Effect of production process and high-pressure processing on viability of *Salmonella spp.* in traditional Italian dry-cured *coppa*

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Abstract

The aim of the study was to investigate the combined effect of the manufacturing process followed by HPP treatment on the inactivation of *Salmonella* spp. in artificially contaminated *coppa* samples, in order to verify the ability of the combined processes to achieve the objective of a 5-log reduction of *Salmonella* spp. needed for exportation to the U.S. Fresh anatomical cuts intended for *coppa* production were supplied by four different delicatessen factories located in Northern Italy. Raw meat underwent experimental contamination with *Salmonella* spp. using a mixture of 3 strains. Surface contamination of the fresh anatomical cuts was carried out by immersion into inoculum containing *Salmonella* spp. The conditions of the HPP treatment were: pressure 593 MPa, time 290 seconds, water treatment temperature 14°C. Surface and deep samples were performed post contamination (T0), end of the cold phase (T1), end of process (Tend), and after HPP treatment (postHPP) and *Salmonella* spp. Enumerated. The results of this study show a significant reduction of *Salmonella* spp. all through the production process (P<0.01) for all companies, followed by an additional reduction of bacterial counts due to HPP treatment (P<0.01), both in superficial and deep contaminations (P<0.01). The superficial overall reduction resulted of 1.58 to 5.04 log CFU/g during the production process. HPP treatment resulted in a significant (P<0.01) superficial and deep decrease in *Salmonella* spp. enumeration varying from 0.61 to 4.01 log and from 1.49 to 4.13 log. According to the data presented in this study, only the combined approach of *coppa* manufacturing process followed by HPP treatment always led to a 5-log reduction of Salmonella spp. required by USDA/FSIS guidelines.

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Introduction

Coppa is a typical Italian cured pork meat product obtained from the cervical muscles of the neck of heavy pigs. The traditional areas of production are the provinces of Parma and Piacenza (Emilia Romagna region, Northern Italy), however it is produced with different recipes in many other Italian regions. Few data exist in literature on the characteristics and on product processing and the most relevant information can be found in the PDO specifications (http://www.salumidoppiacentini.com/coppa-d-p/index.jspeldoc?IdC=160&IdS=168&tipo_cliccato=0&tipo_padre=0&nav=1&css=generico_dop.css&menu=1; http://www.coppadiparmaigp.com/disciplinare-di-produzione-igp-coppa-parma/) or in the few published papers (Busconi *et al.*, 2014; Zanardi *et al.,* 2000). *Coppa* is a product consisting of a whole piece of meat, whose manufacturing process includes some peculiar phases. After deboning, half-slicing, and trimming the anatomical cut, salting is carried out: a mixture of salt, additives, and spices is distributed all over the meat, the composition of the ingredients varies according to the tradition and the recipes of production. Meat is then massaged manually or by a meat tumbling machine in order to ensure the homogeneous distribution of the mixture. Generally, one or two salting processes are carried out and followed by storage at low temperatures for a few days on steel trays (cold rest). At the end of the rest period, the meat cuts are wrapped in natural or synthetic casings, tied with a string, and then hung for several days in a drying chamber where they are exposed to higher temperatures and lower relative humidity, in order to reduce moisture. Finally, the ripening takes place for several weeks at a lower temperature and higher relative humidity than drying, until the product reaches the desired characteristics. Dry-cured meat products contamination by food-borne pathogens as *Salmonella* spp. and *L. monocytogenes* may result from superficial contamination of the fresh anatomical cuts. It can occur both during slaughtering and production and/or from cross-contamination in case of manipulation by contaminated operators or contact with contaminated equipment or surfaces.

Both EU and U.S. regulation requires ready-to-eat (RTE) meat products to be free from *Salmonella* (European Commission 2005; Todd *et al.*; 2004, FSIS 2017). In addition, Salmonella Compliance Guidelines for small and very small meat and poultry establishments that produce RTE products (FSIS, 2017) recommended that enterprises producing RTE meat must validate their processes in order to achieve at least a 5-log reduction of *Salmonella* spp. (FSIS, 2017). Establishments most often achieve the target by cooking, but they can use other lethality treatments such as fermentation, drying, salt curing, alternative processing technologies or a combination of these (FSIS, 2017); the same requirement is due for exportation of meat products to the U.S. (Italian Ministry of Health, 2015).

