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Shelf life estimation of refrigerated vacuum packed beef accounting for uncertainty

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ABSTRACT

This study estimates the shelf life of vacuum packed beef meat (three muscles: striploin (*longissimus thoracis et lumborum*, LTL), tenderloin (*psoas major*, PM) and outside chuck (*trapezius thoracis*, TT)) at refrigeration temperatures (0 °C–10 °C) based on modelling the growth of two relevant groups of spoilage microorganisms: lactic acid bacteria (LAB) and *Enterobacteriaceae*. The growth models were developed combining a two-step and a one-step approach. The primary modelling was used to identify the parameters affecting the growth kinetics, guiding the definition of secondary growth models. For LAB, the secondary model included the effect of temperature and initial pH on the specific growth rate. On the other hand, the model for *Enterobacteriaceae* incorporated the effect of temperature on the specific growth rate and the lag phase; as well as the effect of the initial pH on the specific growth rate, the lag phase and the initial microbial count. We did not observe any significant effect of the type of muscle on the growth kinetics. Once the equations were defined, the models were fitted to the complete dataset using a one-step approach. Model validation was carried out by cross-validation, mitigating the impact of an arbitrary division between training and validation sets. The models were used to estimate the shelf life of the product, based on the maximum admissible microbial concentration (7 log CFU/g for LAB, 5 log CFU/g for Enterobacteriaceae). Although LAB was the dominant microbiota, in several cases, both LAB and *Enterobacteriaceae* reached the critical concentration practically at the same time. Furthermore, in some scenarios, the end of shelf life would be determined by *Enterobacteriaceae*, pointing at the potential importance of non-dominant microorganisms for product spoilage. These results can aid in the implementation of effective control measures in the meat processing industry.

1. Introduction

Brazil is recognised as one of the biggest beef producers worldwide, with beef production constituting one of the main pillars of the national economy (Ministério da Agricultura, Pecuária e Abastecimento (MAPA), 2020). As such, one of the focus areas of the beef industry is guaranteeing the quality of the products during their whole shelf life.

Fresh meat is a highly perishable food due to high content in nutrients and physicochemical properties that enable growth of spoilage microorganisms (Fung et al., 2010; Gram et al., 2002; Nychas et al., 2007, 2008). It is common within the beef industry to use refrigeration and packaging technologies to extend the product shelf life, as the combination of these two technologies mitigate the growth of spoilage organisms. A diverse microbiota (mainly bacteria) is reported on meat after the slaughter of bovines (Broda et al., 2009; Dillon and Board, 1991; Jay et al., 2005; Kaur et al., 2021; Hwang et al., 2020). Factors such as temperature, packaging atmosphere, meat pH and microbial interactions, can affect microbial composition during shelf-life (Doulgeraki et al., 2012; Kaur et al., 2021).

Vacuum packaging and refrigeration temperatures (from −1.5 °C to 4 °C) limit the rapid development of *Pseudomonas* spp., *Brochothrix thermosphacta*, *Acinetobacter* spp., *Shewanella* spp., among others (Gill and Newton, 1978; Labadie, 1999; Pennacchia et al., 2011; Wang et al., 2017). On the other hand, these conditions allow other microorganisms

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presenting anaerobic and microaerophilic metabolisms, such as lactic acid bacteria to grow (Macedo et al., 2011). Some studies also highlight the growth of *Enterobacteriaceae*, *Bacillus* spp., *Clostridium* spp. under chilled conditions (Broda et al., 1996; Dainty and Mackey, 1992; Gill, 2004; Gribble et al., 2014; Shao et al., 2021). Among these, *Enterobacteriaceae* deserve special attention due to their high spoilage potential. In addition, *Enterobacteriaceae* have a variable behaviour that depend on the storage temperature, meat pH, and also varies among species (Brightwell et al., 2007).

Besides the extrinsic factor temperature, meat pH is an intrinsic factor that significantly impacts microbial growth and, consequently, the shelf life of the product (Gill, 2004). The normal pH of beef is usually between 5.4 and 5.5, but it can vary depending on several factors. Meat cuts with pH values ≥6.1 results in a defect called dark, firm and dry (DFD) (Abril et al., 2001; Mekonnen, 2015). DFD cuts allow fast bacterial growth, resulting in the reduction of the product's shelf life (Mills et al., 2014). Likewise, from a sensorial point of view, the ultimate pH of beef also affects attributes such as tenderness and colour, which are essential for the acceptability of the product by the consumers (Wicks et al., 2019). Therefore, the pH of the meat is commonly used as an indicator of the product quality.

Several authors have proposed a division of beef cuts in three pH ranges: normal (5.4–5.8), intermediate (5.8–6.1) and high (> 6.1) (Gill and Newton, 1979; Hood and Tarrant, 1980; Weglarz, 2010). The Brazilian meat industry generally follows this division to classify the quality of carcasses for export. Nevertheless, the Brazilian legislation does not establish a microbiological quality criterion for chilled beef considering the pH after slaughtering. Accordingly, the exports are based exclusively on the sanitary requirements of the country of destination (Circular N° 096/2004/DCI/DIPOA, Brazil; Resolution 833 Exenta, 2002, Chile).

Microbiological and physicochemical processes comprise the main causes of spoilage of beef meat. In this sense, predictive microbiology can be a useful tool to understand the most relevant factors affecting spoilage, and to estimate product's shelf life. This subfield of food microbiology is dedicated to the development and validation of mathematical models that describe the response of microbial populations to extrinsic and intrinsic factors from farm to fork (Pérez-Rodríguez and Valero, 2013). Shelf life of meat is defined on the basis of a range of increasingly subjective factors, such as colour, texture, firmness, odour, flavour and tenderness. Perceptible alterations in these factors (e.g., off-odour, off-flavour, meat discolouration), that are considered unacceptable by consumers, determine the shelf life of meat. These sensory alterations mainly occur due to considerable microbial growth and the progress of their metabolic activities, which cause a premature end of the product's shelf life. This fact is usually influenced by favourable conditions of, among others, temperature, pH, and packaging atmosphere (Sumner et al., 2021). Recent studies have shown that the quality traits provide convincing evidence to determine the shelf life of meat along with the microbial population, based on the analysis of the content of nitrogenous compounds, pH, moisture loss, colour or organoleptic traits of meat (Chen et al., 2020; Frank et al., 2019; Tayengwa et al., 2020).

Therefore, under the hypothesis that the length of the product shelf life is affected by a maximum microbial concentration, predictive microbiology can be used to estimate a food product shelf life (Koutsoumanis et al., 2021). In this work, a predictive modelling approach was employed to estimate the shelf life of vacuum-packaged beef meat, using an extensive dataset gathered within the Brazilian food chain.

2. Materials and methods

2.1. Sample collection

Three different types of muscles: striploin (longissimus thoracis et lumborum, LTL), tenderloin (psoas major, PM) and outside chuck (trapezius thoracis, TT) were obtained directly from a cattle slaughterhouse from São Paulo, Brazil. Different muscles were selected based on their potential pH differences (Abril et al., 2001) and on the basis of potential variation of microbial contamination due to handling during slaughtering operations (De Filippis et al., 2013).

Meat collection was basically divided into four stages:

- The first stage was the meat sampling: the meat was collected randomly, by the different lines of the deboning area within 48 h after the slaughter of the animal.
- The second stage was the pH measurement: the pH ($t = 0$) was measured at different points of each muscle to confirm its value using a portable pH meter (Brand Testo 205, Brazil). Based on their pH values, the muscles were grouped following common industry practices, in normal (5.4 — 5.8), intermediate (5.8 — 6.1) and high (> 6.1). The obtained values were considered as the initial pH of beef.
- The third stage was the sample processing: the muscles were transferred to the packing room for fractionation into steaks ($n = 1080$), which were weighed (~ 250 g), vacuum packed, and stored in a cold chamber at 0 °C until their transportation to the laboratory.

Finally, the samples were transported to the laboratory of Quantitative Food Microbiology (FEA, UNICAMP, São Paulo, Brazil), within 24 h after collection, in sealed styrofoam boxes in a ratio of meat to ice around 1:1. Then, the samples were stored in incubators (Eletrolab, Brazil) at four isothermal conditions: 0 °C, 4 °C, 7 °C and 10 °C ± 1 °C.

2.2. Microbiological analysis

In this study, culture-dependent methods were employed for evaluating the growth of LAB and *Enterobacteriaceae* as affected by temperature, pH and type of beef. These data were then used to assess the shelf life of vacuum packed beef. The selection of LAB and *Enterobacteriaceae* as microbial groups monitored for modelling purposes was based on a previous microbial ecology study conducted in the laboratory (data not yet published) that showed that these microorganisms presented a significant growth and role in the spoilage of chilled vacuum packaged beef. Besides, it is known that both microbial groups, LAB and *Enterobacteriaceae*, can grow under chilling and low oxidation-reduction potential (Eh) (anaerobiosis) (Brightwell et al., 2007; Macedo et al., 2011).

