Growth potential of *Listeria monocytogenes* in veal tartare

Simone Stella, Cristian Bernardi, Erica Tirloni

Department of Health, Animal Science and Food Safety, University of Milan, Italy

Abstract

In the present study the growth potential *of Listeria monocytogenes* in veal tartare was evaluated. A challenge test was performed on three tartare batches at 8°C, aiming to evaluate the growth potential of the pathogen. The data indicated the absence of a significant growth (δ<0.5 log cfu/g) during the entire period. When considering intermediate sampling times, an increase of 0.56 log cfu/g was detected after five days of storage in one of the batches. Microflora of veal tartare was dominated by lactic acid bacteria, that increased gradually during the trial, reaching counts up to 7 Log CFU/g in two of the three batches considered. Spoilage bacteria were present (especially *Pseudomonas* spp., yeasts and *Enterobacteriaceae*) but in very low counts and with a limited increase during the period considered. Finally, daily maximum tolerable *L. monocytogenes* counts were calculated to highlight the maximum acceptable load to avoid the overcoming of the legal limit of 100 CFU/g: a total increase of 0.95 log cfu/g in 12 days of shelf-life was estimated, obtaining a "safety initial concentration" at t0 of 10 CFU/g of the pathogen.

Introduction

Tartare is a perishable food generally characterized by a very short, assigned shelf-life. Spoilage may occur during slaughtering and production stages, by several microorganisms. Delhalle *et al*. (2016) identified, *Brochothrix thermosphacta, Lactobacillus algidus, Lactococcus piscium, Leuconostoc gelidum, Photobacterium kishitani, Pseudomonas* spp. and *Xanthomonas oryzae* in Belgian steak tartare. Tirloni *et al*. (2020) evaluated the microbiological shelf-life of steak tartare under refrigeration, showing a gradual increase in Total Viable Count, that reached 7 Log CFU/g after 12 days of storage, with LAB representing the main microflora. Considering potential pathogenic bacteria, the presence of verocytotoxigenic *Escherichia coli, Salmonella enterica* and *Listeria monocytogenes*, has already been found in steak tartare; such contamination could originate from various steps of meat production chain (Rhoades *et al*., 2009). Outbreaks were also reported due to the presence Shiga toxin producing *Escherichia coli* (STEC) O157 (Greenland *et al*., 2009) and *Salmonella* Typhimurium (Roels *et al*., 1997). Moreover, *Listeria monocytogenes* may be of particular concern in this typology of product as its presence in ground meat has been already reported with prevalences from 2.07% to 52% (Bohaychuk *et al*., 2006; EFSA-ECDC, 2017; Fantelli & Stephan, 2001; Scanga *et al*., 2000; Sheridan *et al*., 1994). RASFF Portal (https://webgate.ec.europa.eu/rasff-window/portal/) reports 13 notifications regarding microbial contamination of meat tartare, with *L. monocytogenes* being by far the main concern.

In accordance with European legislation (Reg. EC 2073/2005), RTE food, like steak tartare, must comply with the limit of 100 CFU/g throughout the whole shelf life, if the food business operator is able to demonstrate, with the satisfaction of the competent authority, that the product will not exceed this load until the expiry date. As reported in the guidelines produced by European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm), the growth potential (δ), as the difference between the *L. monocytogenes* concentrations found at the end and at the beginning of the shelf-life, is one of the options to classify the product as able or unable to support *L. monocytogenes* growth (ANSES, 2014).

Aim of the present study was the evaluation of the growth potential of *L. monocytogenes* in veal tartare, according to EURL Lm guidelines to fulfil the food safety criteria for the pathogen as reported in the EU legislation.

Materials and methods

Steak tartare samples

Veal tartare was supplied by a medium scale producer located in Northern Italy. Briefly, veal meat (95%) was minced, adding a mixture of salt, natural flavouring (plant origin extracts), dissolved in tap water; the product was mixed for 2 minutes in a processor and insufflated immediately with CO_2 to assure a fast temperature decrease. After production, each portion, weighing 70g, was vacuum-skin packaged in polystyrene barrier foam trays with permeable intact films (Cryovac Sealed Air Corporation). A best-before date of 12 days at 4°C was assigned by the producer.

Correspondence: Erica Tirloni, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133, Milan, Italy.
Tel.: +39.02.50317855 - Fax: +39.02.50317870.
E-mail: erica.tirloni@unimi.it

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Challenge test

Experimental design

The challenge test was conducted with the aim to investigate the growth potential of *L. monocytogenes* in the product: three independent challenge tests were performed on different batches at constant temperature of 8°C, mimicking a likely thermal abuse (Roccato *et al*., 2017). Samples were analysed at time steps until their expiry date at t_0, t_2, t_5, t_8, t_{10} and t_{12} from inoculation, with t_0 as the day of inoculation.

