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Characterisation of *Listeria monocytogenes* strains isolated from soft and semi-soft cheeses sampled in a region of Italy

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Summary

A total of 47 *Listeria monocytogenes* strains isolated in a survey of cheeses sampled from retail outlets were characterised. Five cheeses (Gorgonzola, Taleggio, Asiago, Crescenza and Brie) were chosen from the most popular soft and semi-soft cheeses consumed in Italy and most commonly contaminated with *L. monocytogenes*. The serotype and antibiotic resistance pattern were determined for each strain and their macrorestriction profile was analysed with pulsed-field gel electrophoresis (PFGE). The main serotypes detected were 1/2a (76.6%) and 1/2c (21.3%). Serotype 1/2b was found in only one sample. A total of 97.9% of strains were resistant to oxacillin (OX), 80.9% to lincomycin (L) and 78.7% to clindamycin (CC). Of these strains, 17% were found to be resistant to two antibiotics (OX-CC or OX-L) while 70.2% were resistant to three antibiotics (OX-CC-L). No strains were susceptible to all the compounds tested. A combined analysis of the macrorestriction profiles *Asc*I and *Apa*I identified eleven pulsotypes divided into three clusters. Two pulsotypes predominated, accounting for 57.4% and 21.3% of the isolated strains. Analysis of the PFGE profiles did not reveal any correlation between pulsotype and type of cheese, producer or retail outlet. A temporal analysis revealed that one pulsotype was persistent throughout the study period, with the exception of August and September, in which time a different pulsotype was detected. This variability suggests the influence of factors affecting the dynamics of the contamination of these products. Large-scale studies could help clarify this phenomenon.

Keywords

Antibiotic resistance, Cheese, Italy, *Listeria monocytogenes*, PFGE, Pulsed-field gel electrophoresis, Serotype.

Introduction

Listeria monocytogenes is the fifth most important cause of zoonoses today and the number of cases of listeriosis in humans has increased in the European Union in recent years (13). As this is a ubiquitous micro-organism that is capable of adapting to a wide range of environmental conditions, *L. monocytogenes* contamination in the food processing environment is a serious risk for ready-to-eat products such as cheeses. Between 1999 and 2001, about half of the outbreaks and sporadic cases of listeriosis in humans were associated with the consumption of cheese. In 2006 in the Czech Republic and 2007 in Norway, two outbreaks associated with cheese consumption caused 78 hospitalisations with 13 deaths (13) and 21 hospitalisations with 5 deaths (14), respectively. According to the results of the pilot project for

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L. monocytogenes surveillance conducted by the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'* (*Istituto G. Caporale*) in 2007, *L. monocytogenes* was isolated in 4.5% of Gorgonzola samples and 5.9% of Taleggio samples (23), in contrast with 1.4% reported by European Food Safety Authority (EFSA) for other Italian cheeses (12). Contamination levels were also high (up to 460 most probable number per gram [mpn/g] in Taleggio) – higher than the food safety limit fixed by European Commission regulation EC 2073/2005 (8).

The presence of *L. monocytogenes* in cheese is mainly environmental in origin and the mechanisms with which it spreads differ according to the production environment (19, 26). The predominance and persistence of specific subtypes suggest the existence of strains that have a particular ability to colonise food-processing environments and survive environmental stress (2, 16). It is still just as difficult to identify the sources of contamination in food processing environments and how they contribute to the contamination of the finished product, as it is to establish the connection between contaminated foods and sporadic cases of *L. monocytogenes* infection (11). Phenotyping methods, such as serotyping, have a low discriminatory capacity and are often time-consuming and laborious. These methods require the constant application of molecular typing techniques that enable correlation of isolated strains and the establishment of connections with particular matrices to evaluate the capacity of *L. monocytogenes* to adapt to specific niches. Although various typing methods have been developed, the gold standard is still pulsed-field gel electrophoresis (PFGE) due to its high reproducibility, robustness and advanced discriminatory capacity (22, 25), which make it a rapid and valid tool in the surveillance of outbreaks and sporadic cases of listeriosis (4, 15, 16, 25).

