Occurrence of *Coxiella burnetii* in goat and ewe unpasteurized cheeses:Screening and genotyping

Alessia Galiero [a,*], Filippo Fratini [a], Cesare Cammà [b], Marco Di Domenico [b], Valentina Curini [b], Irene Baronti [a], Barbara Turchi [a], Domenico Cerri [a]

[a] Department of Veterinary Sciences, University of Pisa, Viale delle Piagge, 2, 56124 Pisa, Italy

[b] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, via Campo Boario, 64100 Teramo, Italy

Abstract

Q fever is a zoonosis caused by Coxiella burnetii which infects humans as well as several animal species; sheep, goats and cattle are the primary animal reservoir. The main route of human exposure to Coxiella burnetii is inha- lation of contaminated aerosols from excreta, especially birth products, while the role of unpasteurized dairy products in the transmission of Q fever to humans remains still controversial.

The aim of this work was to evaluate the presence of Coxiella burnetii in unpasteurized cheese samples (n = 84) by PCR and to genotype the circulating strains by Multispacer sequence typing (MST) analysis.

Coxiella burnetii DNA was detected in 27/84 (32.14%) cheeses and positivity rate of handicraft cheeses reached 17.24%, while positivity rate of non-handicraft cheeses reached 65.38%. In addition, the MST profile of Coxiella burnetii detected in 5 cheese samples have shown the circulation of ST12 and ST32 genotypes in Tuscany.

1. Introduction

Q fever is a zoonosis caused by Coxiella burnetii which infects humans as well as several animal species; sheep, goats and cattle are the primary animal reservoir (OIE, 2015). In these species, infection re- mains often asymptomatic, although abortion, premature birth, weak offspring, infertility, metritis and pneumonia can occur; similarly, in humans infection can be asymptomatic or it can manifest itself in acute or chronic form. Particularly, acute Q fever is characterized by a flu-like disease or by an atypical pneumonia or hepatitis, while chronic disease is accompanied by endocarditis, chronic fatigue syndrome and repeated abortions (Arricau-Bouvery and Rodolakis, 2005).

Infected animals can shed the organism in feces, in milk, in placental membranes and in birth fluids (Van den Brom et al., 2015); in this re- gard, individual goat milk may contain from 102 bacteria/mL to 10- 5 bacteria/mL (Di Domenico et al., 2014). Infected ruminants shed the organism mainly in

birth products, milk and feces, and dust particles contaminated with Coxiella burnetii are the main source of infection through inhalation, not only for animals, but also for human beings (Van den Brom et al., 2015). Moreover, ticks and rodents are natural res- ervoirs of Coxiella burnetii (Pascucci et al., 2015).

In animals Q fever diagnosis is based on specific antibodies finding by ELISA and on PCR detection of Coxiella burnetii's DNA in different biological samples, including placenta, vaginal mucus, milk, colostrum, feces and tissues from aborted fetus (Borriello et al., 2010).

While the main route of human exposure to Coxiella burnetii is inha- lation of contaminated aerosols from excreta, especially birth products, the role of unpasteurized dairy products, such as cheeses, in the trans- mission of Q fever to humans remains still controversial (Capuano et al., 2012; Eldin et al., 2013; Fishbein and Raoult, 1992; Gale et al., 2015; Hatchette et al., 2001; Hirai et al., 2012; Maltezou et al., 2004).

From an epidemiological point of view, to prevent humans from ac- quiring this infection is necessary to know the disease's prevalence in animals; notably, detection of Coxiella burnetii in goat and sheep sam- ples has not been performed before in Tuscany, even though two studies have assessed the presence of this pathogen not only in horses by PCR (Ebani et al., 2015) and in fallow deer by complement fixation test (Giovannini et al., 1988), but also in human beings by serological diag- nosis (Aquilini et al., 2000; Tiscione et al., 1989).

The aim of this study was to detect the occurrence of Coxiella burnetii in Tuscan cheeses not only to evaluate the presence of the in- fection in our territory, but also to know which genotypes are circu- lating. In addition, although the role of dairy products in human infection is controversial, because in Italy goat and sheep milk is usu- ally consumed as dairy products rather than as liquid milk and now- adays the consumers' attitudes shows an increasing trend towards consumption of unpasteurized dairy products (Verraes et al., 2015), it was important to determine the level of contamination of this specific category of products.

2. Materials and methods

2.1. Sample collection

This study was carried out on 84 cheeses produced from unpasteur- ized bulk tank sheep or goat milk in Tuscany and collected during the years 2014–2015; the samples were stored in ice-containing containers and delivered to the laboratory where they were stored at −20 °C until DNA extraction. This sampling secures 95% level of confidence (z value = 1.96; mar- gin of error 10%) for an expected prevalence of 26.78% (Capuano et al., 2012).

Furthermore, the samples were stratified into groups according to species the cheese was produced from, moisture content and manufacturing process as follow:

a) according to samples labeling, specimens were initially stratified into sheep cheeses and goat cheeses.

b) a Rotronic HygroPalm HP23-AW-A meter (Rotronic) was employed to measure the activity water (aw) of the cheese samples; according to aw values, the cheeses were classified as soft cheeses (ripened until 30 days) (aw 0.97–0.99) (18/84), semi-hard cheeses (ripened from 30 to 60 days) (aw 0.96–0.93) (41/84) and hard cheeses (rip- ened more than 60 days) (aw 0.92–0.79) (25/84);

c) according to manufacturing process, samples were grouped into handicraft cheeses (58/84), which were produced from the bulk tank milk coming exclusively from animals bred in the same farm and sold directly to consumer, and into non-handicraft cheeses (26/84) which came from dairies and were sold in supermarkets.

2.2. DNA extraction

For each sample, DNA extraction was performed on 10 g which were firstly transferred aseptically

into a sterile stomacher bag containing 90 mL of diluent (2% w/v sodium citrate) (Sigma-Aldrich, St. Louis, MO, USA). Then, the mixture was homogenized in a Stomacher blender (230 rev/min for 1 min) and DNA isolation was performed on 1.5 mL of the homogenate using a commercially available kit according to the in- structions provided by the manufacturer (RealPure Spin food stool bac- teria, Durviz). The quality of the DNA extracted was evaluated with regard to integrity by submerged gel electrophoresis (0.8% p/v) and then DNA products were stored at −20 °C until performing PCR assays.

2.3. Multiplex PCR

In order to ascertain that cheeses produced from ovine and goat milk were in conformity with the information reported in labels, a multiplex PCR assay, which could differentiate the milk of three closely related species (goat, sheep and cow) in cheeses, was employed (Bottero et al., 2003). Each reaction (final volume 25 μL) consisted of 12.5 μL master mix (2× KAPA Taq ReadyMix with dye master mix kit, Kapa Biosystems, USA), 25 pmol, 30 pmol, 15 pmol of primers, respectively, of bovine, caprine and ovine origin, 5 μL of DNA and 4 μL of PCR grade water (Ultra Pure Water DNase and RNase-free, Biological Industries, Israel). Amplification was performed in a Thermal Cycler-LifePro (Bioer Tech- nology) using the following cycling conditions: 94 °C for 5 min and then 35 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min (Bottero et al., 2003). For each sample, a volume of 12 μL of PCR product was run on a 3.5% agarose gel in Tris-acetate-EDTA (TAE) running buffer. The DNA was identified as belonging to cattle, sheep or goat, depending on whether the size of the PCR product was 256, 172 or 326 base pairs, respectively.

