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Assessing the capacity of growth, survival, and acid adaptive response of *Listeria monocytogenes* during storage of various cheeses and subsequent simulated gastric digestion

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ABSTRACT

Different physicochemical and microbiological characteristics of cheeses may affect *Listeria monocytogenes* potential to grow, survive, or exhibit an acid adaptive response during storage and digestion. The objectives of the present study were to assess: i) the survival or growth potential of *L. monocytogenes* on various cheeses during storage, ii) the effect of initial indigenous microbiota on pathogen growth in comparison to expected growth curves retrieved by existing predictive models, and iii) the impact of habituation on/in cheeses surfaces on the subsequent acid resistance during simulated gastric digestion. Portions of cream (Cottage and Mascarpone), soft (Anthotyros, Camembert, Mastelo®, Manouri, Mozzarella, Ricotta), and semi-hard (Edam, Halloumi, Gouda) cheeses were inoculated with *ca.* 100 CFU/g or cm^2 of *L. monocytogenes* and stored under vacuum or aerobic conditions at 7 °C ($n = 4$). The impact of varying (initial) levels of starter culture or indigenous spoilage microbiota on pathogen growth was evaluated by purchasing cheese packages on different dates in relation to production and expiration date (subsequently reflecting to different batches) mimicking a potential situation of cheese contamination with *L. monocytogenes* during retail display. Values of pH and a_w were also monitored and used to simulate growth of *L. monocytogenes* by existing models and compare it with the observed data of the study. Survival in simulated gastric fluid (SGF) (pH 1.5; HCl; max. 120 min) was assessed at three time points during storage. Mascarpone, Ricotta, Mozzarella, Camembert, and Halloumi supported *L. monocytogenes* growth by 0.5–0.8 log CFU/g or cm^2 *per* day, since low initial levels of total viable counts (TVC) (1.8–3.8 log CFU/g or cm^2) and high pH/a_w values (*ca.* 6.23–6.64/0.965–0.993) were recorded. On Cottage, Anthotyros, Manouri, Mastelo®, Edam, and Gouda, the pathogen survived at populations similar or lower than the inoculation level due to the high reported competition and/or low pH/a_w during storage. *L. monocytogenes* growth was significantly suppressed ($p < 0.05$) on samples purchased close to expiration date (bearing high TVC), compared to those close to production date, regardless of cheese. Cheeses which supported growth of *L. monocytogenes* enabled higher survival in gastric acidity along their shelf-life compared to cheeses which did not support growth. However, even in the latter cheeses (*i.e.*, Cottage, Mastelo®, Gouda), total elimination of a persisting low initial contamination was not always achieved. Such findings may provide useful evidence for assessing the risk posed by various cheeses types in relation to their compliance with food safety regulations.

1. Introduction

Listeria monocytogenes is a ubiquitous contaminant of major safety concern for Ready-To-Eat foods (RTE) and in particular for deli-meats, fish, and dairy products. Sporadic cases and numerous outbreaks of listeriosis associated with dairy products have been reported in different countries of EU and USA. In most of the cases, cream and soft cheeses (fresh and/or whey) have been identified as the source of infection, possibly due to the presence of weaker hurdles during their production compared to semi-hard and hard cheeses *i.e.*, high % moisture and pH (CDC, 2012; EFSA, 2013; RASFF, 2008; WHO/FAO, 2004).

Existing epidemiological data have indicated that foods involved in listeriosis outbreaks should have a contamination level significantly higher than 100 CFU/g, thus the EU, recognizing the risk posed by *L. monocytogenes*, established microbiological safety food criteria for pathogens' growth in RTE foods. Specifically, according to Regulation (EC) 2073/2005, RTE foods which meet the criteria of: i) pH ≤ 4.4, ii) $a_w \le 0.92$, or iii) pH ≤ 5.0 and $a_w \le 0.94$, and have shelf-life lower than 5 days are considered non able to support *L. monocytogenes* growth. For products that may support growth, the Regulation compels the absence of the pathogen *per* 25 g in 5 tested samples after production

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Table 1
Summary of cheese information related to their composition (manufacturer label) and manufacturing process.

