

Effect of high hydrostatic pressure challenge on biogenic amines, microbiota, and sensory profile in traditional poultry- and pork-based semidried fermented sausage

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Abstract: The processing of traditional poultry- and pork-based semidried fermented smoked sausages needs to be modernized to improve product quality and further extend its shelf life. The aim of the present study was to apply different combinations of high pressure (300 to 600 MPa) and time (154 to 1,800 s) on the sausages using an experimental design based on response surface methodology. The chemical, microbial, and sensory characteristics of sausages treated with high-pressure processing (HPP) were investigated. HPP application to semidried fermented sausages resulted in color changes, which could be dependent on the ingredients, formulation, and smoking conditions used. Nevertheless, none of the HPP treatments applied resulted in detectable changes in sensory properties, as tested in a triangle test and confirmed by the analysis of focus groups assessment. Significant differences were detected for lactic acid bacteria (LAB) counts from 344 MPa and 1,530 s onward, with a marked decrease for the combination of 600 MPa and 960 s ($P < 0.05$). Coagulase-negative *staphylococci* showed higher tolerance to the increase in pressure than LAB. HPP induced a microbial reduction on *Enterobacteriaceae*, molds, and yeasts, minimizing the production of the main biogenic amines. However, the polyamines (spermine and spermidine) increased since their metabolic use by microorganisms did not occur. Given the reduction of the main spoilage microbial indicators with no detectable sensory changes observed with the binomial condition of 600 MPa and 960 s, this was chosen as the optimal combination to be further applied.

Keywords: biogenic amines, high-pressure processing, safety, semidried fermented sausage, sensory quality

Practical Application: The results from sensory analysis revealed that any of the HPP treatments applied resulted in detectable changes in sensory properties, as tested in a triangle test and confirmed by the analysis of the focus groups speeches.

1. INTRODUCTION

Traditional fermented sausages are widely appreciated and, all over the world, there are different fermented sausages produced in small processing units and manufactured according to traditional practices specific to each geographic area. In a region in northern Portugal, known as *Trás-os-Montes e Alto Douro*, a poultry- and pork-based semidried fermented sausage named *alheira* stands out for its unique characteristics, which result from the use of particular ingredients and specific manufacturing practices. This sausage can have the status of Protected Geographical Indications (PGI) (EC, 2012), and are mostly made with chicken and pork meats, but veal, turkey, and game meats can also be used as main ingredients. The

improvement in safety and shelf life of sausage will increase the sustainability of small local producers and demand by consumers (Osorio-Arce & Segura-Correa, 2011). However, recently, these poultry- and pork-based semidried fermented sausages were implicated in foodborne outbreaks (RASFE, 2008, 2015). The presence of pathogens like *Clostridium botulinum* and *Listeria monocytogenes* has been reported in this type of sausages (Meloni, 2015).

High-pressure processing (HPP) is a nonthermal, nondestructive, and chemical-free food preservation technology used to enhance the shelf life and assure the safety of food products with minimal effects on nutritive and organoleptic quality. The effect of HPP treatment on shelf life and safety of foods is due to the inactivation of genetic mechanisms of microorganisms and inhibition of enzymes (Osorio-Arce & Segura-Correa, 2011). HPP efficacy depends on many factors, particularly the intrinsic characteristics of the food matrix. Thus, optimization should be performed to achieve the best microbial lethality performance with minimal sensory changes of the meat product (Daher, Le Gourrierec, & Pérez-Lamela, 2017; Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015).

Biogenic amines (BAs) levels have been used as indicators of food safety and as quality indices for good manufacturing practice (Gardini, Zogul, Suzzi, Tabanelli, & Zogul, 2016). BAs are mainly

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generated from amino acid decarboxylation of food-related microorganisms and are usually considered as potential toxic nitrogenous substances in foods (Gardini et al., 2016; Suzzi & Torriani, 2015). Histamine and tyramine are the most toxic and abundant in protein-rich fermented products, including cheeses (Mayer & Fiechter, 2018) and sausages (Ekici & Omer, 2018). Herein, we intend to optimize the combination of time and high pressure applied to the poultry- and pork-based semidried fermented sausage with the goal of controlling microbial hygienic and spoilage indicators without affecting their sensory characteristics.

2. MATERIALS AND METHODS

2.1 Poultry- and pork-based semidried fermented and smoked sausage production

Three poultry- and pork-based semidried fermented and smoked sausage (*Alheira*) batches of the same formulation obtained from a local manufacturer, in different production days, were prepared by a traditional manufacture procedure. Wheat bread (38.2%), shredded cooked pork meat (36.5%), small pieces of pork lard (13.1%), shredded cooked chicken meat (8.3%), olive oil (1.4%), dehydrated garlic (*Allium sativum* L., 1.3%), salt (0.77%), sweet red pepper powder (*Capsicum annuum* L., 0.23%), sodium diacetate (0.12%), hot *piri-piri* powder (0.02%), and the meat cooking water were mixed to form a homogeneous paste. Then, this mixture was stuffed into natural casings with a 40 to 45 mm diameter, forming sausages with a horseshoe shape of 180 to 200 g. *Alheira* samples were smoked for 4 days at 7 to 12 °C and relative humidity of 65% to 80%, with oak wood (*Quercus ilex* L.) smoke. Sausages were vacuum packed (AK Ramon Vac Line Model VP-800 AB, Barcelona, Spain) in polyamide and polyethylene (PA/PE 90) bags (O₂ permeability: <60 cm³/m²/day and 0% RH; CO₂ permeability: <190 cm³/m²/day and 75% RH; N₂ permeability: <12 cm³/m²/day and 75% RH; water-vapor permeability: <3.5 g/m²/day at 23 °C and 85% RH; Termofilm, Vila Nova de Famalicão, Portugal) and stored at 5 °C.

2.2 High-pressure processing treatment

2.2.1 Experimental design. The best HPP conditions of (time [*t*] and pressure [*P*]) were investigated using response surface methodology (RSM). This technique involves a set of statistical and mathematical methods that are used for modeling and optimization of multiple variables to predict the best performance conditions with a minimum number of experiments (Huang, Wu, Lu, Shyu, & Wang, 2017; Sevenich, Rauh, & Knorr, 2016). The experiments were carried out with a central composite rotatable design (CCRD) as a function of *t* and *P* with five levels for each factor, which allowed fit of first- or second-order polynomials to the experimental data points (Rendueles et al., 2011). Twelve experiments were carried out in CCRD to test the various *P* and *t* combinations, considering four factorial points resulting from combinations of levels coded as (+1) and (−1) for both *P* and *t*; four-star points coded as +√2 and −√2 for combinations of *P* and *t*; and three center points, coded as 0 (Table 1). The group not submitted to pressure treatment was used as control.

