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Growth potential of *Listeria monocytogenes* in fresh sauces for pasta

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Abstract

Ready to use fresh sauces are Ready-to-Eat (RTE) products that, based on their composition, may be able to support the growth of *Listeria monocytogenes*. Therefore, it is essential for producers to be able to demonstrate for each production process if i) the substrate allows the growth of *L. monocytogenes* and ii) if, when present, *L. monocytogenes* can reach or exceed the limit of 100 CFU g^{-1} at the end of the shelf life, as stated by Commission Regulation (EC) No 2073/2005.

In the current research, the growth potential (δ) of *L. monocytogenes* was evaluated at two storage temperatures on fresh mushroom and cheese sauces. At 4 °C both sauces showed a $\delta < 0.5$ $\log_{10}$ CFU g^{-1}, while at 8 °C the cheese sauce showed a $\delta > 0.5$ $\log_{10}$ CFU g^{-1}. Our preliminary findings indicate that if the contamination level of *L. monocytogenes* by shipping is below 10 CFU g^{-1}, the contamination level of the product would not exceed 100 CFU g^{-1} by the expiration date.

1. Introduction

The Codex Alimentarius defines Ready-to-Eat (RTE) products "any food which is normally eaten in its raw state or any food handled, processed, mixed, cooked, or otherwise prepared into a form which is normally eaten without further listericidal steps" (CAC, 2007), thus taking into consideration the hazard represented by *Listeria monocytogenes*. This microorganism is a widespread environmental contaminant, due to its extreme adaptability: it can grow at 1.0 °C and pH 4.8 (Farber & Peterkin, 1991). These characteristics make many RTE products potential ideal substrate for *L. monocytogenes* growth and a major source of listeriosis cases (EFSA, 2009 and 2010).

In recent years there has been a raise in demand for RTE foods worldwide, considering the marked change in dietary habits, primarily due to changing lifestyles, increase of consumption of meals away from home, and the needs of single and working consumers who have less time to devote to cooking. However, in Italy, the attachment to the tradition of Italian consumers led to the formulation of many RTE products which imprinted to traditional dishes, such as pasta. These products are nowadays widely distributed on the market and largely consumed (Coldiretti, 2009). Particularly, sales of minimally processed sauces and fresh sauces have reached 46,000 tons and amounted to over 236.5 million Euros in 2009 (Bernieri, 2009).

Minimally processed and fresh sauces possess product characteristics and other intrinsic factors that make them potential ideal substrates for the development of various microorganisms, among which *L. monocytogenes*. Contamination can occur during preparation either as a result of cross contamination due to poor hygienic conditions or of non compliance to correct processing procedures (e.g. use of time–temperature parameters) (Bertollo, Dragoni, Galasso, Gradassi, & Pancioni, 2007). Very few and local studies have evaluated the presence of Listeria spp. in fresh sauces, such as basil pesto sauce (Magistroni, 2006) and cheese and mushroom sauces (Grassi, Nucera, Morra, & Civera, 2011).

The aim of this study was to evaluate and assess the growth potential of *L. monocytogenes* during the commercial life of two fresh sauces to be used as a condiment for pasta. To the best of our knowledge, this is one of first international studies on the behavior of this microorganism in these RTE products.

2. Materials and methods

2.1. Fresh unpasteurized sauces

The growth potential of *L. monocytogenes* was evaluated on two types of fresh unpasteurized sauces: cheese and mushroom sauces. The flow chart and the complete list of ingredients are presented in Figs. 1 and 2: for both the products no acidifying Lactic Acid Bacteria (LAB) were added. Sauces were thermofirmed in 140 g packages, and the producer indicated a shelf life for both products of 31 days with a storage temperature ranging from 2 to 4 °C.

Cheese sauce was prepared using partially skimmed milk mixed with different types of cheeses (soft cheeses, Grana Padano PDO, and Gorgonzola PDO cheeses). Cheeses were heated for 15 min until they reached the fusion temperature of 78 °C and then used in subsequent steps of the production. Mushroom sauce was prepared with different types of fresh edible mushrooms that were cooked at 85 °C for 15 min, prior to being mixed with other ingredients, including grated cheese. Mushrooms were added in two different mixing steps in order to have a selection in texture of the final product. After the heat treatment and the addition of other ingredients the two semi-finished sauces were chilled in a cellar at 3 °C where the products were kept for 12 h.

