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Presence of *Escherichia coli* O157, *Salmonella* spp. and *Listeria monocytogenes* in raw ovine milk destined for cheese production

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Running title: Pathogen presence in raw sheep milk and cheese

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Abstract

Italy is one of the main producers and exporters of cheese made from unpasteurized sheep milk. Since raw milk and its derived products are known sources of human infections, cheese produced from raw sheep milk could pose a microbiological threat to public health. A thorough investigation of pathogen presence in raw ovine milk and its related products could therefore provide useful information to understand the epidemiological state and to plan prevention actions.

Hence, the first objective of the study was to assess the possible presence of *Escherichia coli* O157, *Salmonella* spp. and *Listeria monocytogens* on all the sheep farms (n. 24) in the Marches region (Central Italy) that directly transform raw milk into cheeses. Moreover, a complete three-step multiple platform, consisting of: (a) simultaneous culture-enrichment in a specifically formulated medium, (b) species-specific DNA magnetic isolation and (c) multiplex real-time PCR, was first validated to prove its equivalence with reference methods, then employed for milk and cheese analysis. Ovine milk samples (n. 143) were collected over a three-year period and analysed by both official reference methods and the molecular platform. Cheese samples (n. 5) were also collected from the farms which tested positive for target pathogens in milk and subsequently analysed. *L. monocytogenes* were not detected on any of the farms, while *E. coli* O157 was found on three farms, although only using the molecular method. Four farms tested positive for *Salmonella* spp., and *S. enterica* subsp. diarizonae serovar 61:k:1,5,7 was isolated in one of those cases. This information could be useful in developing preventative measures to guarantee a high level of consumer safety for this specific product line, and the molecular method could be a time-saving and sensitive tool to be used in routine diagnosis.

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1. Introduction

Sheep milk production is a significant economic sector for Southern European countries, and the continent remains a major actor among world sheep cheese producers and exporters despite the expansion of Middle Eastern producers (De Rancourt *et al.*, 2011). Regarding Italy, nearly 400,000 tonnes of sheep milk and 52,000 tonnes of derived cheese were produced in 2013, and this country ranks first among the main exporters of cheese made from sheep milk, with yearly exports of 18,741 tonnes (FAOSTAT, 2015). Within Italy, the Marches region (Central Italy) is an area particularly devoted to sheep milk and cheese production, and many types of cheese are made with unpasteurised sheep milk across Italy. The so-called "typical products", which are often manufactured by the same small farm producing the milk, are popular among consumers and help to preserve local traditions. However, such products can pose a microbiological threat to public health, since raw milk and derived products are well-known sources of human infections (Gould, *et al.*, 2014).

Considering the high level of consumption in Italy of locally produced cheeses and derived products made from raw ovine milk, our knowledge of the presence of *Escherichia coli* O157 in this product line appears to be inadequate. While the role of cattle as a natural reservoir of this pathogen is well recognized, there are very few references relating to other ruminants. Several authors have reported the isolation of this species from the gut content of sheep and goats (La Ragione, *et al.*, 2009, Osman, *et al.*, 2013) and in raw ovine milk (Rey *et al*., 2006; Sanchez *et al*., 2010, Solomakos *et al*., 2009). In Italy, Pepe *et al.* (1998) underlined *E. coli* O157 presence in 2.9% of the ovine carcasses examined, findings which are in line with other studies conducted in Europe and the USA (Pinaka *et al*., 2013, Kilonzo *et al*., 2011). Moreover, goat milk and related dairy products have sometimes been associated with human cases of *E. coli* O157 infection (Caprioli *et al.*, 1998; Allerberger *et al*., 2001; McIntyre *et al*., 2002). In the Lazio region, strains of *E. coli* O157 have been isolated from ovine milk with a prevalence of 0.6% of examined samples (Rubini *et al*., 1999).

