PAPER

INFLUENCE OF WATER ACTIVITY ON *LISTERIA MONOCYTOGENES* GROWTH IN "SALSICCIA SARDA" FERMENTED SAUSAGE

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ABSTRACT

"Salsiccia Sarda" is catalogued as a ready-to-eat food (RTE) for which actual European Legislation proposes microbiological criteria for *L. monocytogenes*. This study evaluates the influence of water activity (a_w) on *L. monocytogenes* growth in 180 "Salsiccia Sarda" samples. A challenge test was performed to determine the *L. monocytogenes* growth potential (δ). The highest values of δ were detected in samples with limited values of a_w, showing that this product is frequently around the limits for the growth of this pathogen. Our results provide some critical information about process parameter combinations that could lead to greater safety of this product and better *L. monocytogenes* control.

Keywords: Challenge test, growth potential, legislation, *Listeria monocytogenes*, ready to eat meat products, "Salsiccia Sarda", water activity

1. INTRODUCTION

The implementation of HACCP (Hazard Analysis and Critical Control Points) system is fundamental to minimize the percentage of microbiological risk associated with food consumption. *Listeria monocytogenes* is a pathogen that may contaminate different foods and many areas of the food processing environment (BARZA, 1985; SCHLECH, 2000; GAULIN *et al*., 2003; MACDONALD *et al*., 2005; VARMA *et al*., 2007; JACKSON *et al*., 2011; AISSANI *et al*., 2012). This pathogen causes listeriosis in humans and animals; human listeriosis can cause serious illness in immunocompromised individuals, pregnant women, newborns and elderly people. There has been a gradual increase in cases of listeriosis over the past 5 years in European Union countries (EFSA, 2014). In Italy, between 1993 and 2000, the number of cases of listeriosis increased (PETRUZZELLI *et al*., 2010). *L.monocitogenes* was mostly found in ready-to-eat foods (RTE) and was responsible for many outbreaks associated with the consumption of RTE meat, poultry, dairy, fish and vegetable products (LIU, 2006; LIANOU and SOFOS, 2007; Chan and WIEDMANN, 2009; EFSA, 2014; CORONEO *et al*., 2016). The possible presence of pathogens is a critical issue when dealing with a wide variety of fermented, dried and semi-dried sausages. These products are classified as ready-to-eat foods (RTE) in which the presence of *L. monocytogenes* can pose a health risk to consumers. With this regard, some recent listeriosis cases are linked to the consumption of RTE meat products (PHAC, 2009). *L. monocytogenes* contamination tends to increase during the production process of meat products because their production requires different handling steps and exposure to contaminated surfaces in the processing environment. Raw meat is an important contamination source and may be contaminated by *L. monocytogenes* from the slaughterhouse environment or during the meat processing. Once the production plant has been contaminated, *L. monocytogenes* can survive on work surfaces and equipment and grow on the meat products due to its high ability to tolerate environmental stress factors (wide ranges of pH and a_w, high salt concentration, presence of nitrite and nitrate, and refrigeration temperature) (PETRUZZELLI *et al*., 2010; MUREDDU *ET AL*., 2014; MELONI *et al*., 2014). Sardinia has a long tradition of quality meat-products and "Salsiccia Sarda" is considered the Sardinian salami par excellence. It is a fermented RTE meat product included on the list of Italian traditional food products. It is made from minced lean pork mixed with different ingredients (salt, pepper, fennel and herbs). The mixture is introduced into a natural pork casing and, subsequently, the products are first heated to 20-22°C for 4-6 hours and then dried for six days in a fermentation chamber. During the first day of drying, the products are stored at 20-22°C and 60% relative humidity. In the subsequent five days of drying, the temperature is gradually reduced to 15°C and the relative humidity is gradually increased to 70%. The product is then dried and finally subjected to ripening for a period ranging from 8 to 25 days. The "Salsiccia Sarda" marketed shows a pH value of 5.28 and a_w ranging from 0.90 to 0.95 (GRECO *et al*., 2005).

Previous studies of traditional fermented meat products showed that the prevalence of *L. monocytogenes* was 10% in France (THÉVENOT *et al*., 2005), 10.6% in Chile (CORDANO and ROCOURT, 2001) and between 13% and 42% in Italy (DE CESARE *et al*., 2007; MELONI *ET AL*., 2009). PETRUZZELLI *et al*. (2010) reported a *L. monocytogenes* prevalence of 45.7% in traditional salami samples from the Marche region (central Italy).

