SHORT COMMUNICATION

EVALUATION OF THE SAFETY OF *MILANO*-TYPE DRY FERMENTED SAUSAGES PRODUCED BY A FAST DRYING TECHNOLOGY

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ABSTRACT

A challenge test based on the inoculum of a multi-strain cocktail of *Listeria innocua* and S*almonella enterica* viable cells was carried out to evaluate the capacity of an accelerated manufacturing technique (including conventional fermentation of the meat batter followed by freezing, slicing and drying) to guarantee the safety of *Milano*-type fermented sausages. The counts of *S. enterica* decreased by 1.5 and 1.6 log CFU/g in the sausages inoculated with 2 and 4 log CFU/g, respectively, while a less notable reduction (0.4 log CFU/g) was recorded for *L. innocua,* independently from the inoculum load. The comparison between the main microbiological and physico-chemical features of non-inoculated fermented sausages, produced through either the accelerated or the traditional process, highlighted significant differences in the percent R.H. and a_w values, as well as pH In both cases, the absence of *Salmonella* spp. and *Listeria monocytogenes* was ascertained. These outcomes encourage further investigation on the fate of these foodborne pathogens during a shelf-life challenge test. No differences were highlighted for the main sensory parameters analyzed.

Keywords: accelerated drying, challenge test, dry fermented sausages, food safety, *Milano*-type fermented sausages

1. INTRODUCTION

Meat fermentation combined with salting, drying, and sometimes smoking dates back to very ancient times; therefore, in most European Countries the production of fermented dry sausages is largely carried out by following traditional procedures (AQUILANTI *et al.*, 2016). As ready-to-eat (RTE) meat products, dry (fermented) sausages must be stable and safe at the end of the production process; therefore, particular attention must be devoted to the control of pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* spp. that are more likely to survive during manufacturing (PETRUZZELLI *et al.*, 2010), thus constituting a risk for consumer health. Safety of fermented dry sausages, as well as stability towards alterations caused by spoilage microorganisms are generally assured by the interaction of various hurdles that include water activity (a_w), acidity (pH), redox potential (Eh), preservatives (e.g., nitrate/nitrite, sorbate, sulfite), and competitive microorganisms, such as lactic acid bacteria. Among these hurdles, low values of a_w and pH are considered the most important for the inhibition and inactivation of pathogenic bacteria, moreover, pH decrease and the water loss occurring during ripening also exerts a fundamental effect on the textural properties (FEINER, 2006; DALZINI *et al.*, 2015). The drying/ripening phase is therefore regarded as crucial in fermented dry sausage production, where it also represents the most time-consuming step. For this reason, in 2004, Comaposada *et al*. (COMAPOSADA *et al*., 2004) proposed a new technique called Quick-Dry-Slice "QDS process ®", which is aimed at accelerating the drying/ripening phase of dry sausages. With such a method, sausages are fermented to the desired pH and then frozen, sliced, and dried using a continuous system based on the application of convective air (COMAPOSADA *et al*., 2008; STOLLEWERK *et al*., 2011).
In the present study a challenge test based on the inoculum of *Listeria* and *Salmonella* viable cells was carried out in order to evaluate the capacity of an accelerated manufacturing technique, similar to the QDS process®, to guarantee the safety of *Milano*-type sausages. The main microbiological and physico-chemical features of the fermented sausages produced through either the accelerated or the traditional process were compared as well.