L. monocytogenes have been found in hard cheeses made from raw or low heat treated sheep milk (EFSA, 2015) in EU countries, including Italy, with high positivity percentages (Ministry of Work, Health and Social Policies, 2009). Moreover, 36 notifications for *Salmonella* and *L. monocytogenes* in milk and milk products were also reported by the RASFF Portal in 2015 (RASFF, 2015).

A more thorough investigation of pathogen presence in raw ovine milk and related products could therefore provide useful information. Hence, the first objective of this study was to determine the true presence of *E. coli* O157, *Salmonella* spp. and *Listeria monocytogens* on ovine milk farms in the Marches region, for which epidemiological data are lacking.

Recent investigations, carried out on bovine raw milk, highlight the greater sensitivity of PCR-based methods for the detection of the cited microbial species compared to conventional reference methods (Amagliani *et al*., 2012; Giacometti *et al*., 2013). These results proved that the analytical system plays a main role in epidemiological investigations. Molecular diagnosis carries the additional advantage of significantly reducing analysis time, particularly if we consider the current trend, which is moving towards the identification of more than one pathogen through the development of multiple analysis platforms (Omiccioli *et al.*, 2009; Sjöling *et al.*, 2015). These systems are attractive and economically advantageous because they reduce the total space requirement for handling large sample numbers, supplies and reagents, as well as the need for manpower.

However, the reliability of PCR detection methods depends, in part, on the purity of the target template and the presence of sufficient numbers of target molecules, especially with such complex matrices as foods containing potentially inhibitory compounds that may affect PCR amplification of the intended target. In the present study, a Magnetic Capture Hybridisation (MCH) assay, which relies on selective isolation of target DNA by hybridisation to oligonucleotide probes linked to magnetic nanoparticles, was proposed. The MCH is a great improvement on the current methodology of DNA purification and provides pure and inhibitor free nucleic acid preparations: specific nucleic acid sequence enrichment would increase PCR sensitivity, removing most of the

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inhibitors of the amplification reaction and excess of non-target DNA. This technology is proposed here in a multiplex format for the simultaneous hybrid capture of target sequences from the three bacterial species currently detected in raw milk samples: *E. coli* O157, *Salmonella* spp. and *L. monocytogenes*.

A complete three-step multiple platform, consisting of: (a) simultaneous culture-enrichment in a specifically formulated medium, (b) species-specific DNA magnetic isolation and (c) multiplex real-time PCR (m-rtPCR) was employed for the detection of the three target pathogens (*E. coli* O157, *Salmonella* spp. and *L. monocytogenes*) and compared with reference methods. The system has been previously tested on artificially contaminated bovine milk samples, proving to be highly specific and sensitive, enabling the detection of as few as 1 CFU of each pathogen in 125-g milk after 18 h of enrichment (Omiccioli *et al.*, 2009) and seafood (Amagliani *et al.*, 2010). In the present work, before its application on field samples of raw ovine milk, the platform was validated on the same matrix artificially contaminated with the three target species at simultaneous levels of 1, 10, 100 CFU/25 g each.

Ovine milk (n. 143) samples, obtained from all the sheep farms in the Marches region which directly transform raw milk into cheeses, were collected during a three-year period and analysed by both official reference methods and the molecular platform. Cheese samples (n. 5) were also collected from the same farms which tested positive for target pathogens in milk and subsequently analysed.

2. Materials and methods

2.1. Milk and cheese sampling and microbiological analyses

Samples of raw milk (n. 143), each in five 25 g-sample units (Italian Republic, 2007), were obtained from all 24 ovine dairy farms in the Marches (Central Italy) that directly transform their own produced milk (Carloni *et al*., 2015). Samples were collected during milking periods over three

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years. Milk was refrigerated for transportation to the laboratory and examined within 24 h. Milk lot numbers were recorded at each sampling in a dedicated sampling form to ensure traceability. In the case of positive results by either microbiological or real-time PCR assays, cheese samples (25 g) made with the same contaminated lot of milk were obtained from the milk farms and tested with both analytical methods.