2.2.2 High-pressure processing conditions. Samples were processed in a pressure vessel submerged in water as the surrounding pressure-transmitting medium (high-pressure food processor, N.C. Hyperbaric, model Wave 6,000/135; Spain) and pressurized under the 12 conditions in study (Table 1), with the initial temperature of the pressure vessel set at 10 °C. Control samples were maintained under atmospheric pressure at 5 °C. Immediately

Table 1—Experimental data obtained for the optimization of pressure and time on semidried fermented smoked sausage processing.

Treatment	Pressure (MPa)	Time (s)
Control	0.101	0
1	300	960
2	344	390
3	344	1,530
4	450	154
5	450	960
6	450	960
7	450	960
8	450	1,800
9	556	390
10	556	1,530
11	600	390
12	600	960

after treatment, all samples were transported in a cooled container (<5 °C) to the laboratory and analyzed.

2.3 Microbial analysis

Microbiological analyses were performed 1 day after the samples treatment in accordance with ISO 6887-1:2017. Microbial determinations were carried out for total mesophilic aerobic (TMA) counts (Tryptone glucose extract agar, Scharlau Chemie, Barcelona, Spain) after incubation at 30 °C for 2 days (ISO 4833-1:2013); total psychrotrophic anaerobic (TPA) counts (Tryptone glucose extract agar, Scharlau Chemie) after incubation at 10 °C under anaerobiosis during 5 days; *Enterobacteriaceae* counts (Violet Red Bile Dextrose agar, Scharlau Chemie) after incubation at 37 °C for 2 days (ISO 21528-2:2017); lactic acid bacteria (LAB) counts (Man Rogosa and Sharpe Agar, Scharlau Chemie) after incubation at 30 °C for 3 days in anaerobiosis (ISO 15214:1998); coagulase-negative *staphylococci* (CNS) (Manitol Salt Agar, Scharlau Chemie) after incubation at 37 °C during 2 days; *Clostridium perfringens* (Tryptose Sulphite Cyclocerine, Scharlau Chemie) after incubation at 37 °C for 2 days (ISO 7937:2004); *Listeria monocytogenes* (ALOA Agar, Biomerieux, France) after incubation at 37 °C for 24 hr (ISO 11290-1:2017); molds and yeasts (Rose Bengal Chloramphenicol Agar, Scharlau Chemie) after incubation at 25 °C for 5 days (ISO 21527-1:2008). All counts were expressed as log colony forming units (CFU)/g.

2.4 Physicochemical analysis

2.4.1 pH determination. The pH was measured in a previously homogenized sample with a portable pH meter (HI9023, Hanna Instruments, Padova, Italy) equipped with a pH electrode (FC 230B, Hanna Instruments), according to ISO 2917:1999. The average of three determinations was retained for further data analyses.

2.4.2 Water activity determination. Water activity (*a_w*) was measured in a homogenized *Alheira* sample using a Rotronic Hygrometer station (Rotronic Hygroskop DT, Ettlingen, Germany) at 23 °C. Three replicate measurements were done and the average was used for further data analyses.

2.4.3 Color evaluation. Control and HPP-treated samples were assessed for objective color using *L* a* b** system just before the package opening. These measures were performed with a colorimeter (Minolta CR-300, Chromometer, Osaka, Japan) using the coordinates of the CIELAB color system (CIE, 1976). A white tile provided by the manufacturer was used to calibrate the

instrument. The average value of L^* , a^* , and b^* from three measurements obtained in each replicate was used for statistical analysis.

2.4.4 Thiobarbituric acid-reactive substances determination. Lipid oxidation in samples was performed by assessing thiobarbituric acid-reactive substances (TBARS), as previously described by Alfaia et al. (2015). The extraction of malondialdehyde (MDA) was performed from 15 g of homogenized meat samples using trichloroacetic acid, propyl galate, and ethylenediaminetetraacetic titriplex acid (EDTA). The MDA reacted with TBA producing a red-colored complex measured in UV/Visible Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Buckinghamshire, UK) at 538 nm wavelength. A standard MDA curve prepared with a solution of 1,1,3,3 tetramethoxypropane at 10^{-8} mol/mL was used to quantify TBARS and results were calculated and expressed in milligrams of MDA *per kilogram* of meat sample (mg MDA/kg meat).

2.4.5 Biogenic amines determination. The extraction, derivatization, and quantification of eight BAs, namely, tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine, were performed according to the method described by Alves et al. (2017). The amines were extracted with perchloric acid and derivatized with dansyl chloride. The chromatographic separations were performed on a reversed-phase column (Thermoscientific RP-18, 5 μ m, 250 \times 5 μ m, Supelco, Bellefonte, PA, USA), with UV detection at 254 nm. Identification of BAs was done by comparison of the BAs retention times with those of standard solutions. The quantification of BAs was carried out using 1,7-diamino heptane (Sigma-Aldrich) as internal standard and the amounts were expressed as mg/kg of BAs.

2.5 Sensory evaluation

To study the possible influence of the different HPP conditions on *Alheira* sensory characteristics, two approaches were used: a triangle test and a qualitative research using the focus group (FG) interview methodology.

2.5.1 Preparation of the samples. *Alheira* is a poultry- and pork-based semidried fermented sausage that is generally consumed after culinary preparation (roasting, frying) and served hot. To avoid biases due to the preparation of samples, the standardized preparation process used by Patarata, Judas, Silva, Esteves, and Martins (2008) was followed, consisting of 2 min in a microwave oven (850 W), 5 min in a conventional oven (220 °C) until reaching 75 °C in the center, equilibration of temperature at 70 °C until presentation (less than 15 min). Each panel member was served with approximately 50 g of sausage. Each sample was assigned a random three-digit number, and the order of presentation was obtained by the ascending order of the random numbers.

2.5.2 Triangle test. A triangle test was carried out according to ISO 4120:2004 to evaluate if HPP treatments cause detectable sensory modifications on *Alheira*. Testing was made with 56 participants (60.7% male; 21 to 68 years old). Each panelist performed nine triangle tests, corresponding to the nine P^*t combinations in one single session, with 15 min of interval between the fifth and the sixth tests. Each test was composed of one piece for each of the triangle points, anonymously coded. Samples from the three production batches were mixed in the tests. The order of presentation of samples with different treatments was randomly defined. Spring water and unsalted crackers were available for mouth cleansing.