2. MATERIALS AND METHODS

2.1. Experimental manufacturing

Two independent manufacturing trials were performed according to the traditional procedure originally described for the production of *Salame Milano* and the modified procedure, including accelerated drying (Fig. 1). In both cases the same recipe was used, including pork shoulder (75%) and belly (25%), minced with a 4-mm plate, and the following ingredients (expressed as g/100 kg of meat): NaCl (2,300), black pepper powder (200), white pepper powder (100), and granulated garlic (20). The recipe also included 100 g of Nitritec 10/90 Sa, 1,000 g of Saltec Whitec-16+Niko Sa, and 400 g of Protec Pork PR 70 Nat Sa (all purchased by Tec-Al, Traversetolo, Italy) that supplied a defined amount of NaCl (1,500 g/100 kg of meat), sodium nitrite (E252), sodium ascorbate (E301), dextrose, and saccharose. The addition of *Lactobacillus sakei* starter cultures (Lyocarni BOM-13-Clerici-Sacco Group, Cadorago, Italy) was carried out according to the manufacturer's directions.
The meat batter destined for the production of *Milano*-type sausages through the accelerated process was divided into three separate batches (1a, 1b, and 1c) in order to carry out the challenge test. Two batches (1a and 1b) were inoculated with the pathogens

under study at two different contamination levels, whereas the third batch (1c) was processed without pathogens. This last batch was taken as the negative control of the challenge test and the final product obtained from it was compared with *Milano*-type fermented sausages produced through the traditional process.
All three meat batter batches were stuffed into 100-mm-diameter collagen casings and subjected to fermentation at 20-25 °C and 60-70% relative humidity (R.H.) for 48 hours. Subsequently, the sausages were placed at -20 °C, and the frozen sausages were sliced.
The slides were placed on a grid and a final drying was achieved in a ripening room with forced ventilation at a temperature of 20-25°C and 50-60% R.H., for approximately 50 minutes; a decrease in humidity of approximately 40% in the product was obtained.
For each batch, six samples weighing 500 g each were produced.

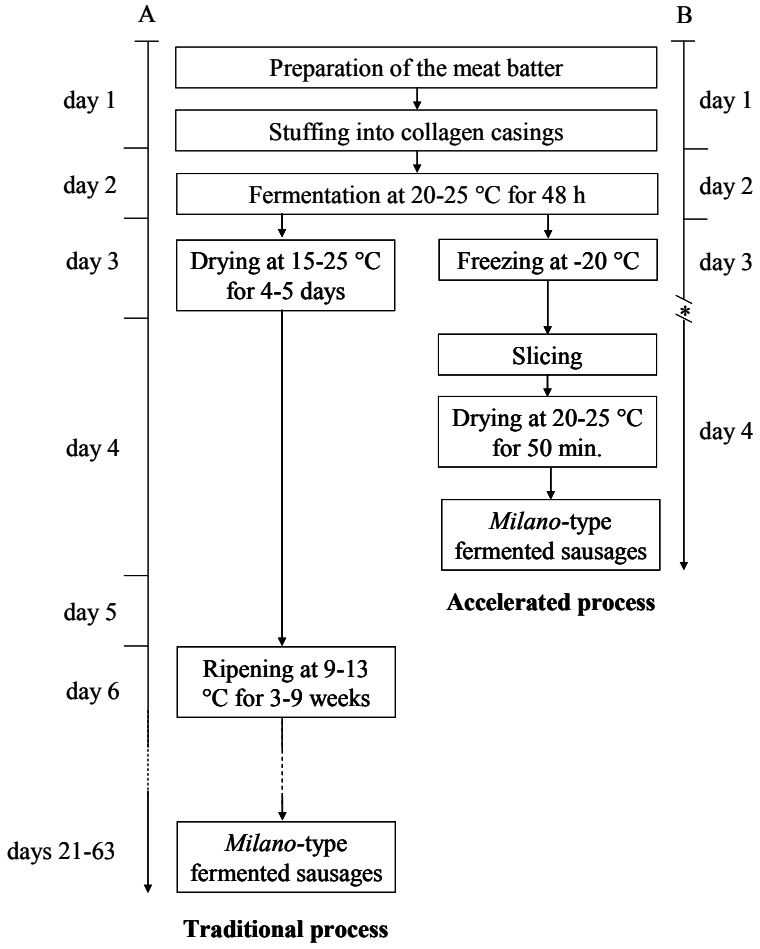

Figure 1. Flow chart of the traditional (A) and accelerated (B) process for manufacturing of *Milano*-type fermented sausages.
*The frozen product is further processed upon market request.

