Microbes and Infection xxx (xxxx) xxx

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Kinetic and proteomic studies in milk show distinct patterns among major *Listeria monocytogenes* clones

Alba Espí-Malillos [a], Carla Palacios-Gorba [a], Inmaculada López-Almela [a], Pilar Ruiz-García [b], María Carmen López-Mendoza [b], Francisco García-Del Portillo [c], M Graciela Pucciarelli [c, d], Juan J. Quereda [a, *]

[a] Grupo de investigación Intracellular Pathogens: Biology and Infection, Departamento Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain
[b] Departamento Producción y Sanidad Animal, Salud Pública Veterinaria y Ciencia y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Cardenal Herrera-CEU, CEU Universities, Valencia, Spain
[c] Laboratory of Intracellular Bacterial Pathogens, National Centre for Biotechnology (CNB)-CSIC, Madrid, Spain
[d] Department of Molecular Biology, Universidad Autónoma de Madrid, Centre of Molecular Biology 'Severo Ochoa' (CBMSO CSIC-UAM), Madrid, Spain

ARTICLE INFO

Article history:
Received 25 October 2023
Accepted 9 February 2024
Available online xxx

Keywords:
Foodborne pathogens
Dairy products
Survival kinetics
Predictive models
Food safety
Ready-to-eat foods

ABSTRACT

Listeria monocytogenes, a contaminant of raw milk, includes hypervirulent clonal complexes (CC) like CC1, CC4, and CC6, highly overrepresented in dairy products when compared to other food types. Whether their higher prevalence in dairy products is the consequence of a growth advantage in this food remains unknown. We examined growth kinetics of five *L. monocytogenes* isolates (CC1, CC4, CC6, CC9, and CC121) at 37 and 4 °C in ultra-high temperature (UHT) milk and raw milk. At 4 °C, hypovirulent CC9 and CC121 isolates exhibit better growth parameters in UHT milk compared to the hypervirulent CC1, CC4, and CC6 isolates. CC9 isolate in raw milk at 4 °C exhibited the fastest growth and the highest final concentrations. In contrast, hypervirulent isolates (CC1, CC4, and CC6) displayed better growth rates in UHT milk at 37 °C, the mammalian host temperature. Proteomic analysis of representative hyper- (CC1) and hypovirulent (CC9) isolates showed that they respond to milk cues differently with CC-specific traits. Proteins related to metabolism (such as LysA or different phosphotransferase systems), and stress response were upregulated in both isolates during growth in UHT milk. Our results show that there is a *Listeria* CC-specific and a *Listeria* CC-common response to the milk environment. These findings shed light on the overrepresentation of hypervirulent *L. monocytogenes* isolates in dairy products, suggesting that CC1 and CC4 overrepresentation in dairy products made of raw milk may arise from contamination during or after milking at the farm and discard an advantage of hypervirulent isolates in milk products when stored at refrigeration temperatures.

1. Introduction

Listeria monocytogenes is a Gram-positive, foodborne pathogen that causes severe invasive infections in humans and animals called listeriosis. The disease is characterized by septicaemia, central nervous system infections, and maternal–neonatal infections [1,2]. Listeriosis is regarded as a serious public health concern due to its high case fatality rate (20–30%) [1].

L. monocytogenes is widely distributed in the environment due to its ability to persist under extreme conditions including cold temperatures, low pH, and high salt environments [3]. In addition to its ability to form biofilms, the ecological range of *L. monocytogenes* enables frequent contamination of foods including dairy, meat, seafood, and fresh products [4]. Dairy products are associated with approximately half of the reported listeriosis outbreaks in Europe and the United States [5–7]. *L. monocytogenes* is a common contaminant of raw milk, being isolated from the bulk tank milk and in-line milk filter socks [8–10]. *L. monocytogenes* can be transmitted to milk in different ways: i) intramammary infection [11]; ii) from faecal or environmental contamination of the udder surface as a consequence of poor

* Corresponding author.
E-mail address: juan.quereda@uchceu.es (J.J. Quereda).

https://doi.org/10.1016/j.micinf.2024.105312

hygiene in the milking parlor [12–14]; and iii) as the result of post-pasteurization contamination due to poor sanitation practices [15].

L. monocytogenes has been classified in four evolutionary lineages, 13 serotypes, more than 170 clonal complexes (CCs), and 300 sublineages (SLs) [16]. Importantly, CCs and SLs of food versus those of clinical isolates exhibit uneven prevalence [17,18]. While lineage II, in particular CC9 and CC121, is mostly associated with food, lineage I isolates, in particular CC1, CC4, and CC6, are hypervirulent and associated with human and animal clinical cases [4,17–19]. Remarkable differences have been observed among CCs when considering their prevalence in different food types. Hypovirulent isolates of CC9 and CC121 are strongly associated with meat products, whereas they are rarely isolated from dairy products (only 6.6% and 4.4% of the CC9 and CC121 food isolates were from this latter type of food) [4]. In sharp contrast, CC1, CC4, and CC6 are associated with dairy products (48.3% of CC1 isolates were from dairy products) [4]. Despite reference studies supporting differences in ecology and virulence among *L. monocytogenes* strains [4,17–19], food safety regulations consider all *L. monocytogenes* strains to be an equally serious threat to public health [20]. The frequent occurrence of *L. monocytogenes* in raw milk [9,21–25], its ability to grow at refrigeration temperatures from a low contamination dose [10], and the striking differences of pathogenic potential and distribution in foods among *L. monocytogenes* isolates [4,17], highlight the importance of investigating the bases of the differences among *L. monocytogenes* isolates for growing in dairy products. A key aspect to decipher is whether overrepresentation of CC1, CC4, and CC6 hypervirulent isolates in dairy products results from their circulation in ruminant farms, or if it reflects advantage of hypervirulent isolates for growing in dairy products. This knowledge will help to better identify food contamination risks and reduce the impact of human listeriosis. The objectives of this study were: (i) to test whether hyper- and hypovirulent isolates grow at different rates in ultra-high temperature (UHT) and raw milk when incubated at host or food preservation temperatures (37 °C or 4 °C, respectively); and (ii) to identify by proteomics factors differentially expressed by hyper-versus hypovirulent isolates in UHT milk.

2. Materials and methods

2.1. UHT and raw milk

To study the growth of *L. monocytogenes*, four 1-L containers of UHT whole milk of the same commercial brand were purchased from a local supermarket. Raw bovine milk was obtained in two 1-L plastic bottles from the Milk Analysis Laboratory of the Institute of Animal Science and Technology of the Polytechnic University of Valencia approximately 24 h after the milking process. The 1-L plastic bottles of raw bovine milk were transported under refrigerated conditions (4–6 °C) to the laboratory and used immediately. Milk samples were analysed to ensure the initial absence of *Listeria* spp.

2.1.1. Detection of Listeria spp. and total aerobic mesophilic bacterial count in raw milk

Testing the absence of *Listeria* spp. in raw milk at the beginning of the experiment was performed according to ISO 11290-1 [26] and ISO 11290-2 [27]. All raw milk samples ($n = 10$) tested negative for *Listeria* spp. The initial mean of total aerobic mesophilic bacterial count of 20,040 CFU/mL (4.30 Log_{10} CFU/mL) at 37 °C condition, and 9,400 CFU/mL (3.97 Log_{10} CFU/mL) at 4 °C condition were performed according to ISO 4833-2 [28].

