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Research

Listeria monocytogenes on cured meat products. A case study on *Speck* (a typical Italian smoked ham) regarding to EC Regulation 2073/2005 requirements

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CONFLICTS OF INTEREST
There are no conflicts of interest for any of the authors.

ABSTRACT:
Ready To Eat (RTE) meat products may be able to support the growth of *Listeria monocytogenes* during their shelf life. In agreement with the European Commission (EC) Regulation N. 2073/2005 on microbiological criteria for foodstuff, the products with pH ≤ 4.4 or a_w ≤ 0.92, those with pH ≤ 5.0 and a_w ≤ 0.94 and those with a shelf-life of less than five days are automatically included in the category of RTE foods that doesn't able to support the growth of *L. monocytogenes*. Otherwise, the manufacturers must demonstrate whether their products not allow the growth of *L. monocytogenes* by use of different approaches based on: i) physical-chemical analyses of product, ii) predictive microbiology and iii) Microbial Challenge Test (MCT), as suggested by EC Regulation 2073/2005. The present study evaluated the three above mentioned approaches on Speck, a traditional smoked dry cured ham produced in South Tyrol (Italy). Both the variability of the physical-chemical properties of Speck (a_w ranging from 0.890 to 0.940 and pH from 5.37 to 6.24) and the use of prediction models did not allow to state whether the product supports the growth of the pathogen and a reliable classification of the product. On the contrary, the MCT carried out showed the *L. monocytogenes* inactivation during shelf life of sliced vacuum-packed or modified atmosphere-packed Speck stored at different temperatures 4°, 8° and 20°C.

KEY WORDS: Speck, dry cured ham, shelf life, *Listeria monocytogenes*, predicted inactivation, microbial challenge test.

1. INTRODUCTION

In 2005, the European Commission (EC) defined a food safety criteria limit of 100 colony forming units (CFU)/g for Ready To Eat (RTE) foods "unable to support the growth of *Listeria monocytogenes*" (EC, 2005). The EC regulation also states that as necessary, Food Business Operators (FBO) shall conduct studies to evaluate the growth of *L. monocytogenes* that may be present in the product, during its shelf life under reasonably foresee able conditions of storage, distribution, and use. Annex II of the regulation text suggests different approaches to classify the RTE product.

The first approach regards the analysis of physical-chemical properties, such as pH, a_w, salt content, concentration of preservatives and type of packaging system, taking into account the storage and processing conditions, the possibilities for contamination and the planned shelf life. Accordingly, the products "with pH ≤ 4.4 or a_w ≤ 0.92, those with pH ≤ 5.0 and a_w ≤ 0.94 and those with a shelf life of less than five days" are automatically included in the category of RTE foods that are unable to support the growth of *L. monocytogenes*.

The second approach is based on the available scientific literature and research data regarding the growth and survival characteristics of the pathogens of concern. Within such approach, the use of predictive mathematical modelling is also allowed, with the growth of pathogen being assessed as a function of relevant critical factors.

As a third approach, the so-called challenge tests have been addressed, where the ability of appropriately inoculated organisms to grow or survive in the product under different storage conditions are investigated (EC, 2005, Scott et al., 2005).

As a rule, when RTE meat products reach the packaging environment in the plants, they should be free of harmful bacteria (Syne et al., 2013); however, the inherent risk of recontamination by *L. monocytogenes* in meat processing environments has been well documented and must be addressed (Gombas et al., 2003, Gómezet al., 2015).

Dry-cured ham is increasingly distributed as a sliced RTE product and post-processing manipulation as slicing or packaging operation scan facilitate *L. monocytogenes* cross-contaminations (Vorst et al., 2006). This bacterium is a ubiquitous psychrotrophic organism able to survive environments with relatively low a_w values, i.e. 0.90 (Nolan et al., 1992). Owing to its complex and versatile physiological adaptation mechanisms, *L. monocytogenes* can persist and often proliferate in contaminated foods under a wide range of antimicrobial conditions, such as low a_w, low pH, and low temperature (Hill et al., 2002).

