

Survival of *Listeria monocytogenes* in Uncooked Italian Dry Sausage (Salami)

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ABSTRACT

This study was undertaken to supplement existing information on the survival of *Listeria monocytogenes* in Italian salami. The fact that Italian salami is frequently consumed by a large number of people poses some serious health implications. Some raw materials have been found to be microbiologically contaminated, for their production occurs without any thermic treatment, and these are in circulation throughout Italy all year round. We selected the product for its microbiological, technological, and commercial characteristics. We analyzed 1,020 samples taken during the autumn and winter 2002 and spring and summer 2003 periods and immediately before selling. The samples were collected from 17 plants with an annual production of between 1 and 2,000 metric tons and with a distribution of products in over 80% of Italy in geographic terms. To detect and enumerate *L. monocytogenes*, we followed International Organization for Standardization (ISO) 11290 part 1 and 2: 1996 (modified using chromogenic medium Agar Listeria according to Ottarviani and Agosti [ALOA]). *L. monocytogenes* was found in 22.7% of samples, but the contamination level was less than 10 CFU/g. Contamination prevalence ranged from 1.6 to 58.3% and was lower than 10% in 5 of the 17 plants checked. The most frequently isolated serotypes were 1/2c, 1/2a, 1/2b, and 4b. Additional studies are necessary to establish if the exposure to a small number of *L. monocytogenes* cells through the consumption of salami represents a significant health risk and, in light of the future introduction of the SANCO/4198/2001 revision 21 "Commission Regulation on Microbiological Criteria for Foodstuffs," is a necessary investigation.

Listeria monocytogenes is a major foodborne bacterial pathogen that causes invasive illness in certain segments of the population, including neonates, pregnant women, immunocompromised people, and the elderly. In healthy people, it may cause a gastrointestinal illness with fever without progression to invasive illness (9, 11, 25). The presence of *L. monocytogenes* in various food products has been extensively investigated. *L. monocytogenes* is resistant to many food-preservation methods (22), and it can colonize food production plants and survive under extreme conditions (18). With particular reference to ready-to-eat (RTE) foods, epidemic and sporadic cases of invasive (17, 20, 21) and noninvasive listeriosis (28) have also been associated with the consumption of meat products. Regarding RTE cured dried meats, a number of factors, including low water activity (a_w), sodium chloride, sodium nitrite, and low pH due to lactic-acid production by starter culture organisms or antagonistic effects or both, ensure the preservation from pathogenic microorganisms. These combined factors control *L. monocytogenes* growth during fermentation and drying, but the organism may survive in the finished product (13, 30). The contamination level of these products is generally low (<100 CFU/g), and to date, there is little or no epidemiological evidence of the involvement of cured dried meats in cases of listeriosis (23).

Nevertheless, the criteria for tolerable levels in RTE have been established because of the public health signifi-

cance of *L. monocytogenes*. For example, in the United States and Italy, the food safety legislation requires 25 g of RTE product to have no bacterium (zero tolerance policy), whereas several other countries have concluded that zero tolerance for certain RTE foods is unrealistic and that it has no significant impact on public health. At present, international criteria for *L. monocytogenes* in foods are being considered by a drafting group within the Codex Committee on Food Hygiene (8); these considerations include a specific reference to risk assessment.

To date, only a few published papers have dealt with parts of a full Codex microbiological risk assessment, and the lack of sufficient data is often a major problem. For *L. monocytogenes*, the most important knowledge lacking is in the area of dose-response models, which are of paramount importance in defining risk-based microbiological criteria. In fact, only a few of the above-mentioned investigations provide complete, valid, and comparable data (15) on *L. monocytogenes* contamination level in the examined foods.

Our objective is to provide risk assessors and policy makers with more accurate estimates of the prevalence and contamination level of this bacterium in high-volume RTE foods, such as salami, that are consumed by a large portion of the population, including at-risk groups, in order to determine consumers' exposure to this risk (32).

