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1 **Association of subclinical mastitis prevalence**

2 **with sheep breeds in Greece**

3
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11
12 Objective was to describe potential associations of subclinical mastitis with sheep breeds in
13 Greece. A countrywide survey (2,198 ewes in 111 farms) was performed. Prevalence of
14 subclinical mastitis was 0.260. Results did not indicate difference in prevalence of subclinical
15 mastitis between farms with pure-bred and farms with cross-bred animals, nor difference in
16 prevalence between farms with Greek pure-bred animals and farms with imported pure-bred
17 animals. Results indicated that prevalence of subclinical mastitis was smaller in farms with
18 Assaf-breed (0.100) and higher in farms with Frisarta-breed (0.625) ($P<0.02$). Prevalence of
19 mastitis was smaller in farms with Greek traditional indigenous breeds (0.221) ($P=0.007$). In
20 a model that included sheep breed and management system in farm, breed emerged of
21 significance for prevalence of subclinical mastitis ($P=0.003$).

22 **Key words:** breed, management system, prevalence, subclinical mastitis, sheep

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26 Predominant type of sheep production in Greece is dairy, with various breeds present
27 around the country. There has been no systematic study of potential association between sheep
28 breeds in the country and mastitis. The genetic background of sheep susceptibility to mastitis has
29 been presented and the role of sheep breeds, carriers of relevant genes, has been mentioned
30 (Bishop, 2015). There is little work worldwide in relation to potential susceptibility of sheep breeds
31 to mastitis: Larsgard and Vaabenoe (1993) have indicated some differences between Norwegian
32 sheep breeds, whilst Burriel (1997) has reported that mule ewes were more susceptible to mastitis
33 than Welsh-Mountain ewes (a traditional indigenous British breed). Objective of the work was to
34 describe potential associations of subclinical mastitis with breeds of sheep in Greece.
35

36 **Materials and methods**

37
38 In total, 111 sheep farms in the 13 administrative regions of Greece were included into the
39 study and visited for collection of samples and information. The investigators visited all farms for

40 sample collection. In each farm, 20 clinically healthy ewes were selected and sampled by use of
41 standardised methods. Bacteriological and cytological examinations were performed in milk
42 samples. Ewes were considered to have subclinical mastitis when a bacteriologically positive milk
43 sample ([a] >10 colonies of the same organism and [b] no more than two different types of colonies)
44 with concurrently increased CMT score (≥ 1) plus neutrophil and lymphocyte proportion ($\geq 65\%$ of
45 all leucocytes) was detected.

46 Mixed-effects logistic regression was employed, using the different farms as 'random effect'.
47 Analysis of variance was employed for performing comparisons between farms in relation to
48 prevalence of subclinical mastitis. Farms with Cephalonia, Crete, Karagouniko, Karystos, Lesvos
49 and Vlahiko breeds were clustered as 'Greek traditional indigenous breeds' and comparisons were
50 repeated. A multivariable model was created using mixed-effects logistic regression with farm as
51 the random effect, which included as variables the management system and the sheep breed.

52 Detailed description of procedures and techniques employed are in Suppl. material 1.
53 Location of farms around the country is shown in Suppl. material 2.

54

55 **Results**

56

57 In total, 2,220 ewes were examined and 2,198 were sampled. Among these, 572 were
58 detected with subclinical mastitis; prevalence was 0.260 (95% C.I.: 0.242-0.279). Prevalence
59 within farm varied from 0.000 to 0.850 (median: 0.250). The most frequently isolated bacteria
60 from ewes with subclinical mastitis were *Staphylococcus* spp. (n = 531) (*Staphylococcus aureus* or
61 coagulase-negative species). Less frequently isolated organisms were *Streptococcus* spp.,
62 *Corynebacterium* spp., *Escherichia coli*, *Micrococcus* spp., *Mannheimia haemolytica* and *Trueperella*
63 *pyogenes*.