2.5.3 Focus group. The objective of the FGs was to detect possible modifications or defects in the HPP-packaged *Alheira*, representing both the visual inspection in a purchase-like situation

and the consumption situation tasting a cooked *Alheira*. Consumption and purchasing intention were inquired. The research was carried out with three FGs, all composed by consumers with no formal experience in sensory analysis and most of them (>95%) regular consumers of *Alheira*. The FG interviews took place in the two biggest Portuguese metropolitan areas: one in Lisbon and two in Oporto. Consumer groups were composed by 10 (eight women and two men; 22 to 59 years), 9 (six women and three men; 45 to 67 years), and 8 (two women and six men; 18 to 29 years) participants. The FG interviews were performed as described by Alfaia et al. (2015). Packaged samples were identified and ordered as above indicated. Samples from the three production batches were used in each session. In the introduction, the operation and the objectives of the FG were explained, and the identification of participants was recorded. In the first part, six individually packaged *alheira* were distributed in the table (two from each batch). The moderator asked participants to describe the packaged semidried fermented sausage and to take particular attention of any aspect they considered unusual. From the attributes pointed out, it was asked which of them they considered positive or negative. Finally, the purchasing intention was recorded. In the second part, cooked samples were presented and participants were asked to describe the aspect, odor, taste/ flavor (explanations were provided on the flavor concept), and texture. Like in packaged samples, the main positive and negative attributes as well as the consumption intention were asked. At the end of the FG interviews the moderator reviewed the main findings of the discussion with the participants. The sessions were audio-recorded. After transcription, the audio files, the content was analyzed for detection of sensory trends for each HPP-treated sausage.

2.6 Statistical analysis

The experimental procedures for the various P^*t combinations were conducted in triplicate with three observations. In a preliminary analysis, these combinations were regarded as individual treatments and the data on microbiological counts, pH, a_w , color, TBARS, and BAs were analyzed with the general linear models (GLM) procedure of statistical analysis software (SAS) (SAS Institute, Cary, NC, USA), considering the effect of treatment after checking for normality and variance homogeneity. Differences between treatment means were tested using Tukey's post hoc test at $P < 0.05$. A second analysis was carried out for the various response variables studied using a response surface approach, where the linear and quadratic effects of P and t , as well as their interactions, were investigated to identify the best combination of factors. A surface plot, described by a second-order polynomial equation, was fitted to each set of experimental data points, using the PROC RSREG of SAS (SAS Institute).

The critical proportion of correct responses in the triangle test was calculated using the sensory discrimination test module from XLStat, using the guessing model and binomial power for a significance level of 5% (XLStat, 2018).

3. RESULTS AND DISCUSSION

3.1 Microbial analysis

The results of the microbial analysis are displayed in Table 2. The dominant microbiota present in unpressurized samples were LAB, CNS, and yeasts, which represent the spontaneous fermentative culture that had a rapid growth under favorable conditions. The counts obtained for CNS and LAB were reflected in the counts obtained for TMA and TPA, respectively. *Enterobacteriaceae*

Table 2—Microbial and physicochemical evaluation of semidried fermented sausage *alheira* submitted to high-pressure processing.

