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Relating physicochemical and microbiological safety indicators during processing of *linguiça*, a Portuguese traditional dry-fermented sausage

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ABSTRACT

Linguiça is a Portuguese traditional fermented sausage whose microbiological quality and safety can be highly variable. In order to elucidate risk factors and the particularities of the manufacturing technology that explain the between-batch variability in total viable counts (TVC), *Enterobacteriaceae*, *Staphylococcus aureus* and *Listeria monocytogenes* in the product; microbiological and physicochemical characterisation of *linguiça* at five stages of production (i.e., raw pork meat, mixed with ingredients, macerated, smoked and ripened) was carried out. A total of six production batches were surveyed from two factories; one utilised curing salts and polyphosphate in their formulation (Factory II). The delayed fermentation in the nitrite-formulated sausages was partly responsible for the increase ($p < 0.01$) in *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* from raw meat (3.21 log CFU/g, 1.30 log CFU/g and 22.2 CFU/g, respectively) to the end of maceration (4.14 log CFU/g, 2.10 log CFU/g and 140 CFU/g, respectively) while the better acidification process in the nitrite-free sausages (Factory I) led to lower counts of *S. aureus* (2.64 log CFU/g) and *L. monocytogenes* (10 CFU/g) in the finished products. In Factory II, although *L. monocytogenes* entered the chain at the point of mixing, it became steadily inactivated during smoking and ripening (<50 CFU/g), despite the initially-delayed fermentation. Nitrite had a strong effect on reducing *Enterobacteriaceae* throughout smoking ($r = -0.73$) and ripening ($r = -0.59$), while it failed to control the growth of *S. aureus*. The main hurdle preventing the development of *S. aureus* in *linguiça* is the pH, and other factors contributing to its control are: longer ripening days ($p = 0.019$), low *S. aureus* in raw meat ($p = 0.098$), properly-washed casings ($p = 0.094$), and less contamination during mixing ($p = 0.199$). In the case of *L. monocytogenes*, at least three hurdles hinder its development in *linguiça*: low a_w ($p = 0.004$), low pH ($p = 0.040$) and nitrite ($p = 0.060$), and other factors contributing to its control are: longer ripening ($p = 0.072$) and maceration ($p = 0.106$) periods, lower a_w at the end of smoking ($p = 0.076$) and properly-washed casings ($p = 0.099$). Results have shown that there is a need to standardise the productive process of *linguiça*, to optimise the initial acidification process, and to reinforce proper programmes of quality control of ingredients and good hygiene practices, so as to minimise the introduction of *Enterobacteriaceae* and pathogens from external sources.

1. Introduction

In Europe, naturally fermented sausages have a long tradition originating from Mediterranean countries that dates back from Roman times. Among the many types of traditional fermented sausages produced in Portugal, *linguiça* is a popular fermented sausage made of raw, salted and unground pork meat which originated in regions with temperate and colder climates — since traditional ripening procedures require low to moderate temperatures. According to the classification of fermented sausages based on microbial stability (Lucke, 2000), *linguiça* belongs to the 'semi-dry, no-mould-growth' category, as defined by its short ripening time (<3 weeks) and final water activity (a_w) of 0.90–0.95. In the production of *linguiça*, diced pork meat is marinated in a mixture of water, salt, regional wine and spices, without addition of starter cultures. After few days of maceration, the mixture is stuffed into pork intestine, and then smoked and dried at low temperatures. However, since these sausages are typically manufactured by traditional customs in regional processing units, their microbiological quality and safety can be variable. The limited research conducted so far on these aspects has highlighted

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that: (i) *linguiça* sausages are not always produced under the best hygiene conditions (Esteves, Saraiva, Fontes, & Martins, 2006); and (ii) there is substantial variability in key process variables such as maceration/smoking/drying time and temperature, and production time (Elias, Fraqueza, & Barreto, 2006). Such deviations may result in both an insufficient acidification and ripening process — therefore not providing the desired microbial stability to the product; and even the presence of foodborne pathogens. To this respect, earlier research has shown that, despite the addition of curing salts and the simultaneous reduction in pH and a_w occurring during fermentation, pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella* may survive in *linguiça* sausages (Ferreira et al., 2007, 2009). A recent meta-analysis (Xavier, Gonzales-Barron, Paula, Estevinho, & Cadavez, 2014) on the incidence of pathogens in traditional Portuguese meat products revealed that the non-compliance to EU microbiological criteria for *L. monocytogenes* (8.3%; 95% CI: 5.1–13.1%) and *Salmonella* spp. (5.7%; 95% CI: 2.8–11.3%) in sausages 'intended to be eaten raw' (including *linguiça*) was considerable higher than EU levels for ready-to-eat products in comparable categories (<1.4%) (EFSA, 2015). Apart from processing faults (i.e., great variability) in the production of *linguiça* sausages, other events contributing to the presence of pathogens are the use of contaminated raw meats and casings as well as cross-contamination from operators and equipment. All these causes need to be evaluated so that control measures leading to the production of safe and homogeneous products can be properly implemented in the production of *linguiça*. Hence, the objectives of this study were three-fold: (i) to reveal eventual particularities of the manufacturing technology that could explain the different levels in total viable counts (TVC), *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* among production batches of *linguiça*; (ii) to elucidate main contamination sources and critical stages; and (iii) to assess overall effects of the different processing stages on microbial counts, and relate them to the physicochemical properties of the product (pH, a_w, moisture, nitrite, nitrate, polyphosphate and sodium chloride concentration).

2. Methodology

2.1. Sampling scheme

Both physicochemical and microbiological extensive longitudinal surveys of *linguiça* along different processing stages from raw meat to final product, as well as microbiological surveys of environmental elements, were carried out in two representative regional factories, located in the Northeast of Portugal. The number of sampling visits was sufficient to gather complete physicochemical, microbial and environmental profile data for six batches of production. None of the factories provided information on the exact formulation of *linguiças*, yet both acknowledged that pork meat was macerated in water, regional red wine, garlic paste, piri-piri, sweet red pepper paste and laurel leaves at low temperature for 2–6 days. One factory added curing salts (nitrite/nitrate) and polyphosphates in the macerating meat (Factory II) while the other did not utilise curing salts (Factory I). Before the day of sausage stuffing, pork small intestine casings are washed and kept in salted water at refrigeration temperature until use. Macerated meat is then stuffed in the natural casings to obtain ~25-cm long horse-shape sausages, and in some occasions, a short maturation period takes place at low temperatures. Vertically-hung sausages in racks are then subject to the drying effect of smoke produced by burning olive and oak tree firewood for ~3 days in a smokehouse, whose ambient temperature is not controlled, yet can be between 40 and 55 °C. Sausages continue to ripen in a refrigerated chamber at low relative humidity, and are packed either under normal atmosphere or vacuum (Fig. 1). Both factories were middle-sized and availed from the following areas: meat cutting, mixing, filling and packaging rooms, smokehouse and refrigerated chamber.

Three production batches of *linguiça* per factory were followed up through systematic sampling of raw meat (n = 3 units per batch), meat mixed with ingredients (n = 3), macerated meat (n = 3), smoked sausage (n = 5) and final product (n = 5). These sampling points along production are hereafter referred to as 'raw meat', 'mixed', 'macerated', 'smoked' and 'ripened', respectively. Within a batch, the day of sampling for both 'raw meat' and 'mixed' belonged to the same day of production (Day 0). However, because the duration periods of the processing stages of maceration (from 2 to 6 days), maturation and smoking (from 3 to 11 days) and ripening (from 3 to 11 days) were variable from batch to batch (even within the same factory), samples were taken always at the end of the processing stage, and the corresponding Day was annotated. In addition, for each of the batches surveyed, a pooled sample of washed casings was extracted (n = 1) on the day that maceration was completed. Samples of raw meat, batter, sausages and casings were collected using sterile equipment and stored aseptically in polyethylene bags.

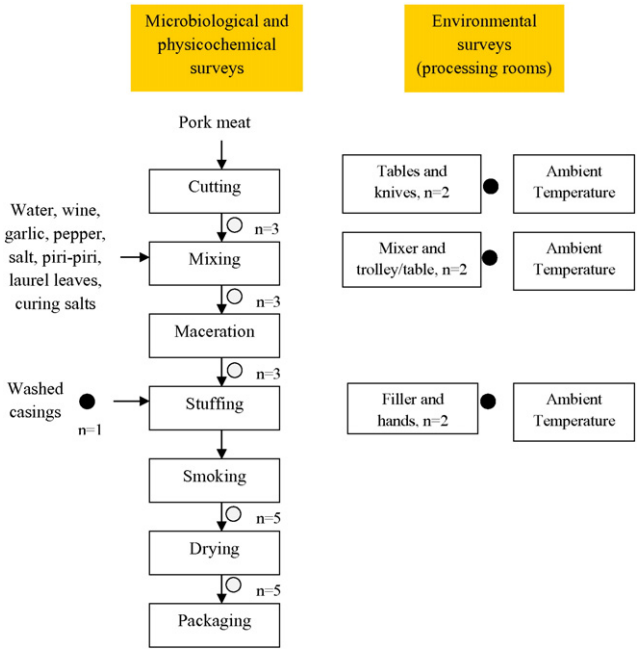

Fig. 1. Flow diagram of *linguiça* sausage processing showing sampling sites for microbiological analyses (TVC, *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* in grey and black circles) and physicochemical analyses (Aw, pH, moisture, sodium nitrite, potassium nitrate, phosphorous and sodium chloride in grey circles) from a production batch.

In the sampling visits, swabs from six environmental elements, namely, table surface, transport trolleys, mixer, filler, knives and operator hands (n = 6) were also taken. The six samples consisted of two environmental elements swabbed during processing from each of the following three rooms: *meat cutting* and *mixing rooms*, sampled on the day of mixing; and *filling room* sampled on the day that maceration was completed. For sampling one environmental element, three 4-mL neutralising buffer swabs were used (Frilabo, Maia, Portugal). As one swab covered an area of ~100-cm^2, the contamination spread over a total area of 300 cm^2 was lifted. In the case of knives and operator hands, both sides were swabbed with three 4-mL buffer swabs, and the areas measured (~200 cm^2). Environmental and product samples were transported to the laboratory in portable, insulated cold-boxes and stored at 4 °C. They were processed before 24 h for microbiological analysis, or were promptly frozen (−18 °C) until use for physicochemical analyses (except pH and a_w, which were measured on the same day of sample collection). Microbiological determinations of TVC, *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* were performed in all samples of raw meat, batter, sausages and environmental elements. In addition, to gain some insight into the evolution of the fermentative microflora, lactic acid bacteria (LAB) and *Micrococcaceae* were quantified only at the beginning of the fermentation process ('Mixed' samples) and at the end of ripening ('Ripened' samples). Physicochemical

determinations in raw meat, batter and sausages encompassed pH, a_w, moisture, sodium nitrite, potassium nitrate, phosphorous and sodium chloride contents.