MATERIALS AND METHODS

Commercial definition and formulation. The Italian salami studied (known as cacciatore) was a type made with lean pork

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TABLE 1. Prevalence values of *L. monocytogenes* and *Listeria* spp. for each plant

Plant no.	Plant size ^a	Casing type	Powdered milk	a _w	No. (%) of samples positive for <i>L. monocytogenes</i> ^b	No. (%) of samples positive for <i>Listeria</i> spp. ^b
1	Large	Natural	Yes	0.87	1 (1.6)	5 (8.3)
2	Large	Artificial	No	0.86	4 (6.6)	10 (16.6)
3	Medium	Natural	Yes	0.92	18 (30.0)	29 (48.3)
4	Medium	Artificial	Yes	0.91	9 (15.0)	14 (23.3)
5	Medium	Natural	Yes	0.92	4 (6.6)	42 (70.0)
6	Large	Artificial	No	0.90	5 (8.3)	32 (53.3)
7	Small	Natural	Yes	0.94	35 (58.3)	25 (41.6)
8	Large	Artificial	Yes	0.85	5 (8.3)	29 (48.3)
9	Large	Artificial	Yes	0.89	8 (13.3)	48 (80.0)
10	Large	Natural	Yes	0.91	10 (16.6)	49 (81.6)
11	Medium	Artificial	No	0.94	33 (55.0)	15 (25.0)
12	Medium	Natural	Yes	0.93	18 (30.0)	18 (30.0)
13	Small	Natural	No	0.91	20 (33.3)	6 (10.0)
14	Medium	Natural	Yes	0.91	19 (31.6)	6 (10.0)
15	Small	Natural	No	0.86	11 (18.3)	37 (61.6)
16	Small	Artificial	Yes	0.94	28 (46.6)	11 (18.3)
17	Small	Natural	No	0.87	4 (6.6)	54 (90.0)

^a Small, 1 to 100 ton/year; medium, 100 to 200 ton/year; large, >201 ton/year.

^b Percentage calculated on 30 samples.

(either refrigerated or frozen), lard, salt, pepper, and garlic. One or more of the following ingredients could also be added: wine, sugar, powdered skim milk, culture starters (in general, combinations of diverse species: *Pediococcus pentasaceus*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Staphylococcus xylosum*, micrococcus), and ascorbic acid. Nitrate or nitrite or both are used as preservatives at concentrations established by 94/34/EC and 95/2/EC (1, 2). After stuffing the sausage mix into natural or artificial casings at <4°C, the product is subjected to drying for 1 to 6 days. During this period, the water content is reduced by storing the product in an environment with a decreasing relative humidity (from 80 to 40%) and decreasing temperature (from 22 to 20°C to 18 to 16°C). This is followed by the maturation stage, during which the reduction of the water content continues, however, at a slower rate. The product is subjected to a relative humidity that decreases from 90 to 60% and application of lower temperatures that vary from 8 to 10°C or 14 to 16°C for 14 to 21 days. The lactic fermentation is initiated in the first 24 h, and this ensures the decrease in pH. The final product, which weighs 150 to 250 g and has a soft consistency, a pH of 5.1 to 5.3, and an a_w of 0.85 to 0.92. (data provided by the manufacturers).

During distribution, the product can be stored at room temperature (18 to 20°C) for a period ranging from 8 to 16 weeks.

Sample collection. The salami samples were collected directly from the 17 largest producers of cacciatore salami, which supply 80% of Italy in geographic terms. Two batches from each plant were collected: one in autumn and winter 2002 and the other in spring and summer 2003. For each of the periods, 30 samples were collected randomly from each of the 17 producers. This was according to the International Commission on Microbiological Specifications for Foods (4), where $n = 30$ and $c = 0$ (the maximum allowable number of sample units yielding unsatisfactory test results). The national regulation stipulates that *L. monocytogenes* must be absent in five sample units ($n = 5$, $c = 0$) at the warehouse where products are stored ready for shipment.

Products with casing not intact were discarded. To identify the sampling batch, we used the procedure reported in the Commission Regulation on Microbiological Criteria for Foodstuffs

(SANCO/4198/2001 revision 21). A total of 1,020 samples were collected from the 17 plants. The samples were transferred to the laboratory the day after they were collected and were maintained at the producer warehouse temperature, which for both seasons ranged from 20 to 25°C; samples were analyzed on the same day they were sent to the laboratory.

Statistical analysis. Pearson's chi-square test for counts and proportions was used to assess the differences in prevalence between the two batches (14). One-way analysis of variance was performed to test differences in prevalence among the three production plant groups, categorized according to size (small: plants producing ≤100 metric tons/year; medium: 101 to 200 metric tons/year; and large: >200 metric tons/year). We also evaluated certain technological factors (the use of sugar or powdered milk or both, natural or artificial casing, and a_w) and productive factors (metric tons per year, expressed as "the size of the plant") with the aim of evaluating statistically significant associations with the presence of *L. monocytogenes* (Table 1). The relationships between the presence of *L. monocytogenes* and plant size, type of casing, use of powdered milk, and a_w were evaluated by using a logistic regression model (Fig. 1).

