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Article

Growth of *Listeria monocytogenes* in Goat's Pasteurized Milk Cheese During Maturation: Its Prediction from a Milk Model Medium

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Abstract

Previous research showed that a strain of *Leuconostoc mesenteroides*, isolated from goat's raw milk cheese, was effective in slowing down the growth and reducing the maximum concentration of *L. monocytogenes* when evaluated in a milk model; and, furthermore, that the extent of inhibition was dependent on the milk initial pH. The objectives of this study were: (1) to determine whether the growth of *L. monocytogenes* in goat's pasteurized milk cheese during maturation could be approximated from growth data obtained in the milk model medium, either in monoculture or in coculture with *L. mesenteroides*; and if so; (2) to model a milk-to-cheese conversion factor (Cf) for *L. monocytogenes* growth rate. Challenge tests were conducted by inoculating *L. monocytogenes* in monoculture and in coculture with *L. mesenteroides* in goat's pasteurized milk adjusted at initial pH levels of 5.5, 6.0 and 6.5. The process of cheesemaking went on, and cheeses were ripened at 12 °C during 12 days. Each experimental growth curve was adjusted to a pH-driven dynamic model where the microbial maximum growth rate is a function of pH. As observed in the milk model medium, in coculture with *L. mesenteroides*, the optimum growth rate (μ_{opt}) of *L. monocytogenes* in maturing cheese was affected by the initial pH of milk: the lowest rate of 0.863 ± 0.042 day⁻¹ was obtained at the initial pH 5.5, in comparison to 1.239 ± 0.208 and 1.038 ± 0.308 day⁻¹ at pH 6.0 and 6.5, respectively. Regardless of the milk initial pH, *L. mesenteroides* did not reduce the maximum load of *L. monocytogenes* in maturing cheeses, as it did in the milk medium. By contrary, at the milk initial pH of 5.5, 6.0, and 6.5, *L. mesenteroides* was able to decrease, on average, 2.2-fold, 1.5-fold and 1.9-fold the μ_{opt} of *L. monocytogenes* in both milk medium and cheese, without significant differences between matrices. Following such validation in goat's cheese, the square-root of milk-to-cheese Cf for *L. monocytogenes* was estimated as 0.751 (SE=0.0108), and type of culture (monoculture, coculture) was not found to affect Cf (p=0.320). In conclusion, this work validated pre-acidification of milk as an efficient strategy that, when combined with the use of a protective culture, can synergically enhance the control of *L. monocytogenes* in cheese.

Keywords: Lactic acid bacteria; *Leuconostoc mesenteroides*; dairy; predictive microbiology; microbial competition; dynamic model

1. Introduction

Goat's milk cheese is among the most widely consumed dairy products worldwide and plays a key role in the Mediterranean diet [1,2]. However, a few microbiological hazards, such as *Listeria monocytogenes*, *Salmonella* spp., enterotoxigenic *Escherichia coli* and *Staphylococcus aureus*, can contaminate goat's milk cheese; particularly if made with raw or unpasteurized milk [3]. The presence of *L. monocytogenes* in cheese is well-documented and may arise from multiple contamination sources throughout production and distribution. Contamination can occur through raw milk, inadequate pasteurization, or post-pasteurization events (i.e., cross-contamination scenarios during cheesemaking, ripening, or storage), as well as improper handling at retail or consumer level—often linked to poor hygiene practices, contaminated equipment, or the documented pathogen's notable resistance to commonly used sanitizing agents [4–6]. The organism's psychrotrophic nature, tolerance to acid and high salt conditions, and ability to form biofilms enable its persistence throughout cheese ripening, storage, and distribution, even at low initial contamination levels [7–10].

According to European Food Safety Authority (EFSA) Zoonoses Report [11], in 2022, the overall occurrence of *L. monocytogenes* in RTE milk products was 0.37%, with average prevalence rates of 0.87% in milk, 0.18% in pasteurized milk cheeses, and 1.3% in cheeses made from raw or low-heat-treated milk. *L. monocytogenes* is the etiological agent of human listeriosis, a foodborne disease that typically manifests as a self-limiting gastrointestinal infection in immunocompetent individuals [12,13]. However, in high risk populations collectively referred to as YOPI (young, old, pregnant and immuno-compromised), the infection may become a systemic invasive disease, leading to life-threatening complications such as septicemia, meningitis, and miscarriage [4,14]. The pathogen's high-risk profile is largely attributed to its ability to cross critical physiological barriers, including the intestinal epithelium, the blood–brain barrier, and the fetoplacental barrier [12,15]. The disease's high fatality rate (20–30%) and severe clinical sequelae further highlight the major public health concern posed by listeriosis [13,16].

Risk assessments have demonstrated that most listeriosis cases are associated with the consumption of high doses of *L. monocytogenes*, particularly when pathogen concentration does not meet the current criteria throughout shelf life (≤ 100 CFU/g) [17,18]. To mitigate this risk, a number of intervention strategies and control measures can be applied during processing, such as the incorporation of bacteriocinogenic lactic acid bacteria (LAB), quick acidification, surface treatments or coating containing antimicrobials, and rigorous sanitation practices [6,19–23].

The use of bacteriocin-producing LAB is considered one of the most efficient strategy for controlling *L. monocytogenes* in cheese because they provide a natural, dual-action approach: they compete for resources while also producing peptides that effectively inactivate the pathogen [24,25]. LAB include genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* species [26]. These microorganisms are recognized by EFSA as bearing a Qualified Presumption of Safety (QPS) status, indicating their safe use in food production [27]. Their intentional application in dairy fermentation is widely recognised because: (i) they act as protective cultures that inhibit spoilage and pathogenic bacteria such as *L. monocytogenes*, through multiple mechanisms, including competition for nutrients, inhibition of pathogen adhesion, competitive exclusion, and production of antimicrobial compounds; and (ii) they enhance the sensory attributes of fermented foods through aroma and flavour development, as well as the synthesis of exopolysaccharides that improve texture [28–31]. As essential microorganisms in fermentation, LAB convert lactose into lactic acid, the primary antimicrobial compound which lowers pH and creates an inhospitable environment for pathogen growth, representing one of the main biopreservation mechanisms in fermented foods [32,33]. Beyond lactic acid, LAB synthesise bacteriocins, hydrogen peroxide, acetoin, carbon dioxide, and different low-molecular-weight metabolites (e.g., diacetyl, reuterin, reutericyclin, fatty acids), all of which enhance food safety and extend shelf life by inhibiting foodborne pathogens and spoilage organisms [29,33]. The anti-listerial activity of LAB may become even more effective when combined

with additional processing hurdles, particularly acid stress, expressed as the local concentration of protons (H^+) or pH, which imposes a major limitation on microbial growth and survival [9,34].

Previous work conducted in a milk model medium demonstrated that the pre-acidification of milk enhances the inhibitory effects of a strain of *Leuconostoc mesenteroides* against *L. monocytogenes* during fermentation, by significantly reducing both the pathogen's growth rate and the maximum concentration [35]. Building upon the kinetic parameters observed in the milk model at different initial pH values, it became essential to validate whether comparable inhibitory effects could be reproduced in an actual cheese matrix during the ripening period.

Thus, this study aims to build on our previous work by determining if such biocontrol capacity of *L. mesenteroides* observed in the milk model can be validated in a dairy product of short maturation such as goat's pasteurized milk cheeses. The objectives of this study were therefore: (1) to determine whether the growth of *L. monocytogenes* in goat's pasteurized milk cheese during maturation could be estimated from growth data obtained in milk model medium, either in monoculture or in coculture with *L. mesenteroides*; and if so; (2) to model a milk-to-cheese conversion factor (Cf) for *L. monocytogenes* growth rate.

