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Behavior of *Listeria monocytogenes* during the Shelf-life of Fresh Refrigerated Pesto Sauce, According to Regulation (EU) 2073/2005 and Subsequent Amendments

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Ligurian pesto is one of the most popular condiments for pasta. For microbiological purposes, refrigerated pesto sauce is a great matter for concern, as it can be contaminated by microorganisms originating from both raw materials and manufacturing processes. *Listeria monocytogenes* is a potential microbiological hazards for this product. The regulatory approach (EU) No. 2073/2005, with subsequent amendments and integrations, takes into consideration the concentration of this pathogen in foods and its growing ability. This study is aimed to assess whether fresh pesto sauce can be included among the Ready-To-Eat (RTE) foods that do not support the growth of *L. monocytogenes*.

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Place and Duration of Study: Quality, Safety and Pre-Industrialization Area, Stazione Sperimentale delle Conserve Alimentari- SSICA, Parma, Italy. Study carried out between October 2021 and June 2023.
Methodology: A screening on 48 different types of commercially available fresh pesto sauces was carried out, evaluating physiochemical parameters (pH and a_w). For three different pesto sauces with physical-chemical characteristics suitable for *L. monocytogenes* growth, microbiological analyses were carried out on both non-inoculated and inoculated samples. The inoculum was made using a mixture of *L. monocytogenes*, to evaluate its behaviour during the pesto shelf-life.
Results: A relevant presence of commercially available pesto sauces supporting the pathogen growth was found. The study highlighted the inability of the inoculated *L. monocytogenes* to grow in the tested pesto sauces at 7°C and 10°C during the labelled shelf-life (that varied from 9 to 33 days).
Conclusion: The results obtained from the study can be helpful to the food companies producing pesto sauces, to predict the behaviour of the microorganism and to correctly classify the product, in accordance with the reference regulation (EU) No. 2073/2005 and its subsequent amendments.

Keywords: Refrigerated pesto sauce; Listeria monocytogenes shelf-life; physical-chemical parameters; EU regulation No. 2073/2005.

1. INTRODUCTION

Ligurian pesto is, together with tomato sauce, one of the most popular sauces for pasta; for years there has been a constant increase in its production and exports. The shelf-life of such product depends on of the intrinsic and extrinsic physical-chemical parameters [pH, water activity (a_w)], storage temperature and heat treatment [1]. Since heat affects product aroma and colour, several food companies that produce pesto sauce preferred the refrigeration as the only preserving system, despite a consequent reduction in the shelf- life of this product.

By a microbiological point of view, refrigerated pesto sauce is a great matter for concern, as it can be contaminated by microorganisms originating from both raw materials and manufacturing processes [2]. Literature data indicate that *Listeria monocytogenes* is a potential microbiological hazard for this product, in addition to the fact that it has been quite frequently isolated from it [3,4,5]. Listeriosis can cause septicemia, meningitis, brain abscesses, local and pregnant female infections, miscarriage, stillbirth, prematurity, and neonatal infection. Generally, the risk of contracting listeriosis is influenced by several factors such as: host sensitivity, microorganism distribution and concentration in foods, and possible growth during shelf-life at refrigeration temperature before the opening of the packed food [6]. For these reasons, the European legislation (EU Regulation No. 2073/2005 and subsequent amendments and integrations) [7] takes into consideration the concentration of *L. monocytogenes* in foods and its growing ability, based on the determination of factors such as pH, a_w, presence of preservatives and storage conditions, intended as time/temperature ratio [8]. Such factors influence the possibility for *Listeria* spp. to survive or to grow in food and are essential for a correct classification of Ready To Eat foods (RTE). Since it is known that the foods involved in the most serious poisonings are typically those in which not only *L. monocytognes* is present, but it can grow to concentrations which represent a risk for the consumer [9], in accordance with the European legislation [7], RTE products other than those for children and for special medical purposes should not have a concentration of *L. monocytogenes* greater than 100 CFU/g at the end of their shelf-life. In particular, for RTE products, a distinction must be made on the basis of physical-chemical parameters, based on *L. monocytogenes* growing ability. Products with pH ≤ 4.2 or with a_w<0.92, products with pH ≤ 5.0 and a_w ≤ 0.94, products with shelf-life of less than 5 days are automatically included in the second group and therefore *L. monocytogenes* presence is allowed at values ≤ 100 Colonies Forming Units (CFU)/g. Since pesto sauce does not always fit in this group, on the basis of the physical-chemical parameters, as indicated in article 3 of European Regulation No. 2073/2005, the operators of the food sector must carry out appropriate scientific studies known as Microbial Challenge Tests (MCT) in order to demonstrate that under the marketing conditions there can be no growth of *Listeria* spp. in the product. The European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm) has recently

