Diez, J., Patarata, L. 2013. Behavior of *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* in *Chouriço de Vinho*, a dry fermented sausage made from wine-marinated meat. *Journal of Food Protection*, 76: 588-94.

Published in final form at:
http://www.ingentaconnect.com/content/iafp/jfp

ABSTRACT

Portuguese *chouriço de vinho* is made by drying roughly minced meat and fat that has been previously marinated with wine (usually red), salt and garlic for 1 to 2 days at a low temperature. This procedure may improve the microbiological safety of the product. The aim of this study was to evaluate the behavior of three pathogens in this product, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus*, to establish the minimum period of drying/maturation to render safe products. The pathogens were inoculated in the *chouriço de vinho* batter. A factorial design was used to study the following variables in the fermentation process: (1) the presence or absence of an indigenous *Lactobacillus sakei* starter culture; (2) the presence or absence of fermentable carbohydrates; and (3) the salt level (1.5% or 3%). The samples were analyzed 24 h after the preparation of the batter (at stuffing); after 7, 15, and 30 days of drying; and after 30 days of storage under a vacuum at 4ºC.

In the early stages of drying, the effect of adding the *L. sakei* starter culture and/or carbohydrates resulted in lower levels of gram-positive pathogens. At all of the conditions studied, the levels of the three pathogens decreased during the drying period. After 15 days of drying, that reduction was approximately 2 log CFU/g in all of the experimental conditions studied. At that sampling time, *L. monocytogenes* was undetectable in the *chouriço de vinho* with *L. sakei* starter culture and carbohydrates. The mean count of *S. aureus* after 15 days of drying was below 1 log CFU/g. After 30 days of drying, no pathogens were detected. The drying period could be shortened to 15 days when considering only the gram-positive pathogens studied and the use of a starter culture and carbohydrates. Due to the low infective dose of the *Salmonella* spp., the product should be considered safe after 30 days, when this pathogen became undetectable.

INTRODUCTION

In Portugal, there are several varieties of *chouriço* that are differentiated mainly by the seasoning, the duration of smoking, and the length of the drying period. *Chouriço* is generally made from roughly minced pork and fat that has been seasoned with salt and variable ingredients that mainly include paprika, garlic, and/or wine. The batter is usually maintained at a low temperature for 24 to 48 h. It is then stuffed into natural thin pork gut and may be cold smoked and matured at a low temperature for 1 to 4 weeks *(3)*. The *chouriço de vinho* (literal translation: wine *chouriço*) is unique because the meat is marinated in wine, usually red, with salt and garlic before stuffing. Due to the intrinsic characteristics of wine, especially its low pH and high amount of ethanol, it is expected to highly inhibit the microbiota of this sausage.

Dry cured meat products, regardless of whether they have been fermented or smoked, have a well-founded reputation as safe products. Their composition, particularly their water activity (a_w) and pH, along with the addition of salt, nitrites, spices, and other ingredients, are hurdles that hinder the growth of foodborne pathogens *(1, 10).* However, the use of poor-quality ingredients, unhygienic facilities and equipment, incorrect food handling, improper manufacturing processes or the absence of Hazard Analysis and Critical Control Points (HACCP) plans can facilitate the survival and/or growth of pathogenic microflora, causing outbreaks *(24)*.

Despite the information available about pathogen control in similar products in other countries, the specificity of the Portuguese *chouriço de vinho* makes its safety difficult to evaluate by comparison or extrapolation. Moreover, because these meat products are made at very different scales, ranging from large industries to small-scale units associated with rural areas, there is significant variability in the procedures and final product characteristics. In particular, the length of drying and the a_w in the final product

will vary with the purpose of delivering juicier *chouriço* and increasing the production yield.

