to liquid smoke. The protein GuaB was separated into two isoforms, with one over-expressed and the other under-expressed.

3.3. Effect of liquid smoke on *hly* expression and hemolytic activity of *L. monocytogenes* EGDe

Expression of the *hly* gene was investigated using a qRT PCR assay after 2h-contact with liquid smoke. Specific amplification of products was visualized using gel electrophoresis (Figure 4) and with melting curve analysis during the real time PCR assays (Figure 5). The LSD test showed no statistical difference in *hly* gene expression between the stress conditions (30 µg ml^{-1} phenol) and the control conditions (no smoke) (data not shown). Two-hour smoke exposure did not affect significantly the *hly* gene expression.
The hemolytic potential of *L. monocytogenes* was evaluated after the same liquid smoke exposure. The results were expressed as described in the Materials and Methods section. When the LB medium contained 3·5 µg ml^{-1} phenol, the hemolytic activity was two times lower than the activity in LB medium. When 7 µg ml^{-1},phenol, the activity was reduced four-fold compared to control and no hemolytic activity was detected with 15 µg ml^{-1} phenol. When cells were stressed with the three phenol concentrations for two hours, then washed and incubated in BHI broth for two more hours, the hemolytic activity was identical that in control conditions (data not shown).

4. Discussion

Liquid smoke and traditional smoke are known to have a bacteriostatic effect (Faith *et al.* 1992; Sunen 1998). But liquid smoke allowed easy manipulation and reproducibility of the experiments. Studies have described inhibition of microorganism growth by liquid smoke or smoke compounds (Faith *et al.* 1992) combined with other treatments of *L. monocytogenes* (Membré *et al.* 1997; Thurette *et al.* 1998; Lebois *et al.* 2004). Our work was focused on the effect of the liquid smoke, hence an optimal temperature and a non-stressing pH were chosen. Different phenol concentrations were assessed and the inhibition of *L. monocytogenes* growth was observed from 15 µg ml^{-1}. These results were in agreement with previous studies where 20 µg ml^{-1} phenol inactivated *L. monocytogenes* at low temperatures in broth (Membré *et al.* 1997) and strongly affected the growth of *Shewanella putrefaciens* at 25°C in a basic medium (Leblanc *et al.* 2000). When *L. monocytogenes* was cultivated in BHI supplemented with 15 µg ml^{-1} phenol, cell number decreased very fast. These results showed that growth and survival of cells were strongly affected by liquid smoke even for concentrations currently found in the smoked salmon produced in Europe (Leroi *et al.* 2001; Cardinal *et al.* 2004). In our experiments, at 37°C with 15 µg ml^{-1} phenol in a rich medium, no growth was detected and cells did not survive beyond 48 h. Whereas, at 8°C with 20 µg ml^{-1} phenol, growth of *L. monocytogenes* in smoked salmon occurs (Cornu *et al.* 2006). The physico-chemical characteristics of the smoked salmon matrix may have a protective effect against phenolic stress. This may also result from a cross-protection from the other stresses experienced by bacteria during processing and storage (Lou and Yousef 1997).

In order to understand the mechanisms used by *L. monocytogenes* in contact with liquid smoke, a proteomic study was performed. More than 50 spots were removed from the 2-DE gels and 17 different proteins regulated after liquid smoke exposure were identified. Five of them had isoforms, showing quantitative variations according to control or treated sample. Interestingly after contact with smoke, ClpP-2 a subunit of the general stress protein ClpP was over-expressed. This serine protease is able to cleave polypeptides shorter than seven amino acids. It is required for a wide variety of functions and is increased in heat shock, salt and ethanol stress as described for *B. subtilis* (Gerth *et al.* 1998). The ClpP serine protease is also required for the virulence of *L. monocytogenes* (Gaillot *et al.* 2000).