Treatment	P/t	TMA	TPA	LAB	CNS	Enteroc	Moulds	Yeasts	L*	d*	β*	a _{ir}	pH	TBARS
Control	0.101/0	7.97 ± 0.71 ^a	7.73 ± 0.55 ^a	7.89 ± 0.62 ^a	5.39 ± 0.65 ^a	3.52 ± 0.76 ^a	2.80 ± 0.42 ^a	5.61 ± 1.30 ^a	58.52 ± 0.31 ^a	9.04 ± 0.10 ^b	25.02 ± 0.59 ^a	0.96 ± 0.006 ^c	5.22 ± 0.27 ^a	1.21 ± 0.23 ^a
1	300/960	6.74 ± 0.20 ^{ab}	5.92 ± 0.12 ^{ab}	6.42 ± 0.16 ^{ab}	3.82 ± 0.74 ^b	0 ^b	0 ^b	0.75 ± 0.30 ^b	53.87 ± 2.93 ^b	8.49 ± 0.67 ^b	21.73 ± 1.81 ^a	0.96 ± 0.008 ^a	5.16 ± 0.35 ^a	1.41 ± 0.11 ^a
2	344/390	6.52 ± 0.25 ^{ab}	5.88 ± 0.33 ^{ab}	6.31 ± 0.19 ^{bc}	3.43 ± 0.45 ^{bc}	0 ^b	0.49 ± 0.85 ^{ab}	0.57 ± 0.98 ^b	52.70 ± 1.80 ^{bc}	9.75 ± 0.57 ^{ab}	22.34 ± 0.11 ^a	0.96 ± 0.004 ^a	5.30 ± 0.42 ^a	1.43 ± 0.15 ^a
3	344/1,530	5.26 ± 0.76 ^{bcd}	4.86 ± 0.42 ^{bc}	5.72 ± 0.50 ^{bcd}	3.10 ± 0.30 ^{bc}	0 ^b	0.63 ± 1.10 ^{ab}	0 ^b	55.47 ± 1.58 ^{ab}	8.82 ± 0.47 ^b	20.50 ± 1.04 ^a	0.96 ± 0.004 ^a	5.07 ± 0.28 ^a	1.34 ± 0.04 ^a
4	450/150	5.85 ± 0.54 ^{bc}	4.76 ± 0.27 ^{bc}	5.61 ± 0.44 ^{bcd}	2.96 ± 0.22 ^{bc}	0 ^b	1.32 ± 0.28 ^{ab}	0 ^b	54.22 ± 1.24 ^{bc}	8.37 ± 0.70 ^b	22.22 ± 1.77 ^a	0.96 ± 0.004 ^a	5.39 ± 0.22 ^a	1.64 ± 0.16 ^a
5	450/960	4.20 ± 0.36 ^{de}	4.44 ± 0.17 ^{bc}	4.72 ± 0.85 ^{bcd}	2.99 ± 0.15 ^{bc}	0 ^b	0.72 ± 1.25 ^{ab}	0.33 ± 0.56 ^b	52.31 ± 1.36 ^{bc}	9.24 ± 0.29 ^{ab}	22.23 ± 0.81 ^a	0.96 ± 0.001 ^a	5.27 ± 0.34 ^a	1.53 ± 0.28 ^a
6	450/960	4.55 ± 0.63 ^{de}	4.19 ± 0.88 ^{bc}	4.48 ± 0.51 ^{de}	2.93 ± 0.10 ^{bc}	0 ^b	1.06 ± 0.92 ^{ab}	0 ^b	52.15 ± 1.85 ^{bc}	9.19 ± 0.66 ^{ab}	23.33 ± 0.27 ^a	0.96 ± 0.004 ^a	5.42 ± 0.16 ^a	1.45 ± 0.18 ^a
7	450/960	4.38 ± 0.58 ^{de}	3.41 ± 1.34 ^{de}	4.26 ± 0.72 ^{de}	2.93 ± 0.16 ^{bc}	0 ^b	0 ^b	0 ^b	52.84 ± 1.48 ^{bc}	9.52 ± 0.71 ^{ab}	22.46 ± 1.25 ^a	0.96 ± 0.004 ^a	5.37 ± 0.36 ^a	1.71 ± 0.42 ^a
8	450/1,800	4.60 ± 0.42 ^{de}	4.36 ± 0.32 ^{bc}	4.55 ± 0.43 ^{bcd}	2.85 ± 0.19 ^{bc}	0 ^b	0.49 ± 0.85 ^{ab}	0.43 ± 0.75 ^b	54.31 ± 1.70 ^{ab}	8.50 ± 1.11 ^b	22.51 ± 1.64 ^a	0.96 ± 0.006 ^a	5.48 ± 0.11 ^a	1.59 ± 0.21 ^a
9	556/390	4.60 ± 1.09 ^{de}	2.62 ± 0.73 ^{de}	2.74 ± 1.26 ^{ef}	2.68 ± 0.63 ^c	0 ^b	0.49 ± 0.85 ^{ab}	0 ^b	54.56 ± 0.59 ^{ab}	9.00 ± 0.82 ^b	20.38 ± 3.61 ^a	0.96 ± 0.003 ^a	5.51 ± 0.11 ^a	1.48 ± 0.35 ^a
10	556/1,530	3.13 ± 0.36 ^e	1.45 ± 1.26 ^{de}	0.32 ± 0.55 ^f	3.03 ± 0.14 ^{bc}	0 ^b	0 ^b	0 ^b	51.56 ± 0.58 ^{bc}	10.07 ± 0.48 ^{ab}	21.84 ± 1.01 ^a	0.95 ± 0.002 ^a	5.18 ± 0.17 ^a	1.77 ± 0.39 ^a
11	600/390	3.29 ± 0.13 ^e	1.22 ± 1.70 ^{de}	0.97 ± 0.87 ^g	3.01 ± 0.03 ^{bc}	0 ^b	0.57 ± 0.98 ^{ab}	0 ^b	54.24 ± 0.97 ^{ab}	8.84 ± 0.33 ^b	21.30 ± 0.45 ^a	0.95 ± 0.004 ^a	5.28 ± 0.18 ^a	1.58 ± 0.26 ^a
12	600/960	3.01 ± 0.16 ^e	0.99 ± 0.98 ^e	0 ^g	2.92 ± 0.17 ^{bc}	0 ^b	0.63 ± 1.10 ^{ab}	0 ^b	49.27 ± 0.31 ^c	10.89 ± 0.37 ^a	23.08 ± 2.64 ^a	0.96 ± 0.002 ^a	5.45 ± 0.06 ^a	1.47 ± 0.53 ^a

Data are expressed as the mean ± standard deviation of the three replicates. Data are expressed in log 10 CFU/g, where "0" refers to "not quantified at 10⁻¹". P, pressure (MPa); t, time (seconds); TMA, total mesophilic aerobic; TPA, total psychrotrophic anaerobic; LAB, lactic acid bacteria; CNS, coagulase negative *Staphylococci*; Enteroc, *Enterococci*; Moulds, moulds; Yeasts, yeasts; L*, water activity; TBARS, thiobarbituric acid reactive substances. a,b,c,d,e Means with different lowercase letters in the same column differ significantly at P < 0.05 (Tukey's test).

and molds were also abundant in the *Alheira* (untreated semidried fermented sausage) with counts of 3.52 ± 0.76 and 2.80 ± 0.42 log CFU/g, respectively. According to the guidelines published by Gilbert et al. (2000), and taking into account that *Enterobacteriaceae* are used as a hygiene indicator, the fermented sausages studied should be classified as acceptable regarding their microbiological quality. Nonetheless, the presence of *Enterobacteriaceae* could be correlated with the production of BAs at favorable temperature regimes (Latorre-Moratalla et al., 2008). These data highlight the need and importance of implementing good manufacturing practices involving adequate hygienic measures in *Alheira*'s manufacture and the use of alternative technologies such as HPP. *L. monocytogenes* and *C. peffringens*, as potential pathogens in this product were not detected in any of the analyzed samples (quantification level <1 log CFU/g).

The application of HPP in the poultry- and pork-based semidried fermented sausage reduced the counts of all the microorganisms analyzed. Gram-negative bacteria were more sensitive to HPP application than the Gram-positives, which is in agreement with other authors (Bravo, de Alba, & Medina, 2014; Huang, Lung, Yang, & Wang, 2014).

HPP treatment was effective in decreasing *Enterobacteriaceae* counts, which was below the quantification limit in all the pressurized samples, even when the lowest pressures and shorter times combinations were applied. The reduction of 3.52 ± 0.76 log CFU/g in *Enterobacteriaceae* counts is relevant because it allows us to infer that HPP helps to improve the hygienic quality of *Alheira*, and consequently, reduce the probability of BAs formation. The HPP also improves the safety of this kind of sausage against a number of important foodborne pathogens from the *Enterobacteriaceae* family, such as *Salmonella*, *Yersinia enterocolitica*, pathogenic *Escherichia coli* (including Enterotoxigenic *Escherichia coli* (ETEC) and Enteropathogenic *Escherichia coli* (EPEC)), and *Shigella* spp. (Latorre-Moratalla et al., 2008). In addition, some *Enterobacteriaceae* are associated with food spoilage, which results in off-flavors, off-odors, color defects, and other organoleptic deviations that will shorten food shelf life.

