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Ecology and Epidemiology of Bovine-Related Coagulase-Negative *Staphylococcus* Species

Anneleen De Visscher

Merelbeke, 2016

Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.

Thomas A. Edison

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Ecologie en Epidemiologie van Boviene Coagulase-Negatieve *Staphylococcus* Species

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Table of Contents

Chapter 1	General introduction	1
Chapter 2	Aims and outline of the thesis	25
Chapter 3	Assessment of the suitability of mannitol salt agar for growing bovine-associated coagulase-negative staphylococci and its use under field conditions	29
Chapter 4	Teat apex colonization with coagulase-negative *Staphylococcus* species throughout lactation and before parturition	49
4.1	Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle	51
4.2	Teat apex colonization with coagulase-negative *Staphylococcus* species before parturition: distribution and species-specific risk factors	73
Chapter 5	Intramammary infection with coagulase-negative *Staphylococcus* species in early lactation and throughout lactation	103
5.1	Intramammary infection with coagulase-negative staphylococci at parturition: species-specific prevalence, risk factors, and impact on udder health	105
5.2	Species group-specific predictors at the cow and quarter level for intramammary infection with coagulase-negative staphylococci in dairy cattle throughout lactation	135
Chapter 6	Coagulase-negative *Staphylococcus* species in bulk milk: prevalence, distribution, and associated subgroup- and species-specific risk factors	151
Chapter 7	General discussion	187
Summary		223
Samenvatting		231
Curriculum vitae and publications		239
Acknowledgements - Dankwoord		247

List of Abbreviations

AFLP	Amplified fragment length polymorphism
BCS	Body condition score
CI	Confidence interval
CNS	Coagulase-negative staphylococci
DHI	Dairy herd improvement
DIM	Days in milk
HF	Holstein Friesian
IMI	Intramammary infection
ITS-PCR	Internal transcribed spacer polymerase chain reaction
LnqSCC	Natural log transformed quarter somatic cell count
MLST	Multi-locus sequence typing
MMUL	Milking machine unit liner
MSA	Mannitol salt agar
MSG	Milker's skin and gloves
OR	Odds ratio
PCR	Polymerase chain reaction
PCR-RFLP	PCR restriction fragment length polymorphism
PFGE	Pulsed-field gel electrophoresis
qSCC	Quarter milk somatic cell count
SCC	Somatic cell count
SE	Standard error
TA	Teat apex
TAC	Teat apex colonization
tDNA-PCR	Transfer RNA intergenic spacer PCR
WGS	Whole genome sequencing

Chapter 1

General Introduction

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Bovine mastitis

Mastitis, an inflammation of the mammary gland, is one of the most prevalent and costly diseases in the dairy industry worldwide. Economic losses are due to factors such as milk production losses, additional treatment, and increased culling (Halasa et al., 2007) and vary between €65 and €182 per cow in the herd per year (Huijps et al., 2008). The disease also affects milk quality and animal welfare (Heringstad et al., 2000) and is associated with the highest antimicrobial drug use on dairy farms (Pol and Ruegg, 2007; Barlow, 2011). Growing concern rises over the use of and resistance to antimicrobials in both human and animal health (Barlow, 2011; WHO, 2014), indicating the need for good prevention rather than treatment programs. Herd-management practices and cow- and quarter-level risk factors, helping to effectively control and especially prevent mastitis are thus necessary (Gruet et al., 2001; Halasa et al., 2007; Piepers et al., 2007; Supré et al., 2011), though depend on the causative agents involved (Barkema et al., 1999b; Smith and Hogan, 2001; Zadoks et al., 2001; Piepers et al., 2011).

Mastitis typically results from bacterial intramammary infection (IMI) and is called clinical or subclinical in the presence or absence of visible symptoms, respectively (Kemp et al., 2008). The causative bacteria can be grouped according to their ecological nature (i.e. habitat) in so-called host(dairy cow)-adapted (e.g. *Staphylococcus aureus*) and environmental pathogens (e.g. *Streptococcus uberis*) (Gruet et al., 2001; Pyörälä and Taponen, 2009; Blowey and Edmonson, 2010). Also, their epidemiology differentiates bacteria in contagious and opportunistic subgroups (Pyörälä and Taponen, 2009). Contagious pathogens (e.g. *S. aureus*) are characterized by a single origin, multiple infected animals in a herd, and a cow-to-cow-transmission through a vector (e.g. the milking machine), whereas opportunistic bacteria (e.g. *Escherichia coli*) have a range of different origins, are not spread among animals and only cause IMI under favoring conditions (e.g. lack of hygiene). This classification, however, is not always straightforward and strain-specific differences exist within bacterial species (Zadoks et al., 2003). More than 100 species and subspecies are able to cause bovine mastitis (Smith and Hogan, 2001). In Flanders, the predominant major pathogen causing clinical mastitis is *S. uberis*, followed by *E. coli, S. aureus* and *Streptococcus dysgalactiae* (Verbeke et al., 2012). Subclinical mastitis is, on the other hand, mainly caused by *S. aureus* and esculine-

positive cocci (Piepers et al., 2007). The latter pathogen distributions are in accordance with those described in other countries and regions (Pitkälä et al., 2004; Tenhagen et al., 2006; Bradley et al., 2007). Yet, the coagulase-negative staphylococci (CNS) are currently the overall most frequently isolated bovine mastitis pathogens in Flanders (Piepers et al., 2007) and other regions and countries (Pyörälä and Taponen, 2009).

Coagulase-negative staphylococci

The heterogeneous group of CNS includes 50 species (www.bacterio.net, 2015). Several commercially available phenotypic identification methods, such as API Staph ID 20 (Zadoks and Watts, 2009; Park et al., 2011), API Staph ID 32 (Thorberg and Brändström, 2000; Taponen et al., 2006; Ruegg, 2009; Sampimon et al., 2009b; Zadoks and Watts, 2009), API Staph Trac-System (Matthews et al., 1990b), BBL Crystal Gram-Positive System (Ruegg, 2009; Zadoks and Watts, 2009), STAPHase, Staph-Ident (Watts et al., 1984), Staph-Zym (Thorberg and Brändström, 2000; Capurro et al., 2009; Sampimon et al., 2009b; Zadoks and Watts, 2009), VITEK 2 (Bal et al., 2010), and VITEK Gram-Positive Identification Card (Matthews et al., 1990b), have been used and described before. These phenotypic systems rely on the evaluation of the expression of several characteristics of the bacterial species, though suffer from a subjective interpretation of the tests, a variable expression, and they are mainly validated for human CNS species (Ruegg, 2009; Zadoks and Watts, 2009). Comparison of those phenotypic methods with the so-called gold standard, i.e. gene sequencing, revealed the necessity of a genotypic method, based on the detection of the bacterial DNA, when identifying CNS species in order to obtain a sufficient accuracy, reproducebility and typeability (Sampimon et al., 2009b; Zadoks and Watts, 2009; Park et al., 2011; Vanderhaeghen et al., 2015). Validation of accurate identification methods was thus recommended (Taponen et al., 2006; Ruegg, 2009; Sampimon et al., 2009b) and performed (Bes et al., 2000; Santos et al., 2008; Capurro et al., 2009; Supré et al., 2009; Piessens et al., 2010; Braem et al., 2011; Park et al., 2011). The implementation of those techniques revealed species-specific traits and characteristics.

As a group, CNS are considered as minor pathogens for udder health (Schukken et al., 2009) though contrasting findings have been reported and differences among

species have been revealed in their impact on udder health. Coagulase-negative staphylococci have also been isolated from mild cases of clinical mastitis (Trinidad et al., 1990; Bradley et al., 2007; Compton et al., 2007; Olde Riekerink et al., 2007; Taponen et al., 2007; Gillespie et al., 2009; Persson Waller et al., 2011; Piessens et al., 2011; Simojoki et al., 2011), but seem to lack the ability to cause severe clinical mastitis (Taponen et al., 2007). However, the latter hypothesis was also countered as a higher body temperature and disturbed clinical condition was occasionally caused by CNS (Jarp, 1991). The impact of CNS IMI on milk production has also been discussed and questioned. Either no impact was observed (Kirk et al., 1996) or even a lower milk production was detected in some studies (Pyörälä and Taponen, 2009; Thorberg et al., 2009; Simojoki et al., 2011), whereas others showed a higher milk production (Compton et al., 2007; Schukken et al., 2009; Piepers et al., 2010).

Besides, potential protective effects of CNS have also been explored. For teat apices colonized with whatever CNS species (Piepers et al., 2011) or with *Staphylococcus chromogenes* (De Vliegher et al., 2003) the protective assumption could be substantiated. Different hypotheses have been suggested to explain the observed findings such as competitive exclusion or an already activated immune response (Rainard and Poutrel, 1988; Matthews et al., 1990a; Piepers et al., 2009b). The production of inhibitory substances (e.g. bacteriocins) has been proposed for *S. chromogenes* (De Vliegher et al., 2004) and noticed for several other staphylococci (Allgaier et al., 1985; Sashihara et al., 2000; Nascimento et al., 2005; Coelho et al., 2007; Wilaipun et al., 2008; Ceotto et al., 2010; Fagundes et al., 2011; Braem et al., 2014).

Prevalence and distribution of coagulase-negative staphylococci

Coagulase-negative staphylococci are the predominant mastitis pathogens in prevalence studies performed in recent years (Barkema et al., 1999a; Pitkälä et al., 2004; Tenhagen et al., 2006; Bradley et al., 2007; Piepers et al., 2007; Sampimon et al., 2009a; Schukken et al., 2009; Reyher et al., 2011). At parturition, CNS have also commonly been observed in milk samples (Kirk et al., 1996; Rajala-Schultz et al., 2005, Parker et al., 2007; Piepers et al., 2011; Rajala-Schultz et al., 2009). A fast decrease in the number of CNS-infected quarters and animals after calving has been

recorded (Myllys, 1995; Piepers et al., 2009a), although differences among CNS species have been noticed (Aarestrup and Jensen, 1997; Taponen et al., 2006).

Accurate studies relying on genotypic identification, as recommended for CNS research, revealed approximately 25 CNS species causing bovine IMI (Santos et al., 2008; Sampimon et al., 2009b; Park et al., 2011; Persson Waller et al., 2011; Piessens et al., 2011; Supré et al., 2011) (Table 1). *Staphylococcus chromogenes* was the predominant species in all except one study (Persson Waller et al., 2011). *Staphylococcus haemolyticus* and *S. epidermidis* were also frequently isolated in all studies. Species other than the aforementioned ones isolated in most studies are *S. cohnii, S. hominis, S. hyicus, S. sciuri, S. simulans,* and *S. xylosus.* Large discrepancies exist between studies in reporting the presence of *S. arlettae, S. auricularis, S. capitis, S. caprae, S. caseolyticus, S. devriesei, S. equorum, S. fleurettii, S. gallinarum, S. lentus, S. nepalensis, S. pasteuri, S. pseudintermedius, S. saprophyticus, S. succinus,* and *S. warneri*. As some species were only recently described (e.g. *S. devriesei,* Supré et al., 2010) this is not unexpected. Furthermore, a herd- or even country-specific microbiota might exist (Piepers et al., 2007; Gillespie et al., 2009; Piessens et al., 2011; Supré et al., 2011) as well as the applied identification method and study design could influence the CNS prevalence and distribution.

The CNS distribution has been unraveled throughout lactation in the aforementioned studies, but the species-specific distribution at parturition needs further exploration.

Relevance of coagulase-negative staphylococci for bovine udder health

The relevance of CNS for bovine udder health has been studied for many years and concerns topics such as their impact on the (quarter milk) somatic cell count and their potential to cause persistent IMI.

A mild to moderate increase in SCC in CNS-infected cows and quarters, as opposed to uninfected cows and quarters, has been observed (Lam et al., 1997; Barkema et al., 1999a; Taponen et al., 2007; Gillespie et al., 2009; Thorberg et al., 2009; Sampimon et al., 2009a; Schukken et al., 2009; Piepers et al., 2010; Sampimon et al., 2010).

Table 1. Identification of coagulase-negative staphylococci (CNS) causing intramammary infection throughout lactation only relying on genotypic species identification (ID)

Reference	Country	N[1]			Species ID[2]	N CNS[3]	Most prevalent CNS species (%)[4]		
		H	A	Q			1st	2nd	3rd
Santos et al., 2008	Brazil	23	54	216[5]	PCR-RFLP	10	*S. chromogenes* (52)	*S. epidermidis* (15)	*S. capitis* (6)
Sampimon et al., 2009b	The Netherlands	/	/	/	*rpoB* sequencing	17	*S. chromogenes* (37)	*S. epidermidis* (13)	*S. xylosus* (9)
Park et al., 2011	USA	/	/	/	*16S* sequencing	11	*S. chromogenes* (72)	*S. xylosus* (9)	*S. haemolyticus* (6)
Persson Waller et al., 2011	Sweden	/	/	/	*tuf* sequencing	11	*S. epidermidis* (31)	*S. chromogenes* (21)	*S. haemolyticus* (14)
Piessens et al., 2011[6]	Belgium	6	60	2580	AFLP	13	*S. chromogenes* (31)	*S. haemolyticus* (26)	*S. epidermidis* (12)
Supré et al., 2011[6]	Belgium	3	89	3064	tDNA-PCR	12	*S. chromogenes* (46)	*S. xylosus* (16)	*S. cohnii* (11)

[1]Number of included herds (H), animals (A) and quarters (Q). [2]Species identification using restriction fragment length polymorphism (PCR-RFLP), sequencing of the *rpoB*, *16s rRNA* or *tuf* gene, amplified fragment length polymorphism (AFLP), or transfer RNA intergenic spacer PCR (tDNA-PCR). [3]Number of different CNS species. [4]Percentage within all isolated CNS species. [5]Only a part of all CNS were retained for species ID. [6]Longitudinal studies.

Especially on low bulk milk SCC herds, CNS have an important contribution to the total number of somatic cells in the bulk milk (Rainard et al., 1990; Piepers et al., 2009a; Pyörälä and Taponen et al., 2009; Schukken et al., 2009; Sampimon et al., 2010). In contrast, some studies did not detect an impact on the SCC (Kirk et al., 1996; Compton et al., 2007) although the latter solely concerned heifers.

Species-specific analysis actually revealed differences among CNS species (Sampimon et al., 2009a; Thorberg et al., 2009; Simojoki et al., 2011; Supré et al., 2011): *S. chromogenes, S. simulans* and *S. xylosus* cause a statistically significant elevation in SCC not different from *S. aureus* in quarters from lactating dairy cows and heifers (Supré et al., 2011), suggesting that those species are more relevant for udder health than others. The latter finding needs further investigation and should be substantiated for IMI at parturition as well. It suggests that prevention of CNS IMI on well-managed dairy farms should at least target these “more relevant” species.

Coagulase-negative staphylococci are able to cause persistent IMI (Rainard et al., 1990) even through the dry period (Rajala-Schultz et al., 2009; Supré et al., 2011) despite a high spontaneous cure rate (Taponen et al., 2006). Differences among species have been observed (Aarestrup and Jensen, 1997; Gillespie et al., 2009; Rajala-Schultz et al., 2009; Thorberg et al., 2009; Piessens et al., 2011), although also similar strains have been isolated from both persistent and transient IMI (Taponen et al., 2007). The latter suggests not only the bacterium plays a role, but also the host-response to IMI.

Different cow-associated habitats harboring coagulase-negative staphylococci

Coagulase-negative staphylococci as a group are considered as opportunistic pathogens, part of the normal skin microbiota (Devriese and De Keyser, 1980; Schukken et al., 2011). Differences in CNS prevalence are observed when exploring various cow-related habitats (e.g. teat skin, teat canal, udder skin, perineum…) (White et al., 1989; Trinidad et al., 1990; Taponen et al., 2008). Research picturing CNS distribution on teat apices is only suggestive and incomplete as species identification lacked accuracy by the use of phenotypic tests in one study (White et al., 1989) and only lactating dairy cows from one herd were included in another study (Taponen et al., 2008). Large studies relying on molecular identification are required to unravel CNS teat apex distribution and to study potential infection sources from

different habitats. We focus on the 2 most important ones: teat apices and the environment.

A higher risk of developing a *S. aureus* IMI at parturition when teat apices were colonized with *S. aureus* was suggested more than 2 decades ago (Roberson et al., 1994). This was not substantiated in a more recent study as different *S. aureus* strains were isolated from IMI and teat skin from lactating dairy cows (Zadoks et al., 2002). Analogous to *S. aureus*, teat skin might act as a reservoir for bovine CNS. Teat apices colonized with CNS as a group or solely *S. chromogenes*, only phenotypically identified, were not associated with IMI in quarters of heifers at parturition (De Vliegher et al., 2003; Piepers et al., 2011). In contrast, highly similar *S. chromogenes* strains were detected in milk and on udder skin of lactating cows (Taponen et al., 2008). Genotypic peri-partum studies are needed to bring further insights.

The cows' environment acts as a potential source for bovine CNS IMI (Matos et al., 1991; Piessens et al., 2011). *Staphylococcus equorum*, *S. cohnii,* and *S. sciuri* are considered as environmental species as they were frequently observed in the cows' environment, i.e. air, slatted floors, sawdust of the cubicles and sawdust of the stock (Piessens et al., 2011), and were hardly isolated from bovine IMI. On the other hand, *S. chromogenes* and *S. epidermidis* were rarely present in the bovine environment, although being a predominant cause of bovine IMI. *Staphylococcus haemolyticus* and *S. simulans* were both present in the environment and causing bovine IMI (Santos et al., 2008; Sampimon et al., 2009b; Park et al., 2011; Persson Waller et al., 2011; Piessens et al., 2011; Supré et al., 2011) and therefore deemed opportunistic in nature. Piessens et al. (2011) found *S. xylosus* only in environmental samples whereas all other aforementioned genotypic studies detected *S. xylosus* in bovine milk samples. The ecological nature of the different CNS species should be further elucidated.

Challenges related to coagulase-negative staphylococcal research

Research relying on genotypic identification demonstrated the abundant presence of diverse CNS species in different bovine habitats, such as the cows' environment (Piessens et al., 2011), milk samples (Santos et al., 2008; Sampimon et al., 2009b; Park et al., 2011; Persson Waller et al., 2011; Piessens et al., 2011; Supré et al.,

2011), and other udder-related habitats (Taponen et al., 2008) indicating their ubiquitous nature. Picturing CNS distribution in those different habitats and investigating potential transmission routes is needed in order to understand variation in epidemiological and ecological nature among species. Identification of associated factors seems a logical next step (Pyörälä and Taponen, 2009).

Species-specific research, however, requires extensive studies including a large number of herds, cows and/or quarters in order to obtain a sufficient number of isolated CNS species. Bulk milk samples have previously been used (Jayarao et al., 2004; Virgin et al., 2009; Olde Riekerink et al., 2010) and allow for including a large number of herds. Yet, numerous bacteria other than CNS are present in bulk milk samples impeding CNS isolation and indicating that bacteria in bulk milk are not solely originating from infected quarters.

Another major challenge turns up when using swabs to explore different habitats (e.g. teat apices) as again mixed cultures are observed, potentially suppressing isolation of CNS (De Vliegher et al., 2003; Taponen et al., 2008).

The use of selective media offers advantages and helps to circumvent the inconveniences of overgrowth and hampered isolation, when using bulk milk samples or swabs (Finegold and Sweeney, 1961). Various media have already been used for the recovery of *S. aureus* and CNS, e. g. Baird Parker agar (Jayarao et al., 2004), Vogel-Johnson agar (Olde Riekerink et al., 2006), DNA-toluidine blue agar (Jarp, 1991; Chapin and Lauderdale, 2007), and different modifications of CHROMagar (Chapin and Lauderdale, 2007; Han et al., 2007; Virgin et al., 2009). These media are mainly recommended for the selective recovery of *S. aureus* though growth of other bacteria was not completely avoided. Chapman (1945) attempted to block the growth of bacteria other than staphylococci by developing a selective medium with a high sodium chloride concentration, i.e. mannitol salt agar (MSA). Nowadays, MSA is commercially available and offers advantages in the recovery of CNS (Kateete et al., 2010). This medium has already been used both in human (Jayaratne and Rutherford, 1999; Shittu et al., 2004; Shittu et al., 2006; Han et al., 2007) and veterinary medicine (Cheng et al., 2010; Addis et al., 2011; Piessens et al., 2011) though no studies have tested the growth of bovine-associated CNS species on this medium and its reliability. The latter should be examined as a prerequisite of any selective medium is the growth of the genera and/or species of interest.

Risk factors for the presence of coagulase-negative staphylococci in different habitats

As prevention and control measures differ among mastitis pathogens (Barkema et al., 1999b; Smith and Hogan, 2001; Zadoks et al., 2001; Piepers et al., 2011), risk factor studies should be conducted for all mastitis-associated bacteria including CNS (Pyörälä and Taponen, 2009).

Several risk factors such as source of drinking water, housing of dry cows, pasture access, monthly measuring of SCC, milk leakage, bulk milk SCC and udder health monitoring by a veterinarian, are associated with IMI caused by CNS as a group (Sampimon et al., 2009a) (Table 2). Poor hygiene, non-clipped udders and no application of teat disinfectants prior to calving were found significant risk factors for CNS-group IMI in heifers (Piepers et al., 2011) (Table 2). An environmental nature of the involved CNS species, which were not differentiated, was assumed in the latter study based on the risk factors that were identified, yet not substantiated.

Additionally, differences between cows and quarters in susceptibility for IMI have been observed (Zadoks et al., 2001; Piessens et al., 2011; Supré et al., 2011) and a herd-dependent CNS microbiota has been reported (Piepers et al., 2007; Gillespie et al., 2009; Piessens et al., 2011; Supré et al., 2011). Research identifying risk factors associated with CNS IMI both throughout lactation and at parturition is required and should therefore be conducted at the quarter and cow level as well as at the herd level.

Conclusion

Many efforts are ongoing to enlarge our knowledge of the various CNS species belonging to the heterogeneous group of bacteria. The species-specific prevalence and distribution of several habitats need to be further explored using adequate semi-selective media and should rely on genotypic identification methods. The impact of the different species should also be investigated and substantiated. Furthermore, risk factor studies at the species or subgroup level should be conducted in order to gain more insight in the ecology and epidemiology of bovine-related CNS species.

Table 2. Factors significantly associated with the likelihood of intramammary infection (IMI) caused by coagulase-negative staphylococci (CNS) as a group both in early lactation and throughout lactation

Independent factor	Categories	OR[1]	Reference
IMI in early lactation			
Cow level			
Hygiene[2]	Good vs. poor	1.9	Piepers et al., 2011
Teat dipping prior to calving[2]	No vs. yes	0.4	Piepers et al., 2011
Udder clipping prior to calving[2]	No vs. yes	0.4	Piepers et al., 2011
IMI throughout lactation			
Herd level			
Pasture access during outdoor season	No vs. yes vs. restrained vs. **part of the season restricted**[3]	NA[4]	Sampimon et al., 2009a
Percentage of stalls contaminated with milk	Continuous	NA	Sampimon et al., 2009a
Dry cows housed in two groups	Yes vs. no	NA	Sampimon et al., 2009a
Source of drinking water used	Tap water vs. **ditch water** vs. own well water source vs. combination of tap water with ditch or own well water source vs. other	NA	Sampimon et al., 2009a
	Tap water vs. ditch water vs. own well water source vs. combination of tap water with ditch or own well water source vs. **other**	NA	Sampimon et al., 2009a
Bulk milk SCC (cells/mL)	<150,000 vs. 150,000 - 250,000 vs. **> 250,000 cells/mL**	NA	Sampimon et al., 2009a
Measuring cow SCC at milk recording	No vs. yes	NA	Sampimon et al., 2009a
Udder health monitoring by veterinarian	No vs. yes	NA	Sampimon et al., 2009a

[1]Odds ratio. All *P*-values are < 0.05. [2]Only heifers were included. [3]If > 2 categories: the significant category is represented in bold. [4]Not available.

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Chapter 2

Aims and Outline of the Thesis

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The *general aim* of this thesis was to broaden our knowledge of the ecological nature and the epidemiological behavior of bovine-related coagulase-negative *Staphylococcus* species (CNS). We also aimed to obtain better insights in the significance for udder health of the different species belonging to this heterogeneous group of bacteria.

The *specific aims* of this thesis were:

- To evaluate mannitol salt agar as a convenient medium for bovine CNS recovery, i.e. being able to circumvent overgrowth and hampered CNS isolation from overcrowded habitats, such as teat apices and bulk milk (**Chapter 3**).
- To describe the prevalence and the distribution of bovine-related CNS species in different habitats, i.e.:
  - teat apices of lactating dairy cows and heifers and extramammary habitats close to the udder (**Chapter 4.1**)
  - teat apices of non-lactating dairy cows and end-term heifers prior to calving (**Chapter 4.2**)
  - quarter milk samples of fresh dairy cows and heifers (**Chapter 5.1**)
  - bulk milk samples (**Chapter 6**).
- To identify risk factors associated with the presence of bovine-related CNS species in different habitats,i.e.:
  - teat apices of dry dairy cows and end-term heifers prior to calving (**Chapter 4.2**)
  - quarter milk samples of fresh dairy cows and heifers (**Chapter 5.1**)
  - quarter milk samples of lactating dairy cows and heifers (**Chapter 5.2**)
  - bulk milk samples (**Chapter 6**).
- To examine the impact of different bovine-related CNS species on udder health, i.e.:
  - the effect on the quarter SCC in early lactation (**Chapter 5.1**)
  - the effect on the quarter SCC throughout lactation (**Chapter 5.2**).

Chapter 3

Assessment of the Suitability of Mannitol Salt Agar for Growing Bovine-Associated Coagulase-Negative Staphylococci and its Use under Field Conditions

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Abstract

This study aimed at testing the applicability of mannitol salt agar (MSA), a medium generally used in human medicine for differentiating *Staphylococcus aureus* from coagulase-negative staphylococci (CNS), for culturing bovine-associated CNS species. All test isolates from a comprehensive collection of well-identified CNS species, including both reference strains and field isolates, were able to grow. Subsequently, bulk milk samples and teat apex swabs were used to examine the capability of MSA for yielding CNS under field conditions. Sixty-nine and 47 phenotypically different colonies were retrieved from bulk milk and teat apices, respectively. The majority of isolates from teat apices were staphylococci, whereas in bulk milk, staphylococci formed a minority. After 24h of growth, recovery of separate colonies of CNS was much more convenient on MSA compared to a non-selective blood agar. The results of this study indicate that MSA is a suitable medium for both growth and recovery of bovine-associated CNS.

Key words

Coagulase-negative *Staphylococcus* species, CNS, Mannitol salt agar, Dairy cows, Bulk milk

Introduction

In many countries, coagulase-negative staphylococci (CNS) have become the most common cause of subclinical mastitis in dairy cows (e.g. Piepers et al., 2007; Sampimon et al., 2009a; Schukken et al., 2009). In addition, CNS are abundantly present both in the cows' environment (Taponen et al., 2008; Piessens et al., 2011) and on their teat apices (De Vliegher et al., 2003; Braem et al., 2013). Increasing evidence exists that the different CNS species vary in virulence and epidemiological behavior (Taponen et al., 2006; Park et al., 2011; Piessens et al., 2011, 2012a; Sampimon et al., 2011). More research is needed to understand the herd-specific distribution of CNS species, to identify species-specific infection sources and transmission routes and to picture differences in virulence characteristics (Pyöräla and Taponen, 2009; Supré et al., 2011).

Current microbiological techniques in mastitis research mostly rely on differential non-selective (blood) isolation media, also for recovery of CNS (Rajala-Schultz et al., 2009; Persson Waller et al., 2011). Studies focusing on CNS might benefit from adequate selective isolation media as a range of bacteria other than CNS could be cultured from different bovine-related niches (Piessens et al., 2011; Braem et al., 2013). Recent studies have made use of mannitol salt agar (MSA) to grow CNS (Piessens et al., 2011; Quirk et al., 2012; Braem et al., 2013), although it has not been shown that all bovine-associated CNS grow on this medium. MSA was developed in 1945 (Chapman, 1945). Most bacteria other than staphylococci are not able to grow on this agar due to the high sodium chloride concentration (7.5%) (Chapman, 1945; Finegold and Sweeney, 1961; Shittu et al., 2006). Currently, MSA is commercially available and recommended for the recovery of staphylococci as the mannitol fermentation offers advantages in the differentiation of *Staphylococcus aureus* and CNS species (Kateete et al.; 2010). Mannitol salt agar has so far mainly been used for selective growth of human *S. aureus* and human CNS (Shittu et al., 2004, 2006; Han et al., 2007), but also to follow desirable growth of CNS during meat fermentations (Janssens et al., 2012). Obviously, a prerequisite for any kind of selective medium is that the ability to grow the desired species is proven both from storage (e.g. -80°C) and from the source material. As phenotypic identification techniques developed for human CNS isolates have been shown to lack accuracy for bovine- (Sampimon et al., 2009b; Zadoks and Watts, 2009) and caprine- (Koop et

al., 2012) associated CNS, differences in growth capabilities between human and the most common bovine-associated CNS on MSA should be anticipated. This study aimed at evaluating the growth capabilities of the most common CNS species, using both reference strains and field isolates, originating from dairy cows and their environment on MSA. In addition, the applicability of MSA for routine recovery of CNS species was assessed, using bulk milk samples and swabs of teat apices.

Materials and methods

Assessment of growth of CNS species on MSA

In a first experiment, the 25 most frequently isolated CNS species from bovine intra- and extra-mammary sites were assembled (Table 1). Of each species, both a reference strain and a field isolate were included (n_{total} = 50). All field isolates were previously described in published studies and were identified to the species level using either transfer RNA intergenic spacer PCR (tDNA-PCR) and/or *rpoB* sequencing (n = 18) (Supré et al., 2009, 2011 and unpublished data), amplified fragment length polymorphism (AFLP) genotyping (n = 5) (Piessens et al., 2010, 2011), $(GTG)_5$-PCR fingerprinting (n = 1) (Braem et al., 2011, 2013) or *rpoB*, *tuf* and *16S rRNA* sequencing (n = 1) (Taponen et al., 2006, 2012). All 50 isolates were stored at -80°C in either Microbanks (Microbank^{TM}, Pro-lab diagnostics, Bromborough, UK) or brain heart infusion with 15% (w/v) glycerol (Oxoid, Basingstoke, UK). For performing of the test, the isolates were plated directly (one quadrant per isolate) on MSA (Chapman medium, Oxoid) and on Columbia agar with sheep blood (Oxoid), the latter being used as a non-selective medium. Plates were aerobically incubated at 37°C. Growth was examined after 24h and 48h, after which incubation for another 24h at room temperature was initiated. If no growth could be detected on MSA in this first attempt, the procedure was repeated on both agars.

Subsequently, in a second experiment, the same procedure was performed for 10 isolates of the six and four CNS species that have most frequently been isolated at our laboratory from milk and teat apices, respectively (n_{total} = 100) (Table 2). All these isolates were previously collected as part of different field studies (Piessens et al., 2011; Supré et al., 2011; Braem et al., 2013) and were identified to the species level using tDNA-PCR (all *Staphylococcus chromogenes, S. cohnii, S. equorum, S. haemolyticus, S. simulans* and *S. xylosus*), AFLP genotyping (all *S. epidermidis*) or

$(GTG)_5$-PCR fingerprinting (all *S. saprophyticus*). All 100 isolates were stored at -80 °C in either Microbanks or brain heart infusion with 15% (w/v) glycerol. The test was performed as described above. If no growth could be detected on MSA the procedure was repeated once on both agars.

Suitability of MSA for CNS recovery in field applications

A bulk milk sample was collected on 20 randomly selected Flemish dairy herds by the Milk Control Centre (MCC, Lier, Flanders). Each milk sample (10 µl) was plated on MSA, and Columbia agar with sheep blood (Oxoid). The resulting 40 plates were aerobically incubated at 37°C and read after 24h and 48h. All colonies were phenotypically assessed by size, shape, smoothness, opacity, color, butyrous consistency, mannitol fermentation, hemolysis and lustre of the colonies. From all colony types represented by at least two colonies, one colony was picked up and subcultured on esculin blood agar (Oxoid) (one quadrant per isolate) to obtain pure cultures. All purified isolates were subjected to a catalase test and all catalase-positive isolates were examined using Gram-staining. Gram-positive cocci were then subjected in parallel to DNase and coagulase tests. For all isolates that were DNase-positive and coagulase-positive, mannitol fermentation was tested and a polymyxin test was performed, in order to further distinguish *S. aureus* (coagulase-positive, DNase-positive, mannitol salt-positive and polymyxin-resistant) and non-*S. aureus* staphylococci (coagulase-negative and/or DNase-negative, mannitol salt-positive or -negative and polymyxin-sensitive) (Finegold and Sweeney, 1961; Hogan et al., 1999; Kateete et al., 2010). All coagulase- or DNase-negative bacteria were considered to be non-*S. aureus* staphylococci and were further referred to as CNS, despite a minority being coagulase-positive. CNS were identified to the species level using tDNA-PCR followed by capillary electrophoresis as described by Supré et al. (2009). If no identification could be obtained, the isolates were subjected to sequencing of the *rpoB* gene. Sequencing of the *16S rRNA* gene was performed if amplification of the *rpoB* gene failed.

In addition, swabs were collected from teat apices of three dry cows and two heifers one month before parturition as described by De Vliegher et al. (2003). All 20 swabs were plated on MSA and Columbia agar with sheep blood (half a plate per swab). Plates were examined and isolates were subjected to further analysis as described above.

Results

Assessment of growth of CNS species on MSA

Results of the growth assessment experiments are shown in Tables 1 and 2. In the first experiment, only the *S. equorum* reference strain and the *S. hominis* and *S. lugdunensis* field isolates showed delayed growth (after 24h) on MSA in comparison with Columbia agar with sheep blood on which growth was detected within 24h. The *S. devriesei* reference strain grew within 24h on MSA but only after a second attempt. In contrast, on Columbia agar with sheep blood, growth was detected within 24h after the first attempt. In the second experiment, only a single *S. chromogenes* isolate and two *S. haemolyticus* isolates originating from milk showed delayed growth (after 24h) in comparison with Columbia agar with sheep blood (within 24h). A single *S. xylosus* isolate from milk grew within 24h on MSA but only after a second attempt. On Columbia agar with sheep blood, that isolate grew within 24h after the first attempt. Among the isolates originating from teat apices, only a single *S. haemolyticus* isolate failed to grow on MSA, even when the test was repeated. However, when that *S. haemolyticus* isolate was picked up from Columbia agar with sheep blood after 24h incubation, and then plated on MSA, growth was also detected on MSA within 24h.

Suitability of MSA for CNS recovery in field applications

Concerning the bulk milk samples, no further analysis was done of colonies growing on Columbia agar with sheep blood, as colonies could not be conveniently picked up, due to overgrowth or a too dense configuration after 24h incubation. On all MSA plates, in total 69 phenotypically different colony types were recovered after 48h. From all these types at least two representative colonies were apparent. Per plate, one to five different types were present.

Table 1. Origin and growth of reference strains and field isolates, respectively, of 25 coagulase-negative *Staphylococcus* species frequently isolated from cows' milk or environment, on mannitol salt agar (aerobic incubation at 37°C)

CNS species	Reference strain			Field isolate		
	Origin	Strain number[1]	Growth	Origin	Strain number[2]	Growth
S. agnetis	Bovine milk	CCUG 59809[T]	24h	Bovine milk	ST 1	24h
S. arlettae	Poultry skin	LMG 19114[T]	24h	Sawdust	VP 0428	24h
S. auricularis	Human ear	ATCC 33753[T]	24h	Milker's hand	KS 536	24h
S. capitis	Human skin	ATCC 49326[T]	24h	Milker's hand	KS 547	24h
S. chromogenes	Bovine skin	NCTC 10530[T]	24h	Bovine teat apex	KS 81	24h
S. cohnii	Human skin	DSM 20260[T]	24h	Bovine teat apex	KS 567	24h
S. devriesei	Bovine teat apex	CIP 110234[T]	24h[3]	Teat cup	KS 447	24h
S. epidermidis	Human nose	LMG 10474[T]	24h	Milker's hand	KS 414	24h
S. equorum	Surface of cheese	DSM 15097 [T]	24h-48h	Teat cup	KS 402	24h
S. fleurettii	Caprine cheese	CCM 4922[T]	24h	Teat cup	KS 339	24h
S. gallinarum	Poultry skin	CCM 3572[T]	24h	Stable floor	VP 0303	24h
S. haemolyticus	Human skin	CCM 2737[T]	24h	Bovine teat apex	KS 516	24h
S. hominis	Human skin	CCM 2732	24h	Teat cup	KS 338	24h-48h
S. hyicus	Porcine skin	NCTC 10350[T]	24h	Bovine milk	KS 167	24h
S. kloossii	Skin squirrel	DSM 20676 [T]	24h	Bovine teat apex	GB G057	24h
S. lentus	Caprine udder	ATCC 29070[T]	24h	Stable floor	VP 273	24h
S. lugdunensis	Human axillary lymph node	ATCC 43809[T]	24h	Teat cup	KS 699	24h-48h
S. nepalensis	Caprine nasal mucosa	DSM 15150 [T]	24h	Milker's hand	KS 774	24h
S. pasteuri	Human vomit	ATCC 51129[T]	24h	Bovine milk	KS 219	24h
S. sciuri	Skin eastern gray squirrel	ATCC 29062[T]	24h	Teat cup	KS 491	24h
S. simulans	Human skin	CCM 2705[T]	24h	Bovine teat apex	KS 129	24h
S. succinus	Dominican amber	LMG 22185[T]	24h	Stable floor	VP 613	24h
S. vitulinus	Ground lamb	CCM 4511[T]	24h	Stable floor	VP 105	24h
S. warneri	Human skin	ATCC 27836[T]	24h	Milker's hand	KS 412	24h
S. xylosus	Human skin	CNRS N850267	24h	Bovine milk	KS 13	24h

[1]CCUG = Culture Collection, University of Göteborg, Göteborg, Sweden; LMG = Laboratory of Microbiology, Ghent University, Ghent, Belgium; ATCC = American Type Culture Collection, Rockville, MD, USA; NCTC = National Collection of Type Cultures, London, UK; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Weringerode, Germany; CIP = Collection de l' Institut Pasteur, Paris, France; CCM = Czech Collection of Microorganisms, Prague, Czech Republic; CNRS = Centre National de Référence des Staphylocoques, Lyon, France; [T] = type strain. [2]ST = Taponen et al., 2006; VP = Piessens et al., 2011; KS = Supré et al., 2011 (bovine milk) and Supré et al., unpublished results (milker's hand, bovine teat apex, teat cup); GB = Braem et al., 2013. [3]Growth within 24h (37°C) at second attempt.

Only 20 of the 69 colony types were identified as CNS: *S. equorum* (n = 8), *S. sciuri* (n = 3), *S. haemolyticus* (n = 2), *S. epidermidis* (n = 2), single isolates of *S. capitis*, *S. fleurettii*, *S. saprophyticus*, *S. warneri* and one unidentifiable *Staphylococcus* sp.. Bacteria other than CNS were identified as esculin-positive streptococci (n = 23), *Bacillus* spp. (n = 8), *Corynebacterium* spp. (n = 8) (identified using *16S rRNA*), *S. aureus* (n = 5), *Jeotgallicoccus psychrophilus* (n = 1) (identified using *16S rRNA*) and four unidentifiable isolates.