One vacuum packed steak was opened for each sampling point, and a portion of 10 g of meat were extracted aseptically for microbiological analysis. The samples were homogenised in 90 ml of Ringers Solution Quarter Strength (recipe prepared with laboratory ingredients based on Da Silva et al. (2017)) for 2 min at room temperature using a stomacher (Laboratory Blender Seward Stomacher 400, England) (Ercolini et al., 2006). Afterwards, ten-fold serial dilutions were made in Ringers Solution Quarter Strength, and aliquots of 1 ml of the appropriated dilutions were plated, twice, for enumeration of LAB and *Enterobacteriaceae*.

Lactic acid bacteria enumeration was performed by pour-plating with a double-layer in De Man Rogosa and Sharpe Agar (MRS Agar, Oxoid, Basingstoke, Hampshire, England). The plates were incubated at 30 °C for 48 h before enumeration. Following a similar plating methodology, Violet Red Bile Glucose Agar (VRBG Agar, Kasvi, Brazil) was used to enumerate *Enterobacteriaceae* after incubation at 30 °C for 24 to 48 h. The results were expressed in log CFU/g. Gram staining, and tests of catalase and oxidase were used as confirmatory for LAB and *Enterobacteriaceae*, respectively.

2.3. Modelling of microbial growth and data analysis

The Baranyi model (Baranyi and Roberts, 1994) was used as the primary model to describe the relationship between the microbial count of either microorganism ($N(t)$) and the storage time (t) at a constant temperature. This model is shown in Eq. 1, where μ is the maximum specific growth rate (ln CFU/h), N_0 is the initial microbial count (CFU/

g), N_{max} is the microbial count in the stationary growth phase (CFU/g) and h_0 is the "work to be done" (unitless) that is related to the duration of the lag phase (λ) by the identity $h_0 = \mu \cdot \lambda$. The model was fitted by least-squares of the natural logarithm of the microbial concentration.

$$lnN = lnN_0 + \mu \cdot A(t) - ln\left(1 + \frac{e^{\mu \cdot A(t)} - 1}{e^{lnN_{max} - lnN_0}}\right) \quad (1)$$

$$A(t) = t + \frac{1}{\mu} ln\left(e^{-\mu t} + e^{-h_0} - e^{-\mu t - h0}\right)$$

The influence on microbial growth of temperature, pH and type of muscle was analysed using secondary models. The relationship between μ and storage temperature (T) was described using the Ratkowsky model (Ratkowsky et al., 1982). In eq. (2), T_{min} (°C) is the (theoretical) minimum temperature enabling growth and b is the slope of the relationship between $\sqrt{\mu}$ and temperature.

$$\sqrt{\mu} = b(T - T_{min}) \quad (2)$$

On the other hand, there is no broadly accepted secondary model to describe the impact of pH and type of muscle on microbial growth. The same applies to the relationship between temperature and the other model parameters of the Baranyi model, such as N_0, λ (or h_0) and N_{max} (Augustin et al., 2000; Delignette-Muller et al., 2005).

Although there were pH differences at the beginning of the experiment among the meat cuts, making it possible to categorise the meats in three groups (pH < 5.8, between 5.8 and 6.1, > 6.1), these differences vanished shortly at the beginning of the storage. In other words, the pH of meat cuts with initial pH < 5.8 was not smaller than the pH of meats from the other groups during the whole experiment (supp. Fig. 1). Consequently, the effect of pH on the growth kinetics was based only on the initial pH of the meat, not on measurements made during the storage period.

In order to identify parsimonious models that represent the bacterial kinetics, secondary models were proposed based on descriptive statistics. The Baranyi growth model was fitted to different subsets of the data using the R package *growthrates* (Petzoldt, 2020). The subsets were the result of grouping the data by initial pH of the meat and/or type of muscle (both described as categorical variables):

A) Considering the global dataset for each temperature condition (no separation by pH or type of muscle);
B) Considering the temperature and the initial pH of beef;
C) Considering the temperature and the type of muscle;
D) Considering the temperature, initial pH of beef and type of muscle.

The effect of the initial pH of beef and type of muscle was evaluated visually using scatter plots and/or boxplots of the parameter estimates for the different datasets. The conclusions of this exploration were supported by ANOVA analysis with the model parameters as response variable and the selected factor(s) (temperature, pH and/or type of muscle) as explanatory variables. The plots are included as supplementary material to this article.

None of the growth curves observed for LAB had a lag phase (checked both visually and by model fitting). Hence, the "work to be done" was fixed to zero ($h_0 = 0$). Furthermore, we did not observe any effect of temperature, pH or type of muscle on the parameters N_0 and N_{max} were observed (supp. Fig. 2). Therefore, no secondary model was used for these parameters. Regarding the maximum specific growth rate, the effect of the initial pH of beef on the growth of both microorganisms was modelled using a categorical variable. Initially, the meat cuts were divided in three groups according to their initial pH, as commonly done in industry. However, no significant differences were observed between the model parameters estimated for meat cuts with pH between 5.8 and 6.1 and those with pH > 6.1 (ANOVA; $P > 0.05$). Therefore, both ranges were merged, and the effect of pH was modelled using a qualitative variable with two categories (pH_{high}, pH_{low}) depending on whether the initial pH was higher or lower than 5.8. No effects of the type of muscle on any of the model parameters were observed.

Therefore, the most parsimonious model among those tested for the growth of LAB was the one that included the effect of the pH on both parameters of the Ratkowsky model (b and T_{min}), as shown in Eq. (3) where the values $b_{[i]}$ and $T_{min[i]}$ depend on whether the initial pH equals pH_{high} or pH_{low}. To improve identifiability, the value of $b^2_{[i]}$ was estimated instead of $b_{[i]}$.

$$\sqrt{\mu} = b_{[i]}\left(T - T_{min_{[i]}}\right) \quad (3)$$

Regarding *Enterobacteriaceae*, the exploratory analysis resulted in the same model for μ (Eq. 3). Unlike for LAB, the growth curves for *Enterobacteriaceae* had a noticeable lag phase for the meats with initial pH < 5.8. The sequential modelling approach showed that the work to be done (h_0) depended on the storage temperature (supp. Fig. 3). Therefore, a linear relationship between them has been proposed as shown in Eq. (4), where $h_{T0[low]}$ and $a_{[low]}$ are respectively the intercept and the slope of the line.

$$h_0 = h_{T_0[low]} - a_{[low]} \cdot T; \text{if initial pH} < 5.8 \quad (4)$$

$h_0 = 0$; otherwise.

Moreover, a relationship between the initial count of *Enterobacteriaceae* (N_0) and the initial pH of the meat cuts was observed (ANOVA; $P > 0.05$). Considering that meat cuts with lower initial pH had higher N_0, this may be due to microbial growth lowering the meat pH, although this needs to be validated using independent complementary studies. Therefore, N_0 was described as a categorical variable, $N_{0[i]}$, which can take two values depending on whether the initial pH is lower than 5.8 or not.

$$N_0 = \begin{cases} N_{0[low]}, & pH_0 < 5.8 \\ N_{0[high]}, & pH_0 \geq 5.8 \end{cases} \quad (5)$$

Once the complete growth model (primary and secondary models) was defined, it was fitted to the complete dataset for either LAB or *Enterobacteriaceae* following a one-step approach using non-linear regression (*nls* function in R) using the Gauss-Newton algorithm with default control parameters. The goodness of the fit of each model was evaluated using the Root Mean Square Error ($RMSE = \sqrt{(logN_{fit} - logN_{obs})2/n}$; where n is the number of observations). All the calculations were implemented in R version 3.5.3 (R Core Team, 2016). The code is available upon request from the corresponding author of the manuscript.

2.4. Model (cross-)validation

In order to avoid an arbitrary division between data used for model fitting and for model validation, the growth models were validated by cross-validation (Balsa-Canto et al., 2020; James et al., 2013). Two types of cross-validation were used in this study. The first one was based on the type of meat cut. According to the exploratory analysis, the type of meat cut did not affect the growth kinetics of either microorganism. Therefore, the models were fitted to the data obtained for two of the meat types, using the remaining data for validation. In order to avoid an arbitrary division between training and validation sets, this process was applied three times by cross-validation (i.e., using each one of the meats for validation once).

The second method of cross-validation used in this study was k-fold cross-validation. Briefly, this approach begins by dividing the data in k groups by random sampling. Next, the growth model is fitted k times, each time removing one of the k groups from the dataset. The model validation is performed by comparing the predictions of each fitted model against the data in the group not included in model fitting. Both

for the validation of the LAB and *Enterobacteriaceae* models, the data was divided in $k = 5$ groups. The analysis was repeated for different values of k without observing any relevant impact on the conclusions of the analysis (not shown).

2.5. Shelf life estimation

Consumer shelf life can be defined on subjective sensory factors such as colour, texture, firmness, odour, flavour, and tenderness. Obvious alterations of these factors, which are considered unacceptable by consumers, define the end of the shelf life of the product. These sensory alterations occur, especially, as a result of considerable microbial growth and the development of their metabolic activities, that are usually influenced by favourable conditions of temperature, pH, and packaging atmosphere (Sumner et al., 2021).

