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Proceeding Paper

Effect of Lemon Balm and Spearmint Extracts on the Survival of *S. aureus* in Goat's Raw Milk Cheese †

Beatriz Nunes Silva [1,2,3,4], Sara Coelho-Fernandes [1,2], José António Teixeira [3,4], Vasco Cadavez [1,2] and Ursula Gonzales-Barron [1,2,*]

1 Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança—Campus de Santa Apolónia, 5300-253 Bragança, Portugal
2 Laboratório para a Sustentabilidade e Tecnologia em Regiões de Montanha, Instituto Politécnico de Bragança—Campus de Santa Apolónia, 5300-253 Bragança, Portugal
3 CEB—Centre of Biological Engineering, University of Minho—Campus Gualtar, 4710-057 Braga, Portugal
4 LABBELS—Associate Laboratory, 4710-057 Guimarães, Portugal
* Correspondence: ubarron@ipb.pt; Tel.: +35-12-7330-3325
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Abstract: A previous investigation from our research group has revealed that lemon balm and spearmint hydroethanolic extracts present high inhibitory capacity in vitro against *S. aureus*. Raw milk cheeses have exhibited moderate prevalence of *S. aureus*, thus imposing a safety issue for consumers. In this sense, our work aimed to evaluate the antibacterial effect of lemon balm and spearmint extracts against *S. aureus* in goat's raw milk cheeses during maturation, and to characterise the survival kinetic parameters of this pathogen by extended Bigelow models.

Keywords: log–decay model with tail; Bigelow model; *D*-value; z_{pH}; dairy; predictive microbiology

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1. Introduction

A meta-analysis study by Gonzales-Barron et al. [1] revealed *S. aureus* as the pathogen of highest incidence in goat raw milk (35.2%; 95% CI: 23.2–49.3%), and the overall occurrence of *S. aureus* in goat's milk cheeses to be noticeably high (16.0%; CI: 7.92–29.8%). Moreover, cheeses made of raw milk, regardless of their origin, presented an even higher prevalence of *S. aureus* (38.7%; 95% CI: 9.28–79.6%). These concerning prevalence values underscore the importance of improving cheese manufacture to control *S. aureus* development. For this, biopreservative agents such as plant extracts can be used [2].

Previous investigation from our research group has demonstrated the antimicrobial capacity of several plant extracts against *S. aureus* in vitro by the determination of their minimum inhibitory concentration (MIC) [3]. Lemon balm extract resulting from hydroethanolic (70% *v*/*v*) solid–liquid extraction presented a MIC of 2.5 mg/mL against *S. aureus*, whereas the equivalent extract obtained from spearmint showed an MIC of 1.25 mg/mL against this pathogen [3]. Such outcomes suggest the potential of lemon balm and spearmint extracts to be incorporated into foods as preservatives against microbial spoilage.

In this sense, the objective of this work was to assess the antimicrobial effect against *S. aureus* of lemon balm and spearmint extracts in goat's raw milk cheeses, when directly incorporated into curd, and to characterise the survival kinetic parameters of this pathogen by means of an extended Bigelow model. With this approach, values of decimal reduction time (*D*) can be described as a function of pH and incorporation of plant extract, and the inactivation parameters of *S. aureus* may help on the optimisation of the manufacturing process to ensure the microbial safety of cheeses.

2. Materials and Methods

2.1. Plant Material and Extraction Procedure

Dried lemon balm and spearmint aerial parts were provided by Pragmático Aroma Lda. (“Mais Ervas”, Trás-os-Montes, Portugal) and mechanically ground. Extracts were obtained using ethanol 70% (*v*/*v*) as solvent in a shaking water bath (150 rpm) at 60 °C for 90 min. The sample/solvent ratio was 1:20. The mixtures were filtrated (7–10 µm), and the ethanolic fraction was evaporated. The remaining aqueous fraction was frozen and lyophilised.

2.2. Inoculation of S. aureus in Milk and Cheese Production

Staphylococcus aureus ATCC 6538 kept on a fresh slant was cultivated twice at 37 °C, 200 rpm, for 16 h, first on tryptic soy broth (TSB) and then on tryptic soy broth with pH adjusted to 6.34, to mimic goat’s milk pH. On the day of inoculation, the second subculture was centrifuged at 10,640× *g* at 4 °C for 10 min, for removing debris and residual culture media. After centrifugation, the supernatant was discarded, and pellets were washed with sterile 0.9% physiological solution. Centrifugation and washing procedures were repeated twice and cells were re-suspended in sterile 0.9% physiological solution to reach ~7 log CFU/mL.

Lab-scale cheeses were prepared by adding the rennet (0.75 mL/L milk) and inoculum (5 mL/L milk) to milk at ~34 °C. Through this procedure, each cheese reached a *S. aureus* target concentration of 4 to 5 log CFU/g, depending on the milk’s initial contamination. After 30 min at 34 °C, curdled milk was cut and drained, and 1% (*w*/*w*) of lyophilised spearmint or lemon balm extract was added to the curd and mixed, while an inoculated control without extract was kept. The curd was then placed in 50 mL tubes and centrifuged at 6000 rpm at 20 °C for 3.5 min. The supernatant (whey) was removed, and cheeses of ~5 g were cut from the compacted curd and placed in a 15% brine solution for 10 min for salting. Finally, the weight in g of each cheese was annotated and cheeses were kept in a climate-controlled chamber (10 °C, 98% RH) for fermentation and maturation to take place for 15 days.