2.2. L. monocytogenes isolates

Five L. *monocytogenes* isolates belonging to distinct CCs (SL1/CC1, SL219/CC4, SL6/CC6, SL9/CC9, and SL121/CC121) were used in the present study (Table S1). All isolates were maintained at −80 °C in glycerol. Before the start of the experiment, each isolate was plated onto Brain Heart Infusion (BHI) agar and incubated at 37 °C for 48 h. A single colony of each isolate was transferred aseptically into 2 mL of BHI broth and incubated at 37 °C overnight for 16 h with agitation at 250 rpm.

2.3. Analysis of growth parameters

The growth of *L. monocytogenes* was analysed at 37 °C during distinct post-inoculation times, spanning up to 32 h, for both UHT and raw milk samples. *L. monocytogenes* growth at 4 °C was monitored at distinct post-inoculation times up to 43 days in UHT milk and up to 35 days in raw milk. An initial target inoculum level of 2.3 Log_{10} CFU/mL (≈200 CFU/mL) was used for all isolates. The growth study was performed by using four biological replicates per isolate and type of milk for both 37 and 4 °C conditions. Three technical replicates were also plated per isolate and type of milk on each sampling day. For enumeration at the established times, 2 mL of samples were plated into 6 agar plates when *L. monocytogenes* counts were ≤100 CFU/mL. For the enumeration of counts >100 CFU/mL, samples were appropriately diluted in phosphate-buffered saline (PBS) and plated in triplicate on BHI agar and Microinstant® *Listeria* Ottaviani and Agosti agar plates (for UHT milk and raw milk, respectively). The plates were incubated at 37 °C for 48 h after which they were enumerated. Results were expressed as Log_{10} CFU/mL. Additionally, the pH was measured on each replicate sample at distinct post-inoculation times. The pH was measured using the sensION™ + pH_3 pH meter equipped with an electrode XS SEMI-MICRO pH 0–14, which was calibrated with technical buffers on each sampling day.

2.4. Growth fitting curves

The growth patterns of the different *L. monocytogenes* isolates in UHT and raw milk were constructed by plotting Log_{10} CFU/mL of samples against the hours or days of the study. The experimental data were fitted to the modified Gompertz equation [29] using the statistical software Statgraphics Centurion XV.II (Starpoint Inc., USA). The model equation was:

$$\mathrm{Log_{10}\,N(t)} = \mathrm{Log_{10}N_0} + \mathrm{C{*}exp}\left(-\exp\left(\left(\frac{2.718{*}\mu_{max}}{C}\right){*}(\lambda - t) + 1\right)\right)$$

Where $N(t)$ represents *L. monocytogenes* isolates concentration (CFU/mL) at a particular time; N_0 the initial *L. monocytogenes* isolates concentrations (CFU/mL); C the difference between the curve asymptotes (Log_{10}(CFU/mL)), corresponding to the difference between the maximum and the minimum Log_{10} count reached; μ_{max} the maximal growth rate (Log_{10}(CFU/mL)/hour or day); λ the lag phase (hours and days at 37 and 4 °C, respectively); and t the particular time (hours and days at 37 and 4 °C, respectively).

The goodness of fit of the models was assessed by means of the standard error of the estimate (RMSE), which shows the standard deviation of the residuals, and the adjusted determination coefficient (R^2adj), which indicates the percentage with which the model explains the variability of microbial concentration over time.

2.5. Proteomics of cell wall and membrane/cytosol subcellular fractions prepared from L. monocytogenes CC1 and CC9 isolates

2.5.1. Bacterial isolates, cultures, and media

L. monocytogenes CC1 and CC9, with genomes completely sequenced [17,30] were grown in BHI broth and commercial whole UHT cow milk at 4 °C in static conditions for 12 days. An inoculum of 10 mL was grown at 37 °C without shaking overnight from a fresh single colony. For BHI cultures, 0.5 mL of the inoculum was transferred to 25 mL of sterile BHI at 37 °C without shaking until an optical density (OD_{600}) of 0.25 was achieved. Bacteria growth in BHI was monitored by optical density at 600 nm. Next, 10 mL of the culture were transferred into 100 mL of fresh BHI and incubated for 12 days at 4 °C in static conditions until an optical density (OD_{600}) of 1.2 ± 0.3 was achieved. For UHT milk cultures, 7 mL of the first inoculum grown in BHI were transferred into 140 mL of commercial UHT milk for 6 h at 37 °C without shaking. Next, 60 mL were transferred into 240 mL of UHT milk and incubated for 12 days at 4 °C in static conditions and were stopped at a cell density of $\approx 7 \times 10^8$ CFU/mL. Bacterial growth in whole milk was determined by colony count on BHI agar plates.

2.5.2. Isolation of bacteria from milk

Trisodium citrate (1 M) was added at a final concentration of 0.25 M to the cultures of *L. monocytogenes* grown in UHT milk, which were maintained at 4 °C for 10 min under these conditions before centrifugation. This treatment, adapted from that described previously [31], limits casein precipitation in milk samples. Bacteria were harvested by centrifugation (10,000×*g*, 15 min, 4 °C) and resuspended in 10 mL of PBS pH 7.4 containing a cocktail of protease inhibitors and DNAse 100 mg/mL. Pellets were then resuspended in 25 mL of cold PBS and were overlaid on 15 mL of 45% (w/v) sucrose prepared in deionized sterile H_2O in a 50 mL conical centrifuge tube. The gradients were centrifuged for 40 min at 10,000×*g* at 4 °C and washed six times with 20 mL of cold PBS. This procedure was adapted from that described previously [32]. Supernatant was carefully removed, and the pellets were stored at −80 °C until processed to obtain peptidoglycan-enriched material.

2.5.3. Preparation of peptidoglycan-enriched material from Listeria

The preparation of peptidoglycan from *L. monocytogenes* was performed as previously described [33]. Briefly, stored bacterial pellets at −80 °C were resuspended in 25 mL of PBS. Bacteria were lysed in three passes through a French press. Unbroken cells were removed by centrifugation (5,000×*g*, 5 min, 4 °C), and cell envelopes were obtained by centrifugation of supernatant at a high speed (140,000×*g*, 1 h, 4 °C). The pellet containing the envelopes was resuspended in 1.5 mL of PBS (pH 7.4) and gently mixed with 1.5 mL of boiling 8% Sodium Dodecyl Sulfate (SDS). Boiling conditions were maintained for 2 h and the material was further incubated overnight at room temperature in shaking conditions. The SDS-insoluble material (pellet), enriched in peptidoglycan and strongly-associated proteins, was collected by centrifugation at high speed (300,000×*g*, 20 min, 22 °C) and washed four times with 2.5 mL of warm distilled water. The supernatant, containing the membrane/cytosol fraction, was analysed using iTRAQ® 8-plex with LC-MS/MS. The washed material (pellet) was finally resuspended in 300 μL of 50 mM ammonium bicarbonate. Trypsin digestion was performed in shaking conditions overnight at 37 °C. Peptides were separated from the undigested macromolecular peptidoglycan by centrifugation (300,000×*g*, 30 min, 22 °C). The supernatant containing the peptide mixture was lyophilized and kept at −20 °C.