Bacterial strains and inoculation

Samples used for the challenge tests were inoculated with a mixture composed by three strains of *L. monocytogenes* (strains code 12MOB045LM, 12MOB085LM, 12MOB089LM), selected according to the EURL guidelines (ANSES, 2013) from the panel supplied by the National Reference Laboratory (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, I). The choice was based on their ability to grow in a substrate comparable to veal tartare (strains isolated from meat and able to grow at low pH and low temperature). The strain stocks were kept frozen at -80°C in Microbank Cryogenic vials (Pro-Lab Diagnostics U.K., Merseyside, UK). From

each stock culture, a loop was transferred to Brain Heart Infusion broth (BHI) (Oxoid) incubated at 37°C for 24h. The cultures were then inoculated again in BHI broth at 8°C to pre-adapt the cultures to the temperature of the challenge test. Cultures were then harvested in late exponential growth phase, defined as a relative change in absorbance of 0.05-0.2 at 540 nm (Jenway 6105, Staffordshire, UK), as already described (Tirloni *et al*., 2019). Cell concentrations were assessed by contrast microscopy at 1000x magnification (Motic, B310, Wetzlar, Germany), and then, precultures of the three isolates were diluted in sterile saline (0.85% NaCl) to reach the same concentrations and mixed together in equal volume. A target concentration of 4.2 Log CFU/ml was used, in order to reach a concentration of ~2 log CFU g^{-1} in the final product with a limited inoculum volume (about 0.7% of the product weight). The bacterial suspension was added to the veal tartare mass and mixed; then, the product was divided in portions (70 g) that were vacuum packaged and incubated at 8°C. Samples were analysed according to the experimental plan. Blank samples were also prepared by inoculating the same volume of sterile saline in the whole mass; at the above reported sampling times, these samples were submitted to the evaluation of natural microflora, pH and aw.

Microbiological analyses

Detection of *Listeria monocytogenes* was performed according to AFNOR method on the product before inoculation (AFNOR BRD 07/4–09/98). Inoculated tartare samples were submitted to *L. monocytogenes* enumeration in triplicate. Briefly, the whole product share (70 g) was 5-fold diluted in pre-chilled sterile saline (0.85% NaCl and 0.1% peptone) and homogenized for 60 s in a Stomacher 400 (Seward Medical, London, UK). Appropriate 10-fold dilutions were then made with pre-chilled sterile saline and *L. monocytogenes* was enumerated by spread plating on Rapid L'mono agar (Generon, Modena, Italy), then incubated at 37°C for 48 h (AFNOR BRD 07/05–09/01 method).

Blank samples were analysed in order to enumerate total mesophilic and psychotropic viable counts (TVC) (ISO 4833-1:2013), *Enterobacteriaceae* (ISO 21528-2:2017 method), *Pseudomonas* spp. (ISO 13720:2010 method), Lactic Acid Bacteria (LAB) (ISO 15214:1998 method), yeasts and moulds (ISO 21527-1:2008 method), and spores of sulphite-reducing *Clostridia* (ISO 15213:2003, after pasteurization of the dilutions).

At each sampling time, pH was measured by a pH meter (Amel Instruments, Milan, I): the sample was mixed with distilled water (max 1/2 w/w, in order to obtain a sufficiently fluid consistence), according to the MFHPB-03 method (Health Canada, 2014); three independent measurements were performed on each sample. Aw was also determined at each sampling time (Hygrolab Rotronic, Michell Italia, Rho, I).

Growth potential

The results obtained from the enumeration of *L. monocytogenes* were Log-transformed and used to calculate the trend of the concentration in the contaminated samples. According to EURL Lm guidelines, the growth potential (δ) of *L. monocytogenes* was calculated as the difference between the logarithmic medians of the counts detected, respectively, at the end and at the beginning of the challenge test. Once the values were calculated for each of the 3 batches analysed, the highest δ value was chosen. Food is considered able to support *L. monocytogenes* growth when this δ value is greater than 0.5.

Calculation of specific daily increase

Calculation of specific daily increase was made according to Tirloni *et al*. (2020). Briefly, the differences between the median values for each period (t_2-t_0, t_5-t_2, t_8-t_5, t_{10}-t_8 and t_{12}-t_{10}) were determined for each batch. For each period, the highest difference among the three batches was chosen and used for the calculation of the specific daily increase. Finally, the values obtained were used to build a curve, determining the daily tolerable count in order to avoid the overcoming of the limit of 100 CFU/g.