In Italy, there are several type of RTE meat product, as salami, sausages, semi-dry sausages and cured ham. Speck is a traditional dry-cured ham produced in South Tyrol, northern Italy and regulated by the European Union under the Protected Geographical Indication (PGI) status (Disciplinare di produzione della Indicazione Geografica Protetta). This product is lightly smoked and therefore represents a synthesis between the smoked bacon of the Alps and the Mediterranean ham. Speck is normally associated to the typical "*baffa*" (ham) presentation, although recent consumer trends have guided Speck producers to roll out formats that more closely meet the various requirements of the market. Speck may be released for consumption loose, packaged under-vacuum, or in a modified atmosphere and it may be whole, cut or sliced.

Therefore, the aim of this study was to evaluate if Speck is able to support the growth of *L. monocytogenes* during its shelf life. For this purpose, a combined approach of three criteria was used: i) the collection and the analysis of physical-chemical properties of product at the end of manufacturer process (beginning the shelf life), ii) the use of predictive mathematical models to evaluate the survival/inactivation of *L. monocytogenes*, based on physical-chemical data collected, and then iii) the microbial challenge test (MCT) by artificial inoculation of Speck with *L. monocytogenes* strains, in order to evaluate its behaviour during the shelf life of product packed under vacuum or in modified atmosphere and stored at different temperatures.

2. MATERIALS AND METHODS

2.1. Physical-chemical analyses (first approach)

Speck samples for physical-chemical analyses were collected from local manufacturers just after the production process and were characterized by a final weight loss of about 37%. Assuming that different types of packaging (under-vacuum or under modified atmosphere) and cutting (whole or sliced) can influence the physical-chemical properties of the products, two types of Speck were used in this study: vacuum-packed (VP) samples (whole, 1kg) and modified atmosphere (30% CO_2 and 70% N_2) packed samples (MAP) (sliced, 100 g). A total of 80 packs (40/type of packaging) were analyzed. Physical and chemical analyses were performed on approximately 10 g of each sample. The water activity (a_w) was measured at 25°C by means of an a_w recorder (AquaLabseries 3, Model TE Decagon Devices Inc., Pullman, USA) in agreement with ISO/FDIS 21807 (ISO/FDIS, 2004). The pH was measured using a HI 223 Calibration check™ Microprocessor pH meter (Hanna Instrument, USA) equipped with a Gel-Glass electrode (Hamilton, Switzerland).

2.2. Predicting inactivation model (second approach)

To predict the logarithmic reduction of *L. monocytogenes,* a "Non-Thermal Survival Model" in ComBase (www.combase.cc) was used. Considering physiological state, temperature, pH and a_w as constant variables, the predictive model defines the inactivation rate expressed as *D*-value in broth at the same physical-chemical properties of Speck product.

2.3. Microbial Challenge test (MCT)

2.3.1. Product characterization (third approach)

Twenty-five packs Specks with a_w value ≥ 0.93 were collected from local manufacturers just after production process. Since the naturally microbiota can influence the growth rate of *L. monocytogenes*, a characterization of the products as related to their producers was made to evaluate the variability of typical bacteria counts (Cornu et al. 2011). The products were analysed to evaluate typical microbiota as total count, lactic acid bacteria (LAB), *Staphylococcus* Non Coagulase Positive (NCP), *Enterobacteriaceae*, yeasts and the absence of any typical *Listeria* colonies. Parameters as pH and a_w were also noticed. The analyses were performed according to Frustoli et al., (2010).