2.2. Microbiological analysis

For both type of sauces, 110 samples were collected at the producing plant and were analyzed in the laboratory. In particular, five samples for each sauce were analyzed within 48 h from packaging, to exclude the presence of *L. monocytogenes* using method ISO 11290-1 (ISO, 2004a). The remaining 100 samples

were inoculated with 1 ml of a PBS bacterial suspension in order to reach a final concentration on the product of 103 CFU g^{-1}. Such concentration was preferred over the one recommended of 102 UFC g^{-1} (AFSSA, 2008) in order to effectively detect and enumerate Listeria colonies, considering that no data were available on similar products challenge test in literature. However it may still represent a "worst case scenario" of contamination, thus overestimating the pathogen growth. Considering the growth variability among strains, a mixed bacterial suspension was prepared using three different *L. monocytogenes* strains isolated from foods and previously characterized as serotypes 4b/4e (isolated from a soft cheese), 1/2a (from PDO Gorgonzola cheese) and 3b (from a meat product). Contaminated trays were then stored for the duration of the test at two different temperatures, 4 °C and 8 °C: the latter was chosen in order to simulate the temperature kept in household fridges when compared to the 4 °C of distribution.

2.3. Enumeration of *L. monocytogenes*

After having excluded the presence of *L. monocytogenes*, the *Listeria* suspension was inoculated in the collected samples and this day was considered as T_0. A quantitative analysis for *L. monocytogenes* enumeration (ISO, 2004b) was performed on five trays for each type of sauce; the analyses were repeated at regular intervals (twice a week) until the end of sauces' shelf-life. Enumeration of LAB was also carried out at regular intervals (8, 18, 25 and 31 days), and counts were performed using MRS agar (Oxoid) in micro-aeropyilic conditions at 37 °C for 48 h.

Contextually to bacterial enumeration, measurement of aw (AquaLab CX-3) and pH (pH meter 744 - Metrohm) was performed for all tested samples.

For each time point, results of the five samples were aggregated and reported as the median concentration of microorganisms expressed in log10 CFU g^{-1}. In order to evaluate *L. monocytogenes* growth potential, δ was calculated at each time point, with negative results indicating a decrease in microbial load.

3. Results and discussion

Considering the current large distribution and consumption of RTE and minimally processed sauces, the producer, amongst other measures, is recommended to perform laboratories analyses, defined by Commission Regulation (EC) No 2073/2005 as challenge tests, in order to document the ability of a food to support the growth of *L. monocytogenes*. Guidelines and procedures for performing challenge tests are available in a dedicated document produced by the Agence Française de Sécurité Sanitaire des Aliments (AFSSA, 2008). It must be noted that for this study it was not possible to test three batches of the same product or three similar sauces produced in the same plant. However, our findings may offer useful insights to producers willing to perform challenge tests, providing them with an effective experimental design which needs only to be implemented with the adequate number of aliquots for each product or a single aliquot of three similar products (AFSSA, 2008). Moreover, the presented assay was performed for a period of time longer than the shelf-life provided by the producer as to mimic the microbial dynamics in case consumers would hold and consume the product beyond its shelf life. Moreover, this assay was performed considering the temperature recommended by the producer (4 °C) and 8 °C, since more than 50% of the European household fridges hold temperatures beyond 6–8 °C (EFSA, 2008).

Our findings showed initial aw values of 0.97 for both products, and these remained essentially unchanged until the end of the study. pH was different between the two products: 5.68 for cheese and 5.25 for mushrooms sauce. The difference increased at each time period with final values 5.18 for cheese sauce and 4.46 for mushroom sauce, when kept at 8 °C. For both the sauces the samples stored at 8 °C showed a lower pH than the ones stored at 4 °C (Tables 1 and 2).

Enumerations of *L. monocytogenes* and LAB over time are reported in Tables 1 and 2 for cheese and mushrooms sauce, respectively. In the cheese sauce, *L. monocytogenes* load was between 3.28 and 3.49

log10 CFU g^{-1} on day 1. Successively, a slight increase was observed up until day 18 for products stored at 4 °C (δ max = 0.21), barring a stead decrease until the end of commercial life. For products stored at 8 °C, bacterial development was greater, as evidenced by δ max = 1.02 at day 14.