Cheese	Type	Source of milk	Starter culture	Milk pasteurization	Ripening	Min. % fat in dry matter	% fat	Max. % moisture	% salt	Shelf-life (at 0–4 °C)
Cottage	Cream	Cow	Yes	Yes	No	21.0	4.0	75.0	1.0	7–10 days
Mascarpone	Cream	Cow	No	Yes	No	84.0	42.0	46.0	0.1	3 months
Ricotta	Soft (whey)	Sheep	No	No	No	44.0	12.0	70.0	0.3	30 days
Mozzarella	Soft	Cow	Yes	Yes	No	44.0	18.0	58.0	0.2	28 days
Camembert	Soft	Cow	Yes	Yes	Yes	50.0	22.3	52.0	1.7	6 months
Anthotyros	Soft (whey)	Sheep/goat	No	Yes	No	70.0	15.0	65.0	0.0	20 days
Manouri	Soft (whey)	Sheep/goat	No	Yes	No	70.0	47.5	60.0	2.1	6 months
Mastelo®	Soft	Cow	No	Yes	No	46.0	29.0	54.0	2.0	3 months
Halloumi	Semi-hard	Cow/sheep/goat	No	Yes	Yes	43.0	25.0	46.0	3.0	12 months
Edam	Semi-hard	Cow/goat	Yes	Yes	Yes	45.0	28.3	42.0	1.5	4 months
Gouda	Semi-hard	Cow	Yes	Yes	Yes	50.3	30.0	43.3	1.9	4 months

and requires the products would not exceed 100 CFU/g at the moment of consumption. Moreover, Codex Alimentarius similarly specifies absence of the pathogen in 5 samples of 25 g (CAC, 2007). Considering the aforementioned legislated criteria, *L. monocytogenes* constitutes a major risk for cheese making industry, since it is well-known that this organism is able to grow or survive over a wide pH range as well as at high salt concentrations, factors that significantly vary among different type of cheeses (*i.e.*, pH: 5.00–6.50, a_w: 0.94–0.99, moisture: soft, semi-hard, hard) (Cataldo et al., 2007; Rogga et al., 2005; Ryser et al., 1985; Ryser and Marth, 1987). Moreover, given that cheese is prepared from small traditional factories up to large industrial plants, except for the latter legislated factors, others such as levels of indigenous microbiota (determined by the type and concentration of starter cultures, the use of pasteurized milk, and/or GHPs) and processing/storage temperature may potentially affect the survival or growth of *L. monocytogenes*. However, existing quantitative information on the impact of the above factors, alone or in combination on the survival or growth of *L. monocytogenes* during storage of a variety of cheese types is rather limited (Tiwari et al., 2014; Valero et al., 2014).

The ability of *L. monocytogenes* to survive or grow on/in cheese, given the aforementioned variability in physicochemical factors (pH and/or a_w), may lead to long-term exposure of the pathogen to acid and/or osmotic stresses, possibly triggering an adaptive response (Farber and Peterkin, 1991; Ferreira et al., 2001; Lou and Yousef, 1997). For example, the habituation of *L. monocytogenes* on/in cheeses with pH close to sub-optimal values (*i.e.*, 5.0–5.5) may activate acid tolerance response (ATR) mechanisms, rendering cells highly resistant to subsequent lethal pH (Davis et al., 1996; Gahan et al., 1996; O'Driscoll et al., 1996). Thus, the pathogen has a high chance of surviving gastric passage, although stomach acidity is considered a major defence barrier against foodborne infections. Indeed, according to Gahan and Hill (2005), this "stress hardening" response is likely essential for infection and probably the bacterial gene expression during passage through the stomach will influence subsequent survival in the gastrointestinal tract. Several reports have assessed the survival of *L. monocytogenes* on RTE meat products after gastrointestinal simulation (Barmpalia-Davis et al., 2009; Formato et al., 2007; Peterson et al., 2007; Stopforth et al., 2005). However, data related to the influence of cheese as a food matrix and of storage time (during the shelf-life of the product) on the ability of *L. monocytogenes* to survive gastrointestinal simulation are limited (Dikici and Calicioglu, 2013; Schvartzman et al., 2011).

Considering the above, the objectives of the present study were: i) to assess the ability of various cheese types for potential growth or survival of *L. monocytogenes* during storage, ii) to evaluate the effect of initial indigenous microbiota on pathogen growth in comparison to expected growth curves retrieved by existing predictive models, and iii) to examine the impact of habituation on/in cheeses on the subsequent acid resistance during simulated gastric digestion.