Statistical analysis

Data from challenge tests were submitted to one-way ANOVA using PRISM graph pad 6. The threshold for statistically significant differences was settled at $P<0.05$.

Results

Microbiological analyses on blank samples

The results of the microbiological and chemical-physical analyses of blank samples from batches 1, 2 and 3 are reported in Table 1. The mesophilic TVC of veal tartare at t0 ranged from 4.38 to 5.29 Log CFU/g. Variable counts were expected in this product typology: indeed, the production process includes cuts handling by the operators during the deboning and sectioning phases, and meat grinding: such phases likely contribute to the presence of high bacterial loads in the product. TVC gradually increased in all the batches reaching up to 6-7 Log CFU/g. The same trend was observed for psychrotrophic TVC; no statistical difference was revealed in the whole period considered among mesophilic and psychrotrophic TVC. LAB represented the main microflora of the product, with an increase in batches 1 and 3 during the 12 days-storage, from ~3 Log CFU/g to ~7 Log CFU/g.

Pseudomonas spp. at the beginning of the trial ranged between 3.08 and 4.08 Log CFU/g with a slight increase until the end of the trial, reaching values between 4 and 5 Log CFU/g. *Enterobacteriaceae* were detected in very low counts for all the period considered; the same trend was observed for *Brochothrix thermosphacta*, with no or very limited increase during the trials. Yeasts showed very limited loads in all the three batches for all the sampling times considered. Moulds and *Clostridia* were always below the detection limit until the end of the trial (2 and 1 Log CFU/g, respectively).

The pH showed a decrease in all the batches: starting from an initial value of 5.36-5.54, typical of fresh meat, pH reached values between 4.94-5.10, owing mainly to the activity of LAB. In any case, the pH values observed during the trial were not sufficient to inhibit the growth of the pathogen. Aw showed values in the range 0.98-0.99 in all the batches for all the sampling times, as expected for a vacuum-packed meat preparation.

Growth potential of *L. monocytogenes* in veal tartare

The eventual presence of *L. monocytogenes* in non-inoculated samples was first investigated. None of the batches resulted to be naturally contaminated: this is a mandatory condition for performing a valid growth potential determination, according to the EURL guidelines (ANSES, 2014).

To fulfil the requirements of European legislation, the ability to support/not support the growth of *L. monocytogenes* by a RTE food like veal tartare should be investigated. Table 2 shows the results of *L. monocytogenes* counts performed in the three different batches, including the respective median values, while Table 3 shows the delta (δ) values calculated between consecutive sampling times and between each sampling time and t_0 (day of inoculation). The inoculation level resulted in agreement with EURL guidelines (at t_0: 1.40-1.88 Log CFU/g).

Considering the batch with the highest growth during the entire period considered (batch 1), a growth potential ($\delta = t_{12} - t_0$) of 0.15 Log CFU/g was obtained: in accordance with EURL guidelines, this value

indicates the absence of significant growth of the pathogen (δ<0.5 Log CFU/g). As suggested by EURL guidelines, it is convenient to consider intermediate sampling times, with the aim to highlight an eventual initial growth followed by a decrease in *L. monocytogenes* counts; if we consider each sampling time, the results indicated the absence of a significant growth in batches 2 and 3, while in batch 1 a maximum increase of +0.56 Log CFU/g was detected at t_5 (Table 3). Thus, the product should be considered able to support the growth of *L. monocytogenes*.

Calculation of specific daily increase

Data obtained from the challenge tests, considering the highest median increase among the batches for each inter-sampling period (worst-case scenario), allowed to estimate a total maximum increase of +0.98 Log CFU/g, reached after 10 days of storage (+0.33, +0.23, +0.22, +0.20, -0.03 for the five-time intervals). Thus, a starting concentration of at least 1.02 Log CFU/g (10 CFU/g) would be necessary at t_0 to overcome the threshold limit of 2 Log CFU/g (Figure 1).

Discussion and conclusions

Veal tartare is a very perishable product due to the stages that characterize its production; it is also a product of concern in terms of presence of potential pathogenic microorganisms including *L. monocytogenes*, as it is not supposed to be eaten after a decontamination phase. In this study, the evaluation of the growth potential of *L. monocytogenes* according to EURL Lm guidelines was performed. First, a high starting microflora concentration level was highlighted: this microflora was mainly

Table 1. Microbial and chemical-physical characterization of blank samples during the challenge tests.