In the mushroom sauce, characterized by greater initial acidity (day 1: pH 5.25), the δ value at day 18 was below 0.5 $\log_{10}$ CFU g^{-1}, both at 8 °C than 4 °C.

Both kind of sauces, even while representing a suitable substrate for the growth of *L. monocytogenes* (based on their intrinsic parameters), showed a δ less than 0.5 $\log_{10}$ CFU g^{-1} at 4 °C and in the time points considered. In particular for the mushroom sauce, the absence of difference between the two storage temperatures indicates that the product is suited for distribution and storage in household fridges without hindering product safety. In fact, even when sauces were stored at 8 °C, the low levels of pH and the high levels of LAB flora were able to effectively control the growth of Listeria. This would also explain the ten-fold higher δ value at 4 °C than 8 °C at day 18, probably related to the fact pH and LAB flora were not able to inhibit Listeria at lower refrigeration temperatures. However, as the uncertainty in the enumeration analysis is approximately 0.25 $\log_{10}$ CFU g^{-1}, the difference may have been overestimated.

On the other hand, cheese sauce may become a substrate allowing Listeria growth when stored at 8 °C (δ max = 1.02 $\log_{10}$ CFU g^{-1}). However, estimating an initial contamination of 0.7 $\log_{10}$ CFU g^{-1} (5 CFU g^{-1}) and considering the δ max of 1.02, the maximum observable contamination could reach 1.72 $\log_{10}$ CFU g^{-1} (52 CFU g^{-1}) with storage at 8 °C, well below the limit of 100 CFU g^{-1} during the commercial life of the product, as indicated in Commission Regulation (EC) No 2073/2005.

Moreover, it is important to point out that these sauces undergo a heated production step that may guarantee that initial contamination, if present, would be controlled. However, it must be emphasized the paramount importance of the correct application of GMP/GHP and HACCP procedures, in particular by checking raw materials and controlling and monitoring the critical steps of cheese fusion and mushroom cooking in order to control for *L. monocytogenes*. The listericidal effect of the heat treatment must always be kept under control mostly for cheese sauce, in which one of the ingredients is represented by Gorgonzola PDO cheese, that has been frequently associated with the presence of *L. monocytogenes* (Lomonaco et al., 2009; Manfreda, De Cesare, Stella, Cozzi, & Cantoni, 2005).

Our findings show the effectiveness of acidification in controlling the growth of Listeria in fresh sauces, even in conditions mimicking slight thermal abuse. The results may be attributed to the high level of LAB present in the sauces. Unfortunately records of LAB levels in different production batches were not available at the producer; however the quality control records showed that at day 0, the yearly average total aerobic mesophylic counts were 3 log CFU g^{-1} and 4 log CFU g^{-1} for mushroom and cheese sauces, respectively. One of the microbial groups most represented in such counts could belong to LAB. In fact, for the cheese sauce, thermo-resistant or thermophylic LAB may be part of the flora present in raw materials and cheese. These species can drastically decrease after thermal treatment but after few days at refrigeration temperature they can multiply, as observed in other studies on pasteurized as well as plasticized (“pasta filata”) cheeses (Fitzsimons, Cogan, Condon, & Beresford, 2001; Terrosu et al., 2008). Considering the mushroom sauce, the high levels of LAB present in the final product may be related to the contamination of raw mushrooms, or, more probably, by the addition of grated cheeses after heat treatment. In fact, literature shows that LAB were present in low numbers (less than 3 log CFU g^{-1}) in 20 of the 22 mushroom species tested (Venturini, Reyes, Rivera, Oria, & Blanco, 2011). However it needs to be also mentioned that the high LAB levels may be also attributed to a contamination from the production environment after heat treatment, which may occur during processing prior to packaging.

Finally, the good acidification of the product makes it possible to use low levels of additives for further acidification, as the LAB contribute to great extent to product acidification, limiting *Listeria* growth. However this observation highlights the need for further studies on the identification and typing of the

predominant LAB species in these two products, in order to better characterize the microflora of such sauces.

