Listeria monocytogenes in Gorgonzola: subtypes, diversity and persistance over time

Listeria monocytogenes in Gorgonzola: Subtypes, diversity and persistence over time

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Abstract

L. monocytogenes represents a primary concern in the production of Gorgonzola, a Protected Designation of Origin (PDO) Italian blue-veined cheese produced only in the Piedmont and Lombardy regions. *L. monocytogenes* isolates (N = 95) obtained from Gorgonzola rinds, paste, and production/ripening environments were serotyped and then genotyped using Pulsed Field Gel Electrophoresis (PFGE). The goal of this study was to investigate the variability of *L. monocytogenes* PFGE-types across different PDO Gorgonzola manufacturers (N = 22). The majority of the strains (88%) were serotyped as 1/2a. PFGE identified 2 major pulse-types grouping 62 strains, detected from different plants and years, suggesting the presence of persistent and niche-adapted *L. monocytogenes*. In 9 plants, environmental strains shared the same pulse-types with strains from rinds or paste, suggesting a possible transmission pathway. Encouragingly, *L. monocytogenes* was retrieved from only 1 paste, indicating that production processes were under control in 21 plants. In the remaining plant, un-effective pasteurization or cross-contamination during production processes could be the cause of the contamination. Consequently, it is imperative that producers operate under the total respect of the Good Manufacturing Practices and following the principles of the Hazard Analysis Critical Control Point plans, in order to contain contamination throughout the whole processing.

1. Introduction

Listeria monocytogenes is a potentially dangerous foodborne pathogen, which has been isolated from different food products and has been associated with several outbreaks of human listeriosis, sometimes involving large numbers of consumers. The high risk groups are identified as YOPI (Young, Old, Pregnant, Immuno-compromised), in which *L. monocytogenes* can cause septicaemia and meningitis with high mortality rates.

In Italy, listeriosis was made a notifiable disease in 1991; in 1993 *L. monocytogenes* food surveillance program was established. Between 1993 and 2000, 283 cases occurred; however, the specific food responsible for the infection was identified on only a few occasions (Franciosa et al., 1998 and Aureli et al., 2000).

The transmission of *L. monocytogenes* from foods to humans is mostly related to those products, which do not receive any heat treatment by the consumer, so-called ready-to-eat (RTE), which include cheeses among others. The presence of *Listeria* sp. in cheeses can be due to different factors: e.g. unsatisfactory pasteurization treatment or contamination after heat treatment (i.e. in the ripening rooms or during transportation from the processing plant to ripening facilities). In particular, contamination may be enhanced by improper sanitizing procedures and/or development of resistance to chemicals routinely used for sanitization and/or biofilm formation. Moreover, some properties of *L. monocytogenes*, such as tolerance to low pH values (up to 4.4), to high salt concentrations and the ability to grow at refrigeration temperatures, allow the survival of this pathogen during dairy production processes. It is, therefore, critical to prevent *L. monocytogenes* contamination in dairy processing plants by tightening hygienic control measures during the whole cheese production chain.

L. monocytogenes represents a primary concern in the production of Gorgonzola, a Protected Designation of Origin (PDO) Italian blue-veined cheese produced only in the Piedmont and Lombardy regions (Pinto and Reali, 1996 and Bottarelli et al., 1999). In fact, the ubiquitous nature of the pathogen and its adaptability to different niches poses a risk for food processing industry which has been struggling to eradicate *L. monocytogenes* from processing plant environments. As a result, even cheese made from pasteurized milk can become contaminated post-pasteurization. In particular, Gorgonzola is made from pasteurized cow's milk, but the fungal maturation and the pH fluctuations during ripening can allow the survival and reproduction of Listeria sp., mostly on rinds, contaminated as a result of cross-contaminations from the ripening environments or during handling (Carminati et al., 2004). Nonetheless, the presence of the pathogen on rinds cannot be considered a direct risk for the consumer, since rinds are not edible (UE Notice No 2008/C 111/17, 2008) and do not usually allow the spread of the pathogen to the cheese paste (Manfreda et al., 2005). Indeed, a recent survey evidenced that Gorgonzola paste was never contaminated with the bacterium, even if the rind was (GOLIS project, 2004).