Part of fermentative microbiota (LAB and yeast) of HPP-treated poultry- and pork-based semidried fermented sausage was affected when the binomial combinations involving higher pressures were applied, independently of the holding time (Figure 1A). Significant differences were detected for LAB counts from 344 MPa and 1,530 s onward with a marked decrease for the P*t combination of 600 MPa and 960 s (P < 0.05). The increase in time of pressurization to 1,530 s with low pressures (344 MPa) was able to reduce significantly the LAB counts in sausages when compared with control (P < 0.05). At moderate pressures (450 MPa), the LAB counts on these fermented sausages were similar independently of the time of application (P > 0.05). Only with pressures higher than 556 MPa, the LAB counts reduction was 5 log CFU/g. The combination of 556 MPa with a longer time (1,530 s) induced a notable reduction in LAB counts (P < 0.05). In the HPP-treated semidried fermented sausages, a reduction of CNS counts was observed, by approximately 2 log CFU/g, for the lowest pressures and shorter time combinations tested (Figure 1B). However, CNS showed higher tolerance to the increase in pressure than LAB. In fact, CNS presented similar counts in treated fermented sausages with the application of the lowest and the highest pressure values. In fact, the barotolerance of certain CNS strains, namely, *Staphylococcus aureus*, is referred described by several authors, and their inactivation by HPP is highly dependent on the food matrix (Han et al., 2011; Jofré, Aymerich, Bover-Cid, & Garriga, 2010).

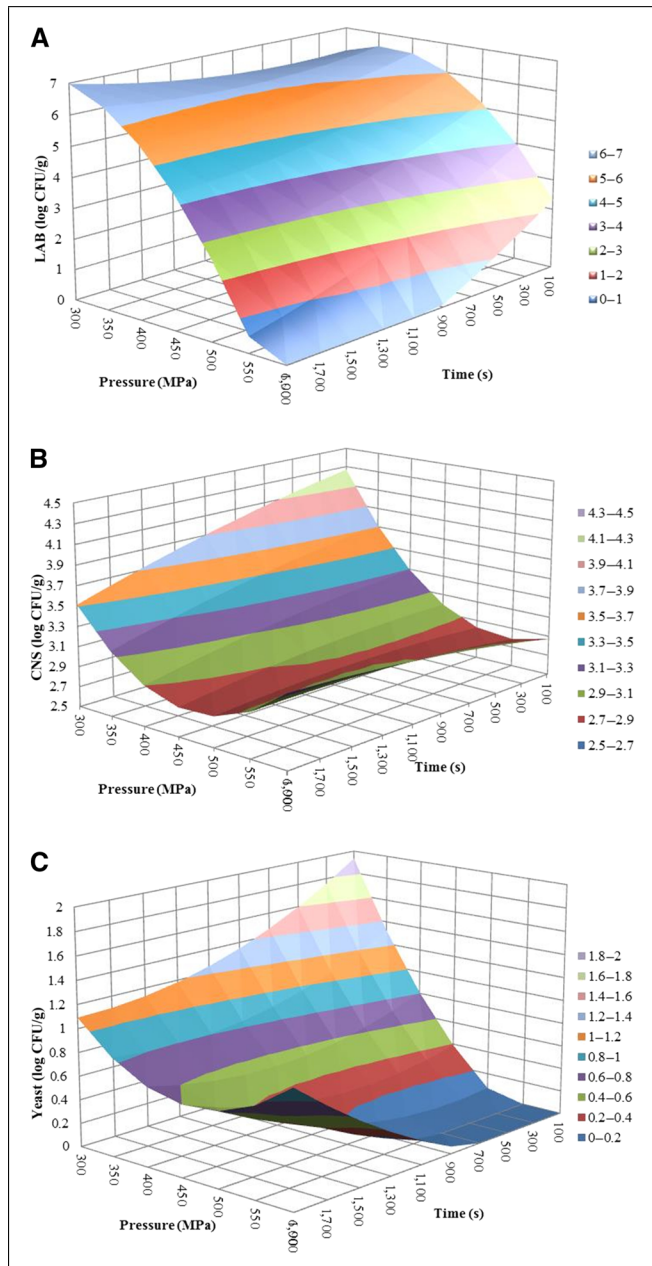


Figure 1—Response surface of binomial conditions of pressure (P) versus time (t) on semidried fermented sausage LAB (A), CNS (B), and yeast (C) counts.

CNS converts amino acids and free fatty acids into powerful aroma constituents, which are essential for the characteristic taste notes of fermented sausages, and the maintenance of these counts may be important in safeguarding the organoleptic characteristics of HPP-treated sausages (Baylis, Uyttendele, Joosten, & Davies, 2011).

Yeast counts in HPP-untreated samples were 5.61 ± 0.30 log CFU/g. The application of HPP significantly reduced yeast counts (by approximately 5 log CFU/g) in all treatments tested ($P < 0.05$) and the levels were below the quantification limit when pressures upon 556 MPa were applied (Figure 1C). In addition to LAB and CNS, yeast and mold could also play an important role as fermentative microbiota, and their lipolytic and proteolytic activities influence the development of the organoleptic characteristics of these fermented smoked sausages (Han et al., 2011; Jofré et al., 2010).

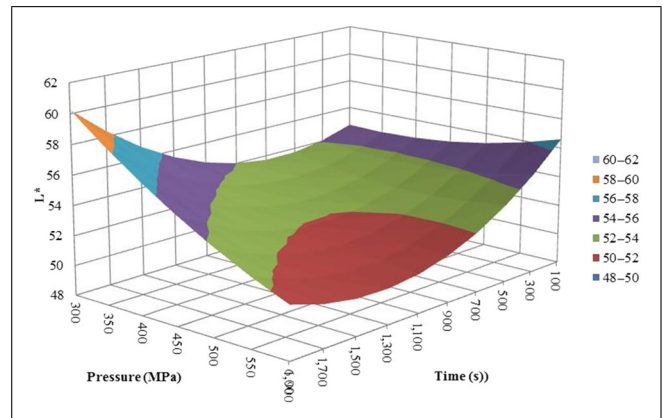


Figure 2—Response surface of binomial conditions of pressure (P) versus time (t) on semidried fermented sausage lightness (L^*).

However, the effect of HPP on spoilage yeasts and molds has not been extensively studied as for other microorganisms. According to Holck, Axelsson, McLeod, Rode, and Heir (2017), yeasts and molds are considered to be relatively sensitive to HPP, with some species exceptions. In our study, mold counts obtained in unpressurized semidried fermented sausage samples were 2.80 ± 0.42 log CFU/g. The application of HPP reduced the counts of these microorganisms but with a more erratic pattern than that observed for yeast. These findings could be explained by the fact that molds are described as having an intermediate sensibility for HPP, mainly because their mycelia are particularly susceptible, while the spores are notably resistant to HPP treatment (Ojha et al., 2015). The control of yeast and mold counts by HPP application in this type of sausage is particularly important because they could participate in its spoilage, shortening poultry- and pork-based semidried fermented sausage shelf life. Superficial molds are not admitted in the poultry- and pork-based semidried fermented sausages *Alheira* and they are a criterion for its depreciation, in contrast to other European fermented sausages where their presence confers protection and oxidation stability to the product (Holck et al., 2017).