In addition, while the processes of meat cutting, mixing and stuffing took place, the ambient temperature and relative humidity of each of these rooms were recorded by a thermo-hygrometer transmitter (TFA® Dostmann, Wertheim, Germany) at five different sites within a room (Fig. 1). Average ambient temperatures of these three rooms were then calculated for each of the production batches.

2.2. Microbiological analyses

In the laboratory, all sausage casings were removed using sterilised instruments to produce sausage samples. For the microbial determinations, 25 g of sample was taken aseptically, diluted in 225 mL sterile buffered peptone water (BPW, VWR Chemicals Prolabo, Portugal) and homogenised for 2 min (Stomacher 400, Seward, UK). For the analysis of an environmental element, the solutions from the three swab tubes were mixed, and a 4 mL-volume was taken and diluted in 36 mL BPW. For casings, ~10 g was weighed, and washed and shaken in 90-mL BPW. Appropriate decimal dilutions were prepared. For TVC, 1-mL volumes from sampling dilutions were spread onto Aerobic Count Plate Petrifilm™ disks (3M Health Care, St. Paul, USA), and incubated at 30 °C for 72 h. For *Enterobacteriaceae*, 1-mL volumes were spread onto *Enterobacteriaceae* Count Plate Petrifilm™ disks (3M Health Care, St. Paul, USA), and incubated at 37 °C for 24 h. For *S. aureus*, 1-mL volumes were spread on Petrifilm™ Staph Express Count (3M Health Care, St. Paul, USA), incubated at 37 °C for 24 h, and coagulase-positive colonies confirmed with Petrifilm™ Staph Express Disk, according to the manufacturer's instructions. Lactic acid bacteria (LAB) counting was performed on Man, Rogosa and Sharpe (MRS) agar (Liofilchem, Italy) overlaid with 5 mL agar 0.8%, incubated at 30 °C for 48-72 h. Micrococcaceae were counted on Baird–Parker agar (VWR Chemicals Prolabo, Portugal), incubated at 37 °C for 48 h.

For the microbiological analysis of *L. monocytogenes*, 25 g of sample was homogenised in 225 mL of Half Fraser Base CM0895 (Oxoid, Hampshire, UK). The enumeration was performed according to the ISO 11290-2:1998/Amd. 1:2004(E) procedure (ISO, 1998). After incubation of the initial suspension for 1 h at 20 °C, a 0.1-mL volume was surface-inoculated on Oxoid Chromogenic Listeria Agar (OCLA, Oxoid) and incubated at 37 °C for 24 h. The samples with no growth were analysed for detection of *L. monocytogenes* according to the ISO 11290-1:1996/Amd.1:2004(E) procedure (ISO, 1996a). The initial suspension was supplemented with SR 166 selective supplement (Oxoid), incubated at 30 °C for 24 h and streaked on OCLA (incubated at 37 °C for 24 h). If no growth was detected, 0.1 mL of the same initial supplemented suspension was transferred into 10-ml Fraser Broth supplemented with SR 166 (Oxoid), incubated at 37 °C for 48 h and streaked onto OCLA (incubated at 37 °C for 24 h). The colonies that grew on OCLA were confirmed with additional tests of haemolysis, catalase reaction, Gram stain and motility. The presumptive colonies of *Listeria* spp. were confirmed using API® *Listeria* (bioMérieux, Marcy l'Etoile, France) biochemical strips according to manufacturer's instructions. The microbiological determinations per sample were carried out in duplicate. Microbial results were expressed in log CFU/g (products and casings) and log CFU/cm^2 (environmental elements) for all microbial groups with exception of *L. monocytogenes*, where CFU/g and CFU/cm^2 were instead used.

2.3. Physicochemical analyses

The pH of samples was measured directly in the centre of the samples with a pH-meter HI8424 (Hanna Instruments, Portugal) while a_w was measured using a HygroPalm AW1 (Rotronic International, Portugal). Moisture and sodium chloride content (NaCl) were quantified according to the International Organization for Standardization (ISO) recommended standards 1442:1997 (ISO, 1997) and 1841–1:1996 (ISO, 1996b), respectively. Nitrites and nitrates were quantified according to ISO 2919:1975 and ISO 3091:1975 (ISO, 1975a, 1975b), and expressed as sodium nitrite ($NaNO_2$) and potassium nitrate (KNO_3), respectively. Total phosphorus was quantified following AOAC 969.31 (AOAC, 1995) and the molecular absorption spectrophotometric method from SMEWW 4500P-E (Clesceri, Greenberg, & Trussell, 1998). Phosphorous was expressed as phosphorus pentoxide (P_2O_5). All physicochemical determinations were made in triplicate for each sample. Due to the typical loss of water content during the manufacturing process, the concentrations of all chemical compounds were also expressed in dry matter (dm).

2.4. Statistical analyses

Considering that data originated from longitudinal surveys in *actual* processing conditions – rather than experimental set-ups under laboratory controlled conditions, the objective of the statistical analysis was not to construct predictive models of microbial counts during processing, but *to infer* on (i) the influence of the changing physicochemical characteristics and environmental contamination on the evolution of the hygiene indicators and pathogens in *linguiça* sausages; and (ii) to identify, among all measured variables, the factors that contributed the most to the survival of pathogens in *linguiça*. Variables defined for data analyses encompassed microbial groups (*TVC*, *Entero*, *Staphy* and *Listeria*) and physicochemical properties (*pH*, a_w, *moisture*, *NaCl*, $NaNO_2$, KNO_3 and P_2O_5), as mentioned above. In addition, other variables were defined using the data generated in a production batch: *Factory* (either Factory I or II), *Day* (day of sampling at the end of a processing stage, as detailed in Subsection 2.1), *Stage* (a categorical variable encompassing all of the sampling points; namely, meat, mixed, macerated, smoked and ripened), *TVCCasings*, *EnteroCasings*, *StaphyCasings* and *ListeriaCasings* (mean counts of mesophilic bacteria, *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* in casings, calculated for every batch), *MeanTVCEnv*, *MeanEnteroEnv*, *MeanStaphyEnv* and *MeanListeriaEnv* (mean environmental contamination for each bacterial group calculated as the grand average of all the environmental samples within a batch), *RoomTVCEnv* (mean TVC counts of the environmental elements sampled in the cutting, mixing and filling room within a batch), *RoomEnteroEnv* (mean *Enterobacteriaceae* counts of the environmental elements sampled in the cutting, mixing and filling room within a batch), *RoomStaphyEnv* (mean *S. aureus* counts of the environmental elements sampled in the cutting, mixing and filling room within a batch), *RoomListeriaEnv* (mean *L. monocytogenes* counts of the environmental elements sampled in the cutting, mixing and filling room within a batch), and *RoomT* (mean ambient temperature of the meat cutting, mixing and filling rooms within a batch). Due to the typical moisture loss during the manufacturing process, the concentrations of all chemical compounds in the meat/sausage samples were converted to dry matter (dm). Variables expressed in dm were defined as: *NaCldm*, $NaNO_2dm$, KNO_3dm and P_2O_5dm. Three types of statistical analysis were then carried out, as described below.

2.4.1. Analysis I: associations between physicochemical properties and microbial counts along processing

The objective of these analyses was to appraise the particularities in the evolution of the physicochemical parameters that could partially explain the batch-specific differences in microbial concentrations along production. The following linear models

$$TVC_{ijk} = \beta_0 + \beta_{1i} Day(Stage_i) + \beta_2 a_w + \beta_3 pH + \beta_4 NaNO_2dm + \beta_5 pH \times NaNO_2dm + \varepsilon_{j(k)} \quad (1)$$

$$Entero_{ijk} = \beta_0 + \beta_{1i} Day(Stage_i) + \beta_2 a_w + \beta_3 pH + \beta_4 NaNO_2dm + \beta_5 pH \times NaNO_2dm + \varepsilon_{j(k)} \quad (2)$$

$$Staphy_{ijk} = \beta_0 + \beta_{1i} Day(Stage_i) + \beta_2 a_w + \beta_3 pH + \beta_4 NaNO_2dm + \beta_5 pH \times NaNO_2dm + \varepsilon_{j(k)} \quad (3)$$

were adjusted separately to the TVC, *Enterobacteriaceae* and *S. aureus* data sets. The covariance of the error term $\varepsilon_{j(k)}$ is unstructured and allows for dependence of the observations within batches of production k, yet nested within factories j. Likewise, because the day (*Day*) at which a processing stage i (*Stage*) ended was different from batch to batch, a nested term $Day(Stage_i)$ was pondered as an attempt to account for the effect of stage duration. Stage was included in the linear models to extract the individual effects of mixing with other ingredients, maceration, smoking and ripening; and, in this manner, to evaluate the effects of a_w, pH and nitrite ($NaNO_2$) *only in a global way*. As the interaction between pH and nitrite proved to be significant, the term was included in all models above.

As the untransformed *L. monocytogenes* data (CFU/g) was over-dispersed (variance ≫ mean), consisting of low microbial counts and large proportion of zero counts (non-detections), a Poisson-gamma (negative binomial) count data model was opted for. Earlier, Gonzales-Barron, Cadavez, and Butler (2014) demonstrated that this type of count data models along with their zero-modified counterparts are much more suitable for inferential assessment than normality-based regression models when analysing over-dispersed microbiological data. Thus, in order to appraise the same fixed effects as in Eqs. (1)–(3), yet accounting for the non-detections, a regression model based on the Poisson-gamma distribution was fitted to the *L. monocytogenes* data,

$$Listeria_{ijk} = exp\{\beta_0 + \beta_{1i}Day(Stage_i) + \beta_2 a_w + \beta_3 pH + \beta_4 NaNO_2 dm + \beta_5 pH \times NaNO_2 dm\} + exp(\varepsilon_{j(k)}) \quad (4)$$

where the errors $\varepsilon_{j(k)}$ follow a gamma distribution $(1/\theta, \theta)$ with expected value 1 and dispersion parameter θ. For a detailed description of the Poisson-gamma regression and fitting procedures for microbial data of low counts, refer to Gonzales-Barron et al. (2014).