High hydrostatic pressure (HPP) is a non-thermal food preservation technology applied to enhance the microbiological safety and to extend the shelf life of the treated food while keeping the organoleptic and nutritional characteristics unaltered. HPP has been considered to be the main emergent preservation technology with more prospects for its application in the meat industry (Hugas *et al.,* 2002); it is mainly

used as a final sanitization measure after production and/or packaging procedures. HPP has been successfully applied for the treatment of a wide variety of food such as jams, fruit sauces, yogurt, beef, fruit and vegetable juices, processed poultry products, oysters, cheese and carpaccio (Tao *et al.,* 2016). Several treated RTE dry-cured meat products such as ham and salami are currently available on the market in Europe, U.S.A., Japan, Canada (Tao *et al.,* 2016).

The aim of the present study was to investigate the combined effect of the manufacturing process followed by HPP treatment on the inactivation of *Salmonella* spp. in artificially contaminated *coppa* samples, in order to verify the ability of the combined processes to achieve the objective of a 5-log reduction of *Salmonella* spp. needed for exportation to the U.S.

Materials and Methods

Inoculum composition

The *Salmonella* spp. inoculum culture was prepared using a mixture of 3 strains: 118174/1 (monophasic *S.* Typhimurium) isolated from fresh pork sausage, 106463/1 (*S.* Derby) isolated from fresh swine meat, and the reference strain *S.* Typhimurium ATCC 14028 according to Bonilauri *et al.* (2019). 100 µl of a stock culture (stored in 20% glycerol at -80°C), each strain was transferred to 10 ml Brain Heart Infusion (BHI) broth and incubated for 24 h at 30°C. Subsequently, an aliquot of 100 µl was transferred to 1000 ml BHI broth and incubated at 30°C for 72 h to reach the stationary phase.

Just before the use, the 3 subcultures of *Salmonella* spp. were combined in equal volume (one liter each) in order to obtain a multi-strain cocktail of about 109 colony forming units (CFU)/ml and the resulting mixed culture was checked by enumeration on selective agar.

Samples contamination and production process

Fresh anatomical cuts intended for *coppa* production were supplied by four different small delicatessen factories located in Northern Italy herein named A, B, C and D. Raw meat (weight between 2.5 and 3 kg) underwent experimental contamination with *Salmonella* spp. Surface contamination of the fresh anatomical cuts was carried out by immersion into inoculum containing *Salmonella* spp. The immersion lasted for 10 minutes and was followed by drying for dripping at room temperature for 30 minutes.

The four production processes were carried out in IZSLER laboratories following the producers' standard protocols as summarized in Table 1. One (company A and D) or two salting (companies B and C) were comprised, salting mixtures being supplied by the four companies. In all the protocols meat samples underwent one or more steps in the meat tumbling machine in order to get a homogenous distribution of the salting mixture. After the salting step, *coppa* samples were singularly packed in synthetic casings and at the end of the maturation period, were separately transferred to nylon-polyethylene bags and vacuum sealed. *Coppa* samples underwent processing steps according to the producer's specification (see Table 1): a resting phase (14 to 32 days at 1-8°C), a drying phase (3 to 7 days at 12-20°C), and a ripening phase (40 to 69 days at 14-18°C).

HPP treatment

For each contamination study, 5 vacuum-packed *coppa* samples were exposed to HPP treatment and 5 samples acted as control. The level of contamination before HPP was 1.56 – 5.09 log CFU/g in the superficial samples and 1.60 – 3.06 log CFU/g in the deep samples (see Table 2 Tend values). The conditions of the HPP treatment were: pressure 593 MPa, time 290 seconds, water treatment temperature 14°C, product temperature during treatment 4°C (Bonilauri *et al.*, 2019). The pressure-holding treatment time in this study did not include the pressure increase time or the decompression time. The water temperature during the process started from 14°C, grew until 32°C during the treatment, and immediately returned to 14°C after the end of pressure stress.

Sampling procedure

The protocol of this study included both analysis on the surface and in depth of *coppa* samples. For superficial sampling, three squares with a length of approximately 3x3 cm and a thickness of about 0.3 cm enough to get a final weight of 25 g, were excided from apical, central and terminal positions of each *coppa*. Deep sampling was carried out after immersion of *coppa* samples in boiling water for 60 seconds. A sample unit of 25 g from the depth of *coppa* was then extracted.

