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Proceeding Paper

Staphylococcus aureus Inactivation in a Non-Ready-to-Eat Sausage during Maturation: A Dynamic Model †

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Abstract: The aim of this study was to model the effect of annatto (*Bixa orellana*) extract against *S. aureus* (SA) in a Portuguese non-ready-to-eat meat product (*alheira* sausage) during maturation. *Alheira* batter was prepared with 0.0%, 0.5%, 1.0%, or 1.5% of lyophilised annatto extract and stuffed in pre-washed natural casings following inoculation with SA, then hung in a climatic-controlled chamber at 10 °C for 13 days. For every treatment, a dynamic model was adjusted and adequately fitted to all survival curves with residuals and root mean square errors between 0.0008–0.0016 and 0.029–0.040, respectively, producing significant parameter estimates. Therefore, the addition of annatto extracts significantly shortened the shoulder phase and decreased the time to achieve one log reduction, which, in practical terms, corresponded to up to 1.35 [SE = 0.08] log CFU/g reduction by the end of the 13-day maturation.

Keywords: *alheira*; modelling; Bigelow; safety; pathogens; annatto; biopreservative

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1. Introduction

Bixa orellana, commonly known as annatto, is one of the oldest known natural dye yielding plants native to Central and South America [1]. The Annatto seeds and their extracts have been used as a colouring agent to provide orange/red hues to fat-rich products, and recently, it has also demonstrated to possess inhibitory activity against some pathogens. *Alheira* is a non-ready-to-eat sausage from Northern Portugal, which has shown moderate occurrence and survivability of *Staphylococcus aureus* during processing [2]. The objective of this study was to dynamically model the effect of annatto extract against *S. aureus* in *alheira* during maturation.

2. Materials and Methods

2.1. Seeds Material and Extraction Procedure

The annatto seeds purchased in a local market (Peru) were mechanically ground and submitted to hydroethanolic extraction by dynamic maceration of 1 g seeds material with 30 mL 80% ethanol (*v*/*v*) for 1 h at room temperature. The mixtures were filtrated (7–10 µm), and the ethanolic fraction was evaporated. The remaining aqueous fraction was frozen and lyophilised.

2.2. Antimicrobial Activity of the Annatto Extract

The extracts were screened against *Escherichia coli* (ATCC 25922), *S. aureus* (ATCC 6538), *Listeria monocytogenes* (WDCM 00019), *Salmonella enterica* subsp. *enterica* serovar

Typhimurium (ATCC 43971), and *Bacillus cereus* (ATCC 14579). Minimum inhibitory concentration (MIC, mg/mL) was determined by the serial microdilution method previously described [3,4]. Antimicrobial solutions were prepared in concentrations ranging from 32 to 0.031 mg/mL in sterile Mueller–Hinton broth or tryptic soy broth (TSB). A total of 100 µL of broth was placed in a sterility control (SC) well and 50 µL in a control well of a microplate; then, volumes of 50 µL of each antimicrobial solution were added to different wells. The bacterial suspension was vortexed and 50 µL was immediately added to each well except for SC. The assay was performed in triplicate. The microplates were incubated at 37 °C for 16 to 20 h; then, 30 µL resazurin 0.02% was added to all wells and incubated for 2–4 h until colour change.

The reading of the MICs was performed in the columns where there was no colour change, and the content of each well was directly plated with concentrations greater than or equal to the MIC in Mueller–Hinton agar or tryptic soy agar (TSA), depending on the microorganism studied. TSB and TSA were media used only for *L. monocytogenes* assays. The plates were incubated at 37 °C for 24 h, and, finally, the minimum bactericidal concentration (MBC) reading was taken as the lowest concentration where there was no microbial growth on plates.

2.3. Inoculation of S. aureus in Alheiras

Staphylococcus aureus (ATCC 6538) kept on a fresh slant was cultivated overnight in brain heart infusion broth (BHI) at 37 °C. On the day of inoculation, the inoculum was prepared from a second subculture in early stationary phase (~9.0 log CFU/mL) and diluted in physiological water to reach 7.0 log CFU/mL.