The relative importance of fermentation for the safety of *chouriço de vinho* is not well documented. Previous studies *(3, 28)* observed that this product had a pH of approximately 5.2 and high levels of lactic acid bacteria counts (> 8 log CFU/g), suggesting the presence of natural fermentation phenomena. A controlled fermentation step in the manufacturing process of *chouriço de vinho* using starter cultures and carbohydrates is possible because the sensory effects are tolerable, if they are detected at all, because this product is highly flavored with wine, garlic, and smoke *(27)*. Additionally, a high variability in the salt levels in this type of product has been observed, with a mean salt level of 4% but with samples from small producers showing values as high as 9% *(30)*. The current trend of reducing salt intake for health reasons is incompatible with these levels. However, there is a safety risk associated with the reduction of salt due to the relationship between the high salt level and the low a_w, which is a key step for preservation.

The correct application of food safety tools used to ensure that dry-cured sausages are biologically safe, namely HACCP, implies that the control measures have high efficacy. Thus, the objective of this study was to evaluate the behavior of three pathogens of high concern in this product, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus*, that are inoculated in the *chouriço de vinho* batter during the drying/maturation period to establish the minimum drying period to manufacture safe products. The variability in the fermentation process was taken into consideration by evaluating the effect of the presence of an indigenous *Lactobacillus sakei* starter culture and/or fermentable carbohydrates. Due to the variability in salt levels observed in *chouriço de*

vinho, two different levels of salt (1.5% and 3% added) were evaluated in the experiment.

MATERIAL AND METHODS

Microorganisms and growth conditions. Four indigenous strains of each pathogen, *Salmonella* spp., *L. monocytogenes*, and *S. aureus*, were previously isolated from *chouriço* or its production environment and identified by a species-specific PCR technique *(29)*. The experiments were conducted with a cocktail of each pathogen composed of the four indigenous strains obtained from *chouriço* during different processing phases or preparation environments (laboratory collection) and one reference strain: *Salmonella enterica* subsp. *enterica* CECT 4155 (CECT, Valencia, Spain), *L. monocytogenes* NCTC 7973 (NCTC, Salisbury, UK) and *S. aureus* ATCC 25923 (LGC, Barcelona, Spain). The pathogens were grown on brain heart infusion (BHI, Biokar. Beauvais, France) and incubated for 24 h at 37ºC (*Salmonella* spp. and *S. aureus*) or 24 h at 30ºC (*L. monocytogenes*).

A total of 12 indigenous strains (laboratory collection) of lactose-fermenting lactic acid bacteria (LAB) isolated from *chouriço* made from wine-marinated meat, previously identified as *L. sakei (27)* and *L. sakei* ATCC 15521, were used to study their antagonistic activity against pathogens. The LAB were grown in Man-Rogosa-Sharpe broth or agar (MRS, Biokar) and incubated at 30ºC.

Preparation of the cultures. All strains of the pathogens and the *L. sakei* were maintained at -18°C and were cultured twice in the broth culture described above before use. The cultures for inoculation were grown individually overnight, harvested by

centrifugation, washed twice and suspended in 0.85% NaCl to obtain an optical density of 0.5 (600 nm, 10 mm).

Antagonistic activity against foodborne pathogens. The spot on agar method *(32)* was used to determine the antagonism of the LAB activity against the pathogens. Thirteen strains of *L. sakei* were tested individually against a cocktail of five strains of *Salmonella* spp., *L. monocytogenes* or *S. aureus*. A cell suspension in 0.85% NaCl of each *L. sakei* strain was dotted (10 µl) onto the surface of MRS agar with only 0.2% glucose (MRS 0.2) and was anaerobically incubated for 24 h at 30°C. For *L. monocytogenes*, *Salmonella* spp. or *S .aureus*, 100 µl of the cell suspension cocktail to be tested for sensitivity (approximately 8 log CFU/mL) was inoculated in 7 mL of melted BHI supplemented with 0.7% agar (BHI soft agar) and poured over the plate on which the producer was grown. After anaerobic incubation for 24 h at 30°C, the plates were checked for inhibition zones. The results were recorded as follows: **-** no inhibition zone; **(+)** inhibition zone under 5 mm; **+** inhibition zone over 5 mm; and **++** inhibition zone over 10 m. The pH of the supernatant of the 24h growth of *L. sakei* strains in MRS broth only with 0.2% of glucose was recorded. As the pathogens were used in a cocktail of five strains (four indigenous plus one reference strain), the potential cross-inhibition between the strains was previously screened using the spot on agar method described above by replacing MRS 0.2 with BHI agar for all the possible combinations of the strains of pathogens tested.