Microbiological analyses were carried out by validated ELFA (Enzyme Linked Fluorescent Assay, bioMérieux, Marcy l'Etoile, France) for *Salmonella* spp. (VIDAS *Salmonella* SLM; AFNOR BIO n. 12/16-09/05), *L. monocytogenes* (VIDAS *L. monocytogenes* II LMO2; AFNOR BIO n.12/11-03/04) and *E. coli* O157 (VIDAS *E. coli* O157 ECO; AFNOR BIO n.12/8-07/10). Confirmation was made according to ISO 6579 (ISO 6579:2008 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.) for *Salmonella* spp.; by colony isolation on Oxford and ALOA agar (Biolife, Milan, Italy), with subsequent biochemical (API Listeria, bioMérieux) and β-haemolysis tests on Blood agar base n.2 (Biolife) for *L. monocytogenes*; by colony isolation on MacConkey agar with Sorbitol, Cefixime, and Tellurite (CT SMAC; Biolife) and serotyping by monovalent sera anti-O157 (Siemens Healthcare, Marburg, Germany) and anti-H7 (Statens Serum Institut, Copenhagen, Denmark) for *E. coli* O157.

2.2. Validation of the molecular method and statistical analysis

The entire molecular platform was validated with artificially contaminated samples of ovine milk following the method reported in detail below.

2.2.1. Preparation of artificially contaminated milk samples

Reference strains of the three bacterial target organisms (*S. enteritidis* ATCC 13076, *L. monocytogenes* ATCC 7644 and *E. coli* O157 ATCC 35150) were separately grown in TSB to optical densities (A_{600}) of 0.25 for *S. enteritidis*, 0.08 for *L. monocytogenes* and 0.27 for *E. coli* O157, corresponding to 10^8 CFU/ml; serial 10-fold dilutions in the same medium were prepared

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and the bacterial number in the cultures was estimated by standard plate counts. Milk aliquots of 25 g were artificially contaminated with 0-1-10-100 CFU (each level in 30 replicates) of each species simultaneously and diluted 1:10 in Multipathogen Enrichment Medium (MEM, Diatheva, Fano, Italy). After incubation at 35°C for 18 ± 2 h, the bacterial growth was determined by plating on selective media (ALOA agar, CT-SMAC and Salmonella Chromogenic Medium).

After enrichment, 10 ml-aliquots were processed for DNA isolation by MCH.

2.2.2. Magnetic Capture Hybridisation

SiMAG-Amino-modified magnetic nanoparticles from Chemicell (Berlin, Germany) were covalently conjugated with NH_2- oligonucleotides (capture probes LP, SP or EP) (Omiccioli *et al.*, 2009) specifically designed for the three target pathogens. Probe attachment and capture efficiency tests were conducted as previously described (Amagliani *et al.*, 2006). Immobilisation process yield and efficiency were indirectly estimated by subtraction of unbound probes (measured spectrophotometrically by A_{260} readings and quantified through standard curves) from the starting amount used in the functionalisation protocol (5 µmol). Hybridisation efficiency was also determined by using fluorescently labeled probes with oligonucleotide sequences complementary to LP, SP and EP (hybridisation probes Lhyb, Shyb and Ehyb). These probes were used in hybridisation tests, then detached by denaturation and quantified spectrofluorimetrically.

A mixture of LP-, SP- and EP-modified nanoparticles was prepared by combining equivalent volumes of each type, and 150 µg per sample was used for the simultaneous specific isolation of DNA of the three bacterial species in a multiplex MCH (mMCH) assay. Briefly, 10 ml-aliquots of milk sample enrichments were centrifuged at 6000 *g* for 20 min at 4°C and bacterial pellets resuspended in 450 µl lysis buffer (0.05 M Tris-HCl pH 8, 0.05 M EDTA pH 8, 0.5% v/v Triton X-100) with 9 µl RNase A 10 mg/ml, 80 µl Proteinase K 10 mg/ml, for enzymatic digestion at 37°C for 30 min. A subsequent centrifugation at 12,000 *g* for 10 min was required to eliminate the fat

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layer on the surface. After denaturation at 100°C for 10 min, mMCH was carried out as previously described (Amagliani *et al.*, 2010).