2.3.2.Listeria strains and inoculum preparation

A mix of two *L. monocytogenes* reference strains (Scott A and ATCC7644) and three *L. monocytogenes* wild strains isolated from speck and obtained from the SSICA collection was used in this study. For inoculum preparation, frozen suspensions were streaked on Tryptone Soya Agar (TSA; Oxoid, UK) and TSA plates were incubated at 37°C for 24 h; the strains were confirmed on ALOA (Biolife, Italy). Single colonies were transferred to tubes containing 10 ml Tryptone Soya Broth (TSB; Oxoid, UK) and allowed to grow at 37°C for 24 h. Afterwards a second subculture was inoculated in fresh TSB broth and incubated at 8°C. Cell counts were performed at days 2, 4 and 5 to determine the beginning of the stationary phase. A temperature profile of 24 h at 37°C followed by a subculture of 4 days at 8°C was used in order to obtain early stationary phase cells in a condition representing a cold environment. Afterwards, strains were mixed in equal proportion; the mixed culture was diluted in Pepton Physiological Solution (PPS 0.1% (w/v) peptone (Oxoid) and 8.5% (w/v) NaCl (Sigma Aldrich)) to an inoculation level of 3.0×10^4 cfu/ml.

2.3.3 Samples preparation and sampling time

Each Speck was aseptically sliced (30 g/pack) and the samples were spiked with 100 µl of *L. monocytogenes* mix, in order to reach a final concentration of about 10^2 cfu/g. To simulate a contamination during cutting and slicing, the inoculum was spread over the slice surface with a sterile plastic handle until its absorption. Inoculated slices were vacuum-packed (VP) or modified atmosphere-packed (MAP - 30%CO_2 and 70% N_2) in plastic bags (PET/PE with oxygen permeability <50 $cm^3/m^2/24$ h and water vapor permeability <15 $mg/m^2/24h$). Contaminated samples were stored for 180 days (VP) and 120 days (MAP) at dynamic temperature profiles to evaluate six different shelf lives (SL) (Table 1). For each study, nine samples were collected at five sampling times for a total of 270 analyses. The enumeration of *L. monocytogenes* was carried out according to UNI EN ISO 11290-2 (ISO, 2005; Scotter et al., 2001). When microbial numbers decreased to below the detection limit (<1.0 log cfu/g), samples were analyzed for the presence of *L. monocytogenes,*according to UNI EN ISO 11290-1 (ISO, 2005).

Table 1. Time/temperature profiles of six different shelf lives (SL) retained to evaluate the behaviour of *L. monocytogenes* on Speck vacuum packed (VP) or modified atmosphere (MAP) packed.

Shelf life	Packaging	Time/Temperature
SL1	VP	180 days/4°C
SL2	VP	30 days/4°C and 150 days /8°C
SL3	VP	31 days/4°C and 150 days /20°C
SL4	MAP	120 days/4°C
SL5	MAP	30 days/4°C and 90 days /8°C
SL6	MAP	60 days/4°C and 60 days /8°C

2.4. Data analyses

For physical-chemical analyses SPSS (V.13.0- SPSS inc., Chicago IL.) was used to calculate mean value and standard deviation of a_w and pH. Boxplot analysis, useful to highlight outliers, was performed to evaluate the distribution of pH and a_w values in Speck.

Microbial counts were transformed to logarithms before median, means and standard deviations were computed, and counts were reported as log cfu/g. When *Listeria* counts in Speck samples decreased to below the threshold of numeration (< 1.0 log cfu/g), values of 1.0 and 0.0 were respectively used for positive and negative samples, for the statistical elaboration of the results using SPSS 13.0.

