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HOW THE COLD CHAIN IMPACTS THE SHELF LIFE OF A READY-TO-EAT FOOD REGARDING *LISTERIA MONOCYTOGENES*

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ABSTRACT

For RTE foods that are able to support the growth of *L. monocytogenes*, a European Regulation (n°2073/2005) specifies that the 100-CFU/g limit “applies if the manufacturer is able to demonstrate that the product will not exceed the limit of 100 CFU/g throughout the shelf-life”. Many factors can interfere on the evolution of the pathogen (time-temperature history, pH, a_w , associated microflora,...). The objective of this work was to demonstrate the impact of the cold chain on the shelf-life of a deli meat, sliced cooked ham. Sliced cooked ham data were obtained from challenge-tests with artificially inoculated packages with *L. monocytogenes*. These data are useful for estimating growth parameters and model validation. We tested different scenarios of storage temperature: from theoretical to more realistic scenarios based on data from a survey carried out in France. The variability of temperatures as well as characteristics of the product, and initial contamination level at the end of the manufacturing line, were taken into account in predictive models to calculate the time to reach the regulatory limit and to determine the shelf life. We showed that shelf-life determination is strongly dependant of the scenario chosen to simulate the cold chain.

1. INTRODUCTION

Listeria monocytogenes is a Gram-positive non sporeforming bacillus. It is a psychrotrophic bacteria: it may grow from the temperature -2°C to the temperature $+45^{\circ}\text{C}$. It may be responsible for listeriosis which occurs in two forms: non-invasive forms and invasive forms. Non-invasive forms are essentially febrile gastroenteritidis but they are rare. The main symptoms of invasive forms are bacteraemia or septicaemia, meningitis, meningoencephalitis, rhomencephalitis, brain abscess. Invasive forms concern also pregnant women: the infection may lead to flu-like symptoms but also to more severe symptom such as miscarriage, stillbirth, premature delivery, or infection of the newborn.

Food contamination by *L. monocytogenes* can occur for many foods at all stages of the food chain. But it is a real concern for ready-to-eat (RTE) foods because: (i) they can be contaminated by *L. m.*, (ii) they can support the growth of the bacteria and (iii) they will not receive a heat-treatment (ensuring the destruction of the bacteria) before consumption. Main RTE foods known as being potentially affected by this problem are processed meats, soft and semi-soft cheeses, seafoods such as smoked fishes (Mejlholm et al., 2010) but other RTE foods, e.g. fruits such as cantaloupe, might be implicated. European regulation (EC) N°2073/2005 (Anonymous, 2005) lays down the microbiological limit for the RTE foods; it demands 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 up to 100 CFU/g in “products placed on the market during their shelf-life”. Thus, food business operators shall conduct studies to investigate compliance with the criteria throughout the shelf-life. Annex II of this regulation

lists the tools to assess the microbiological shelf life. Studies can integrate physico-chemical characteristics, data from durability studies and challenge-tests and also results from predictive microbiology. Whatever the studies used, they shall take into account the inherent variability linked to the product, related to the micro-organisms and to the processing and storage conditions.

Many factors can affect the evolution of *L. monocytogenes*: ingredients, pH, a_w , conservatives, redox potential, associated microflora, structure, gas atmosphere, process and temperature during the cold chain. Among these factors, the temperature of the cold chain is a key point to take into consideration regarding the shelf-life of RTE foods. It includes production warehouse, transportation, distribution warehouse, retail storage, display cabinet, shopping basket and domestic refrigerator

Recently, several research teams proposed stochastic modeling approaches to describe the distribution of *L. monocytogenes* contamination in RTE foods throughout their shelf life (Couvert et al., 2010; Koutsoumanis and Angelidis, 2007). Couvert et al. (2010) proposed a model designed to include the main sources of variability leading to a scattering of natural contaminations observed in food portions: the variability of the initial contamination, the variability of the biological parameters such as cardinal values and growth parameters, the variability of individual cell behaviours, the variability of pH and water activity of food as well as portion size, and the variability of storage temperatures.

