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Effect of *Mycoplasma agalactiae* mastitis on milk production and composition in Valle dell Belice dairy sheep

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ABSTRACT

Contagious agalactia (CA), a disease caused by *Mycoplasma agalactiae* and other pathogenic mycoplasmas, is a well-known multietiological syndrome affecting dairy breeds of sheep and goats in the Mediterranean basin. The aim of this work was to study the effect on milk production and composition of mastitis caused by *M. agalactiae* in Valle del Belice dairy sheep. All ewes were manually milked twice daily and the milk from both daily milking was analysed for milk composition and somatic cell counts. Moreover the morning milk samples were collected aseptically from each animal for bacteriological analyses. A mixed linear model was utilised to consider milk production and composition between animals infected by CA and healthy animals. After bacteriological investigation using both cultural and molecular methods, 37 ewes were found to be infected by *M. agalactiae* while 50 uninfected ewes were randomly selected from the same herds to compare milk production and composition between infected and healthy animals. Statistical analyses showed that the infection with *M. agalactiae* had a significant effect on yield and some milk components. In particular, infected ewes showed lower milk production with lower lactose content and higher somatic cell counts. The implementation of disease control programmes based on rapid laboratory diagnosis and modern control methods is desirable for Mediterranean endemic areas.

HIGHLIGHTS

- Contagious agalactia is caused by *M. agalactiae* and affects small ruminant dairy farms in the Mediterranean basin.
- Contagious agalactia is endemic in many countries and has a severe health and economic impact.
- Effect on milk production and composition of mastitis caused by *M. agalactiae*.

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Introduction

In small ruminants, diseases caused by *Mycoplasma* generally cause a short septicaemia with secondary localisation of the pathogen in organs such as the udder, respiratory tract, joints, genital tract and conjunctiva. Among these, two diseases are prominent for their pathogenicity and socio-economic implications and for this reason are included in the OIE International Animal Code List: contagious caprine pleuropneumonia (CCPP) and contagious agalactia (CA). CCPP is caused by *M. capricolum* subsp. *capripneumoniae*, which mainly affects goats; it is

widespread in African and Asian countries but has not recently been reported in Europe. CA is caused mainly by *M. agalactiae* and affects small ruminant dairy farms in the Mediterranean basin; three other mycoplasmas cause a clinically similar but sporadic syndrome. CA, characterised by mastitis, arthritis and keratoconjunctivitis, was recognised in sheep and goat by Bridre and Donatien (1925) who for the first time were able to isolate and grow the causative microorganism. Since 1932 the disease has been endemic in Sicily and inactivated vaccines produced by Regional Istituti Zooprofilattici Sperimentali IZS have been used

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to control the disease (Stazzi and Mirri 1956). CA has a high economic impact for sheep and goat farmers due to its high morbidity and impact on milk production (Nicholas et al. 2008). The favourable conditions of most Mediterranean livestock management particularly in southern Italy which comprises: extensive farming, shared pasture, selection of breeds with high milk production, manual milking, mixed breeding of sheep and goats in the same farm and uncontrolled exchange of animals between farms exacerbate the disease. The incubation period of CA ranges from 6 to 30 days and there can be an acute and sub-acute form (Nicolet 1994). The acute syndrome is rare and is characterised by high fever, neurological signs, tremors and death. The subacute form is typical of endemic areas and is characterised by mastitis, keratoconjunctivitis and arthritis. Infection of the udders evolves as interstitial mastitis, initially seen as a swelling of the udder, increased udder temperature and pain during milking, followed by a drastic fall in production and quality of milk (Nicholas 2002). Arthritis and keratoconjunctivitis are normally observed in 5–10% of cases.

The culture and isolation of mycoplasma is laborious, and many serological tests are non-specific and/or poorly sensitive (Razin 1994). Nowadays, molecular techniques have the advantage that they are rapid, more sensitive, specific and efficient compared to the conventional methods (Magistrado et al. 2001; Keramas et al. 2004). In Sicily CA is responsible for about 40% of mastitis cases in sheep and goats and after brucellosis is one of the most widespread problems in small ruminants.