Five batches of *alheiras* proxy were prepared by soaking sliced wheat white bread (22%) in hot boiled water (60%) for 20 min. After breaking down, garlic powder (1%), red pepper powder (1%), table salt (1%), and finely-shredded cooked chicken meat (10%) were added and mixed to form a well-integrated batter. Separately, virgin olive oil (5%) was heated to ~50 °C and added with 0.0, 0.5, 1.0, or 1.5% (*w*/*w*) of the lyophilised annatto extract, and incorporated, still warm, into the batter. The batter was mixed throughout and stuffed in pre-washed natural pig casings to produce mini-*alheiras* of approximately 80 g. The weight in g of each *alheira* was annotated (W). Mini-*alheiras* were then inoculated by individually syringing a volume of 10W µL of the inoculum into the test units. Through this standardised procedure, each "mini-*alheira*" reached a *S. aureus* target concentration of ~5.0 log CFU/g. The fifth batch of *alheira* was identically produced, but without *S. aureus* inoculation to act as negative control, in order to characterise the evolution of the physicochemical properties of the *alheiras* during maturation when produced at laboratory-scale. Mini-*alheiras* were hung in a climate-controlled chamber (10 °C, 85% RH) for fermentation/maturation to take place over 13 days [5].

2.4. Microbiological and Physicochemical Analyses

All analyses were conducted on days 0 (day on which the *alheiras* were inoculated, before maturation), 2, 5, 7, 10, and 13. For the microbiological determinations, only inoculated *alheiras* were analysed. For every test unit, appropriate serial dilutions were prepared by homogenising the entire *alheira* content (without the casing) in 100 mL of buffered peptone water (Liofilchem, Roseto degli Abruzzi, Italy) for 90 s. In order to determine the concentration of lactic acid bacteria (LAB), 1-mL aliquots of the dilutions were pour-plated in MRS agar (Liofilchem, Italy), overlaid with 1.2% bacteriological agar (Liofilchem, Italy), and incubated at 30 °C for 48 h. Calculations were based on confirmed bacteria [5]. For the counts of *S. aureus*, 0.1-mL aliquot was plated on Baird–Parker agar (Liofilchem, Italy), supplemented with Egg Yolk Tellurite (Liofilchem, Italy), following the ISO norm [6]. Typical colonies were counted after 48 h following incubation at 37 °C. For every treatment, two runs were conducted, and microbiological determinations were performed in triplicate.

Physicochemical analyses were carried out for the non-inoculated *alheiras*, and comprised the measurement of pH, water activity (a_w), and weight loss. Weight loss was also recorded in the inoculated *alheira* units. The pH measurement was carried out in duplicate per test unit, using a pH meter (Hanna Instruments, model HI5522, USA) equipped with a HI1131 glass penetration probe. In order to measure a_w, the *alheira* content was transferred into the cuvette of an Aqualab meter (model 4TE Decagon, USA), and the value was recorded after measurement stabilisation. This was repeated twice times per test unit.

2.5. Statistical Analysis

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

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

$$\frac{dC_c}{dt} = -kC_c$$

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

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

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 is the decimal reduction time (day) at the constant temperature T (10 °C) and at the pH of the *alheira*; pH_{ref} is the reference pH (set to 7.0); z_{pH} is the distance of pH from pH_{ref}, which leads to a ten-fold change in decimal reduction time; and *Dref* is the decimal reduction time at pH_{ref} (day). Statistical analysis was conducted in R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

3. Results and Discussion

3.1. Antimicrobial Activities of the Annatto Extract

The results of the MIC obtained against *E. coli*, *L. monocytogenes*, *S.* Typhimurium, *Bacillus cereus*, and *S. aureus* are presented in Table 1.

Table 1. Antimicrobial activities of the hydroethanolic extract of annatto.

