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ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

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ΜΑΣΤΙΤΙΔΑ ΣΕ ΠΡΟΒΑΤΙΝΕΣ ΠΡΟΚΑΛΟΥΜΕΝΗ ΑΠΟ *STAPHYLOCOCCUS* SPP.: ΠΑΡΟΥΣΙΑΣΗ ΝΕΟΤΕΡΩΝ ΚΛΙΝΙΚΩΝ, ΕΠΙΔΗΜΙΟΛΟΓΙΚΩΝ, ΔΙΑΧΕΙΡΙΣΤΙΚΩΝ, ΜΙΚΡΟΒΙΟΛΟΓΙΚΩΝ ΚΑΙ ΖΩΟΝΟΤΙΚΩΝ ΔΕΔΟΜΕΝΩΝ ΚΑΙ ΑΞΙΟΛΟΓΗΣΗ ΕΝΟΣ ΚΑΙΝΟΤΟΜΟΥ ΕΜΒΟΛΙΟΥ ΓΙΑ ΤΗΝ ΠΡΟΛΗΨΗ ΤΗΣ ΝΟΣΟΥ

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ΔΙΔΑΚΤΟΡΙΚΗ ΔΙΑΤΡΙΒΗ

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The method of Science is the method of bold conjectures
and ingenious and severe attempts to refute them.

Karl Popper

The method of Science requires an exquisite balance
between a fierce ambition to discover the truth and
a ruthless skepticism toward your own work.

Gary Gaubes

Η διατριβή είναι αφιερωμένη στην οικογένειά μου

ΠΕΡΙΛΗΨΗ

Η παρούσα διατριβή αποσκοπούσε (α) στη μελέτη της υποκλινικής μαστίτιδας σε εκτροφές προβάτων στην Ελλάδα, ειδικότερα στη διερεύνηση του ποσοστού προσβολής στις προβατίνες, στην ταυτοποίηση των αιτιολογικών παραγόντων, στη συστηματική εξέταση στελεχών σταφυλοκόκκων από περιστατικά της νόσου, στην αξιολόγηση της ανοσολογικής ανταπόκρισης στις σταφυλοκοκκικές λοιμώξεις του μαστικού αδένα και στην ανάλυση παραγόντων προδιάθεσης για την υποκλινική μαστίτιδα, (β) στη μελέτη της υποκλινικής μαστίτιδας από στελέχη σταφυλοκόκκων που σχημάτιζαν βιομεμβράνες, ειδικότερα στη διερεύνηση του ποσοστού προσβολής στις προβατίνες και στην ανάλυση παραγόντων προδιάθεσης για τη συγκεκριμένη λοίμωξη, (γ) στη μελέτη του πιθανού ρόλου των περιβαλλοντικών παραγόντων στην εκδήλωση της μαστίτιδας, ειδικότερα στην αξιολόγηση της συσχέτισης του ποσοστού προσβολής της υποκλινικής μαστίτιδας με περιβαλλοντικούς (κλιματολογικούς και τοπογραφικούς) παράγοντες και στην ταυτοποίηση περιοχών όπου υπάρχει αυξημένος κίνδυνος εκδήλωσης υποκλινικής μαστίτιδας και (δ) στην αξιολόγηση ενός εμβολίου για τη σταφυλοκοκκική μαστίτιδα στις προβατίνες, το αντιγόνο του οποίου βασιζόταν σε στέλεχος *S. aureus*, που σχημάτιζε βιομεμβράνη.

Η διατριβή χωρίζεται σε τρία κεφάλαια και ακολουθεί η Γενική Συζήτηση.

Στο Κεφάλαιο Ι, ανασκοπείται η σχετική βιβλιογραφία. Το Κεφάλαιο υποδιαιρείται σε δύο τμήματα. Στο τμήμα Α, ανασκοπείται η βιβλιογραφία σχετικά με τη σημασία των σταφυλοκόκκων στη μαστίτιδα των προβάτων. Στο τμήμα Β, ανασκοπείται η βιβλιογραφία η σχετική με τους παράγοντες προδιάθεσης στη μαστίτιδα.

Στο Κεφάλαιο ΙΙ, παρουσιάζονται τα ευρήματα εκτενούς μελέτης της υποκλινικής μαστίτιδας σε πρόβατα στην Ελλάδα.

Συλλέχθηκαν δείγματα γάλακτος από 2.198 προβατίνες σε 111 εκτροφές με συνολικό πληθυσμό 35.925 ζώων, στις 13 διοικητικές περιφέρειες της Ελλάδας, για βακτηριολογική και κυτταρολογική εξέταση. Το ποσοστό προσβολής της υποκλινικής μαστίτιδας ήταν 0,260. Κύριοι αιτιολογικοί παράγοντες ήταν οι σταφυλόκοκκοι (*Staphylococcus* [*S.*] *aureus* και πηκτάση-αρνητικοί σταφυλόκοκκοι), που αποτελούσαν 0,699 όλων των απομονωθέντων μικροβιακών στελεχών. Το ποσοστό προσβολής της σταφυλοκοκκικής υποκλινικής μαστίτιδας ήταν 0,191. Σε πολυπαραγοντική ανάλυση των παραγόντων προδιάθεσης, ο κύριος παράγοντας που συσχετίστηκε με το ποσοστό προσβολής της υποκλινικής μαστίτιδας ήταν το σύστημα διαχείρισης της εκτροφής (στις εκτροφές με σύστημα ημιεντατικής διαχείρισης βρέθηκε το μεγαλύτερο ποσοστό προσβολής) (P = 0,008).

Άλλοι παράγοντες στο πολυπαραγοντικό μοντέλο ήταν το στάδιο της γαλακτικής περιόδου (σε προβατίνες στον 2ο μήνα της γαλακτικής περιόδου βρέθηκε το μεγαλύτερο ποσοστό προσβολής) (*P* = 0,151) και η πραγματοποίηση απολύμανσης των θηλών του μαστού μετά το άρμεγμα (*P* = 0,054). Τα αποτελέσματα δεν έδειξαν διαφορές στο ποσοστό προσβολής υποκλινικής μαστίτιδας μεταξύ εκτροφών με καθαρόαιμα έναντι εκτροφών με μη καθαρόαιμα πρόβατα, ούτε διαφορές μεταξύ ελληνικών και εισαγόμενων καθαρόαιμων φυλών προβάτων. Το μικρότερο ποσοστό προσβολής υποκλινικής μαστίτιδας βρέθηκε σε εκτροφές με πρόβατα φυλής Assaf (0,100) και το μεγαλύτερο σε εκτροφές με πρόβατα φυλής Φριζάρτα (0,625) (*P* < 0.02). Το ποσοστό προσβολής υποκλινικής μαστίτιδας βρέθηκε ότι ήταν πιο μικρό σε εκτροφές με εγχώριες ελληνικές καθαρόαιμες φυλές προβάτων (0,221) (*P* = 0,007). Σε πολυπαραγοντική ανάλυση, όπου ελήφθησαν υπόψη ως μεταβλητές η φυλή των προβάτων και το σύστημα διαχείρισης της εκτροφής, η φυλή συσχετίστηκε με το ποσοστό προσβολής της υποκλινικής μαστίτιδας (*P* = 0,003). Ο σχηματισμός βιομεμβράνης αξιολογήθηκε σε 708 στελέχη σταφυλοκόκκων με εφαρμογή μικροβιολογικών και μοριακών μεθόδων. Από αυτά τα στελέχη, 262 χαρακτηρίστηκαν ως θετικά στο σχηματισμό βιομεβράνης, 227 ως μετρίως θετικά και 219 ως αρνητικά. Τα γονίδια που ανιχτεύτηκαν συχνότερα ήταν τα *eno* και *icaB*, βρέθηκε δε ότι τα στελέχη *S. aureus* διέθεταν περισσότερα γονίδια από τους πηκτάση-αρνητικούς σταφυλοκόκκους, ενώ περισσότερα γονίδια ανιχνεύθηκαν επίσης σε σταφυλοκόκκους που σχημάτιζαν βιομεμβράνες. Υποκλινική μαστίτιδα προκαλούμενη αποκλειστικά από σταφυλοκόκκους που σχημάτιζαν βιομεμβράνη, διαγνώστηκε σε 337 προβατίνες: το ποσοστό προσβολής στον πληθυσμό ήταν 0,153. Σε πολυπαραγοντική ανάλυση των παραγόντων προδιάθεσης, οι κύριοι παράγοντες που συσχετίστηκαν με το ποσοστό προσβολής της υποκλινικής μαστίτιδας από στελέχη σταφυλοκόκκων που σχημάτιζαν βιομεμβράνη, ήταν ο τρόπος αρμέγματος (μεγαλύτερο ποσοστό προσβολής σε εκτροφές όπου γινόταν με άρμεγμα με τα χέρια) (*P* = 0,017) και το σύστημα διαχείρισης της εκτροφής (μεγαλύτερο ποσοστό προσβολής σε ημιεντατικές εκτροφές) (*P* = 0,045). Στη συνέχεια, έγινε αξιολόγηση της ευαισθησίας 142 στελεχών σταφυλοκόκκων από περιστατικά υποκλινικής μαστίτιδας σε πρόβατα, σε διάφορους αντιμικροβιακούς παράγοντες. Συνολικά, 0,415 των στελεχών ήταν ανθεκτικά και 0,056 αυτών ήταν πολυανθεκτικά. Επιπλέον, η συχνότητα ανθεκτικών στελεχών ήταν μεγαλύτερη σε στελέχη πηκτάση αρνητικών σταφυλοκόκκων (0,470) από στελέχη *S. aureus* (0,185). Παρατηρήθηκε ανθεκτικότητα συχνότερα στην πενικιλλίνη (0,225 των στελεχών), την τετρακυκλίνη (0,183) και την αμπικιλλίνη (0,169) (*P* < 0,032). Επιπλέον, βρέθηκε ότι στελέχη που σχημάτιζαν βιομεμβράνες ήταν ανθεκτικά στην τετρακυκλίνη πιο συχνά από στελέχη που δεν σχημάτιζαν (0,206 έναντι 0,000) (*P* = 0,013). Η παρουσία του γονιδίου *tekK* συσχετίστηκε με την παρουσία του γονιδίου *icaA* στα ίδια στελέχη (*P* = 0,029). Από τα ανθεκτικά στελέχη, τα περισσότερα (0,766 αυτών) απομονώθηκαν στην περίοδο

αμέσως μετά τον τοκετό σε αντίθεση με τα ευαίσθητα στελέχη (0,577 αυτών) (*P* = 0.035), επίσης δε περισσότερα ανθεκτικά στελέχη (0,234 αυτών) σε σχέση με ευαίσθητα (0,099 αυτών) απομονώθηκαν σε εκτροφές που εφάρμοζαν ενδομαστική χορήγηση αντιμικροβιακών στο τέλος της γαλακτικής περιόδου (*P* = 0,045). Τα περισσότερα στελέχη *S. aureus* κατατάχθηκαν στον τύπο ST133 (0,593 αυτών) και τα περισσότερα στελέχη *S. epidermidis* στους τύπους ST100, ST142 ή ST152 (σε κάθε τύπο 0,190 αυτών). Δεν βρέθηκε συσχέτιση των τύπων των μικροβιακών στελεχών με την ανθεκτικότητα αυτών σε αντιμικροβιακούς παράγοντες. Κατά την εξέταση του γονιδιώματος ενός στελέχους *S. lentus*, βρέθηκε ότι το γονίδιο *ermG* ήταν μέρος του τρανσποζόνιου Tn917 ενσωματωμένο στο χρωμόσωμα. Επίσης, βρέθηκε ένα μικρού μεγέθους πλασμίδιο σε στέλεχος *S. hominis* με το γονίδιο *ermC*. Τέλος, βρέθηκαν πλασμίδια (pSau-2716Lar, PSau-3893Lar) με το γονίδιο *tetK* σε ένα στέλεχος *S. aureus* και ένα *S. epidermidis*. Έγινε ανίχνευση αντισωμάτων έναντι των σταφυλοκόκκων που σχημάτιζαν βιομεμβράνες σε δείγματα αίματος από 355 προβατίνες σε 71 εκτροφές. Η συχνότητα οροθετικότητας ήταν μεγαλύτερη σε προβατίνες ειδικά εμβολιασμένες έναντι μαστίτιδας από σταφυλοκόκκους που σχημάτιζαν βιομεμβράνες (0,750) έναντι μη εμβολιασμένων ζώων (0,458). Η μέση τιμή του τίτλου αντισωμάτων σε εμβολιασμένα ζώα (13,64) ήταν μεγαλύτερη από αυτήν σε μη εμβολιασμένα ζώα (7,39). Δεν υπήρχε συσχέτιση μεταξύ της ταυτότητας των στελεχών σταφυλοκόκκων από δείγματα γάλακτος και του τίτλου αντισωμάτων στα δείγματα αίματος από τα ίδια ζώα. Στα εμβολιασμένα ζώα, υπήρχε συσχέτιση (*r* = 0,922) μεταξύ του συνολικού αριθμού των σχετιζόμενων με τον σχηματισμό βιομεμβρανών γονιδίων στα στελέχη σταφυλοκόκκων και των τίτλων αντισωμάτων στα δείγματα αίματος. Επιπλέον, σε στελέχη σταφυλοκόκκων από οροθετικές προβατίνες ανιχνεύτηκαν περισσότερα γονίδια (διάμεση τιμή 4) απ' ό,τι σε στελέχη από οροαρνητικές προβατίνες (διάμεση τιμή 3). Σε πολυπαραγοντική ανάλυση των παραγόντων κινδύνου, ο εμβολιασμός βρέθηκε ως ο μόνος παράγοντας για τη συχνότητα οροθετικότητας των ζώων έναντι στελεχών σταφυλοκόκκων που σχημάτιζαν βιομεμβράνες. Τα χωρικά δεδομένα θέσης των εκτροφών καταγράφηκαν στο πεδίο με τη χρήση φορητών μονάδων 'Global Positioning System Garmin'. Οι γεωγραφικές αναφορές επιλύθηκαν σε επίπεδο εκτροφής. Σε πολυπαραγοντικό μοντέλο ανάλυσης, οι δύο περιβαλλοντικές μεταβλητές που σχετίζονταν με αυξημένο ποσοστό προσβολής υποκλινικής μαστίτιδας ήταν η ελάχιστη θερμοκρασία του ψυχρότερου μήνα του χρόνου (συντελεστής: -0,084, *P* = 0,014) και η μέση θερμοκρασία 30 ημέρες πριν από τη δειγματοληψία (συντελεστής: 0,031, *P* = 0,029). Στη συνέχεια, τα δεδομένα αναλύθηκαν με προτυποποίηση οικολογικών βιοθέσεων (Ecological Niche modelling, ENM). Πραγματοποιήθηκαν δύο διαφορετικές αναλύσεις: μία για την υποκλινική μαστίτιδα ανεξαρτήτως αιτιολογικού παράγοντα και μία για την υποκλινική μαστίτιδα από σταφυλοκόκκους που σχημάτιζαν βιομεμβράνη. Κατασκευάστηκε ένα πρότυπο στο οποίο οι εκτροφές προβάτων χωρίστηκαν σε δύο συστάδες, σύμφωνα με το ποσοστό

προσβολής υποκλινικής μαστίτιδας: οι εκτροφές με ποσοστό προσβολής στα ανώτερα τρία τεταρτημόρια του συνόλου των εκτροφών σε κάθε κατηγορία χρησιμοποιήθηκαν ως σημεία εμφάνισης της νόσου στη διαδικασία δημιουργίας των οικολογικών βιοθέσεων (‘μολυσμένες εκτροφές’), ενώ οι εκτροφές με ποσοστό προσβολής στο κατώτερο τεταρτημόριο του συνόλου των εκτροφών σε κάθε κατηγορία αποτέλεσαν τα (ψευδο)αρνητικά σημεία. Διαφορές στους περιβαλλοντικούς παράγοντες που κυριαρχούσαν στις τοποθεσίες των υπό μελέτη εκτροφών, ταυτοποιήθηκαν σε 13 παράγοντες ανάλογα με το σύστημα διαχείρισης που εφαρμοζόταν σε κάθε εκτροφή. Επίσης, όταν οι εκτροφές σε κάθε σύστημα διαχείρισης εξετάστηκαν χωριστά, αποκαλύφθηκαν διαφορές μεταξύ τους εντός κάθε συστήματος διαχείρισης, καθώς και μεταξύ των δύο νοσολογικών καταστάσεων. Ο παράγοντας με την υψηλότερη σχετική συμβολή στην ανάλυση ήταν η απόσταση από άλλες εκτροφές προβάτων. Άλλοι παράγοντες σημαντικοί στα μοντέλα πρόγνωσης ήταν το υψόμετρο, η μέγιστη θερμοκρασία του θερμότερου μήνα και η συνολική βροχόπτωση του ξηρότερου μήνα. Η επαλήθευση του μοντέλου αποκάλυψε ότι ≥ 0,760 των μολυσμένων εκτροφών βρίσκονταν σε τοποθεσίες που είχαν ήδη προβλεφθεί ως υψηλού κινδύνου.

Στο Κεφάλαιο ΙΙΙ, μετά από σύντομη ανασκόπηση για τον εμβολιασμό έναντι της μαστίτιδας σε πρόβατα (τμήμα Α), παρουσιάζεται μία κλινική μελέτη (τμήμα Β) και μία πειραματική μελέτη (τμήμα Γ) για αξιολόγηση ενός εμβολίου έναντι της σταφυλοκοκκικής μαστίτιδας σε πρόβατα. Το αντιγόνο του εμβολίου βασιζόταν σε στέλεχος *S. aureus*, που σχημάτιζε βιομεμβράνη.

Η κλινική δοκιμή (Μέρος Β) πραγματοποιήθηκε σε πέντε εκτροφές γαλακτοπαραγωγών προβάτων, με 316 προβατίνες στην ομάδα V (εμβολιασμένες) και 307 στην ομάδα C (μάρτυρες), οι οποίες μελετήθηκαν σε όλη την διάρκεια της γαλακτικής περιόδου. Πραγματοποιήθηκαν δύο εμβολιασμοί κατά τη διάρκεια του τελευταίου σταδίου της εγκυμοσύνης των προβατίνων. Αρχίζοντας 15 ημέρες μετά τον τοκετό και στη συνέχεια σε μηνιαία μεσοδιαστήματα, πραγματοποιήθηκαν έως 9 δειγματοληψίες γάλακτος για βακτηριολογικές και κυτταρολογικές εξετάσεις. Τα στελέχη σταφυλοκόκκων εξετάστηκαν για δημιουργία βιομεμβράνης. Συλλέχθηκαν επίσης δείγματα αίματος για προσδιορισμό αντισωμάτων. Σταφυλόκοκκοι αποτελούσαν 0,564 και 0,761, αντίστοιχα, των συνολικών στελεχών από προβατίνες στις ομάδες V και C, αντίστοιχα. Σταφυλόκοκκοι ως αιτιολογικοί παράγοντες της μαστίτιδας απομονώνονταν σπανιότερα από τις προβατίνες V (0,053) συγκριτικά με τις προβατίνες C (0,103) ($P < 0,001$). Μεταξύ αυτών, μικρότερο ποσοστό στελεχών σταφυλοκόκκων από τα ζώα της ομάδας V σχημάτιζε βιομεμβράνη από στελέχη από τα ζώα της ομάδας C: 0,532 έναντι 0,749 των στελεχών ($P < 0,001$). Επίσης, σταφυλόκοκκοι που σχημάτιζαν βιομεμβράνη απομονώθηκαν σπανιότερα ως αιτιολογικοί παράγοντες μαστίτιδας από προβατίνες της ομάδας V (0,023) συγκριτικά με τις προβατίνες της ομάδας C (0,060) ($P < 0,001$). Οι τιμές των αντισωμάτων αντί- exopolysaccharide poly-N-acetylglucosamine (PNAG) ήταν υψηλότερες στις V

προβατίνες απ' ό,τι στις προβατίνες C (P < 0,001). Ο κίνδυνος εκδήλωσης μαστίτιδας, σταφυλοκοκκικής μαστίτιδας και μαστίτιδας από σταφυλοκόκκους που σχημάτιζαν βιομεμβράνη ήταν μικρότερος στις προβατίνες V από αυτόν στις προβατίνες C: 0,367, 0,171 και 0,080 έναντι 0,443, 0,309 και 0,189, αντίστοιχα (P < 0,030). Η συγκεντρωτική αποτελεσματικότητα του εμβολίου ήταν 0,446 για τη σταφυλοκοκκική μαστίτιδα, 0,577 για τη μαστίτιδα από σταφυλοκόκκους που σχημάτιζαν βιομεμβράνη, 0,331 για τις σταφυλοκοκκικής αιτιολογίας ενδομαστικές λοιμώξεις και 0,515 για τις ενδομαστικές λοιμώξεις από σταφυλοκόκκους που σχημάτιζαν βιομεμβράνη.

Στην πειραματική μελέτη (Μέρος Γ), σε εμβολιασμένες ή μη προβατίνες έγινε ενδομαστικός ενοφθαλμισμός με *S. chromogenes* (n = 17 και 8, αντίστοιχα). Επίσης περιλήφθηκε κατάλληλος τύπος μαρτύρων (n = 12). Τα ζώα μελετήθηκαν κατά τη διάρκεια μίας γαλακτικής περιόδου με ποικιλία μεθόδων. Όλες οι ενοφθαλμισμένες προβατίνες ανέπτυξαν μαστίτιδα. Στις εμβολιασμένες προβατίνες, η διάρκεια της μαστίτιδας ήταν μικρότερη (4 έναντι 17,5 ημέρες) (P = 0,022), η συχνότητα των περιστατικών ενδομαστικής λοίμωξης ήταν μικρότερη (0,325 έναντι 0,528) (P < 0,001) και ο συνολικός αριθμός σταφυλοκόκκων στα δείγματα γάλακτος ήταν μικρότερος (P < 0,01). Επίσης, βρέθηκαν διαφορές στις αναλογίες μεταξύ ενοφθαλμισμένων και μη μαστικών αδένων (μετά από σχετικούς μετασχηματισμούς) στην ένταση της φωτεινότητας στο μαστικό παρέγχυμα κατά την υπερηχογραφική εξέταση και στην παραγωγή γάλακτος. Η διάμεση τιμή της βαρύτητας των ιστοπαθολογικών ευρημάτων στο μαστικό παρέγχυμα των εμβολιασμένων ζώων (0,5) ήταν μικρότερη απ' ό,τι στα μη εμβολιασμένα ζώα (2) (P = 0,005).

Τα συμπεράσματα που προκύπτουν από τα ευρήματα αυτής της διατριβής, είναι τα παρακάτω.

(α) Τα αποτελέσματα της εκτενούς μελέτης σε όλη τη χώρα επιβεβαίωσαν τη σημασία της υποκλινικής μαστίτιδας ως συχνό πρόβλημα των προβάτων σε εκτροφές γαλακτοπαραγωγικής κατεύθυνσης. Το ποσοστό προσβολής ήταν 26,0%. Οι σταφυλόκοκκοι αποτελούσαν τους πιο σημαντικούς αιτιολογικούς παράγοντες της νόσου. Το σύστημα διαχείρισης στην εκτροφή ταυτοποιήθηκε ως ο σημαντικότερος παράγοντας προδιάθεσης για την υποκλινική μαστίτιδα. Παράγοντες σχετιζόμενοι με τα ζώα, π.χ., το στάδιο της γαλακτικής περιόδου, επίσης αποτελούσαν πράγοντες προδιάθεσης για τη νόσο. Η εφαρμογή στοχευμένων μεθόδων διαχείρισης υγείας του μαστού, π.χ., η εφαρμογή αντισηψίας στις θηλές μετά το άρμεγμα, επίσης σχετίζονταν με μειωμένο ποσοστό προσβολής της νόσου. Υπήρχε επίσης συσχέτιση της υποκλινικής μαστίτιδας με τη φυλή των ζώων.

(β) Αναγνωρίστηκε μία νέα νοσολογική οντότητα: μαστίτιδα προκαλούμενη από στελέχη σταφυλοκόκκων (ανεξαρτήτως είδους) που σχημάτιζαν βιομεμβράνη. Το ποσοστό προσβολής της

νόσου ήταν 15,5%. Το άρμεγμα με τα χέρια ταυτοποιήθηκε ως ο σημαντικότερος παράγοντας προδιάθεσης για τη νόσο.

(γ) Η συχνότητα απομόνωσης στελεχών σταφυλοκόκκων ανθεκτικών σε αντιμικροβιακούς παράγοντες ήταν περιορισμένη. Η ανθεκτικότητα αφορούσε κυρίως στην πενικιλλίνη, την τετρακυκλίνη και την αμοξυκιλλίνη. Η αναλογία ανθεκτικών στελεχών ήταν μεγαλύτερη μεταξύ στελεχών πηκτάση-αρνητικών παρά μεταξύ στελεχών *S. aureus*. Ανθεκτικά στελέχη απομονώθηκαν πιο συχνά από εκτροφές στις οποίες γινόταν χορήγηση αντιμικροβιακών παραγόντων στο τέλος της γαλακτικής περιόδου, καθώς και στην περίοδο αμέσως μετά τον τοκετό. Γονίδια ανθεκτικότητας σχετίζονταν με κινητά γενετικά στοιχεία στα στελέχη σταφυλοκόκκων.

(δ) Τα περισσότερα στελέχη *S. aureus* ταυτοποιήθηκαν στον τύπο ST133 και τα περισσότερα στελέχη *S. epidermidis* στους τύπους ST100, ST142 και ST152.

(ε) Τα αντισώματα κατά των στελεχών σταφυλοκόκκων που σχημάτιζαν βιομεμβράνες, παράγονταν κυρίως ως συνέπεια του εμβολιασμού της μαστίτιδας από αυτά τα στελέχη. Όμως, τίτλοι αντισωμάτων μπορούσαν επίσης να βρεθούν και ως συνέπεια σχετικής ενδομαστικής λοίμωξης. Ο προσδιορισμός του τίτλου των αντισωμάτων μπορεί να χρησιμοποιηθεί ως μέθοδος αξιολόγησης της έκτασης της σταφυλοκοκκικής μαστίτιδας στις εκτροφές.

(ζ) Τα ευρήματα τεκμηρίωσαν, για πρώτη φορά, τη σημασία των περιβαλλοντικών παραγόντων ως παραγόντων προδιάθεσης για τη μαστίτιδα των προβατίνων. Επίσης για πρώτη φορά, καταρτίστηκαν προγνωστικά πρότυπα για την υποκλινική μαστίτιδα λαμβάνοντας υπόψη περιβαλλοντικές παραμέτρους. Τα ευρήματα μπορούν επίσης να χρησιμοποιηθούν σε προγράμματα πρόληψης της μαστίτιδας, για τον καθορισμό στρατηγικών για την πρόληψη της νόσου, προσαρμοσμένες στις ανάγκες των περιοχών και των εκτροφών, ανάλογα με τους κυρίαρχους περιβαλλοντικούς κινδύνους.

(η) Το σύνολο των ευρημάτων παρείχε σαφή στοιχεία σχετικά με την πολυπαραγοντική φύση της μαστίτιδας στα πρόβατα και τις αλληλεπιδράσεις διαχείρισης - περιβάλλοντος - ζώων.

(θ) Το υπό αξιολόγηση εμβόλιο, το αντιγόνο του οποίου βασιζόταν σε στέλεχος *S. aureus* που σχημάτιζε βιομεμβράνη, παρείχε κάποιου βαθμού προστασία έναντι της σταφυλοκοκκικής μαστίτιδας. Το αποτέλεσμα ήταν πιο σαφές σε ενήλικες προβατίνες και κατά τους πρώτους τέσσερις μήνες της γαλακτικής περιόδου. Το αποτέλεσμα δεν επηρεαζόταν από το σύστημα διαχείρισης, τη διαδικασία αρμέγματος, το ιστορικό μαστίτιδας στην εκτροφή ή τη φυλή των ζώων. Το εμβόλιο μείωσε τη βαρύτητα των ενδομαστικών λοιμώξεων και περιόρισε τις σχετικές αλλοιώσεις στο μαστικό παρέγχυμα και τη διάδοση του παθογόνου παράγοντα. Τα αντισώματα που ανιχνεύθηκαν σε εμβολιασμένες προβατίνες μετά τη χορήγηση του εμβολίου, φαίνεται ότι συνέβαλαν σε μείωση του πληθυσμού των υπεύθυνων βακτηρίων.

(ι) Για τον έλεγχο της μαστίτιδας στις προβατίνες, ο εμβολιασμός δεν πρέπει να θεωρείται ως το μόνο μέσο για την πρόληψη της μαστίτιδας στο πλαίσιο της διαχείρισης της υγείας του μαστού. Άλλα μέτρα (π.χ., ορθή αρμεκτική πρακτική, κατάλληλη συντήρηση του αρμεκτικού συστήματος, απομάκρυνση προβατίνων, χορήγηση αντιμικροβιακών παραγόντων στο τέλος της γαλακτικής περιόδου) πρέπει να συμπεριληφθούν και μπορούν να δράσουν συνεργειακά για βελτίωση της πρόληψη της μαστίτιδας.

Με βάση την κείμενη νομοθεσία και μετά από σχετική απόφαση στη με αριθμό 54/06.07.2017 συνεδρίαση της Γενικής Συνέλευσης Ειδικής Σύνθεσης του Τμήματος Κτηνιατρικής του Πανεπιστημίου Θεσσαλίας, η συγγραφή της διατριβής έγινε στην αγγλική γλώσσα.

Δημοσιεύσεις σχετιζόμενες με την παρούσα διατριβή

Στις παρακάτω επιστημονικές δημοσιεύσεις παρουσιάζονται τμήματα της παρούσας διατριβής:

I. N.G.C. Vasileiou, D.C. Chatzopoulos, D.A. Gougoulis, S. Sarrou, A.I. Katsafadou, V. Spyrou, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2018) "Slime-producing staphylococci as causal agents of subclinical mastitis in sheep" *Veterinary Microbiology*, 224:93–99.

II. N.G.C. Vasileiou, P.J. Cripps, K.S. Ioannidi, D.C. Chatzopoulos, D.A. Gougoulis, S. Sarrou, D.C. Orfanou, A.P. Politis, T. Calvo Gonzalez-Valerio, S. Argyros, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2018) "Extensive countrywide field investigation of subclinical mastitis in sheep in Greece" *Journal of Dairy Science*, 101:7297–7310.

III. N.G.C. Vasileiou, D.A. Gougoulis, V. Riggio, K.S. Ioannidi, D.C. Chatzopoulos, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2018) " Association of subclinical mastitis prevalence with sheep breeds in Greece" *Journal of Dairy Research*, 85:317–320.

IV. A. Giannakopoulos[+], N.G.C. Vasileiou[+], D.A. Gougoulis, P.J. Cripps, K.S. Ioannidi, D.C. Chatzopoulos, C. Billinis, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2019) "Use of geographical information system and ecological niche modelling for predicting potential space distribution of subclinical mastitis in ewes" *Veterinary Microbiology*, 228:119–128.

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V. N.G.C. Vasileiou, A. Giannakopoulos, P.J. Cripps, K.S. Ioannidi, D.C. Chatzopoulos, D.A. Gougoulis, C. Billinis, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2019) "Study of potential environmental factors predisposing ewes to subclinical mastitis in Greece" *Comparative Immunology, Microbiology and Infectious Diseases*, 62:40–45.

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ΤΡΙΜΕΛΗΣ ΣΥΜΒΟΥΛΕΥΤΙΚΗ ΕΠΙΤΡΟΠΗ

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MASTITIS IN EWES ASSOCIATED WITH *STAPHYLOCOCCUS* SPP.: NEW CLINICAL, EPIDEMIOLOGICAL, MANAGEMENT, MICROBIOLOGICAL AND ZOONOTIC FINDINGS AND EVALUATION OF A NOVEL VACCINE AGAINST THE DISEASE

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ABSTRACT

Specific objectives of the present thesis were as follows: (i) the study of subclinical mastitis in ewes in sheep flocks in Greece, specifically to investigate prevalence of subclinical mastitis, to identify aetiological agents involved, to evaluate in depth staphylococcal isolates recovered, to assess the immunological response in staphylococcal mammary infections and to study factors potentially predisposing ewes to subclinical mastitis, (ii) the investigation of subclinical mastitis caused by biofilm-forming staphylococci, specifically to investigate prevalence of the infection and to identify potential risk factors for this condition, (iii) the detailed assessement of the possible role of environmental factors in development of mastitis, specifically to associate the prevalence of subclinical mastitis with environmental (climatic and topographic) factors and to identify regions potentially of high risk for increased frequency of subclinical mastitis and (iv) the evaluation of a vaccine against staphylococcal mastitis in ewes; the staphylococcal antigen in the vaccine is based on a bacterin of *S. aureus* strain, expressing the exopolysaccharide poly-N-acetylglucosamine (PNAG), which is involved in biofilm formation by these bacteria.

The thesis is divided into three chapters followed by the General Discussion.

In Chapter I, the relevant literature is reviewed. The Chapter is subdivided into two Parts. In Part A, the literature on the role of staphylococci in ovine mastitis is reviewed. In Part B, the literature, in predisposing factors for mastitis in ewes is presented.

In Chapter II, the findings of a large investigation in subclinical mastitis in Greece are presented.

Milk samples were collected from 2,198 ewes in 111 farms with a total population of 35,925 ewes, in all 13 administrative regions of Greece, for bacteriological and cytological examination. Prevalence of subclinical mastitis was 0.260. Main aetiological agents were staphylococci (*Staphylococcus* [*S.*] *aureus* and coagulase-negative species), which accounted for 0.699 of all isolates recovered; prevalence of staphylococcal mastitis was 0.191. In a multivariable mixed-effects analysis, the primary factor found to be associated with increased prevalence of subclinical mastitis was the management system practiced in flocks (in flocks under semi-intensive system, there was the highest prevalence) (P = 0.008). Other factors that were included in the multivariable model were the stage of lactation period (ewes in 2nd month post-partum showed highest prevalence) (P = 0.151) and application of post-milking teat dipping (P = 0.054). Results did not indicate difference in prevalence of subclinical mastitis between farms with pure-bred and farms with cross-bred animals,

nor difference in prevalence between farms with Greek pure-bred animals and farms with imported pure-bred animals. Results indicated that prevalence of subclinical mastitis was smaller in farms with Assaf-breed (0.100) and higher in farms with Frisarta-breed (0.625) ($P < 0.02$). Prevalence of mastitis was smaller in farms with Greek traditional indigenous breeds (0.221) ($P = 0.007$). In a model that included sheep breed and management system in farm, breed emerged of significance for prevalence of subclinical mastitis ($P = 0.003$). Then, biofilm formation was evaluated in 708 staphylococcal isolates recovered during the study. Isolates were studied by means of microbiological and molecular methods. Of these isolates, 262 were characterised as biofilm-forming, 227 as weak biofilm-forming and 219 as non biofilm-forming. Most frequently detected genes were *eno* and *icaB*; *S. aureus* possessed more genes than coagulase-negative isolates; greater number of genes was detected in biofilm-forming than in weak biofilm-forming or non biofilm-forming isolates. Subclinical mastitis caused specifically by biofilm-forming staphylococci was detected in 337 ewes: prevalence in population sampled was 0.153. A multivariable mixed-effects model revealed that milking mode (highest prevalence in hand-milked flocks) ($P = 0.017$) and flock management system (highest prevalence in semi-intensive flocks) ($P = 0.045$) were the two factors associated with increased prevalence of mastitis in flocks. Then, susceptibility to antimicrobial agents of 142 staphylococcal isolates obtained during the study was studied. In total, 0.415 of these were resistant and 0.056 multi-drug resistant. More coagulase-negative staphylococci (0.470) were resistant than *S. aureus* (0.185) isolates. Resistance was greater to penicillin (0.225), tetracycline (0.183) or ampicillin (0.169) ($P < 0.032$). More biofilm-forming (0.206) isolates were resistant to tetracycline than non biofilm-forming (0.000) ones ($P = 0.013$). Presence of *tetK* was associated with presence of *icaA* in the same isolates ($P = 0.029$). Further, 0.766 of resistant isolates *versus* 0.577 of susceptible ones were recovered immediately *post-partum* ($P = 0.035$) and 0.234 of resistant isolates *versus* 0.099 of susceptible ones were recovered in farms that practiced routine administration of antimicrobial agents at the end of a lactation period ($P = 0.045$). Most *S. aureus* (0.593) were classified in ST133 and most *S. epidermidis* were classified in ST100, ST142 or ST152 (0.190 each). There was no association of ST with resistance. Whole genome sequencing showed that, in a *S. lentus* strain, the *ermB* gene was part of transposon Tn*917* integrated into the chromosome; also, a small plasmid was observed in an *ermC*-carrying *S. hominis* strain and, finally, in a *S. aureus* and a *S. epidermidis* strains, small *tetK*-carrying plasmids (pSau-2716Lar, pSau-3893Lar) of 4.439 kb were found. Blood samples for detection of anti-PNAG antibodies were collected from 355 ewes in 71 flocks. Prevalence of seropositivity was higher among ewes vaccinated against staphylococcal mastitis (0.750) than non-vaccinated ones (0.458). Mean antibody titres in samples from vaccinated ewes (13.64) were significantly higher than in samples

from non-vaccinated ones (7.39). There was no association between identity of staphylococcal isolates recovered from milk samples and antibody titres in blood samples from respective ewes. Among vaccinated ewes, there was correlation (r = 0.922) between total number of genes relevant to biofilm-formation in mastitis staphylococcal isolates and antibody titres in blood samples from respective ewes; further, isolates from ewes with seropositive samples had higher median number of genes (4) than isolates from ewes with seronegative samples (3). In a multivariable mixed-effects model, vaccination emerged as the only significant factor for the prevalence of seropositivity of anti-PNAG antibody titres. Data on farm location were collected in the field using hand-held Global Positioning System Garmin units. The geo-references were resolved to specific farm level. In a multivariable mixed-effects analysis, the two environmental variables found to be associated with increased prevalence of subclinical mastitis were the minimum temperature of coldest month (coefficient: -0.084 ± 0.033, P = 0.014) and the mean temperature for 30 days prior to sampling date (coefficient: 0.031 ± 0.014, P = 0.029). Collected data were analysed by an Ecological Niche Model under the framework of a geographic information system. Two separate analyses were performed: one for subclinical mastitis independently of causal agent (prevalence in population sampled: 0.260) and one for subclinical mastitis caused specifically by biofilm-forming staphylococci (prevalence in population sampled: 0.153). A model was constructed in which sheep farms were divided into two clusters, according to prevalence of subclinical mastitis: farms in the upper three quartiles of prevalence were used as occurrence points for the Ecological niche modelling procedure ('infected farms'); farms in the lower quartile of prevalence within each category were (pseudo)negative points. Significant differences in environmental parametres prevailing in locations of farms into the study, were identified for up to 13 parametres between locations of farms according to management system applied in farms. When farms in each management system were considered separately, differences became evident between farms in each management system, as well as between the two infections. The factor with the highest relative contribution in the analyses was the distance from other sheep farms; other factors also of importance in the predictive models were the altitude, the maximum temperature of warmest month and the total precipitation of driest month. Verification of the model revealed that ≥ 0.760 of infected farms' were located in areas predicted as high risk for prevalence of subclinical mastitis or biofilm staphylococcal subclinical mastitis.

In Chapter III, after a brief review regarding vaccination against mastitis in ewes (Part A), a clinical trial (Part B) and an experimental study (Part C) to evaluate a vaccine against staphylococcal mastitis in ewes are presented. The staphylococcal antigen in the vaccine was based on a bacterin of *S. aureus* strain expressing PNAG.

The clinical trial (Part B) was performed in five dairy sheep farms, with 316 ewes in group V(accinated) and 307 in group C(ontrol) studied throughout a lactation period. Two administrations of the vaccine were performed during the last stage of gestation of ewes. Starting 15 days after lambing and at monthly intervals thereafter, up to 9 milk samplings were performed for bacteriological and cytological examinations. Staphylococcal isolates recovered were examined for biofilm formation. Blood samples were collected for measurement of IgG PNAG-specific antibodies. The most frequently isolated bacteria were staphylococci: 0.564 and 0.761, respectively, of total isolates recovered from ewes of group V and C, respectively; staphylococci as causal agents of mastitis were isolated less frequently from V (0.053) than in ewes in C (0.103) ($P < 0.001$). Among mastitis-associated staphylococcal isolates recovered from V ewes, a smaller proportion was biofilm-forming than among ones from C: 0.532 *versus* 0.749 of isolates ($P < 0.001$); biofilm-forming staphylococci as causal agents of mastitis were isolated less frequently from ewes in group V (0.023) than in ewes in group C (0.060) ($P < 0.001$). Anti-PNAG-specific antibody values increased in V ewes and were higher than in C ($P < 0.001$); a greater proportion of ewes with low antibody titres developed staphylococcal mastitis (0.414) than of V ewes with high antibody titres (0.170). Incidence risk of mastitis, staphylococcal mastitis and biofilm-associated staphylococcal mastitis was smaller in V than in C: 0.367, 0.171 and 0.080 *versus* 0.443, 0.309 and 0.189, respectively ($P < 0.030$). The first case of staphylococcal mastitis occurred later in V than in C: 3rd *versus* 2nd sampling point. Overall, efficacy of the vaccine was 0.446 for staphylococcal mastitis, 0.577 for biofilm-associated staphylococcal mastitis, 0.331 for staphylococcal intramammary infection and 0.515 for biofilm-associated staphylococcal intramammary infection.

In the experimental study (Part C), vaccinated or unvaccinated ewes were challenged with intramammary inoculation of *S. chromogenes* (n = 17 and 8, respectively); appropriate controls were also included (n=12). Animals were monitored during a lactation period by an array of methods. All inoculated ewes developed mastitis. In vaccinated ewes, duration of mastitis was shorter (4 *versus* 17.5 d) ($P = 0.022$), frequency of incidents of mammary infection was smaller (0.325 *versus* 0.528) ($P < 0.001$) and bacterial counts ($P < 0.01$) were lower. There were also differences in ultrasonographic grey-scale ratios and milk yield ratios between vaccinated and unvaccinated ewes. Also, in vaccinated ewes, there was an increase in anti-PNAG antibody titres. Median score for histopathological findings in mammary tissue samples from vaccinated (0.5) was smaller than in samples from unvaccinated (2) ewes ($P = 0.005$).

The conclusions from the results of the present thesis are summarised herebelow.

(a) The results of the countrywide investigation confirmed the significance of subclinical mastitis as a frequent problem of dairy sheep. Prevalence of subclinical mastitis was 26.0%. Staphylococci were

the primary aetiological agents of the disease. Management system practiced in farms was identified as the most important factor predisposing to subclinical mastitis. Animal-related factors, e.g., stage of lactation period, may also play a role in development of mastitis. There was also evidence of associations of subclinical mastitis with breed. Application of targeted health management methods, e.g., post-milking teat dipping, may be associated with reduced prevalence of subclinical mastitis.

(b) A new disease entity has been recognised: mastitis caused by biofilm-forming staphylococci. The disease is caused by staphylococcal isolates independently of their species. Prevalence of the disease was 15.5% on national basis. Hand-milking was identified as the most important factor predisposing to that infection.

(c) Prevalence of staphylococcal isolates resistant to antimicrobial agents was limited. Resistance referred mainly to penicillin, tetracycline and amoxycillin. Prevalence of resistance was higher among coagulase-negative isolates than among *S. aureus* isolates. Detection of resistant isolates was more frequent among isolates recovered from flocks that practiced routine administration of antimicrobial agents at the end of a lactation period and among isolates recovered immediately *post-partum*. Resistance genes were found to be associated with mobile genetic elements.

(d) Most *S. aureus* isolates were classified in ST133 and most *S. epidermidis* were classified in ST100, ST142 or ST152.

(e) Anti-PNAG antibodies were induced mainly consequently to anti-staphylococcal mastitis vaccination. Antibody titres could also increase during mammary infection by biofilm-forming staphylococci. Evaluation of antibody titres can be potentially used as a screening technique for establishing extent of staphylococcal mastitis in flocks.

(f) The findings have documented, for the first time, the significance of environmental factors predisposing ewes to mastitis. Also for the first time, predictive models for subclinical mastitis in ewes taking into account environmental parametres have been provided. The findings also can be used in mastitis prevention schemes, in order to establish strategies that will help to control mastitis, tailored according to needs of regions and farms, as per environmental risks.

(g) The entirety of findings has provided clear evidence regarding the multifactorial nature of mastitis in sheep: in mastitis, there are clear management – environment – animal interactions.

(h) The vaccine under evaluation, which was based on a bacterin of *S. aureus* strain expressing PNAG, could lead to some protection against staphylococcal mastitis. The effect was more pronounced in adult ewes and during the first four months of lactation period; it was independent of management system, milking procedure, reported history of mastitis or animal breed. The vaccine reduced the severity of the infection and limited damage to the mammary parenchyma and

dissemination of the causal pathogen. Anti-PNAG antibodies were detected in vaccinated ewes after administration of the vaccine and might have led in clearance of causal bacteria.

(i) For control of mastitis in ewes, vaccination should not be considered as the only means for reducing mastitis within an udder health program; other udder health management measures (e.g., correct milking routine, appropriate milking system maintenance, culling of ewes with long-standing infections, administration of antimicrobials at the end of a lactation period) should be included and can work synergistically to improve control of mastitis.

Publications associated with the present thesis

The following scientific papers presenting facets of the present thesis, are available:

I. N.G.C. Vasileiou, D.C. Chatzopoulos, D.A. Gougoulis, S. Sarrou, A.I. Katsafadou, V. Spyrou, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2018) "Slime-producing staphylococci as causal agents of subclinical mastitis in sheep" *Veterinary Microbiology*, 224:93–99.

II. N.G.C. Vasileiou, P.J. Cripps, K.S. Ioannidi, D.C. Chatzopoulos, D.A. Gougoulis, S. Sarrou, D.C. Orfanou, A.P. Politis, T. Calvo Gonzalez-Valerio, S. Argyros, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2018) "Extensive countrywide field investigation of subclinical mastitis in sheep in Greece" *Journal of Dairy Science*, 101:7297–7310.

III. N.G.C. Vasileiou, D.A. Gougoulis, V. Riggio, K.S. Ioannidi, D.C. Chatzopoulos, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2018) " Association of subclinical mastitis prevalence with sheep breeds in Greece" *Journal of Dairy Research*, 85:317–320.

IV. A. Giannakopoulos[+], N.G.C. Vasileiou[+], D.A. Gougoulis, P.J. Cripps, K.S. Ioannidi, D.C. Chatzopoulos, C. Billinis, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2019) "Use of geographical information system and ecological niche modelling for predicting potential space distribution of subclinical mastitis in ewes" *Veterinary Microbiology*, 228:119–128.

+: these authors have contributed equally and their names are listed alphabetically

V. N.G.C. Vasileiou, A. Giannakopoulos, P.J. Cripps, K.S. Ioannidi, D.C. Chatzopoulos, D.A. Gougoulis, C. Billinis, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2019) "Study of potential environmental factors predisposing ewes to subclinical mastitis in Greece" *Comparative Immunology, Microbiology and Infectious Diseases*, 62:40–45.

VI. N.G.C. Vasileiou, S. Sarrou, C. Papagiannitsis, D.C. Chatzopoulos, E. Malli, V.S. Mavrogianni, E. Petinaki, G.C. Fthenakis (2019) "Antimicrobial agent susceptibility and typing of staphylococcal isolates from subclinical mastitis in ewes" *Microbial Drug Resistance*, doi: 10.1089/mdr.2019.0009.

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GENERAL INTRODUCTION

Preface - Objectives of the thesis

Mastitis in sheep

General classification of mammary infections

In ewes, mammary infections could be best classified according to their aetiology. That way, the following diseases can be distinguished: (i) bacterial mastitis (hereafter termed 'mastitis'), (ii) mycoplasmal mastitis (often termed contagious agalactia) and (iii) lentiviral mammary infection.

Further, mastitis can be classified with regard to presence of clinical signs, the course of the disease or occurrence within the production cycle.

With regard to presence of clinical signs, mastitis can be classified as (i) clinical, which refers to clinically available abnormalities in the mammary glands (including the mammary secretion), and (ii) subclinical, which occurs in the absence of any clinically evident changes. Changes occurring in subclinical mastititis refer to presence of pathogens in milk, presence of inflammatory reaction and changes in milk composition. These can only be detected by means of specific paraclinical and laboratory tests.

According to the course of the disease, mastitis can be classified as (i) hyper-acute, (ii) acute, (iii) subacute and (iv) long-standing. Finally, when taking into account the time-point of occurrence within the production cycle, mastitis can be classified as (i) occurring during the lactation period and (ii) occurring during the dry-period.

Early history of mastitis work

Mastitis in sheep has first been studied towards the end of the 19th century by the French veterinarian and microbiologist Edmund Nocard. Nocard (1887) described the experience of a practicing veterinarian of the time as follows: "*This disease is rightly considered to be the plague of the cheese-making flocks; it is not rare to see one tenth of the animals affected by the terrible evil; those, which recover, are invariably lost for milk- production. All the treatments have been tried equally unsuccessfully; this, what the shepherds have found the best to save the animal, is to cut open the udder in different directions from the start of the disease and to treat the wounds with any detergent lotion. The majority of veterinarians of the country consider this disease as a simple mastitis, caused by the milk engorgement and the knocks given on the mammary gland by the milkers; but the owners refuse to believe in these possible causes of the disease and the majority of them thinks that this mastitis is related to anthrax. This opinion should be rejected, because the*

animals never exhibit, during their life or after their death, the signs or lesions of anthrax; microscopic examination of the blood has never allowed me to see bacteria …" (Fthenakis 1988).

Nocard (1887) described staphylococcal mastitis with pathological and microbiological examinations. He isolated from the milk of an affected ewe the microorganism and presented its morphological, cultural and biochemical characteristics. The causal agent has been identified as *Staphylococcus* [*S.*] *aureus*. Also, he studied the effects of experimental intramammary inoculation of mammary secretion from an affected mammary gland, by means of which he successfully reproduced a pathological condition similar to the natural disease. Thereafter, Bridre (1907) undertook a systematic investigation of the disease in dairy flocks in France. He found that the incidence of the disease was approximately 5%, with a fatality rate of 20%. Further, he attempted to protect sheep by means of immunisation.

Thereafter, Leyshon (1929), working in Great Britain, has described the first case of mastitis caused by *Mannheimia haemolytica*. Further publications regarding mastitis have been produced thereafter, in most cases originating from regions where sheep are maintained for dairy production.