2. Materials and Methods

2.1. Bacterial Strains and Culture Preparation

Previous investigations by our research group demonstrated that a strain of *Leuconostoc mesenteroides*, previously isolated from artisanal goat's raw milk cheeses, exhibits anti-listerial and acidifying activities, first observed in vitro [36,37], and later corroborated in challenge tests with *L. monocytogenes* in a milk model medium (i.e., heat-treated reconstituted milk) [35]. The strain was identified using 16S rRNA gene sequencing and selected as the bioprotective culture for the present study. Stock cultures were stored at $-80^{\circ}C$ in Man, Rogosa and Sharpe (MRS) broth (Liofilchem, Roseto degli Abruzzi, Italy) supplemented with 25% glycerol as a cryoprotectant. A loop of the stock culture was initially inoculated into MRS broth and incubated at $30^{\circ}C$ for 24 hours. Subsequently, two successive subcultures were prepared by inoculating 100 μL of the subculture into 10 mL of MRS broth and incubated under the same conditions. Finally, 500 μL of each subculture was inoculated into 200 mL of MRS broth and incubated at $30^{\circ}C$ for 18 hours to achieve a final concentration of approximately 10^7 log CFU/mL. The *L. monocytogenes* strain ATCC 19111, obtained from the culture collection of the Polytechnic Institute of Bragança stock collection, was used. A loop of the stock culture, maintained on Brain Heart Infusion (BHI) agar (Liofilchem, Roseto degli Abruzzi, Italy) slants at $4^{\circ}C$, was inoculated into 10 mL of BHI broth and incubated overnight at $37^{\circ}C$ in an orbital shaker at 200 rpm to achieve an inoculum concentration of approximately 10^5 CFU/mL. Pre-activation was performed in 5 mL of BHI broth under same conditions. Cell concentrations were verified by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Peak Instruments Inc., Version 1701, Houston, TX, USA).

2.2. Manufacture of the Cheese Matrix: Bacterial Strains Inoculation in Milk

Laboratory-scale cheeses were manufactured using pasteurized goat's milk (thermally treated at $65^{\circ}C$ for 30 minutes), to eliminate native microflora and thereby avoid potential effects of microbial heterogeneity on the behavior of *L. monocytogenes* and *L. mesenteroides* [38]. After pasteurization, the pH of milk was adjusted to 5.5, 6.0 or 6.5 using 0.1M HCl or NaOH solutions. The subsequent milk inoculation was carried out according to the experimental design: a total of six challenge test treatments were produced, comprising three monoculture treatments of *L. monocytogenes* at three milk initial pH levels; and three coculture treatments of *L. monocytogenes* and *L. mesenteroides* at three milk initial pH levels. Challenge tests were conducted in duplicate in batches of one-litre milk. Accordingly, in every batch of the monoculture trials, *L. monocytogenes* was inoculated at a target concentration of 10^5 CFU/mL; and with *L. mesenteroides* at a target concentration of 10^7 CFU/mL, in the coculture trials. Using a water bath, each milk batch was maintained at $34 \pm 1^{\circ}C$; and commercial

rennet (0.75 mL/L milk) was then added to initiate coagulation. After 30 minutes, the curd was cut, drained, and transferred into 50 mL Falcon tubes, followed by centrifugation at 6000 rpm for 3.5 minutes at 20 °C. The whey was discarded, and the compacted curd was cut into model cheeses (~5 g each). Salting was the next stage and consisted of immersing cheeses in brine (7.5% NaCl w/v) at a cheese-to-brine ratio of approximately 90 g:1.5 L, for 10 minutes at 25 °C [39]. Finally, the cheeses were weighed (g) and stored at 12°C and 98% relative humidity in a climate-controlled chamber (Aralab, FitoClima 300 ECP 20, Portugal) for 12 days to allow fermentation and maturation.

2.3. Microbiological and Physicochemical Analyses

Microbiological and physicochemical analyses were performed throughout the ripening period, starting on day 0 (day of cheese elaboration) and ending on day 12. For microbiological analyses, serial dilutions were prepared by homogenising each cheese sample in 50 mL sterile Buffered Peptone Water (BPW) (Liofilchem, Roseto degli Abruzzi, Italy) for 60 seconds using a Bag Mixer 400 (Interscience, Saint Nom la Bretèche, France). Appropriate decimal dilutions were then prepared in sterile BPW for microbial enumeration. *L. monocytogenes* was enumerated by plating a 0.1 mL aliquots onto Listeria Palcam agar (Liofilchem, Roseto degli Abruzzi, Italy), added with Listeria Palcam supplement (Liofilchem, Roseto degli Abruzzi, Italy), following the standard ISO method [40]. Typical colonies were counted after incubation at 37 °C for 24 hours. For LAB enumeration, 1 mL aliquots of the dilutions were inoculated into MRS agar (Liofilchem, Roseto degli Abruzzi, Italy), overlaid with 10 mL of 1.2% bacteriological agar (Liofilchem, Roseto degli Abruzzi, Italy), according to ISO method [41]. Plates were incubated at 30 °C for 48 hours, and colonies with typical LAB morphology were counted.

Physicochemical analyses included measurements of pH and water activity (A_w), both performed in duplicate. The pH was determined using a FiveGo pH meter F2 coupled with a LE438 IP67 probe (Mettler-Toledo, Greifensee, Switzerland). A_w was determined using an Aqualab 4TE water activity meter (Decagon, San Francisco, CA, USA).

2.4. Modelling Approach

2.4.1. Modelling the Growth of *L. monocytogenes* in Goat's Milk Cheese During Maturation, and as Affected by *L. mesenteroides*

A total of nine experimental growth curves were obtained: three of *L. monocytogenes* in monoculture, three of *L. monocytogenes* in coculture, and three of *L. mesenteroides* in coculture. The pH-driven dynamic model introduced in Loforte et al. [35] was chosen for modelling these data sets, as it was proven to outperform the microbial Jameson-effect model, and was validated using multiple growth parameters of *L. monocytogenes* in raw and heat-treated milk obtained from the literature [35].

Thus, each of the nine growth curves was adjusted to a pH-driven dynamic model of the form,

$$\frac{dY}{dt} = \mu_{\max} \left(1 - \frac{\exp(Y)}{\exp(Y_{\max})} \right)$$

$$\mu_{\max} = \mu_{\text{opt}} (1 - 10^{pH_{\min} - pH}) \quad (\text{Equation (1)})$$

where Y represents the counts of *L. monocytogenes* or *L. mesenteroides* in time (log CFU/g, base e) whereas $Y_{\max}$ their maximum population densities (log CFU/g). The maximum specific growth rate of *L. monocytogenes* or *L. mesenteroides* ($\mu_{\max}$ in day⁻¹) is set to depend on the matrix pH. The parameter μ_{opt} is the optimum growth rate of *L. monocytogenes* or *L. mesenteroides* in cheese; and $pH_{\min}$ is the minimum pH for growth of *L. monocytogenes* or *L. mesenteroides*. $pH_{\min}$ was set to 4.303 for *L. monocytogenes*, and to 4.000 for *L. mesenteroides* [35].

Model 1 include ordinary differential equations (ODE) that do not have an analytical solution but can be solved with numerical methods. Numerical optimisation consists of searching for the most suitable parameters of the dynamic models such that the residual sum of squares (RSS) of the errors is minimised. The 4th order Runge-Kutta method was adopted to solve ODE while the unknown kinetic parameters were estimated by least-square optimization, using the deSolve and FME

packages implemented in the R software. The kinetic parameters of Equation 1 was estimated using the ‘nlme’ package.

The goodness of fit was assessed for each of the nine growth curves through the estimation of the mean absolute error (MAE) and root mean square error (RMSE), defined as,

$$MAE = \frac{\sum |Y_{obs\ i} - Y_{fit\ i}|}{n}$$
 (Equation (2))

$$RMSE = \sqrt{\frac{\sum (Y_{obs\ i} - Y_{fit\ i})^2}{df}}$$
 (Equation (3))

in addition to the variance of the model residuals. $Y_{fit\ i}$ and $Y_{obs\ i}$ denote each of the i -th concentration of *L. monocytogenes* or *L. mesenteroides* fitted by the dynamic model and its corresponding observation, respectively. The degree of freedom (df) is calculated as ‘ $n - np$ ’, where n is the number of observations of an experimental growth curve and np is the number of parameters of the fitted model.