Studies to date that have characterised *L. monocytogenes* strains that have been isolated from cheese describe PFGE profiles (pulsotypes) in relation to the type of product examined (10, 19, 22) but have not examined any association between the strains isolated from different types of cheese. This study used serotyping, evaluation of antimicrobial resistance profiles and PFGE to characterise strains of *L. monocytogenes* isolated from five of the most popular soft and semi-soft cheeses consumed in Italy. Samples were taken from retail outlets in Abruzzo region so as to evaluate distribution and establish any possible association between the strains isolated and the type of product, retail outlet and sampling period.

Materials and methods

Samples

As part of the *Istituto G. Caporale* pilot project for the surveillance of *L. monocytogenes*, 47 *L. monocytogenes* strains isolated from soft and semi-soft cheeses taken from 39 retail outlets in Abruzzo between March 2005 and October 2006 were analysed. These consisted of 21 strains isolated from Gorgonzola, 21 from Taleggio, 3 from Brie, 1 from Crescenza and 1 from Asiago.

The strains underwent serotyping, analysis of antibiotic resistance and PFGE.

Serotyping

Strains were serotyped using the method described in the United States Food and Drug Administration *Bacteriological analytical manual* (3), using commercial sera for somatic (O) and flagellar (H) antigens (Denkan Seiken Co. Ltd, Tokyo).

Antibiotic resistance

Antibiotic susceptibility was tested on Mueller-Hinton agar according to Kirby-Bauer method (20), using the antibiotics presented in Table I.

The results were interpreted in accordance with the criteria of the Clinical and Laboratory Standards Institute (6, 7). The resistance profile of the strains was thus reported according to the abbreviation for the antibiotics to which they showed resistance.

Pulsed-field gel electrophoresis

L. monocytogenes strains were characterised using PFGE using the PulseNet protocol (5), involving the restriction enzymes *Asc*I and

Table I
Antibiotics used for the Mueller-Hinton agar diffusion test according to Kirby-Bauer
Cut-off values and concentration ranges as suggested by the Clinical and Laboratory Standards Institute

Antibiotic	Abbreviation	Cut-off value (mm) R≤	Concentration range tested (µg)
Ampicillin	AM	19*	10
Cephalothin	KF	14	30
Chloramphenicol	C	12	30
Clindamycin	CC	14	2
Enrofloxacin	ENO	16	5
Erythromycin	E	13	15
Gentamicin	GM	12	10
Lincomycin	L	9	2
Oxacillin	OX	10	1
Penicillin	P	19*	10 units
Streptomycin	S	11*	10
Tetracycline	TE	14	30
Trimethoprim + sulphamethoxazole	SXT	10	1.25/23.75
Vancomycin	VA	9	30

* values indicated by Becton Dickinson, approved by the Food and Drug Administration

*Apa*I and *Salmonella* serotyped *Braenderup* (H9812) as the standard. Bacterial suspensions were included in agarose, lysed, washed and digested with the restriction enzymes. The digested samples underwent electrophoresis in SeaKem Gold agarose 1% (BIOSPA, Milan) in the Chef Mapper XA (BioRad Inc, Hercules, California) at 6 V/cm with a pulse time between 4 sec and 40 sec for 22 h.

Analysis of pulsed-field gel electrophoresis profiles

The PFGE profiles were analysed using BioNumerics software version 4.0 (Applied Maths, Kortrijk). The similarities between the macrorestriction profiles (MRPs) were calculated using the Dice coefficient (17), applying an optimisation coefficient and band tolerance of 1.2% for both enzymes. Clustering was performed and dendrograms generated using the unweighted pair group method with arithmetic mean (UPGMA). Clustering reliability was estimated by calculating the cophenetic correlation coefficient for each dendrogram.

Results

Serotypes

Serotyping was successful for all 47 strains. The serotypes detected were as follows:

- 1/2a (76.6%)
- 1/2c (21.3%)
- 1/2b (2.1%).

Serotype 1/2a was observed in 76.2% of the strains isolated from Gorgonzola, 85.7% from Taleggio and 66.7% isolated from Brie. Serotype 1/2c was detected in 23.8% of the strains isolated from Gorgonzola, 14.3% of those isolated from Taleggio and was the only serotype isolated from Asiago and Crescenza. Serotype 1/2b was detected only in one sample of Brie (Table II).

Antimicrobial resistance

Five resistance profiles were identified in the 47 strains analysed, namely: the most common was OX-CC-L, followed by OX, OX-CC, OX-L and L (Table II). A total of 97.9% of strains were resistant to OX, 80.9% to L and 78.7% to CC. No resistance was observed in AM, KF, GM, E, ENO, SXT, VA, C, P, S or TE.