Additionally, in an attempt to discern the physicochemical parameters (as variables *pH*, a_w, *moisture*, *NaCldm*, $NaNO_2dm$, KNO_3dm and P_2O_5dm) having a greater impact on the microbial counts *reached at the end of every processing stage*, factor analyses were performed separately on sixteen data subsets partitioned by microbial group (4) and processing stage (i.e., mixed, macerated, smoked and ripened). Results from these factor analyses reveal associations between microbial loads and physicochemical parameters by processing stage; and so they complement the findings from models in Eqs. (1)–(4). A maximum likelihood method to extract two factors with *varimax* rotation was specified.

2.4.2. Analysis II: impact of processing days and environmental contamination on microbial counts along production

The objective of these statistical analyses was to evaluate the mean effects of the duration of a processing stage and the environmental contamination/temperature of a processing room on the microbial concentrations along production. The longitudinal models of the form,

$$TVC_{ijk} = \beta_0 + \beta_{1ij}\left\{Stage_i \times Factory_j\right\} + \beta_{2ij}\left\{Day(Stage_i) \times Factory_j\right\} + \varepsilon_{j(k)} \quad (5)$$

$$Entero_{ijk} = \beta_0 + \beta_{1ij}\left\{Stage_i \times Factory_j\right\} + \beta_{2ij}\left\{Day(Stage_i) \times Factory_j\right\} + \varepsilon_{j(k)} \quad (6)$$

$$Staphy_{ijk} = \beta_0 + \beta_{1ij}\left\{Stage_i \times Factory_j\right\} + \beta_{2ij}\left\{Day(Stage_i) \times Factory_j\right\} + \varepsilon_{j(k)} \quad (7)$$

were fitted to the counts of TVC (Eq (5)), *Enterobacteriaceae* (Eq. (6)) and *S. aureus* (Eq. (6)) as response variables. The categorical variable $Stage_i$ along with the nested variable $Day(Stage_i)$ were included in the model so as to estimate the mean increase or decrease in microbial concentration per day of maceration, smoking and ripening (represented by the fixed-effects β_{2ij} in Eqs. (5)–(7)). As the stage-specific day slopes β_2 may differ between factories, the categorical variable $Factory_j$ was allowed to enter in interaction with both $Stage_i$ and $Day(Stage_i)$.

For the *L. monocytogenes* data, a Poisson-gamma regression model was adjusted, although with a slightly different structure. Since for Factory I, *L. monocytogenes* counts took values of either 0 or 50 CFU/g, it was not possible to estimate the effect of day β_2 per factory. Thus, the terms $Factory_j$ in interactions with $Stage_i$ and $Day(Stage_i)$ were dropped from the model, and its parameter estimates can be assumed to be applicable to both factories.

$$Listeria_{ijk} = exp\{\beta_0 + \beta_{1i}\{Stage_i\} + \beta_{2i}\{Day(Stage_i)\}\} + exp(\varepsilon_{j(k)}) \quad (8)$$

To assess the effects of environmental contamination and ambient temperature on the TVC counts along processing, the variables *MeanTVCEnv*, *RoomTVCEnv* and *RoomT* were added one by one to Eq. (5) and their significance tested. Likewise, the significances of the corresponding environmental variables for *Enterobacteriaceae* (*MeanEnteroEnv*, *RoomEnteroEnv* and *RoomT*), *S. aureus* (*MeanStaphyEnv*, *RoomStaphyEnv* and *RoomT*) and *L. monocytogenes* (*MeanListeriaEnv*, *RoomListeriaEnv* and *RoomT*) were tested by linearly adding them one by one to Eqs. (6), (7) and (8), respectively.

2.4.3. Analysis III: factors favouring the growth/survival of Enterobacteriaceae and pathogens during processing

The objective of the last type of statistical analysis was to pinpoint the main (risk) factors that contributed to the growth or survival of *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* in *linguiça* sausages at the end of smoking and ripening. Considering all the information extracted from the surveys, the factors likely to have an effect on the final microbial counts were defined, as follows (independent variables): raw meat pH, concentrations of nitrite and nitrate added at maceration, *Enterobacteriaceae*/*S. aureus*/*L. monocytogenes* in raw meat, *Enterobacteriaceae*/*S. aureus*/*L. monocytogenes* right after mixing with ingredients, mean *Enterobacteriaceae*/*S. aureus*/*L. monocytogenes* from environmental elements, *Enterobacteriaceae*/*S. aureus*/*L. monocytogenes* in casings, a_w/pH/moisture/NaCl at the end of maceration, a_w/pH/moisture/NaCl at the end of smoking, a_w/pH/moisture/NaCl at the end of ripening, duration of maceration, duration of smoking, duration of ripening, mean temperature of mixing room and mean temperature of filling room. For every bacterial group, two separate stepwise variable selection analyses were performed using the microbial concentration at the end of smoking or at the end of ripening as dependent variables, and all of the factors specified above as independent variables. The significance level for an effect to enter and to stay in the model was set to 0.20. All models were adjusted in R version 2.14.2 (R Development Core Team).

3. Results and discussion

The factory surveys evidenced a great variability in microbial counts and their patterns both among production batches and between industries, partly due to the fact that these regional industries produce *linguiça* sausages under variable manufacturing processes. As an example, Fig. 2 shows both the variability in TVC and *L. monocytogenes* among samples during *linguiça* processing, and the variability in the duration of the processing stages. Notice that, depending on the batch, smoking was completed on the 5th, 9th, 10th, 11th or 15th day of production, while the total processing time (see "ripening" in Fig. 2) took between 8 to 21 days.

3.1. Physicochemical changes along linguiça processing

The divergences in the physicochemical properties between factories (Table 1) did not only originate from the distinct manufacture processes (i.e., Factory I had a longer production process) but also from the addition of higher concentrations of salt and additives (nitrite, nitrate and commercial sausage sodium polyphosphate by Factory II). The addition of these additives in the production process of Factory II can be

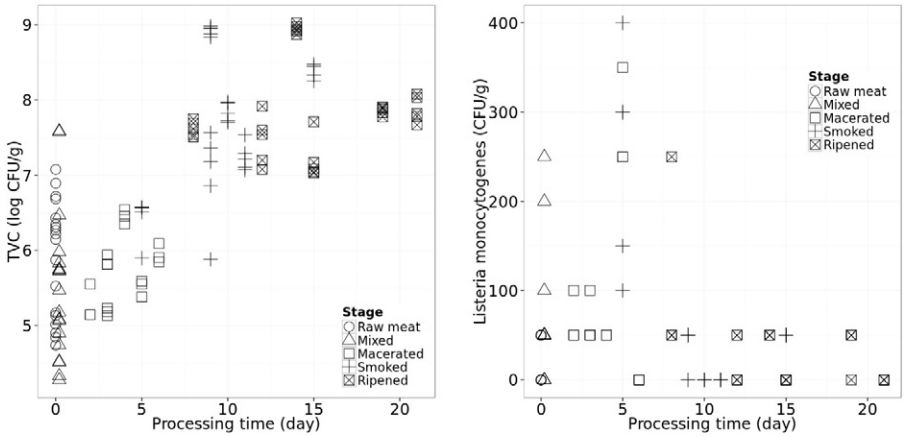

Fig. 2. Evolution of total viable counts (TVC, left) and *L. monocytogenes* (right) in meat during production of *linguiça* dry-fermented sausages. Each marker represents one sample's measurement.

verified by the significant increase in $NaNO_2$ (11.15 mg/kg wb), KNO_3 (143.5 mg/kg wb) and P_2O_5 (0.591% wb) in the mixture in comparison to the previous sampling point, raw meat (0.046 mg/kg wb, 4.884 mg/kg wb and 0.454% wb, respectively) (Table 1). In the subsequent sampling stages of macerated meat, and smoked and ripened sausages, the concentrations (wet basis) of the above additives significantly increased in each of the factories, although this is only a result of the significant moisture loss taking place progressively during smoking and ripening (from 71.3% and 68.2% moisture at the end of maceration to 40.2% and 52.7% in final products from Factories I and II, respectively). When the evolution of nitrite and nitrate was instead analysed in dry basis, a progressive increase in nitrite could be observed during fermentation and ripening of the sausages of low nitrite concentration (Fig. 3, Factory I), due to the action of the catalase-positive cocci which are the main agents of nitrate reduction (Lucke, 2000; Sebranek & Bacus, 2007). However, in the sausages formulated with high concentrations of nitrite and nitrate, such reduction was not evident (Fig. 3, Factory II). A plausible explanation for this is that the low amount of nitrate reduced by *Micrococcus* may have been concealed by the high variability in the nitrite/nitrate concentrations observed among samples from Factory II. Such high variation in nitrite/nitrate concentrations among the 'mixed' and 'macerated' samples occurred as a consequence of a poor dispersion of the additives in the mixture (raw data not shown).

Table 1
Evolution of physicochemical and chemical characteristics of *linguiça* dry-fermented sausages along processing per factory and overall. Means and standard deviations (in brackets) are shown.