Aetiology

Mastitis is caused by a variety of pathogenic bacteria. In ewes that are milked, the most frequent aetiological agents of clinical mastitis is *S. aureus*, which accounts for causing approximately 70% of cases. In ewes that suckle lambs, *S. aureus* and *M. haemolytica* are the major pathogens, together accounting for approximately 80% of cases. The most frequent causal agents of subclinical mastitis are coagulase-negative staphylococci, which are responsible for over 75% of cases of the infection (Gelasakis et al. 2015). A variety of other bacteria can be implicated as causal agents of mastitis; these include *Escherichia coli*, streptococci, *Trueperella pyogenes*, *Corynebacterium* spp. etc. (Gelasakis et al. 2015).

Importance

The adverse financial effects of mastitis are summarised below:

- Death of affected ewes.
- Loss of affected mammary glands and consequent early culling of ewes.
- Replacement costs, due to early culling of affected ewes.
- Veterinary expenses.
- Reduced milk production by affected ewes.
- Downgrading of the quality of milk produced, due to changes in its composition.

- Discard of milk unsuitable for human consumption, due to increased content of bacteria, bacterial toxins or drug residues.
- Increased mortality and reduced growth rate of lambs of affected ewes.

Moreover, in ewes, mastitis is of significant welfare concern (European Food Safety Authority 2014). Clinical mastitis is a disease that leads to anxiety, restlessness, changes in feeding behaviour and pain (Fthenakis and Jones 1990c). Even in subclinical mastitis, normal behavioural patterns of sheep are modified (Gougoulis et al. 2008a, 2010), hence raising welfare concerns.

Objectives of the thesis

Various factors have been implicated to contribute in the virulence of staphylococci in the mammary gland and to participate in the pathogenesis of mastitis. Slime production is an important virulence factor, contributing to biofilm formation by staphylococci, which thus survive and disseminate outside the mammary gland or adhere on epithelial cells therein. Staphylococcal adhesion on host mammary epithelial cells is a multidimensional process, regulated by diverse surface proteins termed 'microbial surface components recognising adhesive matrix molecules'. Formation of biofilms contributes to expansion of the multiplying bacteria, offers reduced susceptibility to antimicrobial agents and promotes bacterial survival from mammary defences (Clarke and Foster 2006, Melchior et al. 2006, Otto 2008). The main constituents of biofilm matrix are polysaccharides and peptides (Perez et al. 2009).

The general objective of the thesis is to increase available knowledge that would lead in effective control of the disease, particularly of staphylococcal mastitis. Specific objectives are as follows.

- The study of subclinical mastitis in Greece, specifically (i) to investigate prevalence of subclinical mastitis, (ii) to identify aetiological agents involved, (iii) to evaluate in depth staphylococcal isolates recovered, (iv) to assess the immunological response in staphylococcal mammary infections and (v) to study factors potentially predisposing ewes to subclinical mastitis.
- The investigation of subclinical mastitis caused by biofilm-forming staphylococci, specifically (i) to investigate prevalence of the infection and (ii) to identify potential risk factors for this condition.
- The detailed assessement of the possible role of environmental factors in development of mastitis, specifically (i) to associate the prevalence of subclinical mastitis with environmental

(climatic and topographic) factors and (ii) to identify regions potentially of high risk for increased frequency of subclinical mastitis.

- The evaluation of a vaccine against staphylococcal mastitis in ewes. Clinical work was performed in five dairy sheep farms (clinical study) and in the departmental facilities (experimental study). The staphylococcal antigen in the vaccine is based on a bacterin of *S. aureus* strain, expressing the exopolysaccharide poly-N-acetylglucosamine (PNAG), which is involved in biofilm formation by these bacteria.

The present thesis has been carried out at the Department of Obstetrics and Reproduction of the Veterinary Faculty of the University of Thessaly. A large countrywide investigation was performed in 111 flocks across Greece, in all the 13 administrative regions of the country. Field studies were performed in five dairy sheep farms located in Thessaly. Part of laboratory work was performed in the Department of Microbiology of the Faculty of Medicine of the University of Thessaly and at the laboratories of Laboratorios Hipra in Spain.

Research work started in 2014 and was carried out until early 2017; it was followed by analysis of results and writing up of the thesis. The thesis was financially supported by departmental funds and funds provided by Laboratorios Hipra.

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CHAPTER I

REVIEW OF THE LITERATURE

A. ROLE OF STAPHYLOCOCCI IN OVINE MASTITIS

Introduction

In general, the onset and outcome of staphylococcal infections are dependent on the combination of the virulence of the invading isolate and the defence abilities of the host. Protection of a host against staphylococci is to a large degree dependent upon (a) the integrity of the skin and mucous membranes, which form an important barrier to entrance of staphylococci into the body, and (b) the number and functionality of leucocytes, which are important for phagocytosing and destroying the invading bacteria (Murray et al. 2008).

Staphylococcal infections are usually preceded by colonisation and are characterised by intense tissue inflammation. They occur when the bacteria enter through skin or mucous membrane breakdowns (e.g., injuries, ulcerations, surgical incisions). The invading bacteria multiply locally and produce enzymes and toxins, leading in tissue destruction, influx of polymorphonuclear cells, severe tissue damage and abscess formation, which characterise the staphylococcal infections (Lowy 1998). By means of this mechanism and in accord with the characteristics of staphylococci, local infections occur initially at the point of entry. Thereafter, more severe infections, consequently to bacterial dissemination in tissues or haematogenously can occur at other areas of the body of the host.

Staphylococci have been isolated from various sites of the body of healthy sheep (Table I.i). In sheep, the most important problem caused by staphylococci is mastitis, in clinical or subclinical form. Moreover, the bacteria have been implicated also in various other disorders (Table I.ii), the significance of which varies. Some of these occur frequently, e.g. impetigo or abscess disease, some sporadically, e.g., vaginal infections or abortion, whilst some others have been described only experimentally, e.g., osteomyelitis or rhinosinusitis.

Table I.i. List of body sites of healthy sheep, from which staphylococci have been isolated.

Site	References
Skin	Kayalvizhi et al. (2008), Gbolagunte and Hambolu (2010)
Nasal cavity	Queen et al. (1994), Vautor et al. (2009), Tesfaye et al. (2013), Rahimi et al. (2015)
Respiratory tract	Barbour et al. (1997), Glendinning et al. (2017)
Vagina	Manes et al. (2010), Silva et al. (2011)
Teat duct	Fragkou, Mavrogianni et al. (2007)
Preputial cavity	Gouletsou et al. (2006)

Table I.ii. List of disorders of sheep (other than mastitis), in which staphylococci have been implicated.

Disorder	References
Septicaemia	Elidrissi et al. (1992), Jonkam et al. (2007)
Dermatitis / Impetigo	Scott et al. (1980), Fraser et al. (1982), Synge et al. (1985), Hajtos et al. (2000), Koutinas et al. (2007)
Wound infections	Tiwari et al. (2016)
Interdigital dermatitis	Azizi et al. (2011)
Furunculosis	Yeruham et al. (2002)
Necrotic dermatitis	Jubb et al. (1985), Bath et al. (2011)
Abscess disease	de la Fuente et al. (1985, 1997), Moller et al. (2000), Alharbi (2011)
Lymphadenitis	Ribeiro et al. (2011), Chikhaoui and Khoudja (2013)
Granulomas ('botryomycosis')	Jubb et al. (1985).
Vaginal infections	de Paula Vasconcelos et al. (2016), Manes et al. (2010)
Uterine infections	Regassa and Noakes (1999)
Abortion	Lehman and Elze (1997), Edwards et al. (2008)
Orchitis	Gouletsou (2005)
Epididymitis	Jubb et al. (1985), Gouletsou and Fthenakis (2015)
Scrotal pyocele	Lacasta et al. (2009)
Tick pyaemia	Gillespie and Timoney (1981), Anderson (1983), Jubb et al. (1985), Webster and Mitchell (1986)
Experimental rhinosinusitis	Ha et al. (2007)
Respiratory infection	Tibbo et al. (2001), Enkhbaatar et al. (2008)
Lung abscesses	Azizi et al. (2013)
Experimental osteomyelitis	Kaarsemaker et al. (1997)
Neonatal polyarthritis	Jubb et al. (1985)
Keratoconjunctivitis	Egwu and Faull (1993)

The microorganism

Classification - Taxonomy

Taxonomically, the genus *Staphylococcus* [*S.*] is classified in the bacterial family Staphylococcaceae (order: Bacillales, class: Bacilli, phylum: Firmicutes, domain: Bacteria). Currently, the genus includes over 50 species, with many of them also having subspecies. Apart from speciation, various other taxonomic schemes have been proposed for staphylococci, which are based on phenotypic characteristics or molecular findings.

A well-known approach refers to the reaction of staphylococcal species to the coagulase test, which identifies whether a staphylococcal isolate produces the exoenzyme coagulase: staphylococcal species are classified as coagulase-positive or coagulase-negative, with a third class, coagulase-variable, having been established recently. Another approach refers to natural susceptibility of the species to novobiocin: staphylococcal species are classified as novobiocin-sensitive or novobiocin-resistant. Further, clustering into groups based on results of 16s rRNA sequencing, has also been performed and 11 groups have been distinguished (Table I.iii).

Isolation and identification

The procedure of isolation of staphylococci starts with culture of the clinical samples, which in mastitis are, most often, milk samples collected from ewes under examination or from bulk milk tank. Less often, other types of samples may need to be processed, e.g., swabs from mammary lesions or udder skin, material from teat duct or mammary tissue samples (Mavrogianni et al. 2007, Fragkou et al. 2014).

Table I.iii. Clustering of staphylococcal species[1] into 11 groups based on results of 16s rRNA sequencing.

Group	Species
S. aureus	*S. anaerobius* (coagulase +ve), *S. argenteus*, *S. aureus* (coagulase +ve), *S. schweitzeri*, *S. simiae* - possible classification: *S. aureus* subsp. *anaerobius* and *S. aureus* subsp. *aureus* (both coagulase +ve)
S. auricularis	*S. auricularis*
S. carnosus	*S. carnosus*, *S. condimenti*, *S. massiliensis*, *S. piscifermentans*
S. epidermidis	*S. capitis*, *S. caprae*, *S. epidermidis*, *S. saccharolyticus*
S. haemolyticus	*S. devriesei*, *S. haemolyticus*, *S. hominis* (subspecies *hominis* novobiocin sensitive – subspecies *novobiosepticus* novobiocin resistant)
S. hyicus - intermedius	*S. agnetis*, *S. chromogenes*, *S. cornubiensis*, *S. felis*, *S. delphini* (coagulase +ve), *S. hyicus* (coagulase +ve), *S. intermedius* (coagulase +ve), *S. lutrae* (coagulase +ve), *S. microti*, *S. muscae*, *S. pseudintermedius* (coagulase +ve), *S. rostri*, *S. schleiferi* subsp. *coagulans* (coagulase +ve) and subsp. *schleiferi* (coagulase –ve)
S. lugdunensis	*S. lugdunensis*
S. saprophyticus (novobiocin-resistant species)	*S. arlettae*, *S. caeli*, *S. cohnii*, *S. edaphicus*, *S. equorum*, *S. gallinarum*, *S. kloosii*, *S. nepalensis*, *S. saprophyticus*, *S. succinus*, *S. xylosus*
S. sciuri (novobiocin-resistant, oxidase-positive species)	*S. fleurettii*, *S. lentus*, *S. sciuri*, *S. stepanovicii*, *S. vitulinus* (synonum to *S. pulvereri*)
S. simulans	*S. simulans*
S. warneri	*S. pasteuri*, *S. warneri*
Not clustered in any group	*S. argensis*, *S. petrasii*, *S. pettenkoferi*

1. Reports regarding other species, e.g., *S. leei* (coagulase +ve), *S. lyticans*, S. *pseudolugdunensis*, have also been published, but their valid taxonomic status has not yet been confirmed.

There is no need for enrichment of samples. These can be inoculated directly onto conventional (e.g., blood agar) or selective media (e.g., Chapman's medium) and the plates should be incubated at 35 °C to 37 °C for up to 48 h (Fragkou et al. 2014); if nothing had grown, media can be reincubated for a further 24 h. Due to the tolerance of staphylococci in increased salt concentrations (as high as 10%), mannitol salt agar is an appropriate selective medium (e.g., Chapman's medium) for their isolation. Chromogenic agar can also be used for isolation of *S. aureus* isolates (Ariza-Miguel et al. 2015). False negative results of culture process may always occur and should be taken into account. Prolonged transportation time or inappropriate maintenance of samples can trigger bacterial survival mechanisms, e.g., biofilm formation. In such cases, the bacteria would not produce obvious colonies when transferred onto agar plates (Veeh et al. 2003,

Skrlin 2016). Further, in long-standing infection, staphylococci may not grow on solid media (Ehrlich et al. 2014).

Evaluation of colonial appearance is the first step for identification of staphylococcal isolates. Staphylococcal species usually form distinctive colonies on sheep blood agar, which are smooth and butyrous, with low convex profile and an entire edge (Winn and Koneman 2006). Most *S. aureus* isolates produce pale light yellow colonies, due to increased concentration of staphyloxanthin, a carotenoid pigment with a notable role in pathogenicity of the bacteria (Lan et al. 2010). Other species, most often, produce white-coloured colonies.

Microscopically, Gram stain would confirm that an isolate is a Gram-positive microorganism. Staphylococcal colonies are differentiated from other Gram-positive bacteria (e.g., streptococci, enterococci) by means of the catalase test, which detects presence of cytochrome oxidase enzymes, with staphylococci showing a positive reaction in the test. This is followed by the coagulase test, which serves to distinguish coagulase-positive isolates (*S. anaerobius*, *S. aureus*, *S. delphini*, *S. hyicus*, *S. intermedius*, *S. lutrae*, *S. pseudintermedius* and *S. schleiferi* subsp. *coagulans*).

Thereafter, identification to species level can be performed by means of commercial assays, which use modified carbohydrate fermentation tests, adaptations of standard bacteriological identification biochemical tests (Donay et al. 2004), or by means of molecular identification techniques, like the Polymerase Chain Reaction (PCR) or Matrix-Assisted Laser Desorption / Ionisation - Time of Flight (MALDI-TOF), which can analyse protein profile of bacterial cells (Katsafadou et al. 2015).

Virulence factors

Pathogenicity of staphylococcal infections is based on the virulence factors of the bacteria that allow them to survive within the host and cause relevant damage. The bacteria carry a wide array of potential pathogenicity factors, being capable to (i) adhere on host tissues, (ii) avoid, overcome or invade host immune system and (iii) release harmful toxins or enzymes (Sahuquillo Arce et al. 2013). During infections, the bacteria are able to produce the respective virulence determinants in a sequence tightly coordinated by relevant regulatory systems. The expression of both virulence factors and regulatory mechanisms is controlled by specific virulence genes. Factors contributing to the pathogenicity of staphylococci can be classified as (i) bacterial cell surface components (adherence factors) and (ii) secreted variants.

Adherence factors include proteins and antigens, which mainly act during the early phase of infection. Their principal function is to facilitate attachment of the bacteria onto the host cell surface,

simultaneously leading to the cascade host immune response evasion (Foster and Höök 1998). Various capsular polysaccharides, located on the bacterial cell wall, would be involved in the inhibition of phagocytosis by neutrophils, while teichoic acids are implicated to bacterial adherence of mucosal surface. Most of these are microbial surface components recognising adhesive matrix molecules. These molecules recognise the most essential components of the extra-cellular matrix, including fibrinogen, fibronectin and collagens. Among others, staphylococcal protein A (SpA), fibronectin-binding protein A and fibronectin-binding protein B (FnbpA and FnbpB), collagen-binding protein and clumping factor A and B proteins have also been determined to play a keynote role in the virulence of staphylococci (Palmqvist et al. 2002, Williams et al. 2002, Heying et al. 2007).

Slime production by staphylococcal isolates contributes to biofilm formation by these bacteria and plays an important role in the pathogenesis of staphylococcal infections and mastitis specifically (Schönborn et al. 2017), particularly at the early stage of the infection, when the bacteria adhere on the mammary epithelial cells. Genes *icaA*, *icaB*, *icaC*, *icaD* are responsible for production of icaADBC-encoded polysaccharide intercellular adhesion and ica-independent chemically diverse slime (Cramton et al. 1999, 2001). More specifically, the *icaA* gene product is a transmembrane protein, which for optimal action requires presence of the *icaD* gene product. *icaC* gene encodes a product contributing to chain formulation of N-acetyl-glucosamine oligomers produced by the *icaAD* combination (Gerke et al. 1998); this product is also considered to be involved in translocation of the polysaccharide on cell surface (Gerke et al. 1998). The *icaB*-encoded protein is considered responsible for the bacterial evasion process from the phagocytosis process (Vuong et al. 2004, Cerca et al. 2007). Moreover, the *clfa* gene encodes a surface protein, demonstrated as clumping factor A, which has a crucial role in bacterial initial attachment and evasion of host immune responses (Stutz et al. 2011). The *bap* gene also encodes an important surface protein, termed 'biofilm associated protein'; beyond its contribution to initial bacterial attachment, it has also been considered that this protein is capable to induce a polysaccharide intercellular adhesion / poly-N-acetyl glucosamine-independent biofilm formation process, especially on abiotic surfaces (Latasa et al. 2006). Finally, the *eno* gene encodes a laminin-binding surface protein (Carneiro et al. 2004).

Secreted virulence factors are mainly presented during the late phase of infection and usually have more distinct role on microbial pathogenicity (Otto 2013). Based on their principal activity, secreted virulence determinants are further classified in four categories: (i) super-antigens, (ii) cytolytic toxins, (iii) exoenzymes and (iv) miscellaneous proteins. Tissue shock syndrome toxin-1 (TSST-1) and enterotoxins are considered as the most prominent superantigens, usually causing clinical conditions of increased severity. Cytolytic toxins (i.e., *α*-haemolysin, *β*-haemolysin, *γ*-haemolysin, toxins of the leucocidin family) are capable to form pores to host cell wall, causing the

osmotic leakage of cell content and, therefore, lysis of the cell; cytolysis provides to the bacteria the required nutrients for further growth. The extracellular enzymes are produced by most staphylococcal isolates and aim to inactivate host antimicrobial mechanisms, allowing the bacterial dissemination. These include various lipases, nucleases and proteases (i.e., hyaluronidase, serine, cysteine, staphylokinase). Finally, many other proteins have been shown to have a further impact on the host immune system; staphylococcal complement inhibitor protein, extracellular fibrinogen binding protein, chemotaxis inhibitory protein and formyl peptide receptor-like protein-1 inhibitory protein are the ones most frequently detected (Otto 2013).

Synthesis of above factors occurs during the two growth phases of the bacteria. During the early phase, cell wall-associated factors facilitate tissue attachment and evasion of host defence system, allowing staphylococci to accumulate and, possibly, also form a biofilm, and the late phase, during which bacteria secrete a spectrum of exoproteins, including proteinases, haemolysins and super-antigens, whilst, at the same time, cell wall-associated factors are downregulated, leading to enhancement of the biofilm and bacterial dissemination within the mammary gland (Novick 2003, Wang and Muir 2016). The production of the various virulence factors of the bacteria is controlled by various mechanisms, following the general strategy of the microbial adhesion, invasion and proliferation. The function of regulation systems is in response of bacterial cell density (quorum-sensing) and environmental factors (e.g., nutrient availability, pH, temperature, oxygen tension). Moreover, it is also noteworthy that a virulence determinant may be under the influence of several regulatory systems that act synergistically to ensure the appropriate conditions for bacterial survival (Wang and Muir 2016).

In vitro studies have shown that regulation mechanisms can be classified into two major groups: (i) the two-component regulatory systems and (ii) the global transcriptional regulators (Cheung and Zhang 2002, Cheung et al. 2004). At nucleotide level, the two-component regulatory systems include the staphylococcal accessory element (*sae* locus) and the accessory gene regulator (*agr* locus). The *sae* locus codes the expression of several virulence determinants, mainly contributing to bacterial adhesion and host immune evasion. The *agr*-coded genes seem to promote the expression of bacterial exoproteins (e.g., TSST-1, enterotoxins, serine proteinase), simultaneously reducing the synthesis of cell-wall associated proteins (e.g. SpA, FnbpA, FnbB). This system was initially described in *S. aureus* isolates, in which four distinct allelic variants, *agrA*/*B*/*C*/*D*, have been sequenced. Subsequently, presence of homologous *agr*-like loci in other staphylococcal species have been detected (Dufour et al. 2002). Most *S. aureus* isolates have a third significant group of virulence genes regulators, usually referred as sigma factors (σ).

Mechanisms of resistance to antimicrobial agents

A major attribute of most staphylococcal species (including *S. aureus*) is their extended capacity to develop rapidly resistance to antimicrobial agents. Nowadays, multi-drug resistant staphylococcal isolates (e.g., *S. aureus*, *S. epidermidis*, *S. intermedius*) are commonly recovered from human or animal clinical samples (Mediavilla et al. 2012).

Antimicrobial tolerance or resistance is linked to the genetic background of each individual isolate and develops through mutations and rearrangements within the staphylococcal genome or by acquisition of resistance determinants. The wide spectrum of staphylococcal genetic variants and the increased pressure contribute significantly in antimicrobial-resistance formation. Most resistance determinants of staphylococcal genetic material are located in highly volatile areas (e.g., genomic islands, plasmids) promoting the occurrence of mutations leading to resistance (Foster 2017). Further, during co-infections, genetic transfer may also take place and essential resistant components may easily be transferred horizontally. Finally, genetic transfer of resistant genetic elements among bacteria belonging to different species has also been recorded (Haaber et al. 2017).

In general, mechanisms of resistance against antimicrobials developed by staphylococci are as follows: (i) relevant modulations of cell wall permeability, (ii) enzymatic inactivation of the antimicrobial agent, (iii) modification of the antimicrobial target and (iv) activation of relevant bacterial efflux pumps (McCallum et al. 2010). Depending on specific antimicrobial agents, one or more of these mechanisms may be involved (Pantosti et al. 2007).

β-lactams and glycopeptides are antimicrobial agents that inhibit the formation of staphylococcal cell wall. The penicillin-binding protein (PBP) 1 and PBP 2 are the target of *β*-lactams, which inhibit the function of PBPs and therefore the formation of an intact cell wall. To overcome the effects of *β*-lactams, methicillin-resistant *S. aureus* (MRSA) isolates produce a fifth additional PBP named PBP 2a, encoded by the *mecA* gene, which has reduced affinity with *β*-lactams and remains active under the presence of *β*-lactams (Hackbarth and Chambers 1993, Lovering et al. 2012). On the other hand, glycopeptides (e.g., vancomycin) attach to the dipeptide D-ala/D-Ala, and inhibit the function of PBPs. *S. aureus* isolates with intermediate susceptibility to vancomycin have a remarkably modified architecture on their cell wall, almost disappearing the crucial targets of glycopeptides (McGuinness et al. 2017). Regarding resistance to tetracyclines, the main mechanism is associated with the energy-dependent efflux of tetracycline encoded by the *tetA*(K) and *tetA*(L) genes (Khosravi et al. 2017). Finally, with regard to acquisition of resistance to aminoglycosides, resistant isolates are capable to release cytoplasmic aminoglycoside modifying enzymes, which

inhibit ribosome binding. Finally, resistance to macrolides and to lincosamides is mainly due to ribosomal modification encoded by the *erm* genes.

Implication of staphylococci in mastitis in ewes

Implication in clinical mastitis

The incidence risk of clinical mastitis in ewes is considered to be less than 7% throughout a lactation period (Bergonier and Berthelot 2003, Arsenault et al. 2008). *S. aureus* is considered as the primary causal agent of clinical mastitis. Field reports have indicated that staphylococci have been isolated from up to 70% of cases of clinical mastitis in investigations in dairy production flocks (Bergonier and Berthelot 2003, Mork et al. 2007). In meat production flocks, staphylococci have been reported to be a less frequent aetiological agent, but still responsible for up to 40% of cases of the disease (Arsenault et al. 2008). Other coagulase-positive staphylococcal species associated with clinical mastitis in ewes include the species *S. anaerobius*, *S. hyicus*, *S. intermedius* and *S. schleiferi* (Table I.iv).

Coagulase-negative staphylococcal have also been isolated from cases of clinical mastitis, although much less frequently than *S. aureus*. These include *S. epidermidis*, *S. saprophyticus*, *S. simulans*, *S. xylosus* and *S. warneri* (Table I.iv). Further, Fthenakis and Jones (1990c) have reported experimentally induced clinical mastitis caused by *S. chromogenes*.

Implication in subclinical mastitis

Frequency of subclinical mastitis is much higher than that of clinical disease. Criteria employed for definition of subclinical mastitis are important in determining its frequency, as different criteria would lead to significant differences in findings between studies. Here, it is noteworthy to cite a report of epidemic occurrence of subclinical mastitis in a flock, where prevalence of the disease has been reported to be 94%, with all cases caused by coagulase-negative staphylococci (Fthenakis et al. 2004).

Table I.iv. Selected references regarding implication of non-*S. aureus* staphylococcal isolates in mastitis or mammary carriage in ewes.

Staphylococcal species	References
S. anaerobius	de la Fuente et al. (1993)
S. auricularis	Fthenakis et al. (1994), Ariznabarreta et al. (2002), Kirecci et al. (2009)
S. capitis	Hariharan et al. (2004), Kirecci et al. (2009), Pilipcincova et al. (2010)
S. caprae	Kirecci et al. (2009), Onni et al. (2010), Pilipcincova et al. (2010)
S. chromogenes	Fthenakis and Jones (1990c), Fthenakis et al. (1994), Winter et al. (2002), Leitner, Chaffer et al. (2003), Mavrogianni et al. (2004), Cuccuru et al. (2011), Persson et al. (2017)
S. cohnii	Kirecci et al. (2009)
S. epidermidis	Fthenakis and Jones (1990c), Burriel (1997b), Saratsis et al. (1998), Winter and Colditz (2002), Winter et al. (2002), Leitner, Chaffer et al. (2003), Cuccuru et al. (2011)
S. equorum	Deinhofer and Pernthaner (1993), Hariharan et al. (2004), Persson et al. (2017)
S. haemolyticus	Deinhofer and Pernthaner (1993), Winter and Hofer (1996), Kirecci et al. (2009), Persson et al. (2017)
S. hominis	Ariznabarreta et al. (2002), Pilipcincova et al. (2010)
S. hyicus	Fthenakis et al. (1994), Winter et al. (1999), Ozenc et al. (2011)
S. intermedius	Ariznabarreta et al. (2002), Ergun et al. (2009), Kirecci et al. (2009)
S. lentus	Deinhofer and Pernthaner (1993), Winter and Hofer (1996), Ariznabarreta et al. (2002), Tejada et al. (2012)
S. lugdunensis	Deinhofer and Pernthaner (1993)
S. saprophyticus	Fthenakis et al. (1994), Ergun et al. (2009), Kirecci et al. (2009)
S. sciuri	Kirecci et al. (2009), Tejada et al. (2012)
S. simulans	Fthenakis and Jones (1990c), Fragkou, Mavrogianni et al. (2007), Onni et al. (2010), Cuccuru et al. (2011)
S. warneri	Fthenakis et al. (1994), Winter and Hofer (1996), Rupp et al. (2009)
S. xylosus	Fthenakis and Jones (1990c), Fthenakis et al. (1994), Deinhofer and Pernthaner (1993), Ozenc et al. (2011), Persson et al. (2017)

There is a clear consensus in the literature that coagulase-negative staphylococcal species are the primary aetiological agents of subclinical mastitis and can constitute up to 70% of bacterial isolates from cases of subclinical disease (Fthenakis 1994, Pengov 2001, Albenzio et al. 2002). The more frequent staphylococcal species recovered from cases of subclinical mastitis were *S. chromogenes*, *S. epidermidis*, *S. simulans* and *S. xylosus*. Other species recovered as aetiological agents of the disease less often include *S. auricularis*, *S. capitis*, *S. caprae*, *S. cohnii*, *S. equorum*, *S. haemolyticus*, *S. hominis*, *S. lentus*, *S. saprophyticus*, *S. sciuri*, *S. schleiferi*, *S. warneri* (Table I.iv).

S. aureus has also been recovered as aetiological agent of subclinical mastitis, but much less frequently than from cases of clinical disease. Only sporadically, *S. aureus* may account for as many as 40% of isolates from cases of subclinical mastitis (Al-Majali and Jawabreh 2003).

Mammary carriage

The term mammary 'carriage' (or 'carrier state') (Verhoeven et al. 2014) is used to describe presence of bacteria in the udder with no increased somatic cell numbers (i.e., in the absence of inflammation). The term refers to bacterial flora present in the teat duct or teat cistern (Fragkou, Mavrogianni et al. 2007, Mavrogianni et al. 2007).

The significance of mammary carriage is that the microorganisms might become pathogenic under the effect of various factors, which decrease defensive efficacy of hosts or promote pathogenicity of bacteria. In a previous experimental study, Fragkou, Mavrogianni et al. (2007) have shown that, as the consequence of factors reducing efficacy of teat defences, staphylococcal carriage could multiply, ascend to the mammary parenchyma and, ultimately, cause clinical mastitis.

In people, nasal carriage of *S. aureus* has been associated with subsequent disease, especially by antimicrobial-resistant isolates (Seybold et al. 2011, Datta et al. 2014, McLaws 2015); carriage can lead in disease by facilitating new invading bacteria to fully expressing their pathogenicity or even by participating in the infectious process themselves in order to establish the pathological findings. Indeed, it is possible that pathogenicity of carriage staphylococci would increase, resulting in disease. In contrast, in sheep, Vautor et al. (2009) have found that most staphylococcal nasal carriage isolates were of different type than isolates recovered from udder samples. Potentially, this may indicate a small possibility for nasal carriage isolates to cause mastitis.

Cases of false positive mammary carriage refer to isolation of bacteria at the very early stage of infection before an inflammatory reaction would be elicited (Fthenakis and Jones 1990c), as well as in cases of contamination of milk samples during collection. Nevertheless, with experienced staff and strict maintenance of aseptic sampling conditions, contamination would be minimal; for example, Rovai et al. (2014), in a field investigation, have found that only 2% of milk samples from ewes were contaminated.

Characteristics of isolates with regard to virulence factors

Research work in typing *S. aureus* isolates from cases of clinical mastitis has reported varying results regarding presence of *agr* locus, but with no particular consistence in the findings. Vautor et al. (2007) have indicated that 70% of isolates belonged to agr group III and 13% to agr group I. In contrast, de Almeida et al. (2013) have indicated that isolates from clinical mastitis belonged mostly to agr group I (38%) and less often to agr group II (19%), whilst isolates from subclinical mastitis

were equally distributed to these two groups (38% in each). Finally, Bar-Gal et al. (2015) have reported that all *S. aureus* isolates from mastitis, clinical or subclinical, belonged to agr group I.

Further, there is conflict in the international literature regarding significance of biofilm-forming staphylococcal isolates from cases of mastitis in ewes. This has been reported to vary greatly, from 0% (Azara et al. 2017) to 26% (Vautor et al. 2007) to 40 - 43% (Ergun et al. 2012) to 91 - 98% (Tel et al. 2012).

Examination of staphylococcal isolates by Multi Locus Sequence Typing also revealed varying and inconsistent findings. Porrero et al. (2012) have examined *S. aureus* isolates from clinical mastitis and have revealed that they belonged to types ST9, ST133, ST1739 and ST2011. de Almeida et al. (2013) have reported that *S. aureus* isolates from clinical mastitis belonged to types ST750, ST1728, ST1729 and ST1730, whilst those from subclinical mastitis to types ST750, ST1728 and ST1729. Finally, Bar-Gal et al. (2015) have reported that *S. aureus* isolates from clinical or subclinical mastitis belonged to types ST133 and ST522. In general, *S. aureus* ST133 is most often isolated from samples of sheep origin (McMillan et al. 2016).

In studies, which had investigated production of virulence factors by staphylococcal isolates (*S. aureus* or coagulase-negative isolates) from cases of mastitis, various factors or genes encoding thereof have been detected: tissue shock syndrome toxin-1 (Orden, Goyache et al. 1992, Scherrer et al. 2004), leucocidin (Burriel and Dagnall 1997), Panton-Valentine toxin (Unal and Cinar 2012), exfoliative toxins (Mariutti et al. 2015), haemolysins (Azara et al. 2017) and autolysin (Azara et al. 2017). Presence of above factors was, in general, more prevalent in *S. aureus* than in coagulase-negative isolates, which can explain the increased pathogenicity of the former bacteria.

Resistance to antimicrobial agents of isolates from cases of mastitis in ewes

There is one rule for the effective treatment of mastitis: the combination of speed and efficacy. As soon as first signs of mastitis are diagnosed, treatment should be initiated (Mavrogianni et al. 2011). Effective antimicrobial agents should be administered. Ideally and to preserve susceptibility of pathogens to available drugs, treatment should be performed by means of a narrow spectrum agent, specifically effective against the causal agents. Drug administration follows identification of causal agent and establishment of its patterns of antimicrobial susceptibility by means of the relevant technique (Mavrogianni et al. 2011).

Evidence from around Europe does not indicate significant problems of reduced susceptibility to antimicrobial agents of staphylococcal isolates from cases of mastitis in sheep. Vautor et al. (2007)

have reported only sporadic resistance in *S. aureus* isolates recovered in France. Onni et al. (2011), in Italy, have also found limited resistance in *S. epidermidis* isolates, bar to penicillin, for which a resistance rate of 38% was recorded. Similar results have been observed in Turkey, where in coagulase-negative isolates from subclinical mastitis only resistance to *β*-lactams was noteworthy (43%), whilst there was much smaller frequency of resistance to tetracycline (11%) and even less to other agents (Ergun et al. 2012). Further work in Turkey corroborated above findings: rate of resistance to penicillin was 27% and to tetracycline 8% (Unal et al. 2012). Finally, Martins et al. (2017) have published similar to previous ones results regarding susceptibility patterns of coagulase-negative isolates from cases of subclinical mastitis: 17% of isolates were resistant to penicillin and 11% to tetracycline. A different result has been reported by Azara et al. (2017), who found increased resistance to tetracycline only (50%) of *S. aureus* isolates from clinical mastitis. In contrast to above results, in Brasil, Franca et al. (2012) have reported increased frequency of resistance to amoxicillin, erythromycin, lincomycin, streptomycin and tetracycline (>35% of staphylococcal isolates tested).

Whilst above results are indicative of the possibility for effective treatment in cases of mastitis, *in vitro* results of antimicrobial susceptibility do not always correspond with results of *in vivo* administration of an antimicrobial agent. Various reasons may account for this discrepancy, e.g., pharmacokinetic limitations during clinical application, inappropriate treatment regime or iatrogenic infection occurring during treatment (Mavrogianni et al. 2011).

Staphylococcal mastitis in ewes

Sources of infection

Staphylococci usually disseminate into the mammary gland from the hands of milkers (Marco Melero 1994). Use of bare hands during milking contributes to transfer of bacteria from hands of people to the teat, which at that time is open, whence they can enter into the teat duct; if local defences would be compromised they may invade the mammary gland.

Other potential sources of bacteria include the nasopharynx of sucking lambs (Gougoulis et al. 2008b), staphylococcal flora present in the teat duct (Fragkou, Mavrogianni et al. 2007) and isolates present on the udder skin (Mavrogianni et al. 2007). In fact, Albenzio et al. (2003) have considered that lambs' mouths and milkers' hands were the major sources of ewe udder and milk contamination, whilst the least numbers of staphylococci and streptococci were isolated from the teat cups. During suckling, the teat orifice comes into contact with the wall of the buccal cavity of

lambs. As Laukova and Marounek (1992) have isolated staphylococci form the upper alimentary tract of lambs, it becomes evident that the bacteria can transmit to the teat during sucking. As the lower part of the teat comes into contact with the pharynx of the lamb (Titchen 1977), the bacteria are attached thereon, subsequently entering into the duct; perhaps the tongue of the lamb may 'push' the bacteria upwards into the duct. Isolation of the microorganism after only a short (1 min.) sucking activity indicates the speed by which the whole process can take place (Gougoulis et al. 2008b). Staphylococci have been found as resident flora of the teat duct of healthy ewes (Fragkou, Mavrogianni et al. 2007, Mavrogianni et al. 2007). When the microbial equilibrium is disrupted for any reason, it is possible that pathogenicity of the flora organisms would increase, leading to invasion of the mammary parenchyma and disease. Further, with relation to staphylococci present on the udder skin, possible physicochemical changes occurring in the skin (e.g., as the result of bad weather conditions or teat disinfectants) chapped udder skin, may contribute to increasing susceptibility of the mammary gland. In the epidermis, the process of drying decreases lipid content, which contains antibacterial fatty acids, bacteriostatic salts and proteins, as well as immunoglobulins (Noble and Summerville 1974). Additionally, the reduced hydration of chapped skin, alters skin microflora, consequently decreasing resistance to bacterial colonisation (Fox et al. 1991). Also, chapping removes the acid mantle and increases teat surface area, due to excoriations and fissuring, thus providing additional surface for bacterial attachment. All these increase bacterial populations on the udder skin, thus increasing risk of infection of the mammary parenchyma (Mavrogianni et al. 2007).

Course of the disease

The general principles of mammary defences apply for staphylococcal infections. After successful ascent of staphylococci into the teat cistern and the mammary parenchyma, leucocytes constitute the main line of defence against staphylococci, in sequence macrophages, neutrophils and lymphocytes; these are developed immediately upon bacterial entry into the mammary gland, 2 - 4 hours after that and 3 - 4 days later, respectively (Fthenakis and Jones 1990c, Persson-Waller et al. 1997, Fragkou et al. 2010). Increased permeability of the blood-milk barrier by various mechanisms (e.g., by modulating claudins at the tight mammary junctions) allows blood constituents and molecules to enter into the infected mammary gland. The diametre of mammary vessels and the blood volume therein increase soon after infection, leading to the transportation of increased amount of blood constituents into the mammary gland (Barbagianni 2016). That way, the inflammatory response is developed and sustained. Neutrophils phagocytose bacteria and proceed

to perform intracellular killing by rapid release of reactive oxygen species: superoxide radicals and hydrogen peroxide ('respiratory burst') (Van Oostveld et al. 2002). Neutrophils also release various proteins with clear antibacterial activity, e.g., cathelicidins, which become available at the mammary parenchyma (Brown and Holden 2002, Katsafadou 2017).

Interleucins play an important role in the host response, as they regulate the influx of leucocytes in milk after bacterial invasion. Albenzio et al. (2012) have reported the relationships between bacteria, leucocytes and interleucins produced after bacterial invasion (TNF-α, IL-8, IL-1β, IL-10, IL-12). Concentrations of TNF-α and IL-12 were higher after invasion of pathogenic bacteria (including staphylococci); IL-8 was associated with somatic cell counts and pathogenic bacteria. Regarding IL-1β, Albenzio et al. (2012) have not observed any differences after intramammary staphylococcal challenge, whilst other authors had (Winter and Colditz 2002, Winter et al. 2003).

Production of virulence factors by the invading staphylococci leads, principally, in destruction of mammary epithelial cells and leucocytes, as well as of blood vessels within the mammary parenchyma and consequentially in intramammary haemorrhages (Fthenakis and Jones 1990c). Further, biofilm formation allows rapid expansion of the bacteria, which thus become difficult to counteract. Occasionally, pathogens that had not been eliminated by the mammary defences, may pass into the blood circulation. Further, staphylococci can survive into leucocytes, especially if these were not fully functioning, e.g., due to lack of energy in the immediately post-partum period (Fthenakis et al. 2015). This could lead in development of bacterial accumulations and abscessation within the mammary parenchyma, whence a recrudescence of the infection can ensue (Fthenakis and Jones 1990c).

The various virulence factors in mastitis-associated staphylococci have not been detected consistently in all isolates evaluated; there were significant differences even among the various species of the microorganism. This underlines the importance of the host defences in the development and outcome of an intramammary infection. Potential development of clinical mastitis or limitation of the infection to subclinical mastitis depends upon the combination of virulence factors of the invading bacteria and the efficacy of the defence mechanisms of the host. There is now evidence confirming that cellular defences are the determinant host factor that may limit a virulent isolate to only cause subclinical mastitis (Barbagianni et al. 2015, Mavrogianni et al. 2017). Cell defences are non-specific, acting against all invading bacteria, including staphylococci. A factor that can affect efficacy of cell defences is sheep breed (Portolano et al. 2005, Riggio and Portolano 2015). Potentially, subclinical mastitis can revert to clinical, if defences of the host would be impeded. Complete recovery requires a fully functioning defence system of the animals, coupled with an effective treatment course. Other possible outcomes include the development of a long-standing

('chronic') infection with periodic flare-ups of clinical disease, the necrosis of mammary parenchyma, leading in partial or extensive tissue slough-off, the formation of intramammary abscesses with presence of staphylococci therein and the recrudescence of disease.

Public health considerations

Staphylococcal enterotoxins are in a family of over 20 exotoxins, which are related and have a sequence homology (Pinchuk et al. 2010). The enterotoxins can cause significant diseases in people, among these food poisoning and toxic shock syndrome. Enterotoxins are mainly produced by *S. aureus*, although other staphylococcal species may also show enterotoxigenic properties. Staphylococcal food poisoning is an intoxication occurring consequently to consumption of foods containing sufficient amounts of one (or more) pre-formed enterotoxin(s) (Argudin et al. 2010). Hence, it becomes evident that if milk collected from sheep would be subjected to appropriate thermal processing leading in staphylococcal killing, there would be no real danger of enterotoxin poisoning from dairy products from that milk. In contrast, the danger would increase sharply in case of consumption of unprocessed milk, given the frequency of staphylococcal mammary infections, especially as production of enterotoxin has been detected in *S. aureus* isolates from intramammary infections (Orden, Cid et al. 1992) or farm milk tank (Scherrer et al. 2004) of sheep.

For the same reasons, dairy products were not considered to be implicated in the dissemination of antimicrobial resistance to consumers of milk. Nevertheless, recent studies have documented that cell-free genetic material of staphylococci resistant to antimicrobial agents, which was not destroyed during thermal processing of milk and could be transferred to people (Wang et al. 2012, Schwarz et al. 2017). Given that resistance genes could be incorporated in other bacterial species (e.g., *Streptococcus* spp., *Acinetobacter* spp.), which are part of the normal bacterial flora of people, that way dissemination of resistance genes can occur, potentially making staphylococcal isolates as 'stores' of resistant genes and dairy products a means for their transfer.

B. PREDISPOSING FACTORS FOR MASTITIS IN EWES

Introduction

Various factors predispose animals to mastitis and increase risk of its incidence. Research in predisposing factors of ovine mastitis can contribute in elucidation of mechanisms of the disease and thus in development and implementation of control measures, which take into account conditions occurring in sheep farms. A basic classification scheme of predisposing factors includes animal- and non-animal-related predisposing factors (Table I.v).

Table I.v. Classification of factors that may predispose ewes to mastitis.

Non animal-related factors	Animal-related factors
Environmental and climatological factors	Anatomic factors
Housing	Genetic background
Nutrition	Litter size – Ethological factors
Milking practices	Number and stage pf lactation period
	Health problems

Non animal-related factors

Environmental and climatological factors

Various insects, for example the fly *Hydrotoea irritans*, may transfer bacteria from teats of ewes with mastitis (i.e., with increased bacterial numbers) to teats of healthy ewes (Jensen and Swift 1982, Jones 1990). Dirt and faeces on teat skin can increase risk of mastitis by environmental pathogens (Jones 1990).

The potential effects of climatological factors in mastitis development were first indicated by Clark (1972), who mentioned that mastitis was occurring more frequently after gusts of cold winds or grazing in wet and muddy pastures, in rainy weather. A possible association between adverse weather conditions and mastitis in sheep has also been reported in the 1980s (Anon. 1988).

Sevi et al. (2001) have reported that provision of shade helped minimising the impact of thermal stress in the immune function and udder health of ewes. High environmental temperatures can lead in reduced leucocyte counts in sheep (El-Tarabany et al. 2017). Also, in such cases,

leucocytes have impaired function (e.g., Lacetera et al. 2005; Lecchi et al. 2016). As leucocytes play a significant role in protecting ewes against mastitis, their reduced number and inefficiency would account for the high incidence in farms with extreme temperature measurements. Moreover, an indirect effect of other factors associated with increased environmental temperatures, e.g., reduced feed intake limiting energy availability to defence systems (Barbagianni et al. 2015), should not be ruled out.

Decreased temperatures can also be implicated in favouring development of mastitis. Fox and Norell (1994) and Zucali et al. (2011) have reported that, in cattle, exposure to low temperatures increased staphylococcal colonisation on teat skin. This may be the result of teat chapping occurring in such conditions. An increased incidence of chapped teats in cold weather has been associated with intramammary infections (Fox and Hancock 1989). Burriel (1997a) has reported that colder and wetter weather prolonged survival of *M. haemolytica* in the environment of sheep, which in turn could increase cumulative bacterial counts, contributing to easier transmission from animal to animal. Physicochemical changes in chapped teat skin contribute to the increased susceptibility (Mavrogianni et al. 2006). In the epidermis, the process of drying results in a decrease in lipids (including antibacterial fatty acids), bacteriostatic salts and proteins (e.g., teat duct keratin), as well as immunoglobulins (Noble and Somerville 1974). Additionally, the reduced hydration of chapped skin leads to changes in skin microflora and consequent decreased resistance to bacterial colonisation (Fox and Cumming 1995). Finally, chapping removes the acid mantle and, as the result of excoriation and fissuring, increases the teat surface area, thereby providing additional space for bacterial attachment (Sieber and Farnsworth 1984). In cold weather, when lambs suck with increased frequency (Slee and Springbett 1986) and when teats may become chapped (Fox and Hancock 1989), it would seem likely that lambs contribute to the transfer of bacteria to teat skin, documented (Gougoulis et al. 2008b, Fragkou et al. 2011) as the means of bacterial transfer leading to colonisation, entry into the teat duct and mastitis.

With regard to precipitation, Zafalon et al. (2016) have reported higher frequency of subclinical mastitis in ewes during rainiest periods. This possibly occurred because of difficulties in maintaining hygiene in the environment of animals.

Despite environmental factors being outside of the control of farm managers, these findings can be valuable in establishing principles of udder health management in sheep flocks. During periods when environmental conditions are similar to those considered of high risk for mastitis development, preventive measures should be applied meticulously to minimise risk of mammary infections.

Housing

Poor housing practices (e.g., overstocking, limited ventilation, irregular manure removal) can contribute to increased bacterial populations within animal barns, thus increasing risk of mastitis development; for example, Caroprese (2008) has reported that when < 7 m^3 per sheep were available within animal house, increased risk of mastitis was found. Indrebo (1991) has studied specific aspects of sheep housing in relation to mastitis and reported that mastitis was prevalent in sheep houses with slatted floors. In contrast however, Cooper et al. (2016) have reported that concrete and soil were associated with higher incidence rate of clinical mastitis.

Further, frequent manure removal from farm buildings has been associated with reduced mastitis incidence. Possibly, this was the consequence of improved air quality within the barns, e.g., reduced ammonia concentration (Sevi et al. 2003).

Nutrition

Balanced nutrition of lactating ewes supports mammary health through the immunological competence of the animals (Caroprese et al. 2015). Nutritional errors may, through various pathways, affect the immune abilities of the ewes and predispose the animals to mastitis (Waage and Vatn 2008).

With regard to energy, it has been reported that pregnancy toxaemia has a marked effect on the immune functions of affected ewes and can predispose ewes to mastitis in the immediately post-partum period (Barbagianni et al. 2015). Increased ketone body blood concentrations have been found to lead to reduced blastogenesis and proliferation of T lymphocytes (Franklin et al. 1991), whilst lymphocytes of ketomaemic animals had reduced mitogenic responses than lymphocytes of healthy cows (Kandefer-Szerszen et al. 1992). Sartorelli et al. (1999) have shown that increased concentrations of *β*-hydroxybutyrate could adversely affect particle uptake by neutrophils; Hoeben et al. (1997) reported that increased concentrations of *β*-hydroxybutyrate led to an inhibitory effect on the respiratory burst activity of neutrophils, by inhibiting the generation of superoxide anions; Grinberg et al. (2008) have shown that increased concentrations of *β*-hydroxybutyrate suppress formation of extracellular traps by neutrophils, which leads to impaired pathogen killing. All above pathways contribute in impairing significant mammary defence mechanisms.

Vitamin A deficiency has been found to lead to increased incidence risk of mastitis and increased milk somatic cell counts (Koutsoumpas et al. 2013). This may be due to an impairment of

mammary epithelia resulting from the deficiency, which facilitates mammary invasion and multiplication by pathogens.

Giadinis et al. (2011) have also indicated a possible predisposing role of selenium deficiency in mastitis. Reduced selenium concentration may lead to impeded cellular defense, which in turn can result in a higher incidence of clinical mastitis. Selenium-containing proteins, mainly GSH-P_x and thioredoxin reductase, are involved in the metabolism of cellular peroxides; therefore, selenium deficiency impairs the ability of immune cells to produce the respiratory burst (Morgante et al. 1999, Rooke et al. 2004).

Zinc deficiency can adversely affect keratin formation in the teat; this would lead in limited protection of ewes from entrance of bacterial pathogens and increased potential for mastitis development. Saianda et al. (2007) have indicated that administration of a zinc proteinate feed supplement to ewes resulted in reduced bacterial adhesion on mammary epithelial cells, possibly due to maintaining epithelial integrity in treated ewes.

Fthenakis et al. (2004) have presented a case of > 90% prevalence of staphylococcal mastitis in ewes provided with a feed with excessive (20%) gossypol content. Various pathways leading in immunocompromise of affected ewes have been postulated in that case (Fthenakis et al. 2004).

It is generally believed that presence of aflatoxins in feed might lead in higher incidence of mastitis. However, Kourousekos (2011), who reviewed the relevant literature, discussed that a likelihood of potential aflatoxin effects on udder health could not be supported by the available evidence.

Milking practices

Various practices during the milking routine, for example, wrong milking order of ewes incorrect teat preparation (e.g., incomplete dirt removal) or stressing animals (impairing oxytocin release and complete emptying of mammary glands), can predispose to mastitis. Also, the practice of providing feed during milking may contribute; after milking and feeding, ewes would lay on the ground, thus facilitating bacterial entrance through the open teat orifice and leading in mammary infections.