Concerning the samples from teat apices, colonies from 16 Columbia agar with sheep blood plates could not be conveniently picked up, due to overgrowth or a too dense configuration after 24h. On one Columbia agar with sheep blood plate, no growth could be observed after 48h. On three out of 20 Columbia agar with sheep blood plates, separate colonies could be picked up. However, these were not included for further analysis due to the lack of complete data for Columbia agar with sheep blood plates. On all MSA plates, in total 47 phenotypically different colony types could be recovered after 48h. From all these types at least two representative colonies were apparent. Per plate, one to three different types were present. CNS represented the majority (n = 34) of these colony types: *S. chromogenes* (n = 9), *S. fleurettii* (n = 8), *S. haemolyticus* (n = 7), *S. equorum* (n = 6), single isolates of *S. capitis* and *S. caseolyticus* (reclassified as *Macrococcus caseolyticus*, Kloos et al., 1998) and two unidentifiable *Staphylococcus* spp.. Bacteria other than CNS were identified as *Bacillus* spp. (n = 9), esculin-positive streptococci (n = 3) and *Corynebacterium* sp. (n = 1) (identified using *16S rRNA*).

Discussion

MSA has mainly been used for the selective isolation of human-associated staphylococci. While some previous studies have illustrated the suitability of MSA as a selective medium for non-human CNS isolates (Cheng et al., 2010; Addis et al., 2011), our study is the first to test a large representative and diverse collection of reliably identified bovine-associated CNS species.

Our results show that MSA is a highly suitable medium for the growth and recovery of CNS originating from cows and their environment. Considering that growth could not be detected for all isolates within 24h, aerobic incubation at 37°C for 48h is recommended. With CNS being increasingly recognized as relevant

bacteria for udder health and recent efforts directed at unraveling the species- and strain-specific epidemiology, these results are highly valuable for future studies.

Table 2. Origin and growth of the six and four most frequently isolated coagulase-negative *Staphylococcus* species from milk and teat apices, respectively, on mannitol salt agar (aerobic incubation at 37°C)

CNS species and	Different isolates									
	1	2	3	4	5	6	7	8	9	10
Milk										
S. chromogenes	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h-48h
S. cohnii	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h
S. epidermidis	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h
S. haemolyticus	24h	24h	24h	24h	24h	24h-48h	24h-48h	24h	24h	24h
S. simulans	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h
S. xylosus	24h	24h	24h	24h	24h	24h	24h	24h[1]	24h	24h
Teat apices										
S. cohnii	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h
S. equorum	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h
S. haemolyticus	-[2]	24h	24h	24h	24h	24h	24h	24h	24h	24h
S. saprophyticus	24h	24h	24h	24h	24h	24h	24h	24h	24h	24h

[1]Growth within 24h (37°C) at second attempt. [2]No growth.

The delayed growth of both the reference strain of *S. equorum* and a field isolate of *S. hominis* corresponds well with the earlier described poor growth of different isolates of these species originating from horses and humans, respectively (Kloos and Schleifer, 1975; Schleifer et al., 1984). However, caution is needed in comparing the results as different media were used. As the human reference strain (in this trial) and different human isolates of *S. lugdunensis* showed good growth within 24h incubation on 10% NaCl agars (Freney et al., 1988), the slow growth of the single bovine-associated field isolate of this species was rather unexpected. It is unclear whether this discrepancy can also be attributed to host species-related differences. The failure of one of the tested *S. xylosus* isolates and the reference strain of *S. devriesei* to grow at the first attempt was most probably due to coincidence. Indeed, in a previous study *S. xylosus* was shown to grow even on media containing up to 10% NaCl (Schleifer and Kloos, 1975). Still, it must be noted that these isolates originated from humans. The failure to grow of a single *S. haemolyticus* isolate

originating from a teat apex should not be a reason for concern, as all other isolates grew very well and among bovine-associated *S. haemolyticus* isolates, a high genetic diversity has been observed (Piessens et al., 2012b).

In the field application study, analysis was performed on one representative colony per phenotypically distinguished colony type. The possibility exists that in this way colonies with similar phenotypic characteristics actually were different CNS species, leading to an underestimation of the true CNS diversity in the samples. Therefore, not having investigated all grown colonies is a limitation of the current study. However, as the purpose was to test the practical use and applicability of MSA for recovery of the most common bovine-associated CNS species, picking up all present colonies was not feasible. Obviously, having to do so would also be highly unfeasible in routine practice or in future research studying CNS in bulk milk, on teat apices, or other niches. Yet, in future work this inconvenience could be overcome at least partially by repeated sampling of the niche being studied. On the other hand, it was observed that different colony types sometimes belonged to the same species. Also, differences in biochemical characteristics (e.g. mannitol fermentation) between the reference strain and field isolates and among field isolates of the same species (see Supplementary Table S1), were noted, suggesting strain differences within species. Another drawback of our study is the lack of a "gold standard" reference medium in the field application study. This means we are not able to conclude that MSA grew the full range of CNS species potentially present in the samples. In this study, Columbia agar with sheep blood, a medium frequently used in daily practice, was mainly included to show the applicability and advantages of MSA in field applications compared to this non-selective medium. Columbia agar was not intended to be the "gold standard" and could not be used as such due to overgrowth. The use of serial dilutions as was done by Jayarao et al. (2004) could have been a partial solution, but this holds the risk that species that are present in very low numbers are disregarded through such an approach. Overall, it must be acknowledged that a straightforward method for detecting all species present in any kind of sample is still lacking. This might be a relevant topic for future research. Nonetheless, our results illustrate that MSA is far better suited for detection of bovine-associated CNS.

It was not surprising that bacteria other than staphylococci grew on MSA in the performed studies as also in previous studies growth of *Corynebacterium* spp.

(Braem et al., 2013), esculin-positive streptococci (Chapman, 1945; Braem et al., 2013) and *Bacillus* spp. (Braem et al., 2013; Han et al., 2007) has been observed. Yet, this appeared not to be an inconvenience for the isolation of separate CNS colonies and should therefore not hamper the use of MSA as an isolation medium for bovine-associated CNS.

Conclusions

The most common CNS species from dairy cows and dairy cows' environment were illustrated to grow well on MSA. As phenotypically different colonies on MSA could be picked up easily, MSA clearly offers advantages in comparison with non-selective media. This study shows that MSA is a practical and appropriate medium to further unravel the epidemiology and behavior of the different bovine-associated CNS species from different intra- and extra-mammary niches.

Appendix A. supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.rvsc.2013.05.015.

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Chapter 4

Teat Apex Colonization with Coagulase-Negative *Staphylococcus* Species throughout Lactation and before Parturition

Chapter 4.1

Further Evidence for the Existence of Environmental and Host-Associated Species of Coagulase-Negative Staphylococci in Dairy Cattle

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Abstract

Coagulase-negative staphylococci (CNS) are abundantly present in the dairy farm environment and on bovine skin and mucosae. They are also the most prevalent bacteria causing bovine intramammary infections (IMI). Reservoirs and transmission routes of CNS are not yet fully unraveled. The objectives of this study were to explore the distribution of CNS in parlor-related extramammary niches and to compare it to the distributions of CNS causing IMI in those herds. Niches that were targeted in this study were cows' teat apices, milking machine unit liners, and milker's skin or gloves. Each of the three herds had its own CNS microbiota in those niches. The most prevalent species in the parlor-related extramammary niches were *Staphylococcus cohnii*, *S. fleurettii*, and *S. equorum* in the first, second, and third herd, respectively, whereas *S. haemolyticus* and *S. sciuri* were found in all herds. *Staphylococcus cohnii* and *S. fleurettii*, as well as *S. haemolyticus,* which was present in each herd, were also frequently found in milk samples. By contrast, *S. chromogenes*, *S. simulans*, and *S. xylosus* favored the mammary gland, whereas *S. equorum* was more common in the parlor-associated niches. Within each herd, species distribution was similar between teat apices and milking machine unit liners. In conclusion, some of the extramammary niches related to the milking process might act as infection sources for IMI-causing CNS. This study provides further evidence that the group of CNS species is comprised of environmental, opportunistic and host-adapted species which differ in ecology.

Key words

Coagulase-negative *Staphylococcus* species, Dairy cow, Intramammary infection, Parlor-associated extramammary niche

Introduction

Interest in coagulase-negative staphylococci (CNS) increased over the past years both in human (Rasmussen et al., 2000; Dekio et al., 2005) and in veterinary medicine (Taponen et al., 2007; Gillespie et al., 2009). Effort is ongoing to unravel the characteristics of CNS as a group (Sampimon et al., 2009; Piepers et al., 2010), and the specific characteristics of the different species (Simojoki et al., 2011; Supré et al., 2011). Recently, we reported that several CNS species were abundantly present in the cows' environment (air, slatted floor, and sawdust), but rarely associated with intramammary infections (IMI) (Piessens et al., 2011). These "environmental CNS" include *Staphylococcus equorum*, *S. fleurettii*, and *S. sciuri.* Other species, such as *S. haemolyticus* and *S. simulans*, were commonly found in the barn environment and were also isolated from quarters with IMI. These species are considered to be "opportunistic pathogens" (Piessens et al., 2011). *Staphylococcus chromogenes* was commonly associated with IMI, but rarely found in the barn environment, implying that other reservoirs potentially play a role in its epidemiology (Piessens et al., 2011). *Staphylococcus cohnii* and *S. xylosus* are also frequently isolated from infected quarters in some herds (Supré et al., 2011), but their sources are as yet unidentified.

The udder contains a natural barrier mechanism to separate and protect the mammary gland from the environment. Potential extramammary sources of infection that have not been examined in previous studies include the cows' teat apices (TA), the milking machine unit liners (MMUL) and milkers' skin. Previous work suggests that the presence of *Staphylococcus aureus* on teat skin can be associated with the risk of infection in early lactation (Roberson et al., 1994), while milking machine unit liners also play a role in *S. aureus* transmission (Zadoks et al., 2002). Milkers' skin can carry *S. epidermidis*, which may cause IMI in cows (Thorberg et al., 2006). By contrast, presence of CNS (Piepers et al., 2011) and *S. chromogenes* (De Vliegher et al., 2003) on TA pre-partum was not associated with IMI (Piepers et al., 2011) and even associated with a decreased risk of *S. chromogenes* IMI in heifers in early lactation (De Vliegher et al., 2003). However, little is known about the distribution of CNS species on TA, MMUL, and milkers' skin or gloves (MSG) and their possible role as source of CNS IMI.

The aims of this study were (1) to map the distribution of the parlor-associated CNS species, based on a molecular identification technique, (2) to compare the distribution of parlor-associated species and CNS species causing IMI in these herds, and (3) to explore whether TA, MMUL, and the milker could play a role in CNS transmission in these herds.

Material and methods

Herds, animals, and teat apices

A single cross-sectional sampling was performed in 3 commercial Flemish dairy herds in February 2008. The herds were part of a longitudinal study (starting in September 2007, ending in January 2009) to determine the impact of CNS species-specific IMI on quarter milk somatic cell count (Supré et al., 2011). Herd characteristics and udder health practices are described in Table 1.

Per herd, 25 animals were included in the longitudinal study on IMI and 6 of the 25 animals were randomly selected for the current study, with stratification by parity, i.e. 2 animals in their first, second, or higher lactation, respectively. Teat apices (n = 72) were sampled both pre- and post-milking, with the exception of one animal in herd 3 that was only sampled pre-milking (n_{TA} = 140). Sterile cotton swabs were used (Copan Diagnostics Inc., CA, USA). The sampling protocol was as follows: cleansing of the teat with an individual dry paper cloth, pre-milking swabbing of the teat apex, disinfection of the teats with alcohol, attachment of the milking cluster, milking, removal of the milking cluster, post-milking swabbing of the teat apex, and finally post-milking teat dipping. Contact with milk was avoided during swabbing. After sampling, swabs were placed in sterile tubes and immediately stored at 4°C.

Extramammary niches

All MMUL (40 on farms 1 and 2, 48 on farm 3) (n = 128) were sampled before the milking session started and, with the exception of one unit on farm 3, again after milking of all cows was finished, prior to cleaning of the milking machine (n_{MMUL} = 252). The inside of all liners was sampled by rubbing and turning the swab and again contact with milk was avoided.

Table 1. Overview of herd characteristics and udder health management practices of the three participating herds

	Herd 1	Herd 2	Herd 3
Herd characteristics[1]			
Number of lactating cows	43.5 (39 - 48)[2]	48.9 (43 - 53)	65.7 (61 - 70)
Mean milk yield (kg/day)	30.9 (28.5 - 32.4)	32.3 (30.1 - 34.3)	31 (29.4 - 31.8)
Mean 305d-yield (in kg)	9,921.6 (9,551 - 10,189)	10,194.7 (9,975 - 10,396)	10,180.2 (9,978 - 10,367)
Herd SCC (x 1000 cells/mL)[3]	152.9 (98 - 220)	195.9 (133 - 323)	186.7 (103 - 298)
Udder health management			
Gloves during milking	1 of 2 milkers	All milkers	1 of 2 milkers
Iodine post-milking teat desinfection	Dip	Dip	Spray
Dry-off management			
Long-acting antimicrobial	Cloxacillin	Cefquinome	Nafcillin / penicillin / streptomycin
Internal teat sealer	Yes	Yes	Yes
Routine culling of chronically infected cows	No	Yes	No
Calving pen			
Separate straw box	Yes	Yes	Yes
Used for sick cows	Yes	No	Yes
Bedding material	Sawdust	Sawdust	Sawdust
Cleaning of slatted floor	2x per day	Not regularly	2x per day

[1]Measured through the Dairy Herd Improvement (DHI) program. [2](Minimum - maximum). [3]Average of the monthly herd somatic cell count (SCC), measured through the DHI program.

Gloves or bare hands of the milkers (2 on farms 1 and 3, 1 on farm 2) and the bends of their elbows were sampled both before and after milking, after wiping them with a dry paper cloth as described before (Thorberg et al., 2006). Thus, the group of MSG samples comprised of 20 samples from hands or gloves and 20 samples from elbows (n_{MSG} = 40).

Identification of CNS species

All swabs were streaked onto a Columbia agar with 5 % ovine blood (Oxoid, Basingstoke, UK). After aerobic incubation at 37 °C for 24h, plates were examined. CNS were presumptively identified following NMC procedures (Hogan et al., 1999). All colonies per morphotype were counted. One colony was picked up from every

morphotype with ≥ 5 colonies, allowing for isolation of more than one morphotype per swab, and was streaked onto Columbia blood agar (Oxoid) to obtain pure cultures. After 18 - 24h aerobic incubation at 37°C, individual colonies were checked for purity and stored at -80°C until further analysis. DNA was prepared according to Unal et al. (1992). All CNS were identified to the species level using transfer RNA-intergenic spacer PCR (tDNA-PCR) (Supré et al., 2009). If no identification could be obtained, isolates were subjected to sequencing of the *rpo*B-housekeeping gene as described before (Supré et al., 2009). Results were recorded as presence or absence of a species in a sample, regardless of the number of times this particular species was isolated in that sample.

Descriptive and statistical analysis

The distribution of each identified CNS species in the parlor-associated extramammary niches was described and counted within each herd. The distribution of CNS species in the extramammary niches and the distribution of CNS species from IMI in the same herds, based on culture results from 3 consecutive samplings, were displayed next to each other (Supré et al., 2011). The latter was done to explore whether the parlor-associated extramammary niches could act as infection source or fomite for IMI in these herds.

McNemar's Tests were performed in SPSS (SPSS Statistics 22, SPSS Inc., Chicago, IL, USA) to determine associations between presence of CNS before and after milking from TA and in MMUL, respectively. Associations between presence of CNS on hands or gloves and in elbow bends were checked using a Fisher Exact Test (SPSS Statistics 22, SPSS Inc., Chicago, IL, USA).

Results and discussion

In previous studies, the distribution of CNS species in milk samples and the barn environment had been described (Piessens et al., 2011; Supré et al., 2011), but potential niches of CNS in the milking parlor, which plays a crucial role in mastitis epidemiology and control, had not been explored in depth. In this study, we focused on niches related to the milking process in order to broaden our knowledge of the epidemiology of CNS species.

For precise identification of CNS species, we used a molecular identification technique, tRNA-intergenic spacer PCR (Supré et al., 2009). This method showed high typeability for CNS from extramammary sites in this study. Only 1.4% of 288 CNS, isolated from TA, in MMUL, and on MSG, could not be identified to the species level using this method. The remaining isolates were subjected to sequencing of the *rpoB* gene. To our knowledge, only one other molecular study compared CNS isolated from parlor-associated niches and from infected quarters (Taponen et al., 2008).

In total, 432 extramammary samples were collected. At least one CNS species was found in 46.8% of those samples and 19 different CNS species were isolated in this cross-sectional study. The most common species were *S. cohnii* (23.3% of all extramammary CNS isolates), *S. haemolyticus* (17.4%), *S. equorum* (16.0%), *S. fleurettii* (15.3%) and *S. sciuri* (10.4%). The three herds had distinct extramammary microbiota (Figure 1A). *Staphylococcus cohnii* was the dominant species in herd 1 (46.8% of all extramammary CNS isolates in that herd), *S. fleurettii* in herd 2 (68.3% of all extramammary CNS isolates in that herd), and *S. equorum* in herd 3 (47.6% of all extramammary CNS isolates in that herd). Herd-specific species distribution was also described for CNS isolated from sawdust, floors, and air, in particular for *S. fleurettii* but not for *S. cohnii* and *S. equorum* as those species were isolated in all herds in that study (Piessens et al., 2011). In contrast, *S. haemolyticus* and *S. sciuri* were present in the extramammary niches in all herds in the present study (Figure 1), which corresponds well with the findings for those species in environmental samples in the study of Piessens et al. (2011).

High numbers of CNS isolates were recovered from teat apices. Of the 140 TA samples 59.3% were CNS-positive. All cows had at least one TA that harbored CNS. The number of different species isolated per TA ranged from 1 to 4, while up to 6 were found per cow. *Staphylococcus cohnii* was found in high proportion on TA in herd 1 (50.0% of the TA isolates in that herd) but it was absent from TA in the other herds (Figure 1). *Staphylococcus fleurettii* was frequently and only isolated from TA in herd 2 (72.4% of the TA isolates in that herd). *Staphylococcus equorum* was commonly found in herd 3 (50.0% of the TA isolates in that herd). *Staphylococcus haemolyticus* and *S. sciuri* were the only species isolated from TA in all herds (Figure 1).

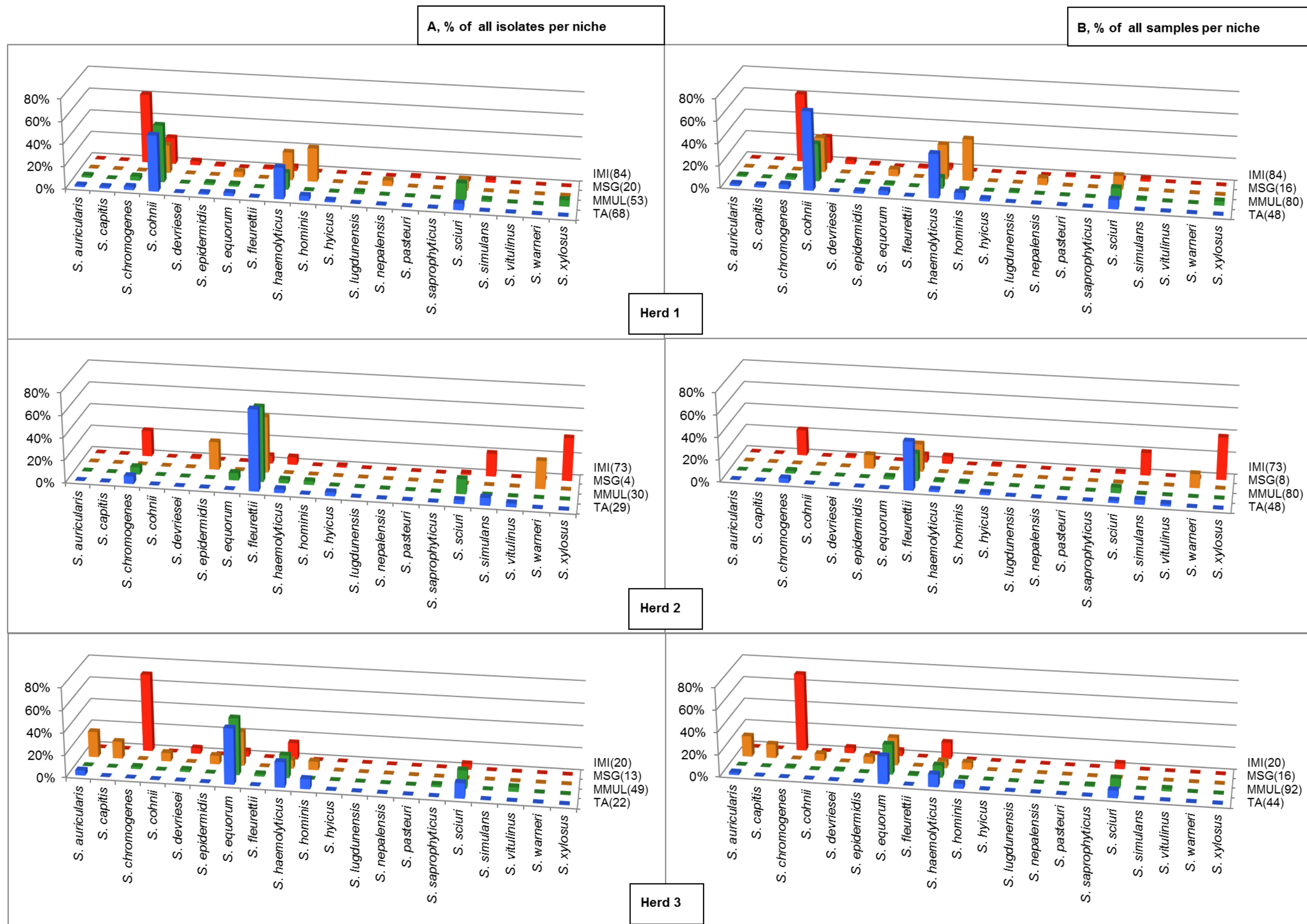
A, % of all isolates per niche
B, % of all samples per niche
Herd 1
Herd 2
Herd 3
80%
60%
40%
20%
0%
S. auricularis
S. capitis
S. chromogenes
S. cohnii
S. devriesei
S. epidermidis
S. equorum
S. fleurettii
S. haemolyticus
S. hominis
S. hyicus
S. lugdunensis
S. nepalensis
S. pasteuri
S. saprophyticus
S. sciuri
S. simulans
S. vitulinus
S. warneri
S. xylosus
IMI(84)
MSG(20)
MMUL(53)
TA(68)
IMI(84)
MSG(16)
MMUL(80)
TA(48)
IMI(73)
MSG(4)
MMUL(30)
TA(29)
IMI(73)
MSG(8)
MMUL(80)
TA(48)
IMI(20)
MSG(13)
MMUL(49)
TA(22)
IMI(20)
MSG(16)
MMUL(92)
TA(44)

Figure 1. Distribution of the coagulase-negative *Staphylococcus* species in 3 Flemish dairy herds in parlor-associated extramammary niches [teat apices (TA), blue; milking machine unit liners (MMUL), green; milkers' skin and gloves (MSG), orange] and intramammary infections (IMI), red, expressed as a percentage of isolates relatively to the total number of isolates within the respective niche (A) (total sum of percentages is 100) and as a percentage of samples positive for a specific CNS species relatively to the total number of collected samples (i.e. including negative samples, total sum of percentages > or < 100 as one teat apex can harbor more different species) within the respective niche (B). Total number of isolates or samples per source per herd is shown in brackets.

Other species found on TA were *S. auricularis*, *S. chromogenes*, *S. hominis*, *S. hyicus*, and *S. simulans*. *Staphylococcus capitis*, *S. epidermidis* and *S. vitulinus* were only found in a small proportion of TA samples (Figure 1). Presence of CNS species on TA may be the result of colonization of the skin, of contamination with environmental CNS such as those from bedding material, or of exposure of teats to milk during milking. *Staphylococcus xylosus*, previously considered as a typical teat skin colonizer (Devriese and de Keyser, 1980) was not isolated from TA in our study (Figure 1B) nor isolated from TA by Braem et al. (2013). *Staphylococcus simulans* and *S. sciuri* were frequently isolated from sawdust and floors on Flemish dairy farms (Piessens et al., 2011) and were expected to be present on TA due to close contact with sawdust and floors. Like *S. xylosus*, however, *S. simulans* was not very common in the current study, whereas *S. sciuri* was present on TA in all herds. The differences between studies may be due to the fact that microbiota are herd-specific. The relationship between detection of CNS on TA and in milk is a "chicken-and-egg" relationship. Milk can act as a source of CNS for teats during milking and the teat microbiota can serve as a source of CNS that cause IMI. In herd 1, *S. cohnii* was common on TA and in IMI. Other species, e.g. *S. haemolyticus* in herd 1, *S. fleurettii* in herd 2 and *S. equorum* in herd 3 were far more common on TA than in milk, implying that milk is unlikely to have been the source of TA contamination during milking. Rather, these species may be natural colonizers of teat skin, as observed for *S. xylosus* in other studies (Devriese and de Keyser, 1980; Taponen et al., 2008). In addition, the likelihood of presence of CNS on teat skin was significantly lower after milking than before milking ($P < 0.001$) (Table 2), suggesting that CNS are washed

away from TA rather than deposited on TA during milking. Finally, *S. chromogenes*, the most frequently isolated species from infected quarters, was only occasionally isolated from TA (2.9% of all TA swabs), providing further support to the notion that milk is not a frequent source of teat skin contamination. *Staphylococcus chromogenes* could not be isolated from teat apices by Braem et al. (2013) either. In other studies, isolation of *S. chromogenes* from teat apices or udder skin was more common than in the current study (De Vliegher et al., 2003; Taponen et al. 2008; Bexiga et al., 2014). Again, herd differences in TA colonization by different CNS species may explain the discrepancies between studies.

Of the 252 MMUL samples, 36.5% were found CNS-positive. In herd 1, 46.3% of the MMUL were CNS-positive, 31.3% in herd 2 and 32.6% in herd 3. An efficient cleaning procedure of the milking machine in between milkings was evidenced by the absence of CNS in all but one pre-milking MMUL swabs (Table 2), from which *S. xylosus* was recovered. Taponen et al. (2008) reported a low number of CNS-positive samples after washing of the MMUL as well. After the milking process, a significantly higher proportion of MMUL (73.4%) were CNS-positive (Table 2). Within each herd, the CNS species distribution in MMUL samples was very similar to the distribution in TA samples (Figure 1A). The latter finding can be explained by direct contact between teat skin and liners. For example, as *S. equorum* and *S. fleurettii* are not frequently isolated from milk in herd 3 and herd 2, respectively (Figure 1A), contact with contaminated milk is an unlikely explanation for the presence of CNS in the MMUL. The likelihood of presence of CNS on teat apices was significantly lower after milking than before milking (P < 0.001), while the reverse was true for MMUL (Table 2) (P < 0.001). This suggests that CNS are washed off the teats during milking and transferred to MMUL. This could increase the risk of exposure to CNS for the cows being milked last as compared to those that are milked first.

Of the 40 collected samples from MSG, 67.5% were positive. All milkers were CNS-positive on at least one of the sampled sites, both pre- and/or post-milking. Eleven species were isolated from MSG, with *S. auricularis*, *S. cohnii, S. equorum*, S*. haemolyticus*, and *S. hominis* the predominant species (Figure 1 and Table 3). *Staphylococcus capitis*, *S. epidermidis*, *S. fleurettii*, *S. nepalensis*, *S. sciuri*, and *S. warneri* were isolated less frequently.

Table 2. Number of coagulase-negative *Staphylococcus* isolates recovered before (b) and after (a) milking from the parlor-associated niches (teat apices, milking machine unit liners, and milkers' skin and gloves) in 3 Flemish dairy herds

Species	Teat apices													Milking machine unit liners													Milkers' skin and gloves							Total	
	Herd 1				Herd 2				Herd 3				S[1]	Herd 1				Herd 2				Herd 3				S	Herd 1		Herd 2		Herd 3		S	n	%
	b	n_c[2]	a	n_c	b	n_c	a	n_c	b	n_c	a	n^c		b	n_{cl}[3]	a	n_{cl}	b	n_{cl}	a	n_{cl}	b	n_{cl}	a	n_{cl}		b	a	b	a	b	a			
S. auricularis	1	1							1	1			2			1	1									1					1	2	3	6	2.1
S. capitis	1	1											1													0					2		2	3	1.0
S. chromogenes	1	1	1	1	1	1	1	1					4			2	2			2	2			1	1	5							0	9	3.1
S. cohnii	19	6	15	6									34			27	10									27	1	4			1		6	67	23.3
S. devriesei													0											1	1	1							0	1	0.3
S. epidermidis	1	1											1			1	1									1			1		1		2	4	1.4
S. equorum	1	1	1	1					10	6	1	1	13			1	1			2	2			25	10	28	1				2	2	5	46	16.0
S. fleurettii					19	6	2	2					21							20	8			1	1	21			1	1			2	44	15.3
S. haemolyticus	13	6	6	4	1	1			4	4	1	2	25			8	5			1	1			10	6	19	1	4			1		6	50	17.4
S. hominis	1	1	2	2					2	2			5							1	1					1	3	3				1	7	13	4.5
S. hyicus			1	1	1	1							2													0							0	2	0.7
S. lugdunensis													0			1	1									1							0	1	0.3
S. nepalensis													0													0	1						1	1	0.3
S. saphrophyticus													0											1	1	1							0	1	0.3
S. sciuri	2	2	2	2	1	1			3	3			8			8	7			4	3			8	6	20		2					2	30	10.4
S. simulans					1	1	1	1					2			1	1									1							0	3	1.0
S. vitulinus					1	1							1											2	2	2							0	3	1.0
S. warneri													0													0				1			1	1	0.3
S. xylosus													0	1	1	2	2									3							0	3	1.0
Total	40		28		25		4		20		2		119	1		52		0		30		0		49		132	7	13	2	2	8	5	37	288	100.0

[1]Subtotal.[2]Number of cows. [3]Number of clusters.

Unlike MMUL, hands or gloves and elbow bends had similar levels of CNS detection before and after milking (60% and 70%, respectively for hands or gloves, and 70% for elbow bends regardless of sampling time). The distribution of CNS species on MSG differed among the three herds (Figure 1 and Table 3). Three species, *S. hominis* (herd 1 and 3), *S. epidermidis* (herd 2 and 3), and *S. capitis* (herd 3) were solely isolated from elbow bends. For *S. hominis*, the association with isolation from elbows as opposed to hands was positively significant (P = 0.033) whereas the number of isolates from the other species was too low for meaningful significance testing. According to the interpretation of Thorberg et al. (2006), species that are predominantly found in the elbow bend belong to the human microbiota. The association between those species and human skin is not exclusive, because the same species were detected on TA or in MMUL, albeit at low prevalence (1.0 - 4.5%, Table 2). *Staphylococcus nepalensis*, *S. sciuri*, and *S. warneri* were detected on bare hands or gloves but not in elbow bends. Again, the number of isolates from those species was too low for significance testing. Throughout the 13 months of sampling in the study herds (in the longitudinal study for detection of IMI), *S. nepalensis* and *S. warneri* were never isolated from milk, potentially excluding contamination of the hands or gloves via contact with milk. The two species were not detected on TA or in MMUL either. In a study on different farms, both species were occasionally found in the barn environment (Piessens et al., 2011) suggesting that their presence on hands is the result of environmental contamination, although the direction of transmission (hand to environment or environment to hand) cannot be determined with certainty. In previous studies, *S. warneri* was isolated from human forehead (Dekio et al., 2005) and the human nasal cavity (Rasmussen et al., 2000) from non-farmers, showing that *S. warneri* is a natural component of the human skin microbiota. Other species, notably *S. auricularis*, *S. cohnii*, *S. equorum*, *S. fleurettii*, and *S. haemolyticus* were isolated both from elbow bends and from hands or gloves. These species (except *S. auricularis*) also represent the majority of TA and MMUL isolates (Figure 1). In case of IMI in those study herds, transmission of *S. cohnii*, *S. equorum*, *S. fleurettii*, and *S. haemolyticus* possibly occurred during the milking process, either via MMUL or via the hands or gloves of the milkers. Another hypothesis is the existence of a shared microbiota between cows and humans. An organ(skin)-specificity rather than host-specificity of certain CNS species might occur, as was described for *S. aureus* strains (Zadoks et al., 2002). However, these

particular CNS species have to our knowledge, not been reported in human studies (Rasmussen et al., 2000; Dekio et al., 2005). Thorberg et al. (2006) hypothesized that *S. epidermidis* might be transmitted from humans to bovines, but this species was not frequently found in our study nor in that of Taponen et al. (2008). However, *S. epidermidis* was causing a substantial number of IMI in another study (Piessens et al., 2011), emphasizing once more that the distribution of CNS species can be herd-specific.

During swabbing of TA care was taken to avoid contact with milk because cross contamination of skin and milk samples could lead to apparent overlap in species or strain distributions, as previously described for *S. aureus* (Zadoks et al., 2002). Also, milk samples were collected aseptically and IMI definitions were based on multiple milk samplings. Therefore, we assume to have reduced the risk of misclassification due to contamination of either sample type. Comparison of the CNS species distribution in TA, MMUL and MSG with the distribution among IMI can help to shed light on the role of parlor associated extramammary sources in causing IMI. For some species, such as *S. cohnii* in herd 1, *S. fleuretti* in herd 2, and *S. equorum* in herd 3, TA, MMUL and MSG are likely sources or fomites for IMI, given that the prevalence in the extramammary sources is higher than the prevalence among IMI isolates (Figure 1B and Figure 2). On the other hand, some herds had a high number of IMI due to certain CNS species (e.g. *S. simulans* and *S. xylosus* in herd 2, and *S. chromogenes* in all herds) whereas these species were only rarely isolated from TA, MMUL or MSG (Figure 1B and Figure 2). *Staphylococcus cohnii*, found in 23% of IMI and 77% of extramammary samples, can be considered as an opportunistic pathogen based on the distribution in herd 1. *S. haemolyticus*, considered as an opportunistic species by Piessens et al. (2011), and *S. sciuri* appeared to be present in all herds (Figure 1, this study and Piessens et al., 2011) and followed a similar distribution as *S. cohnii* in herd 1 (Figure 1A). *Staphylococcus equorum* was rarely isolated from IMI but commonly found in MMUL and on TA in herd 3. Based on these data this species can be considered as an environmental organism (Figure 2). This is in agreement with the study of Piessens et al. (2011), who commonly found this species in the barn environment but rarely isolated it from the mammary gland. Similar findings were obtained for *S. fleurettii* (Figure 1, herd 2, this study and Piessens et al., 2011).

Table 3. Number of coagulase-negative *Staphylococcus* isolates recovered from samples from elbow bends (n = 10 elbows, 20 samples), bare hands (n = 4 hands, 8 samples) or gloves (n = 6 gloves, 12 samples)

Species	Elbow bends				Bare hands				Gloves				Total			
	Herd 1	Herd 2	Herd 3	S[1]	Herd 1	Herd 2	Herd 3	S	Herd 1	Herd 2	Herd 3	S	Herd 1	Herd 2	Herd 3	Total
S. auricularis			2	2		nd[2]	1	1				0			3	3
S. capitis			2	2		nd		0				0			2	2
S. cohnii	3			3	1	nd	1	2	1			1	5		1	6
S. epidermidis		1	1	2		nd		0				0		1	1	2
S. equorum			1	1	1	nd		1			3	3	1		4	5
S. fleurettii		1		1		nd		0		1		1		2		2
S. haemolyticus	3			3	1	nd	1	2	1			1	5		1	6
S. hominis	6		1	7		nd		0				0	6		1	7
S. nepalensis				0		nd		0	1			1	1			1
S. sciuri				0	1	nd		1	1			1	2			2
S. warneri				0		nd		0		1		1		1		1
Total	12	2	7	21	4	nd	3	7	4	2	3	9	20	4	13	37

[1]Subtotal. [2]Not determined as gloves were worn by the milker.

Interestingly, CNS species with a more substantial impact on udder health (i. e. *S. chromogenes*, *S. simulans*, and *S. xylosus*; Supré et al., 2011) were rarely isolated from the parlor-associated niches (Figure 2). *Staphylococcus chromogenes* was also seldom observed in the barn environment (Piessens et al., 2011) whereas it was the most common species among isolates from IMI and is genetically less diverse than *S. haemolyticus* (Piessens et al., 2012). Persistent *S. chromogenes* IMI occur (Supré et al., 2011), providing a potential reservoir of IMI for other animals. Based on the latter, *S. chromogenes* would be a primarily udder-adapted pathogen. However, assuming that cow-to-cow transmission of udder-adapted (contagious) pathogens takes place via MMUL or MGS, no transmission route for this species could be identified in this study. Furthermore, strain typing studies of *S. chromogenes* provide only limited support for contagious transmission as considerable within-herd strain heterogeneity can be observed (Zadoks et al., 2011; Bexiga et al., 2014). In our study, *S. simulans* and *S. xylosus* behaved primarily as udder-associated bacteria. This niche association was also suggested for *S. simulans* by other researchers (Taponen et al., 2008; Simojoki et al., 2011; Bexiga et al., 2014), although previous studies isolated *S. simulans* and *S. xylosus* from the barn environment (Piessens et al., 2011) and teat skin (Devriese and Keyser, 1980), respectively, rather than from IMI.

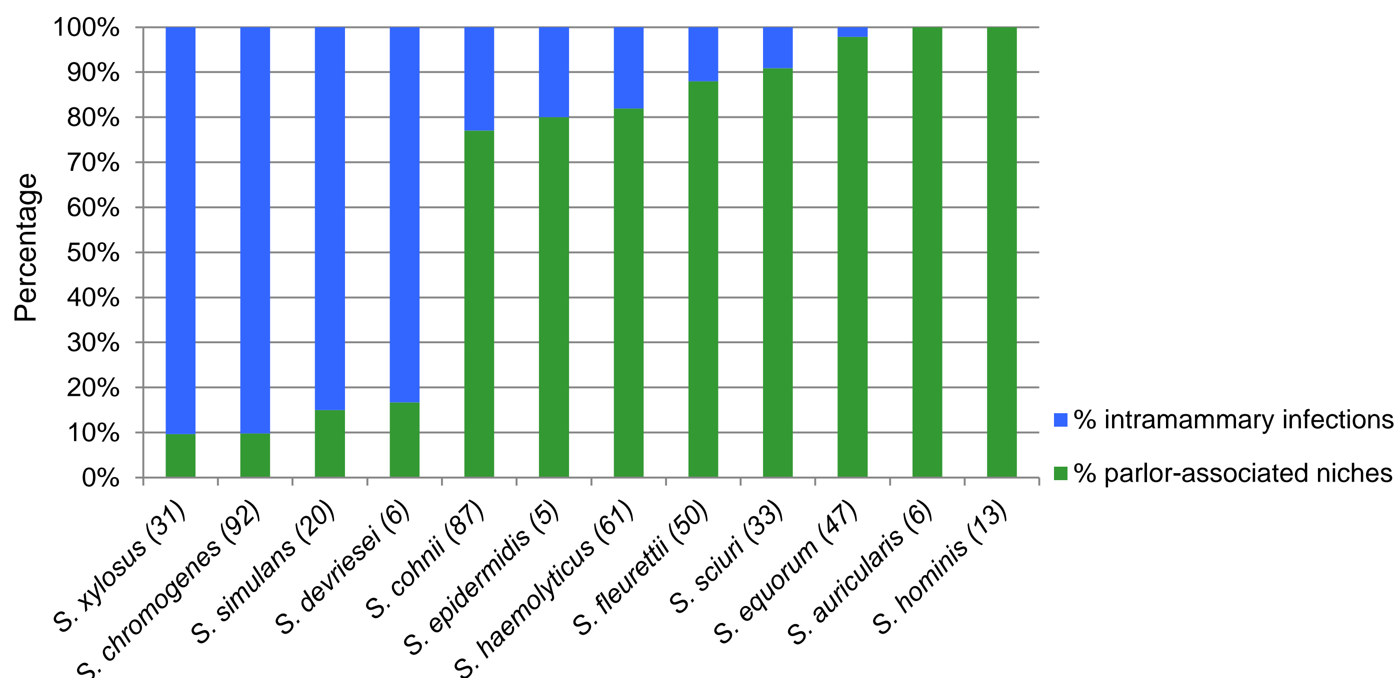

Figure 2. Distribution of the most common coagulase-negative *Staphylococcus* species isolated from intramammary infections (blue) or found in parlour-associated extramammary niches (green) per species.