The objective of this work was (i) to illustrate how physico-chemical characteristics as well as data from challenge-test can be combined together to predict *L. monocytogenes* fate and to determine the shelf-life of a RTE for an identified hazard and (ii) to assess the impact of the different possible scenarios chosen to mimic storage temperature on the determination of shelf-life.

2. DATA NECESSARY FOR SHELF-LIFE DETERMINATION

2.1 Physico-chemical characteristics and initial contamination level of the considered RTE food

Cooked ham was used to illustrate the impact of time-temperature history chosen to simulate the real cold chain and its impact on shelf life establishment. This product was chosen because it is one of the most consumed RTE foods. Physico-chemical characteristics can be variable between products of a company. We directly inspired from a recent study (Ifip, 2010) where these characteristics were measured on different cooked ham to estimate this variability (Table 1). According to recent data obtained in the framework of a EU-wide survey of *L. monocytogenes* in RTE foods (Thisted Lambertz et al., 2012), prevalence as well as the initial contamination in cooked ham is very low, 1% and always <1 cfu/g respectively. Thus it was assumed that only a few cells per pack of sliced cooked ham are present in the early stage of the cold chain. Parameters assumed to describe these low levels of contamination are given in Table 1. Variability of all these inputs parameters was modeled with normal distribution.

Table 1. Parameters of Normal distribution used to describe variability of physico-chemical characteristics of cooked ham and initial concentration of *L. monocytogenes*.

Parameters	Mean	Standard deviation
pH	6.1	0.1
a_w	0.978	0.005
N0 [in $\log_{10}(\text{cfu/g})$]	-2	0.3

2.2 Microbiological challenge-tests

All tests were performed with one batch of cooked ham from one producer. The cooked ham was packed under modified atmosphere (50% CO₂ and 50% N₂) and its shelf-life was 31 days.

Challenge tests used a reference strain of *L. monocytogenes* of the national French program in predictive microbiology Sym'Previus, isolated from industrial meat food environment and stored at -80°C (n° 352 Aériel 67-France). One physiological state was used in this study: cells at the end of the exponential growth phase. Two subcultures of *L. monocytogenes* were grown in Brain Heart Infusion (BHI) at 37°C for respectively 16 h and for 8 h. A third subculture was carried out at 8°C for 6 days, in Brain Heart Infusion broth to obtain, for the inoculum 10⁹ CFU/ml in the late exponential growth phase.

Samples of 10 g of product were inoculated with 0.5 ml of the diluted subculture to obtain an initial concentration of approximately 10^2 CFU/g at the surface of the foodstuff. Control samples were constituted without inoculation with *L. monocytogenes*.

Vacuum-packed (50% CO₂; 50% N₂) artificially contaminated cooked ham samples and control samples were stored at four temperature levels and four studied periods: 5°C/39 days, 8 °C/28 days, 12°C/12 days and 15°C/12 days.

Natural lactic acid flora [(1ml was plated into MRS (according to NF V 04-503)] and aerobic microorganisms (1ml was plated into PCA, 30°C three days, pH [Hanna instruments HI 213 model (5g); according to NF V04-108),], and a_w (GBX-FA-St 1 model; NF ISO 21807: 2004) were quantified at three days during challenge testing to characterize the variability of the physico-chemical characteristics and natural flora of the batch of cooked ham.

Twelve growth curves of *L. monocytogenes* in mixed-culture (potentially presence of natural flora like lactic acid flora) were obtained at four levels of temperature (5°C ; 8°C ; 12°C ; 15°C) under modified atmosphere (50% CO₂ and 50% N₂). *L. monocytogenes* was quantified at regular time intervals to obtain data points representing all of the parts of the growth curve (lag, exponential and stationary phases). 10g samples were homogenized with 90ml of tryptone salt solution using a stomacher blender. Ten-fold serial dilutions were carried out and 0,1ml of the appropriate dilution was plated onto Compass *L. monocytogenes* [Biokar Laboratories) (37°C; 48h)] for the quantification of *L. monocytogenes*. The enumerations of *L. monocytogenes* were performed according to the ISO 11290-2 standard (Anonymous, 1998) on three samples at 10 or 11 different times during the lag, the exponential and the stationary phases of the growth curve.