Valle del Belice sheep are the most commonly reared dairy breed in Sicily with about 105,000 heads with an average milk production of 198 litres for lactation (AIA 2015). The main use of the milk from Valle del Belice breed is for the production of traditional raw milk cheeses namely Pecorino Siciliano and PDO Vastedda del Belice at farm level by small local dairies or by cheese factories. Studies on the effects of CA on milk yield and composition in small ruminants are scarce. In one study on the same sheep breed a reduction of 17% of milk yield and an alteration of milk chemical composition was observed (Todaro et al. 2015). In clinically affected herds, a significant increase in somatic cell counts from bulk milk tank in sheep and goat was observed (Gonzalo et al. 2005; Contreras et al. 2008). In a study on the effects of *Mycoplasma spp.* in herds without clinical signs of CA, no significant differences in goat milk quality were found (de la Fe et al. 2009). The aim of this work was

Table 1. Numbers of ewes and test-day observations according to herd.

Herd	Total N° ewes	Case	Control	N° TD	Mean N° of TD per ewe
1	215.0	12.0	12.0	113.0	4.7
2	225.0	13.0	23.0	269.0	7.5
3	169.0	12.0	15.0	150.0	5.6
Total	609.0	37.0	50.0	532.0	–

TD: test day.

to study the effect on milk production and composition of mastitis caused by *M. agalactiae* in Valle del Belice dairy sheep.

Materials and methods

Animals and milk sampling

Animals chosen for this study belonged to three herds of Valle del Belice dairy sheep located in the Agrigento district of southern Sicily. These three herds were chosen as they only raised ewes belonging to the Valle del Belice dairy sheep breed selected for milk production. The study was conducted to assess the effect on milk production and composition of a subclinical form of mastitis due to *M. agalactiae*. The procedures involving animal sample collection followed the recommendation of Directive 2010/63/EU. Sampling was carried out by trained technicians within the frame of milk recording, hence no permission from the animal research ethics committee was necessary. The breeding system in all farms was extensive, with an average lactation length of nine months (270 days), with animals fed on pasture and with some feed integration. Milk samples (50 mL) were collected at approximately monthly intervals from October 2017 until June 2018. All ewes were manually milked twice daily and the milk from both daily milkings were analysed. Milk composition, in particular fat (FAT), protein (PRT), casein (CAS), lactose percentage (LCT) and somatic cells count (SCC) were determined using a Combifoss FT+ (Foss Electric Hillerød, Denmark) and were calculated as the weighted average of the morning and evening milking, where weighting is according to the corresponding daily milk yield (MY). At the morning milking, a milk sample (50 mL) from both half-udders was collected aseptically from each animal for bacteriological and molecular analyses as described below. All milk samples were kept refrigerated during transportation to the laboratory and processed within 24 h. On the basis of both cultural and real-time PCR methods, ewes were classified as infected if *M. agalactiae* was detected in the milk at least one or more times during the lactation otherwise animals were classified as healthy. Ewes

positive by bacteriological analysis for pathogens other than *M. agalactiae* were excluded from the survey. At the end of the lactation, 532 test day observations from 87 ewes (37 cases and 50 controls) were retained for statistical analysis (Table 1).

Bacteriological analysis

The culture and isolation assays were carried out according to standard procedure (Nicholas and Baker 1998). Briefly, 300 μ l of milk samples were incubated in tubes with 3 ml of mycoplasma medium (Oxoid[®] Mycoplasma Broth Base) after enrichment with yeast extract, porcine serum (at 10%) and antibiotics, until the stationary growth phase. Each sample was diluted in 5 log steps from 10^{-1} to 10^{-6} to eliminate the risk of contamination. All the broths were incubated at 37°C with 10% CO₂ for over 4 days until evidence of mycoplasma growth, that is indicated by a fine cloudiness or opalescence (OIE 2008). The samples were then spotted (10 μ l) onto agar media (Oxoid[®] Mycoplasma Agar Base) and incubated at 37°C with 10% CO₂ for 48–72 h. The agar medium was examined for typical mycoplasma ‘fried-egg’ colonies using a stereomicroscope (Nikon SMZ800N). Moreover all milk samples were examined for other mastitis agents by conventional techniques, on 5% sheep blood agar plates, incubated at 37°C, and examined after 10–24 h and 36–48 h incubation (Riggio et al. 2010). Several bacteriological colonies were seen, mainly of the genera *Staphylococcus*, *Streptococcus*, *Pasteurella*, *Escherichia* and *Pseudomonas*. Animals positive for bacteria other than *M. agalactiae* were not considered in this study.