3. RESULTS AND DISCUSSION

3.1. Physical-chemical analyses

Overall, the mean of a_w and pH values in Speck were equal to 0.917 ± 0.011 and 5.89 ± 0.18 respectively. The a_w is a well known parameter in meat microbiology, for its ability to predict the microbial stability and correlate with the potential microbial growth and metabolic activity (Leistner & Rodel, 1981; Gould, 1985; Glass & Doyle, 1991; Sabatakou et al., 2001; Hew et al., 2005). In our study the average a_w was lower than the limit of 0.920 stated by the EC Regulation 2073 (EC, 2005), then it strongly suggest that Speck can be included in the category of ready-to-eat foods unable to support the growth of *L. monocytogenes*. As shown in the boxplot of these variables (Figure 1), the 50% of the samples was contained in the central box. The a_w interquartile ranged between 0.912 and 0.925, and the pH interquartile ranged between 5.79 and 6.02. However, the analysed samples showed a broad range of a_w and pH values (a_w ranging from 0.890 to 0.940 and pH from 5.37 to 6.24) (Figure 1). Therefore, based only on physical-chemical properties, it unequivocally cannot bestated whether Speck is unable to support the growth of *L. monocytogenes*.

3.2. Predicting inactivation

Based on the variability of the pH and a_w values recorded, a predictive approach seemed to be appropriate for better evaluating the safety of Speck as a meat matrix that do not allow preventing the growth of *L. monocytogenes*. The predictions were run at 4°C, 8°C and 12°C according to Audits International (1999), Sergelidis et al. (1997), Samelis et al. (2002), USDA-FSIS (2003), Godwin et al. (2007), EU CRL (2008), ANSES (2014) since they have documented the handling abuses of RTE meat products in terms of storage temperature and packaging conditions of left overs, respectively during product shipment and home storage. Results (Table 2) indicated that *L. monocytogenes* was unable to growth at a_w values of 0.93 and pH 6.18 (95th percentile extrapolated values from the box plots analysis – see Figure 1). In agreement with Porto-Fett et al. (2008), the *D*-values reveal that the higher the storage temperature, the greater the rate of inactivation; in fact high temperatures are known to accelerate death at lethal pH or a_w compared to refrigeration temperatures (Gounadaki et al., 2007).

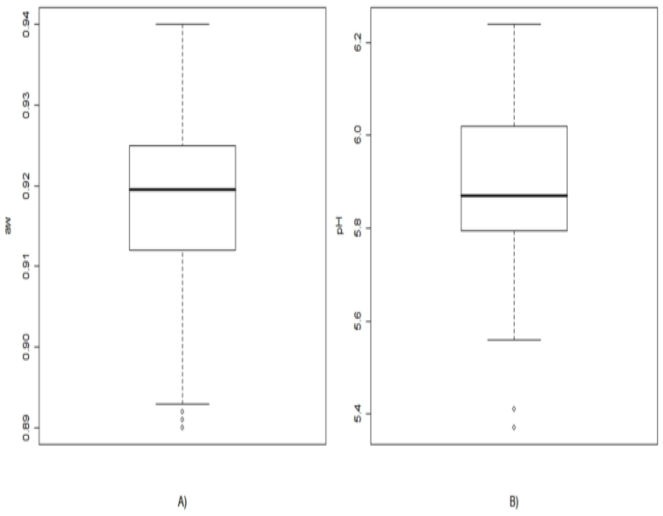

Figure 1. Boxplot diagram with outliers (o) of a_w (A) and pH (B) values in Speck.

Boxplots provide a summary of the statistical information: the bottom of the box is at the first quartile (Q1),that corresponding to 25th percentile, and the top is at the third (Q3), that corresponding to 75th percentile,the horizontal line within the box corresponds to the median value and the vertical lines are an index of data variability. The whiskers are the lines that extend from the top and bottom of the box to the lowest and highest observations that are inside the region, defined by the following limits: lower limit, Q1-1.5 (Q3-Q1), upper limit: Q3+1.5 (Q3-Q1). Outliers are points outside of the lower and upper limits and are plotted with circles (Tukey, 1977).

Table 2. Predicted inactivation of *L. monocytogenes* (physiological state 1.2e-2) in cultured media with a_w 0.93, pH 6.18 at different temperature/time conditions.

