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Proceeding Paper

Describing the Fate of Autochthonous Lactic Acid Bacteria in Artisanal Goat's Raw Milk Cheeses during Storage: An Omnibus Modelling Approach †

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Abstract: Artisanal fermented products constitute a worthwhile source of autochthonous lactic acid bacteria (LAB) with biopreservative, technological and organoleptic potential. This study aimed to characterize the fate of autochthonous LAB in artisanal goat's raw milk fresh cheeses at different temperatures by using an omnibus (one-step) modelling strategy. Lab-scale cheese prototypes (10 g) were artisanally elaborated from goat's raw milk and stored at 4, 12, 18 and 25 °C. LAB were enumerated (ISO:15214) during cheeses shelf-life. Growth models were fitted to data, producing maximum growth rates averaged 0.345 ± 0.162 and 3.338 ± 0.907 ln CFU/g/d at 4 and 25 °C, respectively, and population increases averaged 3.388 ± 0.203 and 7.877 ± 1.480 ln CFU/g. The omnibus modelling approach allowed defining a general model that describes the autochthonous LAB growth with a correlation coefficient of 0.991 between experimental and estimated data. These results contribute to the better understanding of autochthonous LAB in artisanal cheeses for their further use as potential new generation biopreservatives.

Keywords: biopreservation; predictive microbiology; fermentation; validation

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1. Introduction

In recent years, consumer demands for food quality throughout the product shelf-life have been rising. This quality is understood as the maintenance of their sensorial properties, for instance, avoiding processes of lipid oxidation or color losses. Additionally, food quality also includes ensuring microbial stability, e.g., preventing food spoilage. However, food industry efforts are especially aimed to minimize the emergence of outbreaks caused by foodborne pathogens [1]. In Spain, the last food safety alerts have been particularly related to the presence of *Listeria monocytogenes* or *Salmonella* spp. in ready-to-eat (RTE) meat products (e.g., cured and cooked sausages), fish (e.g., smoked fish) and dairy products (e.g., fresh cheeses) [2–9].

Current consumer preferences to "green label foods" are displacing the traditional utilization of synthetic preservatives in foods [10]. In this sense, biopreservation is based on the use of natural substances derived from bacteria, fungi, plants or animals, with the

aim of extending the shelf-life of food products while guaranteeing their safety [11]. Lactic acid bacteria (LAB) are microorganisms naturally present in numerous raw materials (e.g., milk or meat) and commonly used in industrial fermentation processes. They are considered as Generally-recognized-as-safe (GRAS) microorganisms and awarded with Qualified Presumption of Safety (QPS) by the Food and Drug Administration (FDA) and European Union [12,13]. Briefly, the production of certain metabolites helps to inhibit the growth of spontaneous microorganisms (ensuring microbiological safety) and optimize fermentation processes (i.e., providing uniformity and quality in the final product).

Artisanal fermented products have been identified as reservoirs of LAB with high-quality potential for their application in foods. The selection of "novel starters" with already well-suited level of adaptation to the physicochemical product conditions results promising for their future utilization as starter cultures.

Characterizing LAB behaviour in the product through the use of predictive microbiology models is fundamental for a better understanding of the impact they may have on the final product's quality. Typically, a primary model is fitted to growth data obtained over time at constant temperatures. The estimated parameters may be applied to a secondary model fitting which describes the effect of environmental conditions on the growth rate [14]. In the case of the one-step or omnibus modelling, both fitting stages are performed at the same time, under a mixed-effects nonlinear regression involving all the experimental conditions and the residual errors linked to them. This is an advantageous approach, since it permits the variability not explained by the environmental conditions to be accommodated by the model parameters [14].

Therefore, this study aimed to characterize the fate of autochthonous LAB, by using an omnibus (one-step) modelling strategy, in goat's raw milk fresh cheeses artisanally manufactured and stored at different temperatures.

