Quantifying the bioprotective effect of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* on vacuum packaged hot-smoked sea bream

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ABSTRACT

In this study, the bioprotective potential of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* CTC1034 was evaluated on vacuum-packed hot-smoked sea bream at 5 ºC and dynamic temperatures ranging from 3 to 12 ºC. The capacity of three microbial competition interaction models to describe the inhibitory effect of *L. sakei* CTC494 on *L. monocytogenes* was assessed based on the Jameson effect and Lotka-Volterra approaches. A sensory analysis was performed to evaluate the spoiling capacity of *L. sakei* CTC494 on the smoked fish product at 5 ºC. Based on the sensory results, the bioprotection strategy against the pathogen was established by inoculating the product at a 1:2 ratio (pathogen:bioprotector, log CFU/g). The kinetic growth parameters of both microorganisms were estimated in mono-culture at constant storage (5 ºC). In addition, the inhibition function parameters of the tested interaction models were estimated in co-culture at constant and dynamic temperature storage using as input the mono-culture kinetic parameters. The growth potential (δ log) of *L. monocytogenes*, in mono-culture, was 3.5 log on smoked sea bream during the experimental period (20 days). In co-culture, *L. sakei* CTC494 significantly reduced the capability of *L. monocytogenes* to grow, although its effectiveness was temperature dependent. The LAB strain limited the growth of the pathogen under storage at 5 ºC (< 1 log increase) and at dynamic profile 2 (< 2 log increase). Besides, under storage at dynamic profile 1, the growth of *L. monocytogenes* was inhibited (< 0.5 log increase). These results confirmed the efficacy of *L. sakei* CTC494 for controlling the pathogen growth on the studied fish product. The Lotka-Volterra competition model showed slightly better fit to the observed *L. monocytogenes* growth response than the Jameson-based models according to the statistical performance. The proposed modelling approach could support the assessment and establishment of bioprotective culture-based strategies aimed at reducing the risk of listeriosis linked to the consumption of RTE hot-smoked sea bream.

1. INTRODUCTION

Smoked fish products are sold as ready-to-eat (RTE) foods characterized by a relatively long refrigerated shelf-life when packaged under vacuum (Hwang, 2007). These seafood commodities are popular, but they are also considered among the top risk foodstuffs since they can be contaminated with foodborne pathogens and no cooking is applied before consumption (Ghanbari et al., 2013). At present, the microbiological concerns in the EU associated with extended shelf-life refrigerated RTE foods are focused on psychrotrophic foodborne pathogens such as *Listeria monocytogenes* (EFSA BIOHAZ Panel, 2018).

Biopreservation, also called bioprotection, is a biocontrol approach to enhance product safety and shelf-life using microorganisms selected for their antimicrobial properties, so called protective cultures (Leroi et al., 2015). Lactic-acid bacteria (LAB) are considered a new generation of food additives and the basis of food biopreservation (Said et al., 2019). Protective cultures are considered by the regulatory agencies as 'new' food additives, meaning that they require market authorization for their technological use in foods. However, most LAB are Generally Recognized as Safe (GRAS) and many LAB species (including *Lactobacillus sakei*) have been granted by EFSA with the Qualified Presumption of Safety (QPS) status (EFSA, 2018). In the EU, microorganisms with the latter food-grade standard do not need to undergo a further safety assessment other than to provide evidence of efficacy and to satisfy the specified qualifications, if applicable, for its market approval. Two recent studies have proved that the antilisterial sakacin K-producing *Lactobacillus sakei* strain CTC494 (from meat origin) is effective to inhibit *L. monocytogenes* in filleted sea bream and cold-smoked salmon under refrigerated storage (Aymerich et al., 2019; Costa et al., 2019). Nevertheless, the inhibitory capacity of this bioprotective LAB strain has not been tested in other fish products where the differences in product's characteristics and formulations might either favor its inhibition thanks to

the antimicrobial hurdle combinations (Leistner, 2000) or hinder the ability of the strain to inhibit *L. monocytogenes* (Tahiri et al., 2009; Vasilopoulos et al., 2010).

Quantifying microbial interaction in food can be highly complex and often overlooked in predictive microbiology studies (Powell et al., 2004). Most of the competitive growth models available in literature are based on two approaches: one based on the Jameson effect phenomenon (i.e. nutrient competition) (Jameson, 1962) and the other using the general Lotka-Volterra competition model (i.e. predator-prey model) (Powell et al., 2004; Valenti et al., 2013). Both mathematical models represent a simultaneous deceleration of bacterial populations. The inhibition of *L. monocytogenes* by endogenous LAB usually responsible for spoilage has been studied and modelled in minimally processed fish products (Mejlholm et al., 2015; Mejlholm and Dalgaard, 2015, 2007). In this regard, most of the published microbial interaction models aim at describing competition between background microbiota and microbial pathogens, rather than to characterize the performance of bioprotective bacteria with specific antagonistic activities, that are normally added at higher levels than the natural background (spoilage) microbiota (Cornu et al., 2011). To the best author's knowledge, studies having quantified the bioprotective effect of bacteriocin-producing LAB cultures through the development and implementation of predictive models are scarce. The first attempt to model the inhibitory effect of a bacteriocinogenic LAB strain against *Listeria* in fish was made by Costa et al. (2019), in which model parameters were derived from experiments in a fish-based broth and then validated on fresh filleted sea bream.

The objective of this study was (i) to evaluate the bioprotective potential of *L. sakei* CTC494 against *L. monocytogenes* on hot-smoked sea bream under constant and dynamic storage temperature conditions and (ii) to evaluate the capacity of three microbial

interaction models based on the Jameson effect and Lotka-Volterra approaches to describe the inhibitory effect of *L. sakei* CTC494 on *L. monocytogenes*.