Considering the economic importance of Gorgonzola (CPGC, 2007) and its worldwide commercialization, it is very important to understand how *L. monocytogenes* survives, replicates and contaminates production environments and possibly final products.

Discriminative molecular subtyping methods have been used for the characterization of *L. monocytogenes* in order to hypothesize routes of transmission (Wiedmann, 2002). Phenotypic typing methods, such as sero- and phage-typing have low discriminatory power and may require labor intensive protocols. For these reasons, molecular subtyping methods have been extensively applied for tracking *L. monocytogenes*. Although new methods are being investigated and developed, Pulsed-Field Gel Electrophoresis (PFGE) is still currently the 'gold standard' subtyping method for *L. monocytogenes*, due to its high reproducibility, robustness and high discriminating power (Graves and Swaminathan, 2001 and Hyytiä-Trees et al., 2007).

The application of typing methods for the identification of contamination pathways can point out critical control points (CCPs) in the production chain. These CCPs may lead to the identification of possible means to prevent or reduce the contamination of the final product (Gudmundsdóttir et al., 2005, Leite et al., 2006 and Ho et al., 2007).

The goal of this study was to investigate the presence of *L. monocytogenes* PFGE types in the PDO Gorgonzola production region in order to get preliminary data on types distribution across producers.

2. Materials and methods

2.1. Samples

A panel of 95 *L. monocytogenes* strains was analyzed in this study. Strains were obtained from 22 Gorgonzola cheese processing plants during a 4 year period, from 2004 to 2007. Processing plants were located in Piedmont (N = 13) and in Lombardy (N = 9). *L. monocytogenes* strains were isolated from Gorgonzola rinds (N = 45), cheese paste (N = 1), and in environments and working surfaces in contact

with the products (N = 49). For each sample the sampling year, sample typology and processing plant were recorded (Table 1).

2.2. *L. monocytogenes* isolation and identification

Detection of *L. monocytogenes* was performed using the AFNOR V08/055 procedure (1997). The strains were identified based on colony morphology and results of conventional biochemical tests: Gram-staining, catalase test, beta-haemolysis, carbohydrate utilisation and Camp test. Positive colonies were further confirmed by API-Listeria (BioMerieux) and then tested by *L. monocytogenes* specific PCR assay, amplifying a 274 bp. fragment of the pfrA gene (D'Agostino et al., 2004).

2.3. Serotyping

Serotyping was performed following the scheme established by Seeliger and Hohne (1979). Strains were serotyped using antisera against somatic (O) and flagellar (H) antigens according to manufacturer instructions (Denka Seiken, Tokyo, Japan) with minor modifications: for determination of H antigens, isolates were passed three to four times through semi-solid BHI broth containing agar 0.2% w⁄v at 25 °C.

2.4. Pulsed-field gel electrophoresis (PFGE)

Genomic DNA from *L. monocytogenes* strains was prepared in agarose plugs as described in Graves and Swaminathan (2001). DNA cleavage was achieved by restriction enzyme digestion of agarose-immobilised DNA using AscI and ApaI (New England Biolabs, Beverly, MA). Restricted samples were electrophoresed on 1% SeaKem Gold agarose in 0.5× TBE at 6 V/cm on a Chef DR III system (Bio-Rad, Hercules, CA, USA). A linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C and ± 60° were applied for 21 h.

Fingerprints were analysed with Bionumerics software (v 2.0, Applied Maths, Kortrijk, Belgium); similarity between fingerprints was determined by the Dice coefficient using an optimization value of 1.2% for AscI and 1.4% for ApaI. Band position tolerance was 1.4% for both the enzymes. The results were then combined and a dendrogram was generated by the Complete Linkage algorithm (Aldenberger and Blashfield, 1984). In order to identify identical PFGE types, a Dice coefficient similarity of 100% was used.