The results of the response surface approach are shown in Figure 1 for the various treatment combinations. Considering the reduction observed in LAB, the binomial conditions with higher pressures (556 and 600 MPa) were considered the most adequate to guarantee higher stability of the poultry- and pork-based semidried fermented sausage. Since higher pressures provide a higher reduction in all spoilage and even technological groups of microorganisms, avoiding excessive fermentation during product storage, the 600 MPa and 960 s condition was chosen as the more appropriate. Furthermore, and according to Rendueles et al. (2011), this high pressure will guarantee the elimination of pathogenic microorganisms, such as *L. monocytogenes* and *C. botulinum*, thus improving product safety.

3.2 Physicochemical analysis

Color measurements, pH, a_w , and TBARS are presented in Table 2. Regarding sausage color, the application of HPP induced a significant decrease in L^* values for high-pressure values and longer time (Figure 2), which resulted in a darkening of the samples ($P < 0.05$). The minimum values of L^* were reached for the 600 MPa and 960 s combination. The redness (a^* values) of poultry- and pork-based semidried fermented sausage was also affected by HPP treatment, when using the same combination (600 MPa and 960 s). On the other hand, “yellowness” (b^* value) of sausages

was similar for all the treatments tested ($P > 0.05$). In contrast to our results, Holck et al. (2017)) observed an increase in lightness and a decrease in redness in HPP-treated products. In the present study, the semidried fermented sausage color could be dependent on the ingredients, formulation, and smoking conditions used, and thus, the slight differences observed may not have a real impact on the product. It has been reported that fresh meat is severely affected when HPP is applied while the changes in cured meat products are considered acceptable but depending on the water content and a_w value of the product (Possas, Valdramidis, García-Gimeno, & Pérez-Rodríguez, 2019). The changes induced by HPP in color meat products could be attributed to globin denaturation, heme group displacement or release, ferrous myoglobin oxidation to ferric metmyoglobin, and denaturation of myoglobin ferric species (Argyri, Papadopoulou, Nisiotou, Tassou, & Choriantopoulos, 2018). The fermented sausage under study has a high content of other ingredients in addition to meat and, unlike what is observed in other kinds of sausages, the color changes may be related with its starch and fat content. Also, cooked meats have been used instead of raw, so the cooking process had denatured the protein.

The mean pH, a_w , and TBARS values in *Alheira* samples of the control group were 5.22 ± 0.27 , 0.96 ± 0.006 , and 1.21 ± 0.23 mg MDA/kg, respectively. Similar values of pH and a_w were reported in fermented sausages (Bajovic, Bolumar, & Heinz, 2012; Ferrini, Comaposada, Arnau, & Gou, 2012); however, these parameters *per se* do not assure the poultry- and pork-based semidried fermented sausage microbiological stability. It will be needed to preserve this meat product at refrigeration temperatures (<5 °C) up to the time of consumption (Argyri et al., 2018). Moreover, the HPP process was applied in the final product, where most of the changes derived from the fermentation process had already occurred with a slight decrease in pH due to lactate production. No significant differences on pH or a_w values were observed in HPP-treated sausages relative to the control samples ($P > 0.05$). The impact of HPP on the water binding properties of meat products is still unclear, with contradictory effects of HPP on water binding properties (reviewed by Hygreeva & Pandey, 2016). However, it is known that HPP has the capacity to change the conformational structure of proteins improving water binding capacity in pressurized samples (Martínez et al., 2017). In this semidried fermented smoked sausage (*Alheira*), no major changes in water holding capacity are expected with pressurization because proteins were previously denatured by cooking.

The lipid oxidation of *Alheira* was measured using the TBARS assay. The quality of meat products is greatly affected by lipid oxidation, and it has been shown that HPP may trigger lipid oxidation depending on temperature, pressure level, duration of the process, presence of oxygen, and unsaturated fatty acids content (Stratakos, Delgado-Pando, Linton, Patterson, & Koidis, 2015). Furthermore, the type of packaging (e.g., the exclusion of oxygen due to vacuum) can also decrease lipid oxidation in meat products (Leistner, Rödel, & Krispien, 2013). In our study, the TBARS values were low in all samples, and thus, the application of HPP did not promote lipid oxidation ($P > 0.05$). Although Bajovic et al. (2012) stated that critical pressure levels can induce lipid oxidation at 300 and 600 MPa in fresh meat, none of the pressure and time combinations applied to this semidried fermented smoked sausage seem to result in high levels of lipid oxidation.

The levels of BAs in the poultry- and pork-based semidried fermented sausage are shown in Table 3. The low amounts of BAs in the non-HPP-treated sausages showed that spontaneous fermentative microbiota were unable to produce BAs under the

Table 3—Biogenic amines contents (mg/kg) of semidried fermented sausage *alheira* submitted to high-pressure processing.