Physico-chemical property	Stage	Factory I (no additives)	Factory II (with additives)	Both factories
pH	Raw meat	6.133 (0.034)^a	6.073 (0.067)^a	6.103 (0.059)^a
	Mixed	5.710 (0.034)^b	6.233 (0.067)^b	5.972 (0.059)^b
	Macerated	5.632 (0.034)^b	6.209 (0.067)^b	5.921 (0.059)^b
	Smoked	5.358 (0.026)^c	5.772 (0.052)^c	5.565 (0.046)^c
	Ripened	5.364 (0.026)^c	5.513 (0.052)^d	5.439 (0.046)^d
a_w	Raw meat	0.977 (0.004)^a	0.978 (0.004)^a	0.978 (0.004)^a
	Mixed	0.966 (0.004)^b	0.957 (0.004)^b	0.961 (0.004)^b
	Macerated	0.964 (0.004)^b	0.965 (0.004)^b	0.965 (0.004)^b
	Smoked	0.931 (0.003)^c	0.941 (0.003)^c	0.936 (0.003)^c
	Ripened	0.913 (0.003)^d	0.933 (0.003)^d	0.923 (0.003)^d
Sodium nitrite (mg/kg wb)	Raw meat	0.064 (0.054)^a	0.046 (1.351)^a	0.055 (1.046)^a
	Mixed	0.076 (0.054)^a	11.15 (1.351)^b	5.616 (1.046)^b
	Macerated	0.094 (0.054)^a	7.565 (1.351)^c	3.830 (1.046)^b
	Smoked	0.558 (0.041)^b	6.601 (1.046)^d	3.580 (0.811)^b
	Ripened	0.879 (0.041)^c	6.238 (1.046)^d	3.559 (0.811)^b
Potassium nitrate (mg/kg wb)	Raw meat	4.831 (1.000)^a	4.884 (7.086)^a	4.857 (21.22)^a
	Mixed	7.979 (1.000)^b	143.5 (7.086)^b	75.77 (21.22)^b
	Macerated	8.484 (1.000)^b	154.9 (7.086)^c	81.72 (21.22)^b
	Smoked	12.64 (0.774)^c	211.4 (5.488)^c	112.1 (16.44)^c
	Ripened	14.58 (0.774)^d	240.8 (5.488)^c	127.7 (16.44)^c
Phosphate as P_2O_5 (% wb)	Raw meat	0.378 (0.020)^a	0.454 (0.022)^a	0.416 (0.033)^a
	Mixed	0.341 (0.020)^a	0.591 (0.022)^b	0.466 (0.033)^a
	Macerated	0.329 (0.020)^a	0.601 (0.022)^b	0.465 (0.033)^a
	Smoked	0.466 (0.015)^b	0.761 (0.017)^c	0.614 (0.026)^b
	Ripened	0.578 (0.015)^b	0.844 (0.017)^d	0.711 (0.026)^c
Sodium chloride (% wb)	Raw meat	0.089 (0.071)^a	0.104 (0.072)^a	0.096 (0.084)^a
	Mixed	1.126 (0.071)^b	1.692 (0.072)^b	1.409 (0.084)^b
	Macerated	1.178 (0.071)^b	1.784 (0.072)^b	1.481 (0.084)^b
	Smoked	1.627 (0.055)^c	2.307 (0.056)^c	1.967 (0.065)^c
	Ripened	2.044 (0.055)^d	2.619 (0.056)^d	2.332 (0.065)^d
Moisture (% wb)	Raw meat	60.00 (1.779)^a	68.70 (1.701)^a	64.35 (1.609)^a
	Mixed	72.32 (1.779)^b	66.83 (1.701)^a	69.57 (1.609)^b
	Macerated	71.34 (1.779)^b	68.24 (1.701)^a	69.79 (1.609)^b
	Smoked	49.50 (1.378)^c	58.14 (1.317)^b	53.82 (1.247)^c
	Ripened	40.27 (1.378)^d	52.76 (1.317)^c	46.51 (1.247)^d

Different superscript letters indicate differences ($P < 0.05$) of least square means sequentially between stages.

The higher retention of moisture in the sausages produced by Factory II – and hence, their higher mean a_w at the end of smoking (0.941) and ripening (0.933) – can be explained by both the shorter ripening period in Factory II and the use of polyphosphate in their formulation, which is known to increase the water-binding capacity of fermented meats (Pearson & Gillett, 1996). Although polyphosphates have the advantages of preventing auto-oxidation and decreasing purges in vacuum–packaged products (hence, improving yields), it has a pH rising effect in cured meats (viz. the increase in water-binding occurs as a consequence of the polyphosphates acting as polyelectrolytes to increase ionic strength; this frees some of the negatively-charged sites on the proteins so they can bind more water; Pearson & Gillett, 1996). This explains the difference in acidification patterns between nitrite-free sausages (Factory I) and nitrite-formulated sausages (Factory II): while the *linguiça* sausages from Factory I presented a neat decline in pH from raw meat until the end of ripening, the ones produced by Factory II underwent overall a significant increase in pH (6.233) at the point of mixing with additives, which was sustained until the end of maceration (6.209). Retarded by the addition of curing salts and polyphosphates in the formulation of Factory II, the sausages' pH only started dropping from smoking onwards, although their mean values at the end of smoking (5.772) and ripening (5.513) remained higher than the sausages' pH from Factory I (5.358 and 5.364, respectively; Table 1). To this respect, Lucke (2000) pointed out that a high pH of the mixture leads to higher final pH values, hence favouring the growth of acid-sensitive undesired microorganisms, and making the process unsafe. If acid production starts too late or proceeds too slowly, which occurred in the batches of Factory II, pathogens like *Salmonella* spp. or *S. aureus* may grow. Taking into account that (i) *linguiças* (from both factories) are characterised by their low acidity (pH ~ 5.4; SD = 0.05), and that (ii) growth of the aforementioned pathogens is suppressed only at pH < 5.3; the time and temperature at which the macerating meat remains above this pH is critical (ICMSF, 2005).

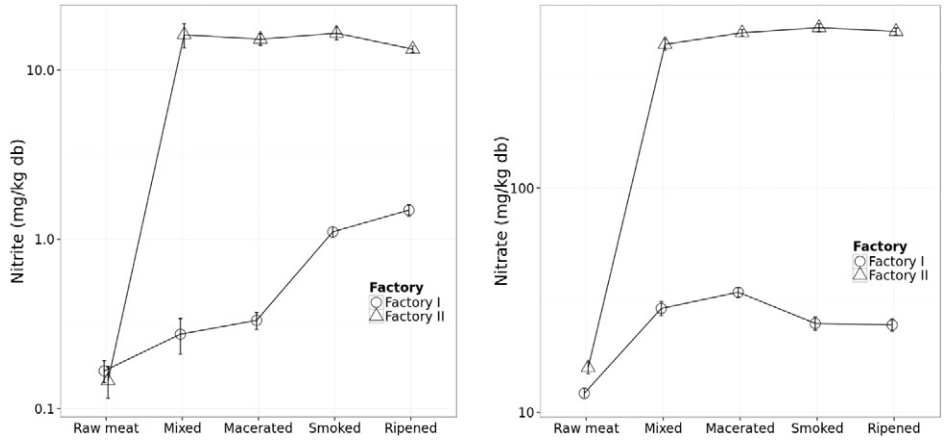

Fig. 3. Factory-specific evolution of nitrite (left) and nitrate (right) concentrations (mg/kg db) in meat during production of *linguiça* dry-fermented sausages. Each marker represents mean and standard error from the production batches.

Good manufacturing practise guidelines (AMS, 1997) have been developed, which limit the time the sausage meat is exposed to temperatures >15 °C before pH 5.3 is reached. Thus, *linguiça* sausages should also be ripened and stored below 15 °C.

Other European fermented semi-dry sausages characterised by their low acidity are the Portuguese *chouriço de vinho* (pH ~ 5.2; Linares, Garrido, Martins, & Patarata, 2013) and *salpicão* (pH = 5.3–5.7; Ferreira et al., 2007), the Spanish *salchichón* (pH = 5.2–5.6; Casquete et al., 2012) and *botillo* (pH ~ 5.5; García-Fontán, Lorenzo, Martínez, Franco, & Carballo, 2007), and the Venetian sausage (pH = 5.6–5.7; Comi et al., 2005). It was also observed that the raw meats used in *linguiça* processing were of high pH (6.103, SD = 0.06; Table 1), which may contribute to problems early in fermentation. Ideally, pork meat should have a normal pH (5.5–5.8). The pH decrease caused by the production of organic acids allows the meat to liberate its moisture in a quicker and more uniform way. Whilst the evolution of a_w could be considered as normal in both factories (Table 1), it was after stuffing that a significant drop took place (due to the lower relative humidity, higher temperatures during smoking and steady dehydration during cold ripening). Still, Factory II produced sausages of higher a_w (0.941 after smoking and 0.933 after ripening) than Factory I (0.931 and 0.913, respectively), which stemmed from the addition of polyphosphate, and also the shorter processing time of Factory II. *Linguiça* sausages were dried to an overall moisture content of 46%, which corresponded to a moisture loss of ~30%, and a final a_w of 0.92. These levels of moisture and a_w per se should contribute to the protection of the product against undesirable microorganisms (García-Fontán et al., 2007).

In relation to the permissible amounts of additives in this type of sausages (Anonymous, 2011), their concentrations in sausages from Factory I were below the limits (sodium nitrite E250: 150 ppm; potassium nitrate E252: 150 ppm; and polyphosphates E452: 5000 ppm expressed as P_2O_5). However, it is clear that, in Factory II, nitrates (143.5 ppm) and polyphosphates (5910 ppm; Table 1) were added to meet the maximum legal limit. Nevertheless, since *linguiça* is a semi-dry sausage produced in a short processing time, the use of high concentrations of nitrates is in fact not needed. As Sebranek and Bacus (2007) pointed out, nitrates are normally added in long, slow curing processes that necessitate a long-term reservoir for nitrite to be slowly released over the course of the process.

3.2. Total viable counts and fermentative microflora along linguiça processing

The general evolution patterns of TVC in *linguiça* were different between factories, although, overall, increases occurred during smoking and ripening (Fig. 4; and in Table 2, notice significant positive intercepts Day(Smoked) and Day(Ripened)). Such increase in TVC arises from the growth of lactic acid bacteria (LAB), which rapidly becomes the main microbial group as fermentation proceeds. In Factory I, LAB increases from 4.44 log CFU/g right after mixing to 8.75 log CFU/g in the ripened sausages, while in Factory II from 4.07 to 7.83 log CFU/g (results not shown). An indication that, from maceration onwards TVC approached LAB counts is given by the strong linkage between the TVC and pH patterns in sausages from Factory II. Said otherwise, only after maceration, the production rate of organic acids was high enough to cause a significant drop in pH (Table 1), and this was accompanied by the significant increase in TVC (partly comprised of LAB) right after maceration (from 5.5 to 8.01 log CFU/g in the finished product; Fig. 4). The delay in acid production (viz. improper fermentation) observed in the manufacture process of Factory II also had an impact on the *Micrococcaceae* population. Because of the sustained higher pH levels of the *linguiças* from Factory II, the acid-sensitive *Micrococcaceae* became more competitive and reached 2-log higher counts (from 3.86 at the point of mixing to 5.93 log CFU/g in ripened sausages; results not tabulated). On the contrary, in the sausages processed with a good acidification profile (Factory I), *Micrococcaceae* only increased ~1-log (from 3.47 at mixing to 4.56 log CFU/g in ripened sausages). Micrococci are responsible for the reduction of nitrate to nitrite, and for the formation of the cured meat colour and flavour, but they are inhibited by low pH and low temperature.