3. Results and discussion

3.1. *L. monocytogenes* detection and serotyping

All 95 strains identified as *L. monocytogenes* by biochemical tests were confirmed with PCR.

All *L. monocytogenes* strains were typeable and four O:H serotypes were identified. Serotype 1/2a represented 88% of the isolates (N = 84), serotypes 3a and 1/2b each accounted for 5% (N = 5 each)

whereas serotype 4b was observed only in one isolate. Based on these results, serotype 1/2a was the most frequently isolated from PDO Gorgonzola producing plants as reported in recent researches (Carminati et al., 2004, Manfreda et al., 2005 and Gianfranceschi et al., 2007). However, this may be a concern considering that serotype 1/2a was responsible for a case of listeriosis related to Gorgonzola consumption (Gianfranceschi et al., 2006).

3.2. PFGE

All 95 *L. monocytogenes* strains were typeable with both AscI and ApaI. The combination of the patterns generated by the two enzymes divided the isolates into 29 profiles. The PFGE-generated dendrogram (Fig. 1) allowed the identification of 3 isolates sharing 0% similarity with the remaining 92, which were similar at the 21% level. Those 92 isolates were divided into 21 unique profiles (indicated with isolates' ID No.) and 5 profiles (indicated with letters A–E) grouping the remaining 71 strains (Fig. 1 and Table 2).

In particular, profiles A and B contained only two isolates each, whereas profiles C, D and E contained 45, 17 and 5 isolates, respectively (Table 2). Moreover, the similarity between profile C and D was 97% and between these two profiles and profile E was more than 78% (Fig. 1).

The isolates belonging to profiles C and D, showed only one or two bands difference. This might indicate one of them is a mutant variant of the other. In fact, in each of the 5 PFGE profiles, except for A, isolates were detected in different years and from different processing plants (Table 2). However, only profiles C and D were recovered over a three and four year period from 14 and 5 different processing plants, respectively (Table 2).

The results suggested the presence of persistent and widely diffused *L. monocytogenes* strains, contaminating different plants over time. Persistent strains in food processing plants have already been reported by other researches (Vogel et al., 2001 and Leite et al., 2006). Some authors showed the persistency of a strain in a dairy plant for more than 7 years (Unnerstad et al., 1996). In addition, the presence of profile C in Gorgonzola producing plants located in 7 provinces, far apart from each other (from 37 up to 150 km) and without sharing the same dairy farms, may suggest that this genotype is widespread in the Piedmont–Lombardy region.

The presence of low strain heterogeneity in *L. monocytogenes* strains isolated from cheese rinds and Gorgonzola producing environments was already observed by Carminati et al. (2004) who studied 42 manufacturers in the same study area. These authors found the pathogen in 57.1% of the producing plants (44.8% of cheese rinds), and all the serotype 1/2a strains were grouped by Random Amplified Polymorphic DNA into 5 genotypes. Similarly, Manfreda et al. (2005), were able to isolate the pathogen over a one year period from a single Gorgonzola producer and ribotyping evidenced a low genetic heterogeneity of strains isolated from cheese and environment. The different subtyping techniques (this study; Manfreda et al., 2005 and Carminati et al., 2004) allowed the identification of low genotypic variability in Gorgonzola associated strains, thus suggesting that some *L. monocytogenes* strains may be well established in the environmental niche of Gorgonzola production. In fact, no identical patterns were found in *L. monocytogenes* strains retrieved from other foods or cheeses, when the genotype of the

recurrent strains (A and B profiles) was compared with the other pulse-types present in the laboratory's internal PFGE-database (data not shown).

The presence of a relatively highly clonal population of *L. monocytogenes* in Gorgonzola environment may be explained by two different hypotheses: (i) the use of the same processing conditions (dry salting, low temperatures of ripening, rise of pH, washing of the rind with brine) may select clones able to out-compete others (Carminati et al., 2004); (ii) the isolation procedures could suppress or favour only certain clones (Loncarevic et al., 1996).