"Salsiccia Sarda" is catalogued as a ready-to-eat food (RTE) for which actual European Legislation, Regulation (EC) 2073/2005 as amended by Regulation (EC) 1441/2007 (European Commission, 2005; European Commission, 2007), specifies microbiological criteria for *L. monocytogenes*. According to these regulations, the *L. monocytogenes* growth is not supported in RTE products with pH <4.4 or $a_w \leq 0.92$ or with pH ≤5.5 and $a_w \leq 0.94$. For RTE products that meet these conditions, a criterion of risk acceptability was established

of 100 CFU (Colony-Forming Unit)/g during the shelf-life. Although several studies (MELONI *et al*., 2009; MELONI *et al*., 2012) have shown the presence of *L. monocytogenes* in 42% of fermented "Salsiccia Sarda", the contamination levels are always lower than 100 CFU/g. Many factors affect the growth capacity of *L. monocytogenes* in foods and the intrinsic and extrinsic properties (i.e. pH, NaCl content, a_w, food composition, competing microflora, antimicrobial constituents naturally present, growth temperature, atmospheric gases) are certainly the most important (BEAUFORT *et al*., 2008).
During the production process of fermented meat, *L. monocytogenes* can survive due to its ability to tolerate low pH conditions and high salt concentrations (FARBER and PETERKIN, 1991). Its survival is also linked to the absence of specific procedures in the production process. One of the very important factors that influences the growth/survival of *L. monocytogenes* is the a_w. Its variation inhibits part of the aerobic flora and a selection of the lactic flora (LAB) causing a decrease in pH during production. Thus, careful monitoring of the a_w parameter, together with the proper choice of the ripening time, are essential for the microbial safety and stability of fermented sausages which, if marketed with a low maturation level, may be microbiologically unsafe. The aim of the present study was to assess the presence of *L. monocytogenes* in 84 naturally contaminated samples of "Salsiccia Sarda" over a seven month period. Moreover the growth of *Listeria monocytogenes* in 180 experimentally spiked samples of "Salsiccia sarda" was evaluated using a full factorial experimental design. This design evaluated the impact of storage times and temperatures, level of ripening, packaging conditions and type of sausage.

2. MATERIALS AND METHODS

During the period between December 2014 and June 2015, a total of 84 "Salsiccia Sarda" samples, collected from local manufacturing plants in Sardinia, were examined. All of the samples were transported under a controlled temperature (4°C) and were subjected to analysis at the Laboratory of Food Hygiene at the University of Cagliari which operates in conformity with European standard UNI CEI EN ISO/IEC 17025:2005.

2.1. Microbiological analyses

The presence/absence of *L. monocytogenes* was investigated using the international standard method UNI EN ISO 11290-1:2005. Twenty-five grams of "Salsiccia Sarda" samples were suspended in 225 mL of Half Fraser Broth (Microbiol Diagnostici, Uta, Cagliari, Italy) incubated at 30°C ± 1 °C for 24h ± 2 h (primary enrichment). Afterwards, 0.1 mL of the primary enrichment was transferred into a 10 mL tube containing Fraser broth (Microbiol Diagnostici, Uta, Cagliari, Italy) and incubated at 37°C ± 1 °C for 48h ± 2 h (secondary enrichment). After incubation, primary and secondary enrichment broths were streaked onto Agar Listeria Ottaviani Agosti (ALOA, Microbiol Diagnostici, Cagliari, Italy) and Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) Agar (Microbiol Diagnostici, Cagliari, Italy) plates and incubated at 37°C, respectively. From the positive sample plates, up to 5 presumptive colonies were subcultured on Tryptone Soy Yeast Extract Agar (TSYEA, Microbiol Diagnostici, Cagliari, Italy) and incubated at 37°C for 24 h. Species confirmation was obtained with the following tests: Gram staining, catalase and oxydase test (Microbiol Diagnostici, Cagliari, Italy), haemolytic activity, CAMP tests on sheep blood agar (Microbiol Diagnostici, Cagliari, Italy) and the biochemical test API Listeria® (BioMérieux, Marcy-l´Etoile, France). In all biochemical reactions the reference strain *L. monocytogenes* ATCC 35152 was used as positive.

2.2. Molecular investigation

Detection of *L. monocytogenes* was performed according to the previously published PCR protocols described by CORONEO *et al*. (2016).
The DNA extraction was performed using the DNeasy Merikon food kit (Qiagen, Hilden, Germany). Following the manual indications, twenty-five gram samples of "Salsiccia sarda" were suspended in 225 mL of Half Fraser Broth (Microbiol Diagnostici, Cagliari, Italy), and incubated at 30°C ±1°C for 24h ± 2 h. After the pre-enrichment step, 1mL of each sample was taken and centrifugated for three minutes at 11,000 x g. The *L. monocytogenes* DNA was detected using the Merikon *L. monocytogenes* kit (Qiagen, Hilden, Germany). *L. monocytogenes* ATCC 35152 was used as PCR-positive control in all amplifications and molecular grade water as negative control. The reaction was carried out with the StratageneTM Mx3005PqPCR (Stratagene, La Jolla, CA, USA) as it follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60 °C for 23 s and extension at 72°C for 10 s.