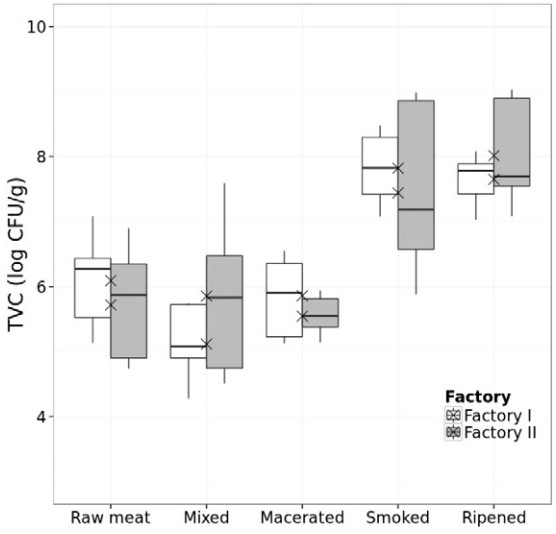

Fig. 4. Factory-specific total viable counts in meat along the different processing stages of *linguiça* dry-fermented sausages. Data dispersion is represented by a boxplot; median and mean are represented by the mid-horizontal line and cross, respectively.

Table 2
Parameter estimates of the generalised linear model assessing the overall effects of processing stage, a_w, pH and sodium nitrite concentration (mg/kg db) on the total viable counts (TCV) and *Enterobacteriaceae* counts (log CFU/g) in *linguiça* during production.

Effect	TVC		*Enterobacteriaceae*	
	Estimate (St. error)	Pr > |t|	Estimate (St. error)	Pr > |t|
Intercept	−6.439 (4.132)	0.122	−30.51 (7.191)	<.0001
Day				
Mixed	−2.337 (1.103)	0.037	−1.184 (1.920)	0.539
Macerated	-0.072 (0.056)	0.208	-0.045 (0.098)	0.642
Smoked	0.146 (0.024)	<.0001	0.126 (0.043)	0.004
Ripened	0.108 (0.018)	<.0001	0.001 (0.032)	0.774
a_w	18.63 (4.380)	<.0001	40.09 (7.623)	<.0001
pH	−0.919 (0.307)	0.003	−0.868 (0.534)	0.107
pH × nitrites	−0.448 (0.129)	0.001	0.742 (0.225)	0.001
Nitrites	2.839 (0.743)	<.0001	−4.258 (1.292)	0.001
Covariance				
Batch (factory)	0.352		1.065	
BIC	241		360	

Some interesting findings were also obtained from the generalised linear models. Overall, the mixing of raw meat with ingredients brought about a significant decrease in TVC counts ($p = 0.037$ in Table 2), likely due to the inhibitory compounds from spices and the viability and/or culturability of the microorganisms (Ducic, Blagojevic, Markov, & Velicanski, 2014). Linares et al. (2013) demonstrated the antibacterial activity of garlic when mixed with wine for macerating pork meat in the fabrication of a Portuguese *chouriço* type. Considering only the nitrite-formulated sausages (Factory II), a slight decrease in TVC was observed during maceration (from 5.85 log CFU/g after mixing to 5.55 log CFU/g after maceration), which could be further attributed to the negative, stress-mediated effects of the salt-nitrite combination. The same inhibitory effect of nitrite on TVC in macerating meat was supported by the factor analysis outcomes showing the inverse correlation between TVC and nitrites ($r = -0.983$ in Table 3). However, from the end of maceration (where TVC begins to approximate better the LAB counts), the effect of nitrite on TVC was no longer inhibitory. As the nitrite (in dry basis) in sausages concentrated, TVC continued to increase ($p < .0001$ in Table 2), which suggested that the concentration of nitrite applied (up to 11 ppm) had no inhibitory effect on LAB development. It is known however that at input levels of 150 ppm, sodium nitrite may slightly slow down the lactic acid formation (Lucke, 2000). Interaction between pH and nitrite ($p = 0.001$; Table 1) was also extracted by this model and the others, since the stability of nitrite is pH dependent. Phosphate addition to the cure increased the pH of the meat to a modest extent, and thereby, caused greater nitrite retention during processing. On the other hand, the bacteriostatic effect of nitrite is increased as the pH is lowered. The significant negative association between pH and TVC ($p = 0.003$ in Table 2) suggests that in general as pH decreases (viz. fermentation taking place), TVC has an increasing trend, yet this is more evident during maceration ($r = -0.964$ in Table 3) and smoking ($r = -0.872$) and slows down during ripening ($r = -0.535$). This pattern is in agreement with the usual development of LAB in dry-fermented sausages, which displays a rapid increase to ~8 log CFU/g during fermentation and then stabilises along ripening and storage (Hospital, Hierro, & Fernández, 2014).

There was also a tendency that sausages with higher a_w had also higher TVC ($p < .0001$ in Table 2), and this was particularly true in ripened sausages ($r = 0.655$; Table 3). Moreover, and also related to the water content in sausages, the factor analyses revealed that the higher the polyphosphate concentration during mixing ($r = 0.860$) and the higher the moisture during maceration ($r = 0.842$ in Table 3), the higher the TVC at the end of such processing stages. From the longitudinal analysis shown in Table 4, the number of days that smoking and ripening took place in both factories had, as expected, significant effects on the increase in TVC, and these were factory-specific. For instance, in sausages from Factory II, the average increase in TVC was 0.380 ($p = 0.001$) per day of smoking, and lower at 0.184 ($p = 0.025$) per day of ripening (Table 4). This is true because the ripening temperature allows LAB growth and the a_w of *linguiça* did not fall below the minimum a_w for LAB growth (a_w ~ 0.91 depending on the strain; Lucke, 2000). On a batch basis, there was no association between environmental contamination (TVC counted from environmental elements) and TVC levels in sausages (Table 4). This was not an unexpected outcome as from maceration onwards, the mesophiles counts in sausages provide an

Table 3
Synopsis of the factor analyses performed per bacterial group and processing stage, showing the physicochemical parameters that best correlated with the factor having the highest loading on the bacterial group. Coefficients of correlation with such factor are shown in brackets. The communalities (h) for the bacterial group are also indicated.

Bacterial group	Stage			
	Mixed	Macerated	Smoked	Ripened
TVC	TVC (0.489) pH (0.817) P_2O_5 (0.860)	TVC (0.887) pH (−0.964) Nitrites (−0.983) Nitrates (−0.998) Moisture (0.842)	TVC (0.917) pH (−0.872) Day (0.613)	TVC (0.789) a_w (0.655) pH (−0.535)
	$h_{TVC} = 0.244$	$h_{TVC} = 0.787$	$h_{TVC} = 0.842$	$h_{TVC} = 0.703$
Enterobacteriaceae	Entero (0.471) pH (0.909)	Entero (0.590) pH (0.574) P_2O_5 (0.860)	Entero (0.720) Aw (0.814) Nitrites (−0.731) Nitrates (−0.533) P_2O_5 (0.851)	Entero (0.736) pH (−0.761) Nitrites (−0.590) Nitrates (−0.536) Day (0.730)
	$h_{Entero} = 0.241$	$h_{Entero} = 0.353$	$h_{Entero} = 0.703$	$h_{Entero} = 0.544$
S. aureus	*S. aureus* (0.259) a_w (−0.312)	*S. aureus* (0.292) NaCl (0.759)	*S. aureus* (0.946) a_w (−0.767) Moisture (−0.864) Day (0.548)	*S. aureus* (0.642) pH (0.618) Nitrites (0.665) Nitrates (0.757) Day (−0.960)
	$h_{S.\ aureus} = 0.241$	$h_{S.\ aureus} = 0.241$	$h_{S.\ aureus} = 0.793$	$h_{S.\ aureus} = 0.412$
L. monocytogenes	*L. mono* (0.596) pH (0.738) P_2O_5 (0.824)	*L. mono* (0.742) pH (0.980) Nitrites (0.978) Nitrates (0.979) P_2O_5 (0.824)	*L. mono* (0.621) a_w (0.850) pH (0.810) Nitrites (0.770) Nitrates (0.791) P_2O_5 (0.710) Day (−0.705)	*L. mono* (0.495) a_w (0.550) pH (0.523) Nitrites (0.834) Nitrates (0.860) P_2O_5 (0.857) Day (−0.885)
	$h_{L.\ mono} = 0.364$	$h_{L.\ mono} = 0.724$	$h_{L.\ mono} = 0.705$	$h_{L.\ mono} = 0.246$

indication of LAB levels, and not of contamination. In relation to ambient temperatures, a higher temperature in the maceration room was associated ($p = 0.016$ in Table 4) to greater TVC counts.

3.3. Enterobacteriaceae counts along linguiça processing

Initial *Enterobacteriaceae* numbers in raw pork (3.205–3.675 log CFU/g; Fig. 5) were comparable between factories, and in the range usually reported as a raw material for this type of traditional products (González & Díez, 2002). Although in all batches surveyed, *Enterobacteriaceae* was inactivated during ripening, their evolution patterns were quite dissimilar between factories (Fig. 5). In Factory II, a significant increase in this hygiene indicator was observed from raw meat (3.205 log CFU/g) to meat in batter (3.954 log CFU/g), which held high until the end of maceration (4.14 log CFU/g). This occurred due to at least two causes: the higher meat pH due to the addition of polyphosphate, and the cross-contamination from environment during mixing. The first cause is explicated by the positive association between pH and *Enterobacteriaceae* at the end of mixing ($r = 0.909$) and at the end of maceration ($pH = 0.574$; Table 3). The second cause was deduced from the stepwise variable selection analysis (Table 7), which demonstrated that contaminated environment was a strong factor ($p = 0.068$ in Table 7) determining the final high *Enterobacteriaceae* counts in *linguiça*. In Factory I, which did not add polyphosphates, *Enterobacteriaceae* decreased significantly from raw meat (3.675 log CFU/g) to meat in batter (2.505 log CFU/g). This may be due to the effect of antimicrobial compounds present in spices (Linares et al., 2013) strengthened in an environment of lower acidity.

