Investigation on the microbiological hazards in an artisanal salami produced in Northern Italy and its production environment in different seasonal periods

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Abstract

In the present study, the occurrence of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp*. and *Escherichia coli* VTEC was investigated in two batches of artisanal Italian salami tested in winter and summer. Moreover, enumerations of total bacterial count, lactic acid bacteria and *Enterobacteriaceae* were performed as well as monitoring of water activity and pH. Samples were taken from raw materials, production process environment, semi-finished product and finished products. The results revealed an overall increase of total bacterial count and lactic acid bacteria during the ripening period, along with a decrease of *Enterobacteriaceae*, pH and water activity. No significant difference was observed between the two batches. The enterobacterial load appeared to decrease during the maturation period mainly due to a decrease in pH and water activity below the limits that allow the growth of these bacteria.

E. coli VTEC, *Salmonella* spp. or *L. monocytogenes* were not detected in both winter and summer batches. However, *Klebsiella pneumoniae* was detected in both summer and winter products. Except for one isolate, no biological hazards were detected in the finished salami, proving the efficacy of the ripening period in controlling the occurrence of microbiological hazard in ripened salami. Further studies are required to assess the virulence potential of the *Klebsiella pneumoniae* isolates.

Introduction

Salami typically refers to ready-to-eat dry fermented sausages made by mixing raw pig meat and fat, in addition to other ingredients such as pepper, garlic and/or fennel. A wide variety of Salami recipes exists in Italy and it is produced by local, small scale, family-based food companies (Comi *et al*., 2005; Halagarda and Wójciak, 2022).

Consumer perception and interest in artisanal and/or traditional food has changed in the 21st century. Artisanal foods have come to be perceived as more genuine and nutritious compared to their industrial counterparts, with small scale products with high quality and low availability often generating a positive image of health and ethicality (Almli *et al.*, 2011; Roccato *et al.,* 2017).

On the other side, standardization of productive parameters and automation are more challenging in artisanal food. A less standardized process can suggest a potential variability in the product's intrinsic proprieties, which often depend on the type and number of bacteria in the food. Microorganisms can persist in the production environment on both non-food contact and food contact surfaces and raw materials. A deficiency in hygiene procedures and sanitary training of the production staff could lead to cross contaminations from the environment and/or food operators to the product in all production processing steps (Halagarda and Wójciak, 2022; Omer *et al*., 2018; Roccato *et al*., 2017; Thévenot *et al*., 2005).

Salami and other dry fermented pig meats have a long history of safe consumption. However, meat products and ready-to-eat foods are one of the food vehicles most commonly associated with *Salmonella* human cases. According to the European One Health 2020 Zoonosis Report, after *Campylobacter*, *Salmonella* was the second most reported zoonosis in food in 2019, with 120,946 human cases. Pig meat and products thereof are a growing concern to food authority being one of the most frequently identified food vehicle in 2020 (EFSA and ECDC, 2021).

Escherichia coli O157 has been identified as etiologic agent in different salami outbreaks occurred in Italy. One of these, occurred in the Veneto region in 2004 due to a salami made by pig meat only (Williams *et al*., 2000; Conedera *et al*., 2007). *E. coli* O157 was the most common serotype associated to foodborne illnesses in European countries in the period between 2013 and 2014 (Omer *et al*., 2018).

Other biological hazards linked to meat-associated outbreaks, less frequently reported, are *Listeria monocytogenes* and *Staphylococcus aureus*. *L. monocytogenes* has been detected in France in 2005 in a dry sausage processing plant, its equipment, and the final product. It has also been observed that the contamination rate increases during the production. (Thévenot *et al*., 2005).

Staphylococcus spp. such as *S. xylosus* or *S. scuiri* can be present in salami ingredients and can be either naturally present or used as starters to help the fermentation process (Comi *et al.*, 2005). On the contrary, the presence of *S. aureus* must be avoided although it has been found in minced meat (Armany *et al*., 2021).

Hurdles technology is a method that can help reduce microbiological hazards in

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Key words: Italian salami, Artisanal food, Artisanal salami, Microbiological hazard.

