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Modelling the fate and serogroup variability of persistent *Listeria monocytogenes* strains on grated cheese at different storage temperatures

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ABSTRACT

Processed cheese from cow's milk is one of the most consumed dairy products worldwide. Since this product is defined as ready-to-eat, foodborne pathogens such as *Listeria monocytogenes* can represent a health concern for susceptible populations. In this study, the individual and combined kinetic behaviour of four L. *monocytogenes* serogroups (namely, 1/2a, 1/2b, 1/2c and 4b) persistently isolated from a Spanish cheesemaking factory was modelled on grated cheese at different isothermal conditions (4 and 12 °C) during a 120-days period. The serogroup variability was characterized over the storage time by the isolation and identification of the different serogroups in the cocktail containing the four strains. This processed cheese did not support the growth of L. *monocytogenes* during storage. Survival patterns described by the log-linear type model indicated a high variability of L. *monocytogenes* serotypes at the tested temperatures: L. *monocytogenes* serogroup 4b showed a more rapid decrease rate at 4 °C than at 12 °C, while the opposite trend was found for the rest of serogroups and the L. *monocytogenes* cocktail containing all the strains. Survival rate of L. *monocytogenes* serogroup 1/2c at 4 °C was 0.007 log CFU/d being the most resistant serotype while at 12 °C, serogroup 1/2a showed the lowest survival rate (0.011 log CFU/d), thus showing a prolonged survival at this temperature. This study highlights the potential implications of L. *monocytogenes* contamination in processed cheese and shows that serogroup variability should be considered when evaluating survival patterns in contaminated products. Finally, the predictive models developed here could be useful to assist food operators to set specific storage conditions and formulations to avoid L. *monocytogenes* growth and survival in grated cheeses.

1. Introduction

Cheese consumption has grown in EU in recent years largely due to the improvement in the process quality and the increase of the number of European Quality Labels. For example, cheese is the second most consumed dairy product in Spain and represents > 20% of the dairy products consumed by Spanish households in 2015, totalling 8 Kg per capita (MAPAMA, 2017), with an inter-annual increase of 1.4% in 2016 (Mercasa, 2017). Herd certification programs adapted Hazard Analysis Critical Control Point (HACCP) systems and systematic microbiological quality control throughout the supply chain (European Union, 2004) have been developed to guarantee the safety of cheese. However, cheese has been identified in risk assessment as a food of greater concern to public health due to listeriosis (Arrese and Arroyo-Izaga, 2012). L. *monocytogenes* can contaminate cheeses made from either raw or pasteurized milk, and listeriosis has become the emblematic example of severe illness transmitted by dairy products and particularly cheeses (Montel et al., 2014). Several L. *monocytogenes* outbreaks have been reported linked to cheese (Martinez-Ríos and Dalgaard, 2018), and recently, the Spanish Agency for Consumer Affairs, Food Safety and Nutrition (AECOSAN) has warned about a case of meningitis in Madrid (Spain) implicated to the consumption of cheese in Spain (AECOSAN, 2018).

Predictive microbiology can help towards a better understanding of the behaviour of foodborne pathogens, by mathematically modelling reproducible patterns that can be used to predict microbial growth or survival over a range of conditions (Pérez-Rodríguez and Valero, 2013). Various mathematical models have been recently published for L. *monocytogenes* in dairy products, such as milk (Koutsoumanis et al., 2010), smear soft cheese (Ferrier et al., 2013), mold ripened cheese

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(Lobacz et al., 2013), mature raw sheep milk cheese (Valero et al., 2014), soft blue-white cheese (Rosshaug et al., 2012) and in other cheese types or cheese model systems (Demers-Mathieu et al., 2013; Schvartzman et al., 2010; Spanu et al., 2015). Grated cheeses are widely commercialized in the market as side food ingredients for several composite dishes. These cheeses are subjected to different handling processes in dairy industries (slicing, packaging etc.), making them more prone to eventual bacterial contamination. Little scientific information is currently available on L. *monocytogenes* behaviour in such types of cheeses, and there is a lack of data on genotypic variation in L. *monocytogenes* isolated from the processing environment of dairy industries and how they can persist over long storage periods.

In this study, the kinetic behaviour of four cheese-borne, persistent L. *monocytogenes* strains belonging to the 4 major genetic groups of this species (1/2a – sequence type (ST) 204-, 1/2b –ST 5-, 1/2c –ST9- and 4b –ST6-) was modelled on grated cheese at different isothermal conditions (4 and 12 °C). Finally, L. *monocytogenes* variability was characterized over the storage time through the isolation and identification of the most persistent serotypes.

2. Material and methods

2.1. Grated cheese samples

Processed grated cheese from cow's milk was used in this study. Cheese samples were provided by a local producer, packaged in modified atmosphere (50% CO_2 + 50% N_2). This type of cheese contains a moderate concentration of fat and proteins (approximately 21% each). The shelf-life of the commercial portions of this type of products usually is 3 months (90 days). The main ingredients are cow's milk cheese, butter, milk proteins, potato starch and modified potato starch, whey powder, melting salts, cream, salt, food preservative (E-202), and citric acid. Ten 25-g cheese samples were tested to confirm the absence of L. *monocytogenes* using the ISO 11290-1 detection method (ISO, 2017a). The pH and a_w values were measured on three non-inoculated samples every week along the timeframe of the study. The a_w was measured at 25 °C using an Aqualab water activity meter (Aqualab model Series 4 Decagon Devices Inc., Pullman, WA), whereas the pH was measured after blending 10 g of cheese in 90 ml of distilled and deionised water using a pH meter Crison Basic 20+ equipped with an electrode pH 0–14 (Crison Instruments, S.A., Barcelona, Spain).