The following predictors were used: (i) plant size: three groups, defined according to the annual production in metric tons; (ii) a_w: mean of 10 sample measurements for each plant; (iii) powdered milk: binary variable, presence or absence; and (iv) casing type: binary variable, use of natural or artificial casing.

The procedure was stepwise selection, so we performed an analysis of deviance for the sequential addition of each variable by specifying the chi-square test to test differences between models (12, 24). The steps were modelling with logistic regression, fitting full model, and fitting reduced model, which led to the choice of the final equation that much better reproduced the observed prevalence values of *L. monocytogenes*. *P* values of <0.05 were considered significant. All analyses were performed with R Project for Statistical Computing, R version 2 (General Public License, Boston, 1991, available at: www.r-project.org).

Sample preparation. To minimize the potentially inhomogeneous distribution of *Listeria* spp. in the product, after having

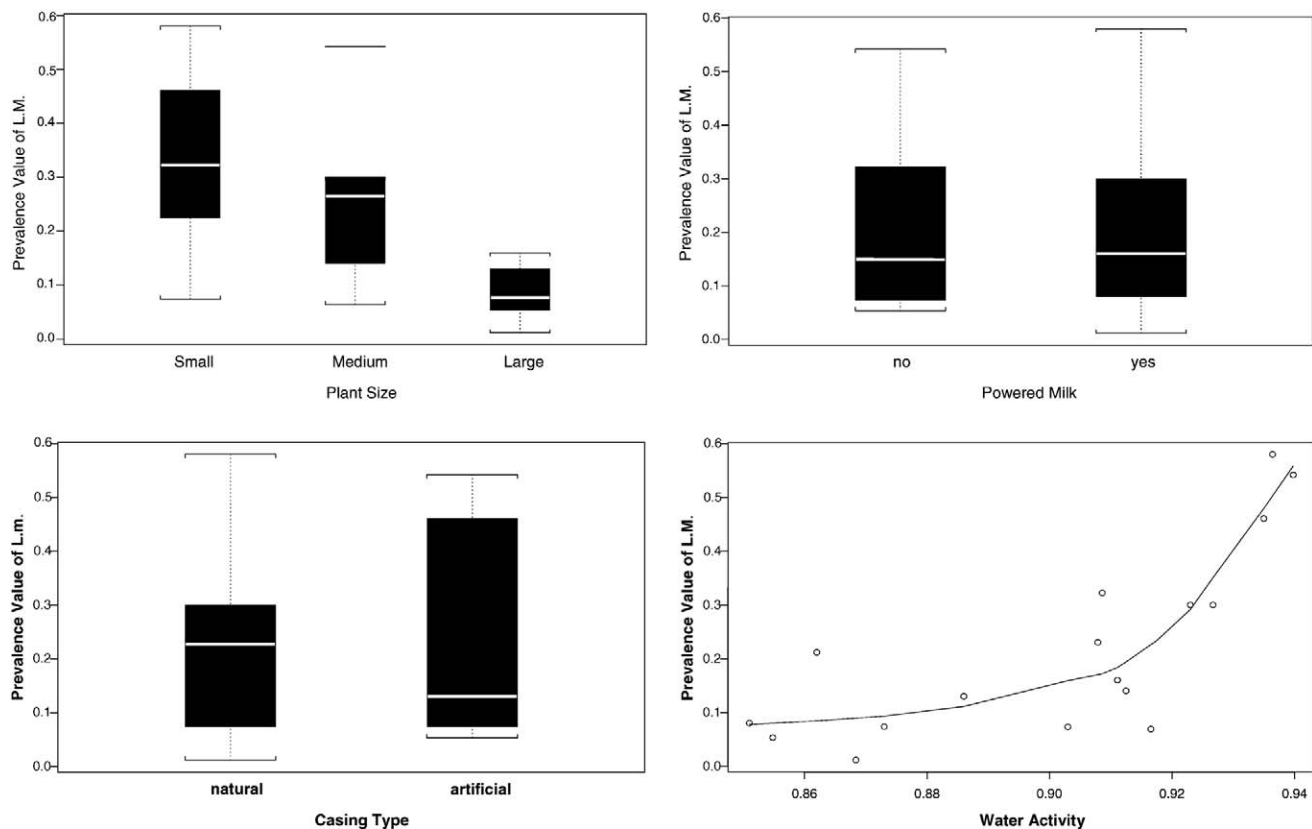


FIGURE 1. Scatter plots and box plots of *L. monocytogenes* against considered explanatory variables.