Treatment	Pressure (MPa)	Time (s)	Tryptamine	Phenylalanine	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine
Control	0	0	22.08 ± 9.71 ^a	15.12 ± 4.03 ^a	40.76 ± 22.84 ^a	84.81 ± 33.96 ^a	10.53 ± 9.94 ^a	168.76 ± 19.11 ^a	49.61 ± 4.71 ^a	87.72 ± 3.76 ^a
1	960	300	13.01 ± 1.42 ^b	3.17 ± 1.68 ^b	20.06 ± 6.94 ^{bc}	26.86 ± 13.07 ^{bc}	2.10 ± 0.80 ^b	16.86 ± 9.22 ^{bc}	59.28 ± 1.58 ^{ab}	142.59 ± 19.83 ^{bd}
2	390	344	17.81 ± 2.85 ^{ab}	1.93 ± 0.61 ^b	25.47 ± 3.76 ^c	34.62 ± 4.10 ^{bc}	2.43 ± 0.84 ^b	23.60 ± 14.54 ^{bc}	67.67 ± 10.11 ^b	146.39 ± 16.40 ^d
3	1,530	344	13.27 ± 2.15 ^b	2.14 ± 0.82 ^b	20.18 ± 2.24 ^{bc}	37.94 ± 19.70 ^{bc}	1.43 ± 1.25 ^b	24.15 ± 15.90 ^{cc}	61.43 ± 4.81 ^b	126.40 ± 14.94 ^{bc,c}
4	154	450	12.27 ± 1.74 ^b	1.87 ± 1.54 ^b	15.15 ± 3.92 ^{bc}	23.84 ± 11.33 ^{bc}	1.64 ± 1.42 ^b	36.25 ± 24.41 ^c	62.11 ± 4.12 ^b	134.61 ± 10.89 ^{bd,e}
5	960	450	12.48 ± 0.98 ^b	1.82 ± 1.27 ^b	14.99 ± 2.96 ^{bc}	19.25 ± 12.85 ^b	2.12 ± 0.28 ^b	19.81 ± 23.47 ^{bc}	61.90 ± 4.56 ^b	129.95 ± 1.86 ^{bc,d,e}
6	960	450	13.97 ± 2.92 ^b	1.45 ± 1.22 ^b	14.92 ± 2.34 ^{bc}	13.96 ± 2.22 ^{bc}	1.09 ± 0.95 ^b	9.15 ± 0.47 ^b	57.91 ± 3.23 ^{ab}	116.95 ± 2.76 ^c
7	960	450	13.67 ± 2.42 ^b	1.65 ± 1.42 ^b	15.67 ± 2.80 ^{bc}	25.85 ± 7.88 ^b	1.17 ± 1.01 ^b	14.53 ± 3.05 ^{bc}	56.88 ± 1.94 ^{ab}	121.13 ± 4.52 ^{c,e}
8	1,800	450	12.62 ± 0.63 ^b	1.21 ± 0.66 ^b	13.19 ± 1.34 ^b	15.84 ± 3.56 ^b	1.88 ± 0.50 ^b	26.00 ± 10.71 ^{bc}	58.89 ± 4.56 ^{ab}	122.75 ± 6.71 ^{c,e}
9	390	556	13.52 ± 1.18 ^b	1.79 ± 1.57 ^b	13.14 ± 3.02 ^b	13.56 ± 2.05 ^b	0.65 ± 1.13 ^b	12.19 ± 9.96 ^b	63.15 ± 7.91 ^b	132.22 ± 11.08 ^{bc,d,e}
10	1,530	556	18.49 ± 9.66 ^{ab}	2.84 ± 1.05 ^b	21.26 ± 5.12 ^{bc}	31.18 ± 7.78 ^{bc}	2.41 ± 0.42 ^b	10.85 ± 3.28 ^b	66.86 ± 9.77 ^b	129.90 ± 6.99 ^{bc,d,e}
11	390	600	14.02 ± 1.33 ^b	1.39 ± 0.16 ^b	16.96 ± 2.36 ^{bc}	22.11 ± 1.83 ^{bc}	0.50 ± 0.87 ^b	11.54 ± 4.31 ^b	66.18 ± 11.53 ^b	123.82 ± 8.39 ^{c,e}
12	960	600	15.08 ± 3.15 ^{ab}	1.47 ± 1.25 ^b	13.54 ± 1.03 ^{bc}	17.96 ± 3.70 ^{bc}	1.07 ± 0.99 ^b	12.26 ± 4.73 ^b	58.69 ± 5.43 ^{ab}	123.46 ± 8.52 ^{c,e}

Data are expressed as the mean ± standard deviation of the three replicates. Data are expressed in mg/kg of *Alheira*.
a,b,c Means with different lowercase letters in the same column differ significantly at $P < 0.05$ (Tukey's test).

Table 4—Results of triangle test of semidried fermented sausage *alheira* submitted to high-pressure processing. Main findings obtained with the focus groups for the packaged *alheira* and during the tasting of the prepared product.

Treatment	Triangle test ^a (n = 56)	Focus group (n = 10, 9, 8)	
		Packaged	Tasting after preparation
Control (0.101/0)	NA	Whitish; flat; seems to have air pouches; pasty texture	Smells very well majority; the taste could be more intense; well balanced in meat; cannot feel the pieces of meat in the mouth (unpleasant); short flavor (low in garlic and acidity); too much fat
1 (300/960)	0.30 (0.723) ^b	Normal, meat visible; heterogeneous aspect	Good smell; slightly pasty; it is possible to feel the pieces of meat
2 (344/390)	0.32 (0.624)	Normal, thinner; hardy; lot of meat	Very good, both in smell and taste (not unanimous); pasty and lumpy texture; too much fat
3 (344/1,530)	0.34 (0.512)	Normal; not correctly filled, holes inside; homogeneous aspect	Smells well, pasty texture; it is more difficult to feel the meat pieces; buy unanimous; too much smoke aroma, covering the other aromas
4 (450/150)	0.43 (0.087)	Normal, slightly stiffer; pasty; excessively homogeneous; it is not possible to see pieces of meat (which is appreciated)	The smell is less intense, more piquant and slightly pastier; good balance in the taste; more meat than others, it is possible to feel different textures in the mouth (not unanimous)
5, 6, 7 (450/960)	0.31 (0.624)	Normal; pale color; less filled; many pleats (can be from the vacuum), seems soft; very homogeneous aspect, we cannot feel the pieces	Flavor and smell different; smoky aroma (eventually too intense); the taste has something different, it seems to have a different seasoning (not unanimous); very well balanced aroma and taste (not unanimous); very pasty and greasy
8(450/1,800)	0.38 (0.298)	Normal, slightly pale (compared to the product bought by some participants) pasty texture; seems to have air pouches inside	More intense flavor; more and bigger pieces of meat (not unanimous); slightly piquant; clammy and lumpy texture; too much fat; greasy texture and flavor; the bread dominate the flavor
9 (556/390)	0.32 (0.624)	Normal, soft; pasty (too much more for some participants); stains related to pieces of meat; seems to be incorrectly filled	Smell and flavor similar to the others; very pleasant flavor; pasty and clammy texture (not unanimous)
10 (556/1,530)	0.39 (0.209)	Normal, soft; pasty; the casing seems to be thicker; it is possible to see pieces of meat	Slightly more pasty and clammy than the other (not unanimous); it is possible to feel the meat pieces (not unanimous) slightly lumpy; very good; buy unanimous
11 (600/390)	0.30 (0.727)	Similar to the others (majority); paler colors; casing is opaque, it seems to be excessively filled; the casing seems to have roughness; it is possible to see whitish stains in the casing	Smell well; short smell (missing more garlic); lots of fat and bread (motif for not buying); very pasty texture, slightly liquid; the texture has something that is not usual and that is not pleasant; good persistency of taste; variable buying intention
12 (600/960)	0.45 (0.051)	Normal, correctly filled; it is possible to see air inside the sausage, seems to not be correctly filled; buy unanimous	Smooth and pleasant smell; pasty; do not feel the pieces of meat in the mouth; piquant; lumpy and clammy texture (not unanimous); can feel pieces of fat, that is unpleasant; majority buy

NA, not applicable.