The effects of stuffing and smoking on *Enterobacteriaceae* were opposite between factories. In Factory II, the numbers of *Enterobacteriaceae* decreased significantly from 4.137 log CFU/g before stuffing to 2.523 log CFU/g after smoking (Fig. 5). Smoking and temperature of smoking have been shown to have antimicrobial effects on pathogenic members of the *Enterobacteriaceae* family such as *Escherichia coli* O157:H7, *Salmonella* Newport and *Yersinia enterocolitica* (Hajmeer et al., 2011). Smoke contains volatile antimicrobial compounds such as short-chain fatty acids and aldehydes, and, after smoking, growth of microorganisms on the sausage surface is inhibited for some time. However, the hurdle that largely hindered the activity of *Enterobacteriaceae* is the nitrite used in the formulation of Factory II. This is confirmed by the significant inverse effect of nitrite (-4.258; $p = 0.001$ in Table 2) on *Enterobacteriaceae* as estimated by the generalised linear model. The factor analyses (Table 3) further revealed that it was mainly during smoking ($r = -0.731$) and, to a less extent, during ripening ($r = -0.59$) that nitrite exerted its inhibitory effect on this hygiene indicator. While the means by which nitrite achieves microbial inhibition is still not clear, it has been proposed that nitrous acid and/or nitric oxide may be responsible for the inhibitory effects of nitrite (Sebranek & Bacus, 2007). An opposite effect of stuffing and smoking was noticed in the sausages from Factory I, where after maceration (3.623 log CFU/g), *Enterobacteriaceae* numbers significantly increased to 4.381 log CFU/g in smoked *linguiça*. In this case, the competitiveness of *Enterobacteriaceae* could have been prompted by the lack of nitrite, cross-contamination during stuffing (from operators and/or equipment) and the introduction of *Enterobacteriaceae* through casings. Notice that, on a batch level, it was found that cross-contamination from environment ($p = 0.181$) and casings ($p = 0.122$) were moderate determinants of the *Enterobacteriaceae* levels in smoked and ripened *linguiça*, respectively (Table 7).

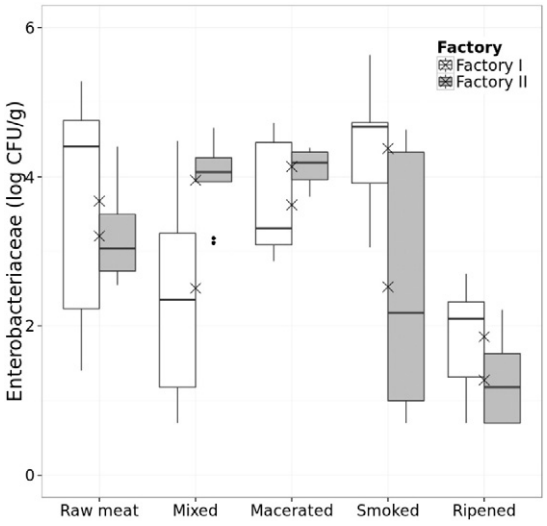

Fig. 5. Factory-specific *Enterobacteriaceae* concentration in meat along the different processing stages of *linguiça* dry-fermented sausages. Data dispersion is represented by a boxplot; median and mean are represented by the mid-horizontal line and cross, respectively.

During drying (i.e., decrease in a_w during smoking and ripening), *Enterobacteriaceae* were generally inactivated, as hinted by both the highly significant direct effect of a_w (40.09; $p < .0001$ in Table 2), and the positive correlation between *Enterobacteriaceae* and Aw measured in the smoked sausages ($r = 0.814$ in Table 3). As a consequence, the moisture-related variable polyphosphate was positively associated to *Enterobacteriaceae* at the end of maceration ($r = 0.860$) and at the end of smoking ($r = 0.851$). Macerated meat and smoked sausages with higher polyphosphate concentration (i.e., enhanced moisture retention capability) had a tendency to harbour more *Enterobacteriaceae*. From our surveys' data, the effect of decreasing pH on the overall counts of *Enterobacteriaceae* along *linguiça* processing was not as marked as the decreasing a_w. While the generalised linear model (Table 2) suggested that overall there is no pH effect ($p = 0.107$), the factor analyses indicated that (i) during mixing and maceration, the higher the pH the higher the *Enterobacteriaceae* numbers (due to the addition of polyphosphates as explained above); and (ii) in ripened sausages the lower the pH, the higher the *Enterobacteriaceae* numbers ($r = -0.761$ in Table 3). The latter statement is however an artefact introduced by Factory II, since the addition of nitrite and polyphosphates in their formulation caused, respectively, the *Enterobacteriaceae* to drop and the pH to remain higher. In reality, there is no true association between *Enterobacteriaceae* and pH in ripened sausages.

From the longitudinal analysis, the duration of smoking significantly affected the survival of *Enterobacteriaceae*, with an average of 0.732-log decrease per day ($p < .0001$) when nitrite is added in the formulation (Table 4). Furthermore, on a batch basis, higher levels of *Enterobacteriaceae* environmental contamination in the mixing room ($p = 0.07$) and higher ambient temperatures in the cutting ($p = 0.032$), mixing ($p =$

Table 4
Influence of processing days (Day) and environmental parameters (batch contamination level and room temperature) on the total viable counts and *Enterobacteriaceae* counts (log CFU/g) recovered from *linguiça* during production.

Effect	TVC		*Enterobacteriaceae*	
	Estimate (St. error)	Pr > |t|	Estimate (St. error)	Pr > |t|
Day				
Factory I				
Maceration	0.183 (0.114)	0.115	−0.202 (0.246)	0.416
Smoking	0.156 (0.051)	0.004	0.053 (0.110)	0.631
Ripening	0.119 (0.044)	0.010	0.206 (0.195)	0.350
Factory II				
Maceration	0.040 (0.205)	0.844	−0.123 (0.137)	0.373
Smoking	0.380 (0.105)	0.001	−0.732 (0.070)	<.0001
Ripening	0.184 (0.079)	0.025	0.072 (0.053)	0.184
Environmental contamination				
Mean	0.393 (0.258)	0.130	2.303 (0.510)	<.0001
Cutting room	−0.210 (0.146)	0.160	0.140 (0.411)	0.226
Mixing room	0.131 (0.182)	0.475	2.700 (1.454)	0.070
Maceration room	−0.120 (0.287)	0.678	−0.662 (0.539)	0.226
Temperature				
Cutting room	0.014 (0.047)	0.765	0.109 (0.050)	0.032
Mixing room	0.082 (0.056)	0.147	0.149 (0.059)	0.014
Maceration room	0.100 (0.040)	0.016	0.100 (0.041)	0.018

0.014) and maceration ($p = 0.018$) rooms were associated to higher numbers of *Enterobacteriaceae* in the final product (Table 4). By bringing together all the above findings on *Enterobacteriaceae* in *linguiça*, we can rank the factors that favoured the higher counts of *Enterobacteriaceae* in smoked or ripened sausages, as follows (Table 7): high a_w at the end of smoking ($p = 0.046$), cross-contamination from environment ($p = 0.068$), high ambient temperature during maceration ($p = 0.077$), high pH at the end of maceration ($p = 0.109$), high numbers of *Enterobacteriaceae* in casings ($p = 0.122$), and lack of nitrites in formulation ($p = 0.181$). Notice that, among all these determining factors, nitrite ranked the lowest, because even without the addition of nitrite, the sausages produced by Factory I presented final level of *Enterobacteriaceae* (1.854 log CFU/g) comparable to those of Factory II (1.271 log CFU/g with nitrite added; Fig. 5). As ripening develops, the hurdles of pH and a_w drop, competition with LAB, low oxygen tension and the presence of salt were sufficient to inhibit *Enterobacteriaceae* in the sausages without nitrite.

3.4. S. aureus counts along linguiça processing

The evolution patterns of coagulase-positive *S. aureus* in *linguiça* were factory-specific (Fig. 6). This pathogen was invariably isolated from raw meats in numbers below 3 log CFU/g. While in Factory I, the overall numbers survived from raw meat (2.445 log CFU/g) to the end of maceration (2.364 log CFU/g), in Factory II, despite the addition of nitrite, a significant increase was observed from raw meat (1.292 log CFU/g) until the end of mixing (2.296 log CFU/g) and those levels persisted until the end of maceration (2.100 log CFU/g). This finding underscores that mixing is a critical point as, at this stage, contamination was likely to be introduced from handlers, poorly sanitised equipment/utensils and contaminated spices, particularly when they are grounded. The results from the generalised linear model showed that *S. aureus* continued to grow as (dry basis) nitrite concentration increased along processing ($p = 0.001$; Table 5), which implies that nitrite had no inhibitory effect on this pathogen. Moreover, in the nitrite-formulated sausages (Factory II), *S. aureus* increased significantly during smoking (up to 3.013 log CFU/g) and during ripening (up to 3.419 log CFU/g; Fig. 6). Inoculating *S. aureus* cells in fresh sausages, Correia, Pereira, Pinto, Barcellos, and Bersot (2014) observed that nitrite at the highest concentration of 200 ppm did not control their growth. Interestingly, the factor analysis revealed some positive association ($r = 0.665$; Table 3) of nitrites and *S. aureus* counts during ripening, which, although can be only coincidental or confounding, one cannot dismiss the fact that curing salts may promote the growth of staphylococci under anoxic conditions (in the core of the sausages) as a function of their nitrite- and nitrate- reducing systems. However, under aerobic conditions (close to the sausage surface), reductase activity is diminished (Hospital et al., 2014).

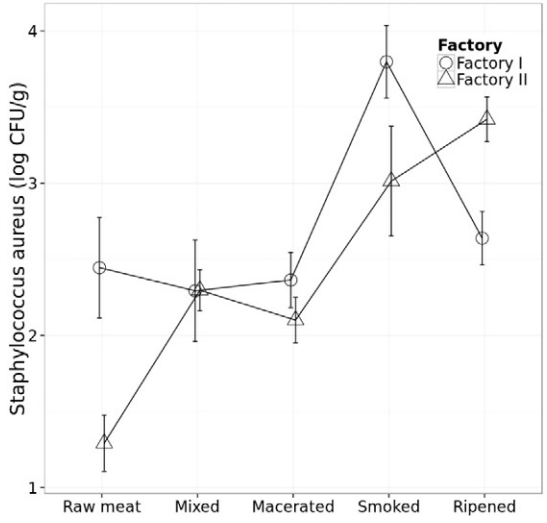

Fig. 6. Factory-specific profiles of *S. aureus* concentration in meat during production of *linguiça* dry-fermented sausages. Each marker represents mean and standard error from the production batches.