2.2. Challenge test

Four strains were used in the challenge test: the *Listeria innocua* ATCC 33090 reference strain was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA), whereas the three *Salmonella enterica* serovars, namely, *Salmonella* Pomona IZSUM 76/13, *Salmonella* Derby IZSUM 31/13, and *Salmonella* Agona IZSUM 39/13, previously isolated from meat products, were obtained from the culture collection of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (Perugia, Italy). All of the strains were stored at -80 °C.
The strain of *Listeria innocua* (as a surrogate of *L. monocytogenes*) and the three low-pathogenicity serovars of *Salmonella enterica* were chosen to allow the challenge test to be safely carried out at the factory. Before being inoculated into the meat batter, each strain was individually subcultured twice in Brain Heart Infusion (BHI, Biokar, Beauvais, France) at 37 °C for 24 h and grown overnight in the same substrate, harvested by centrifugation, washed twice, and resuspended in 0.85% NaCl. Two batches (1a and 1b) were inoculated with the strain cocktail at 2 and 4 Log colony forming units (CFU)/g, respectively. The third non-inoculated batch (1c) was taken as a negative control. Triplicate samples were withdrawn from the three batches along the production timeline: after meat batter preparation (T0), after fermentation and freezing (T1), and after slicing and drying (T2). Counting of *Listeria innocua* was carried out in accordance with the UNI EN ISO 11290-2: 2005 standard method, whereas *Salmonella* spp. viable counts were carried out in CHROMagar *Salmonella* plus (Sharlab, Barcelona, Spain) with incubation at 37 °C for 48 h. The pH values were determined according to ISO 2917:1999 using a portable pH meter (Seven Multi, Mettler Toledo); a_w was determined in accordance with the ISO 21807:2004 standard method using an Aqualab 3TE device (Decagon Devices, Inc. Pullaman, Washington, USA).

2.3. Microbiological analyses

Viable counts were carried out on triplicate samples as follows: total mesophilic aerobes in accordance with UNI EN ISO 4833-1:2013; lactic acid bacteria in accordance with ISO 15214:1998; *Enterobacteriaceace* in accordance with AFNOR BIO 12/21–12/06 (TEMPO, BioMérieux); yeasts on Wallerstein Laboratory Nutrient (WLN) agar (OSIMANI *et al.*, 2009); moulds on Rose-Bengal Agar with Chloramphenicol Selective Supplement (Oxoid) at 22 °C for 3-5 days. The presence of *Listeria monocytogenes* and *Salmonella* spp. was assessed in accordance with AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively.

2.4. Physico-chemical analyses

Physico-chemical analyses were carried out on triplicate samples, as follows: moisture as percent R.H., in accordance with AOAC Official Method 950.46; percent ashes, in accordance with AOAC Official Method 935.42; nitrates and nitrites, via high-performance anion exchange chromatography, using Dionex DX-500 chromatography system (Dionex, Sunnyvale, CA, USA).

2.5. Sensory analyses

A preliminary acceptance test to compare the end products obtained through the accelerated technique and the traditional process was carried out. To this aim, eight panelists familiar with the taste/consumption of fermented dry sausages were chosen

among the employees of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche of Perugia (Italy). The following sensory parameters were assessed: odour (meat, animal, spicy, other); aroma (meat, animal, spicy, other); salinity (form low to high); sourness (from low to high); bitterness (from low to high); spicy (from low to high); colour intensity (from pink to dark red); colour uniformity; consistency (from low to high); elasticity (from low to high); hardness (from low to high); moisture (from low to high); chewiness (from low to high). The parameters ranked from low to high were assessed using a 8-point hedonic scale, ranging from 0 (low) to 7 (high).

2.6. Statistical analyses

The data collected were subjected to one-way analysis of variance (ANOVA) using JMP statistical software version 11.0.0 (SAS Institute Inc., Cary, NC, USA), and differences were considered non-significant at $P > 0.05$.