2. Materials and Methods

2.1. Lab-Scale Cheese Manufacturing

The production of goat's raw milk fresh cheeses in the laboratory was adapted to the practices and procedures of an artisanal cheesemaker from Málaga (Andalusia, Spain). The ingredients mainly consisted of goat's milk, salt (NaCl, 2% *v*/*v*), calcium chloride ($CaCl_2$, 0.28% *v*/*v*) and rennet (0.28% *v*/*v*), supplied by the producer. Starter cultures were not used for cheese elaboration. First, goat's milk was pasteurized at 72 °C for 15 s at the industry, refrigerated for transportation (< 5 °C) and pre-heated to 30 °C before cheese production in laboratory. The $CaCl_2$ was added, and milk was left for fermentation during 1 h at 32 °C. Subsequently, the commercial liquid rennet was added for coagulation (40 min at 32 °C). After that, the curd was cut and agitated for 10–15 min. The curd was transferred to disinfected molds and pressed for 50–60 min to partially remove the whey. The cheese was incorporated into Falcon tubes to be centrifugated (15,000 rpm for 10 min), later removing the remaining exceeding whey (supernatant). The final lab-scale samples were weighed to reach 10 ± 0.1 g per tube. Finally, samples were stored at different conditions; standard refrigeration temperature of 4 °C for 20 days and abuse temperature conditions of 12, 18 and 25 °C for 15, 10 and 5 days, respectively.

2.2. Validation Experiments

The production of 1 kg fresh cheeses was totally developed in the producer facilities, following the same manufacturing procedure described in the previous section. The cheeses were aired in a conditioned chamber (<9 °C) and vacuum-packaged before of their transportation to the laboratory (<5 °C). Under sterilized conditions, 10 g cheeses were weighted and placed into Falcon tubes. In this case, the storage conditions were fixed at 8 and 15 °C for 15 days. Furthermore, dynamic temperature conditions were tested considering the temperature profile of a production-to-consumption flowchart, designed based on the real information available in the FRISBEE tool [15].

2.3. Microbial Analysis

Successive control points for the microbial enumeration were strategically set throughout the sample shelf-life, according to the storage temperature and the expected LAB growth behavior. Two entire lab-scale cheese samples (10 g) for each temperature condition were analyzed in duplicate per control point. A first dilution was performed by adding the sample into 90 mL of 1% peptone water using a dilutor system (IUL Instruments®, Barcelona, Spain). In cases where high microbial loads were anticipated, ten-fold serial dilutions were conducted in a 0.8% (*w*/*v*) saline solution. Finally, microbial enumeration was carried out following the ISO:15214 standard, incubating at appropriate conditions (10% CO_2 anaerobic atmosphere at 30 °C).

2.4. Statistical Analysis

All the data processing and modelling procedure was developed in R software [16]. The growth of autochthonous LAB in raw goat milk fresh cheeses was described by an omnibus model that coupled a primary model for growth to data-driven polynomial secondary models. The omnibus model was constructed by using the "*predmicror*" R package [17]. Huang's growth equation was the primary model chosen [18]:

$$Y_{ij} = Y_{0j} + Y_{\max j} - \ln(e^{Y_0} + (e^{Y_{max}} - e^{Y_0})\, e^{-\mu_{max}\,\beta\,(t)}) + \varepsilon_{ij}\ ;$$

$$\sqrt{\mu_{\max j}} = (a_0 + u_j) + a_1 * Temperature$$

where Y_{ij} represents LAB concentrations (ln CFU/g) at times *i*, and at the different conditions *j*; and Y_{0j} is the initial microbial concentration (ln CFU/g) at each condition *j*. The environmental condition *j* is defined solely by the temperature of incubation. Since the initial LAB concentration in milk exhibited slightly differences across repetitions, the mean initial microbial concentration Y_0 was adjusted to account for random effects u_j that varied according to the conditions. Y_{max} denotes the maximum population density (ln CFU/g), which is also affected by random deviations due to condition *j*. The maximum growth rate (ln CFU/g/d) of LAB is represented by μ_{max}, and its square root transformation is assumed to be linearly affected by temperature. The terms a_0 and a_1 referred to the intercept and slope of this relationship, respectively. The goodness of fit was assessed by graphs of normality of residuals and by the determination of correlation coefficients of observed values versus the fitted values ($R_{obs\text{-}fit}$); whereas remaining heteroscedasticity was evaluated by the graph of fitted values versus normalized residuals ($R_{fit\text{-}residuals}$).