2.2.3. Multiplex real-time PCR analysis

DNA-nanoparticle hybrids (5 µl) were then amplified with the Multipathogen FLUO kit (Diatheva), a m-rtPCR assay for the simultaneous detection of the three target species, with internal amplification control (IAC). The amplification was carried out in a RotorGene 6000 (Corbett Research, Sydney, Australia) with the following thermal protocol: 10 min at 95°C, 40 cycles of 20 s at 95°C and 1 min at 63°C. Fluorescence acquisition was set on Green (495-520 nm), Red (647-667 nm) and Orange (590-610 nm) channels for the detection of *Salmonella* spp., *L. monocytogene*s and *E. coli* O157 respective signals, with Yellow channel (538-554 nm) for the IAC.

2.2.4. Statistical analysis

A comparison of results with the ELFA method was statistically analysed through a 2 x 2 table by K statistics (Mackinnon, 2000).

2.3. Molecular analysis of milk and cheese

Milk samples of 25 g were culture enriched in 225 ml MEM for 18 ± 2 h at 35°C, then 10 ml-aliquots were analysed as described above for validation with artificially contaminated milk. When there were discrepancies between ELFA and m-rtPCR results, 1 ml-aliquots of selective enrichment broths (CT-MAC, Salmonella Xpress 2, Fraser Broth) of VIDAS procedures were used for mMCH and subsequent m-rtPCR.

Cheese samples (25 g) were collected from farms which tested positive for target pathogens in milk and analysed as above.

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3. Results

3.1. Magnetic Capture Hybridisation

Capture probe sequences and structures have been previously described and their specificity, both *in silico* and experimentally, has already been assessed (Omiccioli *et al.*, 2009). Here they were used for nanoparticle functionalisation through a well-established protocol (Amagliani *et al*., 2006), and their performances were evaluated (Tab. 1). Results of 7 immobilisation batches indicated good reproducibility and similar values among the three sequences. LP ranged from 690.5 to 788.1 pmol/mg of nanoparticles, SP from 602.4 to 735.5 pmol/mg, EP from 513.2 to 649.4 pmol/mg. Hybrid capture proved to be reliable among various batches and different sequences. The efficiency percentage was always considerably higher than 60%, with EP-conjugated nanoparticles being the most efficient, despite showing the smallest amount of immobilised probe. Thanks to the similarity of efficiency values, the combination in equal amounts of the three nanoparticle types to be used in a mMCH was possible.

\3.2. Validation of the molecular method and statistical analysis

Concentrations of bacterial cultures used for artificial contamination of milk samples were approximately as expected, with *S. enteritidis* ATCC 13076 being 0.99 x 10^8 CFU/ml, *L. monocytogenes* ATCC 7644 1.34 x 10^8 CFU/ml and *E. coli* O157 ATCC 35150 0.62 x 10^8 CFU/ml.

Sheep milk samples of 25 g were artificially contaminated with 0-1-10-100 CFU of each bacterial species simultaneously (each level in 30 replicates). In order to ensure validation of the method with unequivocally contaminated samples, after enrichment in MEM, aliquots were plated on selective agar to assess bacterial growth. Bacterial presence of all three species was detected in every replicate of levels 10 and 100 CFU, and in 40% of level 1 CFU. Therefore, only positive replicates of level 1 CFU, along with all replicates of levels 0-10-100 CFU, were used for bacterial DNA

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isolation by mMCH and subsequent m-rtPCR. Zero-level samples tested negative in m-rtPCR, while all contaminated replicates tested positive for each pathogen. The IAC was correctly amplified, confirming the effective application of mMCH to such a complex matrix as sheep milk. A comparison of the performance of ELFA and the molecular assay was carried out for levels 0-1-10-100 CFU. The relative accuracy, sensitivity and specificity of the molecular assay were all 100%, demonstrating that it is in complete agreement with the ELFA assays; therefore, it should be considered as an alternative method equivalent to ELFA.

