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# A mathematical modeling approach to the quantification of lactic acid bacteria in vacuum-packaged samples of cooked meat: Combining the TaqMan-based quantitative PCR method with the plate-count method

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ABSTRACT

The TaqMan-based quantitative Polymerase Chain Reaction (qPCR) method and the Plate Count (PC) method are both used in combination with primary and secondary mathematical modeling, to describe the growth curves of *Leuconostoc mesenteroides* and *Weissella viridescens* in vacuum-packaged meat products during storage under different isothermal conditions. Vacuum-Packaged *Morcilla* (VPM), a typical cooked blood sausage, is used as a representative meat product, with the aim of improving shelf-life prediction methods for those sorts of meat products. The standard curves constructed by qPCR showed good linearity between the cycle threshold ($C_T$) and $\log_{10}$ CFU/g, demonstrating the high precision and the reproducible results of the qPCR method. The curves were used for the quantification of *L. mesenteroides* and *W. viridescens* in artificially inoculated VPM samples under isothermal storage (5, 8, 13 and 18 °C). Primally, both the qPCR and the PC methods were compared, and a linear regression analysis demonstrated a statistically significant linear correlation between the methods. Secondly, the Baranyi and Roberts model was fitted to the growth curve data to estimate the kinetic parameters of *L. mesenteroides* and *W. viridescens* under isothermal conditions, and secondary models were used to establish the dependence of the maximum specific growth rate on the temperature. The results proved that primary and secondary models were adequate for describing the growth curves of both methods in relation to both bacteria. In conclusion, the results of all the experiments proved that the qPCR method in combination with the PC method can be used to construct microbial growth kinetics and that primary and secondary mathematical modeling can be successfully applied to describe the growth of *L. mesenteroides* and *W. viridescens* in vacuum-packaged *morcilla* and, by extension, other cooked meat products with similar characteristics.

## 1. Introduction

Lactic acid bacteria (LAB) are considered a majority bacterial group in the spoilage of vacuum-packaged cooked meat products (Björkroth et al., 1998; Chenoll et al., 2007). LAB growth causes particular types of spoilage in some foods, such as a decrease in pH, sour odors, off-flavors, vacuum-loss, white exudate production, and slime production (Egan, 1983; Santos et al., 2003; Schillinger and Lücke, 1987), reducing the shelf-life of these products and, consequently, leading to food waste and significant economic losses for the food industry. Thus, the evaluation of the behavior of LAB during the shelf-life of vacuum-packaged cooked meat products is a relevant issue.

Blood sausages are popular cooked meat products known in many parts of the world as *morcilla* (Cachaldora et al., 2013), *prieta* (Gonzalez-Schnake and Nova, 2014), *morcela de arroz* (Pereira et al., 2015), and *sanganel* (Iacumin et al., 2017). This product was selected, because it is a typical cooked meat product with a complex matrix that makes it more susceptible to deterioration than other meat products. Moreover, vacuum-packaged *morcilla de Burgos* (VPM), produced in northern Spain, is a typical blood sausage that is widely studied, due to its microbiota and product spoilage characteristics (Diez et al., 2008a, 2009a, 2009b; Koort et al., 2006; Santos et al., 2005a). Its microbial population can include particular bacterial species (e.g. *Weissella cibaria*, *Leuconostoc lactis*, *Leuconostoc citreum* and *Lactobacillus sakei*), in

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which *Leuconostoc mesenteroides* and *Weissella viridescens* (formerly, *Lactobacillus viridescens*) (Collins et al., 1993) have been reported as the main LAB responsible for spoilage (Diez et al., 2008b, 2009c; Santos et al., 2005b). VPM was therefore selected for the shelf-life tests that measured the growth of *L. mesenteroides* and *W. viridescens*.

LAB were quantified during the storage of meat products with two culture-based techniques. Firstly, the Plate Count (PC) method, performed with de Man, Rogosa and Sharpe (MRS) agar (De Man et al., 1960), and subsequently identified by biochemical, morphological, and physiological tests, that are considerably time-consuming, labor-intensive, and may yield uncertain results (Chenoll et al., 2007). Secondly, molecular approaches, mainly quantitative Polymerase Chain Reaction (qPCR) based procedures were used, which can detect a specific bacterium in a food matrix. Recently, qPCR has been combined with culture-based methods for quantification of bacteria in foods (Ilha et al., 2016; Kim et al., 2017; Luedtke and Bosilevac, 2015; Papić et al., 2017). The main advantages of qPCR are the high specificity and selectivity, where the exponential amplification of target specific DNA is measured using dual-labeled fluorescence probes (TaqMan™ technology) (Laube et al., 2010). TaqMan-based qPCR method can obtain quantitative data through the construction of standard curves from serially diluted known-amount standards (Rodríguez-Lázaro and Hernández, 2013). Few studies have used standard curves for the detection and the quantification of LAB in meat products (Elizaquível et al., 2008; Gómez-Rojo et al., 2015; Martín et al., 2006). In the study reported by Elizaquível et al. (2008), a new qPCR procedure, developed for the specific detection and quantification of *L. mesenteroides* in meat products, targeted the 23S rRNA gene. Later on, Gómez-Rojo et al. (2015) developed a specific qPCR assay for the detection and quantification of *W. viridescens* in blood sausage.

Predictive microbiology is an important tool to describe the behavior of spoilage organisms and the progression of spoilage processes in foods (McMeekin and Ross, 1996). According to Whiting and Buchanan (1993), the traditional approach in predictive microbiology follows a two-step process: primary and secondary modeling. In the first step, primary models describe the microbial dynamics over time under constant environmental and culturing conditions (McKellar and Lu, 2004). Sigmoidal type models (e.g. Baranyi and Roberts model (Baranyi and Roberts, 1994)) are commonly used as a primary model for fitting microbial growth data, in order to determine the kinetic parameters, such as maximum specific growth rate, and lag phase duration (e.g., Dalcanton et al., 2013; Longhi et al., 2014; Menezes et al., 2018). In the second step, an appropriate secondary model is used to describe the effect of environmental changes (e.g. temperature) on the parameters of the primary models (Huang, 2017; Ross and Dalgaard, 2004).

Predictive models have been used to describe the growth of spoilage LAB in foods under different temperature conditions, which has a significant influence on the kinetics of microbial growth (Longhi et al., 2013; Tarlak et al., 2018), such as LAB natural microbiota in vacuum-packaged cooked sliced ham (Menezes et al., 2018) and vacuum-packaged raw beef (Li et al., 2013), *Lactobacillus plantarum* in vacuum-packaged cooked chopped pork (Dalcanton et al., 2013), *L. mesenteroides* in chicken breast, turkey breast and ham (Zurera-Cosano et al., 2005, 2006), and *W. viridescens* in commercial vacuum-packaged sliced ham (Silva et al., 2017; Longhi et al., 2018). However, there are no reports of the use of a qPCR method, performed at different growth phases and applying mathematical modeling, for the quantification of *L. mesenteroides* and *W. viridescens* in cooked meat products and, more particularly, in blood sausages. Therefore, the main purpose of this research is to advance mathematical modeling from molecular data for describing the growth of *L. mesenteroides* and *W. viridescens* in VPM in storage under constant temperature conditions. Additionally, a comparison between the primary and secondary modeling acquired by both the qPCR and the PC methods will be performed. The study will assess the performance of mathematically modeled descriptions of the microbial spoilage of VPM.