3. RESULTS AND DISCUSSION

Salame Milano is a dry fermented sausage originally produced in the geographical area around the city of Milan (Italy). This denomination is included on the official list of Italian traditional products published yearly by the Italian Ministry of Agriculture and Forestry (*G.U. Repubblica Italiana* no. 147, 27/06/2013 Suppl. Ord. No. 52). Today, *Milano*-type fermented sausages are produced in various Italian areas according to the traditional process for *Salame Milano*. Both products are characterized by a bright red colour, a homogeneous "grain of rice" (compact but not elastic) texture, and a sweet-delicate flavour. All of these features are obtained after a long ripening period that varies from 3 to 9 weeks, depending on the product size (20-60 cm of length and 6-11 cm of width). However, recent studies (ARNAU *et al.*, 2007; STOLLEWERK et al., 2011) suggested the possibility of shortening the drying period of fermented sausages, thus reducing the cost of drying facilities (capital and labour) and increasing both profit margin and product competitiveness. Conversely, safety concerns are always to be faced when food processes are shortened. Accordingly, in this study, a challenge test was carried out to investigate the capacity of pathogens to survive during an accelerated production of *Milano*-type dry fermented sausages. A comparison between the main microbiological and physico-chemical features of the dry fermented sausages produced through the traditional protocol for *Salame Milano* and the accelerated technique was carried out as well.
The results of the challenge test during the production of the *Milano*-type fermented sausages are reported in Fig. 2.
It is worth noticing that the samples withdrawn after fermentation and freezing (T1) had microbial counts of *Salmonella* spp. that showed a significant decrease of 1.5 or 1.6 log CFU/g in the sausages inoculated with 2 and 4 log CFU/g, respectively. Afterwards, in both cases, the viable counts did not change significantly, and values of 0.7 ± 0.04 and 2.7 ± 0.04 log CFU/g were recorded in the final samples withdrawn after slicing and drying (T2). A similar behaviour was highlighted with regard to the inoculated *L. innocua* cells, although the decrease recorded from the T0 to the T1 samples (0.4 log CFU/g) was less notable and the final (T2) counts (1.4 ± 0.60 and 3.7 ± 0.01 log CFU/g) were higher than those measured for *Salmonella*. The non-inoculated control batch (1c) was always negative for the presence of both *Salmonella* and *Listeria* viable cells.