In the nitrite-free *linguiças*, *S. aureus* equally increased significantly after stuffing and smoking (up to 3.797 log CFU/g; Fig. 6). Once again, further contamination could have been introduced from operators' poor hygienic handling during stuffing and through contaminated casings. In fact, the batch-level analysis evidenced that contaminated casings determined to a great extent ($p = 0.094$; Table 7) the numbers of *S. aureus* in the final product. Other factors such as smoke and low a_w failed to control the growth of *S. aureus* in *linguiça*. The generalised linear model demonstrated that a_w had overall no effect ($p = 0.204$; Table 5) on *S. aureus*. This result was anticipated as *S. aureus* is little affected by salt (Bang, Hanson, & Drake, 2008), and can hence survive in low a_w. The factor analyses showed specifically that, after smoking, the lower the moisture ($r = -0.864$) – and hence the lower the a_w ($r = -0.767$) – the higher the *S. aureus* in sausages (Table 3). Furthermore, regardless of the presence of nitrite, the extent of increase in *S. aureus* depended upon the duration of smoking. Whilst the average increase in *S. aureus* was 0.247-log per day ($p = 0.005$) in sausages from Factory I, the mean increase in the sausages from Factory II was higher at 0.489-log per day ($p < .0001$; Table 6). *S. aureus* developed during smoking because of the high ambient temperatures and the a_w permissible for growth. Lucke (2000) highlighted the importance to use ageing temperatures below 15 °C until the a_w has decreased to below 0.90. During the lag-phase of the development of LAB, *S. aureus* may reach high numbers in the outer layers of the sausage if fermentation temperatures are too high. Generally speaking, the factory surveys have shown that this halophilic bacterium may, as a whole, survive and grow during mixing ($p = 0.057$), maceration ($p = 0.048$) and smoking ($p < .0001$) (Table 5).

During ripening of *linguiça*, *S. aureus* could either grow (0.170-log increase per day in sausages from Factory II; $p = 0.006$) or be inactivated (0.205-log decrease per day sausages from Factory I; $p = 0.007$ from Table 6). In the nitrite-free *linguiças* from Factory I, this pathogen decreased up to a mean of 2.638 log CFU/g (Fig. 6). The longer ripening period of the batches from Factory I and the better acidification process contributed to the greater log reduction of *S. aureus* in their products (Table 6). In Factory II, the ripening effect was the opposite: *S. aureus* slightly increased in the nitrite-formulated sausages throughout ripening (up to a mean of 3.419 log CFU/g in the finished product). The higher pH of these sausages had a key role in the survival of this pathogen. The generalised linear model indicated that the decrease in pH along processing had a significant inhibitory effect on *S. aureus* ($p = 0.008$; Table 5). The opposite is also true, and, more specifically, it was during ripening that the positive correlation between pH and *S. aureus* ($r = 0.618$) was evidenced by the factor analysis (Table 3). Thus, the higher pH values of the sausages produced by Factory II explicated, to a great extent, why

Table 5
Parameter estimates of the generalised linear model assessing the overall effects of processing stage, a_w, pH and sodium nitrite concentration (mg/kg db) on *Staphylococcus aureus* and *Listeria monocytogenes* concentration (log CFU/g) in *linguiça* during production.

Effect	*Staphylococcus aureus*		*Listeria monocytogenes*	
	Estimate (St. error)	Pr > |t|	Estimate (St. error)	Pr > χ^2
Intercept	−10.93 (9.443)	0.250	−21.37 (13.78)	0.120
Day				
Mixed	3.422 (1.773)	0.057	−9.144 (5.066)	0.071
Macerated	0.165 (0.082)	0.048	−0.583 (0.288)	0.043
Smoked	0.177 (0.045)	<.0001	−0.200 (0.087)	0.021
Ripened	0.047 (0.032)	0.142	−0.157 (0.067)	0.019
a_w	−8.006 (6.257)	0.204	53.49 (18.37)	0.004
pH	3.439 (1.278)	0.008	−4.266 (1.500)	0.040
pH × Nitrites	−0.848 (0.240)	0.001	1.242 (0.602)	0.039
Nitrites	5.058 (1.403)	0.001	−6.505 (3.476)	0.061
Covariance				
Batch (factory)	0.505		114.2	
BIC/Deviance	245		1.15	

Table 6
Influence of processing days (Day) and environmental parameters (batch contamination level and room temperature) on the counts of *Staphylococcus aureus* (log CFU/g) and *Listeria monocytogenes* (CFU/g) recovered from *linguiça* during production.

Effect	*S. aureus*		*L. monocytogenes*	
	Estimate (St. error)	Pr > |t|	Estimate (St. error)	Pr > χ^2
Day				
Factory I				
Maceration	−0.183 (0.187)	0.332	–[a]	–[a]
Smoking	0.247 (0.084)	0.005	–	–
Ripening	−0.205 (0.072)	0.007	–	–
Factory II				
Maceration	0.134 (0.098)	0.182	0.170 (0.382)[b]	0.656
Smoking	0.489 (0.056)	<.0001	−0.165 (0.080)	0.040
Ripening	0.170 (0.057)	0.006	−0.144 (0.083)	0.083
Environmental contamination				
Mean	–[c]	–[c]	0.350 (0.495)	0.479
Cutting room	–	–	–[d]	–[d]
Mixing room	–	–	–	–
Maceration room	–	–	–	–
Temperature				
Cutting room	0.068 (0.048)	0.181	−0.056 (0.107)	0.598
Mixing room	−0.113 (0.089)	0.208	−0.048 (0.069)	0.481
Maceration room	0.046 (0.033)	0.162	−0.593 (0.072)	<.0001

[a] Since for Factory I, *L. monocytogenes* counts were either 0 or 50 CFU/g, the model assessing the effect of processing days was fitted to the combined data from the two factories.
[b] Results applicable to both factories.
[c] The model could not estimate these parameters as *S. aureus* mean environmental contamination was not different from batch to batch.
[d] The model could not estimate these parameters because environmental data partitioned by room rendered *L. monocytogenes* mean concentrations having very low batch-to-batch variation.

S. aureus was not inhibited during ripening. Similarly, Bang et al. (2008) observed that *S. aureus* could increase up to ~2-log units during the drying step in sausages whose final pH was relatively high (~5.6). Summarising, in factory II, an improper fermentation took place (viz. production of lactic acid started too late and/or proceeded too slowly), which prompted the increase in *S. aureus* during smoking and ripening.

In this way, the main hurdle hindering the development of *S. aureus* in *linguiça* is the pH, and hence, the initial activity of the LAB. To inhibit *S. aureus*, normally present in raw meat and ingredients, a rapid pH drop in the sausages is needed early in fermentation. The time and temperature at which the fermenting meat remains above pH 5.3 determines the extent of growth of *S. aureus*. Thus, the time and temperature to reach pH 5.3 is critical for controlling the growth of *S. aureus* (AMS, 1997). *Linguiça* sausage is not formulated with starter cultures, which otherwise could ensure a predictable rate of pH fall for the control of *S. aureus*. Nevertheless, the products from both factories had less than 4 log CFU/g *S. aureus* (Fig. 6), which are levels at which no enterotoxin formation can occur (critical concentration is ~7 log CFU/g according to Bang et al., 2008). Proper fermentation (rapid pH decline) and low temperatures play critical roles in maintaining the safety of *linguiça*. In addition, other risk factors controlling the growth of *S. aureus* in smoked or ripened *linguiças*, as inferred from the surveys' data, are ranked as follows (Table 7): days of ripening (the longer the ripening, the greater the inhibition when pH is low; $p = 0.019$), contaminated casings ($p = 0.094$), contaminated raw meat ($p = 0.098$), days of smoking (the longer the smoking, the greater the growth; $p = 0.129$) and *S. aureus* concentration after mixing ($p = 0.192$). Bear in mind that the extent of '*S. aureus* after mixing' strongly depends upon the contamination of ingredients, handling during mixing and cross-contamination from equipment. Thus, quality ingredients, hygienic preparation and good equipment sanitisation are three factors that are implicitly included in this analysis.

3.5. *L. monocytogenes* counts along linguiça processing

Because *L. monocytogenes* was present in the meat samples at low concentrations and with uneven distribution; their recoveries by sampling were associated with high variability. For this reason, statistical analyses for *L. monocytogenes* was based on the Poisson-gamma regression models, whereby concentrations (response variable) are not log-transformed but used directly as CFU, and zero counts (absence in 25 g) also entered the models as such (For an in-depth discussion on the advantages of applying such Poisson-gamma count data models in the analysis of low-counts microbial data, see Gonzales-Barron et al., 2014). While the initial contamination by *L. monocytogenes* in diced pork was comparable and low in the two factories (~20 CFU/g), the evolution pattern was very different between factories and among batches (Fig. 7). In batches from Factory I, the levels of this pathogen remained generally low throughout processing, because of the rapid acidification profile of these sausages and, possibly, a better equipment and facilities hygiene, in view that this pathogen is predominantly an environmental contaminant. In batches from Factory II, however, a significant increase in *L. monocytogenes* occurred during mixing (up to a mean of 100 CFU/g), and subsequently during maceration (up to a mean of 140 CFU/g). While further contamination may have entered at the point of mixing through poorly-disinfected equipment, working surfaces and the addition of spices; the statistical analysis revealed that the high pH and the presence of polyphosphate created the appropriate conditions for *L. monocytogenes* to grow during mixing and maceration. In both processing stages, the polyphosphate concentration (only added in Factory II) and *L. monocytogenes* were positively correlated ($r = 0.824$ in both cases; Table 3), meaning that in samples with higher P_2O_5 concentration, higher counts of *L. monocytogenes* were recovered. As polyphosphates, in turn, retain moisture in the meat by maintaining a higher pH, higher *L. monocytogenes* numbers were also associated with higher pH in mixed meat ($r = 0.738$), and in macerated meat ($r = 0.980$; Table 3). These findings are in agreement with those of Samelis, Metaxopoulos, Vlassi, and Pappa (1998) who recovered listeriae from fermented sausages until the fourth day of processing in batches characterised by higher pH values. ICMSF (2005) warned that, if there is a fermentation delay, *L. monocytogenes* can grow in the sausage mix,

Table 7
Main risk factors (process variables, intrinsic characteristics, environmental and raw materials contamination) contributing to the growth/survival of *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* in sausages at the end of smoking and ripening, as pinpointed by stepwise variable selection analyses.