**Table 1**
Bacterial strains used in this study.

| Species | Strain | Origin |
|---|---|---|
| *Leuconostoc mesenteroides* (*LM*) | CECT 219[T] (ATCC 8293) | Fermenting olives |
| | 66[a] | Blood sausage |
| | $L_1$[b] | Blood sausage |
| | $L_8$[b] | Blood sausage |
| | $L_{35}$[b] | Blood sausage |
| *Weissella viridescens* (*WV*) | CECT 283[T] (ATCC 12706) | Cured meat products |
| | 132[a] | Blood sausage |
| | $W_1$[b] | Blood sausage |
| | $W_8$[b] | Blood sausage |
| | $W_{35}$[b] | Blood sausage |

[T] Type strain; CECT, Spanish Type Culture Collection or ATCC, American Type Culture Collection.
[a] Strains isolated and identified by Restriction Fragment Length Polymorphism (RFLP) in studies reported by Santos et al. (2005b).
[b] Strains isolated and identified by RFLP in studies reported by Diez et al. (2009a).

## 2. Material and methods

### 2.1. Bacterial strains

Ten different strains of two microbial species, *L. mesenteroides* (*LM*) and *W. viridescens* (*WV*), were applied in this study, as listed in Table 1. The use of a strains cocktail provided more realistic results (closer to natural microbiota), as the strains presented different physiological conditions. To do so, two strains of reference cultures were supplied by the Spanish Type Culture Collection (CECT) (Valencia, Spain) and eight strains of food isolates were collected from VPM (Diez et al., 2009a; Santos et al., 2005b). The strains identified by *L* and W (Table 1) were isolated at different sausage storage times (e.g. $W_8$ and $L_8$ strains were isolated after eight days of storage at 4 °C). Thus, we collected a cocktail of eight native strains that appeared in the product in different batches, undergoing different phases of deterioration, so that there is a greater representation of spoilage. Suspensions of the ten strains were individually maintained frozen at −80 °C in MRS (De Man et al., 1960) broth (Sigma-Aldrich™) supplemented with 30% glycerol.

### 2.2. Inoculum preparation

Preparation of the inoculum involved frozen suspensions of each strain that were thawed, with 100 μL and transferred to tubes containing 10 mL of MRS broth. They were then incubated at 30 °C for 24 h and subcultured on the following day. The subculture was grown for 18 h until a stationary growth phase ensued (see previous results). Two cocktails, one containing all five strains of *LM* and one containing all five strains of *WV* were prepared by mixing 2 mL of each strain to obtain two inoculum sizes of $10^9$ CFU/mL.

### 2.3. Artificially inoculated VPM sample preparation

Sliced *morcilla* samples (approximately 20 g) were received from a Burgos manufacturer on the day of production and stored at 4 °C. Then, individual samples were vacuum-packaged and pasteurized in a thermostatic bath with horizontal stirring (Unitronic 320 DR, JP Selecta, Spain) at 75 °C for 10 min, to eliminate the natural microbiota of the product (Diez et al., 2009a, 2009b). After the pasteurization treatment, each sample was opened and artificially inoculated with 200 μL of inoculum containing approximately $10^5$ CFU/mL of either *LM* or *WV* (in duplicate) with the aim of reaching an approximate concentration of $1 \times 10^3$ CFU/g. Then, the samples were vacuum-packaged again and sterile water (200 μL) was added to the non-inoculated control samples

(negative control), in duplicate.

### 2.4. Storage conditions and growth measurements

Artificially inoculated VPM samples were isothermally incubated at 5, 8, 13, and 18 °C. The four different temperatures were tested to reflect the different storage conditions of refrigerated foods. The temperatures of 5 and 8 °C were chosen, based on Breen et al. (2006) and Hassan et al. (2014), respectively, who considered that they represented the average temperatures of domestic refrigerators. The moderate temperature abuse of 13 °C was chosen based on Johnson et al. (1998). Gilbert et al. (2007) and Brennan et al. (2013) considered that 18 °C was the maximum temperature of domestic refrigerators (serious abuse of temperature). The temperature around the samples was recorded on a EBI 20-TH1 data logger (Ebro, Germany). Microbial growth was measured in duplicate with both the qPCR and the PC method until the stationary growth phase.

### 2.5. Quantification of LM and WV

Artificially inoculated VPM samples were analyzed throughout the storage period at each experimental temperature. Twenty grams of product were placed into a sterile plastic bag with a filter. Ringer's solution (180 mL) was added to each bag and the samples were homogenized for 2 min with a Stomacher (Smasher™ Lab Blender, Weber Scientific, USA). Further steps after this initial suspension were undertaken as follows.

### 2.6. Quantification by the PC method

Decimal dilutions of Ringer's solution were prepared for microbiological analysis and 100 μL aliquots of the appropriate dilutions were plated on MRS Agar in duplicate at 30 °C for 48 h, using the method specified by the International Organization for Standardization – ISO 15214:1998 (Anonymous, 1998). Non-inoculated control samples were analyzed throughout the experiments. An average CFU/g (and deviation) of two independent samples of each growth curve point was calculated and used to determine the growth kinetics. The initial count (day 0) of strains in VPM was determined by PC on MRS after inoculation.

### 2.7. Quantification by the qPCR method

#### 2.7.1. Genomic DNA extraction

DNA from artificially inoculated VPM was extracted in duplicate using cell suspension of 40 mL of homogenate, which was centrifuged (Centrifuge 5810R, Eppendorf) at 300 ×*g* for 10 min (4 °C). The cells were subsequently collected by centrifugation at 5000 ×*g* for 15 min (4 °C) and their genomic DNA was isolated using the GenElute™ Kit (Bacterial Genomic DNA Kit) (Sigma-Aldrich, Saint Louis, USA) as per the manufacturer's recommendations for Gram-positive bacteria. The DNA extracted was resuspended in 100 μL of the recommended buffer and spectrophotometrically quantified (BioTek™ Epoch).

#### 2.7.2. Amplification conditions

The qPCR reactions were performed in MicroAMP® Optical Eight-tube Strips (Applied Biosystems, Foster City, CA, USA) using the StepOne™ Real-Time PCR detection system (Applied Biosystems).

Primers and TaqMan probes were used, as described by Elizaquível et al. (2008) and Gómez-Rojo et al. (2015), to identify the presence of both *LM* and *WV* in VPM by qPCR and for their quantification (Table 2). Amplification reactions were performed in duplicate in a final volume of 25 μL containing 12.5 μL of 1 × FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany), the primer pair, the TaqMan probe, *nuclease-free water,* and 2 μL of template DNA. The amplification reactions of *LM* were: 10 min at 95 °C, followed by 45 cycles for 15 s at 95 °C, 1 min at 60 °C and a final extension for 5 min at 72 °C. The amplification reactions of *WV* were: 5 min at 95 °C, followed by 45 cycles for 1 min at 95 °C, 30 s at 64 °C, 30 s at 72 °C, and a final extension for 10 min at 72 °C.

#### 2.7.3. Standard curves for VPM

Standard curves were calculated for separate quantification of *LM* and *WV* in VPM. Artificially inoculated samples were prepared as follows: 40 g of *morcilla* were aseptically cut into slices and vacuum-packaged in an EVT-7CD packaging machine (Tecnotrip, Tarrasa, Spain). The packages containing the VPM samples were pasteurized (described in Section 2.3), to eradicate all contaminating microorganisms; shown in previous studies as an effective treatment for that purpose (Diez et al., 2009a, 2009b). After the pasteurization treatment, the samples were homogenized in 360 mL of Ringer's solution in a sterile plastic bag with a filter (Interscience, BagPage®, France) for 2 min with a Stomacher (Smasher™ Lab Blender, Weber Scientific, USA). The resulting mixture was taken from the filter side and distributed in aliquots of 40 mL that were inoculated with 10-fold serial dilutions of the inoculum of *LM* (corresponding to a final concentration $1.3 \times 10^2$ to $1.3 \times 10^8$ CFU per gram) or *WV* (corresponding to a final concentration from $4.3 \times 10^1$ to $4.3 \times 10^8$ CFU per gram).