Overall, based on the requisite that an absolute compliance to rigorous processing hygiene procedures still has to be met by producers, our findings, even if preliminary, showed that although fresh sauces may be potential favorable substrate for *L. monocytogenes*, the amount of contamination never exceeded 100 CFU g^{-1} during storage and distribution. This holds true even when products are not kept at strict 4 °C temperature, a situation that is likely to occur during household fridge storage. Further studies performed on more batches are needed in order to better evaluate product and environment variability and clearly define the growth potential of *L. monocytogenes* in these sauces. However, the presented preliminary data are currently needed, given the lack of similar information in literature and could be considered as a guidance for producers willing to perform challenge tests on their products.

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Table 1. Results from the analyzed cheese sauce: i) evolution of the microbial flora (*L. monocytogenes* (L.m.) and LAB - lactic acid bacteria) during storage and ii) growth potential (δ) of *L. monocytogenes* when stored at 4 °C and 8 °C. All values are expressed as median $\log_{10}$ UFC g^{-1} and reported with the related range (min–Max). Day 1 is the first day after the inoculum of *L. monocytogenes*.

Days	4 °C						8 °C					
	L.m.	min–Max	δ (t_x – t1)	LAB	min–Max	pH	*L.m.*	min–Max	δ (t_x – t1)	LAB	min–Max	pH
1	3.49	3.44–3.57				5.72	3.28	3.11–3.60				5.68
4	3.50	3.28–3.52	0.01			5.68	3.59	3.40–3.70	0.31			5.66
8	3.64	3.15–3.84	0.15	5,1	4.00–6.68	5.70	3.87	3.78–3.90	0.59	6.7	6.68–6.88	5.54
11	3.50	2.94–3.98	0.01			5.64	4.04	3.29–4.28	0.76			5.40
14	3.63	3.52–4.15	0.14			5.62	4.30	3.85–4.41	1.02			5.32
18	3.70	3.75–4.58	0.21	6.5	3.70–7.61	5.50	3.88	2.92–4.26	0.60	8.6	4.56–8.94	5.12
22	3.38	2.48–3.50	−0.11			5.59	3.52	3.00–4.70	0.24			5.18
25	3.17	2.70–3.32	−0.32	7.3	6.56–8.00	5.45	3.43	2.90–3.74	0.15	8.5	8.41–8.60	5.23
28	3.07	2.90–3.86	−0.42			5.45	3.48	3.26–4.54	0.20			5.10
31	2.90	2.30–3.67	−0.59	7.6	7.23–8.23	5.43	3.47	3.32–400	0.19	8.6	8.45–8.67	5.18

t_x = day of sampling.
t_1 = Day 1.

Table 2. Results from the analyzed mushroom sauce: i) evolution of the microbial flora (*L. monocytogenes* (L.m.) and LAB - lactic acid bacteria) during storage and ii) growth potential (δ) of *L. monocytogenes* when stored at 4 °C and 8 °C. All values are expressed as median $\log_{10}$ UFC g^{-1} and reported with the related range (min–Max). Day 1 is the first day after the inoculum of *L. monocytogenes*.

Days	4 °C						8 °C					
	L.m.	min–Max	$\delta(t_x - t1)$	LAB	min–Max	pH	*L.m.*	min–Max	$\delta(t_x - t1)$	LAB	min–Max	pH
1	3.49	3.00–3.65				5.25	3.43	3.18–3.61				5.25
4	3.42	3.30–3.65	−0.07			5.20	3.48	3.30–3.60	0.05			5.23
8	3.67	2.88–3.80	0.18	6.0	4.0–6.53	5.23	3.48	3.40–3.75	0.05	6.4	6.30–6.60	5.19
11	3.86	3.73–3.90	0.37			5.14	3.79	3.73–4.28	0.36			5.14
14	3.58	3.20–3.65	0.09			5.17	3.39	2.62–4.23	−0.04			4.91
18	3.91	3.15–4.00	0.42	6.3	4.48–6.85	5.17	3.47	3.08–4.30	0.04	8.8	8.58–8.93	4.38
22	2.84	2.48–3.90	−0.64			5.18	3.47	2.48–3.87	0.04			4.20
25	2.30	<2.00–3.83	−1.18	6.6	5.00–6.88	4.76	3.20	<2.00–3.32	−0.23	8.4	8.38–8.52	4.63
28	1.69	<2.00–3.40	−1.79			4.71	3.30	<2.00–3.45	−0.13			4.50
31	2.16	<2.00–3.78	−1.88	7.6	6.30–8.14	4.71	3.32	<2.00–3.22	−0.11	8.7	7.80–8.85	4.46

t_x = day of sampling.
t_1 = Day 1.