One could argue only a limited number of cows (n = 18) and herds (n = 3) were included in the current study. Still, a vast number of CNS isolates were collected and identified using tDNA-PCR, a validated and accurate molecular identification technique, giving a precise picture of their distribution in parlor-related extramammary niches. We acknowledge the limited number of herds and animals narrows the conclusions that can be drawn from the study. However, research based on such precise molecular data as in our study, on possible infection sources of IMI-causing CNS, will help to further increase our understanding of the epidemiology and ecology of CNS species and will generate new hypotheses, useful for larger future studies. Eventually, it can be helpful to determine prevention and control measures for IMI with the species that have a more substantial impact on udder health, such as *S. chromogenes*, *S. simulans*, and *S. xylosus* (Supré et al., 2011).

Conclusion

Exploring distributions of CNS reveals that each herd has its own CNS microbiota in the extramammary niches. The most prevalent species in the extramammary samples were *S. cohnii*, *S. fleurettii*, and *S. equorum* in herd 1 - 3, respectively, while *S. haemolyticus* and *S. sciuri* were present in all herds. TA and MMUL shared similar CNS distributions within each herd. The MSG seems to harbor a specific microbiota, although some species might be shared between human skin and the other niches sampled in this study. *Staphylococcus chromogenes*, *S. simulans*, and *S. xylosus* favor the mammary gland and were barely found on TA, MMUL, and MSG, while *S. equorum* and *S. fleurettii* preferred the extramammary niches. *Staphylococcus cohnii*, *S. haemolyticus*, and *S. sciuri* were considered as less adapted to one of the compartments in particular.

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Chapter 4.2

Teat Apex Colonization with Coagulase-Negative *Staphylococcus* Species before Parturition: Distribution and Species-Specific Risk Factors

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Abstract

Coagulase-negative staphylococci (CNS) are the main cause of bovine intramammary infections and are also abundantly present in extramammary habitats such as teat apices. Teat apex colonization (TAC) with CNS has already been explored in lactating dairy cows at the species level, whereas this is not true for dry cows and end-term heifers. Therefore, the aim of this observational study was to describe CNS TAC in non-lactating dairy cows and end-term heifers in Flemish dairy herds and to identify associated risk factors at the herd, cow and quarter level. All CNS were molecularly identified to the species level using transfer RNA intergenic spacer PCR (tDNA-PCR) and sequencing of the *16S rRNA* gene, allowing for species-specific statistical analyses using multivariable, multilevel logistic regression. *Staphylococcus devriesei*, *S. chromogenes*, *S. haemolyticus* and *S. equorum* were the most frequently isolated species. *Staphylococcus chromogenes* was the sole species colonizing teat apices of cows and heifers in all herds whereas large between-herd differences were observed for the other species. Teat apices of red and white Holstein Friesians, of quarters dried-off without an internal teat sealer, and swabbed in months with lower precipitation and higher ambient temperature were significantly more likely to be colonized by *S. devriesei*. Slightly dirty teat apices and teat apices swabbed in months with lower precipitation had higher odds of being colonized by *S. chromogenes* whereas teat apices sampled in months with lower precipitation and higher ambient temperature were more likely to be colonized by *S. haemolyticus*. Dirty teat apices and teat apices swabbed in months with lower ambient temperature in combination with low precipitation had higher odds of being colonized by *S. equorum*. Diverse factors explaining CNS TAC, yet mostly related to humidity, ambient temperature and hygiene, substantiate differences in epidemiological behavior and ecology between species

Key words

Teat apex, Coagulase-negative *Staphylococcus* species

Introduction

Besides being the most common cause of intramammary infections in dairy cows in many regions and countries (Vanderhaeghen et al., 2015), coagulase-negative staphylococci (CNS) are abundantly present in extramammary habitats such as sawdust and air (Piessens et al., 2011) and also colonize dairy cows' teat apices (Taponen et al., 2008; Braem et al., 2012; De Visscher et al., 2014). In fact, the majority of teat apices colonizing microbiota belong to the group of the CNS (Braem et al., 2013). Differences in ecological and epidemiological characteristics among bovine-associated CNS species have been revealed (Vanderhaeghen et al., 2015), yet many aspects remain undetermined. The presence of CNS on teat apices of lactating dairy cows and heifers has already been explored at the species level (Taponen et al., 2008; Braem et al., 2013; De Visscher et al., 2014; Vanderhaeghen et al., 2015) but little is known about CNS teat apex colonization (TAC) in dry cows and end-term heifers. A high prevalence of CNS colonized teat apices in pregnant dairy heifers before calving has been described (De Vliegher et al., 2003; Piepers et al., 2011) but those studies either solely concerned heifers, studied CNS as a group, or focused specifically on *Staphylococcus chromogenes* only using non-molecular identification. Large observational studies describing the species distribution on teat apices in non-lactating dairy cows and end-term heifers and identifying factors associated with their presence are needed to add to our understanding of the role of CNS species in bovine udder health (Taponen et al., 2008; De Visscher et al., 2014; Vanderhaeghen et al., 2015). Although we have started to learn about the relation between CNS TAC and IMI, and associations seem to be present (Leroy et al., 2015), potential protective aspects of TAC remain to be studied (Vanderhaeghen et al., 2014).

Therefore, this study aimed to determine and to describe: (1) the species-specific prevalence of CNS colonization of teat apices of dry cows and end-term heifers before calving, (2) the CNS distribution, (3) associated herd-, cow-, and quarter-level risk factors, and (4) the variance components of CNS TAC.

Materials and methods

Herds and cows

Thirteen commercial Flemish dairy herds were included, all of them participating in the DHI program (CRV, Arnhem, The Netherlands), being a first selection criterion. Other inclusion criteria were no pre-partum antibiotic treatment of heifers and the use of artificial insemination as accurate expected calving dates were demanded. The majority of farmers (n = 8) only housed dairy cattle, whereas the others also farmed pigs (n = 1) or beef cattle (n = 4). The DHI-records and bulk milk quality data of the Milk Control Centre Flanders (MCC Flanders, Lier, Belgium) allowed for calculating the herd size and the bulk milk SCC, respectively, at the start of the sampling period in July 2012. The entire sampling period lasted until February 2013. Before the start of the study an average of 57 cows and heifers were in lactation (range = 30 - 95) per herd (arithmetic mean of the 6 last test-day samples). The geometric mean bulk milk SCC was 201,000 cells/mL (range = 79,000 cells/mL - 310,000 cells/mL). On 7 of the farms, dry cows were housed on concrete slatted floor with cubicles with mats (n = 4), with mattresses (n = 2) or without bedding (n = 1). The other 6 farms kept the dry cows on straw, either in a deep litter barn (n = 5) or in deep litter boxes with a bottom layer of sand and a full concrete floor (n = 1). Pregnant heifers were typically housed on a concrete slatted floor in cubicles (n = 11) with mats (n = 5), with mattresses (n = 5) or without bedding (n = 1). The other 2 farms housed the pregnant heifers either in a deep litter barn with straw (n = 1) or in deep litter boxes with a bottom layer of sand and straw (n = 1). On the majority of farms, dry cows and pregnant heifers (n = 12 and n = 11, respectively) were kept on pasture between May and September. Seven of the herds housed the dry cows separated from the lactating cows before calving, whereas in the majority of herds (n = 10) the pregnant heifers were housed together with the lactating cows.

On each farm, 12 pregnant heifers and dry cows (total n = 156) were selected according to the proportion of lactating cows and heifers present in the herd at the start of the study, reflecting the parity distribution in the herd, resulting in a total of 53 pregnant heifers and 103 dry cows (range = start of 2nd lactation - start of 10th lactation).

Table 1. Overview of all herd-, cow-, and quarter-level risk factors potentially associated with (species-specific) teat apex colonization with coagulase-negative staphylococci

Risk factor	Recording method	Description	Categories considered in the final model
Herd level			
Herd type	Questionnaire	Dairy cattle only or with pigs or beef cattle	Dairy cattle only vs. dairy cattle with other animals
Herd size	DHI-records	Mean number of animals in lactation at onset of sampling period[1]	Smaller (< 60 lactating cows) vs. larger (≥ 60 lactating cows) herd[2]
Bulk milk SCC	MCC-records	Bulk milk SCC at onset of sampling period[3]	Lower (< 196 x 10³ cells/mL) vs. higher bulk milk SCC (≥ 196 x 10³ cells/mL)[2]
Cow level			
Housing	Questionnaire	Housing of dry cows and pregnant heifers	Cubicles vs. deep litter
Pasture access	Questionnaire	Pasture access during outdoor season	No pasture vs. pasture
Contact[4]	Questionnaire	Contact with lactation cows before parturition	No contact vs. contact
Breed	DHI-records	Breed	Black and white HF[5] vs. red and white HF
Parity	DHI-records	Parity starting at calving	First lactation vs. second lactation or older
Vitamins	Questionnaire	Supplementation with minerals and vitamins before parturition	No supplementation vs. supplementation
Antimicrobials	Questionnaire	Antimicrobials used at drying-off	No vs. narrow-spectrum vs. broad-spectrum[6]
Teat sealer	Questionnaire	Application of an internal teat sealer at drying-off	No teat sealer vs. teat sealer
Teat dip	Questionnaire	Teat dipping / spraying before parturition	No dipping / spraying vs. dipping / spraying
BCS	Visual	Five-point scale[7] at sampling	< 2.5 vs. 2.5-3 vs. > 3
Hygiene	Visual	Hygiene of mammary gland and teats at sampling[8]	Very clean vs. slightly dirty vs. dirty
Temperature	RMI[9]	Monthly ambient temperature (°C) at sampling	Low vs. high[10]
Precipitation	RMI	Monthly precipitation (L/m²) at sampling	Low vs. high[10]
Quarter level			
Quarter position	Visual	Position of the quarter	Front vs. hind quarter
Teat apex condition	Visual	Teat apex condition at sampling	Good condition vs. protuberant teat end vs. invaginated teat end
Teat skin condition	Visual	Teat skin condition at sampling	Little grooves vs. many grooves

[1]Arithmetic mean based on 6 last DHI-records before the onset of the study (July 2012). [2]Categorization based on median value of all mean values of all herds. [3]Geometric mean based on 6 last records of the Milk Control Centre Flanders before the onset of the study. [4]Housing of dry cows and end-term heifers together with lactating animals. [5]Holstein Friesian. [6]Antimicrobials with a narrow spectrum include cloxacillin benzathine, cefalexine, and cefalonium whereas cefquinome is a broad-spectrum antibiotic. [7]Edmonson et al. (1989). [8]Categorization based on a 5-point scale (Reneau et al., 2005). [9]Royal Meteorological Institute of Belgium. [10]Categorization based on median value of all monthly records during 1 year (March 2012 until February 2013).

The majority of the selected animals were black and white Holstein Friesian (HF) (85%, n = 132) and 15% were red and white HF (n = 24). Eighty-five percent (n = 132) of the animals were supplemented with minerals and vitamins before parturition. On all farms, blanket dry cow treatment was applied using either cloxacillin benzathine (47% of the 103 dry cows, n = 48 cows) or cephalosporins with a broad or Gram-positive spectrum (40%, n = 41 cows and 10%, n = 10 cows, respectively). Dry cow treatment information was lacking for a few cows only (3%, n = 3) as these cows were purchased when dry. One cow did not receive antimicrobials at drying-off due to a sudden drop in milk production. Only a minority of the dry cows received an internal teat sealer (39% of the 103 dry cows, n = 40) at drying-off. Iodine teat dip was applied to 38 dry cows and end-term heifers (24%) before parturition.

Samples and data collection

To determine TAC before parturition, swabs of all teat apices of the 153 cows and heifers (total n = 624) were collected 14 days before expected calving date. Visible soil and manure were first removed. Further, a dry cotton swab (Copan, Novolab, Belgium) was rotated gently on the teat apex as described by De Vliegher et al. (2003). Swabs were transported under cooled condition (4°C) to the Mastitis and Milk Quality Research Lab (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium).

Several herd- and cow-level factors, potentially associated with CNS (species-specific) TAC, were either calculated based on DHI-records or on data of the Milk Control Centre Flanders (MCC Flanders, Lier, Belgium) or collected before the onset of the study via a questionnaire (Table 1). Other potential cow- and quarter-level factors were recorded at sampling (teat swabbing) (Table 1).

Laboratory analyses

All swabs were plated on mannitol salt agar (MSA) (Oxoid, Erembodegem, Aalst, Belgium) (one plate per swab) (De Visscher et al., 2013). Plates were aerobically incubated at 37°C and examined after 24h and 48h. One colony of all phenotypically different colony types was picked up and subcultured on esculin blood agar (Oxoid) (one quadrant per isolate) to obtain pure cultures for subsequent analysis. All potential CNS isolates were stored at -80°C or immediately identified to the species

level using transfer RNA intergenic spacer PCR (tDNA-PCR). If no identification could be obtained, sequencing of the *16S rRNA* gene was performed (Supré et al., 2009).

Descriptive and statistical analyses

Observations were checked for unlikely values before analyses were performed. Complete data were available from 596 teat apices from 96 dry cows and 53 end-term heifers.

Logistic multilevel regression models were fit using MLwiN 2.16 (Centre for Multilevel Modeling, University of Bristol, Bristol, UK) applying reweighted iterative generalized least squares and 1st order penalized quasi-likelihood. As quarters were spatially clustered within cows and cows within herds, analyses were run with cow and herd as random effects.

Five different binary outcome variables at the quarter level were used: (1) teat apex colonized with CNS (whatever species) versus teat apex not colonized with CNS and (2 to 5) teat apex colonized with 1 of the 4 most frequently isolated CNS species: (2) *S. devriesei*, (3) *S. chromogenes*, (4) *S. haemolyticus,* and (5) *S. equorum*, versus teat apex not colonized with CNS or colonized with (an)other CNS species.

First, univariable associations were examined between the outcome variables and the independent factors (Table 1) with statistical significance assessed at $P < 0.15$. Second, Spearman rank correlation coefficients were calculated among the significant independent variables to avoid multicollinearity in the next steps. A correlation coefficient ≥ |0.6| among 2 factors led to the selection of 1 of the 2 variables for further analysis. In a third step, multivariable models were fit for all outcome variables using backward stepwise elimination. Statistical significance was assessed at $P < 0.05$. Biological relevant interaction terms were tested between all remaining statistically significant factors and kept in the final multivariable model when significant ($P < 0.05$). A Bonferroni-correction was applied to adjust for multiple comparisons. The observational-level standardized residuals were plotted against the observational-level predicted values to test the adequacy of all final models.

Table 2. Species distribution of coagulase-negative staphylococci (CNS) colonizing teat apices (n = 624) from 103 dry cows and 53 end-term heifers on 13 Flemish dairy herds

Species	Herd 1		Herd 2		Herd 3		Herd 4		Herd 5		Herd 6		Herd 7		Herd 8		Herd 9		Herd 10		Herd 11		Herd 12		Herd 13	
	$\%_{DC}$[1]	$\%_{H}$[2]	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$	$\%_{DC}$	$\%_{H}$
S. devriesei	25.0	6.3	28.6	45.0	20.8	37.5	31.3	18.8	21.4				18.8	25.0	21.9	12.5	38.9	50.0	14.3	10.0		6.3	37.5	6.3	5.0	
S. chromogenes	9.4		10.7	25.0		8.3	34.4	62.5	7.1	10.0	19.4	25.0	21.9	18.8	6.3	37.5	5.6	33.3	21.4	10.0	9.4	31.3	28.1	6.3	17.5	25.0
S. haemolyticus			21.4	25.0	12.5	12.5	18.8	56.3	28.6		8.3	25.0	34.4	50.0	9.4	6.3	38.9		35.7	20.0	3.1		12.5	6.3		50.0
S. equorum	31.3	68.8			20.8	20.8			3.6	45.0	22.2		3.1	6.3	18.8				10.7	30.0	12.5	6.3	6.3	6.3	17.5	
S. auricularis	6.3		3.6	25.0	4.2	8.3	3.1	12.5	7.1		5.6	16.7	18.8	56.3	15.6	12.5	5.6			5.0	3.1				7.5	
S. sciuri			7.1								30.6		3.1	6.3	9.4		13.9	25.0		10.0	3.1				22.5	37.5
S. cohnii		31.3			16.7	25.0		6.3				8.3	15.6	6.3					3.6		3.1					
S. xylosus			3.6			25.0			10.7			8.3	3.1	6.3			13.9	25.0		5.0	3.1				2.5	12.5
S. saprophyticus					16.7	4.2								18.8							3.1				2.5	
S. vitulinus			3.6								11.1						5.6	8.3								
S. arlettae										5.0			12.5	6.3												
S. succinus			3.6				3.1								3.1		2.8	8.3							2.5	
S. caseolyticus	3.1	6.3									2.8					6.3					6.3					
S. capitis									3.6						3.1				7.1					6.3		
S. simulans																	8.3		3.6							
S. hyicus				10.0		4.2																				
S. epidermidis									7.1								2.8									
S. agnetis	6.3																									
S. kloossii						4.2								6.3												12.5
S. fleurettii											5.6															
S. warneri							3.1																			
S. pasteuri																							3.1			
S. pseudintermedius														6.3												
S. gallinarum																									2.5	

[1]Percentage of teat apices of dry cows colonized with a specific species within each herd. [2] Percentage of teat apices of end-term heifers colonized with a specific species within each herd.

In addition, the Hosmer-Lemeshow goodness-of-fit test and 2 residual log pseudo-likelihood statistics were assessed on the fixed effect models only (SAS 9.3, SAS Institute Inc., Cary, NC, USA) (Dohoo et al., 2009). The test was for none of the final models statistically significant indicating good fit of our models. Data are presented as odds ratio's (OR) and 95% confidence intervals. The proportion of variation for the outcome variables at the herd, cow and quarter level was estimated based on the null and final models by assuming that the variance at the quarter level was $\pi^2/3$ (Dohoo et al., 2001) as described by Piepers et al. (2011).

Results

Descriptive analyses

The majority of teat apices (87%, n = 540) yielded growth on MSA and per plate 0 to 4 phenotypically different colony types were present. Different colony types sometimes appeared to belong to the same CNS species after tDNA-PCR or *16S rRNA* gene sequencing, eventually resulting in 619 CNS isolates available for further analysis. Seventy-two percent (n = 448) of all teat apices were colonized with CNS and harbored 1 up to 3 different CNS species. Twenty-four different CNS species were identified.

The overall most frequently isolated species was *S. devriesei* (19% of all teat apices colonized, n = 120 teat apices), followed by *S. chromogenes* (17%, n = 107), *S. haemolyticus* (17%, n = 107), *S. equorum* (13%, n = 81), *S. auricularis* (8%, n = 49), *S. sciuri* (7%, n = 41), *S. cohnii* (4%, n = 25) and *S. xylosus* (4%, n = 25) (Table 2). Isolates (n = 390) other than CNS were phenotypically or genotypically (i.e. using tDNA-PCR or *16S rRNA* gene sequencing) identified as *Staphylococcus aureus* (n = 4), *Arthrobacter* sp. (n = 1), *Bacillus* spp. (n = 307), *Jeotgallicoccus* spp. (n = 28), other Gram-positive cocci such as *Aerococcus* spp. (n = 12), *Enterococcus* sp. (n = 1) or *Streptococcus* spp. (n = 5), Gram-negative bacteria such as *Pseudomonas* spp. (n = 16), *Psychrobacter* spp. (n = 4) or other (n = 7), and fungi (n = 5).

On each herd, between 6 (herd 12) and 12 (herd 7) different CNS species were isolated from the teat apices. *Staphylococcus chromogenes* was the sole species colonizing teat apices in all 13 herds whereas *S. devriesei*, *S. haemolyticus,* and *S. auricularis* were colonizing teat apices on all farms except one.

Table 3. Univariable multilevel logistic regression models[1] for teat apex colonization with coagulase-negative staphylococci (CNS) as a group or with 1 of the 4 most frequently isolated CNS species, respectively

Risk factor	CNS (n = 448)					*S. devriesei* (n = 120)					*S. chromogenes* (n = 107)					*S. haemolyticus* (n = 107)					*S. equorum* (n = 81)				
	N_n[2]	$\%_n$[2]	N_{CNS}[3]	$\%_{CNS}$[3]	*P*[4]	N_{no}[5]	$\%_{no}$[5]	N_s[6]	$\%_s$[6]	*P*	N_{no}	$\%_{no}$	N_s	$\%_s$	*P*	N_{no}	$\%_{no}$	N_s	$\%_s$	*P*	N_{no}	$\%_{no}$	N_s	$\%_s$	*P*
Herd level																									
Herd type					0.84					0.15					0.33					0.12					0.31
Dairy cattle	105	59.7	279	62.3	Ref.[8]	297	58.9	87	72.5	Ref.	310	60.0	74	69.2	Ref.	303	58.6	81	75.7	Ref.	347	63.9	37	45.7	Ref.
With other[7]	71	40.3	169	37.7		207	41.1	33	27.5		207	40.0	33	30.8		214	41.4	26	24.3		196	36.1	44	54.3	
Herd size					0.70					0.43					0.32					0.12					0.44
Smaller	88	50.0	200	44.6	Ref.	239	47.4	49	40.8	Ref.	247	47.8	41	38.3	Ref.	254	49.1	34	31.8	Ref.	241	44.4	47	58.0	Ref.
Larger	88	50.0	248	55.4		265	52.6	71	59.2		270	52.2	66	61.7		263	50.9	73	68.2		302	55.6	34	42.0	
Bulk milk SCC					0.73					0.48					0.33					0.49					0.94
Lower	85	48.3	203	45.3	Ref.	242	48.0	46	38.3	Ref.	230	44.5	58	54.2	Ref.	229	44.3	59	55.1	Ref.	255	47.0	33	40.7	Ref.
Higher	91	51.7	245	54.7		262	52.0	74	61.7		287	55.5	49	45.8		288	55.7	48	44.9		288	53.0	48	59.3	
Cow level																									
Housing					0.85					0.74					0.31					0.57					0.69
Cubicles	115	65.3	285	63.6	Ref.	326	64.7	74	61.7	Ref.	325	62.9	75	70.1	Ref.	326	63.1	74	69.2	Ref.	343	63.2	57	70.4	Ref.
Deep litter	61	34.7	163	36.4		178	35.3	46	38.3		192	37.1	32	29.9		191	36.9	33	30.8		200	36.8	24	29.6	
Pasture access					0.21					0.09					0.29					1.00					0.73
No	12	6.8	48	10.7	Ref.	59	11.7	1	0.8	Ref.	46	8.9	14	13.1	Ref.	53	10.3	7	6.5	Ref.	51	9.4	9	11.1	Ref.
Yes	164	93.2	400	89.3		445	88.3	119	99.2		471	91.1	93	86.9		464	89.7	100	93.5		492	90.6	72	88.9	
Contact					0.15					0.14					0.28					0.03					0.06
No	74	42.0	202	45.1	Ref.	226	44.8	50	41.7	Ref.	236	45.6	40	37.4	Ref.	235	45.5	41	38.3	Ref.	240	44.2	36	44.4	Ref.
Yes	102	58.0	246	54.9		278	55.2	70	58.3		281	54.4	67	62.6		282	54.5	66	61.7		303	55.8	45	55.6	
Breed					0.86					0.12					0.22					0.41					0.39
Black and white HF[9]	153	86.9	375	83.7	Ref.	438	86.9	90	75.0	Ref.	432	83.6	96	89.7	Ref.	443	85.7	85	79.4	Ref.	453	83.4	75	92.6	Ref.
Red and white HF	23	13.1	73	16.3		66	13.1	30	25.0		85	16.4	11	10.3		74	14.3	22	20.6		90	16.6	6	7.4	
Parity					0.43					0.45					0.06					0.84					0.33

1st lactation	56	31.8	156	34.8	Ref.	174	34.5	38	31.7	Ref.	167	47.7	45	42.1	Ref.	174	33.7	38	35.5	Ref.	178	32.8	34	42.0	Ref.
≥ 2nd lactation	120	68.2	292	65.2		330	65.5	82	68.3		350	67.7	62	57.9		343	66.3	69	64.5		365	67.2	47	58.0	
Vitamines					0.14					0.24					0.16					0.51					0.75
No	15	8.5	81	18.1	Ref.	67	13.3	29	24.2	Ref.	87	16.8	9	8.4	Ref.	82	15.9	14	13.1	Ref.	75	13.8	21	25.9	Ref.
Yes	161	91.5	367	81.9		437	86.7	91	75.8		430	83.2	98	91.6		435	84.1	93	86.9		468	86.2	60	74.1	
Anti-microbials[10]					0.39					0.06					0.20					0.76					0.46
No	58	34.3	158	35.7	Ref.	178	36.2	38	31.7	Ref.	171	33.7	45	42.9	Ref.	178	35.2	38	35.5	Ref.	180	33.9	36	44.4	Ref.
Narow	70	41.4	162	36.6		200	40.7	32	26.7		198	39.1	34	32.4		194	38.4	38	35.5		205	38.6	27	33.3	
Broad	41	24.3	123	27.8		114	23.2	50	41.7		138	27.2	26	24.8		133	26.3	31	29.0		146	27.5	18	22.2	
Teat sealer[10]					0.26					0.13					0.96					0.31					0.56
No	127	75.1	325	73.4	Ref.	349	70.9	103	85.8	Ref.	374	73.8	78	74.3	Ref.	372	73.7	80	74.8	Ref.	395	74.4	57	70.4	Ref.
Yes	42	24.9	118	26.6		143	29.1	17	14.2		133	26.2	27	25.7		133	26.3	27	25.2		136	25.6	24	29.6	
Teat dip					0.59					0.33					0.31					0.82					0.44
No	133	75.6	339	75.7	Ref.	385	76.4	87	72.5	Ref.	394	76.2	78	72.9	Ref.	385	74.5	87	81.3	Ref.	406	74.8	66	81.5	Ref.
Yes	43	24.4	109	24.3		119	23.6	33	27.5		123	23.8	29	27.1		132	25.5	20	18.7		137	25.2	15	18.5	
BCS[11]					0.22					0.87					0.57					0.60					/
< 2.5	8	4.7	12	2.7	Ref.	18	3.6	2	1.7	Ref.	16	3.1	4	3.7	Ref.	19	3.7	1	0.9	Ref.	20	3.7	0	0	
2.5-3	158	92.4	406	91.2		455	91.5	109	91.6		464	91.2	100	93.5		465	91.4	99	92.5		490	91.2	74	93.7	
> 3	5	2.9	27	6.1		24	4.8	8	6.7		29	5.7	3	2.8		25	4.9	7	6.5		27	5.0	5	6.3	
Hygiene[12]					0.04					0.27					0.01					0.76					<0.001
Very clean	67	40.6	133	30.0	Ref.	149	30.5	51	42.9	Ref.	175	34.8	25	23.8	Ref.	168	33.5	32	29.9	Ref.	193	36.5	7	8.9	Ref.
Slightly dirty	69	41.8	179	40.4		202	41.3	46	38.7		186	37.0	62	59.0		204	40.7	44	41.1		218	41.2	30	38.0	
Dirty	29	17.6	131	29.6		138	28.2	22	18.5		142	28.2	18	17.1		129	25.7	31	29.0		118	22.3	42	53.2	
Temperature					0.05					0.03					0.36					0.03					<0.001
Low	15	8.5	53	11.8	Ref.	66	13.1	2	1.7	Ref.	61	11.8	7	6.5	Ref.	67	13.0	1	0.9	Ref.	36	6.6	32	39.5	Ref.
High	161	91.5	395	88.2		438	86.9	118	98.3		456	88.2	100	93.5		450	87.0	106	99.1		507	93.4	49	60.5	
Precipitation					0.06					0.06					0.03					0.01					<0.01
Low	127	72.2	365	81.5	Ref.	381	75.6	111	92.5	Ref.	397	76.8	95	88.8	Ref.	391	75.6	101	94.4	Ref.	447	82.3	45	55.6	Ref.
High	49	27.8	83	18.5		123	24.4	9	7.5		120	23.2	12	11.2		126	24.4	6	5.6		96	17.7	36	44.4	

Quarter level																									
Quarter position					0.26					0.51					0.74					0.31					0.52
Front	94	53.5	218	48.7	Ref.	249	49.4	63	52.5	Ref.	257	49.7	55	51.4	Ref.	263	50.9	49	45.8	Ref.	274	50.5	38	46.9	Ref.
Hind	82	46.6	230	51.3		255	50.6	57	47.5		260	50.3	52	48.6		254	49.1	58	54.2		269	49.5	43	53.1	
Teat end condition[11]					0.55					0.64					0.11					0.83					0.61
Good	149	87.1	373	83.8	Ref.	423	85.1	99	83.2	Ref.	437	85.9	85	79.4	Ref.	432	84.9	90	84.1	Ref.	458	85.3	64	81.0	Ref.
Protuberant	8	4.7	36	8.1		32	6.4	12	10.1		34	6.7	10	9.3		34	6.7	10	9.3		39	7.3	5	6.3	
Invaginated	14	8.2	36	8.1		42	8.5	8	6.7		38	7.5	12	11.2		43	8.4	7	6.5		40	7.4	10	12.7	
Teat skin condition[11]					0.49					0.83					0.07					0.49					0.12
Little grooves	90	52.6	249	56.0	Ref.	263	52.9	76	63.9	Ref.	272	53.4	67	62.6	Ref.	270	53.0	69	64.5	Ref.	308	57.4	31	39.2	Ref.
Many grooves	81	47.4	196	44.0		234	47.1	43	36.1		237	46.6	40	37.4		239	47.0	38	35.5		229	42.6	48	60.8	

[1]Cow and herd included in all models as random effects to correct for potential clustering of quarters within cows and cows within herds. [2]Number and percentage of teat apices not colonized with CNS. [3]Number and percentage of teat apices colonized with CNS. [4]*P*-value for the overall effect. [5]Number and percentage of teat apices not colonized with CNS or colonized with a CNS species other than *S. devriesei*, *S. chromogenes*, *S. haemolyticus,* or *S. equorum*, respectively. [6]Number and percentage of teat apices colonized with *S. devriesei, S. chromogenes. S. haemolyticus,* or *S. equorum*, respectively. [7]Dairy cattle with pigs or beef cattle. [8]Reference category per risk factor. [9]Holstein Friesian. [10]Missing data from 3 dry cows. [11]Missing data from 2 dry cows. [12]Missing data from 4 dry cows.

Staphylococcus equorum, *S. xylosus*, *S. sciuri* and *S. cohnii* could not be isolated from teat apices in 3, 4, 5 and 6 herds, respectively (Table 2, Figure 1). Species other than the aforementioned ones were only found in the minority of herds (Table 2).

The majority of the dry cows and pregnant heifers (95%, n = 98 out of 103 dry cows and 94%, n = 50 out of 53 heifers, respectively) had at least 1 teat apex colonized with CNS. Seventy-four percent (n = 156) of all teat apices of the end-term heifers (n = 212) harbored CNS whereas 71% (n = 292) out of 412 teat apices of the dry cows were colonized with CNS. Teat apices of the pregnant heifers were mainly colonized by *S. chromogenes* (55% heifers, n = 29 out of 53 heifers and 21% teat apices, n = 45 out of 212 teat apices), followed by *S. haemolyticus* (42% heifers, n = 22 and 18% teat apices, n = 38), *S. devriesei* (40% heifers, n = 21 and 18% teat apices, n = 38) and *S. equorum* (32% heifers, n = 17 and 16% teat apices, n = 34). *Staphylococcus epidermidis*, *S. fleurettii*, *S. gallinarum*, *S. pasteuri, S. simulans,* and *S. warneri* were not observed on teat apices from these young animals. *Staphylococcus devriesei* was the most frequently isolated species from the teat apices of the dry cows (44% dry cows, n = 45 out of 103 dry cows and 19% teat apices, n = 82 out of 412 teat apices), but the dominance of 1 species was less pronounced as observed for the heifers as other species were also often identified (*S. haemolyticus*: 39% dry cows, n = 40 and 17% teat apices, n = 69; *S. chromogenes*: 38% dry cows, n = 39 and 15% teat apices, n = 62; *S. equorum*: 28% dry cows, n = 29 and 11% teat apices, n = 47). Three species recovered from the heifers, *S. hyicus*, *S. kloossii* and *S. pseudintermedius*, could not be isolated from the teat apices of the dry cows.

Risk factors

A first reduction based on unconditional associations revealed 4, 5, 5, 4 and 5 herd-, cow- and/or quarter-level factors to be associated with TAC with CNS, *S. devriesei*, *S. chromogenes*, *S. haemolyticus* or *S. equorum*, respectively (Table 3). No significant associations were identified for bulk milk SCC, housing, teat dip, BCS and quarter position. Strong correlations between 2 or more factors were not observed. The final multilevel, multivariable logistic regression models are shown in Table 4.

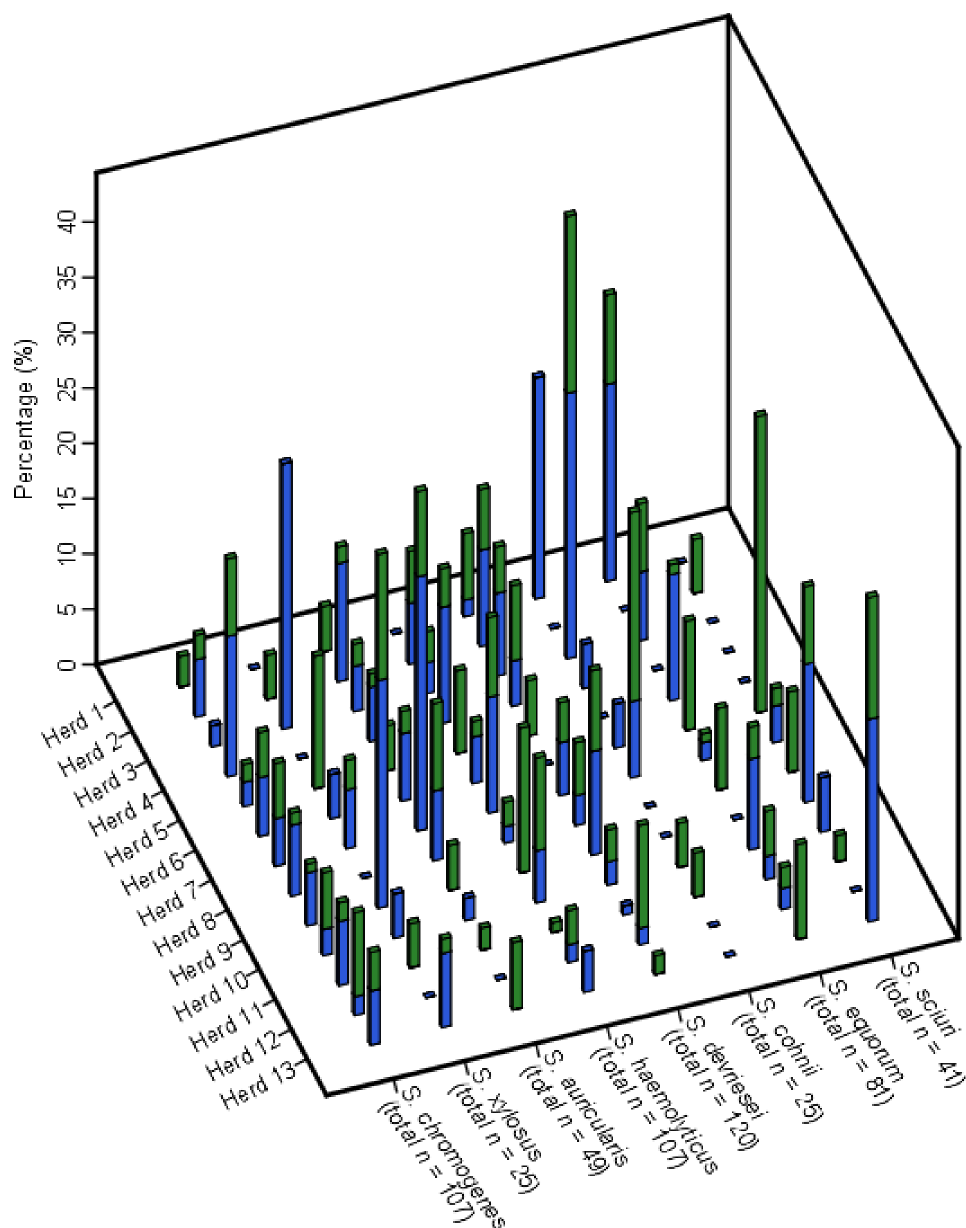

Figure 1. Distribution of coagulase-negative *Staphylococcus* species on teat apices in 13 Flemish dairy herds in accordance with the proportion of teat apices from end-term heifers (blue) and dry dairy cows (green), respectively, per herd, colonized with a certain species relative to the total number of isolates over all herds per species.

Teat apices of red and white HF dry cows and pregnant heifers were more likely to be colonized with *S. devriesei* (OR = 2.22; 95% CI: 1.02-4.84) as opposed to teat apices from black and white HF. The application of an internal teat sealer significantly decreased the odds of being colonized with *S. devriesei* (OR = 0.45; 95% CI: 0.21-0.95). Dirty teat apices were significantly more likely to be colonized

with CNS in general (OR = 2.91; 95% CI: 1.47-5.77) and *S. equorum* in particular (OR = 5.24; 95% CI: 1.87-14.66) than very clean teat apices. Slightly dirty teat apices had higher odds of being colonized with *S. chromogenes* (OR = 2.39; 95% CI: 1.30-4.40) as opposed to very clean teat apices. A higher ambient temperature significantly increased the odds of being colonized with *S. devriesei* (OR = 6.63; 95% CI: 1.37-32.13) or *S. haemolyticus* (OR = 10.89; 95% CI: 1.27-93.52) whereas the opposite was true for *S. equorum* (OR = 0.30; 95% CI: 0.12-0.76) with the association even being more pronounced in case of low precipitation than in case of high precipitation (significant interaction term, see Figure 2). Teat apex colonization with *S. chromogenes* was not associated with ambient temperature. Teat apices swabbed in months with high precipitation were less likely to be colonized with CNS in general (OR = 0.45; 95% CI: 0.24-0.84), and with *S. devriesei* (OR = 0.35; 95% CI: 0.14-0.86), *S. chromogenes* (OR = 0.44; 95% CI: 0.21, 0.94) and *S. haemolyticus* (OR = 0.23; 95% CI: 0.08-0.67) in particular than teat apices swabbed in months with low precipitation. For *S. equorum*, the latter was only true in case of low ambient temperature and when no Bonferroni-correction was applied (OR = 0.21; 95% CI: 0.05-0.93) (Table 4, Figure 2). In case of high ambient temperatures, teat apices swabbed in months with high precipitation were even more likely to be colonized with *S. equorum* than teat apices swabbed in months with low precipitation (Figure 2).

The highest variability was observed at the quarter level for all dependent variables. For the outcome variable *S. chromogenes* TAC, the variation at the cow level in the null model was much more pronounced as opposed to the herd level whereas this discrepancy was less pronounced for *S. haemolyticus* or *S. devriesei* and even absent for *S. equorum* (Table 5). For the latter species, the variation occurred in an almost equal amount at the cow and herd level.

Discussion

This is the first large species-specific observational study that describes the prevalence and distribution of CNS colonizing teat apices from dry cows and end-term heifers and simultaneously identifies risk factors for TAC prior to calving at different levels of the data hierarchy.

