Persistent and non-persistent strains of *Listeria monocytogenes*: A focus on growth kinetics under different temperature, salt, and pH conditions and their sensitivity to sanitizers

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Keywords:
Listeria monocytogenes
Persistent
Growth
Microtiter plate assay
Stress
Temperature
Sanitizers
Hydrogen peroxide
Quaternary ammonium compounds

Abstract

This study aimed to investigate the effect of different conditions, including temperature (37 ºC, 22 ºC, and 4 ºC), NaCl concentrations (2.5%, 4%, and 8%), and acidity (pH 1⁄4 5), on the growth response of persistent and non-persistent isolates of *Listeria monocytogenes*. The resistance to two common sanitizers (ben- zalkonium chloride and hydrogen peroxide) was also investigated. A selected group of 41 persistent and non-persistent *L. monocytogenes* isolates recovered from three cheese processing plants during a previous longitudinal study was assembled. Average lag time was similar for persistent and non-persistent isolates grown at 37 ºC, 22 ºC and 4 ºC, but significantly shorter ($p < 0.05$) for persistent isolates grown at 2.5%, 4% and 8% NaCl, and at pH 5. Average growth rates were significantly higher ($p < 0.05$) for persistent than for non-persistent isolates when grown at 22 ºC, 2.5%, 4% and 8% NaCl, and at pH 5. These results suggest that persistent strains may be better adapted to grow under stressful conditions frequently encountered in food processing environments than non-persistent strains. No relation between persistence and resistance to the tested sanitizers was found.

1. Introduction

The source of almost all human listeriosis cases is the ingestion of contaminated foods by *Listeria monocytogenes* (Farber and Peterkin, 1991; McLauchlin et al., 2004). Despite all efforts to decrease its

incidence, as a consequence of food safety regulations and improvements in food safety practices, invasive listerisosis is still considered the most important cause of death from foodborne infections in industrialised countries (EFSA, 2015). Cross-contamination of food products by the equipment and the gen- eral environment of food processing plants, during the different processing stages or preparation of final products, is a major problem (Lappi et al., 2004; Thévenot et al., 2005; Almeida et al., 2013; Ferreira et al., 2011). A particular feature that makes the control of *L. monocytogenes* difficult to achieve in the processing environment is the capacity of this bacterium to survive (and in specific circumstances even grow) under various conditions, such as a wide pH range (4.7 - 9.2), high salt concentrations (up to 10% wt/vol), and low temperatures (0.5 - 9.3 ^{o}C) (McClure et al., 1989; Petran and Zottola, 1989; Phan-Thanh, 1998; Walker et al., 1990).

Repeated isolation of strains of *L. monocytogenes* showing a specific molecular subtype (e.g. by pulsed-field gel electrophoresis or ribotyping characterization) over an extended period of time in the same processing plant for several months or years has been reported (Almeida et al., 2013; Ferreira et al., 2011; Lappi et al., 2004; Lundén et al., 2003a; Miettinen et al., 1999; Norton et al., 2001). These strains are considered to be an in-house persistent strain. In contrast, non-persistent strains, present distinct molec- ular subtypes and are occasionally isolated.

L. monocytogenes is frequently exposed to various stressing agents during food processing or cleaning and disinfection pro- cedures. The effects of such environmental stresses on this path- ogen are of great interest as they could influence its response and ability to persist in these environments, and thus contribute to defining conditions for better control in food processing plants. The aim of the present study was to evaluate the effect of different temperatures, various concentrations of NaCl, and a moderately acidic condition, on the specific growth response of a selected group of persistent and non-persistent *L. monocytogenes* isolates recovered from three cheese processing plants. The resistance of these strains to two common disinfectants (benzalkonium chloride and hydrogen peroxide) was also investigated.