Table II
Serotype distribution and antibiotic resistance profiles in different types of cheese

Cheese	Serotype (%)			Resistance profile (%)				
	1/2	1/2 b	1/2 c	L	OX	OX CC	OX CC L	OX L
Asiago (*n* = 1)	–	–	100	–	–	–	100	–
Brie (*n* = 3)	66.7	33.3	–	–	–	–	100	–
Crescenza (*n* = 1)	–	–	100	–	–	–	100	–
Gorgonzola (*n* = 21)	76.2	–	23.8	4.8	–	4.8	81	9.5
Taleggio (*n* = 21)	85.7	–	14.3	–	23.8	14.3	52.4	9.5
Total	76.6	2.1	21.3	2.1	10.6	8.5	70.2	8.5

n number of strains
L lincomycin
OX oxacillin
CC clindamycin

A total of 17% of strains were found to be resistant to two antimicrobials (OX-CC and OX-L) while 70.2% (found in all five cheeses tested), were resistant to three antimicrobials (OX-CC-L); 12.7% of strains were resistant to a single antimicrobial (OX or L). No strains were susceptible to all the compounds tested. The distribution of the various resistance patterns with respect to type of cheese is presented in Table II.

Pulsed-field gel electrophoresis

All 47 strains were successfully typed with *Asc*I and *Apa*I. Both enzymes showed the same discriminatory capacity, with nine macro-restriction profiles identified for both restriction with *Asc*I and restriction with *Apa*I. Combined analysis produced 11 different pulsotypes (A-M), demonstrating a similarity of 66.7% (Fig. 1). The most commonly isolated pulsotypes were B (57.4%), I (21.3%) and A (4.3%). All of the remaining pulsotypes were only associated with a single strain.

Analysis of the dendrogram also meant that the strains could be grouped into three main clusters, namely: I (33 strains), II (2 strains) and III (10 strains), with a similarity value of 72.9%. Two strains (pulsotypes L and M), with similarity values of 66.7% and 67%, respectively, were located at the edges of two clusters. Cluster I, with a similarity of 85.5%, consisted of six pulsotypes (A-F), including pulsotypes A and B with a similarity of 96.2%, and C and D with 96.7% similarity. Cluster III consisted of a single pulsotype corresponding to profile I, which comprised 10 isolates with 100% similarity.