published the new revision of the "EURL Lm TECHNICAL GUIDANCE DOCUMENT on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to *Listeria monocytogenes*" [10]: Such document covers the technical aspects related to *L. monocytogenes* assessment in RTE foods.

In this perspective and based on both the increase in the consumption of fresh refrigerated pesto and the recent recalls [11] of some types of pesto due to the presence of *L. monocytogenes*, this study was aimed to verify whether fresh pesto sauce can be included among the RTE foods that do not support the growth of the above mentioned pathogen according to Regulation (EU) No. 2073/2005 and subsequent amendments.

2. MATERIALS AND METHODS

2.1 Sample collection and chemical analyses

A screening was carried out on 48 different types of commercially available fresh pesto sauces, selected by their physico-chemical parameters (pH and aw). The pH was measured using a Mettler Toledo™ pH meter (S210 Model, Thermo Fisher Scientific Instrument, Waltham, MA, USA) equipped with a glass, ceramic electrode (Hamilton, Franklin, MA, USA), whereas water activity (aw) was measured at 25 °C by means of the aw meter AquaLab, series 4, Model TE (Decagon Devices, Inc., Pullman, USA) in accordance with the ISO 18787 [12].

2.2 Microbiological analyses

2.2.1 Fresh pesto sauces

Microbiological analyses were carried out on three different fresh commercial pesto sauces without garlic selected by the screening of the market (section 2.1), produced by sector companies which supplied three different batches just after production.

The three pesto sauces, named A, B and C, had the following composition and shel-life:

A: Protected designation of origin (PDO) Genoese basil 30%, olive oil, PDO "Grana Padano" cheese, extra virgin olive oil, salt, pine nuts. Shelf- life: 9 days between +1°C and + 4°C.

B: PDO Genoese basil 31%, extra virgin olive oil, PDO "Parmigiano Reggiano" cheese, pine nuts 17%, PDO "Pecorino Romano", sea salt, ascorbic acid. Shelf- life: 21 days between 0°C and +4°C.

C: Ligurian basil, pine nuts, PDO "Parmigiano Reggiano" cheese, olive oil, salt, ascorbic acid. Shelf- life: 33 days between 0°C and +4°C.

2.2.2 Non-inoculated samples

For the initial and final characterization of the non-inoculated pesto sauces, all analyses were conducted in accordance with International Organization for Standardization (ISO). Twenty-five grams of each sample were aseptically transferred into a plastic one-chamber filter stomacher bag (Interscience, Macerata, Italy) and homogenized in 225 mL of peptone salt solution for 3 min using a Stomacher400 circulator (Seward, London, UK), according to ISO 6887-1 [13]. After decimal serial dilution, 1.0 mL of each sample was transferred onto Plate Count Agar (Oxoid, Cambridge, UK) to carry out the total microbial count at 30°C (ISO 4833-1:2013/Amd 1: 2022) [14], onto Violet Red Bile Lactose Agar (Oxoid, Cambridge, UK) for *Enterobacteriaceae* count (ISO 21528-2:2017/EC 1:2018) [15], onto DeMan, Rogosa and Sharpe Agar (Oxoid, Cambridge, UK) for Mesophilic Lactic Acid Bacteria count (ISO 15214:1998 (E) [16]. Yeasts and moulds count was performed by means of an internal method which involves direct surface plating on Malt Extract Agar plates (Oxoid, Cambridge, UK) incubated at 25°C for 5 days; presence or absence of *Listeria* spp. was performed by adding 225 mL of Fraser broth base (Oxoid, Cambridge, UK) at 25 g of sample according to ISO 11290-1 [17]. Physico-chemical parameters (pH and a_w) have also been evaluated as described in section 2.1.