2 MATERIAL AND METHODS

2.1. Bacterial strains

The selected bacterial strains used in this work were the bioprotective culture *L. sakei* CTC494 and the target pathogen *L. monocytogenes* CTC1034. This pathogenic strain was used in a previous work as a reference strain to study the antagonism of bacteriocin-producing LAB, including *L. sakei* CTC494 (Garriga et al., 2002). Both microorganisms were stored at -80 ºC in the appropriate culture broth with 20% (v/v) glycerol. Before inoculation, a fresh culture was prepared for each strain and a well-isolated colony was used to perform two consecutive 24 h-subcultures, which were grown in de Man, Rogosa and Sharpe broth (MRS, Oxoid, UK) for *L. sakei* at 30 ºC with 10% CO_2 and Brain Heart Infusion (BHI, Oxoid, UK) for *L. monocytogenes* at 37 ºC. This resulted in a cell density of *ca.* 10^8 CFU/mL and 10^9 CFU/mL for *L. sakei* and *L. monocytogenes*, respectively.

2.2. Preparation of hot-smoked sea bream

Gilthead sea bream fishes (*Sparus aurata*) from marine aquaculture were collected and processed by the Andalusian Aquaculture Technology Centre (CTAQUA, Cádiz, Spain) following an industrial hot smoking process. First, fish samples were manually scaled, gutted, filleted and bled in ice-water. Then, fillets were brined in a NaCl/sugar solution (ratio 3:1) for 2 hours. After that, fish fillets were removed from the brine solution, washed with water and introduced in a smoking oven (till the fish core temperature reached 65°C during 30 min). After cooling at room temperature for 15 min, smoked fillets were vacuum-packed and transferred to the laboratory the day after processing in polystyrene boxes under cold conditions.

2.3. Sensory assessment

A sensory analysis was carried out to evaluate the impact of the bioprotective culture on the organoleptic characteristics of hot-smoked sea bream during refrigerated storage based on an acceptance test. For that, 10 g-fish portions were surface inoculated with the *L. sakei* CTC494 as described below (section 2.4) at three initial cell densities (*ca*. 2 log CFU/g, 4 log CFU/g and 6 log CFU/g) and stored at 5 ± 0.5 ºC under vacuum packaging conditions for 18 days. A control batch was prepared without inoculating bacteria.

The sensory evaluation was performed based on the work by Yanar et al. (2006), who evaluated the shelf-life of hot smoked tilapia stored at 4 ºC. For that, 5 panelists were trained according to the standard EN ISO 8586:2012 method. The descriptors were generated by open discussion and consensus in a previous session using fish samples stored under the same experimental condition applied in this study. The descriptors retained were general appearance (score of the overall appearance), intensity of odour (score of the overall odour) and texture to the touch. The assessors scored control and inoculated samples for the appearance, odour and texture characteristics using a 9-point hedonic scale. A score of 7–9 denoted ‘‘very good’’ quality, a score of 4.0–6.9 ‘‘good’’ quality, and a score of 1.0–3.9 indicated “unacceptable” quality. The sensory assessment was performed on the storage days 4, 7, 11, 14 and 18. At each evaluation point, one fish portion from each assayed condition was individually served under white light on conventional petri dishes, labeled with three-digit random numbers.

The obtained sensory scores for each attribute were plotted against time and sensory deterioration rates were obtained by fitting a linear model. Deterioration rates (slopes) for inoculated and control samples were statistically compared by performing t-test ($p \leq 0.05$) using the statistical software package SPSS 25.0 (Chicago, Illinois, USA).

2.4. Challenge tests

In the first trial, the growth of mono-cultured and co-cultured *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on smoked sea bream was evaluated under constant refrigerated storage (5 ± 0.5 ºC) for 20 days. Four batches were prepared: (1) non-inoculated product (control); (2) inoculated with *ca*. 2 log CFU/g *L. monocytogenes*; (3) inoculated with *ca*. 4 log CFU/g *L. sakei* and (4) inoculated with a mixture of *L. monocytogenes* and *L. sakei* at a ratio 1:2 (i.e. 2 log CFU/g *L. monocytogenes* and 4 log CFU/g *L. sakei*). The inoculation ratio pathogen:bioprotector used in batch 4 was selected based on the results previously obtained from the sensory analysis (section 2.3).

Prior to inoculation, smoked fish fillets were cut into portions of *ca.* 5 cm^2 and 10 g using a sterile scalpel. This fish portion was considered the analytical fish sample. The caudal region of the fish fillet was discarded from the analysis to avoid experimental variability. Smoked sea bream portions were surface inoculated on the flesh side at 1% (v/w) from the appropriate decimal dilution using a L-shaped sterile spreader. For experiments in co-culture (batch 4), the product was first inoculated with the pathogen and left for 10 min in the safety cabinet to allow cell attachment. The LAB strain was then spread, followed by a cell attachment period as the pathogen case. This inoculation procedure (Aymerich et al., 2019) was used to mimic a post-processing contamination scenario in which the fish product is contaminated by the pathogen after the hot-smoking process and then the bioprotective culture is applied. After inoculation, fish portions were individually vacuum-packed in polyamide-polyethylene plastic bags (Sacoliva, Barcelona, Spain) and stored as described above.

In the second trial (approx. 2 months later) with a new batch of hot-smoked sea bream, the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* was evaluated in co-culture at two different dynamic temperature profiles, which were designed to simulate the fluctuating conditions of the cold-chain distribution and storage of smoked fish

products in Spain. Profile 1 had a mean temperature of 6.3 ºC and range between 3.6 to 12.8 ºC while profile 2 had 7.6 ºC of mean temperature and range between 3.3 to 11.8 ºC. Microorganisms were co-inoculated on the sample's surface and vacuum packaged as described before for batch 4. An additional non-inoculated (control) batch was also studied.

The challenge tests were conducted in Hot-Cold incubators containing programmable time-temperature profiles (Selecta, Barcelona, Spain). Incubation temperatures were measured continuously throughout the experiments by data loggers (MicroLite, Fourier Technologies, Israel). For experiments, 2 samples (duplicate) were analysed for microbiological and/or physicochemical determinations at different sampling points. The experiments were repeated twice in different days.

2.5. Microbiological analyses

Fish portions of 10 g were aseptically transferred into a sterile stomacher bag and homogenized for 60 s (masticator, IUL Instruments, Barcelona, Spain) with 0.1% sterile peptone water (Oxoid, UK).