2. Materials and methods

2.1. Bacterial isolates and inoculum preparation

Forty-one *L. monocytogenes* isolates representing persistent and non-persistent isolates were selected from Listeria Research Center from Escola Superior de Biotecnologia (LRCESB) culture collection. The selection of isolates was based on the PFGE typing results of a previous longitudinal study by Almeida et al. (2013) that evaluated during a four-year period the contamination by *L. monocytog*enes

in the environment, raw material and final-products of cheese- processing plants. PFGE data provided evidence for persistence of *specific L. monocytogenes* strains, defined by the repeated isolation of L. monocytogenes isolates with identical molecular subtypes on different dates, in an artisanal producer of raw ewe's milk cheeses (APC), and in a small-scale industrial cheese producer (SSI). For this study persistent isolates were selected with PFGE types Da, Db, and Dc (recurrently isolated during 15, 9 and 8 months, respectively) from APC producer, and PFGE types Ka, Kb, and E (recurrently isolated over a period of four, three and four years, respectively) from SSI producer; one representative isolate of each PFGE type was selected per each sampling date, where one or more sample(s) were contaminated by *L. monocytogenes* with a specific persistent PFGE type (Table 1). Ten non-persistent strains, i.e. strains with unique PFGE types and that were isolated only once from samples of the two processing plants were also included in this study (Table 1). Stock cultures were grown and kept in tryptic soya broth with yeast extract 0.6% w/v (TSBYE, Merck, Darmstadt, Germany) supplemented with 30% (w/v) of glycerol at 80 ºC. Before use, frozen stocks were streaked onto tryptic soya agar with yeast extract 0.6% w/v (TSAYE, Lab M, Bury, United Kingdom) and incu- bated at 37 ºC, overnight. A single colony was inoculated into 10 ml of TSBYE and incubated overnight at 37 ºC. The cultures were then sub-cultured in 10 mL of TSBYE (1% v/v) and incubated in the same conditions.

The bacterial growth parameters, including the length of lag phase and growth rate, of 31 persistent and ten non-persistent *L. monocytogenes* strains as function of temperature, salt concentra- tion, and pH, were evaluated (Table 2). As previously reported by other studies, a considerable inter-strain variability of the growth behaviour was observed (reviewed by Lianou and Koutsoumanis, 2013); as expected, the lower the temperature of incubation the more extended was the length of the lag phase. No differences were observed in average lag time among persistent and non-persistent isolates grown in TSBYE at 37 ºC, 22 ºC and 4 ºC (Table 2). However, significant differences (p ¼ 0.017) were observed in average growth rates among persistent and non-persistent isolates grown in TSBYE at 22 ºC (Table 2). With the increase in NaCl concentration, *L. monocytogenes* isolates presented longer lag times and lower growth rates (Table 2). Persistent *L. monocytogenes* isolates grown in TSBYE supplemented with NaCl (concentrations of 2.5%, 4% and 8%) presented a significantly shorter average lag time (p ¼ 0.001, p ¼ 0.004 and p ¼ 0.002, respectively) and a significantly higher average growth rate (p ¼ 0.000, p ¼ 0.000 and p ¼ 0.027, respectively) when compared to non-persistent isolates. When grown under moderate acidic conditions (i.e. TSBYE adjusted to pH 5) at 37 ºC, persistent isolates presented an average lag time significantly shorter (p ¼ 0.000) and average growth rates significantly higher (p ¼ 0.002) than non-persistent strains (Table 2).

Table 1
Characteristics of *L. monocytogenes* isolates used in this study.