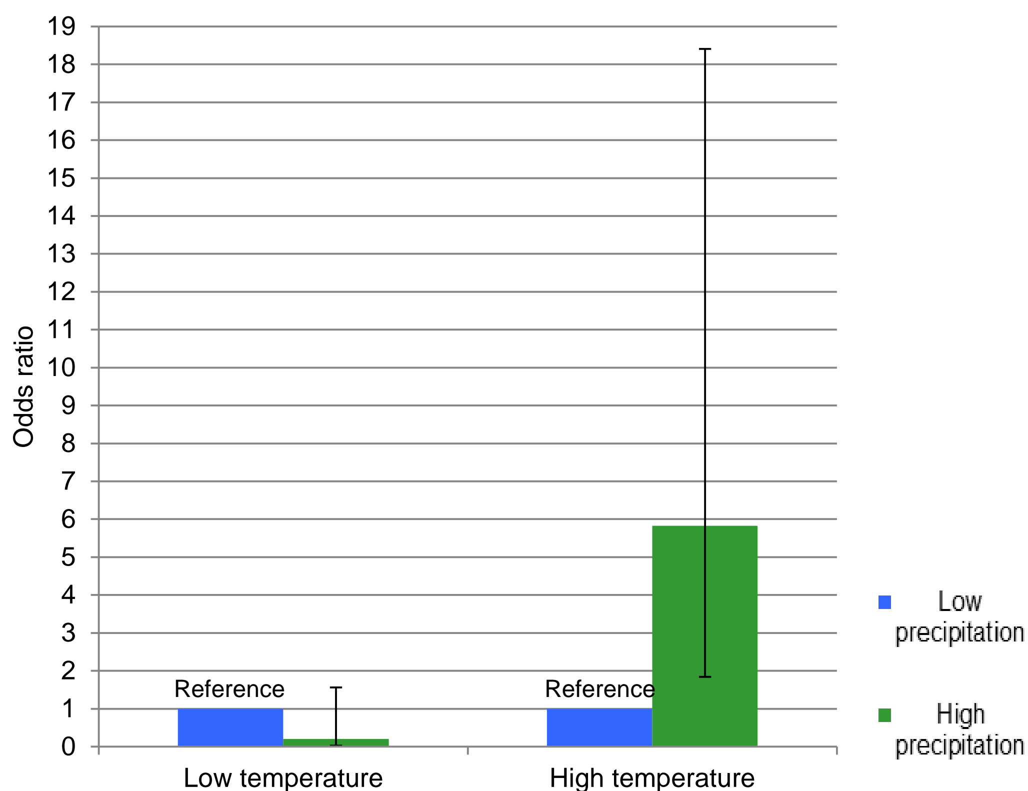

Figure 2. Interaction term between temperature and precipitation visualized using odds ratio's and 95% confidence intervals for teat apices colonized with *Staphylococcus equorum* versus not colonized or colonized with (an)other coagulase-negative *Staphylococcus* species (as shown in Table 4).

A sufficient number of herds, reflecting different management strategies, and the use of molecular speciation allowed us to precisely picture the species-specific CNS distribution on the teat apices. Similar to what was reported in lactating cows (Braem et al., 2013; De Visscher et al., 2014), the species distribution strongly varied among the different herds (Figure 1). None of the species was found on all farms except for *S. chromogenes*, which corresponds well with an earlier study reporting a herd-level prevalence of *S. chromogenes* colonized heifers of at least 10% (De Vliegher et al., 2003).

Table 4. Final multivariable multilevel logistic regression models[1] describing potential herd-, cow- and quarter-level risk factors associated with teat apex colonization with coagulase-negative staphylococci (CNS) as a group or with 1 of the 4 most frequently isolated species, respectively

Risk factor[2]	CNS (n = 448)						*S. devriesei* (n = 120)						*S. chromogenes* (n = 107)					
	β[3]	SE	OR[4]	95% CI		*P*[5]	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*
Intercept	0.84	0.27	2.32	1.37	3.94		-3.13	0.80	0.04	0.01	0.21		-1.90	0.27	0.15	0.09	0.26	
Breed						NS[6]						0.05						NS
Black and white HF[7]							Ref.[8]	—	—	—	—							
Red and white HF							0.80	0.40	2.22	1.02	4.84							
Teat sealer						NS						0.04						NS
No							Ref.	—	—	—	—							
Yes							-0.80	0.38	0.45	0.21	0.95							
Hygiene						0.01						NS						<0.01
Very clean	Ref.	—	—	—	—								Ref.	—	—	—	—	
Slightly dirty	0.43	0.28	1.54	0.89	2.67								0.87	0.31	2.39	1.30	4.40	
Dirty	1.07	0.35	2.91	1.47	5.77								-0.04	0.38	0.96	0.46	2.03	
Temperature						NS						0.02						NS
Low							Ref.	—	—	—	—							
High							1.89	0.81	6.63	1.37	32.13							
Precipitation						0.02						0.02						0.03
Low	Ref.	—	—	—	—		Ref.	—	—	—	—		Ref.	—	—	—	—	
High	-0.81	0.32	0.45	0.24	0.84		-1.05	0.45	0.35	0.14	0.86		-0.82	0.39	0.44	0.21	0.94	
Temperature*Precipitation[9]						NS						NS						NS

[1]Cow and herd included in all models as random effects to correct for potential clustering of quarters within cows and cows within herds. [2]Only cow-level factors were identified. [3]Regression coefficient. [4]Odds ratio. [5]*P*-value for the overall effect. [6]Not significant. [7]Holstein Friesian. [8]Reference category per risk factor. [9]Interaction term between temperature and precipitation.

Table 4 continued. Final multivariable multilevel logistic regression models[1] describing potential herd-, cow- and quarter-level risk factors associated with teat apex colonization with coagulase-negative staphylococci (CNS) as a group or with 1 of the 4 most frequently isolated species, respectively

RF	*S. haemolyticus* (n = 107)						*S. equorum* (n = 81)					
	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*
Intercept	-3.85	1.10	0.02	<0.01	0.18		-0.96	0.71	0.38	0.09	1.54	
Breed						NS						NS
Black and white HF												
Red and white HF												
Internal teat sealer						NS						NS
No												
Yes												
Hygiene						NS						<0.01
Very clean							Ref.	—	—	—	—	
Slightly dirty							0.61	0.52	1.84	0.66	5.13	
Dirty							1.66	0.53	5.24	1.87	14.66	
Temperature						0.03						0.01
Low	Ref.	—	—	—	—		Ref.	—	—	—	—	
High	2.39	1.10	10.89	1.27	93.52		-2.88	0.56	0.06	0.02	0.17	
Precipitation						0.01						0.83
Low	Ref.	—	—	—	—		Ref.	—	—	—	—	
High	-1.46	0.54	0.23	0.08	0.67		-1.58	0.77	0.21	0.05	0.93	
Temperature*Precipitation						NS						<0.001

[1]Cow and herd included in all models as random effects to correct for potential clustering of quarters within cows and cows within herds. [2]Only cow-level factors were identified. [3]Regression coefficient. [4]Odds ratio. [5]*P*-value for the overall effect. [6]Not significant. [7]Holstein Friesian. [8]Reference category per risk factor. [9]Interaction term between temperature and precipitation.

The prevalence of teat apices colonized with CNS as a group and with *S. chromogenes* in particular was higher than those observed in previous studies focusing on pre-partum TAC with CNS as a group (Piepers et al., 2011) and *S. chromogenes* (De Vliegher et al., 2003), respectively. These differences might partially be explained by using a more suitable and appropriate medium in our study (De Visscher et al., 2013).

A lower percentage of teat apices was colonized with CNS in our previous work (De Visscher et al, 2014) than in the current study in which only teat apices prior to calving were swabbed. Routinely cleaning of the teats before cluster attachment, a flushing effect of the milking process (De Visscher et al., 2014), and the application of post-milking teat disinfection (Piessens et al., 2012; Quirk et al., 2012) may at least partly explain this discrepancy. Further, a higher number of species and some remarkable diversity in species distribution were found in our pre-partum study compared with the studies concerning lactating cows (Braem et al., 2013; De Visscher et al., 2014). *Staphylococcus devriesei*, the predominant species in the current study, was only rarely isolated in one (Braem et al., 2013) and even absent in another study (De Visscher et al., 2014), both focusing on TAC in lactating dairy cows. Although less pronounced as for *S. devriesei*, the same was true for *S. auricularis* (Braem et al., 2013; De Visscher et al., 2014). In turn, *S. cohnii* (Braem et al., 2013; De Visscher et al., 2014), *S. saprophyticus*, *S. simulans* (Braem et al., 2013), *S. xylosus* (Taponen et al., 2008) and *S. fleurettii* (De Visscher et al., 2014) were less commonly isolated from teat apices from non-lactating cows and heifers as opposed to teat apices from lactating dairy cows. The differences between lactating and non-lactating animals are less consistent for *S. chromogenes*, the second most frequently isolated species. *Staphylococcus chromogenes* could not or hardly be isolated from teat apices from lactating dairy cows in some studies (Braem et al., 2013; De Visscher et al., 2014), but was often isolated from bovine teat skin in some other single-dairy-herd studies (White et al., 1989; Taponen et al., 2008). *Staphylococcus haemolyticus*, *S. equorum* (Braem et al., 2013; De Visscher et al., 2014; Vanderhaeghen et al., 2015), and to a lesser extent also *S. sciuri* (Braem et al., 2013; De Visscher et al., 2014) were frequently isolated species from teat apices from both lactating dairy cows and non-lactating cows and heifers. Again, our findings suggest that herds have various CNS microbiota colonizing different habitats implying the potential role of management strategies. Although we have included

herd-level factors in the current study, the number of herds was too small to identify important ones. Other studies specifically designed for analyzing herd-level variables, are needed.

Teat apices of red and white HF as opposed to teat apices of black and white HF dry cows and pregnant heifers were more likely to be colonized with *S. devriesei*. Also, the application of an internal teat sealer, a management tool useful in preventing environmental pathogens invading quarters during the dry period (Huxley et al., 2002), significantly decreased the odds of being colonized with *S. devriesei* in this study. No well-defined interpretation can explain these observed associations, yet they merit further study. *Staphylococcus devriesei* was also significantly more present on teat apices in months with low precipitation and high ambient temperatures comparable to what was observed for *S. haemolyticus*. Because hygiene did not influence TAC with *S. devriesei* nor with *S. haemolyticus* in the current study, an environmental nature of *S. devriesei* and *S. haemolyticus* could be questioned as previously reported for *S. haemolyticus* by Leroy et al. (2015). Strain-typing is necessary to come to better conclusions. As staphylococci prefer a higher temperature to grow (Prescott et al., 2002) and dryness might cause imperceptible teat end lesions, forming a good matrix for expanding the present CNS microbiota, warmth and low precipitation could provide *S. devriesei* and *S. haemolyticus* with good growth conditions.

On the other hand, TAC with *S. equorum* was significantly associated with hygiene, with dirty teat apices being more colonized as opposed to very clean teat apices. In addition, teat apices were more likely to be colonized with *S. equorum* when swabbed under humid conditions in combination with a higher ambient temperature. The latter climatic conditions could support the bacterial load from the environment and thus could increase the exposure of teat apices to *S. equorum* (Smith et al., 1985). Earlier studies also observed a more pronounced presence of *S. equorum* in extramammary habitats (Piessens et al., 2011; De Visscher et al., 2014) as opposed to milk (Supré et al., 2011; Piessens et al., 2011; Fry et al., 2014) and our current findings seem to confirm the environmental nature of *S. equorum*.

Staphylococcus chromogenes was rarely isolated from the bovine environment (Piessens et al., 2011), but was predominantly present in bovine IMI (Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014).

Table 5. Variance components at the herd, cow, and quarter level of the null and final models for teat apex colonization with coagulase-negative staphylococci (CNS) as a group or with 1 of the 4 most frequently isolated species, respectively

	CNS (n = 448)			*S. devriesei* (n = 120)			*S. chromogenes* (n = 107)			*S. haemolyticus* (n = 107)			*S. equorum* (n = 81)		
	Var.est.[1]	± SE[2]	%	Var.est.	± SE	%	Var.est.	± SE	%	Var.est.	± SE	%	Var.est.	± SE	%
Null model															
Herd	0.38	0.22	8.6	0.81	0.43	15.9	0.24	0.18	5.8	0.73	0.40	14.6	1.20	0.63	21.4
Cow	0.74	0.25	16.8	0.98	0.34	19.3	0.64	0.30	15.4	0.97	0.35	19.4	1.11	0.44	19.8
Quarter	3.29		74.6	3.29		64.8	3.29		78.9	3.29		65.9	3.29		58.8
Total variance	4.41		100	5.08		100	4.17		100	4.99		100	5.60		100
Final model															
Herd	0.41	0.23	9.5	0.06	0.13	1.4	0.19	0.16	4.8	0.40	0.27	8.7	0.56	0.37	12.7
Cow	0.62	0.25	14.4	1.02	0.34	23.3	0.51	0.29	12.8	0.91	0.35	19.8	0.57	0.39	12.9
Quarter	3.29		76.2	3.29		75.3	3.29		82.5	3.29		71.5	3.29		74.4
Total variance	4.32		100	4.37		100	3.99		100	4.60		100	4.42		100

[1]Variance estimate. [2]Standard error.

An earlier study (De Vliegher et al., 2003) and the current pre-partum one observed *S. chromogenes* TAC in all included herds. The latter observations all indicate a host-adapted nature of *S. chromogenes*. On the other hand, teat apices from lactating dairy cows and heifers were hardly colonized with *S. chromogenes* (Taponen et al., 2008; Braem et al., 2013; De Visscher et al., 2014). This can partly be explained by a smaller number of included herds, different genotypic methods, or both, yet needs further study.

Most of variation of the outcome variables in the null models resided at the quarter level as reported before for CNS IMI (Piepers et al., 2011; Passchyn et al., 2014). As no quarter-level factors were identified and the variation explained in the final models was low, as seen before (Piepers et al., 2011; Verbeke et al., 2012; Passchyn et al., 2014), factors others than the studied ones play a key role in explaining the likelihood of colonization (or infection). Focusing on teat dimensions for instance might be useful in improving udder health (Zwertvaegher et al., 2013) and a potential pathway is diversity among cows and heifers due to their distinct genetic background (Detilleux, 2009; Verbeke et al., 2012). Differences in susceptibility for CNS IMI among cows and quarters could also rely on differences in immune status (Piepers et al., 2009), which is also partially genetically determined. Still, calculation of the variance components for binary outcome variables is not as straightforward as it is for continuous outcomes (Dohoo et al., 2009) making comparisons with that level not appropriate. Still, we hypothesize that factors at the quarter level rather than at the cow and herd level play a more important role for *S. chromogenes* TAC than for TAC with the other 3 species. For TAC with *S. chromogenes,* more variation was present between cows than between herds when compared with the other species, which is most likely related to the fact that *S. chromogenes* was the sole species colonizing teat apices in all included herds. Regarding the proportion of variation being present at the herd or cow level, analogous patterns could be observed for *S. devriesei* and *S. haemolyticus*. Remarkably, for TAC with *S. equorum* an almost equal amount of the total variation occurred at the herd and cow level despite being an environmental species.

Conclusions

Staphylococcus devriesei, *S. chromogenes*, *S. haemolyticus,* and *S. equorum* were the predominant species colonizing teat apices from dry dairy cows and end-term heifers before calving. *Staphylococcus chromogenes* was present on teat apices in all herds whereas large herd differences were observed for other species. Diverse factors explaining CNS species-specific TAC, yet mostly related to humidity, ambient temperature, and hygiene, substantiate differences in epidemiological behavior and ecology between species. An environmental nature for *S. equorum* is suggested whereas *S. haemolyticus* and *S. devriesei* seem to act as cow-adapted bacteria, as *S. chromogenes* does.

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Chapter 5

Intramammary Infection with Coagulase-Negative *Staphylococcus* Species in Early Lactation and throughout Lactation

Chapter 5.1

Intramammary Infection with Coagulase-Negative Staphylococci at Parturition: Species-Specific Prevalence, Risk Factors, and Impact on Udder Health

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Abstract

Coagulase-negative staphylococci (CNS) are the main cause of bovine intramammary infections (IMI) in many countries. Despite a high prevalence of CNS IMI at parturition, species-specific risk factor studies, relying on accurate identification methods, are lacking. Therefore, this observational study aimed at determining the prevalence and distribution of the different CNS species causing IMI in fresh heifers and dairy cows in Flemish dairy herds and identifying associated species- and subgroup-specific risk factors at the herd, cow, and quarter level. The impact on udder health was investigated as well. *Staphylococcus chromogenes, S. sciuri,* and *S. cohnii* were the most frequently isolated species. The only CNS species causing IMI in fresh heifers and dairy cows in all herds was *S. chromogenes* whereas large between-herd differences in distribution were observed for the other species. Quarters from heifers (versus multiparous cows) and quarters with an inverted teat end (versus good condition) had higher odds of being infected with *S. chromogenes, S. simulans,* and *S. xylosus* and with *S. chromogenes* solely. Pre-partum teat apex colonization with *S. chromogenes* increased the likelihood of *S. chromogenes* IMI in the corresponding quarters at parturition. Quarters with dirty teat apices were more likely to be infected with an environmental CNS species supporting the environmental nature of *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri*. Three species (*S. chromogenes*, *S. simulans,* and *S. xylosus*) were associated with a higher quarter somatic cell count at parturition as opposed to uninfected quarters.

Key words

Dairy cattle, Mastitis, Coagulase-negative *Staphylococcus*, Risk factor

Introduction

Coagulase-negative staphylococci (CNS) are the most prevalent cause of bovine intramammary infections in many countries and are most typically found in milk samples of fresh heifers (De Vliegher et al., 2012). Recent work has shown CNS are, in many aspects, not a homogeneous group (Vanderhaeghen et al., 2014, 2015). As *Staphylococcus chromogenes* is the most prevalent species causing bovine IMI and is rarely isolated from the bovine environment (Piessens et al., 2011, 2012), a host-adapted nature is assumed. In turn, *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* are more commonly present in environmental habitats (Piessens et al., 2011) than in milk (Piessens et al., 2011; Supré et al., 2011; Fry et al., 2014), indicating an environmental ecology. Furthermore, *S. chromogenes*, *S. simulans,* and *S. xylosus* have a more substantial impact on udder health than other species. They can cause a considerable increase in quarter SCC (Supré et al., 2011; Fry et al., 2014). A species-specific distribution of CNS in fresh cows and heifers from different Flemish dairy herds has not yet been described.

Despite the high prevalence of CNS IMI, only a few studies focused on the identification of associated risk factors. One recent study identified CNS group-specific predictors using molecular identification. Yet, the study solely concerned CNS IMI throughout lactation (De Visscher et al., 2015). Other studies only included fresh heifers (Piepers et al., 2011; Verbeke et al., 2012; Passchyn et al., 2014) or were conducted at the CNS group level (Sampimon et al., 2009; Piepers et al., 2011; Verbeke et al., 2012; Passchyn et al., 2014). A need exists to identify species-specific risk factors for CNS IMI. However, large studies are required to reach a sufficient number of isolates per species for this purpose. In the absence of adequate numbers it is defendable to create subgroups of species, e.g. based on a common impact on udder health, a common ecological nature, or a common epidemiological behavior. This approach at least avoids studying CNS as a group, as was commonly done before and respects recent findings indicating that CNS are not a homogenous group.

This observational study aimed at (1) determining the species-specific prevalence and distribution of CNS IMI in fresh heifers and cows in Flemish dairy herds, (2) assessing the variance components of subgroup- and species-specific IMI, and (3) identifying associated subgroup- and species-specific herd-, cow- and quarter-level

risk factors. In addition, (4) the impact on the quarter milk SCC in early lactation was studied for a number of species.

Material and methods

Herd and cows

Thirteen commercial Flemish dairy herds were included. Herd inclusion criteria were (1) participation in the DHI program in Flanders on an annual basis with an interval of 4 to 6 weeks between 2 test-days (CRV, Arnhem, The Netherlands), (2) no pre-partum antibiotic treatment of heifers, and (3) the use of artificial insemination in order to predict the expected calving date as accurate as possible. On each farm, 12 end-term heifers and dry cows per herd (total n = 156) were randomly selected in accordance with the proportion of lactating heifers and cows at the start of the study period (July 2012), resulting in a total of 53 end-term heifers and 103 dry cows. The total study period lasted till February 2013. Detailed herd and cow information can be found elsewhere (De Visscher et al., 2016).

Samples and data collection

Within four days after parturition quarter milk samples (total n = 624) were aseptically collected following the guidelines of the National Mastitis Council (Hogan et al., 1999) for bacteriological culturing and determination of the quarter milk SCC (qSCC). Milk samples were transported under cooled conditions (4°C) to the Mastitis and Milk Quality Research Lab (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium). Quarter milk SCC was immediately determined using a Direct Cell Counter (DCC, Delaval, Belgium).

Various herd- and cow-level risk factors potentially associated with CNS subgroup- or species-specific IMI at parturition were recorded or collected via a questionnaire at the start of the study period (July 2012) (Table 1). The DHI-records allowed for calculating the herd size. An average of 57 cows and heifers were in lactation (range = 30 - 95) per herd (arithmetic mean of the 6 last test-day samples) at the start of the sampling period.

Table 1. Overview of herd-, cow-, and quarter-level variables investigated as risk factors for intramammary infection with (subgroups of) coagulase-negative staphylococci (CNS) at parturition in a cohort of 53 heifers and 103 dairy cows in 13 Flemish dairy herds

Independent variable	Recording method	Description	Breakdown categories in final model
Herd level			
Herd size	DHI-records	Mean number of cows in lactation at start of sampling period[1]	Smaller (< 60 lactating cows) vs. larger (≥ 60 lactating cows) herd[2]
Bulk milk SCC	MCC-records[3]	Mean bulk milk SCC at start of sampling period[4]	Lower bulk milk SCC (< 200 x 10³ cells/mL) vs. higher bulk milk SCC (≥ 200 x 10³ cells/mL)[5]
Cow level			
Housing	Questionnaire	Housing of a specific dry cow or pregnant heifer	Cubicles vs. deep litter
Pasture access	Questionnaire	Pasture access during outdoor season	No pasture vs. pasture
Contact	Questionnaire	Contact with lactation cows before parturition	No contact vs. contact
Breed	DHI-records	Breed	Black and white HF[6] vs. red and white HF
Parity	DHI-records	Parity starting at parturition	Second lactation or older vs. first lactation
Vitamines	Questionnaire	Supplementation with minerals and vitamins before parturition	No supplementation vs. supplementation
Antimicrobials	Questionnaire	Antimicrobials used at drying-off	No vs. narrow-spectrum vs. broad-spectrum[7]
Teat sealer	Questionnaire	Application of an internal teat sealer at drying-off	No teat sealer vs. teat sealer
Teat disinfection	Questionnaire	Teat dipping / spraying before parturition	No dipping / spraying vs. dipping / spraying
Calving pen	Questionnaire	On straw or on pasture	Straw vs. pasture
Ease of calving	Questionnaire	Progress of calving	Unassisted vs. easy pull vs. hard pull
BCS	Visual	Five-point scale[8] at sampling	< 2.5 vs. 2.5-3 vs. > 3
Hygiene	Visual	Hygiene of mammary gland and teats at sampling[9]	Very clean vs. slightly dirty vs. dirty
Temperature	RMI[10]	Monthly ambient temperature (°C) at sampling of a specific dry cow or pregnant heifer	Low vs. high[11]
Precipitation	RMI	Monthly precipitation (L/m²) at sampling of a specific dry cow or pregnant heifer	Low vs. high[11]

Quarter level			
Quarter position	Visual	Position of the quarter	Front vs. hind quarter
Teat apex condition	Visual	Teat apex condition at sampling	Good condition vs. protuberant teat end vs. inverted teat end[12]
Teat skin condition	Visual	Teat skin condition at sampling	Little grooves vs. many grooves[13]
Teat apex colonization	Swabbing and genotypic analysis[14]	Species-specific teat apex colonization 14 days before expected calving date	Colonized with a certain CNS species vs. colonized with another CNS species vs. not colonized

[1]Arithmetic mean based on 6 last DHI-records before the start of the study (July 2012). [2]Categorization based on median value of all mean values of all herds. [3]Milk Control Centre Flanders, Lier, Belgium. [4]Geometric mean based on 6 last records of the Milk Control Centre Flanders before the start of the study. [5]Categorization based on Schukken et al. (2009). [6]Holstein Friesian. [7]Antimicrobials with a narrow spectrum include cloxacillin benzathine, cefalexine, and cefalonium whereas antibiotics with a broad spectrum only include cefquinome. [8]Edmonson et al. (1989). [9]Categorization based on a 5-point scale (Reneau et al., 2005). [10]Royal Meteorological Institute of Belgium. [11]Categorization based on median value of all monthly records from one year (March 2012 till February 2013), i.e. 10°C and 59.35 L/m². [12]Categorization based on a visually scoring system of Neijenhuis et al. (2000) and recoded afterwards. [13]Teat skin condition was scored visually into “little grooves”, i.e. normal, smooth, soft, healthy, shallow grooves, and “many grooves”, i.e. more dry, rough, and with deeper grooves. [14]De Visscher et al., 2016.

Categorization of the herd size was based on the median value of all calculated aforementioned arithmetic means: smaller herds, i.e. < 60 lactating animals, and larger herds, i.e. ≥ 60 lactating animals. Bulk milk SCC was available through the bulk milk quality data of the Milk Control Centre Flanders (MCC Flanders, Lier, Belgium) and recoded into lower bulk milk SCC, i.e. < 200,000 cells/mL, and higher bulk milk SCC, i.e. ≥ 200,000 cells/mL according to Schukken et al. (2009).

At each sampling of fresh cows and heifers (i.e. within 4 days after calving), other potential cow- and quarter-level variables were recorded (Table 1). The Royal Meteorological Institute of Belgium measures a monthly ambient temperature (°C) and precipitation (L/m²). Classification of temperature and precipitation was based on the median of all monthly values during the study period (from July 2012 till February 2013), i.e. 10°C and 59.35 L/m², respectively. Scoring of teat apex condition was performed based on a visual scoring system (Neijenhuis et al., 2000) and recoded afterwards into good condition, a protuberant teat end and an inverted teat end. Teat skin condition was scored visually into “little grooves”, i.e. normal, smooth, soft, healthy, shallow grooves, and “many grooves”, i.e. more dry, rough, and with deeper grooves. Fourteen days before expected calving date, swabs of teat apices (n = 624) have been collected. All swabs were plated on mannitol salt agar (MSA) as described by De Visscher et al. (2016) and examined as described below in order to identify the CNS species colonizing teat apices before parturition.

Laboratory analyses

All quarter milk samples (n = 624) were plated on MSA (Oxoid, Erembodegem, Aalst, Belgium) (one quadrant per milk sample) and aerobically incubated at 37°C (De Visscher et al., 2013) to recover CNS. Plates were examined after 24h and 48h. All phenotypically different colony types were counted and 1 colony per colony type was picked up and sub-cultured on esculin blood agar (Oxoid) (one quadrant per colony) to obtain pure cultures. All potential CNS isolates were stored at -80°C for subsequent analysis or immediately identified to the species level using transfer RNA intergenic spacer PCR (tDNA-PCR) or sequencing of the *16S rRNA* gene if no identification was obtained (Supré et al., 2009).

All quarter milk samples were additionally plated on esculin blood agar (Oxoid) and MacConkey agar (Oxoid) and examined according to the guidelines of the National Mastitis Council (Hogan et al., 1999). This information was used only to

exclude quarters also infected with any major pathogen (*Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus uberis,* other esculin-positive streptococci, *Escherichia coli*, *Klebsiella, Pseudomonas,* yeast and molds) from the subsequent statistical analyses.

Definition quarter IMI status

Quarters were considered to be infected with a specific CNS species when ≥ 1 colony forming unit per 0.01 mL milk of this species was observed on MSA, according to the definition of Dohoo et al. (2011), in order to maximize the sensitivity. Quarters infected with ≥ 3 genotypically different CNS species or with (a) major pathogen(s), were excluded from the subsequent statistical analyses.

Descriptive and statistical analysis

Descriptive data analysis. The prevalence and herd-specific distribution of all CNS species at parturition were described.

Risk factors. Before analyses were performed, observations were checked for unlikely values. Complete data were available from 608 quarters from 99 fresh cows and 53 fresh heifers. Dry cow treatment information was lacking for 3 few cows only as these cows were purchased when dry. Some data were missing due to loss of one questionnaire. However, data belonged to different quarters, cows, and herds.

The proportion of variation for the outcome variables at the herd, cow, and quarter level was estimated for the null models. The variance at the quarter level was assumed $\pi^2/3$ (Goldstein et al., 2002) as described by Piepers et al. (2011).

Logistic multilevel regression models were fit (MLwiN 2.16, Centre for Multilevel Modeling, University of Bristol, Bristol, UK) as follows:

$Y_{ijk} \sim \text{Binomial}(\pi_{ijk})$

$\text{Logit}(\pi_{ijk}) = \beta_{0ijk} + \beta_1 X_{ijk} + v_{0k} + u_{ojk}$

$\text{Var}(Y_{ijk} \mid \pi_{ijk}) = \pi_{ijk}(1 - \pi_{ijk})$ with $v_{0k} \sim \text{Normal}(\mu_k, \sigma_k)$ and $u_{ojk} \sim \text{Normal}(\mu_{jk}, \sigma_{jk})$

where Y_{ijk} is the infectious status of quarter *i*, from cow *j*, from herd *k* and π_{ijk} the probability of this quarter (1) being infected at parturition with *S. chromogenes, S. simulans* or *S. xylosus,* the so-called more relevant CNS species or uninfected or infected with another CNS species, (2) being infected or not at parturition with a so-called host-adapted species *S. chromogenes,* and (3) being infected or not at parturition with a so-called environmental species, i.e. *S. cohnii, S. equorum, S.*

saprophyticus, or *S. sciuri.* Y_{ijk} is a function of the explanatory variable X through the logit function, and approximately follows a binomial distribution; β_{0ijk} is the intercept, i.e. the baseline probability of infection when all predictors are equal to zero; β_1 is the regression coefficient for explanatory variable X; v_{0k} and u_{ojk} are the herd and cow random effects, respectively, and approximately follow normal distributions with respective variances σ_k and σ_{jk}. Reweighted iterative generalized least squares and 1st order penalized quasi-likelihood estimation methods were applied.

Initially, univariable models examining associations between the outcome variables and the independent variables (potential risk factors, see Table 1) were fit and used as a first selection of variables. Statistical significance was assessed at $P < 0.15$. Afterwards, Spearman correlation coefficients among the statistically significant variables were calculated to identify multi-collinearity in the multivariable models. One out of 2 variables was selected, according to biological relevance, for further analysis if a correlation coefficient ≥ |0.6| was calculated. Next, multivariable models were fit for the significant variables from the univariable analysis using backward stepwise elimination with statistical significance assessed at $P < 0.05$. Biological relevant interaction terms were tested between all remaining statistically significant risk factors and kept in the final multivariable model when significant ($P < 0.05$). Also, confounding was investigated. A factor was considered a confounder if its removal caused a relative change > 25% in the regression coefficients of the remaining variables or with a regression coefficient between -0.4 and 0.4 if and absolute change > 0.1 was observed (Noordhuizen et al., 2001).

In order to test the fitness of all final models, the observational-level standardized residuals were plotted against the observational-level predicted values. Additionally, Hosmer-Lemeshow goodness-of-fit tests were assessed on the fixed effect models only (SAS 9.3, SAS Institute Inc., Cary, NC, USA) (Dohoo et al., 2009). The test was statistically significant for none of the final models indicating good fit of our models.

Odds ratios (OR) and 95% confidence intervals were calculated to present the magnitude of the associations.

Impact on quarter milk somatic cell count. A linear mixed regression model was fit (MLwiN 2.16, Centre for Multilevel Modeling, University of Bristol, Bristol, UK), to determine the association between subgroups of CNS species and the natural log transformed qSCC, as follows:

$$Y_{ijk} = \beta_{0ijk} + \beta_1 X_{1ijk} + \beta_2 X_{2ijk} + v_{0k} + u_{ojk} + \varepsilon_{ijk}$$

where Y_{ijk} is the natural log transformed qSCC of quarter *i*, from cow *j*, from herd *k*. Y_{ijk} is a function of the explanatory variables X_1 and X_2 and approximately follows a normal distribution; X_1 is the fixed effect of the infectious status of the quarter (3 levels: uninfected, infected with the less relevant CNS, infected with the more relevant CNS species, i.e. *S. chromogenes, S. simulans,* or *S. xylosus*); X_2 is the fixed effect to adjust for the day of sampling (3 levels: 1st day, 2nd day, 3rd day or later); β_{0ijk} is the intercept (overall mean); β_1 and β_2 are the regression coefficients for explanatory variables X_1 and X_2, respectively; v_{0k} and u_{ojk} are the herd and cow random effects, respectively, and approximately follow normal distributions with respective variances σ_k and σ_{jk} and ε_{ijk} is the random error term, assumed to be normally distributed with mean 0 and variance σ^2. Reweighted iterative generalized least squares were applied. Quarters infected with a major pathogen were excluded from this dataset.

Results

Distribution

Thirty-four percent (n = 211 out of 624 quarters) of all quarter milk samples collected at parturition yielded growth on MSA. Per plate 0 to 5 phenotypically different colony types were present. After tDNA-PCR and/or sequencing of the *16S rRNA* gene, a number of different colony types represented the same CNS species resulting in 19 different species and 191 CNS isolates available for further analysis.

Twenty-six percent of all quarters (n = 163 out of 624 quarters) was infected at parturition with 1 or 2 different CNS species. *Staphylococcus chromogenes* (13% of all quarters and 41% of all isolates, n = 79) was the predominant species, followed by *S. sciuri* (4% and 13%, respectively, n = 25), *S. cohnii* (3% and 11%, respectively, n = 20), *S. equorum* (2% and 7%, respectively, n = 14), *S. xylosus* (2% and 7%, respectively, n = 13), and *S. haemolyticus* (1% and 5%, respectively, n = 9). Phenotypic or genotypic identification revealed 84 non-CNS-isolates on MSA belonging to the phyla *Firmicutes* (*Aerococcus* spp., n = 3; *Bacillus* spp., n = 43; *Jeotgallicoccus* sp., n = 1; *S. aureus*, n = 5; *Streptococcus* spp., n = 22) and *Proteobacteria* (*Pseudomonas*, n = 8; *Psychrobacter*, n = 2).

In each herd, between 3 (herd 9) and 10 (herd 6) different CNS species were isolated. The only CNS species causing IMI at parturition in all herds was *S.*

chromogenes. *Staphylococcus sciuri* and *S. xylosus* were infecting quarters in 10 herds, whereas *S. cohnii* and *S. equorum* both caused IMI in 7 herds. *Staphylococcus haemolyticus* could only be isolated from IMI in 6 herds. Other species were only causing IMI on a minority of farms (Table 2 and Figure 1).

The majority of fresh heifers (74%, n = 39 out of 53 heifers) were diagnosed with a CNS IMI at parturition in at least 1 quarter whereas only half of the multiparous cows were infected with CNS (46%, n = 47 out of 103 multiparous cows). Thirty-seven percent (n = 79 out of 212 quarters) and 20% (n = 84 out of 412 quarters) of all quarters of heifers and older cows, respectively, had a CNS IMI. *Staphylococcus hyicus* only caused IMI in quarters from heifers whereas *S. auricularis*, *S. capitis*, *S. devriesei*, *S. hominis*, *S. lentus*, *S. pasteuri*, *S. vitulinus,* and *S. warneri* were only isolated from IMI from quarters from multiparous cows (Table 2). All aforementioned species were, however, only rarely present in quarter milk at parturition. A considerable higher percentage of heifers had quarters infected with the so-called more relevant CNS (*S. chromogenes*, *S. simulans,* and *S. xylosus*) (60% heifers, n = 32 out of 53 heifers and 28% quarters, n = 60 out of 212 quarters) as opposed to multiparous cows (27% cows, n = 28 out of 103 older cows and 8% quarters, n = 35 out of 412 quarters). An almost equal amount of heifers and multiparous cows harbored an environmental species (30% heifers, n = 16 out of 53 heifers and 11% quarters, n = 23 out of 212 quarters versus 28% cows, n = 29 out of 103 older cows and 9% quarters, n = 38 out of 212 quarters). In contrast, *S. chromogenes* was predominantly isolated from quarters from heifers as opposed to multiparous cows (51% heifers, n = 27 out of 53 heifers and 25% quarters, n = 53 out of 212 quarters versus 19% cows, n = 20 out of 103 older cows and 6% quarters, n = 26 out of 412 quarters) (Table 2).

Risk factors

For “IMI with the relevant species” and “IMI caused by *S. chromogenes* only”, no variation occurred at the herd level whereas for “IMI with the environmental species”, variation was observed at all three levels in the null model (Table 3).

Fitting the univariable models revealed 7 cow-level and 2 quarter-level risk factors to be associated with IMI at parturition both with the more relevant species as well as with *S. chromogenes* only.

Table 2. Species distribution of coagulase-negative staphylococci causing intramammary infection at parturition isolated from 624 quarters from 53 fresh heifers and 103 fresh multiparous cows in 13 Flemish dairy herds

Species	Herd 1		Herd 2		Herd 3		Herd 4		Herd 5		Herd 6		Herd 7		Herd 8		Herd 9		Herd 10		Herd 11		Herd 12		Herd 13		Total	
	$\%_C$[1]	$\%_H$[2]	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	$\%_C$	$\%_H$	N	%
S. chromogenes	6 .3	18 .8	3 .6	20.0	8 .3	25 .0		31 .3	3 .6	30.0	11 .1	25 .0	3 .1	25 .0	6 .3	18 .8	5 .6	16 .7	10 .7	20.0	6 .3	56 .3	12 .5	12 .5	5 .0	25 .0	79	41.4
S. sciuri	3 .1		3 .6	25 .0	4 .2				7 .1	10.0	5 .6	16 .7	6 .3	6 .3		12 .5	2 .8				3 .1				2 .5	12 .5	25	13.1
S. cohnii	12 .5		7 .1	5 .0	4 .2	4 .2	15 .6			5 .0	8 .3								3 .6	5 .0							20	10.5
S. equorum	3 .1	12 .5							7 .1		5 .6		6 .3		3 .1				3 .6	10.0		6 .3					14	7.3
S. xylosus				5 .0		4 .2	3 .1	6 .3	7 .1				3 .1	6 .3		6 .3	2 .8			5 .0		6 .3			2 .5		13	6.8
S. haemolyticus		6 .3	3 .6	5 .0			3 .1				2 .8		3 .1										6 .3	6 .3			9	4.7
S. arlettae				5 .0									9 .4	12 .5													6	3.1
S. simulans	3 .1						3 .1								3 .1					5 .0						12 .5	5	2.6
S. saprophyticus	3 .1											8 .3	6 .3														4	2.1
S. devriesei											2 .8		3 .1						3 .6								3	1.6
S. vitulinus							3 .1				2 .8																2	1.0
S. epidermidis															3 .1									6 .3			2	1.0
S. hominis							6 .3																				2	1.0
S. lentus	6 .3																										2	1.0
S. auricularis													3 .1														1	0.5
S. capitis																							3 .1				1	0.5
S. hyicus					4 .2																						1	0.5
S. warneri											2 .8																1	0.5
S. pasteuri											2 .8																1	0.5

[1]Percentage of quarters of fresh cows infected with a specific species within each herd. [2] Percentage of quarters of fresh heifers infected with a specific species within each herd.