2.4. End point PCR

PCR was performed on DNA extracted from all the samples (n =84) using 2× KAPA Taq ReadyMix

with dye master mix kit (Kapa Biosystems, USA), with primers QBT-1 5′-TATGTATCCACCGTAGCCAGTC-3′ and QBT-2 5′-CCCAACAACACCTCCTTATTC-3′ (Hoover et al., 1992); these primers amplify a region (687 bp) of the IS1111 sequence. Each reaction (final vol- ume 25 μL) consisted of 12.5 μL master mix (2× KAPA Taq ReadyMix with dye master mix kit, Kapa Biosystems, USA), 0.6 μM of each primer, 5 μL of DNA and 4.5 μL of PCR grade water (Ultra Pure Water DNase and RNase- free, Biological Industries, Israel). Amplification was performed in a Ther- mal Cycler-LifePro (Bioer Technology) using the following cycling condi- tions: 95 °C for 5 min and then 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min (Lamas et al., 2009). Furthermore, a positive control (Nine Mile strain, ATCC VR-615) and a negative control (Ultra Pure Water DNase and RNase-free, Biological Industries, Israel) were incorporated in each assay. For each sample a volume of 12 μL of PCR product was run on a 1.5% agarose gel in Tris-acetate-EDTA (TAE) running buffer.

2.5. Real time PCR

Real time PCR assay was also used to test all the specimens (n = 84). A fragment (86 bp) of Coxiella burnetii IS1111 element was amplified by the primers CoxbS (5′-GATAGCCCGATAAGCATCAAC-3′) and CoxbAs (5′-GCATTCGTATATCCGGCATC-3′) (Panning et al., 2008) and the probe FAM-TGCATAATTCATCAAGGCACCAATGGT-TAMRA (Di Domenico et al., 2014). Each reaction consisted of 10 μL master mix (2 × TaqMan Fast Universal PCR Master Mix), 300 nM of the probe, 900 nM of each primer, 5 μL of DNA and PCR grade water to a final vol- ume of 20 μL. Amplification was performed in a 7900HT Fast Real time PCR System (Applied Biosystems) using the following thermal profile: initial denaturation at 95 °C for 20 s, followed by 35 cycles of 95 °C for 1 s, 60 °C for 20 s.

2.6. MST analysis

Molecular characterization of Coxiella burnetii DNA was performed by MST assay as previously described (Di Domenico et al., 2014). Briefly, each reaction consisted of 1 × PCR Buffer II (Applied Biosystems),
200 nM of each primer (Glazunova et al., 2005), 200 μM dNTPs (Promega), 2.5 mM MgCl2 Solution (Applied Biosystems), 0.03 U/μL AmpliTaqGoldTM (Applied Biosystems), 5 μL of DNA and PCR grade water to a final volume of 50 μL. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: initial denaturation of 10 min at 95 °C, followed by 45 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C, and exten- sion for 30 s at 72 °C, with a final extension at 72 °C for 7 min. The reac- tion mix for the Cox56 and Cox57 spacers PCRs were modified as previously described by Di Domenico et al. (2014). PCR products were purified using the Expin™ PCR SV Kit (GeneAll) and sequenced by using BigDye Terminator v.3.1 (Applied Biosystems) and the 3130 XL Genetic Analyzer (Applied Biosystems). Raw sequence data were as- sembled using DNAStar Navigator and the sequences were compared with those reported in the reference database available on the website http://ifr48.timone.univmrs.fr/mst/coxiella_burnetii/strains.html.

2.7. Statistical analysis

The statistical significance of the differences in positivity rates among the different stratification groups of cheeses was tested with the chi-squared test; statistical analyses were performed with the soft- ware R v.3.2.3 (R Foundation for Statistical Computing, Vienna, Austria), available by means of the link https://cran.r-project.org, and differences were considered significant if associated with a p-value b 0.05. In addition, Z-test for Proportions-Independent Groups was carried out using a free online software available at http://www. socscistatistics.com/tests/ztest/Default2.aspx.

3. Results

3.1. Multiplex PCR, end point PCR and Real time PCR

On the basis of the multiplex PCR results (Fig. 1) (Tables 1 and 2), cheese samples tested were stratified as follow: 9/84 (10.71%) were from goat milk, 60/84 (71.42%) from sheep milk, 1/84 (1.19%) from bo- vine milk, 9/84 (10.71%) from goat and sheep milk, 4/84 (4.76%) from sheep and cow milk, 1/84 (1.19%) from cow, goat and sheep milk.

The analysis performed by the end point PCR (Fig. 2) and the Real time PCR indicated the presence of Coxiella burnetii's DNA in 20 (23.8%) and in 27 (32.14%) respectively, of the 84 samples which were tested (Tables 1 and 2). On the basis of Real time PCR results, positive samples were stratified as indicated in Table 3.

Comparing the results between handicraft and non-handicraft

cheeses, the latter showed a higher risk of infection ($\chi 2 = 16.934$; p- value = 0.00003871). Furthermore, not only the difference between goat and ewe cheeses was not significant ($\chi 2 = 2.2627$; p-value = 0.1325), but also that among soft cheeses, semi-hard and hard cheeses ($\chi 2 = 5.3361$; p-value = 0.2545).

Furthermore, the results highlighted that 33.33% (8/24) of farms and 83.33% (10/12) of dairies were positive to Coxiella burnetii.

3.2. MST analysis

A complete MST genotype was obtained only from five cheese sam- ples: one cheese produced from goat and ewe milk, one cheese pro- duced from ovine and bovine milk and three ewe cheese samples. All these samples showed Real time PCR Ct values lower than 32. The sam- ple 2F, although had a Ct lower than 32, was not genotyped because it originated from the same farm of the specimen 1F.

Two different sequence types, ST12 and ST32, were identified (Tables 1 and 2).

4. Discussion

Applying the multiplex PCR to the DNA extracted from the cheeses to verify the label statements, it can be noted that the information pro- vided by labeling was confirmed for 67 samples, while it was untruthful for 17 cheeses. Particularly, in one cheese labeled as goat, only sheep and bovine milk was detected and in another six specimens, declared as produced from goat milk, a mixture of goat and ovine milk was found; similarly, in two cheeses, labeled as sheep, only goat milk was highlighted, and in other one cheese, labeled as sheep, only bovine milk was detected. In addition, in seven cheeses, labeled as sheep, it was highlighted a mixture of ovine milk with bovine milk (n = 3) or with goat milk (n = 3) and one sample was found to be produced from all the three species milk. The finding of cow milk alone or in asso- ciation with small ruminant's milk could be due to the fact that the former is often employed to adulterate cheeses produced from ewe and goat milk because of its lower price and its availability throughout the year (Špoljarić et al., 2013).

Furthermore, although the primers used in the endpoint PCR and in the Real time PCR amplified a sequence of the same IS1111 region of Coxiella burnetii's genome, the fact that 27/84 (32.14) samples were positive to Real time PCR, while, out of them, only 20/84 (23.80%) were positive also to the endpoint PCR assay, could be probably due to the fact that Real time PCR is characterized by higher sensitivity (Valasek and Repa, 2005). In fact, the samples, which were recorded as false negative by the end point PCR assay, had high Ct values and, consequently, a low amount of Coxiella burnetii's DNA.