2.4.2. Prediction of the Growth of *L. monocytogenes* in Goat’s Milk Cheese from a Milk Model Medium

As indicated earlier, previous research focused on determining the kinetic parameters of *L. monocytogenes* in monoculture and in coculture with selected LAB, including *L. mesenteroides*, in reconstituted milk growth medium. Such outcomes were employed in this study and brought together with the growth results in cheese, in order to determine whether the growth of *L. monocytogenes* in cheese during maturation could be approximated from data obtained in milk model. The kinetic parameters of *L. monocytogenes* in the milk model, which are reutilised in the present validation study are summarized in Table 1; and comprises estimates of $\mu_{opt\ LM}$ and $Y_{max\ LM}$ in in monoculture and coculture, at three different milk initial pH levels [35].

Table 1. Kinetic parameters (and standard errors) of *Listeria monocytogenes* (maximum microbial concentration, Y_{max} in log CFU/g (base e), and optimum growth rate, μ_{opt} in day⁻¹) in a milk model medium (i.e., heat-treated reconstituted milk) adjusted to different initial pH levels, in monoculture and coculture with *Leuconostoc mesenteroides*, obtained from Loforte et al. [35].

Milk initial pH	Parameters	Monoculture	Coculture
5.5	μ_{opt}	3.201 (0.060)	1.469 (0.205)
	Y_{max}	20.85 (0.060)	15.05 (0.367)
6.0	μ_{opt}	3.416 (0.177)	2.293 (0.284)
	Y_{max}	21.10 (0.212)	16.32 (0.204)
6.5	μ_{opt}	3.432 (0.073)	1.552 (0.132)
	Y_{max}	21.31 (0.085)	16.91 (0.132)

The prediction analysis from milk model to cheese was then developed in four steps:

A. The effect of the growth medium (goat’s milk cheese and milk) on the kinetic parameters growth rate and maximum concentration of *L. monocytogenes* was graphically assessed for both monoculture and coculture experiments. This allowed to compare the effect of the milk initial pH on the kinetic parameters in the two food matrices.

B. Reduction ratios (RR) of the optimum growth rate of *L. monocytogenes* due to the action of *L. mesenteroides* were calculated in the milk model ($RR_{\mu_{opt\ milk}}$) and in pasteurized goat’s milk cheese ($RR_{\mu_{opt\ cheese}}$), as follows,

$$RR_{\mu_{opt\ milk}} = \frac{\mu_{opt\ LM\ monoculture\ in\ milk}}{\mu_{opt\ LM\ coculture\ in\ milk}}$$
 (Equation (4))

$$RR_{\mu_{opt\ cheese}} = \frac{\mu_{opt\ LM\ monoculture\ in\ cheese}}{\mu_{opt\ LM\ coculture\ in\ cheese}}$$
 (Equation (5))

Six $RR_{\mu_{opt}}$ were obtained, considering that growth rates were obtained at three milk initial pH levels. Similarly, the six reduction ratios of the maximum concentration of *L. monocytogenes* due to the presence of *L. mesenteroides* were calculated as,

$$RR_{Y_{\max} \text{ milk}} = \frac{Y_{\max} \text{ LM monoculture in milk}}{Y_{\max} \text{ LM coculture in milk}} \quad (\text{Equation (6)})$$

$$RR_{Y_{\max} \text{ cheese}} = \frac{Y_{\max} \text{ LM monoculture in cheese}}{Y_{\max} \text{ LM coculture in cheese}} \quad (\text{Equation (7)})$$

The effect of *L. mesenteroides* on the growth rate and the maximum concentration of *L. monocytogenes* was considered as significant if $RR_{\mu \text{ milk}}$, $RR_{\mu \text{ cheese}}$ and $RR_{Y_{\max} \text{ milk}}$, $RR_{Y_{\max} \text{ cheese}}$, respectively, were significantly higher than 1.0. In order to perform such evaluation, standard errors of RR were estimated using the approximation of Stuart & Ord (1998) for the standard deviation of a ratio of two random variables of known standard deviations.

C. A linear model of the form,

$$\sqrt{\mu_{\text{opt cheese}}}_{ic} = \text{CultureType} + \sqrt{Cf} \cdot \sqrt{\mu_{\text{opt milk}}}_{ic} + \varepsilon_{ic} \quad (\text{Equation (8)})$$

was adjusted, where $\sqrt{\mu_{\text{opt cheese}}}_{ic}$ is the square-root of the optimum growth rate of *L. monocytogenes* in cheese obtained at *i*-th milk initial pH (5.5, 6.0 or 6.5) and the *c*-th culture type (monoculture or in coculture with *L. mesenteroides*); $\sqrt{\mu_{\text{opt milk}}}_{ic}$ is the square-root of the optimum growth rate of *L. monocytogenes* in milk obtained at *i*-th milk initial pH and the *c*-th culture type; $\sqrt{Cf}$ is the square-root of the conversion factor for growth rate from milk medium to cheese matrix; and ε_{ic} the model's residuals. A non-significant *CultureType* covariate would mean that there is no effect of the type of culture, and that the linear model could be further simplified. A significant $\sqrt{Cf}$ can be interpreted as evidence that growth data obtained in a milk model medium can be employed to predict the pathogen's kinetics in goat's milk cheese.

D. The final validation was carried out in a separate growth data set. We used, as comparator, growth data (microbial concentrations) of *L. monocytogenes* in cheese made of goat's milk with adjusted milk pH of 6.0, inoculated with *L. mesenteroides*; and left to mature at 12 °C. The growth prediction was worked out through Monte Carlo [42,43] simulation using as input parameters: (1) the estimated growth rate of *L. monocytogenes* in the milk model medium adjusted at pH 6.0 (milk initial pH) in coculture with *L. mesenteroides*; (2) the conversion factor modelled as a normal distribution with mean $\sqrt{Cf}$ and its standard error; and (3) the cheese pH profile during maturation for solving the pH-driven dynamic model (Eq. 1). In this way, the experimental growth in an actual food matrix, cheese, was compared against the dynamic prediction obtained using data from milk medium.

All statistical analyses were carried out in the R software [44] using the packages ggplot2, matrixStats, deSolve and FME.

3. Results and Discussion

3.1. *Listeria Monocytogenes* Growth in Cheese During Ripening

The pH-driven dynamic model effectively described the growth of *L. monocytogenes* in goat's milk cheese, in monoculture, during ripening, as suggested by the significant kinetic parameters at the three initial pH values (Table 2). The goodness-of-fit were comparable among the three curves, with RMSE values of 0.5658, 0.5806, and 0.3851 for milk initial pH of 5.5, 6.0 and 6.5, respectively. As summarised in Table 2 and Figure 1 (blue lines), μ_{opt} of *L. monocytogenes* in maturing cheese was barely affected by the milk initial pH, whereas the maximum population density ($Y_{\max}$) was significantly moderated by the milk initial pH (Figure 1). Yet, interestingly, both μ_{opt} and $Y_{\max}$ followed the same pH-effect trend, reaching higher values at the milk initial pH of 6.0 (1.886 ± 0.465 day⁻¹ and 19.45 ± 0.821 log CFU/g, respectively), and lower values at the pH of 5.5 (1.805 ± 0.177 day⁻¹ and 17.67 ± 0.800 log CFU/g, respectively). The increases in *L. monocytogenes* maximum load capacity ($Y_{\max}$) in cheese at initial pH 6.0 and 6.5 correspond to approximately 1.8 and 1.2 log CFU/g relative to pH 5.5, highlighting the key role of acidity in limiting pathogen proliferation.