In machine milked-ewes, various inappropriate practices can increase the risk for mammary infection, due to bacterial entrance into the mammary gland and/or compromise of teat defence mechanisms. Bacterial entrance is associated with factors like reverse milk flow, impacts or reverse pressure gradient (Romero et al. 2019); this can be further increased in cases of milking machine malfunction, which may affect teat status, causing lesions predisposing to mammary infection

(Fragkou, Papaioannou et al. 2007). Moreover, various errors in milking system management, which include application of clusters on wet and slippery teats, use of damaged clusters, incorrect pressure or pulsation of the system, errors in post-milking cleaning of the system (e.g., suboptimal temperature of cleaning water, omission of detergents) or leaks in air or milk pipes, would significantly increase risk for mastitis development in ewes (Romero et al. 2019). Application of automated cluster removal has been found to limit vacuum drops in short milk tubes and oedema at teat end after milking (Bueso-Rodenas et al. 2015) and may thus contribute in maintaining mammary health.

In hand-milked ewes, staphylococci from hands of milkers can be transmitted to teats of ewes (Marco Melero 1994). Likely as a consequence of that, hand-milking has been found to result in increased bacterial colonisation within the teat duct (Mavrogianni et al. 2007).

The potential effects of post-milking teat dipping in ewes have not been studied as extensively as in cows (Bergonier et al. 2003). Incorrect application of teat dipping can also predispose to mastitis: use of dense solutions may cause teat chapping, which increases risk of mammary infection (Mavrogianni et al. 2006, Fragkou, Papaioannou et al. 2007), whilst contaminated products may result in mastitis by infrequent pathogens (e.g., *Serratia marcescens*) (Tzora and Fthenakis 1998).

Finally, in contrast to traditional views, there is now evidence that the drying-off process (i.e., abrupt or progressive) does not influence incidence risk of mastitis. Hence, it should not be regarded as a risk factor for mastitis development (Petridis et al. 2012, 2013).

Animal-related factors

Anatomic factors

Various factors, which modify the normal morphology and standard conformation of the udder can predispose ewes to mastitis. For example, udders with irregular conformation and large pendulous udders (‘baggy udders’) would predispose to mastitis, possibly because the tips of the teats may touch the soil, that way bacteria entering more easily into the teat duct (Fthenakis 1992b, Larsgard and Vaabenoe 1993), whilst ewes with firm udders have been found to produced milk with lower somatic cell counts (Huntley et al. 2012). The teat length and diametre can also play a role in the infection of mammary glands; long teats are considered to contribute in minimising mammary infections, as in these teats bacteria need more time to ascend and, therefore, the defences of the

teat (Mavrogianni et al. 2005) can counteract effectively bacterial infections. In wider teats, infection can take place more easily and are considered to contribute to increased risk of mastitis. Bad conformation of teats (e.g. misplaced teats, small-sized teats), can predispose ewes to mastitis, particularly in flocks in which mechanical milking is applied. Such teats often lead to poor milkability of ewes, due to difficulties in application of milking machine clusters, because they cannot fit properly therein and thus are subjected to inappropriate pressure and manipulation during milking, which increase risk of bacterial infection. Finally, supernumerary teats predispose to mastitis, as they provide more portals for bacterial invasions of the mammary glands.

Genetic background

Differences in breed susceptibility to mastitis have been identified. This has been documented in ewes of high milk production. For example, the Frisarta breed, a high milk yield breed, has been found to be susceptible to clinical mastitis after bacterial deposition into the teat, whilst Karagouniko breed ewes have been found to be resilient to inoculation (Fragkou, Skoufos et al. 2007). Similar results have been suggested for meat-producing flocks by Ridler (2008) in New Zealand.

The various anatomical characteristics that may contribute to increased or decreased incidence of mastitis often have a genetic background, hence, through their heritability genetic factors influence mastitis development (Legarra and Ugarte 2005). Further, Barillet et al. (2001), McLaren et al. (2018) and Oget et al. (2019) have mentioned that cell content in milk can be employed for selection of animals potentially resistant to mastitis, whilst Riggio et al. (2010) have proposed the use of infection status (defined as presence of at least 5 colony-forming-units per 10 μL of milk) for this purpose. By using these criteria, differences in outcomes have been found in lines of sheep considered to be resistant or susceptible to mastitis. Barillet et al. (2001) have concluded that selection for mastitis resistance based on somatic cell counts was feasible, although results obtained during the first lactation period would rather not be included in relevant analyses.

Also, transcriptome differences between resistant and susceptible lines were related to transcriptional activity within milk leucocytes; RARα, TP53, AHR were considered as transcriptional factors of interest, both because of their differential expression and their interaction with a larger number of regulated genes between lines (Oget et al. 2019).

Candidate genes implicated in innate immunity and thus resistance to mastitis included *SOCS2*, *CTLA4*, *C6*, *C7*, *C9*, *PTGER4*, *DAB2*, *CARD6*, *OSMR*, *PLXNC1*, *IDH1*, *ICOS*, *FYB* and *LYFR* in Chios breed (Banos et al. 2017). Fragkou et al. (2010) have attributed the breed differences

in susceptibility of mastitis to differences in lymphocyte activity and proposed a potential genetic background to the infection, given that in cattle the genetics of bovine leucocyte antigen (*BoLA*) has been linked to resistance to mastitis (Takeshima and Aida 2006).

It is interesting that in the past Traoré et al. (2008) have hinted at a possible link between resistance to mastitis and gastrointestinal nematodes. More recently, experimental and field studies have provided evidence of an association between gastrointestinal parasitic infections and mastitis in ewes (Mavrogianni et al. 2017, Kordalis et al. 2019).

Litter size – Ethological factors

Increased litter size has been positively associated with development of mastitis (Waage and Vatn 2008, Prpic et al. 2016). As there is no genetic association between number of ovulations and mastitis susceptibility (Oget et al. 2019), a reason for this should be considered the higher chance for bacterial transfer from lambs mouths to the teat duct (Gougoulis et al. 2008b, Fragkou et al. 2011). Moreover, ewes bearing multiple foetuses have increased energy needs during the last stage of gestation (Russell 1984), which can predispose to pregnancy toxaemia and mastitis immediately post-partum (Barbagianni et al. 2015).

In a detailed study, using molecular techniques, it has been found that identical *M. haemolytica* were recovered from the tonsils of lambs and the teat duct of their dams, results that proved the transmission of bacteria during sucking. Further, it has been found that during mastitis sucking patterns of offspring of affected ewes were modified and became irregular, whilst cross-sucking with other ewes was also recorded (Gougoulis et al. 2008a). This indicates that lambs may also transmit bacteria to ewes other than their dams.

Number and stage of lactation period

There is evidence that increased age and number of lactation period have been associated with higher incidence of mastitis (Watkins et al. 1991, Ahmad et al. 1992, Fthenakis 1994, Sevi et al. 2000, Leitner et al. 2001). More specifically, the probability of the mammary gland remaining uninfected is reduced as the lactation period advances (Mavrogianni et al. 2007). Fragkou et al. (2010) have postulated that this might be the effect of lymphocytic mammary defences becoming defective with age (Kraft et al. 1987).

In ewes, frequency of clinical mastitis immediately post-partum is not as high as in cows (Bergonier and Berthelot 2003). At that stage of the lactation period, *Escherichia coli* predominates

as a causal agent (Jones and Lanyon 1987). Clinical mastitis can occur more often when lambs are taken away from their dams and milking starts (Fthenakis and Jones 1990a, Bergonier and Berthelot 2003). Thereafter, staphylococci are the primary causal agents of the infection.

Health factors

Parasitic (nematodes, trematodes) infections may predispose to increased incidence risk of mastitis. Proposed pathogenetic mechanisms include the energy depletion by helminthes affecting leucocytic function in affected animals, thus impeding efficient mammary defences (Mavrogianni et al. 2017, Kordalis et al. 2019). Moreover, specifically in trematode infections, increased *β*-hydroxybuturate blood concentrations, consequently to the liver damage caused by these parasites, can have a direct effect in function of leucocytes (Mavrogianni et al. 2014).

Contagious echtyma (orf) and *Papilloma Virus* infections in teats have a significant predisposing role in mastitis. These epitheliotropic viruses hamper relevant local defences (specifically, the lymphoid nodules at the border between teat duct-teat cistern) (Mavrogianni et al. 2006) and thus facilitate bacterial multiplication at the teat and invasion into the mammary parenchyma. Other teat disorders (e.g., chapped teats) (Fragkou, Papaioannou et al. 2007) or staphylococcal dermatitis of udder skin (impetigo) may also predispose to mastitis; in these cases, increased risk for infection is related to high bacterial accumulation in udder and teat skin.

Pregnancy toxaemia can also predispose to mastitis in the immediately post-partum period of the subsequent lactation period. Dystocia has also been associated with mastitis in the subsequent lactation period (Waage and Vatn 2008); in this case, the association may be based on the same background as pregnancy toxaemia, given that the disorder often results in dystocia (Barbagianni et al. 2015).

CHAPTER II

A STUDY OF SUBCLINICAL MASTITIS IN EWES IN GREECE

A. INTRODUCTION

In a recent literature review (Gelasakis et al. 2015), it has been reported that field investigations on mastitis in ewes were limited in terms of number of animals sampled and of number and geographical extent of farms. In the 15 papers reviewed therein, which described investigations in 10 countries, median per study number of animals sampled was 380, number of farms was 11, and number of milk samples examined was 703 (Gelasakis et al. 2015).

This chapter presents results of an extensive and detailed study of subclinical mastitis in ewes across Greece. The investigation included 111 farms located in all 13 administrative regions of Greece; total ewe population in these flocks was approximately 35,000 animals. In Greece, sheep production is the predominant form of agriculture, with over 95% of ewes farmed for dairy production. Objectives of the work presented in this chapter were (i) to investigate prevalence of subclinical mastitis, (ii) to identify aetiological agents involved, (iii) to evaluate in depth staphylococcal isolates isolated and to assess the immunological response in staphylococcal mammary infections, (iv) to study factors potentially predisposing ewes to subclinical mastitis, (v) to investigate prevalence of subclinical mastitis caused by biofilm-forming staphylococci, (vi) to identify potential risk factors for this condition, (vii) to associate the prevalence of subclinical mastitis with environmental (climatic and topographic) factors and (viii) to identify regions potentially of high risk for increased frequency of subclinical mastitis.

B. MATERIALS AND METHODS

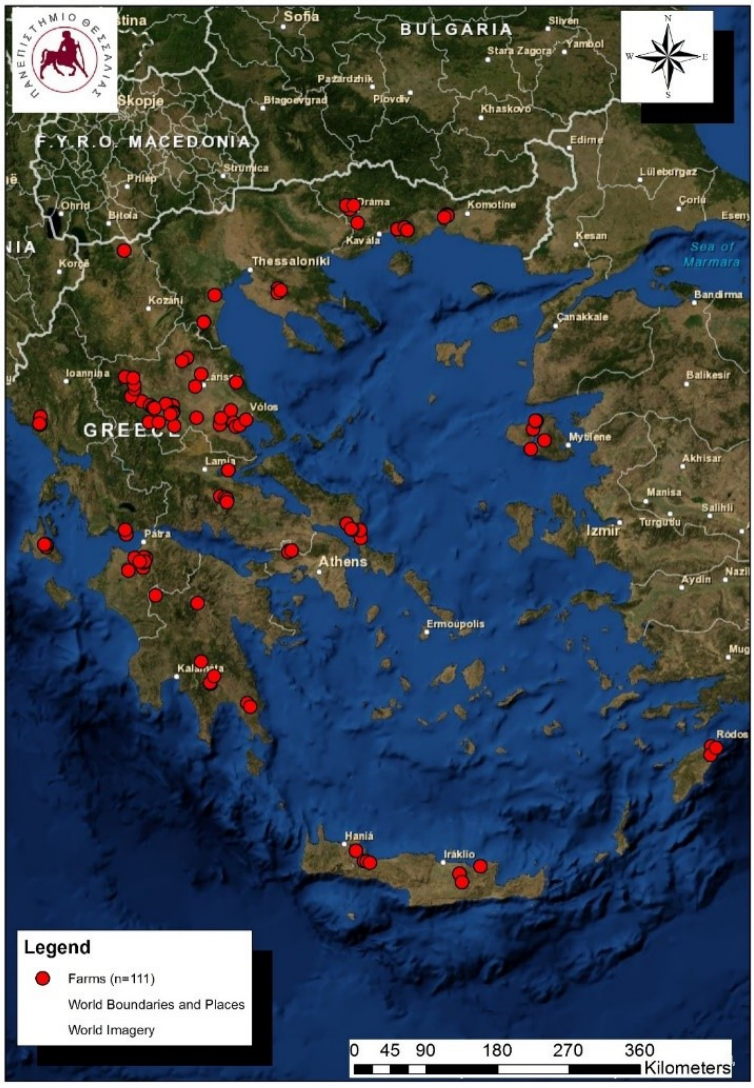
Sheep farms

In total, 111 sheep farms in the 13 administrative regions of Greece were included into the s*tudy and visited for collection of samples and information. Veterinarians active in small ruminant health management around Greece, were contacted by telephone and asked if they wished to collaborate in the investigation. In total, 25 veterinarians were contacted; of these, 23 (0.92) had agreed to collaborate. Farms were selected by the collaborating veterinarians on convenience basis (willingness of farmers to accept a visit by University personnel for sample collection). All farms in the study were visited for sample collection. Location of farms around the country is shown in Figure II.1.

At start of each visit, breed of animals in the farm was recorded and an interview was carried out with the farmer to obtain various information regarding health management in the flock. Further, the veterinarian was asked whether ancillary tests for diagnosis of mastitis were performed in samples from animals in the farm (possible answers: [*Yes* / *No*]).

Data on farm location were collected in the field using hand-held Global Positioning System Garmin units. The geo-references were resolved to specific farm level. ArcGIS V.10.1 GIS software (ESRI; Redlands, Ca, USA) was employed for description and analysis of spatial information.

Figure II.1. Location of the 111 sheep farms around Greece, which were included in the investigation.



*

Animal sampling

In each farm, 20 clinically healthy ewes (*secundiparae* or older) were selected for sampling. For selection of animals, farmers had been asked to remove from the main flock, *primiparae* ewes and ewes with known udder abnormalities. The remaining animals were walked to the milking area and 20 ewes were selected by using an electronic random number generator

(www.randomresult.com) among the first 50 (farms with >100 ewes, n=99) or 30 (farms with ≤100 ewes, n=12) animals that walked therein.

A standardised clinical examination of the udder (observation, palpation, comparison between glands) was performed (Fthenakis 1994, Mavrogianni et al. 2005). The first two squirts of secretion were drawn on the gloved hand of an assisting investigator and assessed. Disposable, non-sterile latex gloves (Alfa Gloves; Karabinis Medical SA, Peania, Greece) were worn during sampling, which were changed after procedures in each animal were completed and before moving to the next one. If udder abnormalities (e.g., abnormal secretion, mammary nodules [i.e., firm space-occupying structures], papilloma-type lesions) were present, the ewe was excluded from sampling. Animals found with abnormalities and excluded, were not replaced.

The orifice, edge and lower half of the body of the teat were disinfected by single-use sterile gauzes, onto which povidone iodine 7.5% (Betadine surgical scrub®; Mundipharma Medical Company, Basel, Switzerland) had been poured, followed by wiping off by means of a new sterile gauze; different gauzes were used for each teat. 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 directly onto a paddle for performing the California Mastitis Test (CMT).

Finally, in each flock, from 5 ewes, selected at random among the 20 ewes from which milk samples were collected, blood samples were also collected from the jugular vein. Samples were collected into plain tubes. They were centrifuged for serum collection within 2 h after collection.

All samples were stored into portable refrigerators with ice packs and transported by car. For samples collected in islands, airplane (farms in Crete, Lesvos or Rhodes) or boat (farms in Cephalonia) transportation, as accompanying luggage (always ice-packed), was also involved.

Standard microbiological examinations

Primary isolation and initial identification

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 48 h. If nothing had grown, media were re-incubated for another 24 h. Bacterial identifications were performed by using standards methods (Barrow and Feltham 1993, Euzeby 1997).

Identification of coagulase-negative staphylococci to species level

In total, 115 coagulase-negative staphylococcal isolates (91 from cases of subclinical mastitis [0.200 of such isolates] and 24 from cases of mammary carriage [0.145 of such isolates]) recovered in pure culture during the study, were selected at random and identified to species level by using the Vitek® 2 automated system (BioMerieux, Marcy-l'-Étoile, France). For selection of isolates for speciation among all those recovered, an electronic random number generator was employed.

Cytological examination

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). Five degrees of reaction ('negative', 'trace', 'l', '2', '3') were described (Schalm et al. 1971). Milk smears were also produced and dried.

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 (0.203 of all samples). 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× and counting at least 100 leucocytes.

Examination of staphylococcal isolates for *in vitro* biofilm formation and relevant gene detection

All staphylococcal isolates recovered during the field investigation were tested for *in vitro* biofilm formation.

Culture appearance on Congo-red agar

Culture appearance on Congo Red agar plates was used for evaluation of biofilm formation by the staphylococcal isolates. All isolates were cultured on Congo Red agar plates (BioPrepare

Microbiology, Athens, Greece) for aerobic incubation at 37 °C for 24 hours (Freeman et al. 1989). In all batches, *Staphylococcus* [*S.*] *aureus* ATCC25923 was added as positive control and *S. epidermidis* ATCC12228 was added as negative control.

Microplate adhesion method

All isolates were tested by means of the microplate adhesion technique. The technique described by Fabres-Klein et al. (2015), which had been based on the work of Vasudevan et al. (2003), was followed. Each bacterial isolate (quantity contained into a 0.01 mL microbiological loop) was inoculated into a tube containing 1 mL tryptic soy broth for 16 h incubation at 37 °C. Then, a volume of the inoculated broth was aspirated and diluted 1:40 into tryptic soy broth containing 0.25% glucose. A volume of 0.2 mL was placed into each well ('U-shaped' bottom) of a 96-well sterile microplate (Kisker Biotech; Steinfurt, Germany), which was subsequently incubated at 37 °C for 24 h. The following were included on each microplate as controls: (i) three wells with 'negative control', i.e., with inoculum from the *S. epidermidis* as above, and (ii) three wells with 'positive control', i.e., with inoculum from the *S. aureus* as above. Subsequently, the microplate was washed off thrice with 0.2 mL of sterile PBS (pH 7.4) on each occasion and dried at 45 °C for 20 min. Subsequently, 0.05 mL of 1% crystal violet was added into each well and the microplate was left for 15 min., after which the microplate was washed off again thrice with 0.2 mL of sterile distilled water on each occasion and dried at 45 °C for 20 min. Subsequently, 0.2 mL of 100% ethanol was added into each well and the content of well was mixed. A volume of 0.15 mL was aspirated from each well and transferred to a new microplate for reading, which was carried in absorbance at 630 nm in a microplate reader (BioTek™ ELx800™ Absorbance Microplate Reader; Thermo Fisher Scientific, Waltham, USA).

In each testing lot, three repeat evaluations of each isolate (three triplicates) were performed. Moreover, each isolate was evaluated in three separate testing occasions, with respective work performed on different dates. That way, three sets of results, each of three triplicates, had become available for each isolate.

Detection of genes associated with biofilm formation

In 116 of above isolates (*S. aureus* n = 19, coagulase-negative staphylococci n = 97 – from cases of subclinical mastitis n = 96; from cases of mammary carriage n = 20), PCR was used to detect the following genes: *icaA*, *icaB*, *icaC*, *icaD*, *bap*, *eno* and *clfa*.

For DNA template preparation, 1 mL of a bacterial cell suspension to a final turbidity equivalent to 1.0 Mc Farland standard, was centrifuged at 13,000 rpm for 10 min. The pellet was re-suspended in 0.1 mL of lysis buffer (50 mM Tris - HCl [pH 7.5], 1% Triton X-100, 1 mM EDTA and 200 mg mL^{-1} proteinase K) The mixture was incubated for 3 h at 37 °C and then boiled (100 °C) for 10 min. and finally clarified by centrifugation (Petinaki et al. 2001).

Presence of the above seven genes was detected by simple PCR assays. Details of primers and conditions employed are in Table II.i (Tristan et al. 2003, Cucarella et al. 2004, Kiem et al. 2004). Amplification was performed in a PT-100 Thermocycler (MJ Research Inc., St Bruno, Canada). Reactions were performed in a total volume of 0.05 mL PCR mixture, containing 0.045 mL of Platinum PCR SuperMix (Applied Biosystems, Foster City, USA) and approximately 150 ng of the extracted DNA. The thermal cycling procedure consisted of a pre-denaturation step at 94 °C for 5 min., 30 cycles of denaturation at 94 °C for 1 min., annealing for 1 min. and extending at 72 °C for 1 min. and a final elongation step at 72 °C for 7 min. Subsequently, 0.005 mL of each product was analysed by electrophoresis on 1.5% agarose gel, previously stained with ethidium bromide, and observed at ultraviolet light. Each product equal in size to the expected amplicon was considered as positive.

Table II.i. Primers used and work conditions undertaken for detection of *icaA*, *icaB*, *icaC*, *icaD*, *bap*, *eno* and *clfa* genes in staphylococcal isolates recovered from milk samples of ewes.

Gene	Primer sequence	Concentration (μM)	Product size (bp)	AT[1] (ºC)	Reference
icaA	Fw-TCTCTTGCAGGAGCAATCAA Rv-TCAGGCACTAACATCCAGCA	0.2	188	55.5	Arciola et al. 2001
icaB	Fw-AGAATCGTGAAGTATAGAAAATT Rv-TCTAATCTTTTTCATGGAATCCGT	0.2	900	55.0	Kiem et al. 2004
icaC	Fw-ATGGGACGGATTCCATGAAAAAGA Rv-TAATAAGCATTAATGTTCAATT	0.2	1100	55.0	Kiem et al. 2004
icaD	Fw-ATGGTCAAGCCCAGACAGAG Rv-CGTGTTTTCAACATTTAATGCAA	0.2	198	55.5	Arciola et al. 2001
bap	Fw-CCCTATATCGAAGGTGTAGAATTG Rv- GCTGTTGAAGTTAATACTGTACCTGC	0.2	971	62.0	Curacella et al. 2001
eno	Fw- ACGTGCAGCAGCTGACT Rv-CAACAGCATYCTTCAGTACCTTC	0.2	302	55.0	Tristan et al. 2003
clfa	Fw-ATTGGCGTGGCTTCAGTGCT Rv-CGTTTCTTCCGTAGTTGCATTTG	0.2	288	55.0	Tristan et al. 2003

1. AT: annealing temperature.

Examination of staphylococcal isolates for susceptibility / resistance to antimicrobial agents and relevant gene detection

In 142 staphylococcal isolates (*S. aureus* n = 27, coagulase-negative staphylococci n = 115; from cases of subclinical mastitis n = 118; from cases of mammary carriage n = 24), susceptibility testing was performed to 11 antimicrobial agents (ampicillin, azithromycin, cefoxitin, clarithromycin, clindamycin, erythromycin, fosfomycin, fucidic acid, penicillin, tetracycline, trimethoprime-sulfamethoxazole) by VITEK 2. All erythromycin-resistant isolates were then tested by the disk diffusion-test, in order to differentiate the inducible MLS_B from the M-phenotype.

All isolates, regardless of their phenotype, were also tested by PCR for the following genes: *ermA*, *ermB*, *ermC*, *ermT*, *ermY*, *lnuA*, *lnuB*, *lnuC*, *lsaA*, *mphC*, *msrA*, *tetK*, *tetL*, *tetM*, *tetS*, *tetT* and *vgaA*. DNA template preparation was made as described above.

Presence of the above 17 genes was detected by simple PCR assays. Details of primers and conditions employed are in Table II.ii. Amplification was performed in a Veriti 9902 Thermocycler (Applied Biosystems, Foster City, USA).

Examination of staphylococcal isolates using Multi Locus Sequence Typing

In total, 27 *S. aureus* and 22 *S. epidermidis* isolates were typed by using Multi Locus Sequence Typing (MLST) (http://saureus.mlst.net and http://sepidermidis.mlst.net).

Illumina sequencing

In total, 10 isolates (from cases subclinical mastitis n = 7, from cases of mammary carriage n = 3), selected as representative of the species identified and the various resistance genes detected, were subjected to whole genome sequencing.

Table II.ii. Primers used and work conditions undertaken for detection of 17 resistance-associated genes in staphylococcal isolates recovered from milk samples of ewes.

Gene	Primer sequence	Concentration (μM)	Product size (bp)	AT[1] (°C)	Reference
ermA	Fw-TCTAAAAAGCATGTAAAAGAA Rv-CTTCGATAGTTTATTAATATTAGT	0.2	645	55.0	Alos et al. 2000
ermB	Fw-GAAAAGGATCTCAACCAAATA Rv-AGTAACGGTACTTAAATTGTTTAC	0.2	639	55.0	Alos et al. 2000
ermC	Fw-TCAAAACATAATATAGATAAA Rv-GCTAATATTGTTTAAATCGTCAAT	0.2	649	52.0	Alos et al. 2000
ermT	Fw-GGTGTAATTATGTAACCGCCA Rv-ACTTCCTGTAGCTGTGCTTTC	0.2	218	52.0	Alos et al. 2000
ermY	Fw-ATTACTTCGAAACGTAATATAGAT Rv-ATAGCTATTGAAAAGAGACAAGA	0.2	~ 700	60.0	Matsuoka et al. 2003
lnuA	Fw- GGTGGCTGGGGGGTAGATGTATTAACTGG Rv-GCTTCTTTTGAAATACATGGTATTTTTCGA	0.2	323	55.0	Lina et al. 1999
lnuB	Fw-CCTACCTATTGTTTGTGGAA Rv-ATAACGTTACTCTCCTATTC	0.2	944	50.0	Bozdogan et al. 1999
lnuC	Fw-GTAGATGCTCTTCTTGGAT Rv-TTCTACCGGAAAACAATCC	0.2	246	55.0	Gravey et al. 2013
lsaA	Fw-GATCAGGCAAATATCACGATG Rv-CTTCATGAAATGTGCATCATGC	0.2	~ 1400	60.0	Malbruny et al. 2011
mphC	Fw-ATCTCATTGAATGAATCAGGAC Rv-CTACTCTTCCATACCTAACTC	0.2	~ 850	60.0	Matsuoka et al. 2003
msrA	Fw-GCAAATGGTGTAGGTAAGACAACT Rv- ATCATGTGATGTAAACAAAAT	0.2	399	55.0	Alos et al. 2000
tetK	Fw-TATTTTGGCTTTGTATTCTTTCAT Rv-GCTATACCTGTTCCCTCTGATAA	0.2	1159	59.0	Trzcinski et al. 2000

tetL	Fw-ATAAATTGTTTCGGGTCGGTAAT Rv-AACCAGCCAACTAATGACAATGAT	0.2	1077	59.0	Trzcinski et al. 2000
tetM	Fw-GAACTCGAACAAGAGGAAAGC Rv-ATGGAAGCCCAGAAAGGAT	0.2	740	54.0	Olsvik et al. 1995
tetS	Fw-ATCAAGATATTAAGGAC Rv-TTCTCTATGTGGTAATC	0.2	573	55.0	Gevers et al. 2003
tetT	Fw-CAGTGCGAATATAAGGACACGTC Rv-CAAGCCTTCTCTACAGCATC	0.2	644	61.0	Clermont et al. 1997
vgaA	Fw-CTTGTCTCCTCCGCGAATAC Rv-AGTGGTGGTGAAGTAACACG	0.2	659	55.0	Soltani et al. 2000

1. AT: annealing temperature.

Genomic DNAs from the wild isolates were extracted using the DNASorb-B Kit (Sacace Biotechnologies, Como, Italy). Multiplexed DNA libraries were prepared, using the Nextera XT Library Preparation Kit, and 300-bp paired-end sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, USA) using the MiSeq v. 3 600-cycle Reagent Kit. Initial paired-end reads were quality trimmed, using the Trimmomatic tool v. 0.33 (Bolger et al. 2014), with a sliding window size of 4 bp, required average base quality ≥ 17 and minimum read length of 48 bases. Then, reads were assembled by use of the de Bruijn graph-based *de novo* assembler SPAdes v. 3.9.1 (Bankevich et al. 2012), using k-mer sizes 21, 33, 55, and 77.

For sequence analysis and annotation, the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST), the ISfinder database (https://www-is.biotoul.fr), and the open reading frame finder tool (www.bioinformatics.org/sms) were utilised. Comparative genome alignments were performed using the Mauve v. 2.3.1 program (Darling et al. 2010). Antimicrobial resistance genes were identified using the ResFinder 3.1 tool (https://cge.cbs.dtu.dk/services/ResFinder/), using an identity threshold of > 90% (Zankari et al. 2012).

Detection of anti-PNAG specific antibodies in blood samples

In blood serum samples, measurement of IgG poly-N-acetyl b-1,6 glucosamine (PNAG)-specific antibodies was performed. Serum samples were diluted to 1:100. Diluted samples were assayed by an indirect ELISA in plates coated with purified PNAG (Prenafeta et al. 2010). This was confirmed with a specific monoclonal anti-deacetylated PNAG that had been kindly provided by Dr Gerard Pier. In brief, 96-well plates were coated with the purified PNAG and blocked with a StabilCoat Immunoassay Stabilizer (SurModics, Eden Prairie, USA). Wells were incubated with the diluted samples; protein G conjugated with peroxidase (Pierce; Thermo Fisher Scientific, Waltham, USA) was used to detect bound total IgG antibodies (Prenafeta et al. 2010).

Known positive and negative control serum samples were included on each assay plate. Finally, wells were incubated with a chromogenic substrate for the peroxidase; absorbance of the reaction was measured as optical density in a microplate reader at 405 nm (Sunrise equipped with Magellan 3.11 software; Tecan, Männedorf, Switzerland). Each sample was assayed twice, by using above methodology and controls.

Data related to environmental parametres

Climatic variables were derived from the WorldClim website (http://www.worldclim.org), version 1.4. (Hijmans et al. 2005, WorldClim – Global Climate Data 2019). ArcGIS 10.1 GIS software (ESRI, Redlands, CA, USA) was used to create environmental layers (n = 19) (*clima1*, …, *clima19*) for the analysis. Wind speed was retrieved from the GeoPortal of the Hellenic Regulatory Authority for Energy (www.rae.gr). Altitude was extracted from a digital elevation model (*dem*) with a spatial resolution of 1 square kilometre, presented in the CGIAR Consortium for Spatial Information (2018). Distances between small ruminant farms (*farmdis*) were taken from Greek public authority sources, specifically the Greek Payment Authority and Control Agency for Guidance and Guarantee Community Aid (2018) and the Hellenic Statistical Authority (2018). Land uses and habitat types were derived from the Corine Land Cover 2000 database (European Environment Agency 2018).

Data management and analysis

Classification of farms according to management system

Classification of farms in management system was performed according to the criteria of the the European Food Safety Authority (2014), as intensive, semi-intensive, semi-extensive, extensive, shepherding or mixed. As no farms under shepherding or mixed system were found, these possible results were omitted from all analyses.

Combination of results of culture appearance on Congo Red agar plates and microplate adhesion method

In evaluating results of culture appearance on Congo Red agar plates, biofilm-forming isolates (‘+’) were indicated by black colonies with dry crystalline consistency. Dark-coloured colonies with no dry crystalline culture appearance were assigned an intermediate result (‘±’), whilst colonies that remained pink, were considered to reflect non biofilm-forming isolates (‘-’) (Freeman et al. 1989).

In evaluating results of the microplate adhesion method, initially, median optical density (OD) result of the three triplicates on each of the three testing lots was calculated. Then, for each testing lot, isolates with median OD ≥ [0.85 × median OD of the positive control strain] were assigned value

1 and isolates with median OD < [0.85 × median OD of the positive control strain] were assigned value 0. Finally, the three assigned values (0 or 1) of each testing lot for each isolate under evaluation were added. Sum ranged from 0 (i.e., in all three testing lots median, of three triplicates, OD was < [0.85 × median OD of the positive control strain]) to 3 (i.e., in all three testing lots median, of three triplicates, OD was ≥ [0.85 × median OD of the positive control strain]).

Subsequently, results of the two methods (culture appearance on Congo Red agar and microplate adhesion) were combined as detailed in Table II.iii. Based on that, staphylococcal isolates were characterised as biofilm-forming, weak biofilm-forming or non biofilm-forming.

Table II.iii. Characterisation of staphylococcal isolates recovered from milk samples of ewes classified according to biofilm formation, by combination of results of culture appearance on Congo Red agar and microplate adhesion method.

		Sum of results of each of three separate testing lots for each isolate in the microplate adhesion method[1]			
		0	1	2	3
Culture appearance on Congo Red agar	-	non BF[2]	non BF	non BF	weak BF
	±	non BF	non BF	BF	BF
	+	weak BF	BF	BF	BF

1. Sum ranged from 0 (i.e., in all three testing lots median, of three triplicates, OD was < [0.85 × median OD of the positive control strain]) to 3 (i.e., in all three testing lots median, of three triplicates, OD was ≥ [0.85 × median OD of the positive control strain]) (OD: optical density).
2. BF: biofilm-forming

Classification of isolates with regard to susceptibility / resistance to antimicrobial agents

The interpretation of the results of susceptibility / resistance testing was based on the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org). Based on the testing results, the isolates were classified as resistant, intermediate or susceptible to each antimicrobial agent according to EUCAST criteria. Multi-drug resistant (MDR) isolates were those found resistant to at least three different classes of antimicrobial agents (Magiorakos et al. 2012).

Optical density during measurement of anti-PNAG specific antibodies in blood samples

Results of antibody measurements in serum samples were expressed as OD values. The mean OD value of the two duplicate examinations obtained during the PNAG-specific antibodies assay in the total IgG measurements, was converted into a RIPC (relative index percent) by using the following formula:

RIPC = [OD value sample - OD value negative control] / [OD value positive control - OD value negative control] × 100.

A cut-off point of 6.0 RIPC for the anti-PNAG-specific total IgG in serum had been established in accordance with results obtained during validation of the assay (Prenafeta et al. 2010), as follows: cut-off = (mean RIPC from negative serum samples + 2sd) + [(mean RIPC from negative serum samples + 2sd) × (variation coefficient of the technique)].

Values above that cut-off point were considered as 'positive', while values below that were considered as 'negative'.

Definitions

Subclinical mastitis was defined in ewes in which a bacteriologically positive milk sample ([i] > 10 colonies of the same organism and [ii] no more than two different types of colonies) with concurrently increased CMT score (≥ 'l') plus neutrophil and lymphocyte proportion (≥ 65% of all leucocytes) was detected (Fragkou et al. 2014).

The term mammary 'carriage' (or 'carrier state') was used to describe presence of bacteria in the mammary gland in the absence of inflammation. Mammary carriage was defined in ewes in which a bacteriologically positive milk sample with no increased CMT score (≤'trace') or neutrophil and lymphocyte proportion (< 65% of all leucocytes) was detected. Isolation of ≤ 10 colonies of an organism or isolation of over two different types of colonies from a sample was considered to indicate contamination.

Staphylococcal subclinical mastitis and staphylococcal mammary carriage were considered among above ewes, respectively, in which staphylococci were identified in milk samples. Cases of staphylococcal subclinical mastitis or of staphylococcal mammary carriage found to be caused by isolates forming biofilm were termed 'biofilm staphylococcal subclinical mastitis' or 'biofilm staphylococcal mammary carriage'; for that, biofilm forming and weak biofilm forming isolates were

taken into account together. All above definitions referred to ewes (hence, animals with both glands affected were counted as one case).

Quantitative information on cellular content of ewes' milk

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 *R2* was 83.4%.

Statistical analyses

Data were entered into Microsoft Excel and analysed using STATA15 (Statacorp Inc., Texas, USA) and IBM SPSS Statistics (ver. 21) (IBM; Armonk, NY, USA).

Prevalence of mastitis outcomes

Basic descriptive analysis was performed. The outcomes of 'subclinical mastitis', 'staphylococcal subclinical mastitis', 'biofilm staphylococcal subclinical mastitis', 'mammary carriage', 'staphylococcal mammary carriage' and 'biofilm staphylococcal mammary carriage' were calculated. Prevalence and exact binomial confidence intervals (CI) were obtained.

In order to estimate the prevalence of subclinical mastitis amongst all the ewes in the study farms, we declared farms to be strata and sampling weights to be [*1/(number of ewes on the farm)*] and used Jackknife analysis with STATA's 'SVY proportion' command.

Analysis for predictors for subclinical mastitis, staphylococcal subclinical mastitis, mammary carriage and staphylococcal mammary carriage

A preliminary assessment of the importance of predictors was performed by using 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'. The same procedure was then followed for

evaluation of significance of predictors for staphylococcal subclinical mastitis, as well as for mammary carriage in farms into the study. In the above computations, specifically for evaluation of the significance of stage of the lactation period, the following categories were taken into account: 1st month after lambing, 2nd month after lambing, 3rd and 4th month after lambing and 5th to 8th month after lambing. No other categories were collapsed during the analysis. Further, specifically for evaluation of significance of administration of antimicrobial agents at end of lactation period, the procedure was repeated by taking into account only farms, in which ewes were at the 1st month of lactation period. Moreover, specifically for evaluation of significance of anti-mastitis vaccination, four (4) farms in which inappropriate vaccination schedules were performed, were excluded from calculations. Finally, a further model was constructed to evaluate whether anti-mastitis vaccination status might have affected the possible effect of other predictors.

A multivariable model was created using mixed-effects logistic regression with farm as the random effect, and initially offering to the model all variables which achieved a significance of $P < 0.2$ in the univariable analysis. Variables were removed from this initial model by backwards elimination. The *P* value of removal of a variable was assessed by the likelihood ratio test, and for those with a *P* of > 0.2 the variable with the largest probability was removed. This process was repeated until no variable could be removed with a *P* value of > 0.2. The final multivariable model required the following variables: (i) management system in flock, (ii) stage of lactation period in ewes in flock and (iii) application of post-milking teat dipping.

Analysis for predictors for biofilm staphylococcal subclinical mastitis and biofilm staphylococcal mammary carriage

A preliminary assessment of the importance of predictors was performed by using 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'. The same procedure was then followed for evaluation of significance of predictors for biofilm staphylococcal subclinical mastitis in farms into the study.

A multivariable model was created using mixed-effects logistic regression with farm as the random effect, and initially offering to the model all variables which achieved a significance of $P < 0.2$ in the univariable analysis. Variables were removed from this initial model by backwards elimination. The *P* value of removal of a variable was assessed by the likelihood ratio test, and for those with a *P* of > 0.2 the variable with the largest probability was removed. This process was repeated until no variable could be removed with a *P* value of > 0.2. The final multivariable model

required the following variables: (i) management system in flock and (ii) milking mode applied in flock.

Analysis for breed as predictor of subclinical mastitis

A preliminary assessment of the importance of breed 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 'random effect'.

The following comparisons were made by using analysis of variance between farms in relation to this outcome: (i) farms with pure-bred animals *versus* farms with cross-bred animals, (ii) farms with Greek pure-bred animals *versus* farms with imported pure-bred animals, (iii) farms with imported pure-bred animals versus all other farms (i.e., farms with Greek pure-bred animals and farms with cross-bred animals), (iv) 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 (v) 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).

Analysis of results related to frequency of susceptibility / resistance to antimicrobial agents

As no intermediate isolates were found, this possible result was omitted from all analyses. The frequency of resistance to antimicrobial agents among isolates (i) from different sources (subclinical mastitis or mammary carriage) or (ii) of different species (*S. aureus* or coagulase-negative species) was evaluated in tables of cross-categorised frequency data by use of the Pearson chi-square test or the Fisher exact test, as appropriate. Frequencies of resistance to the individual antimicrobial agents were also compared. The same tests were also employed to compare frequencies of resistance and biofilm formation in the studied isolates; findings according to origin of

isolates or staphylococcal species were also assessed. Frequencies of detection of resistance genes and genes associated with biofilm formation were also evaluated for possible associations.

For isolates from cases of subclinical mastitis, the frequency of resistance to antimicrobial agents was evaluated in tables of cross-categorised frequency data by use of the Pearson chi-square test or the Fisher exact test, as appropriately, against the various husbandry factors practiced in the farm where isolated.

Analysis of results related to staphylococcal sequence types

For *S. aureus* and *S. epidermidis*, the frequency of the various Sequence Types (STs) identified was evaluated for possible association with biofilm formation or resistance to antimicrobial agents. Further, the possible association of presence of resistance with STs was also investigated.

Analysis of results of anti-PNAG antibody titres

During preliminary assessment of the data, it became evident that some ewes (n = 60) had been vaccinated against biofilm staphylococcal mastitis by means of a licenced commercially-available vaccine. Hence, as antibody titres could be the result either of vaccination or of mammary infection, which were two conflicting factors, apart from analysis of data from all animals, separate analyses for samples from vaccinated and non-vaccinated ewes were also performed. Prevalence and exact binomial CI for the various outcomes were obtained.

Antibody titres were compared between vaccinated and non-vaccinated ewes, as well as between ewes with mastitis, mammary carriage or no infection by using analysis of variance. The various associations were evaluated in tables of cross-categorised frequency data by use of the Pearson chi-square test or the Fisher exact as appropriate. Comparisons of numerical parametric or non-parametric data were performed by means of the Student's t-test or the Mann-Whitney test, as appropriate. Analysis of correlation was performed between total number of genes relevant to biofilm formation detected in staphylococcal isolates and anti-PNAG antibody titres found in blood samples from respective ewes.

An initial assessment of the importance of predictors for seropositivity was performed by using cross-tabulation with the Pearson chi-square test and with simple logistic regression without random effects.

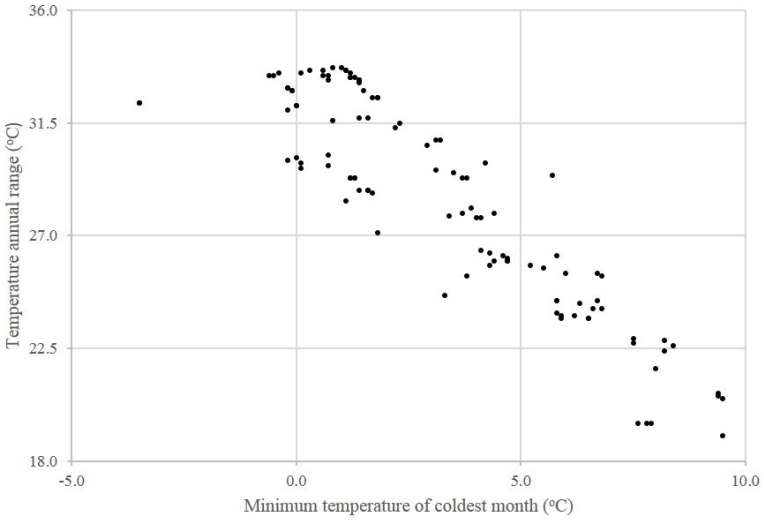
A multivariable model was created using mixed-effects logistic regression with farm as the random effect, and initially offering to the model all variables which achieved a significance of $P < 0.2$ in the univariable analysis. Variables were removed from this initial model by backwards elimination. The P value of removal of a variable was assessed by the likelihood ratio test, and for

those with a *P* of > 0.2 the variable with the largest probability was removed. This process was repeated until no variable could be removed with a *P* value of > 0.2. The final multivariable model required the following variables: (i) management system in flock and (ii) milking mode applied in flock and (iii) anti-staphylococcal mastitis vaccination performed in flock.

Analysis for environmental parametres as predictors of subclinical mastitis

Checks for multicollinearity revealed that the minimum temperature of coldest month and the annual range in temperature were very highly correlated (r = -0.909, R^2 = 0.830) (Figure II.2): the multivariable analyses for possible predictors therefore used the minimum temperature of coldest month and excluded the annual temperature range. A preliminary assessment of the importance of predictors was performed using by cross-tabulation 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'.

Figure II.2. Scatter plot of results of temperature annual range against minimum temperature of coldest month in 111 sheep farms in Greece.



A multivariable model was created using mixed-effects logistic regression with farm as the random effect, and initially offering to the model all variables which achieved a significance of $P \leq 0.2$ in the univariable analysis. Variables were removed from this initial model by backwards elimination. The *P* value of removal of a variable was assessed by the likelihood ratio test, and for those with a *P* of > 0.2 the variable with the largest probability was removed. This process was repeated until no variable could be removed with a *P* value of > 0.2. The final multivariable model

required the following variables: (i) mean temperature of coldest month and (ii) mean temperature for 30 days prior to sampling date.

Analysis for predicting potential space distribution of subclinical mastitis

Two separate analyses were performed: one for subclinical mastitis independently of causal agent and one for biofilm staphylococcal subclinical mastitis. For each analysis, initially all farms into the study (n = 111) were taken into account; this was followed by taking into account results of farms under intensive (n = 26), semi-intensive (n = 57) or semi-extensive / extensive (n = 28) management system separately.

Within each category, farms were divided into two clusters, according to prevalence of subclinical mastitis. Sheep farms classified in the upper three quartiles of prevalence (in analysis for subclinical mastitis: n = 78 [all farms in the study], n= 19 [farms in intensive management], n = 38 [farms in semi-intensive management], n = 21 [farms in semi-extensive / extensive management system] – in analysis for biofilm staphylococcal subclinical mastitis: n = 75, n= 20, n = 31, n = 18, respectively) were used as occurrence points for the Ecological niche modelling procedure ('infected farms'); farms classified in the lower quartile of prevalence within each of above categories were used as (pseudo)negative points. Maximum entropy modelling (MaxEnt software ver. 3.3.3) was used to predict the appropriate ecological niches for infected farms (Phillips et al. 2006). The 'bias file' was included in the analysis, in order to represent the sampling effort and to reduce sampling bias. The goodness of fit of the model predictions was evaluated by the mean area under the curve (AUC) of the receiver operating characteristic curve (ROC). The Jackknife procedure was used to reduce the number of environmental variables to only those that showed a substantial influence on the model.

After completion of the analysis and construction of the predictive maps, the model was verified by checking which of the 'infected farms' were indeed located in areas predicted as high risk for prevalence of subclinical mastitis.

Statistical significance

Statistical significance was defined at $P < 0.05$.

Nucleotide sequence accession numbers and genome sequencing data

The nucleotide sequences of the erm-like carrying fragments described in the study have been deposited in GenBank, under accession numbers MG557993, MH423313, MH423314, MH423315, MH431899 and MH465666. Raw genome sequencing data of the staphylococcal isolates were deposited in ENA under accession number PRJEB31299.

C. RESULTS

Prevalence and aetiology of subclinical mastitis and staphylococcal subclinical mastitis

In the 111 farms, there were 35,925 ewes. Ewe population per farm varied from 40 to 1,500 (mean: 323 ± 21.5) animals. In total, 2,220 ewes were examined. Among these, in 9 farms, in 22 animals, which the respective farmers believed to be normal, clinically evident mammary abnormalities were detected; prevalence was 0.010 (95% CI: 0.007 - 0.015). These included 5 animals with abnormal secretion and 17 animals with intramammary nodules or hardness.

Then, 2,198 ewes with clinically normal udders were sampled. Among these, 572 ewes were detected with subclinical mastitis; its prevalence in the population sampled was 0.260 (95% CI: 0.242 - 0.279). Within farms into the study, its prevalence varied from 0.000 to 0.850 (median value among farms: 0.250). The estimated overall proportion of ewes with subclinical mastitis in the study farms combined was 0.250 (95% CI: 0.157 - 0.344). In 430 ewes (0.752 of ewes with subclinical mastitis), bacteriologically positive milk samples with concurrently increased CMT score plus neutrophil and lymphocyte proportion were detected in only one mammary gland and in 142 ewes in both mammary glands (0.248 of ewes with subclinical mastitis), i.e., prevalence in the population sampled was 0.064 (95% CI: 0.055 - 0.076)].

In total, 760 bacterial isolates were obtained from ewes with subclinical mastitis, i.e., 1.33 isolates per ewe and 1.06 isolates per sample. The most frequently isolated bacteria from ewes with subclinical mastitis were *Staphylococcus* spp. (n = 531; *S. aureus* and coagulase-negative species), which accounted for 0.699 of isolates from ewes with subclinical mastitis. In total, 419 ewes were detected with staphylococcal subclinical mastitis; its prevalence in the population sampled was 0.191 (95% CI: 0.175 - 0.208). Within farms in the study, its prevalence varied from 0.000 to 0.750 (median value among farms: 0.158). Overall, staphylococcal subclinical mastitis accounted for 0.733 of all cases of subclinical mastitis.

Other frequently isolated bacteria were *Streptococcus* spp., *Corynebacterium* spp., *Escherichia coli*, *Micrococcus* spp., *Mannheimia haemolytica* and *Trueperella pyogenes*. Frequency of the various bacterial isolates from ewes with subclinical mastitis is in Table II.iv. Among coagulase-negative staphylococcal species, *S. chromogenes* was the one most often identified. Other

frequently identified species were *S. epidermidis* and *S. simulans*. Frequency of the various coagulase-negative staphylococcal isolates identified is in Table II.v.

Table II.iv. Frequency of bacterial isolates recovered from milk samples from ewes with subclinical mastitis or mammary carriage in Greece.

	Number of isolates (frequency of isolation)[1]	
Microorganism	Isolates from subclinical mastitis	Isolates from mammary carriage[2]
Coagulase-negative staphylococci	454 (0.597)	166 (0.597)
S. aureus	77 (0.101)	11 (0.040)
Streptococcus spp.	36 (0.047)	1 (0.004)
Corynebacterium spp.	27 (0.036)	40 (0.144)
Escherichia coli	26 (0.034)	10 (0.036)
Micrococcus spp.	20 (0.026)	15 (0.054)
Mannheimia haemolytica	19 (0.025)	2 (0.007)
Trueperella pyogenes	19 (0.025)	2 (0.007)
Acinetobacter spp.	16 (0.021)	5 (0.018)
Bacillus spp.	12 (0.016)	17 (0.061)
Enterococcus spp.	9 (0.012)	0 (0.000)
Other[2]	45 (0.059)	9 (0.032)
Total	760	278

1. Results presented on animal basis.
2. *Klebsiella* spp., *Kokuria* spp., *Proteus* spp., *Peptococcus* spp., *Pseudomonas* spp., *Rhodococcus* spp., *Burkholderia* sp.