The collected data were fitted to the logistic with delay primary model (Rosso et al., 1996) using the software Sym'Previus to estimate the growth parameters of *L. monocytogenes* under four temperature environments. These parameters are the initial contamination (N_0), the maximum contamination (N_{max}) levels, the maximum growth rate (μ_{max}), and the lag time (lag). For each growth curve, the maximum growth rate (μ_{max}), and the lag time (lag) were used to estimate μ_{opt} and lag_{min} which are the maximum specific growth rate and lag time values when T, pH and a_w are set to their optimal values. μ_{opt} and lag_{min} were calculated as follows:

$$\mu_{opt} = \frac{\mu_{max}}{\gamma(T)\gamma(pH)\gamma(a_w)}$$

$$lag_{min} = \gamma(T)\gamma(pH)\gamma(a_w) \cdot lag$$

Where $\gamma(\cdot)$ are gamma factors that help to quantify effect of environmental factors (see also above for further precision). The optimal growth rate μ_{opt} and the minimal lag time lag_{min} depend on both strain and food matrix (Couvert et al., 2010; Pinon et al., 2004).

2.3 Scenario chosen for simulating temperature in cold chain

Six different time/temperature scenarios were chosen to simulate the real temperature of RTE in the cold chain. Scenarios #1, #2 and #3 considered simple time/temperature histories of the products. For scenario #1, a constant temperature of 4°C, the regulatory temperature of storage at the stage of direct delivery or catering for perishable foodstuffs preservation (Anonymous, 2009), was chosen. Scenarios #2 and #3 were directly inspired by published European recommendations to conduct challenge-test experiments (EU Community Reference Laboratory for *Listeria monocytogenes*, 2008). For these scenarios three steps were distinguished: the time and temperature history (TTH) for products before they were at retail, the TTH in display cabinet at retail, and the TTH for consumer refrigerator (Table 2).

For the other 3 scenarios, we took data from a study realized in France by ANIA and Cemagref (former name of Irstea) in 2002 (ANIA, 2004). The originality of this study was to follow the product temperature continuously, from the end of production until its consumption. The methodology has consisted in putting a temperature recorder in the product at the end of the production and asking consumers to return it after removing the packaging. In this study, people handling the products (neither professional nor consumer) didn't know

that the product temperature was being recorded, which makes our data more close to reality. This study focused on three types of products: dairy products, ready meals and pre-packaged meat. We have chosen in this paper time/temperature data corresponding to foods products that are close to cooked ham. In scenario #4, we have chosen a circuit whose average temperature was 4°C, but with fluctuations throughout the chain. Scenario #5 is a time temperature scenario with a relatively low average temperature (1.7°C) and scenario #6 has an average temperature of 7°C, with fluctuations.

Table 2. Scenarios of time/temperature chosen for establishing shelf-life*.

Scenario	From facilities to retail	Retail	Consumer
#1		4°C until shelf-life	
#2	4°C for 5 days	8°C for 5days	8°C until shelf-life
#3	8°C for 5 days	12°C for 5days	12°C until shelf-life
#4	3 time-temperature records		
#5			
#6			

* time when 5% of contaminated cooked ham have a concentration above 100 cfu/g

3. SHELF-LIFE DETERMINATION

3.1 Modelling growth of *L. monocytogenes* according to product characteristics and storage conditions

The model used to predict the growth of *L. monocytogenes* is fully described in Couvert et al. (2010). This model is available on a website (www.symprevis.net).