Table 2. Monoculture kinetic parameters of *Listeria monocytogenes* (initial and maximum microbial concentration, Y_0 , Y_{max} in log CFU/g (base e), and optimum growth rate, μ_{opt} in day⁻¹) in cheese produced with goat’s pasteurized milk adjusted to different initial pH levels, as estimated by the pH-driven dynamic model. Residuals (σ^2), root mean square error (RMSE) and mean absolute error (MAE) are shown.

Initial pH of milk	<i>L. monocytogenes</i>			Fit quality
	Parameters	Mean (SE)	Pr > t	
5.5	Y_0	13.07 (1.132)	0.055	σ^2 : 0.4268
	μ_{opt}	1.805 (0.177)	0.040	RMSE: 0.5658
	Y_{max}	17.67 (0.800)	0.106	MAE: 0.4002
6.0	Y_0	12.72 (1.163)	0.048	σ^2 : 0.4495
	μ_{opt}	1.886 (0.465)	0.002	RMSE: 0.5806
	Y_{max}	19.45 (0.821)	0.027	MAE: 0.4109
6.5	Y_0	12.73 (0.771)	0.038	σ^2 : 0.1976
	μ_{opt}	1.845 (0.275)	0.024	RMSE: 0.3851
	Y_{max}	18.87 (0.545)	0.018	MAE: 0.2724

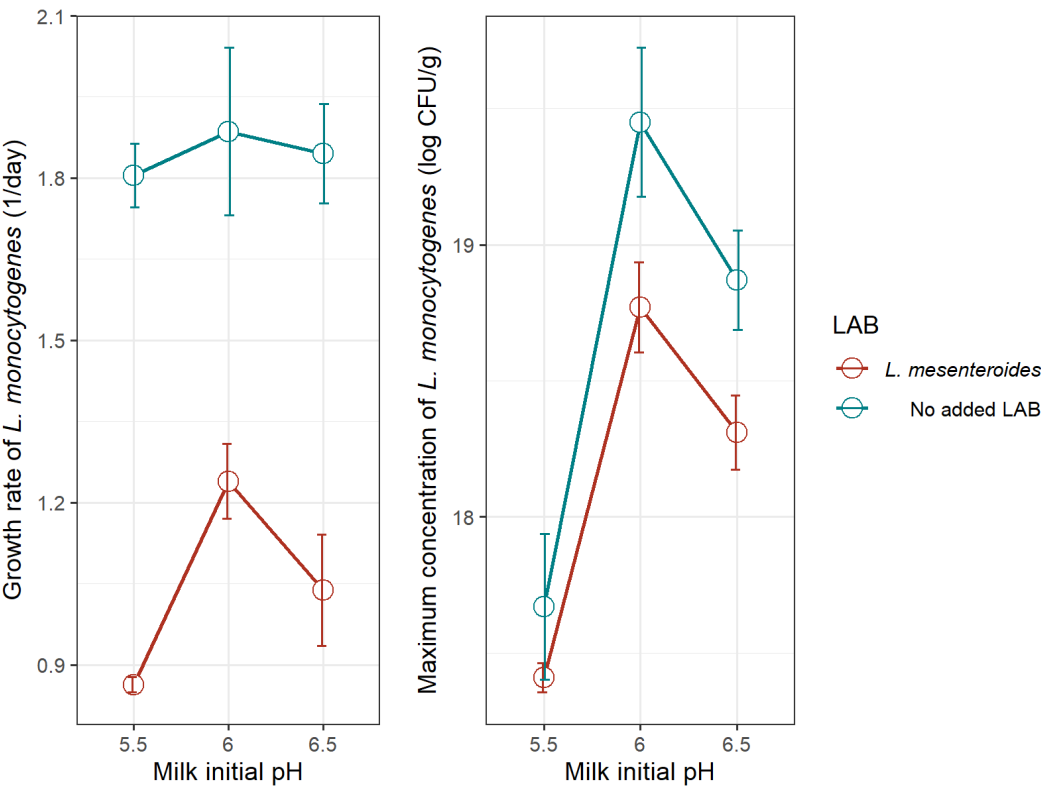


Figure 1. Effect of *L. mesenteroides* on the optimum growth rate (left) and maximum concentration of *L. monocytogenes* (right) in cheeses elaborated with pasteurized goat’s milk adjusted at different pH, as estimated by the pH-driven dynamic model. Bars represent $\pm$ standard error of the parameter estimate.

The influence of pH on the growth of *L. monocytogenes* corroborates the cardinal pH model proposed by Rosso et al. [45], which postulates pH as a critical environmental parameter affecting microbial proliferation, whereby growth rates decline as pH move away from the organism’s optimal range. Most bacteria typically thrive near neutral pH ranges, whereas acidic environments restrict both microbial growth and community diversity [20]. *L. monocytogenes* can grow and survive across a broad pH range (4.4–9.6), with an optimum range between 6 and 8 [46]. However, under acid conditions (e.g., pH <5.5), the higher concentration of hydrogen ions (H⁺) reduces intracellular pH and impairs the pathogen’s ability to maintain cellular homeostasis due to the influx of protons and

anions into the cytoplasm, which disrupts essential metabolic functions and can damage proteins, nucleic acids, and cell membranes [34,47–49].

The inhibitory effect of acidic environments on *L. monocytogenes* has been well documented by other authors. For instance, Martín et al. [50] observed that lower pH in BHI broth led to reduced pathogen counts, with the most pronounced growth restriction occurring at pH 4.5 — close to the lower pH limit for growth (~ pH 4.2) [51]. At such acidity levels, *L. monocytogenes* may remain viable but in a non-proliferative state [50]. Tirloni et al. [23], using a cardinal parameter model for *L. monocytogenes* growth in fresh *Ricotta* cheese, reported a ~ 23% reduction in the maximum specific growth rate ($\mu_{\max}$: from 0.197 h⁻¹ to 0.151 h⁻¹) as pH decreased from 6.61 to 5.49. Likewise, Kapetanakou et al. [52] reported that pH strongly influences *L. monocytogenes* survival in different cheeses types: higher pH cheeses [e.g., *Mascarpone* (6.45), *Ricotta* (6.64), *Camembert* (6.30), and *Halloumi* (6.60)] supported more pathogen growth compared to lower pH varieties [e.g., *Cottage* (5.03), *Edam* (5.58), and *Gouda* (5.53)], highlighting acidity as a natural hurdle. Supporting these findings, Leong et al. [53] observed more pronounced microbial growth in cheeses with higher pH (5.41–6.50) compared to those ranging from 4.29 to 5.40. Although in a food product different from cheese, soymilk, Ariahu et al. [54] demonstrated that higher initial pH (~6.8) values increased both the exponential growth rate and the maximum load capacity of *L. monocytogenes*. It is worthy to point out that in the previous examples, it was the pH of cheese — as a final product, which was demonstrated to effectively slow down the growth of *L. monocytogenes* during shelf life. In our study, it was the pH of milk — as raw material, which was changed in order to assess its effectiveness as a pre-fermentation intervention strategy to control this pathogen in cheesemaking.

The growth kinetics parameters of *L. monocytogenes* and *L. mesenteroides* in goat's milk cheese from the coculture experiments at different milk initial pH values (5.5, 6.0 and 6.5) are presented in Table 3. Overall, for all the growth curves, the model estimated parameters with high precision, as implied by the highly significant p-values. The goodness-of-fit measures were comparable among all the growth curves, with RMSE values ranging between 0.0312 and 0.4335. It was found that, regardless of the milk initial pH (5.5, 6.0 and 6.5), *L. mesenteroides* was able to reach higher $Y_{\max}$ (20.49 ± 0.231 ; 22.00 ± 3.964 ; 21.57 ± 0.187 log CFU/g, respectively) than *L. monocytogenes* (17.41 ± 0.163 ; 18.77 ± 0.497 ; 18.31 ± 0.407 log CFU/g, respectively) by a factor of about 1.2 in the three cases. This phenomenon is very likely to obey to the Jameson-effect microbial competition [55,56], which is often a non-specific interaction between two microbial populations resulting from the depletion of a common nutrient or the accumulation of waste products, although it can also be caused by specific inhibitors or environmental changes, such as acidification, produced by the dominant species (i.e., *L. mesenteroides*) [57].