64 Of the 111 farms, 58 included pure-bred animals (33 with Greek breeds: Cephalonia n=2,
65 Chios n=13, Crete n=4, Frisarta n=2, Karagouniko n=3, Karystos n=1, Lesvos n=5, Vlahiko n=3,
66 and 25 with imported breeds: Assaf n=2, Lacaune n=23). The other 53 farms included cross-bred
67 animals. In farms with intensive management system, pure-bred animals prevailed (17/26), of
68 which most were imported (11/17). Pure-breeds also prevailed in semi-extensive or extensive
69 management system (16/28), but most were Greek breeds (14/16). In farms with semi-intensive
70 management system, cross-breeds prevailed (32/57). Details of breeds in farms are in Suppl.
71 material 3.

72 Difference in prevalence of subclinical mastitis between farms with pure-bred and farms
73 with cross-bred animals (0.276 and 0.243, respectively) was not significant ($P=0.144$). Difference
74 in prevalence of subclinical mastitis between farms with Greek pure-bred animals and farms with
75 imported pure-bred animals (0.284 and 0.265, respectively) was also not significant ($P=0.240$).
76 Not significant was also the difference in prevalence between farms with imported pure-bred
77 animals and all other farms (0.265 and 0.259, respectively) ($P=0.125$). Similarly, differences in
78 prevalence between farms with Greek pure-bred animals, farms with imported pure-bred animals

79 and farms with cross-bred animals (0.284, 0.265 and 0.243, respectively) were not significant
80 ($P=0.123$).

81 When farms with the various pure-breeds were considered, it became evident that
82 prevalence of subclinical mastitis was significantly smaller in farms with Assaf-breed sheep and
83 significantly higher in farms with Frisarta-breed sheep ($P<0.02$ for both comparisons). Further,
84 there was significantly smaller prevalence in farms with Karystos-breed sheep ($P=0.045$) and a
85 tendency for higher prevalence in farms with Chios-breed sheep ($P=0.125$). When farms with the
86 six Greek traditional indigenous breeds were clustered together, it emerged that prevalence of
87 subclinical mastitis was significantly smaller in that cluster ($P=0.007$). All other evaluations did
88 not yield significant differences ($P>0.250$). Details are in Table 1 and Figure 1.

89 Sheep breed emerged from the multivariable mixed-effects model as the significant factor
90 for the prevalence of subclinical mastitis ($P=0.003$). There was a trend for contribution by the
91 management system ($P=0.087$); interactions between breed and management system were not
92 important ($P=0.845$). Results were similar when calculations were performed, after including farms
93 under semi-extensive and extensive management in one cluster ($P=0.007$, $P=0.060$, $P=0.768$,
94 respectively).

95

96 **Discussion**

97

98 Lacaune- and Chios-breed animals are popular in the country. These are sheep with high
99 milk production, thus of importance in the dairy production systems applied in the country, which
100 explains the higher proportion of farms with these breeds. The findings indicate increased
101 penetration of imported breeds, which, in recent years, have been favoured by Greek farmers.
102 Lacaune and Assaf predominate among imported sheep breeds in Greece, as they are animals of
103 increased milk production, higher than indigenous Greek breeds. These animals cannot be
104 adapted to the environment, which is reflected in them being included in farms managed
105 intensively or semi-intensively, where they are sheltered and their needs, especially nutritional
106 requirements, can be controlled and covered. Nevertheless, uncontrolled imports may increase
107 risk for transmission of diseases to the indigenous sheep population; for example, in Spain a large
108 proportion of Assaf animals have been found to be infected with *Small Ruminant Lentivirus*
109 (Minguijon et al., 2015), which may lead to transmission of the pathogen to uninfected flocks in
110 Greece after import. Traditional breeds have also been identified, these being of limited
111 geographical distribution and, mainly, in flocks managed under the semi-extensive or extensive
112 system, which constitute the traditional shepherding forms in the country. These are low-input
113 breeds, with very good adaptability to environmental conditions and able to make excellent use of
114 natural resources and locally produced feedstuffs, which explains their increased frequency in
115 farms managed semi-extensively or extensively (Georgoudis et al., 2011). There is evidence
116 regarding genetic relationship between animals of those breeds (Ligda et al., 2009; Georgoudis et
117 al., 2011), thus lending support to clustering these breeds for the statistical analysis.