Assays were performed in two independent experiments, calculating the mean Cycle Threshold ($C_T$) and Standard Deviation (SD) for each point of the curve in six qPCR runs. Standard curves were constructed by plotting mean $C_T$ values against $\log_{10}$ CFU of *LM* or *WV* per gram of artificially inoculated VPM. In addition, *nuclease-free water and a* non-inoculated portion of VPM samples were analyzed as a negative control in duplicate by qPCR. Only data with a coefficient of variation (CV) < 33% were considered (Žel et al., 2012), for the calculation of standard curve equation. Amplification efficiencies were determined using Eq. (1), where *E* is the amplification efficiency (e.g. 100% amplification efficiency has $E = 1$ (Bustin et al., 2009; Rutledge and Côté, 2003)). Limit of detection (LOD) and limit of quantification (LOQ) were determined as described by Papić et al. (2017).

$$E = 10^{-(1/\text{slope})} - 1 \quad (1)$$

#### 2.7.4. qPCR method applied to artificially inoculated VPM

Total DNA was isolated from 1 mL of each initial suspension, as described in Section 2.5. The qPCR method was then performed with the extracted DNA as the template (see above Section 2.7.1). Separate bacterial counts (CFU/g) of *LM* and *WV* in VPM during storage, estimated by qPCR, were determined using the equation proposed by Ilha et al. (2016) (Eq. (2)), where *A* is the CFU per reaction well, obtained from the $C_T$ of the DNA sample using the standard curve ($C_T$ versus $\log_{10}$ CFU); *B* is the extracted DNA concentration (ng/μL); *C* is the total volume of extracted DNA (μL); *D* is the template DNA mass in the reaction well (ng); and, *E* is the sausage mass (g) used for DNA extraction.

$$\text{Bacterial count (CFU/g)} = \frac{(A)(B)(C)}{(D)(E)} \quad (2)$$

### 2.8. Statistical analysis for the comparison between the qPCR and the PC methods

Microbial count data were logarithmically transformed ($\log_{10}$) and linear regression trend lines (Passing and Bablok, 1983) were evaluated for the results of the qPCR and PC using MedCalc v17.1 (MedCalc Software, Belgium). Coefficients of determination ($R^2$) of the linear regressions were obtained. Limits of agreement of the different quantification methods were evaluated using GraphPad Prism 7.03 (GraphPad Prism® Software, USA) according to the statistical approaches proposed by Bland and Altman (1986). *p-Values* ≤ 0.05 were considered statistically significant.

**Table 2**
Oligonucleotide primers used in the qPCR amplifications.

| Species | Gene | Primer | Sequence (5′ → 3′) | Position | Amplicon size (pb) | Reference |
|---|---|---|---|---|---|---|
| *LM* | *23S rRNA* | LcmesS | CCA GTT GTA ATG CGT TAT TAC C | 1956 | 130 | Elizaquível et al. (2008) |
| | | LcmesA | CAC AGC TTG TCC TTA TAG AAA A | 2082 | | |
| | | TaqMan probe | FAM-TTCACTCTTTTCAAGACTTACTG-MGB | 2031 | | |
| *WV* | *RecN* | WvrecNF | CGC AAA CAC AAC AAG CCT AT | 908 | 91 | Gómez-Rojo et al. (2015) |
| | | WvrecNR | TGT TGA GCA AGT TCC AAA GC | 998 | | |
| | | TaqMan probe | FAM-CCGTGCCCTGACGTTAGCCA-BHQ1 | 930 | | |

### 2.9. Mathematical modeling

#### 2.9.1. Primary models

The Baranyi and Roberts (BAR) primary model was fitted to the growth data of both *LM* and *WV* in VPM obtained by qPCR and PC under isothermal conditions. The BAR model is one of the most biologically-based growth models used in the literature and has previously been used by various authors (e.g. Gospavic et al. (2008), Lytou et al. (2016) and Tremarin et al. (2015)). The BAR model (Baranyi and Roberts, 1994) at constant environmental conditions is given by Eqs. (3), (4), and (5), where $F(t)$ is the adjustment function, $y(t)$ is the logarithm of the microbial concentration [$y = \log_{10}(N(\mathrm{CFU/g}))$] at time $t$ (h), $y_0$ is the logarithm of initial microbial concentration, $y_{max}$ is the logarithm of maximum population, $\mu_{max}$ is the maximum specific growth rate (1/h), $\lambda$ is the lag phase duration (h), and $h_0$ is the parameter related to the physiological state of the cells (dimensionless).

$$y(t) = y_0 + \mu_{max}F(t) - \ln\left\{1 + \frac{e^{\mu_{max}F(t)} - 1}{e^{y_{max}-y_0}}\right\} \quad (3)$$

$$F(t) = t + \left(\frac{1}{\mu_{max}}\right)\ln[e^{(-\mu_{max}t)} + e^{(-h_0)} - e^{(-\mu_{max}t-h_0)}] \quad (4)$$

$$h_0 = \mu_{max}\lambda \quad (5)$$

#### 2.9.2. Secondary models

The Exponential and Ratkowsky square root (Ratkowsky et al., 1982) secondary models (Eqs. (6) and (7), respectively) were used to describe the effect of the temperature on the maximum specific growth rate ($\mu_{max}$), where $a$ (1/h), $b$ (1/°C) and $c$ (1/(°C √h)) are empirical parameters, $T$ is the storage temperature (°C), and $T_{min}$ is the theoretical minimum growth temperature (°C).

$$\mu_{max} = ae^{(bT)} \quad (6)$$

$$\sqrt{\mu_{max}} = c(T - T_{min}) \quad (7)$$

#### 2.9.3. Numerical analyses

The fitting procedure of the primary and secondary models was performed in Matlab R2013a (MathWorks®, Natick, USA). The parametric estimation for the BAR model was performed in two-steps modeling (TSM), as proposed by Amézquita et al. (2005), Baranyi et al. (1995) and Menezes et al. (2018).

#### 2.9.4. Assessment of model performance

Model performance was evaluated with the Adjusted Coefficient of Determination ($R^2_{adj}$), the Root-Mean-Square-Error (RMSE, $\log_{10}$ CFU/g), the Bias factor ($B_f$) and the Accuracy factor ($A_f$), calculated by Eqs. (8) to (11), respectively, where $n$ is the number of observations, $p$ is the number of model parameters, $y_{obs}$, $y_{pred}$, and $\bar{y}$ are the observed values, predicted values, and average values, respectively. According to Ross (1996), the $B_f$ value is a measure of average variation between the observed and predicted values, and the $A_f$ value measures the average difference between the observed and the predicted values. A value of $A_f = 1$, $B_f = 1$, $R^2_{adj} = 1$, and $RMSE = 0$ indicates that there is a perfect agreement between all the observed and predicted values.

$$R^2_{adj} = 1 - \left[\frac{n-1}{n-(p+1)}\right]\left\{1 - \left[\frac{\sum_{i=1}^{n}(y_{pred} - y_{obs})^2}{\sum_{i=1}^{n}(y_{obs} - \bar{y})^2}\right]\right\} \quad (8)$$

$$RMSE = \sqrt{\frac{\sum(y_{pred} - y_{obs})^2}{n-p}} \quad (9)$$

$$B_f = 10^{\left[\frac{\sum\log(y_{pred}/y_{obs})}{n}\right]} \quad (10)$$

$$A_f = 10^{\left[\frac{\sum|\log(y_{pred}/y_{obs})|}{n}\right]} \quad (11)$$

## 3. Results and discussion

### 3.1. qPCR standard curves for LM and WV in VPM

Representative amplification plots corresponding to standard curves of *WV* and *LM* are shown in Figs. 1a and 2a, respectively. The standard curves of *WV* and *LM* are graphically represented in Figs. 1b and 2b, respectively, as a regression curve (with the linear equation and coefficient of determination) in the plot of mean $C_T$ values against $\log_{10}$ CFU. The non-inoculated control recorded an absence of LAB colonies on MRS after 48 h of incubation, moreover no amplification signal was observed. The amplification efficiencies ($E$), calculated using cell cocktail suspensions as a template, were 101% and 93% for *LM* and *WV*, respectively. All the standard curves showed a good linearity between $\log_{10}$ CFU/g and $C_T$ with a $R^2$ value of 0.99 (Figs. 1b and 2b). The relevant coefficients of variation (CV < 33%) (from $C_T$ values of two independent experiments in six reactions, presented in Table 3), ranging from 0.58 to 6.55%, demonstrated the high precision and reproducible results of the qPCR method. Based on the information described above, those results confirm the reliability of the qPCR method for the quantification of both *LM* and *WV* in VPM.