Two isoformes of the ferritin protein (Lmo0943) were identified as under-expressed after contact with liquid smoke. Ferritin-like compounds can induce detoxification and protect bacteria from iron overload and against oxidative stress (Smith 2004). It is also important for the virulence of *L. monocytogenes*, and ferritin could play a direct or indirect role in the production and/or stability of Listeriolysin O (LLO) (Dussurget *et al.* 2005). The theoretical isoelectric point of the LLO precursor is 8.23 and the isoelectric points of the isoforms have been reported as 6·3, 6·9 and 7·6 (Folio *et al.* 2004). In our experimental conditions, we have used pH 4-7 IPG strips allowing a good resolution in an area containing the great majority of *L. monocytogenes* protein spots. However, such a gradient was not suited to the separation of the different isoforms of LLO and, consequently, we were unable to visualize the expression of this protein under the smoke stress.

Expression of proteins involved in different metabolic pathways was affected by the liquid smoke: (i) two proteins involved in metabolism of coenzymes and prosthetic groups (Lmo2101 and FolD), (ii) two proteins involved in glycolytic pathways (PdhA, Lmo1055), (iii) one protein involved in metabolism of carbohydrates (Lmo1086) and (iv) one protein involved in metabolism of nucleotides (GuaB).

Interestingly, the redox-sensing transcriptional repressor Rex (Lmo2072) was over-expressed after smoke exposure. The Rex protein regulates transcription of respiratory genes in response to the intracellular $NADH/NAD^+$ redox poise (Sickmier *et al.* 2005).

The under-expression of two proteins (ferritin and Lmo2101) that protect against oxidative stress showed that the mechanism used by *L. monocytogenes* against the smoke during the 2h-contact was quite different from the mechanism developed during an oxidative stress.

The Lmo0355 protein involved in membrane bioenergetics was absent in the control condition but present after the stress condition. The Lmo2829 protein involved in the metabolism of lipids was over-expressed after the stress condition. Regarding these results, it appeared that the cell membrane could be affected by the smoke. Similar results were observed with *Pseudomonas putida* after phenol exposure (Santos *et al.* 2004) where four proteins involved in lipid metabolism were up-regulated. However, in this case, under the phenol stress, the expression of several proteins involved in the oxidative stress was increased. This showed that the stress induced by smoke could not be reduced just to the phenol stress. Smoke is composed of formaldehydes, phenols and derivatives, organic acids, carbonyl compounds (Guillén and Manzanos 1996; Guillén and Ibargoitia 1998). So, even if the

antimicrobial properties of the smoke are commonly attributed to the phenol, other compounds and physicochemical characteristics are probably involved in the growth inhibition, as previously observed (Cornu *et al.* 2006), but also in the response of micro-organisms to this stress.
As ferritin is known to regulate some virulence factors, we investigated the gene expression and activity of one of the major virulence factors, Listeriolysin O (LLO). The gene expression was not affected by the treatment, but the hemolytic activity of *L. monocytogenes* was proportional to the concentration added. This phenomenon was reversible and the liquid smoke may not act on gene expression but rather on the stability or activity of LLO as previously observed after heat-shock treatment (Sampathkumar *et al.* 1999). Assessment of the pathogenic potential of *L. monocytogenes* grown with liquid smoke using *in vitro* cell-culture tests could elucidate the effect of smoke on virulence.

Liquid smoke added to a rich medium strongly inhibited the growth and survival of *L. monocytogenes*. During a short and quite strong treatment, *L. monocytogenes* did not develop an adaptive physiological response and different metabolic pathways seem to have been affected. Liquid smoke probably acts on the cell membrane and directly or indirectly on the stability of LLO. This could result in attenuation of the virulence of *L. monocytogenes* grown with liquid smoke.

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Table 1 Proteins down-regulated after exposure of *L. monocytogenes* EGDe to liquid smoke.