118 Bacteriological examination of milk samples is employed for diagnosis of subclinical
119 mastitis, as it is considered to provide precise and exhaustive information on infected mammary
120 glands and pathogen involved. However, it is difficult to implement at a large scale and also has
121 various limitations. Moreover, bacterial shedding is variable and levels may sometimes be too low
122 to be detected by conventional techniques (Rupp and Foucras, 2010). Simple, indirect methods
123 have also been widely applied, based on evaluation of inflammation. The ones most frequently
124 used are somatic cell counting and various indirect tests for their measurement. A difficulty in
125 using somatic cell counting is that factors known to influence somatic cell counting have different
126 magnitude in healthy and infected animals (Detilleux and Leroy, 2000). Further, there is difference
127 in types of cells in mammary secretion, which can provide an indication regarding the
128 inflammation. Indeed, the associations between bacteria and somatic cells, particularly of the
129 various types of leucocytes, can be used to better define the disease (Albenzio et al., 2009). The
130 definition of subclinical mastitis used in this study (i.e., combination of a bacteriologically positive
131 milk sample with increased CMT score plus high proportion of neutrophil and lymphocyte) takes
132 that into account and was adopted to overcome shortcomings of the methods described previously.

133 Present results have indicated increased prevalence of subclinical mastitis in Friesarta-
134 breed farms. Animals of the breed are high-yielding, but, in general, considered to be particularly
135 susceptible to diseases, e.g., respiratory infections. Increased susceptibility to mastitis can be
136 attributed to breed-specific impaired local defence mechanisms in the udder (Fragkou et al., 2007;
137 2010). Present findings provide field corroboration to the experimental evidence. Traditional Greek
138 sheep breeds have shown reduced frequency of subclinical mastitis. In a broader sense, resistance
139 could be defined as the ability to avoid any infection and/or the quick recovery from an infection
140 (Rupp and Boichard, 2003) and involves different components: avoiding entry of the pathogen into
141 the teat, mounting an immune response capable of limiting its development in the mammary gland
142 and clearing the infection, as well as controlling the pathogenic effects of the infection, such as
143 tissue damage (Rupp and Foucras, 2010). In Karagouniko ewes, lymphoid follicles have been
144 identified in the teat duct and have been repeatedly shown to play a clear protective role against
145 invading pathogens (Fragkou et al., 2010). Higher allocation of resources to defence mechanisms
146 of ewes afforded by low milk production of these animals can also play a predominant role and
147 contribute to efficient counteraction against invading mammary pathogens. A tendency of
148 increased prevalence of subclinical mastitis in Chios-breed sheep has also emerged. Possible
149 reasons could be the bad udder conformation, which hinders correct milking and contributes to
150 infections (Gelasakis et al., 2012), and the innate peri-parturient immunosuppression associated
151 with macrophage and neutrophil function (Theodorou et al., 2007). Previous studies on other
152 breeds (e.g., Latxa and Sarda) have indeed shown favourable correlations between SCC and udder
153 conformation (Legarra and Ugarte, 2005; Sechi et al., 2007), suggesting that udders with what is
154 perceived to be a good shape would be less affected by subclinical mastitis. In addition, udders
155 with bad conformation can predispose to development of mastitis (Gelasakis et al., 2012). Further,
156 differences in somatic cell counts in milk of healthy animals recorded between sheep breeds (Rupp

157 and Foucras, 2010) can reflect the immunological competence of the respective mammary glands
158 against invading microorganisms and the final result (Albenzio et al., 2012).

159 In cows, there are many studies detailing genetic resistance to mastitis (discussed by
160 Fragkou et al., 2007). Differences to various defence determinants of susceptible/resistant
161 animals have been reported, e.g. number of blood polymorphonuclear cells after calving,
162 lactoferrin concentration, production of immunoglobulins, production of complement fragment
163 C5a, production and mobilization of cytokines. There is also information regarding genetic control
164 of lymphocyte mobilization and role, e.g. heritability (h^2) of T-cell proliferation ranges between h^2
165 = 0 to 0.40, genetic mechanisms have been identified for production of T-cell and B-cell receptor
166 phenotypes.