For non-inoculated (control) samples, the growth of aerobic mesophilic viable count (MVC), endogenous LAB counts and *Listeria* investigation (presence/absence) was evaluated during storage at the tested temperatures. MVC counts were determined by pour plating decimal dilutions from homogenized samples in Plate Count Agar (PCA, Oxoid) incubated 48 h at 37 °C. For LAB enumeration, de Man, Rogosa, Sharpe agar (MRS, Oxoid) was used, which was incubated 48 h at 33 °C with 10% CO_2. The presence/absence of *L. monocytogenes* was carried out following the method EN ISO 11290-1.

For inoculated samples, the enumeration of *L. monocytogenes* was carried out using Oxford agar (Oxoid) containing Oxford selective supplement (SR0140, Oxoid). Counts were obtained after incubating the plates for 24–48 h at 37 °C. LAB were enumerated as for non-inoculated samples.

2.6. Physicochemical analyses

The physiochemical characteristics of hot-smoked sea bream (i.e. pH and a_w) were determined by analysis of 2 fish portions (10 g each). pH measurement was performed for samples from batch 1 (non-inoculated) and batch 3 (inoculated with *ca*. 4 log CFU/g *L. sakei*) on the days of the sensory analysis (section 2.3) using the pH meter Edge HI2020 (HI11310 electrode, Hanna Instruments, USA) by homogenizing the fish portion with distilled water at ratio 1:1.

The determination of a_w was performed with the AquaLab 4TE a_w meter (Decagon Devices Inc., WA, USA) for non-inoculated samples at the start of the challenge tests.

2.7. Modelling microbial interaction

The inhibition of *L. monocytogenes* by the bioprotective *L. sakei* CTC494 was modelled following the mathematical approach proposed by Costa et al. (2019). This consists of (i) the estimation of the growth parameters lag phase, maximum specific growth rate and maximum population density of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in mono-culture, (ii) the estimation of the inhibition function parameters of three existing microbial competition interaction models using as input the mono-culture kinetic parameters and (iii) the statistical analysis of the performance of the tested models to describe experimental observations.

2.7.1. Primary growth kinetic parameters and secondary model

The observed growth data for mono-cultured *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on hot-smoked sea bream obtained at constant storage temperature (5 ºC) were used to fit the primary growth model of Baranyi and Roberts (1994) and the kinetic parameters lag phase (λ, h), maximum specific growth rate ($\mu_{max,}$ 1/h) and maximum population density (N_{max}, log CFU/g) were determined for each microorganism. The model was fitted to the experimental data using the program DMFit for Excel v.3.5 (IFR, Norwich, UK).

Growth rate at different temperatures was predicted using the secondary model for μ_{max} as a function of temperature published by Costa et al. (2019). This model was developed in fish juice of sea bream for the same bacterial strains used in this study and was validated on fresh filleted sea bream stored under isothermal and non-isothermal conditions. The model corresponded to a square root type equation (Ratkowsky et al., 1982) with parameter values b and T_{min} being 0.028 and – 4.5 ºC, respectively for *L. sakei* CTC494, and 0.026 and – 3.4 ºC, respectively for *L. monocytogenes* CTC1034. Since an effect of the fish matrix and vacuum conditions on μ_{max} was expected, an adjustment factor was applied to simulate the microbial interaction on smoked sea bream. The adjustment factor for μ_{max} of each microorganism was calculated as the ratio between the μ_{max} values obtained in this study from mono-culture experiments at 5 ºC in smoked sea bream and in fish juice by Costa et al. (2019).

2.7.2. Microbial interaction models

Three existing microbial competition models were evaluated to assess their ability to describe the growth response of the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* at isothermal and non-isothermal storage conditions: the Jameson effect model, a modified version of the Jameson effect model and the Lotka-Volterra model.

The generic system of equations (Eq. 1) of the tested interaction models, applied in their implicit form to co-cultured *L. sakei* CTC494 *(*subscript '1') and *L. monocytogenes* (subscript '2'), can be expressed as:

$$\begin{cases} \frac{dN_1(t)}{dt} = N_1 \cdot \mu_{max1} \cdot \left(\frac{Q_1}{1+Q_1}\right) \cdot f_1(t) \\ \frac{dN_2(t)}{dt} = N_2 \cdot \mu_{max2} \cdot \left(\frac{Q_2}{1+Q_2}\right) \cdot f_2(t) \\ \frac{dQ_1}{dt} = Q_{1\,t-1} \cdot \mu_{max1} \\ \frac{dQ_2}{dt} = Q_{2\,t-1} \cdot \mu_{max2} \\ Q_0 = \frac{1}{e^{(\mu_{max} \cdot \lambda)} - 1} \end{cases} \quad (1)$$

where N is the cell concentration (CFU/g) at time t, μ_{max} is the maximum specific growth rate (1/h), Q and Q_0 is a measure of the physiological state of cells at time t and t = 0, respectively, f(t) is an inhibition function and λ (h) the lag phase duration of the cells.

The different microbial competition models were tested based on the generic system of equations in Eq. (1) and using the corresponding inhibition function f(t) described in either Eq. (2), or (3) or (4) for each type of model.

The empirical Jameson effect model is based on the assumption that all bacterial populations stop growing when the dominant culture reaches its maximum population density (Giménez and Dalgaard, 2004). In this case, the inhibition function f(t) is defined as:

$$\begin{cases} f_1(t) = \left(1 - \frac{N_1}{N_{max1}}\right) \cdot \left(1 - \frac{N_2}{N_{max2}}\right) \\ f_2(t) = \left(1 - \frac{N_2}{N_{max2}}\right) \cdot \left(1 - \frac{N_1}{N_{max1}}\right) \end{cases} \quad (2)$$

where N_{max} is the maximum cell density (CFU/g) for each population with subscript '1' and '2' for *L. sakei* CTC494 and *L. monocytogenes*, respectively, and other parameters are as indicated in Eq. (1).