Microorganism	Medium	MIC (mg/mL)	MBC (mg/mL)
Escherichia coli	MHB	0.25	1
Listeriamonocytogenes	TSB	0.125	0.33
Salmonella Typhimurium	MHB	1	2
Bacillus cereus	MHB	0.015	1
Staphylococcus aureus	MHB	8	>32

The MIC is defined as the lowest concentration of an antimicrobial agent that completely inhibits growth of the organism in the microdilution wells, as detected by the unaided eye [7]. In this study, the extract examined showed promising results, with MIC values below 8 mg/mL against all pathogens.

While MIC is the lowest concentration of an antibacterial agent necessary to inhibit visible growth, minimum bactericidal concentration (MBC) is the minimum concentration of an antibacterial agent that results in bacterial death.

The most susceptible pathogen was *B. cereus*, as it presented the lowest MIC (0.015 mg/mL), followed by *Listeria monocytogenes* (0.125 mg/mL), *E. coli* (0.25 mg/mL), and *Salmonella* spp. (1 mg/mL). The most resistant bacterium was *S. aureus*, since it presented a MIC value of 8 mg/mL, meaning that higher concentrations of annatto are needed to inhibit this pathogen, compared to the remaining ones.

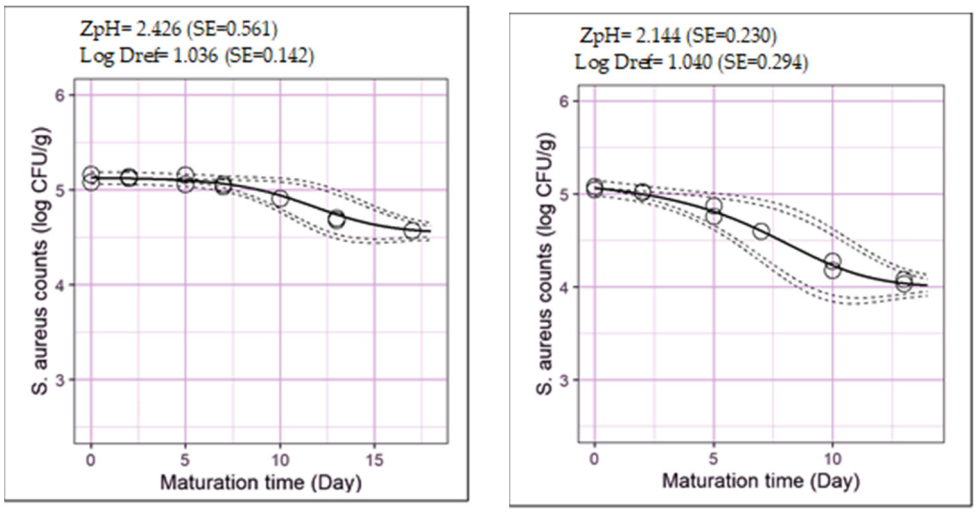
It can be noted that *Listeria monocytogenes* presented the lowest MBC value (even if not the lowest MIC). This means that *Listeria monocytogenes* can be killed by a lower concentration of annatto extract in comparison to the other pathogens, which would need higher concentrations. These outcomes reinforced the overall good antimicrobial capacities pointed out for annatto extract.

3.2. Effect of the Annatto Extract on S. aureus

The addition of 0.50–1.50% annatto extract to the *alheira* batter affected the kinetics of *S. aureus* during maturation (Figure 1). The higher the extract dose added, the greater the inactivation of *S. aureus*. When no annatto extract was incorporated, *S. aureus* concentration dropped in 0.55 log CFU/g after 16 days of maturation, whereas when *alheira* was formulated with 0.50, 1.00, and 1.50% annatto extract, the drop in *S. aureus* mean concentration was gradually higher, in 1.05, 1.20, and 1.35 log CFU/g, respectively.