Figure 1 displays the coculture growth kinetic parameters of *L. monocytogenes* alongside the monoculture counterparts, for comparison. There, it is noticeable that the presence of *L. mesenteroides* affected the growth kinetics of *L. monocytogenes* in maturing cheese, consistently reducing both μ_{opt} and $Y_{\max}$ by comparison to the monoculture, regardless of the milk's initial pH. Such reductions, due to LAB addition, can be regarded as clearly different, as implied by the non-superimposed standard error bars in Figure 1. Furthermore, the pH-effect patterns of *L. monocytogenes* μ_{opt} and $Y_{\max}$, in coculture with *L. mesenteroides* (red lines), mimicked those of *L. monocytogenes* alone (blue lines). It was also noticeable, that unlike the monoculture *L. monocytogenes* μ_{opt} , when *L. mesenteroides* was added to milk, the pathogen's μ_{opt} became significantly affected by the initial pH (notice the non-superimposed standard error bars). Thus, it can be said that the significantly lowest μ_{opt} value of *L. monocytogenes* in coculture was encountered at the milk initial pH of 5.5 (0.863 ± 0.042 day⁻¹). Likewise, the pathogen's lowest $Y_{\max}$ (17.41 ± 0.163 log CFU/g) was encountered at the initial pH of 5.5 when milk was added with the LAB culture. Once again, the fact that the highest *L. monocytogenes* μ_{opt} in cheese was observed at the midpoint milk pH of 6.0 is supported by the cardinal pH model theory [58,59].

Table 3. Coculture kinetic parameters of *Listeria monocytogenes* and *Leuconostoc mesenteroides* (initial and maximum microbial concentration, Y_0 , Y_{max} in log CFU/g (base e), and optimum growth rate, μ_{opt} in day⁻¹) in cheese produced with goat’s pasteurized milk adjusted to different initial pH levels, as estimated by the pH-driven dynamic model. Residuals (σ^2), root mean square error (RMSE) and mean absolute error (MAE) are shown.

Initial pH of milk	<i>L. monocytogenes</i>			<i>L. mesenteroides</i>	
	Parameters	Mean (SE)	Pr > t	Mean (SE)	Pr > t
5.5	Y_0	13.08 (0.049)	<0.001	16.88 (0.205)	<0.001
	μ_{opt}	0.863 (0.042)	<0.001	0.646 (0.087)	0.005
	Y_{max}	17.41 (0.163)	<0.001	20.49 (0.231)	<0.001
	Fit quality				
	σ^2	0.0012		0.0259	
	RMSE	0.0312		0.1468	
	MAE	0.0239		0.1280	
6.0	Y_0	12.45 (0.479)	<0.001	15.91 (0.525)	<0.001
	μ_{opt}	1.239 (0.208)	<0.001	0.713 (0.082)	0.030
	Y_{max}	18.77 (0.497)	<0.001	22.00 (3.964)	0.011
	Fit quality				
	σ^2	0.1390		0.1709	
	RMSE	0.3404		0.3780	
	MAE	0.2468		0.3446	
6.5	Y_0	12.56 (0.615)	<0.001	15.54 (0.268)	<0.001
	μ_{opt}	1.038 (0.308)	0.043	1.009 (0.091)	<0.001
	Y_{max}	18.31 (0.407)	<0.001	21.57 (0.187)	<0.001
	Fit quality				
	σ^2	0.2256		0.0636	
	RMSE	0.4335		0.2336	
	MAE	0.3697		0.1780	

This pattern showed in Figure 1 illustrates a clear interaction between acidity and microbial competition; whereby more acidic conditions enhance the inhibitory effect of *L. mesenteroides* on *L. monocytogenes*. This observation aligns with the systematic review and meta-analysis earlier conducted by Loforte et al. [60], which demonstrated that LAB’s antagonistic effects against *L. monocytogenes* are significantly strengthened in low pH environments (pH < 5.5). Intrinsic factors such as pH, organic acid concentration, and LAB metabolic activity act synergistically through multiple mechanisms, including acidification, competitive exclusion, and bacteriocin synthesis, creating an hostile environment that restricts both the survival and proliferation of *L. monocytogenes* [9,20,25]. The critical role of environmental pH is highlight by the pathogen’s minimum growth threshold, ranging from pH 4.34 to 5.93, which means foods matrices with lower pH values can effectively suppress its growth [61]. Moreover, acidic conditions favour acid-tolerant LAB, enhancing their ecological competitiveness while simultaneously constraining pathogenic microorganisms [62].

L. mesenteroides subsp. *mesenteroides* is among the main *Leuconostoc* species naturally present in milk and dairy matrices, where it plays a key technological role by producing gas (mainly CO₂) and flavour compounds characteristic of fermented products [63,64]. Beyond its technological relevance, other authors [65–68] demonstrated that *L. mesenteroides* exhibits strong antagonistic activity against *L. monocytogenes*, mainly through the synthesis of bacteriocins such as leucocin K7 and mesentericin Y105. Experimental studies provide clear evidence for this inhibitory capacity: Wadhawan et al. [65] reported complete inhibition of *L. monocytogenes* 2203 growth by *L. mesenteroides* isolated from the surface microbiota of wooden cheese ripening boards. Similarly, Borges et al. [69] showed that *L. mesenteroides* subsp. *mesenteroides* SJRP55 reduced *L. monocytogenes* counts by 0.7 log CFU/mL in fermented cream after 28 days of storage when cocultured with *Lactococcus* spp., whereas *Lactococcus*

alone did not suppress the pathogen. In another study, a bacteriocin-producing *L. mesenteroides* isolated from traditional Chinese tofu exhibited strong anti-listerial activity in milk [66]. The combined effects of acidification, nutrient competition, and bacteriocin production create a hostile environment that restricts *L. monocytogenes* survival and growth [9,25]. The intentional use of LAB strains with well-known antimicrobial activity represents a promising biopreservation strategy for fermented dairy products, including cheese [55]. Such an approach enhances food safety, extends shelf life, and meets the growing consumer demand for minimally processed and naturally preserved foods [70].

3.2. Prediction of the Growth of *L. monocytogenes* in Goat’s Pasteurized Milk Cheese from a Milk Model Medium

3.2.1. Comparison of *L. monocytogenes* Growth Kinetics in the Milk Model and Goat’s Pasteurized Milk Cheese in Monoculture and Coculture

The comparison of *L. monocytogenes* μ_{opt} in monoculture and coculture with *L. mesenteroides* in the milk model and goat’s pasteurized milk cheese at different milk initial pH values (5.5, 6.0, and 6.5) reveals clear matrix and pH dependent effects (Figure 2). In both matrices, milk and cheese, the monocultured *L. monocytogenes* consistently exhibited higher μ_{opt} (left panel) when compared to coculture (right), highlighting the inhibitory effect of *L. mesenteroides*, regardless of the milk initial pH level. As earlier discussed, *L. monocytogenes* growth is notably reduced at pH 5.5, reinforcing acidity as a stringent barrier to microbial proliferation in both matrices. Figure 2 also illustrates that the μ_{opt} estimates are systematically lower in cheese than in the milk model, reflecting the intrinsic structural constraints of the cheese matrix—namely reduced moisture, and limited nutrient diffusion, all of which restrict pathogen growth [71–73]. The solid nature of cheese imposes diffusion rate limitations that restrict bacterial proliferation [71]. Taken together, these observations demonstrate a synergistic effect whereby acidity, LAB-mediated competition, and cheese solid structure impose additional barriers to *L. monocytogenes* proliferation. These findings emphasize the importance of physicochemical factors in controlling pathogen behavior during cheeses maturation and highlight the strong bioprotective potential of *L. mesenteroides* under acidic conditions characteristic of fermented dairy systems.