removed aseptically with a sterile lancet the salami casing, the entire product (150 or 200 g: the weight of the salami varied with each producer), except for a 10-g portion from the center of the salami, which was used for determining a_w , was mixed with an equal volume of 0.8% NaCl solution in sterile Stomacher 400 closure bag and blended for 2 min with a Stomacher 400 circulator (PBI International, Milan, Italy) at 230 rpm.

Detection and enumeration. To detect and enumerate *L. monocytogenes*, we followed the procedures specified by the International Organization for Standardization (ISO) 11290 part 1 and 2: 1996 (3), modified (use of chromogenic medium, Agar Listeria according to Ottarviani and Agosti [ALOA]) (ISO 11290 part 1 and 2, amendment 1, 2004) (7).

The detection method (ISO 11290-1) and the enumeration method (ISO 11290-2) were carried out on the same test sample: 200 ml of primary enrichment medium (Fraser broth, Oxoid, Basingstoke, UK) without selective agents was added to a 50-ml sample of salami homogenate (equivalent to 25 g of salami, plus 25 ml of primary enrichment medium), blended for 2 min with a Stomacher 400 circulator (PBI International), and incubated at 20°C for 1 h. Given the possibility of detecting a low number of *L. monocytogenes* in this product, we chose to distribute 1 ml of the initial suspension over the surface of three dishes (90 mm) of the agar medium (ALOA agar, Biolife, Milan, Italy) so as to obtain a detection limit of 10 CFU/g instead of 100 CFU/ml. The selective agent (half-Fraser supplement, Oxoid) was added to the suspension once the test portion for enumeration was done. For the detection procedure, after having added the selective agent, we performed streaking as per ISO Part 1 on the PALCAM agar. Five characteristic colonies grown on PALCAM or ALOA or both per sample were subjected to a hemolysis test involving incubation on tryptone soya agar plus 5% sheep blood

(Oxoid) at 37°C for 24 h. Only one hemolytic colony was subjected to biochemical tests for species identification with the API Listeria kit (bioMérieux, Marcy l'Etoile, France) for the carbohydrate utilization.

Serotyping. We used a commercial Listeria antisera kit (Seiken, Oxoid, Madrid, Spain) in accordance with the manufacturer's instructions, with the following exception: to detect the H antigen using the test tube method, we passed the microorganism through a semiliquid (0.25% agar) brain heart infusion (Oxoid) and incubated this at 25°C for 48 h prior to inoculation in liquid brain heart infusion. The Seiken kit was checked against ATCC strains of *L. monocytogenes* of known serotypes: ATCC 19111 (serotype 1), ATCC 19112 (serotype 2), ATCC 19113 (serotype 3), ATCC 19114 (serotype 4a), ATCC 19115 (serotype 4b) ATCC 19116 (serotype 4c), ATCC 19117 (serotype 4d), and ATCC 19118 (serotype 4e). Serotyping was performed on all of the strains isolated from the various samples analyzed (16).

a_w assay. For each batch, five samples were randomly extracted; from each of these samples, a 10-g portion was extracted from the center part of the product, and the AW Sprint TH-500 (Novasina, Switzerland) was used to measure a_w at room temperature following ISO/DIS 21807, 2002 procedure (5).

RESULTS

Of the 1,020 salami samples, 232 (22.7%) were positive, constituting a relatively high prevalence, which varied greatly with each producer (range: 1.6 to 58.3%). Only for five of the producers was the prevalence lower than 10% (Table 1). The prevalence of contamination decreased significantly ($P < 0.05$) with the increasing producer plant size

TABLE 2. Mean prevalence values of *L. monocytogenes* for each plant group

Factors	Values		
Production range/year (metric tons)	1–100	100–200	>201
No. of plants	5	6	6
Total production/year (metric tons)	206	609	3,644
Mean prevalence by group (%)	32.6	28.0	9.1
Plant size	Small	Medium	Large

(Tables 1 and 2). For all samples, the contamination level was lower than 10 CFU/g. Of the 13 known *L. monocytogenes* serotypes (Table 3), 9 were isolated (1/2a, 1/2b, 1/2c, 4b, 4c, 4a, 4ab, 4e, and 4d). The most frequently isolated serotypes were 1/2c (53% of positive samples), 1/2a (22%), 1/2b (8%), and 4b (12%). The products of all but one of the producers were contaminated by more than one serotype, and the producers in which the greatest number of serotypes was isolated were those that had the highest contamination level. The serotypes isolated in autumn and winter 2002 were the same as those isolated in spring and summer 2003 (data not shown).