P/NP[a]	Isolate code	Producer[b]	Year	Month	Origin	PFGE type	Geno-serogroup[c]
P	1604	APC	2005	Jun	Cheese	Da	IVb
	1635	APC	2005	Jul	Raw milk	Da	IVb
	1675	APC	2005	Oct	CW zone, floor	Da	IVb
	1732	APC	2006	Jan	Production zone, drain	Da	IVb
	1757	APC	2006	Feb	Cheese	Da	IVb
	1816	APC	2006	Sept	Production zone, drain	Da	IVb
	1384	APC	2005	Feb	Whey	Db	IVb
	1634	APC	2005	Jul	Entrance, floor	Db	IVb
	1674	APC	2005	Oct	Milk reception, floor	Db	IVb
	1700	APC	2005	Nov	Production zone, floor	Db	IVb
	1606	APC	2006	Jun	Cheese	Db	IVb
	1728	APC	2006	Jan	Cheese	Dc	IVb
	1727	APC	2006	Jan	Shipping zone, floor	Dc	IVb
	1777	APC	2006	Mar	Cheese	Dc	IVb
	1797	APC	2006	Jun	Trolley	Dc	IVb
	1279	SSI	2004	Oct	Cheese washing zone, sink	E	IIa
	1597	SSI	2005	May	Shipping zone, table	E	IIa
	1659	SSI	2005	Aug	Cheese washing zone, floor	E	IIa
	2123	SSI	2007	Sept	Cheese washing zone, drain	E	IIa
	798	SSI	2003	Jun	Cheese	Ka	IIb
	868	SSI	2003	Nov	Cheese washing zone, sink	Ka	IIb
	925	SSI	2004	Jan	Cheese	Ka	IIb
	1155	SSI	2004	Jun	Cheese washing zone, floor	Ka	IIb
	1592	SSI	2005	May	Cheese	Ka	IIb
	2116	SSI	2007	Jul	Cheese washing zone, drain	Ka	IIb
	1034	SSI	2004	Apr	Cheese washing zone, drain	Kb	IIb
	1108	SSI	2004	May	Cheese	Kb	IIb
	1598	SSI	2005	May	Cheese washing zone, drain	Kb	IIb
	1696	SSI	2005	Oct	Cheese	Kb	IIb
	1716	SSI	2005	Nov	Cheese washing zone, brush	Kb	IIb
	2047	SSI	2007	Feb	Cheese washing zone, drain	Kb	IIb
NP	1712	APC	2006	Dec	CW zone, floor	A	IIa
	1499	APC	2005	Feb	Cheese	B	IIa
	929	APC	2004	Feb	Cheese	C	IIa
	1559	SSI	2005	Apr	Shipping zone, table	Ja	IIb
	747	SSI	2003	May	Cheese	Ha	IIb
	812	SSI	2003	Jul	Cheese washing zone, sink	Hb	IIb
	832	SSI	2003	Aug	Cheese washing zone, sink	La	IIb
	930	SSI	2004	Mar	Cow's raw milk	F	IIa
	994	SSI	2004	Feb	Goat's raw milk	G	IIa
	1302	SSI	2004	May	Cheese washing zone, drain	M	IIb

[a] P, persistent; NP, non-persistent.
[b] APC, artisanal cheese producer; SSI, small-scale industrial cheese producer.
[c] Geno-serogroup IVb (serotypes 4b, 4d, and 4e), geno-serogroup IIa (serotypes 1/2a and 3a) and geno-serogroup IIb (serotypes 1/2b, 3b, and 7).

2.2. Growth response at various NaCl concentrations, at pH 5.0 and at different temperatures

The growth of the selected *L. monocytogenes* isolates was monitored under different conditions of temperature, NaCl, and pH. For NaCl experiments TSBYE was supplemented with NaCl (Merck, Darmstadt, Germany) at a final concentration of 2.5, 4 and 8% (w/v). For the pH experiment TSBYE was adjusted to pH 5 with 0.1 M HCl solution. An overnight culture of each isolate was diluted to achieve inoculation levels of approximately 10^4 Colony Forming Units (CFU)/mL and inoculated at 1% (v/v) in three wells of a sterile 96-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), previously filled with 200 mL of the specific broth for each experi- ment. Three wells with sterile culture media were included as controls for temperature, NaCl and pH experiments. Plates were incubated at 37 ºC and also at 22 and 4 ºC for temperature exper iments. The Optical Density (OD) at 665 nm was registered at 60 min intervals using a Microplate Reader (Model 680, Bio-Rad, Marnes-la-Coquette, France). Three independent replicates were performed for each assay.

2.3. Susceptibility of *L. monocytogenes* strains to sanitizers

Two disinfectants, benzalkonium chloride (BC) (Sigma Chemical Co., St. Louis, MO, USA) and hydrogen peroxide (Aga, Prior Velho, Portugal), were used in the study. Different concentrations of disinfectant solutions were prepared by dissolving the agents in sterile distilled water immediately prior to testing; 1.5 and 0.75% (v/ v) of hydrogen peroxide and 500, 50 and 25 ppm of benzalkonium chloride (BC).