The modified Jameson effect model was based on a modification of the inhibition function $f_2(t)$ in Eq. (2), in which the maximum density of population 1 (i.e. *L. sakei* CTC494; N_{max1}) is replaced by a critical population density, being typically lower than its N_{max1} (Cornu et al., 2011; Le Marc et al., 2009). This parameter describes the concentration value of the population 1 that results in the stop of the growth of population 2. This critical concentration value can be related to the production of an inhibitory substance, at a certain level, able to inhibit growth of the other population. In addition, if $N_{CPD1} < N_1 < N_{max1}$, then $f_2(t) < 0$, which describes a decline of population 2.

$$f_2(t) = \left(1 - \frac{N_2}{N_{max2}}\right) \cdot \left(1 - \frac{N_1}{N_{CPD1}}\right) \quad (3)$$

where N_{CPD1} is the critical population density of population 1 (i.e. *L. sakei* CTC494; CFU/g) and other parameters are as indicated in Eqs. (1) and (2).

Finally, it was used the classical predator-prey (Lotka-Volterra) model for interspecific bacterial competition (Dens et al., 1999; Powell et al., 2004), which includes two coefficients of interaction (α_{12} and α_{21}) measuring the competitive effect of species 1 on species 2 and vice-versa. In our study, the competition term of *L. monocytogenes* on *L. sakei* (α_{21}) was fixed to zero since we assumed that the pathogen did not influence growth of *L. sakei* CTC494 due to the higher concentration, shorter lag time and faster growth of the LAB strain (Mejlholm and Dalgaard, 2015; Møller et al., 2013). Therefore, the tested model includes one inhibition function $f_1(t)$ formulated as:

$$f_1(t) = \left(1 - \frac{N_1 + \alpha_{12} \cdot N_2}{N_{max1}}\right) \quad (4)$$

where α_{12} is the competition term of *L. sakei* CTC494 on *L. monocytogenes* and other parameters are as indicated in Eqs. (1) and (2).

Simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* was described by estimating the inhibition parameter for each tested model, using as input the kinetic parameters estimated by the Baranyi and Roberts primary model from mono-culture data (λ, N_{max}), together with the square root function for μ_{max} reported by Costa et al. (2019) to account for the temperature effect. The parameter estimations were performed in Microsoft Excel with Solver add-in tool using numerical integration with a time step of 0.5 h (Microsoft Corp., Redmond, WA, USA). The interaction factors (CPD_1, α_{12}) were estimated by regression analysis (MS Excel) using kinetic parameters from mono-culture experiments and the secondary model by Costa et al. (2019).

2.7.3. Interaction models prediction performance

The capacity of the prediction of the tested models under constant and dynamic temperature storage conditions was assessed by the statistical indexes Root Mean Squared Error (RMSE) and corrected Akaike Information Criterion (AICc) calculated as shown in Eqs. (5) and (6), respectively.

$$RMSE = \sqrt{\frac{\sum(fitted-observed)^2}{n-p}} \quad (5)$$

$$AICc = n \cdot ln\left(\frac{RSS}{n}\right) + 2 \cdot (p+1) + \frac{2\cdot(p+1)\cdot(p+2)}{n-p-2} \quad (6)$$

where n is the number of observations and p is the number of model parameters to be estimated in Eqs. (5) and (6) and *RSS* is the residual sum of squares in Eq. (6).

In addition to the statistical performance described above, the Acceptable Simulation Zone (ASZ) method was used to evaluate the prediction capacity of the interaction models

at dynamic storage temperatures. The acceptable interval was defined as ± 0.5 log-units from the simulated growth of *L. sakei* or *L. monocytogenes*. The simulations were considered acceptable when at least 70% of the observed counts were within the ASZ (Oscar, 2005).

3 RESULTS AND DISCUSSION

3.1. Sensory evaluation of the bioprotective L. sakei *CTC494 on smoked sea bream*

The average sensory scores and sensory deterioration rates obtained for the three evaluated attributes during storage at 5 ºC for non-inoculated (control) and inoculated samples are showed in Fig. 1 and Table 1, respectively. Overall, no significant differences ($p > 0.05$) were found between the spoilage rates obtained for control and inoculated samples with 2 and 4 log CFU/g of *L. sakei* CTC494 throughout the storage period, with sensory scores denoting, in general, "very good" or "good" quality (4-9). On the contrary, sensory scores were < 4 at 11 days for samples inoculated with *L. sakei* CTC494 at 6 log CFU/g for the attribute appearance (Fig. 1A). Moreover, spoilage rates were significantly higher ($p \leq 0.05$) at inoculation level of 6 log CFU/g, indicating that such a high inoculation level may cause undesired changes to the hot-smoked sea bream as compared to the samples of lower inoculum sizes. Although the smoked fish product was not tested for its taste, it can be assumed that the product was not significantly acidified due to the addition of the LAB strain at levels ≤ 4 log CFU/g, as indicated by the recorded pH values at the end of the storage period for control and inoculated samples at 4 log CFU/g *L. sakei*, which corresponded to 6.0 ± 0.1 and 5.9 ± 0.1, respectively.

Our results are consistent with those reported by Costa et al. (2019), who also sensory validated the application of *L. sakei* CTC494 on fresh fish fillets under modified atmosphere packaging (MAP) and found that at levels of 2 and 4 log CFU/g the sensory characteristics were statistically similar to the non-inoculated product. Based on these results, the bioprotection strategy against the pathogen was established in this study by inoculating the product with 4 log CFU/g of *L. sakei* CTC494 at the beginning of the experiments.

3.2. Evaluating the bioprotective effect of L. sakei *CTC494 against* L. monocytogenes

3.2.1. Mono-culture growth on hot-smoked sea bream

Regarding the microbiological analysis of endogenous microbiota of hot-smoked sea bream, the concentration of endogenous LAB and MVC obtained at the starting day for control samples were below the detection level (< 1 log CFU/g) and remained below this level for LAB and < 2 log CFU/g for MVC during the evaluated storage period at both constant and dynamic temperature conditions (data not shown). In addition, *L. monocytogenes* was not detected in any control sample. The initial a_w and pH of non-inoculated (control) samples corresponded to 0.96 ± 0.007 and 6.0 ± 0.1, respectively.