Distribution of pulsotypes among the different serotypes

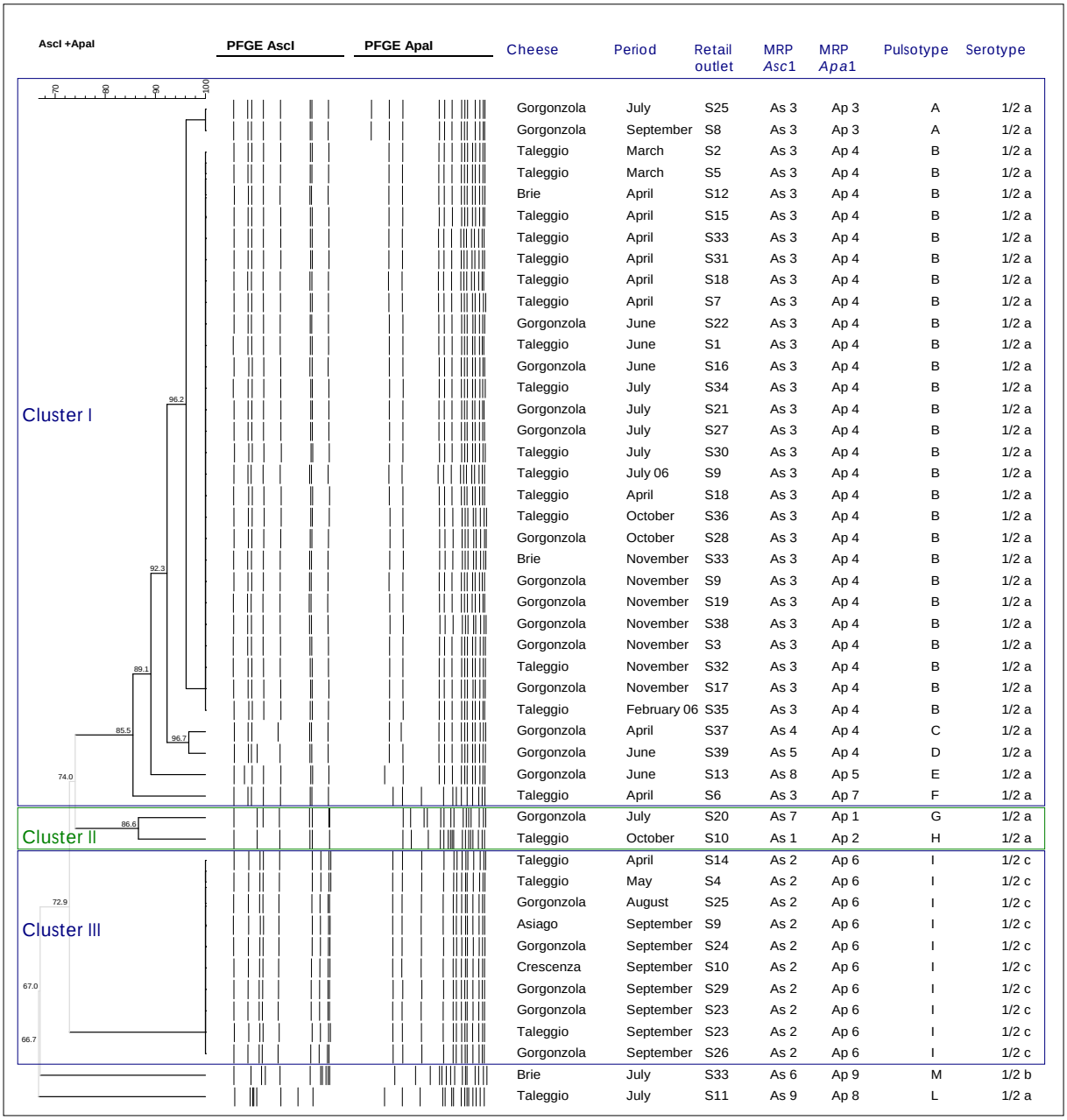
Correlation of the PFGE and serotyping results revealed that Cluster I included 91.7% of strains with serotype 1/2a, Cluster II consisted of two serotype 1/2a strains while all serotype 1/2c strains were found in Cluster III. The strains excluded from the three clusters belonged to serotypes 1/2a and 1/2b.

Pulsotype distribution among the different type of cheese

The distribution of the various pulsotypes with respect to type of cheese is given in Table III. Cluster 1 consisted of strains isolated from Gorgonzola, Taleggio and Brie. These strains corresponded to a single pulsotype (B). Cluster II comprised strains isolated from Gorgonzola and Taleggio, while Cluster III consisted of strains isolated from Gorgonzola, Taleggio, Asiago and Crescenza.

Temporal distribution of pulsotypes

The pulsotype distribution was analysed at four different times, namely: March-April, June-July, August-September and October-November. The genetic profile analysis showed that the Cluster I represented the 91.7%, 76.9% and 90% of *L. monocytogenes* strains isolated in March-April, June-July and October-November, respectively. Within the Cluster I, pulsotype B was identified at a