Isolates were cultured in TSBYE at 37 ºC for 18 h to achieve a bacterial level of approximately 10^9 CFU/mL. Cells were then centrifuged (5500 rpm, 5 min), re-suspended in phosphate- buffered saline (PBS, pH 7.4, Sigma), and 500 mL of the suspension were added to 4.5 mL of each disinfectant solution at different concentrations, mixed with a vortex and incubated at 22 ºC for 5 and 20 min. For controls PBS was used in place of disinfectant. After each exposure time, 1 mL of each suspension was transferred into a new sterile tube and 1 mL of catalase solution (0.2 mg/mL) or Contact D/E Neutralizing (Difco, Franklin Lakes, NJ, USA) was added to neutralize hydrogen peroxide and BC, respectively. The suspen- sions were incubated for 10 min at 22 ºC; serially diluted in sterile 1⁄4 strength Ringer's solution (LAB M) and were plated on TSAYE by the drop count technique (Miles and Misra, 1938). After incubation at 37 ºC for 24 h bacterial colonies were counted and CFU/mL deter- mined. Each disinfectant concentration was tested in triplicate and two independent replicates were performed. The log reduction after exposure to each disinfectant concentration was calculated by subtracting the average Log CFU/mL for disinfectant-exposed cells from the average Log CFU/mL for unexposed control cells. The standard deviation was calculated by using the Log reduction values obtained in the independent replicates.

2.4. Statistical analysis

Pairwise multiple comparisons were carried out to detect sig- nificant differences between persistent and non-persistent *L. monocytogenes* strains in terms of (i) lag time and growth rate at different temperatures (37 ºC, 22 ºC, and 4 ºC), concentrations of NaCl (2.5%, 4%, and 8%), and at acid growth condition (pH 5), and (ii) log reduction after 20 min exposure to hydrogen peroxide (1.5% v/v) and BC (50 ppm). Student's t-test was used when normality of data was verified. Alternatively, the non-parametric ManneWhitney test was used when data were not normally distributed. Normality of data sets was assessed by using KolmogoroveSmirnov test. The significance level assumed in all situations was 5%. All calculations were carried out using IBM SPSS® Statistics® 20 for Windows® (SPSS Inc., Chicago, USA).

3. Results and discussion

3.1. Specific growth response of persistent and non-persistent *L. monocytogenes* strains to different temperatures, NaCl concentrations, and pH conditions

The bacterial growth parameters, including the length of lag phase and growth rate, of 31 persistent and ten non-persistent *L. monocytogenes* strains as function of temperature, salt concentration, and pH, were evaluated (Table 2). As previously reported by other studies, a considerable inter-strain variability of the growth behaviour was observed (reviewed by Lianou and Koutsoumanis, 2013); as expected, the lower the temperature of incubation the more extended was the length of the lag phase. No differences were observed in average lag time among persistent and non-persistent isolates grown in TSBYE at 37 ºC, 22 ºC and 4 ºC (Table 2). However, significant differences (p 1⁄4 0.017) were observed in average growth rates among persistent and non-persistent isolates grown in TSBYE at 22 ºC (Table 2). With the increase in NaCl concentration, *L. monocytogenes* isolates presented longer lag times and lower growth rates (Table 2). Persistent *L. monocytogenes* isolates grown in TSBYE supplemented with NaCl (concentrations of 2.5%, 4% and 8%) presented a significantly shorter average lag time (p 1⁄4 0.001, p 1⁄4 0.004 and p 1⁄4 0.002, respectively) and a significantly higher average growth rate (p 1⁄4 0.000, p 1⁄4 0.000 and p 1⁄4 0.027, respectively) when compared to non-persistent isolates. When grown under moderate acidic conditions (i.e. TSBYE adjusted to pH 5) at 37 ºC, persistent isolates presented an average lag time significantly shorter (p 1⁄4 0.000) and average growth rates significantly higher (p 1⁄4 0.002) than non-persistent strains (Table 2).