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Proceeding Paper

Effect of Sage (*Salvia officinalis* L.) Extract on the Survival of *Staphylococcus aureus* in Portuguese *Alheira* Sausage during Maturation †

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Abstract: The objective of this study was to assess the effect of sage (*Salvia officinalis* L.) extract on the survival of *Staphylococcus aureus* in a Portuguese non-ready-to-eat meat product (*alheira* sausage). *Alheira* batter was produced, mixed with 0.0%, 0.5%, 1.0%, or 1.5% of lyophilised sage extract, and stuffed in natural casings. Sausages were then individually inoculated with *S. aureus* and left to ferment/mature at 10 °C/85% RH for 10 days. Sage extract was found to inactivate *S. aureus* ($p < 0.001$) with no significant differences between doses. At the 10th day of maturation, *S. aureus* decreased in 1.146 log CFU/g (SE = 0.065 log CFU/g) in *alheiras* mixed with 0.5–1.0% sage extract. Nonetheless, this extract retarded the growth of indigenous lactic acid bacteria during maturation. The higher the dose, the greater the effect ($p < 0.001$).

Keywords: antioxidant; antimicrobial; preservative; fate study; challenge study; pathogen

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1. Introduction

Sage (*Salvia officinalis* L.) is a plant of the *Lamiaceae* family, native to the Mediterranean, with excellent antioxidant and antimicrobial properties [1]. In turn, *Alheira* is a non-ready-to-eat sausage produced in northern Portugal, which has in the past shown low-to-moderate prevalence of *Staphylococcus aureus* [2]. Sojic et al. [3] incorporated sage essential oil and sage extract in raw pork sausages and documented their capacity not only to retard lipid oxidation but also to be effective against aerobic microbial growth. Thus, this study was performed to evaluate the antimicrobial effect of sage extract against *S. aureus* in *alheira* sausages during the critical stage of fermentation/maturation.

2. Materials and Methods

2.1. Plant Material and Extraction Procedure

Dried sage aerial parts provided by Pragmático Aroma Lda. ("Mais Ervas", Trás-os-Montes, Portugal) were mechanically ground and submitted to hydroethanolic extraction by dynamic maceration of 1 g plant 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. Antioxidant and Antimicrobial Activity of the Sage Extract

The antioxidant activity was measured through two in vitro assays, using previously described methodologies [4], namely, the thiobarbituric acid reactive substances (TBARS) formation inhibition assay and the oxidative haemolysis inhibition assay (OxHLIA). The extract's capacity to inhibit the formation of TBARS was assessed using porcine brain cells as oxidizable biological substrates and the results were expressed as the extract concentration (μg/mL) required to inhibit 50% of the lipid peroxidation (IC_{50}). The extract's capacity to inhibit the oxidative haemolysis was tested using sheep blood erythrocytes as ex vivo models and the extract concentration able to promote a Δt haemolysis delay of 60 and 120 min was calculated based on the half haemolysis time (Ht_{50} values) of the haemolytic curves of each extract concentration. The results were expressed as the extract concentration (μg/mL) required to keep 50% of the erythrocyte population intact for 60 and 120 min (IC_{50}).

The extracts were screened against *Escherichia coli* (ATCC 25922) and *S. aureus* (ATCC 11632). Minimum inhibitory concentration (MIC, mg/mL) was determined by the serial microdilution method previously described [5,6].

2.3. Inoculation of S. aureus in Alheiras

Staphylococcus aureus subsp. *aureus* Rosenbach (ATCC 11632) 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) 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 sage 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 80–90 g. The weight in g of each *alheira* was annotated (W). Mini-*alheiras* were then inoculated by individually syringing a volume of 10 W 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 for 10 days.