Bacterial group	Stepwise-selected variables	Partial R^2	F-value	Pr > F
Enterobacteriaceae	Smoked			
	a_w end of smoking (+)[a]	0.396	10.81	0.046
	Maceration temperature (+)	0.582	5.58	0.077
	pH end of maceration (+)	0.494	3.90	0.109
	Environment (+)	0.395	2.61	0.181
	Nitrites added (−)	0.395	2.61	0.181
	Ripened			
	Environment (+)	0.369	7.79	0.068
	Casings (+)	0.488	3.82	0.122
S. aureus	Smoked			
	Raw meat (+)	0.650	5.66	0.098
	Days of smoking (+)	0.590	4.32	0.129
	S. aureus after mixing (+)	0.483	2.81	0.192
	Ripened			
	Days of ripening (−)	0.877	21.38	0.019
	Casings (+)	0.660	5.83	0.094
	S. aureus after mixing (+)	0.473	2.69	0.199
L. monocytogenes	Smoked			
	a_w end of smoking (+)	0.585	5.63	0.076
	Casings (+)	0.236	2.71	0.099
	Days of maceration (−)	0.434	3.07	0.155
	Ripened			
	Days of ripening (−)	0.202	3.22	0.072
	Days of maceration (−)	0.520	4.34	0.106
	Moisture end of ripening (+)	0.476	2.37	0.124
	Nitrites added (−)	0.398	2.64	0.179

[a] Positive (+) or negative (−) association between variables.

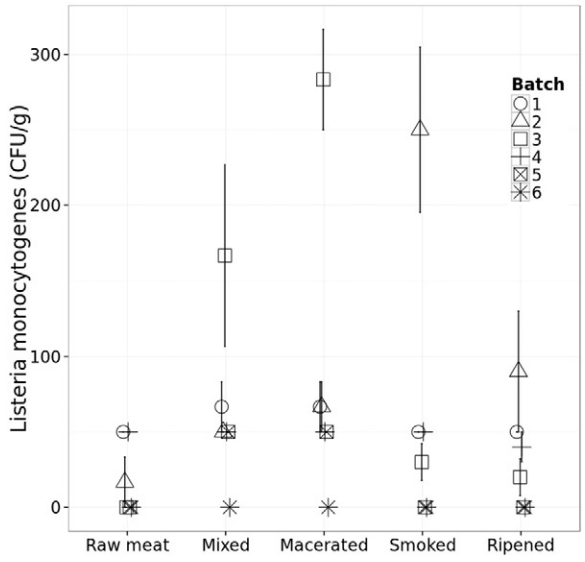

Fig. 7. Batch-specific *L. monocytogenes* concentration in meat along the different processing stages of *linguiça* dry-fermented sausages. Each marker represents the within-batch mean and standard error. Batches 1–3 correspond to Factory II, while the others to Factory I.

which actually took place in batches from Factory II. Despite the low ambient temperature during maceration, *L. monocytogenes* was viable for growth at this stage (notice the inverse association of Temperature–Macerating room with $p < .0001$ in Table 6).

In both factories, *L. monocytogenes* was inactivated during smoking and ripening (Fig. 7). In Factory II, the numbers were reduced up to a mean of 110 CFU/g at the end of smoking and <50 CFU/g at the end of ripening. The reduction of this pathogen following stuffing has been previously reported (Casquete et al., 2012; Thevenot, Delignette-Muller, Christieans, & Vernozy-Rozand, 2005). Hajmeer et al. (2011) found smoking to be effective against *L. monocytogenes* in inoculated Portuguese *linguiça*, causing reduction of up to 5 log, presumably because of the high temperatures employed. In our work, the Poisson-gamma regression evidenced the inhibitory effect of nitrite ($p = 0.061$ in Table 5) on *L. monocytogenes* along processing. Nitrite has been found to contribute to the inhibition of *L. monocytogenes* in dry-fermented sausages at input levels of ~150 ppm (Sebranek & Bacus, 2007; Lucke, 2000). Earlier, McClure, Kelly, and Roberts (1991) had shown that at pH 5.3, even 50 ppm nitrite exerted substantial inhibition of *L. monocytogenes* in tryptone soy broth. In addition, low a_w and pH turned out to be significant hurdles for controlling this pathogen, as implied by the Poisson-gamma regression (Table 5). As pH ($p = 0.040$) and a_w ($p = 0.004$) decreased along processing, *L. monocytogenes* was recovered in steadily lower numbers. This was also confirmed by the factor analysis which displayed positive correlations between a_w and *L. monocytogenes* in both smoked sausages ($r = 0.850$) and ripened sausages ($r = 0.550$; Table 3), and positive correlations between pH and *L. monocytogenes* in both smoked sausages ($r = 0.810$) and ripened sausages ($r = 0.523$; Table 3). The same factor analyses interestingly evidenced that the addition of polyphosphates had somehow a deleterious effect on the control of *L. monocytogenes* throughout processing: notice that in all processing stage mixing ($r = 0.824$), macerating ($r = 0.824$), smoking (0.710) and ripening ($r = 0.857$; Table 3), the samples with higher *L. monocytogenes* counts presented also higher P_2O_5 concentration. The duration of smoking and ripening (hence, the batch duration) had also a meaningful impact on the final counts of *L. monocytogenes*. Batches of either short smoking period ($r = -0.705$) or short ripening period ($r = -0.885$; Table 3) were associated with greater survival of *L. monocytogenes* in smoked or ripened sausages, respectively. The longitudinal analysis summarised that while smoking caused a significant reduction in the counts of *L. monocytogenes* by a factor of 0.847 per day ($\exp(-0.165)$; $p = 0.040$ in Table 6), the reduction during ripening was by a factor of 0.865 per day ($\exp(-0.144)$; $p = 0.083$ in Table 6). During smoking and ripening, the environment became increasingly unfavourable for *L. monocytogenes*, and so their numbers became to decline due to many hurdles such as low pH, low a_w, nitrite, competition with other microorganisms and the presence of antilisterial compounds produced by LAB. As opposed to *S. aureus* which survives better in *linguiça*, there are many hurdles preventing the viability of *L. monocytogenes* in this product. Considering that the small number of cells (<50 CFU/g) found in the final product cannot multiply, if *linguiça* was produced avoiding fermentation faults, the risk of listeriosis from consuming *linguiça* would be very low. Factors contributing to a greater inactivation of *L. monocytogenes* in smoked or ripened linguiças, as identified in this study, are ranked as follows (Table 7): longer ripening period ($p = 0.072$), low a_w at the end of smoking (which is associated to longer smoking periods; $p = 0.06$), washed casings ($p = 0.099$), longer maceration time ($p = 0.106$), low moisture at the end of ripening (which is associated to longer ripening periods; $p = 0.124$) and addition of nitrite ($p = 0.179$).

4. Conclusions

Linguiça is a Portuguese semi-dry fermented traditional sausage whose microbiological quality and safety have been little investigated. The mixing stage can be deemed as a critical point as *Enterobacteriaceae*, *S. aureus* and *L. monocytogenes* increased significantly until the end of this stage in the batches from Factory II, which raised concerns in relation to their good manufacture/hygiene practices and equipment/utensils sanitisation. Analyses of sausages from Factory II, formulated with nitrite and polyphosphate to meet the maximum legal limits (150 ppm and 5000 ppm, respectively), proved that their fermentation process was not optimal. The delayed fermentation, and higher pH level, was partly responsible for the increase in *Enterobacteriaceae* and pathogens' counts during maceration. The better acidification process of sausages, attained in factory I, led to lower counts of *S. aureus* (2.6 log CFU/g) and *L. monocytogenes* (10 CFU/g) in the final products. Nitrite had a strong effect on reducing *Enterobacteriaceae* throughout smoking and maceration, and contributed also to the control of *L. monocytogenes*. *S. aureus* was not affected whatsoever by nitrite, as suggested by their significant increase in numbers during smoking and ripening (up to 3.4 log CFU/g) in the nitrite-formulated sausages. *S. aureus* growth arouse due to improper fermentation (Factory II) that kept the fermenting meats above pH 5.3 for too long a time. In Factory II, although *L. monocytogenes* cells entered the chain at the point of mixing, most likely through contaminated environments, the pathogen became steadily inactivated throughout smoking and ripening, despite the delayed fermentation. Likewise, *Enterobacteriaceae* counts decrease in both nitrite-free and nitrite-formulated sausages during ripening, mainly because of sausage dehydration and low pH. The main hurdle hindering the development of *S. aureus* is the pH, and so a rapid pH drop in the sausages is necessary early in fermentation. Other factors contributing to the control of *S. aureus*, as determined from our data, are: low *S. aureus* in raw meat, shorter smoking period (as high temperatures prompt their growth), low *S. aureus* in casings and longer ripening days (if sausage pH below 5.3). In the case of *L. monocytogenes*, at least three hurdles (tested in this study) prevent its viability: low a_w, low pH and nitrite. Factors that contribute to controlling *L. monocytogenes* are: longer ripening and smoking periods, washed casings and nitrite. Finally, lower *Enterobacteriaceae* counts in the final product were strongly associated with high a_w at the end of smoking, cross-contamination from environment, high ambient temperature during maceration, high pH at the end of maceration, high numbers of *Enterobacteriaceae* in casings, and no added nitrite.

With regard to the pathogenic load in the finished products, *S. aureus* were below 4 log CFU/g (at which no enterotoxin can be produced) while *L. monocytogenes* was high (250 CFU/g) in only one sample out of 30 (in the other samples, either 0 or <50 CFU/g were recovered).

Thus, from the surveys, it can be concluded that, in the first place, there is a need to standardise the productive process of *linguiça*, since currently, the high variability identified, even within batches from the same industry, is greatly responsible for the unpredictable quality and safety of these products. The time production of *linguiça* varied from 8 to 21 days. Proper programmes of quality control of ingredients and sanitisation of equipment and facilities should be reinforced as well as better practices of manufacture and hygienic handling, so as to minimise the introduction of *Enterobacteriaceae* and pathogens from external sources. Care should be also taken in enhancing the washing/treatment of casings as they have been found to be a moderate factor ($p \sim 0.10$) determining the final counts of *Enterobacteriaceae* and pathogens. While the addition of nitrate is not necessary in this type of semi-dry fermented sausages as processing time is relatively short, polyphosphates may result in delayed fermentations, and hence their use should be avoided. While *linguiça* is not formulated with starter cultures, further work should be carried out towards this direction as a strategy to ensure a rapid pH drop that could prevent the growth of *S. aureus*.

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