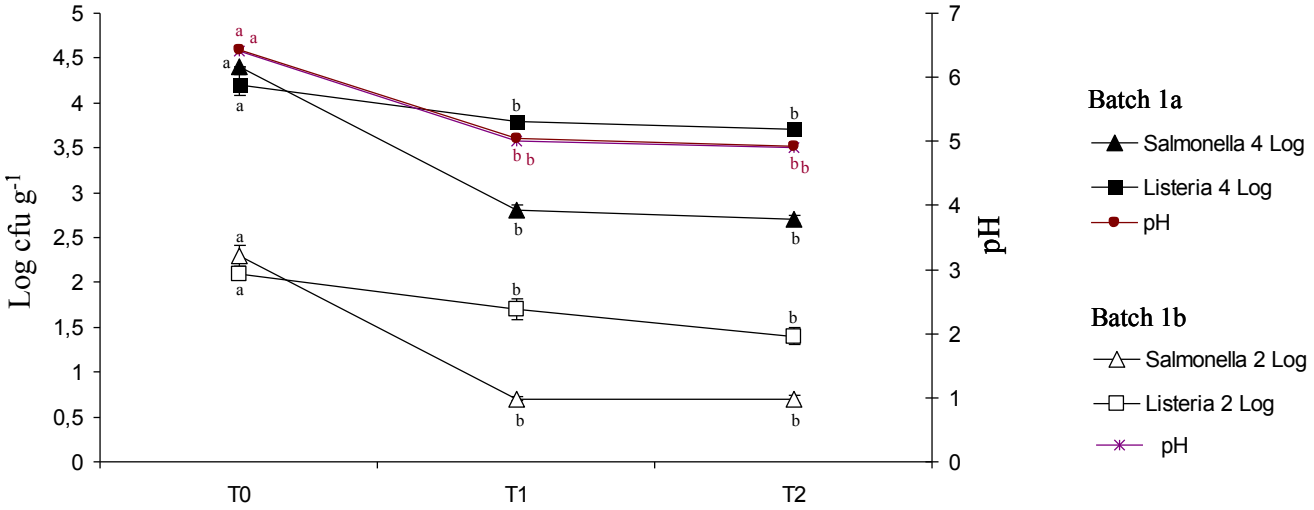

Figure 2. Viable counts of inoculated L*isteria innocua* and *Salmonella* spp. strains and pH values measured during the challenge test carried out along the accelerated process for manufacturing of *Milano*-type fermented sausages.
Process steps: after meat batter preparation (T0), after fermentation and freezing (T1, from day 1 to day 3), after slicing and drying (T2, day 4).
Two batches (1a and 1b) were inoculated with the strain cocktail (*Listeria innocua* and *Salmonella* spp.) at 2 and 4 Log colony forming units (cfu) g^{-1}, respectively, at T0.
Within each curve, means with different letters are significantly different $P < 0.05$, data observed are represented with standard deviation as error bar.

As reported by STOLLEWERK *et al*. (2011), the persistence of *Salmonella* could be related to its adaptation towards acidic environments. Indeed, it is well known that *Salmonella* acid-adapted cells may show increased resistance to organic acids such as those produced by lactic acid bacteria in fermented dairy products. These cells are capable of surviving better than non-adapted ones during fermentation of dairy products stored at 5 °C, possibly as a consequence of adaptive responses to other stresses, including heat shock, oxidative stress, and osmotic stress (LEYER *et al*., 1992). Conversely, different authors (COLE *et al*., 1990; SHABALA *et al*., 2001) have reported that *L. monocytogenes* can survive in stressful environments, such as those characterized by low temperature and high acidity and salt contents. On this topic, MATARAGAS *et al*. (2015) recently found that environmental conditions that prevail during sausage manufacturing may stimulate the expression of general- and/or specific-stress response genes and the intensiveness of these stresses may have an impact on their expression. According to the previous statements, the results of the present challenge test revealed the capacity of both inoculated *Salmonella enterica* serovars and *L. innocua* cells to survive during the accelerated process for the manufacturing of the *Milano*-type fermented sausages. However, the results of our test were more encouraging (especially for *Listeria*) if compared to those obtained by DALZINI *et al.* (2015) in a challenge test during a conventional process for the production of semi-dry low-fat salami. This finding was likely due to the pH (4.91 ± 0.02 and 4.93 ± 0.05 at 2 and 4 log CFU/g, respectively) and the a_w (0.90 ± 0.0) values reached in our experimental conditions, which were much lower when compared to those attained by DALZINI *et al.* (2015). Conversely, the final a_w values obtained through the accelerated process were similar to those reported by ZANARDI *et al.* (2002) for *Milano*-type sausage (0.89) produced in a traditional way.

The main microbiological and physico-chemical features of the *Milano*-type sausages produced through the accelerated technique (batch 1c) were analysed in comparison with the corresponding product obtained (after 3 weeks of ripening) through the traditional process, and the results are reported in Table 1.

The counts of total mesophilic aerobes and lactic acid bacteria were similar in both types of products, independently of the manufacturing process adopted. Because lactic acid bacteria possibly represent a significant fraction of the total mesophilic population, this finding suggests that the accelerated technique does not interfere with the growth of the pro-technological bacteria that was added as starter cultures in either the traditional or the accelerated process. In both cases, the values recorded for LAB counts were in line with those (8 Log CFU/g) reported by REBECCHI *et al.* (1998) for the same type of fermented dry sausages obtained through a traditional process.

By contrast, the absence of a conventional ripening phase was responsible for the significantly lower values found for yeasts and moulds in the samples of sausages obtained through the accelerated process because a conventional, long-lasting ripening is required to allow these "cosmetic" microorganisms to grow on the sausages surface (AQUILANTI *et al.*, 2007). Similarly, when the *Milano*-type sausages were produced through the accelerated technique, the counts of *Enterobacteriaceae* were significantly higher because the conventional ripening is usually responsible for the abatement of such an acid-sensitive microbial population. As is well known, the load of *Enterobacteriaeceae* is currently used as an index of enteric contamination in meat (PETRUZZELLI *et al*., 2016) and may also imply the presence of pathogens (BROWN *et al*., 2000). However, neither the traditional products nor those obtained through the accelerated technique were found to be positive for *Salmonella* spp. or *Listeria monocytogenes*.