Once the fitted models were validated, they were used to estimate the shelf life of the meat products analysed in this study. This was done under the hypothesis that the end of shelf life is reached when the maximum threshold of the specific spoilage microorganisms is achieved. This is a reasonable hypothesis for meat products, whose spoilage is mostly associated with microbial growth (Bruckner et al., 2013; Dougeraki et al., 2012). A concentration of 7 log CFU/g was considered as the concentration of LAB defining the end of the product shelf life (Kreyenschmidt et al., 2010), and 5 log CFU/g for *Enterobacteriaceae* (Regulations, S.I. n^{o} 243/1996 — European Communities Minced Meat and Meat preparations). The growth models developed in the study were used to estimate the storage time required to reach these concentrations. Uncertainty was included by forward uncertainty propagation by Monte Carlo simulation (Garre et al., 2017; Vásquez and Buuschaert, 2014). Briefly, normal distributions for each fitted parameter were defined based on the parameter estimates and their standard errors. Then, a random sample of the parameters was generated, resulting in a family of growth curves. For each one of them, the storage time required to reach the target microbial count (7 log CFU/g for LAB; 5 log CFU/g for *Enterobacteriaceae*) was estimated by linear interpolation. The convergence of the algorithm was assessed by repeating the calculations for different seeds of the pseudo-random number generator, requiring 5000 Monte Carlo iterations per condition.

3. Results

3.1. Growth of LAB in refrigerated vacuum packed beef

3.1.1. Model fitting for growth of LAB

Fig. 1 illustrates the growth of LAB observed in vacuum packed beef at storage temperatures between 0 °C and 10 °C. The concentration of LAB reached values higher than 7 log CFU/g at 836 h and 386 h (0 °C), 432 h and 205 h (4 °C), 294 h and 141 h (7 °C) and 213 h and 103 h (10 °C) at low (< 5.8) and high pH (> 5.8), respectively (Supplementary Tables 1 and 2). Nevertheless, the growth kinetics depended on both the storage temperature and the initial pH of the meat cuts. Meats with initial pH higher than 5.8 had faster LAB growth than meats with lower initial pH. This is evidenced by the former reaching the stationary growth phase approximately at the middle of the experiment for every temperature, whereas meat cuts with initial pH lower than 5.8 do not show a clear stationary phase (although the microbial count seems to stabilise at concentrations close to the N_{max} observed for meats with initial pH > 5.8).

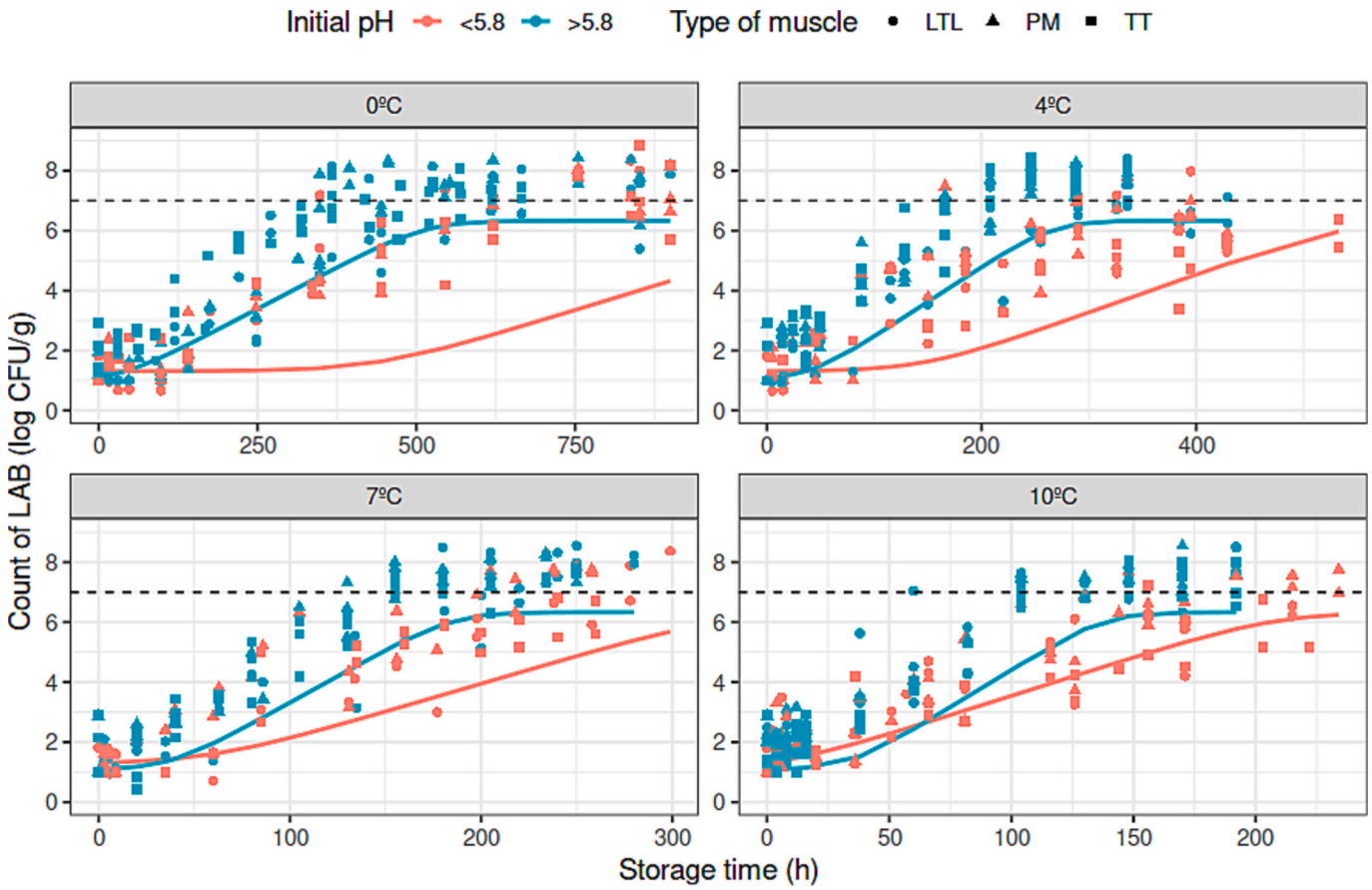

Fig. 1. Growth of LAB in refrigerated vacuum packed beef meat. The dots represent the observed microbial counts, whereas the lines the fitted models (using a one-step approach). The colours represent the initial pH of the meat, and the shapes the type of muscle. The data has been divided in subplots according to the storage temperature.

Table 1 reports the model parameters of the growth model for LAB in refrigerated vacuum packed beef meat fitted using a one-step approach. As already mentioned, the model did not consider a lag phase because the microbial population entered directly in the exponential growth phase (Fig. 1). Moreover, it accounts for the effect of temperature and initial pH on the maximum specific growth rate, but not on N_{max} nor N_0. As illustrated in Fig. 1, the model fitted the data effectively, with the fitted curve describing the overall trend observed in the experiments (RMSE = 0.87 log CFU/g). Note that the estimated values of T_{min} (−10.2 °C at low pH; −10.7 °C for high pH) is a notional estimate and thus a theoretical value that should not be considered representative of the minimum temperature allowing bacterial growth.

3.1.2. Model (cross-)validation for LAB

In order to validate the model, we used two types of cross–validation, using two different criteria for dividing the data. Fig. 2 illustrates the results when the division is based on the type of meat cut. As shown in Fig. 2A, there are no significant differences between the parameter estimates obtained for each validation set. Fitting the model to the complete dataset (red in Fig. 2), results in parameter estimates that can be seen as an average of those obtained when the model is fitted omitting the data of one type of muscle. Furthermore, the model fitted to the complete dataset has slightly smaller parameter uncertainty because it uses every point in the dataset. Furthermore, as shown in Fig. 2B, the fitted curves are practically the same regardless of the data used for model fitting. As expected based on the parameter estimates being "an average" of those obtained in cross-validation, the model fitted to the whole dataset is approximately in the mean of those fitted to each validation set. Nonetheless, there are small differences due to model non-linearities and the data not being perfectly balanced.

In a similar way, Fig. 3 illustrates the result of the cross-validation when the model is validated by 5-fold cross-validation. The results are similar to those obtained when cross-validation is based on the type of meat cut, with the variation in parameter estimates being smaller than parameter uncertainty. However, for 5-fold cross-validation the variation between sets is smaller and more balanced than when it is based on the type of meat cut. This is due to k-fold cross-validation dividing the data by random sampling, not any specific attribute of the data (e.g., the type of meat cut). Consequently, it is reasonable that k-fold validation will result in smaller deviations in the parameter estimates between the different models than when cross-validation is based on some attribute of the data (e.g., by strain or sampling date).