Protein spot name*	Gene name	SWISS-PROT protein accession	Protein description	Mass (kDa)†	pI†	Peptides matched‡	Sequence coverage (%) §
Lmo0227	*lmo0227*	Lmo0227	Conserved hypothetical protein	36·8	6·1	10/93	29
Lmo0811	*lmo0811*	Lmo0811	Similar to carbonic anhydrase	27·2	4·6	13/59	55
Fri (a)	*flp*	Lmo0943	Non-heme iron-binding ferritin	16	4·7	10/49	64
Fri (b)						10/50	64
Lmo1052	*pdhA*	Lmo1052	Similar to pyruvate dehydrogenase (E1 alpha subunit)	41·2	6	10/138	33
Lmo1086	*lmo1086*	Lmo1086	Similar to CDP-ribitol pyrophosphorylase	26·7	6·0	10/71	37
Lmo1360	*folD*	Lmo1360	Highly similar to methylenetetrahydrofolate dehydrogenase and methenyltetrahydrofolate cyclohydrolase	30·9	5·2	8/96	42
Lmo2101 (a)	*lmo2101*	Lmo2101	Similar to a protein required for pyridoxine synthesis	31·7	5·1	17/88	44
Lmo2101 (b)						7/103	21
Lmo 2020**	*divIVA*	Lmo2020	Similar to cell division initiation protein (septum placement)	20·3	4·5		
GuaB (a)	*guaB*	Lmo2758	Similar to inosine-monophosphate dehydrogenase	52·5	6·1	27/74	58

* (a), (b) represent isoform proteins.
† Theoretical values.
‡Number of peptides identified vs total number of peptides used for database query.
§ Percentage of amino acids sequence covered by matching peptides.
** Lmo2020 protein was identified using 2-DE database (Folio *et al*. 2004); http://www2.clermont.inra.fr/proteome/cartesref.htm

Table 2 Proteins up-regulated after exposure of *L. monocytogenes* EGDe to liquid smoke.

Protein spot name*	Gene name	SWISS-PROT protein accession	Protein description	Mass (kDa)†	pI†	Peptides matched ‡	Sequence coverage (%) §
Lmo0355	*lmo0355*	Lmo0355	Similar to flavocytochrome C fumarate reductase chain A	54·4	5·7	10/120	28
Lmo0437	*lmo0437*	Lmo0437	Conserved hypothetical protein	30·4	5·1	8/114	29
Lmo0796	*lmo0796*	Lmo0796	Conserved hypothetical protein	19·3	4·5	4/32	31
Lmo1055	*pdhD*	Lmo1055	Highly similar to dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase complex	49·5	5·0	7/62	19
Lmo1657	*tsf*	Lmo1657	Translation elongation factor	32·6	4·8	10/42	44
Lmo2072	*lmo2072 (rex)*	Lmo2072	Similar to a putative DNA binding proteins	24·2	6·0	10/59	43
ClpP-2(a)	*clpP-2*	Lmo2468	ATP-dependent Clp protease proteolytic subunit	21·6	4·8	8/41	42
ClpP-2(b)						6/39	28
GuaB (b)	*guaB*	Lmo2758	Similar to inosine-monophosphate dehydrogenase	52·5	6·4	9/64	22
Lmo2829 (a)	*lmo2829*	Lmo2829	Similar to yeast protein Frm2p involved in fatty acid signaling	22·2	4·7	10/56	53
Lmo2829 (b)						5/43	29

* (a), (b) represent isoform proteins.
† Theoretical values.
‡ Number of peptides identified vs total number of peptides used for database query.
§ Percentage of amino acids sequence covered by matching peptides.