Table 1. Microbiological and physico-chemical features of the *Milano*-type fermented sausages produced through either the traditional or the accelerated process.

	Traditional process	**Accelerated process**
Microbiological parameters (Log cfu g^{-1})		
Total mesophilic aerobes	8.4±0.46 [a]	8.4±0.02 [a]
Enterobacteriaceae	< 1	1.4±0.02 [a]
Lactic acid bacteria	8.4±0.18 [a]	8.4±0.01 [a]
Yeasts	4.2±0.08 [a]	2.2±0.46 [b]
Moulds	4.8±0.03 [a]	1.7±0.05 [b]
Salmonella spp.	Absent in 25 g	Absent in 25 g
Listeria monocytogenes	Absent in 25 g	Absent in 25 g
Physico-chemical parameters		
R.H. (%)	31.2±0.62 [b]	33.0±0.21 [a]
Ashes (%)	5.6±0.06 [a]	5.0±0.15 [b]
Nitrates (mg/kg)	178.0±6.00 [a]	174.0±9.05 [a]
Nitrites (mg/kg)	n.d.	n.d.
pH	5.6±0.03 [a]	5.0±0.05 [b]
a_w	0.86±0.01 [b]	0.89±0.01 [a]

Values are expressed as means ± standard deviation of triplicate samples.
Within each raw, means with different letters are significantly different $P < 0.05$
cfu = colony forming units
n.d. = not detectable
d.m. = dry matter

With regard to the results of the physico-chemical analyses (Table 1), as expected, the *Milano*-type fermented sausages produced through the accelerated drying process showed percent R.H. and a_w values higher (and percent ash values lower) than those of the products obtained after a traditional ripening/drying phase. However, the recorded value of a_w (0.89 ± 0.01) was considerably lower than that of *chorizo* obtained through the QDS process ® (STOLLEWERK *et al*., 2011) and was consistent with the values (< 0.90) reported for dry sausages manufactured in the Mediterranean area through a ripening period of at least 4 weeks (AQUILANTI *et al*., 2016). The other significant difference shown by the ANOVA concerns the pH that was higher in the traditionally produced *Milano*-type sausages, most likely due to the more intense oxidative activity of the mould population on lactic acid produced by LAB during carbohydrate fermentation (PAULSEN *et al*., 2011). Regarding the preliminary sensory analyses carried out to compare the end products obtained through the accelerated technique and the traditional process, no differences were highlighted for odour, texture, aroma, appearance, salinity, bitterness and sourness (data not shown). It is worth noting that, for the meat descriptor, fermented sausages produced through the traditional technique were characterized by a more intense flavour and aroma of ripened meat, whereas a flavour of sour meat was mainly perceived in fermented sausages produced through the accelerated technique.

4. CONCLUSIONS

Safety concerns need always to be faced when food processes are supposed to be shortened or modified. As ready-to-eat (RTE) meat products, dry sausages must be stable and safe at the end of the production process; therefore, particular attention must be devoted to the control of pathogenic microorganisms such as *Listeria monocytogenes* and *Salmonella* spp. that are more likely able to survive during manufacturing, thus constituting a risk for consumer health. Execution of challenge tests represents a key step to evaluate the behaviour of foodborne pathogens when innovative products or processes are implemented, and they are especially required when scarce literature is available on the innovation applied, as in the present case. Overall, the reduction of viable cells of *Salmonella* and *Listeria* recorded in the challenge test carried out in our study and the microbiological and physico-chemical characterization of the products obtained were quite encouraging. Further investigation on the fate of these foodborne pathogens during the shelf-life of the product must be envisaged in order to provide more complete information to food business operators who would manufacture fermented dry sausages using an accelerated production process.

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