Samples were incubated at 7°C or 10°C for their corresponding shelf-life.

2.2.3 Preparation of bacterial suspensions

The inoculum mixture was prepared by combining six strains of *L. monocytogenes*, two belonging at two international collections (*L. monocytogenes* ATCC 13932 and *L. monocytogenes* Scott A), one isolated from a vegetable product and belonging to SSICA collection (*L. monocytogenes* SSICA 36) and three belonging to the EURL Lm European collection (European Union Reference Laboratory for *Listeria monocytogenes*), isolated from food products and selected for their ability to grow at low a_w, pH and temperature values (*L. monocytogenes* 12MOB047LM, *L.*

monocytogenes 12MOB050LM and *L. monocytogenes* 12MOB051LM).

The selected strains were grown individually from cryobeads stored at -20°C in 10 mL of non-selective Brain Heart Infusion (BHI; Oxoid, Cambridge, UK) Broth at 37°C for 24 hours. Then they were transferred into 10 mL of BHI and incubated at 7°C for 3 days. The bacterial cultures were prepared in order to obtain for each strain a concentration of about 8 Log CFU/mL; the concentration of bacterial cultures was verified by counting them on Agar Listeria Ottaviani & Agosti (Biolife, Milan, Italy) plates incubated at 37°C for 24 hours under aerobic conditions according to ISO 11290-2:2017 method [18]. Before their use, the individual strains cultures were combined in equal volumes, in order to obtain a final concentration varying from 50 to 200 CFU/g. Each batch of pesto sauce was microbiologically and chemically characterized and aseptically divided in sterile jars containing 25 g of product. The samples were then inoculated with the mixture of *L. monocytogenes* strains; three samples for each batch were analysed in order to verify the initial concentration of the pathogen in the product (T_0).

2.2.4 Inoculated samples

The analyses to evaluate *L. monocytogenes* behaviour during the shelf-life of inoculated pesto sauces were carried out on three sample for each batch. *Listeria* spp count was performed by serial dilution and direct surface plating on Agar Listeria Ottaviani & Agosti (ALOA; Biolife, Milan, Italy) plates [18], for a total of 72 analyses.

For each batch, by means of Microbial Challenge Tests (MCT), the growth potential (δ) was calculated according to the formula: $\delta = Log_{max} - Log_I$, where Log_{max} is the highest average value recorded during the shelf-life and Log_i is the average value at time 0. The growth potential obtained for the three lots is the highest value observed.

Samples were incubated at 7°C or 10°C for their corresponding shelf-life, as required by “EURL Lm TECHNICAL GUIDANCE DOCUMENT” [10].

3. RESULTS AND DISCUSSION

3.1 Sample collection and chemical analyses

The evaluation of different types of fresh pesto sauces available on the market showed that most products had the same recipe (the only exception being the presence of garlic); two of the types tested were defined as "Bio", the most used cheese was "Grana Padano" and basil was predominantly “PDO” certified. Most of the investigated pesto sauces had pH values between 5.00 and 5.40 (44%), or between 4.60 and 4.90 represented (30%), whereas sauces with pH range between 4.20 and 4.50 were the 26%.

The most detected a_w values were between 0.90 and 0.92 (46%), pesto sauces with a_w range between 0.93 and 0.95 were the 40%, whereas products with a_w values between 0.86 and 0.89 represented the 12%. Only few samples had values equal to 0.96 (2%).

On the basis of the physical-chemical parameters, the processing of the data allowed to make a distinction between the pesto sauces in which the growth of *L. monocytogenes* is possible and those in which it is not, according to regulation (EU) No. 2073/2005 and subsequent amendments and integrations [8].