Other species of *Listeria* (*L. innocua* and *L. welshimeri*) were also present in the products of all 17 producers, although only in eight of the producers was the prevalence of these species very high (>50%) (Table 1). The simultaneous presence of *L. monocytogenes* and other *Listeria* species, in particular *L. innocua*, would have resulted in a considerable number of false negatives if we had not chosen to use a culture medium (ALOA) capable of differentiating *L. monocytogenes* from other *Listeria* species. *L. innocua* tends to dominate *L. monocytogenes* during the selective enrichment stages and thus masks the presence of small numbers of colonies of *L. monocytogenes* on the isolation media (26, 27, 33). The mean prevalence of *L. monocytogenes* did not differ significantly (23.5 versus 21.9%, respectively) when the autumn and winter 2002 batch was compared with the spring and summer 2003 batch. The data were subjected to the Pearson's chi-square test, the result of which was not significant ($P = 0.65$) (14). This indicates a constant persistence of *L. monocytogenes* contamination in the two seasonal periods. Therefore, we considered the prevalence as the number of positive samples on the total of samples analyzed (Tables 1 and 2).

Before fitting the model to data, it was useful to investigate graphically the relationship of each considered explanatory variable to the prevalence value of *L. monocytogenes*. Preliminary plots were characterized as follows: plant size indicated strong location shifts with respect to the percentage of *L. monocytogenes*, unlike both casing type and powdered milk, which do not show such a shift in location, whereas a_w shows a strong nonlinear relationship against *L. monocytogenes*. Fitting the model to the data, it was necessary to determine whether the contribution (weight) of each single linear predictor was significant or not. In the model with four explanatory variables, we ob-

TABLE 3. Serotype distribution for each plant

Plant no.	No. of strains of:								
	1/2a	1/2b	1/2c	4a	4ab	4b	4c	4d	4e
1			1						
2	1		3						
3	3	11	4						
4	1		8						
5	1	2	1						
6	2		3						
7	18	4	10	2					1
8			5						
9			2			5		1	
10	1	1	7		1				
11	11		9			12	1		
12			4		2	11	1		
13	9		11						
14			19						
15		1	9		1				
16	4		23		1				
17			4						

served that both powered milk and casing type were not significant ($P > 0.05$), whereas plant size and a_w were significantly high ($P < 0.0001$). Therefore, we used a model in which the number of parameters was reduced from four to two (plant size and a_w). Comparing the fitted values of this model with the experimentally observed values, we can see that the used model was appropriate (Fig. 2). The best model for observed prevalence values of *L. monocytogenes* had this form:

$$\begin{aligned} \text{logit}(p) &= \ln\left(\frac{p}{1-p}\right) \\ &= \beta_0 + \beta_1(\text{plant size}) + \beta_2(a_w) \end{aligned} \quad (1)$$

Finally, these results show that only explanatory variable a_w is related to the survival probability of *L. monocytogenes*.

DISCUSSION

To date, the evaluations of the risk related to processed meat (6) have been based on studies that did not take into consideration the various production processes that could influence the behavior of *L. monocytogenes* (10), the various detection methods (which differ in terms of sensitivity and specificity), and the sampling plans (15).