2.4. Microbiological and Physicochemical Analyses

All analyses were conducted on days 0 (day in which the *alheiras* were inoculated, before maturation), 2, 4, 6, 8, and 10. For the microbiological determinations, only inoculated *alheiras* were analysed. For every test unit, appropriate serial dilutions were prepared by homogenising for 2 min the entire *alheira* content (without the casing) in 100 mL of buffered peptone water (Liofilchem, Roseto degli Abruzzi, Italy). To determine the concentration of lactic acid bacteria (LAB), 1-mL aliquot of the dilutions were pour-plated in MRS agar (Liofilchem, Italy), overlaid with 1.2% bacteriological agar (Liofilchem, Roseto degli Abruzzi, Italy), and incubated at 30 °C for 48 h. Calculations were based on confirmed bacteria [7]. For the counts of *S. aureus*, 0.1-mL aliquot was plated on Baird–Parker agar (Liofilchem, Roseto degli Abruzzi, Italy), supplemented with egg yolk ellurite (Liofilchem, Roseto degli Abruzzi, Italy), following ISO norms [8]. Typical colonies were counted after 48 h following incubation at 37 °C. For every treatment, two runs were conducted, and microbiological determinations were done 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, Rhode Island, USA) equipped with a HI1131 glass penetration probe. To measure a_w, the *alheira* content was transferred into the cuvette of an Aqualab meter (model 4TE Decagon, Washington, USA), and the value was recorded after measurement stabilisation. This was repeated twice per test unit.

2.5. Statistical Analysis

Results of antioxidant and antimicrobial activities of the extract are presented as mean ± standard deviation (SD) values. Statistical differences of the mean pH, a_w, and weight loss of the *alheiras* during maturation were obtained through one-way analysis of variance (ANOVA) with time as a factor, followed by Tukey's comparison of means (α = 0.05). The effect of sage extract on *S. aureus* and LAB concentration was assessed by two-way analyses of variance (sage concentration and day as factors), followed by Tukey's comparisons of means between concentrations. Statistical analysis was conducted in R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria).

3. Results and Discussion

3.1. Antioxidant and Antimicrobial Activities of the Sage Extract

The results of the TBARS formation and oxidative haemolysis inhibition assays (OxHLIA) and the MIC obtained against *E. coli* and *S. aureus* are presented in Table 1. These outcomes reinforced the high antioxidant and antimicrobial capacities already pointed out for sage extracts. In our study, the antioxidant capacity of the sage extract measured by TBARS was better than similarly-produced sage extracts from Brazil (EC_{50} = 398 µg/mL), which significantly inhibited lipid oxidation in poultry pâtés during storage [9]. Lower values were obtained in OxHLIA, indicating that the extract has better antioxidant capacity against free radical-induced oxidative damage of erythrocyte membranes than against the formation of malondialdehyde and other reactive substances generated in vitro from lipid peroxidation of the porcine brain cells used in the assay. Based on previous studies, the antioxidant activity of sage extracts can be attributed to the presence of phenolic compounds, mainly rosmarinic acid and derivatives of caffeic acid [10].

Table 1. Antioxidant and antimicrobial activities of the hydroethanolic extract of sage (*n* = 3).

Activity	Mean ± SD
TBARS [a] (IC_{50}, µg/mL)	206 ± 5
OxHLIA[b] (IC_{50}, µg/mL)	
Δt 60 min	23.9 ± 0.9
Δt 120 min	56.0 ± 2.0
MIC—*E. coli* (mg/mL)	1.250 ± 0.00
MIC—*S. aureus* (mg/mL)	0.625 ± 0.00

[(a)] Trolox IC_{50} value: 5.4 ± 0.3 µg/mL. [(b)] Trolox IC_{50} values: 21.8 ± 0.3 µg/mL (Δt = 60 min) and 43.5 ± 0.8 µg/mL (Δt = 120 min).

The antibacterial activity assay showed that *E. coli* was more resistant (MIC = 1.250 mg/mL) to the sage extract than *S. aureus* (0.625 mg/mL) (Table 1). This was not unexpected, since Gram-negative bacteria are generally more resistant to bioactive compounds due to the hydrophilic surface of their outer membrane [1]. Yet, the aforementioned Brazilian study [8] found that their extract did not inhibit *S. aureus* ATCC 25923 at the concentrations tested (MIC > 5 mg/mL).