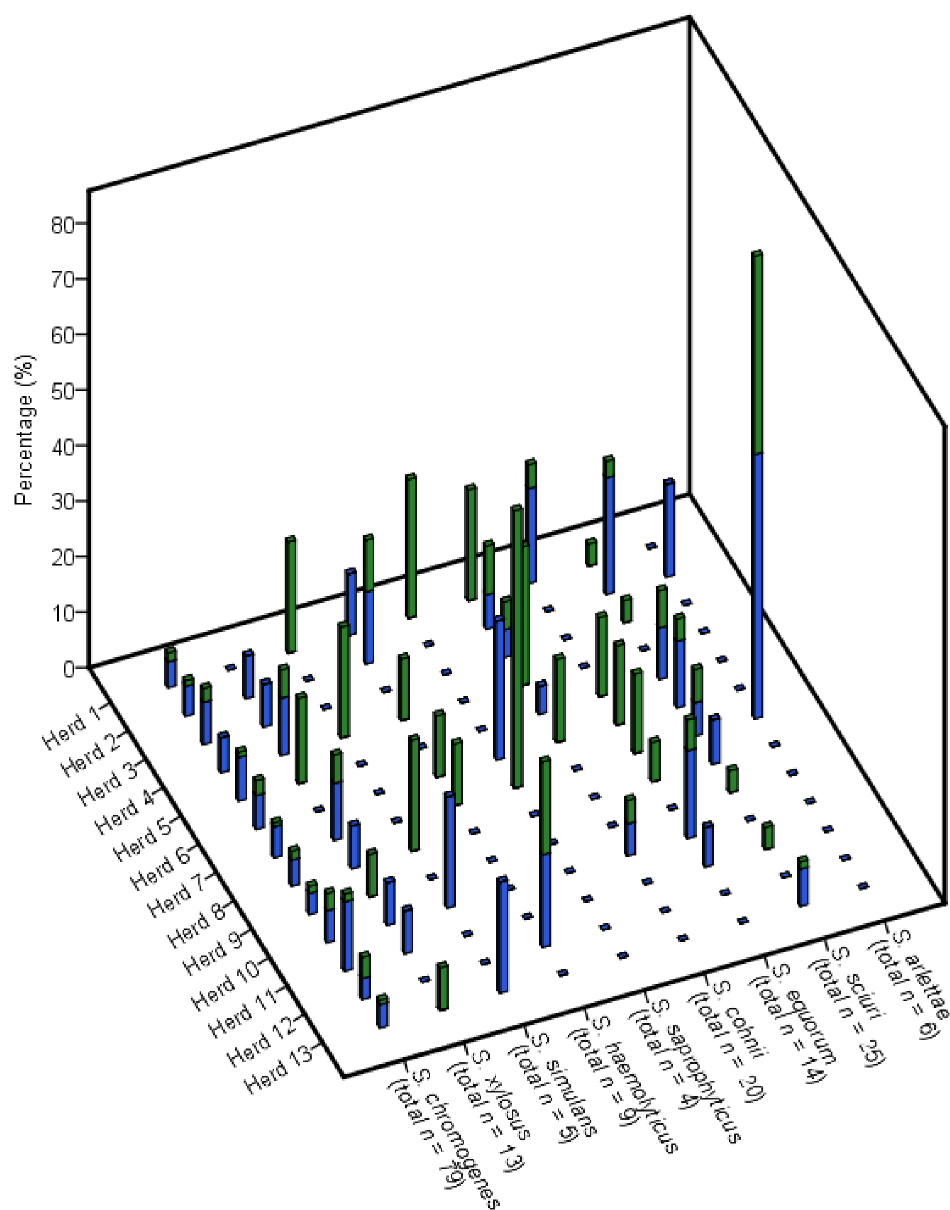

Figure 1. Species distribution of the most frequently isolated coagulase-negative staphylococci (CNS) causing intramammary infections (IMI) at parturition in 13 Flemish dairy herds: percentages of quarters positive for a certain CNS species per herd among the total number of quarters positive for a certain CNS species are shown. Percentages are divided according to the proportion of IMI from fresh heifers (blue) and fresh dairy cows (green), respectively, caused by a certain species per herd.

Only 3 cow-level factors were unconditionally associated with IMI with the so-called environmental species (Table 4). The risk factors “antimicrobials” (reflecting whether or not narrow- or broad-spectrum antibiotics are used at drying-off) and “parity” were correlated (Spearman rho = 0.85). The latter was expected as no antibiotics were administered to end-term heifers which is in contrast with the multiparous cows, all except one receiving antibiotics at drying-off. Only “parity” was used in the subsequent models.

Table 3. Variance components at the herd, cow, and quarter level of the null models for intramammary infections with (subgroups of) coagulase-negative *Staphylococcus* (CNS) species[1]

	Staphylococcus chromogenes, S. simulans, S. xylosus (n = 95)[1]			*Staphylococcus chromogenes* (n = 79)[1]			*Staphylococcus cohnii, S. equorum, S. saprophyticus, S. sciuri* (n = 61)[1]		
	Var.est.[2]	± SE[3]	%	Var.est.	± SE	%	Var.est.	± SE	%
Herd	0.00	0.00	0.0	0.00	0.00	0.0	0.30	0.25	6.9
Cow	1.23	0.38	27.2	1.73	0.51	34.5	0.78	0.44	17.8
Quarter	3.29	-	72.8	3.29	-	65.5	3.29	-	75.3
Total Variance	4.52		100	5.02		100	4.37		100

[1]*Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus*, represent the CNS species being more relevant for udder health; *S. chromogenes* is representative for the host-adapted species; and *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* represent the environmental species. [2]Variance components. [3]Standard Error.

Table 5 presents results from the final multilevel, multivariable logistic regression models. Quarters from heifers were more likely to be infected with the more relevant species and with *S. chromogenes*, representing the host-adapted species, as opposed to multiparous cows (OR = 3.9; 95% CI: 2.21-7.04 and OR = 4.2; 95% CI: 2.08-8.33, respectively). Quarters with an inverted teat end had increased odds of being infected with the more relevant species and solely *S. chromogenes* as opposed to quarters with a good teat end condition (OR = 2.8; 95% CI: 1.36-5.91 and OR = 3.7; 95% CI: 1.52-8.79, respectively). Quarters with teat apices colonized with *S. chromogenes* prior to calving had higher odds of being infected at parturition with this same species (OR = 3.3; 95% CI: 1.41-7.50). Quarters with dirty teat apices were more likely to be infected with an environmental CNS species (OR = 6.4; 95% CI: 1.31-30.90) as opposed to quarters with clean teats. In contrast, IMI with *S.*

chromogenes, representing the host-adapted species, were not significantly associated with the hygiene of the teats.

Post-hoc power and sample calculation

A post-hoc power calculation has been made: 624 quarters were included in the study and a prevalence of 26% was observed. The more relevant species, the host-adapted species, and the so-called environmental species were frequently isolated subgroups of CNS species: 51%, 41%, and 33%, respectively, resulting in approximately 53 to 83 quarters infected with those isolates. With 53 positive quarters, the logistic regression model had 80% power to detect an association corresponding to an odds ratio of 2.3 ($\alpha = 0.05$) whereas with 83 positive quarters, the logistic regression model had 80% power to detect an association corresponding to an odds ratio of 2.1 ($\alpha = 0.05$).

Quarter milk somatic cell count

The geometric mean qSCC at parturition were 218 x 10^3 cells/mL (range = 8 - 5,596 x 10^3 cells/mL), 173 x 10^3 cells/mL (range = 20 - 5,887 x 10^3 cells/mL) and 442 x 10^3 cells/mL (range = 37 - 5,093 x 10^3 cells/mL) for the uninfected quarters (n = 409), the quarters infected with the less relevant CNS (n = 58) and the quarters infected with the more relevant CNS species (*S. chromogenes*, *S. simulans,* and *S. xylosus*) (n = 86), respectively. Quarters infected at parturition with the more relevant CNS species had a significantly higher qSCC as opposed to uninfected quarters, i.e. 528 x 10^3 cells/mL versus 240 x 10^3 cells/mL (LSM = 6.3 vs. LSM = 5.5, $P < 0.001$). The qSCC at parturition of quarters infected with a less relevant CNS species was not different from the qSCC of uninfected quarters, i.e. 235 x 10^3 cells/mL versus 240 x 10^3 cells/mL (LSM = 5.5 vs. LSM = 5.5, $P < 0.001$) (Table 6).

Discussion

This large observational study describes the species-specific prevalence and distribution of CNS IMI immediately after parturition in both heifers and multiparous cows in a number of Flemish dairy herds and substantiates the relevance for udder health of some of the CNS species.

Table 4. Univariable, multilevel logistic regression models[1] for intramammary infection at parturition with (subgroups of) coagulase-negative *Staphylococcus* (CNS) species[2]

Independent variable	*Staphylococcus chromogenes, S. simulans, S. xylosus* (n = 95)[2]					*Staphylococcus chromogenes* (n = 79)[2]					*Staphylococcus cohnii, S. equorum, S. saprophyticus, S. sciuri* (n = 61)[2]				
	N_{nCNS}[3]	N_{CNS}[4]	OR[5]	95% CI		N_n[6]	N_{CNS}	OR	95% CI		N_n	N_{CNS}	OR	95% CI	
Herd level															
Herd size															
Smaller	244	44	Ref.[7]			214	40	Ref.			214	27	Ref.		
Larger	285	51	0.99	0.55	1.79	247	39	0.83	0.42	1.65	247	34	1.2	0.47	3.04
Bulk milk SCC															
Lower	280	56	Ref.			241	47	Ref.			241	34	Ref.		
Higher	249	39	0.82	0.45	1.48	220	32	0.77	0.39	1.53	220	27	0.80	0.32	2.03
Cow level															
Housing															
Cubicles	328	72	Ref.*			280	60	Ref.*			280	46	Ref.*		
Deep litter	201	23	0.53	0.28	1.01	181	19	0.52	0.25	1.09	181	15	0.50	0.22	1.13
Pasture access															
No	44	16	Ref.*			32	15	Ref.*			32	9	Ref.		
Yes	485	79	0.44	0.18	1.06	429	64	0.31	0.11	0.86	429	52	0.46	0.14	1.47
Contact															
No	246	30	Ref.*			215	25	Ref.*			215	26	Ref.		
Yes	283	65	1.9	1.75	1.98	246	54	1.8	0.91	3.68	246	35	1.2	0.58	2.51
Breed															
Black and white HF[8]	447	81	Ref.			384	66	Ref.			384	58	Ref.*		
Red and white HF	82	14	0.91	0.40	2.09	77	13	0.90	0.35	2.32	77	3	0.27	0.07	1.05
Parity															
≥ 2nd lactation	377	35	Ref.*			328	26	Ref.*			328	38	Ref.		

1st lactation	152	60	4.3	2.41	7.64	133	53	5.0	2.55	9.95	133	23	1.5	0.75	2.81
Vitamines															
No	85	11	Ref.			77	9	Ref.			77	8	Ref.		
Yes	444	84	1.4	0.58	3.17	384	70	1.4	0.54	3.78	384	53	1.4	0.38	5.02
Antimicrobials[9]															
No	156	60	Ref.*			137	53	Ref.*			137	23	Ref.		
Narrow-spectrum	215	17	0.20	0.10	0.41	187	13	0.17	0.08	0.40	187	24	0.69	0.33	1.46
Broad-spectrum	148	16	0.28	0.13	0.59	128	11	0.23	0.09	0.56	128	13	0.73	0.29	1.82
Teat sealer[9]															
No	373	79	Ref.*			330	65	Ref.*			330	42	Ref.		
Yes	146	14	0.44	0.21	0.95	122	12	0.52	0.21	1.24	122	18	1.1	0.46	2.44
Teat disinfection															
No	404	68	Ref.			351	56	Ref.			351	49	Ref.		
Yes	125	27	1.3	0.68	2.57	110	23	1.4	0.65	3.01	110	12	0.91	0.37	2.27
Calving pen															
Straw	512	92	Ref.			448	76	Ref.			448	61	Ref.		
Pasture	17	3	1.0	0.19	5.29	13	3	1.6	0.26	9.94	13		/[10]	/	/
Ease of calving															
Unassisted	260	40	Ref.*			231	36	Ref.			231	25	Ref.		
Easy pull	204	32	1.1	0.56	2.07	175	25	0.96	0.45	2.06	175	27	1.3	0.65	2.63
Hard pull	65	23	2.4	1.04	5.29	55	18	2.1	0.81	5.63	55	9	1.4	0.50	3.80
BCS[11]															
< 2.5	45	7	Ref.			35	7	Ref.			35	9	Ref.		
2.5-3	453	83	1.1	0.38	3.40	397	68	0.80	0.24	2.67	397	51	/	/	/
> 3	28	4	0.84	0.15	4.83	27	4	0.63	0.09	4.32	27		/	/	/
Hygiene[11]															
Very clean	70	6	Ref.			68	4	Ref.*			68	2	Ref.*		
Slightly dirty	222	34	1.7	0.57	5.24	197	29	2.3	0.61	8.39	197	21	1.2	0.47	3.04
Dirty	234	54	2.6	0.88	7.63	194	46	0.90	0.45	1.78	194	37	6.4	1.31	30.90
Temperature															
Low	84	20	Ref.			72	17	Ref.			72	13	Ref.		

High	445	75	0.74	0.35	1.60	389	62	0.72	0.30	1.76	389	48	0.71	0.30	1.66
Precipitation															
Low	385	71	Ref.			338	59	Ref.			338	42	Ref.		
High	144	24	0.87	0.44	1.70	123	20	0.92	0.42	2.01	123	19	1.4	0.66	2.83
Quarter level															
Quarter position															
Front	261	51	Ref.			230	41	Ref.			230	28	Ref.		
Hind	268	44	0.83	0.52	1.31	231	38	0.92	0.55	1.54	231	33	1.2	0.66	1.99
Teat end condition[11]															
Good	461	64	Ref.*			403	51	Ref.*			403	47	Ref.		
Protuberant	10	3	1.8	0.37	8.95	9	3	1.8	0.31	10.54	9		/	/	/
Inverted	55	27	3.6	0.45	1.46	47	25	4.2	1.76	9.82	47	13	/	/	/
Teat skin condition[11]															
Litte grooves	300	51	Ref.			266	43	Ref.			266	28	Ref.		
Many grooves	226	43	1.1	0.63	2.05	193	36	1.2	0.60	2.29	193	32	1.4	0.70	2.66
Teat apex colonization															
Not colonized	152	24	Ref.*			133	20	Ref.*			133	22	Ref.		
Colonized with another species[12]	274	40	0.96	0.52	1.79	261	32	0.89	0.44	1.81	218	25	0.56	0.28	1.12
Colonized with the same species[13]	103	31	1.9	0.98	3.80	67	27	3.0	1.34	6.58	110	14	0.66	0.29	1.49

[1]Cow and herd included in all models as random effects to correct for potential clustering of quarters within cows and cows within herds. [2]*Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus*, represent the CNS species being more relevant for udder health; *S. chromogenes* is representative for the host-adapted species; and *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* represent the environmental species. [3]Number of quarters uninfected with CNS or infected with species other than the one of the subgroup at parturition. [4]Number of IMI at parturition caused by different subgroups of CNS, respectively. [5]Odds ratios are presented and * indicates an overall *P*-value < 0.15. [6]Number of quarters uninfected with CNS at parturition. [7]Reference category per risk factor. [8]Holstein Friesian. [9]Missing data from 3 dry cows. [10]Model did not fit due to too low numbers. [11]Missing data from 1 dry cow. [12]Colonized with species other than the ones of the subgroups, respectively. [13]Colonized with species of the different subgroups, respectively.

For the first time potentially associated subgroup- and species-specific risk factors for IMI at calving were investigated. Including a large number of quarters and the use of molecular speciation provide valuable and precise information on CNS IMI in fresh cows and heifers, and adds to the existing knowledge on the ecology and epidemiology of bovine-associated CNS.

The percentage of CNS-infected quarters of fresh heifers (37%) approached the 36% (Rajala-Schultz et al., 2004) and 35% (Piepers et al., 2010) of previous studies, exceeded the 10% of Compton et al. (2007), but was lower than the high prevalence of 74% reported by Taponen et al. (2007). In the latter study, however, samples were collected at the day of calving. The prevalence of CNS-infected quarters of multiparous cows (20%) was almost identical to the 16% (Rajala-Schultz et al., 2004) and 20% (Taponen et al., 2007) reported by other research groups.

The most prevalent species at parturition was *S. chromogenes*, confirming earlier reports (Taponen et al., 2007; Rajala-Schultz et al., 2009). *Staphylococcus sciuri* was the second most frequently isolated species, but was hardly isolated in previous genotypic work, collecting milk samples within 2 weeks after calving (Fry et al., 2014). The same was true for *S. equorum*. *Staphylococcus cohnii*, *S. xylosus,* and *S. haemolyticus* were commonly identified by the current study and by Fry et al. (2014). We rarely observed *S. simulans* and *S. epidermidis*, which might be related to the limited number of herds in our study (n = 13) compared to the Fry et al. (2014) study (n = 89).

Some data were missing though this was not due to bias and data belonged to different quarters, cows, and herds. It is not as straightforward as it is for continuous outcomes to calculate the variance components for binary outcome variables (Dohoo et al., 2009) making comparisons with that level not entirely correct. Yet, no variation occurred between herds in the models looking at IMI with the relevant species (mainly *S. chromogenes*, though) and with *S. chromogenes* only, which can be explained by the fact that *S. chromogenes* caused IMI in all herds. In contrast, variation resided both between cows and between herds in the models with IMI with the environmental species as outcome variables, indicating a more random infection process for those species. No herd-level independent factors could be identified in the current study due to the number of herds included (n = 13), being too low for herd-level studies as no variation at the herd level could be observed.

Table 5. Final multivariable, multilevel logistic regression models[1] describing herd-, cow- and quarter-level risk factors associated with intramammary infections at parturition with (subgroups of) coagulase-negative *Staphylococcus* (CNS) species[2]

Independent variable	*Staphylococcus chromogenes, S. simulans, S. xylosus* (n = 95)[2]						*Staphylococcus chromogenes* (n = 79)[2]						*Staphylococcus cohnii, S. equorum, S. saprophyticus, S. sciuri* (n = 61)[2]					
	β[3]	SE	OR[4]	95% CI		*P*[5]	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*
Intercept	-2.62	0.22				<0.001	-3.08	0.38				<0.001	-3.64	0.78				<0.001
Herd level[6]																		
Cow level																		
Parity						<0.001						<0.001						NS[7]
≥ 2nd lactation	Ref.[8]	—	—	—	—		Ref.	—	—	—	—							
1st lactation	1.37	0.30	3.9	2.21	7.04		1.43	0.35	4.2	2.08	8.33							
Hygiene						NS						NS						0.05
Very clean													Ref.	—	—	—	—	
Slightly dirty													1.37	0.81	3.9	0.80	19.38	
Dirty													1.85	0.81	6.4	1.31	30.90	
Quarter level																		
Teat end condition						0.02						0.01						NS
Good	Ref.	—	—	—	—		Ref.	—	—	—	—							
Protuberant	0.44	0.82	1.6	0.31	7.73		0.66	0.92	1.9	0.32	11.78							
Inverted	1.04	0.38	2.8	1.36	5.91		1.30	0.45	3.7	1.52	8.79							
Teat apex colonization						NS						<0.01						NS
Not colonized							Ref.	—	—	—	—							
Colonized with another species[9]							-0.02	0.38	0.99	0.47	2.07							
Colonized with the same species[10]							1.18	0.43	3.3	1.41	7.50							

[1]Cow and herd included in all models as random effects to correct for potential clustering of quarters within cows and cows within herds. [2]*Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus*, represent CNS species being more relevant for udder health; *S. chromogenes* is representative for the host-adapted species; and *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* represent the environmental species. [3]Regression coefficient. [4]Odds ratio. [5]*P*-value for the overall effect. [6]No herd-level variables were identified. [7]Not significant. [8]Reference category per risk factor. [9]Colonized with species other than the ones of the subgroups, respectively. [10]Colonized with species of the different subgroups, respectively.

A post-hoc calculation has been made and revealed a power of 80% to detect associations corresponding to an odds ratio between 2.1 and 2.3 ($\alpha = 0.05$) in case of 83 or 53 infected quarters, respectively.

Table 6. Final multilevel linear regression model[1] describing subgroups of coagulase-negative *Staphylococcus* species (CNS) at parturition associated with the natural log transformed quarter SCC

Independent variable	β^2	SE	LSM	95% CI		*P*-value[3]
Intercept	5.77	0.16				<0.001
Infectious status[4]						<0.001
Negative	Ref.					
Less relevant CNS	-0.03	0.15	5.5	5.14	5.78	
More relevant CNS	0.79	0.14	6.3	5.99	6.55	
Days in milk						<0.001
1st day	Ref.					
2nd day	-0.15	0.23	5.9	5.53	6.23	
3rd day or later	-0.72	0.18	5.3	5.08	5.54	

[1]Cow and herd included in the model as random effects to correct for potential clustering of quarters within cows and cows within herds. [2]Regression coefficient. [3]*P*-value for the overall effect. [4]*Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus*, represent the CNS species being more relevant for udder health. CNS species other than the 3 aforementioned ones are considered less relevant for udder health.

Supplementation with vitamins, ease of calving (Passchyn et al., 2014) and teat dipping before parturition (Piepers et al., 2011) were associated with the likelihood of CNS IMI in heifers, but were not important in the current study. The same was true for pasturing during outdoor season (Sampimon et al., 2009). On the other hand, housing hygiene increasing the odds of CNS IMI in heifers at parturition (Piepers et al., 2011), was significantly associated with IMI with the so-called environmental CNS species. The latter observation again reinforced the environmental ecology of *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri*, and emphasizes the value of studying CNS at the subgroup- or species-level. The more relevant species were significantly more frequently observed in milk from quarters with an inverted teat end. Teat end shape has previously been linked to a higher prevalence of IMI (Seykora and McDaniel, 1985). Milk deposits on the teat end most likely provide a good growth substrate for bacteria and a larger diameter of the streak canal, associated with inverted teats, allows easier access for bacteria.

Teat apex colonization with *S. chromogenes* significantly increased the odds of *S. chromogenes* IMI at parturition, a phenomenon that was not observed for the other species (data not shown). The latter illustrates the host-adapted nature of *S. chromogenes* as teat apices might act as a habitat for host-adapted species. This has been suggested before for *S. aureus* IMI at parturition (Roberson et al., 1994) and for *S. chromogenes* IMI throughout lactation (Taponen et al., 2008). An earlier study from our group focusing on *S. chromogenes,* not using molecular speciation, did not demonstrate this link (De Vliegher et al., 2003). To come to better conclusions, strain-typing of all isolates should be performed, and is currently ongoing.

It has previously been shown that CNS-infected quarters have a higher SCC as opposed to negative control quarters (Taponen et al., 2007; Gillespie et al., 2009; Schukken et al., 2009). However, recent research reported an impact on the qSCC depending on the CNS species involved (Sampimon et al., 2009; Thorberg et al., 2009; Simojoki et al., 2011). Our findings confirm this variation between species. Actually, the more relevant species, *S. chromogenes*, *S. simulans,* and *S. xylosus* induced a higher qSCC at parturition, as was reported before (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2015). We also confirmed that quarters infected with species other than the so-called relevant ones have a qSCC that is not different from the qSCC of uninfected quarters (Supré et al., 2011; Fry et al., 2014). A limitation of CNS research is the fact that the sensitivity can only reach a maximum of 86.7% (Dohoo et al., 2011), indicating some quarters classified as negative ones can be CNS-infected. The latter can explain the rather high qSCC of the negative quarters observed in this study. However, in order to avoid an impact of a major pathogen on the qSCC, all quarters infected with a major pathogen were removed from the dataset.

Conclusions

Staphylococcus chromogenes, S. sciuri, and *S. cohnii* were the predominant species causing IMI in fresh heifers and dairy cows. The only CNS species isolated from milk in all herds was *S. chromogenes*; the presences of other species differed by herd. The environmental nature was supported for *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* whereas the host-adapted nature of *S. chromogenes*

was substantiated. Pre-partum teat apex colonization with *S. chromogenes* increased the likelihood of *S. chromogenes* IMI in the corresponding quarters at parturition. The more relevant species increased the quarter SCC at parturition as opposed to uninfected quarters.

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Chapter 5.2

Species Group-Specific Predictors at the Cow and Quarter Level for Intramammary Infection with Coagulase-Negative Staphylococci in Dairy Cattle Throughout Lactation

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Abstract

Coagulase-negative staphylococci (CNS) are frequently isolated from quarters with subclinical mastitis, teat apices and the cows' environment. Virulence, ecology, epidemiological behavior, and effect on udder health vary between the different species. *Staphylococcus chromogenes*, *S. simulans* and *S. xylosus* are frequently present in milk and have a more substantial effect on quarter milk somatic cell count than other species. Therefore, these species are considered as the “more relevant” CNS. As species-specific factors associated with CNS intramammary infection (IMI) have not yet been identified and susceptibility for IMI differs between cows and quarters, this study aimed to identify predictors for CNS IMI at the cow and quarter level (some of them changing over time) with a specific focus on the aforementioned more relevant CNS. Precise data were available from a longitudinal study (3,052 observations from 344 quarters from 86 dairy cows belonging to three commercial dairy herds). All CNS were molecularly identified to the species level, and multivariable, multilevel logistic regression models taking into account the longitudinal nature of the data, were fit to study the likelihood of infection. *Staphylococcus chromogenes, S. xylosus* and *S. cohnii* were the most frequently isolated species from CNS IMI in older cows whereas *S. chromogenes*, *S. xylosus* and *S. simulans* were the main species found in IMI in heifers. Quarters from heifers (as opposed to multiparous cows), from heifers and multiparous cows in third or fourth month in lactation (as opposed to early lactation, < 60 days in milk), and with an increasing quarter milk SCC were more likely to be infected with the more relevant CNS species. Quarter milk SCC was identified as the sole statistically significant predictor for IMI with other CNS species, although the size of the effect was lower [odds ratio of 1.6 (1.4 - 1.9) as compared with 2.1 (1.8 - 2.5)] than the effect for IMI with the more relevant CNS. As a strong herd effect was present, studying herd-level predictors is warranted.

Key words

Predictors, Coagulase-negative *Staphylococcus* species, Intramammary infection

Introduction

Coagulase-negative staphylococci (CNS) are the most frequently isolated bacteria from subclinical mastitis cases in dairy cows (Makovec and Ruegg, 2003; Piepers et al., 2007; Sampimon et al., 2009a). In addition, they are abundantly present in the cows' environment (Piessens et al., 2011) and on their teat apices (Taponen et al., 2008; Braem et al., 2012). Several factors associated with CNS-group IMI have been identified (Sampimon et al., 2009a; Piepers et al., 2011), but in none of the studies were CNS accurately identified to the species level and one study only included heifers. As species-specific differences in protective effects, (presumed) virulence, antimicrobial resistance, persistence, and effect on udder health have been revealed (Sampimon et al., 2011; Vanderhaeghen et al., 2014, 2015), studying CNS as a group is no longer recommended. Rather, accurate species-level studies or studies at the subgroup level, when analyses at the species level are hindered by small numbers, are needed.

Intramammary infection with *Staphylococcus chromogenes*, *S. simulans* and *S. xylosus* is associated with a more pronounced increase in SCC compared with other species (Supré et al., 2011; Fry et al., 2014), suggesting these species have a more substantial effect on udder health and are for that reason considered as more relevant than others. Differences in susceptibility for CNS IMI between cows and quarters exist as some cows in a specific herd have no CNS infected quarters whereas others have multiple quarters infected with CNS (Piessens et al., 2011; Supré et al., 2011), indicating the likelihood of infections is explained not only by CNS traits but also by host factors.

The aim of this study was to identify cow- and quarter-level predictors for IMI throughout lactation caused by the more relevant CNS species for udder health, *S. chromogenes*, *S. simulans* and *S. xylosus*, as well as other species.

Material and methods

Data were obtained from a longitudinal study conducted between September 2007 and January 2009 in 3 commercial dairy herds in Flanders (Belgium). On all farms, cows were housed in freestall barns with cubicles with sawdust bedding and a concrete slatted floor. Postmilking teat disinfection and blanket dry cow therapy

combined with application of an internal teat sealant were applied. Detailed herd information can be found elsewhere (Supré et al., 2011; De Visscher et al., 2014). Single quarter milk samples were aseptically collected every month for determining the quarter milk SCC (qSCC) and for bacteriological culturing. Several potential cow- and quarter-level predictors were recorded at each sampling occasion, some of them changing over time (observation level, lowest level of the dataset; Table 1).

Table 1. Overview of all potential cow-, quarter- and observation-level predictor variables (independent variables)

Independent variable	Recording method	Description	Breakdown of categories in final model
Cow level			
Breed	DHI-records	Two breeds	Black and white HF[1] vs. red and white HF
Quarter level			
Quarter position	Visual	Position of the quarter	Front vs. hind quarter
Observation level			
Parity[2]	DHI-records	Parity at test-day	1st lactation vs. 2nd lactation or older
Milk yield[3]	DHI-records	Milk yield at test-day	Low[4] (< 33.35 kg) vs. high (≥ 33.35 kg)
Stage of lactation[3]	DHI-records	Stage in lactation at test-day	1 - 60 DIM vs. 61 - 120 DIM vs. 121 - 180 DIM vs. > 180 DIM
BSC[3]	Visual and palpation	Five-point scale[5]	< 2.5 vs. 2.5 - 3.5 vs. > 3.5
Quarter milk SCC[6]	Fossomatic 5000/FC[7]	Quarter SCC at test-day	Continuous
Teat skin condition[6]	Visual and palpation	Nine-point scale[8]	Without cracks vs. with cracks
Teat apex condition[6]	Visual and palpation	Nine-point scale[8]	Without cracks vs. with cracks

[1]Holstein Friesian. [2]Cow-level variable potentially changing during the study. [3]Cow-level variable changing during the study. [4]Categorization based on median value of all test-day records of all cows. [5]Edmonson et al. (1989). [6]Quarter-level variable changing during the study. [7]Foss, Hillerød, Denkmark. [8]De Vliegher et al. (2003).

Animals with a test-day milk yield ≥ 33.35 kg/day (i.e. the median value of all test-day records of all animals) were defined as high producing, whereas animals with a test-day milk yield < 33.35 kg/day were defined as low producing. Stage in lactation was categorized as described by Zadoks et al. (2001): 1 = 1 - 60 DIM; 2 = 61 - 120 DIM; 3 = 121 - 180 DIM; 4 ≥ 180 DIM. Body condition score of each animal was scored on a 5-point scale divided into quarter-point increments as described by

Edmonson et al. (1989). Recoding was applied for further analyses (1 = ≤ 2.5; 2 = 2.5 - 3.5; 3 = ≥ 3.5). The teat skin and teat apex condition were determined based on a 9-point scale visual scoring system (De Vliegher et al., 2003) and recoded (0 = without cracks; 1 = with cracks). Quarter milk SCC was determined at the Flanders Milk Control Centre (Lier, Belgium) using a Fossomatic 5000/FC (Foss Electric, Hillerød, Denmark). Bacteriological culturing of all milk samples was performed following National Mastitis Council guidelines (Hogan et al., 1999). All CNS were identified to the species level using transfer RNA intergenic spacer PCR (tDNA-PCR) (Supré et al., 2009) and IMI status were assigned based on subsequent samplings as described by Supré et al. (2011). *Staphylococcus chromogenes*, *S. simulans*, and *S. xylosus* were considered as CNS species with a more substantial effect on udder health (Supré et al., 2011; Fry et al., 2014) and referred to as “more relevant” species throughout the paper; all other CNS species originating from milk in this study are referred to as “less relevant” species. Before statistical analysis was conducted, observations were revised and checked for unlikely values. Complete data were available from 344 quarters from 86 cows, making 3,052 observations in total. Logistic mixed regression models were fit in SAS 9.3 (SAS Institute Inc., Carry, NC, USA). Clustering of observations occurred at multiple levels. Repeated observations were clustered in time within quarters, whereas quarters within cows and cows within herds were spatially clustered. For those reasons, analyses were run with herd and sampling forced into all models as fixed effects and quarter as random effect (REPEATED statement). Cow as random effect was omitted because of non-convergence of the models when combined with a quarter random effect. The contribution of each of the random effects was estimated (VARCOMP procedure) and revealed that quarter accounted for more variability than cow (Zadoks et al., 2001). Compound symmetry was used as the covariance structure for within-quarter correlation (Barkema et al., 1997; Zadoks et al., 2001). Two different binary outcome variables at the quarter level were used: (1) IMI with the more relevant CNS, and (2) IMI with the less relevant CNS, both versus being non-infected. Initially, univariable associations were tested between the 2 binary outcome variables and all predictor variables at the cow, quarter and observation level. Statistical significance in this step was assessed at $P < 0.15$. Second, Spearman correlation coefficients were calculated among all statistically significant predictor variables to check for multicollinearity. If 2 predictor variables had a correlation coefficient ≥ |0.60|, only

one was selected for further analyses. In the third step, multivariable models were fit for the 2 outcome variables using backward stepwise elimination. Statistical significance in this step was assessed at $P < 0.05$. Interaction terms were tested between all remaining statistically significant predictors. The goodness-of-fit of the final models was evaluated by plotting the observational-level standardized residuals against the observational-level predicted values.

Results

On average, 53 lactating cows were present in the herds (range = 39 - 70) with an average of 29 animals per herd being included in the study, resulting in a total of 3,052 analyzed milk samples. One hundred twenty-eight IMI with the more relevant CNS and 48 IMI with the less relevant CNS were available for analysis. Overall, *S. chromogenes* caused most IMI (n = 83, 47% of all IMI) followed by *S. xylosus* (n = 28, 16%), *S. cohnii* (n = 19, 11%), *S. simulans* (n = 17, 10%), *S. haemolyticus* (n = 11, 6%), and *S. fleurettii* (n = 6, 3%). Sixty-nine percent (n = 121) of all CNS IMI (n = 176) originated from quarters of cows in second or higher lactation. However, only 0.07% of all quarters of older cows were infected with CNS, whereas 16.4% of all quarters of heifers were infected. The majority of CNS IMI from quarters of heifers (93%, n = 50) and multiparous cows (64%, n = 77) were caused by the more relevant species. *Staphylococcus simulans* was mainly isolated from IMI in quarters of heifers (82%, n = 14). Coagulase-negative staphylococci IMI in quarters of multiparous cows were most frequently caused by *S. chromogenes* (43%, n = 52), *S. xylosus* (18%, n = 22), and *S. cohnii* (15%, n = 18). *Staphylococcus chromogenes* (56%, n = 31), *S. simulans* (26%, n = 14), and *S. xylosus* (11%, n = 6) were the majority of CNS IMI in quarters of heifers. Overall, CNS species were equally collected in any stage of lactation. *Staphylococcus chromogenes* (71%, n = 12), *S. haemolyticus* (12%, n = 2), *S. xylosus* (12%, n = 2), and *S. sciuri* (6%, n = 1) were the only species causing IMI in the first two months after parturition (1 to 60 DIM).
The more relevant CNS species were mostly isolated from IMI in quarters of heifers in a later stage of lactation (> 180 DIM), whereas IMI in quarters of older cows caused by those relevant species were equally present in any stage in lactation. Most of the less relevant CNS (77%, n = 36) were isolated from IMI in quarters with a qSCC < 200,000 cells/mL, whereas for the more relevant CNS this was only 45%.

A first reduction based on the univariable models revealed 5 and 4 predictor variables associated with IMI ($P < 0.15$) with the more relevant CNS and the less relevant CNS, respectively (Table 2). As no correlation coefficient ≥ |0.6| between the statistically significant predictors was calculated, all independent variables were added in the multivariable models.

The final multivariable, multilevel models are presented in Table 3. Quarters from heifers (as opposed to multiparous cows), from heifers and multiparous cows in third or fourth month in lactation (as opposed to early lactation, < 60 DIM), and with an increasing qSCC were more likely to be infected with the more relevant CNS. Quarter SCC was identified as the sole statistically significant predictor for IMI with other CNS species, although the size of the effect was lower [odds ratio of 1.6 (1.4 - 1.9) as compared with 2.1 (1.8 - 2.5)] than for IMI with the relevant species. None of the tested interaction terms was significant. Statistically significant herd effects (herd forced in all models to correct for clustering of cows within herds) for both IMI with the more and less relevant CNS species, respectively, were present.

Discussion

This is to our opinion, the first study identifying CNS group-specific predictor variables in a longitudinal setting using precise molecular techniques for CNS speciation. Before the current study, past investigations examined factors associated with CNS IMI, but researchers either did not differentiate CNS species or used inaccurate phenotypic identification methods (Sampimon et al., 2009b). Several studies demonstrated a high prevalence of CNS around calving. Still, to our knowledge, in none of the other studies was the prevalence of CNS IMI in early lactation compared with the prevalence of CNS IMI later in lactation.

We confirmed that heifers are more likely to be infected with (relevant) CNS in comparison with multiparous cows (Sampimon et al., 2009a). Quarters from heifers and cows in peak lactation (61 - 120 DIM) were more likely to be infected with the more relevant species despite applying post-milking teat disinfection and using dry cow antibiotics in all 3 herds. This might support the assumption of CNS being able to develop resistance to antibiotics and teat disinfectants (Sampimon et al., 2011; Piessens et al., 2012) .

Table 2. Univariable mixed logistic regression models[1] for intramammary infection (IMI) with coagulase-negative staphylococci (CNS) considered more[2] or less relevant for udder health

Predictor variable	Non-infected		IMI with more relevant CNS			IMI with less relevant CNS		
	N	%	N	%	*P*-value	N	%	*P*-value
Quarter position					0.266			0.087
Front quarters	1,007	50.0	56	44.1	Ref.[3]	18	38.3	Ref.
Hind quarters	1,005	50.0	71	55.9		29	61.7	
Breed					0.959			0.434
Black and white HF[4]	1,915	95.2	124	97.6	Ref.	46	97.9	Ref.
Red and white HF	97	4.8	3	2.4		1	2.1	
Parity					< 0.001			0.353
1st lactation	329	16.4	50	39.4	Ref.	4	8.5	Ref.
2nd lactation or older	1,683	83.6	77	60.6		43	91.5	
Stage of lactation					0.023			0.116
1 - 60 DIM	393	19.5	14	11	Ref.	3	6.4	Ref.
61 - 120 DIM	375	18.6	26	20.5		10	21.3	
121 - 180 DIM	370	18.4	23	18.1		8	17.0	
> 180 DIM	874	43.4	64	50.4		26	55.3	
BCS					0.281			0.878
< 2.5	560	27.8	31	24.4	Ref.	11	23.4	Ref.
2.5 - 3.5	13,00	64.6	74	58.3		31	66.0	
> 3.5	152	7.6	22	17.3		5	10.6	
Milk yield					< 0.001			0.735
Low (< 33.35 kg)	995	49.5	85	66.9	Ref.	26	55.3	Ref.
High (≥ 33.35 kg)	1,017	50.5	42	30.1		21	44.7	
Quarter milk SCC	2,012	−	127	−	< 0.001	47	−	0.005
Teat skin condition					0.014			0.129
Without cracks	1,813	90.1	118	92.9	Ref.	38	80.9	Ref.
With cracks	199	9.9	9	7.1		9	19.1	
Teat apex condition					0.256			0.787
Without cracks	1,378	68.5	97	76.4	Ref.	29	61.7	Ref.
With cracks	634	31.5	30	23.6		18	38.3	

[1]Herd and sampling were forced into all models as fixed effects to correct for potential clustering of cows within herds and for multiple observations per quarter, respectively. [2]*Staphylococcus chromogenes*, *S. simulans*, and *S. xylosus*. [3]Reference category per predictor variable. [4]Holstein Friesian.

A high proportion of the variation in the likelihood of CNS IMI was observed at the quarter level, which is in agreement with the findings of another study conducted by our group (Passchyn et al., 2014). This probably relates to the fact that cows are more likely to be infected with CNS than with major pathogens and that some cows are infected with CNS in 1 quarter, whereas others harbor CNS in up to 4 quarters at

the same time (Makovec and Ruegg, 2003; Piepers et al., 2007; Sampimon et al., 2009a). In contrast, other studies (also) dealing with major pathogens, such as *Staphylococcus aureus* and *Streptococcus uberis,* observed that a higher proportion of the variation in the outcome resided at the cow level (Zadoks et al., 2001; Passchyn et al., 2014).