As shown in Fig. 1, although the m-rtPCR was designed to be qualitative rather than a quantitative assay, a relationship between average Cts and contamination levels can be observed for each pathogen.

3.3. Analysis of milk and cheese field samples

Throughout the three-year study period, at least 4 samplings per farm were obtained, although in some cases sampling frequency was higher, thanks to farmer cooperation. A total of 715 25 g-sample units, corresponding to 143 samples in quintuplicate, were examined.

3.3.1. Microbiological analyses of milk

Negative results were always obtained through ELFA assays for *E. coli* O157 and *L. monocytogenes*. *Salmonella* spp. was found on two farms (farm 5, sample 23600; farm 26, samples 6827, 7820 and 8960), although the microorganism was isolated only from farm 26. Serotyping according to the White-Kauffmann-Le Minor scheme, recommended by the EU Reference Laboratory for *Salmonella* spp. and provided by ISO/TR 6579-3:2014, yielded a final identification of *S. enterica* subsp. diarizonae serovar 61:k:1,5,7. These strains, also characterised for antimicrobial susceptibility according to CLSI (Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard- Second Edition M31-A2 and Supplement M31-S1, Zone Diameter and Minimal Inhibitory Concentration

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Interpretive Standards for *Enterobacteriaceae*.; Performance Standards for Antimicrobial Susceptibility Testing M100-S20 Vol. 30 n° 1 January 2010), were susceptible for each of the tested antimicrobials.

3.3.2. Molecular analyses of milk

Samples were analysed pooling the five sample units in a single sample. *L. monocytogenes* were never detected. Three samples tested positive for *E. coli* O157 DNA: 7011 (farm 11); 31401 (farm 6); 28487 (farm 26). *Salmonella* spp. DNA was detected in various milk aliquots. *Salmonella* spp. positive samples were: 23155 (farm 24); 8962 (farm 22); 23600, 24800 and 31242 (farm 5); 6827, 7820 and 8960 (farm 26). In such cases, corrective actions were suggested to farmers (e.g., milking machine sanification, substitution of milking machine filters, exclusion of diarrhetic cases from milking, staff training), and, shortly after the verification of their application, further samples were collected to check for the possible persistence of the contamination.

The molecular analysis of selective enrichments in CT-MAC or Salmonella Xpress 2 (SX2), carried out when discrepancies were observed between ELFA and m-rtPCR (with Ct < 34), always yielded positive results.

The discrepancy between ELFA and m-rtPCR may stem from the contamination level, which is reflected by Ct values. Indeed, samples with consistent results always showed Ct values < 30, in the range between 20.92 and 29.56 (average 26.57), while discordant results showed Ct ranging from 27.69 to 32.40 (average 30.67) for *Salmonella* spp. and from 27.75 to 36.34 (average 32.80) for *E. coli* O157. It should also be noted that the only sample, from which a bacterial strain was isolated, yielded the lowest Ct (20.92). All results are shown in Tab. 2.

3.3.3. Cheese samples

All the farms investigated in the present study produce raw milk cheeses, which are sold to consumers at various ripening times. Cheese from farm 11, produced with *E. coli* O157 m-rtPCR

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positive milk, tested negative for the same pathogen using both ELFA and molecular methods. These results may be accounted for by the fact that the cheese was analysed after 20 days of ripening, and pathogen growth might have been hampered by unfavourable conditions occurring in that matrix due to acidification and ageing. Indeed, factors influencing pathogen survival and growth in cheese are numerous and include acidity, preservatives, temperature, competing flora, water activity, and salt concentration (FDA, 2015).