2.5.4. Protein labelling with iTRAQ® 8-plex reagents and Liquid Chromatography and Mass Spectrometric Analysis (LC-MS)

Proteomics analyses were performed at the Proteomics Core Facility of the National Centre of Biotechnology (CNB−CSIC; Madrid, Spain). Cell wall samples were resuspended in H_2O with 0.5% TFA (Trifluoroacetic acid) and washed with ZipTip C18 resin (Merck Millipore). Membrane/cytosol samples were quantified using Pierce 650 nm reagent digested with trypsin. Tryptic peptides were then quantified with QUBIT and labelled at room temperature for 2 h using the kit iTRAQ® Reagent Multi-plex. All samples were injected into a nano-liquid chromatography (nLC) system Thermo Dionex Ultimate 3000 coupled to a high-speed mass spectrometer Thermo Orbitrap Exploris OE240 operating in Data Dependent Acquisition (DDA) mode and using a 90 min gradient for cell wall samples and 120 min gradient for membrane/cytosol samples. The column used in the reverse phase was a Peptide BEH C18 of 75 μm × 50 cm. Peptides were eluted at a flow rate of 250 nL/min.

The MS/MS spectra were exported to the Mascot generic format (MGF) with the PeakView v7.5 platform, and using MASCOT (Matrix Science, v.2.5), OMSSA (NCBI, v.2.1.9), X!Tandem2 (TheGPM, v.win.13.02.01.1), X!Tandem2 with k-score plugin (LabKey Software, v.2.3−7806), Myrimatch (Vanderbilt University, v.2.1) or MS-GF+ (CCMS-NIGMS, v.Beta v10072). All search engines were then configured to match potential peptide candidates to recalibrated spectra with a mass error tolerance of 10 ppm and fragment ion tolerance of 0.02 Da. For these searches, the predicted proteomes resulting from the annotated genomes of the different whole genome sequenced isolates were used (Table S1). For membrane/cytosol samples, the confidence interval for protein identification was set to ≥95% ($p < 0.05$) and peptides were filtered at a false discovery rate ≤1%. All analyses were conducted using Proteome Discoverer 2.5. software.

2.6. Statistical analysis and bioinformatics programs

The statistical analysis was performed after fitting the experimental *L. monocytogenes* growth data (Log_{10} CFU/mL) in UHT milk and raw milk at 37 °C and 4 °C to the modified Gompertz model to estimate the maximal growth rate (μ_{max}), the lag phase (λ) and maximal final cell density ($Log_{10}Nf_{max}$) of the pathogen. A two-way ANOVA was used to compare the differences of each kinetic parameter between the five different *L. monocytogenes* isolates, and between the two types of milk (UHT and raw milk), at two different temperatures (37 °C and 4 °C), using the IBM SPSS Statistics (Version 27.0.1). The correlation between pH and colony counts and between pH and *L. monocytogenes* isolates were determined using Pearson's and Spearman's bivariate correlation based on the assumption of normality run with the Shapiro−Wilk test. Growth figures were generated with GraphPad Prism (Version 8.0) and Adobe Illustrator (Version 27.0). Volcano plots were created showing the fold change in protein expression on the X-axis versus the significance expressed as the negative logarithm of the corrected *p*-value on the Y-axis.

3. Results

3.1. Growth in UHT milk of hypervirulent and hypovirulent L. monocytogenes isolates from different CCs

All *L. monocytogenes* isolates tested grew rapidly in UHT milk at 37 °C, and the adaptation time to environmental conditions was less than 3 h. The maximal population density was reached after 21−28 h (Fig. 1A).

CC9 hypovirulent isolate showed a significantly shorter lag phase (λ) when compared to the rest of CCs grown in UHT milk at

37 °C (Table 1). In terms of maximal growth rate (μ_{max}) at 37 °C, there were significant differences between isolates of different CCs. The hypervirulent isolate CC1 showed the highest growth rate. CC4 and CC6 exhibited intermediate growth rates, while hypovirulent isolates CC9 and CC121 displayed the lowest growth rates (Fig. 1A, Table 1, Fig. S1). At 37 °C, there were no significant differences in the final cell density between isolates from various CCs, except for CC121, which exhibited a notably elevated final cell density compared to CC1 and CC4, respectively (Fig. 1A, Table 1).

UHT milk at the beginning of the experiment at 37 °C had a normal pH of whole cow's UHT milk (pH 6.82). There was no significant decrease in pH levels (6.83–7.64) during the monitoring times and no association could be determined between CCs or Log_{10} CFU/mL and pH (Table S2).

Regarding refrigeration conditions, all isolates adapted to 4 °C UHT milk before 15h (Fig. 2A). The maximal population density at 4 °C was reached after 28–35 days (Fig. 2A).

Although no statistically significant differences were shown in λ of the distinct isolates grown in UHT milk at 4 °C, the hypovirulent isolates CC121 and CC9 showed shorter λ than the hypervirulent isolates (CC1, CC4, and CC6) (Table 1). For μ_{max} at 4 °C, there were significant differences between the isolates of different CCs. The