More statistically significant predictors for IMI caused by the more relevant CNS were identified than for IMI with the less relevant CNS. The greater number of isolates of relevant CNS species in the dataset likely explains this, although we hypothesize it also reflects differences in virulence and host adaptation.

On farms with excellent udder health, reflected by a bulk milk SCC < 200,000 cells/mL, CNS infections may be an important contributor to the total number of somatic cells in the bulk milk (Schukken et al., 2009). In addition, a high proportion of cows infected with the more relevant CNS might result in an unwarranted increase in the bulk milk SCC in such a herd, also because IMI with the more relevant CNS last longer, on average, than IMI with the less relevant CNS (Supré et al., 2011). We hypothesize that our data are useful for selecting quarters and cows infected with the more relevant CNS in herds in which major pathogens are not an issue. The results suggest that the focus should be on quarters with an elevated qSCC from heifers and on heifers and multiparous cows in peak lactation (61 - 120 DIM). In addition, molecular techniques able to identify CNS at the species level or at least able to distinguish the more relevant from the less relevant CNS would be very valuable when making individual cow-level decisions in those herds. As statistically significant differences were observed between herds in likelihood of CNS infection, species-specific analysis at the herd level is required. This implies setting up studies that include large numbers of herds based on quarter milk samples from an even larger number of cows. Using bulk milk samples to identify herd-level predictor variables for IMI with the more relevant CNS could be helpful to circumvent this inconvenience and should be investigated

Table 3. Final multivariable models describing cow-, quarter- and observation-level predictor variables associated with intramammary infection (IMI) with coagulase-negative staphylococci (CNS) considered more[1] or less relevant for udder health

Predictor variable	IMI with more relevant CNS (n = 128)						IMI with less relevant CNS (n = 48)					
	β[2]	SE	OR[3]	95% CI		*P*-value	β	SE	OR	95% CI		*P*-value
Intercept	- 4.373	0.652	0.013	0.004	0.045	< 0.001	- 5.752	0.763	0.003	0.001	0.014	< 0.001
Sampling[4]	–	–	–	–	–	0.097	–	–	–	–	–	0.006
Herd[5]						< 0.001						0.002
Herd 1	Ref.[6]	–	–	–	–		Ref.	–	–	–	–	
Herd 2	0.048	0.329	1.049	0.550	1.998		- 0.914	0.379	0.401	0.191	0.842	
Herd 3	- 2.299	0.586	0.100	0.032	0.316		- 1.663	0.511	0.190	0.070	0.516	
Parity						< 0.001						
1st lactation	Ref.	–	–	–	–							
2nd lactation or older	- 2.266	0.315	0.104	0.056	0.192							
Stage of lactation						0.020						
1 - 60 DIM	Ref.	–	–	–	–							
61 - 120 DIM	0.679	0.322	1.971	1.049	3.704							
121 - 180 DIM	0.296	0.339	1.345	0.692	2.613							
> 180 DIM	- 0.061	0.301	0.941	0.522	1.698							
Quarter milk SCC	0.758	0.078	2.134	1.832	2.486	< 0.001	0.489	0.088	1.631	1.371	1.939	< 0.001

[1]*Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus.*[2]Regression coefficient.[3]Odds ratio.[4]Forced into the model to correct for multiple observations per quarter.[5]Forced into the model to correct for potential clustering within herds.[6]Reference category per predictor variable.

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Chapter 6

Coagulase-Negative *Staphylococcus* Species in Bulk Milk: Prevalence, Distribution, and Associated Subgroup- and Species-Specific Risk Factors

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Abstract

Coagulase-negative staphylococci (CNS) are the main bovine mastitis pathogens since recent years. This heterogeneous group of species shows a huge variation in species distribution among herds. Identification of in particular herd-level factors would be helpful in further improving our understanding of the differences in ecological and epidemiological nature among the various species. The use of bulk milk samples enables the inclusion of a large(r) number of herds needed to identify herd-level risk factors, and increases the likelihood of recovering enough isolates per species, needed for conducting subgroup- and species-specific analyses, at the same time. This study aimed at describing the prevalence and distribution of CNS species in bulk milk samples in Flemish dairy herds and at identifying associated subgroup- and species-specific herd-level factors. Ninety percent of all bulk milk samples yielded CNS. Both so-called environmental and host-adapted species were recovered. *Staphylococcus equorum* was the predominant species, followed by *S. haemolyticus* and *S. epidermidis*. A seasonal effect was observed for several CNS species. Bulk milk samples from herds with a loose-pack or a tie stall housing system were more likely to yield most CNS species except for *S. epidermidis*, *S. simulans,* or *S. cohnii* compared to herds with a freestall barn. In September, herds where udders were clipped had lower odds of yielding the so-called more relevant CNS species, i.e. *S. chromogenes, S. simulans,* and *S. xylosus,* in their bulk milk than herds where udder clipping was not practiced. Herds participating in a monthly veterinary udder health monitoring program were more likely to yield the more relevant CNS species in their bulk milk. Herds always receiving their milk quality premium or pre-disinfecting the teats before attachment of the milking cluster had lower odds of having *S. equorum* in their bulk milk. Herds not using a single dry cotton or paper towel for each cow during pre-milking udder preparation were more likely to have *S. cohnii*-positive bulk milk. Herds where flushing with hot water or steam of the milking cluster after having milked a cow with a (sub)clinical mastitis was applied, were less likely to yield *S. simulans, S. haemolyticus,* and *S. cohnii* in their bulk milk and tended to be at a lower probability of being positive for *S. aureus* in their bulk milk. Always wearing gloves during milking decreased the odds of having *S. devriesei*-positive bulk milk. Tap water being used as drinking water increased the odds of yielding *S. simulans* in the bulk milk. Further research has to

be carried out to investigate to what extent the observed associations are causal. Contamination of the bulk milk with CNS might occur from environmental transmission for some species, i.e. *S. equorum* or *S. cohnii,* or from within the udder for other species, i.e. *S. simulans.* However, studies collecting bulk milk and quarter milk samples in the same time frame along with environmental samples should be conducted to determine the exact origin of CNS species in bulk milk.

Key words

Bulk milk, Coagulase-negative *Staphylococcus* species

Introduction

Coagulase-negative staphylococci (CNS) have become the main bovine mastitis pathogens in recent years in several regions and countries (Piepers et al., 2007; Schukken et al., 2009; Reyher et al., 2011). Research relying on genotypic identification demonstrated the abundant presence of diverse CNS species in different bovine habitats such as the cows' environment (Piessens et al., 2011), milk samples (Santos et al., 2008; Park et al., 2011; Persson Waller et al., 2011), and other udder-related habitats (Taponen et al., 2008; De Visscher et al., 2014, 2016a). Picturing the CNS prevalence and distribution in those habitats and conducting risk factor studies, as have been done for other mastitis pathogens, is the obvious next step towards a better understanding of the variation in epidemiological and ecological nature among species (Zadoks et al., 2001; Østerås et al., 2006; Fox et al., 2009). Still, species-specific research requires extensive studies including a considerable number of different herds, cows and quarters in order to obtain enough isolates of each (subgroup of) species for further (herd-level) analyses (Vanderhaeghen et al., 2015).

Bulk milk is a convenient matrix as it is readily available as part of the (regulatory) milk quality screening programs in different countries and regions and includes milk of all lactating animals in the herd whose milk is not discarded. Bulk milk has already shown its value for the identification of herd-level management practices associated with the prevalence of *Staphylococcus aureus* (Olde Riekerink et al., 2010). Using bulk milk instead of cow or quarter milk conveniently enables the inclusion of a larger number of herds compared to collecting composite or quarter-level milk samples. Bulk milk has only been used in one recent study, in which CNS were only phenotypically identified to detect genes encoding for several virulence factors (Bertelloni et al., 2015) and in another study evaluating the suitability of mannitol salt agar for CNS recovery in field applications (De Visscher et al., 2013). However, bulk milk has not been applied before as a matrix for determining the herd-level prevalence and distribution of the different CNS species and for identifying associated herd-level factors.

This study aimed (1) to describe the herd-level prevalence and distribution of CNS species in Flemish dairy herds using bulk milk samples, (2) to assess the variation in

the presence of (subgroups of) different CNS species among herds, and (3) to identify herd-level risk factors for the isolation of (subgroups of) CNS species.

Materials and methods

Herds, samples, and data

One hundred commercial Flemish dairy herds were randomly selected from the database of the Milk Control Center Flanders (MCC Flanders, Lier, Belgium) comprising all Flemish dairy producers delivering milk to a dairy factory. The Excel RAND function (Excel 2010, Microsoft Corp., Redmond, WA) was applied for random selection procedures. In the provinces Antwerp, Flemish Brabant, Limburg, East Flanders, and West Flanders, 19, 5, 11, 28, and 37 herds were contacted, respectively, matching the distribution of dairy herds per province in Flanders.

Fifty herds only housed dairy cattle, whereas the other half also farmed pigs, beef cattle, or poultry. Herds had an average milk quota of 449,000 kg/year, ranging between 92,000 kg and 1,500,000 kg. A fishbone milking parlor was the most commonly found milking parlor set-up (n = 52), followed by a tie stall (n = 20), a tandem parlor (n = 18), a side-by-side parlor (n = 6), an automated milking system (n = 2), and a rotary parlor (n = 1). In 1 herd, a fishbone milking parlor was replaced by an automated milking system during the year of sampling (i.e. 2013).

Bulk milk quality data were retrieved from MCC Flanders (Lier, Belgium) and recoded into lower bulk milk SCC, i.e. < 200,000 cells/mL, and higher bulk milk SCC, i.e. ≥ 200,000 cells/mL according to Schukken et al. (2009). MCC Flanders executes the mandatory milk quality screening program in Flanders. Bulk milk samples were collected 3 times with a 3 month-interval in 2013 (March, June, September) as part of this program and used for this study. The geometric mean bulk milk SCC in the month of sampling (March, June, September) was calculated based on 4 weekly records and revealed a minimum, a maximum, and an average of 74,000 cells/mL, 539,000 cells/mL and 235,000 cells/mL, respectively.

Several additional herd-level factors, potentially associated with the presence of (subgroups of) CNS species, were collected through a questionnaire and enclosed the entire study period (i.e. January 2013 till December 2013) (Table 1).

Laboratory analyses

Bulk milk samples were collected at the dairy farm and plated on mannitol salt agar (MSA) (Oxoid, Erembodegem, Aalst, Belgium) (one bulk milk sample per plate) in the lab of MCC Flanders (De Visscher et al., 2013). After 24h aerobic incubation, plates were transported under cooled condition (4°C) to the Mastitis and Milk Quality Research Lab (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium). All phenotypically different colony types were counted, picked up (one colony per colony type) and subcultured on esculin blood agar (Oxoid) to obtain pure cultures. Afterwards, plates were aerobically incubated (37°C) for another 24h and again examined. All recovered isolates suspected of belonging to the group of CNS were stored at -80°C or immediately subjected to species identification using transfer RNA intergenic spacer PCR (tDNA-PCR) or *16S rRNA* gene sequencing if no identification could be obtained (Supré et al., 2009).

Descriptive and statistical analyses

Before any analysis was performed, observations were checked for unlikely values. Complete data were available from 95 herds. Some data were missing, but data belonged to different herds.

The proportion of variation for the outcome variables at the herd, cow and quarter level was estimated for the null models using variance components analysis (MLWiN 2.16, Centre for Multilevel Modeling, University of Bristol, Bristol, UK). The variance at the quarter level was assumed $\pi^2/3$ (Goldstein et al., 2002) as described by Piepers et al. (2011).

Logistic regression models were fit applying reweighted iterative generalized least squares and 1st order penalized quasi-likelihood in MLwiN 2.16 (Centre for Multilevel Modeling, University of Bristol, Bristol, UK). Month of sampling (March, June and September) was forced into all models as fixed effect and herd was added as random effect to account for clustering of observations (3 samplings) within herds. First, separate models were fit to identify risk factors for different subgroups of CNS. Several binary outcome variables were created: the likelihood of the presence of (1) the more relevant CNS for udder health, i.e. *Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus* (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2015), in a bulk milk sample versus CNS-negative bulk milk or bulk milk with (an)other CNS species, (2) the so-called host-adapted CNS species, i.e. *S.*

chromogenes and *S. epidermidis* (Vanderhaeghen et al., 2015), versus CNS-negative bulk milk, and (3) the so-called environmental CNS species, i.e. *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* (Piessens et al., 2011), versus CNS-negative bulk milk. Second, species-specific risk factors were identified for all specific CNS species of which more than 25 isolates were available (presence of such species versus CNS-negative bulk milk) and for *S. aureus* (presence of *S. aureus* versus negative bulk milk).

Univariable models between all outcome variables and the independent factors (Table 1) were fit. Statistical significance in this first step was assessed at $P < 0.15$. Subsequently, Spearman rank correlation coefficients were calculated among all significant independent variables, with a margin of |0.6|, in order to identify multicollinearity in the next steps. Finally, multivariable models were fit using backward stepwise elimination and assessing statistical significance at $P < 0.05$. Biological relevant interaction terms among the remaining statistically significant independent factors were tested and kept in the final models if significant ($P < 0.05$). A factor was considered a confounder if its removal caused a relative change > 25% in the regression coefficients of the remaining variables or with a regression coefficient between -0.4 and 0.4 if and absolute change > 0.1 was observed (Noordhuizen et al., 2001). To correct for multiple comparisons, a Bonferroni-correction was applied. In order to test the goodness-of-fit of all models, the observational-level standardized residuals were plotted against the observational-level predicted values, all revealing good models. Odds ratio's (OR) and 95% confidence intervals were reported.

The relation between the presence of the more relevant CNS species, i.e. *S. chromogenes, S. simulans,* and *S. xylosus* (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2015), and the presence of the major pathogen *S. aureus* in bulk milk was analyzed. A Fisher's exact test (SPSS Statistics 22, SPSS Inc., Chicago, IL, USA) on a contingency table was performed.

Results

Descriptive analysis

All except one of the collected bulk milk samples (n = 299) yielded growth on MSA with a range between 0 and 9 phenotypically different colony types.

Table 1. Overview of all herd-level factors potentially associated with the presence of (subgroups of) coagulase-negative staphylococci in bulk milk samples (n = 300) from 100 Flemish dairy herds

Independent variable	Recording method	Description	Categories considered in final model
Bulk milk SCC	MCC-records[1]	Bulk milk SCC at sampling[2]	Lower (< 200 x 10³ cells/mL) vs. higher bulk milk SCC (≥ 200 x 10³ cells/mL)[3]
Premium	Questionnaire	Receiving premium for good milk quality	No premium vs. premium
Pre-milking udder preparation	Questionnaire	Cleaning the udder before attachment of milking cluster	Dry with 1 cotton or paper towel for each cow / alcohol wipes vs. dry with 1 cotton or paper towel for several cows vs. wet / automatic / no udder preparation
Pre-milking teat disinfection	Questionnaire	Teat disinfection before milking	No disinfection vs. disinfection
Forestripping	Questionnaire	Forestripping before attachment of milking cluster	No forestripping vs. forestripping
Flushing	Questionnaire	Flushing of the teat cup liners with hot water or steam after milking (sub)clinical mastitis cows	No flushing / steaming vs. flushing / steaming
Gloves	Questionnaire	Wearing gloves during the milking process	No / sometimes / automatic milking vs. always
Post-milking teat disinfection	Questionnaire	Teat disinfection after milking	No dipping / spraying vs. dipping / spraying
Housing	Questionnaire	Housing of lactating cows	Freestall barn vs. loose-pack / tie stall
Bedding hygiene	Questionnaire	Cleaning of cubicles at least 2x per day and filling of loose-pack or tie stall at least 2x per day	Bad vs. good
Drinking water	Questionnaire	Source of drinking water	No tap water vs. tap water
Clipping	Questionnaire	Yearly clipping of the udder	No clipping vs. clipping
Treatment	Questionnaire	Treatment of subclinical mastitis during lactation	No treatment vs. treatment
Monitoring	Questionnaire	Monthly monitoring of the udder health by a veterinarian	No monitoring vs. monitoring

[1]Milk Control Centre Flanders, Lier, Belgium. [2]Geometric mean SCC based on weekly records (n = 4) of the Milk Control Centre Flanders in the month of sampling. [3]Categorization based on Schukken et al. (2009).

A number of colony types appeared to belong to the same CNS species or to other genera after tDNA-PCR or *16S rRNA* gene sequencing, generating 572 CNS isolates available for further analysis. Eventually, ninety percent (n = 271) of all bulk milk samples were CNS-positive with 1 up to 6 different CNS species per sample. In total, twenty-five different CNS species were identified (Table 2).

Table 2. Species distribution of coagulase-negative *Staphylococcus* species (CNS) present in bulk milk samples from 100 Flemish dairy herds at three sampling occasions (March - June - September)

Species	March[1]	%[2]	June	%	Sept.	%	Total	%
S. equorum	47	33.6	47	24.1	44	18.6	138	24.1
S. haemolyticus	18	12.9	25	12.8	31	13.1	74	12.9
S. epidermidis	13	9.3	14	7.2	16	6.8	43	7.5
S. simulans	3	2.1	5	2.6	30	12.7	38	6.6
S. cohnii	7	5.0	15	7.7	13	5.5	35	6.1
S. sciuri	8	5.7	8	4.1	18	7.6	34	5.9
S. xylosus	7	5.0	14	7.2	12	5.1	33	5.8
S. chromogenes	11	7.9	9	4.6	12	5.1	32	5.6
S. saprophyticus	0	0.0	14	7.2	14	5.9	28	4.9
S. devriesei	2	1.4	11	5.6	13	5.5	26	4.5
S. fleurettii	7	5.0	6	3.1	4	1.7	17	3.0
S. warneri	4	2.9	4	2.1	6	2.5	14	2.4
S. hominis	5	3.6	4	2.1	4	1.7	13	2.3
S. caseolyticus	1	0.7	5	2.6	5	2.1	11	1.9
S. succinus	1	0.7	5	2.6	2	0.8	8	1.4
S. vitulinus	4	2.9	3	1.5	0	0.0	7	1.2
S. arlettae	0	0.0	2	1.0	3	1.3	5	0.9
S. auricularis	1	0.7	1	0.5	3	1.3	5	0.9
S. agnetis	0	0.0	0	0.0	3	1.3	3	0.5
S. gallinarum	0	0.0	1	0.5	1	0.4	2	0.3
S. pasteuri	0	0.0	1	0.5	1	0.4	2	0.3
S. hyicus	1	0.7	0	0.0	0	0.0	1	0.2
S. lentus	0	0.0	1	0.5	0	0.0	1	0.2
S. rostri	0	0.0	0	0.0	1	0.4	1	0.2
S. nepalensis	0	0.0	0	0.0	1	0.4	1	0.2
Total	140	100	195	100	237	100	572	100

[1]Number of herds (n = 100) yielding a certain CNS species in bulk milk.
[2]Percentage of identified CNS species within month of sampling.

The most commonly isolated species were *S. equorum*, *S. haemolyticus*, *S. epidermidis*, *S. simulans*, *S. cohnii*, *S. sciuri*, *S. xylosus*, *S. chromogenes*, *S. saprophyticus*, and *S. devriesei* (Table 2). Eighty-nine bulk milk samples (n total = 300) (30%) collected in 61 out of 100 herds were *S. aureus*-positive (n March = 45, n June = 21, n September = 23) and ninety-two bulk milk samples (31%) collected in 62 out of 100 herds harbored at least one of the more relevant CNS species for udder health, i.e. *S. chromogenes*, *S. simulans,* and *S. xylosus* (n March = 19, n June = 27, n September = 46). Pathogens other than staphylococci were either phenotypically or genotypically (i.e. using tDNA-PCR or *16S rRNA* gene sequencing) identified as esculin-positive streptococci (n = 156), *Bacillus* spp. (n = 116), *Acinetobacter* spp. (n = 33), *Corynebacterium* spp. (n = 28), *Pseudomonas* (n = 26), Alcaligenaceae (n = 14), *Psychrobacter* spp. (n = 12), *Jeotgallicoccus* spp. (n = 11), and other Gram-negative bacteria (n = 2). Also, fungi (n = 3) and yeasts (n = 3) were identified.

The number of herds (n = 100) yielding a certain CNS species in March and/or June and/or September are shown in Figure 1. *Staphylococcus equorum*, *S. haemolyticus*, *S. epidermidis*, *S. sciuri*, *S. cohnii*, *S. xylosus* and *S. devriesei,* as well as *S. aureus* were on at least one herd isolated on all three sampling occasions in the same herd(s).

Statistical analyses

Variance components. In all null-models, most of the variation in the outcome variables resided at the observation level. The herd level variation was highest for the subgroup of host-adapted CNS (34%) and *S. epidermidis* (36%), *S. xylosus* (32%), and *S. haemolyticus* (32%) solely whereas only 24% of the variation was observed at the herd level for the subgroup of environmental CNS and 13% for *S. aureus*.

Univariable analyses. Univariable analyses revealed in each model 4 factors significantly associated with the presence of different subgroups of CNS species (Table 3). The species-specific univariable analyses showed 5 herd-level factors to be significantly associated with the presence of *S. haemolyticus*, *S. devriesei,* or *S. equorum*, in bulk milk, 4 factors for the presence of *S. epidermidis, S. xylosus,* or *S. sciuri* and 3 factors for the presence of *S. chromogenes*, *S. simulans,* or *S. cohnii* (Table 4). Univariable analyses with *S. saprophyticus* (n = 28) did not fit due to low

numbers as was also observed for some analyses with *S. aureus*. *Staphylococcus aureus* was only significantly associated with 1 independent variable, i.e. flushing (*P* = 0.07) (Table 4). No significant associations were identified with bulk milk SCC, fore-stripping and post-milking teat disinfection. Strong correlations between 2 or more factors or confounding were not observed.

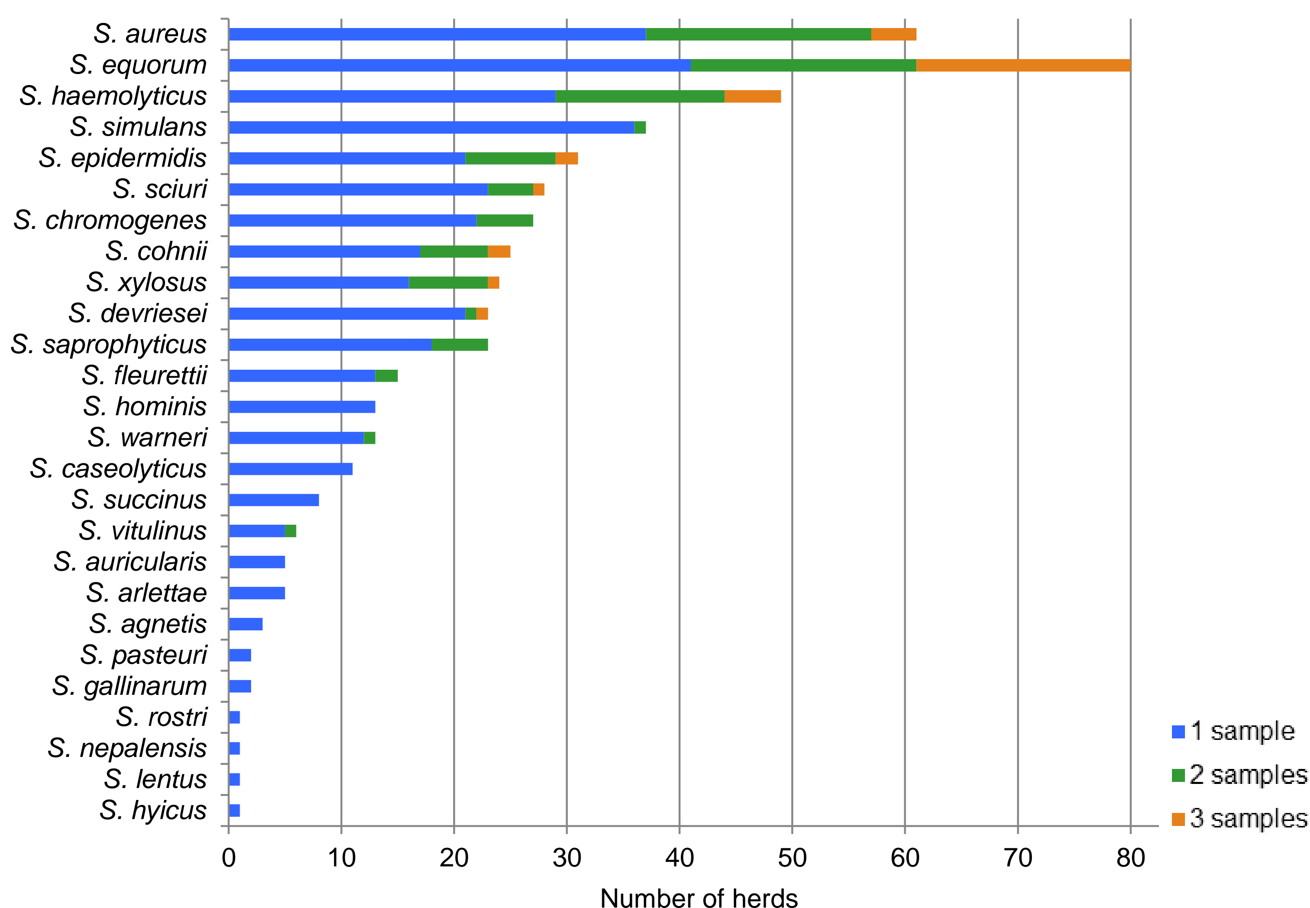

Figure 1. Number of herds (n = 100) yielding a certain coagulase-negative *Staphylococcus* species or *S. aureus* in bulk milk in 1 (blue), 2 (green), or 3 months of sampling (orange) (March - June - September).

Multivariable subgroup-specific risk factors analyses. Table 5 presents the different final multilevel, multivariable logistic regression models for subgroups of species. The more relevant CNS species, i.e. *S. chromogenes, S. simulans,* and *S. xylosus,* as well as the environmental CNS species, i.e. *S. cohnii*, *S. equorum*, *S. saprophyticus*, and *S. sciuri*, were significantly more often observed in bulk milk collected in June and September as opposed to March. Host-adapted CNS, i.e. *S.*

chromogenes and *S. epidermidis,* were equally often cultured from the bulk milk samples collected in March, June and September. Herds with lactating cows and heifers housed in either a loose-pack or a tie stall had higher odds of yielding each of the 3 CNS subgroups in bulk milk with an OR of 2.5 (95% CI: 1.36-4.75) for the more relevant CNS species, 13.1 (95% CI: 1.40-123.75) for the host-adapted CNS species, and 11.4 (95% CI:1.44-90.80) for the environmental CNS species, respectively. In September, herds where udders were clipped had lower odds of yielding the more relevant CNS species in their bulk milk than herds where the udders were not clipped (Figure 2). Herds participating in a monthly veterinary udder health monitoring program were more likely to yield the more relevant CNS species in their bulk milk samples.

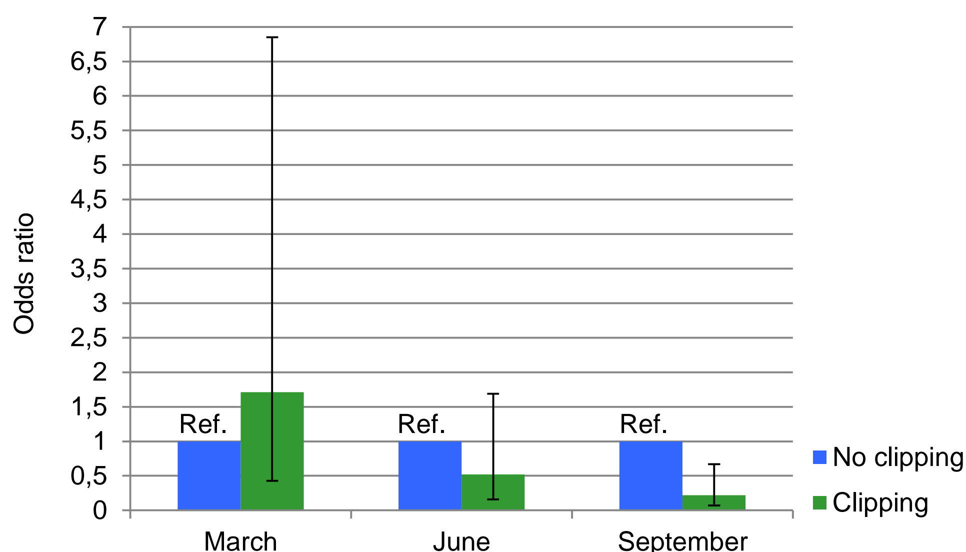

Figure 2. Interaction term between clipping and month of sampling visualized using odds ratio’s with 95% confidence intervals for the presence of the for udder health more relevant coagulase-negative *Staphylococcus* species, i.e. *S. chromogenes, S. simulans,* and *S. xylosus,* in bulk milk samples (n = 300) from 100 Flemish dairy herds (as shown in Table 5).

Multivariable species-specific risk factor analyses. Table 6 shows the different final multilevel, multivariable logistic regression models for individual CNS species. None of the evaluated herd-level factors was significantly associated with the presence of *S. epidermidis* in bulk milk. *Staphylococcus cohnii* was significantly more

often observed in bulk milk collected in June and September as opposed to March. A seasonal effect was also seen for *S. simulans*, *S. devriesei* as well as for *S. sciuri*, as they were all more recovered in bulk milk in September as opposed to March. For the three latter species, no difference was observed among bulk milk samples collected in June and March. *Staphylococcus epidermidis, S. chromogenes*, and *S. equorum* were equally present in the samples collected in March, June and September. Housing cows in in loose-pack stall and a tie stall increased the odds of yielding *S. chromogenes*, *S. xylosus*, *S. haemolyticus*, *S. devriesei*, *S. equorum,* and *S sciuri*. Herds where the milking cluster was not flushed with hot water or steam after having milked a cow with (sub)clinical mastitis were more likely to have *S. simulans-, S. haemolyticus*-, and *S. cohnii*-positive bulk milk. Herds with drinking water being tap water had higher odds of yielding *S. simulans* in their bulk milk. Not or only sometimes wearing gloves during milking increased the odds of having *S. devriesei* in the bulk milk. Herds always receiving their milk quality premium were less likely to yield *S. equorum* in their bulk milk. Teat disinfection before attachment of the milking cluster decreased the odds of yielding S. *equorum* in the bulk milk. Herds using a dry cotton or paper towel for several cows during the pre-milking udder preparation were more likely to have *S. cohnii*-positive bulk milk opposed to those that used a dry cotton or paper towel for each cow.

Fisher's exact test. The presence of the more relevant CNS, i.e. *S. chromogenes, S. simulans,* and *S. xylosus,* was not associated with the presence of the major pathogen *S. aureus* in bulk milk (P = 0.347) (SPSS Statistics 22, SPSS Inc., Chicago, IL, USA).

Discussion

This is the first extensive study describing the prevalence and distribution of CNS species in bulk milk of a large number of samples and dairy herds. The use of MSA as a (quasi-) selective medium along with the molecular speciation of all phenotypically different CNS isolates allowed us to precisely picture the species-specific prevalence and distribution of CNS in bulk milk within 1 year and to identify subgroup- and even species-specific herd-level risk factors whereas previous studies identified herd-level risk factors for CNS as a group (Sampimon et al., 2009; Piepers et al., 2011).

Coagulase-negative staphylococci are prevalent in bulk milk in Flanders (De Visscher et al., 2013), though the species-specific prevalence and distribution greatly varied among herds. *Staphylococcus aureus* and the more relevant CNS species for udder health were recovered in almost equal numbers, however their presence was not associated and no seasonal effect was observed for *S. aureus*. The prevalence of *S. aureus* corresponds well with earlier reported results on the bulk milk prevalence of *S. aureus* (Olde Riekerink et al., 2006, 2010). Bacteria in bulk milk are, however, not solely originating from infected quarters. Environmental contamination or insufficient hygiene and poor preservation conditions can also cause positive bulk milk samples, potentially hampering the interpretation of the data (Elmoslemany et al., 2009a, 2009b), which also applies for the current study. Although our study revealed the presence of both so-called host-adapted and environmental CNS species in bulk milk, studying the presence of CNS in bulk milk samples and the presence in quarter milk and environmental samples at the same time, relying on strain-typing, is warranted to determine the exact origin of the CNS in bulk milk.

A seasonal effect was observed for several species which is in contrast with earlier CNS group-studies (Gillespie et al., 2012). Bad housing conditions and poor hygiene are important factors increasing the total bacterial count in bulk milk (Olde Riekerink et al., 2010). Housing of lactating animals most likely also plays an important role in our study as the presence of only 3 species was not associated with this risk factor, i.e. *S. cohnii*, *S. epidermidis,* and *S. simulans*. The latter might either indicate an ubiquitous nature of those 3 species both in free-stall and loose-pack or tie stall barns (perhaps for *S. cohnii*) or suggest a more cow-dependent presence in bulk milk (perhaps for the cow-adapted CNS *S. epidermidis* and *S. simulans*). Cleaning of the cubicles or loose-pack was not associated with the presence of any CNS species in our study, however, bedding hygiene was not visually checked. Caution is thus needed in drawing conclusions.

Clipping the udder reduced the odds of having bulk milk contaminated with a more relevant CNS species, i.e. *S. chromogenes, S. simulans,* and *S. xylosus* (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2015). A previous study reported a negative association between clipping of the udder prior to calving and CNS IMI at parturition (Piepers et al., 2011). The latter might indicate that the presence of the relevant CNS in bulk milk results from a transmission via the teat skin and teat hairs as well as via quarters infected with those species.

Table 3. Univariable, multilevel logistic regression models[1] for the presence of subgroups[2] of coagulase-negative *Staphylococcus* (CNS) species in bulk milk samples (n = 300)

Independent factor	N_{Herds}	More relevant CNS species (n = 92)[2]					Host-adapted CNS species (n = 70)[2]					Environmental CNS species (n = 201)[2]				
		N_{no}[3]	$\%_{no}$[3]	N_{CNS}[4]	$\%_{CNS}$[4]	*P*[5]	N_n[6]	$\%_n$[6]	N_{CNS}	$\%_{CNS}$	*P*	N_n	$\%_n$	N_{CNS}	$\%_{CNS}$	*P*
Bulk milk SCC	100[7]					0.94					0.88					0.85
Lower	-	74	35.6	32	34.8	Ref.[8]	11	37.9	27	38.6	Ref.	11	37.9	69	34.3	Ref.
Higher	-	134	64.6	60	65.2		18	62.1	43	61.4		18	62.1	132	65.7	
Premium	100					0.18					0.12					0.05
No	19	35	16.8	22	23.9	Ref.	1	3.4	14	20.0	Ref.	1	3.4	45	22.4	Ref.
Yes	81	173	83.2	70	76.1		28	96.6	56	80.0		28	96.6	156	77.6	
Udder preparation	99[9]					0.72					0.18					0.26
Dry 1 for each cow	42	90	43.7	36	39.1	Ref.	8	27.6	27	38.6	Ref.	8	27.6	92	46.2	Ref.
Dry 1 for several cows	21	44	21.4	19	20.7		10	34.5	15	21.4		10	34.5	41	20.6	
Wet / automatic / no	36	72	35.0	37	40.2		11	37.9	28	40.0		11	37.9	66	33.2	
Pre-milking teat disinfection	99[9]					0.30					0.94					0.24
No	79	168	81.6	70	76.1	Ref.	21	72.4	53	75.7	Ref.	21	72.4	169	84.9	Ref.
Yes	20	38	18.4	22	23.9		8	27.6	17	24.3		8	27.6	30	15.1	
Forestripping	99[10]					0.41					0.41					0.86
No	43	93	45.4	37	40.2	Ref.	13	44.8	24	34.3	Ref.	13	44.8	86	43.2	Ref.
Yes	55	112	54.6	55	59.8		16	55.2	46	65.7		16	55.2	113	56.8	
Flushing	100					0.82					0.13					0.23
No	84	174	83.7	78	84.8	Ref.	21	72.4	61	87.1	Ref.	21	72.4	169	84.1	Ref.
Yes	16	34	16.3	14	15.2		8	27.6	9	12.9		8	27.6	32	15.9	
Gloves	99[9]					0.40					0.34					0.08
No	69	147	71.4	61	66.3	Ref.	15	51.7	45	64.3	Ref.	15	51.7	142	71.4	Ref.
Yes	30	59	28.6	31	33.7		14	48.3	25	35.7		14	48.3	57	28.6	
Post-milking teat disinfection	100[11]					0.97					0.48					0.20
No	21	49	23.6	22	23.9	Ref.	4	13.8	16	22.9	Ref.	4	13.8	56	27.9	Ref.

Yes	75	159	76.4	70	76.1		25	86.2	54	77.1		25	86.2	145	72.1	
Housing	100					<0.001					0.02					0.02
Freestall	72	163	78.4	53	57.6	Ref.	28	96.6	47	67.1	Ref.	28	96.6	142	70.6	Ref.
Loose- pack / tie stall	28	45	21.6	39	42.4		1	3.4	23	32.9		1	3.4	59	29.4	
Bedding hygiene	97[12]					0.38					0.06					0.07
Bad	17	33	16.2	18	20.7	Ref.	1	3.4	14	21.2	Ref.	1	3.4	39	19.9	Ref.
Good	80	171	83.8	69	79.3		28	96.6	52	78.8		28	96.6	157	80.1	
Drinking water	100					0.09					0.16					0.18
Other	85	182	87.5	73	79.3	Ref.	28	96.6	58	82.9	Ref.	28	96.6	173	86.1	Ref.
Tap water	15	26	12.5	19	20.7		1	3.4	12	17.1		1	3.4	28	13.9	
Clipping	100					0.03					0.85					0.69
No	41	76	36.5	47	51.1	Ref.	11	37.9	25	35.7	Ref.	11	37.9	82	40.8	Ref.
Yes	59	132	63.5	45	48.9		18	62.1	45	64.3		18	62.1	119	59.2	
Treatment	100					0.22					0.15					0.77
No	61	132	63.5	51	55.4	Ref.	19	65.5	33	47.1	Ref.	19	65.5	128	63.7	Ref.
Yes	39	76	36.5	41	44.6		10	34.5	37	52.9		10	34.5	73	36.3	
Monitoring	100					0.03					0.70					0.90
No	69	152	73.1	55	59.8	Ref.	20	69.0	47	67.1	Ref.	20	69.0	138	68.7	Ref.
Yes	31	56	26.9	37	40.2		9	31.0	23	32.9		9	31.0	63	31.3	

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]*Staphylococcus chromogenes, S. simulans,* and *S. xylosus* represent the CNS species being more relevant for udder health; *S.chromogenes* and *S. epidermidis* are representative for the host-adapted species; and *S. cohnii, S. equorum, S. saprophyticus,* and *S. sciuri* represent the environmental species. [3]Number and percentage of bulk milk samples not infected with CNS or infected with species other than the one of the subgroup. [4]Number and percentage of bulk milk samples positive for the different subgroups of CNS species, respectively. [5]*P*-value for the overall effect. [6]Number and percentage of bulk milk samples not infected with CNS. [7]The specific information differs between months of sampling per herd. [8]Reference category per independent factor. [9]The specific information is missing in 2 months (March - June) from 1 herd. [10]The specific information is missing in 3 months (March - June - September) from 1 herd and differs between months of sampling in 1 herd. [11]The specific information differs between months of sampling in 4 herds. [12]The specific information is missing in 3 months (March - June - September) from 3 herds.