Regardless of the validation approach, Figs. 2 and 3 show that the dataset used to build the model has little influence in the model predictions. Furthermore, the model fitted to the complete dataset is "an average" of the models fitted to smaller datasets and has lower uncertainty. Therefore, it was considered that the model fitted to the complete dataset (i.e., the "Global model") is validated to describe the growth of LAB in refrigerated vacuum packaged beef meat under the conditions included in this study.

Table 1
Estimated parameters of the growth model for LAB in vacuum packaged beef meat under refrigeration conditions (0–10 °C).

Parameter	Estimated value	Standard error
$b2_{[low]}$ (ln CFU/h/°C)	1.5×10^{-4}	1.3×10^{-5}
$b2_{[high]}$ (ln CFU/h/°C)	3.0×10^{-4}	2.6×10^{-5}
$T_{min_{[low]}}$ (°C)	−10.2	0.6
$T_{min_{[high]}}$ (°C)	−10.7	0.6
log N_{max} (log CFU/g)	7.6	0.1
log N_0 (log CFU/g)	1.3	0.0
	RMS = 0.87 log CFU/g	

3.2. Growth of Enterobacteriaceae in refrigerated vacuum packed beef

3.2.1. Model fitting for growth of Enterobacteriaceae

Fig. 4 illustrates the observed microbial growth of *Enterobacteriaceae* in beef meat cuts. Although the initial count of *Enterobacteriaceae* was higher in meat cuts with initial pH < 5.8, the growth was slower than in meats with initial pH > 5.8, and a lag phase was apparent. Therefore, in a similar way as for LAB, the stationary growth phase was reached by the middle of the experiment in meats with initial pH > 5.8 for all the storage conditions tested, whereas the stationary phase was not reached in meat cuts with initial pH < 5.8. This is partly because the growth curves of *Enterobacteriaceae* in meat cuts with initial pH < 5.8 had a lag phase and partly due to a lower growth rate. Regardless of this, at every storage temperature tested, the *Enterobacteriaceae* count exceeded 5 log CFU/g at 397 h (0 °C), 444 h and 221 h (4 °C), 261 h and 155 h (7 °C) and 161 h and 115 h (10 °C) at low (< 5.8) and high pH (> 5.8), respectively, except for storage at 0 °C with initial pH < 5.8 as indicated in Supplementary Tables 1 and 2.

The parameters of the model fitted to the growth data of *Enterobacteriaceae* in vacuum packed beef using a one-step approach are reported in Table 2. Based on the exploratory analysis, this model includes the effect of the initial pH of the meat on the initial microbial count, as well as on the parameters of the Ratkowsky model. Furthermore, it considers a lag phase for meat products with initial pH < 5.8. As illustrated in Fig. 4, these hypotheses are supported by the data, with the fitted model being able to describe the overall trend of the observations, although the RMSE of the model (1.00 log CFU/g) is higher than for LAB. This is reasonable considering that this data set has more noise than the observations for LAB.

3.2.2. Model (cross)validation for growth of Enterobacteriaceae

Figs. 5 and 6 illustrate the results of the cross-validation for the growth model of *Enterobacteriaceae* when the split is based on, respectively, the type of meat or 5-fold cross-validation. The results are similar to those observed for LAB. In general, the uncertainty of the parameter estimates is larger than their variation between the individual models (Figs. 5A and 6A). Furthermore, the growth curve predicted by the model fitted to the complete dataset is quite comparable to the predictions of the individual models (Figs. 5B and 6B).

Only in two cases there are differences between the curve fitted by the global model and the cross-validated ones. Not including the data on trapezius thoracis (TT) for model fitting results in slightly larger estimates for N_{max} and T_{min} (although not significantly different at $\alpha = 0.05$). Therefore, the model fitted to this subset of the data predicts higher microbial concentration during the stationary growth phase than the others. However, removing this information also doubles the uncertainty of parameter ln N_{max} (Fig. 5A). The second case whose fitted curve deviates with respect to the general trend is observed when the model is fitted to the data excluding the data points for longissimus thoracis et lumborum (LTL). In this case, the model predicts faster growth than the rest for meat cuts with initial pH < 5.8. Nevertheless, removing this data also increases in almost 2-fold the uncertainty of parameter $b_{[low]}$; the slope of the Ratkowsky model for meat cuts whose initial pH is in this range. Therefore, in both cases, the omission of the data increases the uncertainty of a parameter that is tightly related to the deviation with respect to the global model. Hence, it is reasonable to attribute these deviations to the noise in the data, not to an effect of the type of muscle in the growth kinetics.

In the case of 5-fold cross-validation, the results are similar as for the other cross-validation method, although the deviations are smaller. As already argued, this is likely due to the random sampling used in k-fold cross-validation. Therefore, regardless of the way the data is split in training and validation sets, the growth model is practically the same. For this reason, it was concluded that the growth model for *Enterobacteriaceae* is validated to describe the growth kinetics of this microorganism in refrigerated vacuum packed meat within the parameters of

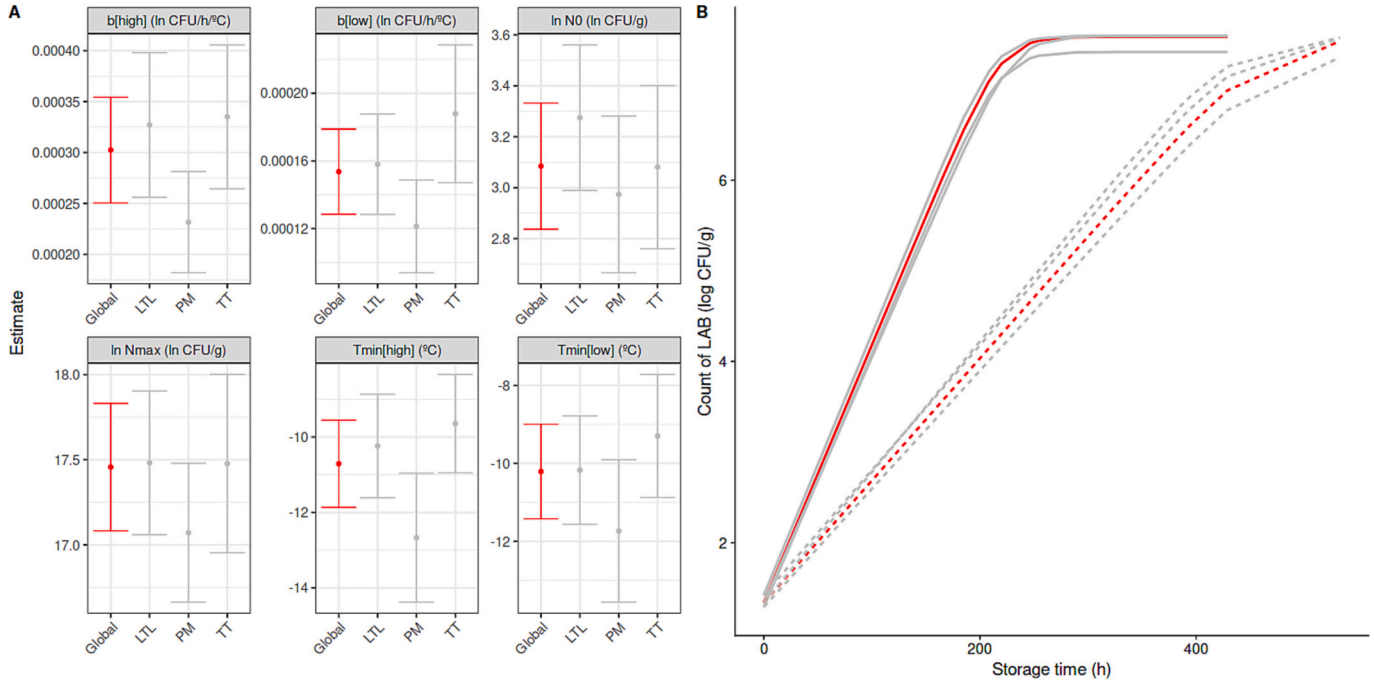

Fig. 2. Cross-validation by type of meat of the growth model for LAB developed in this study. (A) In grey, parameter estimates (estimated value represented as a dot, standard errors as error bars) when the data points belonging to one type of meat are not used for model fitting (x-axis). The parameter estimates when the model is fitted to the complete dataset is shown in red. (B) Growth curves fitted for each sub-model at 4 °C for meats with initial pH > 5.8 (solid lines) and initial pH < 5.8 (dashed lines). The growth curve corresponding to the model fitted to the complete dataset is shown in red.