The LOD were established in 9.4 CFU/reaction ($1.3 \times 10^2$ CFU/g) and 3.1 CFU/reaction ($4.3 \times 10^1$ CFU/g) of *LM* and *WV*, respectively (Table 3), considering the volume of the elution buffer. According to Berdal and Holst-Jensen (2001), LOD is set 5 to 10-fold lower than LOQ in complex samples. Thus, LOQ were established at $1.3 \times 10^3$ CFU/g and $4.3 \times 10^2$ CFU/g of *LM* and *WV*, respectively. Consequently, the following detection ranges in VPM were established: $1.3 \times 10^2$ to the maximum tested level of $1.3 \times 10^8$ CFU/g, and, $4.3 \times 10^1$ to the maximum tested level of $4.3 \times 10^8$ CFU/g, for *LM* and for *WV*, respectively (Table 3). Compared with qPCR developed for pure culture, the sensitivity was greater than the sensitivity levels reported for LAB quantification in meat products, e.g. *Lb. sakei* ($3 \times 10^3$ CFU/g) (Martín et al., 2006), *LM* ($1.8 \times 10^4$ CFU/g) (Elizaquível et al., 2008), and *WV* ($10^3$ CFU/g) (Gómez-Rojo et al., 2015). Thus, it is an applicable tool for the detection and quantification of *LM* and *WV* present in cooked meat products. Nevertheless, the question arises whether those standard curves can be used for LAB quantification in VPM at different growth phases (lag, exponential and stationary) and different storage temperatures. The qPCR and the PC methods were therefore both evaluated

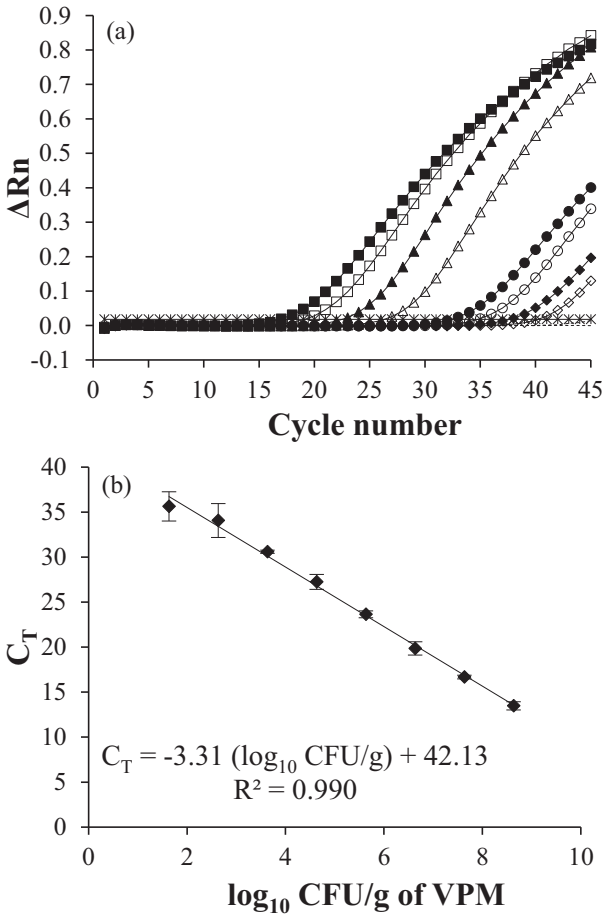


**Fig. 1.** Standard curve obtained for a cocktail of *Weissella viridescens* (*WV*) in VPM. Representative amplification plots. (a) Increase in fluorescence (ΔRn) with respect to the number of qPCR cycles for serial dilutions of a cocktail of *WV* corresponding to $4.3 \times 10^8$ (-■-), $4.3 \times 10^7$ (-□-), $4.3 \times 10^6$ (-▲-), $4.3 \times 10^5$ (-Δ-), $4.3 \times 10^4$ (-●-), $4.3 \times 10^3$ (-○-), $4.3 \times 10^2$ (-♦-), $4.3 \times 10^1$ (-◊-) CFU/g, non-inoculated control sample (–) and threshold (-x-). (b) Standard curve constructed by plotting the mean $C_T$ values for six qPCR runs for two independent amplification against the logarithm of the cell concentration in CFU (determined by PC) of *WV*. The error bars show the standard deviation.

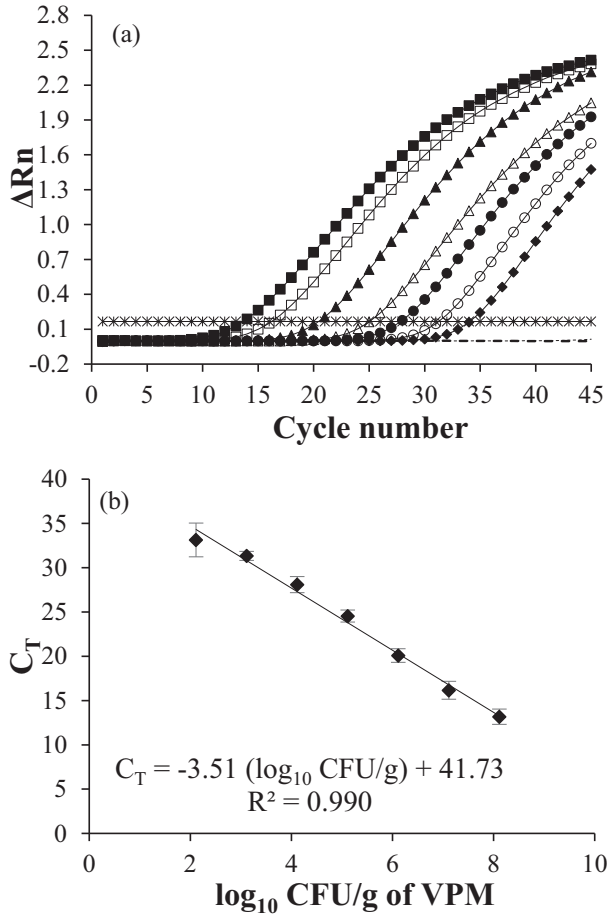


**Fig. 2.** Standard curve obtained for a cocktail of *Leuconostoc mesenteroides* (*LM*) in VPM. Representative amplification plots. (a) Increase in fluorescence (ΔRn) with respect to the number of qPCR cycles for serial dilutions of a cocktail of *LM* corresponding to $1.3 \times 10^8$ (-■-), $1.3 \times 10^7$ (-□-), $1.3 \times 10^6$ (-▲-), $1.3 \times 10^5$ (-Δ-), $1.3 \times 10^4$ (-●-), $1.3 \times 10^3$ (-○-), $1.3 \times 10^2$ (-♦-) CFU/g, non-inoculated control sample (–) and threshold (-x-). (b) Standard curve constructed by plotting the mean $C_T$ values for six qPCR runs for two independent amplification against the logarithm of the cell concentration in CFU (determined by PC) of *LM*. The error bars show the standard deviation.

to quantify *LM* and *WV* in artificially inoculated VPM samples, held in storage under isothermal conditions until the stationary growth phase. Secondly, the BAR model was fitted to the growth curve data, to estimate the kinetic parameters of both *LM* and *WV* under isothermal conditions, and secondary models were used to establish the dependence of the maximum specific growth rate on the temperature.