2.3. Challenge tests

The present study used 180 "Salsiccia Sarda" samples which were produced in Sardinia. The samples were characterized by two different ripening times (i.e., 12 days and 20 days) because these are the levels of ripening most commonly used to meet the market demands. The 180 samples consisted of 90 "Salsiccia Sarda" samples of pure pork (n.45 at 12 days and n.45 at 20 days of ripening) and 90 myrtle flavored "Salsiccia Sarda" samples (n.45 at 12 days and n.45 at 20 days of ripening). The ingredients of each product are shown in Table 1.

Table 1. Ingedients of "Salsiccia Sarda"samples.

Ingredients	"Salsiccia sarda" (%)	"Salsiccia sarda" myrtle flavored (%)
Meat and fat		
Minced lean pork	87.0	87.0
Pork back fat	8.0	8.0
Additive		
Salt	3	3
Dextrose and sucrose	0.736	0.736
Potassio nitrate (E252)	0.024	0.024
Sodium ascorbate (E301)	0.040	0.040
garlic	0.15	0.15
Ground pepper	0.25	0.25
Other spice	0.8	0.8
Myrtle flavor		1
Starter	p	p

p: presence.

In this study the 45 samples tested at each ripening time belonged to three different batches (i.e., 15 samples/batch). "Salsiccia Sarda" samples were artificially contaminated with *L. monocytogenes*. The samples not inoculated were defined as Blank Samples (B_s) and

used to evaluate the natural contamination of "Salsiccia Sarda" with *L. monocytogenes*. The testing points were: T_0 which was the time of inoculation, and T_1, T_2, T_3, T_4, T_5, which were respectively the examination points carried out every 45 days for a total of seven and a half months after inoculation. This storage time has been adopted in order to achieve an extreme condition for the purposes of the research.

2.4. Inoculation of "Salsiccia Sarda", packaging and storage conditions

The challenge test was carried out according to the Technical Guidance Document prepared by EU Community Reference Laboratory (CRL) for *L. monocytogenes* (Beaufort *et al*., 2008). Three strains of *L. monocytogenes* were used in the study. The inoculum was composed by: *L. monocytogenes* reference strain ATCC 35152 obtained from the American Type Culture Collection and two were wild type strains (serovar 1/2a and 1/2c) previously recovered from the "Salsiccia Sarda" samples. The preparation of inoculum has been previously described (CORONEO *et al*., 2016). The level of contamination was approximately 10-100 CFU/g, which was obtained contaminating 10g of Salsiccia slices with 100 μL of inoculum at a concentration of 10^3 CFU/mL. Colony counts were confirmed by Plate Count Agar (PCA, Microbiol, Ca, It).
The inoculated "Salsiccia Sarda" samples (pure pork and myrtle flavored) were packaged under air (n=180) or Modified Atmosphere (MAP) (i.e., 30% CO_2 and 70% N_2) (n=180) and then stored at three different temperatures, 4°C, 8°C and 25°C. The challenge tests were carried out in independent trials for each batch (A, B and C) performed one week apart. A full factorial design of the variables (i) temperature, (ii) time of analysis, (iii) ripening time, (iv) type of packaging and (v) type of salsiccia was accomplished. The related experimental design is reported in Table 2, leading to 144 different combinations of the variables with three replicates.

Table 2. Experimental design for challenge studies.

Variables						
Temperature	4°C		8°C		25°C	
Time of analysis	T_0	T_1	T_2	T_3	T_4	T_5
Level of ripening	12 d				20 d	
Type of packaging	Normal		Modified Atmosphere Packaging (MAP)			
Type of sausage	Normal		Myrtle flavored			

The detection and enumeration of *L. monocytogenes* was conducted according to International Standard methods UNI EN ISO 11290-1:2005 and UNI EN ISO 11290-2:2005. The enumeration of *L. monocytogenes* was performed on an aliquot of the sample homogenized 1/10 with base Fraser Broth (Microbiol Diagnostici, Cagliari, Italy) and incubated at 20°C ± 2°C for 1 h ± 5 min. A volume of 1mL from each suspension was streaked onto three ALOA plates and incubated at 37°C for 24 and 48 hours. Presumptive colonies of *L. monocytogenes* were counted. The final results were expressed as Log_{10}CFU/g.

2.5. Intrinsic properties

For all the samples of "Salsiccia Sarda" intrinsic properties, pH and a_w, were determined. The measurement of pH and a_w was carried out using pH meter Eutech Instruments pH 510 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and AquaLab4TE (Decagon,

Pullman, WA, USA), respectively. The a_w measurement was performed at different points of the product according to the diagram in Fig. 1 and to International Standard methods UNI 11302: 2009.

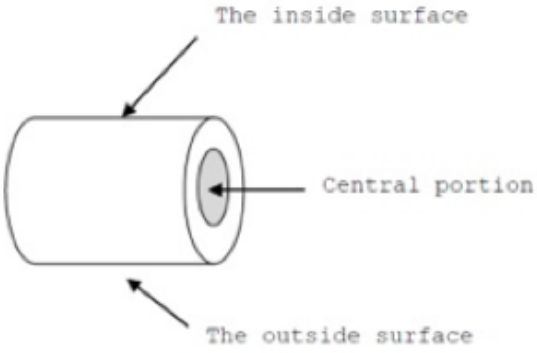

Figure 1. Operation chart used for the a_w measurement in Salsiccia Sarda samples.