As concern as the cheese manufacturing process, comparing the level of cheese PCR-positivity reported in our study, to that of similar re- searches, it can be noted that non-handicraft cheeses were positive at a percentage (17/26, 65.38%) which was higher than that reported by Capuano et al. (2012) (12/100, 12%, p = 0, p b 0.05) at a statistically sig- nificant level. Moreover, handicraft cheeses were positive at a percent- age (10/58, 17.24%) which was lower than that recorded by Capuano et al. (2012) (24/69, 34.78%, p = 0.02642, p b 0.05) and by Eldin et al. (2013) (59/100, 59%, p = 0, p b 0.05). In addition, comparing the results between handicraft and non-handicraft cheeses, the fact non-

218 handicraft cheeses were positive more often than handicraft products could be due to the higher
219 number of animals and flocks from which the milk origi- nated as suggested by Eldin et al. (2013).
220 This hypothesis could also ex- plain the fact that not only the positivity rate of dairies (83.33%) was
221 higher than that of farms (33.33%), but also the positivity at dairy level was higher (66.66%) than
222 that recorded at farm level (21.18%) (Fig. 3 and Fig. 4).
223 Furthermore, because Coxiella burnetii is an obligate intracellular bacterium (OIE, 2015) and,
224 consequently, its multiplication does not occur in milk and in cheese (Gale et al., 2015), the finding
225 that semi- hard cheeses and hard cheeses were positive at higher percentage levels than soft cheeses
226 could be due to the fact that Coxiella burnetii was more concentrated in ripened cheese because of
227 the lower moisture content. The same can be stated regarding Ct values recorded in semi-hard and in
228 hard cheeses, which were lower than those found in soft cheeses, in- dicating a higher number of
229 Coxiella burnetii cells.
230 In addition, our results highlight that ewe cheese samples contained low bacterial burdens, because
231 all Ct values obtained with the Real time PCR were ≥ 30. These results could be due to the fact that
232 shedding of Coxiella burnetii differs in route of excretion and in duration among an- imal species; in
233 fact, sheep tend to shed more Coxiella burnetii cells in feces and in vaginal swabs rather than in milk
234 (Astobiza et al., 2010; Joulié et al., 2015; Rodolakis et al., 2007). In addition, infected sheep shed
235 intermittently Coxiella burnetii in milk during a short period after parturition. Particularly, the study
236 of Astobiza et al. (2010), monitoring by PCR the presence of Coxiella burnetii in individual sheep
237 milk samples for 150 days, highlighted that sheep naturally infected shed the bacteri- um in milk up
238 to 90 days, although the excretion was concentrated in the first month after parturition. Similarly,
239 Rodolakis et al. (2007), ana- lyzing by PCR bulk tank milk produced from small ruminants for 90
240 days after parturition, observed that the shedding of Coxiella burnetii in sheep bulk tank milk could
241 last up to 12 weeks. Unfortunately, the level of Coxiella burnetii shedding in goat milk cannot be
242 compared to that re- corded in sheep specimens, since all cheeses, positive to Coxiella burnetii and
243 declared as produced from goat milk, were actually made from a mixture of goat and sheep milk.

As concern as the potential hazard to human health posed by the presence of Coxiella burnetii in cheese, it should be considered that the transmission of this bacterium from animals to humans mainly occurs by inhalation (Van den Brom et al., 2015). In fact, although the presence of viable Coxiella burnetii in commercially available raw milk was re- ported and there are also reports which suggest that the consumption of unpasteurized infected bovine milk could be the source of human cases of Q fever (Gale et al., 2015; Shah et al., 2015; Signs et al., 2012), the cause-and-effect link between the consumption of infected milk and the development of clinical disease still needs to be proven (EFSA, 2010). Similarly, although the oral transmission of Q fever to humans through ingestion of infected cheeses is nowadays unclear (EFSA, 2010), consumption of infected cheeses cannot be excluded as risk fac- tor for the development of Q fever in human beings; in fact, seropositiv- ity rates were significantly higher among persons who consumed infected unpasteurized goat products compared with those who didn't ingest them (Fishbein and Raoult, 1992; Hatchette et al., 2001; Maltezou et al., 2004). In agreement with the above, in an experimental assay, in which bovine and ewe milk were artificially infected before cheese-making, Babudieri and Moscovici (1950) reported, inoculating cheese samples into guinea pigs, that Coxiella burnetii survived in cheese produced from infected bovine milk for a ripening period of 17 days; on the contrary, no viable Coxiella burnetii was assessed in ewe cheese rip- ened for the same period. Unfortunately, no data are available regarding the genotype of the strain used to contaminate milk, the infecting dose, pH values, moisture or NaCl percentage of the cheeses analyzed. Simi- larly, in the study of Šipka (1958) the viability of Coxiella burnetii was assessed by inoculating cheese samples, produced from bovine milk naturally infected, into guinea pigs; in addition, unlike Babudieri and Moscovici (1950), Šipka (1958) measured pH, moisture and NaCl per- centage of the cheese specimens. With reference to this, it should be noted that Coxiella burnetii was viable until 42 days of ripening in cheeses characterized by a pH values which ranged from 7.34 to 6.34, by a moisture content which ranged from 50.08% to 52.16% and by NaCl percentage which ranged from 7.14% and 8.36%; conversely, Coxiella burnetii was not more detectable on 49 day (pH 6.34, moisture 51.42%, NaCl 8.32%). These two researches indicated that

ripening, which has effect on aw, pH and NaCl percentage, could have an impact on the viability of Coxiella burnetii in cheese. Thus, the cheeses analyzed within the context of our study, which were classified as soft and semi- hard cheeses and which were ripened until 30 days and 45 days, respectively, could constitute a higher source of human exposure to viable Coxiella burnetii. Unfortunately, in the study of Šipka (1958), no infor- mation are available on the strain which was employed to contaminate the milk.

On the contrary, Eldin et al. (2013) and Hirai et al. (2012) assessed that consumption of cheese does not pose a public health risk for trans- mission of Coxiella burnetii because the pathogen was not viable in the retail cheeses analyzed by inoculating samples into mice; notably, out of them, only Hirai et al. (2012) analyzed DNA sequences from positive samples which were divided into the Priscilla group. With reference to this, further studies are needed, because the viability of Coxiella burnetii in cheese could be affected by the strain: in fact, up to now, to our knowledge, no research has been carried out on evaluating how the sur- vival of Coxiella burnetii to pH, aw and ripening in cheeses is affected by the genotype involved.

Under the circumstances, the precautionary principle to prevent human from the infection, achieved by milk's pasteurization before cheese-making and the subsequent use of commercial starters in cheese manufacturing, should be applied; particularly, the minimum time- temperature combinations, which are widely used, 63 °C for 30 min or 72 °C for 15 s, allow to inactivate the pathogen in milk (Juffs and Deeth, 2007).