Figures

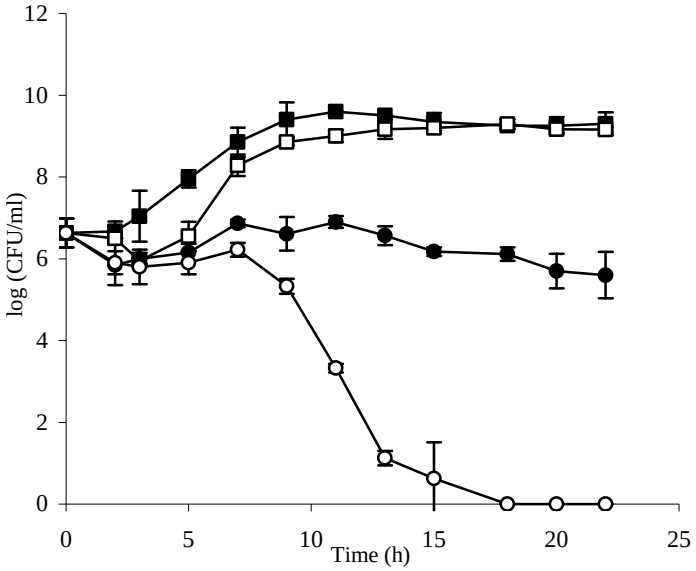


Figure 1

Effect of liquid smoke on *Listeria monocytogenes* EGDe growth in brain heart infusion (BHI) broth at 37°C. Concentrations of liquid smoke applied to the bacteria expressed in phenol concentration (■: 0 µg ml^{-1}, □: 7 µg ml^{-1}, ●: 15 µg ml^{-1}, ○:30 µg ml^{-1}).
Standard deviations were determined from two independent experiments.

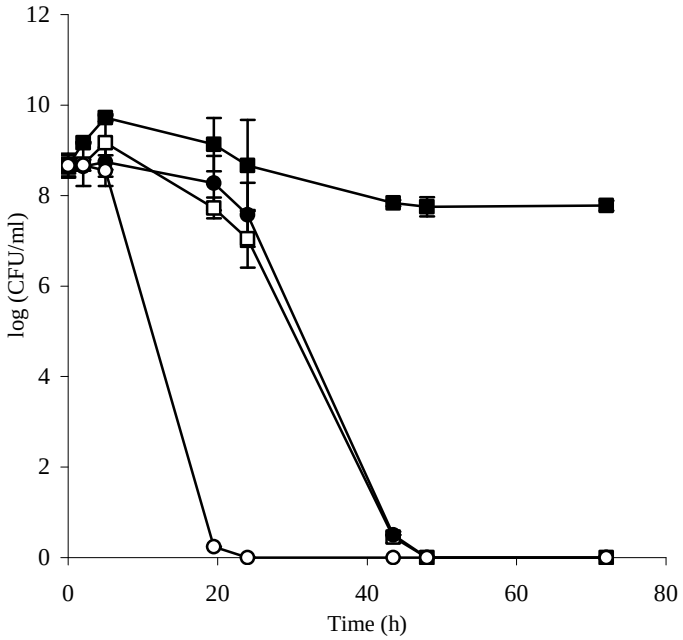
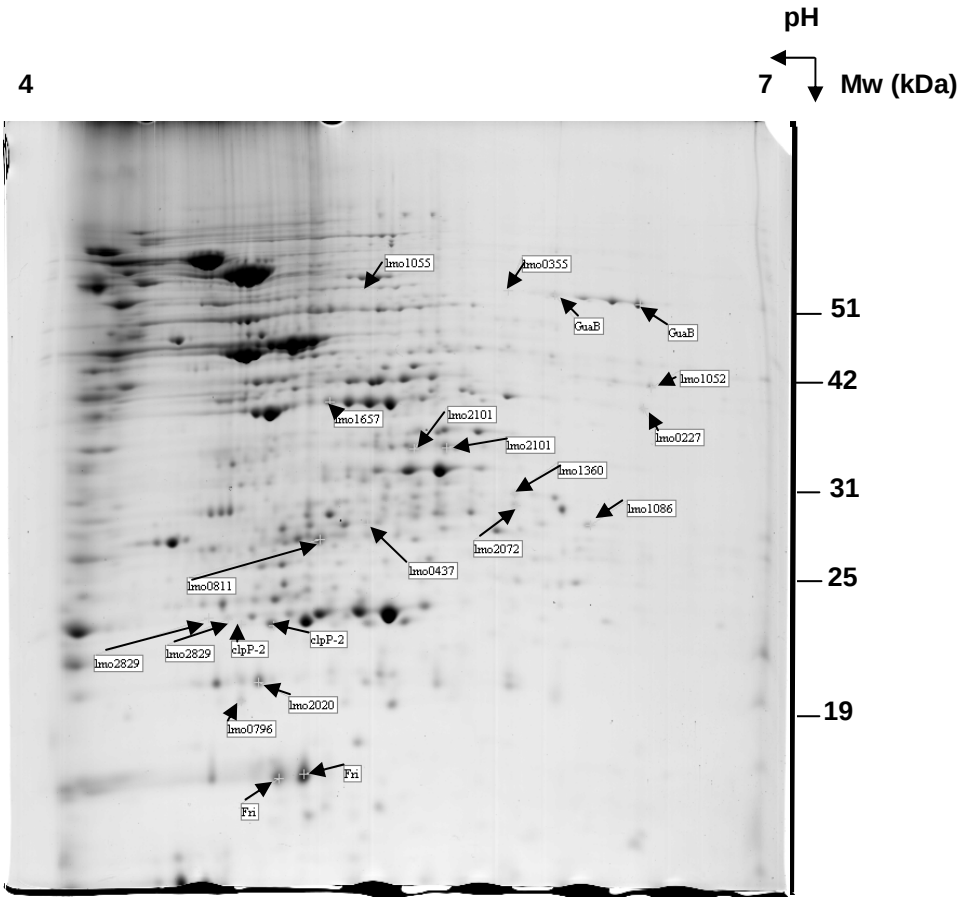