Furthermore it needs to be pointed out that different cleaning strategies were used in the processing plants analyzed in the present study, although the majority of the producers sanitized the environments with peroxides and ammonium compounds. The detection of *L. monocytogenes* from surfaces could be explained by a decreased efficacy of the sanitizing compounds used or faults in the hygienic strategies employed. However, future research is being planned, in order to confirm the ability of these isolates to resist the sanitizing agent and/or to produce stable biofilm on surfaces.

The subtyping of *L. monocytogenes* showed that fourteen plants harboured more than one profile; in particular, for processing plants X and XXII, two of the largest producers, it was possible to detect the presence of 6 and 5 profiles, respectively (Table 3). These manufacturers used to rent some of their ripening rooms to small scale producers, thus allowing the entrance of Gorgonzola wheels produced elsewhere, therefore giving possible access to other *L. monocytogenes* types. However, no definitive conclusions can be drawn on *L. monocytogenes* transmission between producers given the collected data. In fact, the discrepancies between the number of PFGE types across producers is likely due to sampling inadequacy, since few strains per producer were collected each year.

In nine facilities the same profiles were found in cheese rinds and in the environmental samples. The presence of the pathogen on rinds may represent a risk because during storage, rinds may became moist, allowing the transfer of the pathogen to cheese paste (Manfreda et al., 2005).

It has to be noted that only 1 cheese paste tested positive for *L. monocytogenes* during the whole study period indicating that the production processes are under effective control in all the visited plants, except producer XII. The cheese paste shared the same profile with environmental strains (Table 4), thus representing a concern because it may imply that *L. monocytogenes* can be transmitted from the environment to the produced cheese.

However, contamination of the paste or transfer of the pathogen to the paste from the rinds is highly unlikely and depends on the type of growth of *L. monocytogenes* on the rinds (GOLIS project, 2004). In particular, the presence of the pathogen in the paste may be related to un-effective pasteurization or to cross-contamination, during production processes such as piercing, wheel cutting and final portioning. In these processes, *L. monocytogenes* can be transferred from the rind to the paste of the same cheese by the mechanical action of the cutting machine or by cross-contamination of other wheels due to a contamination of the blades or other portioning equipment.

In fact, the portioning procedures cannot be considered low-risk practices because once in the paste, *L. monocytogenes* is able to grow and multiply during the whole shelf-life reaching levels 10–1000 times

higher than the initial contamination (GOLIS project, 2004). However it needs to be emphasized that the presence of the bacterium on the rinds does not necessarily imply a high risk for the consumer. In fact Gorgonzola paste consistently tests negative for *L. monocytogenes* when official controls of the Competent Authority are performed (GOLIS project, 2004).

Moreover, in order to further decrease consumer exposure, the recent EU notice 2008/C 111/17 explicitly stated that Gorgonzola rinds are not edible, information that needs to be clearly presented on the labels/packages.

Nonetheless, Gorgonzola cheese was recently implicated in a listeriosis outbreak in Italy (Gianfranceschi et al., 2006). Consequently, it is imperative that producers operate under the total respect of the Good Manufacturing Practices and following the principles of the Hazard Analysis Critical Control Point (HACCP) plans, in order to contain contamination throughout the whole processing.

In addition, the present study unveils the important role that operators in Gorgonzola producing facilities may play in spreading of the pathogen, as revealed by *L. monocytogenes* strains detected in hallways, wheels of internal transport vehicles, locker rooms and toilets. Therefore, operators training as a critical part in the HACCP procedures becomes essential.

In conclusion, this research supports the importance of a stringent *L. monocytogenes* control strategy including regular environmental and sanitizing programs monitoring in every stage of cheese production. The application of molecular typing techniques for tracing the source of contamination in Gorgonzola production may lead to a better understanding of the routes of *L. monocytogenes* spread thus allowing the planning of powerful preventive measures.