Cheese	Period	Retail outlet	MRP *Asc*1	MRP *Apa*1	Pulsotype	Serotype
Gorgonzola	July	S25	As 3	Ap 3	A	1/2 a
Gorgonzola	September	S8	As 3	Ap 3	A	1/2 a
Taleggio	March	S2	As 3	Ap 4	B	1/2 a
Taleggio	March	S5	As 3	Ap 4	B	1/2 a
Brie	April	S12	As 3	Ap 4	B	1/2 a
Taleggio	April	S15	As 3	Ap 4	B	1/2 a
Taleggio	April	S33	As 3	Ap 4	B	1/2 a
Taleggio	April	S31	As 3	Ap 4	B	1/2 a
Taleggio	April	S18	As 3	Ap 4	B	1/2 a
Taleggio	April	S7	As 3	Ap 4	B	1/2 a
Gorgonzola	June	S22	As 3	Ap 4	B	1/2 a
Taleggio	June	S1	As 3	Ap 4	B	1/2 a
Gorgonzola	June	S16	As 3	Ap 4	B	1/2 a
Taleggio	July	S34	As 3	Ap 4	B	1/2 a
Gorgonzola	July	S21	As 3	Ap 4	B	1/2 a
Gorgonzola	July	S27	As 3	Ap 4	B	1/2 a
Taleggio	July	S30	As 3	Ap 4	B	1/2 a
Taleggio	July 06	S9	As 3	Ap 4	B	1/2 a
Taleggio	April	S18	As 3	Ap 4	B	1/2 a
Taleggio	October	S36	As 3	Ap 4	B	1/2 a
Gorgonzola	October	S28	As 3	Ap 4	B	1/2 a
Brie	November	S33	As 3	Ap 4	B	1/2 a
Gorgonzola	November	S9	As 3	Ap 4	B	1/2 a
Gorgonzola	November	S19	As 3	Ap 4	B	1/2 a
Gorgonzola	November	S38	As 3	Ap 4	B	1/2 a
Gorgonzola	November	S3	As 3	Ap 4	B	1/2 a
Taleggio	November	S32	As 3	Ap 4	B	1/2 a
Gorgonzola	November	S17	As 3	Ap 4	B	1/2 a
Taleggio	February 06	S35	As 3	Ap 4	B	1/2 a
Gorgonzola	April	S37	As 4	Ap 4	C	1/2 a
Gorgonzola	June	S39	As 5	Ap 4	D	1/2 a
Gorgonzola	June	S13	As 8	Ap 5	E	1/2 a
Taleggio	April	S6	As 3	Ap 7	F	1/2 a
Gorgonzola	July	S20	As 7	Ap 1	G	1/2 a
Taleggio	October	S10	As 1	Ap 2	H	1/2 a
Taleggio	April	S14	As 2	Ap 6	I	1/2 c
Taleggio	May	S4	As 2	Ap 6	I	1/2 c
Gorgonzola	August	S25	As 2	Ap 6	I	1/2 c
Asiago	September	S9	As 2	Ap 6	I	1/2 c
Gorgonzola	September	S24	As 2	Ap 6	I	1/2 c
Crescenza	September	S10	As 2	Ap 6	I	1/2 c
Gorgonzola	September	S29	As 2	Ap 6	I	1/2 c
Gorgonzola	September	S23	As 2	Ap 6	I	1/2 c
Taleggio	September	S23	As 2	Ap 6	I	1/2 c
Gorgonzola	September	S26	As 2	Ap 6	I	1/2 c
Brie	July	S33	As 6	Ap 9	M	1/2 b
Taleggio	July	S11	As 9	Ap 8	L	1/2 a

MRP macrorestriction profile

Figure 1
Combined dendrogram of the *Asc*I and *Apa*I macrorestriction profiles
The similarities between the profiles were calculated using the Dice coefficient, with optimisation and tolerance set to 1.2 for both enzymes
Clustering and dendrogram generation were performed using the unweighted pair group method with arithmetic mean (UPGMA)
The cophonetic correlation is 98%

Table III
Percentage distribution of pulsotypes in the different cheeses

Pulse type	Cluster	Asiago (*n* = 1)	Brie (*n* = 3)	Crescenza *n* = 1)	Gorgonzola (*n* = 21)	Taleggio (*n* = 21)	Total
A	I				9.5		4.3
B			66.7		47.6	71.4	57.4
C					4.8		2.1
D					4.8		2.1
E					4.8		2.1
F						4.8	2.1
G	II				4.8		2.1
H						4.8	2.1
I	III	100		100	23.8	14.3	21.3
L						4.8	2.1
M			33.3				2.1

higher frequency (75% in March-April, 57.1% in June-July and 90% in October-November). In contrast, in August-September, 88.9% of strains were grouped in Cluster III (pulsotype I) (Fig. 2).