Furthermore, despite the fact that several human Q fever outbreaks have been recently related to infected sheep and goats (Van den Brom et al., 2015), up to now, in Tuscany, none has analyzed goat and ewe specimens such as tissues, milk samples or cheeses, although the pres- ence of Coxiella burnetii was assessed by immunofluorescence antibody assay (IFA) in foresters (Aquilini et al., 2000) and in livestock operators (Tiscione et al., 1989) and in other animal species; with reference to the latter, Ebani et al. (2015) found 6/160 horses positive to PCR carried on DNA extracted from blood samples and Giovannini et al. (1988) highlighted the presence of 3/43 deer positive to complement

296 fixation test; unfortunately none of them genotyped the circulating strains.
297 In addition, this research, carried out on cheeses, has given us also the opportunity to investigate, on
298 large scale, the level of prevalence of animal infection which can constitute a risk for the development
299 of human Q fever through airborne transmission, not only for personnel handling animals, but also
300 for humans living over a mile from the ani- mal sources (McQuiston and Childs, 2002). In this regard,
301 we have cho- sen to collect cheeses, on one hand because in Italy goat and sheep milk is usually
302 consumed as dairy products rather than as liquid milk, on the other hand because they are more easily
303 available than bulk tank milk samples giving us the opportunity to analyze a larger number of
304 specimens.
305 Furthermore, the MST analysis showed the presence of two MST ge- notypes, ST12 and ST32, which
306 are different from all the sequenced types already described before in Italy
307 (http://ifr48.timone.univmrs.fr/ mst/coxiella_burnetii/strains.html); in fact, up until now, only ST16,
308 ST18 and ST29 MST genotypes were described in human samples and three novel ST profiles, one
309 similar to ST20, one to ST8 and one to ST27 were detected in bovine milk, goat fetus and goat milk,
310 respective- ly (Di Domenico et al., 2014). Particularly, in our study, ST12 and ST32, which belong
311 to Group II and are closely related on the basis of phyloge- netic analysis (Hornstra et al., 2011), were
312 found in ewe cheeses (sam- ple ID 49, 55) produced in two plants located in the same municipality
313 area of Pisa and only 17 km far away from each other. Furthermore, al- though, as reported in the
314 reference database (http://ifr48.timone. univmrs.fr/mst/coxiella_burnetii/strains.html), ST12 was
315 detected in clinical human samples (heart valve, human blood, retrosternal abscess, aneurysm,
316 valvular prosthesis and spleen abscess) from France, Swit- zerland and Senegal, to our knowledge,
317 this is the first time that ST12 profile has been identified in animal samples. This result suggests that
318 sheep could represent an important source of human Q fever in Tuscany (Italy). In fact, although the
319 oral exposure is still controversial, the risk posed from human exposure to Coxiella burnetii through
320 inhalation of aerosol is widely recognized, especially for certain categories at risk, such as farmers
321 and veterinarians or people who are not occupationally exposed, but who live close to infected flocks

(Van den Brom et al., 2015). On the contrary, the zoonotic role of the ST32 genotype has been already assessed because it was identified not only in a goat pla- centa sample in Austria, but it was also detected in human specimens, heart valve and aortic biopsy, respectively in Germany and in France. On the other hand, to our knowledge, our study has highlighted for the first time, the presence of ST32 profile in a sheep specimen. In addi- tion, because a ST32 strain has been also revealed in a cheese produced from a mixture of ovine and bovine milk, further studies are needed to know which strains are circulating in Tuscan cattle; notably, up to now, only the ST13, ST16, ST18, ST20, ST23, ST24 and ST28 strains have been found in bovine samples (http://ifr48.timone.univmrs.fr/mst/coxiella_burnetii/strains.html). Furthermore, because a ST12 strain has been highlighted in a sample produced from a mixture of goat and sheep milk, future research should be carried out on individual goat milk samples collected from the infected farms to evaluate which strains are affecting the Tuscan goat population.

Notably, different human outbreaks were described in several re- gions of Italy (Monno et al., 2009; Santoro et al., 2004; Starnini et al., 2005); particularly, serological and molecular investigations confirmed the presence of Coxiella burnetii in water buffaloes (Galiero, 2007; Perugini et al., 2009), in cattle (Cabassi et al., 2006; Ceglie et al., 2015; Di Domenico et al., 2014; Lucchese et al., 2015; Magnino et al., 2009; Natale et al., 2009; Parisi et al., 2006; Petruzzelli et al., 2013; Torina et al., 2007; Valla et al., 2014; Vicari et al., 2013), in sheep and in goats (Ceglie et al., 2015; Di Domenico et al., 2014; Marenzoni et al., 2013; Masala et al., 2004; Masala et al., 2007; Parisi et al., 2006; Torina et al., 2007). However, only Di Domenico et al. (2014) described the MST pro- files of the strains analyzed, while Ceglie et al. (2015) characterized Coxiella burnetii's strains by multiple locus variable-number tandem re- peat (MLVA) analysis. Notably, the recent outbreaks recorded in Europe were referred to ST8 and ST33 (Santos et al., 2012; Tilburg et al., 2012a). Furthermore, it should be taken into account that, although in our research only one genotype was detected in each non-handicraft cheese sample, it cannot be excluded that non-handicraft cheeses may contain more than one strain of Coxiella burnetii. In fact, on one hand, our finding could be due to the fact that the

dairies collected bulk tank milk from farms located in a limited geographical area where the same ST profile is circulating; on the other hand, as suggested by Pearson et al. (2014), it is more likely that, because MST PCR can genotype strains only when they are present in high amount, additional genotypes, which circulate at very low levels, have not been detected. The same can be stated with regard to handicraft cheeses which may contain more than one strain of Coxiella burnetii; in fact, Bauer et al. (2015) de- tected the presence of ST20 and ST8 in one bovine bulk tank milk sam- ple in Indiana, indicating that coinfection may occur in the same herd. As concern as small ruminant samples, to our knowledge, studies car- ried out by MST analysis demonstrated the presence of only one geno- type per goat bulk tank milk samples and individual milk specimens (Di Domenico et al., 2014; Pearson et al., 2014). The same was highlighted also as concern as tissue specimens which should actually contain higher bacterial burdens than milk; in fact, up to know, the presence of only one genotype was proven per goat tissue and per sheep specimens (Arricau-Bouvery et al., 2006; Di Domenico et al., 2014; Reichel et al., 2012; Santos et al., 2012; Tilburg et al., 2012b). Unfortunately, our results cannot be correlated to human data re- corded in Tuscany, since to the best of our knowledge, in our territory no genotyping data are available about the strains which circulate in humans. Furthermore, additional analyses of samples from a broader range of ruminant species are needed to increase epidemiological data regarding the occurrence of Coxiella burnetii in Tuscany.

In conclusion, more studies should be conducted on the presence of Coxiella burnetii in Italian small ruminants flocks combining epidemio-logical data with genotyping results to better understand which are the major genotypes involved in animal and in humans infections and to clarify the role of small ruminants in human outbreaks.

Conflict of interest statement

The authors declare that they have no conflict of interest.