Parameter	Time					
	t_0	t_2	t_5	t_8	t_{10}	t_{12}
Batch 1						
Mesophilic TVC (Log CFU/g)	4.38	4.60	6.11	6.77	6.81	7.20
Psychrotrophic TVC (Log CFU/g)	4.20	5.78	6.51	7.28	8.00	8.20
LAB (Log CFU/g)	3.30	4.41	6.03	6.30	6.77	6.90
Pseudomonas spp. (Log CFU/g)	3.11	3.00	3.78	5.38	5.05	5.26
Enterobacteriaceae (Log CFU/g)	2.00	2.00	3.38	5.41	5.26	5.61
Brochothrix thermosphacta (Log CFU/g)	2.60	2.30	2.00	2.48	2.00	2.30
Sulphite-reducing clostridia (Log CFU/g)	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Yeasts (Log CFU/g)	<2.00	2.00	2.48	3.11	3.51	3.53
Moulds (Log CFU/g)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
pH	5.36	5.40	5.26	5.16	5.10	4.99
Aw	0.99	0.99	0.99	0.99	0.99	0.99
Batch 2						
Mesophilic TVC (Log CFU/g)	5.29	5.95	5.86	5.96	5.80	5.72
Psychrotrophic TVC (Log CFU/g)	6.00	8.08	8.33	8.36	8.38	8.44
LAB (Log CFU/g)	3.04	3.75	4.16	4.28	4.56	4.30
Pseudomonas spp. (Log CFU/g)	4.08	3.58	3.51	3.32	3.34	3.46
Enterobacteriaceae (Log CFU/g)	2.30	3.00	2.85	3.30	2.00	2.48
Brochothrix thermosphacta (Log CFU/g)	2.70	2.00	2.30	<2.00	<2.00	2.00
Sulphite-reducing clostridia (Log CFU/g)	<1.00	<1.00	<1.00	< 1.00	<1.00	<1.00
Yeasts (Log CFU/g)	<2.00	<2.00	<2.00	2.00	<2.00	<2.00
Moulds (Log CFU/g)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
pH	5.49	5.16	4.98	4.96	4.93	4.94
Aw	0.98	0.99	0.98	0.98	0.98	0.98
Batch 3						
Mesophilic TVC (Log CFU/g)	4.51	4.82	6.59	6.70	6.91	7.29
Psychrotrophic TVC (Log CFU/g)	3.98	4.70	7.72	8.38	8.53	8.60
LAB (Log CFU/g)	2.85	4.01	6.90	7.41	7.33	7.41
Pseudomonas spp. (Log CFU/g)	3.08	3.18	4.02	5.26	4.64	4.08
Enterobacteriaceae (Log CFU/g)	<2.00	2.30	3.70	5.23	4.48	3.30
Brochothrix thermosphacta (Log CFU/g)	2.60	2.48	2.60	3.66	3.32	3.26
Sulphite-reducing clostridia (Log CFU/g)	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Yeasts (Log CFU/g)	2.00	2.00	3.18	3.66	4.00	4.13
Moulds (Log CFU/g)	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
pH	5.54	5.58	5.46	5.22	5.12	5.10
Aw	0.99	0.99	0.99	0.99	0.99	0.98

composed by LAB, that may act also as potential competitors of pathogenic bacteria present.

This was expected in bovine meats stored under vacuum, as already shown in previous studies (Yost and Nattress, 2002; Stella *et al*., 2018). The data obtained agree with what found in a previous study in steak tartare made with adult beef (Tirloni *et al*., 2020), where LAB was the predominant microflora as well. As already reported in previous studies, there are many mechanisms of action (competition for nutrients, production of bacteriocins and organic acids, production of hydrogen peroxide…) that act in the inhibition of pathogens 'growth. To be effective, the presence of LAB, should achieve a "critical" load that allowed to limit the replication of other bacteria present ("Jameson effect") (Jameson, 1962; Gálvez *et al*., 2008). In this study, the growth of LAB determined an acidification of the product, but the values observed were