In this model, each contaminating cell is assumed to be able to grow and the change in the bacterial population coming from each cell is described by a logistic with delay growth model (Rosso et al., 1996). The effect of storage temperature and product characteristics (pH and water activity) on maximum growth rate (μ_{max}) is described with cardinal secondary models and rely on gamma-concept (Zwietering et al., 1996). The growth rate in optimal conditions is one parameter of the model. This growth rate is reduced by gamma-factors, values between 0 and 1, which allow quantifying the effects of environmental factors.

Several sources of variability are included in the model to assess the variability of the fate of microorganisms naturally contaminating food. The variable factors are the initial contamination, the biological parameters (cardinal values, μ_{opt}), the food characteristics (pH, a_w , portion size), and the storage temperature. Cardinal values, that are necessary for $\gamma(\cdot)$ calculation, are already known and implemented in the model (Couvert et al., 2010). Over factors were taken into account according to data and scenario described in part 2. The output of the model is a distribution of the contamination after d days, d is chosen by the user.

3.2 Shelf-life determination

When using a deterministic model, the output of a predictive model is a single value, e.g. after d days the concentration of *L. monocytogenes* is N ufc/g. In this case, the determination of the shelf-life is straightforward; it corresponds to the date when the predicted level N of the pathogen reaches 100 cfu/g.

The application of the probabilistic approach to RTE meat products showed that compliance is anymore a discrete characteristic, i.e. above or below the 100 cfu/g limit after d days for a proportion of consumer unit of sale. For determining the shelf-life, there is a need for translating the safety criteria in probabilistic terms (Koutsoumanis and Angelidis, 2007). We have chosen for illustration purpose to determine the shelf-life according to the following criterions: the probability for

contaminated products to be above 100 cfu/g should be below 5%. It is very important to notice that these criteria do neither reflect author's view nor risk manager's view (Commission of the European Communities or French ministry of Agriculture). This level of probability is under the sole responsibility of food business operators (FBOs). The level chosen here is just used for illustration purpose.

4. RESULTS & DISCUSSION

4.1 Observed growth of *L. monocytogenes* and determination of μ_{opt} and lag_{min}

Growth kinetics of *L. monocytogenes* in vacuum-packed cooked ham inoculated artificially are presented in Figure 1. A significant increase in the concentration of *L. monocytogenes* was observed during the study period at each temperature (5°C; 8°C; 12°C; 15°C). Initial pH values and initial a_w values allow classifying the product in the food category allowing growth of *L. monocytogenes*. Physico-chemical parameters (pH, a_w) did not significantly evolve along the studied periods. Whatever the temperature, other microflora were naturally present on cooked ham. This microflora, natural lactic acid bacteria and other aerobic mesophilic bacteria, did not significantly affect growth potential of *L. monocytogenes* as maximum level reached by *L. monocytogenes* were above 8 log₁₀ (cfu/g). Growth rate and lag time values obtained by fitting the primary model for the four different temperatures permitted to assess four values of μ_{opt} and lag_{min} . Variability of both these parameters were characterized with a Normal distribution defined by mean and standard deviation of the four values obtained. It should be noticed that both the mean and the coefficient of variation of μ_{opt} are consistent with those obtained by Augustin et al. (Augustin et al., 2011) for cooked ham.