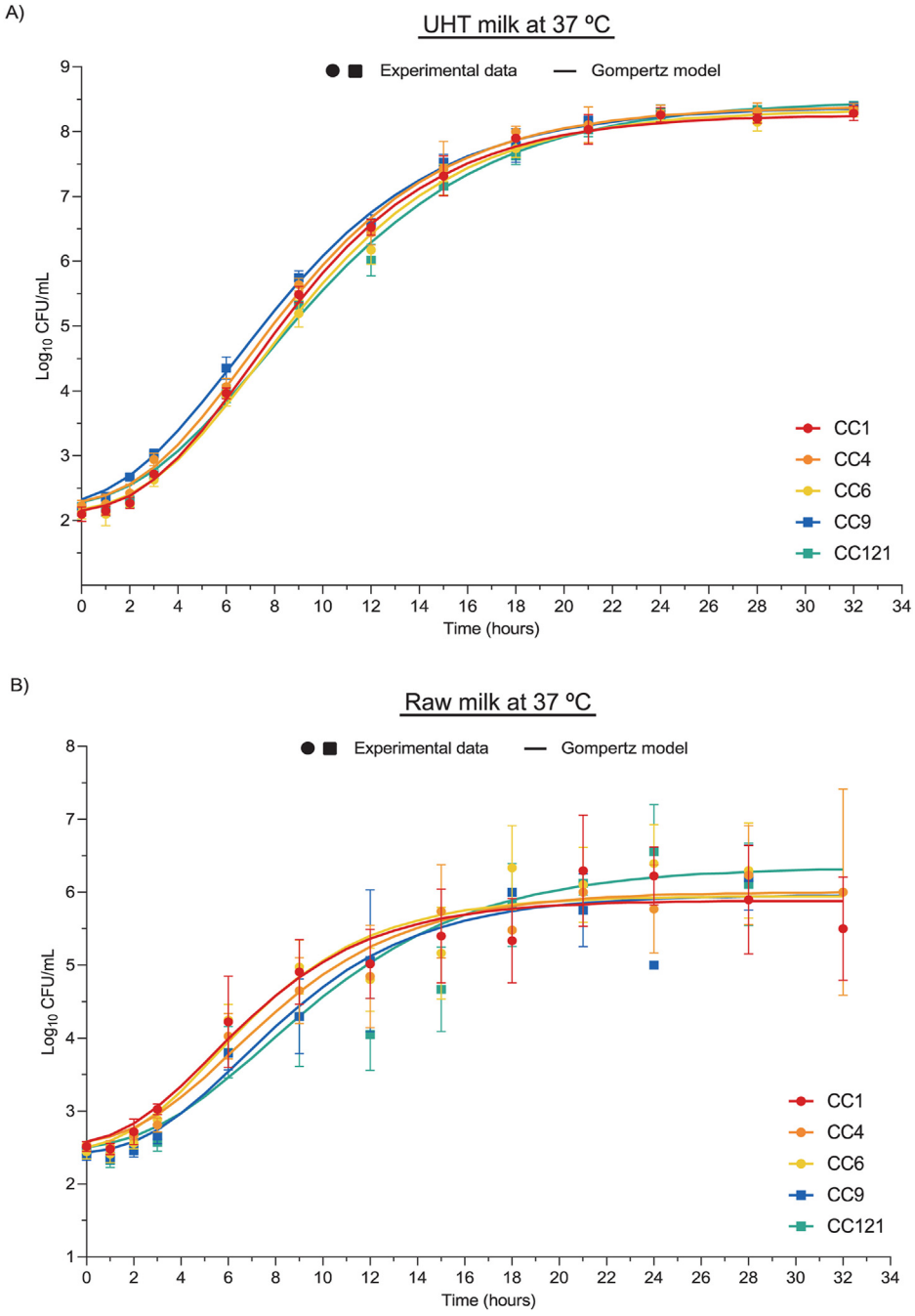

Fig. 1. Growth of *L. monocytogenes* in (A) UHT and (B) raw milk at 37 °C. *L. monocytogenes* CCs were enumerated at 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, and 32 h in both types of milk. Experimental growth data is denoted as (●) for hypervirulent CCs and (■) for hypovirulent CCs. The continuous lines are the fit curves generated by the modified Gompertz model. The mean values and error bars showing standard deviations were performed based on four biological replicates for each CCs and type of milk.

hypovirulent isolates CC9 and CC121 grew at higher rates than the hypervirulent counterparts CC1, CC4, and CC6. CC9 was the fastest growing isolate and CC1 displayed the lowest growth rate (Table 1, Fig. S1). Statistically significant differences in the final cell density at 4 °C were observed among the isolates of distinct CCs. The hypervirulent isolate CC4 had the highest final cell density, followed by the two hypovirulent isolates of CC9 and CC121 (Fig. 2A, Table 1). Isolates CC1 and CC6 reached the lowest final cell densities (Fig. 2A, Table 1).

UHT milk at the beginning of the experiment at 4 °C had a normal pH of whole cow's UHT milk (pH 6.82). No association could be determined between CCs or Log_{10} CFU/mL and pH (Table S4).

Altogether these results demonstrate that hypovirulent isolates grew faster in UHT milk at 4 °C than hypervirulent isolates, and conversely, the hypervirulent isolates exhibit better growth rates in UHT milk at 37 °C than hypovirulent isolates.

3.2. Growth in raw milk of hypervirulent and hypovirulent L. monocytogenes isolates from different CCs

During the first and the third hour of storage, all *L. monocytogenes* isolates grew in raw milk at 37 °C, reaching their maximal population density within 18–24 h (Fig. 1B).

At 37 °C, there were significant differences between the λ of the isolates of different CCs. CC1 showed the shortest λ followed by the other hypervirulent isolates (CC6 and CC4), whereas the hypovirulent isolates CC9 and CC121 showed the longest λ (Table 1). No statistically significant differences were shown in either the μ_{max} or the maximal final concentration of the distinct isolates grown in raw milk at 37 °C.

Raw milk at the beginning of the experiment at 37 °C had a normal pH of cow's raw milk (pH 7.0). No association could be determined between CCs or Log_{10} CFU/mL and pH (Table S3).

All *L. monocytogenes* isolates grew in raw milk at 4 °C after a storage period varying between 8 and 10 days and reached a maximal population density after 21–28 days (Fig. 2B).

At 4 °C, isolates of CC9, CC121, and CC1 showed the shortest λ compared to isolates of CC4 and CC6 (Fig. 2B, Table 1). Regarding the μ_{max} at 4 °C, the hypovirulent isolate of CC9 showed significantly faster growth when compared to the rest of the isolates of different CCs (Table 1, Fig. S1). No significant differences were observed in the maximal final concentration among isolates of different CCs at 4 °C, except for the significant difference found between CC9 and CC121 (Fig. 2B, Table 1).

Raw milk at the beginning of the experiment at 4 °C had a normal pH of cow's raw milk (pH 6.88). No association could be determined between CCs or Log_{10} CFU/mL and pH (Table S5). Altogether these results demonstrated that CC9 isolates grew in raw milk at 4 °C faster and reached higher maximal final cell densities than isolates from the rest of CCs tested.

3.3. Growth comparison between UHT and raw milk of L. monocytogenes isolates

At 37 °C, CC1 and CC6 showed significantly shorter λ when grown in raw milk than in UHT milk. In contrast, CC9 showed significantly longer λ when grown in raw milk than in UHT milk (Table 1). Although no statistically significant differences were observed for μ_{max} between UHT and raw milk at 37 °C, lower μ_{max} in raw milk were observed among isolates of different CCs at 37 °C, except for the hypovirulent isolate of CC9, which grown faster in raw milk (Table 1). For all strains, raw milk reached significantly lower maximal final cell density than UHT milk at 37 °C (Fig. 1B, Table 1).

At 4 °C growth in raw milk had a longer λ than in UHT milk, and in all cases, this delay in the onset of growth was statistically significant (Table 1). Likewise, the maximal final cell density reached was lower in raw milk than in UHT milk, and for all strains, this lower multiplication capacity was statistically significant (Table 1). However, for some isolates, the growth rates were higher in raw milk than in UHT milk. This increase was statistically significant for isolates of CC9 and CC1 (Table 1).

Altogether, the results related to λ and the maximal final cell density show that raw milk restricted *L. monocytogenes* growth compared to that observed in UHT milk.

Table 1
Estimated kinetic parameters of *L. monocytogenes* strains grown in milk obtained from the modified Gompertz model.