3.2. Maturation Process of Lab-Scale Produced Alheiras

Table 2 compiles the variation in pH, a_w, and weight loss occurring in *alheiras* during maturation. As suggested by the pH, the fermentation process was slow during the first two days of maturation, and steadily continued until the end of maturation. On the other hand, the a_w did not change significantly from the fourth day of maturation onwards, whereas from the sixth day, *alheiras* no longer dehydrated significantly (see weight loss). In contrast to the results from a wide sampling of bulk *alheiras* sold in Portuguese fair markets [11], our lab-made *alheiras* compared well to the commercialised ones in terms of pH (5.49; SD = 0.034 versus 4.83; SD = 0.563) and aw (0.9734; SD = 0.0010 versus 0.9870;

SD = 0.0040). For this reason, we were confident that the 80-g mini-*alheiras* produced in the laboratory mimicked the actual ones and could be used as proxy for the inoculation study.

Table 2. Evolution of pH, water activity (a_w), and weight loss of non-inoculated mini-*alheiras* characterising the process of maturation at 10 °C and 85% RH (standard deviation in brackets, $n = 3$).

Maturation Day	pH *	a_w *	Weight Loss (%) *
0	6.03 (0.019) ^{a}	0.9931 (0.0032) ^{a}	-
2	5.98 (0.026) ^{a}	0.9870 (0.0037) ^{b}	12.09 (1.23) ^{a}
4	5.79 (0.026) ^{b}	0.9769 (0.0009) ^{c}	18.16 (0.86) ^{b}
6	5.55 (0.022) ^{b}	0.9751 (0.0082) ^{c}	21.28 (0.68) ^{c}
8	5.49 (0.034) ^{b}	0.9734 (0.0010) ^{c}	22.12 (1.25) ^{c}

(*) Mean values with different superscript letters in a column are significantly different by Tukey's comparison of means ($p < 0.05$).

3.3. Effect of the Sage Extract on S. aureus and Lactic Acid Bacteria in Alheiras

The addition of 0.50–1.50% sage extract to the *alheira* batter affected the kinetics of both *S. aureus* and lactic acid bacteria during maturation (Figure 1). When the *alheiras* were not formulated with sage extract (i.e., control), the inoculated concentration of *S. aureus* (5.0 log CFU/g) persisted throughout maturation despite acidification and dehydration, whereas the indigenous LAB proliferated more rapidly until reaching ~10.5 log CFU/g at the 10th day of maturation. From the *alheiras* added with sage extract, it became evident that the higher the extract concentration, the greater the effect on the populations of *S. aureus* and LAB (Figure 1). While sage extract produced a (desired) inactivation effect of *S. aureus*, it produced a (non-desired) delay in the development of LAB due to an increase in the lag phase and a decrease in the maximum concentration. ANOVA results confirmed that both day of maturation ($p > 0.001$) and sage concentration ($p < 0.001$) exerted significant effects on the two bacterial groups (Table 3).