The kinetic parameters and their standard errors estimated by the Baranyi and Roberts model for experiments in mono-cultures at 5 ºC are showed in Table 2. A long lag phase λ and an exponential growth rate μ_{max} was distinguished for each studied microorganism. *L. sakei* CTC494 reached the stationary phase after approximately 13 days of storage. However, no stationary phase was observed for *L. monocytogenes* CTC1034 at the end of the experimental period (20 days), thus the Baranyi and Roberts model (no asymptote) was used to describe the growth behaviour of *L. monocytogenes* on hot-smoked sea bream. Despite this, the growth potential (δ log) of *L. monocytogenes* was 3.5 log during the storage period, which demonstrates that refrigeration at 5 ºC does not prevent the pathogen growth on the studied fish product. *L. monocytogenes* growth was also observed in other vacuum-packed hot-smoked fish products at refrigeration temperatures (Branciari et al., 2016; Mahmoud et al., 2012; Tosun and Özden, 2014).

The parameter μ_{max} in mono-culture experiments was much higher for *L. sakei* (0.062 1/h) than for *L. monocytogenes* (0.024 1/h). The μ_{max} value obtained for the latter microorganism was lower than that estimated by Costa et al. (2019) for the same strain in

mono-culture in fish juice at 5 ºC (0.0477 1/h), corresponding to a reduction of 50% with respect to the value reported by Costa et al. (2019). These authors found that the μ_{max} value of *L. monocytogenes* in the fish product (i.e. MAP fresh filleted sea bream) presented a reduction of 68 % with respect to that observed in fish juice. Differences in reduction rate could be due to the specific characteristics of the product studied herein. In the work by Costa et al. (2019), the presence of indigenous microbiota in fresh fish fillets that was higher can explain a more reduced pathogen growth when compared to smoked sea bream in our study, where MVC and LAB were in low numbers as a consequence of the hot smoking process. In addition, the presence of CO_2 in MAP fish fillets has been described as an inhibiting factor influencing *L. monocytogenes* growth (Bolívar et al., 2018; Provincial et al., 2013). Regarding this, Tosun and Özden (2014) reported that MAP was most effective in controlling the growth of *L. monocytogenes* in hot-smoked rainbow trout fish fillets at 2 ºC while vacuum packaging had no effect.

3.2.2. Co-culture growth on smoked sea bream

Growth curves of co-cultured *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on smoked sea bream are showed in Fig. 2. The LAB strain exhibited a similar lag phase at 5 ºC and under dynamic temperature profile 1 (around 5 days), which also corresponded to that estimated in mono-culture (Table 2). *L. sakei* CTC494 showed a shorter lag phase under profile 2, probably due to the abrupt temperature rise at 4 storage days. In addition, the microorganism reached similar N_{max} values (*ca*. 8.6 log CFU/g) at all storage temperatures and both (mono and co-) culture conditions. These results suggest, as expected, that the growth of *L. sakei* CTC494 in co-culture was not affected by the presence of *L. monocytogenes*.

On the contrary, *L. sakei* CTC494 significantly reduced the capability of *L. monocytogenes* to grow at the tested inoculation ratio, although its effectiveness was temperature dependent. In this regard, *L. sakei* CTC494 limited the growth of the pathogen under storage at 5 ºC (< 1 log increase) and at dynamic profile 2 (< 2 log increase) throughout the evaluated period (Figs. 2A and 2C, respectively). Besides, under storage at dynamic profile 1 (Fig. 2B), the growth of *L. monocytogenes* was inhibited (< 0.5 log increase).

The competition pattern was similar for all temperature conditions, showcasing a slight *L. monocytogenes* increase, which ceased when the *L. sakei* population approached to its N_{max} (> 8 log CFU/g). This phenomenon would reflect a non-specific interaction described by a potential Jameson effect between populations. At 5 ºC and profile 1, *L. monocytogenes* population showed a remarkable decline after *L. sakei* had reached its N_{max}, but at profile 2 growth of the pathogen slightly continued.

The efficacy of *L. sakei* CTC494 against *L. monocytogenes* has already been proved on fish. Aymerich et al. (2019) reported that the growth of *L. monocytogenes* on three types of cold-smoked salmon inoculated with *L. sakei* CTC494 was completely inhibited after 21 days at 8 ºC under vacuum packaging. In other study, the increase of the pathogen was less than 1 log units on fresh filleted sea bream in the presence of the LAB strain after storage under MAP at isothermal and non-isothermal storage conditions (Costa et al., 2019). The degree of inhibition of *L. monocytogenes* exerted by *L. sakei* CTC494 in different types of fish products illustrates the capacity of this bioprotective culture as a potential antimicrobial agent (AMA) to be used as part of the *L. monocytogenes* control alternatives for RTE food defined by the US Food Safety Inspection Service Listeria rule (FSIS, 2014).

3.3. Microbial interaction on smoked sea bream

The three microbial interaction models were fitted to the co-culture experimental results with Eq. (1) estimating the corresponding inhibition function parameters (Eq. (2), (3) or (4)). The fitted inhibition parameters (CPD_1 and α_{12}) and the goodness-of-fit indexes are presented in Table 3, together with the ASZ values (%) obtained from the prediction capacity evaluation for dynamic storage temperatures.

To consider the effect of food matrix on μ_{max} on the growth simulation at dynamic temperature conditions, the μ_{max} for *L. monocytogenes* estimated from the secondary model (based on fish juice) was adjusted by applying an adjustment factor of 0.50 (section 3.2.1) in the tested microbial interaction models. For *L. monocytogenes*, the Lotka-Volterra model showed, in general, the best fitting according to the statistical indices RMSE and AICc, with values varying from 0.334 to 0.536 and -12.19 to -4.65, respectively. The modified Jameson effect model presented better fitting than the Jameson effect model, except for dynamic profile 2 in which the lowest RMSE value was obtained for the latter model (0.314).

For *L. sakei* CTC494, the three interaction models provided similar fitting, with RMSE values being slightly lower for the Jameson effect model (Table 3). For all tested models, the worst fitting was obtained for profile 2 as indicated by higher RMSE and AICc values (Table 3). In profile 2, it can be also observed that the bioprotector strain exhibited an adaptation delay after the initial lag phase (Fig. 2C). This phenomenon has been previously described as intermediate lag periods induced by abrupt temperature shifts in which cells need to adjust to the changing temperature environment (Swinnen et al., 2005). Intermediate lag periods of significant duration are expected to be present when

the temperature profiles are below the optimal temperature of growth, thus the predictive ability of the secondary models is affected negatively (Longhi et al., 2013). This phenomenon has been previously observed for the same microorganism in fresh fish (Costa et al., 2019) and also for different *Lactobacillus* strains in other food products stored at dynamic temperatures (Longhi et al., 2013; Silva et al., 2017).

