Association of subclinical mastitis prevalence with sheep breeds reared in Greece

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Supplementary material 1. Detailed description of procedures and techniques employed in the study.

1. Sheep farms and animal sampling

In total, 111 sheep farms in the 13 administrative regions of Greece were included into the study and visited for collection of samples and information. Veterinarians active in small ruminant health management around Greece, were contacted and asked if they wished to collaborate in the investigation. In total, 23 veterinarians had agreed to collaborate. Farms were selected by the collaborating veterinarians on convenience basis (i.e., willingness of farmers to accept a visit by University personnel for sampling animals). The principal investigators (NGCV, GCF) visited all farms for sample collection. Farms were classified according to management system followed therein, as intensive (n=26), semi-intensive (n=57), semi-extensive or extensive (n=28), by following the criteria of the European Food Safety Authority (2014).

In each farm, 20 clinically healthy ewes (*secundiparae* or older) were selected at random for sampling. For selection of animals, farmers had been asked to remove *primiparae* ewes and ewes with known udder abnormalities from the main flock. A standardised clinical examination (observation, palpation, comparison between glands) of the udder was performed, always by the principal investigator (NGCV) (Fthenakis, 1994; Mavrogianni et al., 2005) and the first two squirts of secretion were drawn on the gloved hand of an assisting investigator and assessed. All investigators involved in sampling procedures wore disposable, non-sterile latex gloves. If udder abnormalities were recorded during clinical examination, the ewe was excluded from sampling. Animals found with abnormalities and excluded, were not replaced.

Standard methods for aseptic collection of milk samples were followed (Fthenakis, 1994). Then, 10 to 15 mL of secretion were collected into a sterile container; separate samples were collected from each mammary gland into separate containers. Milk samples were then drawn onto a paddle for performing the California Mastitis Test (CMT). For transportation, samples were stored into portable refrigerators with ice packs and transported by car; for samples collected in islands, airplane or boat transportation, as accompanying luggage, was also involved.

2. Paraclinical examinations

Laboratory procedures started within 24 h after collection. Milk samples (10 μL) were cultured using Columbia blood agar plates incubated aerobically at 37 °C for up to 72 h. Bacterial

identifications were performed by using standards methods (Barrow and Feltham, 1993; Euzeby, 1997).

After sample collection, at ewe-side, all samples were tested by use of the CMT. The test was performed as previously described for ewes' milk (Fthenakis, 1995); it was carried out and scored always by the same person, i.e., the principal investigator (NGCV). Five degrees of reaction ('negative', 'trace', '1', '2', '3') were described (Schalm et al., 1971). Milk smears were also produced and dried. The milk smears were stained by the Giemsa method for estimation of leucocyte subpopulations; proportion of leucocyte types therein was calculated by observing at least 10 fields of each milk film under magnification 10×. Subsequently, the Microscopic cell counting method (Mccm) (IDF reference method) (International Dairy Federation, 1984; Contreras et al., 2007; Raynal-Ljutovac et al., 2007) was performed in 894 samples (20.3% of all samples).

3. Data management and analysis

Ewes were considered to have subclinical mastitis when a bacteriologically positive milk sample ([a] >10 colonies of the same organism and [b] no more than two different types of colonies) with concurrently increased CMT score (≥'1') plus neutrophil and lymphocyte proportion (≥65% of all leucocytes) was detected (Fragkou et al., 2014). The definition referred to ewes (hence, animals with both glands affected were counted as one case).

Quantitative information on the cellular content of ewes' milk was obtained by using two sets of data: the CMT results and the results of the Mccm. Although it is generally established that CMT results are reliable proxy measurements for somatic cell counts (SCCs) (Fthenakis, 1995; Gonzalez-Rodríguez and Carmenes, 1996), we further confirmed that in the present study. Following assignment of numerical values to CMT scores (value 0 to score 'negative', value 1 to score 'trace', value 2 to score '1', value 3 to score '2', and value 4 to score '3') and $\log_{10}$-transformations, correlation between CMT scores and Mccm SCCs was r=0.913 (95% CI: 0.902-0.923) (P<0.001) and the corrected R^2 was 83.4%; significance of the difference between r and rho (the correlation hypothesized to exist within the population from which the sample had been drawn) was P<0.001.

For analysis, data were entered into Microsoft Excel and analysed using IBM SPSS Statistics (ver. 21) (IBM; Armonk, NY, USA). The outcome of 'subclinical mastitis' was considered. Exact binomial confidence intervals (C.I.) were obtained. A preliminary assessment of the importance of predictors was performed using by cross-tabulation with the chi-square test, and with simple logistic regression without random effects. Subsequently, mixed-effects logistic regression was employed to perform the same comparisons, using the different farms (n=111) as a 'random effect'. Then, analysis of variance was employed and the following comparisons were made between farms in relation to this outcome:

(a) farms with pure-bred animals *versus* farms with cross-bred animals,

(b) farms with Greek pure-bred animals *versus* farms with imported pure-bred animals,

(c) farms with imported pure-bred animals *versus* all other farms (i.e., farms with Greek pure-bred animals and farms with cross-bred animals),

(d) farms with the various Greek pure-bred animals (in total, 8 breeds), farms with imported pure-bred animals (in total, 2 breeds) and farms with cross-bred animals and

(e) farms with the various pure-bred animals (in total, 10 breeds) between them.