Physicochemical analysis

a_w was measured with AcquaLab, series

Table 1. Main characteristics of the three production processes reproduced in this study.

	Company A	Company B	Company C	Company D
Anatomic cut weight (Kg)	2,7	2,5	3	2,5/3
Number of salting	1	2	2	1
Resting length (days)/temperatures	14/3-5°C	32/3-5°C	27/1-4°C	9/6-8°C
Drying length (days)/temperatures	5/20°C	7/27°C to 14°C	6/22°C to 16°C	3/12-27°C
Ripening length (days)/temperatures	51/15°C	40/14-18°C 24/17-21°C	69/14-16°C	44/14°C to 16°C

Table 2. Experimental scheme including the number of analyzed test units for each processing step, sampling characteristics and scheduled analyses.

Sampling time	Processing step	Test units	Type of Sampling	Analysis
T0	Post-contamination	3	Superficial	*Salmonella* spp. enumeration, pH, a_w
T1	Post-resting	3	Superficial – In deep	*Salmonella* spp. enumeration, pH, a_w
Tend	Post-ripening	5	Superficial – In deep	*Salmonella*spp. enumeration, pH, a_w
THPP	Post-HPP treatment	5	Superficial – In deep	*Salmonella* spp. enumeration

4, Model TE instrument, in accordance with ISO 21807:2004. The pH values were determined with Mettler Toledo LE427 glass electrode probe attached to pHenomenal PC5000 L (VWR) pH/conductivity meter. Weight loss values (expressed as percentage of the initial weight) were determined throughout the production process, on three samples for each contamination study.

Microbiological analysis

Salmonella spp. enumeration, samples were 1/10 diluted and homogenized in Stomacher for 60 s. Ten-fold serial dilutions were pour-plated onto XLD agar and incubated at 37°C for 24 h. Suspected colonies were identified using MicrogenTM *GN-ID* (GNA, Microgen Bioproducts. Ltd., UK). In samples below the quantification limit of 10 Colony Forming Units (CFU)/g, the qualitative analysis was carried out according to ISO 6579:2002/Cor1:2004 (ISO, 2004).

Data analysis

According to EFSA (2010), for statistical analysis, if *Salmonella* spp. was detectable by the presence/absence test but not quantifiable in enumeration analysis (under the limit of quantification: LOQ=10 CFU/g) the value of 9 CFU/g (corresponding to $\log_{10} 9 = 0.95$ log cfu/g) was assigned. If *Salmonella* spp was no detectable by the presence/absence test, the value of 0.03 CFU/g was assigned (corresponding to less than 1 cell on 25g); log10 0.03= -1.52 log CFU/g). To compare the level of pathogens observed during processing steps and post HPP treatment, the two-way ANOVA test was chosen; level 1 was Company productive process (A, B, C, D) and level 2 was productive phases post contamination (T0), end of the resting phase (T1), end of ripening phase (Tend), and after HPP treatment (postHPP). When statistically significant differences were detected, one-way ANOVA and post hoc pairwise comparison across levels were performed by using Tukey's test. Surface and deep contaminations were compared separately.

The statistical analyses were performed by using the computer software program STATA 7.0 (STATA Corporation, College Station, TX, USA). Significance was established at $p < 0.05$.

Results and Discussion

The four production processes were characterized by different numbers of salting, cold and warm phase lengths and temperatures (Table 1) resulting in dry-cured *coppa* with different physicochemical characteristics (a_w ranging from 0.892 to 0.922, pH ranging from 5.66 to 6.61 on the surface and a_w ranging from 0.916 to 0.925, pH ranging from 5.61 to 6.13 in the deep part as reported in Table 3). The pH trend was in line with reported variability (5.5-6.5) cited by the PDO Product specification for *coppa* Piacentina (http://www.salumidoppiacentini.com/coppa-d-p/index.jspeldoc?IdC=160&IdS=168&tipo_cliccato=0&tipo_padre=0&nav=1&css=generico_dop.css&menu=1) Artificial contamination gained at a superficial initial concentration ranging from 6.52 to 7.47 log CFU/g of *Salmonella* spp. (Table 4). A contamination of the inoculated bacteria from the surface to the depth of the anatomical cuts was shown, probably facilitated by the use of the meat tumbling machine in concomitance with salting. In particular at the end of cold progressing phases deep contamination was first examined and reached values comprised among 4.10 to 4.84 log CFU/g of *Salmonella* spp.