Owing to the LAB strain used in our study is a sakacin K producing species, a specific antagonistic effect would be expected due to this potentially inhibitory substance on *L. monocytogenes*. Although the bacteriocin was not quantified to confirm this effect, the fish product and storage temperatures under study might constitute a suitable bacteriocin production-supporting environment. In this regard, Hugas et al. (1998) quantified sakacin K produced by *L. sakei* CTC494 in MRS broth and observed bacteriocin activity at initial pH of 6.0 and 6.5 and higher production at low temperatures (4, 10 and 15 ºC) compared to abuse storage (20, 25 and 30 ºC). In addition, Leroy and De Vuyst (2001) suggested that extremely rich environments (e.g. MRS broth) will not necessarily increase specific bacteriocin production compared to nutrient-depleted environments, as in the present work (i.e. sugar limitation). Apparently, bacteriocin production is stimulated by less favorable growth conditions, such as low temperatures and competing microbiota (Delboni and Yang, 2017).

Regarding the prediction capacity of the tested models under dynamic profile 1, all models were able to describe satisfactory the simultaneous microbial growth, with 75% of the observations within the ASZ, excepting for the Jameson model, for which the value for *L. monocytogenes* was 37.5%. Under dynamic profile 2, none of the models provided reasonable predictions for co-cultured *L. sakei* CTC494 (< 70%). This lack of prediction could be explained by the existence of an intermediate lag phase as mentioned in above

lines. However, all models represented adequately the growth response of *L. monocytogenes*, as confirmed by ASZ values equal to 87.5%.

The estimated value of the CPD_1 parameter included in the modified Jameson effect model remained similar for the different temperatures (Table 3). The value of the competition factor of *L. sakei* CTC494 on *L. monocytogenes* (α_{12}) estimated by the Lotka-Volterra model was different depending on the temperature conditions. At 5 ºC and profile 1, the obtained α_{12} were above 1, which properly describes the observed *L. monocytogenes* population decline when *L. sakei* CTC494 reached its N_{max}. In contrast, at profile 2, α_{12} was < 1, which means that *L. monocytogenes* growth was slowed down as *L. sakei* CTC494 approached to its N_{max}, as can be seen in Fig. 2C. This reflects the different effect of non-isothermal conditions on the interaction between both microorganisms. The Lotka-Volterra's coefficient of interaction (α) of different microbial species in diverse food matrices has also been reported in the literature as a temperature-dependent parameter. Some examples are the values of α estimated at different temperatures for the growth of *L. monocytogenes* in fish juice (α-average = 1.4 between 2.2 and 5 ºC and α-average = 1.6 and 1.8 at 8 and 12 °C, respectively) (Costa et al., 2019), or the polynomial model developed by Møller et al. (2013) which described the effect of temperature on α of the natural microbiota on growth of *Salmonella* spp. in ground pork during storage between 9 ºC to 24 ºC.

Most of the studies dealing with microbial interaction responses in fish products have been performed on mixed cultures based on a pathogen and non-bacteriocinogenic microorganisms, being either endogenous or artificially inoculated (Giménez and Dalgaard, 2004; Koseki et al., 2011; Mejlholm and Dalgaard, 2015, 2007). These studies aimed to describe the maximum population density of the pathogen, for which the classical Jameson effect model is used. Other studies have used the Lotka-Volterra model

as in the case of the work by Giuffrida et al. (2007) that analysed the interaction between *Aeromonas hydrophila* and aerobic natural microbiota on sea bream surfaces considering the complexity of fluctuating environmental conditions and the interspecific bacterial interactions.

4 CONCLUSIONS

The results from this study contribute to extend the application of the bacteriocinogenic strain *L. sakei* CTC494 for controlling growth of *L. monocytogenes* in hot-smoked fish products from Mediterranean aquaculture during refrigerated storage. Results from the challenge tests demonstrated the potential of *L. sakei* CTC494 applied at a dose of 4 log CFU/g to limit or inhibit the growth of *L. monocytogenes* on hot-smoked sea bream under different storage temperatures. Nevertheless, further research is still needed to confirm this inhibitory effect under other possible scenarios such as different temperature profiles (e.g. abrupt temperature changes and abuse temperatures) and process parameters as well as considering batch variability. The proposed modelling approach, based on a validation process in food and the application of adjustment factors to kinetic parameters from the modified Jameson and Lotka-Volterra models, was able to satisfactorily describe the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* in the target fish product. This predictive tool could support the assessment and establishment of bioprotective culture-based strategies aimed at reducing the risk of listeriosis linked to the consumption of RTE hot-smoked sea bream.

Author contributions

Araceli Bolívar: Methodology, Investigation, Writing - Original Draft preparation, Visualization. **Jean Carlos Correia Peres Costa**: Validation, Writing - Review and Editing. **Guiomar D. Posada-Izquierdo**: Writing - Review and Editing. **Sara Bover-Cid**: Writing - Review and Editing. **Gonzalo Zurera**: Validation, Project administration, Funding acquisition. **Fernando Pérez-Rodríguez**: Conceptualization, Writing - Review and Editing, Supervision.

Declaration of interest

None.