Time (days) [a]	Temperature (°C)	D-value (days)	L. monocytogenes log- reduction (log cfu/g)
180	4	66	2.6
180	8	51	3.4
150	12	34	4.3

[a] The days of incubation are in agreement with the shelf life defined by the manufacturer's Speck

Based on the physical-chemical analyses and the predicted inactivation it can be concluded that most of Speck samples tested in this study didnot support the growth of *L. Monocytogenes* in accordance with EC Regulation 2073 (EC, 2005). The results reported in Figure 1 showed that a minority of products was characterized by a_w>0.93 that is the limit considered for growth/not growth for *L. monocytogenes/innocua* in non-thermal survival model (Combase Modelling Toolbox, 2008).

In these cases, inactivation studies may be used to determine if non-thermal technologies or if combinations of pH, a_w, preservatives and long product storage may guarantee sufficient lethality to provide a food product safe (NACMCF, 2009).

3.3. Microbial challenge test

The microbiological and physical-chemical characteristics of Speck samples are summarized in Table 3 and their pH and aw individually plotted in Figure 2 where they range between 5.70-6.00 and 0.931-0.950, respectively. It is noteworthy that the pH/a_w combination of these Speck resulted in a 'growth' response when submitted to the prediction algorithm.

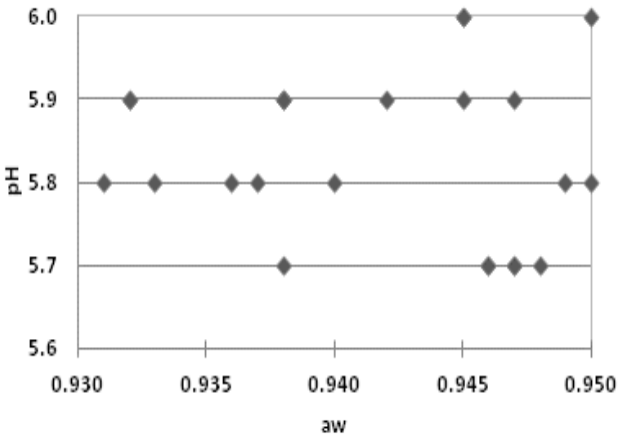

Figure 2. pH/$a_w$values of 25 Speck used to perform the MCTs

Table 3. Microbial (log cfu/g) and chemical-physical properties of Speck used to perform the MCTs.

Test	Method	N	Mean	Std. Dev.
Colony count at 30°C	**ISO 4833-1:2013**	**25**	**5.6**	**0.81**
Mesophiliclactic acid bacteria	**ISO 15214:1998**	**25**	**4.6**	**1.38**
StaphylococcusCNS	**SSICA LM/MP/N.15 2000 Rev.1**	**25**	**4.9**	**0.67**
Enterobacteriaceae	**ISO 21528-2:2004**	**25**	**<1**	**0.00**
Yeasts	**ISO 21527-2:2008**	**25**	**3.3**	**1.07**
aw	**SSICA LM/MP/N.29 REV. 2 2006**	**25**	**0.941**	**0.006**
pH	**SSICA LM/MP/N.30 REV. 0 2000**	**25**	**5.83**	**0.098**

Table 4. *L. monocytogenes* log cfu/g in sliced Speck vacuum packed and modified atmosphere packed during different shelf lives (SL). Data reported the median, mean, standard deviation of log cfu/g measured on 9 samples at each sampling time; no growth were observed for any individual sample.

