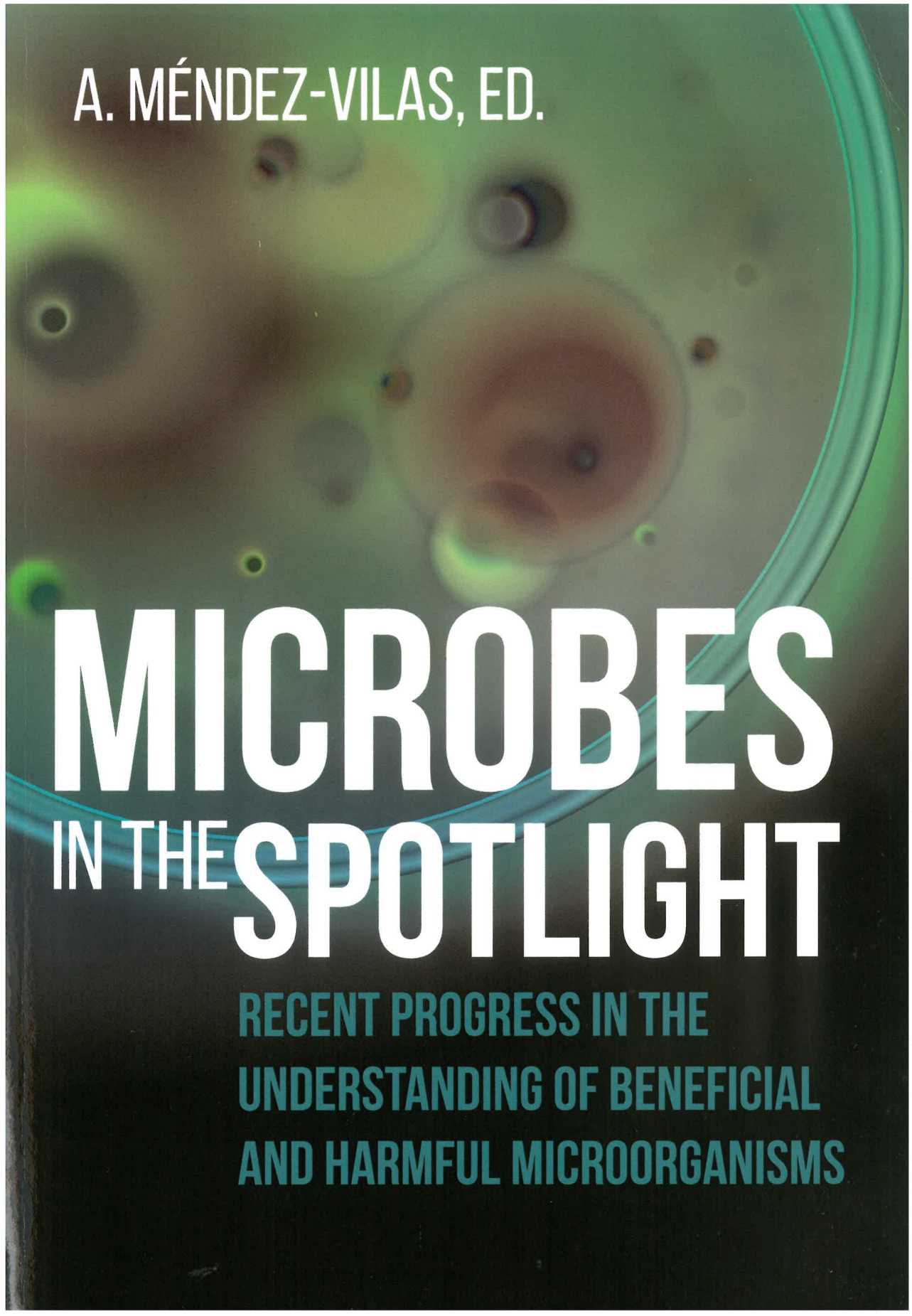
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Influence of starter culture and ripening temperature on survival of *L. monocytogenes* in traditional Portuguese dry-fermented sausages

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Linguiça is a Portuguese traditional dry-fermented sausage whose microbiological quality and safety have been reported to be highly variable. The present study evaluated the influence of the addition of a commercial starter culture and ripening temperature (10° C and 18°C) on the survival of *L. monocytogenes* in *linguiça*. A three-parameter Weibull-based decay omnibus model was fitted to the microbial data originating from four production batches (no starter-10°C; starter-10°C; no starter-18°C and starter-18°C) of *linguiça* inoculated with *L. monocytogenes*. In the batches without starter culture, by the 10th day of ripening, *L. monocytogenes* population decreased in ~1.02 log CFU/g at 10°C and ~1.22 log CFU/g at 18°C of ripening temperature. However, with the addition of commercial starter culture, the pathogen was further reduced in ~1.40 and ~2.70 log CFU/g, respectively. Since, traditionally, *linguiça* is not produced with starter culture, research efforts should be directed towards the preparation of a tailor-made culture as an additional hurdle to control *L. monocytogenes* in *linguiça*.