2.6. Growth potential

The growth potential (δ) of *L. monocytogenes* was determined by the difference between the counts at the end (T_5) ($\log_{10}$CFU/g) and at the beginning (Time "0") ($\log_{10}$CFU/g) of shelf-life. The RTE product was considered as supporting growth of the *L. monocytogenes* when δ was higher than 0.5 $\log_{10}$.The pathogen was considered not able to grow in a RTE product when δ values were negative or lower than 0.5 $\log_{10}$ (BEAUFORT *et al*., 2008).

2.7. Statistical analysis

All tests of the assessment of *L. monocytogenes* growth were run in triplicate and averaged. Means($\bar{x}$) and standard deviations (s) were computed for each experimental condition. The confidence interval is calculated as

$$\mu = \bar{x} \pm t_{0.05,n} SE(\bar{x}) = \bar{x} \pm t_{0.05,n} \sqrt{s^2/n}$$

where n= 3 is the number of replicates. Analyses were performed using Microsoft Excel XP 2010 and Matlab® 2015 equipped with the toolbox Statistics. Correlation amongst the variables has BEEN estimated by resorting to Spearman's rank correlation coefficient (GIBBONS and Wolfe, 2003). This statistic seems to be the most reasonable choice for our data since it is a non-parametric statistic that reveals to be more robust when dealing with non-linear relationships.
A multiway analysis of variance (*N*-way ANOVA) test is accomplished for testing the effects of the factors: i) storage temperature, which assumes three different levels; ii) measuring time, which is available at 5 different levels; iii) time of ripening (data available at two different levels); iv) absence/presence of myrtle (2 levels); v) type of packaging (2 levels); vi) measured pH and vii) measured a_w. With regards to the latter two variables, the measured values of pH and a_w are divided into classes of width 0.1 and 0.05 respectively.

This allows a finite number of levels of such factors (11 for the pH and 27 for the a_w) to be considered for the statistical test.

3. RESULTS AND DISCUSSIONS

3.1. Conventional microbiological analysis

In the present work, natural contamination of the salsiccia samples analyzed was never detected along the seven month observation period. Our result is consistent with some literature in which several Mediterranean-style dried fermented sausages could be included in the category of RTE products that do not favor *L. monocytogenes* growth. There is, however, a great variability according to local traditions that influence fermentation and ripening (HOSPITAL *et al*., 2012; MELONI, 2015). In fact, as reported by PETRUZZELLI *et al*. (2010), a high frequency of isolation of *L. monocytogenes* was found in Ciauscolo salami manufactured in the Marche region. This type of salami is particularly exposed to the risk of contamination because of its peculiarities (short maturation period, high a_w, rare use of additives and starter cultures).
The microbiological results were confirmed by molecular analysis.

3.2. Challenge test

The challenge tests conducted in this study on the sausage samples subjected to different storage and packaging conditions show that *L. monocytogenes* was unable to survive and grow until the end of shelf life in both situations regarding packaging and refrigeration (with a significance level $P<0.05$). However, we also observed an increase of the pathogen concentration in a specific time of the shelf life in samples with a particular level of ripening (12 days), stored in certain packaging conditions (under air) and with a_w values around 0.92.
In fact, the *L. monocytogenes* concentration in "Salsiccia sarda" samples at 12 days of ripening and stored at 4°C increased from 1.66 $\log_{10}$CFU/g at T_0 to 3.9 $\log_{10}$CFU/g at T_2 when a_w values were equal to 0,922± 0.001 (Table 3).

Table 3. Results of spiked samples packaged under air and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.66	5,2±0.22	0.924±0.007	1.6	5.4±0.40	0.924±0.017	1.4	5.3±0.12	0.921±0.005
	T_1	1.48	5.3±0.12	0.931±0.005	1.6	5.7±0.50	0.931±0.000	2.17	5.7±0.07	0.925±0.015
	T_2	3.9	5,5±0.07	0,922±0.001	3.3	5.8±0.12	0.925±0.005	3.3	5.8±0.05	0.923±0.002
	T_3	3.07	5.7±0.42	0.930±0.001	1	5.8.0±0.25	0.909±0.000	<1	5.7±0.30	0.905±0.001
	T_4	<1	5.8±0.32	0.917±0.012	1	5.7±0.40	0.903±0.015	<1	5.9±0.27	0.890±0.015
	T_5	<1	5.8±0.27	0.912±0.010	<1	5.7±0.32	0.901±0.010	<1	5.8±0.25	0.881±0.004
	δ	-1.35			-1.6			-0.70		