References

374 Aquilini, D., Parola, P., Salvo, E., Paladini, A., 2000. Seroepidemiology of the rickettsioses, human
375 granulocytic ehrlichiosis, Lyme disease, Q fever, and tularemia in forestry workers in Tuscany, Italy.
376 J. Spirochetal Tick-borne Dis. 7, 35–41.
377 Arricau-Bouvery, N., Rodolakis, A., 2005. Is Q fever an emerging or re-emerging zoonosis?
378 Vet. Res. 36 (3), 327–349.
379 Arricau-Bouvery, N., Hauck, Y., Bejaoui, A., Frangoulidis, D., Bodier, C.C., Souriau, A., Meyer, H.,
380 Neubauer, H., Rodolakis, A., Vergnaud, G., 2006. Molecular characterization of Coxiella burnetii
381 isolates by infrequent restriction site-PCR and MLVA typing. BMC Microbiol. 6 (1), 1.
382 Astobiza, I., Barandika, J.F., Hurtado, A., Juste, R.A., García-Pérez, A.L., 2010. Kinetics of Coxiella
383 burnetii excretion in a commercial dairy sheep flock after treatment with oxytetracycline. Vet. J. 184
384 (2), 172–175.
385 Babudieri, B., Moscovici, C., 1950. Behaviour of Coxiella burnetii with physical and chemi- cal
386 treatment. Rendiconti - Istituto Superiore Di Sanità 13 (9-10), 739–748.
387 Bauer, A.E., Olivas, S., Cooper, M., Hornstra, H., Keim, P., Pearson, T., Johnson, A.J., 2015. Es-
388 timated herd prevalence and sequence types of Coxiella Burnetii in bulk tank milk samples from
389 commercial dairies in Indiana. BMC Vet. Res. 11 (1), 186.
390 Borriello, G., Iovane, G., Galiero, G., 2010. Q fever in domestic ruminants. Large Animal Rev. 16
391 (6), 273–283.
392 Bottero, M.T., Civera, T., Nucera, D., Rosati, S., Sacchi, P., Turi, R.M., 2003. A multiplex po-
393 lymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. Int.
394 Dairy J. 13 (4), 277–282.
395 Cabassi, C.S., Taddei, S., Donofrio, G., Ghidini, F., Piancastelli, C., Flammini, C.F., Cavirani, S.,
396 2006. Association between Coxiella burnetii seropositivity and abortion in dairy cattle of Northern
397 Italy. New Microbiol. 29 (3), 211–214
398 Capuano, F., Mancusi, A., Casalinuovo, F., Perugini, A., Proroga, Y., Guarino, A., Berri, M., 2012.
399 Real-time PCR-based detection of Coxiella burnetii in cheeses. Eur. Food Res. Technol. 235 (6),

1181–1186.

Ceglie, L., Guerrini, E., Rampazzo, E., Barberio, A., Tilburg, J.J.H.C., Hagen, F., Lucchese, L., Zuliani, F., Marangon, S., Natale, A., 2015. Molecular characterization by MLVA of Coxiella burnetii strains infecting dairy cows and goats of north-eastern Italy. Mi- crobes Infect. http://dx.doi.org/10.1016/j.micinf.2015.09.029.

Di Domenico, M., Curini, V., De Massis, F., Di Provvido, A., Scacchia, M., Cammà, C., 2014. Coxiella burnetii in Central Italy: novel genotypes are circulating in cattle and goats. Vector Borne Zoonotic Dis. 14 (10), 710–715.

Ebani, V.V., Nardoni, S., Bertelloni, F., Rocchigiani, G., Mancianti, F., 2015. Tick-borne infec- tions in horses from Tuscany, Italy. J. Equine Vet. Sci. 35 (4), 290–294.

EFSA, 2010. Scientific opinion on Q fever. EFSA J. 8 (5), 1595.

Eldin, C., Angelakis, E., Renvoisé, A., Raoult, D., 2013. Coxiella burnetii DNA, but not viable bacteria, in dairy products in France. Am.J.Trop. Med. Hyg. 88 (4), 765–769.

Fishbein, D.B., Raoult, D., 1992. A cluster of Coxiella burnetii infections associated with ex- posure to vaccinated goats and their unpasteurized dairy products. Am.J.Trop. Med. Hyg. 47 (1), 35–40.

Gale, P., Kelly, L., Mearns, R., Duggan, J., Snary, E.L., 2015. Q fever through consumption of unpasteurised milk and milk products - a risk profile and exposure assessment. J. Appl. Microbiol. 118 (5), 1083–1095.

Galiero, G., 2007. Causes of infectious abortion in the Mediterranean buffalo. Ital. J. Anim. Sci. 6 (2), 194–199.

Giovannini, A., Cancellotti, F.M., Turilli, C., Randi, E., 1988. Serological investigations for some bacterial and viral pathogens in fallow deer (Cervus dama) and wild boar (Sus scrofa) of the San Rossore Preserve, Tuscany, Italy. J. Wildl. Dis. 24 (1), 127–132.

Glazunova, O., Roux, V., Freylikman, O., Sekeyova, Z., Fournous, G., Tyczka, J., Tokarevich, N., Kovacava, E., Marrie, T.J., Raoult, D., 2005. Coxiella burnetii genotyping. Emerg. In- fect. Dis. 11 (8), 1211–1217.

Hatchette, T.F., Hudson, R.C., Schlech, W.F., Campbell, N.A., Hatchette, J.E., Ratnam, S., Raoult, D., Donovan, C., Marrie, T.J., 2001. Goat-associated Q fever: a new disease in Newfoundland. Emerg. Infect. Dis. 7 (3), 413–419.

Hirai, A., Nakama, A., Chiba, T., Kai, A., 2012. Development of a method for detecting Coxiella burnetii in cheese samples. J. Vet. Med. Sci. 74 (2), 175–180.

Hoover, T.A., Vodkin, M.H., Williams, J.C., 1992. A Coxiella burnetii repeated DNA element resembling a bacterial insertion sequence. J. Bacteriol. 174 (17), 5540–5548.

Hornstra, H.M., Priestley, R.A., Georgia, S.M., Kachur, S., Birdsell, D.N., Hilsabeck, R., Gates, L.T., Samuel, J.E., Heinzen, R.A., Kersh, G.J., Keim, P., Massung, R.F., Pearson, T., 2011. Rapid typing of Coxiella burnetii. PLoS ONE 6 (11). http://dx.doi.org/10.1371/ journal.pone.0026201.

Joulié, A., Laroucau, K., Bailly, X., Prigent, M., Gasqui, P., Lepetitcolin, E., Blanchard, B., Rousset, E., Sidi-Boumedine, K., Jourdain, E., 2015. Circulation of Coxiella burnetii in a naturally infected flock of dairy sheep: shedding dynamics, environmental contam- ination, and genotype diversity. Appl. Environ. Microbiol. 81 (20), 7253–7260.

Juffs, H., Deeth, H., 2007. Scientific evaluation of pasteurisation for pathogen reduction in milk and milk products. Food Standards Australia New Zealand, Canberra, Australia. 1.

Lamas, C.C., Rozental, T., Bóia, M.N., Favacho, A.R.M., Kirsten, A.H., da Silva, A.P.M., de Lemos, E.R.S., 2009. Seroprevalence of Coxiella burnetii antibodies in human immuno- deficiency virus-positive patients in Jacarepaguá, Rio de Janeiro, Brazil. Clin. Microbiol. Infect. 15 (2), 140–141.

Lucchese, L., Capello, K., Barberio, A., Zuliani, F., Stegeman, A., Ceglie, L., Guerrini, E., Marangon, S., Natale, A., 2015. IFAT and ELISA phase I/phase II as tools for the identi- fication of Q fever chronic milk shedders in cattle. Vet. Microbiol. 179 (1), 102–108. Magnino, S., Vicari, N., Boldini, M., Rosignoli, C., Nigrelli, A., Andreoli, G., Pajoro, M., Fabbi, M., 2009. Detection of Coxiella burnetii in bulk milk of dairy cattle from Lombardy,

Italy. Large Animal Rev. 15 (1), 3–6.