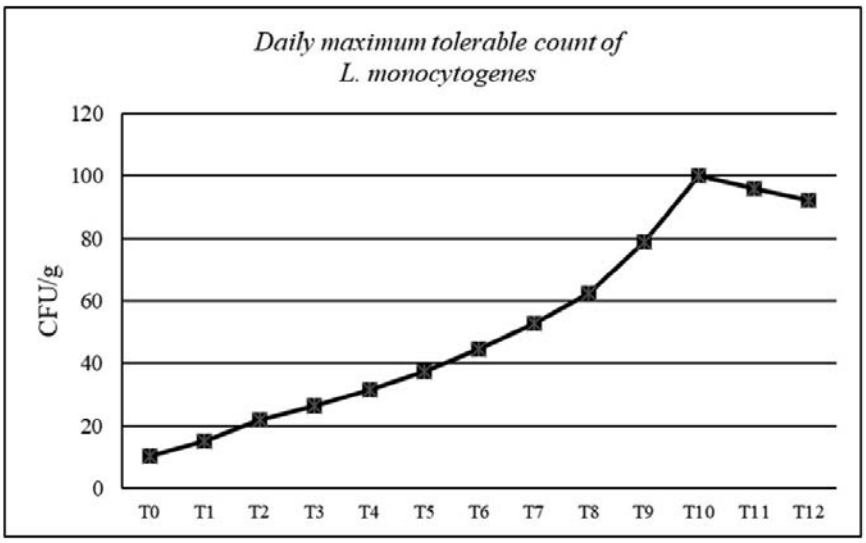

Figure 1. Daily maximum tolerable count of *L. monocytogenes* to not overcome the threshold limit of 100 CFU/g until the end of the assigned shelf-life.

Table 2. *L. monocytogenes* counts in the three batches considered and median values (expressed in Log CFU/g).

	t_0	t_2	t_5	t_8	t_{10}	t_{12}
Batch 1						
A	1.88	2.08	1.98	1.81	1.81	1.85
B	1.60	1.74	2.16	1.88	1.00	1.30
C	1.40	1.93	2.20	2.44	2.04	1.78
Median	1.60	1.93	2.16	1.88	1.81	1.78
Batch 2						
A	1.85	1.93	1.00	1.40	1.60	0.70
B	1.88	1.88	1.48	1.00	1.88	0.60
C	1.74	1.74	1.18	1.54	1.30	1.00
Median	1.85	1.88	1.18	1.40	1.60	0.70
Batch 3						
A	1.78	1.98	1.74	1.70	1.40	1.48
B	1.88	1.60	1.70	1.48	1.54	1.78
C	1.74	1.95	1.54	1.93	1.74	1.48
Median	1.78	1.95	1.70	1.70	1.54	1.48

Table 3. Delta (δ) values calculated as Log CFU/g t – Log CFU/g t_0 and between consecutive times.

δ	t_2-t_0	t_5-t_0	t_8-t_0	t_{10}-t_0	t_{12}-t_0
Batch 1	0.33	0.56	0.27	0.21	0.18
Batch 2	0.03	-0.67	-0.45	-0.24	-1.15
Batch 3	0.18	-0.08	-0.08	-0.23	-0.30
	t_2-t_0	t_5-t_2	t_5-t_8	t_8-t_{10}	t_{10}-t_{12}
Batch 1	0.33	0.23	-0.29	-0.06	-0.03
Batch 2	0.03	-0.70	0.22	0.20	-0.90
Batch 3	0.18	-0.26	0.00	-0.15	-0.07

not sufficiently low to consider pH as a unique effective hurdle for *Listeria* growth.

According to Regulation (EC) 2073/2005 on microbiological criteria, veal tartare cannot be automatically considered as unable to support the growth of *L. monocytogenes* in ready-to-eat products (the concomitant presence of pH below 5.0 and aw below 0.94 should be achieved to give a sure growth prevention). Anyway, the combination of microbiological and chemical-physical factors that characterized the product did not allow the development of the pathogen in two out of the three batches considered. Considering the entire shelf-life assigned, in none of the batches, *L. monocytogenes* grew more than 0.5 Log CFU/g, but considering intermediate samplings (t_5 in batch 1), a significant growth should be considered (+0.56 Log CFU/g), also if occasional. Thus, the product, according to Regulation (EC) 2073/2005 on microbiological criteria, should be classified as able to support the growth of *L. monocytogenes* in this ready-to-eat product. Very similar results were found by in our previous study on steak tartare, where an increase equal to +0.51 Log CFU/g was observed at the same sampling time (five days of storage) (Tirloni *et al*., 2020).

Finally, the calculation of the maximum tolerable load at t_0 in order not to overcome the threshold limit of 100 CFU/g for the entire shelf-life showed the need to assure an initial count equal or lower than 10 CFU/g. Thus, in presence of positive samples of veal tartare along the shelf-life, an enumeration of alive *L. monocytogenes* cells would be suggestable to give a correct information to the Food Business Operator and to the Competent Authority.

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