20 days	T_0	1.48	5.7±0.03	0.881±0.012	1.48	5.6±0.35	0.874±0.010	1.3	5.77±0.30	0.868±0.005
	T_1	1.84	5.7±0.04	0.903±0.224	<1	5.8±0.01	0.872±0,248	<1	5.7±0.01	0.901±0.298
	T_2	<1	5.9±0.27	0.908±0.010	<1	5.9±0.02	0.891±0.298	<1	5.8±0.01	0.901±0.248
	T_3	<1	5.8±0,30	0.884±0.005	<1	5.7±0.27	0.881±0.010	<1	5.9±0.07	0.893±0.005
	T_4	<1	5.6±0.22	0.863±0.124	<1	5.8±0.22	0.865±0.020	<1	5.8±0.010	0.872±0.010
	T_5	<1	5.8±0.27	0.852±0.012	<1	5.8±0.02	0.862±0.007	<1	5.9±0.27	0.861±0.007
	δ	-1.17			-1.00			-0.99		

L.m: *L.monocytogenes* concentration expressed as median $\log_{10}$CFU/g **; δ**: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications.

The *L. monocytogenes* concentration decreased in later observations (T_4 and T_5) due to a decrease in a_w and an increase in pH values (Table 3). The same results were observed in samples at 12 days of ripening stored at 8°C and 25°C. In the salsiccia samples with 20 days of ripening, *L. monocytogenes was* able to survive only in samples stored at 4°C at time T_1, whereas at higher temperatures (8°C and 25°C) it was not detected (Table 3).
In both MAP packed salsiccia samples, at 12 and 20 days of ripening, stored at 4°C, 8°C and 25°C, significant *L. monocytogenes* growth was not observed (Tables 4).

Table 4. Results of spiked samples MAP packaged and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.7	5.3±0.45	0.926±0.022	1.00	5.0±0.42	0.914±0.005	1.4	5.2±0.30	0.912±0.002
	T_1	<1	5.24±0.02	0.924±0.005	<1	5.3±0,05	0.906±0.001	<1	5.0±0.32	0.907±0.002
	T_2	<1	5.53±0.12	0.916±0.010	<1	5.6±0.12	0.910±0.001	<1	5.4±0.27	0.901±0.001
	T_3	<1	5.5±0.15	0.910±0.002	<1	5.8±0.17	0.887±0.005	<1	5.5±0.05	0.893±0.050
	T_4	<1	5.6±0.22	0.909±0.124	<1	5.7±0.22	0.899±0.015	<1	5.6±0.30	0.882±0.012
	T_5	<1	5.4±0.15	0.892±0.007	<1	5.6±0.10	0.880±0.007	<1	5.5±0.27	0.871±0.007
	δ	-1.7			-0.7			-1.1		
20 days	T_0	1.48	5.6±0.32	0.854±0.010	1.48	5.6±0.22	0.882±0.020	1.48	5.7±0.22	0.891±0.020
	T_1	<1	5.7±0.30	0.852±0.010	1	5.7±0.30	0.853±0.010	1	5.7±0.30	0.870±0.010
	T_2	<1	5.6±0.25	0.863±0.348	<1	5.5±0.35	0.864±0.007	<1	5.5±0.32	0.885±0.010
	T_3	<1	5.7±0.22	0.872±0.010	<1	5.8±0.30	0.852±0.010	<1	5.8±0.27	0.874±0.007
	T_4	<1	5.8±0.2	0.864±0.007	<1	5.9±0.17	0.851±0.005	<1	5.7±0.22	0.882±0.010
	T_5	<1	5.8±0.00	0.853±0.149	<1	5.8±0.17	0.850±0.0149	<1	5.8±0.10	0.861±0.224
	δ	-1.25			-0.8			-1.13		

L.m: *L.monocytogenes* concentration expressed as median $\log_{10}$CFU/g ; δ: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications.

In myrtle flavored "Salsiccia sarda" samples at 12 days of ripening and stored at 4°C, the *L. monocytogenes* concentration increased from 1.6 $\log_{10}$CFU/g at T_0 to 2.07 $\log_{10}$CFU/g at T_5. These results show that bacterial survival was greater as evidenced by $\delta = 0.47$ $\log_{10}$CFU/g (Table 5).