Maltezou, H.C., Constantopoulou, I., Kallergi, C., Vlahou, V., Georgakopoulos, D., Kafetzis, D.A.,

452 Raoult, D., 2004. Q fever in children in Greece. Am.J.Trop. Med. Hyg. 70 (5), 540–544.
453 Marenzoni, M.L., De Luca, S., Stefanetti, V., Castelli, L., Bietta, A., Coletti, M., Passamonti, F.,
454 Ranucci, D., Casagrande Proietti, P., 2013. Investigation of Coxiella burnetii occurrence in dairy
455 sheep flocks by molecular analysis in Umbrian area: a pilot study. Large Anim. Rev. 19 (5), 225–229.
456 Masala, G., Porcu, R., Sanna, G., Chessa, G., Cillara, G., Chisu, V., Tola, S., 2004. Occurrence,
457 distribution, and role in abortion of Coxiella burnetii in sheep and goats in Sardinia, Italy. Vet.
458 Microbiol. 99 (3), 301–305.
459 Masala, G., Porcu, R., Daga, C., Denti, S., Canu, G., Patta, C., Tola, S., 2007. Detection of path-
460 ogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR. J. Vet. Diagn. Investig. 19
461 (1), 96–98.
462 McQuiston, J.H., Childs, J.E., 2002. Q fever in humans and animals in the United States.
463 Vector Borne Zoonotic Dis. 2 (3), 179–191.
464 Monno, R., Fumarola, L., Trerotoli, P., Cavone, D., Massaro, T., Spinelli, L., Rizzo, C., Musti, M.,
465 2009. Seroprevalence of Q-fever, brucellosis and leptospirosis in farmers and ag- ricultural workers
466 in Bari, southern Italy. Clin. Microbiol. Infect. 15 (2), 142–143.
467 Natale, A., Busani, L., Comin, A., De Rui, S., Buffon, L., Nardelli, S., Marangon, S., Ceglie, L., 2009.
468 First report of bovine Q-fever in north-eastern Italy: preliminary results. Clin. Microbiol. Infect. 15
469 (2), 144–145.
470 OIE, 2015. Q fever. OIE Terrestrial Manual 2015 (Chapter 2.1.12).
471 Panning, M., Kilwinski, J., Greiner-Fischer, S., Peters, M., Kramme, S., Frangoulidis, D., Meyer, H.,
472 Henning, K., Drosten, C., 2008. High throughput detection of Coxiella burnetii by real-time PCR
473 with internal control system and automated DNA prepara- tion. BMC Microbiol. 8 (1), 1.
474 Parisi, A., Fraccalvieri, R., Cafiero, M., Miccolupo, A., Padalino, I., Montagna, C., Capuano, F.,
475 Sottili, R., 2006. Diagnosis of Coxiella burnetii-related abortion in Italian domestic ru- minants using
476 single-tube nested PCR. Vet. Microbiol. 118 (1), 101–106.
477 Pascucci, I., Di Domenico, M., Dall'Acqua, F., Sozio, G., Cammà, C., 2015. Detection of Lyme

478 Disease and Q Fever Agents in Wild Rodents in Central Italy. Vector Borne Zoonotic Dis. 15 (7),
479 404–411.
480 Pearson, T., Hornstra, H.M., Hilsabeck, R., Gates, L.T., Olivas, S.M., Birdsell, D.M., Hall, C.M.,
481 German, S., Cook, J.M., Seymour, M.L., Priestley, R.A., Kondas, A.V., Clark Friedman, C.L., Price,
482 E.P., Schupp, J.M., Liu, C.M., Price, L.B., Massung, R.F., Kersh, G.J., Keim, P., 2014. High
483 prevalence and two dominant host-specific genotypes of Coxiella burnetii in U.S. milk. BMC
484 Microbiol. 14 (1), 1.
485 Perugini, A.G., Capuano, F., Esposito, A., Marianelli, C., Martucciello, A., Iovane, G., Galiero, G.,
486 2009. Detection of Coxiella burnetii in buffaloes aborted fetuses by IS111 DNA am- plification: a
487 preliminary report. Res. Vet. Sci. 87 (2), 189–191.
488 Petruzzelli, A., Amagliani, G., Micci, E., Foglini, M., Di Renzo, E., Brandi, G., Tonucci, F., 2013.
489 Prevalence assessment of Coxiella burnetii and verocytotoxin-producing Escherichia coli in bovine
490 raw milk through molecular identification. Food Control 32 (2), 532–536.
491 Reichel, R., Mearns, R., Brunton, L., Jones, R., Horigan, M., Vipond, R., Vincent, G., Evans, S.,
492 2012. Description of a Coxiella burnetii abortion outbreak in a dairy goat herd, and as- sociated
493 serology, PCR and genotyping results. Res. Vet. Sci. 93 (3), 1217–1224.
494 Rodolakis, A., Berri, M., Héchard, C., Caudron, C., Souriau, A., Bodier, C.C., Blanchard, B.,
495 Camuset, P., Devillechaise, P., Natorp, J.C., Vadet, J.P., Arricau-Bouvery, N., 2007. Com- parison
496 of Coxiella burnetii shedding in milk of dairy bovine, caprine, and ovine herds. J. Dairy Sci. 90 (12),
497 5352–5360.
498 Santoro, D., Giura, R., Colombo, C., Antonelli, P., Gramegna, M., Gandola, O., Gridavilla, G., 2004.
499 Q fever in Como, Northern Italy. Emerg. Infect. Dis. 10 (1), 159–160.
500 Santos, A.S., Tilburg, J.J.H.C., Botelho, A., Barahona, M.J., Núncio, M.S., Nabuurs-Franssen, M.H.,
501 Klaassen, C.H., 2012. Genotypic diversity of clinical Coxiella burnetii isolates from Portugal based
502 on MST and MLVA typing. Int. J. Med. Microbiol. 302 (6), 253–256.
503 Shah, S.Y., Kovacs, C., Tan, C.D., Pettersson, G., Shrestha, N.K., Lutwick, L., Gordon, S.M., 2015.