Storage temperature (°C)	Kinetic parameter	Milk type	*L. monocytogenes* clonal complex				
			CC1	CC4	CC6	CC9	CC121
37	λ (h)	UHT milk	2.42 ± 0.17 aA	2.29 ± 0.25 aA	2.63 ± 0.17 aA	1.59 ± 0.12 bA	2.27 ± 0.18 aA
		raw milk	1.59 ± 0.45 aB	2.03 ± 0.41 abcA	1.76 ± 0.37 abB	2.75 ± 0.54 cB	2.58 ± 0.36 bcA
	μ_{max} [Log_{10}(CFU/mL)/h]	UHT milk	0.51 ± 0.01 aA	0.50 ± 0.02 abA	0.49 ± 0.02 abA	0.48 ± 0.00 bA	0.44 ± 0.01 cA
		raw milk	0.36 ± 0.08 aA	0.31 ± 0.04 aA	0.37 ± 0.05 aA	0.61 ± 0.51 aA	0.29 ± 0.04 aA
	$Log_{10}Nf_{max}$ [Log_{10}(CFU/mL)]	UHT milk	8.33 ± 0.05 aA	8.39 ± 0.02 aA	8.33 ± 0.07 abA	8.38 ± 0.05 abA	8.48 ± 0.01 bA
		raw milk	5.79 ± 0.49 aB	6.05 ± 0.23 aB	5.80 ± 0.36 aB	5.43 ± 1.13 aB	6.28 ± 0.14 aB
	RMSE	UHT milk	0.167	0.212	0.175	0.131	0.176
		raw milk	0.450	0.439	0.432	0.325	0.481
	R^2adj.	UHT milk	0.995	0.991	0.994	0.996	0.994
		raw milk	0.894	0.904	0.897	0.908	0.900
4	λ (days)	UHT milk	0.48 ± 0.42 aA	0.61 ± 0.42 aA	0.21 ± 0.21 aA	0.12 ± 0.11 aA	0.00 ± 0.00 aA
		raw milk	8.13 ± 0.75 aB	10.74 ± 0.31 bB	10.34 ± 1.35 bB	9.12 ± 0.36 abB	9.88 ± 0.33 abB
	μ_{max} [Log_{10}(CFU/mL)/day]	UHT milk	0.26 ± 0.02 aA	0.32 ± 0.01 bA	0.31 ± 0.01 bA	0.42 ± 0.03 cA	0.38 ± 0.01 dA
		raw milk	0.36 ± 0.02 aB	0.38 ± 0.06 aA	0.33 ± 0.11 aA	0.62 ± 0.10 bB	0.37 ± 0.06 aA
	$Log_{10}Nf_{max}$ [Log_{10}(CFU/mL)]	UHT milk	7.99 ± 0.15 aA	8.83 ± 0.07 bA	7.91 ± 0.09 aA	8.55 ± 0.16 cA	8.59 ± 0.05 bcA
		raw milk	4.85 ± 0.10 abB	4.43 ± 0.11 abB	4.90 ± 0.14 abB	5.03 ± 0.17 aB	4.40 ± 0.51 bB
	RMSE	UHT milk	0.200	0.242	0.278	0.295	0.303
		raw milk	0.126	0.145	0.214	0.178	0.203
	R^2adj.	UHT milk	0.988	0.986	0.979	0.979	0.979
		raw milk	0.987	0.976	0.960	0.978	0.948

Data represents estimated parameter ± standard deviation. Values in the same row followed by the same lowercase letter are not significantly different ($P > 0.05$) and values in the same column followed by the same uppercase letter are not significantly different ($P > 0.05$). Parameters are estimated from the growth curves and each point of the curves was based on four replicate samples in UHT milk and raw milk.

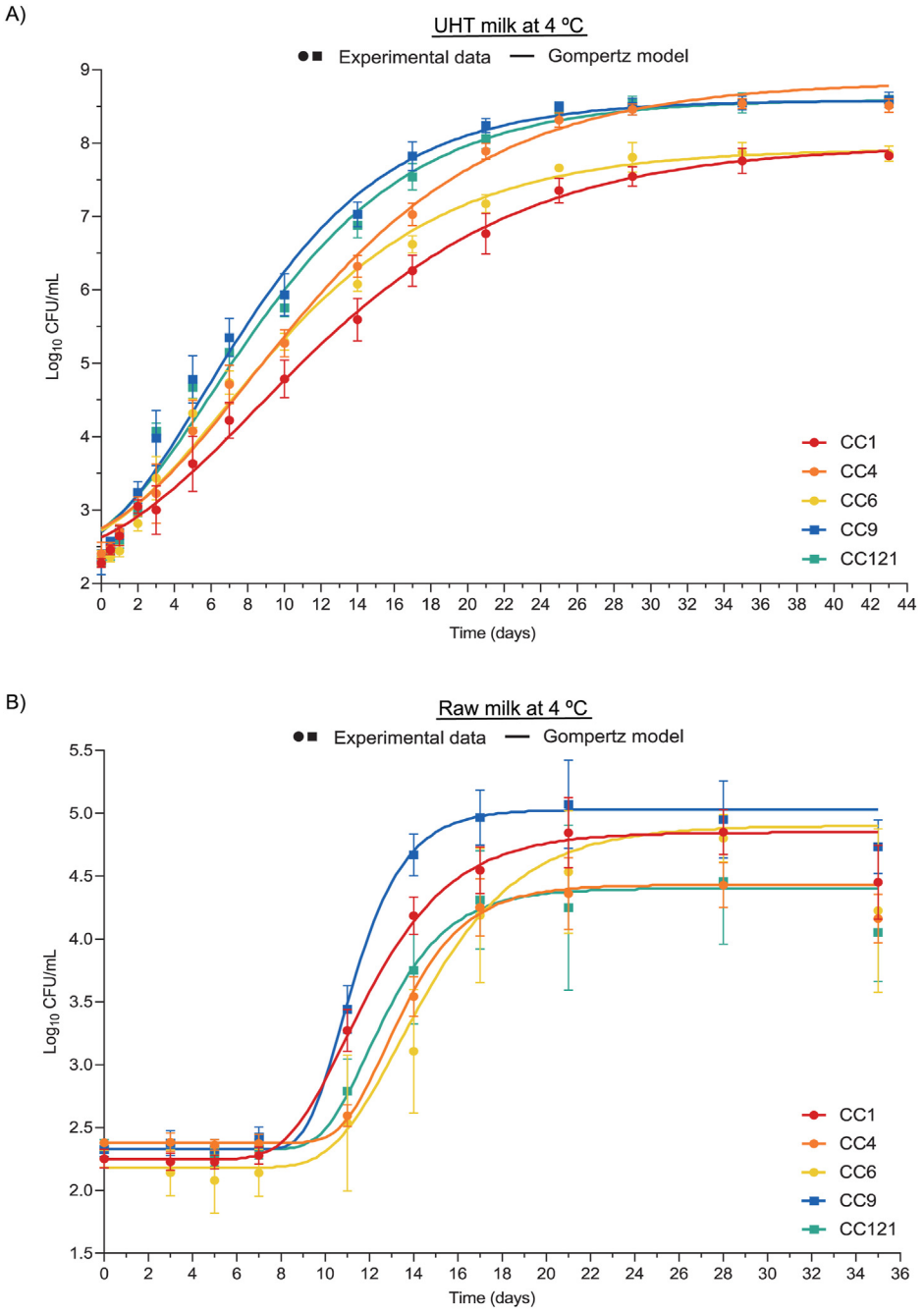

Fig. 2. Growth of *L. monocytogenes* in (A) UHT and (B) raw milk at 4 °C. *L. monocytogenes* CCs were enumerated at 0, 0.5, 1, 2, 3, 5, 7, 10, 14, 17, 21, 25, 29, 35, and 43 days in UHT milk. *L. monocytogenes* CCs were enumerated at 0, 3, 5, 7, 11, 14, 17, 21, 28, and 35 days in raw milk. Experimental growth data is denoted as (●) for hypervirulent CCs and (■) for hypovirulent CCs. The continuous lines are the fit curves generated by the modified Gompertz model. The mean values and error bars showing standard deviations were performed based on four biological replicates for each CCs and type of milk.