Manufacture of *chouriço de vinho*. A factorial design was used to test the influence of the (1) presence or absence of the indigenous *L. sakei* starter culture, (2) presence or absence of 0.75% dextrose plus 0.75% lactose and (3) the level of salt

(1.5% or 3%). Four replicates were conducted for each condition. To screen the studied effects more efficiently, the *chouriço de vinho* was prepared using meat with very low initial contamination, which was obtained by immersing the pork belly in ethanol, burning the surface and aseptically excising the inner part. All 8 batches were manufactured with ground pork belly (15 mm grinder plate; Mainca, Barcelona, Spain), 5% regional red wine (11% ethanol, pH 3.8), 125 mg/kg $NaNO_2$ and 125 mg/kg KNO_3, and the variable ingredients of the batch. The *L. sakei* starter culture, selected from the 13 tested for antagonistic activity, was suspended in 10 mL of 0.85% NaCl to achieve an initial concentration of 6 log CFU/g. The same amount of 0.85% NaCl was added to the samples without the starter culture. Each batch was divided in three portions, and each portion was separately contaminated with the cocktail of *Salmonella* spp., *L. monocytogenes*, or *S. aureus* to achieve an initial value of 2-3 log CFU/g of each pathogen, according to the guidelines for microbial challenge testing *(26).* After mixing (10 min; Mainca, Barcelona, Spain), the batter was rested for 24 h at 4ºC before stuffing. The *chouriços de vinho* were then stuffed into natural thin pork gut, tied in a horseshoe shape, and suspended to drain the excess marinade for 4 h in a drying chamber at 4ºC with 85% relative humidity. They were then smoked for 3 h in a smoking chamber (Begarat, Thermaxs 100EC, Berlin, Germany) with smoke generated from beech wood scraps. The initial temperature of the smoke chamber was 18ºC. From the start of the process, the temperature inside the *chouriços de vinho* was monitored at 10-min intervals and never exceeded 35ºC. The *chouriços de vinho* were then dried at 15ºC in an 85% RH environment (Aralab Fitoclima, Rio de Mouro, Portugal) for 30 days. Samples of the finished *chouriços de vinho* from each batch were vacuum-packed and stored at 4ºC for 30 days. Samples were collected for analysis at stuffing (24 h after

the preparation of the batter); after 7, 15, and 30 days of drying; and after 30 days of storage under vacuum at 4ºC (60 days after stuffing).

Microbial analysis. Ten grams of *chouriço de vinho* was aseptically collected from each batch from the center of the sausage and diluted with 40 ml of buffered peptone water (BPW, Biokar). The sample was homogenized for 2 min in a Stomacher (IUL, Barcelona, Spain). A series of tenfold dilutions was prepared and inoculated in duplicate.

The *Salmonella* spp. counts were obtained after incubation on xylose lysine deoxycholate (XLD, Biokar) at 37ºC for 24 or 48 h, the *S. aureus* counts were obtained after incubation on Baird-Parker agar (BPA, Biokar) at 37ºC for 48 h, and the *L. monocytogenes* counts were obtained after incubation on Oxford agar (OA, Biokar) at 30ºC for 48 h. On the 1st and 7th days, 0.1 mL of the first dilution was plated on the surface of the appropriate medium in 90 mm Petri dishes. To obtain a lower detection limit for the method, on the 15th day, 0.5 mL of the first dilution was plated on the surface of 150 mm Petri dishes. The results are presented as log CFU/g. When the microorganism count was below the detection limit, it was considered to be zero for statistical purposes. The presumptive colonies in each culture medium were kept for confirmation by species-specific PCR. These were suspended in 100 µL sterilized ultra-pure water and stored at -20ºC until the PCR was performed. The cell suspension was used directly in the species-specific PCR reaction *(33)*. The level of each pathogen was corrected by the proportion of presumptive colonies confirmed by PCR.