The comparison of *L. monocytogenes* Y_{max} in the milk model and goat’s pasteurized milk cheese at initial pH values of 5.5, 6.0, and 6.5 reveals clear effects of matrix type and acidity (Figure 3). In both food media, monocultured *L. monocytogenes* reached significantly higher maximum populations at all tested milk initial pH levels than those obtained in coculture with *L. mesenteroides*. Acidic conditions, particularly at pH 5.5, restrict the pathogen’s final population density relative to higher pH values [23,74]. *L. monocytogenes* adapts to acid stress through several homeostasis mechanisms, including the F_0F_1 -ATPase, the glutamic acid decarboxylase (GAD) system, and the arginine and agmatine deiminase, which increase cytoplasmic buffer capacity and help maintain intracellular pH [75–78]. Nevertheless, exposure to acidic conditions increases membrane permeability and disruption of cellular metabolism, ultimately reducing growth rates and population densities [79].

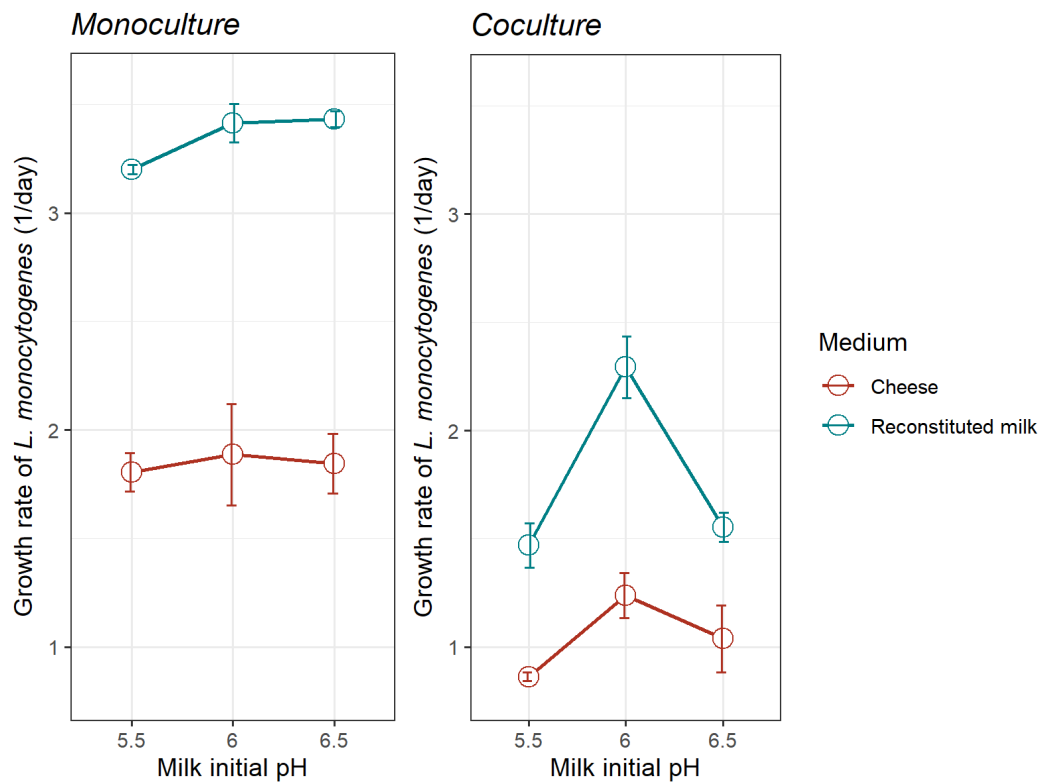


Figure 2. Comparison of the optimum growth rates of *L. monocytogenes* in reconstituted milk model medium and goat's pasteurized milk cheese, obtained in monoculture (left) and in coculture with *Leuconostoc mesenteroides* (right). Bars represent $\pm$ standard error of the estimate.

Unlike the μ_{opt} estimates, where the milk model systematically supported faster *L. monocytogenes* growth in comparison to cheese, regardless of the type of culture (Figure 2); the milk model supported higher Y_{max} of *L. monocytogenes* than cheese only in monoculture (Figure 3). When cocultured with *L. monocytogenes*, the pathogen's reached higher carrying capacity in cheese than in the milk model, this can be explained by the fact that the inhibitory substances or antimicrobial metabolites produced by *L. mesenteroides* are of limited diffusion within the cheese solid matrix [71]. In monoculture, when there is no production of anti-listerial substances, *L. monocytogenes* population was able to reach a greater maximum carrying capacity in the liquid media (i.e., milk), as water availability is higher and nutrient diffusion less limiting [38]. Overall, these Y_{max} patterns highlight that the final pathogen load is determined by the combined influence of acidity, microbial competition, and the physical structure of medium.

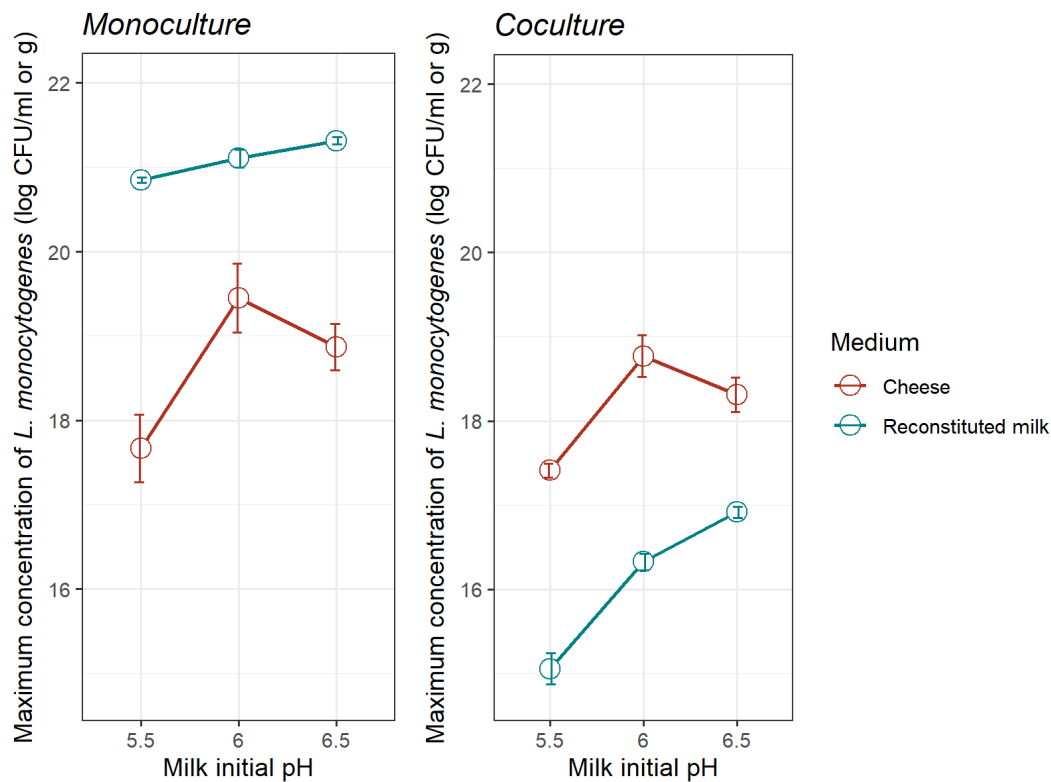


Figure 3. Comparison of the maximum concentration of *L. monocytogenes* in reconstituted milk model medium and goat's pasteurized milk cheese, obtained in monoculture (left) and in coculture with *Leuconostoc mesenteroides* (right). Bars represent ± standard error of the estimate.