The results of this study show a significant reduction of *Salmonella* spp. all through the production process ($P<0.01$) for all companies, followed by an additional reduction of bacterial counts due to HPP treatment ($P<0.01$), both in superficial and deep contaminations ($P<0.01$), in accordance with several other dry-cured meat products in which *Salmonella* spp. decrease resulted equal to 3.28 and 5.5 log in pork loins (Morales-Partera *et al.,* 2017) and ham after 69 days of curing (Reynold *et al.*, 2001) respectively, the observed differences were mainly due to different product characteristics and different production processes.

In detail, the superficial overall reduction resulted of 1.58 to 5.04 log CFU/g during the production process, being 4.66-5.04 log CFU/g for Company A, B and C and significantly lower (1.58 log CFU/g

Table 3. Results of chemico-physical analysis differentiated for manufacturing company carried out in superficial (Sup) and deep (Deep) samples: it is reported the mean value of the obtained measurements followed by the standard deviation into brackets.

	Company A				Company B				Company C				Company D			
	pH_{Sup}	a_{wSup}	pH_{Deep}	a_{wDeep}	pH_{Sup}	a_{wSup}	pH_{Deep}	a_{wDeep}	pH_{Sup}	a_{wSup}	pH_{Deep}	a_{wDeep}	pH_{Sup}	a_{wSup}	pH_{Deep}	a_{wDeep}
T0	5.95 (0.07)	0.997 (0.001)	N.D.	N.D.	6.40 (0.12)	0.997 (0.001)	N.D.	N.D.	5.99 (0.25)	0.994 (0.002)	N.D.	N.D.	5.93 (0.12)	0.995 (0.002)	N.D.	N.D.
T1	6.04 (0.10)	0.959 (0.009)	6.03 (0.16)	0.979 (0.01)	6.20 (0.18)	0.933 (0.008)	5.92 (0.12)	0.973 (0.005)	5.86 (0.13)	0.964 (0.038)	5.62 (0.15)	0.972 (0.002)	5.54 (0.03)	0.974 (0.004)	5.73 (0.14)	0.976 (0.006)
Tend	5.66 (0.18)	0.892 (0.017)	5.61 (0.13)	0.924 (0.01)	6.61 (0.23)	0.922 (0.005)	6.13 (0.16)	0.925 (0.006)	6.05 (0.19)	0.904 (0.019)	5.80 (0.05)	0.916 (0.010)	5.98 (0.12)	0.913 (0.005)	5.76 (0.09)	0.923 (0.004)

N.D.: Not Determined.

Table 4. Mean value log cfu/g (standard deviation) of *Salmonella* spp. (S) enumeration analyses carried out in superficial (Sup) and deep (Deep) Samples.

	Company A		Company B		Company C		Company D	
	S_{Sup}	S_{Deep}	S_{Sup}	S_{Deep}	S_{Sup}	S_{Deep}	S_{Sup}	S_{Deep}
T0	6.60^{A} (0.22)	N.D.	7.35^{A} (0.18)	N.D.	6.52^{A} (0.25)	N.D.	7.47 (0.02)	N.D.
T1	$5.38^{B,x}$ (0.27)	$4.80^{A,x}$ (0.80)	$5.32^{B,x}$ (0.33)	$4.84^{A,x}$ (0.12)	$4.85^{B,x}$ (0.27)	$4.10^{A,x}$ (0.44)	4.81 (0.87)	4.71 (0.97)
Tend	$1.56^{C,x}$ (0.56)	$2.61^{B,y}$ (0.20)	$2.49^{C,x}$ (0.27)	$1.95^{B,x}$ (0.70)	$1.86^{C,x}$ (1.22)	$1.60^{B,x}$ (0.37)	5.89 (1.05)	3.06 (1.20)
THPP	0.95 (0.00)	-1.52 (0.00)	-0.04 (1.36)	0.46 (1.11)	0.95 (0.00)	-0.53 (1.35)	1.88 (0.60)	-1.03 (1.11)

ND: Not Determined; # assumed value: 0.95 when in all replicates pathogen was detected but not countable <10 CFU/g, -1.52 when in all replicates pathogen is not detected, -0.04 when in 3 out of 5 replicates pathogen was detectable but not countable and the last two were not detected, -0.53 when in 2 out of 5 replicates pathogen was detectable but not countable and the last three were not detected, 0.46 when in 4 out of 5 replicates pathogen was detectable but not countable and the last one was not detected, -1.03 when in 1 out of 5 replicates pathogen was detectable but not countable and in 4 replicates were not detected; when assumed value is used no statistical comparison was possible. Different capital letter significant differences between results in different rows. ^{x,y} means differences between surface and deep contamination in rows. Differences between Companies were not significant (see text).