### 3.2. Comparison of growth data from the qPCR and the PC methods

The *LM* and the *WV* cells numbered by qPCR using the standard curves (Figs. 1b and 2b) were compared with the cells numbered by PC on MRS plates in VPM stored at 5, 8, 13, and 18 °C until the stationary growth phase (average values). Linear regression analysis demonstrated a statistically significant linear correlation ($R^2$ values of 0.945 and 0.907 for *LM* and *WV*, respectively; $p < 0.0001$; Fig. 3a and b). Furthermore, Bland–Altman plots were constructed, to investigate whether there was any agreement between the results of both methods (Fig. 4a and b). The statistical analysis revealed that the most samples were inside the 95% confidence interval (CI) limits ( ± 1.96 SD); however, PC gave a higher value mean bias (0.73 $\log_{10}$ CFU for *LM* and 0.59 $\log_{10}$ CFU for *WV*) than the qPCR method. The agreement in results obtained by both methods for artificially inoculated VPM samples was suitable, although the qPCR slightly underestimated the $\log_{10}$ CFU values by PC. This observation has previously been described (Krämer et al., 2011). According to Reichert-Schwillinsky et al. (2009), qPCR quantification standards related to numbers of CFU determined in the culture used for DNA isolation might add a bias to the analysis, depending on the physiological state of the bacterial population present in the culture. LAB physiology will be influenced under stress conditions such as high acidity caused by lactic acid buildup (Even et al., 2002), leading to lower DNA replication in the cells (Grattepanche et al., 2005). However, cells can return to a stress-free physiological state during sample preparation steps for plate counting, thereby leaving the decrease in bacterial counts on agar plates undetected (Grattepanche et al., 2005), which is conducive to a higher discrepancy between culture-based and culture-independent methods. Moreover, the qPCR underestimation could result from incomplete DNA extraction, as described by Achilleos and Berthier (2013), or less efficient amplification of the DNA that was extracted, regardless of the presence of an inhibitor in the sample matrix, as earlier proposed by Kemp et al. (2014), although in our case the extraction method was optimal for DNA extraction from *morcilla* (Gómez-Rojo et al., 2015).

Achilleos and Berthier (2017) studied the quantification of starter strains by growth phase during cheese making. During the exponential

**Table 3**
Quantification results obtained from the qPCR method in artificially inoculated VPM with a cocktail of *Weissella viridescens* (*WV*) and *Leuconostoc mesenteroides* (*LM*).

| CFU/g in VPM[a] | CFU/reaction | $C_T$ (average ± SD)[b] | CV (%)[c] |
|---|---|---|---|
| | *Weissella viridescens* (*WV*) | | |
| $4.3 \times 10^8$ | $3.1 \times 10^7$ | 13.5 ± 0.5 | 3.38 |
| $4.3 \times 10^7$ | $3.1 \times 10^6$ | 16.7 ± 0.2 | 1.23 |
| $4.3 \times 10^6$ | $3.1 \times 10^5$ | 19.9 ± 0.7 | 3.72 |
| $4.3 \times 10^5$ | $3.1 \times 10^4$ | 23.6 ± 0.4 | 1.62 |
| $4.3 \times 10^4$ | $3.1 \times 10^3$ | 27.2 ± 0.8 | 3.04 |
| $4.3 \times 10^3$ | $3.1 \times 10^2$ | 30.6 ± 0.2 | 0.58 |
| $4.3 \times 10^2$ | 31 | 34.1 ± 1.9 | 5.53 |
| $4.3 \times 10^1$ | 3.1 | 35.6 ± 1.6 | 4.56 |
| CFU/g in VPM[a] | CFU/reaction | $C_T$ (average ± SD)[b] | CV (%)[c] |
| | *Leuconostoc mesenteroides* (*LM*) | | |
| $1.3 \times 10^8$ | $9.4 \times 10^6$ | 13.2 ± 0.9 | 6.55 |
| $1.3 \times 10^7$ | $9.4 \times 10^5$ | 16.2 ± 1.0 | 6.25 |
| $1.3 \times 10^6$ | $9.4 \times 10^4$ | 20.1 ± 0.8 | 3.83 |
| $1.3 \times 10^5$ | $9.4 \times 10^3$ | 24.5 ± 0.7 | 2.76 |
| $1.3 \times 10^4$ | $9.4 \times 10^2$ | 28.1 ± 0.9 | 3.23 |
| $1.3 \times 10^3$ | 94 | 31.3 ± 0.5 | 1.62 |
| $1.3 \times 10^2$ | 9.4 | 33.1 ± 1.9 | 5.75 |

[a] Obtained from PC method.
[b] Mean $C_T$ values obtained for six qPCR runs from two independent amplification experiments. SD, standard deviation.
[c] CV: coefficient of variation.

and stationary phases, they observed that the data obtained with the qPCR method were lower than those obtained with the PC method. The aforementioned authors considered that the underestimation was due to biological differences between cells, because both methods are based on highly different principles. Nevertheless, no rule-of thumb has been reported in the literature to explain the good levels of agreement between both methods.

### 3.3. Primary modeling of LM and WV in VPM using the qPCR and the PC methods

The growth curves of a cocktail of five *WV* and five *LM* strains in VPM at 5, 8, 13, and 18 °C were obtained by qPCR and PC methods, as shown in Fig. 5a and b, respectively. The BAR model was separately fitted to the two experimental datasets of *LM* and *WV* growth in VPM and the model parameters were estimated in two steps modeling (TSM) (Amézquita et al., 2005; Baranyi et al., 1995; Menezes et al., 2018). In the first step, the average value ( ± SD) for the $h_0$ parameter was calculated, resulting in $h_0$ = 0.66 ( ± 0.37) (PC method) and $h_0$ = 1.91 ( ± 1.24) (qPCR method) for *LM* and $h_0$ = 0.14 ( ± 0.15) (PC method) and $h_0$ = 1.81 ( ± 1.72) (qPCR method) for *WV*. According to Baranyi et al. (1995), the $h_0$ values depended on the physiological state of the cells and should be the same at all temperatures (considering the same bacterial inoculum and food). In the second step, the BAR model (with a fixed $h_0$ parameter value) was once again separately fitted to the two experimental datasets of *LM* and *WV* growth in VPM and three model parameters (λ, $\mu_{max}$ and $y_{max}$) were estimated. In the BAR model, λ is related to the $h_0$ parameter (Eq. (5)). All the growth data for *LM* and *WV* showed increasing trends with sigmoid growth curves (exhibited lag, exponential and stationary phases) (Fig. 5a and b). Specifically, when incubated at 5, 8, 13, and 18 °C, the BAR model was appropriate for describing the growth of the cocktail of *LM* and *WV* inoculated in VPM samples for both methods (PC and qPCR). The cocktail strains used to develop the predictive models can increase confidence in the ability of the model to predict with accuracy throughout the range of environmental scenarios (De Blackburn, 2006).