Acknowledgments: The authors would thank Dr. Chiara Oliveri for her support in performing the microbiological testing

Contributions: LG, CC, FP, AL, LG, AD, data collection; CC, FP, AL, LG, AD, experimental plan and data analysis; LG, FP, manuscript writing; FP, AD, GM, reviewing and references search.

Conflict of interest: The authors declare no potential conflict of interest.

Funding: This work was funded within the EU H2020 PRIMA Project "Innovative bio-interventions and risk modelling approaches for ensuring microbial safety and quality of Mediterranean artisanal fermented foods".

Availability of data and materials: data and materials are available by the authors.

Received for publication: 2 September 202.
Accepted for publication: 23 JAnuary 2023.

Italian Journal of Food Safety 2023; 12:10831
doi:10.4081/ijfs.2023.10831

food, to achieve microbiological stability and ensure food safety. An example of this methodology is the fermentation process during salami's ripening period. Maturation can play a key role in reducing microbiological risks due to microbial growth barriers. For example, lactic acid bacteria during fermentation causes a decrease in pH and water activity (a_w), making the environment harder for pathogenic bacteria to survive (Kamenik, 2017; Thévenot *et al.,* 2005).

This study aimed to evaluate the occurrence of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella spp*. and *Escherichia coli* VTEC in two batches of artisanal Italian salami tested in the winter and summer. Moreover, enumerations of total bacterial count, lactic acid bacteria and *Enterobacteriaceae* were performed as well as monitoring of water activity and pH. Samples were taken from raw materials, production process environment, semi-finished and finished products.

Materials and methods

Two batches of *salame gentile* produced by an Italian artisanal factory were investigated in July 2020 (summer batch) and November 2020 (winter batch). The product was made mostly by a mixture of ground swine meat, fat and spices (garlic and pepper) stuffed in a natural casing. After the preparation, the product was left to dry for 1 week in a drying chamber (7-8°C during winter, 16-18° during summer), and subsequently in a ripening chamber where it stayed for 28 weeks at 10°C with 70%RH. The sampling plan included for each batch the collection of raw material (minced meat for stuffing) (10 samples), semi-finished products (salami during ripening stage at 3, 10, 18 and 28 weeks) (50 samples), and environmental samples (swabs from stuffing machine, table's surface, walls, and water drainage channel located in each processing area) (80 samples).

On the 140 collected samples, *Listeria monocytogenes*, *Salmonella spp*., *Staphylococcus aureus* and *E. coli* VTEC were investigated by standard protocols (ISO 11290-1,2017; ISO 6579-1,2017; ISO 6888-1/A1,2004; ISO 16654-2,2001). Isolates identification was confirmed by biochemical (RapID™ ONE System, RapID™ STAPH PLUS System, Thermo Scientific™) and/or serological tests (Oxoid™ Salmonella Latex Test, Thermo Scientific™). Specific polymerase chain reaction (PCR) assays were performed to further confirm biochemical tests results as well as VTEC associated virulence genes (Wesley *et al*., 2002; Perelle *et al*., 2004; Chander *et al*., 2011; Brakstad *et al*., 1992). All samples were tested for the enumeration of total bacterial count (ISO 4833-2,2013). Lactic acid bacteria (ISO 15214,1998) and *Enterobacteriaceae* (ISO 21528-2,2017) in raw materials, semi-finished and finished products. Salami samples at all stages were also submitted to physicochemical analyses of pH (ISO 2917,1999) and water activity (ISO 21807,2004). Moreover, for the isolation and identification of bacteria belonging to the *Enterobacteriaceae* family, 25 g of sample were diluted in 225 mL of Buffer Peptone water (BPW, Thermo Scientific, Milan, Italy) and incubated for 24 hours at 37°C. For *Enterobacteriaceae* species identification, BPW pre-enriched cultures were then streaked on MacConkey agar (Thermo Scientific) and incubated for 24 hours at 37°C. Five colonies per plate were harvested and submitted to confirmation by biochemical test and PCR as reported above. In case of confirmation, one isolate per species per sample was retained.

Data obtained were submitted to analysis of variance (ANOVA test) followed by a Scheffé test for post-hoc comparative analysis to detect any significant differences ($P<0.05$) between batches and days of ripening.