Table 4. Univariable, multilevel logistic regression models[1] for the presence of specific coagulase-negative *Staphylococcus* (CNS) species and *S. aureus* in bulk milk samples (n = 300)

Independent factor	N_{Herds}	*S. epidermidis* (n = 43)					*S. chromogenes* (n = 32)					*S. simulans* (n = 38)					*S. aureus* (n = 89)				
		N_n [2]	$\%_n$ [2]	N_{CNS} [3]	$\%_{CNS}$ [3]	*P* [4]	N_n	$\%_n$	N_{CNS}	$\%_{CNS}$	*P*	N_n	$\%_n$	N_{CNS}	$\%_{CNS}$	*P*	N_{not} [5]	$\%_{not}$	N_{au} [6]	$\%_{au}$	*P*
Bulk milk SCC	100[7]					0.84					0.69					0.68					0.91
Lower	-	11	37.9	15	34.9	Ref.[8]	11	37.9	14	43.8	Ref.	11	37.9	12	31.6	Ref.	3	33.3	29	32.6	Ref.
Higher	-	18	62.1	28	65.1		18	62.1	18	56.2		18	62.1	26	68.4		6	66.7	60	67.4	
Premium	100					0.26					0.08					0.65					NC[9]
No	19	1	3.4	6	14.0	Ref.	1	3.4	8	25.0	Ref.	1	3.4	7	18.4	Ref.	0	0	14	15.7	Ref.
Yes	81	28	96.6	37	86.0		28	96.6	24	75.0		28	96.6	31	81.6		9	100	75	84.3	
Udder preparation	99[10]					0.43					0.63					0.29					0.44
Dry 1 for each cow	42	8	27.6	17	39.5	Ref.	8	27.6	11	34.4	Ref.	8	27.6	13	34.2	Ref.	2	22.2	38	42.7	Ref.
Dry 1 for several cows	21	10	34.5	8	18.6		10	34.5	8	25.0		10	34.5	7	18.4		3	33.3	16	18.0	
Wet / automatic / no	36	11	37.9	18	41.9		11	37.9	13	40.6		11	37.9	18	47.4		4	44.4	35	39.3	
Pre-milking teat disinfection	99[10]					0.87					0.86					0.52					0.53
No	79	21	72.4	33	76.7	Ref.	21	72.4	23	71.9	Ref.	21	72.4	31	81.6	Ref.	6	66.7	69	77.5	
Yes	20	8	27.6	10	23.3		8	27.6	9	28.1		8	27.6	7	18.4		3	33.3	20	22.5	
Forestripping	99[11]					0.47					0.32					0.96					0.69
No	43	13	44.8	15	34.9	Ref.	13	44.8	10	31.3	Ref.	13	44.8	17	44.7	Ref.	5	55.6	41	46.6	Ref.
Yes	55	16	55.2	28	65.1		16	55.2	22	68.8		16	55.2	21	55.3		4	44.4	47	53.4	
Flushing	100					0.13					0.30					0.03					0.08
No	84	21	72.4	39	90.7	Ref.	21	72.4	26	81.3	Ref.	21	72.4	33	86.8	Ref.	5	55.6	74	83.1	Ref.
Yes	16	8	27.6	4	9.3		8	27.6	6	18.8		8	27.6	5	13.2		4	44.4	15	16.9	
Gloves	99[10]					0.58					0.32					0.43					0.16
No	69	15	51.7	26	60.5	Ref.	15	51.7	21	65.6	Ref.	15	51.7	25	65.8	Ref.	4	44.4	62	69.7	Ref.
Yes	30	14	48.3	17	39.5		14	48.3	11	34.4		14	48.3	13	34.2		5	55.6	27	30.3	
Post-milking teat disinfection	100[12]					0.77					0.20					0.95					NC
No	21	4	13.8	9	20.9	Ref.	4	13.8	10	31.3	Ref.	4	13.8	6	15.8	Ref.	0	0	18	20.2	Ref.
Yes	75	25	86.2	34	79.1		25	86.2	22	68.8		25	86.2	32	84.2		9	100	71	79.8	

Housing	100					0.06					0.01					0.10					NC
Freestall	72	28	96.6	31	72.1	Ref.	28	96.6	19	59.4	Ref.	28	96.6	26	68.4	Ref.	9	100	74	83.1	Ref.
Loose-pack / tie stall	28	1	3.4	12	27.9		1	3.4	13	40.6		1	3.4	12	31.6		0	0	15	16.9	
Bedding hygiene	97[13]					0.35					0.01					0.38					NC
Bad	17	1	3.4	5	12.2	Ref.	1	3.4	10	33.3	Ref.	1	3.4	5	14.3	Ref.	0	0	6	7.10	Ref.
Good	80	28	96.6	36	87.8		28	96.6	20	66.7		28	96.6	30	85.7		9	100	79	92.9	
Drinking water	100					0.14					0.48					0.02					NC
Other	85	28	96.6	34	79.1	Ref.	28	96.6	29	90.6	Ref.	28	96.6	26	68.4	Ref.	9	100	81	91.0	Ref.
Tap water	15	1	3.4	9	20.9		1	3.4	3	9.4		1	3.4	12	31.6		0	0	8	9.0	
Clipping	100					0.72					0.70					0.65					0.64
No	41	11	37.9	15	34.9	Ref.	11	37.9	14	43.8	Ref.	11	37.9	19	50.0	Ref.	4	44.4	33	37.1	Ref.
Yes	59	18	62.1	28	65.1		18	62.1	18	56.3		18	62.1	19	50.0		5	55.6	56	62.9	
Treatment	100					0.15					0.24					0.61					0.31
No	61	19	65.5	19	44.2	Ref.	19	65.5	15	46.9	Ref.	19	65.5	23	60.5	Ref.	4	44.4	56	62.9	Ref.
Yes	39	10	34.5	24	55.8		10	34.5	17	53.1		10	34.5	15	39.5		5	55.6	33	37.1	
Monitoring	100					0.85					0.51					0.19					0.29
No	69	20	69.0	30	69.8	Ref.	20	69.0	20	62.5	Ref.	20	69.0	23	60.5	Ref.	5	55.6	66	74.2	Ref.
Yes	31	9	31.0	13	30.2		9	31.0	12	37.5		9	31.0	15	39.5		4	44.4	23	25.8	

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]Number and percentage of bulk milk samples not infected with CNS. [3]Number and percentage of bulk milk samples positive for the different CNS species, respectively. [4]*P*-value for the overall effect. [5]Number and percentage of negative bulk milk samples. [6]Number and percentage of bulk milk samples positive for *S. aureus*. [7]The specific information differs between months of sampling per herd. [8]Reference category per independent factor. [9]Non-convergence of the model. [10]The specific information is missing in 2 months (March - June) from 1 herd. [11]The specific information is missing in 3 months (March - June - September) from 1 herd and differs between months of sampling in 1 herd. [12]The specific information differs between months of sampling in 4 herds. [13]The specific information is missing in 3 months (March - June - September) from 3 herds.

Table 4 continued. Univariable, multilevel logistic regression models[1] for the presence of specific coagulase-negative *Staphylococcus* (CNS) species in bulk milk samples (n = 300)

Independent factor	N_{Herds}	*S. xylosus* (n = 33)					*S. haemolyticus* (n = 74)					*S. devriesei* (n = 26)				
		N_n[2]	$\%_n$[2]	N_{CNS}[3]	$\%_{CNS}$[3]	*P*[4]	N_n	$\%_n$	N_{CNS}	$\%_{CNS}$	*P*	N_n	$\%_n$	N_{CNS}	$\%_{CNS}$	*P*
Bulk milk SCC	100[5]					0.30					0.64					0.45
Lower	-	11	37.9	9	27.3	Ref.[5]	11	37.9	22	29.7	Ref.	11	37.9	9	34.6	Ref.
Higher	-	18	62.1	24	72.7		18	62.1	52	70.3		18	62.1	17	65.4	
Premium	100					0.07					0.09					0.08
No	19	1	3.4	9	27.3	Ref.	1	3.4	19	25.7	Ref.	1	3.4	5	19.2	Ref.
Yes	81	28	96.6	24	72.7		28	96.6	55	74.3		28	96.6	21	80.8	
Udder preparation	99[7]					0.38					0.46					0.23
Dry 1 for each cow	42	8	27.6	15	45.5	Ref.	8	27.6	27	36.5	Ref.	8	27.6	12	46.2	Ref.
Dry 1 for several cows	21	10	34.5	7	21.2		10	34.5	14	18.9		10	34.5	4	15.4	
Wet / automatic / no	36	11	37.9	11	33.3		11	37.9	33	44.6		11	37.9	10	38.5	
Pre-milking teat disinfection	99[7]					0.65					0.41					0.61
No	79	21	72.4	23	69.7	Ref.	21	72.4	61	82.4	Ref.	21	72.4	22	84.6	Ref.
Yes	20	8	27.6	10	30.3		8	27.6	13	17.6		8	27.6	4	15.4	
Forestripping	99[8]					0.62					0.47					0.23
No	43	13	44.8	13	39.4	Ref.	13	44.8	27	37.0	Ref.	13	44.8	8	32.0	Ref.
Yes	55	16	55.2	20	60.6		16	55.2	46	63.0		16	55.2	17	68.0	
Flushing	100					0.26					0.02					0.02
No	84	21	72.4	28	84.8	Ref.	21	72.4	67	90.5	Ref.	21	72.4	25	96.2	Ref.
Yes	16	8	27.6	5	15.2		8	27.6	7	9.5		8	27.6	1	3.8	
Gloves	99[7]					0.26					0.11					0.01
No	69	15	51.7	24	72.7	Ref.	15	51.7	55	74.3	Ref.	15	51.7	23	88.5	Ref.
Yes	30	14	48.3	9	27.3		14	48.3	19	25.7		14	48.3	3	11.5	
Post-milking teat disinfection	100[9]					0.32					0.35					0.23
No	21	4	13.8	11	33.3	Ref.	4	13.8	20	27.0	Ref.	4	13.8	9	34.6	Ref.
Yes	75	25	86.2	22	66.7		25	86.2	54	73.0		25	86.2	17	65.4	
Housing	100					<0.001					0.01					<0.01

Freestall	72	28	96.6	12	36.4	Ref.	28	96.6	40	54.1	Ref.	28	96.6	10	38.5	Ref.
Loose-pack / tie stall	28	1	3.4	21	63.6		1	3.4	34	45.9		1	3.4	16	61.5	
Bedding hygiene	97[10]					0.09					0.04					0.12
Bad	17	1	3.4	8	25.0	Ref.	1	3.4	21	29.2	Ref.	1	3.4	6	23.1	Ref.
Good	80	28	96.6	24	75.0		28	96.6	51	70.8		28	96.6	20	76.9	
Drinking water	100					0.23					0.68					0.21
Other	85	28	96.6	28	84.8	Ref.	28	96.6	68	91.9	Ref.	28	96.6	19	73.1	Ref.
Tap water	15	1	3.4	5	15.2		1	3.4	6	8.1		1	3.4	7	26.9	
Clipping	100					0.22					0.20					0.65
No	41	11	37.9	21	63.6	Ref.	11	37.9	41	55.4	Ref.	11	37.9	11	42.3	Ref.
Yes	59	18	62.1	12	36.4		18	62.1	33	44.6		18	62.1	15	57.7	
Treatment	100					0.29					0.61					0.69
No	61	19	65.5	16	48.5	Ref.	19	65.5	47	63.5	Ref.	19	65.5	13	50.0	Ref.
Yes	39	10	34.5	17	51.5		10	34.5	27	36.5		10	34.5	13	50.0	
Monitoring	100					0.11					0.51					0.79
No	69	20	69.0	16	48.5	Ref.	20	69.0	60	81.1	Ref.	20	69.0	20	76.9	Ref.
Yes	31	9	31.0	17	51.5		9	31.0	14	18.9		9	31.0	6	23.1	

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]Number and percentage of bulk milk samples not infected with CNS. [3]Number and percentage of bulk milk samples positive for the different CNS species, respectively. [4]*P*-value for the overall effect. [5]The specific information differs between months of sampling per herd. [6]Reference category per independent factor. [7]The specific information is missing in 2 months (March - June) from 1 herd. [8]The specific information is missing in 3 months (March - June - September) from 1 herd and differs between months of sampling in 1 herd. [9]The specific information differs between months of sampling in 4 herds. [10]The specific information is missing in 3 months (March - June - September) from 3 herds.

Table 4 continued. Univariable, multilevel logistic regression models[1] for the presence of specific coagulase-negative *Staphylococcus* (CNS) species in bulk milk samples (n = 300)

Independent factor	N_{Herds}	*S. cohnii* (n = 35)					*S. equorum* (n = 138)					*S. sciuri* (n = 34)				
		N_n[2]	$\%_n$[2]	N_{CNS}[3]	$\%_{CNS}$[3]	*P*[4]	N_n	$\%_n$	N_{CNS}	$\%_{CNS}$	*P*	N_n	$\%_n$	N_{CN}S	$\%_{CNS}$	*P*
Bulk milk SCC	100[5]					0.32					1					0.30
Lower	-	11	37.9	16	45.7	Ref.[6]	11	37.9	50	36.2	Ref.	11	37.9	9	26.5	Ref.
Higher	-	18	62.1	19	54.3		18	62.1	88	63.8		18	62.1	25	73.5	
Premium	100					0.94					0.04					0.10
No	19	1	3.4	2	5.7	Ref.	1	3.4	34	24.6	Ref.	1	3.4	9	26.5	Ref.
Yes	81	28	96.6	33	94.3		28	96.6	104	75.4		28	96.6	25	73.5	
Udder preparation	99[7]					0.09					0.35					0.61
Dry 1 for each cow	42	8	27.6	21	61.8	Ref.	8	27.6	62	45.6	Ref.	8	27.6	12	35.3	Ref.
Dry 1 for several cows	21	10	34.5	6	17.6		10	34.5	33	24.3		10	34.5	7	20.6	
Wet / automatic / no	36	11	37.9	7	20.6		11	37.9	41	30.1		11	37.9	15	44.1	
Pre-milking teat disinfection	99[7]					0.42					0.08					0.91
No	79	21	72.4	29	85.3	Ref.	21	72.4	122	89.7	Ref.	21	72.4	26	76.5	Ref.
Yes	20	8	27.6	5	14.7		8	27.6	14	10.3		8	27.6	8	23.5	
Forestripping	99[8]					0.72					1.00					0.31
No	43	13	44.8	19	54.3	Ref.	13	44.8	63	46.0	Ref.	13	44.8	10	30.3	Ref.
Yes	55	16	55.2	16	45.7		16	55.2	74	54.0		16	55.2	23	69.7	
Flushing	100					0.02					0.52					0.23
No	84	21	72.4	33	94.3	Ref.	21	72.4	110	79.7	Ref.	21	72.4	29	85.3	Ref.
Yes	16	8	27.6	2	5.7		8	27.6	28	20.3		8	27.6	5	14.7	
Gloves	99[7]					0.96					0.12					0.05
No	69	15	51.7	19	55.9	Ref.	15	51.7	96	70.6	Ref.	15	51.7	27	79.4	Ref.
Yes	30	14	48.3	15	44.1		14	48.3	40	29.4		14	48.3	7	20.6	
Post-disinfection	100[9]					0.27					0.17					0.70
No	21	4	13.8	10	28.6	Ref.	4	13.8	42	30.4	Ref.	4	13.8	6	17.6	Ref.
Yes	75	25	86.2	25	71.4		25	86.2	96	69.6		25	86.2	28	82.4	
Housing	100					0.26					0.03					<0.01

Freestall	72	28	96.6	30	85.7	Ref.	28	96.6	98	71.0	Ref.	28	96.6	19	55.9	Ref.
Loose-pack / tie stall	28	1	3.4	5	14.3		1	3.4	40	29.0		1	3.4	15	44.1	
Bedding hygiene	97[10]					0.09					0.08					0.02
Bad	17	1	3.4	6	17.6	Ref.	1	3.4	28	20.4	Ref.	1	3.4	9	27.3	Ref.
Good	80	28	96.6	28	82.4		28	96.6	109	79.6		28	96.6	24	72.7	
Drinking water	100					0.34					0.17					0.79
Other	85	28	96.6	32	91.4	Ref.	28	96.6	117	84.8	Ref.	28	96.6	31	91.2	Ref.
Tap water	15	1	3.4	3	8.6		1	3.4	21	15.2		1	3.4	3	8.8	
Clipping	100					0.46					0.80					0.21
No	41	11	37.9	10	28.6	Ref.	11	37.9	55	39.9	Ref.	11	37.9	17	50.0	Ref.
Yes	59	18	62.1	25	71.4		18	62.1	83	60.1		18	62.1	17	50.0	
Treatment	100					0.83					0.77					0.49
No	61	19	65.5	23	65.7	Ref.	19	65.5	90	65.2	Ref.	19	65.5	26	76.5	Ref.
Yes	39	10	34.5	12	34.3		10	34.5	48	34.8		10	34.5	8	23.5	
Monitoring	100					0.51					0.80					0.38
No	69	20	69.0	23	65.7	Ref.	20	69.0	101	73.2	Ref.	20	69.0	21	61.8	Ref.
Yes	31	9	31.0	12	34.3		9	31.0	37	26.8		9	31.0	13	38.2	

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]Number and percentage of bulk milk samples not infected with CNS. [3]Number and percentage of bulk milk samples positive for the different CNS species, respectively. [4]*P*-value for the overall effect. [5]The specific information differs between months of sampling per herd. [6]Reference category per independent factor. [7]The specific information is missing in 2 months (March - June) from 1 herd. [8]The specific information is missing in 3 months (March - June - September) from 1 herd and differs between months of sampling in 1 herd. [9]The specific information differs between months of sampling in 4 herds. [10]The specific information is missing in 3 months (March - June - September) from 3 herds.

Nineteen herds did not receive their milk quality premium at least one time during the study period. The main reason for not receiving the milk quality premium in Flemish dairy herds is an elevated coli count (Passchyn et al., submitted). Therefore, it is not that surprising that especially the presence of *S. equorum* in the bulk milk samples was associated with not achieving the milk quality premium. *Staphylococcus equorum* has mainly been isolated from extramammary habitats such as teat apices (Braem et al., 2013; De Visscher et al., 2014, 2016a), milking machine unit liners and milkers' skin and gloves (De Visscher et al., 2014), and the cow's environment (Piessens et al., 2011). In that respect, the *S. equorum* bulk milk contamination in the current study might occur from an environmental transfer via the teat surface. The latter hypothesis is supported by the fact that the presence of *S. equorum* in the bulk milk was also negatively associated with pre-disinfection of the teats. Pre-milking teat disinfection lowered the likelihood of finding CNS in bulk milk in another study as well, which can most probably be attributed to a more careful removal of environmental teat skin organisms before milking (Jayarao et al., 2004).

The use of a single towel for multiple cows enlarged the probability of isolating *S. cohnii* from the bulk milk. The latter practice increases both the risk of contamination of quarters and cows with environmental organisms and the risk of transmission of the more udder-adapted pathogens to uninfected quarters and cows. Also, flushing of the milking cluster with hot water or steam after having milked a cow with (sub)clinical mastitis was negatively associated with the presence of *S. cohnii* in bulk milk. This association suggests the presence of *S. cohnni* in the teat liners which is in accordance with previous work (De Visscher et al., 2014). The latter finding was explained by direct contact between teat skin and liners (De Visscher et al., 2014). *Staphylococcus cohnii* rather seems to be an ecological equivalent of *S. equorum* although in at least some studies *S. cohnii* has been more commonly isolated from bovine milk (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2016b). Bulk milk contamination with this species presumably occurred from an environmental transmission via the teat surface and via the teat liners. However, the presence of *S. cohnii* in the bulk milk via teat liners contaminated with milk residues of *S. cohnii*-infected animals cannot be excluded.

Flushing with hot water or steam after having milked a cow with (sub)clinical mastitis was also negatively associated with the isolation of *S. simulans* and *S. haemolyticus* in the bulk milk. Both species have recently been assumed to be

opportunistic in nature as they have frequently been isolated from bovine IMI as well as from several extramammary habitats including the cow's environment (Piessens et al., 2011; Supré et al., 2011; Vanderhaeghen et al., 2015). The latter findings along with the results from the current study let us assume that the presence of *S. simulans* and *S. haemolyticus* in the bulk milk is rather the result of contamination from infected milk than from an environmental transfer.

Wearing gloves decreased the odds of having *S. devriesei*-positive bulk milk. This is interesting as in recent work *S. devriesei* could be hardly isolated from the milker's hands or gloves (De Visscher et al., 2014). *Staphylococcus devriesei* has been mainly isolated from extramammary habitats including teat apices from dry cows and end-term heifers and the cow's environment (Piessens et al., 2011; De Visscher et al., 2016a) and less from bovine IMI (Piessens et al., 2011; Supré et al., 2011; De Visscher et al., 2016b). Teat apices of lactating animals appear to be not (Taponen et al., 2008; De Visscher et al., 2014) or hardly colonized (Braem et al., 2013) with *S. devriesei*. The presence of this species in the bulk milk might be a result from an environmental transfer via the teat surface along with contamination of the teats via the milker's hands. However, more in-depth research concerning the ecology and epidemiological nature of *S. devriesei* is needed before final conclusions can be drawn.

Quarters infected with CNS are important contributors to the total number of somatic cells in herds with low bulk milk SCC (Piepers et al., 2009; Schukken et al., 2009; Sampimon et al., 2010). Also, mean bulk milk SCC was significantly associated with the mean CNS bulk milk count and isolation of *S. aureus* in bulk milk (Jayarao et al., 2004). In our study, however, no association between the bulk milk SCC and the presence of any of the CNS species or *S. aureus* was revealed. Although post-milking teat disinfection was found to decrease the total bacterial count in bulk milk (Jayarao et al., 2004) and is considered an effective control measure against both the host-adapted and environmental bacteria (Hogeveen et al., 2011), no species-specific associations were observed in this study.

Interestingly, participation in a monthly veterinary udder health program was positively associated with the presence of the for udder health more relevant CNS. A cause-effect reversal is suspected, yet the true nature of this association remains unknown.

Table 5. Multivariable, multilevel logistic regression models[1] for the presence of subgroups[2] of coagulase-negative *Staphylococcus* (CNS) species in bulk milk samples (n = 300)

Independent factor	More relevant CNS species (n = 92)[2] (N_{Herds} = 62)						Host-adapted CNS species (n = 70)[2] (N_{Herds} = 67)						Environmental CNS species (n = 201)[2] (N_{Herds} = 95)					
	β[3]	SE	OR[4]	95% CI		*P*[5]	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*
Intercept	-2.39	0.50				<0.001	0.14	0.43				0.74	0.99	0.33				<0.01
Month of sampling						<0.001						0.38						0.02
March	Ref.[6]	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–	
June	1.20	0.57	3.3	1.08	10.17		0.80	0.70	2.2	0.57	8.66		1.31	0.53	3.7	1.31	10.54	
September	2.63	0.58	13.8	4.46	42.86		0.77	0.65	2.2	0.60	7.72		1.09	0.51	3.0	1.09	8.06	
Housing						<0.01						0.02						0.02
Freestall	Ref.	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–	
Loose-pack / tie stall	0.93	0.32	2.5	1.36	4.75		2.58	1.14	13.1	1.40	123.75		2.44	1.06	11.4	1.44	90.80	
Clipping						0.09						NS[7]						NS
No	Ref.	–	–	–	–													
Yes	0.54	0.58	1.7	0.55	5.32													
Monitoring						0.02						NS						NS
No	Ref.	–	–	–	–													
Yes	0.76	0.32	2.1	1.14	4.00													
Month of sampling x clipping[8]						0.02						NA[7]						NA

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]*Staphylococcus chromogenes, S. simulans,* and *S. xylosus* represent the CNS species being more relevant for udder health; *S. chromogenes* and *S. epidermidis* are representative for the host-adapted species; and *S. cohnii, S. equorum, S. saprophyticus,* and *S. sciuri* represent the environmental species. [3]Regression coefficient. [4]Odds ratio. [5]*P*-value for the overall effect. [6]Reference category per independent factor. [7]Not significant or not applicable. [8]Interaction term between month of sampling and clipping.

Table 6. Multivariable, multilevel logistic regression models[1] for the presence of specific coagulase-negative *Staphylococcus* (CNS) species and *S. aureus* in bulk milk samples (n = 300)

Independent factor	*S. epidermidis* (n = 43) (N_{Herds} = 31)						*S. chromogenes* (n = 32) (N_{Herds} = 27)						*S. simulans* (n = 38) (N_{Herds} = 36)						*S. aureus* (n = 89) (N_{Herds} = 61)					
	β[2]	SE	OR[3]	95% CI		*P*[4]	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*	β	SE	OR	95%CI		*P*
Intercept	-0.16	0.47				0.73	-0.84	0.52				0.10	-2.11	0.85				0.01	2.44	0.53				<0.001
Month of sampling						0.23						0.37						<0.001						0.90
March	Ref.[5]	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–	
June	1.03	0.74	2.8	0.65	12.00		0.67	0.83	1.9	0.38	9.83		1.49	1.15	4.4	0.47	42.14		-0.05	0.92	1.0	0.16	5.74	
September	1.06	0.71	2.9	0.72	11.52		1.04	0.75	2.8	0.65	12.37		4.05	1.05	57.3	7.34	446.79		-0.37	0.82	0.7	0.14	3.43	
Flushing						NS[6]						NS						0.05						NS
No													Ref.	–	–	–	–							
Yes													-1.97	1.00	0.1	0.02	1.00							
Housing						NS						0.01						NS						NS
Freestall							Ref.	–	–	–	–													
Loose-pack / tie stall							2.93	1.17	18.7	1.90	183.89													
Drinking water						NS						NS						0.03						NS
Other													Ref.	–	–	–	–							
Tap water													2.88	1.32	17.8	1.34	237.02							

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]Regression coefficient. [3]Odds ratio. [4]*P*-value for the overall effect. [5]Reference category per independent factor. [6]Not significant.

Table 6 continued. Multivariable, multilevel logistic regression models[1] for the presence of specific coagulase-negative *Staphylococcus* (CNS) species in bulk milk samples (n = 300)

Independent factor	*S. xylosus* (n = 33) (N_{Herds} = 24)						*S. haemolyticus* (n = 74) (N_{Herds} = 49)						*S. devriesei* (n = 26) (N_{Herds} = 23)					
	β[2]	SE	OR[3]	95% CI		*P*[4]	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*
Intercept	-2.18	0.78				0.01	-0.10	0.45				0.83	-2.13	1.01				0.03
Month of sampling						0.07						0.05						0.03
March	Ref.[5]	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–	
June	1.86	0.98	6.4	0.95	43.42		1.29	0.74	3.6	0.85	15.52		1.34	1.33	3.8	0.28	51.67	
September	2.07	0.96	8.0	1.22	51.77		1.57	0.71	4.8	1.20	19.12		3.23	1.27	25.3	2.11	303.48	
Flushing						NS[6]						0.01						NS
No							Ref.	–	–	–	–							
Yes							-2.50	1.02	0.1	0.01	0.61							
Gloves						NS						NS						0.04
No													Ref.	–	–	–	–	
Yes													-2.46	1.18	0.1	0.01	0.87	
Housing						<0.001						< 0.01						0.01
Freestall	Ref.	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–	
Loose-pack / tie stall	4.16	1.21	64.1	5.99	685.15		3.50	1.24	33.3	2.91	380.04		3.78	1.40	43.8	2.84	675.30	

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]Regression coefficient. [3]Odds ratio. [4]*P*-value for the overall effect. [5]Reference category per independent factor. [6]Not significant.

Table 6 continued. Multivariable, multilevel logistic regression models[1] for the presence of specific coagulase-negative *Staphylococcus* (CNS) species in bulk milk samples (n = 300)

Independent factor	*S. cohnii* (n = 35) (N_{Herds} = 24)						*S. equorum* (n = 138) (N_{Herds} = 78)						*S. sciuri* (n = 34) (N_{Herds} = 28)					
	β[2]	SE	OR[3]	95% CI		*P*[4]	β	SE	OR	95% CI		*P*	β	SE	OR	95% CI		*P*
Intercept	-2.49	1.03				0.02	3.72	1.23				< 0.01	-1.72	0.67				0.01
Month of sampling						< 0.01						0.19						0.01
March	Ref. [5]	–	–	–	–		Ref.	–	–	–	–		Ref.	–	–	–	–	
June	3.06	0.98	35.5	5.21	241.98		0.92	0.59	2.5	0.79	8.01		1.46	0.91	4.3	0.72	25.55	
September	2.53	0.94	12.5	2.00	78.15		0.78	0.57	2.2	0.72	6.57		2.27	0.82	9.7	1.95	48.05	
Premium						NS[6]						0.02						NS
No							Ref.	–	–	–	–							
Yes							-2.91	1.22	0.1	0.01	0.59							
Udder preparation						0.04						NS						NS
Dry 1 for each cow	Ref.	–	–	–	–													
Dry 1 for several cows	2.70	1.13	14.8	1.63	134.95													
Wet / automatic / no	0.72	1.12	2.0	0.23	18.18													
Pre-milking teat disinfection						NS						0.02						NS
No							Ref.	–	–	–	–							
Yes							-1.74	0.73	0.2	0.04	0.73							
Flushing						0.01						NS						NS
No	Ref.	–	–	–	–													
Yes	-3.31	1.30	<0.01	<0.01	0.47													
Housing						NS						0.04						< 0.01
Freestall							Ref.	–	–	–	–		Ref.	–	–	–	–	
Loose-pack / tie stall							2.32	1.10	10.2	1.17	88.58		3.57	1.21	35.6	3.33	379.43	

[1]Month of sampling forced in all models as fixed effect and herd as random effect to correct for potential clustering. [2]Regression coefficient. [3]Odds ratio. [4]*P*-value for the overall effect. [5]Reference category per independent factor. [6]Not significant.

As the more relevant CNS, i.e. *S. chromogenes*, *S. simulans,* and *S. xylosus*, have already been associated with an increased quarter SCC and have been shown to cause persistent IMI (Supré et al., 2011; Fry et al., 2014; De Visscher et al., 2015), we hypothesize that herds suffering from a high proportion of cows with an elevated SCC might have decided to monitor their udder health more closely with their veterinarian. An explanation for the association of drinking water with the isolation of *S. simulans* from bulk milk is also difficult. Sampimon et al. (2009) observed a negative association between drinking water and the presence of CNS in quarter milk samples. The positive association in the current study might also be attributed to a cause-effect reversal.

Conclusions

Bulk milk yields a wide range of different CNS species. Host-adapted as well as environmental CNS species are present. Several herd-level risk factors were associated with the presence of subgroups of as well as specific CNS species. Contamination of the bulk milk with CNS might occur from environmental transmission for some species, i.e. *S. equorum* or *S. cohnii,* or from within the udder for other species, i.e. *S. simulans.* However, studies collecting bulk milk and quarter milk samples in the same time frame along with environmental samples should be conducted to determine the exact origin of CNS species in bulk milk.

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Chapter 7

General Discussion

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Introduction

Effort is ongoing to broaden our knowledge of the ecological and epidemiological nature and the relevance for udder health of the bovine-related coagulase-negative *Staphylococcus* species (CNS). This thesis also aimed to do so. Mannitol salt agar was considered as a suitable medium for CNS recovery (*Chapter 3*) as all bovine-associated CNS were able to grow, and was further applied in *Chapter 4.2, 5.1* and *6*. A predominance of *Staphylococcus chromogenes* in quarter milk samples and the recovery in all herds was observed in early lactation (*Chapter 5.1*), whereas bulk milk harboured a wide variety of CNS species (*Chapter 6*). Herd-specific CNS microbiota were detected (*Chapter 4.2, 5.1, 5.2, 6*). Quarters of heifers (*Chapter 5.1, 5.2*) and quarters of animals in peak lactation *(Chapter 5.2*) were more likely to be infected with the so-called more relevant CNS species, i.e. *S. chromogenes*, *S. simulans,* and *S. xylosus*. The udder-adapted, and by extension host-adapted, nature of *S. chromogenes* was substantiated (*Chapter 4.2, 5.1*). Also, the environmental ecology of *S. cohnii* (*Chapter 5.1*)*, S. equorum* (*Chapter 4.2, 5.1*)*, S. saprophyticus* and *S. sciuri* (*Chapter* 5.1) was confirmed whereas a more host-adapted nature was evidenced for *S. devriesei* and *S. haemolyticus* (*Chapter 4.2*). *Staphylococcus chromogenes* was also shown to be a predominant teat apex colonizer prior to calving (*Chapter 4.2*) whereas teat apices of lactating dairy cows and heifers were colonized to a lesser extent with this species (*Chapter 4.1*). Teat apices colonized with *S. chromogenes* prior to parturition had higher odds of having *S. chromogenes* intramammary infection (IMI) in early lactation (*Chapter 5.1*). The higher relevance for udder health of *S. chromogenes*, *S. simulans,* and *S. xylosus* was confirmed as those 3 species were able to increase the quarter SCC both in early and in a later stage in lactation (*Chapter 5.1*, *5.2*) whereas other species were not.

All recovered CNS in this thesis were accurately identified to the species level providing detailed and unique information. The first applied genotypic identification method was transfer RNA intergenic spacer PCR (tDNA-PCR) as this technique was validated for bovine-related CNS species by our research group (Supré et al., 2009), implying all necessary equipment and a comprehensive library were available. If no identification could be obtained, sequencing of the *rpoB* gene was performed. However, the presences of several genera other than the *Staphylococcus* genus (*Chapter 3, 4.2, 5.1, 6*), though often phenotypically indistinguishable from them,

made us prefer sequencing of the *16S rRNA* gene, which relies on universal primers in contrast with the *rpoB* gene. The lower accuracy of our preferred sequencing method did not affect our results as sequencing of the *16S rRNA* gene was mostly performed to identify genera other than the *Staphylococcus* genus and was never used alone, though in combination with tDNA-PCR, for CNS species identification.

Prevalence data and species distribution in Flanders were completed as several unexplored bovine habitats were studied and for the first time, subgroup- and species-specific risk factors and predictors were identified. The results in this work considerably enhance our insights in bovine-associated CNS ecology and epidemiology, and their relevance for udder health. Besides, large collections of CNS species and associated data are available for further investigations. In this final chapter our findings are summarized, compared to other research and discussed.

Future CNS research will benefit from the use of mannitol salt agar

Research intending to examine the prevalence and distribution of specific bacterial species requires the use of a (quasi-)selective medium, allowing the genera / species of interest to grow as well as circumventing suppression by other pathogens at the same time. The latter prerequisite was unconfirmed for CNS and was therefore investigated in *Chapter 3*. The ability of all bovine-associated CNS to grow on MSA was demonstrated, though some aspects should be taken into account. First, a few isolates of some CNS species, i.e. *S. chromogenes*, *S. equorum*, *S. haemolyticus*, *S. hominis,* and *S. lugdunensis*, did not show growth within 24h aerobic incubation at 37°C (*Chapter 3*). A 48h incubation time should thus be respected, especially in those studies that aim at identifying all present CNS species in a specific habitat. In fact, studies using MSA with different incubation conditions (Piessens et al., 2011; El-Jakee et al., 2013; Abbas et al., 2014; Li et al., 2015) might actually have missed the presence of some species in their samples for that reason. Secondly, both growth characteristics and mannitol fermentation seem to strongly vary among isolates within species (*Chapter 3*), emphasizing caution is needed in the interpretation. Therefore, mannitol fermentation cannot be used solely in differentiating bovine *S. aureus* from non-S. *aureus* staphylococci (Cheng et al., 2010; Kateete et al., 2010; Chavhan et al; 2012; Oliveira et al., 2015; *Chapter 3*), as was wrongly assumed before (Chapman, 1945; Addis et al., 2011; Singh et al., 2013). The need for

supplementary methods for phenotypic identification of *S. aureus* does, however, not outweigh the advantages of this conscientiously tested agar for overcrowded bovine-associated habitats. The suitability of MSA for CNS recovery under field conditions was tested as well (*Chapter 3*) and the practicability of this agar in comparison with non-selective media was demonstrated. The results made us decide to use MSA for CNS recovery from all other habitats and matrices in our studies, namely teat apices (*Chapter 4.2*), quarter milk samples (*Chapter 5.1*) and bulk milk samples (*Chapter 6*).

In the aforementioned studies, pathogens other than staphylococci were isolated though they never hindered the recovery of all phenotypically different CNS colony types (Chapman et al., 1945; Graber et al., 2013). Actually, the number of phenotypically different colony types ranged from 0 (no growth) to 4 (teat apices, *Chapter 4.2*), 5 (quarter milk samples, *Chapter 5.1*), and 9 (bulk milk samples, *Chapter 6*). Other pathogens recovered from the studied habitats belonged to the phyla *Firmicutes* (Chapman, 1945; Han et al., 2007; Braem et al., 2013; *Chapter 3*, *4.2*, *5.1*, *6*), *Proteobacteria* (*Chapter 4.2*, 5.1, *6*), and *Actinobacteria* (Braem et al., 2013; *Chapter 3*, *4.2*, *6*) and a minority of isolates were fungi (*Chapter 4.2, 6*).

In conclusion, as MSA is the only thoroughly examined (quasi-)selective agar for the recovery of bovine-associated CNS from multiple bovine-associated habitats and non-selective media are inappropriate for CNS recovery from overcrowded habitats, such as teat apices, bulk milk and manure, future studies should take advantage of this medium. However, if one wants to recover other (mastitis-causing) microbiota besides staphylococci, non-selective media can be used for quarter milk samples while for overpopulated habitats several other (tested) (quasi-)selective media along with MSA should be applied.

Bovine-associated habitats have been further explored

Our work contributed to mapping the species-specific CNS prevalence and distribution of IMI both in early lactation and throughout lactation (Table 1, *Chapter 5.1*). Yet, several other studies have genotypically identified different CNS species. Still, not all of those genotypic studies were set up for determining the CNS species-specific quarter IMI prevalence (Santos et al., 2008; Sampimon et al., 2009b; Park et al., 2011; Persson Waller et al., 2011; Quirk et al., 2012; Fry et al., 2014b; Lange et al., 2015). Additionally, in some other studies not all isolated CNS were retained for

genotypic identification (Santos et al., 2008; Bexiga et al., 2014). In fact, only five studies provided quarter prevalence data to compare with (Figure 1) (Piessens et al., 2011; Supré et al., 2011; Mørk et al., 2012; Tomazi et al., 2014, *Chapter 5.1*).

In almost all studies *S. chromogenes* caused the majority of IMI whereas in some studies *S. epidermidis* (Persson Waller et al., 2011; Bexiga et al., 2014), *S. equorum* (*Chapter 5.1*, data not shown), and *S. xylosus* (Quirk et al., 2012) were mainly isolated. *Staphylococcus chromogenes* was however always very prevalent, just as *S. haemolyticus*, *S. simulans*, *S. cohnii*, *S. sciuri*, *S. saprophyticus,* and *S. capitis* were (Table 1).