The initial counts of the inoculated samples were between 3.11 and 4.18 $\log_{10}$ CFU/g for *LM* and between 2.90 and 3.67 $\log_{10}$ CFU/g for *WV*, considering all the growth data enumerated by both methods. As predicted, the temperature had a strong impact on the bacterial behavior. In this study, the temperature in the incubator did not vary by more than ± 0.3 °C. The stationary phase was reached after almost 3, 7, 15, and 20 days at 18, 13, 8, and 5 °C, respectively, and the maximum bacterial count in all cases was over 9 $\log_{10}$ CFU/g independent of storage conditions (Fig. 5a and b). The maximum specific growth rate ($\mu_{max}$ (1/h)) increased with the temperature and varied between 0.031 1/h and 0.191 1/h for *LM* and between 0.031 1/h and 0.305 1/h for *WV*, and the time lag, λ, decreased from 62.00 h at 5 °C to 3.74 h at 18 °C for *LM*, and, from 39.88 h at 5 °C to 0.60 h at 18 °C for *WV* (Table 4). The $\mu_{max}$ and λ values were similar with those published for *WV* in cultivation broth at temperatures range from 4 °C to 30 °C (Martins et al., 2016), and for *LM* in a culture medium under anaerobic conditions at a temperature range between 10.5 °C and 17.5 °C (Zurera-Cosano et al., 2006). These results demonstrated that VPM is a rich nutrient for LAB growth and that meat products should be stored at lower temperatures, to prevent those bacteria from reaching higher concentrations.

The VPM shelf-life was defined as the time taken to reach $10^7$ CFU/g, a criterion described by several authors for different meat products, as well as in VPM (Diez et al., 2008a, 2009c; Irkin et al., 2011; Slongo et al., 2009; Vermeiren et al., 2005). In this study, VPM samples reached the shelf-life at 6.4 and 5.4 days when artificially inoculated

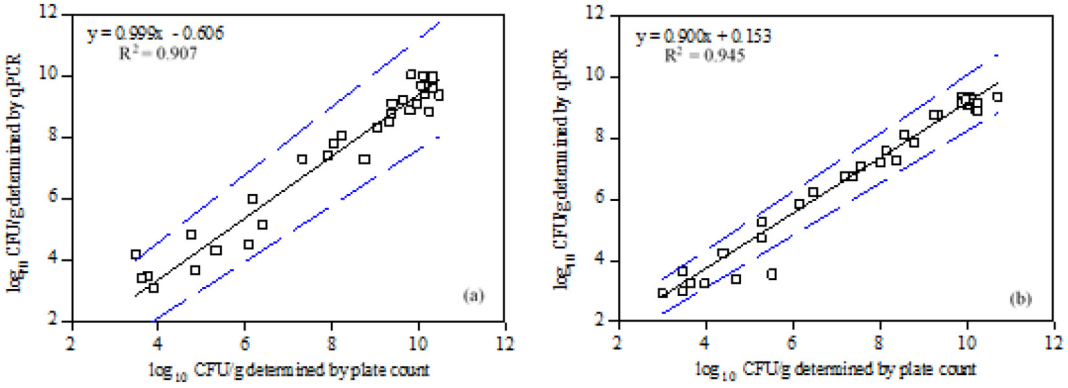


**Fig. 3.** Linear regression analysis of the $\log_{10}$ CFU/g values observed by plate count (PC) and qPCR for quantification of (a) *Weissella viridescens* (*WV*) and (b) *Leuconostoc mesenteroides* (*LM*) and in artificially contaminated VPM samples: linear trend line equation (thick continuous line). Thin blue dotted lines in all two graphs represent confidence interval. $R^2$, coefficients of determination. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

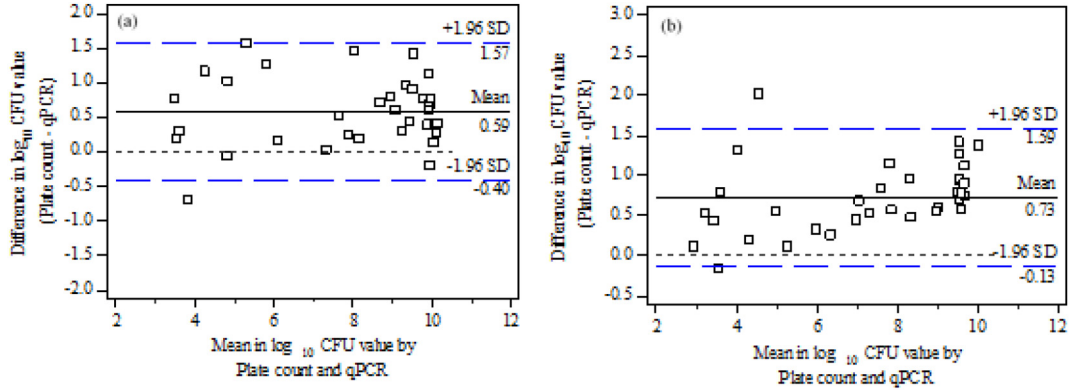


**Fig. 4.** Evaluation of the agreement between plate count PC and qPCR for quantification of *Weissella viridescens* (*WV*) and *Leuconostoc mesenteroides* (*LM*) in artificially contaminated VPM samples using Bland–Altman analysis. (a) Agreement between PC and qPCR for quantification of *WV*. Mean bias 0.59 ( ± 1.96 SD from −0.40 to 1.57 $\log_{10}$). (b) Agreement between PC and qPCR for quantification of *LM*. Mean bias 0.73 ( ± 1.96 SD from −0.13 to 1.59 $\log_{10}$). Line representing zero log difference is shown as a thin dotted line, whereas standard deviations of mean log difference as thin blue dotted lines. SD, standard deviation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

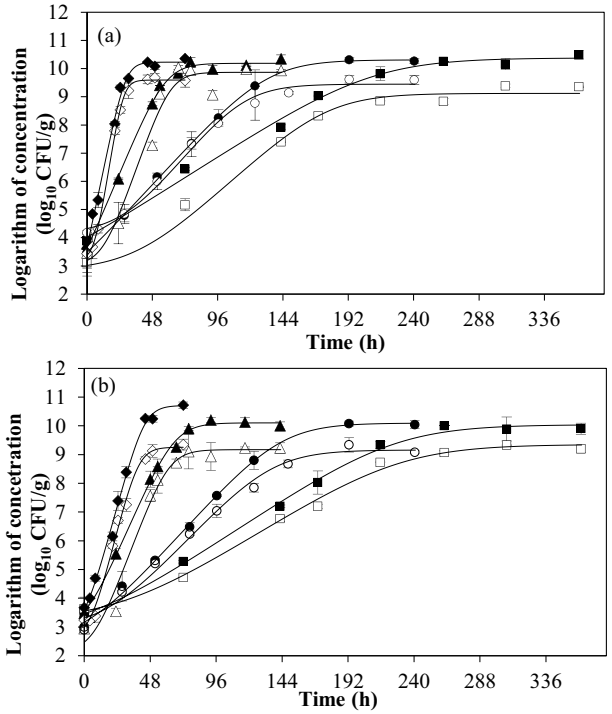


**Fig. 5.** Growth curves of (a) *Weissella viridescens* (*WV*) and (b) *Leuconostoc mesenteroides* (*LM*) at 5 °C (square symbols), 8 °C (circle symbols), 13 °C (triangle symbols) and 18 °C (lozenge symbols) quantified by plate count (PC, filled symbols) and qPCR (unfilled symbols) methods, and the fitting of Baranyi and Roberts model (BAR, continuous lines) to the experimental data obtained in VPM. The error bars shows the standard deviation.