^aTriangle test was not made with packaged samples.^bResults are presented as proportion of correct answers (*p*).

fermentative conditions of sausage manufacture. When compared to the control, HPP treatment reduced the BAs content in sausages, mainly those of tyramine and histamine (Table 3). It seems that HPP treatment minimizes BA production from the amino acids' precursors available due to a decrease in the aminogenic bacteria, namely, *Enterobacteriaceae* and yeasts (Suzzi & Gardini, 2003). The increase in polyamines, spermine, and spermidine, on sausages treated by HPP, could outcome from a reaction of cells to protect DNA from HPP as previously described by Held and Awad (1991). Polyamines are involved in a variety of cellular processes such as gene expression, cell growth, survival, stress response, and proliferation. Reverse genetic studies in microorganisms showed that deletion of the genes in polyamine metabolic pathways or depletion of polyamines have negative effects on cell survival and proliferation (Gevrekci, 2017). The metabolic use of spermine and spermidine was done in a lesser extent once *Enterobacteriaceae*, molds, yeasts, and CNS counts were reduced by HPP application.

3.3 Sensory analysis

Data of the sensory analysis made with the semidried fermented smoked sausage *Alheira* submitted to different conditions of *P* and *t* are presented in Table 4. The results from the triangle test made with the semidried fermented smoked sausage revealed that any of the HPP treatments applied resulted in detectable changes in sensory properties ($P < 0.05$). The proportion of correct responses in the triangle test were between 0.30 and 0.45, with the last value

near the cutoff point of significance ($P < 0.05$), corresponding to the more intense treatment applied (600 MPa and 960 s).

The main findings of the FG interviews are presented in Table 4, both for the product still in the package and for the product tasted after culinary preparation. It was observed that most of the characteristics pointed out by the participants are transversal to all the experimental conditions, and mainly related to the composition of *Alheira* itself, and not putatively to the treatments. From the information obtained with the FG assessing the packaged product, most of the characteristics detected are related to the diameter of the sausage, the transparency or opacity of the casing, the correctness of filling (poor or excessive) and the associated presence of air pouches, the homogeneity and the visualization of meat pieces, and the tactile perception of pasty texture. However, these characteristics did not show any pattern related to the HPP treatment applied, and generally, none of these aspects were considered problematic and did not influence the purchasing intention of the participants. When the participants tasted the cooked samples, the same trend was observed, such that the main aspects stressed out were related to the particular recipe used by this manufacturer and to aspects that are intrinsic to this semidried fermented sausage. Participants had a very variable opinion on the aroma and flavor of *Alheira* sausage, such that for the same sample, even in the same FG, the points of view were, in certain cases, completely different. Some of the participants considered the aroma and flavor very pleasant and adequate, while others were quite disappointed

due to the shortness of these attributes, particularly the flavor of garlic, which is an obligatory seasoning in this sausage. Another characteristic discussed was texture. These poultry- and pork-based semidried fermented sausages had a pasty texture, identified in all the sample groups and almost by all the participants, sometimes with a clammy or lumpy description. In addition, the amount of meat and the perception of fat pieces were mentioned by many participants. In line with the results obtained in the triangle test, we did not find a pattern of characteristics that relates to the HPP treatment. The variability found in this product is natural, mainly due to its traditional manufacturing production methods. The participants' perception was also greatly influenced by its personal experiences with the poultry- and pork-based semidried fermented sausage *Alheira* consumption.

4. CONCLUSION

The poultry- and pork-based semidried fermented smoked sausage has intrinsic characteristics that may not be able to assure its microbial stability at ambient temperature. In spite that meats are boiled at temperatures that could assure the inactivation of most of the vegetative forms of its more problematic pathogens, the addition of the remaining ingredients to the mixture could result in further contamination. The use of HPP in the processing of this semidried fermented smoked sausage showed promising results in improving its microbial characteristics, with minimal impact on product oxidation, color, and sensory characteristics. The counts of *L. monocytogenes* and *C. perfringens* were below the quantification level, thus assuring product safety. The best treatment regarding food spoilage microbiota reduction associated with only a slight modification of sausages lightness and redness, without significant changes in their sensory properties, was 600 MPa for 960 s. Lower pressures (300 MPa and 960 s) are also relevant since they are associated with lower energy consumption and showed a similar reduction of spoilage microbiota (*Enterobacteriaceae*, yeast, and molds) and of CNS compared with treatments that used higher pressures. Therefore, this poultry- and pork-based semidried fermented smoked sausage could benefit from the application of HPP technology, in order to potentially reduce the pathogenic and spoilage microbiota and consequently reach a longer shelf life and an improved safety.

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AUTHOR CONTRIBUTIONS

Maria João Fraqueza and Luis T. Gama were responsible for study conception and design. Ana Borges, Almudena Cozar, Luis Patarata, Cristina Mateus Alfaia, Maria José Fernandes, Maria and Helena Fernandes were responsible for acquisition of data. Ana Borges, Maria João Fraqueza, Luis Patarata, and Luis Telo da Gama were responsible for analysis and interpretation of data. Ana Borges, Almudena Cózar, Luis Patarata, and Maria João Fraqueza drafted the manuscript. Cristina Alfaia and Herminia Vergara Pérez

performed critical revision. More contributions regarding acquisition, analysis, and interpretation of data are as follows:

- Ana Borges, Almudena Cozar, and Maria João Fraqueza did the technological experiments.
- Ana Borges and Maria Helena Fernandes performed the microbial analyses.
- Ana Borges and Luis Patarata performed the sensorial analysis.
- Ana Borges, Almudena Cozar, and Cristina Alfaia performed de BAs analysis.
- Maria José Fernandes and Ana Borges performed the chemical analysis.
- Ana Borges, Maria João Fraqueza, Luis Patarata, and Luis Telo da Gama analyzed the data.
- Luis Telo da Gama and Ana Borges performed the statistical analysis.
- Ana Borges, Maria João Fraqueza, and Luis Patarata interpreted the data.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

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