3. Results and Discussion

3.1. First-Order Modelling

Table 1 summarizes the mean maximum growth rates (μ_{max}), maximum populations (**Y_{max}**) and population increases (ΔY) obtained for the different temperature conditions. The values of μ_{max} steadily increased with temperature from 0.345 ± 0.162 to 3.338 ± 0.907 ln CFU/g/d for 4 and 25 °C, respectively. Particularly, the slope of the relationship between the LAB μ_{max} and temperature increased with the highest temperatures (Figure 1). The Y_{max} values were also higher as the storage temperature increased. However, the ΔY did not display significant differences ($p > 0.05$) between the averaged values obtained for temperatures above 12 °C. LAB growth obtained in this sort of cheeses was substantially higher than in others such as raw sheep milk cured cheeses, where μ_{max} was 0.081 and 0.136 ln CFU/g/d at 12 and 22 °C, respectively [19].

Table 1. Maximum growth rates ($\mu_{max,}$ ln CFU/g/d), maximum population density (Y_{max}, ln CFU) and population increase (ΔY, ln CFU) obtained by primary modelling of LAB growth in fresh lab-scale cheeses stored under different temperature conditions (°C) during their shelf-life. Standard deviation of the replicates is shown in brackets.

Temp (°C)	μ_{max}	Y_{max}	ΔY
4	0.345 (0.162)	13.085 (2.412)	3.388 (0.203)
12	0.833 (0.411)	18.820 (1.998)	7.870 (2.333)
18	2.011 (0.690)	19.907 (1.201)	7.917 (0.952)
25	3.338 (0.907)	20.749 (0.493)	7.877 (1.480)

[1] The experiment was performed by triplicate. Averages were obtained from data collected for each experiment, by duplicate for each cheese sample analyzed per control point (n = 2).

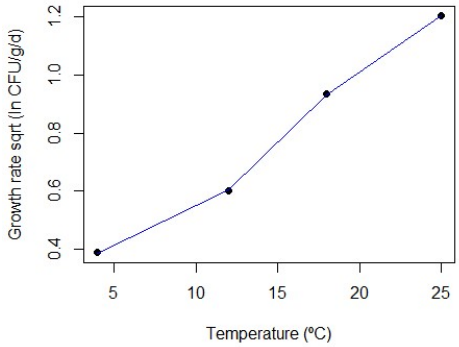
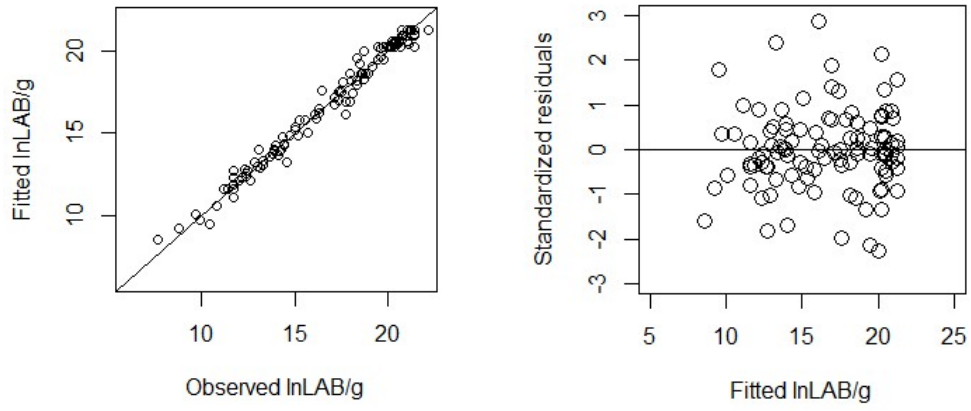


Figure 1. Linear relationship between the square root of the maximum growth rate ($\sqrt{\mu_{max}}$) of LAB in lab-scale fresh cheeses (expressed as ln CFU/g/d) and the temperature (°C) of cheese storage.

3.2. Omnibus Modelling

Estimates of the fixed parameters were determined, where Y_0 was 11.658 ± 0.547 ln CFU/g and Y_{max} was 19.961 ± 0.411 ln CFU/g. The intercept a_0 was estimated at a value of 0.159 ± 0.129, while the slope a_1 at a value of 0.066 ± 0.008. All these estimated where statistically significant ($p < 0.001$).