The samples with counts of *Salmonella* spp. and *L. monocytogenes* below the detection limit at 24 h of incubation were screened for the presence of the pathogen in 25 g as described in ISO 6579 *(17)* and ISO 11290-1 *(12)*, respectively. The

presumptive colonies on selective media were confirmed by the PCR procedure as described above.

The lactic acid bacteria (LAB) counts were obtained on MRS after incubation at 30ºC for 72 h *(15).* The natural microflora of the *chouriço de vinho* (coagulase-negative staphylococci – CNS, *Enterobacteriaceae*, and *Pseudomonas* spp.) were counted to detect any biases that their overgrowth could produce in the experiment. The procedures used are described elsewhere *(33).* The counts of *Enterobacteriaceae* and CNS were corrected by subtracting the number of *Salmonella* spp. and *S. aureus*, respectively, from their counts.

Physicochemical analysis. The chemical composition was determined according to procedures recommended by the ISO for the moisture *(13)*, total fat *(14)*, protein (Kjeldahl N x 6.25) *(18)*, and NaCl *(16).* The results are presented as the mean and standard deviation of 8 repetitions for the moisture, total fat and protein and as 4 repetitions for NaCl. The water activity was measured (only in samples prepared with *L. sakei* starter culture) in a Hygroscope DT apparatus (Rotronic, Zurich, Switzerland) with aWA40 cell maintained at 20±2ºC. The pH was measured directly in the *chouriço de vinho* itself using a pH meter (Crison, Barcelona, Spain) with a penetration probe (Mettler-Toledo, Giesen, Germany).

Data analysis. The effect of the *L. sakei* starter culture, addition of carbohydrates, and salt level was assessed by a factorial analysis of variance. The differences ($P < 0.05$) between the mean values were determined using the Tukey-Kramer test (Statistics software package 7.0, Stat Soft, Inc., Tulsa, USA).

RESULTS AND DISCUSSION

Antagonistic activity against foodborne pathogens. The results of the assay for the antagonistic activity of the strains of *L. sakei* are presented in Table 1. The ability to inhibit *Salmonella* spp. was absent in almost all of the strains tested. The activity of *L. sakei* against the gram-positive pathogens *L. monocytogenes* and *S. aureus* was observed in nearly half of the indigenous strains tested; two strains (Ls 1320 and Ls 1376) were powerful inhibitors of *L. monocytogenes*. The pattern of inhibition for *S. aureus* was similar, but slightly weaker. The tested ATCC *L. sakei* strain did not inhibit any of the tested pathogens. The only strain (Ls 1376) that was able to inhibit the three pathogens tested was selected for the starter culture in the manufacture of *chouriço de vinho*.

Behavior of the pathogens during the drying process. The *chouriço de vinho* prepared in the present study had an average composition of 34.18 ± 1.51% moisture, 24.50 ± 1.72% protein, 37.36 ± 1.72% fat, and 2.46 ± 0.08% or 4.58± 0.34% salt (for samples with the addition of 1.5% or 3% salt, respectively) after 30 days of drying.

One day after the contamination of the batter, which was maintained at 4ºC, the *Salmonella* spp. counts were between 2 and 2.5 log CFU/g (Table 2). None of the effects under study showed any influence on the number of viable *Salmonella* spp. ($P>0.05$). A decrease in the *Salmonella* spp. was evident at the 7^{th} day of drying, and after 15 days of drying, the pathogen level decreased approximately 2 log CFU/g from the initial count. After 30 days, the *Salmonella* spp. count was below the detection limit, and no *Salmonella* spp. were detected after enrichment in 25 g in any sample. Additionally, no viable *Salmonella* spp. were found after 30 days of storage at 4ºC under vacuum package (60 days after stuffing).