Regarding *Salmonella* spp., cheeses from farms 5 and 24 tested negative with ELFA but positive with m-rtPCR from SX2. The presence of *Salmonella* was confirmed in cheese from farm 26 both with ELFA and subsequent tests according to ISO 6579, and PCR-based assay (Tab. 2).

4. Discussion

Food safety is becoming an increasingly important concern for the European Union, as illustrated by the growing list of food safety regulations it has adopted. In particular, zoonoses are recognized as a threat to public health, and the early identification of the responsible microorganism is key to the effective control and prevention of such diseases. Hence, the diagnostic proficiency of laboratories is of paramount importance to ensure the rapid recognition of circulating zoonotic agents and the subsequent risk of an outbreak. Laboratory data on zoonoses allow us to make a more precise diagnosis and to notify interested parties, to implement a rapid response system and to improve our knowledge of pathogen presence in a defined area.

E. coli O157 infections, salmonellosis and listeriosis are common illnesses found worldwide, and constitute a serious threat to food safety. The presence of even small amounts of pathogenic bacteria in milk destined for the production of raw milk lactic cheeses constitutes a risk for consumers. In particular, the presence of viable *E. coli* O157 is of concern to all producers of raw lactic milk cheeses since it can develop an adaptive tolerance response to acid environments, promoting resistance to gastric acid during the digestive process and the survival of an infective dose of

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bacteria (Vernozy-Rozand *et al*., 2005). The need for further investigation of *E. coli* O157 in ovine milk is also justified by a number of experimental studies that have shown the pathogen's ability to survive acidic pH in several fermented dairy products (Gulmez *et al.*, 2003), in white brined cheese (Osaili *et al*., 2014) and in Cheddar cheese after 60 days of ripening (Schlesser *et al*., 2006). The growth of *L. monocytogenes*, *Salmonella* spp. and *E. coli* O157:H7 has recently been detected in cheese during extended storage at 25°C (Leong *et al*., 2014). It is therefore imperative to ensure that milk used in cheese production is of the highest bacteriological quality.

The aim of this investigation was to broaden our knowledge of the presence of *E. coli* O157, *Salmonella* spp. and *L. monocytogenes* in ovine milk in Central Italy. Moreover, we validated and applied a molecular platform for the simultaneous detection of foodborne pathogens, evaluating its possible future use as a time-saving tool in routine diagnosis. A multipathogen detection system, including culture-enrichment, mMCH and m-rtPCR, was applied and compared to ELFA validated assays. Validation of the molecular method by comparison with certified materials and its application to field samples together with reference methods allowed us to define its true effectiveness and reliability in view of its possible future use as an alternative routine diagnostic tool.

The recovery of bacteria from foods, including milk, is often complicated by the very low level of contamination. The use of a proper enrichment medium can therefore improve the sensitivity and reproducibility of the test. The simultaneous approach for pathogen detection must take into account the differences in pathogen growth rates and requirements. Hence, the enrichment medium selected for the first step of the detection protocol ensured the concurrent growth of the mentioned species and was able to enrich a test sample with multiple pathogens, even at very low levels.

The subsequent phase of the molecular protocol provided bacterial DNA selective isolation and concentration by a magnetic hybridisation procedure. Data on the functionalization of magnetic supports indicated that the immobilisation process is highly standardized and provided materials suitable for the intended downstream application with food matrix enrichments. To the best of our

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knowledge, apart from our group's work, the application of MCH for bacterial DNA detection in food has never been reported. However, MCH proved highly effective when directly applied to milk samples (Amagliani *et al*., 2006). Moreover, the magnetic procedure has also been tested in multiplex format (mMCH) for the specific and simultaneous isolation of *E. coli* O157, *Salmonella* spp. and *L. monocytogenes* DNA in artificially and naturally contaminated bovine milk (Omiccioli *et al.*, 2009) and seafood (Amagliani, *et al.*, 2010), showing high sensitivity, even when the pathogens were present in unequal amounts. Here we propose the application of the same method for the simultaneous and specific isolation of bacterial DNA of the target species from raw ovine milk and related products. Validation results proved the equivalence of ELFA validated systems and the multiplex molecular assay. Hence, the molecular assay appears to be suitable for routine use and to support ISO culture methods for preliminary screening in accordance with the EU Regulation 2073/2005 recommendations concerning alternative methods. The molecular assay is a platform that could also be applied to other food product lines.