Shelf life	Time (days)	Median	Mean	Standard Deviation
SL1	0	2.0	2.2	0.52
	30	1.3	1.7	0.85
	75	1.3	1.6	0.68
	150	1.0	1.3	0.60
	180	1.0	1.1	0.19
SL2	0	2.0	2.2	0.52
	30	1.3	1.7	0.85
	75	1.0	1.5	0.74
	150	1.0	1.3	0.54
	180	1.0	1.0	0.11
SL3	0	2.0	2.2	0.52
	30	1.3	1.7	0.85
	75	1.0	1.3	0.47
	150	<1.0[a]	<1.0	0.00
	180	<1.0	<1.0	0.00
SL4	0	1.9	2.1	0.59
	30	1.0	1.7	0.83
	75	1.3	1.7	0.74
	150	1.7	1.6	0.64
	180	1.0	1.5	0.69
SL5	0	1.9	2.1	0.59
	30	1.0	1.7	0.83
	75	1.0	1.6	0.75
	150	1.3	1.6	0.62
	180	1.0	1.3	0.54
SL6	0	1.9	2.1	0.59
	30	1.0	1.7	0.83
	75	1.3	1.7	0.74
	150	1.0	1.4	0.49
	180	1.0	1.5	0.63

The results of the microbial screening showed a high level (5.6 ± 0.8 log cfu/g) of bacterial load, that resulted mainly composed by lactic acid bacteria and Gram positive cocci coagulase negative (CNS); *Enterobacteriaceae* counts were less than 100 cfu/g. No Positive samples for *Listeria* were detected.

No increase in the initial *Listeria* contamination values was recorded at all packaging conditions, times and temperatures for any individual sample. The fate of *L. monocytogenes* in inoculated samples is shown in Table 4. The number of pathogen remained relatively unchanged after 30 days at 4°C; from the 75th day of shelf life, contamination levels average collected and their relative standard deviations showed a steady significant ($p<0.05$) reduction in the initial contamination level at all temperatures for VP samples. As expected (Dourou et al., 2009, Grisenti et al. 2004), the decrease was faster at 20°C than at refrigeration temperatures and *L. monocytogenes* levels decreased to below the level of the detection by both direct plating (< 1.0 log cfu/g) and enrichment (absent in 25 g of products) techniques in any sample after 150 days of storage in SL3. In particular, when samples were stored at 4°C (SL1), *Listeria* concentration decreased by 1.1log cfu/g; when 4°C were combined with 8°C(SL2) its contamination level decreased by 1.1 log cfu/g and when 4°C were combined with 20°C (SL3), its concentration decreased by ≥ 2.0 log cfu/g after 180 days of storage. When samples were packaged in MAP the pathogen number remained at the initial level during all the time of SL4 carried at 4°C and in SL5 and SL6 the level decreased by 0.8 log and 0.7 log cfu/g respectively at the end and after 90 days of shelf life.

Therefore, this study documents the inability of *L. monocytogenes* to grow in Speck even at those a_w and pH values that would enable it to grow, according to the predictive models. In agreement with previous studies carried out on Italian dry cured meat products (Grisenti et al. 2004; Frustoli et. al. 2007; Grisenti et al. 2008; Frustoli et al. 2010), dry-curing and meat maturing result in complex ecosystems where the interaction of the various known or unknown "hurdles" can determine the inhibition of *Listeria* growth. Inactivation of *L. monocytogenes* may be ascribed to the metabolic activity of LAB that can produce lactic acid, diacetyl, ethanol and/or bacteriocins and other metabolic by-products.

Similar findings were reported for other European and US typical meat products (Ingham et al. 2004; Dourou et al. 2009). In other terms, the combination of salting, smoking and drying as practiced with the Italian Speck can be considered an effective antilisterial process (Mittich et al. 2009) as there was no growth of the pathogen when it was inoculated at post-lethality stages and stored at either refrigeration temperature or room temperature. Moreover, during shelf life of SV or MAP packaged Speck a decrease in the number of *Listeria* was observed.

CONCLUDING REMARKS

This study investigated the eventual development of *L. monocytogenes* in a typical Italian meat product by use of three different approaches.

Speck dried hams would seem able to allow the growth of *L. monocytogenes*, the experimental data obtained in this research from an appropriately designed challenge tests demonstrate that the pathogen does never grow, even when their pH and *aw* are combined at their highest values. Moreover, results show a steady decline in the count of the inoculated pathogen throughout the product shelf life, which suggests an inherently lethal role played by the microbiota meat matrix.

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