The model's validity was assessed by measuring the goodness-of-fit. The $R_{obs\text{-}fit}$ was determined, obtaining a value of 0.991, meanwhile $R_{fit\text{-}residuals}$ was 0.042. The standardized residuals were also plotted against the fitted LAB concentration values (Figure 2), where the visual dispersion is an indicative of strong goodness of fit.



(**A**) (**B**)

Figure 2. Omnibus model's goodness-of-fit including the coefficient of correlation of the observed values versus the fitted ln LAB/g (**A**) and the coefficient of correlation of the fitted values versus the model's residuals (**B**).

3.3. Internal Validation

First internal validation was performed by removing from the dataset, used for the omnibus model set up, the growth data obtained for the two middle temperatures (12 and 18 °C) by separated. The model was run for the new dataset (remaining three conditions of temperatures). In case of 12 °C data left out, the prediction made by the model for this temperature presented an optimal bias factor (*Bf*) of 1.100, an accuracy factor (*Af*) of 1.175 and RMSE of 1.360 ln CFU/g. The average mean deviation from the observed data was of 7.326%, the R^2 of 0.715 and the mean square error of 2.550. For the second validation example discarding 18 °C data, the prediction made by the model presented a *Bf* of 1.034, *Af* of 1.165 and RMSE of 1.445 ln CFU/g. For the evaluation of the RMSE in products such as milk, values ranging 0.4 to 0.8 log CFU/mL have been considered as accurate [20]. The average mean deviation from the observed data was 6.756%, with a R^2 value of 0.778.

3.4. External Validation

3.4.1. Fixed Temperatures

LAB growth curves obtained in cheeses produced in industry and stored under the temperatures of 8 and 15 °C were separately fitted to the Huang's model equations. The estimated parameters obtained for the fixed temperatures were compared with the estimates of the model built with the lab-scale cheeses. *Af* and *Bf* obtained values of 1.016 and 1.000, and 1.013 and 1.000, for LAB growth fitting at 8 and 15 °C, respectively. A difference of 0.237 in average was determined between the $\sqrt{\mu_{max}}$ obtained for the industrial cheeses and the lab-scale ones. The slope was modified with a correction factor of 1.235 in the model equation for improving adjustment.

3.4.2. Dynamic Temperatures

All the parameters defining the omnibus model constructed in lab-scale fresh cheeses (Y_{max} and Y_0 equal to 19.960 and 10.683), including the correction factor 1.235, were considered for this validation trial.

As shown in Figure 3, the fitting was nearly optimal (*Af* = 1.037 and *Bf* = 1.019) for the growth curves of autochthonous LAB in fresh cheeses subjected to dynamic temperatures along the cheese production chain, considering abuse conditions. This fact underscores the model's functionality in assessing the behaviour of specific LAB strains in real-life manufacturing scenarios.

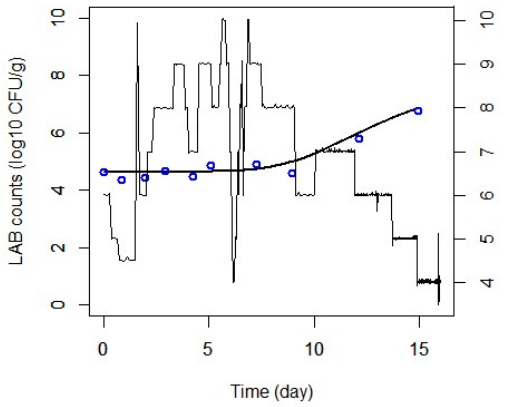


Figure 3. Observed growth data (single replicate) for the autochthonous LAB strains at dynamic temperatures (blue markers) and fitted model predictions from the omnibus modelling (black line).

4. Conclusions

The omnibus modelling allowed to define a general model describing the fate of autochthonous LAB in artisanal raw goat milk cheeses. These results contribute to the better

understanding of the role of autochthonous LAB for their further use as potential new generation biopreservatives. This area of investigation reinforces the quality of these products in the Mediterranean region.

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