2.3. Microbiological and Physicochemical Analyses

The analyses were conducted on days 0 (day of cheese production), 2, 4, 7, 10, 13, and 15. For the microbiological determinations, for every test unit, appropriate serial dilutions were prepared by homogenising the cheese in 50 mL of buffered peptone water for 60 s. In order to determine the concentration of *S. aureus*, 0.1 mL of aliquot was plated on Baird-Parker agar, supplemented with Egg Yolk Tellurite, following ISO norms [4]. Typical colonies were counted after 48 h following incubation at 37 °C.

Physicochemical analyses comprised the measurement of pH, which was carried out using a pH meter (Hanna Instruments, model HI5522, Woonsocket, RI, USA) equipped with a HI1131 glass penetration probe.

2.4. Modelling

For every treatment, a log–decay function with tail in differential form as primary model, with varying *D*-value, coupled to a secondary model Bigelow equation of *D*-value as a function of *pH* (with parameters log D_{ref} at *pH* 7.0 and z_{pH}) was adjusted, as follows:

$$\frac{dN}{dt} = -kN\left(\frac{1}{1+C_c}\right)\left(1-\frac{N_{res}}{N}\right) \quad (1)$$

$$\frac{dC_c}{dt} = -kC_c \quad (2)$$

$$D = \frac{\ln(10)}{k} \quad (3)$$

$$\log D = \log D_{ref} - \left(\frac{pH - pH_{ref}}{z_{pH}}\right)^2 \quad (4)$$

Herein, N is the population density (CFU/g), k is the inactivation rate (1/day), C_c is the physiological state of the cells, N_{res} is the residual population density (CFU/g), D (days) is the decimal reduction time at the constant temperature T (10 °C) and at the pH of the cheese, pH_{ref} is the reference pH (set to 7), z_{pH} is the distance of pH from pH_{ref} which leads to a ten-fold change in decimal reduction time, and D_{ref} is the decimal reduction time at pH_{ref} (days).

3. Results and Discussions

Bigelow-type secondary models were used to describe the inactivation of *S. aureus* in goat's raw milk cheeses during maturation as affected by spearmint and lemon balm extracts. The survival curves of *S. aureus* in cheese without and with plant extracts, as depicted by the dynamic models, are presented in Figure 1 (spearmint) and Figure 2 (lemon balm). The results of the Bigelow parameters for each treatment are shown in Table 1.

The dynamic models adequately fitted the survival curves with root mean square errors (RMSEs) of 0.1172 and 0.0633 for spearmint and lemon balm, respectively (Table 1), producing significant parameter estimates ($p < 0.05$).

Table 1. Effect of adding 1% spearmint extract and 1% lemon balm extract in curd on the Bigelow's inactivation parameters of *Staphylococcus aureus* in goat's raw milk cheese during maturation.

Treatment	Bigelow Parameters	Mean (SE)	Pr (>∣t∣)	Goodness-of-Fit Measures
Spearmint 0% ($C_c(0) = 1.5$)	$\log D_{ref}$	0.932 (0.166)	<0.0001	$S^2 = 0.0017$ RMSE = 0.0403 MAE = 0.0357
	z_{pH}	1.727 (0.392)	0.001	
Spearmint 1% ($C_c(0) = 0.1$)	$\log D_{ref}$	0.621 (0.061)	<0.0001	$S^2 = 0.0147$ RMSE = 0.1172 MAE = 0.0978
	z_{pH}	3.172 (0.660)	<0.0001	
Lemon balm 0% ($C_c(0) = 1.5$)	$\log D_{ref}$	0.996 (0.056)	<0.0001	$S^2 = 0.0015$ RMSE = 0.0374 MAE = 0.0330
	z_{pH}	1.851 (0.007)	<0.0001	
Lemon balm 1% ($C_c(0) = 0.1$)	$\log D_{ref}$	1.189 (0.200)	<0.0001	$S^2 = 0.0042$ RMSE = 0.0633 MAE = 0.0556
	z_{pH}	2.339 (0.835)	0.019	

S^2: variance; RMSE: root mean square error; MAE: mean absolute error.