From this screening it clearly emerges that there is a discrete number (11/48) of pesto sauces in which the growth of the researched pathogen would be possible (Fig. 1). Among them, in five samples the presence of *L. monocytogenes* was detected in 25 g and in one sample the microorganism not only was detectable, but also present at a concentration of 20 CFU/g. It should be underlined that two of these pesto sauces had physical-chemical characteristics suitable for the growth of the pathogen.

3.2 Non inoculated samples

The results of the analyses carried out are shown in Figs. 2-4. The studied pesto sauces showed an initial microbial count of approximately 5-6 Log CFU/g; at 7°C, at the end of the storage, the concentration was substantially unchanged for pesto sauce A, whereas an increase of about 1 and 2 Log CFU/g was observed for pesto sauces B and C, respectively; at 10°C, at the end of storage, an increase of about 1 Log CFU/g was detected for pesto sauce A and of about 2 and 3 Log CFU/g for sauces B and C, respectively (Fig. 2).

A very similar trend was observed for mesophilic lactic acid bacteria count (Fig. 3.), the main constituents of the Total microbial count at 30°C, presumably originating from cheese.

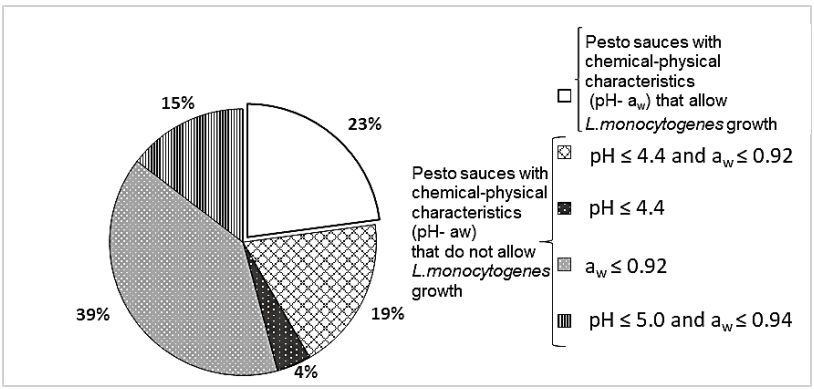

Fig. 1. Percentages of pesto sauces from the market suitable or not for *L. monocytogenes* growth, based on their physical-chemical characteristics

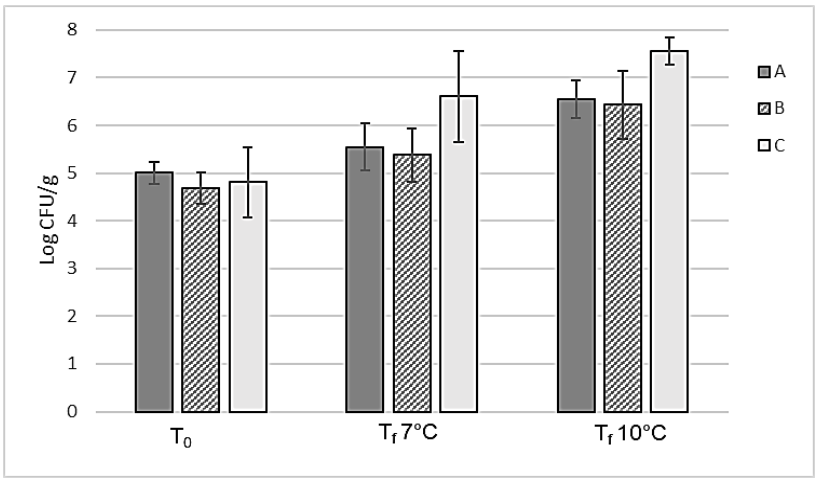

Fig. 2. Total microbial count at 30°C in pesto sauces A, B, C, at time 0 (T_0) and at the end of the shelf- life after storage at 7°C (T_f 7°C) or at 10°C (T_f 10°C). The error bars indicate the Standard deviation for mean values