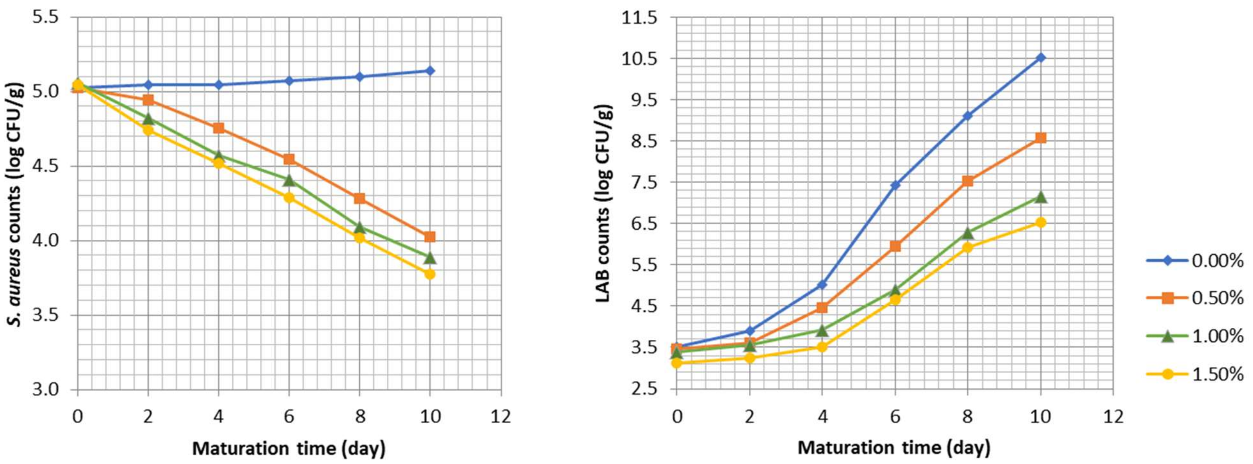

Figure 1. Kinetics of inoculated *S. aureus* and indigenous lactic acid bacteria (LAB) in mini-*alheiras* formulated with 0.0, 0.50, 1.00, and 1.50% (w/w) liophilised sage extract during maturation at 10 °C and 85% RH.

Statistically, and deducing the effect of day of maturation, all extract concentrations (0.5, 1.0, and 1.5%) significantly reduced *S. aureus* counts ($p < 0.001$) in fermenting *alheira* in comparison to the control treatment (0.0%). Nonetheless, as a whole, the three sage extract concentrations did not lead to significantly different reduction levels of *S. aureus*, as can be inferred from three non-significant *p*-values of the comparisons of means (Table 3). At the 10th day of maturation, sage extract added in *alheira* batter at a dose between 0.5–1.0% produced an average reduction of *S. aureus* counts of 1.146 log CFU/g

(SE = 0.065 log CFU/g), whereas adding 0.5% extract caused a non-significantly different reduction of 1.000 log CFU/g (SE = 0.046 log CFU/g) (results not shown). This implies that the lowest extract concentration tested of 0.5% could be sufficient to control *S. aureus* in *alheiras*.

Table 3. Effect of sage extract on the populations of inoculated *S. aureus* and indigenous lactic acid bacteria (LAB) in mini-*alheiras* during maturation at 10 °C and 85% RH, as evaluated by analysis of variance (ANOVA) followed by Tukey's comparisons of means between extract concentrations. CFU: colony forming units.

Bacterium	Test	Mean Difference (log CFU/g)	F/*t* Value	Pr(>F)/Pr(>|*t*|)
S. aureus	ANOVA			
	Day	-	16.79	<0.001
	Concentration	-	23.50	<0.001
	Comparison of means			
	0.5%–0.0%	−0.476	−5.407	<0.001
	1.0%–0.0%	−0.598	−6.786	<0.001
	1.5%–0.0%	−0.672	−7.626	<0.001
	1.0%–0.5%	−0.122	−1.379	0.520
	1.5%–0.5%	−0.196	−2.219	0.136
	1.5%–1.0%	−0.074	−0.841	0.835
LAB	ANOVA			
	Day	-	85.35	<0.001
	Concentration	-	27.27	<0.001
	Comparison of means			
	0.5%–0.0%	−0.983	−3.910	0.002
	1.0%–0.0%	−1.760	−7.003	<0.001
	1.5%–0.0%	−2.085	−8.295	<0.001
	1.0%–0.5%	−0.777	−3.093	0.018
	1.5%–0.5%	−1.102	−4.385	<0.001
	1.5%–1.0%	−0.325	−1.292	0.573

Although the challenge study carried out by Sojic et al. [3] did not assess the survival of *S. aureus* in raw sausage incorporated with sage essential oil and extract, they did observe a decrease in microbial growth of approximately 0.5 and 1.1 log CFU/g in comparison to the control treatment at concentrations of 0.075 μL/g and 0.1 μL/g, respectively.

Opposite to the results found with *S. aureus*, the statistical analysis on LAB counts showed that, on average, the higher the sage extract dose, the lower the LAB populations, except for the *alheiras* added with 1.0% and 1.5% sage extract, which overall did not differ significantly (p = 0.573; Table 3). This could be also visually inferred from Figure 1.

4. Conclusions

This work demonstrated that sage extract has a beneficial effect in controlling *S. aureus* in *alheira* during the critical stage of fermentation/maturation. This outcome is very relevant to local producers since this frequently-found pathogen is mostly introduced to the batter through inadequate handling when mixing and stuffing, particularly when *alheiras* are artisanally produced.

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