2.2. Bacterial strains and preparation of L. monocytogenes inocula

Four strains of L. *monocytogenes* were used in this study: LBMM1009 (serogroup 4b, sequence type ST6), LBMM 1111 (serogroup 1/2a, ST 204), LBMM 1104 (serogroup 1/2b, ST5) and LBMM 1109 (serogroup 1/2c, ST9) strains isolated from the dairy company providing the cheese samples. The four strains were chosen due to previous evidence that they can survive the stressful environment of cheesemaking better than other strains, as they were repetitively isolated in the facility of the cheese company. All strains were maintained at −80 °C in cryovial containing beads and cryopreservatives (Oxoid TP15731 Maintenance Freeze medium, Oxoid, Hampshire, UK). Prior to the start of the experiment, a bead of each strain was surface- plated onto a Petri dish with Brain Heart Infusion (BHI) agar (Beckton, Dickinson and Co.) and incubated at 37 °C for 24 h. Then, a loopful of one colony was transferred aseptically into 10 ml of BHI Broth (Beckton, Dickinson and Co.) and incubated at 37 °C overnight. To determine the initial concentration of each working inoculum, an aliquot was serially diluted, and surface plated onto BHI agar, incubated at 37 °C for 24 h and colonies were counted. Each working inoculum was diluted and mixed to create a final cocktail of the four L. *monocytogenes* strains (approx. 2.5×10^6 CFU/ml at stationary phase). To ensure that the same concentration of each strain was present in the mixed cocktail, individual cultures were plated and microbial concentration was further checked.

2.3. Artificial contamination of cheese samples and storage

Grated cheese was aseptically removed from the commercial bags and transferred into sterile *co*-extruded polyamide/polyethylene packing bags (Industrias Pargón, Salamanca, Spain). Five different scenarios of artificial inoculation were tested: i) inoculation with the L. *monocytogenes* strain LBMM1009 (serogroup 4b), ii) inoculation with the L. *monocytogenes* strain LBMM 1111 (serogroup 1/2a), iii) inoculation with the L. *monocytogenes* strain LBMM 1104 (serogroup 1/2b), iv) inoculation with the L. *monocytogenes* strain LBMM 1109 (serogroup 1/2c), and v) inoculation with a cocktail containing the four L. *monocytogenes* strains. Samples of 25 g of grated cheese were inoculated with 100 µl of PBS containing L. *monocytogenes*. To facilitate a homogeneous distribution of the inoculum, the microbial culture was evenly distributed in ten different surface areas of 10 µl each (for a final concentration of $\sim 1 \times 10^4$ CFU/g). Then, inoculated samples were air-dried in a biosafety cabinet, and subsequently packaged at the same atmosphere above described and stored at two different temperatures (4 and 12 °C).

2.4. Microbiological analyses

Microbiological studies on the presence and enumeration of L. *monocytogenes* at each sampling point were conducted using three independent inoculated cheese samples according to ISO 11290-1 (ISO, 2017a) and ISO 11290-2 (ISO, 2017b), respectively. The analysis period covered 109 and 118 days at 4 °C and at 12 °C, respectively in relation to the established shelf-life by the manufacturer (Beaufort et al., 2014). The quantification limit was 10 CFU/g and the counts from the triplicate samples were expressed as log CFU/g. It was assumed that in case that cheeses samples yielding three successive negative results using the standard detection method were not further analyzed. In parallel enumeration of lactic acid bacteria (LAB) was conducted in each sampling time according to ISO 15214 (ISO, 1998).

2.5. PCR-serogrouping of L. monocytogenes

From the experiment of the L. *monocytogenes* cocktail, twenty colonies with L. *monocytogenes* characteristics grown in the ALOA agar plates were assayed in each sampling event for each grated cheese sample to determine the percentage of each L. *monocytogenes* serogroup. L. *monocytogenes* serogrouping was determined using a multiplex PCR targeting the specific target genes lmo0737, lmo1118, ORF2819, ORF2110 and *Listeria* spp. specific prs published by Doumith et al. (2004) and amended by Leclercq et al. (2011) for PCR IVb-VI.

2.6. Data modelling

The survival patterns of the different L. *monocytogenes* serotypes in the grated cheese samples were constructed by plotting the logarithm of the number of colony-forming units per g of sample (log CFU/g) against the storage time (d). The log-linear model (Eq. (1)) was fitted to survival curves using the GInaFiT add-in for Excel® (Geeraerd et al., 2005).

$$\log N(t) = \log N_0 - k_{\max} \cdot t \quad (1)$$

where $N(t)$ is the number of survival cells (log CFU/g) at time t (d); N_0 corresponds to the initial inoculum level (log CFU/g); and k_{max} is defined as the maximum survival rate (log CFU/d).

On the other hand, growth of LAB was also monitored, and log count data were processed to MS Excel. The Baranyi model (Baranyi and Roberts, 1994) was fitted using DMFit v 3.5 (Institute of Food Research, Norwich, England). Kinetic growth parameters; namely maximum growth rate (μ_{max}, log CFU/d) and maximum population density (*MPD*, log CFU/g) were estimated from the observed data at each condition studied.

For both survival and growth models, Root Mean Squared Errors

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