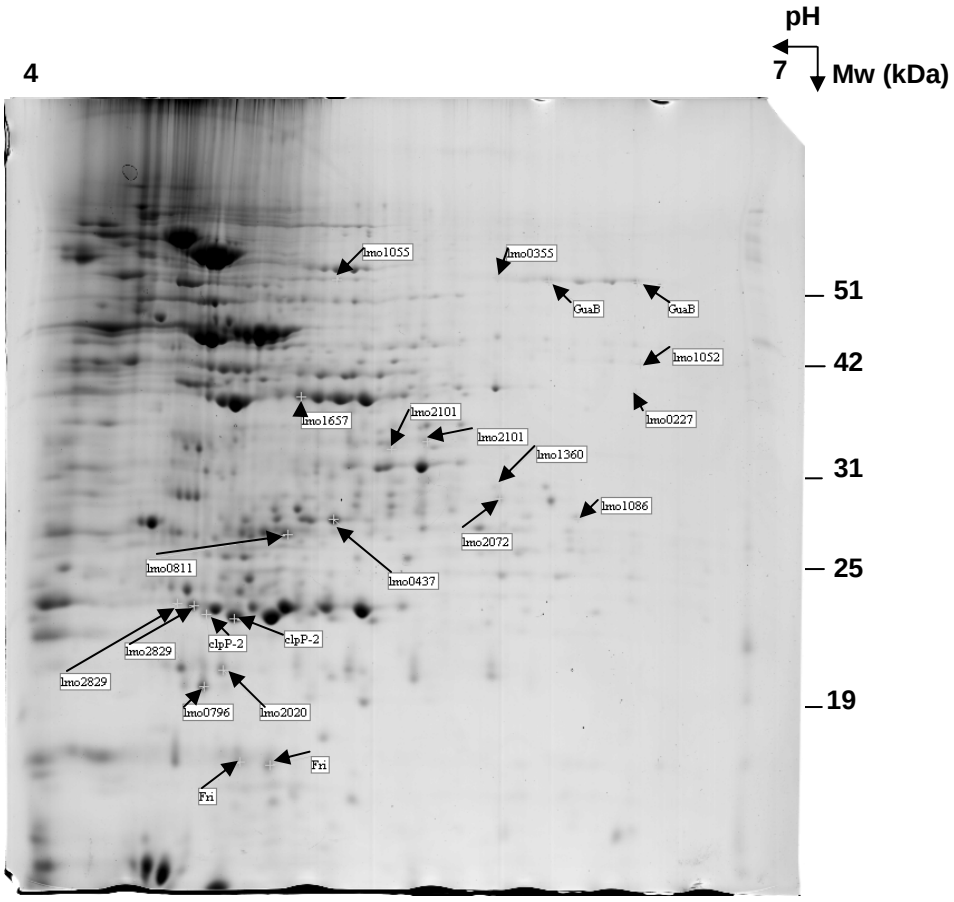
Figure 2

Effect of liquid smoke on *Listeria monocytogenes* EGDe survival in BHI broth at 37°C.
Concentrations of liquid smoke applied to the bacteria expressed in phenol concentration (■: 0 µg ml^{-1}, □: 15 µg ml^{-1}, ●: 22 µg ml^{-1}, ○: 30 µg ml^{-1}).