Fig. 1. Flow diagram for the preparation of mushroom sauce. Ingredients are listed below: champignons, porcini, other edible mushrooms, sunflower oil, onions, Grana Padano PDO cheese, rice flour, parsley, sugar, whey, cream, salt, vegetable fiber, yeast extract, garlic, pepper, acidifier: E330, antioxidant: E300.

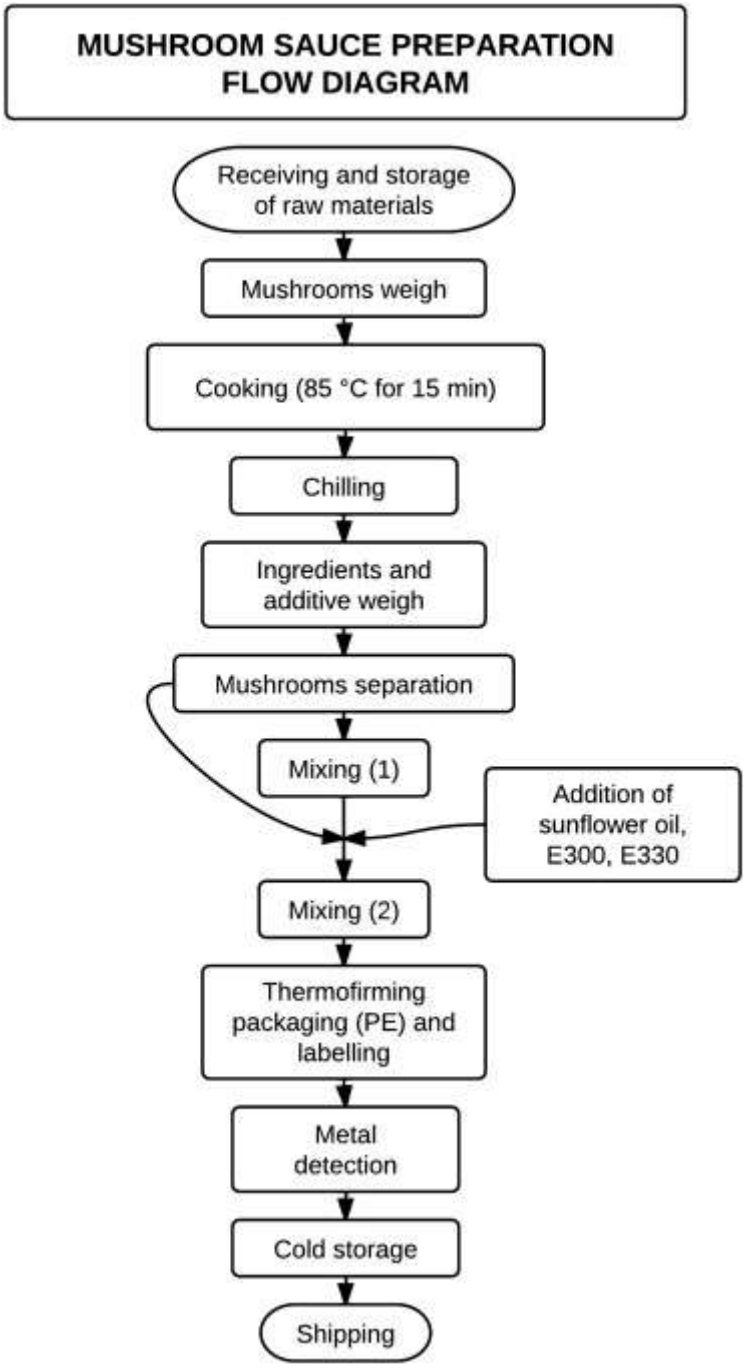

Fig. 2. Flow diagram for the preparation of cheese sauce. Ingredients are listed below: partially skimmed milk, soft cheese, Grana Padano PDO cheese, Gorgonzola PDO blue cheese, sunflower oil, cream powder, milk proteins, rice flour, pepper, salt, acidifier: E330, antioxidant: E300, preservatives: E251.