Results

Investigations of microbiological quality and physico-chemical properties of 2 batches of artisanal salami revealed an overall increase of total bacterial count and lactic acid bacteria during ripening along with a decrease of *Enterobacteriaceae*, pH and water activity (Table 1). No statistically significant differences within the summer and winter batch were observed both considering raw starting materials and final products. In particular, in the summer batch, total bacterial counts enumerations increased from 4.04 to 8.77 $\log_{10}$ CFU/g during ripening along with a lactic acid bacteria increase from 3.37 to 7.78 $\log_{10}$ CFU/g in the raw meat mixture and the final product, respectively. Along with an increase in lactic acid bacteria, a increase of pH was registered during production namely from 5.67 to 6.26. In both batches, *Enterobacteriaceae* decreased from approx. 4 $\log_{10}$ CFU/g to values close to the detection limit, suggesting the efficacy of ripening in controlling the occurrence of pathogens belonging to this taxonomic family. A statistical significant difference within batches was observed during ripening with a higher load of total bacterial count and lactic acid bacteria in the summer batch in comparison to the winter batch (lactic acid bacteria: 8.53 vs 6.59 in the semi-finished product at the drying room, 9.08 vs 8.37 $\log_{10}$ CFU/g at 3 weeks of ripening, 9.34 vs 8.28 $\log_{10}$ CFU/g at 10 weeks and 8.48 vs 7.04 at 18 weeks).

Regarding the environment, an increase of total bacterial count was observed along the rooms of the different production steps with the lowest load in the stuffing room (4.28 $\log_{10}$ CFU/cm^2 in the summer batch) and the highest load in the ripening room (6.47 $\log_{10}$ CFU/cm^2 in the summer batch).

Concerning the investigated biological hazards, *L. monocytogenes*, *Salmonella spp*. and *E. coli* VTEC were never detected. Two *Staphylococcus aureus* isolates were collected from the raw meat mixture of the summer batch and in the semi-finished salami (salami of the drying room) in the winter batch (Table 2). In all samples but the 2 in which *S. aureus* was detected the number of coagulase positive staphylococci was lower than 100 CFU/g. In the 2 named samples this value reached approximately 1000 CFU/g (data not shown). During the identification at species level of isolates belonging to *Enterobacteriaceae*, biological hazards such as *Enterobacter cloacae*, *Citrobacter freundii, Klebsiella pneumoniae* and *Klebsiella oxytoca* were detected all along the artisanal production. A total of 30 isolates were collected (Table 2).

Five isolates of *Enterobacter cloacae* were detected, 2 of which in the summer batch (1 in semi-finished salami at 10 weeks of ripening and 1 in finished salami at 28 weeks of ripening) and 3 in the winter batch (2 in environmental samples of the stuffing room and 1 in semi-finished salami). Regarding *Citrobacter freundii*, 9 isolates were detected, 4 of which in the summer batch (all 4 in semi-finished salami) and 5 in the winter batch (1 in the drains of the drying room and 3 in semi-finished salami). As far as *Klebsiella spp.* is concerned, 1 isolate of *K. oxytoca* was collected from semi-finished salami of the summer batch, whereas 13 isolates of *K. pneumoniae* were collected from both batches. In particular, 5 were isolated from raw meat mixture, 3 from semi-finished product and 5 from the processing environment. Noteworthy, except for one isolate of *Enterobacter cloacae*, no biological hazards were detected in finished salami, suggesting the efficacy of the 28 weeks of ripening in controlling the risk of contamination.

Discussion

In the present study, microbiological hazards were investigated in an artisanal salami produced in Norther Italy and its

Table 1. Microbiological (log10 CFU/g or cm²) and physico-chemical results on raw, semi-finished and finished products as well as on the production environment of a summer and winter batch of artisanal salami.