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Figure 1

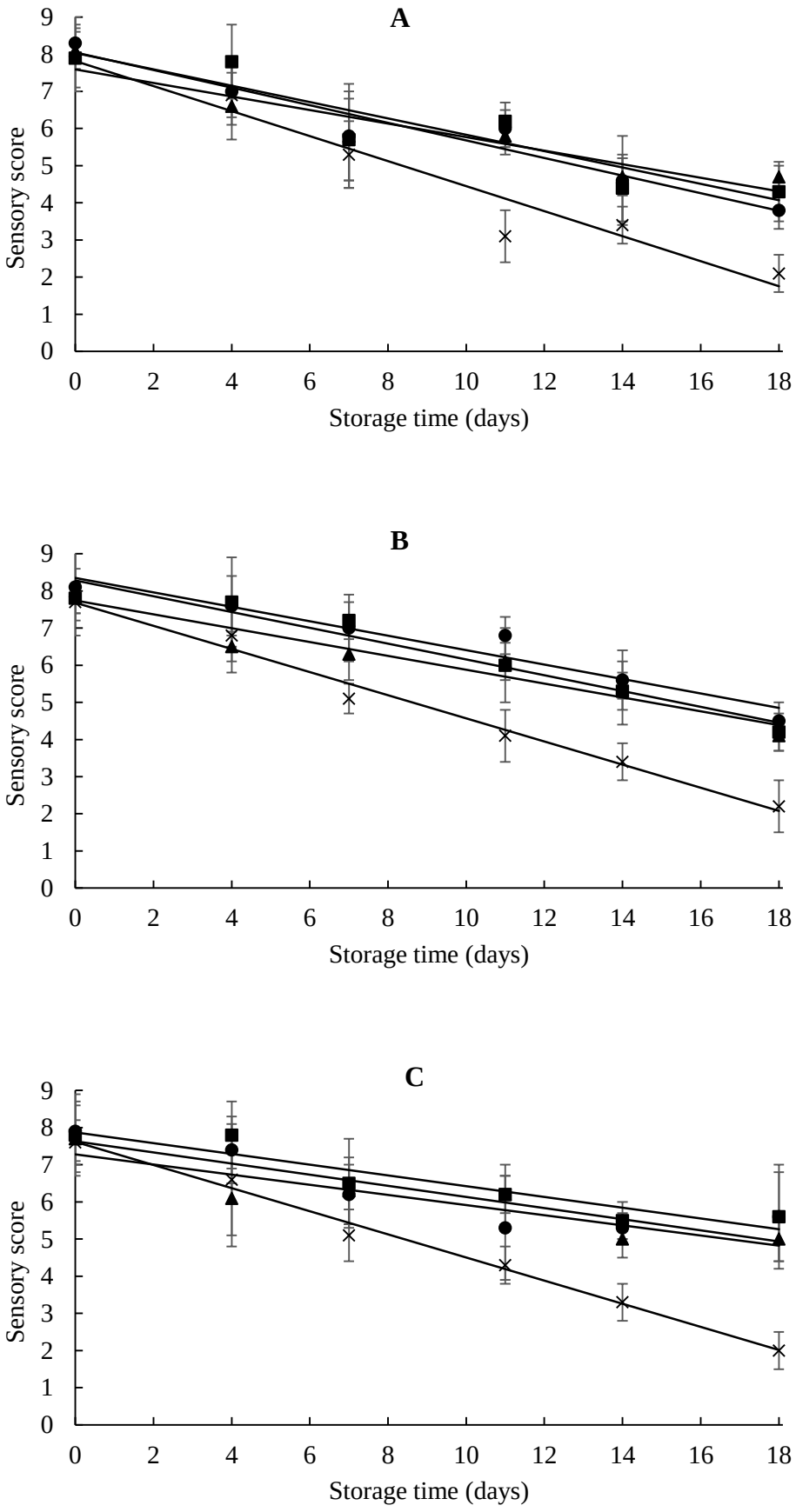
A
B
C
Sensory score
Storage time (days)
0
1
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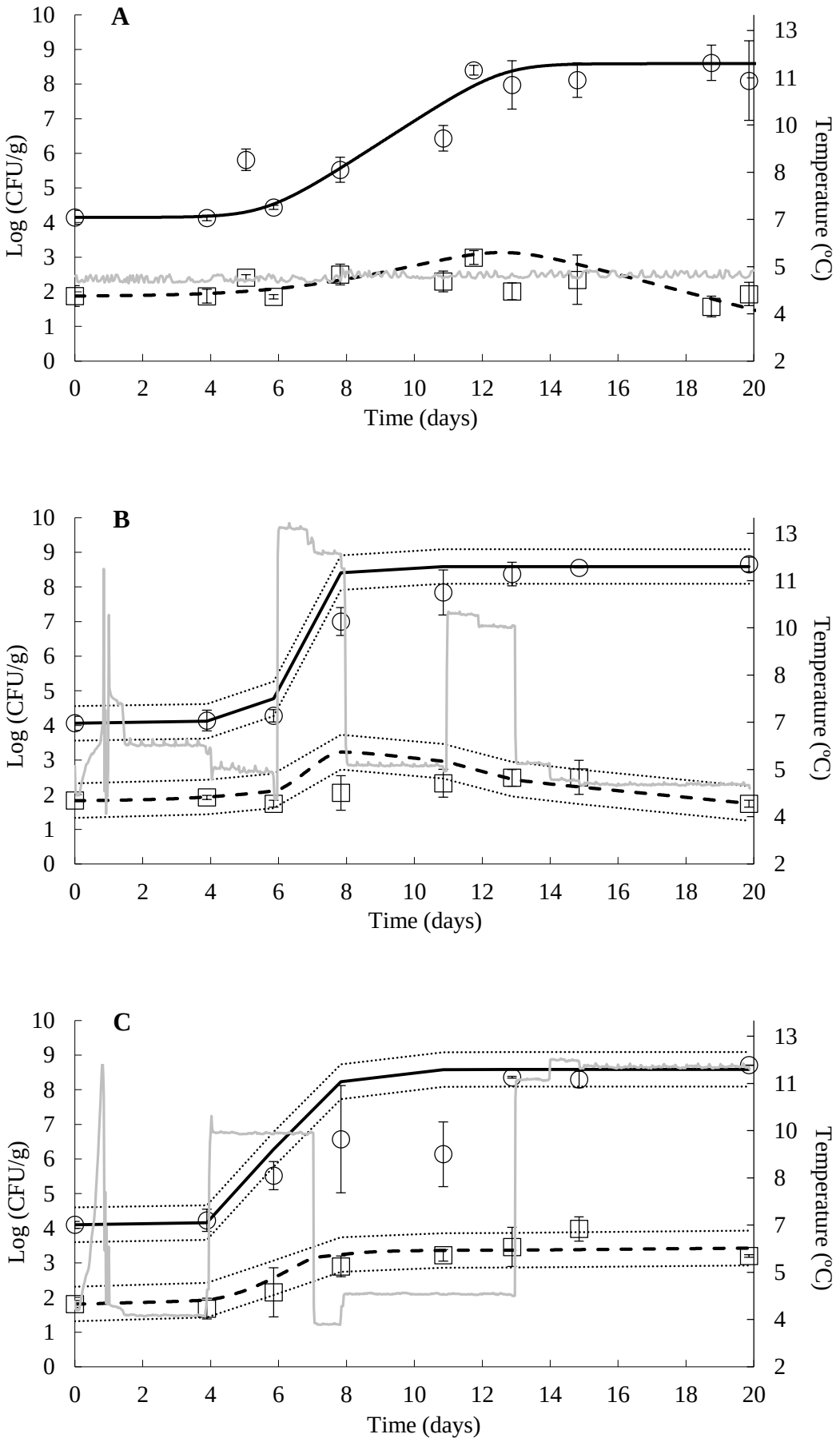
Figure 2