Among ewes sampled, in 219, mammary carriage was detected; its prevalence in the population sampled was 0.100 (95% CI: 0.088 - 0.113). Within farms into the study, its prevalence varied from 0.000 to 0.533 (median value among farms: 0.053). In total, 278 bacterial isolates were obtained from ewes with mammary carriage, i.e., 1.27 isolates per ewe. The most frequently isolated bacteria from ewes with mammary carriage were *Staphylococcus* spp. (n = 177), which accounted for 0.637 of the isolates from ewes with mammary carriage. Overall, staphylococcal mammary carriage accounted for 0.630 of all cases of mammary carriage; its prevalence in the population sampled was 0.063 (95% CI: 0.053 - 0.074). Details of bacteria identified are in Tables II.iv and II.v.

In 361 ewes, only increased CMT score in milk was detected; hence, prevalence in the population sampled was 0.164 (95% CI: 0.149 - 0.180). Within farms into the study, its prevalence varied from 0.000 to 0.450 (median value among farms: 0.150). Of above ewes, 122 (0.338) were in 5th to 8th month after lambing. Further, in 98 ewes of above ewes (0.271), increased CMT score was detected in both glands of the animal; of these 98 animals, 42 (0.429) were in 5th to 8th month after lambing.

Table II.v. Frequency of coagulase-negative staphylococcal species recovered from milk samples from ewes with subclinical mastitis or mammary carriage in Greece.

	Number of isolates (frequency of isolation)	
Staphylococcal species	Isolates from subclinical mastitis	Isolates from mammary carriage
S. chromogenes	21 (0.230)	7 (0.292)
S. epidermidis	18 (0.198)	4 (0.166)
S. simulans	16 (0.176)	0 (0.000)
S. hominis	8 (0.088)	0 (0.000)
S. xylosus	7 (0.077)	5 (0.208)
S. lentus	5 (0.055)	4 (0.166)
S. caprae	3 (0.033)	1 (0.042)
S. haemolyticus	3 (0.033)	1 (0.042)
S. capitis	2 (0.022)	0 (0.000)
S. cohnii	1 (0.011)	0 (0.000)
S. equorum	1 (0.011)	0 (0.000)
S. hyicus	1 (0.011)	0 (0.000)
S. saprophyticus	1 (0.011)	0 (0.000)
S. schleiferi	1 (0.011)	0 (0.000)
S. sciuri	1 (0.011)	0 (0.000)
S. warneri	1 (0.011)	1 (0.042)
S. warneri or *saprophyticus*	1 (0.011)	0 (0.000)
S. vitulinus	0 (0.000)	1 (0.042)
Total	91	24

Predisposing factors for subclinical mastitis and staphylococcal subclinical mastitis

Cumulative information collected by means of the questionnaire in farms in the study are summarised in Table II.vi.

Management system

Farms into the study were dairy production flocks. At the start of each lactation period, lambs sucked their dams for 10 to 50 - 60 days after birth. Subclinical mastitis was detected more frequently in farms managed semi-intensively (prevalence: 0.296) or intensively (prevalence: 0.254) and less frequently in farms managed semi-extensively (0.196) or extensively (0.178); for staphylococcal mastitis, respective prevalence rates were 0.224, 0.179, 0.141 and 0.110 ($P < 0.001$ between all types of management system).

Management system in flocks emerged from the multivariable mixed-effects model as the only significant predictor for the prevalence of subclinical mastitis ($P = 0.008$).

Stage of lactation period

Subclinical mastitis occurred more frequently during the 2nd month of lactation period (prevalence: 0.305) or on 5th month and thereafter (prevalence: 0.292) and less frequently during 3rd and 4th months post-partum (0.246) or on 1st month post-partum (0.223); for staphylococcal mastitis, respective prevalence rates were 0.215, 0.227, 0.178 and 0.159 ($P < 0.005$ between all above periods). Also, it emerged that of the 26 *E. coli* isolates, 21 (0.808) had been recovered during the first month of lactation period.

From the multivariable mixed-effects model, stage of lactation period of ewes in flocks was not found a significant predictor for the prevalence of subclinical mastitis ($P = 0.151$).

Reproductive control

Most farms into the study (n = 81, 0.730) did not practice reproductive control; the few farms that did, used intravaginal progestogen sponges (n = 24) or melatonin implants (n = 6). No association was evident between use of reproductive control and prevalence of subclinical mastitis ($P = 0.55$).

Table II.vi. Information collected by means of interview of farmers in 111 sheep farms visited across Greece.

Question	Possible answers	Results (mean number ± standard error)	
No. of ewes in flock	Number	323.5 ± 21.5	
Question	**Possible answers**	**Results (no. of farms)**	
Production type in flock	Breeding	0	
	Dairy	0	
	Dairy and meat	111	
	Meat	0	
	Wool and hide	0	
Management system in flock	Intensive	26	
	Semi-intensive	57	
	Semi-extensive	22	
	Extensive	6	
	Shepherding	0	
	Mixed	0	
Stage of lactation period in ewes in flock (i.e., at time of the visit)	Month post-partum	1st	38
		2nd	20
		3rd	10
		4th	14
		5th	4
		6th	9
		7th	5
		8th	9
		1st and 2nd	1
		2nd and 4th	1
	Suckling lambs	13	
	Milking	70	
	Suckling lambs and milking concurrently	28	
Use of reproductive control	Yes	30	
	No	81	

Method of reproductive control employed (in flocks that applied such control)	Use of progestogen sponges	24
	Use of melatonin implants	6
Milking technique applied (in dairy / dairy and meat production flocks)	Machine-milking	74
	Hand-milking	37
Application of post-milking teat dipping (in dairy / dairy and meat production flocks)	Yes	12
	No	99
Intramammary administration of antimicrobial agents at end of lactation period	Yes	14
	No	97
Type of intramammary administration of antimicrobial agents at end of lactation period (in flocks that applied such procedure)	Selective	4
	Complete	10
Culling of ewes with history of mastitis during preceding lactation period	Yes	26
	No	85
Application of mastitis preventive measures at end of a lactation period[1]	No measures applied	74
	Culling of ewes	23
	Administration of antimicrobial agents	11
	Culling of ewes and administration of antimicrobial agents	3
Application of anti-mastitis vaccination	Yes	28
	No	83
Mammary pathogens against which vaccination was performed (in flocks in which vaccination was applied)	Staphylococci	28
	Other	0
Use of licensed vaccine or autogenous vaccine (in flocks that applied such procedure)	Licensed vaccine	28
	Autogenous vaccine	0
Vaccination schedule followed (in flocks that applied such procedure)	Licensed schedule	24
	Own schedule	4

1. Information deduced by the investigators after taking into account results of interviews

Milking practices

Differences on prevalence of subclinical mastitis or staphylococcal subclinical mastitis were seen between ewes that were milked (0.282 and 0.207, respectively), ewes that suckled lambs (0.214 and 0.167, respectively), or ewes in which milking and lamb suckling was applied concurrently (0.227 and 0.159, respectively) ($P < 0.03$). Of the 19 *M. haemolytica* isolates, 13 (0.684) had been recovered in flocks, in which ewes were suckling lambs at the time of sampling.

In most farms into the study (n = 74, 0.667), machine-milking was performed. No difference on prevalence of subclinical mastitis was observed between farms in which machine-milking (0.258) or hand-milking (0.285) was performed ($P = 0.49$); however, difference on prevalence of staphylococcal subclinical mastitis was seen between farms in which machine-milking was applied (0.179) and farms in which hand-milking was practiced (0.228) ($P = 0.035$). Machine-milking was applied mainly in farms with intensive (24 in 26 flocks) or semi-intensive (43 in 57 flocks) management ($P = 0.033$).

Most *Streptococcus* (n = 25, 0.694) were isolated in flocks, in which machine-milking was applied.

In only a few farms (n = 12, 0.108) post-milking teat dipping was applied. In those farms, smaller prevalence of subclinical mastitis or staphylococcal subclinical mastitis (0.196 and 0.128, respectively) was recorded than in farms in which the practice was not applied (0.268 and 0.198, respectively) ($P < 0.015$ for both comparisons). Odds ratio for prevalence of subclinical mastitis in farms, in which teat dipping was applied, was 0.665 (0.475 - 0.932, $P = 0.018$), compared to those in which the technique was not being practiced.

From the multivariable mixed-effects model, application of post-milking teat dipping was not found a significant predictor for the prevalence of subclinical mastitis ($P = 0.054$).

Mastitis preventive measures at end of lactation period

In only a few farms (n = 14), administration of antimicrobial agents (complete or selective) was practiced at the end of a lactation period. However, no differences in prevalence of subclinical mastitis or staphylococcal subclinical mastitis were evident between farms in which antimicrobial agents were (0.265 and 0.194, respectively) or were not (0.260 and 0.190, respectively) used; similar findings were observed even when only farms with ewes at the first month of lactation period were taken into account (for farms in which antimicrobial agents were administered: 0.193 and 0.143 – for farms in which antimicrobial agents were not administered: 0.228 and 0.163, respectively). In other

farms (n = 26), ewes that had previously developed mastitis, were culled at end of lactation period. In general, application of any of the above measures did not appear to be associated with prevalence of mastitis ($P > 0.55$ for all comparisons). No difference was evident among farms in the various management systems in intramammary administration of antimicrobial agents at end of lactation period or in anti-mastitis vaccinations ($P > 0.5$).

Anti-mastitis vaccination

Anti-mastitis vaccination was performed in 28 farms by using immunological products licensed in the European Union. Only anti-staphylococcal mastitis vaccines were employed. In 4 farms, inappropriate vaccination schedules (i.e., other than the one licensed) were undertaken.

Prevalence of subclinical mastitis and staphylococcal subclinical mastitis in farms in which vaccination was applied correctly (0.221 and 0.167, respectively), were smaller than in farms in which no vaccination was applied (0.272 and 0.197, respectively). Odds ratio for prevalence of subclinical mastitis in farms, in which vaccination was applied, was 0.758 (0.596 - 0.966, $P = 0.025$), compared to those in which vaccination was not practiced. Finally, when possible effects of vaccination were evaluated in models, which already included other potentially predisposing factors, no interaction of vaccination with any factor was evident ($P > 0.5$).

Post-milking teat dipping and vaccination were applied concurrently in 8 farms; in these, prevalence of subclinical mastitis and staphylococcal subclinical mastitis was found to be smaller (0.181 and 0.123, respectively) than in other farms (0.266 and 0.196, respectively) ($P = 0.019$ and $P = 0.025$); prevalence was also smaller than in farms in which only either of these practices was being applied. Odds ratio for prevalence of subclinical mastitis in farms in which post-milking teat dipping and vaccination were applied concurrently, was 0.608 (0.399 - 0.925, $P = 0.020$), compared to those in which neither practice was applied.

Results of multivariable analysis

The findings regarding the various potential predisposing factors for subclinical or staphylococcal subclinical mastitis, with $P \leq 0.2$ in the univariable models, are summarised in Table II.vii. Results of the multivariable mixed-effects analysis are in Table II.viii.

Table II.vii. Summary of findings regarding the various potential predisposing factors for subclinical or staphylococcal subclinical mastitis (only factors with $P \leq 0.2$ in the univariable models are presented).

Variable	Subclinical mastitis			Staphylococcal subclinical mastitis		
	Prevalence	Odds ratio (95% CI)	*P*	Prevalence	Odds ratio (95% CI)	*P*
Management system practiced in flocks			<0.001			<0.001
Intensive (n=26)	0.254	1.576 (0.944-2.627)	0.082	0.179	1.763 (0.950-3.273)	0.072
Semi-intensive (n=57)	0.296	1.946 (1.193-3.172)	0.008	0.224	3.083 (1.709-5.561)	<0.001
Semi-extensive (n=22)	0.196	1.125 (0.664-1.907)	0.662	0.141	1.321 (0.699-2.497)	0.352
Extensive (n=6)	0.178	reference		0.110	reference	
Stage of lactation period of ewes in flock			0.004			0.001
1st month pp[1] (n=39)	0.223	reference		0.159	reference	
2nd month pp (n=22)	0.305	1.533 (1.170-2.009)	0.002	0.215	1.448 (1.068-1.964)	0.017
3rd and 4th months pp (n=23)	0.246	1.138 (0.871-1.488)	0.343	0.178	1.144 (0.845-1.548)	0.384
5th month pp and thereafter (n=27)	0.292	1.439 (1.118-1.852)	0.048	0.227	1.546 (1.1169-2.046)	0.002
Suckling lambs or milking		0.004				0.028
Ewes suckling lambs (n=13)	0.214	reference		0.167	1.058 (0.707-1.582)	0.786
Milking ewes (n=70)	0.282	1.438 (1.041-1.985)	0.028	0.207	1.383 (1.063-1.799)	0.016
Ewes suckling lambs and milking concurrently (n=28)	0.227	1.337 (1.061-1.686)	0.014	0.159	reference	
Application of post-milking teat dipping			0.014			0.007
Application of post-milking teat dipping (n=12)	0.196	reference		0.128	reference	
No application of post-milking teat dipping (n=99)	0.268	1.504 (1.073-2.107)	0.018	0.198	1.689 (1.133-2.517)	0.010
Anti-mastitis vaccination			0.028			0.217
Vaccination (n=24)	0.221	reference		0.167	reference	
No vaccination (n=83)	0.272	1.319 (1.036-1.679)	0.025	0.197	1.224 (0.935-1.601)	0.142

1. pp: post-partum.

Table II.viii. Results of multivariable analysis for prevalence of subclinical mastitis in ewes in Greece (mixed effects logistic regression with farm as random effect).

Variable	Odds Ratio (95% CI)[1]	*P*
Management system practiced in flocks		0.008
Intensive	2.002 (0.896 – 4.470)	0.090
Semi- intensive	2.481 (1.145 -5.352)	0.021
Semi- extensive	1.292 (0.574 – 2.906)	0.536
Extensive	(reference)	
Stage of lactation period of ewes in flock		0.151
1st month post-partum	(reference)	
2nd month post-partum	1.682 (1.084 – 2.612)	0.020
3rd and 4th months post-partum	1.159 (0.753 – 1.783)	0.502
5th month post-partum and thereafter	1.225 (0.808 – 1.859)	0.339
Application of post-milking teat-dipping		0.054
Application of post-milking teat dipping	(reference)	
No application of post-milking teat dipping	1.695 (0.989 - 2.905)	0.055

Breed as predisposing factor for subclinical mastitis

Of the 111 farms, 58 included pure-bred animals (33 with Greek breeds and 25 with imported breeds). The other 53 farms included cross-bred animals. In farms with intensive management system, pure-bred animals prevailed (17 / 26, 0.654), of which most were imported (11 / 17, 0.647). Pure-breeds also prevailed in semi-extensive or extensive management system (16 / 28, 0.571), but most were Greek breeds (14 / 16, 0.875). In farms with semi-intensive management system, cross-breeds prevailed (32 / 57, 0.561). Details of breeds in farms are in Table ii.ix.

Difference in prevalence of subclinical mastitis between farms with pure-bred and farms with cross-bred animals (0.276 and 0.243, respectively) was not significant (P = 0.144). Difference in prevalence of subclinical mastitis between farms with Greek pure-bred animals and farms with imported pure-bred animals (0.284 and 0.265, respectively) also was not significant (P = 0.240). Not significant was also the difference in prevalence between farms with imported pure-bred animals and all other farms (0.265 and 0.259, respectively) (P = 0.125). Similarly, differences in prevalence between farms with Greek pure-bred animals, farms with imported pure-bred animals and farms with cross-bred animals (0.284, 0.265 and 0.243, respectively) were not significant (P = 0.123).

Table II.ix. Breeds in 111 sheep farms visited across Greece (classified according to management system applied in the 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

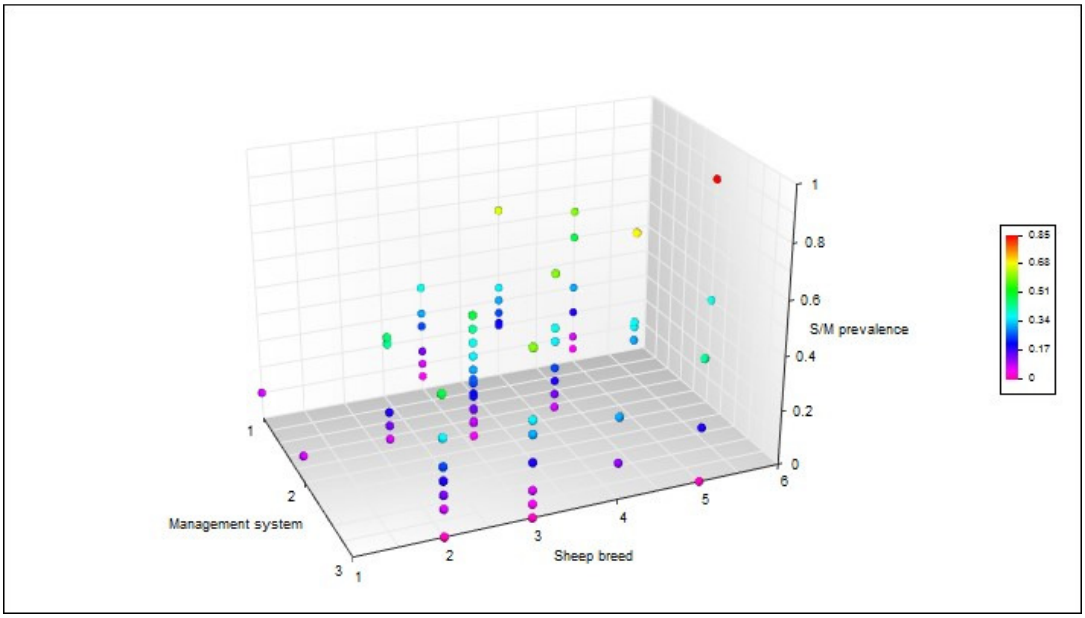
When farms with the various pure-breeds were considered, it became evident that prevalence of subclinical mastitis was significantly smaller in farms with Assaf-breed sheep and significantly higher in farms with Frisarta-breed sheep ($P < 0.02$ for both comparisons). Further, there was significantly smaller prevalence in farms with Karystos-breed sheep ($P = 0.045$) and a tendency for higher prevalence in farms with Chios-breed sheep ($P = 0.125$). When farms with the six Greek traditional indigenous breeds were clustered together, it emerged that prevalence of subclinical mastitis was significantly smaller in that cluster ($P = 0.007$). All other evaluations did not yield significant differences ($P > 0.250$). Details are in Table II.x and Figure II.3.

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

Table II.x. Findings regarding breed as a potential predisposing factor for subclinical mastitis (factors with $P \leq 0.2$ in the univariable models are presented).

Sheep breeds (no. of farms)	Prevalence	Odds ratio (95% CI)	*P*
Greek breeds considered individually			
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
Greek traditional indigenous breeds clustered together			
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
Imported breeds considered individually			
Assaf (n = 2)	0.100	Reference	
Lacaune (n = 23)	0.280	1.554 (0.510 - 4.736)	0.439

Figure II.3. Scatter plot of results of subclinical mastitis prevalence (z axis[1]) against management system applied in farms (x axis[2]) and sheep breed (y axis[3]) in 111 sheep farms in Greece.



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

Significance of mammary carriage

There was a tendency for association between prevalence of subclinical mastitis and mammary carriage in the same farm (r = 0.128, P = 0.09), which was stronger between prevalence of staphylococcal subclinical mastitis and staphylococcal mammary carriage (r = 0.143, P = 0.067).

Prevalence of mammary carriage was smaller in farms in which post-milking teat dipping was applied (0.097) than in farms in which it was not (0.119). Similarly, in farms in which anti-mastitis vaccination was applied, prevalence of mammary carriage and staphylococcal mammary carriage (0.088 and 0.050, respectively) was smaller than in farms in which it was not (0.101 and 0.066, respectively) (for all above comparisons: 0.05 < P < 0.10).

Use of ancillary tests for diagnosis of mastitis

In most farms (n = 89), no routine diagnostic laboratory examinations were performed to assist in diagnosis of mastitis. In these, prevalence of mastitis was smaller (0.251) than in farms in which ancillary tests were performed (0.296) (P = 0.22).

Biofilm formation by staphylococcal isolates

In vitro biofilm formation

Of the 708 staphylococcal isolates, 262 (0.370, 95% CI: 0.335 - 0.406) were characterised as biofilm-forming, 227 (0.321, 95% CI: 0.287 - 0.356) as weak biofilm-forming and 219 (0.309, 95% CI: 0.276 - 0.344) as non biofilm-forming. Of the 531 subclinical mastitis-associated isolates, 199 (0.375) were characterised as biofilm-forming, 173 (0.326) as weak biofilm-forming and 159 (0.299) as non biofilm-forming; respective figures for the 177 mammary carriage-associated isolates were 63 (0.356), 54 (0.305) and 60 (0.339). The results did not provide evidence of association of isolate source with biofilm formation (P = 0.61).

Further, more *S. aureus* isolates were biofilm-forming (79 / 88, 0.898) than coagulase-negative isolates (410 / 620, 0.661) (P < 0.001). Also, more *S. epidermidis* isolates were biofilm-

forming (21 /22, 0.955) than any other coagulase-negative isolates (389 / 598, 0.651) ($P < 0.001$). Details are in Table II.xi.

Table II.xi. Frequency (n) of staphylococcal isolates recovered from milk samples of ewes classified according to biofilm formation.

Species	Biofilm-forming isolates	Weak biofilm-forming isolates	Non biofilm-forming isolates	Total
S. aureus	68	11	9	88
S. chromogens	10	9	9	28
S. epidermidis	17	4	1	22
S. simulans	6	6	4	16
S. xylosus	7	2	3	12
S. lentus	1	4	4	9
S. hominis	5	3	0	8
S. caprae	0	1	3	4
S. haemolyticus	1	3	0	4
S. warneri	2	0	0	2
S. capitis	0	1	1	2
S. cohnii	0	0	1	1
S. equorum	0	1	0	1
S. hyicus	1	0	0	1
S. saprophyticus	0	1	0	1
S. schleiferi	0	0	1	1
S. sciuri	1	0	0	1
S. vitulinus	0	1	0	1
S. warneri or saprophyticus	1	0	0	1
Non-fully identified cnS[1]	142	180	183	505
Total	262	227	219	708

1. Coagulase-negative staphylococci.

Detection of genes associated with biofilm formation

In 108 of 116 isolates (0.931, 95% CI: 0.870 - 0.965), at least one gene was detected. The most frequently detected genes were *eno* and *icaB*, in 74 (0.638, 95% CI: 0.547 - 0.720) and 56 (0.483, 95% CI: 0.394 - 0.573) isolates, respectively. Among subclinical mastitis-associated isolates, genes were detected in 91 (0.948), whilst among carriage-associated in 17 (0.850) isolates ($P = 0.14$). A trend was seen that median number of genes detected in subclinical mastitis-associated isolates was 3 and in carriage-associated ones was 2 per isolate ($P = 0.052$).

S. aureus isolates possessed, on average, more genes (median number 4 per isolate) than coagulase-negative isolates (median number 3 per isolate) ($P < 0.001$).

Genes detected more often in *S. aureus* were *icaB* (0.895 of isolates tested, $P < 0.001$), *icaC* (0.947, $P < 0.001$), *icaD* (0.632, $P = 0.004$) and *eno* (0.842, $P = 0.044$). Among coagulase-negative isolates, genes detected more often in *S. epidermidis* were *icaD* (0.500 of isolates tested, $P = 0.048$), *eno* (0.857, $P = 0.033$) and *clf*a (0.500, $P = 0.002$) and those detected more often in *S. simulans* were *icaB* (0.625, $P = 0.023$) and *icaC* (0.500, $P = 0.021$) genes; other comparisons did not single out increased frequency of any other gene in other species ($P > 0.06$). Details according to staphylococcal species are in Table II.xii.

Table II.xii. Frequency (n) of staphylococcal isolates recovered from milk samples of ewes, in which genes associated with biofilm formation were detected.

	Gene							Median no. of
Staphylococcal species	*icaA*	*icaB*	*icaC*	*icaD*	*bap*	*eno*	*clfa*	genes per isolate
S. aureus (n = 19)	7	17	18	12	5	16	7	4
S. chromogens (n = 26)	13	10	7	7	16	12	5	3
S. epidermidis (n = 14)	8	6	5	7	6	12	7	3
S. simulans (n = 16)	5	10	8	5	5	8	2	3
S. xylosus (n = 9)	2	3	1	2	1	8	1	2
Other fully identified cnS[1] (n = 32)	15	10	7	9	12	18	5	2
Total (n = 116)	50	56	46	42	45	74	27	3

1. *S. capitis*, *S. caprae*, *S. cohnii*, *S. equorum*, *S. haemolyticus*, *S. hominis*, *S. lentus*, *S. saprophyticus*, *S. schleiferi*, *S. sciuri*, *S. vitulinus*, *S. warneri*.

Association between gene detection and *in vitro* biofilm formation

When all isolates were considered, there was positive association ($P < 0.01$) of presence of each of *icaA*, *icaB*, *icaC*, *icaD*, *eno* and *clfa* with *in vitro* biofilm formation. Individual genes associated with *in vitro* biofilm formation were: for *S. aureus icaB*, for *S. chromogenes icaA* and for *S. epidermidis icaB*, *icaC*, *icaD* and *clfa* ($P < 0.045$).

The most common combinations of three genes associated with biofilm formation were *icaB* + *icaC* + *eno* (present in 27 isolates) and *icaB* + *icaD* + *eno* and *icaC* + *icaD* + *eno* (each present in 22 isolates). The most common combination of four genes associated with biofilm formation was *icaB* + *icaC* + *icaD* + *eno* (present in 19 isolates). Presence of any three or four gene combination was more frequent in biofilm-forming than in weak biofilm-forming ($P < 0.03$) or in non biofilm-forming ($P < 0.02$) isolates.

Among biofilm-forming isolates, in two isolates no genes had been detected. Further, among non biofilm-forming isolates, genes had been detected in 22.

There was clear evidence of positive correlation between number of genes per isolates and *in vitro* biofilm formation ($r = 0.532$, $P < 0.001$). Overall, in isolates considered to be biofilm-forming, significantly more genes (median 4 per isolate) were detected than in isolates considered to be weak biofilm-forming (median 3 per isolate) or non biofilm-forming (median 2 per isolate) ($P < 0.002$ for all comparisons). When results of staphylococcal species were considered, there was evidence of correlation between number of genes per isolate and biofilm formation for *S. aureus* (median 4, 4, 1 genes per isolate according to biofilm formation status, respectively), *S. chromogenes* (median 3, 3, 2 genes per isolate, respectively) and *S. epidermidis* (median 5, 1, 1 genes per isolate, respectively) ($P < 0.03$).

Prevalence and potential predisposing factors for biofilm staphylococcal subclinical mastitis

In total, 337 ewes were detected with biofilm staphylococcal subclinical mastitis; its prevalence in the population sampled was 0.153 (95% CI: 0.139 - 0.169). Overall, this accounted for 0.589 of all cases of subclinical mastitis and 0.804 of all cases of staphylococcal subclinical mastitis. Within farms into the study, its prevalence varied from 0.000 to 0.500 (median value among farms: 0.105). Moreover, in 104 ewes biofilm staphylococcal mammary carriage was found (prevalence in population sampled: 0.047 [95% CI: 0.039 - 0.057]).

From results of the univariable analysis, the following factors were found to be associated with increased prevalence of biofilm staphylococcal subclinical mastitis: management system applied in flocks ($P = 0.004$), stage of lactation period of ewes ($P = 0.008$), milking mode applied in ewes ($P = 0.017$) and administration of a vaccine containing *S. aureus* expressing biofilm components ($P = 0.040$). Details are in Table II.xiii.

From the multivariable mixed-effects model, milking mode in ewes ($P = 0.017$) and management system in flocks ($P = 0.045$) emerged as the two significant factors for the prevalence of biofilm staphylococcal subclinical mastitis, with limited interaction ($P = 0.204$).

Table II.xiii. Findings regarding the various potential predisposing factors for biofilm staphylococcal subclinical mastitis (only factors with $P \leq 0.2$ in the univariable models are presented).

Variable	Prevalence	Odds ratio (95% CI)	*P*
Management system practiced in flock			0.004
Intensive (n = 26)	0.158	1.583 (1.076 - 2.329)	0.020
Semi-intensive (n = 57)	0.174	1.776 (1.261 - 2.502)	0.001
Semi-extensive (n = 22)	0.106	reference	
Extensive (n = 6)	0.110	1.044 (0.544 - 2.005)	0.896
Stage of lactation period of ewes in flock			0.008
1st month pp[1] (n = 39)	0.127	reference	
2nd month pp (n = 22)	0.201	1.439 (1.261 - 2.400)	< 0.001
3rd or 4th month pp (n = 23)	0.146	1.179 (0.848 - 1.640)	0.327
5th month pp and thereafter (n = 27)	0.160	1.312 (0.957 - 1.798)	0.092
Milking mode applied in ewes in flock			0.017
No milking (n = 13)	0.155	1.146 (0.788 - 1.666)	0.477
Machine-milking (n = 68)	0.138	reference	
Hand-milking (n = 30)	0.188	1.368 (1.061 - 1.763)	0.016
Administration of a vaccine containing *S. aureus* expressing biofilm components applied in flock			0.042
Vaccination (n = 13)	0.110	reference	
No vaccination (n = 98)	0.159	1.533 (1.016 - 2.312)	0.042

1. pp: post-partum.

Evaluation of staphylococcal isolates for resistance to antimicrobial agents

Resistance to antimicrobial agents

Of the 142 isolates, 59 (0.415) were found to be resistant to at least one antimicrobial agent and 8 (0.056 of all isolates, 0.136 of resistant isolates) were found to be multi-drug resistant. The resistant isolates had been recovered in 45 sheep farms of those visited during the study throughout Greece (Figure II.4). There was no difference in frequency of resistance between isolates from different sources (i.e., mastitis or mammary carriage) ($P > 0.35$). There was clear evidence that more coagulase-negative staphylococcal isolates (n = 54 [0.470]) were resistant than *S. aureus* isolates (n = 5 [0.185]) ($P = 0.007$). When resistance rates between *S. aureus* and specific coagulase-negative staphylococcal species were compared, it emerged that these were significantly greater for *S. chromogenes* ($P = 0.027$), *S. epidermidis* ($P = 0.019$), *S. haemolyticus* ($P = 0.043$), *S. hominis* ($P = 0.006$), *S. lentus* ($P = 0.012$) and *S. xylosus* ($P = 0.019$). When resistance rates among the various coagulase-negative staphylococcal species were compared, it emerged that this was significantly

smaller in *S. simulans* ($P = 0.015$) and greater in *S. hominis* ($P = 0.099$) isolates than in isolates of all other species; no other noteworthy differences were recorded ($P > 0.18$ for all other comparisons). Multi-resistant isolates were identified more frequently only among *S. hominis* ($P < 0.025$ in comparison to other species). Details are in Table II.xiv.

Figure II.4. Location of 45 sheep farms around Greece, in which staphylococcal isolates with resistance to antimicrobial agents had been isolated.

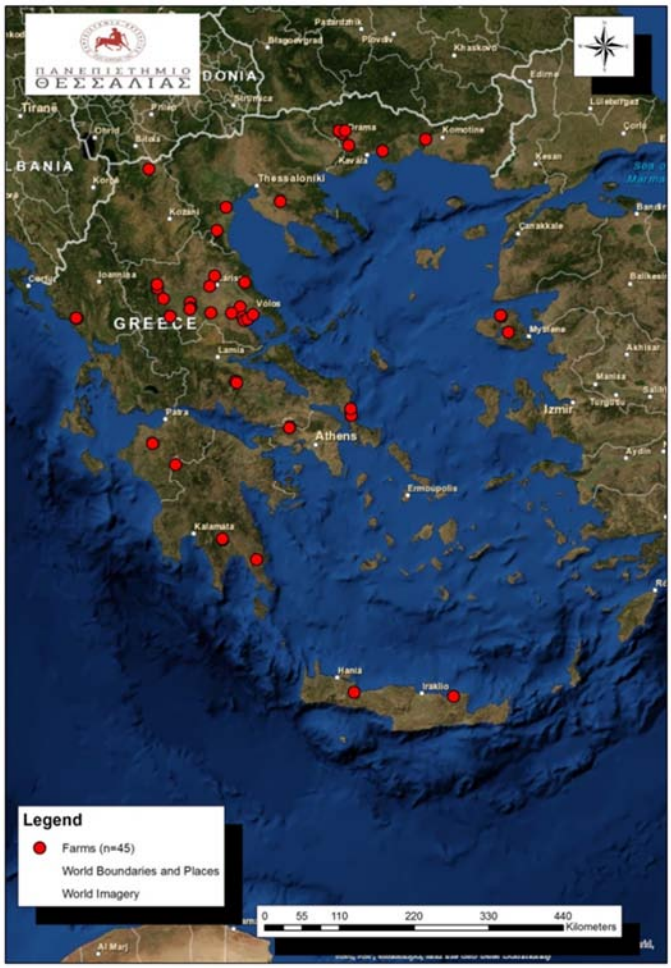


Table II.xiv. Frequency of resistance to antimicrobial agents of staphylococcal isolates from milk samples from ewes.

		Susceptible isolates	Resistance isolates	
			to ≥ 1 agent	to ≥ 3 classes of agents
Origin of isolates				
Subclinical mastitis	118	71 (0.602)	47 (0.398)	6 (0.051, 0.128)[1]
Mammary carriage	24	12 (0.500)	12 (0.500)	2 (0.083, 0.167)
Identity of isolates				
S. aureus	27	22 (0.815)[a,b,c,d,e,f,g]	5 (0.185)[a,b,c,d,e,f,g]	1 (0.037, 0.200)
S. capitis	2	0 (0.000)	2 (1.000)	0 (0.000, 0.000)
S. caprae	4	3 (0.750)	1 (0.250)	0 (0.000, 0.000)
S. chromogenes	28	15 (0.536)[a]	13 (0.464)[a]	0 (0.000, 0.000)
S. cohnii	1	0 (0.000)	1 (1.000)	0 (0.000, 0.000)
S. epidermidis	22	11 (0.500)[b]	11 (0.500)[b]	1 (0.045, 0.091)
S. equorum	1	1 (1.000)	0 (0.000)	0 (0.000, 0.000)
S. haemolyticus	4	1 (0.250)[c]	3 (0.750)[c]	0 (0.000, 0.000)
S. hominis	8	2 (0.250)[d]	6 (0.750)[d]	3 (0.375, 0.500)[a]
S. hyicus	1	1 (1.000)	0 (1.000)	0 (0.000, 0.000)
S. lentus	9	3 (0.333)[e]	6 (0.667)[e]	0 (0.000, 0.000)
S. saprophyticus	1	1 (1.000)	0 (1.000)	0 (0.000, 0.000)
S. schleiferi	1	1 (1.000)	0 (1.000)	0 (0.000, 0.000)
S. sciuri	1	0 (0.000)	1 (1.000)	1 (1.000, 1.000)
S. simulans	16	13 (0.813)[h]	3 (0.187)[h]	0 (0.000, 0.000)
S. vitulinus	1	1 (1.000)	0 (0.000)	0 (0.000, 0.000)
S. warneri	2	2 (1.000)	0 (0.000)	0 (0.000, 0.000)
S. warneri or *saprophyticus*	1	1 (1.000)	0 (0.000)	0 (0.000, 0.000)
S. xylosus	12	5 (0.417)[e]	7 (0.583)[e]	2 (0.167, 0.400)
All coagulase-negative staphylococcal species	115	61 (0.530)[g,h]	54 (0.470)[g,h]	7 (0.061, 0.130)[a]

a-h: same superscript in same column indicates $P < 0.05$.

1. Proportion of all isolates tested, proportion of isolates with resistance to ≥ 1 agent.

Frequency of resistance was greater to penicillin (0.225), tetracycline (0.183) or ampicillin (0.169) ($P < 0.032$ compared to other frequencies). Source of isolates did not account for differences in frequency of resistance to specific antimicrobial agents ($P > 0.12$), bar for resistance to clindamycin, which was more frequent in mammary carriage isolates ($P = 0.004$). When resistance rates to specific antimicrobial agents between *S. aureus* and coagulase-negative staphylococcal species were compared, it emerged that resistance was greater (but not significant) in the latter isolates only against penicillin ($P = 0.088$) or tetracycline ($P = 0.082$). Details are in Table II.xv.

Table II.xv. Frequency of resistance to individual antimicrobial agents of staphylococcal isolates from milk samples from ewes.

		Antimicrobial agents[1]										
	(n)	P	TE	AMP	FOSF	E	CC	FA	AZ	CLA	FOX	SXT
Origin of isolates												
Subclinical mastitis	118	28 (0.237)	20 (0.169)	22 (0.186)	5 (0.042)	8 (0.068)	8[a] (0.068)	7 (0.059)	4 (0.034)	4 (0.034)	3 (0.025)	1 (0.008)
Mammary carriage	24	4 (0.167)	6 (0.250)	2 (0.083)	2 (0.083)	4 (0.167)	7[a] (0.292)	2 (0.083)	0 (0.000)	0 (0.000)	0 (0.000)	0 (0.000)
Identity of isolates												
S. aureus	27	3 (0.111)	2 (0.074)	3 (0.111)	1 (0.037)	2 (0.074)	1 (0.037)	0 (0.000)	2 (0.074)	2 (0.074)	0 (0.000)	0 (0.000)
cnS[2]	115	29 (0.252)	24 (0.209)	21 (0.183)	6 (0.052)	10 (0.087)	14 (0.122)	9 (0.078)	2 (0.017)	2 (0.017)	3 (0.026)	1 (0.009)

a: same superscript in same column indicates $P < 0.01$.

1. AZ: azithromycin, AMP: ampicillin, CC: clindamycin, CLA: clarithromycin, E: erythromycin, FA: fucidic acid, FOSF: fosfomycin, FOX: cefoxitin, P: penicillin, TE: tetracycline, SXT: trimethoprim- sulphamethoxazole.

2. Coagulase-negative staphylococcal species.

In 25 of the 59 isolates (0.424) found to be resistant to antimicrobial agents, no relevant genes have been detected. There was no difference between isolates from subclinical mastitis (21 / 47, 0.446) or mammary carriage (4 / 12, 0.333) ($P = 0.48$). Also, there was no difference between *S. aureus* (2 / 5, 0.400) or coagulase-negative staphylococcal species (23 / 54, 0.426) isolates ($P = 0.65$).

The most frequently detected gene associated with resistance to antimicrobial agents was *tetK* (in 18 isolates). In total, genes of the *tet* operon were detected in 24 isolates; genes encoding resistance to tetracycline (*tekK*, *tetL*, *tetM*, *tetS*, *tetT*) were the most frequently detected. Genes *ermA*, *ermT*, *lnuA* and *lnuC* were not detected in any isolate. Although, on average, more resistance genes were detected in isolates from mammary carriage than in isolates from subclinical mastitis (0.542 *versus* 0.316), differences were not significant ($P = 0.21$). Within the various species, in *S. epidermidis* and *S. hominis* were detected, on average, more resistance genes than in other species (0.818 and 0.875, respectively) ($P < 0.025$). Details are in Table II.xvi.

No associations were found between biofilm formation and resistance among the isolates (resistant and multi-drug resistant), when results of resistance to all antimicrobial agents were taken into account ($P > 0.40$). Results of detailed analysis of resistance to tetracycline revealed some association ($P = 0.082$) for all isolates, independently of source and identity. This was stronger in isolates from subclinical mastitis: 0.206 of the biofilm-forming isolates were resistant to tetracycline, whilst no non biofilm-forming isolate was ($P = 0.013$), as well as in the coagulase-negative staphylococcal isolates: 0.261 of biofilm-forming isolates were resistant to tetracycline *versus* 0.037 of non biofilm-forming isolates ($P = 0.012$). Details are in Table II.xvii. For resistance to other antimicrobial agents, there was no association with biofilm formation ($P > 0.12$ for all comparisons).

Detailed analysis of associations of tetracycline resistance-encoding genes and biofilm formation relevant genes provided evidence of association of presence of *tetK* and *icaA* ($P = 0.029$). Other associations between genes were not identified ($P > 0.22$). Among tetracycline-resistant staphylococcal isolates, no association between *tet* operon and *ica* operon was evident ($P > 0.70$).

Table II.xvi. Frequency of genes associated with resistance to antimicrobial agents detected in staphylococcal isolates from milk samples from ewes.

		Genes[1]											
	(n)	*ermB*	*ermC*	*lnuB*	*lsaA*	*mphC*	*msrA*	*tetK*	*tetL*	*tetM*	*tetS*	*tetT*	*vgaA*
Origin of isolates													
Subclinical mastitis	118	3 (0.025)	3 (0.025)	0	1 (0.008)	2 (0.017)	2 (0.017)	15 (0.127)	3 (0.025)	2 (0.017)	1 (0.008)	3 (0.025)	0
Mammary carriage	24	1 (0.042)	4 (0.167)	1 (0.042)	0	0	0	3 (0.125)	2 (0.083)	1 (0.042)	0	1 (0.042)	1 (0.042)
Identity of isolates													
S. aureus	27	1 (0.037)	0	0	0	0	1 (0.037)	2 (0.074)	1 (0.037)	0	0	0	0
S. capitis	2	0	0	0	0	0	0	1 (0.500)	0	0	0	0	0
S. chromogenes	28	2 (0.071)	0	0	0	0	0	0	1 (0.036)	2 (0.071)	0	0	0
S. epidermidis	22	0	2 (0.091)	1 (0.045)	0	0	0	10 (0.455)	0	0	1 (0.045)	4 (0.182)	0
S. haemolyticus	4	0	0	0	0	0	0	1 (0.250)	1 (0.250)	0	0	0	0
S. hominis	8	0	2 (0.250)	0	0	0	1 (0.125)	3 (0.375)	0	1 (0.125)	0	0	0
S. lentus	9	1 (0.111)	1 (0.111)	0	1 (0.111)	2 (0.222)	0	0	1 (0.222)	0	0	0	1 (0.222)
S. sciuri	1	0	0	0	0	0	0	0	1 (1.000)	0	0	0	0
S. xylosus	12	0	2 (0.167)	0	0	0	0	1 (0.083)	0	0	0	0	0
cnS[2,3]	115	3 (0.026)	7 (0.061)	1 (0.009)	1 (0.009)	2 (0.017)	1 (0.009)	16 (0.139)	4 (0.035)	3 (0.026)	1 (0.009)	4 (0.035)	1 (0.009)

1. Genes *ermA*, *ermT*, *lnuA* and *lnuC* not detected in any isolate.

2. Coagulase-negative staphylococci.
3. In species not mentioned in the table, no genes associated with resistance to antimicrobial agents were detected.

Table II.xvii. Frequency of resistance to tetracycline in relation to biofilm-formation by staphylococcal isolates from milk samples from ewes.

		Biofilm-forming		Non biofilm-forming	
		Resistant	Susceptible	Resistant	Susceptible
Origin of isolates					
Subclinical mastitis	118	20 (0.206)[a]	77 (0.794)[b]	0 (0.000)[a]	21 (1.000)[b]
Mammary carriage	24	5 (0.417)	12 (0.583)	1 (0.143)	6 (0.857)
Identity of isolates					
S. aureus	27	2 (0.077)	24 (0.923)	0 (0.000)	1 (1.000)
Coagulase -ve staphylococci	115	23 (0.261)[a]	65 (0.739)[b]	1 (0.037)[a]	26 (0.963)[b]

a, b: same superscript in same row indicates $P < 0.03$.

Factors associated with presence of resistance in staphylococcal isolates

Resistant isolates were isolated more frequently during the period immediately *post-partum*; 0.766 of resistant isolates *versus* 0.577 of susceptible ones were recovered during that period ($P = 0.035$). Further, more resistant isolates originated from farms that practiced routine administration of antimicrobial agents at the end of a lactation period, i.e., before the dry-period: 0.234 *versus* 0.099 of susceptible isolates ($P = 0.045$). There were no further differences in proportion of susceptible / resistant isolates in relation to other factors evaluated ($P > 0.32$). Details are in Table II.xviii.

Table II.xviii. Factors applied in sheep farms in which staphylococcal isolates were isolated from milk samples collected from ewes therein.

	Susceptible isolates (n = 71)	Resistant isolates (n = 47)
Management system applied in farms		$P = 0.900^2$
Intensive management	16 (0.225)	10 (0.213)
Semi-intensive management	41 (0.578)	29 (0.617)
Semi-extensive or extensive management	14 (0.197)	8 (0.170)
Stage of lactation period in farm during sample collection		$P = 0.035$
1st month *post-partum*	30 (0.423)	11 (0.234)
2nd month *post-partum* and thereafter	41 (0.577)	36 (0.766)
Milking technique		$P = 0.327$
Machine-milking	42 (0.592)	32 (0.681)
Hand-milking	29 (0.408)	15 (0.319)
Application of post-milking teat dipping		$P = 0.438$
Teat-dipping	8 (0.113)	4 (0.085)
No teat-dipping	63 (0.887)	43 (0.915)
Intramammary administration of antimicrobial agents at beginning of dry-period		$P = 0.045$
Administration of antimicrobials	7 (0.099)	11 (0.234)
No administration of antimicrobials	64 (0.901)	36 (0.766)
Anti-staphylococcal mastitis vaccination		$P = 0.841$
Anti-staphylococcal vaccination	20 (0.282)	14 (0.298)
No anti-staphylococcal vaccination	51 (0.718)	33 (0.702)

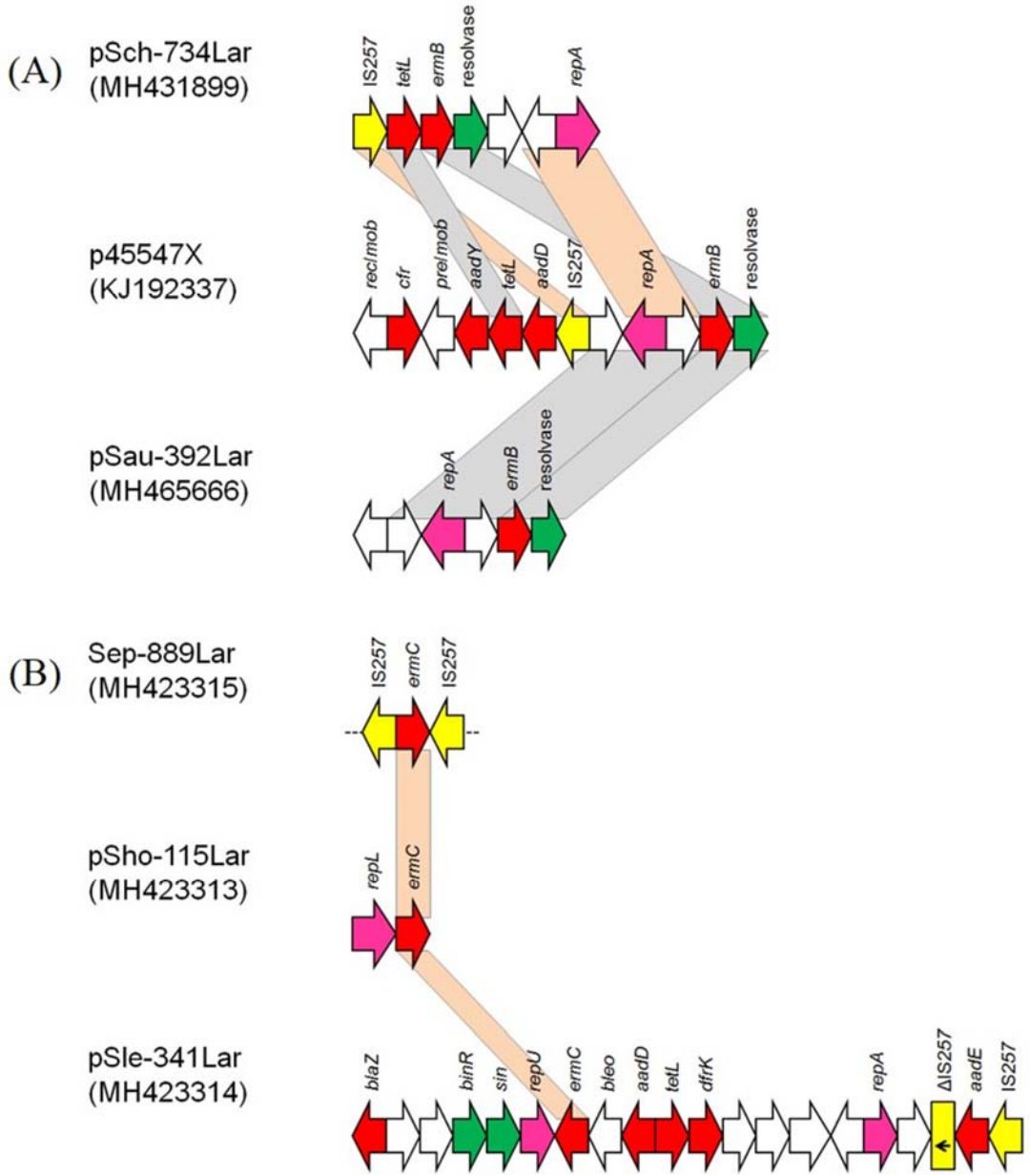
Mobile elements associated with resistance genes

Analysis of whole genome sequencing data showed that in a *S. lentus* strain the *ermB* gene was part of the transposon Tn*917* integrated into the chromosome. Tn*917*, which was originally described from *Enterococcus faecalis* DS16 (Shaw and Clewell 1985), has been mainly associated with the spread of *ermB* (Kadlec et al. 2012). Despite the fact that *ermB* is chromosomally located in most staphylococci isolates, two *ermB*-carrying plasmids (pSau-392Lar and pSch-734Lar) were found in a ST133 *S. aureus* strain (Sau-392Lar) and a *S. chromogenes* strain (Sch-734Lar). Plasmid pSau-392Lar, which was 7,779 bp in size, was highly similar to *ermB*-carrying plasmid p45547X (84% coverage, 99% identity) (Figure II.5), characterised from a ST398 methicillin-sensitive *S. aureus* strain isolated from a human patient in Brazil (Gales et al. 2015) No resistance genes other than *ermB* were identified in pSau-392Lar. Plasmid pSch-734Lar, which was also a derivative of p45547X, was a 10,252-bp molecule. pSch-734Lar also carried the *tetL* gene, conferring resistance to tetracyclines.