Keywords *linguiça*; *chorizo*; fate studies; inoculation; maturation

1. Introduction

Among the many types of traditional fermented sausages produced in Portugal, *linguiça* is a popular fermented sausage made of raw, salted and unground pork meat which originated in regions with temperate and colder climates. However, since these sausages are typically manufactured by traditional customs in regional processing units, their microbiological quality and safety can be variable. The limited research conducted so far on these aspects has highlighted that: (i) *linguiça* sausages are not always produced under the best hygiene conditions [1] and (ii) there is substantial variability in key process variables such as maceration/smoking/drying time and temperature, and production time [2]. To this respect, earlier research has shown that, despite the simultaneous reduction in pH and water activity occurring during fermentation, pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* may survive in *linguiça* sausages [3]. A recent meta-analysis on the incidence of pathogens in traditional Portuguese meat products [4] revealed that the non-compliance to EU microbiological criteria for *L. monocytogenes* (8.3%; 95% CI: 5.1 – 13.1%) in Portuguese sausages 'intended to be eaten raw' (including *linguiça*) was considerable higher than EU levels for RTE products in comparable categories (<1.4%) [5]. Thus, the objective of this study was to evaluate the influence of the addition of a commercial starter culture and ripening temperature (10°C and 18°C) on the fate of *L. monocytogenes* in experimentally-inoculated *linguiça* during maceration and ripening.

2. Methodology

Batches of *linguiça* were prepared by mixing diced raw pork meat with salt (20.0 g/kg meat), dry garlic (4.5 g/kg), sweet pepper (12.5 g/kg), laurel leaves (0.5 g/kg), dextrose (10.0 g/kg), a mix of red/white wine (410 ml/kg) and water (410 ml/kg). The batter was then inoculated with *L. monocytogenes* to reach a concentration of ~6 log CFU/g. The mix was then macerated for 3 days at 4°C, and stuffed into natural pork casings. Sausages were then hung vertically in a climate-controlled chamber for ripening at 10°C or 18°C with 80% relative humidity (RH) during twelve days. In two batches of lab-produced *linguiça*, commercial starter culture was added to the mixture to obtain a concentration of lactic acid bacteria (LAB) of ~5 log CFU/g. Thus, to follow up the survival kinetics of *L. monocytogenes* during maceration and ripening, successive samples were taken from each of the four following treatments or environmental conditions: (i) Without starter and ripened at 10°C; (ii) With starter and ripened at 10°C; (iii) Without starter and ripened at 18°C; and (iv) With starter and ripened at 18°C. The sampling time points were: meat mixed with ingredients (day 0), macerated meat before stuffing (day 3) and during the ripening process (days 5, 7, 9, 11, 12, 13 and 14). On those days, *L. monocytogenes* was enumerated by homogenising 25 g of sample in 225 mL Half Fraser Base CM0895 (Oxoid, Hampshire, UK). The enumeration was performed according to the ISO 11290-2:1998/Amd. 1:2004(E) procedure [6]. After incubation of the initial suspension for 1 h at 20 °C, a 0.1-mL volume was surface-inoculated on Oxoid Chromogenic Listeria Agar (OCLA, Oxoid) and incubated at 37 °C for 24 h. Lactic acid bacteria (LAB)

counting was performed on Man, Rogosa and Sharpe (MRS) agar (Liofilchem, Italy) overlaid with 5 mL agar 0.8%.

The survival of *L. monocytogenes* in *linguiça* was modelled by an omnibus regression, which is a type of multilevel model that simultaneously fits a primary and a secondary predictive model. The chosen primary model was the Weibull decay equation with three parameters: χ (scale), β (shape) and $log\ N_0$ (initial microbial concentration in log CFU/g). In the secondary models, the linear effects of the addition of starter culture (*Starter*) and ripening temperature (T) on the Weibull parameters χ and β were tested. Thus, the observed concentration of *L. monocytogenes* (*log N*) in *linguiça* sausages produced without/without (j) the addition of starter culture, and ripened at the temperature k, was assumed to be a realisation of:

$$\log N_{jk} = \log N_0 - \left(\frac{t}{\chi_k}\right)^{\beta_{jk}} + \varepsilon_{jk}$$

$$Ln\beta_{jk} = a_0 + a_1 Starter_j + a_2 T_k + a_3 Starter_j T_k$$

$$Ln\chi_k = b_0 + b_1 T_k \qquad (1)$$

where ε_{jk} are the residuals normally distributed with mean zero and variance σ^2.