2. Materials and methods

2.1. L. monocytogenes strains and inoculum preparation

Four *L. monocytogenes* strains were used in the present study, with two belonging to serotype 1/2a and two to serotype 4b (isolates of animal origin). All strains were maintained on slants of Tryptic Soy Agar supplemented with 0.6% w/v yeast extract (TSAYE) (Lab M Limited, United Kingdom) and sub-cultured once a month. Each strain was grown separately in Tryptic Soy Broth supplemented with 0.6% w/v yeast extract (TSBYE) (Lab M Limited, United Kingdom) for 24 h at

Table 2
Values of pH and a_w (average ± standard deviation) of studied cheeses purchased close to their production date, inoculated with 100 CFU/g or cm^2 of *L. monocytogenes*, and stored under vacuum or aerobic conditions at 7 °C.

Cheese	pH[a]			a_w[a]		
	Beginning of storage[b]	Middle of storage[c]	End of storage[d]	Beginning of storage[b]	Middle of storage[c]	End of storage[d]
Cottage	5.03 ± 0.00[A]	5.00 ± 0.01[A]	5.00 ± 0.01[A]	0.994 ± 0.001[A]	0.985 ± 0.000[B]	0.983 ± 0.001[B]
Mascarpone	6.45 ± 0.17[A]	6.57 ± 0.01[A]	6.49 ± 0.00[A]	0.988 ± 0.003[A]	0.989 ± 0.004[A]	0.971 ± 0.006[B]
Ricotta	6.64 ± 0.02[A]	6.55 ± 0.00[A]	6.59 ± 0.02[A]	0.990 ± 0.001[A]	0.988 ± 0.001[A]	0.984 ± 0.000[B]
Mozzarella	6.23 ± 0.01[A]	6.19 ± 0.02[AB]	6.14 ± 0.05[B]	0.993 ± 0.003[A]	0.985 ± 0.002[A]	0.986 ± 0.009[A]
Camembert	6.30 ± 0.02[A]	6.27 ± 0.01[A]	6.26 ± 0.06[A]	0.973 ± 0.003[A]	0.969 ± 0.002[A]	0.969 ± 0.003[A]
Anthotyros	6.72 ± 0.01[A]	5.27 ± 0.04[B]	4.84 ± 0.00[C]	0.991 ± 0.001[A]	0.988 ± 0.002[A]	0.985 ± 0.001[B]
Manouri	5.71 ± 0.07[A]	5.56 ± 0.04[A]	5.11 ± 0.33[B]	0.990 ± 0.000[A]	0.990 ± 0.000[A]	0.990 ± 0.000[A]
Mastelo®	6.39 ± 0.04[A]	6.15 ± 0.01[B]	6.11 ± 0.02[B]	0.952 ± 0.000[A]	0.951 ± 0.001[A]	0.949 ± 0.001[A]
Halloumi	6.60 ± 0.03[A]	6.58 ± 0.01[A]	6.58 ± 0.01[A]	0.965 ± 0.004[A]	0.963 ± 0.001[A]	0.955 ± 0.001[B]
Edam	5.58 ± 0.00[A]	5.53 ± 0.03[AB]	5.51 ± 0.01[B]	0.953 ± 0.001[A]	0.952 ± 0.001[A]	0.942 ± 0.001[B]
Gouda	5.53 ± 0.01[A]	5.48 ± 0.01[A]	5.51 ± 0.02[A]	0.967 ± 0.004[A]	0.967 ± 0.001[A]	0.947 ± 0.006[B]

[a] pH and a_w values during storage (uppercase letters), for each cheese, having different letter are significantly different ($p < 0.05$).
[b] Beginning of storage: Day 0.
[c] Middle of storage: Cottage → Day 10; Mascarpone → Day 8; Ricotta → Day 8; Mozzarella → Day 10; Camembert → Day 10; Anthotyros → Day 8; Manouri → Day 8; Mastelo® → Day 14; Halloumi → Day 8; Edam and Gouda → Day 26.
[d] End of storage: Cottage → Day 20; Mascarpone → Day 16; Ricotta → Day 15; Mozzarella → Day 21; Camembert → Day 20; Anthotyros → Day 16; Manouri → Day 15; Mastelo® → Day 30; Halloumi → Day 18; Edam and Gouda → Day 48.