504 Delayed diagnosis of Q fever endocarditis in a rheumatoid arthritis patient. IDCases 2 (4), 94–96.
505 Signs, K.A., Stobierski, M.G., Gandhi, T.N., 2012. Q fever cluster among raw milk drinkers in
506 Michigan, 2011. Clin. Infect. Dis. 55 (10), 1387–1389.
507 Šipka, M., 1958. Survival of Coxiella burneti in cheese. Vet. Glas. 12, 9–12.
508 Špoljarić, J., Mikulec, N., Plavljanić, D., Radeljević, B., Havranek, J., Antunac, N., 2013. Prov- ing
509 the adulteration of ewe and goat cheeses with cow milk using the reference method of isoelectric
510 focusing of γ-casein. Mljekarstvo 63 (3), 115–121.
511 Starnini, G., Caccamo, F., Farchi, F., Babudieri, S., Brunetti, B., Rezza, G., 2005. An outbreak
512 of Q fever in a prison in Italy. Epidemiol. Infect. 133, 377–380.
513 Tilburg, J.J.H.C., Rossen, J.W., Van Hannen, E.J., Melchers, W.J., Hermans, M.H., Van de
514 Bovenkamp, J., Roest, H.J., de Bruin, A., Nabuurs-Franssen, M.H., Horrevorts, A.M., Klaassen, C.H.,
515 2012a. Genotypic diversity of Coxiella burnetii in the 2007–2010 Q fever outbreak episodes in The
516 Netherlands. J. Clin. Microbiol. 50, 1076–1078.
517 Tilburg, J.J.H.C., Roest, H.-I.J., Buffet, S., Nabuurs-Franssen, M.H., Horrevorts, A.M., Raoult, D.,
518 Klaassen, C.H.W., 2012b. Epidemic genotype of Coxiella burnetii among goats, sheep, and humans
519 in the Netherlands. Emerg. Infect. Dis. 18 (5), 887–889.
520 Tiscione, E., Ademollo, B., Donato, R., Roller, S., Signorini, L.F., 1989. Prevalence of antibod- ies
521 against Coxiella burnetii in 2 geographical zones of Tuscany. Annali di Igiene: Medicina Preventiva
522 e di Comunità 1 (5), 1133–1143.
523 Torina, A., Vicente, J., Alongi, A., Scimeca, S., Turlá, R., Nicosia, S., Di Marco, V., Caracappa, S.,
524 De La Fuente, J., 2007. Observed prevalence of tick-borne pathogens in domestic animals in Sicily,
525 Italy during 2003–2005. Zoonoses Public Health 54 (1), 8–15.
526 Valasek, M.A., Repa, J.J., 2005. The power of real-time PCR. Am. J. Physiol. – Adv. Physiol.
527 Educ. 29 (3), 151–159.
528 Valla, G., Bizzarri, D., Ferrari, G., Bussacchini, M., 2014. Prevalence of Coxiella burnetii in bulk
529 milk in herds of dairy cows and possible correlation with Italian reproductive problems. Large Anim.

530 Rev. 20 (2), 51–56.
531 Van den Brom, R., Van Engelen, E., Roest, H.I.J., Hoek, W.D., Vellema, P., 2015. Coxiella burnetii
532 infections in sheep or goats: an opinionated review. Vet. Microbiol. http://
533 dx.doi.org/10.1016/j.vetmic.2015.07.011.
534 Verraes, C., Vlaemynck, G., Van Weyenberg, S., De Zutter, L., Daube, G., Sindic, M., Uyttendaele,
535 M., Herman, L., 2015. A review of the microbiological hazards of dairy products made from raw
536 milk. Int. Dairy J. 50, 32–44.
537 Vicari, N., Faccini, S., Ricchi, M., Garbarino, C., Decastelli, L., Boldini, M., Rosignoli, C., Dalmasso,
538 A., Bronzo, V., Fabbi, M., 2013. Occurrence of Coxiella burnetii in bulk tank milk from northwestern
539 Italy. Vet. J. 192, 529–531.
540

Fig. 1. PCR after agarose gel electrophoresis. Lanes M, molecular size marker (100 bp DNA Ladder H3 RTU, Nippon Genetics Europe GmbH, Germany); lane 1–18: 51–64 samples; cattle milk = 256 bp, sheep milk = 172 bp or goat milk = 326 bp.

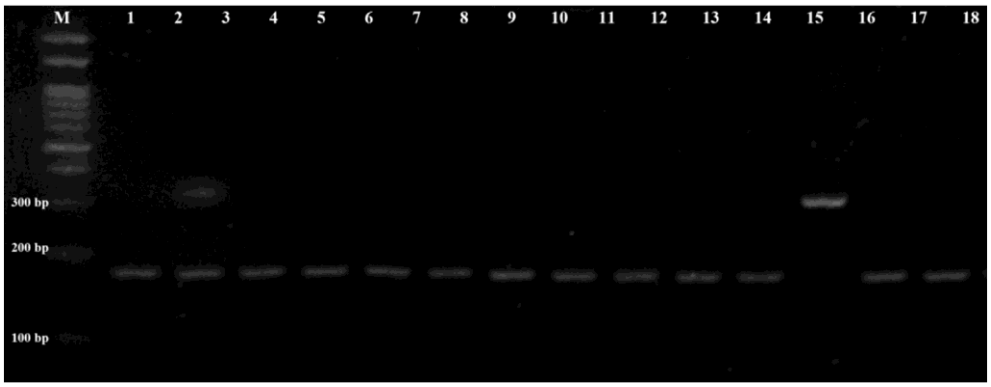


Table 1

Results recorded in unpasteurized cheeses labeled as produced from goat milk.

	Sample ID	Multiplex PCR	Ripening	aw	Farm/dairy code	End point PCR	Real time PCR (Ct)	MST type
Handicraft	62	G	S.	0.97	1	−	−	/
cheeses	80	O/B	S.	0.985	2	−	−	/
	81	G	S.	0.985	3	−	−	/
	82	G	S.	0.979	3	−	−	/
	83	G	S.	0.98	3	−	−	/
	84	G	S.	0.987	3	−	−	/
	85	G	S.	0.997	2	−	−	/
	1F	G/O	S-h.	0.958	4	+	+ (21)	ST 12
	2F	G/O	S-h.	0.942	4	+	+ (26)	/
	33	G/O	S-h.	0.953	7	+	+ (36)	/
	34	G/O	H.	0.925	7	−	−	/
Non-handicraft cheeses	15	G/O	S-h.	0.955	5	+	+ (33)	/
	16	G/O	S-h.	0.956	6	+	+ (32)	/
	72	G	H.	0.875	8	−	−	/

G = cheese produced from goat milk; O/B = cheese produced from a mixture of ovine milk with bovine milk; G/O = cheese produced from a mixture of goat milk with ovine milk; S. = soft cheese; S-h. = semi-hard cheese; H. = hard cheese

Table 2

Results recorded in unpasteurized cheeses labeled as produced from sheep milk.

	Sample ID	Multiplex PCR	Ripening	aw	Farm/dairy code	End point PCR	Real time PCR(Ct)	MST type
Handicraft cheeses	C F	O	S.	0.978	10	—	—	/
	79	G	S.	0.971	12	—	—	/
	12	O/B	S.	0.972	13	+	+ (31)	ST32
	17	B	S.	0.975	10	—	—	/
	56	O	S.	0.975	14	—	+ (38)	/
	21	O	S.	0.976	15	—	—	/
	38	O/B	S.	0.985	16	—	—	/
	59	O	S.	0.971	18	—	—	/
	69	O	S.	0.975	20	—	—	/
	D F	O	S-h.	0.963	9	—	—	/
	D FS	O	S-h.	0.957	9	—	—	/
	20	O	S-h.	0.945	19	—	—	/
	37	O	S-h.	0.948	24	—	—	/
	18	G	S-h.	0.953	10	—	—	/
	39	G/O	S-h.	0.966	12	—	—	/
	41	O	S-h.	0.954	25	—	—	/
	26	O	S-h.	0.949	19	—	—	/
	C FS	O	S-h.	0.954	10	—	—	/
	51	O	S-h.	0.946	29	+	+ (35)	/
	57	O	S-h.	0.957	14	+	+ (37)	/
	58	O	S-h.	0.932	14	—	—	/
	60	O	S-h.	0.95	18	—	—	/
	61	O	S-h.	0.958	18	—	—	/
	63	O	S-h.	0.957	31	—	—	/
	64	O	S-h.	0.93	31	—	+ (38)	/
	65	O	S-h.	0.947	31	—	—	/
	67	O	S-h.	0.942	32	—	+ (38)	/
	68	O	S-h.	0.945	32	—	—	/
	76	O	S-h.	0.933	33	—	—	/
	78	O	S-h.	0.933	33	—	—	/
	25	O	H.	0.797	19	—	—	/
	86	O	H.	0.92	34	—	—	/
	87	O	H.	0.88	34	—	—	/
	88	O	H.	0.901	34	—	—	/
	D ST	O	H.	0.91	9	—	—	/
	C ST	O	H.	0.926	10	—	—	/
	19	O	H.	0.867	10	—	—	/
	22	O	H.	0.883	15	—	—	/
	23	O	H.	0.898	19	—	—	/
	24	O	H.	0.864	19	—	—	/
	42	O	H.	0.862	35	—	—	/
	45	O	H.	0.919	36	—	—	/
	66	O	H.	0.921	32	—	—	/
	73	O	H.	0.903	25	—	—	/