3.4. Identification of proteins differentially produced by CC1 and CC9 isolates grown in milk at 4 °C

Since the highest growth difference in milk was observed at 4 °C among isolates of CC1 and CC9, our goal was to unravel by a proteomics approach involving high resolution mass spectrometry (MS) how these CCs adapted to milk. Regarding membrane and cytosol proteins, the CC1 isolate grown in UHT milk at 4 °C upregulates 32 proteins and downregulates 19 proteins compared to the same bacteria growing in BHI (Figs. 3, 4A, Table S6, Fig. S1). Importantly, in this hypervirulent CC1 isolate, Internalin C2, Internalin D, the wall teichoic acid glycosylation protein GtcA, CC1_01637 (Lmo0576) (which contains 2 mucin binding domains), and CC1_01393 (Lmo0321) (a membrane protein with a SfLAP domain of glycolipid exporters) were upregulated when growing in milk (Fig. 3, Table S6, Fig. S1). In the case of the CC9 isolate grown in milk, proteomics identified 23 upregulated and 20 downregulated proteins compared to growth in BHI (Figs. 3 and 4A, Table S7, Fig. S1). Ten proteins were upregulated in milk compared to BHI in both CC1 and CC9, including important proteins like diaminopimelate decarboxylase (LysA), proteins involved in stress response (CC1_00885 and its homolog in CC9, Lmo2158),

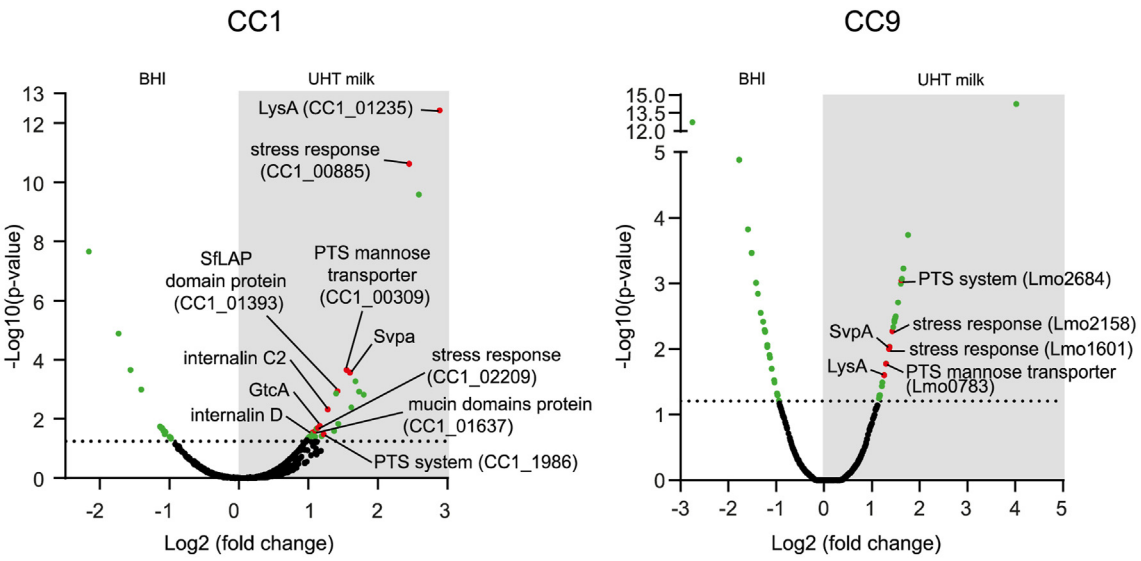

Fig. 3. Volcano plot created by the comparison of *L. monocytogenes* CC1 and CC9 grown in BHI and UHT milk at 4 °C, combining the Log2 fold change and *p*-value (-Log_{10}). The horizontal dashed line represents the adjusted *p*-value threshold <0.05. Proteins upregulated and downregulated in UHT milk at 4 °C compared to samples grown in BHI at 4 °C are highlighted as green and red dots. Red dots include proteins whose biological function is discussed in the text.

components of different phosphotransferase systems and the sortase-B substrate Lmo2185 (Svpa, for surface virulence-associated protein, or Hbp2 for hemoglobin-binding protein) (Figs. 3 and 4A, Table 2, Fig. S1), see Tables S6–S7 for complete lists. Whereas changes for SvpA were similar in CC1 and CC9, that was not the case for LysA, which showed a higher upregulation in CC1 compared to CC9 (Fig. 3). Altogether, these results indicate that among the *L. monocytogenes* isolates tested, there is a *Listeria* CC-specific and a *Listeria* CC-common pattern response to the milk environment (Fig. 4A, Fig. S1).

Regarding cell surface proteins associated to peptidoglycan, proteomics identified in CC1 and CC9 isolates 8 and 4 proteins, respectively, upregulated in bacteria growing in UHT milk (Fig. 4B, Fig. S1, see Tables S8–S9 for complete lists). Interestingly, the protein Lmo1715, which is a predicted class I SAM-dependent methyltransferase, was associated to growth in milk in both CC1 and CC9 isolates (Fig. 4B, Fig. S1, see Tables S8-S9-S10-S11 for complete lists). Other surface proteins identified in the CC1 cell wall subproteome associated to growth in milk included internalins, LPxTG proteins, and the sortase-B substrate Lmo2186 (also known as Hbp1 for hemoglobin-binding protein) (Fig. 4B, Table S9, Fig. S1). Regarding CC9, the surface proteins of the cell wall subproteome associated to growth in milk included Lmo1344 (ComGD, from the competence system), Lmo2305 (which contains a DUF2481 present in Bacteriophage A118 systems) and Lmo0327 (a protein with murein hydrolase activity which encodes an internalin-like protein) [34] (Fig. 4B, Table S8, Fig. S1).

4. Discussion

To the best of our knowledge, only one study has previously evaluated the growth potential of the frequently observed low number of *L. monocytogenes* cells counted in raw milk [10]. Moreover, understanding the growth behaviour of hyper- and hypovirulent *L. monocytogenes* CCs isolates in UHT and raw milk has remained a missing piece to fully understand why the hypervirulent isolates are over-represented in dairy products. A key aspect to decipher is whether the over-representation of hypervirulent isolates in dairy products is a consequence of a growth advantage of these hypervirulent isolates in this type of products.

Here, we investigated the role of temperature and type of milk (UHT versus raw) as determinants influencing the growth of hyper- versus hypovirulent isolates in dairy products. The present study shows that *L. monocytogenes* growth in UHT milk correlates with virulence. Firstly, regarding UHT milk and 37 °C (representing the mammalian host temperature), our results show that the hypervirulent isolate CC1 (which is the CC most associated with dairy products [4]) showed the highest growth rate. CC4 and CC6 exhibited intermediate growth rates, while hypovirulent isolates CC9 and CC121 (which are the CC most prevalent in food [17]) displayed the lowest growth rates. Secondly, regarding UHT milk and 4 °C (representing refrigeration temperature for food preservation), higher growth rates and lower lag phases were found in hypovirulent isolates (CC9 and CC121) when compared to hypervirulent isolates (CC1, CC4, and CC6). The higher growth rate of CC9 and CC121 in UHT milk, and of CC9 in raw milk at food preservation temperatures compared to CC1, CC4 and CC6 hypervirulent isolates, contrasts with the over-representation of these latter isolates in dairy products detected during surveillance of listeriosis [4,35]. Previous studies from our group and others have shown that dairy ruminant farms are a reservoir of hypervirulent isolates, being CC1 and CC4 the most prevalent CCs [36,37]. Moreover, it has been reported that the phylogeography of the hypervirulent *L. monocytogenes* CC1 is linked to cattle global trade and farming [38]. Dairy products may be contaminated by *L. monocytogenes* during or after milking at the farm (10–16% of contaminated bulk tank milk samples in a total of 186 tested samples) [39]. The results shown here together with our previous results [37] therefore suggest that CC1 and CC4 overrepresentation in dairy products made of raw milk may arise from contamination during or after milking at the farm and discard an advantage of hypervirulent isolates in milk products when stored at refrigeration temperatures. This postulate is reinforced by the results of Maury et al. [4], which showed that CC121 and CC9 rank 17 and 20, respectively, in dairy products made of raw milk, whereas in dairy products made of pasteurized/unknown type of milk, these two isolates are the second and the seventh most abundant isolates. Moreover, CC9 and CC121 are associated with dairy products made of non-raw milk, as compared with the rest of the species [4]. Of particular interest was the case of CC4, which showed at 4 °C in UHT milk a growth rate behaviour