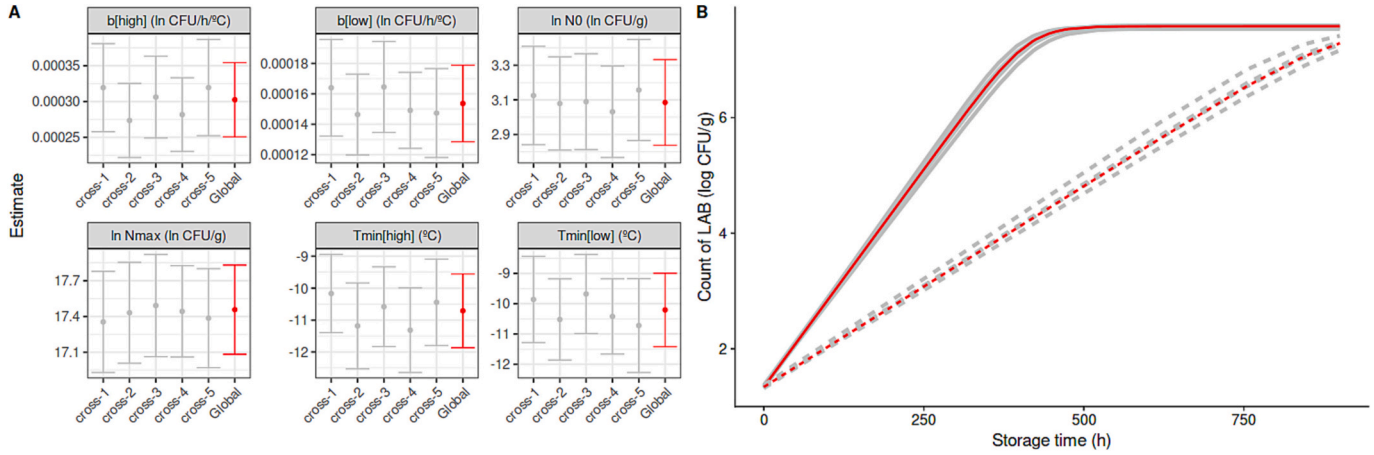

Fig. 3. Cross-validation by 5-fold cross validation of the growth model for LAB developed in this study. (A) Parameter estimates (estimated value represented as a dot, 95 % confidence intervals as error bars) when the data points belonging to one type of meat is not used for model fitting (x-axis). The parameter estimates when the model is fitted to the complete dataset is shown in red. (B) Growth curves fitted for each sub-model at 0 °C for meats with initial pH > 5.8 (solid lines) and initial pH < 5.8 (dashed lines). The growth curve corresponding to the model fitted to the complete dataset is shown in red.

this study.

3.3. Shelf life estimation based on growth models

The growth models developed in this study were used to estimate the shelf life of the beef meats in the study based on a maximum microbial concentration (7 log CFU/g for LAB; 5 log CFU/g for *Enterobacteriaceae*). Fig. 7 illustrates the shelf life estimated based on each spoilage microorganism for different storage temperatures and initial pH of the meat. The estimated product shelf life for each condition is reported as supp. Tables 1 and 2.

A clear effect of the initial pH of the meat on the product shelf life has been observed (supp. Tables 1 and 2). According to the models, meat with initial pH < 5.8 would have a shelf life (34.8 days at 0 °C, 18 days at 4 °C, 10.9 at 7 °C, 6.7 days at 10 °C) almost twice to that estimated for meat cuts with higher initial pH (16.1 days at 0 °C, 8.5 days at 4 °C, 5.9 days at 7 °C, 4.3 days at 10 °C). The calculations also reflect the uncertainty associated with these estimations, including the confidence interval of the mean. In this case, uncertainty was higher for conditions with longer shelf life. For instance, the 90 % confidence interval for meat cuts with initial pH < 5.8 stored at 0 °C spans 3 days, whereas the CI for the same meat stored at 10 °C only spans 0.8 days.

It is worth noting that, although the growth of LAB is faster than the one of *Enterobacteriaceae* for every condition tested (Figs. 1 and 4), this group would not be responsible for spoilage in every refrigeration condition included in the study according to the models. In the case of meat

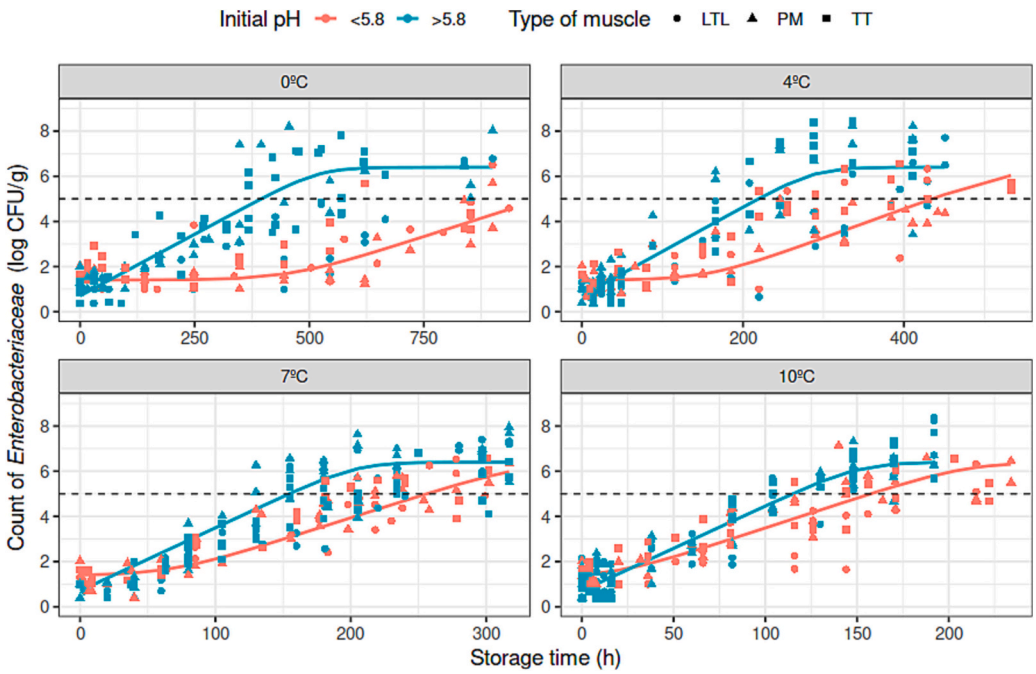

Fig. 4. Growth of *Enterobacteriaceae* in refrigerated vacuum packed beef meat. The dots represent the observed microbial counts, whereas the lines the fitted models (using a one-step approach). The colours represent the initial pH of the meat, and the shapes the type of muscle. The data has been divided in subplots according to the storage temperature.

Table 2
Estimated parameters of the growth model for *Enterobacteriaceae* in vacuum packaged beef meat under refrigeration conditions (0–10 °C).

Parameter	Estimated values	Standard error
$\log N_{0[low]}$ (log CFU/g)	1.4	0.2
$\log N_{0[high]}$ (log CFU/g)	0.7	0.1
$\log N_{max}$ (log CFU/g)	6.4	0.1
$b2_{low}$(ln CFU/h/°C)	1.6×10^{-4}	0.3×10^{-4}
$b2_{high}$(ln CFU/h/°C)	1.8×10^{-4}	0.2×10^{-4}
$T_{min_{[low]}}$ (°C)	−9.8	1.6
$T_{min_{[high]}}$ (°C)	−11.7	0.8
$h0_{[low]}$ (·)	6.9	1.8
a_{low} (1/°C)	0.55	0.17
	RMSE = 1.00 log CFU/g	

with initial pH > 5.8 (Fig. 7B), both microbial populations would reach the critical concentration at practically the same time. On the other hand, for meats with pH < 5.8, the microorganism responsible for the spoilage would vary depending on the storage temperature. For temperatures >5 °C, *Enterobacteriaceae* would define the end of the product shelf life. This change in the main spoiler can be explained based on the secondary models for temperature fitted for each microorganism. As illustrated in Fig. 8, a change in the initial pH of the meat introduces a shift in the secondary model for *Enterobacteriaceae*, but it barely impacts the slope of the relationship. On the other hand, for LAB, a change in the initial pH of the meat affects both the intercept and the slope of the secondary model (the same conclusion can be drawn analysing the values of b and T_{min} reported in Tables 1 and 2). This is the reason why the shelf life estimates based on both indicators are parallel for meats with low initial pH, whereas they cross for meats with high initial pH.

This figure also shows the model parameters reported by previous scientific studies that analysed the growth of LAB and *Enterobacteriaceae* in beef meat. Cayré et al. (2003) reported maximum specific growth rates of 0.412, 0.640 and 1.158 ln CFU/day for LAB at 0 °C, 8 °C and 15 °C respectively. Li et al. (2013) estimated rates of 0.648 and 2.304 ln CFU/day at 7 °C and 10 °C. Regarding *Enterobacteriaceae*, Skandamis and Nychas (2002) studied the growth kinetics of this species in beef packed under modified atmosphere. At 0 °C, they observed a lag phase of 167 h and a maximum specific growth rate of 0.21 ln CFU/day at 0 °C, and at 10 °C they observed a lag phase of 51 h with a maximum specific growth rate of 0.72 ln CFU/day (parameters estimated using ComBase). These values are close to the ranges estimated in this study (Fig. 8). The variation in the results reported in the literature can be attributed to the fact that meat spoilage is strongly affected by variability at different levels. This includes but is not limited to differences in the physico-chemical properties of different meat cuts, the use of different manufacturing practices between industries and the natural variation of the product microbiome. This has been further evidenced in our study, where the initial pH of the meat had a significant impact on the growth kinetics (Figs. 1, 4 and 8). Consequently, the study of the causes of these variations and their quantification, as well as their impact on the product shelf life, are still an active research topic.