Molecular analysis

The DNA was extracted from 1 ml cultures using the InstaGene[™] Matrix (Biorad) according to the manufacturer’s instructions. A Taqman real-time PCR targeting the p40 gene (Oravcovà et al. 2009) was applied for specific detection of *M. agalactiae* in milk samples. According to Oravcovà et al. (2009) the amplification was performed in a 20- μ l reaction volume including 2 μ l of DNA, 1X Sso Fast Probes Supermix (Bio-Rad Laboratories Srl) 300 nM for primers MAP40127F (5′-TCATTACAGCAGTGCCTTTATTAG-3′) and MAP40235R (5′-CACCTAATGCTTGTTTTCAACC-3′) and 200 nM for the 6-carboxyfluorescein [FAM]-labeled MAP40160P probe (5′-FAM-TGTGATGATAAGAACGAAAATTCACAAA-BHQ1-3′). Real-time PCR was conducted in a CFX96 Touch[™] Real-Time PCR Detection System (Biorad) with the following programme: 2 min at 50°C, 10 min at

95°C, and 50 cycles of 15 s at 95°C and 1 min at 60°C. In order to verify the absence of the PCR inhibitors in the DNA templates and to assess the PCR performance of each reaction, an exogenous internal positive control (IPC) was added and coamplified in the same PCR reaction mixture, according to the manufacturer’s instructions (TaqMan[®] Exogenous Internal Positive Control Reagents—VIC[™]Probe—Applied Biosystem).

Statistical analysis

A mixed linear model was fitted to consider milk production and composition between animal infected by CA and healthy animals. SCC values were transformed to log₁₀ prior to statistical analysis. The following mixed model was used:

$$y = Xb + Zu + e$$

where y is the observation vector for MY, FAT, PRT, CAS, LCT and SCC; b is the vector of fixed effects that includes herd (3 levels, from 1 to 3), order of parity (5 levels, from 1 to 5), stage of lactation as covariate, infection state (2 levels, 0 for healthy and 1 for unhealthy); u is the vector of random effect of animals (87 ewes); e is the vector of residuals; X and Z are the incidence matrices of appropriate order relating observations with fixed and random effects, respectively. The random effects were assumed to be normally distributed with zero means and the following covariance structure:

$$\text{Var} \begin{bmatrix} u \\ e \end{bmatrix} = \begin{bmatrix} I\sigma_u^2 & 0 \\ 0 & I\sigma_e^2 \end{bmatrix}$$

For the analysis, the R package nlme version 3.1–137 (Pinheiro et al. 2018) were used. Comparison of milk production and composition between infected and healthy animals was conducted using Fisher’s protected least significant difference (LSD) at the 5% level of probability (Steel and Torrie 1980).

Results

After bacteriological investigation using both culture and real time PCR methods, 37 ewes were found to be infected by *M. agalactiae*. To compare milk production and composition between infected and healthy animals 50 ewes that did not present the infection during lactation were randomly selected from the same herds. The incidence of *M. agalactiae* infection ranged from 5.6 to 7.1% in the three herds considered in this study. Mean, standard deviation and least square means for the traits considered for healthy and infected animals are reported in Table 2. Table 3

Table 2. Descriptive statistics and least significant differences for the milk production traits according to healthy or infected status.

Traits	Mean \pm SD		Difference
	Healthy	Infected	
MY, g	1514.00 \pm 651.26	1327.00 \pm 534.60	186.802*
FAT, %	6.92 \pm 1.25	6.94 \pm 1.20	-0.017
PRT, %	5.75 \pm 0.63	5.74 \pm 0.71	0.004
CAS, %	5.51 \pm 0.54	4.51 \pm 0.58	-0.002
LCT, %	4.50 \pm 0.27	4.38 \pm 0.48	0.110*
SCC, log ₁₀	5.42 \pm 0.66	6.05 \pm 0.66	-0.630*

CAS: casein; FAT: fat; MY: milk yield; PRT: protein; LCT: lactose; SCC: somatic cell counts; SD: standard deviation

* $p < .001$.

Table 3. Significance test from ANOVA for milk production and composition.

Effect	Milk component					
	MY, g	FAT, %	PRT, %	CAS, %	LCT, %	SCC, log ₁₀
herd	0.0173	0.0110	ns	ns	0.0111	0.0016
op	0.0015	0.0487	0.0016	<0.0001	0.0081	0.0001
stl	<0.0001	<0.0001	0.0002	0.0003	<0.0001	0.0259
inf	0.0056	ns	ns	ns	ns	<0.0001

CAS: casein; FAT: fat; inf: infection state; LCT: lactose; MY: milk yield; op: order of parity; PRT: protein percentage; stl: stage of lactation; SCC: log₁₀ somatic cell counts.

shows the analysis of variance for milk production and composition. Stage of lactation contributed significantly to variations of all the variables. Herd effect was significant for all the variables except for PRT and CAS. Infection state fixed effect was significant only for MY and SCC although for LCT a p value of .07 was found. Least significance differences for the considered traits considering the infection state are reported in Table 2. Statistical analyses revealed that the infection with *M. agalactiae* has an effect on milk yield and constituents, namely for LCT and SCC. In particular infected ewes showed a lower milk production (about 187g), lower lactose content (-0.11%) and a higher level of somatic cell count (0.63 log cells/mL, 859,000 cells/mL in terms of geometric mean).