In the present study, the analysis of field samples of raw sheep milk revealed the absence of pathogens or their presence at very low levels. Previous investigations of the same farms (Carloni *et al*., 2015) showed good hygienic conditions and the proper application of good farm management practices.

After a three-year sampling period involving 715 sample units from 24 raw sheep milk farms, only one farm tested positive (ELFA and m-rtPCR with pathogen isolation) for *S. enterica* subsp. diarizonae 61:k:1,5,7 in milk and derived cheese. This result confirmed sheep, also in Central Italy, as a potential reservoir for *S. enterica* subsp. diarizonae 61:k:1,5,7, in accordance with previous investigations (Alvseike *et al*., 2004; Davies *et al*., 2001; Zweifel *et al.*, 2004). However, although foodborne infections in humans caused by this serovar from domestic animals such as sheep have occasionally been described (Davies *et al*., 2001), it is not included among the five serovars with public health significance (Commission Regulation (EC) No 1003/2005), and there were no reports of its presence in food or animals in the last EFSA Report on zoonoses (EFSA, 2015).

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Concerning *E. coli* O157, results of the molecular approach indicate occasional pathogen presence at very low levels. Hence, sheep milk and cheese producers must remain vigilant with this food matrix, paying particular attention to hygienic aspects of production. However, positive results obtained only by the molecular method and not by ELFA should be considered with caution, since the results of biomolecular diagnoses should always be confirmed by pathogen isolation to prove its vitality (UNI EN ISO 20838:2006).

m-rtPCR analysis of cheese samples produced with milk which tested PCR positive and ELFA negative for *E. coli* O157 did not confirm the presence of the pathogen. It can be hypothesized that unfavorable conditions, due to acidification and ripening, hindered pathogen growth, especially if we consider the low starting concentration of the pathogen.

4. Conclusions

This study aimed to enhance our knowledge of pathogen presence in raw ovine milk and its derived products, thus yielding useful epidemiological data. Such data can be used by the National Public Health Service to implement an effective control strategy to ensure the safety of consumers, especially as regards possible environmental contamination during and after milking.

Thanks to its intrinsic properties of high specificity and sensitivity, along with its rapidity, molecular diagnosis through validated methods could provide a very useful tool in early warning systems, thus offering improved and cost effective public health protection.

Figure caption

Fig. 1. Molecular assay validation. Average ± SD of Ct obtained through m-rtPCR of artificially contaminated samples (n. 30 for 0-10-100 CFU and n. 12 for 1 CFU of each bacterial target species). Contamination levels are expressed as log CFU/25 g.

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Tab. 1. Efficiency tests of functionalised nanoparticles. Immobilization process yield and hybrid capture efficiency. LP and Lhyb, *L. monocytogenes* capture probe and hybridization probe; SP and Shyb, *Salmonella* spp. capture probe and hybridization probe; EP and Ehyb, *E. coli* O157 capture probe and hybridization probe.

Immobilization process yield			
	LP	**SP**	**EP**
Average ± SD (%)			
starting amount (5 µmol)	100	100	100
unbound probe	24.3 ± 2.8	25.6 ± 4.4	35.4 ± 3.0
covalently coupled probe	75.7 ± 2.8	74.4 ± 4.4	64.6 ± 3.0
Average ± SD (pmol/mg)			
coupled probe/mg nanoparticles	737.6 ± 36.1	695.7 ± 47.4	596.6 ± 44.9
Total Average ± SD coupled probe in 5 mg nanoparticles	3687.9 ± 180.3	3478.4 ± 236.9	2983.0 ± 224.6
Hybrid capture efficiency			
	Lhyb	**Shyb**	**Ehyb**
Average ± SD (%)	68.8 ± 15.1	63.1 ± 6.5	69.5 ± 7.3

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Tab. 2. Summary of results of sheep milk and cheese field samples analyses with both ELFA and the molecular assay. Only results of positive samples are reported.