	Sample	Summer Batch	Winter Batch
Total Bacterial Count			
Product	Raw meat	4.04±0.03^{a}	4.69±0.25^{a}
	Salami within the drying room	8.58±0.11^{cd}	6.78±0.12^{b}
	Salami at 3 weeks of ripening	8.96±0.18^{cde}	8.31±0.12^{cd}
	Salami at 10 weeks of ripening	9.75±0.80^{e}	8.35±0.16^{cd}
	Salami at 18 weeks of ripening	9.13±0.10^{de}	8.72±0.13^{cde}
	Salami at 28 weeks of ripening	8.77±0.27^{cde}	8.25±0.07^{c}
Environment	Water drainage channel swab within the mixture room	7.68±0.08^{f}	5.79±0.49^{de}
	Table swab within the mixture room	4.28±0.46^{bc}	4.07±0.05^{abc}
	Filler stuffer machine swab	3.25±0.86^{ab}	2.66±0.46^{a}
	Drying room environmental swab	4.18±0.35^{bc}	4.25±0.16^{bc}
	Water drainage channel swab within the drying room	6.80±0.05^{ef}	6.85 ± 0.20^{ef}
	Environmental swab within the ripening room	6.47±0.18^{def}	5.19±0.71^{cd}
	Water drainage channel swab within the ripening room	6.82±0.36^{ef}	7.04±0.64^{ef}
Lactic acid bacteria			
Product	Raw meat	3.37±0.06^{a}	3.13±0.27^{a}
	Salami within the drying room	8.53±0.07^{def}	6.59±0.08^{b}
	Salami at 3 weeks of ripening	9.08±0.31^{ef}	8.37±0.33^{de}
	Salami at 10 weeks of ripening	9.34±0.45^{f}	8.28±0.18^{de}
	Salami at 18 weeks of ripening	8.48±0.35^{de}	7.04±0.07^{bc}
	Salami at 28 weeks of ripening	7.78±0.49^{cd}	7.83±0.11^{cd}
Enterobacteriaceae			
Product	Raw meat	4.63±0.35^{fg}	3.19±0.13^{fg}
	Salami within the drying room	4.56±0.14^{fg}	5.49±0.29^{fg}
	Salami at 3 weeks of ripening	4.11±0.10^{ef}	4.84±0.55^{ef}
	Salami at 10 weeks of ripening	4.41±0.10^{f}	2.18±0.77^{f}
	Salami at 18 weeks of ripening	<1.00+0.00^{a}	1.60±0.50^{a}
	Salami at 28 weeks of ripening	1.09±0.24^{a}	<1.00±0.00^{a}
pH			
Product	Raw meat	5.67 ± 0.02^{c}	5.73 ± 0.06^{c}
	Salami within the drying room	5.28 ± 0.15^{a}	5.58 ± 0.00^{bc}
	Salami at 3 weeks of ripening	5.41 ± 0.01^{ab}	5.46 ± 0.00^{ab}
	Salami at 10 weeks of ripening	5.41 ± 0.03^{ab}	5.77 ± 0.01^{c}
	Salami at 18 weeks of ripening	5.92 ± 0.03^{d}	5.95 ± 0.04^{d}
	Salami at 28 weeks of ripening	6.26 ± 0.01^{e}	6.02 ± 0.03^{d}
aw			
Product	Raw meat	0.9816±0.0047^{f}	0.9810±0.0017^{f}
	Salami within the drying room	0.9632±0.0035^{e}	0.9717±0.0000^{ef}
	Salami at 3 weeks of ripening	0.9597±0.0775^{e}	0.9597±0.0000^{e}
	Salami at 10 weeks of ripening	0.9498±0.0006^{d}	0.9125±0.0036^{c}
	Salami at 18 weeks of ripening	0.8647±0.00939^{a}	0.9102±0.0020^{c}
	Salami at 28 weeks of ripening	0.8843±0.0030^{b}	0.8844±0.0029^{b}

^{a,b,c,d,e,f}Same letters in different columns indicate different significance.

Table 2. Number of foodborne pathogens identified in raw materials, semi-finished and finished products as well as the processing environment of salami in the two tested batches.