3. Results and Discussion

When statistically comparing the microbial populations between the sampling point right after mixing (Day 0) and after maceration at 4ºC (Day 3), no significant changes in the concentrations of *L. monocytogenes* and LAB were encountered in any of the four environmental conditions. However, during ripening, LAB counts rapidly increased until reaching ~8 log CFU/g (not shown) while *L. monocytogenes* decreased steadily. The pace at which the pathogen's concentration declined depended strongly upon the environmental condition (Fig. 1).

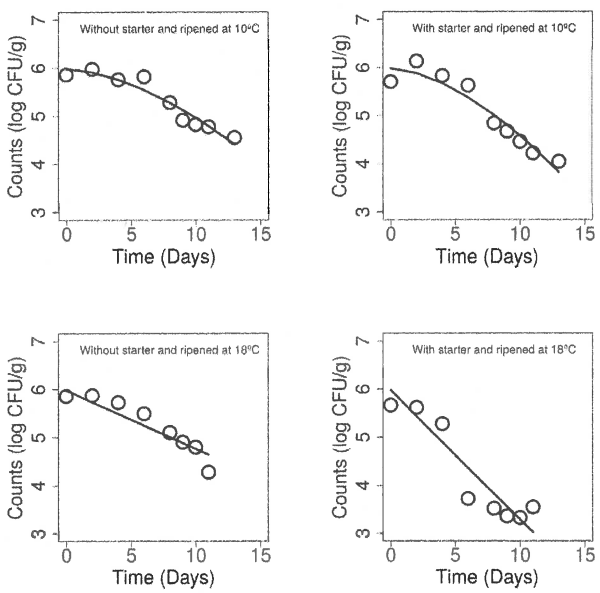

Fig. 1 Survival of inoculated *L. monocytogenes* in *linguiça* dry-fermented sausages along ripening for the four lab-scale production batches, as described by the three-parameter Weibull-based omnibus model.

The Weibull-based omnibus model demonstrated that both the use of starter culture ($p<0.001$) and, to a lesser extent, the ripening temperature ($p=0.15$) affected the concavity (shape parameter β) of the survival curves (Table 1), meaning that a faster decay in *L. monocytogenes* can be expected in *linguiça* formulated with commercial starter culture and ripened at a higher temperature (18ºC). The significant interaction term between the use of starter and ripening temperature (Table 1) indicated that, for the higher ripening temperature (18ºC), the reducing effect of the starter culture on *L. monocytogenes* was further boosted. Such enhanced decay in *L. monocytogenes* can be appreciated in Fig. 1 (see environmental condition "with starter and ripened at 18ºC").

In the Weibull model, the scale parameter χ can be interpreted as the time to reach the first decimal reduction. Yet, contrarily to the shape parameter β, the scale parameter χ was only affected by the ripening temperature and not by the use of starter culture. Hence, a higher ripening temperature ($p=0.045$) reduced the time to reach the first decimal reduction of *L. monocytogenes* in *linguiça*. However, the application of higher ripening temperatures cannot be pondered as a mechanism for controlling the development of *L. monocytogenes* in *linguiça* as other pathogens such as *Staphylococcus aureus* or *Salmonella* spp. may have their growth kinetics improved at 18ºC.

Table 1 Parameter estimates of the Weibull-based omnibus model predicting the non-log-linear decline of *L. monocytogenes* in *linguiça* dry-fermented sausage as a function of ripening temperature (ºC) and added starter culture.

Parameters	Mean	Standard error	Pr > |t|
Predictors of Ln β			
a_0 (Intercept)	2.290	0.099	<.001
a_1 (Starter)	-0.193	0.086	0.035
a_2 (Temperature)	-0.188	0.123	0.149
a_3 (Starter×Temperature)	-0.608	0.194	0.004
Predictors of Ln χ			
b_0 (Intercept)	0.497	0.219	0.033
b_1 (Temperature)	-0.509	0.241	0.045
Log N_0	5.983	0.111	<.001
Variance			
s^2 (residual)	0.056		BIC:34

The omnibus regression model also enabled to predict that in batches of *linguiça* without starter culture, by the 10th day of ripening, *L. monocytogenes* population could be reduced in 1.02 log CFU/g at 10ºC and 1.22 log CFU/g at 18ºC of ripening temperature. With the addition of the tested commercial starter culture, the levels of reduction could be significantly higher at 1.40 and 2.70 log CFU/g when sausages are ripened at 10ºC and 18ºC, respectively. Earlier research consisting of surveys of pathogens in *linguiça* processing plants evidenced that, currently, *L. monocytogenes* can be recovered in the final product in concentrations normally equal or lower than 50 CFU/g (~1.70 log CFU/g) [7]. Thus, the use of starter cultures should be contemplated as an additional hurdle that can further bring down such levels of *L. monocytogenes*. Since *linguiça* is a traditional product that undergoes fermentation with autochthonous bacteria, as a next step, research should be directed towards the preparation of a tailor-made starter culture.

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