Standard deviations were determined from three independent experiments.



a)



b)

Figure 3

Protein synthesis patterns of *L. monocytogenes* EGDe in control conditions a) and after smoke exposure (30 µg ml^{-1} phenol) b). Proteins were separated in pH 4-7 immobilized pH gradient gel. 2D gels were stained using colloidal Coomassie blue. Black arrows indicate differentially expressed proteins.

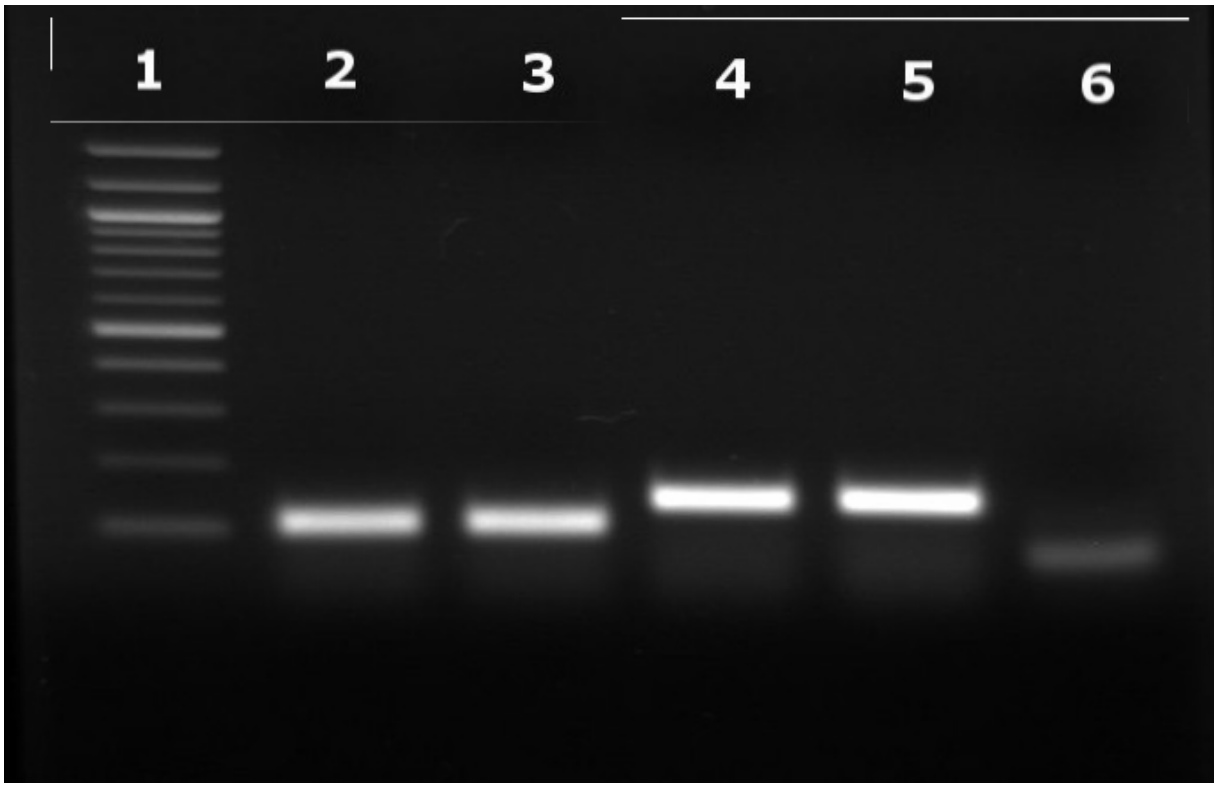
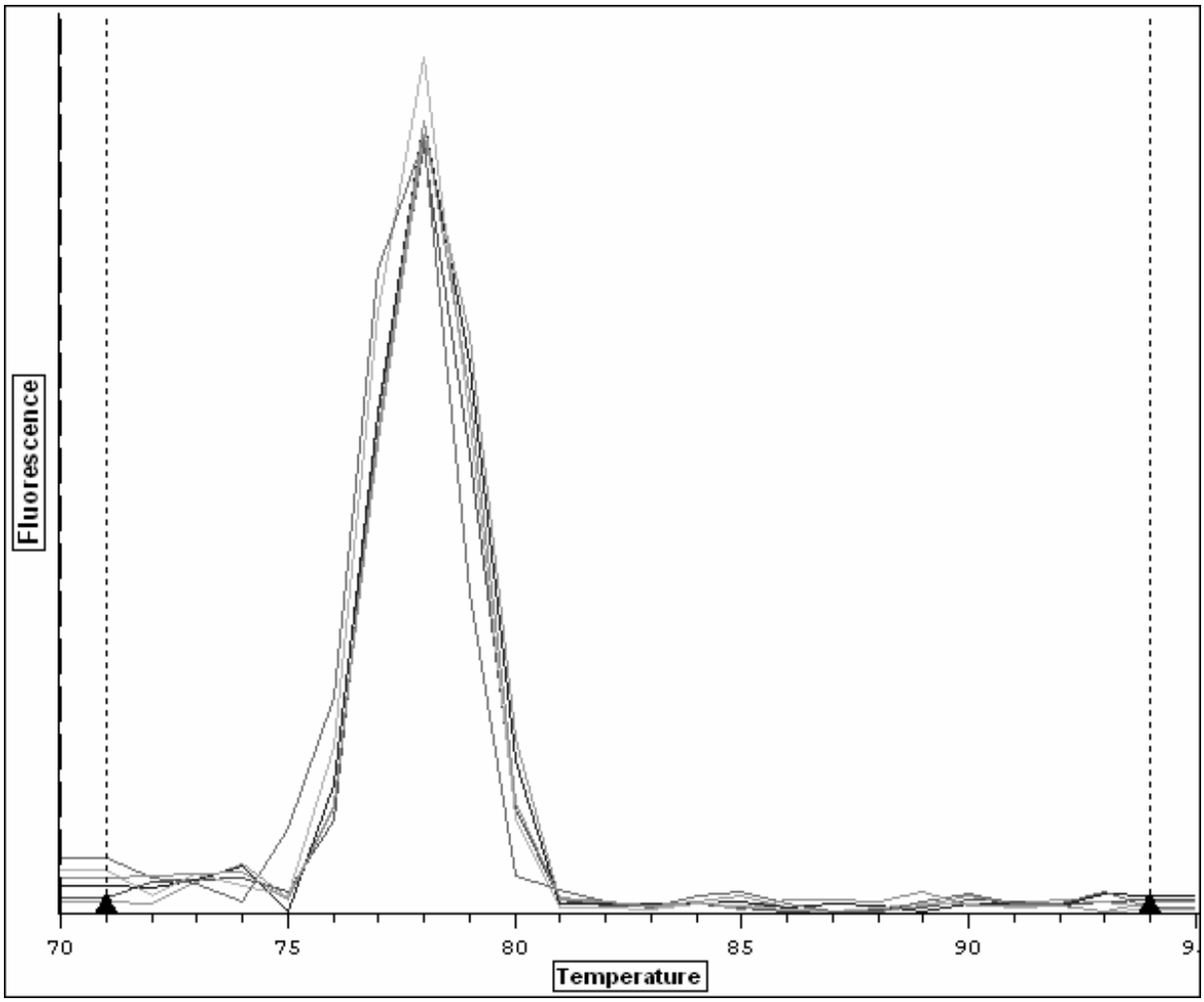
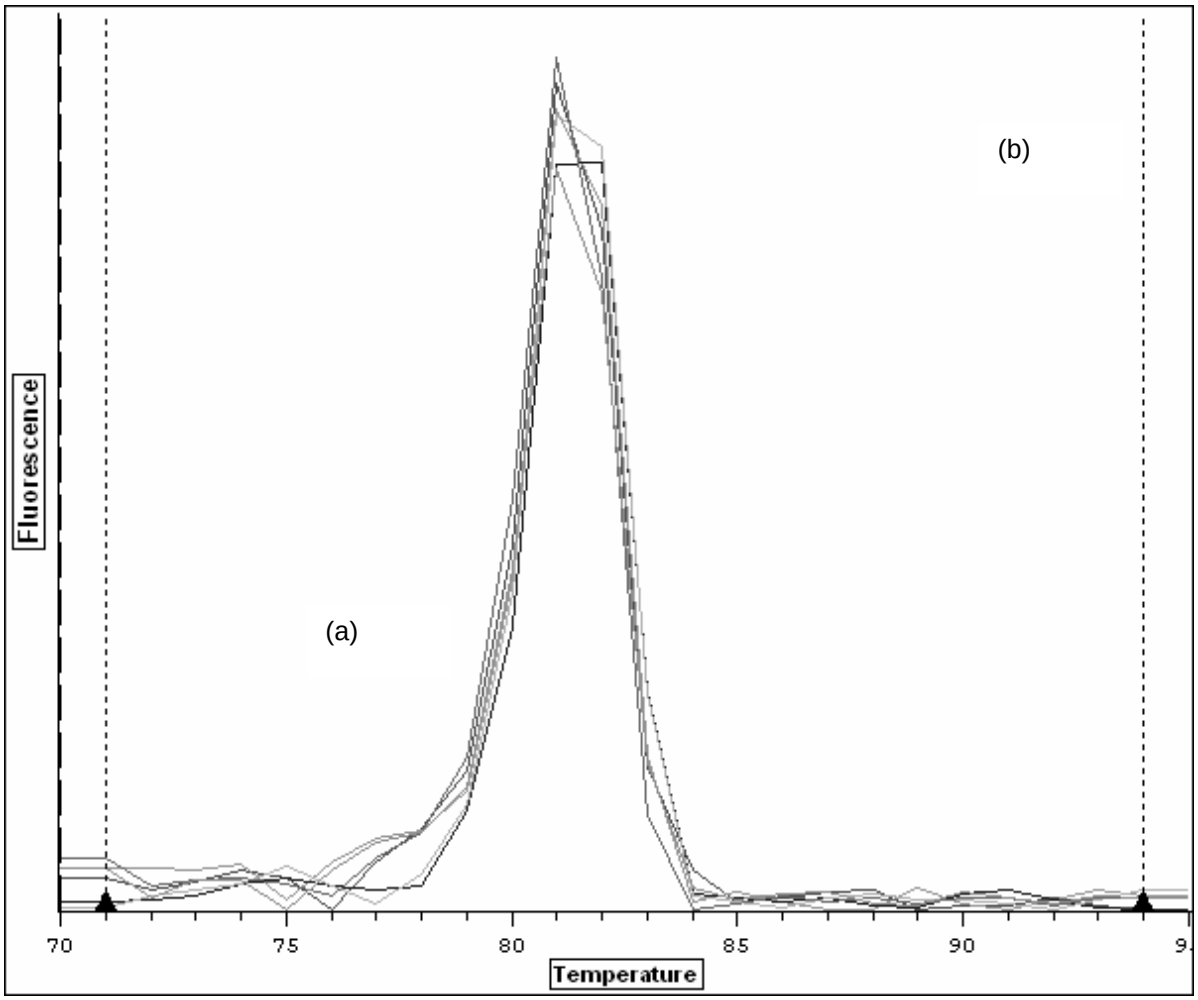


Figure 4

Reverse Transcription quantitative PCR products obtained with primers specific to *hly* gene and *gap* gene. Lane 1: 100 bp ladder; Lane 2: *hly* cDNA amplification (control conditions); Lane 3: *hly* cDNA amplification (stress conditions); Lane 4: *gap* cDNA amplification (control conditions); Lane 5: *gap* cDNA amplification (stress conditions). Lane 5: negative control (no cDNA)



a)



b)

Figure 5

Melting curves corresponding to amplification of *hly* (a) and *gap* (b) cDNA.