Further, sequencing data showed that a small plasmid (pSho-115Lar) of ~2.5 kb was observed in an *ermC*-carrying *S. hominis* strain (Sho-115La). Plasmid pSho-115Lar comprised the *repL* and *ermC* genes. Previous studies have reported the presence of small (2.3–2.5 kb) *ermC*-carrying plasmids, like pNE131 (Lampson and Parisi 1986), in staphylococcal isolates (Wendlandt et al. 2014). However, in a *S. lentus* strain (Sle-341Lar), the *ermC* gene was carried on a plasmid (pSle-341Lar), which was 20,992 bp in size. Plasmid pSle-341Lar, which exhibited limited nucleotide similarity to previously characterised plasmids, carried additional genes for resistance to *β*-lactams (*blaZ*), aminoglycosides (*aadD* and *aadE*), trimethoprim (*dfrK*) and tetracyclines (*tetL*) (Figure II.5). In a *S. epidermidis* strain, the *ermC* gene was surrounded by two copies of the IS*257* element in parallel orientation, forming a composite transposon (Papagiannitsis et al. 2018).

Finally, *de novo* assembly and analysis of sequencing data showed that, in a *S. aureus* and a *S. epidermidis* strains, small *tetK*-carrying plasmids (pSau-2716Lar and pSau-3893Lar) of 4.439 kb were found. Both plasmids, which comprised the *repC*, *pre* and *tetK* genes, were identical to small *tetK*-carrying plasmids, like pT181 (Khan and Novick 1983), previously characterised from staphylococcal isolates. Details of genes detected during whole genome analysis of these strains are in Table II.xix.

Figure II.5. Linear maps of mobile elements carrying erm-like genes.



Arrows show the direction of transcription of open reading frames (ORFs), while truncated ORFs appear as rectangles (arrows within rectangles indicate the direction of transcription); replicons of the plasmids are shown in pink, resistance genes are shown in red and insertion sequence elements and transposases are shown in yellow and green, respectively; homologous segments (representing ≥ 99% sequence identity) are indicated by light gray shading, while orange shading shows inverted homologous segments

Table II.xix. Genes associated with resistance to antimicrobial agents detected in staphylococcal isolates from milk samples from ewes.

	Class of antimicrobial agents						
	aminoglycosides	*β*-lactams	fosfomycin	MLS_B[1]	tetracycline	trimethoprim	fluoroquinolones
Origin: subclinical mastitis							
S. aureus	*ermB*	*tetK*	*norA*				
S. chromogenes	*ermB*	*tetL*					
S. hominis	*blaZ*	*fosA*	*emrC*				
S. hominis	*aadK*	*blaZ*	*fosA*	*emrC*			
S. lentus	*mphC*	*mphC*, *emrB*					
S. lentus							
S. xylosus	*mphC*						
Origin: mammary carriage							
S. chromogenes				*ermB*	*tetL*		
S. epidermidis	*aadE*, *aadD2*	*blaZ*	*fosA*	*emrC*, *lnuB*	*tetK*	*dfrG*	
S. lentus	*aadK, aadD2*	*blaZ*		*vgaA*, *ermC*	*tetL*	*dfrK*	

1. macrolides, lincosamides, streptogramine B

MLST staphylococcal types

Most *S. aureus* isolates (14 of the 27 isolates examined [0.519]) were classified in ST133. Other types identified were ST2011 (3 isolates), ST30 (2 isolates) and ST5, ST7, ST130, ST581, ST700 and ST2111 (1 isolate in each); two new types were identified: ST 4332 and ST 4333. Most *S. epidermidis* isolates were classified in ST100, ST142 or ST152: 4 isolates in each one. Other types identified were ST59, ST 153, ST200, ST225, ST226, ST315, ST454 (1 isolate in each); two new types were identified: 678 (2 isolates) and 711.

Most *S. aureus* isolates (n = 2) resistant to antimicrobial agents were ST133 (0.400 of resistant isolates). Further, most *S. epidermidis* resistant isolates were ST142 (n = 3) or ST 152 (n = 3) (together, 0.545 of resistant isolates). However, there was no significant pattern of association of strain type with resistance to antimicrobial agents ($P > 0.09$).

Finally, there was no pattern of association of strain type with biofilm formation.

Virulence-associated genes in *S. aureus*

Analysis of whole genome sequencing data by VirulenceFinder 1.5 tool (Zankari et al. 2012) showed that in a ST133 *S. aureus* isolate the following virulence genes were present: *hlgA*, *hlgB*, *hlgC*, *lukD*, *lukE*, *sec*, *sel*, *tst*, *aur*, *splA* and *splB*. These could be implicated in colonisation and dissemination within the mammary gland of the host (Bonar et al. 2015).

Detection of anti-PNAG antibodies in blood samples

Staphylococcal subclinical mastitis and seropositivity

In total 90 cases of subclinical mastitis were detected in the 355 ewes. Among these, 44 cases of biofilm staphylococcal subclinical mastitis were detected (0.489 of all cases of subclinical mastitis). Prevalence of this infection was 0.124 (95% CI: 0.094 – 0.162). Of these, 38 were detected in non-vaccinated (prevalence: 0.129) and 6 in vaccinated ewes (prevalence: 0.100). Moreover, 8 cases of biofilm staphylococcal mammary carriage were also detected (prevalence: 0.023, 95% CI: 0.011 – 0.044); all were in non-vaccinated ewes (prevalence: 0.027). Details are in Table xx.

Table II.xx. Association of anti-PNAG antibody titres in blood with isolation of biofilm-forming staphylococci from mastitis in ewes in a field investigation in Greece.

		Cases of mammary infection with biofilm-forming staphylococci			
		subclinical mastitis	mammary carriage	no mammary infection	total
Non-vaccinated ewes					
Cases with anti-PNAG antibody titres	+	24	6	105	135
	-	14	2	144	160
	total	38	8	249	295
Vaccinated ewes					
Cases with anti-PNAG antibody titres	+	5	0	40	45
	-	1	0	14	15
	total	6	0	54	60

Anti-PNAG antibody titres above the cut-off point (6.0 RIPC) were detected in samples from 180 ewes, i.e., prevalence of seropositivity among all ewes sampled was 0.507 (95% CI: 0.455 – 0.559). Prevalence of seropositivity was significantly higher among vaccinated (0.750) than non-vaccinated (0.458) ewes ($P < 0.001$) (Table II.xx). Further, median prevalence of seropositivity in farms in which vaccination had been performed (0.800) was higher than in farms in which vaccination had not been performed (0.400) ($P < 0.001$). Among non-vaccinated ewes, there was evidence that biofilm staphylococcal subclinical mastitis was associated with seropositivity ($P = 0.021$) (Table II.xx).

Mean antibody titres in samples from vaccinated ewes (13.64 ± 1.45) were significantly higher than in samples among non-vaccinated ones (7.39 ± 0.56) ($P < 0.001$). Within vaccinated and non-vaccinated ewes, there was no difference among ewes with mastitis, mammary carriage or no infection ($P > 0.09$). In contrast, antibody titres in samples from vaccinated ewes with no infection (13.59 ± 1.55) were significantly higher than in non-vaccinated ewes with mastitis (8.71 ± 0.88) ($P = 0.004$). Details are in Table II.xxi.

Table II.xxi. Mean anti-PNAG antibody titres in blood of ewes in a field investigation in Greece.

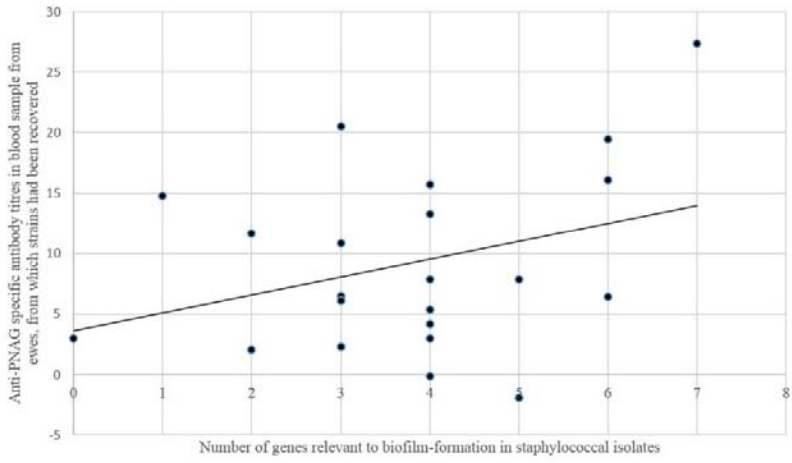
	Cases of mammary infection with biofilm-forming staphylococci			
	subclinical mastitis	mammary carriage	no mammary infection	total
vaccinated ewes	14.08 ± 4.11^{a}	-	$13.59 \pm 1.55^{b,d}$	13.64 ± 1.45^{c}
non-vaccinated ewes	$8.71 \pm 0.88^{a,d}$	9.01 ± 1.04	7.13 ± 0.65^{b}	7.39 ± 0.56^{c}

a: $P = 0.13$, b: $P < 0.001$, c: $P < 0.001$, d: $P = 0.004$, for comparisons between results with same superscript.

There was no association between identity of staphylococcal isolates recovered from milk samples and antibody titres in blood samples from respective ewes. Median values of antibody titres were as follows for the main mastitis causal staphylococcal species: 11.99 for ewes from which *S. aureus* was recovered, 12.65 for ewes from which *S. epidermidis* was recovered and 9.04 for ewes from which *S. simulans* was recovered ($P > 0.85$).

There was a trend for correlation between total number of genes relevant to biofilm formation in staphylococcal isolates recovered from milk samples and antibody titres in blood samples from respective ewes: $r = 0.401$ ($P = 0.065$) (Figure II.6); the correlation was stronger when only isolates recovered from vaccinated ewes were taken into account: $r = 0.922$ ($P = 0.035$). Isolates from ewes with seropositive samples had higher median number of genes (4) than isolates from ewes with seronegative samples (3), but difference was not significant ($P = 0.14$). In no case, presence of a particular gene relevant to biofilm formation in staphylococcal isolates was associated with seropositivity or antibody titres in blood samples from respective ewes ($P > 0.075$ for all comparisons).

Figure II.6. Number of genes relevant to biofilm-formation in staphylococcal isolates recovered from mammary infections in relation to anti-PNAG antibody titres in blood samples from respective ewes ($r = 0.336$; $P = 0.063$).



Factors associated with seropositivity

From results of the univariable analysis of predictors among ewes in the study, management system applied in flock ($P = 0.011$), milking mode applied in animals ($P = 0.010$) and anti-

staphylococcal mastitis vaccination (P = 0.005) were found to be associated with increased prevalence of seropositivity (Table II.xxii).

From the multivariable mixed-effects model, vaccination (P = 0.019) emerged as the only significant factor for the prevalence of seropositivity of anti-PNAG antibody titres.

From results of the multivariable mixed-effects model analysis of predictors only among the non-vaccinated ewes in the study, no factor was found of significance for the prevalence of seropositivity of anti-PNAG antibody titres among these ewes (P > 0.22) (Table II.xxiii).

Table II.xxii. Findings regarding potential predisposing factors for prevalence of seropositivity of anti-PNAG antibody titres (only factors with $P \leq 0.2$ in the univariable models are presented).

Variable	Prevalence	Odds ratio (95% CI)	*P*
Management system practiced in flock			0.011
Intensive (n = 19)	0.611	2.233 (1.217 – 4.096)	0.010
Semi-intensive (n = 36)	0.450	1.165 (0.684 – 1.986)	0.574
Semi-extensive or extensive (n = 16)	0.413	reference	
Milking mode applied in ewes in flock			0.010
Machine-milking (n = 46)	0.535	1.783 (1.145 – 2.776)	0.011
Hand-milking (n = 25)	0.392	reference	
Administration of a vaccine containing *S. aureus* expressing biofilm components applied in flock			0.005
Vaccination (n = 12)	0.600	2.262 (1.269 – 4.032)	0.006
No vaccination (n = 59)	0.451	reference	

Table II.xxiii. Findings regarding potential predisposing factors for prevalence of seropositivity of anti-PNAG antibody titres in non-vaccinated ewes (only factors with $P \leq 0.2$ in the univariable models are presented).

Variable	Prevalence	Odds ratio (95% CI)	*P*
Management system practiced in flock			0.150
Intensive (n = 16)	0.538	1.860 (0.956 – 3.618)	0.068
Semi-intensive (n = 30)	0.433	1.224 (0.675 – 2.218)	0.506
Semi-extensive or extensive (n = 13)	0.385	reference	
Milking mode applied in ewes in flock			0.038
Machine-milking (n = 30)	0.487	1.665 (1.028 – 2.696)	0.038
Hand-milking (n = 21)	0.371	reference	

Significance of environmental parametres

Environmental parametres in sheep farms

Significant differences ($P \leq 0.04$) in environmental parametres prevailing in locations of farms into the study, were identified for 13 parametres between locations of farms with intensive management and farms with semi-extensive / extensive management and for 11 parametres between locations of farms with semi-intensive management and farms with semi-extensive / extensive management. No differences ($P > 0.13$) were evident for any parametre between locations of farms with intensive management and farms with semi-intensive management. Results are in Table xxiv. Further, no differences were seen in altitude (194 ± 30 m, 190 ± 22 m, 151 ± 26 m for farms with intensive, semi-intensive or semi-extensive / extensive management, respectively) or distance between sheep farms (328 ± 39, 313 ± 26, 370 ± 50 m, respectively) ($P > 0.085$).

Table II.xxiv. Environmental conditions (mean ± standard error of the mean) prevailing in 111 sheep farms visited across Greece.

Code	Environmental variable	Farm management system		
		Intensive (n = 26)	Semi-intensive (n = 57)	Semi-extensive / extensive (n = 28)
clima1	Annual mean temperature (ºC)	15.4 ± 0.2^{A}	15.9 ± 0.2^{B}	16.8 ± 0.3^{A,B}
clima2	Mean diurnal temperature range (ºC)	10.9 ± 0.2^{A}	10.1 ± 0.2^{B}	8.7 ± 0.4^{A,B}
clima3	Isothermality (*clima2* / *clima7* × 100)	35.2 ± 0.4^{A}	34.5 ± 0.3^{a}	33.0 ± 0.3^{a,A}
clima4	Temperature seasonality (ºC)	6799 ± 128^{A}	6553 ± 97^{a}	6122 ± 141^{a,A}
clima5	Maximum temperature of warmest month (ºC)	32.2 ± 0.4	31.8 ± 0.3	30.8 ± 0.3
clima6	Minimum temperature of coldest month (ºC)	1.8 ± 0.3^{A}	3.0 ± 0.4^{B}	5.1 ± 0.6^{A,B}
clima7	Temperature annual range (ºC)	30.4 ± 0.6^{A}	28.9 ± 0.5^{B}	25.8 ± 0.8^{A,B}
clima8	Mean temperature of wettest quarter (ºC)	9.0 ± 0.4^{a}	9.6 ± 0.3	10.5 ± 0.4^{a}
clima9	Mean temperature of driest quarter (ºC)	23.9 ± 0.2	24.2 ± 0.2	24.7 ± 0.2
clima10	Mean temperature of warmest quarter (ºC)	15.4 ± 0.2^{A}	15.9 ± 0.2^{a}	16.8 ± 0.3^{a,A}
clima11	Mean temperature of coldest quarter (ºC)	6.9 ± 0.3^{A}	7.8 ± 0.3^{B}	9.3 ± 0.4^{A,B}
clima12	Total annual precipitation (mm)	643 ± 30	654 ± 19	651 ± 29
clima13	Total precipitation of wettest month (mm)	98 ± 7	106 ± 4	121 ± 9
clima14	Total precipitation of driest month (mm)	14 ± 1^{A}	12 ± 1^{B}	7 ± 1^{A,B}
clima15	Precipitation seasonality (%)	46.4 ± 2.9^{A}	53.5 ± 2.5^{B}	66.6 ± 3.8^{A,B}
clima16	Total precipitation of wettest quarter (mm)	263 ± 18	282 ± 12	318 ± 23
clima17	Total precipitation of driest quarter (mm)	60 ± 3^{A}	56 ± 4^{B}	36 ± 5^{A,B}
clima18	Total precipitation of warmest quarter (mm)	64 ± 4^{A}	12 ± 1^{B}	7 ± 1^{A,B}
clima19	Total precipitation of coldest quarter (mm)	244 ± 18	261 ± 11	289 ± 20

^{a,b} between columns: $0.01 < P \leq 0.04$, ^{A,B} between columns: $P < 0.01$.

Significance of environmental parametres for predisposing to subclinical mastitis

During univariable analysis, without consideration of farm as 'random effect', there was evidence of association of some climatic factors with mastitis prevalence; specifically, these were temperature-related parametres: annual mean temperature (P = 0.050), mean diurnal temperature range (P = 0.033), minimum temperature of coldest month (P = 0.031), temperature annual range (P = 0.029), as well as the annual mean wind speed (P < 0.001). Further, a location factor was also relevant, specifically microhabitat type in the area (P = 0.001). For other factors, there was little evidence of association with prevalence of subclinical mastitis. Details are in Tables II.xxv and II.xxvi.

The final multivariable mixed-effects model required the following variables: (a) minimum temperature of coldest month and (b) mean temperature for 30 days prior to sampling date. From the model (P = 0.028), both the minimum temperature of coldest month (coefficient: -0.084 ± 0.033, P = 0.014) and the mean temperature for 30 days prior to sampling date (coefficient: 0.031 ± 0.014, P = 0.029) were significantly associated with prevalence of subclinical mastitis (Figure II.7).

Table II.xxv. Continuous environmental variables used in analysis for potential predisposing factors for subclinical mastitis.

Variable	Source	Mean (±standard error)	P[1]
Annual mean temperature (ºC)	WorldClim Database	16.0 ± 0.1	0.050
Mean diurnal temperature range (ºC)	WorldClim Database	9.9 ± 0.2	0.033
Maximum temperature of warmest month (ºC)	WorldClim Database	31.6 ± 0.2	0.162
Minimum temperature of coldest month (ºC)	WorldClim Database	3.2 ± 0.3	0.031
Temperature annual range (ºC)	WorldClim Database	28.5 ± 0.4	0.029
Mean temperature for 30 days prior to sampling date (ºC)	WorldClim Database	16.3 ± 0.7	0.071
Total annual precipitation (mm)	WorldClim Database	650.5 ± 13.8	0.356
Annual mean wind speed (m s^{-1})	ArcGIS-HRAE	4.1 ± 0.1	<0.001
Mean wind speed for 30 days prior to sampling date (m s^{-1})	ArcGIS-HRAE	7.9 ± 0.3	0.190
Altitude (m)	DEM	181.1 ± 14.4	0.081
Distance from small ruminant farms (m)	ArcGIS- GPACAP	332.6 ± 21.2	0.920
Distance from public health agencies (m)	ArcGIS- GPACAP	8288.4 ± 398.6	0.047

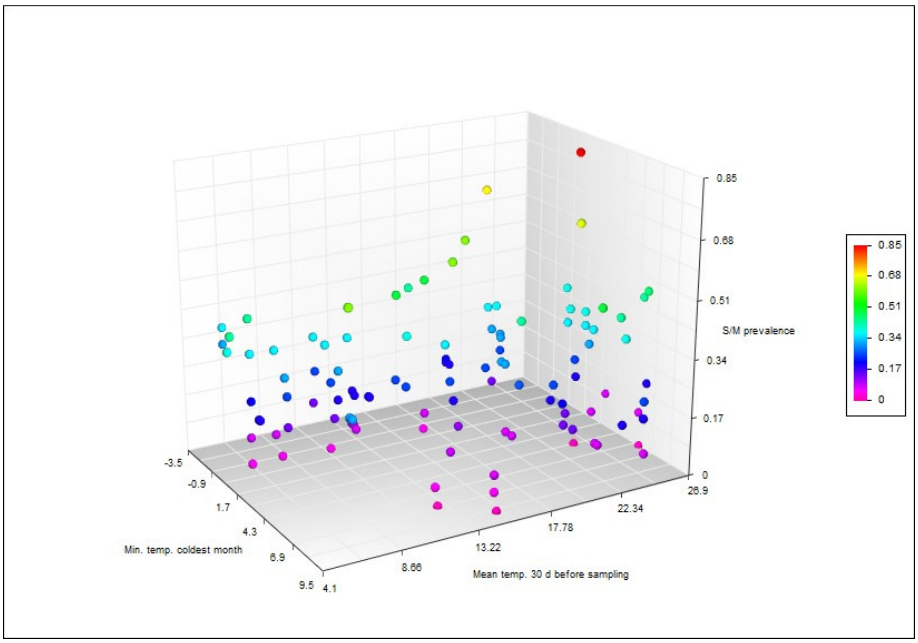
1. P derived during univariable model analysis.

Table II.xxvi. Categorical environmental variables used in analysis for potential predisposing factors for subclinical mastitis.

Variable	Source	Main categories (proportion)	*P*[1]
Land use	ArcGIS-Corine LC (EEA)	Cultivations (0.65), shurbland (0.13), pastures (0.12)	0.132
Microhabitat type	ArcGIS-Corine LC (EEA)	Permanently irrigated land (0.20), complex cultivation patterns (0.18), non-irrigated arable land (0.14), sclerophyllous vegetation (0.09), natural grasslands (0.08), beaches, sands and dunes (0.06)	0.001

1. *P* derived during univariable model analysis.

Figure II.7. Three-dimensional scatter plot of results of subclinical mastitis prevalence [S/M prevalence', z axis) against the two significant environmental factors: minimum temperature of coldest month (ºC) ['Min. temp. coldest month', x axis] and mean temperature of 30 days prior to sampling (ºC) ['Mean temp. 30 d before sampling', y axis].



Predictive Ecological Niche Models

In both analyses, with all farms considered in the analysis, the variable with highest gain when used in isolation, was distance between sheep farms, which appeared to have the most useful information by itself; the same variable was also the one that decreased gain the most when omitted.

Also in both analyses, other variables with increased gain when used in isolation, were maximum temperature of warmest month, altitude and total precipitation of wettest month. Other variables that decreased gain when omitted, were maximum temperature of warmest month, as well as altitude and total precipitation of driest month (subclinical mastitis) and total precipitation of driest month and mean temperature of driest quarter (biofilm staphylococcal subclinical mastitis). The contribution of the variables studied in the MaxEnt model for all farms in the study, is in Table xxvii. Jackknife of regularised training gain test for subclinical mastitis and biofilm staphylococcal subclinical mastitis in these farms is in Figure II.8. Regularised training gain (sum of the likelihood of the data plus a penalty function) was 1.269 and 1.190, training AUC was 0.906 and 0.905 and unregularised training gain was 1.469 and 1.407, for subclinical mastitis and biofilm staphylococcal subclinical mastitis, respectively.

Verification of the model indicated that 61 of 78 (0.782) and 66 of 75 (0.880) 'infected farms' were located in areas predicted as high risk for prevalence of subclinical mastitis or biofilm staphylococcal subclinical mastitis, respectively.

When farms in each management system were considered separately, differences became evident between farms in each management system, as well as between the two infections. Factors with highest relative contribution in each analysis, for farms under intensive, semi-intensive or semi-extensive / extensive management system were, respectively: maximum temperature of warmest month (in both analyses), total precipitation of driest month and maximum temperature of warmest month (for each of the two analyses) and mean temperature of driest quarter (in both analyses). Relative contributions of the variables studied to the MaxEnt model for sheep farms in each management system are in Table xxviii. Jackknife of regularised training gain test for subclinical mastitis in these farms are in Figure II.9. Environmental variables with highest gain when used in isolation, in each analysis, for farms under intensive, semi-intensive or semi-extensive / extensive management system were, respectively: maximum temperature of warmest month and annual mean temperature (for each of the two analyses), annual mean temperature and maximum temperature of warmest month (for each of the two analyses) and mean temperature of driest quarter (in both analyses). Details are in Table xxix and Figure II.10.

Verification of the model indicated that, in total, 61 of 78 (0.782) and 62 of 75 (0.827) 'infected farms' were located in areas predicted as high risk for prevalence of subclinical mastitis and biofilm staphylococcal subclinical mastitis, respectively (Table xxix).

Table II.xxvii. Estimation of relative contributions of the variables to the MaxEnt model for subclinical mastitis and biofilm staphylococcal subclinical mastitis in all sheep farms.

		Subclinical mastitis		Biofilm staphylococcal subclinical mastitis	
		% contribution	Permutation importance	% contribution	Permutation importance
clima1	Annual mean temperature	1.2	1.2	0.2	0.0
clima2	Mean diurnal temperature range	0.3	0.0	0.2	0.7
clima3	Isothermality	0.0	0.1	0.7	1.4
clima4	Temperature seasonality	2.4	1.6	3.0	1.0
clima5	Maximum temperature of warmest month	22.1	16.5	15.9	23.0
clima6	Minimum temperature of coldest month	0.0	0.0	0.0	0.0
clima7	Temperature annual range	1.1	0.4	0.3	0.2
clima8	Mean temperature of wettest quarter	0.0	0.3	0.0	0.2
clima9	Mean temperature of driest quarter	1.9	2.6	3.0	2.3
clima10	Mean temperature of warmest quarter	0.0	0.1	0.0	0.0
clima11	Mean temperature of coldest quarter	0.0	0.0	0.0	0.0
clima12	Total annual precipitation	1.2	1.4	0.8	1.0
clima13	Total precipitation of wettest month	3.1	4.1	3.6	2.8
clima14	Total precipitation of driest month	2.9	8.7	2.7	8.7
clima15	Precipitation seasonality	0.1	0.2	0.0	0.7
clima16	Total precipitation of wettest quarter	0.0	0.0	0.0	0.0
clima17	Total precipitation of driest quarter	0.0	0.0	0.0	0.0
clima18	Total precipitation of warmest quarter	0.1	1.3	0.0	0.0
clima19	Total precipitation of coldest quarter	0.1	0.0	0.1	0.8
farmsdis	Distance from sheep farms	55.7	51.4	59.3	54.8
dem	Altitude	7.8	10.1	10.1	2.4

Table II.xxviii. Estimation of the relative contributions of the variables to the MaxEnt model for subclinical mastitis and biofilm staphylococcal subclinical mastitis, in sheep farms according to management system.

	Management system in farm					
	Intensive management		Semi-intensive management		Semi-extensive / extensive management	
Environmental variable	% contribution	Permutation importance	% contribution	Environmental variable	% contribution	Permutation importance
Subclinical mastitis						
Distance from sheep farms	41.8	28.0	56.3	48.0	44.6	34.7
Altitude	11.6	30.4	4.8	4.3	11.1	18.5
Maximum temperature of warmest month	24.7	0.0	29.8	15.3	0.0	0.0
Total precipitation of driest month	8.5	25.6	0.0	0.2	6.9	28.0
Annual mean temperature	6	0.9	0.6	2.6	12.6	0
Mean temperature of driest quarter	0.3	0.0	0.3	1.3	12.9	4.8
Total precipitation of coldest quarter	0.0	0.0	2.4	12.3	1.2	0.0
Minimum temperature of coldest month	1.3	9.4	0.2	1.0	0.1	0.0
Mean diurnal temperature range	1.4	4.2	1.8	3.9	0.0	0.5
Biofilm staphylococcal subclinical mastitis						
Distance from sheep farms	44.7	27.8	59.6	58.2	41.5	31.1
Altitude	20.2	43.4	5.1	18.4	16.2	11.2
Total precipitation of driest month	1.8	18.4	2.3	2.7	10.2	41.3
Maximum temperature of warmest month	12.9	0.0	25.6	4.5	0.3	1.4
Total precipitation of wettest quarter	0.0	0.0	0.2	0.1	6.2	6
Mean diurnal temperature range	9.5	1.5	0.5	0.8	0.0	0.0
Mean temperature of warmest quarter	1.3	0.0	0.0	0.0	8.2	0.1
Total annual precipitation	0.2	0.0	0.0	0.0	8.1	0.8
Temperature seasonality	0.9	0.3	2.4	3.7	0.0	0.0
Annual mean temperature	5.8	0.0	0.0	0.0	0.8	0.2
Total precipitation of coldest quarter	0.0	0.0	1.7	3.6	0.0	0.0

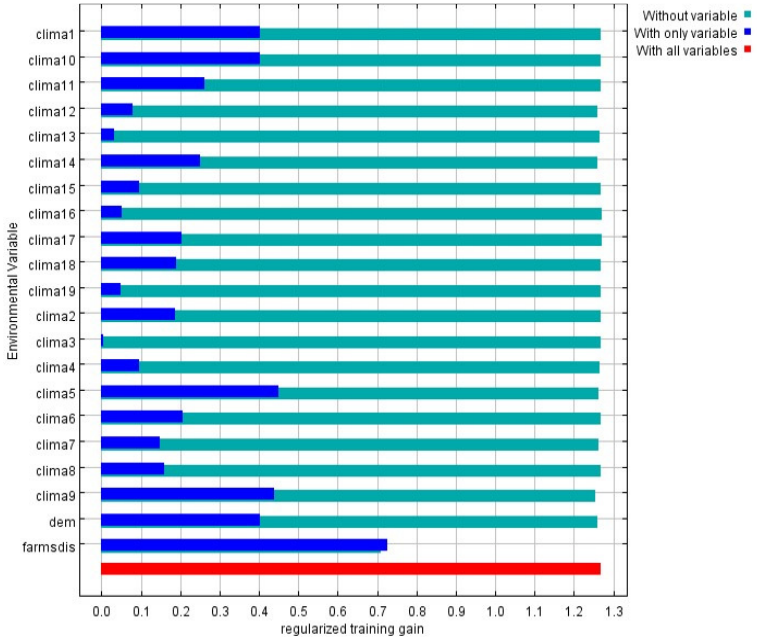
Table II.xxix. Details of variables with highest gain and with most decreasing gain, as well as verification outcomes for subclinical mastitis and biofilm staphylococcal subclinical mastitis, according to management system in sheep farms.

	Mangement system in farm					
	Intensive management		Semi-intensive management		Semi-extensive / extensive management	
Environmental variable	Subclinical mastitis	Biofilm staphylococcal subclinical mastitis	Subclinical mastitis	Biofilm staphylococcal subclinical mastitis	Subclinical mastitis	Biofilm staphylococcal subclinical mastitis
Environmental variables with highest gain when used in isolation	Distance from sheep farms	Distance from sheep farms	Distance from sheep farms	Distance from sheep farms	Distance from sheep farms	Distance from sheep farms
Environmental variables that decreased gain the most when omitted	Altitude	Altitude	Distance from sheep farms	Distance from sheep farms	Distance from sheep farms	Altitude
Regularised training gain	1.490	1.489	1.410	1.474	1.106	1.030
Training AUC	0.948	0.953	0.930	0.939	0.918	0.922
Unregularised training gain	1.920	1.975	1.744	1.855	1.522	1.478
Verification results[1]	0.842	0.900	0.763	0.871	0.762	0.944

1. Proportion of 'infected farms' located in areas predicted as high risk for prevalence of subclinical mastitis or biofilm staphylococcal subclinical mastitis.

Figure II.8. Jackknife of regularised training gain test for all farms in relation to prevalence of (a) subclinical mastitis or (b) biofilm staphylococcal subclinical mastitis in sheep in Greece.

(a)



(b)

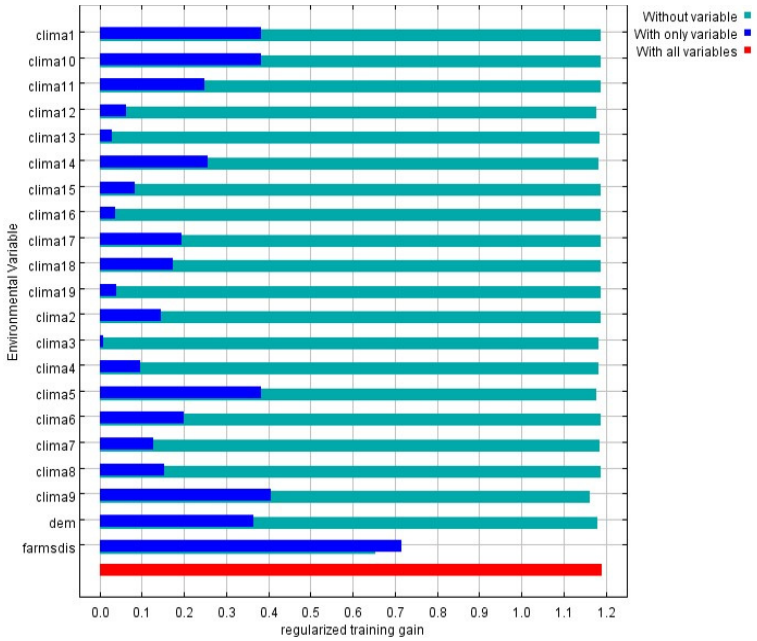
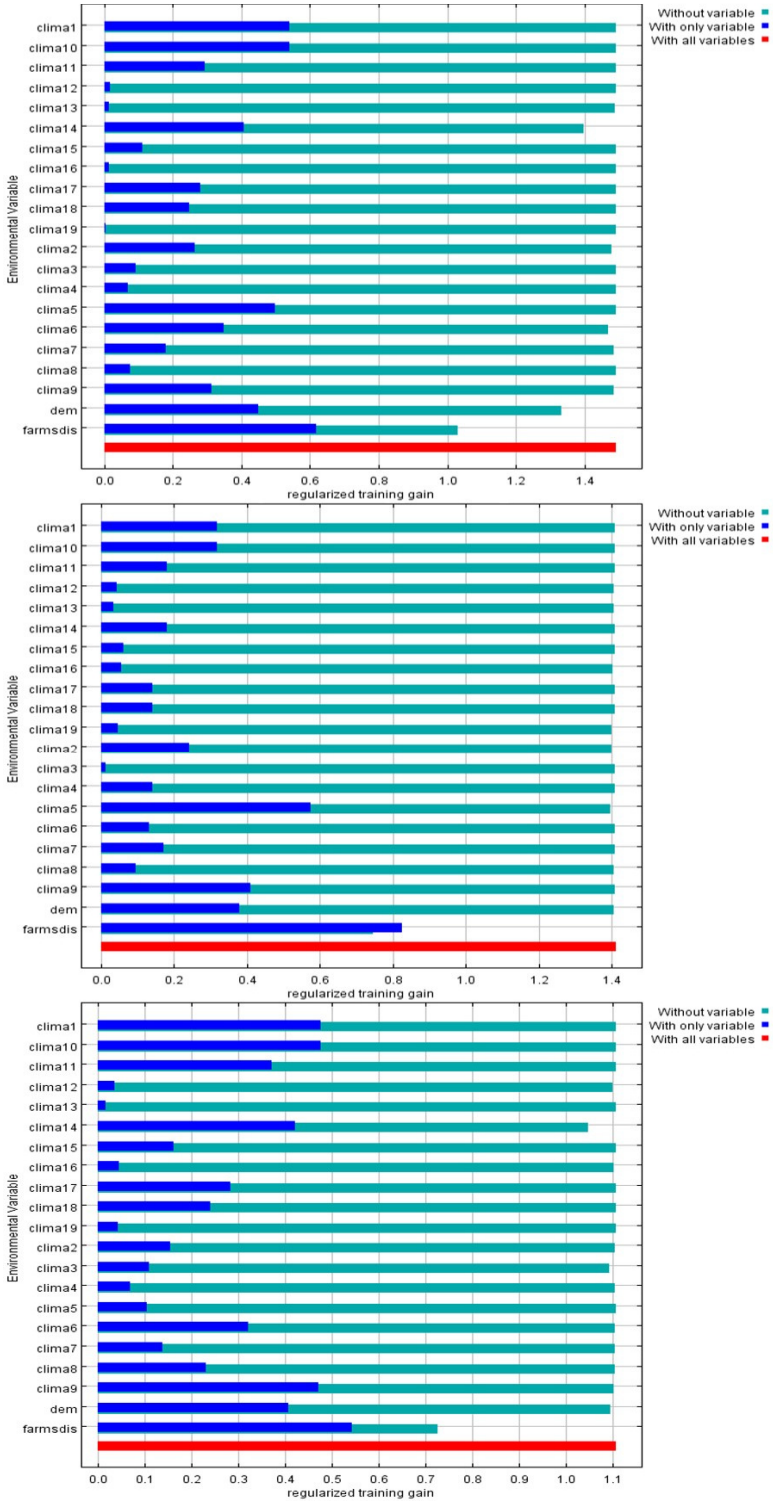


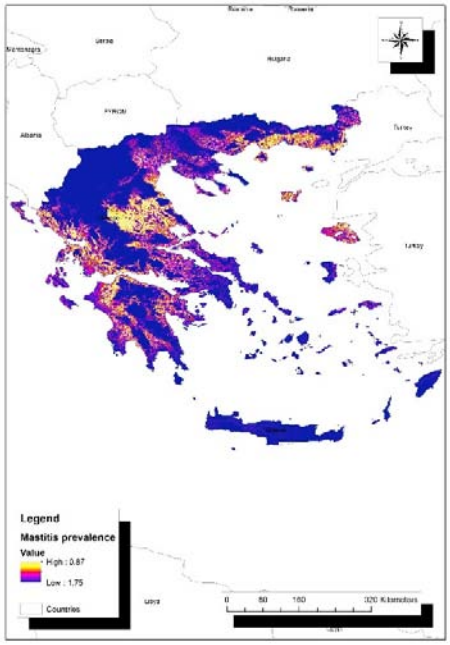
Figure II.9. Jackknife of regularised training gain test for farms in relation to prevalence of subclinical mastitis in ewes in Greece.



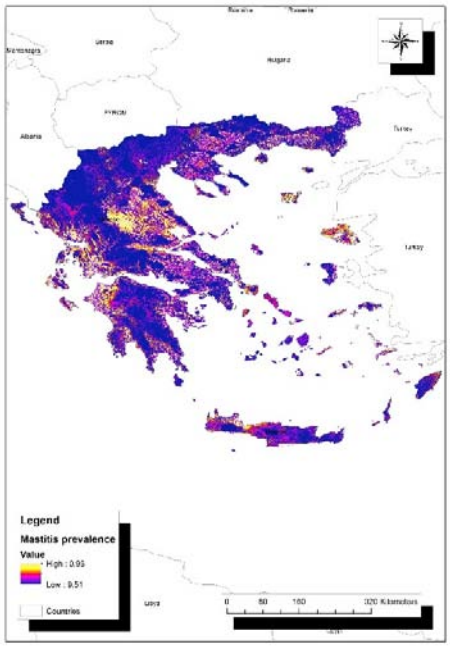
From top to bottom: farms with intensive management system, farms with semi-intensive management system, farms with semi-extensive / extensive management system.

Figure II.10. Maps of Greece showing the potential location of areas of high risk for subclinical mastitis in sheep farms: (a) farms with intensive management, (b) farms with semi-intensive management, (c) farms with extensive / semi-extensive management.

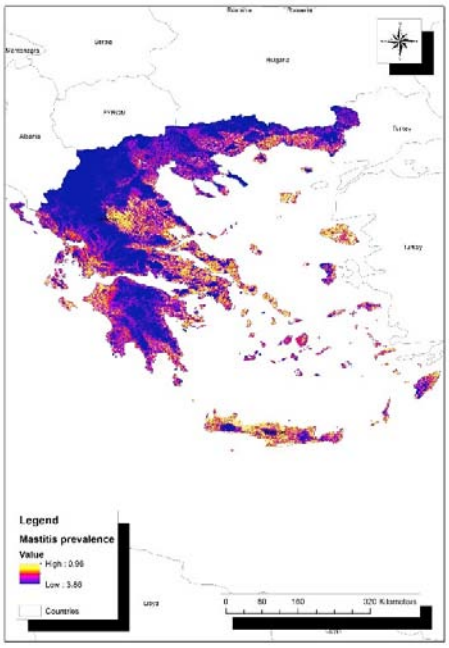
(a)



(b)



(c)



CHAPTER III

EVALUATION OF A NOVEL VACCINE AGAINST STAPHYLOCOCCAL MASTITIS IN EWES

A. VACCINATION AGAINST BACTERIAL MASTITIS IN EWES

Introduction

Vaccinations constitute an integral part of health management programs in sheep flocks. Vaccines have contributed to prevention, control and eradication of diseases more than any other tool available to veterinary medicine (Kyriakis 2015). Classical vaccine development and production technologies have been used successfully for decades against a number of bacterial or viral diseases of animals. Various types of vaccines exist, which, among others, include ones consisting of whole cell cultures (attenuated or inactivated), toxoid vaccines, DNA vaccines or their combinations (Kyriakis 2015).

Vaccination is an active process, where the immunological system of the animal is requested to mount an adequate response against the antigen administered. The basis of vaccination is the enhancement of acquired/specific immunity in the mammary gland. Vaccination aims at recognition of specific determinants of a pathogen that activate a selective response leading to bacterial elimination.

Investigation of immunological responses in the mammary gland of ewes against causal organisms and clarification of the role of the various immunological components supports attempts to immunise ewes against mastitis, by enhancing immunity and potentiating responses to treatment with antimicrobials.

Vaccination against mastitis

Anti-staphylococcal vaccines

Initially, inactivated vaccines containing whole bacterial cells and/or staphylococcal toxoids have been developed and used for prevention of mastitis (e.g., Watson 1988). These products offered mostly reduction in severity of clinical signs, rather than reduction in incidence risk of the disease.

The first attempt to develop a staphylococcal subunit vaccine, for intramuscular administration, was presented by Amorena et al. (1994). The product included inactivated *Staphylococcus* [*S.*] *aureus* and *S. simulans* whole cells, as well as *S. aureus* exopolysaccharide antigens presented within liposomes. The product has been found to lead in reducing incidence risk

of the disease after experimental staphylococcal infections; however, when the exopolysaccharide component had been omitted, no protection was evident against *S. aureus* isolates (Amorena et al. 1994).

In another study, the importance of adjuvants, specifically mineral oil or carbopol, within a vaccine containing inactivated S. aureus cells and toxoids has been assessed (Tollersrud et al. 2002). The vaccine was developed for administration into the supramammary lympho nodes.

Hadimli et al. (2005) have evaluated a staphylococcal whole cell inactivated vaccine, containing *S. aureus* and three coagulase-negative species, for subcutaneous administration in the draining region of the supramammary lymph nodes. Nevertheless, the vaccine did not offer significant protection against the disease.

Perez et al. (2009) have described the induction of antibodies against PNAG, which is the main component of extracellular biofilm matrix of staphylococci. Based on that principle, a vaccine has been produced, which made use of cell-free surface polysaccharide in various vehicles, bacterial unbound cells or bacterial cells embedded in their biofilm matrix in various adjuvants and which, in cows, has been found to elicit exopolysaccharide specific antibody response offering a protection against *S. aureus* mastitis (Prenafeta et al. 2010). The vaccine contributed in prevention of biofilm formation by staphylococcal isolates, thus limiting their expansion and dissemination within and outside the mammary gland. Ultimately, the vaccine has been licenced in the European Union (Vimco®; Laboratorios Hipra, Girona, Spain) for intramuscular administration.

The efficacy of a multivalent whole cell staphylococcal vaccine for subcutaneous administration has been assessed by Alekish et al. (2018). The authors have reported that it led to a non-significant reduction in new cases of mastitis in vaccinated animals, with no effects in milk production or composition.

It is also noteworthy that often autogenous staphylococcal vaccine are produced. Such vaccines are for limited use in specific flocks and do not have a wider applicability. There are no reports of their efficacy, as, obviously, they are produced for ‘tailor-made’ administration in flocks with perceived problem of staphylococcal mastitis, without first being evaluated for efficacy. Autogenous vaccines would be effective against one or a limited number of staphylococcal isolates and might cause adverse reactions, especially if oily adjuvants are added. Azara et al. (2017) have studied and presented the detailed characteristics of staphylococcal isolates included in anti-mastitis autologous vaccines produced in Italy, but not the clinical significance of these vaccines.

Other vaccines

Vaccines developed against *M. haemolytica* mastitis have included an autogenous vaccine developed by Kabay and Ellis (1989), which, after single intraperitoneal administration, led in reduction of incidence risk of mastitis in a non-dairy sheep flock. More recently, it has been reported that intramammary administration of a vaccine already licenced against respiratory infections might seemingly express a possible protective effect against *M. haemolytica* mastitis (Parrott 2017).

The vaccine discussed hereabove as evaluated by Alekish et al. (2018) also included *Streptococcus* spp. and *Trueperella pyogenes* antigens, which have contributed in the effect previously presented. Finally, Leitner and Krifucks (2007) have presented a murine model for development and initial assessment of a vaccine specific for prevention of *Pseudomonas*-associated mastitis; that vaccine was produced specially for use in flocks with that particular problem.

Objective of the work

The objective of the work was to evaluate efficacy of a vaccine against staphylococcal mastitis in ewes under clinical and experimental conditions. The clinical study was performed in five dairy sheep farms. The experimental work was performed in sheep housed at the departmental facilities; it was performed under a licence for experimental procedures obtained from the local offices of the Hellenic Ministry of Agricultural Development and Food, at the Department of Obstetrics and Reproduction of the Veterinary Faculty of University of Thessaly, according to EU regulations.

The staphylococcal antigen in the vaccine under evaluation was based on a bacterin of *S. aureus* strain expressing PNAG (Perez et al. 2009, Prenafeta et al. 2010).

B. EVALUATION OF THE EFFICACY OF THE TEST VACCINE IN A RANDOMISED PLACEBO-CONTROLLED CLINICAL STUDY

Materials and methods

Number of animals in the study

The study was designed with mixing vaccinated and control ewes in a 1:1 ratio within participating farms. As ewe was to be the unit of vaccination, sample size calculations were performed at ewe level (Schukken et al. 2014). Previous data in Greece had indicated that the incidence risk of new cases of mastitis of staphylococcal aetiology in ewes was approximately 13.0% per lactation period (Fthenakis and Jones 1990a, Fthenakis 1994). The size needed for each group was therefore 278 ewes, in order to reject the null hypothesis that incidence risk for vaccinated and control ewes was equal (i.e., efficacy of the vaccine was 0%) with a probability of 0.80. The type I error probability associated with this test of this null hypothesis was 0.05. Subsequently, because of the within-ewe dependency due to mixing (Halloran et al. 1997, Schukken et al. 2014), required sample size was increased by 20%, thus resulting in 334 ewes in each group and total study size population of 668 ewes.

Sheep farms

The trial started in January 2015, with data gathering in farms taking place until July 2016. Farm selection and inclusion in the study were based on convenience, i.e., the willingness of the farmers to collaborate and receive regular visits for examination and sampling of animals in their farm. All farms were located in the region of Thessaly in central Greece. In all farms, standard basic health management practices (anti-clostridial and anti-*Brucella melitensis* vaccinations, administrations of anthelmintic products) were performed regularly. No specific udder health management measures (e.g., teat-dipping, administration of antimicrobials at end of lactation period) were employed in any farm in the study. Details of farms are in Table III.i.

In farms A, P and S, all available ewes were enrolled in the study. In each of farms K and D, 160 ewes were selected for enrolment by using an electronic random number generator (www.randomresult.com) as they walked into the shed. In all farms, a centralised randomisation

schedule in a 1:1 ratio was performed by another investigator for allocation of ewes in groups: V (for vaccination) or C (controls). After allocation in a group, a standardised detailed clinical examination was performed to assess the general health of the animals (Lovatt 2010). Animals with clinically evident problems were excluded. Finally, 348 ewes remained in group V (326 adults and 22 ewe-lambs) and 344 in group C (318 adults and 26 ewe-lambs) (Table III.i). Ewes in each farm were identified using neck straps and plastic tags with unique farm-specific serial numbers. Farmers and farm personnel were not aware of the vaccination status of the animals.

In the three farms, in which machine-milking was applied, the scheduled periodic maintenance evaluations were performed by technical staff of the manufacturer at the set intervals. No manufacturing-related problems were detected in the milking system of any farm.

Vaccination schedule and administration

A vaccine licensed in European Union countries against clinical staphylococcal mastitis in ewes (Vimco®; Laboratorios Hipra, Girona, Spain) was used (batches: 69VW-1, 75LQ-2, 75LQ-1, 77XW-1, 76CN-1, 79PJ-2, 77XW-1). Vaccination was carried out during the last stage of gestation of ewes, by following the licensed schedule of the product. The initial administration was performed 110 days after introduction of rams into the flock of ewes (i.e., 6 to 5 weeks before expected start of lambing season) and was followed by a repeat administration 21 days later. A timeline of the study is in Figure III.1.

For transportation of vaccine from the laboratory to the sheep farms, vials were stored in portable refrigerators with ice packs. Transportation was performed by car.

Allocation concealment was performed. Group allocation of ewes was announced after clinical examination of each animal and its preparation for vaccine or placebo administration.

Administration was performed after clipping the wool in the neck region of the animal. A dose of 2 mL of the product was injected intramuscularly. Control animals were injected intramuscularly with 2 mL of normal saline. A plastic syringe and a hypodermic needle were used and discarded after a single administration. First injection to each animal was performed at the left side of the neck; repeat injection was performed at the right side.

The only other vaccine that had been administered to the ewes during the study period was an anti-clostridial vaccine, which had been given two weeks prior to the initial administration of the vaccine under evaluation. The vaccine was injected subcutaneously in the area behind the front leg of the sheep.

Table III.i. Details of five sheep farms in Greece during evaluation of the test vaccine against staphylococcal mastitis.

Farm	Management system	Total no. of ewes in farm	Ewes into the study (no.)				Ewes that entered into lactation period (no.)				Milking procedure	Reported history of mastitis	Predominant breed
			adult ewes		*primiparae* ewes		adult ewes		*primiparae* ewes				
			V[1]	C[1]	V	C	V	C	V	C			
A	Intensive	140	54	51	15	14	48	45	15	14	Machine-milking	Yes[3]	Chios
D	Semi-intensive	180	80	80	0	0	76	74	0	0	Machine-milking	No	Friesian
K	Semi-intensive	250	80	80	0	0	71	72	0	0	Hand-milking	No	Chios
P	Semi-intensive	80	35	35	4	4	35	35	4	4	Hand-milking	Yes[3]	Karagouniko
S	Intensive	160	77	72	3	8	64	55	3	8	Machine-milking	No	Lacaune
	Total	810	326	318	22	26	294	281	22	26			

1. V: vaccinated ewes, C: unvaccinated control ewes.

2. Reported (as perceived by farmer) increased (> 0.15) incidence risk of clinical mastitis during the preceding lactation period.