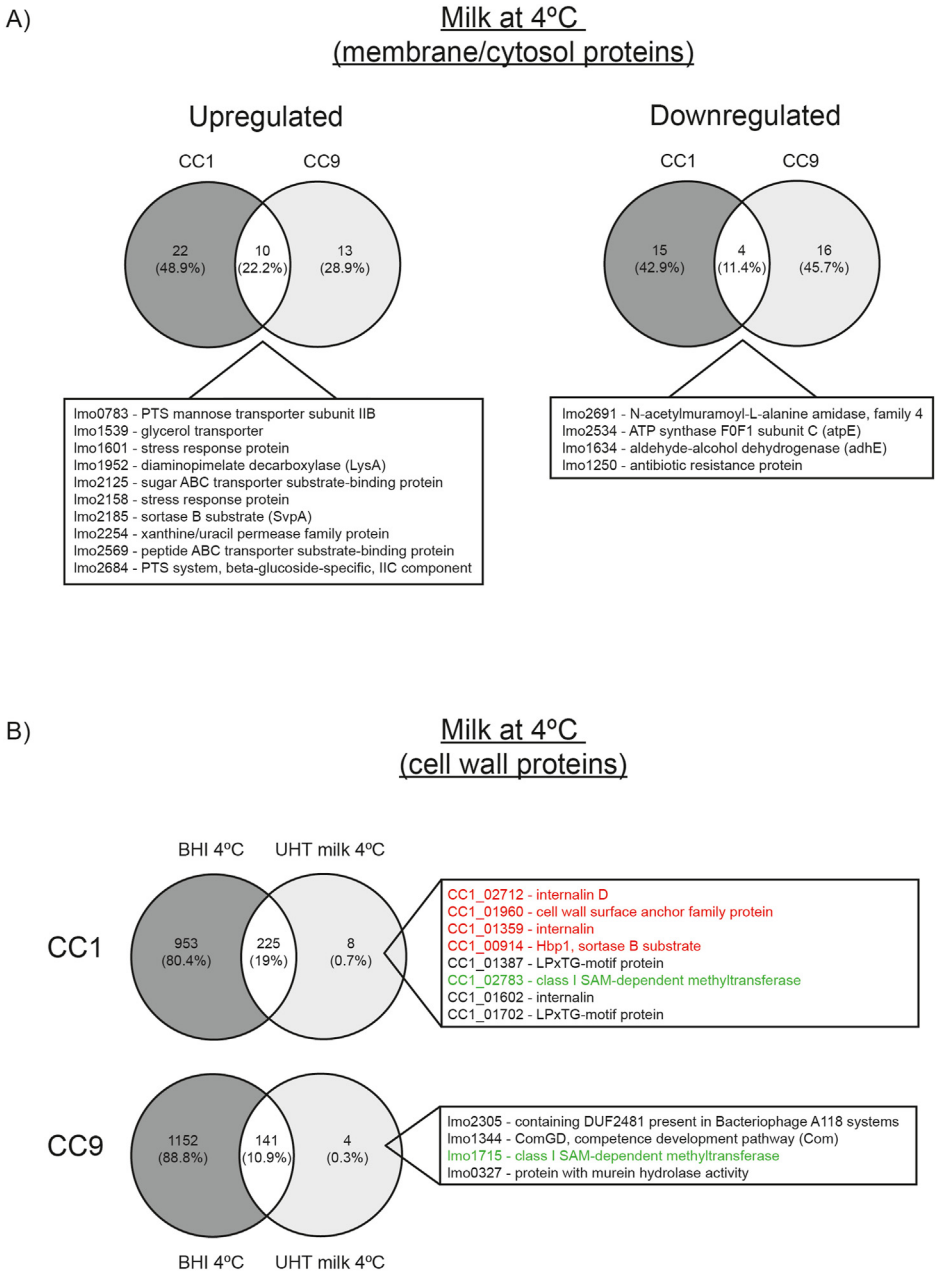

Fig. 4. Venn diagram showing (A) upregulated and downregulated *L. monocytogenes* CC1 and CC9 membrane/cytosolic proteins in UHT milk at 4 °C and (B) cell wall proteins of *L. monocytogenes* CC1 and CC9 grown in BHI and UHT milk at 4 °C. Venn diagrams show the dispersion of the identified cell wall proteins of each strain grown in each condition. Proteins from CC1 with no homology (sequence similarity search using BLAST algorithm) in the CC9 isolate are highlighted in red. When the same protein is present in both strains, it is highlighted in green.

between hypervirulent isolates CC1/CC6 and hypovirulent isolates CC9/CC121, and also had the highest final cell density of all the isolates tested. This CC4 isolate harboured LIPI-4, a cluster of six genes annotated as a cellobiose-family phosphotransferase system [17]. Whether this behaviour is related to the presence of LIPI-4 remains to be elucidated and warranties further investigation.

Despite the importance of dairy products linked to *L. monocytogenes* outbreaks, no studies have examined changes in protein content in conditions modelling these food products. This is relevant considering that *L. monocytogenes* remodels to a large extent the proteome in different environments [40–42]. To our knowledge, this study is the first showing that there is a *Listeria* CC-specific and a *Listeria* CC-common response to the milk environment. Our results show several proteins upregulated during *L. monocytogenes* growth in UHT milk at 4 °C like LysA and SvpA. Of note, LysA catalyses the final step in the diaminopimelate biosynthesis pathway of bacteria, whose final product is L-lysine. In bacteria, the essential amino acid L-lysine is an important precursor for the synthesis of the peptidoglycan cell wall, virulence factors, and housekeeping proteins. Indeed, diaminopimelate decarboxylase is being investigated as a target for novel antibacterial agents [43]. Other membrane/cytosol proteins upregulated in CC1 and CC9 in milk conditions include Lmo2158 and Lmo1601, both involved in stress response [44,45], and Lmo0783 and Lmo2684, both components of different phosphotransferase systems. Regarding specific responses, CC9 upregulated in milk three more components of

Table 2
CC1 and CC9 *L. monocytogenes* membrane/cytosolic proteins upregulated in UHT milk at 4 °C compared to samples grown in BHI at 4 °C. In the case of proteins exclusively expressed in UHT milk by both strains, the first Fold change (Log_2) corresponds to *L. monocytogenes* CC1 and the second one to *L. monocytogenes* CC9.