Table 5. Results of spiked samples myrtle flavored packaged under air and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.6	5.4±0.12	0.923±0.005	1.6	5.5±0.45	0.927±0.017	1.74	5.4±0.27	0.916±0.007
	T_1	1.48	5.3±0.47	0.920±0.020	2.5	5.8±0.15	0.921±0.012	<1	5.6±0.50	0.908±0.124
	T_2	3.17	5.7±0.12	0.931±0.005	3.9	5.9±0.07	0.929±0.005	<1	5.6±0.05	0.907±0.050
	T_3	2.25	5.6±0.15	0.930±0.001	1	5.7±0.17	0.908±0.001	<1	5.8±0.17	0.896±0.007
	T_4	2.3	5.5± 0.30	0.922±0.015	1	5.8±0.15	0.905±0.017	<1	5.7± 0.15	0.891±0.020
	T_5	2.07	5.6±0.22	0.917±0.010	<1	5.7±0.02	0.891±0.010	<1	5.8±0.12	0.883±0.124
	δ	0.47			-1.1			-1.26		
20 days	T_0	1.48	5.4±0.02	0.903±0.017	1.48	5.4±0.30	0.920±0.348	1.3	5.3±0.22	0.920±0.015
	T_1	<1	5.7±0.02	0.901±0.007	<1	5.4±0.22	0.901±0.273	<1	5.9±0.27	0.902±0.010
	T_2	<1	5.6±0.22	0.891±0.422	<1	5.2±0.25	0.885±0.248	<1	5.7±0.42	0.894±0.005
	T_3	<1	5.8±0.27	0.882±0.012	<1	5.4±0.22	0.863±0.075	<1	5.8±0.27	0.882±0.012
	T_4	<1	5.7±0.30	0.844±0.005	<1	5.5±0.30	0.857±0.012	<1	5.7±0.22	0.821±0.015
	T_5	<1	5.6±0.22	0.825±0.012	<1	5.7±0.20	0.842±0.007	<1	5.8±0.12	0.790±0.007
	δ	-1.00			-0.70			-1.05		

L.m: *L.monocytogenes* concentration expressed as median $\log_{10}$CFU/g ; δ: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications.

In the spiked samples myrtle flavored MAP packaged and tested at 12 days of ripening, the *L. monocytogenes* concentration increased from 1.78 $\log_{10}$CFU/g at T_0 to 3.58 $\log_{10}$CFU/g at T_1, then decreased to 1.78 $\log_{10}$CFU/g at T_2. In all other types of samples *L. monocytogenes* survival and growth was not observed (Tables 6).

3.3. Statistical analysis

The previous considerations can be ascertained in a more rigorous manner by resorting to a multiway ANOVA test. This test has been carried out on the data in order to assess which factors significantly affect the *L. monocytogenes* growth. The results are reported in Table 7 where the statistically significant factors are highlighted with an asterisk. It appears that the growth is strongly influenced by the factors (i) time of analysis (*P*-value = 1.8555e-7) and (ii) a_w (*P*-value = 4.9e-3). It should be noted that the factor "Level of ripening" shows a p-value = 0.0622.

Table 6. Results of spiked myrtle flavored samples MAP packaged and tested at 12 and 20 days of ripening.

	Storage temperature									
	4°C				8°C			25°C		
	Time	L.m	pH	a_w	L.m	pH	a_w	L.m	pH	a_w
12 days	T_0	1.78	5.4±0.35	0.927±0.012	1.4	5.2±0.07	0.916±0.002	1.84	5.4±0.01	0.918±0.004
	T_1	3.58	5.8±0.02	0.929±0.015	1	5.5±0.22	0.897±0.002	<1	5.8±0.17	0.909±0.006
	T_2	1.95	5.8±0.12	0.926±0.001	<1	5.6±0.01	0.889±0.001	<1	5.7±0.15	0.900±0.0002
	T_3	<1	5.7±0.17	0.914±0.001	<1	5.7±0.17	0.862±0.015	<1	5.8±0.5	0.770±0.05
	T_4	<1	5.8±0.15	0.906±0.020	<1	5.6±0.20	0.875±0.010	<1	5.6±0.25	0.821±0.006
	T_5	<1	5.7±0.10	0.895±0.005	<1	5.8±0.22	0.868±0.007	<1	5.7±0.22	0.810±0.03
	δ	-1.17			-1.30			-1.26		
20 days	T_0	1.3	5.2±0.35	0.921±0.002	1.48	5.3±0.22	0.916±0.012	1.3	5.3±0.22	0.918±0.005
	T_1	<1	5.3±0.27	0.901±0.010	<1	5.4±0.25	0.915±0.000	<1	5.8±0.32	0.902±0.012
	T_2	<1	5.8±0.25	0.896±0.015	<1	5.6±0.20	0.880±0.002	<1	5.7±0.27	0.894±0.012
	T_3	<1	5.7±0.35	0.886±0.017	<1	5.8±0.22	0.878±0.010	<1	5.6±0.32	0.883±0.015
	T_4	<1	5.9±0.30	0.884±0.012	<1	5.7±0.25	0.874±0.007	<1	5.7±0.27	0.876±0.012
	T_5	<1	5.7±0.25	0.867±0.012	<1	5.6±0.17	0.863±0.124	<1	5.8 ±0.01	0.862±0.075
	δ	-0.5			-1.30			-0.7		

L.m: *L.monocytogenes* concentration expressed as median $\log_{10}$CFU/g ; δ: growth potential calculated as the difference between the median L.m (log10CFU/g) at T_5 and the median (log10CFU/g) at T_0; T_0: the time of inoculation; T_1, T_2, T_3, T_4, T_5: the examination points carried out every 45 days for a total of seven and a half months after inoculation. Data are shown as mean $(\bar{x}) \pm t_{0.082} SE(\bar{x})$ of three different replications

Table 7. Effect of the variables on the *L. monocytogenes* concentration in "Salsiccia Sarda".