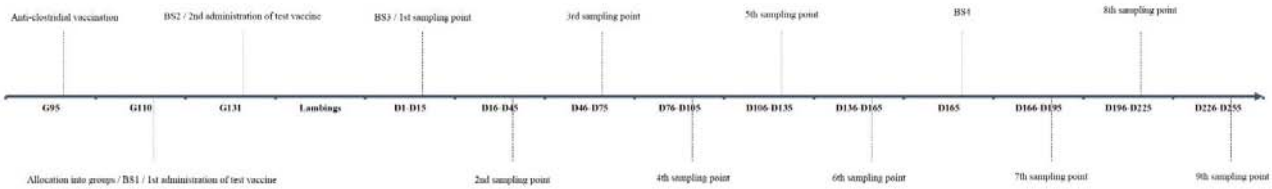
Examination of animals and samplings

After vaccine administration, animals were observed by the farmer for any systemic or local reactions. If any was recorded, a detailed examination was performed; subsequent examinations were performed every three days until the reaction had disappeared. In case of local reactions, the size, nature and duration of presence were described after clinical examination (observation, palpation) of the region of vaccine injection.

After lambing, clinical examination of animals was routinely performed within the first 15 days and then at monthly intervals. In total, depending on the farm, up to 9 milk samplings were performed (Figure III.1). On each sampling point, milk samples were collected from all ewes (both groups), which were in lactation period.

A standardised clinical examination of the udder (observation, palpation, comparison between glands) was performed as previously described (Chapter II, section B), and the first two squirts of secretion were drawn on the gloved hand of an assisting investigator and assessed.

Figure III.1. Timeline diagram of vaccinations and sampling points during evaluation of the test vaccine against staphylococcal mastitis.



G: day of gestation, D: day post-partum, BS1-4: blood sampling, 1st-9th sampling point: milk sampling.

The orifice, edge and lower half of the body of the teat were disinfected by single-use sterile gauzes, onto which povidone iodine 7.5% solution (Betadine surgical scrub®; Mundipharma Medical Company, Basel, Switzerland) had been poured, followed by wiping off by means of a new sterile gauze; different gauzes were used for each teat. 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 directly onto a paddle for performing the California Mastitis Test (CMT).

Jugular blood samples were collected in tubes with no anticoagulant, on four occasions throughout the study, always from the same V and C randomly selected ewes (n = 10 from each group) in each farm. Samples were collected immediately before initial and repeat vaccine

administration (sampling points BS1 and BS2, respectively), as well as three weeks and six months after repeat vaccination (sampling points BS3 and BS4, respectively) (Figure III.1).

For transportation of all samples to the laboratory, they were stored in portable refrigerators with ice packs. Transportation was performed by car.

Laboratory examinations

Microbiological examinations

Laboratory procedures started within 5 h after collection. Milk samples from each mammary gland were processed separately. Samples (10 μL) were cultured using Columbia blood agar plates incubated aerobically at 37 ºC for 48 h. If nothing had grown, media were reincubated for another 24 h. Bacterial identifications were performed by using standard, established techniques: morphological characteristics of colonies, Gram stain and biochemical tests (Barrow and Feltham 1993, Euzeby 1997).

Cytological examination

At ewe-side, all milk samples were tested by use of the CMT, as previously described (Chapter II, section B). Smears were also produced from all milk samples and dried. These were stained by the Giemsa method for estimation of leucocyte subpopulations, as previously described (Chapter II, section B). Subsequently, the Microscopic cell counting method (Mccm) was performed in 1,006 milk samples, as previously described (Chapter II, section B).

Examination of staphylococcal isolates for in vitro *biofilm formation and relevant gene detection*

All staphylococcal isolates recovered from farms A, D, K and P were tested for *in vitro* biofilm formation by a combination of culture appearance on Congo Red agar and microplate adhesion method. The methodology of the work and the techniques employed have been described previously (Chapter II, section B).

Subsequently, isolates (n = 31), selected at random, were tested for presence of genes associated with biofilm formation (*icaA*, *icaB*, *icaC*, *icaD*, *bap*, *eno* and *clfa*) by using PCR, as described previously (Chapter II, section B).

Detection of anti-PNAG antibodies in blood samples

Blood samples were centrifuged for serum collection in preparation for measurement of IgG PNAG-specific antibodies. The technique and procedures described previously (Chapter II, section B) were used.

Data management and analysis

All data were entered into Microsoft Excel and analyzed using IBM SPSS Statistics (ver. 21) (IBM; Armonk, NY, USA).

Definitions

The following outcomes were studied: 'mastitis', 'staphylococcal mastitis', 'biofilm staphylococcal mastitis', 'staphylococcal mammary carriage', 'staphylococcal intramammary infection (IMI)' and 'biofilm staphylococcal intramammary infection (IMI)'. All outcomes referred to ewes (hence, animals with both glands affected were counted as one case).

Mastitis included clinical and subclinical mastitis. Clinical mastitis was defined in ewes with presence of abnormal gross findings in a mammary gland (including changes in secretion) (Fragkou et al. 2014); subclinical mastitis was defined as previously (Chapter II, section B). Also, staphylococcal subclinical mastitis and biofilm staphylococcal subclinical mastitis, mammary carriage, staphylococcal mammary carriage were defined as previously (Chapter II, section B).

Recurrence of staphylococcal mastitis in a ewe was defined when a ewe with staphylococcal mastitis changed to not having staphylococcal mastitis and then again to having it.

Staphylococcal intrammamary infection (IMI) was defined in ewes, in which a staphylococcal positive milk sample was detected, independently of cell content therein. Cases of staphylococcal IMI caused by isolates forming biofilm, were termed biofilm staphylococcal IMI.

Moreover, for the purposes of this study, the following definitions were used to compare the likelihood of occurrence of the various outcomes (disease or infection). Incidence rate was defined as the proportion of animals at risk, which developed the condition when the time at risk was the same in each group. Incidence risk was defined as the proportion of animals at risk, which developed the condition when the time at risk differed between animals (for example, during an entire lactation which varied between individuals).

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. 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 $r = 0.905$ (95% CI: 0.893 - 0.915) ($P < 0.001$) and the corrected R^2 was 81.4%.

Analysis of results related to bacterial isolates

Evaluations of associations regarding vaccination status of ewes from which isolates had been recovered, were performed by use of the Pearson chi-square test or the Fisher exact test as appropriate.

Optical density during measurement of anti-PNAG specific antibodies in blood samples

Results of antibody measurements in serum samples were expressed as optical density (OD) values. Data were processed as described previously (Chapter II, section B).

Analysis of results related to antibody titres

Linear mixed models were used in analysis to account for repeated measures of anti-PNAG antibody titres over the course of the study. Time points of collecting data were selected as within-subjects variables and group allocation as between-subject factor. Independent variables (fixed effects) included study group (V or C), sampling point (BS1 - BS4) and a sampling point by study group interaction.

Association of frequency of staphylococcal and biofilm staphylococcal mastitis cases with positive or negative anti-PNAG antibody titres was evaluated by use of Fisher exact test. Mean anti-PNAG antibody titres between ewes that did or did not develop mastitis were compared by use of Student's t-test.

Modelling for analysis of mastitis occurrence

For estimation of incidence rates, we took into account that a ewe might change from having to not having mastitis and *vice-versa*; during the interval between sampling points, it was not possible to know what had happened between the two sampling points, i.e. how many cases of mastitis and 'cures' there might have occurred. Therefore, the following definitions were made (Mavrogianni et al. 2007).

• On a particular sampling point, a ewe was defined as being 'at risk of developing mastitis', if it had no mastitis on the previous sampling point.

• On the subsequent sampling point, this ewe could be either 'healthy' (in which case it was still at risk to developing mastitis) or 'with mastitis' (in which case it was not at risk).

• On subsequent sampling points, if this ewe was 'healthy', then it was again 'at risk'.

• If a ewe was detected to be healthy on one sampling point but with mastitis on the next one, then mastitis was considered to have taken place half-way between the two sampling points; if a ewe was detected with mastitis on one sampling point but healthy on the next one, then mastitis was deemed to have been eliminated half-way between the two sampling points.
• If a ewe was detected with mastitis on two consecutive sampling points, then it was considered to have been with mastitis throughout the time between those two time points; conversely, if a ewe was found healthy on two consecutive sampling points, then it was considered to have been healthy throughout the time between those two time points.

Based on the above, it was possible to calculate incidence rates of mastitis at each sampling point. Further, it was possible to estimate the length of time for which a ewe was at risk before it developed mastitis, as well as the length of time that a ewe had mastitis.

Finally, the lactation period of ewes into the study was divided in two stages: the first included the first four sampling points and the second the subsequent sampling points (from 5^{th} up to 9^{th}).

Analysis of results related to mastitis occurrence

For calculations, all animals in group V or C (i.e., in all farms), as well as animals in group V or C in each of the five farms were taken into account. Then, calculations were made for clusters of farms, according to following variables: (a) intensive or semi-intensive management system, (b) hand- or machine-milking or (c) reported or not history of clinical mastitis. Comparisons were performed between groups V and C for: (a) animals in the study (i.e., in all farms), (b) animals in individual farms or (c) animals within a cluster of farms.Association of mastitis with mammary carriage in ewes of group V or C was evaluated in a table of cross-categorised frequency data by use of the Pearson chi-square test or the Fisher exact test as appropriate. Comparison of the time when first case of mastitis outcomes occurred in V and C ewes, was performed by the Mann-Whitney test for non-parametric data. Association of mastitis cases with stage of lactation (1^{st} or 2^{nd}) in ewes of group V or C was also evaluated by use of the Pearson chi-square test.

Vaccine efficacy

Efficacy of the vaccine under evaluation was calculated as [(incidence rate on day i in group C - incidence rate on day i in group V ewes) / incidence rate on day i in group C ewes] (Centers for Disease Control and Prevention 2017). Efficacy was calculated for mastitis, staphylococcal mastitis, biofilm staphylococcal mastitis, staphylococcal IMI and biofilm staphylococcal IMI on each of the nine sampling points.

Statistical significance

In all analyses, statistical significance was defined at $P < 0.05$.

Results

Post-vaccination adverse reactions

In total, adverse reactions were recorded in two group V ewes (incidence rate: 0.006, 95% CI: 0.002 – 0.021). No reactions were recorded in group C animals (incidence rate: 0.000, 95% CI: 0.000 – 0.011) ($P = 0.25$ between groups). The reactions referred to development of a hard nodule 3 cm in diametre, at the site of injection, 2 and 3 days after initial vaccination. The lesions progressively subsided within three weeks.

Monitoring of ewes and sample collection during the study

Of the 692 ewes into the study, 623 entered into lactation period for sampling (0.090, range among flocks: 0.813 – 1.000) (Table III.i); of these 316 were in group V (0.908) and 307 in group C (0.892) ($P = 0.25$ between groups). Reasons for which animals did not enter into the lactation period were: sale, culling, death, abortion or failure to lamb.

On average, animals were monitored for 154 days for V and 152 days for C group ($P = 0.34$). Adult ewes were monitored for a longer period than *primiparae* ewes: 154 and 134 days, respectively ($P = 0.003$). In total, 3,637 milk samplings were performed: 1,853 in V and 1,784 in C ewes. A total of 362 blood samples were collected: 180 from V and 182 from C ewes. Details are in Tables III.ii and III.iii.

Bacterial isolation

In total, 428 bacterial isolates were recovered from ewes with mastitis. The most frequently isolated bacteria were *Staphylococcus* spp. (n = 290), accounting for 0.564 and 0.761, respectively, of total isolates recovered from group V and C, respectively ($P < 0.001$). Staphylococci as causal agents of mastitis were isolated less frequently from ewes in group V (from 99 of 1,853 samplings; 0.053) than in ewes in group C (from 184 of 1,784 samplings; 0.103) ($P < 0.001$) (Tables III.iv, III.v,

III.vi). Other bacteria isolated included *Corynebacterium* spp., *Streptococcus* spp., *Acinetobacter* spp., *Escherichia coli* and *Mannheimia haemolytica*.

Further, 167 bacterial isolates were obtained from ewes with mammary carriage. Again, the most frequently isolated bacteria were *Staphylococcus* spp. (n = 98), accounting for 0.538 and 0.632 of total isolates recovered from group V and C, respectively ($P = 0.11$) (Table III.iv).

There was no difference in frequency of *S. aureus* isolates among staphylococcal isolates between the two groups: 0.041 *versus* 0.058 of isolates for group V and C, respectively ($P = 0.33$).

Table III.ii. Details of monitoring of ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

Farm	Ewes that entered into lactation period (no.)		Mean days in the lactation period		Milk samplings performed in ewes[3]		Blood samples collected from ewes	
	Group V[1]	Group C[1]	Group V	Group C	Group V	Group C	Group V	Group C
A	48 / 15[2]	45 / 14	221 / 154	205 / 141	389 / 88	343 / 76	39	39
D	76 / 0	74 / 0	182 / -	179 / -	516 / -	495 / -	37	38
K	71 / 0	72 / 0	164 / -	162 / -	436 / -	446 / -	40	38
P	35 / 4	35 / 4	68 / 68	68 / 68	105 / 12	105 / 12	29	30
S	64 /3	55 / 8	133 / 148	121 / 143	290 / 17	263 / 44	35	37
	294 / 22	281 / 26	155 / 138	153 / 130	1,736 / 117	1,652 / 132	180	182

1. V: vaccinated ewes, C: unvaccinated control ewes.
2. m / n: adult ewes / *primiparae* ewes.
3. No. of ewes sampled on each sampling point presented in Table III.iii.

Table III.iii. Number of ewes sampled in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Sampling point									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	Total
Group V[1]										
Adult ewes	287	279	269	223	198	175	132	124	49	1,736
Primiparae ewes	22	21	21	17	15	14	7	0	0	117
Total	309	300	290	240	213	189	139	124	49	1,853
Group C[1]										
Adult ewes	271	272	264	214	201	167	121	105	37	1,652
Primiparae ewes	26	24	23	19	19	17	4	0	0	132
Total	297	296	287	233	220	184	125	105	37	1,784

1. V: vaccinated ewes, C: unvaccinated control ewes.

Table III.iv. Frequency of bacterial isolates recovered from milk samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Number of isolates (frequency of isolation) from:			
	mastitis	mammary carriage	mastitis	mammary carriage
Microorganism	Group V[1]		Group C[1]	
Coagulase-negative staphylococci	97 (0.536)	42 (0.525)	175 (0.709)	54 (0.612)
S. aureus	5 (0.028)	1 (0.013)	13 (0.053)	1 (0.012)
Non staphylococcal isolates[2]	79 (0.436)	37 (0.463)	59 (0.239)	32 (0.367)
	181	80	247	87

1. V: vaccinated ewes, C: unvaccinated control ewes.
2. Main bacteria isolated: *Corynebacterium* spp., *Streptococcus* spp., *Acinetobacter* spp., *Escherichia coli*.

Table III.v. Frequency of staphylococcal isolates recovered from milk samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Number of isolates (frequency of isolation) from intramammary infections:	
Microorganism	Group V[1]	Group C[1]
Coagulase-negative staphylococci	139 (0.959)	229 (0.942)
S. aureus	6 (0.041)	14 (0.058)

1. V: vaccinated ewes, C: unvaccinated control ewes.

Table III.vi. Details of bacterial isolation from milk samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Group V[1]						Group C[1]					
Isolates:	from mastitis (no.)			from mammary carriage (no.)			from mastitis (no.)			from mammary carriage (no.)		
Farm	Sa[2]	cnS[2]	other[2]	Sa[2]	cnS[2]	other[2]	Sa[2]	cnS[2]	other[2]	Sa[2]	cnS[2]	other[2]
A	0	11	26	0	1	4	2	25	7	0	12	6
D	0	12	10	0	9	14	1	34	11	0	4	5
K	3	47	24	0	22	18	0	68	31	0	22	20
P	0	6	4	0	1	1	2	11	2	0	3	1
S	2	21	15	1	9	0	8	37	8	1	13	0
	5	97	79	1	42	37	13	175	59	1	54	32

1. V: vaccinated ewes, C: unvaccinated control ewes.
2. Sa: *S. aureus*, cnS: coagulase-negative staphylococci, other: non-staphylococci.

Biofilm formation by staphylococcal isolates

In vitro *biofilm formation*

Of the 296 isolates tested, 188 (0.635, 95% CI 0.579 – 0.688) were characterised as biofilm-forming. Of the 222 mastitis-associated isolates that were tested, 149 (0.671) were characterised as biofilm-forming; respective figure for the 74 isolates from mammary carriage was 39 (0.527). The results have not indicated an association of the isolate source (mastitis or mammary carriage) with biofilm formation in isolates from group V ewes (0.532 *versus* 0.576, respectively; $P = 0.42$); there was nevertheless an association of the isolate source (mastitis or mammary carriage) with biofilm formation among isolates from group C ewes (a higher proportion of isolates from cases of mastitis was biofilm-forming than of isolates from mammary carriage: 0.748 *versus* 0.488, respectively; $P = 0.001$).

Overall, amongst mastitis-associated staphylococcal isolates recovered from V group ewes a smaller proportion was biofilm-forming than amongst those from C group ewes: 0.532 (42 / 79) *versus* 0.749 of isolates (107 / 143), respectively ($P < 0.001$). No such difference was seen among mammary carriage isolates: 0.576 *versus* 0.488, respectively ($P = 0.30$) (Tables III.vii, III.viii). Biofilm-forming staphylococci as causal agents of mastitis were isolated less frequently from ewes in group V (from 42 of 1,853 samplings; 0.023) than in ewes in group C (from 107 of 1,784 samplings; 0.060) ($P < 0.001$).

Table III.vii. Frequency of biofilm-forming staphylococcal isolates recovered from milk samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Proportion of biofilm-forming isolates among staphylococcal isolates from:			
	mastitis	mammary carriage	mastitis	mammary carriage
Farm	Group V[1]		Group C[1]	
A	0.636 (7 / 11)	1.000 (1 / 1)	0.889 (24 / 27)	0.833 (10 / 12)
D	0.833 (10 / 12)	0.556 (5 / 9)	0.686 (24 / 35)	0.250 (1 / 4)
K	0.460[a] (23 / 50)	0.545 (12 / 22)	0.765[a] (52 / 68)	0.318 (7 / 22)
P	0.333 (2 / 6)	1.000 (1 / 1)	0.538 (7 / 13)	0.667 (2 / 3)
	0.532[a] (42 / 79)	0.576 (20 / 33)	0.748[a] (107 / 143)	0.488 (20 / 41)

a = $P < 0.01$ for same superscript in the same row for comparison within the same farm:
[1]V: vaccinated ewes, C: unvaccinated control ewes.

Table III.viii. Details of isolation of biofilm-forming staphylococcal isolates from milk samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Group V[1]				Group C[1]			
Isolates:	from mastitis (no.)		from mammary carriage (no.)		from mastitis (no.)		from mammary carriage (no.)	
Farm	Sa[2]	cnS[2]	Sa[2]	cnS[2]	Sa[2]	cnS[2]	Sa[2]	cnS[2]
A	0	7	0	1	2	22	0	10
D	0	10	0	5	1	23	0	1
K	3	20	0	12	0	52	0	7
P	0	2	0	1	1	6	0	2
	3	39	0	19	4	103	0	20

1. V: vaccinated ewes, C: unvaccinated control ewes.
2. Sa: *S. aureus*, cnS: coagulase-negative staphylococci.

Detection of genes associated with biofilm formation

In isolates from mastitis cases, median number of *ica* operon genes was 1 in isolates from group V and 2 in those from group C ewes ($P = 0.040$). Median number of all genes associated with biofilm formation was 2 in isolates from group V and 3 in those from group C ewes ($P = 0.28$) (Table III.ix).

Table III.ix. Frequency of genes associated with biofilm-formation in staphylococcal isolates recovered from milk samples from ewes, during evaluation of the test vaccine against staphylococcal mastitis.

	Gene								
Group	*icaA*	*icaB*	*icaC*	*icaD*	ica[2]	*bap*	*eno*	*clfa*	median
V[1]	0.250	0.250	0.333	0.250	0.583	0.333	0.917	0.250	2
C[1]	0.368	0.368	0.526	0.474	0.737	0.105	0.684	0.421	3

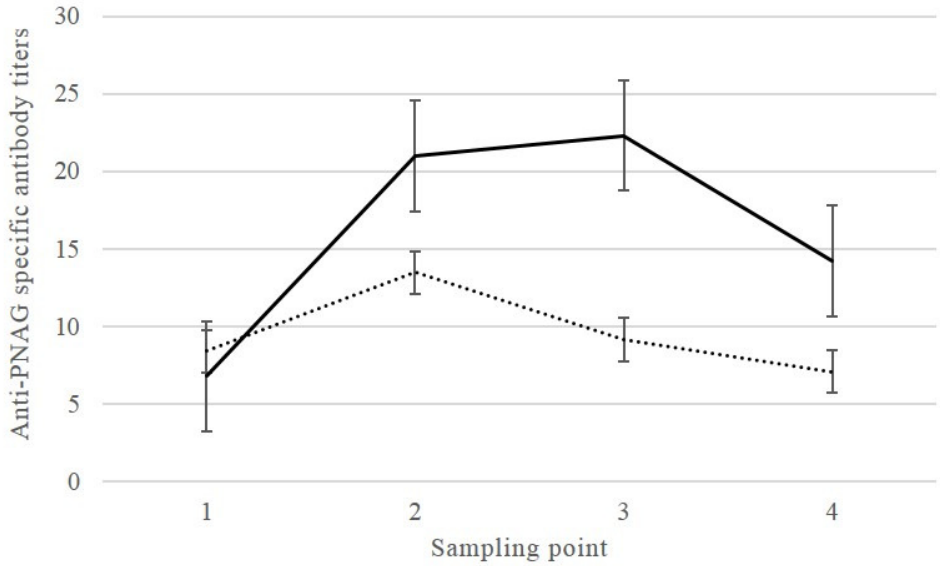
1. V: vaccinated ewes, C: unvaccinated control ewes.
2. At least one gene in the ica operon

Detection of anti-PNAG antibodies in blood samples

Anti-PNAG-specific antibody values increased in group V ewes ($P < 0.001$ between sampling points for V group) and were higher than in C group ones ($P < 0.001$ between groups). Moreover, on sampling points BS3 and BS4, a greater proportion of group V ewes had increased antibody values (0.939 and 0.636, respectively) than of group C ewes (0.396 and 0.375, respectively) ($P < 0.001$ and $P = 0.031$, respectively) (Figure III.2, Table III.x).

There was an association of anti-PNAG antibody titres with risk of mastitis development: a greater proportion of ewes with RIPC < 6.0 on sampling point BS3 (independently of group) subsequently developed staphylococcal or biofilm staphylococcal mastitis (12 / 29 and 9 / 20, 0.414 and 0.450) than of group V ewes with RIPC > 6.0 on the same occasion (8 / 47 and 2 / 38, 0.170 and 0.053, respectively) ($P = 0.020$ and $P < 0.001$, respectively). Further, a greater proportion of ewes with RIPC < 6.0 on sampling point BS3 subsequently developed biofilm IMI (9 / 20, 0.450) than of group V ewes with RIPC > 6.0 on the same occasion (4 / 38, 0.105) ($P = 0.004$); however, no such association with staphylococcal IMI was found (13 / 29 *versus* 14 / 47) ($P = 0.14$).

Figure III.2. Mean values of anti-PNAG-specific antibody titres in blood samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.



Continuous line: V group (vaccinated ewes), dotted line: C group (unvaccinated control ewes).

Table III.x. Mean values of anti-PNAG-specific antibody values in blood samples from ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Group V[1]				Group C[1]			
	Sampling point							
Farm	BS1	BS2	BS3	BS4	BS1	BS2	BS3	BS4
A	3.71	25.95^{c}	21.15^{c}	8.81^{a}	0.96	5.68^{c}	2.03^{c}	0.00^{a}
D	5.33	21.72	18.06^{b}	14.43	8.97	16.36	11.48^{b}	8.38
K	5.09	24.67^{b}	29.48^{c}	21.10	3.47	3.45^{b}	3.49^{c}	7.17
P	5.33	21.72	18.06	na[2]	3.71	25.95	21.15	na[2]
S	5.11	11.65^{b}	15.67^{c}	5.88^{c}	3.27	3.22^{b}	1.95^{c}	0.38^{c}
	6.76	20.97	22.30	14.21	8.37	13.45	9.13	7.06

a: 0.026 < *P* < 0.05, b: 0.01 < P < 0.025, c: *P* < 0.01 for same superscript in the same row for comparison between groups within the same farm at the same sampling point.
1. V: vaccinated ewes, C: unvaccinated control ewes.
2. na: not applicable.

Occurrence of mastitis

When all animals in the study were taken into account, the incidence risk of mastitis, staphylococcal mastitis and biofilm staphylococcal mastitis were smaller in group V than in group C

ewes: 0.367 (95% CI: 0.316 – 0.422), 0.171 (95% CI: 0.133 – 0.216) and 0.080 (95% CI: 0.053 – 0.121) *versus* 0.443 (95% CI: 0.389 – 0.499), 0.309 (95% CI: 0.260 – 0.363) and 0.189 (95% CI: 0.144 – 0.243), respectively ($P = 0.027$, < 0.001, < 0.001, respectively). In all farms, the incidence risk of staphylococcal mastitis and biofilm staphylococcal mastitis were smaller in group V than in group C ewes (Tables III.xi., III.xii). Further, it emerged that incidence risk of staphylococcal mastitis and biofilm staphylococcal mastitis also was always smaller in group V than in group C ewes in all clusters evaluated (Table III.xiii).

In adult ewes, the incidence risk of mastitis, staphylococcal mastitis and biofilm staphylococcal mastitis were smaller in group V than in group C ewes: 0.371, 0.173, 0.065 *versus* 0.473, 0.313, 0.164, respectively ($P < 0.007$). In contrast, in *primiparae* ewes, no such difference was seen in the various mastitis outcomes between the two groups: 0.318, 0.136, 0.045 *versus* 0.115, 0.115, 0.000, respectively ($P > 0.085$).

Only 8 cases of clinical staphylococcal mastitis were recorded throughout the study, of which 1 was recorded in a group V ewe and 7 were recorded in group C ewes ($P = 0.004$). Of these cases, 5 were caused by biofilm-forming staphylococci, none in group V ewes ($P = 0.003$ when compared to cases in group C).

Incidence rates of mastitis and staphylococcal mastitis were smaller in group V than in group C up to the 4th and 6th sampling point, respectively. Incidence rate of biofilm staphylococcal mastitis was smaller in group V throughout the study (Figure III.3, Table III.xiv). The incidence rates during the 1st stage of lactation differed between V and C ewes for all outcomes: 0.319, 0.184 and 0.054 *versus* 0.525, 0.375 and 0.161 for mastitis, staphylococcal mastitis and biofilm staphylococcal mastitis, respectively ($P < 0.001$); in contrast, no differences were recorded between the two groups during the 2nd stage of lactation: 0.343, 0.187 and 0.060 *versus* 0.388, 0.227 and 0.073, respectively ($P > 0.22$).

The first case of staphylococcal and biofilm staphylococcal mastitis occurred later in V (median value: 3rd sampling point) than in C (median value: 2nd sampling point) ewes ($P < 0.003$). Also, a smaller proportion of the cases of staphylococcal mastitis in V ewes occurred during the 1st stage of the lactation period than those in C ewes: 0.593 (32 / 54) *versus* 0.853 (81 / 95) of ewes, respectively ($P < 0.001$).

Table III.xi. Mastitis occurrence in ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

Farm	Ewes with mastitis	Ewes with staphylococcal mastitis	Sampling points with mastitis from which staphylococci were isolated	Sampling point in which staphylococcal mastitis first occurred[1]	Ewes with biofilm staphylococcal mastitis	Ewes with staphylococcal mammary carriage	Ewes with recurrent staphylococcal mastitis
Group V[2] (adult ewes / *primiparae* ewes)							
A	13 / 5	7 / 1	9 / 1	6.0 / 7.0	5 / 1	1 / 0	0 / 0
D	15 / -	6 / -[3]	12 / -	3.5 / -	3 / -	7 / -	0 / -
K	47 / -	19 / -[3]	50 / -	3.0 / -	9 / -	22 / -	4 / -
P	10 / 0	6 / 0	6 / 0	2.5 / na[4]	2 / 0	1 / 0	0 / 0
S	24 / 2	13 / 2	19 / 2	4.0 / 2.5	na[4]	9 / 0	2 / 1
	109 / 7	51 / 3	96 / 3	3.0 / 4.0	19 / 1	40 / 0	6 / 1
Group C[2] (adult ewes / *primiparae* ewes)							
A	16 / 0	12 / 0	27 / 0	2.0 / na	12 / 0	7 / 2	2 / 0
D	23 / -	16 / -[3]	34 / -	3.5 / -	9 / -	3 / -	4 / -
K	53 / -	31 / -[3]	66 / -	1.0 / -	19 / -	22 / -	10 / -
P	13 / 1	11 / 1	12 / 1	2.0 / 2.0	6 / 0	2 / 1	0 / 0
S	28 / 2	22 / 2	41 / 3	1.0 / 4.0	na[4]	10 / 3	5 / 0
	133 / 3	92 / 3	180 / 4	2.0 / 2.0	46 / 0	44 / 6	21 / 0

1. Median value.
2. V: vaccinated ewes, C: unvaccinated control ewes.
3. no *primiparae* ewes enrolled in the study in this farm.
4. na: not applicable.

Table III.xii. Frequency of outcomes in ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

Farm (number of ewes in lactation period)	Mastitis	Staphylococcal mastitis	Biofilm staphylococcal mastitis
	Number of cases[1] (incidence risk[2])		
Group V[3]			
A (n = 63)	18 (0.286)	8 (0.127)	6 (0.095)[a]
D (n = 76)	15 (0.197)	6 (0.079)[c]	3 (0.039)
K (n = 71)	47 (0.662)	19 (0.268)[b]	9 (0.127)[b]
P (n= 39)	10 (0.256)[a]	6 (0.154)[b]	2 (0.051)[a]
S (n = 67)	26 (0.388)	15 (0.224)[b]	na[4]
	116 (0.367)[a]	54 (0.171)[c]	20 (0.080)[c]
Group C[3]			
A (n = 59)	16 (0.271)	12 (0.203)	12 (0.203)[a]
D (n = 74)	23 (0.311)	16 (0.216)[c]	9 (0.122)
K (n = 72)	53 (0.736)	31 (0.431)[b]	19 (0.264)[b]
P (n = 39)	14 (0.359)[a]	12 (0.308)[b]	6 (0.154)[a]
S (n = 63)	30 (0.476)	24 (0.381)[b]	na[4]
	136 (0.443)[a]	95 (0.309)[c]	46 (0.189)[c]

a = $0.026 < P < 0.05$, b = $0.01 < P < 0.025$, c = $P < 0.01$ for same superscript in the same column for comparison within the same farm.

1. Number of ewes with respective outcome during the lactation period (animals were taken into account only once, irrespective of new cases that they might had developed).

2. The proportion of animals at risk, which developed the condition when the time at risk differed between animals (for example, during an entire lactation which varied between individuals).

3. V: vaccinated ewes, C: unvaccinated control ewes.

4. na: not applicable.

Table III.xiii. Frequency of outcomes in clusters of sheep farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

Farm (number of ewes in lactation period)	Mastitis	Staphylococcal mastitis	Biofilm staphylococcal mastitis
Number of cases[1] (incidence risk[2])			
Intensive management system (flocks A and S)			
V (n = 130)	44 (0.338)	23 (0.177)[b]	6 (0.095)[a]
C (n = 122)	46 (0.377)	36 (0.295)[b]	12 (0.203)[a]
Semi-intensive management system (flocks D, K and P)			
V (n = 186)	72 (0.387)[a]	31 (0.167)[c]	14 (0.075)[c]
C (n = 185)	90 (0.486)[a]	59 (0.319)[c]	34 (0.184)[c]
Hand-milking (flocks K and P)			
V (n= 110)	57 (0.518)	25 (0.227)[c]	11 (0.100)[c]
C (n = 111)	67 (0.603)	43 (0.387)[c]	25 (0.225)[c]
Machine-milking (flocks A, D and S)			
V (n = 206)	59 (0.286)	29 (0.141)[c]	9 (0.065)[c]
C (n = 196)	69 (0.352)	52 (0.265)[c]	21 (0.158)[c]
Reported history of clinical mastitis[3] (flocks A and P)			
V (n = 102)	28 (0.275)	14 (0.137)[a]	8 (0.069)[c]
C (n = 98)	30 (0.306)	24 (0.245)[a]	18 (0.184)[c]
No reported history of clinical mastitis[3] (flocks D, K and S)			
V (n = 214)	88 (0.411)[a]	40 (0.187)[c]	12 (0.082)[c]
C (n = 209)	106 (0.507)[a]	71 (0.340)[c]	28 (0.192)[c]

a = 0.026 < P < 0.05, b = 0.01 < P < 0.025, c = P < 0.01 for same superscript in the same column for comparison within the same cluster.

1 Number of ewes with respective outcome during the lactation period (animals were taken into account only once, irrespective of new cases that they might had developed).

2 Number of ewes with respective outcome during the lactation period / number of ewes that entered in lactation period.

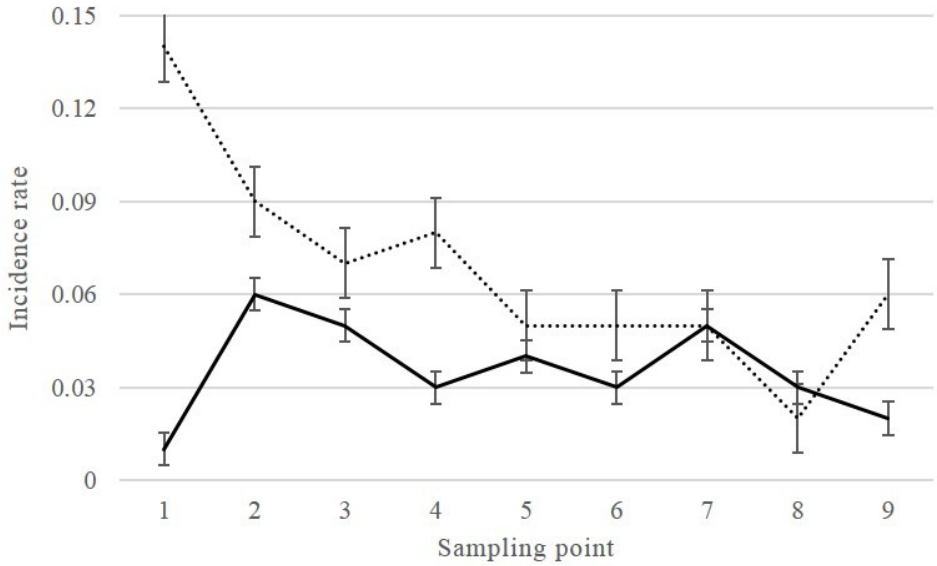
3 Reported (as perceived by farmer) increased (> 0.15) incidence risk of clinical mastitis during the preceding lactation period.

Table III.xiv. Details of incidence rates[1] of mastitis in ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Sampling point								
Outcome	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Group V[2]									
Mastitis	0.055	0.068	0.068	0.066	0.074	0.051	0.086	0.076	0.043
Staphylococcal mastitis	0.013	0.057	0.047	0.031	0.039	0.034	0.055	0.025	0.022
Biofilm staphylococcal mastitis	0.000	0.020	0.018	0.040	0.050	0.060	0.031	0.080	0.022
Staphylococcal mammary carriage	0.029	0.070	0.070	0.013	0.049	0.023	0.031	0.080	0.000
Group C[2]									
Mastitis	0.155	0.109	0.091	0.081	0.064	0.077	0.070	0.082	0.171
Staphylococcal mastitis	0.139	0.084	0.068	0.076	0.050	0.047	0.053	0.020	0.057
Biofilm staphylococcal mastitis	0.064	0.033	0.023	0.038	0.015	0.060	0.035	0.010	0.029
Staphylococcal mammary carriage	0.017	0.018	0.027	0.014	0.050	0.053	0.018	0.020	0.029

1. The proportion of animals at risk, which developed the condition when the time at risk was the same in each group.
2. V: vaccinated ewes, C: unvaccinated control ewes.

Figure III.3. Incidence rates[1] of staphylococcal mastitis in ewes in five farms throughout a lactation period, during evaluation of the test vaccine against mastitis.



Continuous line: V group (vaccinated ewes), dotted line: C group (unvaccinated control ewes).
1. The proportion of animals at risk, which developed the condition when the time at risk was the same in each group.

The median duration of staphylococcal and biofilm staphylococcal mastitis was 30 days for both groups ($P > 0.40$). Further, staphylococcal mastitis recurrence was observed in 7 of the 54 V ewes (0.130) and in 21 of the 95 C ewes (0.221) ($P = 0.12$).

Incidence risk of staphylococcal mammary carriage for ewes in group V and C was 0.127 *versus* 0.163, respectively ($P = 0.099$). The proportion of group V ewes with mammary carriage, which developed mastitis, was lower than for group C ewes: 0.350 of group V (14 / 40) *versus* 0.580 of group C (29 / 50), respectively, for staphylococcal mastitis ($P = 0.025$) and 0.100 (1 / 10) *versus* 0.583 (7 / 12), respectively, for biofilm staphylococcal mastitis ($P = 0.026$).

Incidence risks of staphylococcal IMI and biofilm staphylococcal IMI were smaller in group V than in group C ewes: 0.253 (95% CI: 0.208 – 0.304) and 0.082 (95% CI: 0.057 – 0.118) *versus* 0.378 (95% CI: 0.326 – 0.433) and 0.169 (95% CI: 0.130 – 0.217), respectively ($P < 0.001$ and $P = 0.001$, respectively). Incidence risk of staphylococcal IMI and biofilm staphylococcal IMI were 0.262, 0.085 *versus* 0.388, 0.182, respectively ($P \leq 0.001$) for adult ewes and 0.136, 0.045 *versus* 0.269, 0.038, respectively ($P > 0.22$) for *primiparae* ewes. The first case of staphylococcal IMI occurred later in V (median value: 3rd sampling point) than in in C (median value: 2nd sampling point) ewes ($P < 0.001$). Duration of IMIs was the same for ewes in both groups: 30 days. Finally, IMI recurrence

was observed in 17 of the 80 V ewes (0.213) and in 29 of the 116 C ewes (0.250) ($P = 0.54$) (Tables III.xv, III.xvi).

Efficacy of the test vaccine

Overall, efficacy of the vaccine was 17.2% for mastitis, 44.6% for staphylococcal mastitis and 57.7% for biofilm staphylococcal mastitis. Moreover, efficacy of the vaccine against staphylococcal IMI was 33.1% and against biofilm staphylococcal IMI was 51.5% overall. Efficacy was greater during the 1st stage of lactation period (39.2%, 50.9% and 66.5%, for mastitis, staphylococcal mastitis and biofilm staphylococcal mastitis, respectively) than in the 2nd stage (always < 18% for all outcomes) ($P < 0.001$ for comparisons between the two stages). On the various sampling points, the efficacy of the vaccine was up to 65.0% for mastitis, up to 91.0% for staphylococcal mastitis and up to 100% for biofilm staphylococcal mastitis (Table xvii).

Table III.xv. Occurrence of staphylococcal and biofilm staphylococcal intramammary infection (IMI) in ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

Farm	Ewes with staphylococcal IMI	Sampling points with IMI from which staphylococci were isolated	Sampling point in which IMI first occurred[1]	Ewes with biofilm staphylococcal IMI	Ewes with recurrent IMI
Group V[2] (adult ewes / *primiparae* ewes)					
A	8 / 1	9 / 1	6.0 / 7.0	5 / 1	0 / 0
D	13 / -	17 / -	4.0 / -	7 / -	0 / -
K	29 / -	117 / -	2.0 / -	10 / -	12 / -
P	7 / 0	7 / 0	2.0 / na[4]	3 / 0	1 / 0
S	20 / 2	37 / 3	4.5 / 2.5	na	3 / 1
	77 / 3	187 / 4	3.0 / 4.0	25 / 1	16 / 1
Group C[2] (adult ewes / *primiparae* ewes)					
A	18 / 2	35 / 2	2.0 / 4.0	16 / 1	4 / 0
D	18 / -	44 / -	2.5 / -	9 / -	5 / -
K	37 / -	137 / -	1.0 / -	19 / -	14 / -
P	12 / 1	15 / 2	2.0 / 1.0	7 / 0	2 / 1
S	24 / 4	77 / 8	1.5 / 3.5	na	2 / 1
	109 / 7	308 / 12	2.0 / 3.0	51 / 1	27 / 2

1. Median value.
2. V: vaccinated ewes, C: unvaccinated control ewes.
3. no *primiparae* ewes enrolled in the study in this farm.

4. na: not applicable.

Table III.xvi. Details of incidence rates[1] of staphylococcal and biofilm staphylococcal intramammary infection (IMI) in ewes in five farms throughout a lactation period, during evaluation of the test vaccine against staphylococcal mastitis.

	Sampling point								
Outcome	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Group V[2]									
Staphylococcal IMI	0.042	0.061	0.068	0.048	0.089	0.063	0.078	0.025	0.022
Biofilm staphylococcal IMI	0.016	0.027	0.018	0.040	0.015	0.011	0.031	0.017	0.022
Group C[2]									
Staphylococcal IMI	0.162	0.102	0.072	0.081	0.054	0.089	0.061	0.041	0.086
Biofilm staphylococcal IMI	0.068	0.047	0.027	0.033	0.025	0.012	0.026	0.010	0.029

1. The proportion of animals at risk, which developed the condition when the time at risk was the same in each group.
2. V: vaccinated ewes, C: unvaccinated control ewes.

Table III.xvii. Efficacy of the test vaccine against staphylococcal mastitis in ewes in five farms throughout a lactation period.

	Sampling point								
Outcome	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Mastitis	0.646	0.383	0.251	0.191	0.000	0.331	0.000	0.660	0.746
Staphylococcal mastitis	0.907	0.316	0.317	0.599	0.204	0.276	0.000	0.000	0.620
Biofilm staphylococcal mastitis	1.000	0.383	0.211	0.885	0.668	0.034	0.109	0.169	0.239
Staphylococcal IMI	0.741	0.402	0.056	0.407	-0.648	0.292	-0.279	0.390	0.744
Biofilm staphylococcal IMI	0.765	0.426	0.333	0.879	0.400	0.083	-0.192	-0.070	0.241

C. EVALUATION OF THE EFFICACY OF THE TEST VACCINE IN AN EXPERIMENTAL STUDY

Materials and methods

Experimental design

Animals

Thirty-seven (37) Chios-cross ewe-lambs were used in the study. Before enrolment in the study, a standardised detailed clinical examination was performed to assess the general health of the animals. Animals were allocated into four groups (with two of the groups further subdivided into two subgroups each) as shown in Table III.xviii. Allocation was by using a random number generator to achieve complete randomisation. Animals were identified using neck straps and plastic tags with unique serial numbers.

Animals in each group (B, D) or subgroup (A1, A2, C1, C2) were penned together and separately from animals in other groups / subgroups. Reproductive control (latitude of location: N 39.37 °, month of application: October) was performed by intravaginal insertion of progestogen sponges and injection of equine chorionic gonadotropin (300 i.u.). Animals were mated by rams of known fertility and repeatedly examined ultrasonographically to confirm pregnancy and its normal progress (Valasi et al. 2017). Standard health management procedures were performed in ewes (Fthenakis et al. 2012); netobimin (dose rate: 20 mg kg bw^{-1}) was administered orally to all animals on the 98th day of pregnancy.

Vaccination schedule and administration

A vaccine licensed in European Union countries for protection of ewes against clinical staphylococcal mastitis (Vimco®; Laboratorios Hipra, Girona, Spain) was used (batches: 69VW-1, 77XW-1). Vaccination was carried out during the last stage of gestation of ewes, following the licensed schedule of the product. The initial administration was performed on the 112th day after ram introduction (i.e., 5 weeks before expected lambing) and was followed by a repeat administration 21 days later.

Table III.xviii. Design of an experimental study of evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup	n	Vaccination	Intramammary inoculation with *S. chromogenes*
A1	8	+	10th day post-partum
A2	9	+	50th day post-partum
B	6	+	-
C1	4	-	10th day post-partum
C2	4	-	50th day post-partum
D	6	-	-

+: performed, -: nor performed.

Vaccine (groups A and B) or placebo (groups C and D) administration was performed after clipping the wool in the neck region of the animal. A dose of 2 mL of the product (A and B) or normal saline (C and D) was injected intramuscularly. A plastic syringe and a hypodermic needle were used and discarded after a single administration. First injection to each animal was performed at the left side of the neck; repeat injection was performed at the right side.

The other vaccines that had been administered to the animals during the study period, were a vaccine against bacterial respiratory infections (on the 6th week of pregnancy) and an anti-clostridial vaccine (on the 98th day of pregnancy, i.e. 14 days prior to the initial administration of the vaccine under evaluation). These vaccines had been injected subcutaneously in the area behind the front leg of the sheep.

Inoculations

After lambing, animals in group A or C were challenged (day D0) with *S. chromogenes* (biofilm-forming strain 6684, isolated from ewe' milk), that had been isolated from a case of mastitis in a ewe. Animals in subgroups A1 or C1 were challenged on the 10th day after lambing (D0) and animals in subgroups A2 or C2 were challenged on the 50th day after lambing (D0).

For inoculation, the challenge isolate was grown on Columbia blood agar and checked for purity; then it was inoculated into Soy-broth (BioMerieux) and incubated aerobically at 37 ^{o}C for 5 h. Serial dilutions of the broth culture into phosphate-buffer-saline (PBS), pH 7.3 were carried out; finally, 0.2 mL of the desired dilution was withdrawn with a syringe. The inoculum contained 4.88×10^4 to 5.22×10^5 c.f.u., as estimated by the method of Miles and Misra (1938).

To ensure sterile conditions, on the day before inoculation the hairs of the teats were clipped by using fine scissors and the skin of the udder and teats was scrubbed by using chlorhexidine; then, on the day of inoculation, iodine povidone solution was used. The ewes were completely milked out;

they were inoculated directly into the mammary gland cistern (*sinus lactiferous*). The challenge mammary gland was chosen randomly, at the toss of a coin.

A sterile plastic fine catheter (Abbocath, Abbot) 20 G was inserted into the teat; a syringe containing the inoculum was attached to the catheter and the bacterial suspension was injected. The same technique was used to inject 0.2 mL of PBS into the contralateral mammary cistern of each ewe, in order to be used as control.

Examination of animals and collection of samples

Clinical examinations

After each vaccine administration, animals were observed for any systemic or local reactions.

A general clinical examination of animals was performed 2 days after lambing and then at 5-day intervals. Before inoculation, on D-4 and D-1, a standardised detailed clinical examination of the udder (observation, palpation, comparison between glands) was performed as described previously (Chapter III, section B).

After challenge, detailed clinical examinations as above were performed daily on D1 to D7, D10, D14, D17 and thereafter weekly to D52 for groups A and C. For groups B and D (10th day after lambing = D0), examinations were performed on above days, as well on D36, daily on D39 to D47, D50, D54, D57 and thereafter weekly to D92. Lambs were with their dams throughout the study.

Ultrasonographic examination of udder

A standardised B-mode ultrasonographic examination of the udder was performed (Barbagianni et al. 2017). The examination was always performed after emptying the udder, with the animal in the standing position and restrained inside a crate, using the support of an assistant. Hair on the udder was fully clipped. An ultrasound scanner (MyLab® 30; ESAOTE SpA, Genova, Italy), fitted with a linear transducer with 7.5 - 12.0 MHz imaging frequency was used. Coupling gel was applied. The probe was placed on the caudal surface of the udder and moved around it.

Initially, the uninoculated side of the udder was imaged. The transducer was placed in a position perpendicular to the long axis and dorsal sections of the mammary parenchyma were taken, starting from the upper part downwards. In each mammary gland, three images were saved for further processing; the first image was taken before the branching of the external pudental artery (*arteria pudenda externa*), the second when the distance between branches of the external pudental artery was ~1 cm and the third image was taken immediately before the gland cistern (*sinus lactiferous*) became visible. For this procedure, 40 - 60 mm scanning depth and 10.0 or 12.0 MHz

frequency were used. The imaging procedures were repeated for the inoculated mammary gland. B-mode images were frozen and saved on the equipment hard-disk for performing subsequently appropriate measurements and data analysis.

Collection of milk samples

On each of the above occasions, milk samples were collected from all animals. The procedures described previously were followed (Chapter III, section B).

Milk yield measurements

On eight occasions for groups A and C (D-1, D10, D17 and thereafter weekly to D52) and on 16 occasions for groups B and D (D-1, D10, D17 and thereafter weekly to D38, and then on D39, D45, D50, D52, D57 and thereafter weekly to D92), measurements of milk yield of all ewes were performed.

Measurements were carried in the morning and performed as described by Fthenakis et al. (2005). Ewes were injected intramuscularly with 5 i.u. oxytocin and were hand-milked out. Their lambs were separated from their dams. Four hours later, they were injected again with 5 i.u. oxytocin and once more were hand-milked out. The milk was collected into a plastic container. To avoid misreading by foam formation and for accuracy of results, the milk was slowly poured into a volumetric glass container graduated to 2 mL and allowed still for 5 min. before reading.

Collection of blood samples

Blood samples (for serum extraction) were collected from all animals on seven occasions during the study. Samples were collected as follows: (i) before initial vaccination, (ii) before booster vaccination, (iii) three weeks after booster vaccination (5th – 7th day post-partum), (iv) on D-1, (v) on D14, (vi) on D31 and (vii) on D52.

Mammary tissue biopsy

In ewes of groups A (4 of each of A1 and A2) and C (2 of each of C1 and C2), mammary tissue biopsy was carried out after the end of the study monitoring period, on D53. The operation was carried out under sedation and local anaesthesia by means of lidocaine (Galatos 2011). All surgical procedures were performed under strict aseptic conditions. A biopsy of the parenchyma of both mammary glands was performed (Fthenakis and Jones 1990c); two cubes of tissue, approximately 1 × 1 × 1 cm, were removed from the caudal part of each mammary gland. After obtaining the tissue samples, the wounds were repaired.

Laboratory examinations

Bacteriological examination of milk and tissue samples

Milk samples (10 μL) were cultured using Columbia blood agar plates incubated aerobically at 37 °C for 48 h. If nothing had grown, media were re-incubated for another 24 h. Presence of bacterial colonies morphologically similar to those of *S. chromogenes*, i.e., smooth, entire, glistening, with yellow or yellow-orange pigment, recovered in pure culture, were considered to be those of the challenge organism. Moreover, counts of the organism in milk samples were performed by following the method of Miles and Misra (1938).