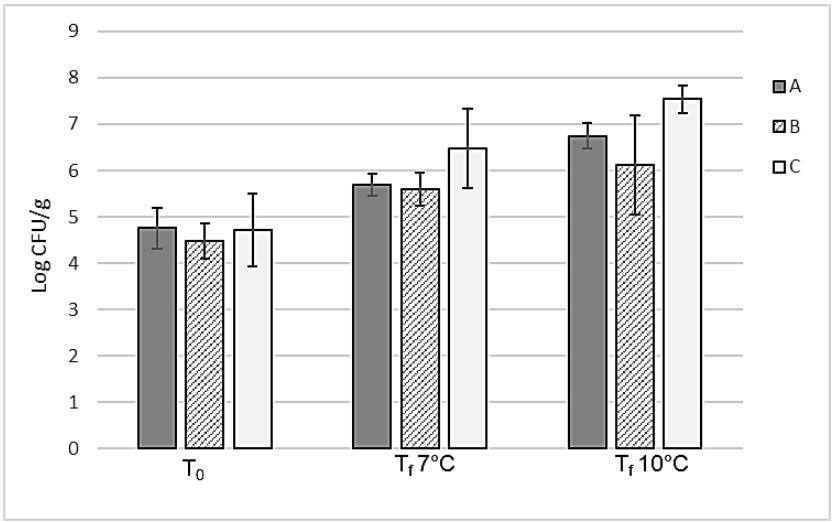

Fig. 3. Mesophilic lactic acid bacteria count in pesto sauces A, B, C, at time 0 (T_0) and at the end of the shelf- life after storage at 7°C (T_f 7°C) or at 10°C (T_f 10°C). The error bars indicate the Standard deviation for mean values

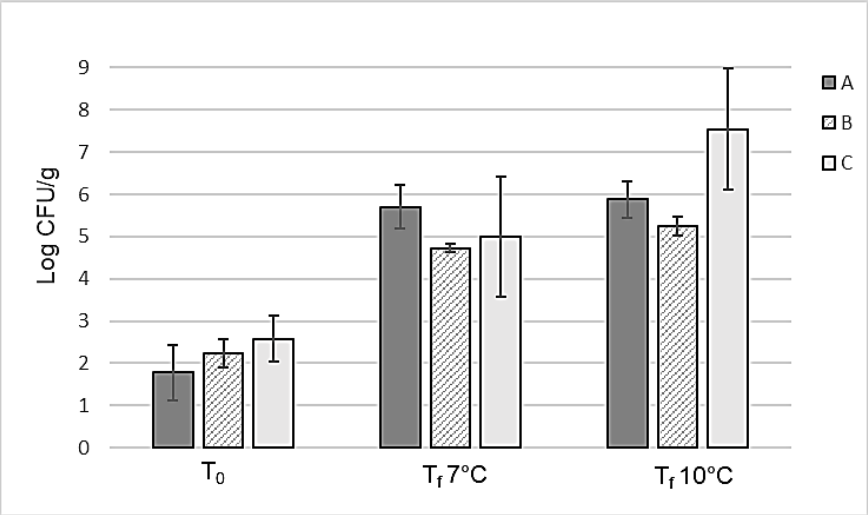

Fig. 4. Yeasts count in pesto sauces A, B, C, at time 0 (T_0) and at the end of the shelf- life after storage at 7°C (T_f 7°C) or at 10°C (T_f 10°C). The error bars indicate the Standard deviation for mean values

Yeasts were initially (T_0) present at concentrations of about 2-3 Log CFU/g, depending on the pesto sauce; at the end of the storage, a considerable increase were observed for all sauces, maybe due to the shelf-life of the product and to the storage temperature, as reported also by Zardetto et al. [19]. In pesto sauce A and B, at 7°C and at 10°C, yeasts reached a concentration of about 6 and 5 Log CFU/g, respectively. In pesto sauce C the concentration was about 5 and 7 Log CFU/g at the end of storage at 7°C and at 10°C, respectively, with a great variability between the different batches (Fig. 4.).