167 Mastitis is a prime target disease to develop breeding for resistance and produce mastitis-
168 resistant sheep (Davies, 2009; Bishop, 2015), as in sheep genomic selection has been shown to
169 have good accuracy for mastitis resistance (Duchemin et al., 2012). The findings have provided
170 evidence of associations of subclinical mastitis with breed, which have only rarely been reported.
171 In Greece, the only breeding program for genetic control of diseases has been that for scrapie.
172 Certainly, it is more difficult to select for resistance to mastitis, which is a polygenic trait,
173 therefore, selection for a complex of traits is necessary, where many genes with small effects are
174 involved. Given the significance of the sheep industry in the country and the importance of
175 mastitis as a limiting factor in milk production, there is a need to consider genetic improvement
176 for reduced susceptibility to mastitis, as a sustainable means to control of the disease.

177

178 **Acknowledgments**

179

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181 for this work has been provided by Laboratorios HIPRA S.A.

182

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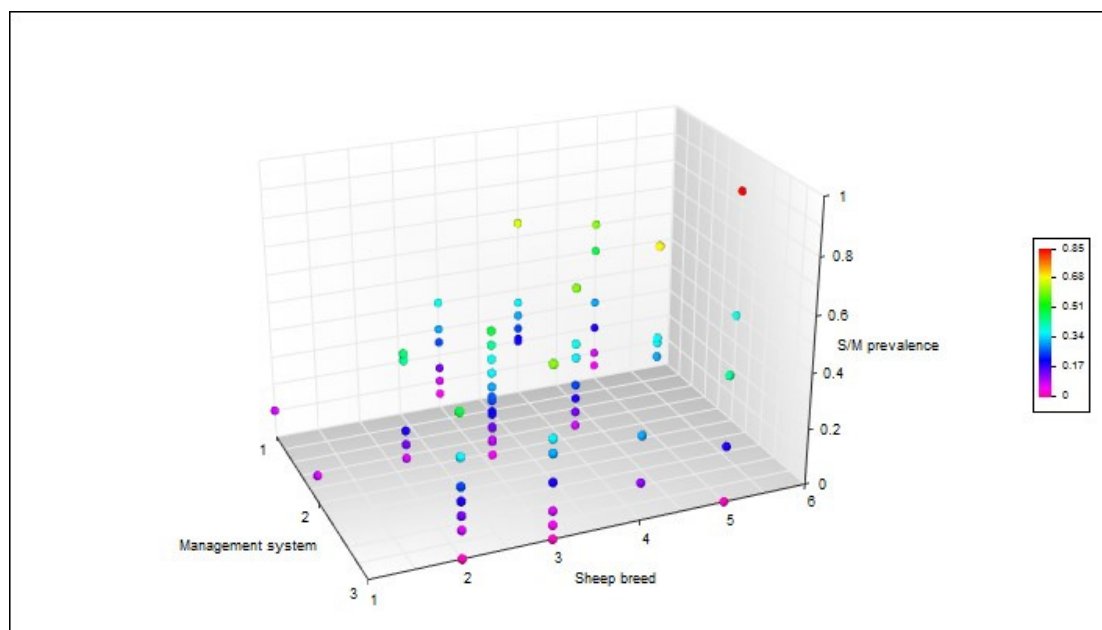
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239 **Figure 1.** Scatter plot of results of subclinical mastitis prevalence (z axis) against management
 240 system applied in farms (x axis) and sheep breed (y axis) in 111 sheep farms in Greece.



241 Management system: 1: intensive, 2: semi-intensive, 3: semi-extensive or extensive.
 242 Sheep breed: 1: Assaf, 2: Greek traditional indigenous breeds, 3: Cross-breeds, 4: Lacaune, 5: Chios, 6:
 243 Frisarta.
 244 Subclinical mastitis prevalence: Colour map indicates prevalence of subclinical mastitis.
 245
 246
 247

248 **Table 1.** Importance of breed in prevalence of subclinical mastitis in sheep in Greece.