Strain/s	Gene	Homologe in EGDe (CC9)	Description	Abundance Ratio (Log_2)
exclusively expressed in UHT milk by CC1	CC1_02534	lmo1718	hypothetical protein	0.99
	CC1_00790	lmo1349	glycine cleavage system P protein, subunit 1_gcvP1	1
	CC1_02538	lmo1959	ferrichrome-binding protein	1.02
	CC1_00561	lmo2458	phosphoglycerate kinase_pgk	1.02
	CC1_01639	lmo0574	glycosyl hydrolase, family 1	1.04
	CC1_00354	lmo0720	hypothetical protein	1.07
	CC1_01834	lmo1469	30S ribosomal protein S21_rpsU	1.07
	CC1_01637	lmo0576	hypothetical protein	1.1
	CC1_00810	lmo1368	DNA repair protein RecN_recN	1.1
	CC1_01182	lmo1902	3-methyl-2-oxobutanoate hydroxymethyltransferase_panB	1.1
	CC1_02257	lmo1649	hypothetical protein	1.14
	CC1_00297	lmo0794	hypothetical protein	1.15
	CC1_02712	lmo0263	internalin D_inlD	1.22
	CC1_02711	lmo0263	internalin C2_inlC2	1.28
	CC1_02513	lmo1739	amino acid ABC transporter ATP-binding protein	1.37
	CC1_01393	lmo0321	putative membrane protein	1.42
	CC1_00740	lmo1301	hypothetical protein	1.43
	CC1_01984	lmo2682	potassium-transporting ATPase subunit A_kdpA	1.62
	CC1_01833	lmo1470	16S ribosomal RNA methyltransferase RsmE	1.68
	CC1_00469	lmo2549	wall teichoic acid glycosylation protein GtcA_gtcA	1.73
	CC1_02567	lmo1994	LacI family transcriptional regulator	1.8
	CC1_01695	lmo0520	hypothetical protein	2.59
exclusively expressed in UHT milk by CC9 (EGDe)	lmo0481		similar to unknown proteins	1.48
	lmo0515		conserved hypothetical protein	1.47
	lmo0610		similar to internalin proteins. Putative peptidoglycan bound protein (LPXTG motif)	1.45
	lmo0781		similar to mannose-specific phosphotransferase system (PTS) component IID	1.49
	lmo0845		similar to *B. subtilis* YxjH and YxjG proteins	1.47
	lmo0880		similar to wall associated protein precursor (LPXTG motif)	1.37
	lmo1628		highly similar to tryptophan synthase (beta subunit) (trpB)	1.21
	lmo2357		similar to unknown protein	1.63
	lmo2391		conserved hypothetical protein similar to *B. subtilis* YhfK protein	1.3
	lmo2649		similar to hypothetical PTS enzyme IIC component	1.36
	lmo2708		similar to PTS system, cellobiose-specific enzyme IIC	1.22
	lmo2743		similar to transaldolase	1.47
	lmo2834		similar to oxidoreductases	4.02
exclusively expressed in UHT milk by both strains (CC1 and EGDe (CC9))	CC1_00309	lmo0783	PTS mannose transporter subunit IIB	1.55/1.3
	CC1_01761	lmo1539	glycerol transporter	1.4/1.66
	CC1_02209	lmo1601	general stress protein	1.14/1.37
	CC1_01235	lmo1952	diaminopimelate decarboxylase_lysA	2.89/1.26
	CC1_02269	lmo2125	sugar ABC transporter substrate-binding protein	1.42/1.49
	CC1_00885	lmo2158	hypothetical protein (stress response)	2.45/1.43
	CC1_00913	lmo2185	hypothetical protein	1.6/1.37
	CC1_00985	lmo2254	xanthine/uracil permease family protein	1.19/1.55
	CC1_00449	lmo2569	peptide ABC transporter substrate-binding protein	1.02/1.26
	CC1_01986	lmo2684	PTS system. Beta-glucoside-specific. IIC component	1.12/1.62

different phosphotransferase systems, namely Lmo0781, Lmo2649, Lmo2708. The phosphotransferase systems play roles in many aspects of bacterial physiology, including sugar transport, biofilm formation and virulence, among others [46,47]. Because these systems are known to mediate sugar uptake, the upregulation of a higher number of proteins of these systems in CC9 could explain its higher growth rate in milk. Since no eukaryote has yet been shown to possess a protein constituent of this system, it therefore appears as a potential target of antimicrobial agents [47]. An important protein upregulated in milk in both strains was the sortase B substrate SvpA. SvpA is an important virulence factor required for the intracellular survival of *L. monocytogenes* [48]. SvpA also plays a physiological role in iron transport [49]. Interestingly, *lmo2185* (coding for SvpA), *lmo2186*, and *srtB* (coding for Sortase B) genes form an operon regulated by the iron-responsive transcriptional repressor Fur and is induced under iron-deficient conditions such as milk [50]. Importantly, our results show that proteins upregulated by hyper- and hypovirulent CCs (diaminopimelate decarboxylase or phosphotransferase systems) could be targeted with novel antibacterial agents to restrict *L. monocytogenes* growth in milk products.

This study paves the way for deciphering the dynamics and drivers of dairy product contamination by hypervirulent *L. monocytogenes* CCs. We believe this information will help infection control policies to reduce the burden of listeriosis.

Author contribution

JJQ conceived the study. ILA, PRG, MCLM, MGP, FGDP, JJQ designed experiments. AEM, CPG, ILA, MGP performed experiments. AEM, CPG, ILA, PRG, MCLM, MGP, FGDP, JJQ analysed the data. AEM, CPG, ILA, JJQ prepared the figures and wrote the original manuscript draft. AEM, CPG, ILA, PRG, MCLM, MGP, FGDP, JJQ reviewed and edited the manuscript. ILA, JJQ supervised the work.

JJQ was involved in funding acquisition. All the authors read and approved the manuscript.

Funding

This work was supported by Generalitat Valenciana (Project reference AICO/2021/278) (JJQ), the Spanish Ministry of Science and Innovation (Project references PID2022-137961OB-I00 (JJQ), PGC2018-096364-B-I00/AEI/10.13039/501100011033/FEDER (MGP)), and Universidad CEU Cardenal Herrera (Programa INDI 22/44) (JJQ). J.J. Quereda is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Science, Innovation, and Universities (RYC-2018-024985-I). Alba Espí-Malillos and Carla Palacios-Gorba are supported by a Predoctoral contract from the Universidad Cardenal Herrera-CEU. The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD048639 and 10.6019/PXD048639. All data generated and analysed during this study have been incorporated into this article.

Ethics statement

No animal experiments were performed.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgement

We thank to Marc Lecuit and Alexandra Moura (Institut Pasteur, France) for providing the reference strains and genome annotations used in this study. We would also like to thank the collaboration of Elena Escolar Saval from the Milk Analysis Laboratory of the Institute of Animal Science and Technology of the Polytechnic University of Valencia who contributed providing the raw milk samples and Mila Mateos Otero for helpful discussions. The technical assistance of Alberto Paradela (Proteomic Facility of the National Center of Biotechnology, CNB) is also acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105312.

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