In sauces B and C *Enterobacteriaceae* were initially present at concentrations of 1-2 Log CFU/g, depending on the batches, whereas they were below the analytical limit in product A (data not reported). At the end of the shelf-life, in all types of sauces and at both storage temperatures, they were below the analytical limit (1 CFU/mL); they seemed to disappear over time, as observed by Vicini et al. [20]. Moulds concentration was always below the analytical limit in all pesto sauces for both storage temperatures.

L. monocytogenes was undetectable in 25 g of product in all sauces analysed at T_0.

The pH at T_0 was 5.50 in pesto sauce A, 5.10-5.32 in sauce B and 5.00-5.13 in sauce C. It remained unchanged in all samples at both storage temperatures, the only exception being the pesto sauce C, where it slightly decreased at the end of the shelf-life at 10°C, reaching a value of 4.92; no changes in a_w values were observed.

3.3 Inoculated samples

The results of the Microbial Challenge Tests (MCT) carried out are shown in Table 1. *L. monocytogenes* behaviour was assessed in pesto sauces A, B, C, since these sauces showed pH and a_w values supporting the growth of the studied microorganism.

In pesto sauce A the initial average inoculum concentrations of *L. monocytogenes* ranged between 1.85 and 2.22 Log CFU/g, with a standard deviations (sd) lower than 0.20, confirmed the inoculum correctness.

Pesto sauce A had pH and a_w values suitable for *L. monocytogenes* growth. However, the growth dynamics of the pathogen, highlighted in the study, indicate that the sauce does not represent a favourable substrate for the development of the microorganism during the shelf-life at the tested storage temperatures (δ <0.5 log CFU/g) [10]. It could be supposed a competitive action against *L. monocytogenes* from the naturally present microbiota consisting of lactic acid bacteria and yeasts which can grow in the product during its shelf-life, as reported by numerous scientific studies [21-24].

The initial inoculum concentration of *L. monocytogenes* in pesto sauce B varied between

Table 1. Results for *L. monocytogenes* inoculated in pesto sauces A, B, C during shelf-life at a storage temperatures of 7°C or 10°C

A							
Storage temperature 7°C							
batch	**T=0**	**T=3 days**	**T=7 days**	**T=10 days**	**δ batch**		**δ Pesto sauce**
	Mean ± sd*						
1	1.85±0.14	1.7 ±0.18	1.36±0.32	1.42±0.10	1.75 -1.85 = - 0.1		0.12
2	1.97±0.12	1.94±0.06	2.09±0.12	1.99±0.13	2.09 - 1.97 = 0.12		
3	2.22± 0.08	1.7 ±0.26	1.88±0.35	2.02±0.11	2.02 - 2.22 = -0.20		
Storage temperature 10°C							
1	1.85±0.14	1.72±0.10	1.50±0.17	1.33±0.35	1.72 - 1.85= -0.13		0.27
2	1.97±0.12	2.05±0.18	2.24±0.05	2.08±0.04	2.24 - 1.97 = 0.27		
3	2.22±0.08	1.93±0.24	2.00±0.27	2.02±0.29	2.02- 2.22= - 0.20		
B							
Storage temperature 7°C							
batch	T=0	T=7 days	T=14 days	T=21 days	T=28 days	δ batch	δ Pesto sauce
	Mean ± sd*						
1	2.06±0.11	2.08±0.06	2.16±0.15	2.09±0.08	2.14±0.11	2.16-2.06=0.1	0.10
2	1.74±0.13	1.64±0.05	1.68±0.04	1.40±0.17	1.39±0.36	1.68-1.74=-0.06	
3	2.36±0.03	1.87±0.11	2.05±0.10	2.10±0.18	1.77±0.07	2.10-2.36=-0.26	
Storage temperature 10°C							
1	2.06±0.11	1.91±0.18	2.04±0.23	2.12±0.07	1.86±0.07	2.12-2.06=0.06	0.06
2	1.74±0.13	1.50±0.1	1.48±0.10	0.80±0.17	1.40±0.17	1.50-1.74=-0.24	
3	2.36±0.03	1.97±0.18	2.00±0.04	2.02±0.15	1.70±0.20	2.02-2.36=-0.34	
C							
Storage temperature 7°C							
batch	T=0	T=10 days	T=20 days	T=30 days	T=40 days	δ batch	δ Pesto sauce
	Mean ± sd*						
1	1.76±0.15	1.10±0.17	0.90±0.17	0.70±0.00	0.70±0.00	1.10-1.76= -0.66	-0.66
2	1.95±0.05	1.22±0.45	1.10±0.17	1.00±0.30	0.80±0.17	1.22-1.95= -0.73	
3	2.04±0.07	1.22±0.45	1.20±0.17	0.70±0.00	0.70±0.00	1.22-2.04= -0.82	
Storage temperature 10°C							
1	1.76±0.15	1.00±0.30	0.80±0.17	0.70±0.00	0.70±0.00	1.00-1.76= -0.76	-0.57
2	1.95±0.05	1.38±0.60	1.03±0.58	1.03±0.58	0.70±0.00	1.38-1.95= -0.57	
3	2.04±0.07	1.38±0.60	1.16±0.41	0.70±0.00	0.70±0.00	1.38-2.04= -0.66	