3.2.2. Reduction Ratio of the Optimum Growth Rate of *L. monocytogenes* Due to the Presence of *L. mesenteroides* in the Milk Model and in Pasteurized Goat's Milk Cheese

The RR assessment was carried out to determine whether the reductions in *L. monocytogenes* μ_{opt} and Y_{max} due to competition with *L. mesenteroides* are statistically different between the milk model and cheese. The reduction ratios (RR) of *L. monocytogenes* μ_{opt} in the milk model and goat's pasteurized milk cheese at initial pH levels of 5.5, 6.0, and 6.5 consistently demonstrated the significant inhibitory effect exerted by *L. mesenteroides*; as implied by all the RR standard error bars laying well above the cut-off of 1.0 (no effect) (Figure 4). During milk fermentation or cheese maturation, *L. mesenteroides* was able to reduce by at least 1.5-fold the growth rate of *L. monocytogenes*. At the lowest evaluated milk initial pH of 5.5, the competition with the LAB reduced the growth rate of the pathogen in goat's milk cheese by half (RR=2.10) (Figure 4).

Furthermore, the μ_{opt} RR patterns for the milk model and cheese did not differ significantly (overlapping standard error bars in Figure 4). This would suggest that the inhibitory capacity of *L. mesenteroides* on *L. monocytogenes* μ_{opt} could be only minimally influenced by the matrix-specific physicochemical differences. This reinforces the strong bioprotective potential of *L. mesenteroides*, whose antimicrobial activity, driven by organic acid production, competitive exclusion, and bacteriocin synthesis, limits pathogen proliferation in both liquid and solid dairy products [66,80]. Moreover, and very relevant from the viewpoint of predictive microbiology, the non-significant effect of matrix on the μ_{opt} RR establishes that the growth rates of *L. monocytogenes* obtained from a milk liquid model can be used to predict the pathogen's development in a more complex solid matrix such as cheese.

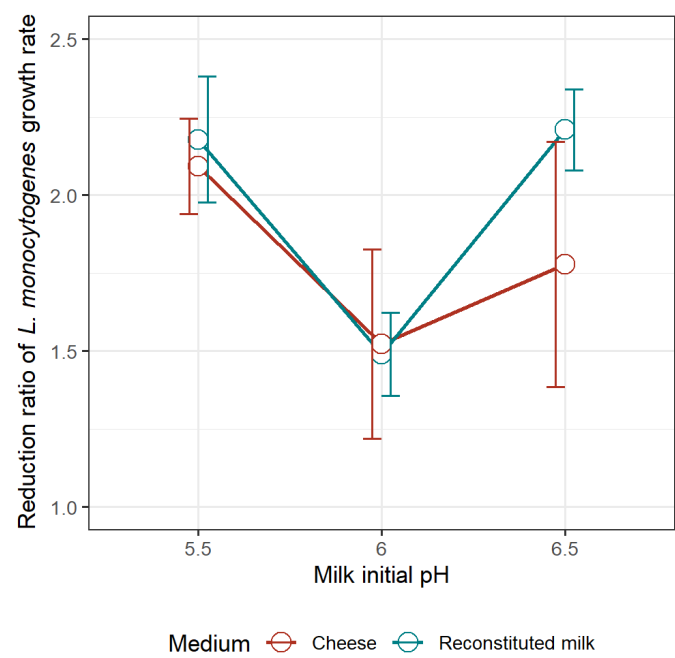


Figure 4. Reduction ratio of the optimum growth rate of *L. monocytogenes* due to the presence of *L. mesenteroides* in reconstituted milk model medium and in goat’s pasteurized milk cheese, as affected by the initial pH of milk. Bars represent ± standard of the estimate.

An analogous RR assessment for Y_{max} is shown in Figure 4. Unlike the outcomes found for *L. monocytogenes* μ_{opt} , there is a significant difference between the milk model and cheese in the level of reduction in the maximum load of *L. monocytogenes* due to competition with *L. mesenteroides* (Figure 5). In the milk model, as the initial pH decreased, *L. mesenteroides* progressively reduced the pathogen’s maximum load. This is consistent with the fact that acidification limits the pathogen’s carrying capacity [23].

However, whereas in milk the RR of Y_{max} was significantly higher than the cut-off value of 1.0, in cheese the mean RR was only marginally higher than 1.0, and, furthermore, the initial pH of milk was not demonstrated to moderate the level of reduction in the maximum load of *L. monocytogenes* due to competition. In other words, while *L. mesenteroides* exerts some inhibitory effect, it may not significantly reduce the final population density of *L. monocytogenes* during cheese maturation. This discrepancy likely reflects the distinct structural properties of the two matrices: liquid milk enables efficient diffusion of antimicrobial metabolites (e.g., lactic acid, bacteriocins) produced by *L. mesenteroides*, thereby enhancing inhibition, whereas the solid cheese matrix imposes diffusion limitations that restrict the spread and activity of these inhibitory compounds, ultimately diminishing their effect on the final pathogen load [9,71]. Although *L. mesenteroides* effectively suppressed *L. monocytogenes* growth rate in cheese, it did not reduce the pathogen’s maximum load capacity to the same extent as in milk.

3.2.3. Model Validation

Given the absence of a matrix effect, at least statistically, on the extent of reduction in the growth rate of *L. monocytogenes* by the LAB action, there was a theoretical basis to determine a conversion factor (Cf) for estimating the optimum growth rate of *L. monocytogenes* in goat’s milk cheese from the optimum growth rate in the milk model. This can be understood as the validation of a model obtained from broth in actual food.

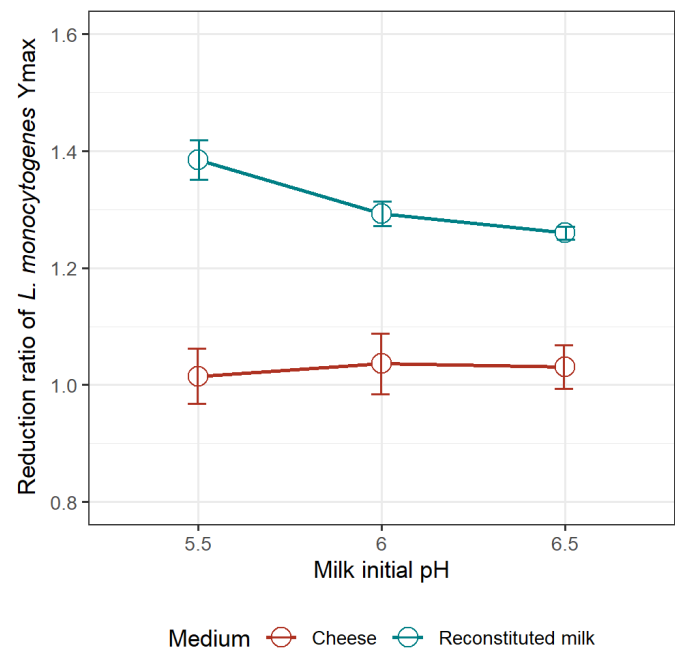


Figure 5. Reduction ratio of the maximum concentration of *L. monocytogenes* due to the presence of *L. mesenteroides* in reconstituted milk model medium and in goat’s pasteurized milk cheese, as affected by the initial pH of milk. Bars represent ± standard error of the estimate.

According to Equation (8), a significant $\sqrt{Cf}$ can be interpreted as evidence that growth data obtained in the milk liquid medium can be employed to predict the pathogen’s kinetics in goat’s milk cheese. The estimated value ($\sqrt{Cf} = 0.751 \pm 0.0108$) indicates that the pathogen grows in cheese at approximately 56% (0.751^2) of the rate observed in milk (Figure 6), a reduction likely driven by intrinsic matrix characteristics such as lower moisture content, higher salt content, and diffusion rate limitations [71]. In practical terms, the determination of Cf enables prediction of *L. monocytogenes* behavior in cheese based on milk data, reducing the number of experimental trials required in the cheese matrix. Moreover, the linear regression suggested that type of culture (monoculture and coculture) did not significantly influence the conversion factor ($p = 0.320$), supporting its applicability. However, the non-significance of this term must be cautiously interpreted, since only six data points were available for fitting this model, and in addition the data were heavily clustered by culture type (Figure 6).