Source	Sum of Squares	Degree of freedom	Mean Square	F-test	Prob>F[a]
Temperature	1.2143e+06	2	6.0717e+05	0.7063	0.4943
Time of analysis	3.6114e+07	5	7.2228e+06	8.4016	1.8555e-7*
pH	6.9740e+06	10	6.9740e+05	0.8112	0.6180
a_w	4.3245e+07	26	1.6633e+06	1.9347	0.0049*
Type of sausage	3.6356e+03	1	3.6356e+03	0.0042	0.9482
Level of Ripening	3.0113e+06	1	3.0113e+06	3.5026	0.0622
Type of packaging	1.0974e+06	1	1.0974e+06	1.2765	0.2594
Error	2.6049e+08	303	8.5969e+05		
Total	3.8097e+08	349			

[a] To determine a significant influence of these variables on the *L. monocytogenes* growth we use N-way ANOVA tests with a significance level $P<0.05$.

Thus, although it cannot be considered as relevant for a significance level of the test a=0.05, we cannot exclude, at least for the data here investigated that it might have some impact on the process. On the other hand, the other variables (i.e. packaging, pH, temperature and absence/presence of myrtle) do not seem to significantly affect the *L. monocytogenes* concentration.

Fig. 2 reports the *L. monocytogenes* concentration with respect to the a_w for the 5 different levels of time. It appears that the microbial concentration reveals a sudden increase for a_w values close to 0.92 at each level of time. The region close to the critical value is zoomed in on in the insets. Incidentally, it was found that the microbial growth may take place even at values slightly less than 0.92 (see insets on Fig. 2C and 2E).

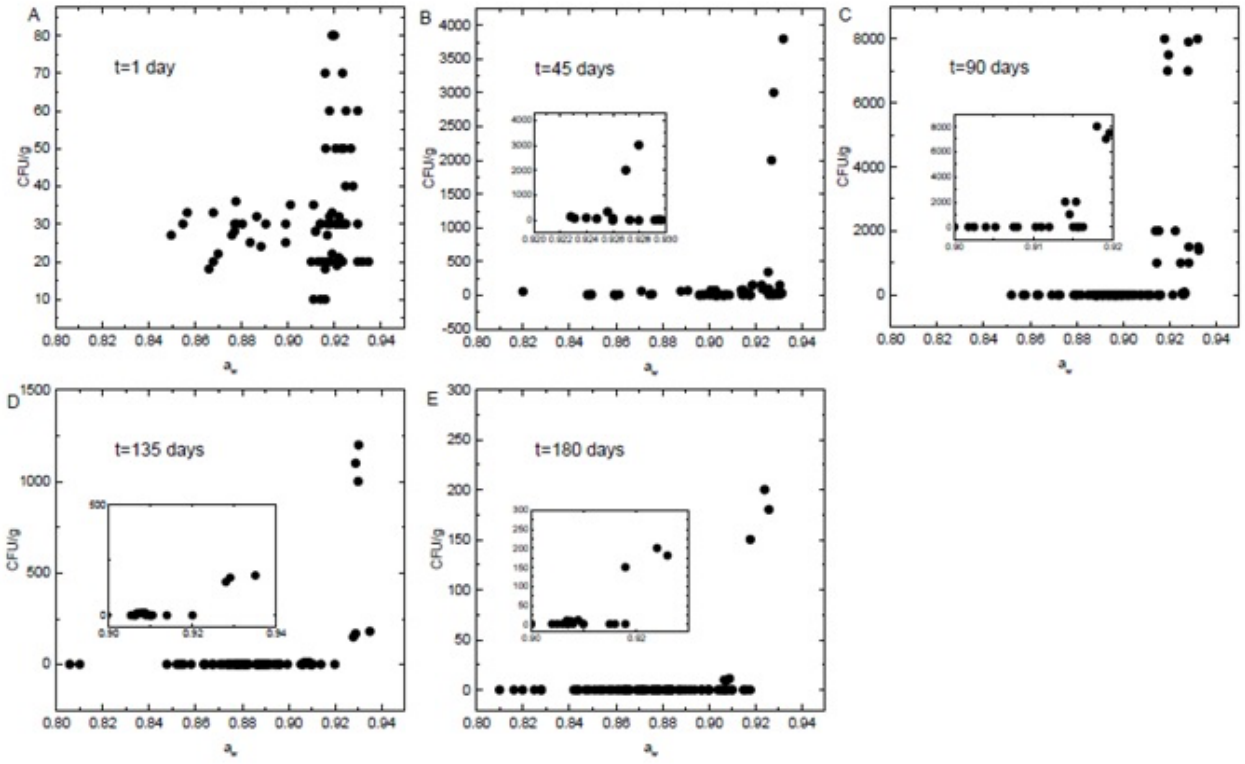

Figure 2. *L. monocytogenes* concentration with respect to the a_w for the different levels of time.