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Table 1. List of *L. monocytogenes* (N = 95) subtyped in this study

ID no.	Source[a]	Processing plant	Year	*N* [b]
1–3	C	I	2005	3
4,5	C	II	2005	5
6,7	E	II	2004	
8	C	II	2004	
9,10	E	III	2005	2
11	E	IV	2005	2
12	C	IV	2005	
13	C	V	2005	1
14	C	VI	2005	1
15–20	C	VII	2005	10
21–24	E	VII	2005	
25	E	VIII	2004	2
26	C	VIII	2004	
27	E	IX	2005	1
28–33	C	X	2007	12
34–39	E	X	2007	
40–42	E	XI	2005	5
43,44	C	XI	2005	
45–47	E	XII	2005	4
48	C	XII	2005	
49,50	E	XIII	2005	2
51–57	E	XIV	2005	11
58–61	C	XIV	2005	
62–65	C	XV	2005	4
66,67	C	XVI	2005	2
68–71	E	XVII	2004	4
72–79	C	XVIII	2004	12
80–83	E	XVIII	2004	
84	E	XIX	2004	1
85,86	E	XX	2004	2
87,88	E	XXI	2004	5
89–91	C	XXI	2004	
92–95	E	XXII	2007	4

[a] C, cheese sample; E, environmental sample.

[b] Number of strains per processing plant.

Table 2. Distribution of the five major PFGE profiles (boxes in Fig. 1) within Gorgonzola processing plants

PFGE profile (*N*)	Sample ID	Producer	Year
A (2)	25,26	VIII	2004
B (2)	14	VI	2005
	20	VII	2005
C (45)[a]	6,7,8	III	2004
	15,16,18,19, 21,22,23,24	VII	2005
	28,29	X	2007
	41,43,44	XI	2005
	46,47,48	XII	2005
	50	XIII	2005
	58	XIV	2005
	62,63,64	XV	2005
	66,67	XVI	2005
	69,70,71	XVII	2004
	72,73,74,75,76,77,78,79,80,81,82,83	XVIII	2004
	84	XIX	2004
	85,86	XX	2004
	90	XXI	2004
D (17)	1	I	2005
	17	VII	2005
	31,33,34,35,36	X	2007
	45	XII	2005
	51,52,53,55,56,57,59,60,61	XIV	2005
E (5)	42	XI	2005
	87,88,89,91	XXI	2004

[a] Included 45 *L .monocytogenes* isolated from 14 plants in 2004, 2005 and 2007.

Table 3. Number of PFGE profiles in each processing plant. Profiles were defined by combining both *Asc*I and *Apa*I pulse-types.

Producer	PFGE profiles
I	3
II	3
III	2
IV	2
V	1
VI	1
VII	3
VIII	1
IX	1
X	6
XI	3
XII	2
XIII	2
XIV	3
XV	2
XVI	1
XVII	2
XVIII	1
XIX	1
XX	1
XXI	2
XXII	5

Table 4. Manufacturers where *L. monocytogenes* contamination was observed from more than one sampling location

Producer	Contaminated locations
II	Rind, wheel of internal transport vehicle, pasteurizer external surface
VII	Rind, floor drains in ripening room, salting and weighting area
VIII	Rind, brushing machine
X	Rind, floor and floor drain in storage area
XI	Rind, floor drains in ripening buffer room
XII	Cheese, ripening floor and surfaces
XIV	Rind, floor drains in salting area, holing room and floor in locker room
XVIII	Rind, floor of ripening room, floor drains in locker room and in hallway
XXI	Rind, salting area surfaces, floor in men's restrooms

Fig. 1. Dendrogram generated from the PFGE profiles (AscI + ApaI) of 95 *L. monocytogenes* isolates. The dendrogram was obtained with Bionumerics software (v2.0, Applied Maths, Kortrijk, Belgium) using the complete linkage algorithm (Aldenberger and Blashfield, 1984).