To allow risk assessors to use the data of our study, we adopted certain precautions. First, we used qualitative and quantitative analysis methods that were validated (26, 27) and issued by international organizations (ISO). Furthermore, the sampling plan was implemented by considering the number of samples capable of determining the most probable prevalence in the products used, following the sampling plans of International Commission on Microbiological Specifications for Foods and selecting case 14, in relation to the degree of risk and conditions in the use of the food products in which the pathogen does not multiply (4). The choice was influenced by the fact that the data published on the behavior of *L. monocytogenes* in sa-

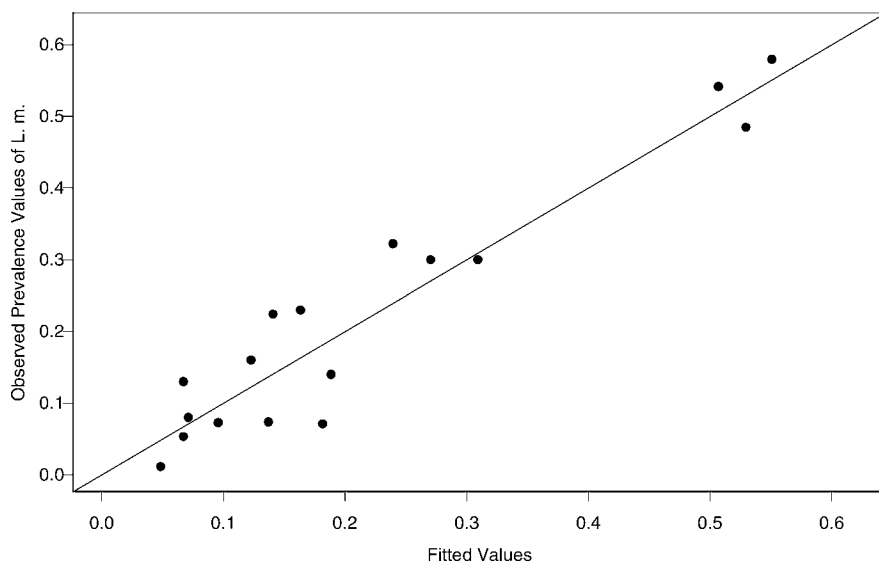


FIGURE 2. Goodness of fit of the final reduced logistic regression model 1.

lami indicate that it survives but does not multiply during its commercial life (13, 30).

The results of this study show that although the contamination levels are low in the final product (<10 CFU/g) cacciatore salami can be contaminated by *L. monocytogenes* and thus represents a source of exposure for consumers, in particular, those at high risk. Even so, the significance of exposure remains to be evaluated, for until now contamination levels lower than 10^3 CFU/g have never been documented for foods implicated in cases of invasive listeriosis (6). Furthermore, in our samples, serotype 1/2c was prevalent (53%), whereas the clinical strains isolated from cases of listeriosis in Italy in the same period (2002 to 2003) mostly belong to serotypes 1/2a (44%), 1/2b (20%), and 4b (17%), and serotype 1/2c has been found in only 2.5% of the strains analyzed (data not published).

In our study, overall contamination persisted in nearly analogous proportion, in the two periods in which the samples were collected ($P > 0.10$), demonstrating the difficulty in controlling this pathogen in production plants. Because this type of salami is frequently contaminated both with *L. monocytogenes* and other *Listeria* spp., it is evident that in this food these microorganisms are frequently introduced into the processing line by raw materials, surfaces, and the environment. The molecular typing of the isolated *L. monocytogenes* strains will be a useful tool that could be used to establish whether the presence of the microorganism in the production environment is endemic. The persistence of *L. monocytogenes* and the subsequent contamination of the product have been documented in dairy, poultry, and pork processing plants. Persistent environmental colonization may represent a source of repeated processing contamination of a product with a specific strain, potentially leading to multiple cases or outbreaks of foodborne listeriosis. Consequently, the elimination of persistent *L. monocytogenes* contamination should become a high priority for food processing plants (19). The production technology for salami has been widely studied (10); in particular, the factors that positively influence the formation processes of the positive organoleptic characteristics, the control of unwanted sec-

ondary fermentation, and the control of the growth and survival of known food pathogens have been clarified. According to our results (Table 1), the slow reduction of free water in order maintain a product that is softer to touch and for eating should be significantly associated with a more extensive contamination of the product (10). In fact, of the many ways used to preserve food products, lowering a_w is one of the most widely used (29, 31).

European regulations oblige the food business operators to conduct inspections on the production process and the finished products before commercialization so as to ensure conformity to current policy. The percent differences in contamination in respect to the size of the production plant could be attributable to a less attentive and scrupulous application of the sanitization procedures in the equipment and production environment, since an effective application of the hazard analysis critical control point system entails costs that could be too high for small plants.

Additional studies will be able to establish if the exposure to few cells of *L. monocytogenes* through the consumption of this type of salami represents a health risk for the Italian population, particularly for the population groups considered to be at risk.

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