A
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Log (CFU/g)
Temperature (°C)
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0
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Figure 1. Sensory scores obtained for the attribute appearance (A), odour (B) and texture (C) for vacuum-packed hot-smoked sea bream samples non-inoculated (●) and inoculated at different initial levels of *L. sakei* CTC494: 2 log CFU/g (■), 4 log CFU/g (▲) and 6 log CFU/g (×) under storage at 5 ºC. Data point are the mean values from five panelists with vertical lines denoting ± the standard deviation.

Figure 2. Experimental growth data of *Listeria monocytogenes* CTC1034 (□) and *Lactobacillus sakei* CTC494 (○) on vacuum-packed hot-smoked sea bream during storage at 5 ºC (A) and under dynamic profile 1 (B) and 2 (C). Dashed and solid lines stand for the fit by the Lotka-Volterra model for *L. monocytogenes* and *L. sakei*, respectively. Dotted fine lines define the acceptable simulation zone (ASZ) in (B) and (C). The recorded storage temperature is shown as grey solid line. Growth data points are the mean values from two independent trials with vertical lines showing ± the standard deviation.

Table 1

Sensory deterioration rates (slope ± standard error) obtained for vacuum-packed hot-smoked sea bream samples non-inoculated (control) and inoculated at different initial levels of *L. sakei* CTC494 (2, 4 and 6 log CFU/g) under storage at 5 ºC. Values for the same attribute with different uppercase letters are significantly different ($p \leq 0.05$).

Initial level (log CFU/g)	**Appearance**	**Odour**	**Texture**
Control	-0.237 ± 0.03^{A}	-0.195 ± 0.03^{A}	-0.150 ± 0.04^{A}
2	-0.220 ± 0.05^{A}	-0.213 ± 0.03^{A}	-0.145 ± 0.03^{A}
4	-0.182 ± 0.03^{A}	-0.186 ± 0.03^{A}	-0.136 ± 0.03^{A}
6	-0.337 ± 0.04^{B}	-0.331 ± 0.02^{B}	-0.311 ± 0.01^{B}

Table 2

Estimated lag time (λ), maximum specific growth rate (μ_{max}), maximum population density (N_{max}) ± associated standard error for the individual growth curves of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on vacuum-packed hot-smoked sea bream at 5 ºC.

	λ (hours)	μ_{max} (1/h)	N_{max} (log CFU/g)	Adj. $R^{2,a}$
L. sakei CTC494	133.1 ± 33.7	0.0620 ± 0.006	8.59 ± 0.17	0.977
L. monocytogenes CTC1034	160.3 ± 39.5	0.0240 ± 0.001	ne[b]	0.954

[a] Adjusted coefficient of determination of the fitted Baranyi and Roberts model (Adj. R^2).
[b] The Baranyi and Roberts model (no asymptote) was fitted to *L. monocytogenes* growth data (ne).

Table 3

Estimated inhibition parameters (CPD_1, α_{12}) and goodness-of-fit of the three microbial interaction models used to describe the bioprotective capacity of *L. sakei* CTC494 against *L. monocytogenes* CTC1034 on vacuum-packed hot-smoked sea bream stored at different temperatures.

Microorganism	Temp. (ºC)	n[a]	Jameson model		Modified Jameson model				Lotka-Volterra model			
			RMSE[b]	ASZ (%)[c]	CPD_1[d]	RMSE	AICc[e]	ASZ (%)	α_{12}[f]	RMSE	AICc	ASZ (%)
L. sakei CTC494	5.0	11	0.611	–	8.28	0.676	-1.39	–	2.019	0.642	-5.32	–
	Profile 1[g]	8	0.595	75.0	8.44	0.688	3.72	75.0	1.418	0.638	-1.87	75.0
	Profile 2[h]	8	1.087	62.5	8.59	1.256	13.34	62.5	0.989	1.163	7.74	62.5
L. monocytogenes CTC1034	5.0	11	0.879	–	–	0.506	-7.78	–	–	0.480	-11.71	–
	Profile 1	8	0.932	37.5	–	0.579	0.95	75.0	–	0.536	-4.65	75.0
	Profile 2	8	0.314	87.5	–	0.361	-6.59	87.5	–	0.334	-12.19	87.5

[a] Number of observations (n).
[b] Root Mean Squared Error (RMSE).
[c] Percentage (%) of observed values falling within the acceptable simulation zone (ASZ), defined as the simulated value ±0.5 log-units.
[d] Critical population density for *L. sakei* (CPD_1, log CFU/g) estimated by the modified Jameson effect model fitting Eqs. (1) and (3).
[e] Corrected Akaike Information Criterion (AICc).
[f] Competition factor of *L. sakei* on *L. monocytogenes* (α_{12}) estimated by the Lotka-Volterra model fitting Eqs. (1) and (4).
[g] Dynamic temperature profile ranging from 3.6 to 12.8 ºC (average of 6.3 ºC).
[h] Dynamic temperature profile ranging from 3.3 to 11.8 ºC (average of 7.6 ºC).