4. Discussion

4.1. Growth of LAB and Enterobacteriaceae in refrigerated vacuum packed beef with low and high pH

The growth of LAB in vacuum packed beef stored at refrigerated temperatures have been widely described (Da Silva et al., 2018; Giannuzzi et al., 1998; Leisner et al., 1995; Zamora and Zaritzky, 1985). Under anaerobic conditions, the competitive nature of LAB is based on its antibacterial and antioxidant activities (Signorini et al., 2006; Zhang et al., 2018), which explains its predominance in beef compared to other spoilage populations included the *Enterobacteriaceae* (Macedo et al., 2011). The genera mostly identified as spoilage population in chilled vacuum-packed meat are LAB (*Lactobacillus* spp., *Leuconostoc* spp.) and *Carnobacterium* (Labadie, 1999; Macedo et al., 2011). The initial pH of meat was another factor that also influenced the growth of LAB.

A growth pattern of LAB related to the initial pH of meat that is characterized by rapid growth at higher pH conditions has been described (Blixt and Borch, 2002; Saraiva, 2008; Weglarz, 2010). This LAB response is consistent with the results found in this study since the lower pH condition (< 5.8) contributed to a delayed growth of LAB in

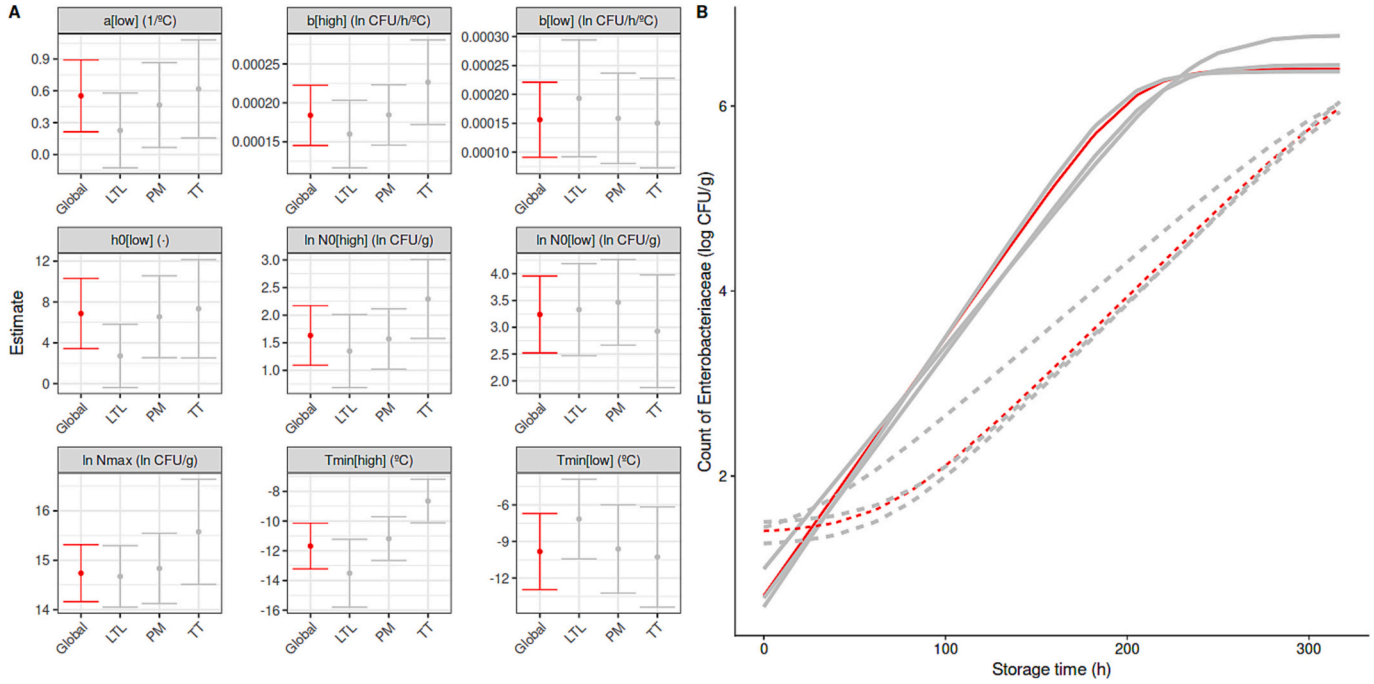

Fig. 5. Cross-validation by type of meat of the growth model for *Enterobacteriaceae* developed in this study. (A) Parameter estimates (estimated value represented as a dot, 95 % confidence intervals as error bars) when the data points belonging to one type of meat are not used for model fitting (x-axis). The parameter estimates when the model is fitted to the complete dataset is shown in red. (B) Growth curves fitted for each sub-model at 7 °C for meats with initial pH > 5.8 (solid lines) and initial pH < 5.8 (dashed lines). The growth curve corresponding to the model fitted to the complete dataset is shown in red.

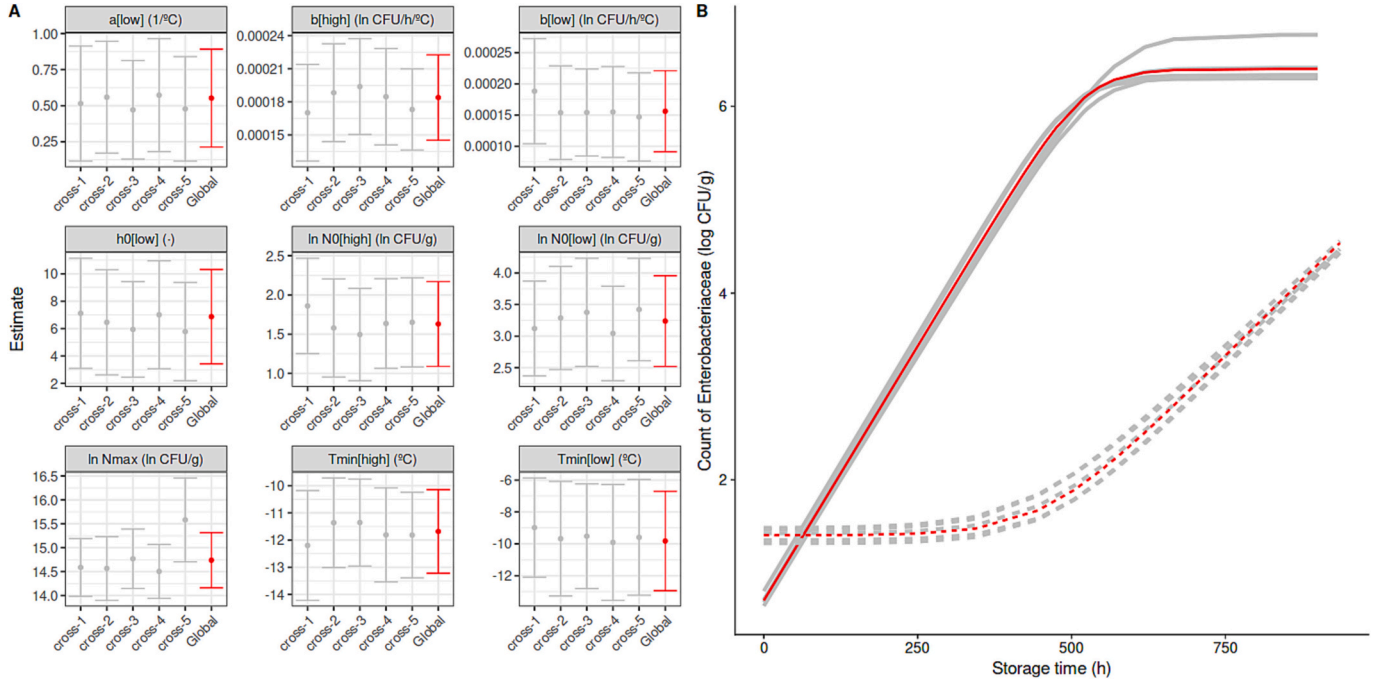

Fig. 6. Cross-validation by 5-fold cross validation of the growth model for *Enterobacteriaceae* developed in this study. (A) Parameter estimates (estimated value represented as a dot, 95 % confidence intervals as error bars) for each k-fold model (x-axis). The parameter estimates when the model is fitted to the complete dataset is shown in red. (B) Growth curves fitted for each sub-model at 0 °C for meats with initial pH > 5.8 (solid lines) and initial pH < 5.8 (dashed lines). The growth curve corresponding to the model fitted to the complete dataset is shown in red.

beef meat compared to the higher pH (> 5.8).