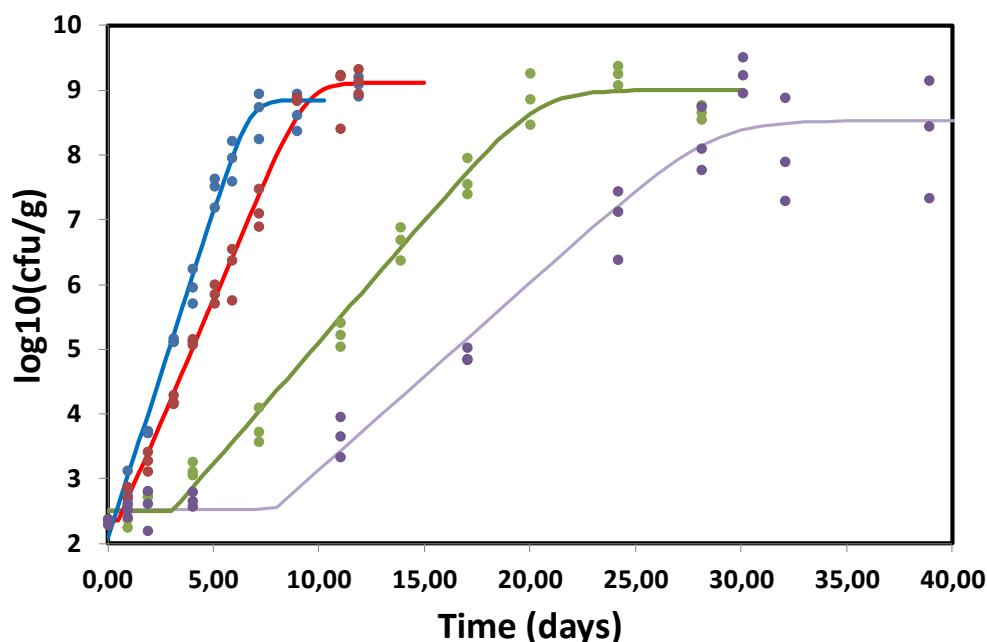


Figure 1. Growth curves of *L. monocytogenes* on sliced cooked ham at 5 (●), 8 (●), 12 (●) and 15°C (●). Continuous line: primary growth model fitted to data.

4.2 Shelf-life comparison

Growth of *L. monocytogenes* is different from one product to another according to product characteristics and biological variability (initial number of cells, lag time of these cells, strains variability). All these sources of variability were taken into account for simulating growth, we used for this data presented in parts 2 and 4.1.

The simulations of growth of *L. monocytogenes* were carried out according to the different scenarios of time-temperature. The results for scenario #1 are shown on Figure 2. After approximately 30 days for some simulations, concentration level reached the limit of 100 cfu/g (Figure 2A). Figure 2B presents the distribution of the levels of contamination after 30 days. Five percent of the products have contamination above 100 cfu/g. According to the rule define above, the shelf-life can be defined as the

time when 5% of contaminated products have concentration above $2 \log_{10}$ (cfu/g); the shelf-life would be 30 days if we consider that product in the cold chain are kept at temperature of this scenario, i.e. the regulatory storage temperature of 4°C .