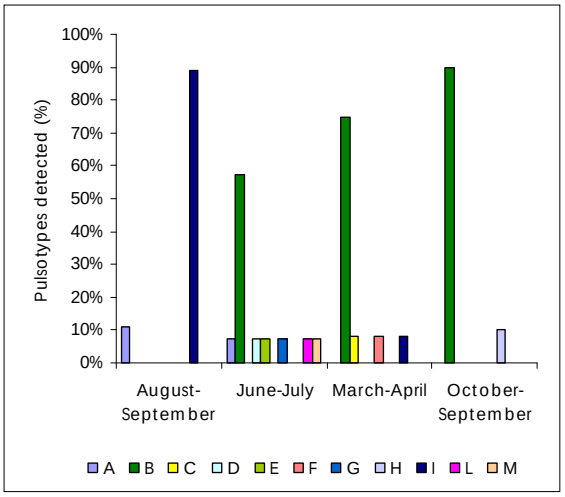


Figure 2
Temporal distribution of pulsotypes

Discussion

This study was designed to reveal any correlation between *L. monocytogenes* strains isolated from five types of soft and semi-soft cheese on retail sale, in addition to the product type, retail outlet and sampling period. The strains were analysed by serotyping, assessment of antimicrobial resistance profiles and PFGE.

Serotype 1/2a predominated, accounting for 76.6% of all *L. monocytogenes* strains isolated, in line with previous studies that also found the predominance of this serotype in strains isolated from cheese and cases of listeriosis associated with the consumption of cheese (16, 22). Some authors consider that this serotype may have particular characteristics which make it more resistant to the conditions prevalent in the production processes (24). Studies of the correlation between serotype and environmental factors revealed that the prevalence of serotype 1/2a in foods may be due to its greater resistance to bacteriocins (6). In contrast with the results of other authors (16, 21, 22), serotype 1/2b was only isolated from a single sample of Brie. However, serotype 1/2c, the prevalence of which in cheese is normally lower than in other matrices (1, 21), was identified in 21.3% of strains.

The results of the antimicrobial resistance tests generally confirmed susceptibility to antimicrobials of choice for treatment of cases of listeriosis (penicillin, ampicillin, gentamicin and trimethoprim in combination with sulphamethoxazole) (9). There was little difference in the resistance profiles found in the different types of cheese, and consequently this was not useful in distinguishing between the strains isolated.

Analysis with respect to retail outlet showed an indiscriminate distribution of the two

principal pulsotypes, namely: B (57.4%) and I (21.3%). Consequently, we could exclude the possibility that the isolated strains might derive from the contamination of utensils or surfaces in retail outlets with a counter service.

The presence of strains with identical macro-restriction profiles isolated from different cheese types could indicate their widespread natural distribution, resulting in their more frequent reintroduction into the processing plants through raw materials (4). This presence could also be attributable to specific characteristics of these strains, such as greater adherence to surfaces and/or resistance to disinfectants that enable them to persist in the production environment, causing long-term contamination affecting different products prepared in the same location.

However, an interesting difference was observed in the prevalence of the two pulsotypes in some of the months in which sampling was performed. A total of 80% (10) of Cluster III strains (profile I), although found in four different types of cheese (Gorgonzola, Taleggio, Crescenza and Asiago) were only isolated in August-September, while the isolated strains were found in Cluster I both before and after this date. This could suggest the presence of a factor that caused the onset of a clone becoming temporarily predominant due to its resistance characteristics or certain environmental conditions. Profile B, found in 57.4% of the strains, was found at different times of year, with the highest frequencies observed in March-April (33.3%), June-July (29.6%) and October-November (33.3%). Some authors have suggested that the isolation of strains with an identical profile at different times of year may be linked to the capacity of this clone to persist in the environment and thereby contaminate different products and/or raw materials (18). Other studies have demonstrated that the finding of strains with identical pulsotypes in different contexts (matrices, producers and different times of year) could indicate the existence of temporally and geographically non-specific strains (4).

Conclusions

An analysis of isolated strains did not reveal any correlation between serotype, pulsotype, cheese type or retail outlet. The prevalent serotypes and pulsotypes were in fact indiscriminately distributed among the samples, with no particular association with any cheese type.

Molecular profiling revealed a prevalence of specific pulsotypes, suggesting the persistence of clones becoming predominant due to their resistance characteristics or to particular environmental conditions.

Further testing is required to further evaluate the capacity of *L. monocytogenes* to persist in the processing environment. Evaluation of adaptivity and cross-adaptivity to cleaning products, degree of adhesion to surfaces and the production of biofilms could help to identify characteristics that would contribute to the discrimination between the strains.

Large-scale, specific studies are therefore necessary to identify the sources and dynamics of *L. monocytogenes* contamination in the production of the most popular – and most frequently contaminated – soft and semi-soft cheeses.

Identifying the sources and their contribution to the contamination of the finished product is a priority that should be established before any effective control measure can be implemented.

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