Foodborne pathogen	Summer batch				Winter batch			
	Raw meat mixture	Semi-finished product	Finished product	Environment	Raw meat mixture	Semi-finished product	Finished product	Environment
Staphylococcus aureus	1					1		
Enterobacter cloacae		1	1			1		2
Citrobacter freundii		4				4		1
Klebsiella oxytoca		1						
Klebsiella pneumoniae	4	1			1	2		5

production environment in two batches processed in summer and winter respectively. The artisanal salami is produced with the addition of natural spices, such as garlic and pepper, thus avoiding the use of nitrites and nitrates. In addition, the artisanal product is characterized by a longer ripening period of about 6 months in comparison to the 3 months of industrial salami. The long period of ripening was associated to an increase in beneficial bacteria, such as lactic acid bacteria (up to 7.8 $\log_{10}$ CFU/g in the finished product) alongside a reduction of *Enterobacteriaceae* (a taxonomic family including foodborne pathogens), water activity, independently from the analyzed batch. In particular, water activity reached values of 0.86 and 0.88 in the summer and winter batch respectively. These values are under the minimum water activity value required for the growth of most bacteria and is close to the limit of 0.83-0.86 reported as the minimum value for *S. aureus* growth (Medveďová and Valík, 2012). Accordingly, among the 30 isolates detected, only one (*Enterobacter cloacae*) was found in the finished salami. Although not found in the final product, the isolation of biological hazards such as *S. aureus*, *E. cloacae*, *Citrobacter freundii* and *Klebsiella spp.* in semifinished salami and the processing environment might represent a point of attention for potential events of cross-contamination which can involve both workers and the salami before commercialization. *S. aureus* is a foodborne pathogen often associated to food of animal origin. Contamination of food occurs due to the presence of the pathogen within raw materials or through the transfer of bacteria to the food during processing (Hennekinne *et al.*, 2012).

E. cloacae is often found in the gastrointestinal tract of animals as well as in soil, water and sewage (Huang *et al.*, 2021). Some isolates were described as associated to nosocomial infections such as pneumonia, urinary tract infections and septicemia, worsen by multidrug resistance of the pathogen to several antimicrobial agents including last-resort carbapenems (Annavajhala *et al.*, 2019). Regarding food, *E. cloacae* was isolated from ready-to eat chicken and beef stews, rice, and pies sold in cafeterias and retails (Nyenje *et al.*, 2012).

Previously considered as environmental contaminant or colonizer with low virulence, *Citrobacter freundii* is now associated to a wide spectrum of infections including urinary tract, surgical wound and bloodstream (Liu *et al.*, 2018). Similar to *E. cloacae*, although to a lesser extent, carbapenem-resistant *Citrobacter freundii* has been described associated to nosocomial infections (Liu *et al.*, 2018). Recently an outbreak in a German hospital was registered with pre sliced vegetables as the potential source of contamination (Pletz *et al.*, 2018).

Klebsiella pneumoniae and *K. oxytoca* are ubiquitous microorganisms which have been associated to nosocomial infections including bronchopneumonia, urinary tract and septicemia. Both species are pathogens of concern due to their multidrug resistance to different antimicrobial agents. Publications on the isolation of resistant *Klebsiella pneumoniae* and *K. oxytoca* in meat and its processing environment, are available (Projahn *et al.*, 2019).

Conclusions

In the artisanal salami of the present study, the ripening period of 28 weeks was associated to an increased load of beneficial bacteria such as lactic acid bacteria and decreased load of *Enterobacteriaceae* up to levels close to the enumeration limit. Moreover, water activity decreased to values considered as not optimal for bacterial growth. Among all samples collected from raw materials, semi-finished and finished products as well as the processing environment, none was positive for *Listeria monocytogens*, *E. coli* VTEC and *Salmonella spp*. However, 30 foodborne isolates identified as *Staphylococcus aureus*, *Enterobacter cloacae*, *Citrobacter freundii*, *Klebsiella pneumoniae* and *K. oxytoca* were collected mostly from semi-finished products and the processing environment. The isolation of these human pathogens in artisanal salami and their environment suggests a higher attention on hygienic practices during food handling such as stuffing, in order to mitigate the risk of emergence of these foodborne isolates which can be transferred to workers as well as to the final product. Further analyses should be performed in order to evaluate the virulence and antimicrobial resistance character of the foodborne isolates.

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