Table 5. Logarithmic unit reductions of *Salmonella* spp. (S) in superficial samples after each sampling step.

	Company A		Company B		Company C		Company D	
	S_{Sup}	S_{Deep}	S_{Sup}	S_{Deep}	S_{Sup}	S_{Deep}	S_{Sup}	S_{Deep}
Resting - Δ(T0-T1)	1.22	N.D.	2.03	N.D.	1.67	N.D.	2.66	N.D.
Drying and Ripening - Δ(T1-T*end*)	3.82	N.D.	2.83	N.D.	3.02	N.D.	-1.08	N.D.
Production process - Δ(T0-T*end*)	5.04	N.D.	4.86	N.D.	4.69	N.D.	1.58	N.D.
HPP - Δ(T*end*-THPP)	0.61	4.13	2.53	1.49	0.88	2.13	4.01	4.09
TOTAL - Δ(T*0*-T*HPP*)	5.65	N.D.	7.39	N.D.	5.57	N.D.	5.59	N.D.

N.D.: Not Determined.

reduction) for company D (see Table 5); for company D a noticeable shorter duration of all the phases was observed; it was reported that *Salmonella* count reduction during seasoning is related not only to a_w reached at the end of the process but in particular to the duration of seasoning (Pin *et al.,* 2011).

In this study, HPP treatment resulted in a significant ($P<0.01$) superficial and deep decrease in *Salmonella* spp. enumeration varying from 0.61 to 4.01 log and from 1.49 to 4.13 log, respectively; the results show that HPP treatment of coppa samples has proven to be effective against both superficial and deep contamination. The generally lower decreases in superficial contamination correlate with the lower a_w values that are proven to have a protective effect on microbial inactivation by HPP (Black *et al.,* 2007; Black *et al.,* 2007*;* Hayman *et al.,* 2008*;* Patterson *et al.,* 2005). HPP has demonstrated to be able to reduce *Salmonella* load in different types of food like raw chicken meat, poultry sausage, RTE meat (Anthoula *et al*., 2018; Hayman *et al.*, 2004; Lerasle *et al*., 2014; Tananuwong *et. al,* 2012; Yuste *et a.,* 2000) and fermented pork sausages (Garriga *et al.,* 2003); in particular in dry-cured ham (Bover-Cid *et al., 2017;* Garriga *et al.,* 2004): when an HPP treatment of 600 MPa for 5 min was used on artificially contaminated sliced cured ham (a_w ca 0.92), the reduction of *S. enterica* ranged from 3.72 to 5.04 log (Bover-Cid *et al.,* 2017). In general, lower values of microbial reductions during HPP treatment were observed in the present study, but the comparison of this kind of data appears to be problematic as regards the possible differences in the characteristics of the treated products, in experimental design, HPP treatment conditions and baroresistance of the strains used for contamination.

The results of this study confirm that HPP treatment can be successfully used as an effective supplemental intervention strategy for controlling *Salmonella* spp. contaminations in dry-cured meat products such as *coppa*. In the case of products intended for exportation to countries with a *zero tolerance* policy for *Salmonella* spp., specifically the United States, HPP treatment used as a final sanitization measure after production, resulted to be a determining factor for the achievement of the USDA/FSIS requisites in establishments B, C and D, resulting particularly relevant in establishment D. According to the data presented in this study, only the combined approach of *coppa* manufacturing process followed by HPP treatment always led to a 5-log reduction of Salmonella spp. required by USDA/FSIS guidelines. Results suggest that the three establishments B, C, D should review their entire production process (especially for establishment D) either by adding the HPP step or, as additional option, by reviewing the time/temperature of the other decontamination steps of resting, drying and ripening.

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