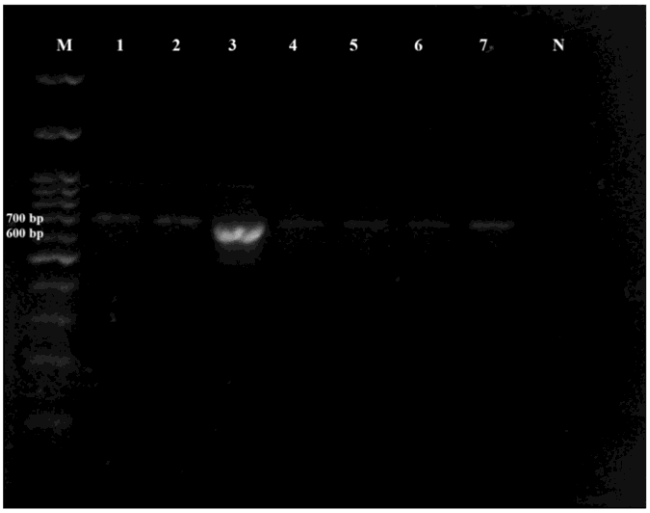
	74	O	H.	0.907	25	—	—	/
	75	O	H.	0.92	25	+	+ (33)	/
	77	O	H.	0.922	33	—	—	/
Non-handicraft cheeses	40	O	S.	0.97	17	—	+ (37.5)	/
	71	G/O	S.	0.976	21	—	—	/
	30	O	S-h.	0.953	11	—	—	/
	32	O	S-h.	0.935	22	+	+ (36)	/
	35	O/G/B	S-h.	0.939	23	+	+ (32)	/
	43	O/B	S-h.	0.969	26	—	+ (35.5)	/
	27	O	S-h.	0.959	27	—	—	/
	46	O	S-h.	0.954	26	—	—	/
	47	O	S-h.	0.967	28	+	+ (34)	/
	49	O	S-h.	0.944	28	+	+ (31)	ST12
	50	O	S-h.	0.951	28	+	+ (34)	/
	52	G/O	S-h.	0.952	30	—	+ (39)	/
	53	O	S-h.	0.939	30	—	—	/
	54	O	S-h.	0.942	30	—	—	/
	13	O	S-h.	0.966	6	+	+ (36)	/
	14	O	S-h.	0.953	26	—	—	/
	70	O	S-h.	0.934	21	+	+ (31)	ST12
	28	O	H.	0.921	26	—	—	/
	29	O	H.	0.908	26	+	+ (35)	/
	31	O	H.	0.925	11	—	+ (39)	/
	44	O	H.	0.893	26	+	+ (32)	/
	48	O	H.	0.819	28	+	+ (32)	/
	55	O	H.	0.928	30	+	+ (30)	ST32

555 O = cheese produced from sheep milk; G = cheese produced from goat milk; O/B = cheese produced from a mixture of ovine milk with bovine

556 milk; B = cheese produced from bovine milk; G/O = cheese produced from a mixture of goat milk with ovine milk; O/G/B = cheese produced

557 from a mixture of ovine, goat and bovine milk; S. = soft cheese; S-h. = semi-hard cheese; H. = hard cheese.

559 Fig. 2. PCR after agarose gel electrophoresis. Lanes M, molecular size marker (100 bp DNA Ladder
560 H3 RTU, Nippon Genetics Europe GmbH, Germany); lane 1: positive control (Nine Mile strain,
561 ATCC VR-615) (687 bp); lane 2–7: 55, 1F, 50, 51, 57, 12 samples; lane N: negative control (Ultra
562 Pure Water DNase and RNase-free, Biological Industries, Israel).



563

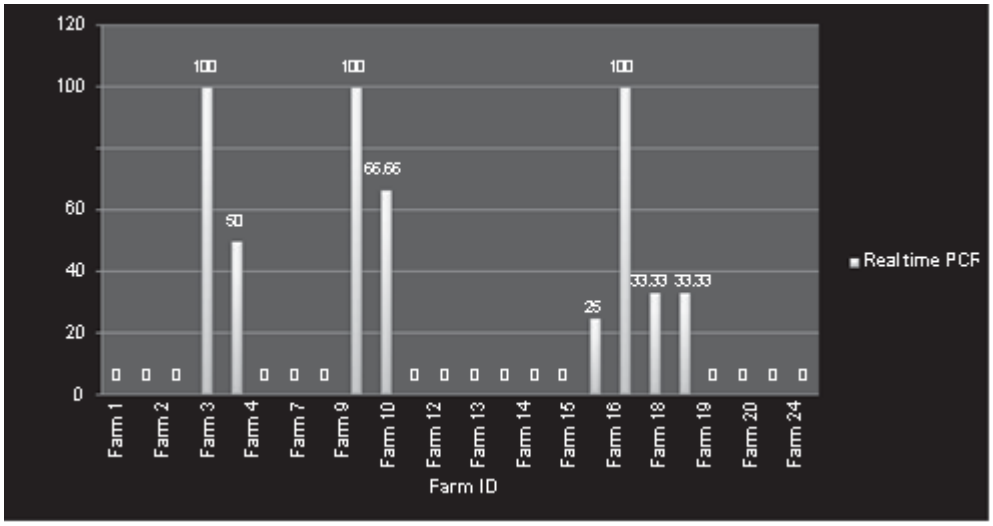
564 Table 3
565 Results recorded by Real time PCR on the cheese samples produced from ewe and goat milk.
566
567
568

	Milk species production						Moisture content			Kind of	
	O	G	G/O	O/B	O/G/B	B	Soft cheese	Semi-hard cheese	Hard cheese	Handicraft	Non-handicraft
Positive samples no.	18	0	6	2	1	0	3	18	6	10	17
Samples tested no.	60	9	9	4	1	1	18	41	25	58	26

569 O = cheese produced from sheep milk; G = cheese produced from goat milk; O/B = cheese produced from a mixture of ovine milk with
570 bovine milk; B = cheese produced from bovine milk; G/O = cheese produced from a mixture of goat milk with ovine milk; O/G/B = cheese
571 produced from a mixture of ovine, goat and bovine milk.
572

573 Fig. 3. Percentage of positivity at farm level by Real time PCR.

574



575

576

577

578

579

Fig. 4. Percentage of positivity at dairy level by Real time PCR