Discussion

This study was based on the evaluation of natural infection due to *M. agalactiae* in three flocks of Valle del Belice dairy sheep. Tests of significance of fixed effects of the mixed model applied in this study showed a significant effect in order of parity and lactation stage for all traits considered, while herd effect was significant for all traits except for protein and casein percentage. The infection status had a significant effect on milk production and somatic cell count, however a P value of 0.07 was found for lactose percentage (Table 3). The percentage of ewes affected was lower than the 7.9% observed by Contreras et al.

(2008) in goat bulk-tank milk and the 11.2% reported by Verbisck-Bucker et al. (2008) in Spanish ibex wild goat. With regard to the effect of losses in milk production, the estimated losses were approximately 12.5% and ranged from 1514g for healthy ewes to 1327g for infected ewes. The lower milk production of infected sheep was similar to results reported by Todaro et al. (2015) who found a decrease of about 17% of milk in experimentally infected ewes of the same sheep breed. Our results showed that infection with *M. agalactiae* had a significant impact on milk production. The loss of milk yield revealed in the present experiments should draw the attention of the dairy sheep industry to the problem of CA since all the milk is used to produce cheese. Surprisingly no significant differences were found for FAT, PRT and CAS amongst healthy and infected animals whereas a lower percentage of LCT and a higher SCC were found for infected ewes.

In the literature, there are discordant data about the effects of CA on milk composition. In general, intramammary infection caused by *M. agalactiae* in endemic countries presents more often as a subacute infection occasionally with subclinical mastitis. Leitner et al. (2003) reported a higher total protein concentration and lower lactose in sheep with subclinical mastitis. Todaro et al. (2015) reported negative effects of *M. agalactiae* on fat and protein content probably due to the lower functionality of the udders of infected ewes. In particular, the authors showed a higher protein percentage (5.66 versus 4.84%) and a lower lactose percentage (3.64 versus 4.66%) in experimentally infected Valle del Belice ewes compared to healthy animals. Fox et al. (2003) reported a lower percentage of fat (3.62 versus 3.69%) and higher percentage of lactose (4.95 versus 4.92%) in farms positive to *M. agalactiae* compared with negative farms, whereas somatic cell counts were not significantly different in mycoplasma positive flocks. The increase in somatic cell counts was observed in different Spanish dairy sheep breeds by Gonzalo et al. (2005) who reported an increase from 5.96 (905×10^3 cells/mL) to 6.06 (1157×10^3 cells/mL) in sheep bulk-tank milk. Similar results were obtained by Contreras et al. (2008) in goat bulk-tank milk. An increase in somatic cell count was also reported in ewes experimentally infected with *M. agalactiae* (Bergonier et al. 1996; Todaro et al. 2015). In a study of bulk tank milk from different goat herds co-infected with various *Mycoplasma spp.*, the presence of different *Mycoplasma spp.* had no effect on fat, protein and lactose (de la Fe et al. 2009). Conversely, the co-infection with different *Mycoplasma*

strains has been shown to affect negatively milk yield and quality in dairy cattle in comparison with single *Mycoplasma spp* (Al-Farha et al. 2017). In our study only infection with *M. agalactiae* was investigated; further research is needed to better understand the effect on milk yield and composition of the co-infection with different *Mycoplasma spp* and other pathogens causing mastitis.

Conclusions

Mycoplasma agalactiae in sheep and goats is the main pathogen of CA in Mediterranean countries. This chronic disease is considered endemic in many countries and has a severe health and economic impact. Statistical analyses revealed that the infection with *M. agalactiae* has an effect on milk yield and constituents. In particular, infected ewes showed lower milk production with lower lactose content and a higher level of somatic cell count. The decrease in milk production and quality has a high economic impact especially for small producers therefore mastitis caused by the *M. agalactiae* should be investigated during routine diagnostic analyses.

Disclosure statement

No potential conflict of interest was reported by the authors.

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