with *LM* and *WV*, respectively, under storage at 5 °C (Table 4). During that period, obvious signs of product spoilage became observable in all samples, such as discoloration, vacuum-loss and sour smell. According to Kalschne et al. (2015), those are the most important changes provided by LAB that affect the purchase decision of a consumer, because of their negative effect on product appearance. Moreover, it should be remembered that temperature variations between 5 °C and 8 °C reduce the product shelf-life from 5.4 to 3.1 days (Table 4). Thus, the maintenance of the cold chain of meat and meat products is important, as temperature abuses result in variations of product quality during distribution and may cause spoilage before the expiration date is reached, leading to food waste and economic loss (Bruckner et al., 2012; Mack et al., 2014). In the study reported by Diez et al. (2009a), the observed shelf-life of VPM was 4 days when artificially inoculated with *LM* and *WV* under storage at 4 °C, due logically to the initial counts (6 $\log_{10}$ CFU/g). According to Santos et al. (2005a), the shelf-life of VPM depended on initial contamination levels and storage conditions. It must be stressed that the sliced VPM used in this study is more susceptible to spoilage and can facilitate more rapid growth of spoilage LAB than unsliced VPM due to the slicing, promotes an increased area of contact, and the direct contact of the inoculated strains with the *morcilla* ingredients, such as blood and condiments. Little et al. (2009) investigated the prevalence of *Listeria monocytogenes* in various ready-to-eat foods and found the highest contamination rates in sliced meats. Besides, the cultures inoculated in the VPM were in optimum growing conditions (inoculum subcultured twice). Under optimum conditions, bacteria are capable of multiplying indefinitely at a very rapid rate, so that their numbers may double every 20 min or so (Vollum et al., 1970). Vermeiren et al. (2005) studied the in vitro and in situ growth characteristics and behavior of spoilage organisms associated with anaerobically stored cooked meat products. These authors reported that the strain *L. mesenteroides* subsp. *mesenteroides*, when subcultured twice (24 h, 30 °C) in culture broth and inoculated on the model cooked ham, obtained excellent growth in both experiments.

### 3.4. Primary model statistical evaluation of LM and WV in VPM

Statistical indexes values ($R^2_{adj}$, RMSE, $A_f$ and $B_f$) obtained by fitting the BAR model to the experimental data obtained by both the qPCR and the PC methods at each temperature are shown in Table 5. The average $R^2_{adj}$ values ( ± SD) were 0.990 ( ± 0.009) and 0.988 ( ± 0.015), while the RMSE values were ≤0.423 and ≤0.553 $\log_{10}$ CFU/g for *LM* and *WV*, respectively, for both methods. Menezes et al. (2018) studied the growth of LAB in vacuum-packed sliced ham with and without oregano essential oil at different temperatures, finding average $R^2$ values of between 0.941 ( ± 0.035) and 0.979 ( ± 0.014) for the BAR model. Slongo et al. (2009) obtained the average $R^2$ value of 0.800 evaluating the growth of LAB in pressurized hams. Feng et al. (2014) found RMSE values ranging from 0.3 to 0.4 $\log_{10}$ CFU/g for vacuum-packaged Irish cooked sausages based on LAB growth. Acceptable $R^2_{adj}$ and RMSE values were therefore found, proving that the BAR model showed an acceptable goodness-of-fit with the data from the observations.

Model performance for the qPCR and for the PC methods was evaluated with two factors: bias ($B_f$) and accuracy ($A_f$). As shown in Table 5, the values of both $A_f$ and $B_f$ were close to 1.0, within an acceptable range and meeting the requirements defined by Ross (1996). For inoculated VPM samples with *LM*, the average $A_f$ values were 1.037

**Table 4**
Growth parameters ( ± 95% confidence interval) estimated by fitting of Baranyi and Roberts (BAR) model (second step, fixed $h_0$) to the experimental data of *Weissella viridescens* (*WV*) and *Leuconostoc mesenteroides* (*LM*) in artificially inoculated VPM at 5, 8, 13 and 18 °C obtained by qPCR and plate count (PC) methods.

| Method | T (°C) | $\mu_{max}$ (1/h) | λ (h) | $y_{max}$ ($\log_{10}$ CFU/g) | Shelf-life (days)[a] |
|---|---|---|---|---|---|
| | | *Weissella viridescens* (*WV*) | | | |
| PC | 5 | 0.031 ( ± 0.005) | 4.55 ( ± 0.59) | 10.38 ( ± 0.37) | 4.2 |
| | 8 | 0.053 ( ± 0.002) | 2.69 ( ± 0.09) | 10.31 ( ± 0.11) | 2.9 |
| | 13 | 0.117 ( ± 0.014) | 1.22 ( ± 0.13) | 10.19 ( ± 0.25) | 1.3 |
| | 18 | 0.236 ( ± 0.029) | 0.60 ( ± 0.07) | 10.23 ( ± 0.31) | 0.6 |
| qPCR | 5 | 0.045 ( ± 0.008) | 39.88 ( ± 7.92) | 9.12 ( ± 0.42) | 5.4 |
| | 8 | 0.061 ( ± 0.007) | 29.63 ( ± 3.66) | 9.45 ( ± 0.29) | 3.1 |
| | 13 | 0.140 ( ± 0.035) | 12.92 ( ± 4.26) | 9.87 ( ± 0.68) | 1.7 |
| | 18 | 0.305 ( ± 0.012) | 5.94 ( ± 3.01) | 9.59 ( ± 0.12) | 0.8 |
| Method | T (°C) | $\mu_{max}$ (1/h) | λ (h) | $y_{max}$ ($\log_{10}$ CFU/g) | Shelf-life (days)[a] |
| | | *Leuconostoc mesenteroides* (*LM*) | | | |
| PC | 5 | 0.032 ( ± 0.003) | 20.54 ( ± 1.73) | 10.04 ( ± 0.28) | 5.5 |
| | 8 | 0.053 ( ± 0.004) | 12.44 ( ± 0.91) | 10.10 ( ± 0.28) | 3.5 |
| | 13 | 0.111 ( ± 0.008) | 5.99 ( ± 0.40) | 10.10 ( ± 0.18) | 1.6 |
| | 18 | 0.177 ( ± 0.019) | 3.74 ( ± 0.36) | 10.71 ( ± 0.45) | 1.0 |
| qPCR | 5 | 0.031 ( ± 0.003) | 62.01 ( ± 7.66) | 9.35 ( ± 0.33) | 6.4 |
| | 8 | 0.052 ( ± 0.008) | 36.67 ( ± 6.16) | 9.16 ( ± 0.46) | 4.0 |
| | 13 | 0.130 ( ± 0.027) | 14.76 ( ± 3.83) | 9.17 ( ± 0.53) | 1.9 |
| | 18 | 0.191 ( ± 0.021) | 10.04 ( ± 1.26) | 9.26 ( ± 0.40) | 1.2 |

[a] Time taken to reach levels of $10^7$ CFU/g.

**Table 5**
Statistical indexes obtained by the fitting of Baranyi and Roberts (BAR) model to the experimental data of *Weissella viridescens* (*WV*) and *Leuconostoc mesenteroides* (*LM*) in artificially inoculated VPM at 5, 8, 13 and 18 °C obtained by qPCR and plate count methods.

| Species | Methods | T (°C) | $R^2_{adj}$ | RMSE ($\log_{10}$ CFU/g) | *Af* | *Bf* |
|---|---|---|---|---|---|---|
| *W. viridescens* | PC | 5 | 0.990 | 0.228 | 1.021 | 1.001 |
| | | 8 | 1.000 | 0.058 | 1.005 | 1.000 |
| | | 13 | 0.993 | 0.198 | 1.016 | 1.000 |
| | | 18 | 0.993 | 0.225 | 1.024 | 1.000 |
| | qPCR | 5 | 0.984 | 0.305 | 1.036 | 1.003 |
| | | 8 | 0.991 | 0.198 | 1.020 | 1.001 |
| | | 13 | 0.952 | 0.553 | 1.053 | 0.999 |
| | | 18 | 0.999 | 0.092 | 1.010 | 1.000 |
| *L. mesenteroides* | PC | 5 | 0.996 | 0.160 | 1.014 | 1.000 |
| | | 8 | 0.997 | 0.147 | 1.018 | 1.001 |
| | | 13 | 0.997 | 0.136 | 1.011 | 1.001 |
| | | 18 | 0.994 | 0.225 | 1.021 | 1.000 |
| | qPCR | 5 | 0.993 | 0.184 | 1.017 | 1.000 |
| | | 8 | 0.986 | 0.273 | 1.038 | 1.004 |
| | | 13 | 0.971 | 0.423 | 1.057 | 0.999 |
| | | 18 | 0.992 | 0.233 | 1.034 | 1.001 |

( ± 0.016) and 1.016 ( ± 0.004) for the qPCR and the PC methods, respectively, while the average $A_f$ values for *WV* were 1.030 ( ± 0.019) and 1.017 ( ± 0.008) for the qPCR and the PC methods, respectively. As shown in Table 5, all average $B_f$ values were equal to 1.000 ( ± 0.002) for both methods, and both types of bacteria. The results indicated that the BAR model fittings neither underestimated nor overestimated the observations of the *LM* and *WV* growth by qPCR and PC methods and that the fitting curves could accurately describe the growth of these bacteria in VPM under isothermal storage. Zurera-Cosano et al. (2005) obtained values of $B_f$ = 1.00 and $A_f$ = 1.13 in their artificial neural network model validation of *LM* in vacuum packaged sliced cooked meat products.