Farm n.	Sample n.	*Salmonella* spp.			*E. coli* O157			*L. monocytogenes*		
		ELFA	m-rtPCR (Ct)		ELFA	m-rtPCR (Ct)		ELFA	m-rtPCR (Ct)	
			MEM	SX2		MEM	CT-MAC		MEM	FB
5	23600	+	29.21	n.d.	-	-	n.d.	-	-	n.d.
	24807 (C)	-	-	-	-	-	n.d.	-	-	n.d.
	24800	-	31.33	27.12	-	-	n.d.	-	-	n.d.
	31242	-	27.69	19.66	-	-	n.d.	-	-	n.d.
	33070 (C)	-	-	24.78	-	-	n.d.	-	-	n.d.
6	31401	-	-	-	-	34.29	n.d.	-	-	n.d.
11	7011	-	-	n.d.	-	27.75	-	-	-	n.d.
	12364 (C)	-	-	n.d.	-	-	-	-	-	n.d.
22	8962	-	32.40	35.00	-	-	n.d.	-	-	n.d.
24	23155	-	31.25	29.04	-	-	n.d.	-	-	n.d.
	24945 (C)	-	-	35.06	-	-	n.d.	-	-	n.d.
26	28487	-	-	-	-	36.34	n.d.	-	-	n.d.
	6827*	+	20.92	n.d.	-	-	n.d.	-	-	n.d.
	9083 (C)	+	27.56	20.24	-	-	n.d.	-	-	n.d.
	7820	+	25.59	n.d.	-	-	n.d.	-	-	n.d.
	8960	+	29.56	n.d.	-	-	n.d.	-	-	n.d.

* Isolation of *S. enterica* subsp. diarizonae 61:k:1,5,7. (C), cheese; n.d., not determined; m-rt-PCR, multiplex real-time PCR; MEM, Multipathogen Enrichment Medium; SX2, Salmonella Xpress 2; CT-MAC, MacConkey broth with Cefixime and Tellurite; FB, Fraser Broth.

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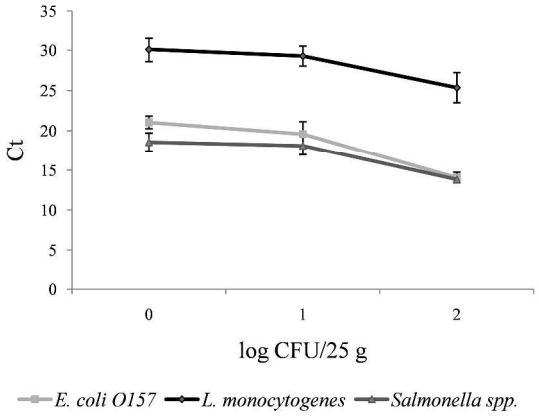

Contamination level (log CFU/25 g)	Ct ± SD	
0	*Salmonella* spp.	18.58 ± 1.13
	E. coli O157	21.08 ± 0.79
	L. monocytogenes	30.10 ± 1.51
1	*Salmonella* spp.	18.13 ± 1.14
	E. coli O157	19.60 ± 1.53
	L. monocytogenes	29.28 ± 1.24
2	*Salmonella* spp.	13.85 ± 0.42
	E. coli O157	14.09 ± 0.67
	L. monocytogenes	25.36 ± 1.87

Fig. 1. Molecular assay validation. Average ± SD of Ct obtained through m-rtPCR of artificially contaminated samples (n. 30 for 0-10-100 CFU and n. 12 for 1 CFU of each bacterial target species). Contamination levels are expressed as log CFU/25 g.
1190x1587mm (96 x 96 DPI)