The growth of *Enterobacteriaceae* in meat with normal pH conditions (<5.8) is affected by factors such as the lactic acid content in the muscle (Grau, 1980), the selective effect exerted by CO_2 when the beef meat is vacuum-packed (Grau, 1980) and the subsequent inhibitory effect caused by the predominance of the LAB during meat storage (Ercolini et al., 2006; Nychas et al., 2008). These could be the reasons why, in this study, the *Enterobacteriaceae* had the higher values of N_0 (~ 3 log CFU/g) but a more extended h_0 at low pH (< 5.8).

On the contrary, at high initial pH of beef (> 5.8), the ideal

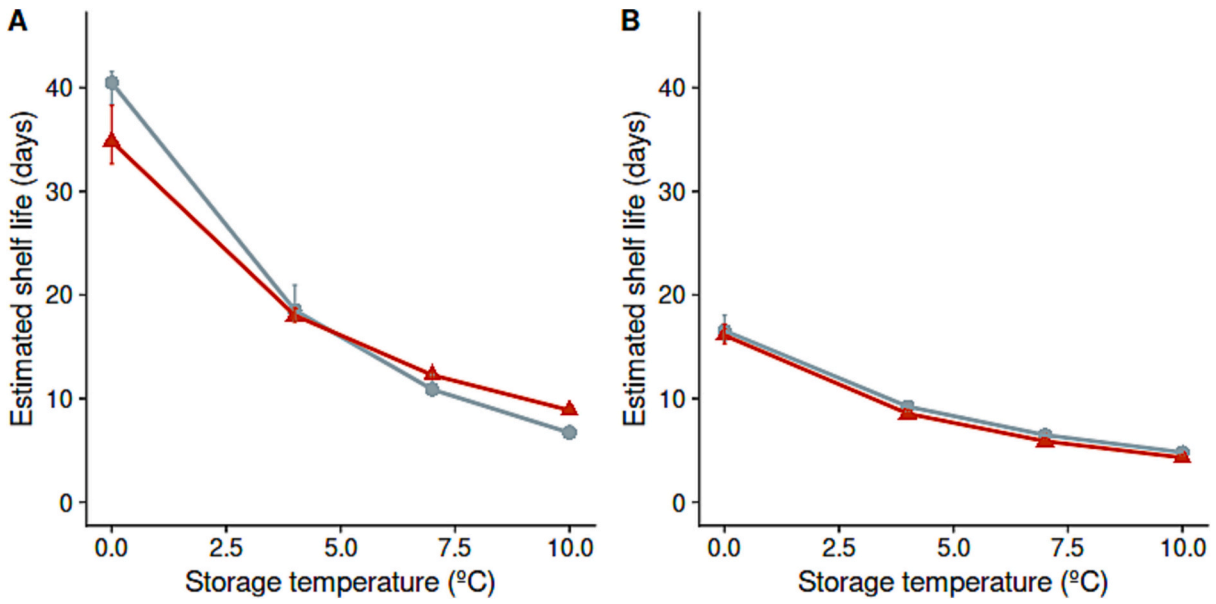

Fig. 7. Estimated shelf life for refrigerated vacuum packed beef meat based on the microbial count of LAB (red triangles) or *Enterobacteriaceae* (grey dots) for meats with initial pH < 5.8 (A) and > 5.8 (B) based on a maximum microbial concentration (5 log CFU/g of *Enterobacteriaceae*; 7 log CFU/g of LAB). The error bars represent 90 % confidence interval of the mean.

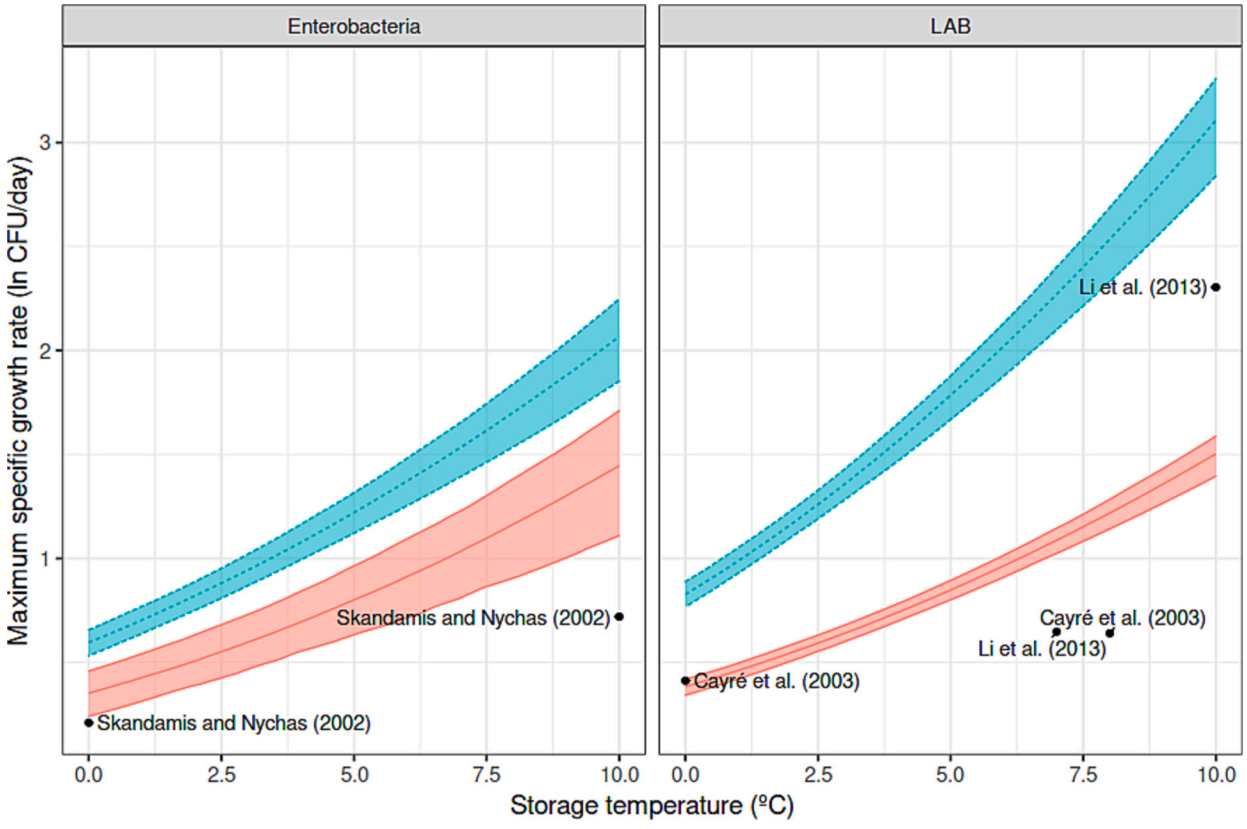

Fig. 8. Estimated growth rate of LAB and *Enterobacteriaceae* in vacuum packed meat with initial pH > 5.8 (blue, dashed lines) and < 5.8 (red, solid line).

conditions for developing of *Enterobacteriaceae* are created. Previous studies indicated that, under these conditions, *Enterobacteriaceae* can reach considerable levels (> 5 log CFU/g) and may, even, be able to compete against LAB (Blixt and Borch, 2002; Coll-Cárdenas et al., 2008; Gill and Newton, 1979). This could explain the findings of this study in meat with high pH. Bacteria from the genera *Serratia*, *Hafnia*, *Enterobacter*, *Rahnella* are commonly identified in meat with pH > 5.8 stored at refrigerated temperatures (Brightwell et al., 2007; Grau, 1983; Macedo et al., 2011). The capability of *Enterobacteriaceae* to grow at refrigerated temperatures have been informed to vary between species (Coll-Cárdenas et al., 2008; Doulgeraki et al., 2012; Gribble et al., 2014; Labadie, 1999), and also is dependent on the initial contamination levels

(Brightwell et al., 2007).

4.2. Relevant aspects for the development and validation of the growth models

Model validation, understood as an assessment of the predictive power for conditions not included in the experimental design, is an essential step in the development of empirical growth models. It must be admitted that model validation is also subjective and open to interpretation. Probably the most common practice in predictive microbiology is the division of the data in two groups: a training set that is used to build the model, and a validation set that is kept for model validation (Hwang and Huang, 2019). When analysed in detail, this approach raises several conceptual concerns. The first one is the multitude of criteria that can be followed to split a dataset in training and validation sets. For instance, in this study, the data was divided by temperatures, meat types, repetitions. Moreover, there were no definite guidelines about the best size for the validation and training sets (e.g., use 1 or 2 temperatures for validation). As evidenced in Figs. 2 and 3, and Figs. 5 and 6, different training sets will result in different parameter estimates and model predictions. Therefore, this approach introduces an element of subjectivity in the model, where a division between training and validation sets that can be ambiguous and subjective has an impact on the fitted model.