The use of an indigenous *L. sakei* strain as a starter culture showed no isolated effect (P>0.05) on the survival of the *Salmonella* spp. during the study period. However, an interaction was observed ($p < 0.05$) between the starter culture and the presence of carbohydrates at 15 days of drying. When the starter culture was used, samples with carbohydrates showed a lower count of *Salmonella* spp. than those without carbohydrates. In samples without the added *L. sakei*, the opposite effect was observed. It is likely that the availability of glucose provided a competitive advantage to the pathogen in the later conditions. No other interaction between the studied effects was significant. The results observed in the present work agree with those reported from a study on Spanish *chouriço* stored at room temperature. In that study, *Salmonella* spp. colonies were undetectable after 5-10 days of storage *(6)*. The reduction in the *Salmonella* spp. level during the maturation period has been associated with the decrease in a_w and pH *(2, 11, 31)*. In the present study, the pH and a_w (Table 3) decreased progressively during the maturation process, which is in agreement with the observed reduction in the *Salmonella* spp. counts.

In the present work, we challenged the *Salmonella* spp. with low levels of inoculation (between 2-2.5 log CFU/g) to simulate a realistic level of contamination of meat; however, further work should be performed to validate the processing of *chouriço de vinho*, particularly whether the process achieves the specific levels of reduction in the *Salmonella* spp. (6.5 log CFU/g) specified in the "Performance Standards for the Production of Processed Meat and Poultry Products" published by the Food Safety and Inspection Services *(7)*.

The behavior of *L. monocytogenes* during the drying process was generally similar to that observed for *Salmonella* spp., with a continuous decrease in the population until it was undetectable in 25 g after 30 days. However, the survival of *L. monocytogenes* was

clearly affected by the addition of the starter culture, which resulted in lower counts ($p<0.05$) observed at 7 and 15 days, resulting in a safer product after shorter a drying period when compared to the condition where no starter culture was included. Several studies have shown that *L. monocytogenes* could be controlled by the use of adequate lactic acid starter cultures *(4, 9)*. The effect of higher levels of salt was negligible, although some authors have reported that NaCl decreases the levels of *L. monocytogenes (25)*.

As observed for the pathogens described above, the level of *S. aureus* decreased during the drying process at basically the same rate and was also undetectable after 30 days. Although this pathogen is considered a biological hazard in dry meat products due to its potential ability to grow in low a_w products, the results of the present study indicate that this pathogen is unable to grow or survive during the manufacturing process. Moreover, because food poisoning by *S. aureus* is caused by the production of enterotoxin, its inability to grow to population levels that can produce enterotoxins at hazardous levels (at least ca. 5 log CFU/g) makes the product safer *(19)*. A similar pattern in the reduction of this pathogen was observed during the processing of Spanish, Turkish and Portuguese sausages *(8, 21, 29)*. The antagonism of *L. sakei* observed in the spot on agar test was also observed in the manufacturing of *chouriço de vinho*, as its addition as a starter culture resulted in a lower count ($p<0.05$) of *S. aureus* at 7 days independently of the addition of carbohydrates or higher amount of salt. Several authors demonstrated the antagonist activity of LAB in dry sausages *(5, 20, 23)*. At 15 days of drying, the effect of the reduced a_w may obscure the effect of the starter culture.