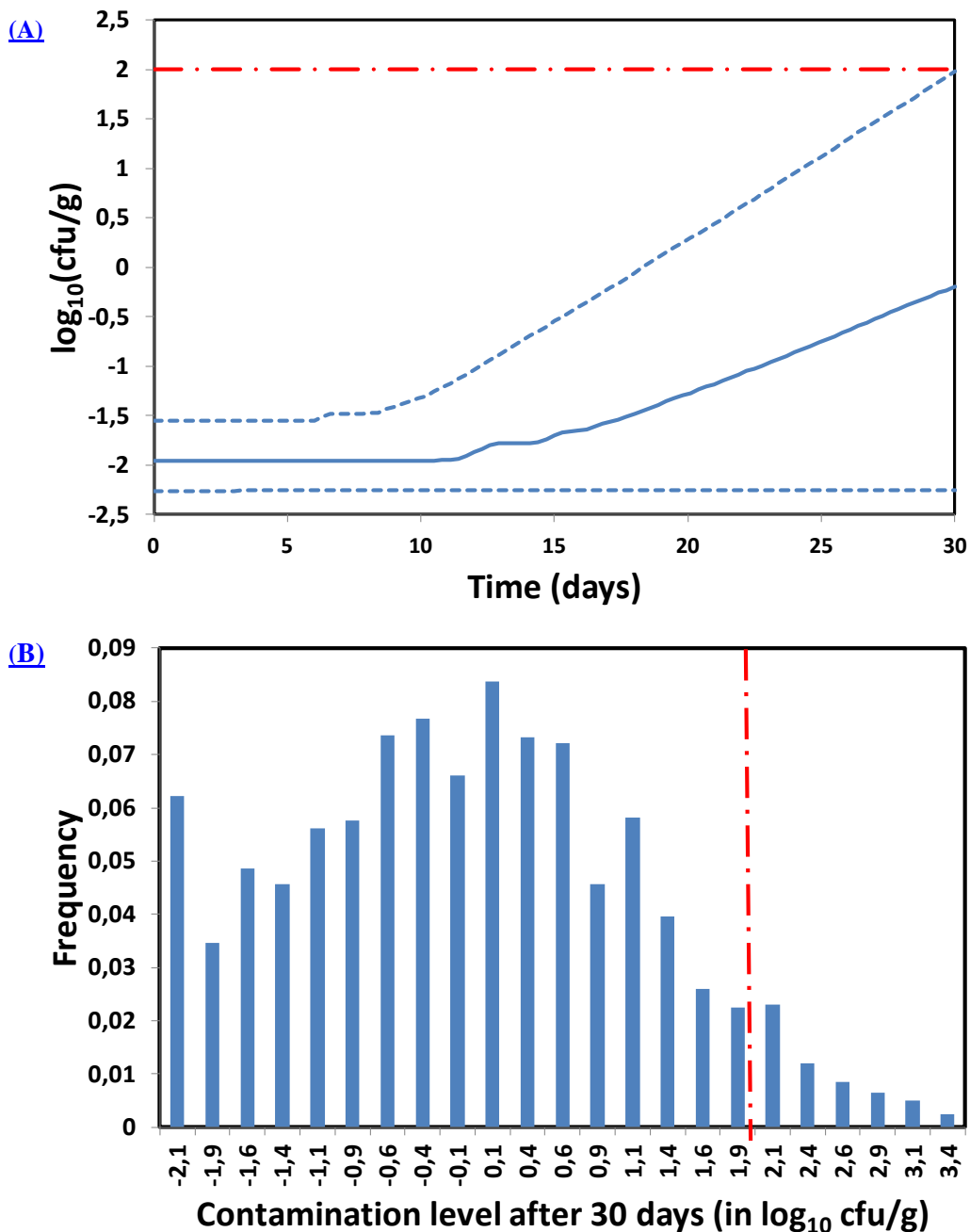


Figure 2. (A) Simulation of growth of *L. monocytogenes* in cooked ham for scenario #1. (—) Median of simulated growth curve, (---) 90% confidence intervals. (B) Distribution on the levels of concentration reached after 30 days for this scenario. (-.-) Limit of contamination

The shelf life for other scenarios was determined in the same way as scenario #1, i.e. as the time when 5% of contaminated products have concentration above $2 \log_{10}$ (cfu/g). Figure 3 presents the shelf lives obtained for the six different scenarios. The shelf-life obtained for scenarios #2 and #3, i.e. temperature based on European recommendations to conduct challenge-test experiments, are respectively, 1.4 and 3 times shorter than for scenario #1.

Scenarios #4, 5 and 6 (measured temperature profiles) conduct to very different shelf lives. A FBO that would consider scenario #5 will set a shelf life twice longer for his product than if he has chosen scenario #1. Scenario #5 highlights the interest of very low temperature for foods storage (Beaufort et al., 2009).

At the opposite if the FBO considers scenario #6, that represents a quite abusive storage temperature, one would set the shelf life that is divided by 3 compared to scenario #1.

It is worth to notice that scenario #4, which is representative of the majority of time temperature histories of Ania study (ANIA, 2004), would give approximately the same shelf life as scenario #2, 4°C for transport and retail and 8°C for consumer phase. Scenario #2 is the mostly used scenario in France to carry out challenge-tests or durability tests. From Figure 3, it can be said that this scenario more or less simulates real median time temperature of products in the cold chain.

