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Loss of viability of *Listeria monocytogenes* in contaminated processed cheese during storage at 4, 12 and 22 °C

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The behaviour of *Listeria monocytogenes* in a processed cheese product was evaluated over time by inoculating the product with three different *L. monocytogenes* strains (Scott A, CA and a strain isolated from processed cheese) at three different inoculation levels (ca. 6×10^5, ca. 6×10^3 and 10^2 CFU/g of cheese or less) and after storage of the contaminated products at 4, 12 or 22 °C. Growth of *L. monocytogenes* was not observed in any of the experimental trials (experiments involving different combinations of strain, inoculum level and storage temperature) throughout the storage period. *L. monocytogenes* populations decreased over time with a rate that was strain- and storage temperature-dependent. Nonetheless, for cheeses that had been inoculated with the higher inoculum and stored at 4 °C viable populations of *L. monocytogenes* could be detected for up to nine months post-inoculation. The *L. monocytogenes* survival curves obtained from the different trials were characterised by a post-inoculation phase during which the populations remained essentially unchanged (lag phase) followed by a phase of logarithmic decline. The duration of the lag phase and the rate of inactivation of *L. monocytogenes* in the different trials were estimated based on data from the linear descending portions of the survival curves. In addition, a non-linear Weibull-type equation was fitted to the data from each survival curve with satisfactory results. The results of the present study emphasize that, according to the definition laid down in the European Union Regulation 1441/2007, the processed cheese product tested in this work should be considered and classified as one that does not support the growth of *L. monocytogenes* under reasonable foreseeable conditions of distribution and storage. However, post-processing contamination of the product should be austerely avoided as the pathogen can survive in the product for extended periods of time, particularly under refrigerated storage (4 °C).

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1. Introduction

Listeria monocytogenes is a food-borne pathogen that is widely distributed in nature and the causative agent of listeriosis, a serious and often life-threatening disease (Ryser and Marth, 2007). Owing to its elaborate physiological adaptation mechanisms, *L. monocytogenes* can survive and even proliferate in a variety of foods under adverse environmental conditions such as low pH, high salinity and low temperature (Angelidis et al., 1999; Hado and Yousef, 2007). Recent epidemiological data from eight European Union (E.U.) Member States have indicated that the incidence of listeriosis in humans has increased or remained relatively high since the year 2000, with the majority of cases concerning the elderly and those with predisposing medical conditions (Goulet et al., 2008).

Beginning 2006, Commission Regulation (EC) 2073/2005 (amended by Commission Regulation (EC) 1441/2007) became effective for all E.U. Member States (European Commission, 2005, 2007). Compared to previously existing legislation, of particular interest are the legislative modifications regarding *L. monocytogenes* in ready-to-eat (RTE) foods. Thus, RTE foods are legislatively distinguished based on the intended target population (i.e., infants or people with special medical conditions versus all other human sub-populations). RTE foods for infants or for special medical purposes are still required to be free of *L. monocytogenes* (absence of the pathogen in 25 g of food in a 10-unit sampling plan). RTE foods other than those intended for infants or special medical purposes are then subdivided into those that are able to support the growth of *L. monocytogenes* and into those that are not. Products "with pH ≤ 4.4 or $a_w \leq 0.92$, products with pH ≤ 5.0 and $a_w \leq 0.94$ and products with a shelf-life of less than five days" are considered as RTE foods that are unable to support *L. monocytogenes* growth. The Regulation also states that "other categories of products can also belong to this category, subject to scientific justification". Finally, the

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L. monocytogenes criteria are adjusted according to their temporal stage in the food chain. Thus, for RTE foods that are able to support the growth of *L. monocytogenes*, the legislation compels the absence of the pathogen (in 25 g) "before the food has left the immediate control of the food business operator, who has produced it", but allows for up to 100 CFU/g in "products placed on the market during their shelf-life". The 100-CFU/g limit also applies throughout the shelf-life of marketed RTE foods that are unable to support *L. monocytogenes* growth. Under the new E.U. legislation, therefore, it is essential for manufacturers of RTE foods to be able to demonstrate to the competent authorities the *L. monocytogenes*-food category in which their products belong to. One way of providing such justification for products that do not meet the fore-mentioned physico-chemical and shelf-life limits is through the conduct of challenge tests. The aim of the current work, therefore, was to characterise a grated processed cheese product with respect to its ability or not to support the growth of *L. monocytogenes*.

Pasteurised processed cheeses are typically produced by blending natural cheeses of different ages and degrees of maturity in the presence of emulsifying salts and other dairy and non-dairy ingredients such as colours, flavourings, fats, spices, salts, acidulants and preservatives. Following the preparation of the desired formulation, the ingredients are mixed under heating. The melted homogeneous mass is then packaged, cooled and stored to yield a product with an extended shelf-life (Kapoor and Metzger, 2008). The minimum heat treatment required by the United States Code of Federal Regulations is 65.5 °C for 30 sec (FDA, 2008).