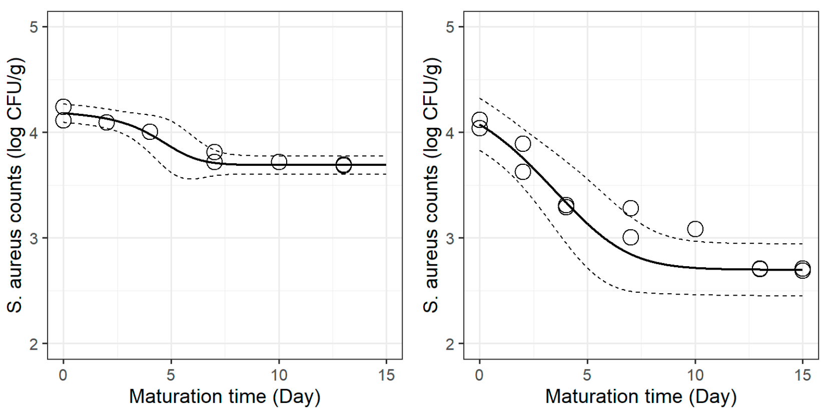

Figure 1. Survival of *S. aureus* in goat's raw milk cheese without (**left**) and with (**right**) spearmint extract, as depicted by dynamic models with 95% confidence intervals (dotted lines).

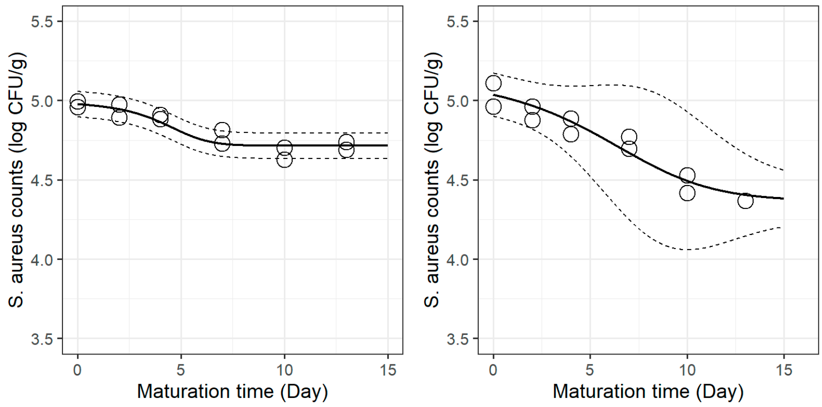

Figure 2. Survival of *S. aureus* in goat's raw milk cheese without (**left**) and with (**right**) lemon balm extract, as depicted by dynamic models with 95% confidence intervals (dotted lines).

From Table 1, the parameter log D_{ref} was affected by the addition of the extracts (0.621 (SE = 0.061) for spearmint; 1.189 (SE = 0.200) for lemon balm) in comparison to the controls (0.932 (SE = 0.166) and 0.996 (SE = 0.056)).

In the case of cheeses with spearmint extract, log D_{ref} was lower than that of the control, implying a greater inactivation rate of the pathogen. The survival curves presented in Figure 1 show that the incorporation of this extract reduced the initial lag phase (the "shoulder") and promoted *S. aureus* inactivation earlier in maturation.

Oppositely, when adding lemon balm extract to the cheese, the estimated log D_{ref} was higher than that of the control, thus suggesting a slower inactivation rate. However, *S. aureus*' inactivation was steadier and more prolonged throughout maturation, compared to the control cheeses, in which *S. aureus*' inactivation phase was rather short and the stationary phase ("tail") was reached sooner (Figure 2).

In both cases, the addition of plant extracts significantly decreased the time to achieve one log reduction, which in practical terms corresponded to up to 1.36 log CFU/g reduction by the end of maturation. Such outcomes support the usefulness of incorporating spearmint and lemon balm extracts to reduce *S. aureus* burden in this dairy product.

The fermentation process was affected by the presence of extracts in the sense that the pH drop in the beginning of the maturation (until day 4) was slower, and the pH value reached after 14 days was slightly higher, compared to the corresponding control group (data not shown). This is reflected by the higher z_{pH} values of the cheeses with spearmint and lemon balm extracts in Table 1 (3.172 (SE = 0.660) and 2.339 (SE = 0.835), respectively) and means that a greater difference between pH and pH_{ref} is necessary to lead to a ten-fold change in D when incorporating plant extracts in cheese, than the one needed for the same variation in D in the controls. This outcome is likely a result of plant extracts affecting, to some extent, the production of organic acids by bacteriocinogenic lactic acid bacteria that drop the pH during fermentation. Nonetheless, after day 4, the pH trend for both treatments and controls were similar.

The results of this work indicate that the main effect of adding 1% lemon balm extract in curd is on *S. aureus*' lag phase and the z_{pH}; while 1% spearmint extract affects lag phase, z_{pH}, and log D_{ref}. Therefore, spearmint extract is more efficient in controlling *S. aureus* in goat's raw milk cheese.

4. Conclusions

Using Bigelow-type secondary models, this work characterised *S. aureus*' survival parameters in goat's raw milk cheese. The results indicate that both parameters, log D_{ref} and z_{pH}, were affected by the addition of extracts. The z_{pH} values are increased by the addition of extracts due to their interaction with the ongoing fermentation.

The dynamic models also demonstrated that the addition of lemon balm and spearmint extracts reduced the time needed to achieve one log reduction in *S. aureus*, thus showing their ability to act as biopreservatives against this pathogen during cheese maturation.

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