249 (a) Greek breeds considered individually

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	P
Cephalonia (n=2)	0.200	10.723 (0.587-195.918)	0.110
Chios (n=13)	0.318	19.164 (1.145-320.750)	0.040
Crete (n=4)	0.218	11.667 (0.671-202.757)	0.092
Frisarta (n=2)	0.625	67.452 (3.803-1,196.329)	0.004
Karagouniko (n=3)	0.233	12.785 (0.727-224.753)	0.082
Karystos (n=1)	0.000	reference	
Lesvos (n=5)	0.242	13.305 (0.776-228.228)	0.074
Vlahiko (n=3)	0.267	15.202 (0.869-265.926)	0.062

250 (b) Greek traditional indigenous breeds clustered together

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	P
Chios (n=13)	0.318	1.640 (1.142-2.355)	0.007
Greek traditional indigenous breeds (n=18)	0.221	reference	
Frisarta (n=2)	0.625	5.865 (2.950-11.660)	<0.001

251 (c) Imported breeds considered individually

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	P
Assaf (n=2)	0.100	Reference	
Lacaune (n=23)	0.280	1.554 (0.510-4.736)	0.439

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

257 1. *Sheep farms and animal sampling*

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

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

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

282 2. *Paraclinical examinations*

283 Laboratory procedures started within 24 h after collection. Milk samples (10 µL) were
284 cultured using Columbia blood agar plates incubated aerobically at 37 °C for up to 72 h. Bacterial
285 identifications were performed by using standards methods (Barrow and Feltham, 1993; Euzeby,
286 1997).

287 After sample collection, at ewe-side, all samples were tested by use of the CMT. The test was
288 performed as previously described for ewes' milk (Fthenakis, 1995); it was carried out and scored
289 always by the same person, i.e., the principal investigator (NGCV). Five degrees of reaction
290 ('negative', 'trace', '1', '2', '3') were described (Schalm et al., 1971). Milk smears were also produced
291 and dried. The milk smears were stained by the Giemsa method for estimation of leucocyte
292 subpopulations; proportion of leucocyte types therein was calculated by observing at least 10
293 fields of each milk film under magnification 10×. Subsequently, the Microscopic cell counting

294 method (Mccm) (IDF reference method) (International Dairy Federation, 1984; Contreras et al.,
295 2007; Raynal-Ljutovac et al., 2007) was performed in 894 samples (20.3% of all samples).

296 3. *Data management and analysis*

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

302 Quantitative information on the cellular content of ewes' milk was obtained by using two
303 sets of data: the CMT results and the results of the Mccm. Although it is generally established
304 that CMT results are reliable proxy measurements for somatic cell counts (SCCs) (Fthenakis,
305 1995; Gonzalez-Rodríguez and Carmenes, 1996), we further confirmed that in the present study.
306 Following assignment of numerical values to CMT scores (value 0 to score 'negative', value 1 to
307 score 'trace', value 2 to score '1', value 3 to score '2', and value 4 to score '3') and \log_{10} -
308 transformations, correlation between CMT scores and Mccm SCCs was $r=0.913$ (95% CI: 0.902-
309 0.923) ($P<0.001$) and the corrected R^2 was 83.4%; significance of the difference between r and ρ
310 (the correlation hypothesized to exist within the population from which the sample had been
311 drawn) was $P<0.001$.

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

- 320 (a) farms with pure-bred animals *versus* farms with cross-bred animals,
- 321 (b) farms with Greek pure-bred animals *versus* farms with imported pure-bred animals,
- 322 (c) farms with imported pure-bred animals *versus* all other farms (i.e., farms with Greek pure-bred
323 animals and farms with cross-bred animals),
- 324 (d) farms with the various Greek pure-bred animals (in total, 8 breeds), farms with imported pure-
325 bred animals (in total, 2 breeds) and farms with cross-bred animals and
- 326 (e) farms with the various pure-bred animals (in total, 10 breeds) between them.

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

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

336 Statistical significance was defined at ≤ 0.05 .

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



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384 **Supplementary material 3.** Breeds in sheep farms in Greece according to management system
 385 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

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