1.74 and 2.36 Log CFU/g. This pesto sauce also showed pH and a_w values suitable for *L. monocytogenes* growth, but the growth dynamics of the pathogen confirm that product does not represent a favourable substrate for the development of the bacterium during shelf-life, at the storage temperatures tested (δ <0.5 log cfu/g). An increase in the microbiota naturally present during storage was also observed for this type of pesto sauce.

The initial contamination of *L. monocytogenes* in pesto C ranged from 1.76 to 2.04 Log CFU/g, with standard deviations less than 0.20. This pesto sauce had a pH value of 5.0-5.1 and aw of 0.95, suitable for *L. monocytogenes* growth; the behaviour of the pathogen indicates that the sauce not only does not represent a suitable substrate for the growth of the bacterium during shelf-life, but determined a slight inactivation of the pathogen during storage at the temperatures tested (δ log CFU/g negative). A large number of published studies demonstrated an antibacterial activity of basil essential oil as reported many authors [25,26], although it cannot be explained why the same inactivating action was not observed for the other types of pesto sauces tested, it could be argued a different origin of the basil used.

The results obtained expressed as Log CFU/g, and the related statistical calculations for the samples incubated at 7°C and at 10°C are reported in Table 1.

Microbial counts were converted into logarithms before means and standard deviations were computed, and counts were reported as Log CFU/g.

4. CONCLUSION

The screening carried out on 48 different types of fresh commercial pesto sauces highlighted a relevant number of pesto sauces with physical-chemical characteristics supporting the growth of *L. monocytogenes*; in five samples, the presence of the pathogen was also detected, in one sample the microorganism being present at a concentration of 20 CFU/g. Therefore, these Ligurian pesto sauces proved suitable to be used as a matrix for assessing *L. monocytogenes* growth.

Refrigerated, non-inoculated pesto sauces tested in this study showed an initial (T_0) Total Microbial Count at 30°C consisting mainly of lactic acid bacteria and yeasts that were present at variable concentrations and only in some cases of *Enterobacteriaceae*. During the storage, an increase in lactic acid bacteria and a considerable increase in yeasts was observed for all pesto sauces, thus being linked to storage time and temperature applied. The pH was generally unchanged, or slightly decreased in one of the tested pesto sauces stored at 10°C.

Refrigerated, inoculated pesto sauces highlighted the inability of the inoculated *L. monocytogenes* to grow at 7°C or 10°C during their shelf-life; in one case a slight inactivation of the pathogen was even observed. It can therefore be assumed that, at the temperatures assayed, all the pesto sauces tested were unable to support the growth of *L. monocytogenes.*

The results obtained from the Microbial Challenge Tests can help the food companies producing pesto sauces to predict the behaviour of the microorganism and correctly classify the product in accordance with the regulation (EU) No. 2073/2005 with its subsequent amendments.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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