In the same way, scenario #3 more or less led to the same results as scenario #6. This indicate that the recommended temperatures in guidance document of European , 8°C for transport and retail and 12°C for consumer phase, is equivalent to the worst case scenario of cold chain temperature.

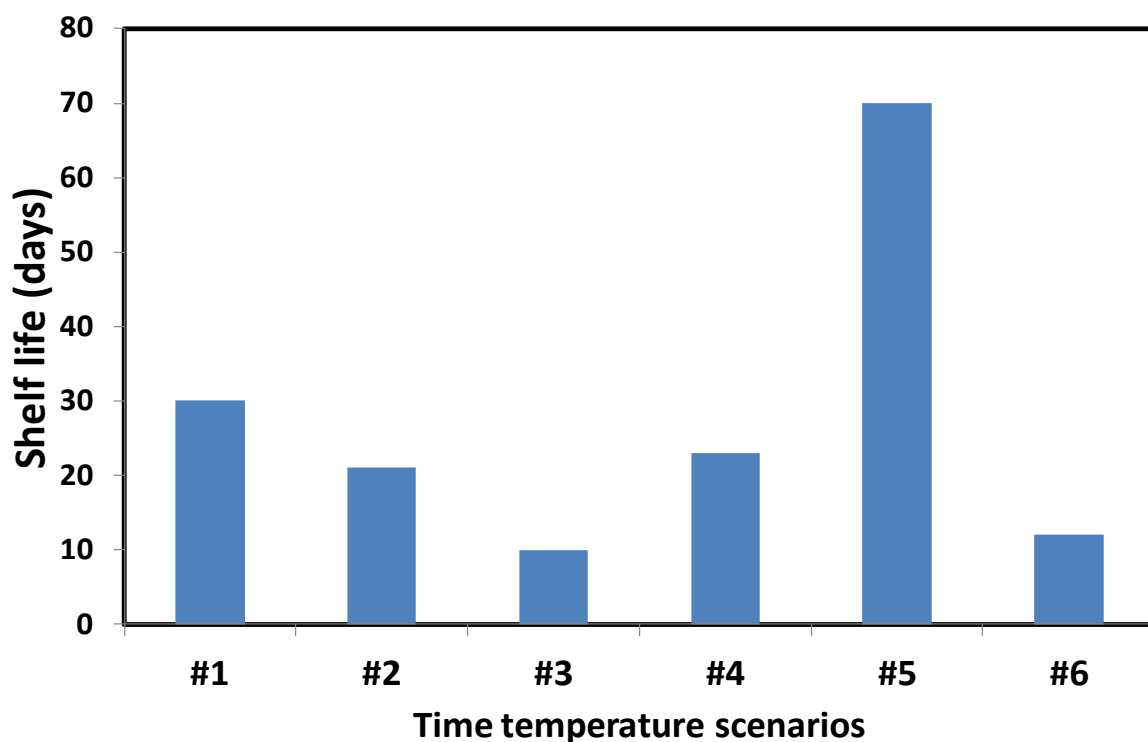


Figure 3. Shelf-life obtained for the six different time-temperature scenarios .

5. CONCLUSION

To determine a shelf-life requires identifying carefully the hazard specific of the foodstuff considered. The approach depends on whether operators are interested in microorganisms implicated of food alteration, fairly easily countable in the food or if they are interested in a germ thus limiting the shelf-life of consumption often hard to detect, like *L. monocytogenes*. Our studies illustrate how to combine both challenge tests and predictive microbiology in compliance with the Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (SANCO/1628/2008). The different case studies are useful for assessment and management of *L. monocytogenes* in processed foods.

The choice of a shelf life is linked to the proportion of packages above the limit and this is under the sole responsibility of FBO and official control institutions. This study illustrated that the shelf-life determination is very dependent of the time/temperature scenario chosen by the FBO. This study also underlines the interest of modelling the itinerary of the products in the different equipments of the cold chain as Duret et al. proposed (Duret et al., 2013).

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