The second limitation of this approach for model validation is the presence of study effects in both the training and validation sets. The term "study effect" is commonly used in meta-analyses to refer to some factors specific to a study and that may introduce a bias in the model parameters or predictions (Felson, 1992). In the case of microbial growth, study effects include, among others, the effect of the experimental protocols (e.g., media preparation, culture preparation, recovery, equipment) and other aspects of data gathering (e.g., microbiome in the geographical area, properties of the meat, industry practices, factors regarding the abattoir). Regardless of how the data from a scientific study is divided between training and validation set, the study effects will be present in every data point. In other words, the model would always be validated for the particular conditions of the study. This is in clear contradiction with the goal of model validation as an assessment of the predictive power of the model for conditions not included in the analysis (i.e., with different study effects).

In this article the application of alternative approaches for model validation based on cross-validation has been illustrated. Instead of fitting the mathematical model to a single dataset, this methodology repeats the fit for several subsets of the data before applying the usual validation criteria, mitigating the subjectivity of the division between the training and validation set. However, this method still requires the definition of the attribute to select for the division. In this article, two types were illustrated: random division (k-fold cross validation) and division by a data attribute (type of meat cut). Both approaches have advantages and disadvantages. From a statistical point of view, a random division of the sets reduces the correlation of the data points within the training and validation sets. On the other hand, this approach is less likely to identify attributes of the data that have a significant impact on the microbial response that would otherwise be identified through a separation by attributes, a result that is often of great interest in growth studies. Nevertheless, this can be mitigated if an individual analysis of the growth curves is performed before model fitting (e.g., using the two-step approach prior to fitting by one-step) to identify factors that may potentially influence the microbial response.

The advantage of using several combinations of training and validation sets instead of one is illustrated in Fig. 6. The plot represents the growth models fitted for *Enterobacteriaceae* when the data from one type of meat is omitted from the analysis. Removing the data on trapezius thoracis (TT) from the training set has a strong impact on the estimated value of N_{max}, affecting model predictions. Therefore, if the common approach for model validation that fits the model to a single training set had been followed, a bias might have been introduced in the parameter estimates. As illustrated here, because cross-validation repeats the fit for several subsets of the data, it mitigates the risk of introducing a bias in parameter estimates due to a singular division of the data in training and validation sets. Note, however, that cross-validation cannot mitigate the second limitation of model validation mentioned above: the prevalence of study effects in both the validation and training data. Because every data point is affected by them, the only way to validate for more general conditions is by including data not gathered in the study. In this study, the data were obtained by random sampling in food industries from a relatively big geographical area through different seasons. Therefore, the study effects should be smaller than in studies where the data is obtained under laboratory conditions using (a cocktail of) reference strains.

Regardless of the approach for model validation, it is important to consider that discrete model predictions (i.e., growth curves) are unlikely to describe the actual microbial response in the food chain. It is well known that genetic differences between strains of the same species will affect their growth and inactivation kinetics (Den Besten et al., 2018). With the current technology, it is impossible to predict the particular strain that will contaminate a food product. Furthermore, empirical models are built based on experimental data, affected by experimental error. Therefore, in order to make more realistic risk and shelf life estimations, parameter estimates and model predictions should account for variability and uncertainty (Garre et al., 2020). As illustrated in this study, the application of Monte Carlo simulations can assist in the calculation of credible (or confidence) intervals for the variables of interest.

Besides model validation, another relevant aspect for model building that was evidenced in this article is how the one-step and two-step model fitting approaches can complement each other. It is generally accepted that the one-step approach results in more robust parameter estimates, accounting better for the uncertainty in the data (Fernández et al., 1999). However, checking the hypotheses of the primary and secondary models with this approach is not straightforward. This may be a minor issue for simple models (e.g., those considering only the effect of temperature), but many case studies aim at describing the effect of other factors that have not been studied in so much detail (e.g., the type of muscle or the initial pH). In these situations, the two-step approach can be insightful. As shown in this article (details in supp. Figs. 2 and 3), fitting a primary model to different subsets of the data can support the definition of the hypothesis of the secondary models, identifying the factors that should be included in the model and the model equations to use (e.g., linear, log-linear). Once the model has been defined based on the two-step method, robust model parameters can be estimated using the one-step approach.

One aspect to consider carefully when comparing the results of this study against others in the literature is the interpretation of the maximum microbial count in the stationary phase (N_{max}). Although there are some conflicting observations in the scientific literature, the effect of bacterial interactions in the stationary phase of mixed microbial cultures has been broadly reported (Bolívar et al., 2021; Cornu et al., 2011; Costa et al., 2020; Gonzales-Barron et al., 2020; Guillier et al., 2008). In many cases, mixed cultures show the Jameson effect: every species enters the stationary phase at approximately the same time, even when they would be able to reach higher concentrations in monoculture. Hence, the value of N_{max} observed in this study for *Enterobacteriaceae* may not be due to the carrying capacity of the media for this species. As shown in Figs. 1 and 4, both populations reach the stationary phase at approximately the same time. Hence, it is feasible that the observed stationary phase of *Enterobacteriaceae* is the result of the Jameson effect.

Another aspect of the model worthy of discussion is the impact of pH on the growth kinetics. Although the initial pH of the meat cuts was different, it converged to similar values before the end of the exponential growth phase in this study. Therefore, the effect of the pH on the

bacterial kinetics is unlikely to be due to the direct effect of this factor. Instead, the initial pH of the meat can be an indicator of other aspects that may influence microbial growth, such as the composition of the microbiome or the physical properties of the meat and substrate availability. Also in this line, in this empirical study significant differences in the growth kinetics of meat products with pH higher or lower than 5.8 have been identified (Fig. 8). However, it is unreasonable to assume that a change from pH 5.79 to pH 5.81 will have a dramatic effect in the growth kinetics, and that the effect of pH will be limited to this narrow pH range. Therefore, future scientific studies should focus on the effect of the initial pH of the meat on the microbial growth kinetics.

4.3. The limitations of the estimates of product shelf life

Shelf life estimates based on the growth kinetics of spoilage species are strongly dependent on the maximum microbial concentration marking the end of the product shelf life. Admittedly, there are still large knowledge gaps regarding these limits. The results of experimental studies focused on product spoilage can be affected by a variety of factors (e.g., bacterial strains, type of product, definition of spoilage) in a manner that is not well understood yet. This makes it hard to compare between studies and extrapolate their empirical results. Therefore, due to their relevance for the estimated shelf life, the association between microbial concentration and food spoilage should be a main aim of future scientific studies, as a way to reduce the uncertainty in shelf life estimation. In this sense, it is worth noting that the confidence intervals in Fig. 7 only include the uncertainty of the mean of the microbial concentration. As illustrated in Figs. 1 and 4, the inherent variability between individual replicates is large, and would introduce a relevant increase in the variance of the shelf life estimates. On top of that, a precise representation of uncertainty should account for the uncertainty in the microbial concentrations determining food spoilage.

When designing studies to associate microbial concentration and product spoilage, it is important to account for the fact that, unlike for food safety where a disease is directly linked to a microbial species (e.g., listeriosis and *Listeria monocytogenes*), food spoilage can be caused by different species (or combinations of them) whose predominance on food and contribution to sensory changes depend on extrinsic and intrinsic factors such as the storage temperature, meat pH, packaging atmosphere, microbial interactions, among others (Kaur et al., 2021; Zhang et al., 2015). Therefore, the fact that a microbial species is dominant in a population does not necessarily imply it is the one responsible for food spoilage; non-dominant species can also be relevant. As illustrated in this study, according to the fitted models, both LAB and *Enterobacteriaceae* would reach their critical concentration at approximately the same time. This makes it impossible to identify which of these species would actually be responsible for the spoilage of the product without additional, independent, experiments (or, if spoilage is the result of the combination of both species). Furthermore, according to the model prediction herein, for meat with low initial pH, the microbial species causing the spoilage of the product would depend on the storage temperature. This points out at the complexity of the association between the concentration of spoilage microorganisms and the spoilage of a food product, which is still to be unravelled.

Regardless of these limitations, the results of this study are of relevance for the beef industry, especially those operating in Brazil. The results of this study emphasise the impact of the initial pH of the meat on the product shelf life. However, no significant differences between the growth rates on meats with pH $>$ 6.1 and pH between 5.8 and 6.1 were observed. These results could point out the need to revise the categorization commonly used by meat industries. Moreover, the description of product spoilage based on the principles from predictive microbiology also needs the incorporation of variability and uncertainty (Den Besten et al., 2018; Koutsoumanis et al., 2021). Therefore, the results of this study could be part of future meta-regression models (Van Asselt and Zwietering, 2006), contributing to the implementation of more general models for the microbial response accounting for different sources of variability.

5. Conclusions

Predictive microbiology can assist in shelf life estimation of food products. Nevertheless, there are still challenges when it comes to model building, model validation and the association between maximum microbial counts and product spoilage. In this article, the shelf life of vacuum packed refrigerated meat was estimated based on mathematical models describing the growth of LAB and *Enterobacteriaceae*. The methodology for model building illustrated in this study (the use of cross-validation and the incorporation of uncertainty in model predictions) can be used as a reference in future studies. Furthermore, the conclusions of the model can be of use for the beef industry, as well as for the design of future studies; especially the fact that spoilage may not always be caused by the dominant microbial species.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110345.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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