Tissue samples were washed with PBS, to remove mammary secretion, and, then, were homogenised (10 g of tissue sample with 50 mL of sterile PBS blended for 3 min.) in a tissue blender (Mixwel; Alliance Bio Expertise, Guipry, France). The mixture was cultured and bacterial counts therein were performed, also by following the method of Miles and Misra (1938).

In 68 of the 272 isolates (0.250) recovered from milk samples and in all (11) isolates recovered from tissue samples, which had been considered to be the challenge organism, detailed identification was performed routinely by using the Vitek® 2 automated system (BioMerieux, Marcy-l'-Étoile, France). Selection of the isolates was performed at random, by using an electronic random number generator (www.randomresult.com).

Cytological examination of milk samples

All milk samples were tested by use of the Microscopic cell counting method (Mccm) (IDF reference method) (International Dairy Federation 1984) for cell counting therein. Smears were also produced from milk samples and dried; these were stained by the Giemsa method for estimation of leucocyte subpopulations. The proportion of leucocyte types was calculated by observing at least 10 fields of each milk film under magnification 10× and counting at ≥ 100 leucocytes.

Detection of anti-PNAG antibodies in blood samples

Blood samples were centrifuged for serum collection in preparation for measurement of IgG PNAG-specific antibodies. The technique and procedures described previously (Chapter II, section B) were used.

Histopathological examination of tissue samples

Tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. Haematoxylin and eosin (HE) standard staining procedures were performed for histopathological studies.

Data management and analysis

Clinical scores

During the clinical examination, clinical scores were produced, by using a scheme (Fthenakis 2000) that took into account systemic (attitude, appetite, temperature) and local mammary (secretion, temperature, pain, oedema, size, colour) signs. For each sign, a score from 0 to 4 was employed; in the udder, separate scores were given to the inoculated and the contralateral mammary gland. Hence, possible scores ranged from 0 to 12 for systemic signs and from 0 to 24 for mammary signs. At the end, a total score with possible values ranging from 0 to 60 was produced by adding together scores given for systemic signs and for mammary signs in each gland. Details are in Table xix.

Definitions of mastitis

Mastitis included clinical and subclinical mastitis. Clinical mastitis was defined in ewes with presence of abnormal gross findings in a mammary gland (including changes in secretion) (Fragkou et al. 2014); subclinical mastitis was defined in ewes in which a bacteriologically positive (with the challenge organism) milk sample with concurrently increased cell counts ($\geq 0.5 \times 10^6$ cells mL^{-1}) plus neutrophil and lymphocyte proportion (≥ 65% of all leucocytes) was detected (Fragkou et al. 2014). Recurrence of staphylococcal mastitis in a ewe was defined when a ewe with mastitis changed to not having mastitis and then again to having it. Mastitis definitions referred to ewes. An incident of mammary infection (IMI) was defined, when the above findings referred to individual mammary glands. Therefore, on a sampling point, if both mammary glands of a ewe were affected, one case of mastitis and two IMIs were calculated.

For the analysis, the post-challenge experimental period was divided in three stages: S1 (that included acute post-inoculation period: sampling points D1, D2, D3), S2 (that included subacute post-inoculation period: sampling points D4 to D10) and S3 (that included chronic post-inoculation period: sampling points D14 to D52).

Table III.xix. Description of local and systemic reaction scores assigned during clinical examination of ewes for evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

	Local (mammary) reactions						Systemic signs		
Score	Secretion	Temperature	Pain	Oedema	Size	Colour	Attitude	Appetite	Rectal temperature
0	normal	normal	none	none	normal	normal	normal	normal	≤ 39.9 °C
1	flakes	warm +	slight	slight	10-35% increase	pink	slight depression	slight reduction	40.0 – 40.4 °C
2	clots	warm ++	moderate	moderate	36-65% increase	tomato red	moderate depression	moderate reduction	40.5 – 40.9 °C
3	serous	warm +++	marked	marked	66-100% increase	dark red	marked depression	almost absent	41.0 – 41.4 °C
4	blood	cold	severe	widespread	decrease	blue to black	severe depression	completely absent	≥ 41.5 °C

Evaluation of ultrasonographic images

During the ultrasonographic examination, images of mammary parenchyma were recorded. Stored images of mammary parenchyma were processed by means of ImageJ software (National Institutes of Health, Rockville Pike, MD, USA), which can edit, process and analyse grey-scale images, by calculating area and pixel value statistics to produce intensity values (National Institutes of Health 2013). In an image processing context, grey-scale analysis referred to the image's overall pixel grey intensity values (Ojala et al. 2002). For analysis of grey-scale, intensity values of each of the three images stored from each mammary gland on each occasion were taken into account together. Areas with vessels or ductal formations were not taken into account for the grey-scale analysis. Results were expressed on a 0 (black) to 255 (white) scale.

For analysis of results of grey-scale measurements, data were normalised by calculating the following ratio: $[GS_i / GS_c]_n / [GS_i / GS_c]_{D-1}$, where GS_i: grey-scale of inoculated mammary gland, GS_C: grey-scale of contralateral mammary gland, n: day of measurement after challenge (i.e., D1, D2, D3 etc.), D-1: day before challenge.

Milk yield ratios

For analysis of results of milk yield measurements, data were normalised by calculating the following ratio: $[MY_i / MY_c]_n / [MY_i / MY_c]_{D-1}$, where MY_i: milk yield of inoculated mammary gland, MY_C: milk yield of contralateral mammary gland, n: day of measurement after challenge (i.e., D10, D17, D24 etc.), D-1: day before challenge.

Optical density during measurement of anti-PNAG specific antibodies in blood samples

Results of antibody measurements in serum samples were expressed as optical density (OD) values. Data were processed as described previously (Chapter II, section B).

Histopathological scores

A scoring system previously developed and described (Fragkou, Mavrogianni et al. 2007) was used and numerical values were assigned for the histopahtological findings in the tissue samples. A 0 to 4 scale was used, as detailed in Table xx. Separate scores were assigned for the tissue sample collected from each mammary gland.

Table III.xx. Description of scores given for histopathological findings in the mammary parenchyma of ewes during evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Score	Description
0	Normal
1	Presence of a few, scattered leucocytes
2	Presence of increased numbers of leucocytes clustered in the intra- and inter-alveolar area
3	Diffuse presence of leucocytes, extravasation and destruction of epithelial cells
4	Haemorrhages, destruction of alveoli and loss of the internal architecture of the parenchyma

Statistical analysis

Data were entered into Microsoft Excel and analysed using SPSS v. 21 (IBM Analytics, Armonk, USA). Basic descriptive analysis was performed.

Evaluations of associations and frequencies were performed by use of the Pearson chi-square test or Fisher exact test as appropriate. Comparison of clinical scores between groups was performed by use of the Mann-Whitney test; comparisons of clinical scores between stages within the same group was performed by the Kruskal-Wallis test. The Mann-Whitney non-parametric test was used to compare duration of mastitis between groups.

Comparisons of bacterial counts in milk between inoculated and contralateral glands within the same group, between inoculated and contralateral glands of different groups and between stages of the study were performed by means of analysis of variance. To account for the matched nature of bacterial counts in samples from inoculated and contralateral glands, the difference between the inoculated and the contralateral gland was calculated for each examination point; comparisons between group A and group C were performed by using the Wilcoxon sign-rank test for each stage of the post-challenge experimental period.

Comparisons of grey-scale ratios and milk yield ratios between groups were also performed by analysis of variance, with experimental group and day of the study as factors.

Linear mixed models were used in analysis to account for repeated measures of anti-PNAG antibody titres over the course of the study. Time points of collecting data were selected as within-subjects variables and group allocation as between-subject factor. Independent variables (fixed effects) included study group, sampling point and a sampling point by study group interaction.

The Mann-Whitney test was also used for comparison of results of histopathological evaluations between groups.

Analysis of correlation was performed between the various parametres assessed during the study among ewes in groups A and C. The results at the various sampling points were used for this

analysis. Specifically for analysis of the results of tissue samples, the sum of bacterial counts and of histopathological scores of the two mammary glands was used; for each of the other parametres, the mean of the values obtained throughout the lactation period was used for the analysis.

Initially, comparisons were performed between subgroups A1 and A2 and between C1 and C2; evaluations did not reveal any significant differences between subgroups. Hence, subgroups were taken together and their results combined with relation to assessment of outcomes.

In all analyses, statistical significance was defined at $P < 0.05$.

Results

Post-vaccination adverse reactions

After vaccination or placebo administration, no adverse reactions were recorded in any ewe. Incidence rate of adverse reaction was 0.000 (95% CI: 0.000 - 0.143).

Development of mastitis and clinical findings

All animals in groups A and C developed mastitis after the intramammary challenge. Among group A ewes, 5 (0.294) developed systemic clinical signs and 10 (0.588) developed clinical signs in the inoculated gland. Respective figures for group C were 4 (0.500) and 8 (1.000) ewes (for comparisons between groups A and C: $P = 0.28$ for presence of systemic signs, $P = 0.040$ for presence of mammary signs). No mastitis developed in any ewe in group B or D ($P < 0.02$ when compared with groups A or C). Details are in Table III.xxi.

Table III.xxi. Details of mastitis development during evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	Ewes with mammary infection in		Ewes with clinical signs		Duration of mastitis (d)	Ewes with recurrent mastitis
	inoculated side	contralateral side	systemic	mammary		
A1	8	0	4	4	4.5 (1 – 52)	3
A2	9	0	1	6	4.0 (1 – 52)	8
A	$17^{a,b}$	0^{a}	5	$10^{a,b}$	4.0^{a} (1 – 52)	11
B	$0^{a,c}$	0^{b}	0	$0^{a,c}$	0 (0 – 0)	0
C1	4	3	3	4	17.5 (11.5 – 52)	3
C2	4	2	1	4	17.5 (2 – 52)	2
C	$8^{c,d}$	$5^{a,b,c}$	4	$8^{c,d}$	17.5^{a} (2 – 52)	5
D	$0^{b,d}$	0^{c}	0	$0^{b,c}$	0 (0 – 0)	0

a-d: $P < 0.05$ for differences between values with similar letters within the same column.
1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.

Throughout the study, median total clinical score was 2.0 for group A ewes and 5.5 for group C ewes ($P = 0.025$). For group A, but not for group C, clinical scores decreased progressively during the study ($P = 0.018$ and $P = 0.47$, respectively, between the three stages). Details are in Table III.xxii. One group C ewe died on D2; the results of post-mortem examination revealed that the inoculated mammary gland was enlarged, oedematous and hyperaemic; the ipsilateral lymph node was enlarged; there was also evidence of a septicaemic carcass with peritonitis.

Table III.xxii. Median (min. – max.) total clinical scores (systemic plus mammary signs in inoculated and contralateral side of udder) during clinical examination of ewes for evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	All study[2]	S1 of study	S2 of study	S3 of study
A1	5 (0 – 7)	2.5 (0 – 5)	1 (0 – 2)	0.5 (0 – 3)
A2	1 (0 – 5)	1 (0 – 5)	0 (0 – 0)	0 (0 – 0)
A	2^{a} (0 – 7)	2 (0 – 5)	0 (0 – 2)	0 (0 – 3)
B	0^{b} (0 – 0)	0^{a} (0 – 0)	0 (0 – 0)	0 (0 – 0)
C1	5.5 (4 – 10)	3.5 (0 – 10)	0 (0 – 2)	2 (0 – 2)
C2	4.5 (1 – 14)	2.5 (0 – 14)	2 (0 – 2)	0 (0 – 1)
C	$5.5^{a,b,c}$ (1 – 14)	$3.5^{a,b}$ (0 – 14)	0 (0 – 2)	1 (0 – 2)
D	0^{c} (0 – 0)	0^{b} (0 – 0)	0 (0 – 0)	0 (0 – 0)

[a-c]. $P \leq 0.05$ between respective values within the same column.
1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.
2. S1: D1 - D3, S2: D4 - D10, S3: D14 - D52.

The duration of mastitis was shorter in group A (4 days) than in group C (17.5 days) ewes (P = 0.022). There was no difference in recurrence of mastitis between ewes in group A (11 ewes) or C (5 ewes) (P = 0.63).

In group A ewes, the relative frequency of IMIs was smaller than in group C ewes (P < 0.001). For the inoculated glands, no significant difference was evident between the two groups during stages 1 and 3 (P > 0.07), but only during stage 2 (P < 0.001); for the contralateral glands, smaller relative frequencies were seen in group A ewes in the three stages (P < 0.03 for all comparisons). In group C, IMIs were also recorded in 5 non-inoculated mammary glands (P = 0.001 *versus* group A). In group A, inoculated mammary glands were more likely to develop IMI than their own within-ewe non-inoculated glands (P < 0.001); in group C, no such difference was found (P = 0.10). relative frequencies of IMIs decreased during the study; in stage 3, they were smaller than in the other two stages (P < 0.025 for all comparisons). Details are in Table III.xxiii.

Table III.xxiii. Relative frequency of incidents of mammary infection in ewes recorded during evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	All study[2]	S1 of study	S2 of study	S3 of study
inoculated mammary glands				
A1	0.673	0.875	0.757	0.510
A2	0.630	0.889	0.578	0.556
A	$0.649^{a,b,c}$	$0.882^{a,b,p,q}$	$0.650^{a,b,c,p}$	$0.536^{a,b,q}$
B	$0.000^{a,d}$	$0.000^{a,c}$	$0.000^{a,d}$	$0.000^{a,c}$
C1	0.817	1.000	1.000	0.607
C2	0.872	1.000	0.933	0.762
C	$0.841^{b,d,e}$	$1.000^{c,d,p}$	$0.971^{b,d,e,q}$	$0.673^{c,d,p,q}$
D	$0.000^{c,e}$	$0.000^{b,d}$	$0.000^{c,e}$	$0.000^{b,d}$
contralateral mammary glands				
A1	0.000	0.000	0.000	0.000
A2	0.000	0.000	0.000	0.000
A	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}
B	0.000^{b}	0.000	0.000^{b}	0.000^{b}
C1	0.216	0.083	0.250	0.250
C2	0.213	0.182	0.067	0.333
C	$0.215^{a,b,c}$	0.130^{a}	$0.171^{a,b,c}$	$0.280^{a,b,c}$
D	0.000^{c}	0.000	0.000^{c}	0.000^{c}

a-g. $P \leq 0.05$ between respective values with similar letters within the same column in each table.
p,q. $P \leq 0.05$ between respective values with similar letters within the same row in each table.
1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.
2. S1: D1 - D3, S2: D4 - D10, S3: D14 - D52.

Bacterial counts in milk

Bacterial counts were smaller in samples from vaccinated (group A) than control (group C) ewes for samples collected from the inoculated ($P < 0.001$) and the contralateral ($P < 0.01$) mammary glands (Figure III.4). There were also differences in bacterial counts in the milk of inoculated mammary glands (groups A and C) when compared to counts in the milk of non-inoculated animals (groups B and D), in which counts were always 0 ($P < 0.01$ for all comparisons). Details are in Table III.xiv.

Within the inoculated groups (A and C), bacterial counts in samples from the inoculated glands were greater than counts in samples from the contralateral glands ($P < 0.025$ for all comparisons). Also within these groups, no differences were seen in bacterial counts between the three stages ($P > 0.22$ for all comparisons).

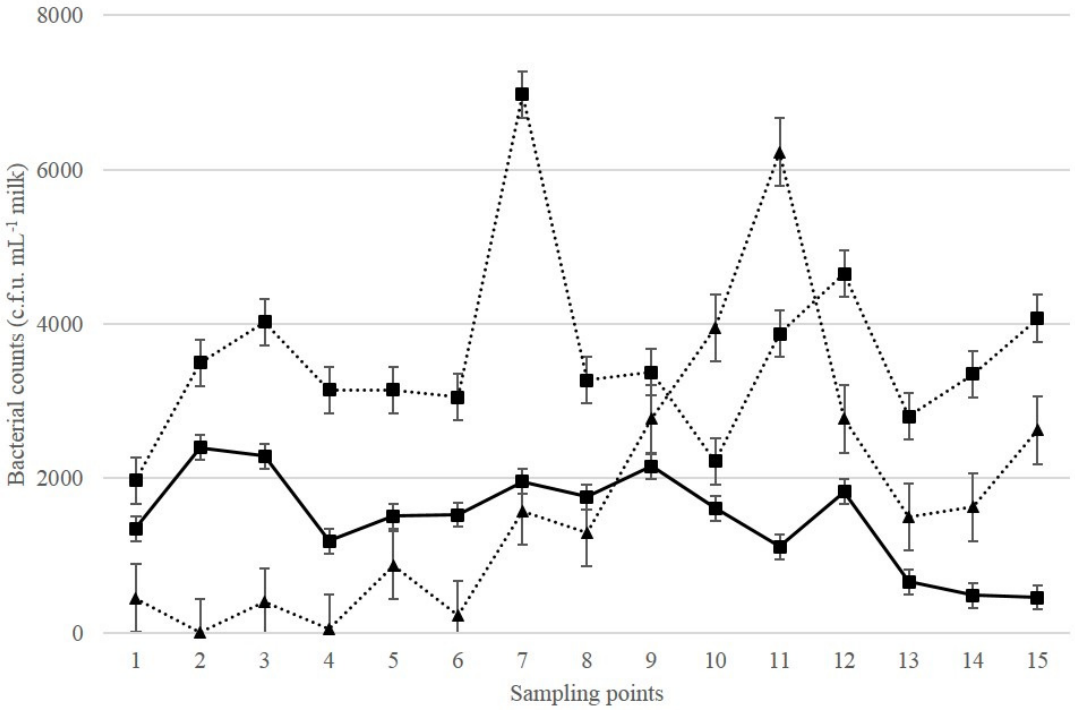
All isolates recovered from milk samples and examined microbiologically were confirmed as *S. chromogenes*.

Table III.xxiv. Mean (± standard error of the mean) bacterial counts (× 10^2 c.f.u.) in milk from ewes during evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	All study[2]	S1 of study	S2 of study	S3 of study
inoculated mammary glands				
A1	12.0 ± 1.5	11.4 ± 2.5	14.3 ± 3.1	8.9 ± 1.8
A2	15.8 ± 0.9	18.1 ± 2.6	18.2 ± 0.9	13.0 ± 1.3
A	14.1 ± 0.8^{a,b,c}	14.8 ± 1.9^{a,b,c}	17.4 ± 1.4^{a,b,c}	11.2 ± 1.1^{a,b,c}
B	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,d}	0.0 ± 0.0^{a,d}
C1	36.0 ± 4.7	33.5 ± 9.0	41.8 ± 10.3	32.9 ± 5.9
C2	35.3 ± 4.3	29.8 ± 8.3	36.6 ± 9.6	36.7 ± 5.4
C	35.6 ± 3.1^{b,d,e}	31.7 ± 5.9^{b,d,e}	39.2 ± 6.9^{b,d,e}	34.8 ± 4.0^{b,d,e}
D	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{c,e}	0.0 ± 0.0^{c,e}
contralateral mammary glands				
A1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
A	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
B	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
C1	24.2 ± 4.5	5.7 ± 3.6	14.6 ± 5.6	39.1 ± 6.8
C2	10.9 ± 3.7	0.0 ± 0.0	1.5 ± 1.2	22.3 ± 6.9
C	17.6 ± 3.0^{a,b,c}	2.8 ± 1.9^{a,b,c}	8.1 ± 3.2^{a,b,c}	30.7 ± 5.0^{a,b,c}
D	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}

a-g. $P \leq 0.05$ between respective values with similar letters within the same column in each table.
p,q. $P \leq 0.05$ between respective values with similar letters within the same row in each table.
1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.
2. S1: D1 - D3, S2: D4 - D10, S3: D14 - D52.

Figure III.4. Mean (± standard error of the mean) bacterial counts in milk from ewes throughout a study of evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.



Solid line: inoculated glands of group A (vaccinated ewes), dotted line with triangular points: inoculated glands of group C (non-vaccinated ewes), dotted line with square points: contralateral glands of group C (non-vaccinated ewes); for contralateral glands of group A and both glands of groups B and D all results were equal to 0.

Ultrasonographic examination of udder

There were differences in grey-scale ratios of ewes between the four groups ($P < 0.001$). Between group differences were significant for A1, B1 and D1 *versus* C1 ($P < 0.01$) and for A2, B2 and D2 *versus* C2 ($P < 0.01$). Details are in Table III.xxv.

Milk yield measurements

There were differences in milk yield ratios of ewes between the four groups ($P < 0.001$). Between group differences were significant for A1, B1 and D1 *versus* C1 ($P < 0.01$) and for A2, B2 and D2 *versus* C2 ($P < 0.01$). Details are in Figure III.5 and in Table III.xxvi.

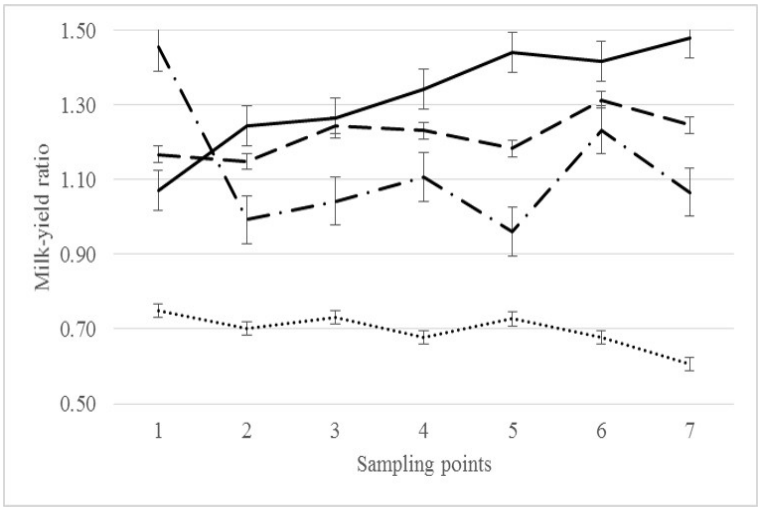
Table III.xxv. Mammary parenchyma grey-scale ratios in groups of ewes throughout a study for evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	Day after inoculation														
	D1	D2	D3	D4	D5	D6	D7	D10	D14	D17	D24	D31	D38	D45	D52
A1	0.85 ± 0.04	0.84 ± 0.03	0.82 ± 0.04	0.85 ± 0.05	0.85 ± 0.04	0.85 ± 0.03	0.92 ± 0.05	0.87 ± 0.03	0.92 ± 0.04	0.98 ± 0.04	0.91 ± 0.05	0.92 ± 0.05	0.95 ± 0.04	1.01 ± 0.04	1.11 ± 0.07
B[2]	0.92 ± 0.09	0.88 ± 0.08	0.92 ± 0.09	0.94 ± 0.09	0.91 ± 0.09	0.94 ± 0.09	0.93 ± 0.09	0.93 ± 0.09	0.89 ± 0.08	0.91 ± 0.08	0.90 ± 0.09	0.88 ± 0.09	0.88 ± 0.08	0.93 ± 0.09	0.90 ± 0.08
C1	0.58 ± 0.03	0.58 ± 0.01	0.61 ± 0.02	0.64 ± 0.05	0.70 ± 0.02	0.69 ± 0.07	0.73 ± 0.02	0.76 ± 0.05	0.81 ± 0.02	0.84 ± 0.04	0.98 ± 0.03	1.14 ± 0.04	1.27 ± 0.03	1.29 ± 0.06	1.44 ± 0.05
D[2]	1.00 ± 0.01	1.02 ± 0.02	1.01 ± 0.02	1.00 ± 0.03	0.99 ± 0.03	1.01 ± 0.03	1.02 ± 0.02	1.04 ± 0.01	1.00 ± 0.02	1.04 ± 0.03	0.98 ± 0.03	1.02 ± 0.04	0.98 ± 0.02	1.00 ± 0.04	1.02 ± 0.05
A2	0.89 ± 0.02	0.91 ± 0.02	0.89 ± 0.03	0.90 ± 0.03	0.92 ± 0.02	0.89 ± 0.02	0.92 ± 0.02	0.93 ± 0.02	0.93 ± 0.02	0.93 ± 0.02	0.94 ± 0.02	0.97 ± 0.02	0.95 ± 0.01	0.98 ± 0.02	1.01 ± 0.03
B[3]							1.06 ± 0.05	1.02 ± 0.03		1.07 ± 0.03	1.05 ± 0.02	1.07 ± 0.03	1.06 ± 0.03	1.04 ± 0.03	1.04 ± 0.02
C2	0.55 ± 0.06	0.58 ± 0.04	0.57 ± 0.07	0.57 ± 0.04	0.61 ± 0.04	0.70 ± 0.06	0.73 ± 0.06	0.78 ± 0.05	0.82 ± 0.08	0.85 ± 0.08	1.16 ± 0.02	1.39 ± 0.03	1.36 ± 0.03	1.46 ± 0.02	1.46 ± 0.03
D[3]							1.03 ± 0.04	1.04 ± 0.05		0.97 ± 0.06	0.99 ± 0.04	0.96 ± 0.07	0.98 ± 0.06	1.01 ± 0.03	0.99 ± 0.05

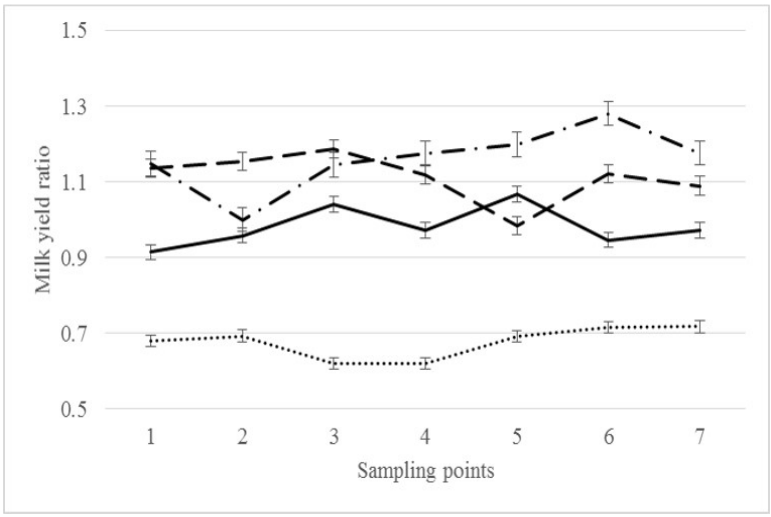
1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.
2. Period from day 9 to day 62 post-partum.
3. Period from day 49 to day 102 post-partum.

Figure III.5. Milk yield ratios in groups of ewes throughout a study for evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

(a) Groups A1, B[1], C1, D[1]



(b) Groups A2, B[2], C2, D[2]



Solid line: group A (vaccinated, inoculated ewes), dashed line: group B (vaccinated, non-inoculated ewes), dotted line: group C (non-vaccinated, inoculated ewes), dashed-dotted line: group D (non-vaccinated, non-inoculated ewes).

1. Period from day 9 to day 62 post-partum.
2. Period from day 49 to day 102 post-partum.

Table III.xxvi. Milk yield ratios in groups of ewes throughout a study for evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	Day after inoculation						
	D10	D17	D24	D31	D38	D45	D52
A1	$1.07^{a} \pm 0.10$	$1.24^{a} \pm 0.16$	$1.26^{a} \pm 0.17$	$1.34^{a} \pm 0.19$	$1.44^{a} \pm 0.21$	$1.42^{a} \pm 0.21$	$1.48^{a} \pm 0.23$
B[2]	$1.17^{b} \pm 0.10$	$1.15^{b} \pm 0.12$	$1.24^{b} \pm 0.11$	$1.23^{b} \pm 0.13$	$1.18^{b} \pm 0.10$	$1.31^{b} \pm 0.18$	$1.25^{b} \pm 0.14$
C1	$0.75^{a,b,c} \pm 0.12$	$0.70^{a,b,c} \pm 0.13$	$0.73^{a,b,c} \pm 0.13$	$0.68^{a,b,c} \pm 0.14$	$0.73^{a,b,c} \pm 0.13$	$0.68^{a,b,c} \pm 0.18$	$0.61^{a,b,c} \pm 0.15$
D[2]	$1.45^{c} \pm 0.55$	$0.99^{c} \pm 0.16$	$1.04^{c} \pm 0.13$	$1.11^{c} \pm 0.17$	$0.96^{c} \pm 0.09$	$1.23^{c} \pm 0.13$	$1.07^{c} \pm 0.04$
A2	$0.91^{a} \pm 0.09$	$0.96^{a} \pm 0.06$	$1.04^{a} \pm 0.10$	$0.97^{a} \pm 0.08$	$1.07^{a} \pm 0.16$	$0.95^{a} \pm 0.08$	$0.97^{a} \pm 0.08$
B[3]	$1.14^{b} \pm 0.12$	$1.15^{b} \pm 0.14$	$1.19^{b} \pm 0.12$	$1.12^{b} \pm 0.14$	$0.98^{b} \pm 0.10$	$1.12^{b} \pm 0.09$	$1.09^{b} \pm 0.10$
C2	$0.68^{a,b,c} \pm 0.07$	$0.69^{a,b,c} \pm 0.10$	$0.62^{a,b,c} \pm 0.03$	$0.62^{a,b,c} \pm 0.06$	$0.69^{a,b,c} \pm 0.02$	$0.72^{a,b,c} \pm 0.07$	$0.72^{a,b,c} \pm 0.08$
D[3]	$1.15^{c} \pm 0.08$	$1.00^{c} \pm 0.11$	$1.15^{c} \pm 0.16$	$1.18^{c} \pm 0.18$	$1.20^{c} \pm 0.18$	$1.28^{c} \pm 0.24$	$1.18^{c} \pm 0.20$

a,b,c. $P < 0.01$ between respective values with similar letters within the same column in each table.
1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.
2. Period from day 9 to day 62 post-partum.
3. Period from day 49 to day 102 post-partum.

Detection of anti-PNAG antibodies in blood samples

After vaccination, there was an increase in antibody titres in ewes of groups A and B ($P < 0.001$) compared to pre-vaccination titres. In contrast, no changes in antibody titres throughout the study were evident in ewes of groups C and D ($P > 0.17$). Antibody titres in ewes of group A (vaccinated) were higher than those in group C (non-vaccinated) ($P < 0.001$). There were no differences between groups A and B and between groups C and D ($P > 0.28$). Details are in Table III.xxvii.

Among vaccinated ewes (groups A and B), values varied from -6.24 to 69.64; values below 6.0 were recorded in 7 samples (0.061 of all) ($P < 0.001$ compared to groups C and D). Among challenged non-vaccinated ewes (group C), values varied from -4.94 to 23.12; values above 6.0 were recorded in 10 samples (0.476 of all). During the same period, among non-challenged non-vaccinated ewes (group D) values varied from -4.17 to 51.95; values above 6.0 were recorded in 6 samples (0.333 of all) ($P = 0.28$ compared to group C).

Table III.xxvii. Mean (± standard error of the mean) values of anti-PNAG-specific antibody titres in blood samples from ewes throughout a study for evaluation of the efficacy of the test vaccine against staphylococcal mastitis in sheep.

Group or subgroup[1]	Sampling points										
	112th day of pregnancy	133rd day of pregnancy	5th – 7th day post-partum	9th day post-partum	24th day post-partum	41st day post-partum	49th day post-partum	62nd day post-partum	64th day post-partum	82^{2t} day post-partum	102nd day post-partum
A1	7.7 ± 4.2	25.5 ± 5.4	34.7 ± 6.6	36.3 ± 6.9	29.7 ± 5.1	26.9 ± 5.5	-	25.0 ± 5.1	-	-	-
A2	4.3 ± 1.6	26.6 ± 4.6	26.2 ± 5.7	-[2]	-	-	26.1 ± 4.9	-	21.7 ± 4.4	26.2 ± 4.6	26.0 ± 6.0
B	4.0 ± 4.6	26.2 ± 6.4	31.6 ± 6.4	30.0 ± 6.0	27.6 ± 7.0	26.2 ± 5.7	-	24.8 ± 6.7	-	-	-
C1	2.0 ± 2.7	1.4 ± 2.5	3.4 ± 4.6	4.2 ± 5.0	5.8 ± 5.2	4.8 ± 4.5	-	5.1 ± 4.2	-	-	-
C2	5.5 ± 4.1	4.7 ± 2.7	9.3 ± 4.3	-	-	-	8.9 ± 3.8	-	9.4 ± 6.0	8.3 ± 5.2	8.6 ± 4.7
D	2. ± 2.9	3.5 ± 4.5	3.8 ± 5.2	4.1 ± 4.2	4.1 ± 3.5	8.1 ± 7.1	-	11.1 ± 8.4	-	-	-

1. Groups A and B: vaccinated, groups C and D: non-vaccinated – groups A and C: challenged with *S. chromogenes*, groups B and D: not challenged.
2. not performed.

Findings in tissue samples

Bacteria were isolated from tissue samples of the inoculated mammary gland of 2 group A (0.250) and 4 group C (0.800) ewes ($P = 0.086$). Bacteria were also isolated from tissue samples of the contralateral mammary gland only of 3 group C (0.600) ewes ($P = 0.035$ between groups). Median bacterial counts in tissue samples from group A ewes (0 cfu g^{-1}, 0 – 4) were smaller than median bacterial counts in tissue samples from group C ewes (6.5 cfu g^{-1}, 0 – 75) ($P = 0.041$).

Median (min. – max.) score for histopathological findings in tissue samples from inoculated glands of group A was smaller than that for group C ewes: 1 (0 – 2) *versus* 2 (2 – 3) ($P = 0.014$); similar findings were recorded for tissue samples from contralateral glands: 0 (0 – 0) *versus* 1 (0 – 2) ($P = 0.012$), as well as when results for all tissue samples (i.e., from both glands) were considered: 0.5 (0 – 2) *versus* 2 (0 – 3) ($P = 0.005$).

In tissue samples, salient lesions included lymphocytic infiltration, alveolar destruction, which was particularly marked in ewes of group C, presence of collapsed alveoli and fibrous tissue proliferation; in group C ewes, the glandular elements have been replaced to a large extent by fibrous tissue. In tissue samples from the udder of the ewe that died 2 days post-challenge (group C2), massive neutrophilic infiltration was present with degeneration of alveolar epithelial cells, destruction of alveoli and extensive extravasation throughout the mammary parenchyma.

Correlations

There was an inverse correlation between antibody titres in blood and bacterial counts in milk ($P < 0.001$). Further, bacterial counts in milk had a positive correlation with clinical scores, IMIs and histopathological scores and inverse correlation with milk yield ratios ($P \leq 0.001$). Details of the significance of correlation between the various parametres assessed are in Table III.xxviii.

Table III.xxviii. Significance (P values) of correlation between parametres[1] assessed throughout a study for evaluation of the efficacy of the test999999 vaccine against staphylococcal mastitis in sheep.

	Parametre							
Parametre	Clinical scores	Mammary infections	Bacterial counts in milk	Grey-scale ratios	Milk yield ratios	Anti-PNAG antibody titres	Bacterial counts in tissues	Histopathol o-gical scores
Clinical scores	•	< 0.001	0.001	< 0.001	0.044	0.248	0.001	0.230
Mammary infections		•	< 0.001	0.001	0.124	< 0.001	0.059	< 0.001
Bacterial counts in milk			•	0.032	< 0.001	< 0.001	0.005	0.001
Grey-scale ratios				•	0.003	0.061	0.002	0.28
Milk yield ratios					•	0.009	0.061	0.34
Anti-PNAG antibody titres						•	0.032	0.004
Bacterial counts in tissues							•	0.123
Histopathological scores								•

Red-shaded cells indicate the *P* value of an inverse correlation.

1. Analysis of correlation in the evaluated parametres was performed among ewes in groups A (vaccinated and challenged with *S. chromogenes*) and C (non-vaccinated and challenged with *S. chromogenes*).

GENERAL DISCUSSION

Introduction

The thesis describes an extensive field investigation of subclinical mastitis in ewes, one of the largest ever on worldwide basis. Sheep farms in all regions of Greece were included into the study; that way, conditions prevailing throughout the country had been taken into account and factors of regional importance weighed less. Application of consistent methodologies for examination and sampling of animals, as well as performance of all tasks always by the same investigator has contributed to minimise possible bias in selection of farms. During this investigation, various factors have been evaluated for their significance in predisposing ewes to mastitis.

Subsequently to the field investigation, extensive laboratory work has been performed to characterise the staphylococcal isolated recoved from the field. As a result, a new disease entity has been identified: 'biofilm staphylococcal mastitis'. The term refers to cases of the infection, caused specifically by bacteria producing slime and forming biofilm and is independent to species of the organisms. The identification of the disease was then corroborated by the detection of antibodies specific against this virulence factor.

Finally, a novel vaccine, the antigenic phase of which induces antibodies against biofilm formation by staphylococci, has been tested in a large clinical study and in experimental work. The results indicate that, whilst protection was afforded to vaccinated ewes, further preventive measures to control mastitis would be necessary.

Subclinical mastitis in ewes in Greece

Prevalence of subclinical mastitis

Present results

Overall prevalence of subclinical mastitis has been calculated to be 0.260. In this study, the primary interest was to report the prevalence of mastitis within farms and not to extrapolate to the entire population of ewes in Greece. For this, it was possible to estimate the overall prevalence of subclinical mastitis in the farms used, by declaring the farms to be strata; by using this approach, estimated overall proportion of ewes with subclinical mastitis in the study farms combined was calculated to be 0.250.

The results confirm subclinical mastitis as a frequent problem in sheep farms, the importance of which is further underlined by its financial significance. In dairy flocks, significance of subclinical mastitis becomes easily understood, as it has a well-documented detrimental effect on milk yield

(causing up to 50% reduction) (Saratsis et al. 1999, Giadinis et al. 2012) and composition (leading to changes adversely affecting cheese-making properties) (Fragkou et al. 2008, Marti de Olives et al. 2013, Abdelgawad et al. 2016). The importance of subclinical mastitis in meat-type production flocks is not straightforward; in such flocks, it can lead to growth retardation and suboptimal bodyweight of lambs (Fthenakis and Jones 1990b, Arsenault et al. 2008). Further, subclinical mastitis can often develop to clinical mastitis (Fthenakis and Jones 1990c), which has been singled out as the most important welfare problem of ewes across all production types and management systems (European Food Safety Authority 2014). The study has also indicated variations between farms: within farm prevalence varied from 0% to 85%. This large range is consistent with the multifactorial nature of mastitis. In view of that, results according to management system applied in farms have been produced and differences were found to be significant; more intensive management has been associated with increased prevalence of subclinical mastitis. The finding that management system emerged of high significance in the multivariable analysis, further supports this presentation.

Comparison with previous findings

Previous relevant findings in Greece and other countries

Investigations into frequency of subclinical mastitis are of interest, because laboratory examination is required for accurate diagnosis; hence, it cannot be seen under clinical conditions and its financial losses cannot be clearly appreciated. In Greece, in a field investigation performed 25 years ago, smaller prevalence of subclinical mastitis (up to 17%) has been recorded (Fthenakis 1994). A significant change in the sheep industry in Greece during the last 25 years has been its increasing intensification, with involvement of high-yielding breeds (e.g., Lacaune, Friesarta) (Zygoyiannis 2006, Gelasakis et al. 2012). Further, practices consistent with intensification (e.g., increased stocking rate) have been associated with increased bacterial presence in milk (Albenzio et al. 2004, Caroprese 2008), hence may also contribute in the increased prevalence of subclinical mastitis in farms under intensive or semi-intensive management. Although specific measures for prevention of mastitis have not been found to be widely implemented, nevertheless, the findings indicate a clear increase in their application; at the time of the previous investigation (Fthenakis 1994), such measures were virtually non-existent in sheep farms in Greece.

In France, Bergonier et al. (2003) have reported that post-milking teat dipping was being performed only in a minority of sheep farms (20%), whilst administration of antimicrobial agents at end of lactation period was more widely practiced (70% of farms). It is noteworthy that in a recent study, sheep farmers in Greece considered administration of antimicrobial agents at end of lactation

period of little significance for the welfare of their animals (Gougoulis et al. 2017), which is consistent with the current small prevalence of the practice.

The results are also of significance to other countries in the region, where similar production type farms exist, especially given that, for example, in France, no relevant recent studies have been found (Gelasakis et al. 2015). In Italy, reports involved work in limited number of flocks (1 to 15), with widely varying results (10 to 50% frequency of bacterial isolation) (Gelasakis et al. 2015). In Turkey, prevalence of subclinical mastitis in one study (16 flocks) has been found to be 18.5% (Ergun et al. 2009). In Spain, in an older study (Las Heras et al. 1999), prevalence of subclinical mastitis was found to be 34%. Of course, a direct comparison of results of field studies is always difficult, as different designs and techniques would have been applied, which might account for differences in results between studies.

Significance of diagnostic criteria

Bacteriological examination of milk samples is employed for diagnosis of subclinical mastitis, as it is considered to provide precise and exhaustive information on infected mammary glands and pathogen involved. However, it is difficult to implement at a large scale and also has various limitations. Moreover, bacterial shedding is variable and levels may sometimes be too low to be detected by conventional techniques (Rupp and Foucras 2010).

The significance of definition criteria is evidenced when a recent study by Dore et al. (2016) is considered; these authors published a meta-analysis of findings among Italian state veterinary laboratories, taking into account isolation of one colony of 'primary' pathogens (e.g., *S. aureus*) or five colonies of coagulase-negative staphylococci (from 10 μL) to indicate bacteriologically positive samples (i.e., broader criteria than the ones in the present study) and reported 45% frequency of bacteriologically positive samples (i.e., greater than in the present study) (Dore et al. 2016). Moreover, Las Heras et al. (1999) took into account isolation of two colonies of bacteria to indicate bacteriologically positive samples (again broader criteria than the ones in the present study) and reported 34% average prevalence of subclinical mastitis.

Simple, indirect methods have also been widely applied, based on evaluation of inflammation. The ones most frequently used are somatic cell counting and various indirect tests for their measurement. A difficulty in using somatic cell counting is that factors known to influence somatic cell counting have different magnitude in healthy and infected animals (Detilleux and Leroy 2000). Further, there is difference in types of cells in mammary secretion, which can provide an indication regarding the inflammation. Indeed, the associations between bacteria and somatic cells, particularly of the various types of leucocytes, can be used to better define the disease (Albenzio et al. 2009). The definition of subclinical mastitis used in this study (i.e., combination of a

bacteriologically positive milk sample with increased CMT score plus high proportion of neutrophil and lymphocyte) takes that into account and was adopted to overcome shortcomings of the methods described previously.

Aetiology of subclinical mastitis

Significance of causative bacteria

Staphylococci (*S. aureus* and coagulase-negative species) have been identified as the primary aetiological agents of subclinical mastitis. These results are broadly similar to those reported before, although in previous studies frequency of identification of *S. chromogenes* has been smaller, in contrast to our results, in which that species have been identified as the most frequent coagulase-negative staphylococci. *S. aureus* has traditionally been considered as a cause of clinical mastitis and less frequently of subclinical (Fthenakis 1994, Las Heras et al. 1999). *S. aureus* isolation might also indicate an early stage of clinical mastitis, although it may also be the result of effective host defences, which allowed only limited invasion that might have resulted in subclinical mastitis alone.

The frequent isolation of *Streptococcus* from ewes in machine-milked flocks indicates a potential predisposing role for increased prevalence of subclinical mastitis caused by that organism, and can be aligned to the findings of Marogna et al. (2010) who have reported an association between machine-milking of ewes and increased frequency of clinical mastitis of streptococcal aetiology. The frequent isolation of *Mannheimia haemolytica* from ewes suckling lambs provides field corroboration of experimental results that we have published before (Gougoulis et al. 2008b, Fragkou et al. 2011), indicating transmission of the organism from the tonsils of lambs to the teat of ewes during suckling of lambs. *M. haemolytica* is the most frequent causal agent of mastitis in meat production flocks (Gelasakis et al. 2015), which obviously results from transmission of the organism during suckling. Further, frequent mastitis caused by *Escherichia coli* during the first month *post-partum* might have resulted from uterine fluid contamination (Ioannidi et al. 2015). Environmental sources can also contribute to coliform infections, as, during that period, sheep are housed for longer periods and stocking densities are increased, which, in association with incorrect ventilation and high relative humidity, may lead in mastitis (Bergonier et al. 2003, Caroprese 2008). Other organisms isolated have been identified as sporadic aetiological agents of mastitis during lactation period in ewes. The significance of the various bacteria, *Corynebacterium* (Tel and Bozkaya 2012, Dore et al. 2016), *Micrococcus* (Keisler et al. 1992, Dore et al. 2016), *Trueperella* (Saratsis et al. 1998, Ribeiro et al. 2015), *Enterococcus* (Sanciu et al. 2013), has been discussed in case reports from around the world.

Significance of mammary carriage

Mammary 'carriage', in most cases, refers to bacterial flora present in the teat duct or teat cistern (Fragkou, Mavrogianni et al. 2007, Mavrogianni et al. 2007). Occasionally, the term may also refer to results of sampling a ewe at the early stage of infection before an inflammatory reaction was elicited (Fthenakis and Jones 1990c). The same outcome would also occur in cases of contamination of milk samples during collection; nevertheless, with experienced staff and strict maintenance of aseptic sampling conditions, that is minimal; for example, Rovai et al. (2014), in a field investigation, have found that only 2% of milk samples from ewes were contaminated.

In the present study, some association of mammary carriage with prevalence of subclinical mastitis has been found and was stronger for staphylococci. This may possibly indicate that mammary carriage organisms might become pathogenic under the effect of various factors, which decrease defensive efficacy of hosts or promote pathogenicity of bacteria; that has been documented with coagulase-negative staphylococci in a previous experimental study (Fragkou, Mavrogianni et al. 2007).

Significance of bacteriologically negative – cytologically positive samples

Observation of cytologically positive samples, which were not bacteriologically positive, may be indicative of receding cases of subclinical mastitis, in which bacterial numbers were reduced or had fully disappeared, as the result of effective defenses of the host animals. In fact, in previous experimental studies of subclinical mastitis, increased cell content without bacterial isolation has been often recorded subsequently to the 20th day post-challenge (Fthenakis and Jones 1990b, c, Gougoulis et al. 2008a). Systemic and local efficient defenses in ewes contribute to achieving 'self-cure', especially when bacterial isolates have reduced pathogenicity.

The importance of effective defense mechanisms in 'self-cure' in staphylococcal mastitis has been underlined by Ali-Vehmas et al. (1997), who reported that subsequently to selenium supplementation (which contributes to improved cellular defense mechanisms) led to increased 'self-cure' and halving the prevalence rate of mammary glands producing bacteriologically positive milk samples (Ali-Vehmas et al. 1997). Of course, infection by low virulence isolates can also result in transient bacterial isolation followed by longer-lasting increased somatic cell counts (Fthenakis and Jones 1990c). Further, one should also take into account that cytologically positive samples in ewes after the middle of a lactation period (particularly in ewes with bilaterally increased scores) may possibly reflect a 'natural' increase in cellular content in milk of ewes, as the lactation period advances, a finding that has been previously reported by Fthenakis (1996).

Potential predisposing factors for subclinical mastitis

Management system practiced in flocks

In the multivariable analysis, flock management emerged as the only significant factor affecting prevalence of subclinical mastitis. This finding may reflect that 'management system' would include various aspects of flock husbandry and organisation, e.g., milking routine, animal breed, housing facilities, nutrition, which have been considered to affect development of mastitis in ewes (Gelasakis et al. 2015, Fthenakis et al. 2017). 'Management system' encompasses a variety of factors, which, to varying extent each one, contribute to subclinical mastitis or its control.

Stage of lactation period

Differences in prevalence of subclinical mastitis were found between the various stages of lactation period. During the first month post-partum, which coincided with the period of lamb suckling, prevalence of mastitis was smaller than average. Suckling of lambs has been associated with transmission of bacteria; the possibility to transmit *M. haemolytica* from tonsils of lambs to teat duct has been discussed hereabove. Later, a sharp increase was recorded during the 2nd month, when lambs were taken away from their dams and full scale milking started. In a previous study in Greece, the same period had been identified as the one with the highest incidence risk of clinical mastitis (Fthenakis and Jones 1990a). One may suggest that the abrupt change stressed the mammary gland, on the one hand increasing intramammary infections and on the other impeding local defences, ultimately resulting in increased mastitis prevalence, as discussed by Mavrogianni et al. (2007). Subsequently, the gland returns to smaller rate of infections and mastitis, followed by a new increase towards end of lactation period, as chances of infection become perennial and frequency of mastitis increases (Fthenakis 1994).

Animal breed

Sheep breeds in Greece

Lacaune- and Chios-breed animals are popular in Greece. These are sheep with high milk production, thus of importance in the dairy production systems applied in the country, which explains the higher proportion of farms with these breeds. The findings indicate increased penetration of imported breeds, which, in recent years, have been favoured by Greek farmers. Lacaune and Assaf predominate among imported sheep breeds in Greece, as they are animals of increased milk production, higher than indigenous Greek breeds. These animals cannot be adapted to the

environment, which is reflected in them being included in farms managed intensively or semi-intensively, where they are sheltered and their needs, especially nutritional requirements, can be controlled and covered. Nevertheless, uncontrolled imports may increase risk for transmission of diseases to the indigenous sheep population; for example, in Spain a large proportion of Assaf animals have been found to be infected with *Small Ruminant Lentivirus* (Minguijon et al. 2015), which may lead to transmission of the pathogen to uninfected flocks in Greece after import.

Traditional breeds have also been identified, these being of limited geographical distribution and, mainly, in flocks managed under the semi-extensive or extensive system, which constitute the traditional shepherding forms in the country. These are low-input breeds, with very good adaptability to environmental conditions and able to make excellent use of natural resources and locally produced feedstuffs. This explains their increased frequency in farms managed semi-extensively or extensively (Georgoudis et al. 2011). There is evidence regarding genetic relationship between animals of those breeds (Ligda et al. 2009, Georgoudis et al. 2011), thus lending support to clustering these breeds for the statistical analysis.

Sheep breed as predisposing factor to mastitis

Present results have indicated increased prevalence of subclinical mastitis in Friesarta-breed farms. Animals of the breed are high-yielding, but, in general, considered to be particularly susceptible to diseases, e.g., respiratory infections. Increased susceptibility to mastitis can be attributed to breed-specific impaired local defence mechanisms in the udder (Fragkou, Skoufos et al. 2007, Fragkou et al. 2010). Present findings provide field corroboration to the experimental evidence. Traditional Greek sheep breeds have shown reduced frequency of subclinical mastitis. In a broader sense, resistance could be defined as the ability to avoid any infection and/or the quick recovery from an infection (Rupp and Boichard 2003) and involves different components: avoiding entry of the pathogen into the teat, mounting an immune response capable of limiting its development in the mammary gland and clearing the infection, as well as controlling the pathogenic effects of the infection, such as tissue damage (Rupp and Foucras 2010). In Karagouniko ewes, lymphoid follicles have been identified in the teat duct and have been shown to play a clear protective role against invading pathogens (Fragkou et al. 2010).