Subsequently, farms with the Greek breeds Cephalonia, Crete, Karagouniko, Karystos, Lesvos and Vlahiko were considered together in a cluster termed 'Greek traditional indigenous breeds' (n=18 farms), as initial comparison between those farms did not show significant difference. Then, comparisons between the various breeds were repeated with smaller number of breeds (in total, 3 Greek pure-breeds and 5 breeds in total).

Finally, a multivariable model was created using mixed-effects logistic regression with farm as the random effect, which included as variables the management system in farms and the sheep breed. The analysis was repeated by considering farms under semi-extensive and extensive management clustered together (i.e., using 3 categories in the management system).

Statistical significance was defined at ≤ 0.05.

4. References

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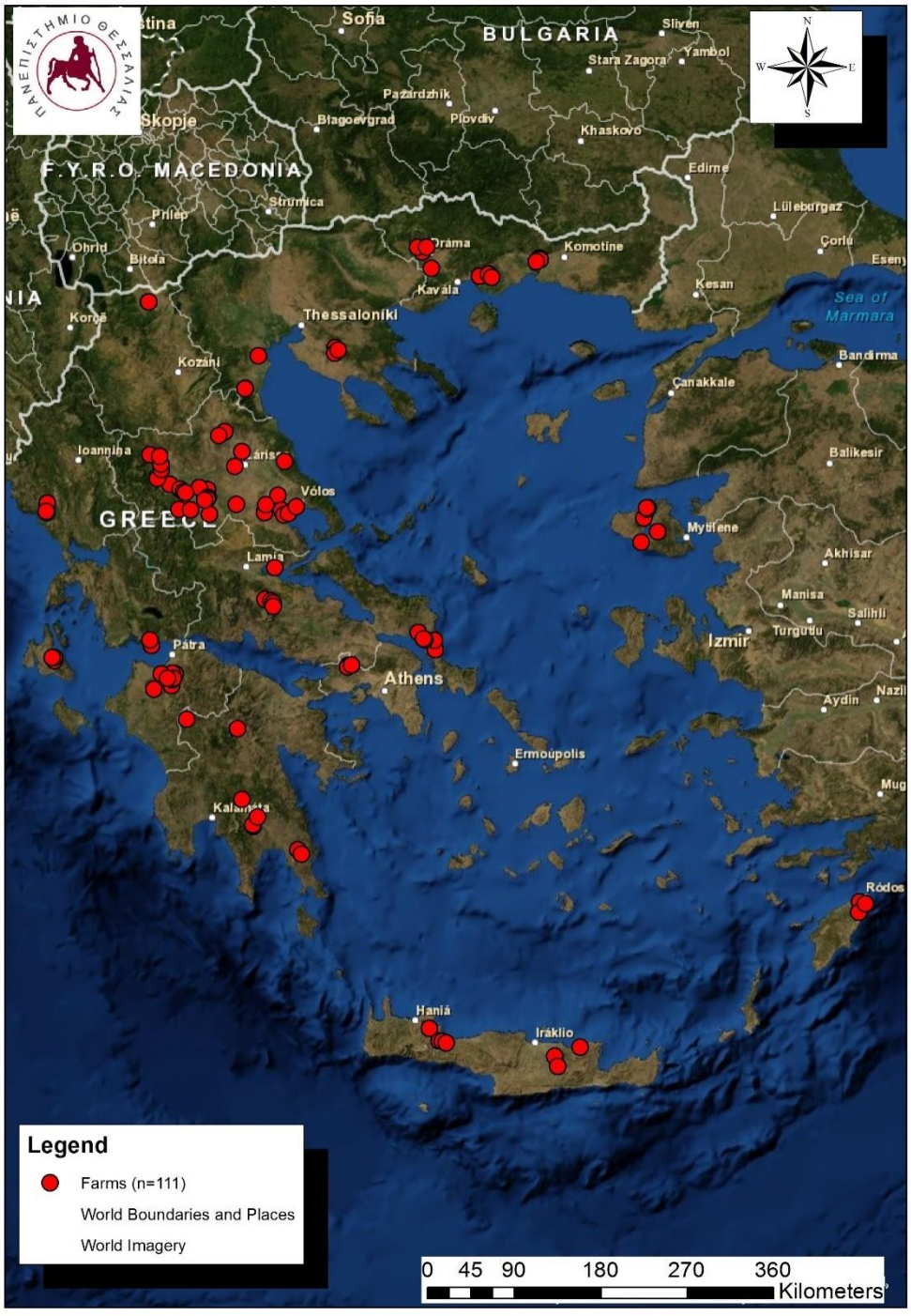
Supplementary material 2. Location of 111 farms included in the study around Greece.



Supplementary material 3. Breeds in sheep farms in Greece according to management system applied in farms.

Sheep breeds	Management system (no. of farms)			Total
	Intensive	Semi-intensive	Semi-extensive or extensive	
1. Pure-breeds	17	25	16	58
1.1. Greek breeds	6	13	14	33
1.1.1. Cephalonia		1	1	2
1.1.2. Chios	6	4	3	13
1.1.3. Crete			4	4
1.1.4. Frisarta		2		2
1.1.5. Karagouniko		2	1	3
1.1.6. Karystos			1	1
1.1.7. Lesvos		4	1	5
1.1.8. Vlahiko			3	3
1.2. Imported breeds	11	12	2	25
1.2.1. Assaf	1	1		2
1.2.2. Lacaune	10	11	2	23
2. Cross-breeds	9	32	12	53
Total	26	57	28	111