The Spearman correlation coefficient between *L. monocytogenes* concentration and a_w is computed at each different time and the corresponding point estimations are reported in Table 8 together with their P-values. For comparison, the corresponding Pearson correlation coefficients and the related P-values are reported in Table 8.

Table 8. The Spearman correlation coefficient values between *L. monocytogenes* concentration and a_w at each different time of analysis.

	Spearman coefficient		Pearson coefficient	
time	r	p-value	r	p-value
1	0.1683	0.1575	0.1844	0.1211
45	0.3716	0.0013	0.2322	0.0497
90	0.6977	9.7e-12	0.4559	5.71e-5
135	0.5919	5.43e-8	0.3434	0.0034
180	0.4272	1.82e-4	0.3447	0.0030

Calculation of the correlation coefficient at time T_6 is meaningless since all the measurements of concentration are zero. It was found that the correlation coefficient is always significantly greater than zero except for the initial time. This further confirms that the a_w is the main factor affecting the growth process. As a final remark, *L. monocytogenes* was never detected in Blank samples (BS) during the challenge test.
These results are consistent with previous studies that have shown a *L. monocytogenes* growth in samples of fermented sausages contaminated with about 5 $\log_{10}$CFU/g (SPERANDII *et al*., 2015). Some manufacturers tend to reduce the ripening period to respond to market needs (HOSPITAL *et al*., 2012; MELONI, 2015). These products with early ripening, as highlighted by our study, may have a_w levels close to 0.92-0.94. This can increase risks associated with the *L. monocytogenes* growth during shelf-life, even in the presence of competitive microflora. Our study showed that, in the presence of an improper ripening time, initial low levels of contamination in the product could however lead to a concentration that is potentially harmful to human health during the first 45 days of storage.

3.4. Intrinsic properties

Among the intrinsic properties, our results showed that the evolution of a_w values is the same of that of other typical Italian fermented meat products (GRECO *et al*., 2005; PETRUZZELLI *et al*. 2010; MELONI *et al*., 2012; MATARAGAS *et al*. 2015a, b). The a_w decreased constantly for both situations regarding packaging and storage temperatures. Its initial values at T_0 were 0.924±0.003 for salsiccia samples at 12 days of ripening when packaged under air and stored at 4°C and at 8°C and 0.921±0.002 for those stored at 25°C. These values decrease to the time T_5 with values equal to 0.912±0.010, 0.901±0.010, 0.881±0.004 for samples stored at 4°C, 8°C and 25°C respectively (Table 3). In general, the sausages with 12 days of ripening showed the average a_w levels typical of products able to support the *L. monocytogenes* growth in all storage conditions (at 4°C, 8°C and 25°C) (Table 3,5)
In salsiccia samples with 20 days of ripening the a_w values are uniform in the products in both situations regarding packaging and storage temperatures. The initial values at T_0 show a decrease of about 0.03-0.08 units at T_5 time ((Table 3, 4, 5, 6). In accordance with other authors (MELONI *et al*., 2014) our results confirm, with reasonable certainty, the safety of products with a longer ripening time.
As far as pH is concerned, our results have showed that the analyzed samples have similar values to those found in most Mediterranean-style fermented sausages (MELONI, 2015) and were close to 5.4-5.8 for both situations regarding packaging and storage temperatures. As reported in other studies (VERMEULEN *et al*., 2007; VERMEULEN *et al*., 2009; MATARAGAS *et al*., 2015b;) the pH level showed a slight increase (0.3-0.5 units), during the experiment, in all sausages analyzed and for both situations regarding packaging and storage conditions. (Tables 3, 4, 5, 6). In general, the a_w and pH values were always within the limits of growth for *L.monocytogenes*.

4. CONCLUSIONS

Previous studies have been carried out to evaluate the effect on *L. monocytogenes* growth and survival during the production of fermented sausages to evaluate the safety of the process (MATARAGAS *et al*., 2015b). The realization, in this study, of an experimental protocol for a challenge test, specific for this traditional product, "Salsiccia Sarda", has

allowed us to obtain usable results for the definition of adequate product security during the shelf-life. In our study, by resorting to statistical tools, the a_w value measured in these fermented sausages was demonstrated as a critical control point.
Through the challenge test, in conjunction with the a_w measurements at each storage time, it has been possible to show that *L. monocytogenes* is able to replicate in a sausage with 12 days of ripening, only when the values of a_w are not less than 0.92. The use of the correct drying process is necessary to lower the a_w which can minimize the potential for *L. monocytogenes* growth. An intermediate time of ripening from 12 to 20 days could be assumed in the production specifications provided that the critical limit is strictly verified for the measurement of water activity (<0.92).

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