The formulation of the processed cheese product tested in this work includes a specific type of hard cheese, which is ripened for three months, milk casein, butter, sodium chloride (2.8%), sorbic acid (0.11%), phosphates (2.7%) as emulsifiers and water. The mixture is heated (80 ± 1 °C) under continuous mixing for 3–4 min in industrial 200-kg capacity, Stephan-type cookers in batch operation with direct steam injection. The melted homogeneous cheese mass is hot-filled in plastic package (film) in rectangular stainless steel hoops (12-kg block sizes) and then cooled in continuous flow water tunnels for 4–5 h until the cheese temperature reaches 15 °C. The hoops containing cooled cheese are subsequently transferred to 4 °C-cold rooms for at least 3 days for further cooling in order to achieve the desired firmness and texture. In the final step of manufacture, the cooled and solidified cheese is removed from the steel hoops and feeds the grating line where the cheese package is removed and the product is grated. The grated cheese is subsequently packaged under modified atmosphere (30% CO_2 and 70% N_2). Cheese packages are stored at 2–8 °C during storage and distribution. The product, which is commercially sold as Tando® processed cheese, has a shelf-life of 12 months. According to the definitions laid down in Regulation 2073/2005, the product can not be *a priori* classified to the category of RTE foods that are unable to support *L. monocytogenes* growth based on its pH, a_w and its intended shelf-life.

L. monocytogenes is often isolated from the environment (e.g. floors, drains) of cheese companies even when good sanitation and hygiene protocols are in place (Pritchard et al., 1994; Kornacki and Gurtler, 2007). The present study was therefore undertaken to evaluate the behaviour of *L. monocytogenes* when the pathogen is introduced to the processed cheese as a post-processing contaminant and the product is subsequently stored under refrigeration (4 °C) or temperature abuse (12 and 22 °C) conditions. Such a contamination could, in theory, occur after the cooling of the melted cheese mass and before the final packaging, e.g. during the grating process. The influence of strain and level of contamination was also studied by using three strains of *L. monocytogenes* of different origin and serotype and three different levels of initial contamination.

2. Materials and methods

2.1. *L. monocytogenes* strains

Three *L. monocytogenes* strains were used in this study. The first strain, *L. monocytogenes* Scott A (clinical isolate, serotype 4b), was selected as one of the test strains because previous research has shown that strain Scott A is particularly resistant and survives for prolonged periods of time in contaminated dairy products (Papageorgiou and Marth, 1989a,b). The second strain was one of the *L. monocytogenes* strains that were isolated during the investigations of the soft-cheese listeriosis outbreak in Los Angeles in 1986. Previous research has shown that this strain, herein designated as *L. monocytogenes* CA, is less resistant in terms of its ability to survive for extended time periods in contaminated dairy products (Papageorgiou and Marth, 1989a,b). The third strain, designated as *L. monocytogenes* IS951, is a processed cheese isolate. The serotypic group of strains CA and IS951 was determined as described below. Bacterial strains were stored at −70 °C in tryptic soy broth (TSB, Biolife Italiana S.r.l., Milano, Italy) as glycerol (20%) stocks.

2.2. Inoculum preparation

Unless specified otherwise, all media and reagents were obtained from Biolife. After growth on tryptic soy agar (TSA) plates, each strain was grown separately in TSB at 30 °C for 24 h and then sub-cultured in fresh TSB at 30 °C for an additional 20 h. Fully grown (stationary phase) cultures in TSB were then centrifuged (10,000 RPM for 10 min) in an Eppendorf mini spin plus centrifuge (Eppendorf, AG, Hamburg, Germany) at room temperature. The supernatants were discarded and the cells were washed twice (resuspended and centrifuged as described above) in sterile ¼-strength Ringer's solution (LAB M Limited, Lancashire, U.K.). The washed pellets were brought up and serially diluted in Ringer's solution to yield the required inocula ("high", "medium" or "low") as described below.

2.3. Cheese inoculation, packaging and storage

Cheese was aseptically removed from its commercial package (typically plastic bags containing 500 g or 1 kg of grated processed cheese) and 25-g portions were aseptically transferred in 400-mL sterile stomacher bags (BagLight®, Interscience, St. Nom, France). Forty μL of washed and appropriately diluted cell suspension were spread drop-wise, as uniformly as possible, over the cheese mass inside each bag to yield three types of initial inocula. The "high" inoculum was designed to yield ca. 6×10^5 CFU/g, the "medium" inoculum consisted of ca. 6×10^3 CFU/g and the "low" inoculum was intended to be 10^2 CFU/g or less. Control samples for each experiment were inoculated with 40 μL of sterile ¼-strength Ringer's solution. Immediately upon their inoculation, the bags containing inoculated cheese were hand-massaged for 10 s to achieve a more uniform distribution of the inoculum in the grated cheese mass. Subsequently, the bags were heat-sealed in a controlled atmosphere environment (30% CO_2/70% N_2) using a YANG sealer (YANG Technologie Alimentari, Model No Atlantis 25, Vertemate, Italy) in order to mimic the product's commercial atmospheric packaging conditions. Sealed bags were appropriately labelled and stored at 4, 12 or 22 °C in high-precision controlled temperature incubators (Model MIR-253, Sanyo Electric Co., Ltd, Osaka, Japan) for up to one year. Thus, a total of 27 different experimental conditions (trials) were generated and tested over time in duplicate (3 strains × 3 inoculum levels × 3 storage temperatures × 2 replications) resulting in 27 *L. monocytogenes* survival curves.

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