Higher allocation of resources to defence mechanisms of ewes afforded in cases of low milk production by animals can also play a predominant role and contribute to efficient counteraction against invading mammary pathogens. A tendency of increased prevalence of subclinical mastitis in Chios-breed sheep has also emerged. Possible reasons could be the bad udder conformation, which hinders correct milking and contributes to infections (Gelasakis et al. 2012), and the innate peri-parturient immunosuppression associated with macrophage and neutrophil function (Theodorou et

al. 2007). Previous studies on other breeds (e.g., Latxa and Sarda) have indeed shown favourable correlations between somatic cell counts and udder conformation (Legarra and Ugarte 2005, Sechi et al. 2007), suggesting that udders with what is perceived to be a good shape would be less affected by subclinical mastitis. In addition, udders with bad conformation can predispose to development of mastitis (Gelasakis et al. 2012). Further, differences in somatic cell counts in milk of healthy animals recorded between sheep breeds (Rupp and Foucras 2010) can reflect the immunological competence of the respective mammary glands against invading microorganisms and the final result (Albenzio et al. 2012).

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

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

Milking practices

Marco Melero (1994) was the first to identify transmission of staphylococci from hands of milkers to teats of ewes during milking. Mavrogianni et al. (2007) have shown that hand-milking resulted in increased rate of bacterial colonisation within the teat duct; possibly, there were greater probabilities for organisms to colonise and cause mastitis, as in hand-milked animals post-milking teat dipping was not practiced frequently; any impediment of the defence mechanisms (local or systemic) may shift the balance and allow the bacteria to multiply, invade the mammary gland and

cause mastitis. Machine-milking has also been associated with transmission of staphylococci to teats (Bergonier and Berthelot 2003).

In the present study, farms in which machine-milking was applied, were found with smaller mastitis prevalence, likely because of the application of accompanying mastitis preventing measures, specifically post-milking teat dipping, which reduces bacterial populations at the teat. The potential effects of post-milking teat dipping in ewes have not been studied as extensively as in cows; there is only anecdotal evidence regarding effects of teat dipping in ewes (Paape et al. 2001, Bergonier et al. 2003). Present findings indicate that the practice is not widely applied. The results also provide some evidence ($P = 0.054$ in the multivariable model) regarding the beneficial role of the practice in reducing mastitis frequency in ewes. It is noteworthy that post-milking teat dipping was the only management factor, among those examined, that has been included in the multivariable model; omission of the practice has been found that it might result in up to 2.9 times higher prevalence of subclinical mastitis in respective flocks. However, one should be aware that wrong application of the practice can support mastitis development; for example, use of dense solution of a product would cause teat chapping, which can predispose ewes to mastitis (Mavrogianni et al. 2006, Fragkou, Papaioannou et al. 2007), or contaminated products can result in mastitis caused by infrequent pathogens (e.g., *Serratia macrescens*; Tzora and Fthenakis 1998).

Mastitis preventive measures at the end of a lactation period

Intramammary administration of antimicrobial agents at the end of a lactation period is considered to contribute significantly in reducing incidence of mastitis during the dry period, as well as in the first days post-partum (Petridis and Fthenakis 2014), findings that have not been confirmed at the present study. Reasons that might have accounted for this discrepancy could be application of an inappropriate treatment regime by farmers or presence of resistant bacterial isolates in the farms.

Anti-mastitis vaccination

Anti-mastitis vaccination referred to only vaccination against staphylococci. This strategy can be considered as correct given that these bacteria account for the larger proportion of causal agents of mastitis (Lacasta et al. 2015, Fthenakis and Calvo Gonzalez-Valerio 2017). Vaccines active against other pathogens would be of little benefit for general use in flocks (Fthenakis and Calvo Gonzalez-Valerio 2017).

Further, vaccination was found to be performed more frequently than post-milking teat dipping or administration of antimicrobial agents at end of lactation. That would indicate that anti-

mastitis vaccination might be integrated into the health management of the flock conveniently, possibly because farmers prefer the practice and can incorporate it into the general routine of the flocks more easily.

Subclinical mastitis caused by biofilm-forming staphylococci

Biofilm formation by staphylococcal isolates

Detection of *in vitro* biofilm formation may depend upon laboratory conditions, therefore it is advisable to simultaneously carry out genotypic examinations (Götz 2002, Vasudevan et al. 2003). In the present study, phenotypic and genotypic works have been combined. The results have indicated a correlation between detection of genes reported to be associated with biofilm formation (Melchior et al. 2006) and *in vitro* biofilm formation (a 'phenotypic' characteristic). Characterisation of isolates, in which no relevant genes had been detected, as biofilm-forming might indicate that other genes, further to the other studied herein, may be implicated in slime production and biofilm formation (Osman et al. 2015, Khoramrooz et al. 2016); for example, *aap* has been detected specifically in coagulase-negative isolates (Tremblay et al. 2013).

There is conflict in the international literature regarding frequency of biofilm-forming staphylococcal isolates from cases of mastitis in ewes. This varies greatly from 0% (Azara et al. 2017) to 26% (Vautor et al. 2007) to 40 - 43% (Ergun et al. 2012) to 91 - 98% (Tel et al. 2012). These works referred to testing of staphylococcal isolates, without backtracking of laboratory results to be applied in the field.

Present work has included a very large set of staphylococcal isolates (n = 708) from an extensive countrywide investigation across Greece, from different sources and of various staphylococcal species. To the best of our knowledge, this is the largest number of staphylococcal isolates recovered from udder samples from ewes, tested for biofilm formation. The results have indicated that most staphylococcal isolates (70%) formed biofilm, although there were differences related to species, not source, of the isolates. Differences in results between publications may reflect differences in origin of isolates and in laboratory techniques employed.

Similarly to results reported by Simojoki et al. (2012), our results did not provide evidence of difference in biofilm formation according to source of the isolates, i.e., subclinical mastitis or mammary carriage. Indeed, in a previous publication (Fragkou, Mavrogianni et al. 2007), we have reported that coagulase-negative staphylococci present as carriage into the teat duct, were able to cause mastitis, when teat defences became depleted, which confirmed their pathogenicity for the

mammary gland. Hence, although biofilm formation can be important for colonisation of the udder, other factors may be involved in further tissue damage and development of inflammatory reaction.

There are also differing results in the international literature regarding presence of the various genes associated with biofilm formation in the staphylococcal isolates studied. The ica locus has been associated with increased virulence of mastitis-associated staphylococcal isolates (Vasudevan et al. 2003). In the present work, it has been found often in *S. aureus*, *S. epidermidis* and *S. simulans*. Increased adhesion properties can lead in colonisation of the bacteria onto mammary epithelial cells and subsequent damage to the parenchyma, which can account for the increased pathogenicity of *S. epidermidis* and *S. simulans* (among coagulase-negative isolates) for the mammary gland of ewes (Fthenakis and Jones 1990c). Previous workers have reported widely varying frequency of the locus: from 0% (Azara et al. 2017) to 100% (Felipe et al. 2017). Presence of *eno* has also been detected often in staphylococcal isolates: 75% - 89% of *S. aureus* and 93% of coagulase-negative (Azara et al. 2017, Darwish and Asfour 2013) isolates; in the present study, respective rates were 84% and 60%, respectively. Frequency of presence of *clfa* also varies widely: from 2% (Azara et al. 2017) to 37% (present study) to 100% (Felipe et al. 2017) in *S. aureus* and 12% (Felipe et al. 2017) to 21% (present study) in coagulase-negative staphylococci. Finally, in contrast to the findings of Cucarella et al. (2001), who have reported association of presence of *bap* with strong biofilm-forming staphylococci, in the present study no association with results of *in vitro* biofilm formation has been detected for that gene.

There were notable differences in biofilm formation and presence of relevant genes by staphylococcal species. Present results indicate that *S. aureus* and *S. epidermidis* were found to be biofilm-forming more frequently than other species causing mastitis, findings which corroborate those of other workers (O'Gara 2007, Osman et al. 2015).

In view of the above contrasting results regarding potential significance of individual genes, we have opted to study total number of genes and gene combinations in relation to *in vitro* biofilm formation. Our results have indicated that biofilm-forming isolates had a significantly higher number of genes than non-forming ones. This is not surprising, as higher number of genes would reflect more relevant proteins produced by respective isolates, therefore stronger biofilm formation by these. *S. aureus* and *S. epidermidis* isolates were found to have, on average, more genes than other species, which is in line with the increased pathogenicity of these species as mastitis pathogens in ewes.

Moreover, gene combinations associated more frequently with *in vitro* biofilm formation have been found to include genes of the *ica* locus and *eno*. The finding can support an idea that, possibly, in future studies, only these genes need to be tested, in order to confirm biofilm formation.

Mastitis caused by biofilm-forming staphylococcal isolates

This study also reports the prevalence of mastitis caused specifically by biofilm-forming staphylococcal isolates in ewes and presents potentially predisposing factors. Biofilm-forming staphylococci (*S. aureus* or coagulase-negative species) have been identified as the primary aetiological agents of subclinical mastitis in sheep, independently of staphylococcal species, and accounted for almost 60% of all cases of subclinical mastitis in ewes.

In the multivariable analysis, type of milking, specifically hand-milking, has been found to be associated with increased prevalence of biofilm staphylococcal subclinical mastitis. This was unexpected, given that biofilm formation has been identified to be important virulence factor of staphylococcal isolates associated with plastic material (e.g., catheters) (Arciola et al. 2012), hence one would have expected machine-milking to have been associated more often with the problem. Nevertheless, milking systems are thoroughly washed and sanitised as part of the routine applied in milking parlours, which would lead to reduced populations of bacteria in the equipment (Pantoja et al. 2011, Simoes et al. 2015). In contrast, milkers, from whose hands biofilm-forming staphylococci have been frequently isolated (Salgado-Ruiz et al. 2015), may not always clean them properly or wear gloves as part of the milking routine, hence contributing to transmission of these organisms to sheep.

Flock management also emerged as a significant factor affecting prevalence of subclinical mastitis. This finding may reflect the significance of ‘management system’ as a predictor for subclinical mastitis in general as discussed above.

The results support the idea of mastitis caused by biofilm-forming staphylococcal mastitis as a disease entity, of aetiology independent of staphylococcal species, but rather associated with presence of a virulence determinant in the causal organisms.

Susceptibility of staphylococcal isolates to antimicrobial agents

There is one rule for the effective treatment of mastitis in ewes: the combination of speed and efficacy. As soon as the first signs of mastitis are diagnosed, treatment should be initiated with effective antimicrobial agents (Mavrogianni et al. 2011). Ideally, to preserve susceptibility of pathogens to available drugs, treatment should be performed by means of a narrow spectrum agent, specifically effective against the causal agents. Drug administration follows identification of causal

agent and establishment of its patterns of antimicrobial susceptibility by means of the relevant technique (Mavrogianni et al. 2011).

Present results

Results of the present study have indicated limited antimicrobial resistance among *S. aureus* isolates, but a significantly higher frequency among coagulase-negative isolates. *S. aureus* is primarily a cause of clinical mastitis, which is easily diagnosed and immediately treated. In contrast, coagulase-negative staphylococci are the primary causes of subclinical mastitis, which is only occasionally diagnosed and thus remains untreated. Potentially, coagulase-negative staphylococci may be exposed to antimicrobials used in animals for various reasons, which is a well-established factor for development of resistance. Further, the possibility that coagulase-negative staphylococci isolates had been transmitted from farm personnel cannot be ruled out; in corroboration to that, it is noted the isolation of species that are not considered typical causal agents of mastitis in sheep, but rather mainly human pathogens, e.g., *S. hominis* or *S. haemolyticus*, as well as the detection of ST types in *S. epidermidis* more often associated with isolates of human origin.

Comparison with previous findings

The findings are in sharp contrast to those of a similar study performed 20 years in Greece, in which frequency of resistance to antimicrobial agents was significantly higher in *S. aureus* than in coagulase-negative staphylococci (Fthenakis 1998). Compared to the findings of that work, frequency of resistance among *S. aureus* was found to be significantly smaller. This can be the beneficial effect of sustained campaigns for correct use of antimicrobial agents that have been performed in the country by veterinary authorities (e.g., Hellenic Veterinary Association 2018).

Recent evidence from around Europe does not indicate significant problems of reduced susceptibility to antimicrobial agents of staphylococcal isolates from cases of mastitis in sheep. Vautor et al. (2007) have reported only sporadic resistance in *S. aureus* isolates recovered in France. Onni et al. (2011), in Italy, have also found limited resistance in *S. epidermidis* isolates, bar to penicillin, for which a resistance rate of 38% was recorded. Similar results have been observed in Turkey, where in coagulase-negative isolates from subclinical mastitis only resistance to *β*-lactams was noteworthy (43%), whilst there was much smaller frequency of resistance to tetracycline (11%) and even less to other agents (Ergun et al. 2012). Further work in Turkey has corroborated above findings: rate of resistance to penicillin was 27% and to tetracycline 8% (Unal et al. 2012). Finally, Martins et al. (2017) have published results similar to previous ones regarding susceptibility patterns of coagulase-negative isolates from cases of subclinical mastitis: 17% of isolates studied were

resistant to penicillin and 11% to tetracycline. A different result has been reported by Azara et al. (2017), who found increased resistance to tetracycline in only *S. aureus* isolates from clinical mastitis. In sharp contrast to above results, in Brasil, Franca et al. (2012) have reported increased frequency of resistance to amoxicillin, erythromycin, lincomycin, streptomycin and tetracycline (> 35% of staphylococcal isolates tested).

Husbandry factors associated with antimicrobial resistance

The results of the study have also indicated, for the first time, some husbandry factors associated with antimicrobial resistance of mastitis-associated isolates. Administration of antimicrobials at the beginning of the dry-period is a management practice that aims (i) to cure infections which have occurred during the previous lactation period and (ii) to prevent development of new intramammary infections during the 'dry-period' (Fthenakis et al. 2012). The practice is an integral part of udder health management (Petridis and Fthenakis 2014), but the present results indicate that it can also be associated with increased isolation of resistant isolates. To minimise the risk for antimicrobial resistance, the procedure should be preceded by microbiological examination of samples collected from ewes to be treated, which will support selection of the most appropriate agent (Petridis and Fthenakis 2014, Saratsis et al. 1998).

Increased frequency of resistant isolates from samples collected after the 1st month *post-partum* can be the consequence of the increased administration of antimicrobials in flocks during the *post-partum* period. Ewes might be given antimicrobials, especially if obstetrical manipulations had been performed, to prevent potential uterine infections. Lambs can be treated with antimicrobials against respiratory infections, which occur at that age and are the most frequent reason for administration of antimicrobials to lambs (González 2015); it is noteworthy that staphylococci can be transmitted from lambs to the dam's teats during sucking (Laukova and Marounek 1992, Gougoulis et al. 2008b). Hence, bacterial isolates can develop resistance and survive, later in the lactation period causing mastitis.

Some corroboration of the above hypotheses is provided by tetracycline and ampicillin being the antimicrobial agents, against which resistance had been more frequently detected. Oxytetracycline is often administered to ewes as a first-line, broad-spectrum drug, against a variety of infections (e.g., mastitis, metritis), and ampicillin is frequently administered to lambs therapeutically or metaphylactically against infections caused by Gram negative bacteria.

In the case of tetracycline, an association was also found between resistance to the drug with formation of biofilm, results which were found at phenotypic and genotypic level. Formation of biofilms is a virulence mechanism of bacteria, contributing to expansion of the multiplying bacteria,

offering reduced susceptibility to antimicrobial agents and promoting bacterial survival from mammary defences (Clarke and Foster 2006, Melchior et al. 2006, Otto 2008).

Resistance-associated genes

There is little data about presence and distribution of resistance-associated genes of staphylococci isolated from raw milk of ewes. The increased frequency of genes of the *tet* operon has also been reported in similar studies in staphylococci from cows' milk (Memon et al. 2013). Frequency of detection of resistance-associated genes in *S. aureus* was smaller than that indicated in reports regarding isolates from cows' milk (Moslem Parvizi et al. 2012, Silva et al. 2013), which may reflect that sheep farming is, in general, applied under less intensively-managed conditions than cattle. This provides fewer reasons and opportunities than in cattle for antimicrobial use and leads in reduced pressure for development of resistance to these agents; for example, the administration of antimicrobials at the beginning of the dry-period is a standard practice in cattle, whilst in sheep, as seen in present results, only few farms practice the procedure.

Sequencing data

Sequencing data showed that resistance genes were associated with mobile genetic elements. Specifically, *ermC* and *tetK* genes were carried by small plasmids, like pNE131 and pT181 (Lampson and Parisi 1986, Khan and Novick 1983), respectively. Of note was that, in a recent study, which examined MLS_B resistance in staphylococcal isolates from people in Greece, pNE131-like plasmids were found to be the main mobile element involved in the dissemination of *ermC* gene in *S. aureus* isolates of animal origin (Sarrou et al. 2019). Moreover, it is interesting that identical pT181-like plasmids carrying *tetK* have been previously found in a *S. aureus* isolates of human origin in Greece (Sarrou et al. 2019). These findings clearly indicate the important role of mobile genetic elements in the intra- and inter-species dissemination of resistance genes.

MLST staphylococcal types

S. aureus ST133 was the predominant type of that species, as also found in previous studies that had examined isolates of ovine origin (McMillan et al. 2016). The results have also indicated that development of resistance was not associated with any type of *S. aureus* or *S. epidermidis* types.

It is noted that present results present the first description of the clonal diversity of *S. epidermidis* stains recovered from ruminant milk; this may account for the detection of three isolates

that were allocated into new types. Thusfar, of the *S. epidermidis* types that were identified most frequently, only ST100 has been isolated from animals (cattle, pigs) before (Argudin et al. 2015); ST 142 and 152 have only been isolated from human samples (Multi-Locus Sequence Typing 2019).

Detection of anti-PNAG specific antibodies in blood samples

The present study has evaluated prevalence of specific anti-PNAG antibody titres. In the past, in an experimental study, it has been proposed that these could be used only to confirm induction of immunity after vaccination (Perez et al. 2009). The results of the multivariable analysis support that vaccination was the most important factor for increased antibody titres. Increase of antibody titres has indicated development of immune response by vaccinated animals; prevalence of biofilm staphylococcal subclinical mastitis was smaller in these animals.

The association between seropositivity and presence of staphylococcal mastitis in non-vaccinated ewes indicates that antibody titres may also possibly increase as the result of mastitis. This confirms the development of immune response by infected ewes. However, the response likely would not offer a strong protection, as recrudescence or recurrence occurs often in staphylococcal mastitis (Fthenakis and Jones 1990c). The results of the study have further indicated that vaccination-induced titres were significantly higher than those in ewes with mammary infection. Nevertheless, one should also take into account that staphylococci may cause a variety of infections in ewes, e.g., osteomyelitis or vaginal infections (Kaarsemaker et al. 1997; de Paula Vasconcelos et al. 2016), which may also be responsible for some cases of increased antibody titres.

The findings are in line with relevant work in people, in whom anti-PNAG antibody titres have been used to diagnose staphylococcal infections associated with implanted medical devices (Jabbouri and Sadovskaya 2010). Nevertheless, there are difficulties in establishing a clear diagnosis of infected patients, the main reason for that being the lack of a standardised antigen for use in the diagnostic assay (Karamanos et al. 1997, Elliott et al. 2000, Selan et al. 2002). Lack of full protection against staphylococcal reinfections has also been advocated in people with increased antibody titres (Pier 2013).

The present results lend further support to the recognition of biofilm staphylococcal subclinical mastitis as a new disease entity, as discussed above. Additionally to the aetiology of that infection previously described, these results indicate the presence of an immune response specific for those causal agents; they also point out that seroprevalence of the infection is higher than prevalence measured based on microbiological and cytological methods. Antibody titres reflect presence of PNAG in staphylococci causing the infection, independently of the species causing

mastitis, as corroborated by the lack of association between staphylococcal species causing mastitis and antibody titres. PNAG production and biofilm formation have been previously found to be associated with number of relevant genes in the causal staphylococci. The finding of a correlation between antibody titres and number of genes present in staphylococcal isolates recovered from respective ewes, further supports that antibody titres are produced as the result of infection by biofilm-forming isolates.

Significance of environmental factors for mastitis

Environmental factors predisposing to mastitis

This work describes potential significance of environmental factors in predisposing to mastitis. Environmental conditions are considered to be important in the development of diseases with aerogenic transmission (e.g., Q fever [Valiakos et al. 2017]) or of diseases caused by agents with growth dependent upon weather conditions (e.g. trichostrongylid and trematode infections [Taylor et al. 2007]) or by agents transmitted through vectors (e.g., bluetongue [Vasileiou et al. 2016]). Moreover, environmental conditions may also affect susceptible hosts and thus contribute to development of a disease. A relevant example would be foot-rot, a disease in which exposure to environmental conditions devitalising the interdigital skin, is a necessary factor (Sargison 2008). In this case, the environmental factors act mostly on animals, rather than on transmission of causal agents as in previous examples; their effect facilitates agent invasion and multiplication in hosts.

Environmental factors in predisposing to mastitis have attracted little attention in the past, possibly because mastitis is an endemic disease caused by organisms present in farms and on animals, with little aerogenic transmission of causal bacteria.

The results have provided evidence that temperature-related variables can play a role in predisposing animals to mastitis. Results of univariable analysis have hinted at several parametres to be important, whilst results of subsequent multivariable analysis have indicated the lowest temperature of coldest month (negative association) and the mean temperature of the period prior to sampling (positive association) as determinant environmental factors for increased prevalence of subclinical mastitis. The combination of the two factors indicated from the multivariable analysis model points out that extreme temperatures adversely affect occurrence of subclinical mastitis.

High environmental temperatures can lead in reduced leucocyte counts in sheep (El-Tarabany et al. 2017). In such cases, leucocytes have impaired function (e.g., Lacetera et al. 2005, Lecchi et al. 2016). As leucocytes play a significant role in protecting ewes against mastitis, their reduced number and inefficiency would account for the increased prevalence in farms with extreme

temperature measurements. Indeed, there is some evidence indicating that in increased temperatures cows develop mastitis more frequently (Arcaro et al. 2013). Moreover, an indirect effect of other factors associated with increased environmental temperatures, e.g., reduced feed intake which would limit energy availability to defence systems and can predispose to mastitis (Barbagianni et al. 2015), should not be ruled out.

Decreased temperatures can also be implicated in favouring development of mastitis. Fox and Norell (1994) and Zucali et al. (2011) have reported that, in cattle, exposure to low temperatures increased staphylococcal colonisation on teat skin. A possible reason for that may be the chapping occurring in such conditions. An increased incidence of chapped teats in cold weather has been associated with intramammary infections (Fox and Hancock 1989). Leyshon (1929) also reported that mastitis in ewes was particularly prevalent in cold weather. Physicochemical changes in chapped teat skin contribute to the increased susceptibility. As previously mentioned, in the epidermis, the process of drying results in a decrease in lipids (including antibacterial fatty acids), bacteriostatic salts and proteins (e.g., teat duct keratin), as well as immunoglobulins (Noble and Somerville1974). Additionally, the reduced hydration of chapped skin leads to changes in skin microflora and consequent decreased resistance to bacterial colonisation (Fox and Cumming 1995). Finally, chapping removes the acid mantle and, as the result of excoriation and fissuring, increases the teat surface area, thereby providing additional space for bacterial attachment (Sieber and Farnsworth 1984). In cold weather, when lambs suck with increased frequency (Slee and Springbett 1986) and when teats may become chapped (Fox and Hancock 1989), it would seem likely that lambs contribute to the transfer of bacteria to teat skin, documented (Gougoulis et al. 2008b, Fragkou et al. 2011) as the means of bacterial transfer leading to colonisation, entry into the teat duct and mastitis.

Apart from potential effects in ewes, environmental temperatures may also have an effect on staphylococci present on hands of milkers, which can be a source of infection for the mammary gland (Gelasakis et al. 2015). There is some indication that high temperatures favour growth and survival of biofilm forming staphylococcal isolates (Martin et al. 2016) in dairy farms, thus may potentially influence virulence of these organisms and development of mastitis according to climatological circumstances. Thus, common practices, e.g., use of warm water for hand-washing when environmental temperatures are low, may support growth and multiplication of these organisms, that way increasing risk for transmission to susceptible ewes. This can explain from a different viewpoint the association of increased mastitis prevalence with environmental conditions.

Ecological Niche Modelling

Ecological niche modelling has never been used in the study of ruminant mastitis. The methodology has been used rarely in the study of sheep infections. Recently, Valiakos et al. (2017) have reported identification of potential spread of *Coxiella burnetii* infection in central Greece. In earlier studies, Rose and Wall (2011) have employed the methodology to report potential spread of *Lucilia* flies, the causative agent of myiosis, in Great Britain, whilst Kantzoura et al. (2011) have identified increased risk locations for development of fasciolosis in south-east Europe.

Use of Ecological niche modelling has been shown repeatedly to be effective in locating potentially increased risk areas for an infection, as well as to identify environmental / ecological conditions under which it would most likely develop (Peterson et al. 2004, Neerinckx et al. 2008, Williams and Peterson 2009, Donalisio and Peterson 2011, Ellis et al. 2012). A significant aspect of infection dynamics taken into account by the methodology is that environmental conditions can affect its occurrence by acting on all its components: transmission and pathogenesis systems (e.g., virulence and transmission of organisms, susceptibility of hosts). Therefore, by using Ecological niche modelling methodologies, one might be able to locate areas, where sheep can be at higher risk of an infection.

Among the various ecological niche techniques currently available, MaxEnt is considered to use the best algorithm, thus providing the best predictive models (Elith et al. 2006, Zeimes et al. 2012). MaxEnt software has the advantage that it requires only presence data and small number of occurrences. The program can take into account continuous and categorical predictor variables and also includes a regularisation protocol to protect against overfitting; the methodology, in general, shows very good predictive performance.

Areas showing probability over 65% for presence of subclinical mastitis, as shown by the MaxEnt modelling, can be considered to be high risk areas. Models used in the present study have recognised such areas, which were found to be distributed in locations of small or medium altitudes with agricultural activities. Other high risk areas were located along the coastal areas of the country, in the western and eastern areas of the country. The greatest proportion of high risk areas was found in low altitude (< 300 m) and in irrigated, cultivated agricultural areas or in pastures and agroforestry formations.

As described above, in the multivariable analysis it emerged that ‘flock management system’ was a significant factor affecting prevalence of the infection. In view of that finding, it has been decided to separate farms into the study in categories according to management system, i.e., under intensive, semi-intensive or semi-extensive / extensive management system. That way the primary factor responsible to predisposing ewes to mastitis had been eliminated, therefore potential effects of environmental factors would have become more pronounced. It is interesting that different

environmental factors were identified to be of importance in flocks under the various management systems. Obviously, when the 'management system' had been removed, effects of environmental factors came out more clearly.

The results of the two analyses, when all farms had been taken into account, did not reveal noteworthy differences between the two infections in environmental factors for inclusion in the predictive model. Further, when computations were performed separately for each management system, some differences in factors into the predictive model emerged between the two infections, but these did not appear to be striking.

Differences between the two infections point out to a possible effect of climatological factors in the causal bacteria. As mentioned above, there is some indication that high temperatures favour growth and survival of biofilm-forming staphylococcal isolates (Martin et al. 2016) in dairy farms, thus may potentially influence virulence of these organisms and development of mastitis according to climatological circumstances.

The identification of distance from other sheep farms as an important factor in the predictive models, indicates that contact with other sheep populations can contribute to dissemination of pathogens, e.g., through staff visits to farms or movement of animals between farms. The findings of the present study indicate that in sheep (as already documented in cattle) there can be between-animal transmission and sheep populations remain a significant risk factor for prevalence of the disease.

Precipitation has not been associated with mastitis before. Only Raboisson et al. (2012) have provided an indirect link, when they indicated that increased number of rain days in a month were related to increased somatic cell counts in monthly counts in bulk milk of cows. In the present situation, one may suggest that increased rainfall would lead to muddy pastures, which limit grazing of animals and thus modify feeding patterns, or to low temperatures, which lead to increased energy requirements of animals affecting their immune status (Caroprese et al. 2015).

Moreover, environmental conditions are important with regard to effects in land use and outcomes, which are strongly influenced by temperature and precipitation conditions. Especially in grazing animals, these can affect nutrition of animals, which is a documented predisposing factor for mastitis in ewes (Giadinis et al. 2011, Barbagianni et al. 2015), mainly through regulation of immune response of animal. Farms near arable lands can have ready access to quality feedstuffs, whilst in semi-extensive / extensive management systems pasture availability is particularly important to covering nutritional requirements. The significance of altitude as an important factor in the predictive models can be related to the variations in temperature as altitude changes, given that temperature

has been identified to be of importance. Moreover, altitude can also be related to the differences in land use, as flora varies with changes in altitude, which can affect grazing of sheep.

Despite environmental factors being outside of the control of farm managers, the present results can be valuable in establishing principles of udder health management in sheep flocks. Hence, in areas with increased risk for mastitis, as indicated herein, relevant preventive measures for mastitis need to be established and applied appropriately, e.g., teat-dipping, should be performed. In areas with increased risk of biofilm staphylococcal subclinical mastitis, measures specifically against these organisms should be applied, e.g., milkers must follow detailed rules in handling animals (e.g., meticulous hand cleaning, glove wearing), as hand-milking has been associated with increased prevalence of that infection; further, milking systems should be thoroughly washed and sanitised as part of the routine applied in milking parlours to reduce populations of biofil producing staphylococci in the equipment (Pantoja et al. 2011, Simoes et al. 2015). Finally, during periods when environmental conditions are similar to those leading to high risk for mastitis development, preventive measures should be applied meticulously to minimise risk of mammary infections.

Importance of research in factors predisposing to mastitis

It becomes evident that many factors can predispose ewes to mastitis. Studies that have contributed in their identification, have increased available knowledge regarding mastitis in sheep and their value in understanding the disease is important. Nevertheless, many of these have discussed the significance of the various factors in isolation, i.e. without taking into account other factors that may also influence development of mastitis.

In mastitis, there are clear management – environment – animal interactions, hence the significance of the above factors should be considered within that frame. In general, mastitis is considered to be more frequent in dairy flocks; however, that may only reflect the frequent udder monitoring in such farms, rather than indicating a true difference between dairy and non-dairy flocks.

In the present study that had evaluated the importance of various factors and their interactions by using multivariable analysis, it emerged that only the management system applied in farms (European Food Safety Authority 2014) was of importance for mastitis. The results have indicated that mastitis occurred more frequently in farms managed under the intensive or semi-intensive system. As discussed above, ‘management system’ includes various aspects of flock organisation (e.g., housing facilities, nutrition, milking routine, animal breed), which have been

described to affect development of mastitis in ewes, and encompasses a variety of factors, which, to varying extent each one, contribute to subclinical mastitis or its control.

All the above confirm the multifactorial nature of mastitis and indicate that its control should rely on many approaches. The present results have also identified that differences exist in factors predisposing to mastitis according to the causal organism of the disease, as well as even according to virulence factors present in staphylococcal isolates.

Investigations and studies into risk factors are important, because, that way it is possible to achieve their elimination, control or correction. This approach contributes in and supports sustainable prevention of the disease, as it leads in reduced use of antimicrobial agents for treatment of disease cases. Moreover, control measures for mastitis, which attempt to eliminate possible predisposing factors can thus be developed and implemented.

Evaluation of a novel vaccine against staphylococcal mastitis

Evaluation of the test vaccine under clinical conditions

Efficacy of the test vaccine against mastitis

The vaccine under evaluation has been licensed against *S. aureus*-associated clinical mastitis in ewes. Nevertheless, subclinical mastitis occurs more frequently and is caused primarily by coagulase-negative staphylococci. Hence, there is increased interest to investigate potential efficacy of the vaccine against subclinical mastitis.

The present results have indicated that the test vaccine reduced occurrence of staphylococcal mastitis, clinical and subclinical, in ewes. The study was carried out in five dairy sheep farms and included a large number of animals; further, the study extended throughout an entire lactation period. The results were uniform across the five farms, with the incidence risk of mastitis in vaccinated ewes being smaller than in controls. During this study, care was taken to use precise, robust and reproducible definitions of outcome. Nevertheless - and in common with all field studies of this type - it is almost inevitable that the assays had sensitivities and specificities that were < 100%. For example, it is likely that some udders were incorrectly declared to be uninfected and there may also have been false-positives. The expected effect of errors in assays would be to underestimate the true size and statistical significance of effects, but not alter their direction; this in turn means that the differences that were observed between experimental groups may actually be underestimates of the vaccine’s true effect.

In the present study, most mastitis cases were subclinical mastitis (approx. 95% of all cases), which is financially important (Saratsis et al. 1999). Very few cases of clinical mastitis were recorded during the study, most of them in unvaccinated animals. Moreover, significantly fewer coagulase-negative staphylococci have been identified as causal agents of mastitis from vaccinated ewes, thus indicating an immunisation against these bacteria. If identification of coagulase-negative isolates to species level had been performed, some of the various outcomes might have differed; for example, duration of mastitis might have been shorter in vaccinated ewes and frequency of recrudescence smaller, whilst more new intramammary infections might have been recognised. Nevertheless, from the viewpoint of intramammary infections with biofilm-forming isolates, there would be small differences, as the condition has been defined independently of the species involved, but based on biofilm formation by the isolates.

The vaccine has been found effective in adult animals, whilst in *primiparae* ewes no significant differences were seen between vaccinated and control animals. In fact, mastitis incidence risk in young ewes was small. During the study, *primiparae* ewes had a shorter lactation period than older ones. The smaller risk of infection coupled with effective mammary defence mechanisms (Kraft et al. 1987, Fragkou, Mavrogianni et al. 2007) could have accounted for the smaller incidence risk of mastitis in young animals.

In vaccine efficacy studies, in which mixed populations (vaccinated and control individuals) are in contact, there is a bias towards no effect (Halloran et al. 1997). Hence, one may postulate that, possibly, in commercial situations, in farms with only vaccinated ewes, the effects of the vaccine would be more pronounced.

Vaccination has been found to lead to a reduced incidence risk of mastitis in all clusters of farms that were investigated. As discussed above, the management system applied in flocks has been found to be the most significant predictor for subclinical mastitis, with highest risk in flocks under semi-intensive or intensive management. Farms under semi-intensive or intensive management were included in the present study and a higher incidence risk was seen in flocks under semi-intensive management. Reduction of mastitis occurrence after vaccination was seen independently of the management system applied in farms.

Further, hand-milking has been identified as a significant predictor for biofilm staphylococcal staphylococcal mastitis during the field investigation. This was confirmed in the present study: in the cluster of farms applying hand-milking (vaccinated and control ewes), incidence risk was higher than in the cluster with farms applying machine-milking. In any case, independently of the milking procedure applied in flocks, the vaccine was found to confer immunity against mastitis. In relation to the reported history of mastitis, the finding of higher mastitis incidence risk in the cluster with flocks

with ‘no reported history’ underlines that it is difficult for farmers to estimate the true frequency of mastitis; moreover, farmers would only notice clinical manifestations of mastitis, thus missing cases of subclinical mastitis.

In relation to breed, it is noteworthy that the highest incidence risk of mastitis was recorded in a flock with Chios breed ewes, which has been identified as a breed susceptible to infections, with prevalence of subclinical mastitis found to be approximately 32%, as described previously. Although the vaccine offered immunity in ewes in that flock, the highest incidence risk among vaccinated ewes was recorded in the animals of that farm.

Outcomes used in the present study were staphylococcal mastitis / intramammary infection and biofilm staphylococcal mastitis / intramammary infection. The vaccine is licensed against staphylococcal mastitis, but targets specifically cases caused by staphylococcal isolates forming biofilm. Biofilm formation is related to increased adhesion properties by bacteria and can lead in colonisation of the bacteria onto mammary epithelial cells and subsequent damage to the parenchyma, which can account for increased pathogenicity of the respective isolates for the mammary gland of ewes. In the field study, prevalence of biofilm subclinical mastitis was found to be 15.5%. Those results indicated that biofilm-forming staphylococci (independently of species) were the primary aetiological agents of subclinical mastitis in ewes, accounting for 60% of all cases of mastitis. The findings of the vaccine efficacy studies have indicated that efficacy of the vaccine was greater against these organisms than against mastitis caused by staphylococcal species generally (biofilm- or non biofilm-forming isolates) and, of course, mastitis caused by all aetiological agents combined. This was expected, as the vaccine’s antigenic component would act specifically against biofilm formation by staphylococci; thus, biofilm-forming isolates would be cleared more effectively.

The vaccine under evaluation includes a *S. aureus* strain, characterised as strong biofilm-forming, *ica* operon positive and *bap* negative (Perez et al. 2009). This might explain the differences detected in *ica* genes between isolates from vaccinated and isolates from control ewes. One should nevertheless take into account that various other genes have been associated with biofilm formation by staphylococcal isolates, e.g. *aap* in coagulase-negative isolates (Tremblay et al. 2013).

Nevertheless, cases of mastitis occurred even in vaccinated ewes. Mastitis is a multifactorial problem and none of the many approaches used for its control is fully effective. Various factors, unrelated to the vaccine, may be responsible; for example, inappropriate cleaning of milking system by the farm staff after end of the milking routine can lead in multiplication of staphylococci in milking clusters, which increases risk for infection of ewes in the subsequent milking; further, management factors, e.g., nutrition, can influence the immune status of ewes and their response to vaccination (Caroprese et al. 2015, Lacasta et al. 2015). It is noteworthy that in a study performed in cows, in

which a vaccine against mastitis with the same staphylococcal component as this vaccine, had been evaluated, only a moderate reduction in incidence rate of staphylococcal mastitis was also found (Schukken et al. 2014).

Given the results of the Ecological Niche Modelling, possibly in areas with increased risk of biofilm staphylococcal mastitis and based on predicting space distribution of mastitis, administration of the vaccine in ewes located in such areas might be considered as a targeted means for reducing incidence risk of mastitis.

Duration of efficacy of the test vaccine

The effects of vaccination were prominent during the first four months of the lactation period. Efficient control of mastitis during that stage is particularly beneficial, as there is increased risk of infection due to post-partum immunosuppression (Barbagianni et al. 2015, Caroprese et al. 2015). Milk yield of ewes is highest at that stage (Ruiz et al. 2000, Oravcova et al. 2006), therefore control of mastitis benefits ewes in dairy (increased milk production) and meat (optimum growth rate of lambs) production systems. Anecdotal information from the field indicates that in flocks with a long lactation period, a booster (third) administration of the vaccine after the 5th month of lactation period is advocated by practicing veterinarians, as off-license use of the product. The present results may provide some justification in the practice, especially in dairy flocks, in which lactation period may extend to over 7 months. It is noteworthy that Leitner et al. (2011) also reported three administrations of the recombinant Target of RNAIII Activating Protein vaccine, which lends support to a view that, for staphylococcal mastitis, three immunisations might be necessary.

Detection of anti-PNAG antibody titres

Antibody titres in immunised animals were significantly higher than in unvaccinated controls, although the latter ewes' titres might have increased possibly as the result of naturally occurring infections.

Increase of antibody titres in vaccinated ewes confirmed development of immune response by these animals, which, in turn, might have contributed in the more effective clearance of biofilm-forming isolates. Increased antibody titres were recorded for up to 180 days post-booster vaccination, hence, in theory, 155 to 160 days into the lactation period. Nevertheless, it is also possible that staphylococcal infections occurring in vaccinated ewes may be responsible, at least to some extent, for the increased titres rather than these being a long-standing effect of vaccination.

Efficacy of the test vaccine against mammary carriage

No differences were seen between groups of ewes in the presence of staphylococcal mammary carriage. Mammary carriage bacteria might become pathogenic under the effect of various factors, which decrease defensive efficacy of hosts or promote pathogenicity of bacteria; this has been documented with coagulase-negative staphylococci in a previous experimental study (Fragkou, Mavrogianni et al. 2007). The present results indicate that vaccination did not inhibit staphylococcal mammary carriage.

Nevertheless, in vaccinated animals, there was an effect against carriage staphylococci causing mastitis (among group V, fewer ewes with mammary carriage developed subsequently staphylococcal or biofilm staphylococcal mastitis [0.350 and 0.100] than among group C [0.580 and 0.583, respectively]). This may possibly be the effect of limiting biofilm formation by such organisms and subsequent dissemination within the mammary parenchyma, as the result of vaccination.

Evaluation of the test vaccine against experimental staphylococcal mastitis

The results have indicated that the test vaccine has contributed in the reduction of the severity of mastitis in vaccinated ewes after challenge. Ewes were inoculated directly into the mammary gland by a pathogenic *S. chromogenes* isolate. This organism caused mastitis in all challenged ewes; moreover, it also caused bilateral mammary infection in control ewes and even death in one animal, thus confirming its increased virulence and invasiveness in the mammary gland. Coagulase-negative staphylococci have been traditionally considered of 'milder' pathogenic action than *S. aureus*. However, death of ewes inoculated with *S. chromogenes* has been reported before (Fthenakis 1988).

All vaccinated ewes developed mastitis, but its adverse effects were milder in unvaccinated animals, as indicated by a wide array of measures. In vaccinated ewes, no cases of mammary infections in the contralateral mammary glands were recorded; further, grey-scale ratios and milk yield ratios indicated a milder damage in mammary parenchyma. Finally, the histopathological scores were smaller. We postulate that these milder effects were the consequences of the smaller bacterial counts in milk and tissue samples collected from vaccinated ewes, as indicated by the results of the correlation analysis (e.g., inverse correlation with milk yield ratios and positive correlation of bacterial counts in tissue samples and with histopathological scores).

The association of bacterial numbers in milk with adverse effects in the mammary parenchyma has been reported in previous studies performed in mice, an animal species found to be appropriate model for the study of staphylococcal mastitis of ewes (Fthenakis 1988, 1992a). Leitner, Krifucks et al. (2003) showed that the pathogenicity of staphylococcal isolates recovered from cases of mastitis, was related to the bacterial dose inoculated in experimental mice. In later studies, Brouillette et al. (2004) found that the numbers of *S. aureus* recovered from murine mammary tissue samples after intramammary challenge were related to inoculation dose and Chinchali and Kaliwal (2014) reported that histological lesions in mammary glands were related to the challenge dose. Those studies were performed with varying challenge doses. In the present study, although a similar challenge dose was used, at the end bacterial counts in milk and tissue samples differed between vaccinated and control ewes. It may be that the anti-PNAG antibodies found to increase after vaccination, led to the smaller bacterial counts of the challenge isolate post-inoculation. The increase in antibody titres in vaccinated ewes confirms development of immune response by these animals. The inverse correlation of the antibody titres with the bacterial counts in milk and in tissue samples, coupled with the shorter duration of mammary infection in vaccinated ewes also lends support to this hypothesis.

As all vaccinated ewes developed mastitis after challenge, it would not be correct to talk about ‘protective’ antibodies. It might be more appropriate to postulate that the anti-PNAG antibodies led to effective clearance of the challenge isolate, as discussed above. In the experimental design employed, the teat, which has a well-documented effect against bacterial invaders (Mavrogianni et al. 2005, 2006), had been by-passed in order to guarantee development of mastitis in the inoculated ewes. Hence, it was not possible to show effects in development of mastitis in this study. Nevertheless, effective clearance of the challenge isolate, as postulated above, could have led in limiting dissemination of the isolate in the contralateral gland and not causing infection therein.

In cases of mastitis, one should take into account that the damage caused in infected mammary glands, would be reflected in milk yield of the ewes (Fthenakis and Jones 1990b). Moreover, smaller tissue damage in mammary glands during the lactation period can lead in reduced culling rate at the end of the lactation period, when ewes are clinically examined and those with extensive lesions culled (Fthenakis et al. 2012, Petridis and Fthenakis 2014). Hence, there is a benefit with financial consequences in development of milder effects in ewes after infection with a mammary pathogen.

General significance of using the test vaccine against staphylococcal mastitis - Implications for mastitis control in sheep flocks

Staphylococci express a variety of virulence factors, which may not all be targets of one vaccine; no single virulence factor necessary for infection, which can be targeted for vaccine development, is known. The variability of the various staphylococcal antigens further impedes development of relevant vaccines (Golubchik et al. 2013). Moreover, recurrence of staphylococcal mastitis indicates that these bacteria do not induce an immunity pattern in affected animals, which might be defined and employed to design a vaccine. These are reflected in the repeated attempts to develop staphylococcal vaccines for people (Pier 2013), despite effective anti-staphylococcal vaccines being essential for public health.

Targeting PNAG was a good selection for providing antigenic coverage against a major virulence factor of these organisms, with particular significance for the mammary gland. However, the clinical and experimental results in the present study indicate that this does not fully protect vaccinated individuals.

Various attempts have been reported to develop vaccines against staphylococcal mastitis. For example, a product containing inactivated *S. aureus* and *S. simulans* whole cells, as well as *S. aureus* exopolysaccharide antigens presented within liposomes been found to lead in reducing incidence risk of mastitis (Amorena et al. 1994). In another approach, a vaccine including recombinant Target of RNAIII Activating Protein (which in staphylococci is involved in pathogenesis of staphylococcal mastitis), has been employed (Leitner, Lubashevsky et al. 2003, Leitner, Yadlin et al. 2003). More recently, a multivalent whole cell staphylococcal vaccine has been assessed by Alekish et al. (2018), who showed that it led to a non-significant reduction in new cases of mastitis in vaccinated animals, with no effects on milk production or composition.

In the clinical study of the vaccine under evaluation, a reduction of the incidence risk of subclinical mastitis has been found. In the experimental study, a particularly invasive isolate was inoculated, one that even caused bilateral mammary infection in unvaccinated ewes. The differences can reflect the different types of study; under field conditions, various management factors can contribute to limiting or exacerbating the infection (Gelasakis et al. 2015).

The results indicate that vaccination alone could not eliminate staphylococcal mastitis. Hence, vaccine administration would need to be considered within the frame of a complete udder health management and to be combined with other management procedures (e.g., correct milking routine, appropriate milking system maintenance, culling of ewes with long-standing infections,

administration of antimicrobials at the end of a lactation period), in order to eliminate relevant mammary infections. All these can work synergistically to improve control of mastitis.

In cases of mastitis, one should take into account that the damage caused in infected mammary glands, would be reflected in milk yield of the ewes (Fthenakis and Jones 1990b). Moreover, smaller tissue damage in mammary glands during the lactation period can lead in reduced culling rate at the end of the lactation period, when ewes are clinically examined and those with extensive lesions culled (Fthenakis et al. 2012, Petridis and Fthenakis 2014). Hence, there is a benefit with financial consequences in development of milder effects in ewes after infection with a mammary pathogen.

Epilogue

Conclusions

The conclusions from the results of the present thesis are summarised herebelow.

(a) The results of the countrywide investigation confirmed the significance of subclinical mastitis as a frequent problem of dairy sheep.

- Prevalence of subclinical mastitis was 26.0% on national basis in Greece.
- Staphylococci were the primary aetiological agents of the disease.
- Management system practiced in farms was identified as the most important factor predisposing to subclinical mastitis. Animal-related factors, e.g., stage of lactation period, may also play a role in development of mastitis. Application of targeted health management methods, e.g., post-milking teat dipping, may be associated with reduced prevalence of subclinical mastitis.
- There was evidence of associations of subclinical mastitis with breed.

(b) A new disease entity has been recognised: mastitis caused by biofilm-forming staphylococci.

- The disease is caused by staphylococcal isolates independently of their species.
- Prevalence of the disease was 15.5% on national basis in Greece.
- Hand-milking was identified as the most important factor predisposing to that infection.

(c) Prevalence of staphylococcal isolates resistant to antimicrobial agents was limited.

- Resistance referred mainly to penicillin, tetracycline and amoxycillin.
- Prevalence of resistance was higher among coagulase-negative isolates than among *S. aureus* isolates.

- Detection of resistant isolates was more frequent among isolates recovered from flocks that practiced routine administration of antimicrobial agents at the end of a lactation period and among isolates recovered immediately *post-partum*.
- Resistance genes were found to be associated with mobile genetic elements.

(d) Most *S. aureus* isolates were classified in ST133 and most *S. epidermidis* were classified in ST100, ST142 or ST152.

(e) Anti-PNAG antibodies are induced mainly consequently to anti-staphylococcal mastitis vaccination.

- Antibody titres may also increase during mammary infection by biofilm-forming staphylococci.
- Evaluation of antibody titres can be potentially used as a screening technique for establishing extent of staphylococcal mastitis in flocks.

(f) The findings have documented, for the first time, the significance of environmental factors predisposing ewes to mastitis.

- Also for the first time, predictive models for subclinical mastitis in ewes taking into account environmental parametres have been provided.
- The findings also can be used in mastitis prevention schemes, in order to establish strategies that will help to control mastitis, tailored according to needs of regions and farms, as per environmental risks.

(g) The entirety of findings has provided clear evidence regarding the multifactorial nature of mastitis in sheep: in mastitis, there are clear management – environment – animal interactions.

(h) The entirety of findings have indicated that the vaccine under evaluation, which was based on a bacterin of *S. aureus* strain expressing PNAG, could lead to some protection against staphylococcal mastitis.

- The effect was more pronounced in adult ewes and during the first four months of lactation period; it was independent of management system, milking procedure, reported history of mastitis or animal breed.
- The vaccine reduced the severity of the infection and limited damage to the mammary parenchyma and dissemination of the causal pathogen.
- Anti-PNAG antibodies were detected in vaccinated ewes after administration of the vaccine and might have led in clearance of causal bacteria.

(i) For control of mastitis in ewes, vaccination should not be considered as the only means for reducing mastitis within an udder health program; other udder health management measures should be included and can work synergistically to improve control of mastitis.

Prospects

Suggestions for further research, in continuation of the present work, are as below.

- The establishment of targets within sheep farms, for evaluation of udder health management plans.
- The exploitation of potential breed resistance to mastitis for enhancing resistance to the disease.
- The detailed study of staphylococcal isolates from other sources within sheep farms (e.g., milking parlour, raw milk) for establishment of potential sources of infection of ewes.
- The evaluation of the administration of the test vaccine within the lactation period, with a view to study the potential protective effect.

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