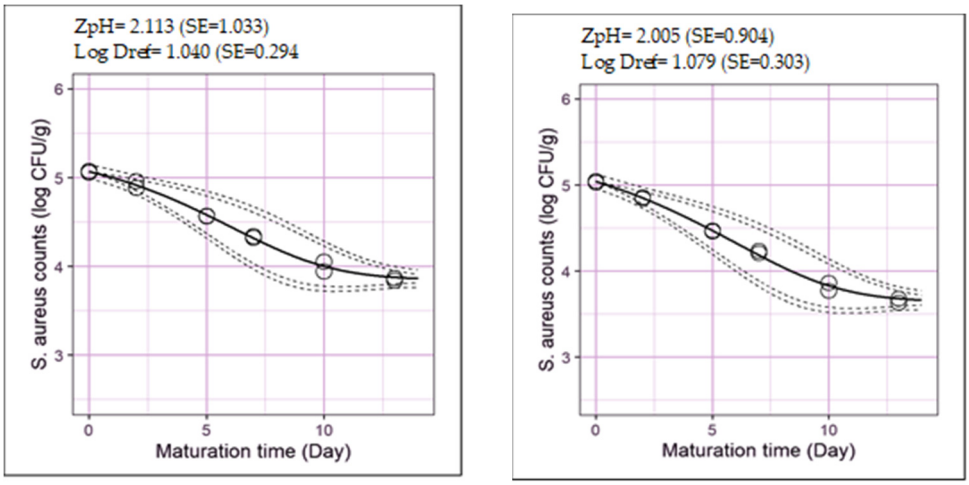
The dynamic model was adequately described to all survival curves with residuals and root mean square errors between 0.0008–0.0016 and 0.029–0.040, respectively, producing significant parameter estimates (Figure 1). The parameter z_{pH} tended to be lower (2.426 [SE = 0.561], 2.144 [SE = 0.230], 2.113 [SE = 1.033]), with increasing doses of annatto extract (0.5, 1.0, and 1.5%, respectively); and all of them were higher than that of the control treatment (2.005 [SE = 0.904]). A higher z_{pH} value means that a greater difference between pH and pH_{ref} is necessary to lead to a ten-fold change in *D* when incorporating annatto extract into *alheira* batter than that needed for the same variation in *D* in the controls. This difference in z_{pH} is likely to be a consequence of annatto extract affecting the production of organic acids by lactic acid bacteria that drop the pH during fermentation.

Furthermore, the survival curves presented in Figure 1 show that the incorporation of this extract reduced the initial "shoulder" and promoted *S. aureus* inactivation earlier in maturation. The addition of annatto extract produced log D* slightly higher (1.040 [SE = 0.064], 1.040 [SE = 0.294], and 1.079 [SE = 0.303]) than the control (1.036 [SE = 0.142]), suggesting that a slightly longer time is needed at a matrix pH of 7.0 to reach the same inactivation level.



(**a**) (**b**)

Figure 1. *Cont.*



(**c**) (**d**)

Figure 1. Kinetics of *S. aureus* in *alheiras* formulated with: (**a**) 0.0%; (**b**) 0.5%; (**c**) 1.0%, and (**d**) 1.5% of annatto extract (*w*/*w*) during maturation at 10 °C and 85% RH. Circle markers represent observations, thick black lines predicted curve, and dotted lines the 95% confidence interval (inner) and 95% prediction interval (outer).

Overall, the addition of annatto extract significantly decreased the time to achieve one log reduction. Such outcome points out the potential of incorporating hydroethanolic annatto extract to reduce the *S. aureus* burden in this Portuguese meat product.

4. Conclusions

This study characterised *S. aureus* survival kinetic parameters in *alheira* sausage, formulated with and without annatto extract, using a dynamic model. Both parameters, log D_{ref} and z_{pH}, were impacted by the addition of annatto extract. The z_{pH} parameter decreased as annatto extract concentration increased, probably due to their interaction with the ongoing fermentation, which slows down the production of lactic acid. The present work has demonstrated that annatto extract has a beneficial effect in controlling *S. aureus* during maturation.

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