The strong agreement between predicted and observed data is evidenced by a high coefficient of determination ($R^2 = 0.9987$), demonstrating a highly precise Cf estimate, which will positively impact on its predictive accuracy. For context, previous predictive microbiology studies have reported concordance rates of approximately 92% and 91.3% between model predictions and experimental data, values considered acceptable indicators of model reliability [71]. Overall, the conversion factor provides a practical and scientifically robust tool to predict *L. monocytogenes* growth kinetics in cheese from milk data.

The validation comparing the experimental growth data of *L. monocytogenes* in goat’s milk cheese at 12 °C with the dynamic prediction, using the growth model in the milk model adjusted at pH 6.0, demonstrates strong agreement (Figure 7). The experimental growth data (blue markers) closely match the model’s predictions (bold blue line), with most data points falling within the 95 % prediction bands, which hints the model’s robustness and ability to predict dynamic growth kinetics in complex cheese matrices.

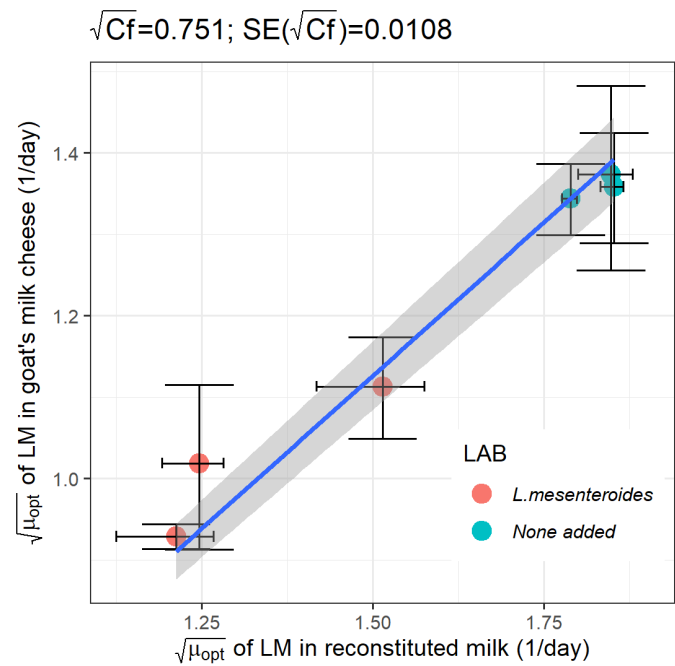


Figure 6. First Validation: Determination of the conversion factor (Cf) for estimating the optimum growth rate of *L. monocytogenes* (LM) in goat's milk cheese from the optimum growth rate in reconstituted milk model medium. Horizontal and vertical bars represent $\pm$ standard error of the optimum growth rate estimates. Mean and standard error of Cf are displayed on the top. The linear regression without intercept produced a residual standard error of 0.04263 on 5 degrees of freedom; and an adjusted R² of 0.9987. Type of culture (no LAB added, coculture with *L. mesenteroides*) was non-significant at $p = 0.32$.

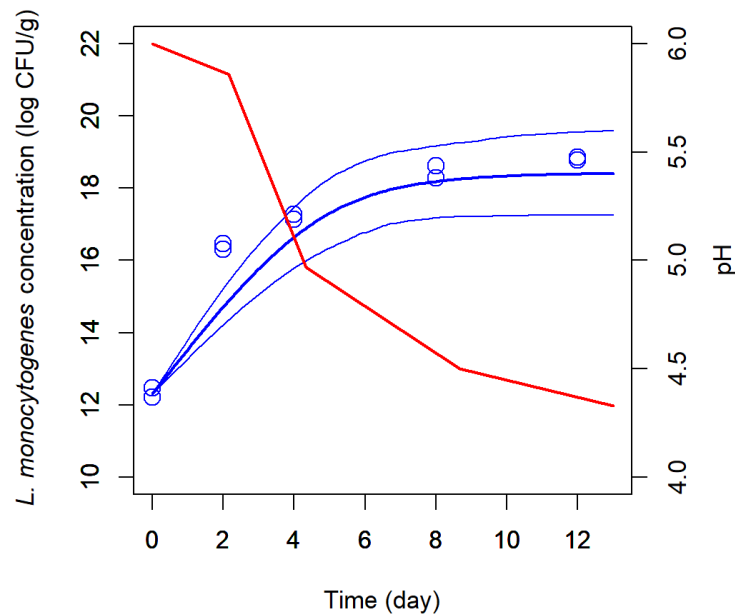


Figure 7. Second Validation: Experimental growth data of *L. monocytogenes* in goat's milk cheese at 12 °C (blue markers) compared against the dynamic prediction (bold blue line), solved using growth data in the milk model medium adjusted at pH 6.0, a normal distribution about the milk-to-cheese conversion factor, and the actual cheese pH profile during maturation (red line). Thin blue lines represent the prediction bands at 95% obtained by Monte Carlo simulation.

The two validations jointly demonstrate two premises: (1) that the growth rate of *L. monocytogenes* determined in the milk model can be used for predicting its growth in pasteurized cheese, if the conversion factor is used; and (2) that the pH-driven dynamic model proposed can represent well the pathogen's kinetic changes in the milk model during fermentation, and in the cheese matrix during maturation.

Such progressive pH decline over time is an important variable to take into account because it is a key environmental constraint that limits *L. monocytogenes* proliferation [22,23,45,81]. Consistently, monitoring pH evolution and ensuring appropriate hygienic practices throughout cheesemaking and ripening are essential indicators of microbial safety [53,82]. Overall, this validation demonstrates that pH-driven dynamic models offer reliable and quantitative tools for predicting *L. monocytogenes* behavior in complex fermenting dairy matrices, supporting their application in microbial risk assessment and product safety optimization.

5. Conclusions

The present study assessed the growth kinetics of *L. monocytogenes* in goat's pasteurized milk cheese, focusing on the inhibitory role of *Leuconostoc mesenteroides* across different milk initial pH conditions; in order to assess the validity of analogous growth parameters previously obtained using a milk model medium. A newly proposed pH-driven dynamic model accurately predicted *L. monocytogenes* growth dynamics in both monoculture and coculture conditions across pH ranges (5.5, 6.0, and 6.5) during cheese maturation. The findings clearly demonstrated that milk initial pH is a pivotal factor driving pathogen behavior, strongly modulating the extent of inhibition and the competitive advantage of LAB, with pH 5.5 showing the strongest suppression of *L. monocytogenes* growth rate in cheese. While *L. mesenteroides* notably reduced the growth rates of *L. monocytogenes* in both liquid and solid matrices; its impact on the pathogen's maximum carrying capacity was by far more pronounced in milk, likely to arise from the better diffusion of the inhibitory compounds in liquid systems. Given the absence of a matrix effect on the competition-mediated reduction in *L. monocytogenes* growth rate, a milk-to-cheese conversion factor ($\sqrt{Cf} = 0.75$) could be estimated and then validated; therefore, setting the ground to reliably extrapolate *L. monocytogenes* growth from a milk broth model to actual cheese. Overall, our findings support the use of *L. mesenteroides* as a natural protective culture along with the pre-acidification of pasteurized milk to synergistically enhance the microbiological safety of cheese. This work provides a practical and scientifically validated tool to streamline experimentation and strengthen risk assessment applications for dairy foods, reducing the need for laborious cheese inoculation experiments. From an industrial perspective, *L. mesenteroides* represents a promising functional adjunct culture for short-fermentation or short-maturation dairy products, such as goat's pasteurized milk cheeses and fermented milks, thereby supporting growing demand for naturally processed, clean-label foods. Overall, this integrated approach combining bioprotective cultures, acidification, and predictive modelling provides a viable, natural strategy to enhance microbial safety in RTE dairy products, positioning *L. mesenteroides* as an effective adjunct culture to reduce listeriosis risk while adhering to low-additive processing trends.

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