### 3.5. Secondary modeling of LM and WV in VPM using PC and qPCR methods

The influence of storage temperature on the maximum specific growth rates ($\mu_{max}$) of *LM* and *WV* in VPM was established with secondary models. The estimated parameter values ( ± 95% confidence interval) of *a*, *b*, *c*, and $T_{min}$ of fitting the Exponential (Eq. (6)) and the Ratkowsky square root (Eq. (7)) secondary models to the $\mu_{max}$ parameter values and the statistical indexes ($R^2_{adj}$ and RMSE), respectively, are shown in Table 6. Likewise, the fitting of secondary models to the $\mu_{max}$ parameter of *LM* and *WV* are shown in Fig. 6a and b, respectively. The results showed that, when VPM was inoculated with the cocktails of either *LM* or *WV* and stored at constant temperatures from 5 to 18 °C, both strains grew as expected, increasing the growth rate with the temperature.

The results of the Ratkowsky square root secondary model represented the temperature dependence of the $\mu_{max}$ parameter from the

**Table 6**
Estimated parameter values ( ± confidence interval) (*a*, *b*, *c* and $T_{min}$) of the fitting of Exponential and Ratkowsky square root secondary models to $\mu_{max}$ parameter values and the statistical indexes ($R^2_{adj}$ and RMSE).

| Species | Methods | Secondary models | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Exponential | | | | Ratkowsky square root | | | |
| | | *a* (1/h) | *b* (1/°C) | $R^2_{adj}$ | RMSE | *c* (1/(°C √h)) | $T_{min}$ (°C) | $R^2_{adj}$ | RMSE |
| *WV* | PC | 0.016 ( ± 0.009) | 0.151 ( ± 0.032) | 0.996 | 0.007 | 0.024 ( ± 0.008) | −1.98 ( ± 4.82) | 0.981 | 0.019 |
| | qPCR | 0.020 ( ± 0.010) | 0.151 ( ± 0.031) | 0.996 | 0.007 | 0.026 ( ± 0.014) | −2.11 ( ± 7.27) | 0.958 | 0.031 |
| *LM* | PC | 0.020 ( ± 0.018) | 0.123 ( ± 0.056) | 0.979 | 0.010 | 0.019 ( ± 0.002) | −4.53 ( ± 1.41) | 0.999 | 0.004 |
| | qPCR | 0.023 ( ± 0.037) | 0.120 ( ± 0.100) | 0.932 | 0.019 | 0.021 ( ± 0.006) | −3.60 ( ± 4.72) | 0.985 | 0.015 |

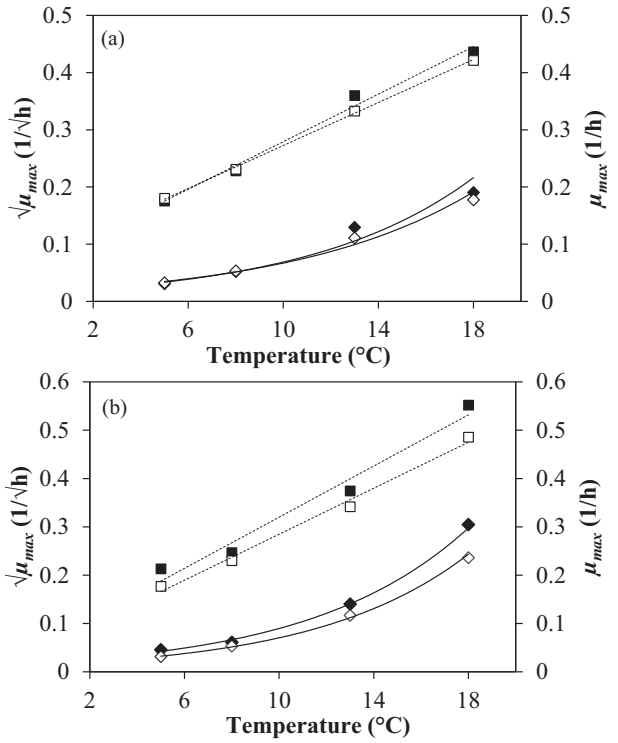


**Fig. 6.** Fitting of secondary models to $\mu_{max}$ parameter data of (a) *Leuconostoc mesenteroides* (*LM*) and (b) *Weissella viridescens* (*WV*) in VPM obtained by qPCR (filled symbols) and PC (unfilled symbols) methods. In all two graphs, dashed line represents the fitting of Ratkowsky square root secondary model (square symbols data) and continuous lines represents the fitting of exponential secondary model (symbols lozenge data).

BAR model for the growth of *LM* better than the results of the Exponential model, yielding $R^2_{adj}$ values of over 0.985 and RMSE values $\leq$0.015 for both the qPCR and the PC methods. In contrast, the results of the Exponential model represented the temperature dependence of the $\mu_{max}$ parameter in relation to *WV* better than the results of the Ratkowsky square root model, yielding $R^2_{adj}$ values equal to 0.996 and RMSE values of 0.007 for both methods, as shown in Table 6.

Finally, the qPCR method was useful for constructing the mathematical model, because this DNA-based technique showed high quantification accuracy for the estimation of growth data of the target bacteria and for the estimation of the growth kinetics of *LM* and *WV* in vacuum-packaged blood sausages and, by extension, other cooked meat products, under isothermal conditions. Microbiologists will therefore have new predictive techniques available to them. They will therefore face new experimental and data-handling challenges, as physiological and molecular information will be increasingly available for incorporation in data-modeling techniques (McMeekin et al., 2008).

## 4. Conclusions

This study has evaluated and combined the qPCR and the PC methods with mathematical models to describe the growth of *L. mesenteroides* and *W. viridescens* in vacuum-packaged *morcilla* (VPM) under isothermal conditions (from 5 to 18 °C). Pooling all the experimental results, the qPCR method and the conventional microbiological PC method yielded similar results and described the growth curves appropriately. Furthermore, the BAR model was successful applied for fitting the molecular data. The exponential and the Ratkowsky square root secondary models accurately represented the dependence of the maximum specific growth rate with the temperature for both species. In conclusion, the qPCR and PC methods combined with the mathematical models provided effective methods for the estimation of the growth kinetics of *L. mesenteroides* and *W. viridescens* under isothermal conditions in vacuum-packaged blood sausage and by extension in other cooked meat products. In addition, the qPCR method has the advantage of measuring the number of a target gene in a large number of samples in less time than the PC method and with high specificity and sensitivity. Further research is required to establish molecular and conventional predictive models from the primary and secondary models discussed in this research. The model must also be applied to other cooked meat products, under non-isothermal conditions, for its validation. The results will help to identify good and bad storage practices and are aimed at improving shelf-life prediction and thereby enhancing spoilage prevention.

## 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.

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