After the marinade period, the count of LAB (Table 2) in the samples inoculated with the indigenous *L. sakei* starter culture was slightly below the target level (4.63±0.34 log CFU/g). The interaction of the culture with the wine and other ingredients and additives

in the marinade was likely responsible for the gap of approximately 1 log CFU/g. In samples without the starter culture, the LAB counts were below the detection limit. During the ensuing period, this population experienced an important increase, which was associated with the increase in the temperature to 35ºC during the smoking process. The samples with the starter culture presented higher counts ($p<0.001$) throughout the experiment, though the absolute difference between inoculated and non-inoculated samples became narrower as the process progressed. At 7 days, when the strongest antagonist effects of the *L. sakei* starter culture against the tested pathogens were observed, the difference in the LAB count in inoculated and non-inoculated samples was approximately 3.8 log CFU/g. At the end of the drying process (30 days), that difference was only approximately 1.2 log CFU/g. The presence of carbohydrates did not affect the growth of LAB, as in four out of the five sampling times studied, there were no significant differences among the samples with or without the glucose and lactose. That difference was significant only at 15 days ($p < 0.001$), with a modest absolute difference of 0.3 log CFU/g. The higher amount of salt was responsible for a slightly lower LAB count (the differences were less than 0.3 log CFU/g), which was statistically significant at days 1, 15 and 60.

The natural microflora growing in *chouriço de vinho*, CNS, *Enterobacteriaceae* and *Pseudomonas* spp., had low counts (below 1.5 log CFU/g, data not presented) during the entire experiment and tended to become undetectable as the process progressed.

The pH of the batter (Table 3) after 1 day of marinade at 4ºC was approximately 5.7 in every experimental condition. As expected, both the use of starter culture or carbohydrates was responsible for lower pH ($p < 0.05$) in the *chouriço de vinho* during the entire experiment. The interaction of both factors was significant at days 1, 15 and 60, reflecting the expected pattern of lower pH in samples containing starter culture and

carbohydrates. Curiously, this pattern of interaction was not observed in the LAB count, indicating that acidification is not completely dependent on the number of LAB.

The water activity at day 1 was 0.96 and 0.97 in samples with or without both carbohydrates and salt. During the process, the small difference of 0.1-0.2 units of a_w was significant between samples with different amounts of salt. At 30 days, the product had an a_w between 0.85 and 0.87, which is in accordance with the characteristics of a similar traditional *chouriço de vinho* previously analyzed *(3)*. However, to achieve that reduced a_w, the length of drying for the traditional product is considerable. Considering the indicators for the preservation of dry meat products based on a_w and pH *(22)*, the *chouriço de vinho* studied here could be marketed earlier, at 15 days based on its reduced a_w, or even earlier, if both the conditions $a_w < 0.95$ and pH < 5.0 are achieved. These conditions were present in the *chouriço de vinho* with carbohydrates or the higher level of salt at 7 days; however, the characteristic increase in pH found thereafter should be taken into account. Moreover, if the raw material has been contaminated with any of the three pathogens studied, particularly with *Salmonella* spp., the microorganisms remain viable in the product, representing a hazard to avoid. At 15 days, the counts of *L. monocytogenes* and *S. aureus* in the *chouriço de vinho* had fallen to approximately 2 log CFU/g in all of the experimental conditions studied, particularly when controlled fermentation was used. Considering that the infective dose of *L. monocytogenes* is high for the general population, and the low counts observed in the present work, the product could be considered finished at the end of this period of drying if it is ensured that it is not consumed by risk groups, namely pregnant women and immunocompromised persons. With respect to *S. aureus*, the product could also be considered safe once the microorganism count fell below the level that produces the toxin at a hazardous level. However, the presence of viable *Salmonella* spp. at 15 days of drying precludes the

commercialization of the described *chouriço*. Based on its microbiological profile, the product should be considered safe only after 30 days of drying, when the *Salmonella* spp. and *L. monocytogenes* were not detected in 25 g and *S. aureus* was below the detection limit. However, to validate the process according to the USDA guidelines *(7)*, a higher level of lethality, namely 6.5 log CFU/g of *Salmonella* spp., should be demonstrated.

ACKNOWLEDGEMENTS

Financial Support. PEst-OE/AGR/UI0772/2011 (CECAV/UTAD) 2011-2012; The authors wish to thank Ms. Carla Araújo for the English editing.

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