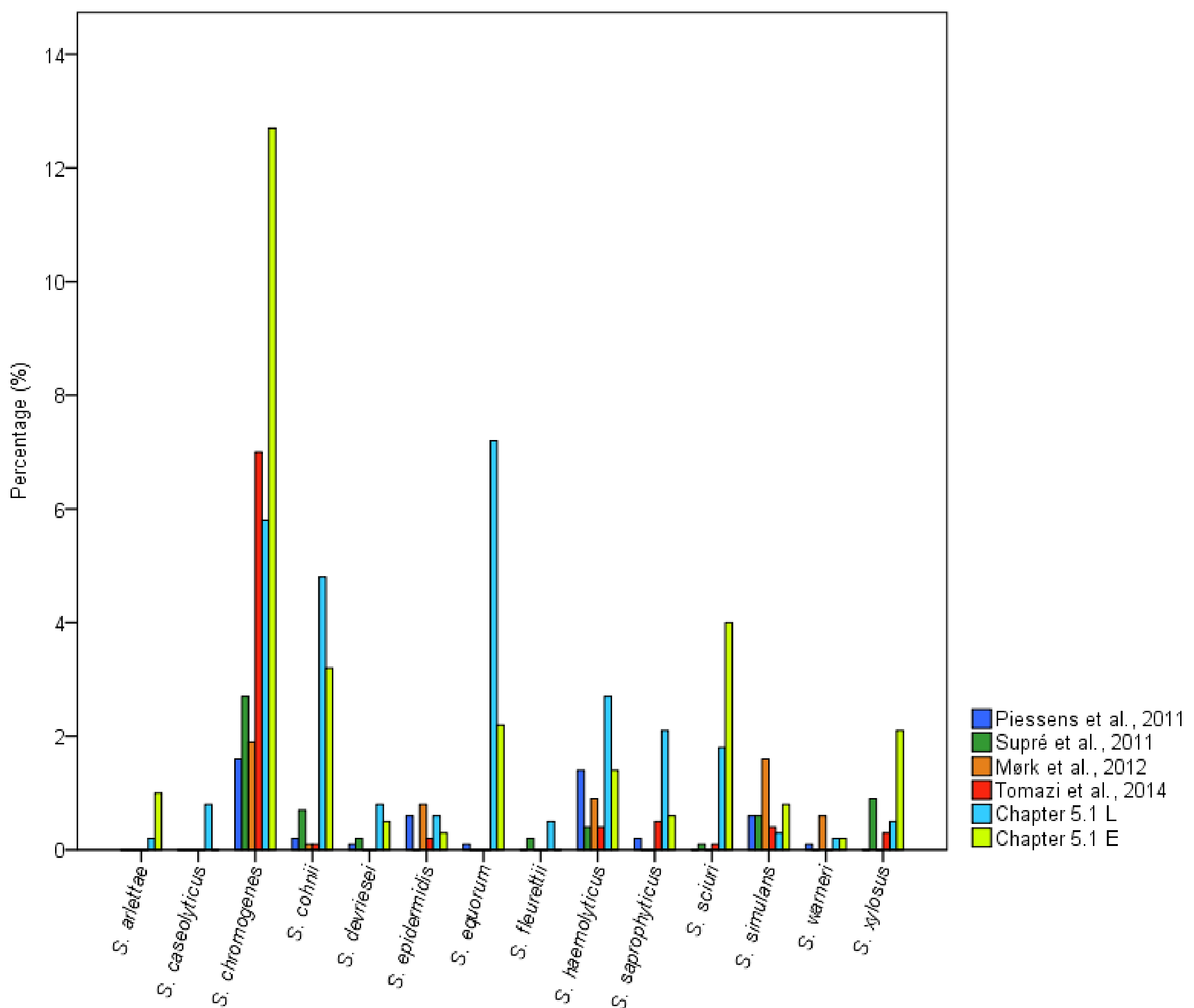

Figure 1. Percentage of bovine quarters infected with frequently observed coagulase-negative *Staphylococcus* species both throughout lactation (Piessens et al., 2011, blue; Supré et al., 2011, green; Mørk et al., 2012, orange; Tomazi et al.,

2014, red; *Chapter 5.1* L, light blue) and in early lactation (*Chapter 5.1* E, yellow-green).

Bovine milk-related species other than the aforementioned ones in the referred genotypic studies are (total n = 28) (in alphabetical order) *S. agnetis, S. arlettae, S. auricularis, S. caprae, S. caseolyticus, S. devriesei, S. fleurettii, S. gallinarum, S. hominis, S. hyicus, S. intermedius, S. lentus, S. nepalensis, S. pasteuri, S. pseudintermedius, S. succinus, S. vitulinus,* and *S. warneri*, substantiating the extent of the *Staphylococcus* group.

Strikingly, the number of different CNS species and the recovery of rarely isolated species varies substantially among studies. The number of included herds obviously plays a role as a low number of herds reduces the likelihood of isolating uncommon species, yet does not precluded it. In some recent studies, a wide range of different CNS species were recovered although only 3 up to 6 herds were included (Piessens et al., 2011; Supré et al., 2011). Actually, the opposite was also true as a low number of different species was observed in a study concerning 21 herds (Tomazi et al., 2014). Another explanation for the diversity might be found in the genotypic identification method applied: it feels as if the use of gene sequencing of the *rpoB*, *16S rRNA* or *tuf* gene (Sampimon et al., 2009b; Park et al., 2011; Persson Waller et al., 2011; Fry et al., 2014b; Lange et al., 2015; *Chapter 5.1*), of tDNA-PCR (Supré et al., 2011; *Chapter 5.1*) and of AFLP (Piessens et al., 2011) as opposed to PCR-RFLP and ITS-PCR (Santos et al., 2008; Quirk et al., 2012; Bexiga et al., 2014; Tomazi et al., 2014) results in the recovery of a higher number of different and more rare species. Sequencing of the *sodA* gene does, however, not fit in the latter hypothesis (Mørk et al., 2012). The performance of PCR-RFLP likely depends on the chosen restriction enzyme, which could be an explanation. Also, the used reference libraries should include a sufficient number of bovine-associated field isolates originating from the different habitats under study per species for optimal species identification (Park et al., 2011; Vanderhaeghen et al., 2015). Isolates with a questionable identification using tDNA-PCR were subjected to sequencing of the *16S rRNA* gene in our studies and were afterwards added to our tDNA-PCR-library in order to continuously preserve and enhance the typeability and accuracy, as recommended (Zadoks and Watts, 2009).

Table 1. Identification of coagulase-negative staphylococci (CNS) causing intramammary infection throughout lactation and in early lactation only relying on genotypic species identification (ID)

Reference	Country	N[1]			Species ID[2]	N CNS[3]	Most prevalent CNS species (%)[4]		
		H	A	Q			1st	2nd	3rd
Throughout lactation									
Santos et al., 2008	Brazil	23	54	216[5]	PCR-RFLP	10	*S. chromogenes* (52)	*S. epidermidis* (15)	*S. capitis* (6)
Sampimon et al., 2009b	The Netherlands	/	/	/	*rpoB* sequencing	17	*S. chromogenes* (37)	*S. epidermidis* (13)	*S. xylosus* (9)
Park et al., 2011	USA	/	/	/	*16S* sequencing	11	*S. chromogenes* (72)	*S. xylosus* (9)	*S. haemolyticus* (6)
Persson Waller et al., 2011	Sweden	/	/	/	*tuf* sequencing	11	*S. epidermidis* (31)	*S. chromogenes* (21)	*S. haemolyticus* (14)
Piessens et al., 2011[6]	Belgium	6	60	2580	AFLP	13	*S. chromogenes* (31)	*S. haemolyticus* (26)	*S. epidermidis* (12)
Supré et al., 2011[6]	Belgium	3	89	3064	tDNA-PCR	12	*S. chromogenes* (46)	*S. xylosus* (16)	*S. cohnii* (11)
Mørk et al., 2012[6]	Norway	4	206	4030	*sodA* sequencing	8	*S. chromogenes* (31)	*S. simulans* (26)	*S. haemolyticus* (15)
Quirk et al., 2012[6]	USA	1	139	/	PCR-RFLP	8	*S. xylosus* (40)	*S. chromogenes* (30)	*S. haemolyticus* (10)
Bexiga et al., 2014[6]	Portugal	4	264	2302[5]	ITS-PCR	5	*S. epidermidis* (34)	*S. simulans* (32)	*S. chromogenes* (14)
Fry et al., 2014b[6]	Canada	89	555	/	*rpoB* sequencing	15	*S. chromogenes* (50)	*S. simulans* (24)	*S. xylosus* (9)
Tomazi et al., 2014	Brazil	21	285	1140	PCR-RFLP	11	*S. chromogenes* (74)	*S. saprophyticus* (6)	*S. haemolyticus* (5)
Lange et al., 2015	Brazil	/	/	/	*16S* sequencing	12	*S. chromogenes* (39)	*S. epidermidis* (13)	*S. haemolyticus* (6)
Chapter 5.1	Belgium	13	156	624	tDNA-PCR	20	*S. equorum* (25)	*S. chromogenes* (20)	*S. cohnii* (16)
In early lactation									
Fry et al., 2014b[6]	Canada	89	555	/	*rpoB* sequencing	18	*S. chromogenes* (43)	*S. simulans* (24)	*S. xylosus* (8)
Chapter 5.1	Belgium	13	156	624	tDNA-PCR	19	*S. chromogenes* (41)	*S. sciuri* (13)	*S. cohnii* (11)

[1]Number of included herds (H), animals (A), and quarters (Q). [2]Species identification using restriction fragment length polymorphism (PCR-RFLP), sequencing of the *rpoB*, *16s rRNA*, *tuf* or *sodA* gene, amplified fragment length polymorphism (AFLP), transfer RNA intergenic spacer PCR (tDNA-PCR) or internal transcribed spacer PCR (ITS-PCR). [3]Number of different CNS species. [4]Percentage within all isolated CNS species. [5]Only a part of all CNS were retained for species identification. [6]Longitudinal studies.

The use of MSA can also explain the observed difference as overgrowth of pathogens other than CNS was circumvented and all phenotypically different colonies were thus picked up and subjected to further analysis. Quirk et al. (2012), however, also used MSA though in combination with another identification method.

Chapter 6 presents the first species-specific herd-level study using bulk milk as an interesting matrix in CNS research and relying on genotypic identification. Both host-adapted CNS species, e.g. *S. chromogenes*, and environmental species, e.g. *S. equorum*, were often recovered from bulk milk. We hypothesize that the predominance of either host-adapted or environmental CNS species in bulk milk is associated with a domination of major pathogens with the same ecology in the herd. In that respect, screening the CNS distribution in bulk milk could provide information on the herd health status and certain management issues, as already seen for so-called contagious major pathogens (Olde Riekerink et al., 2006, 2010; Boss et al., 2011). If this assumption is true, screening of the species-specific CNS distribution in bulk milk could eventually become an efficient instrument to improve udder health and milk quality through an optimised management.

Chapter 4.1 and *Chapter 4.2* add to our knowledge about teat apex colonization (TAC) both prior to parturition and throughout lactation. The predominant species colonizing teat apices of dairy cows and heifers are shown in Figure 2. Differences in CNS prevalence and distribution among lactation studies might be explained by variation in the number of included herds, ranging between 1 (Taponen et al., 2008) and 3 (*Chapter 4.1*), the amount of swabbed teat apices being 65 (Taponen et al., 2008), 72 (Braem et al., 2013) and 140 (*Chapter 4.1*), and the used identification method, i.e. ribotyping (Taponen et al., 2008), $(GTG)_5$-PCR (Braem et al., 2011; Braem et al., 2013) and tDNA-PCR (Supré et al., 2009; *Chapter* 4.1). A larger number of CNS species colonized teat apices prior to parturition (24 species, *Chapter 4.2*) than after calving (in lactation), with 10 (Taponen et al., 2008), 13 (*Chapter 4.1*) and 14 (Braem et al., 2013) different species, respectively, colonizing teat apices. Routinely cleaning of the teat end of lactating dairy cows and heifers as part of udder preparation before milking, the flushing effect of lactation (*Chapter 4.1*), and the application of post-milking teat disinfection (Piessens et al., 2012; Quirk et al., 2012) potentially clarify this discrepancy. Recovery of CNS on MSA (*Chapter 4.2*) and a considerably higher number of included herds likely enlarged the number of detected species as well in our work (*Chapter 4.2*). On the other hand, only limited variation in

the number of different species per teat apex, with a maximum between 3 and 6, was detected among lactating and non-lactating animals (Braem et al., 2013, *Chapter 4.1, 4.2*).

Some species, e.g. *S. auricularis, S. chromogenes,* and *S. devriesei,* never or hardly ever colonized teat apices of lactating animals, whereas the opposite was true for *S. arlettae, S. cohnii*, *S. epidermidis*, *S. fleurettii*, *S. gallinarum*, *S. saprophyticus*, *S. simulans, S. succinus,* and *S. xylosus* (Figure 2).

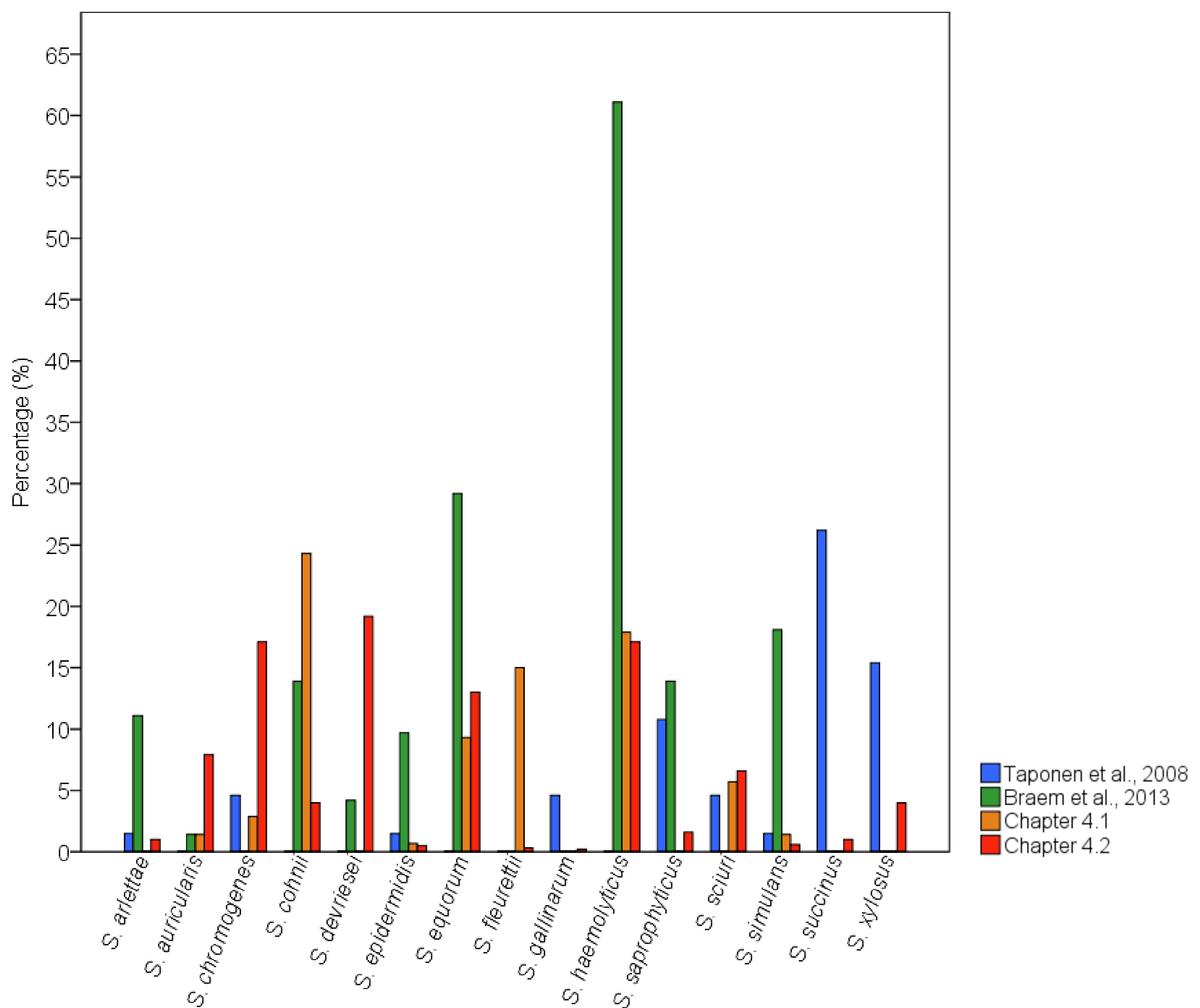

Figure 2. Percentage of teat apices from lactating dairy cows and heifers (Taponen et al., 2008, blue; Braem et al., 2013, green; *Chapter 4.1*, orange) and end-term heifers and dry dairy cows (*Chapter 4.2*, red), respectively, colonized with the predominant coagulase-negative *Staphylococcus* species.

Staphylococcus equorum, *S. haemolyticus,* and *S. sciuri*, though to a lesser extent, frequently colonized teat apices from both lactating and non-lactating animals (Taponen et al., 2008; Braem et al., 2013; *Chapter 4.1, 4.2*), suggesting an omnipresent nature of these particular species. Especially *S. chromogenes* grabbed our attention, being a dominant species (*Chapter 4.2*) and the sole species present on teat apices in all herds pre-partum (De Vliegher et al., 2003; *Chapter 4.2*). Additionally, this species was abundantly present on the udder skin of lactating animals (Taponen et al., 2008). Despite the latter findings, *S. chromogenes* was hardly recovered from teat apices of cows in lactation (Taponen et al., 2008; Braem et al., 2013; *Chapter 4.1*), suggesting a different CNS microbiota on teat skin compared to teat apices.

A herd-specific CNS microbiota exists and demands further study

Staphylococcus chromogenes is highly prevalent in bovine milk in all herds whereas for other species large within-herd differences are observed (Piessens et al., 2011; Supré et al., 2011; Mørk et al., 2012; Bexiga et al., 2014; *Chapter 5.1, 5.2*). Research exploring TAC agrees on the finding that every herd seems to have its own CNS microbiota (Braem et al., 2013; *Chapter 4.1, 4.2*). Also, *S. equorum* was recovered from the bulk milk in almost all herds. Other species such as *S. haemolyticus*, *S. cohnii,* and *S. epidermidis* were in none of the three bulk milk samples detected in some herds whereas in other herds they were isolated from all three bulk milk samples (*Chapter 6*). The observed herd-dependency in the different habitats highlights the potential role of factors such as management and climatic conditions, and requires herd-level analyses to identify variables associated with the presence of CNS (species).

Chapter 6 identifies for the first time herd-level management factors associated with individual CNS species, using bulk milk samples. In contrast with earlier studies (Gillespie et al., 2012), *Chapter 6* revealed a strong seasonal effect of CNS recovery of environmental CNS species, i.e. *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri*, from bulk milk whereas the host-adapted species, i.e. *S. chromogenes* and *S. epidermidis,* were equally prevalent in all seasons. Climatic conditions such as temperature and precipitation are significantly affecting the TAC of several species (*Chapter 4.2*). Contamination of bulk milk with teat apex colonizing species might

clarify the observed seasonal species distribution in bulk milk. Contrastingly, (species-specific) CNS IMI did not depend on climatic conditions (*Chapter 5.1*). Still, no quarter milk samples were available and only 3 bulk milk samples with a 3-month interval were collected (*Chapter* 6) rather than e.g. weekly samples, potentially affecting sensitivity (Godkin and Leslie, 1993; Olde Riekerink et al., 2010). The conclusions from *Chapter 6* remain thus preliminary, as working solely with bulk milk has its limitations, and we look forward to the results from new studies combining the CNS recovery from bulk milk with the CNS isolation from quarter milk samples in the same herds.

Staphylococcus chromogenes **should be easily differentiated from other CNS species**

Our work substantiates the higher relevance for udder health of *S. chromogenes*, put forward by Supré et al. (2011) and Fry et al. (2014b), in early lactation (*Chapter 5.1*) and throughout lactation (*Chapter 5.2*). Still, Bexiga et al. (2014) questioned this species-specific impact, and suggested a cow-dependent response on CNS IMI rather than a species-dependent effect. In that study, however, only 5 different CNS species were identified (Table 1) and the predominant species, *S. chromogenes*, *S. simulans,* and *S. epidermidis,* were isolated in low numbers. The latter might explain why the numerically higher SCC in *S. chromogenes*-infected quarters was not found to be significantly different from the other CNS-infected quarters. In our opinion, enough evidence exists that *S. chromogenes* is more relevant for udder health than other CNS species, although we would not classify it as a major pathogen. The same is likely true for *S. simulans* and *S. xylosus,* yet in the lack of sufficient numbers final conclusions cannot be drawn. Besides the ability to elevate quarter SCC, *S. chromogenes* is able to cause persistent IMI (Taponen et al., 2007; Thorberg et al., 2009; Piessens et al., 2011; *Chapter 5.1*: data not shown). Others have confirmed the persistency potential using pulsed-field gel electrophoresis (Gillespie et al., 2009; Mørk et al., 2012; Bexiga et al., 2014; Fry et al., 2014b) and ongoing strain-typing, using multi-locus sequence typing (MLST), may provide definite conclusions (*Chapter 5.1*). Interestingly, quarters non-cured from *S. chromogenes* in early lactation had a significantly higher quarter SCC as opposed to uninfected quarters (*Chapter 5.1*: data not shown), suggesting at least some *S. chromogenes* strains can survive and

maintain themselves in the mammary gland despite the established host immune response.

Altogether, there are enough reasons to strive in the near future for an easy differentiation of *S. chromogenes* from other CNS in both clinical and subclinical mastitis in routine milk quality laboratories. Differentiation should rely on genotypic methods as differences in phenotypic expression of several characteristics, e.g. DNase reaction and mannitol fermentation, among isolates within species were observed (*Chapter 3*). Eventually, it would allow to quantify the true relevance of *S. chromogenes*. Nowadays, it looks as if prevention of *S. chromogenes* IMI in well-managed dairy herds with an already good udder health should be encouraged to keep bulk milk SCC low. As quarters with *S. chromogenes* colonized teat apices were more likely to be infected with *S. chromogenes* in early lactation and subsequently were more likely to be *S. chromogenes*-infected in that quarter one month later (*Chapter 4.2, 5.1*), prevention of TAC might be the key. This hypothesis should, however, be confirmed by strain-typing, which is ongoing. How to prevent and control TAC with *S. chromogenes* is the next question that needs to be answered. It looks as if teat dipping cannot bring merit as this was not identified as a factor associated with TAC (*Chapter 4.2*). Yet, quarters with inverted teat ends had higher odds of *S. chromogenes* infection (*Chapter* 5.1). Teat characteristics potentially depend on the genetic background, implying genetic selection should be investigated (Detilleux, 2009; Verbeke et al., 2012). Only if *S. chromogenes* TAC during lactation also increases the likelihood of *S. chromogenes* IMI, the role of milking machine settings and the choice of teat cup liners should be scrutinized as well (Bhutto et al., 2010; Zwertvaegher et al., 2013).

Subgroup- and species-specific risk factors have been identified for the first time

Along with the increased prevalence and relevance of CNS IMI, the need for risk factor studies, identifying associations with the presence of CNS in different habitats, arose. Several studies conducted multilevel, multivariable analysis and assessed variance components before (Sampimon et al., 2009a; Piepers et al., 2011; Passchyn et al., 2014), though CNS were always considered as one homogeneous group.

Table 2. Factors significantly associated with the likelihood of intramammary infection (IMI) caused by (subgroups of) coagulase-negative *Staphylococcus* (CNS) species both in early lactation and throughout lactation

Independent factor	Categories	OR[1]	Outcome variable	Reference
IMI in early lactation				
Cow level[2]				
Hygiene[3]	Good vs. poor	1.9*[4]	CNS as a group	Piepers et al., 2011
Teat dipping prior to calving[3]	No vs. yes	0.40*	CNS as a group	Piepers et al., 2011
Udder clipping prior to calving[3]	No vs. yes	0.40*	CNS as a group	Piepers et al., 2011
Supplementation with selenium[3]	Higher vs. lower	0.35*	CNS as a group	Passchyn et al., 2014
Assistance at calving[3]	No vs. yes	4.0*	CNS as a group	Passchyn et al., 2014
Parity	2nd lactation or older vs. 1st lactation	3.9**	More relevant CNS species[5]	***Chapter 5.1***
	2nd lactation or older vs. 1st lactation	4.2**	Host-adapted CNS species[5]	***Chapter 5.1***
Hygiene of udder and teat skin	Very clean vs. slightly dirty vs. **dirty**[6]	6.4*	Environmental CNS species[5]	***Chapter 5.1***
Quarter level				
Teat end condition	Good vs. protuberant vs. **inverted**	2.8*	More relevant CNS species	***Chapter 5.1***
	Good vs. protuberant vs. **inverted**	3.7*	Host-adapted CNS species	***Chapter 5.1***
Teat apex colonization	Non-colonized vs. colonized with another species vs. **colonized with the same species**	3.3*	Host-adapted CNS species	***Chapter 5.1***
IMI throughout lactation				
Herd level				
Pasture access during outdoor season	No vs. yes vs. restrained vs. **part of the season restricted**	NA[7]*	CNS as a group	Sampimon et al., 2009a
Percentage of stalls contaminated with milk	Continuous	NA*	CNS as a group	Sampimon et al., 2009a
Dry cows housed in two groups	Yes vs. no	NA*	CNS as a group	Sampimon et al., 2009a
Source of drinking water used	Tap water vs. **ditch water** vs. own well water source vs. combination of tap water with ditch or own well water source vs. other	NA*	CNS as a group	Sampimon et al., 2009a
	Tap water vs. ditch water vs. own	NA*	CNS as a group	Sampimon et al., 2009a

	well water source vs. combination of tap water with ditch or own well water source vs. **other**			
Bulk milk SCC (cells/mL)	<150,000 vs. 150,000 - 250,000 vs. **> 250,000 cells/mL**	NA*	CNS as a group	Sampimon et al., 2009a
Measuring cow SCC at milk recording	No vs. yes	NA*	CNS as a group	Sampimon et al., 2009a
Udder health monitoring by veterinarian	No vs. yes	NA*	CNS as a group	Sampimon et al., 2009a
Cow level				
Parity	1st lactation vs. 2nd lactation or older	0.10**	More relevant CNS species	***Chapter 5.2***
Stage in lactation	1 - 60 DIM[8] vs. **61 - 120 DIM** vs. 121 - 180 DIM vs. >180 DIM	2.0*	More relevant CNS species	***Chapter 5.2***
Quarter level				
Quarter SCC	Continuous	2.1**	More relevant CNS species	***Chapter 5.2***
	Continuous	1.6**	Less relevant CNS species	***Chapter 5.2***

[1]Odds ratio. [2]No herd level factors were identified. [3]Only heifers were included. [4]*P*-value < 0.05*, *P*-value < 0.001**. [5]*Staphylococcus chromogenes*, *S. simulans,* and *S. xylosus*, represent CNS species being more relevant for udder health; *S. chromogenes* is representative for the host-adapted species; and *S. cohnii*, *S. equorum*, *S. saprophyticus,* and *S. sciuri* represent the environmental species. [6]If > 2 categories: the significant category is represented in bold face. [7]Not available. [8]Days in milk.

Our studies identified risk factors associated with IMI caused by subgroups of species or individual CNS species at first (*Chapter 5.1, 5.2*) (Table 2). The apparently higher susceptibility of heifers for CNS IMI compared to multiparous cows is intriguing and requests further study. Fresh animals also seemed to be more susceptible for CNS IMI (*Chapter 5.1*: 26%) as opposed to animals in a later stage in lactation (Piessens et al., 2011: 6%; Supré et al., 2011: 6%; Tomazi et al., 2014: 7%; Mørk et al., 2012: 13%). Interestingly, the latter seems to mainly concern *S. arlettae*, *S. chromogenes, S. sciuri,* and *S. xylosus* (Figure 1). For other species, the effect of stage in lactation on their occurrence seems to depend on the parity of the animals and vice versa. Throughout lactation, *S. epidermidis* was solely isolated from heifers (*Chapter 5.2*), while shortly after parturition *S. epidermidis* IMI were observed in both heifers and older cows (*Chapter 5.1*). The opposite was true for *S. equorum.* In a later stage in lactation only older cows seemed to be infected, while shortly after parturition this species was recovered from IMI in both heifers and older cows. *Staphylococcus devriesei* and *S. pasteuri* were only isolated from older cows both in early lactation (*Chapter* 5.1) and a later stage in lactation (*Chapter* 5.2), whereas *S. hyicus* was solely isolated from one fresh heifer (*Chapter* 5.1) and one older cow in a later stage in lactation (*Chapter 5.2*).

The ecological and the epidemiological nature of the predominant coagulase-negative staphylococci becomes clear

In recent years, different bovine-associated habitats of CNS species have been studied. In general, this has helped to distinguish within the large group of CNS between the so-called host-adapted species (e.g. *S. chromogenes*, *S. epidermidis*, *S. simulans*) and environmental species (e.g. *S. cohnii*, *S. equorum*, *S. fleurettii*, *S. saprophyticus*, *S. sciuri*). Besides, species can behave in an opportunistic (e.g. *S. equorum*) or contagious (e.g. *S. chromogenes*) manner (Piessens et al., 2012; Vanderhaeghen et al., 2014, 2015).

Our studies (*Chapter 4.1, 4.2, 5.1*) allowed verification of previous findings, as summarized in Figure 3. Indeed, the so-called environmental species were clearly more present in environmental samples, only *S. haemolyticus* interrupts the ascendant trend-line, substantiating its potential opportunistic nature. The

predominance of some CNS species causing IMI is also clearly shown (Figure 3), reinforcing the host-adapted nature of *S. chromogenes* and *S. simulans*. The latter observations along with the obtained results from our risk factor studies and those conducted by other research groups (*Chapter 4.2*, *5.1*) (Table 2) further substantiate the stratification in host-adapted and environmental species. The ecology of *S. xylosus*, a skin-opportunist (Verdier-Metz et al., 2012), still remains to be elucidated (Taponen et al., 2008, Figure 3).

Teat apices of non-lactating animals seem to mainly yield more skin-opportunistic and host-adapted species, such as *S. chromogenes* and *S. xylosus,* and are less subjected to environmental influences (Figure 3). In that respect, the high number of *S. haemolyticus* and *S. devriesei* colonized teat apices of non-lactating cows and heifers suggests a rather host-adapted nature of both species as was also proposed in *Chapter 4.2*. Contrastingly, teat apices of lactating cows and heifers as well as teat skin harbor both environmental and host-adapted species. Different housing conditions and the milking process, influencing teat apex microbiota of lactating animals, most likely explain this discrepancy. Similar strains of *S. chromogenes* were, however, found on the teat skin and in bovine milk, indicating teats are a possible infection source of IMI in the corresponding quarter (Taponen et al., 2008). *Chapter 5.1* additionally revealed a significant association between *S. chromogenes* TAC and IMI as was also recently suggested for *S. haemolyticus* (Leroy et al., 2015). It is very plausible that at least part of the *S. chromogenes* IMI in early lactation originated from the dry period and were established via penetration into the mammary gland of *S. chromogenes* bacteria that first colonized the teat apex.

Up till now, a contagious epidemiology of the host-adapted species has not been proven, in contrast with *S. aureus* (Zadoks et al., 2002; Smith et al., 2005a), as milking machine unit liners seem to be mainly contaminated with the environmental species (Figure 3) and no transmission during milking process was observed (*Chapter 4.1*). However, a contagious behavior was suspected for *S. simulans* and *S. epidermidis* as clonality was shown within herds, i.e. dendrograms demonstrated that isolates were more grouped together per herd (Bexiga et al., 2014). Also, the presence of both host-adapted and environmental species on milkers' skin and gloves suggest contamination rather than transmission through this vector. Strains other than milk-related ones were detected on milkers' skin, justifying our hypothesis (Taponen et al., 2008). Also, human skin yields similar species, such as *S.*

epidermidis, and transmission from humans to cows is suggested rather than among cows through hands (Zadoks et al., 2002; Thorberg et al., 2006; Savijoki et al., 2014; Schmidt et al., 2015).

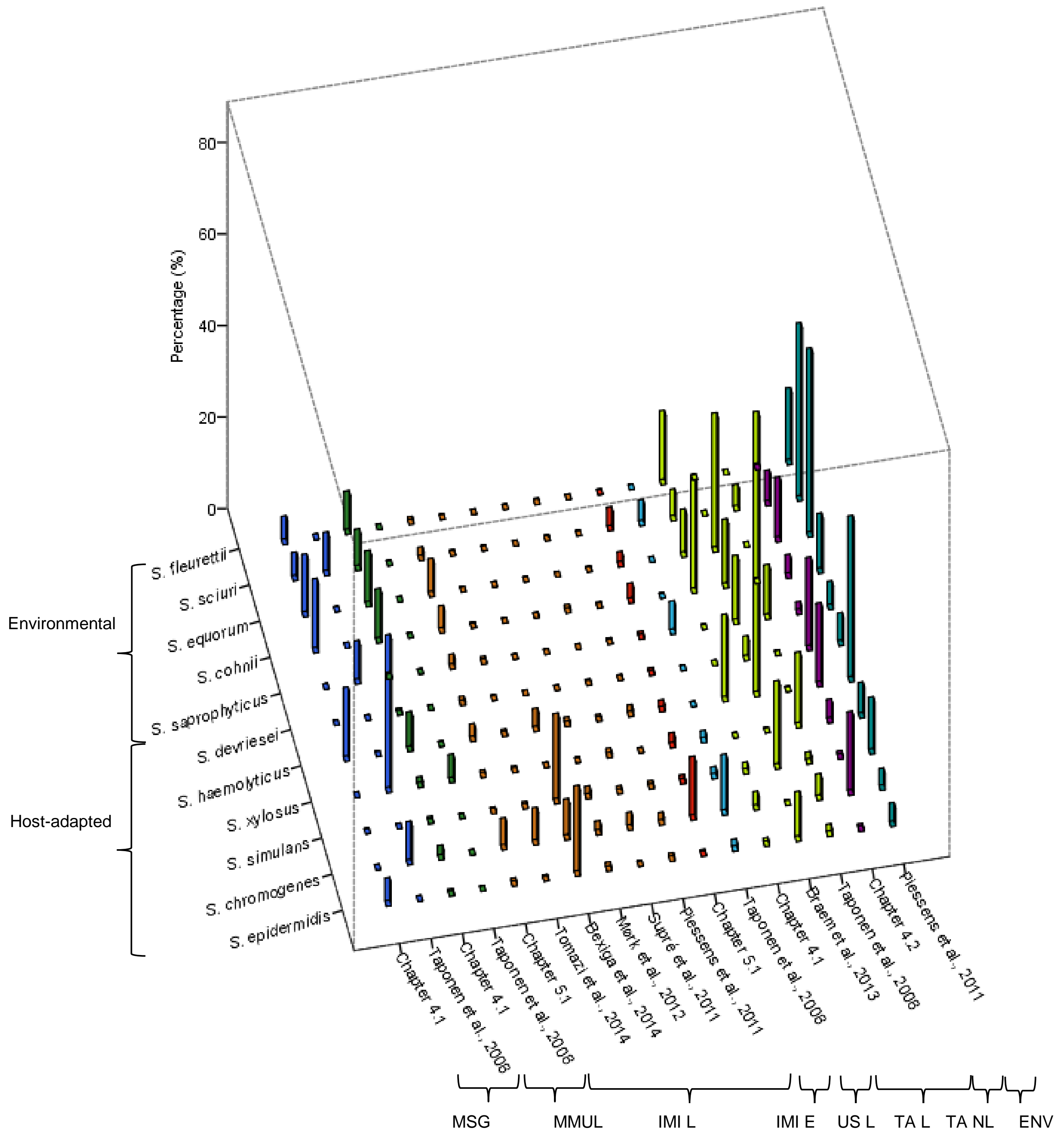

Figure 3. Visualisation of the predominant bovine-associated coagulase-negative *Staphylococcus* species within different habitats: the bovine environment (ENV, blue-green) (Piessens et al., 2011), teat apices of both non-lactating (TA NL, violet)

(*Chapter 4.2*) and lactating animals (TA L, yellow-green) (Taponen et al., 2008; Braem et al., 2013; *Chapter 4.1*), udder skin of lactating cows and heifers (US L, light blue) (Taponen et al., 2008), intramammary infection (IMI) both in early lactation (IMI E, red) (*Chapter 5.1*) and throughout lactation (IMI L, orange) (Piessens et al., 2011; Supré et al., 2011; Mørk et al., 2012; Bexiga et al., 2014; Tomazi et al., 2014; *Chapter 5.1*), milking machine unit liners (MMUL, green) and milkers' skin and gloves (MSG, blue) (Taponen et al., 2008; *Chapter 4.1*). Results are displayed as percentages of habitats (e.g. all swabbed teat apices, all isolated milk samples) harbouring a specific CNS species as reported by different studies. The species are ordered based on their potential ecological nature with *S. fleurettii* and *S. epidermidis* at the ends of the spectrum representing the environmental and host-adapted species, respectively.

Preliminary screening of the species-specific protective effect of teat apex colonization

Protective effects of CNS TAC (De Vliegher et al., 2003; Piepers et al., 2011) and of pre-existing CNS IMI (Poutrel and Lerondelle, 1980; Rainard and Poutrel, 1988; Nickerson and Boddie, 1994; Green et al., 2005) have been suggested. However, a recently conducted meta-analysis showed that the outcome of analyses, i.e. finding protective effects, depends on several factors such as differences in study design (i.e. dose of inoculated major pathogens, size of the study, study duration), IMI definition, and underlying undetected risk. Smaller challenge studies rather than large observational studies, using a higher major pathogen challenge dose, covering a short period, relying on more stringent IMI definitions and with a higher underlying risk for IMI with major pathogens in the population showed more and stronger protective effects (Reyher et al., 2012).

Although it was not a specific aim of this thesis, our data provide a unique opportunity to preliminary analyze whether pre-partum species-specific CNS TAC protects against IMI in early lactation as suggested before by De Vliegher et al. (2003) for *S. chromogenes* and Piepers et al. (2011) for the CNS group (*Chapter 4.2, 5.1*). The number of included herds and sampled animals are comparable with the aforementioned observational studies (De Vliegher et al., 2003: 8 herds - 123 animals; Piepers et al., 2011: 20 herds - 191 animals; *Chapter 5.1*: 13 herds - 156

animals). As the studies from De Vliegher et al. (2003) and Piepers et al. (2011) only concerned heifers and no accurate species identification was performed interpretations of and comparisons with those studies should be done cautiously. The relation between (species-specific) CNS TAC prior to calving, the likelihood of IMI with major pathogens in early lactation, and the likelihood of an elevated quarter SCC early post-partum were analyzed. Pearson Chi² and Fisher's exact tests (SPSS Statistics 22, SPSS Inc., Chicago, IL, USA) on contingency tables were performed for TAC either with all CNS combined and with *S. chromogenes*, *S. devriesei*, *S. equorum,* and *S. haemolyticus* separately (Table 3). Quarters with pre-partum CNS or *S. devriesei* colonized teat apices were less likely to be infected with major pathogens, such as *S. aureus, Streptococcus uberis*, *Streptococcus dysgalactiae, Escherichia coli* and *Pseudomonas*, in early lactation (odds ratio, OR = 0.46; 95% CI: 0.21-1.01 and OR = 0.26; 95% CI: 0.08-0.87, respectively). The latter effect for CNS as a group corresponds well with earlier research (Piepers et al., 2011). Also, a borderline significant protective trend was observed for teat apex colonizaton with *S. haemolyticus* (OR = 0.33; 95% CI: 0.10-1.12). Furthermore, all OR were numerically < 1. More research, taking into account spatial clustering of teats / quarters within cows and cows within herds is, however, needed to substantiate our protective effects. Despite the large number of herds and animals in our studies (*Chapter 4.2, 5.1*), fitting multilevel models, though we acknowledge it is the best choice, was avoided due to a too low number of isolates per species, a consequence of species-level analysis.

Future research

In this thesis, CNS were studied at the subgroup and species level when sufficient numbers were available per species. The latter clearly points out a weakness of the current CNS research as huge datasets are needed to study CNS at the species level. In the future, the availability of high (low-cost) throughput identification systems, such as MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time-Of-Flight Mass Spectrometry) (Barreiro et al., 2010; Huber et al., 2011; Frey et al., 2013; Moser et al., 2013; Björk et al., 2014; El-Behiry et al., 2014; Tomazi et al., 2014; Schmidt et al., 2015) will help to circumvent this issue.

Table 3. Association between pre-partum teat apex colonization and the likelihood of intramammary infection (IMI) with major pathogens in early lactation, and the likelihood of an elevated quarter milk SCC in early lactation, respectively

Teat apex colonization	IMI major pathogens						IMI Gram-positive major pathogens						IMI Gram-negative major pathogens						Quarter milk SCC[1]					
	N_n[2]	N_i[3]	*P*[4]	OR[5]	95% CI		N_n	N_i	*P*	OR	95% CI		N_n	N_i	*P*	OR	95% CI		N <200	N ≥ 200	*P*	OR	95% CI	
CNS			0.05	0.46	0.21	1.01			0.21	0.51	0.18	1.50			0.17	0.44	0.15	1.30			0.40	0.80	0.47	1.36
N_n	55	10					55	5					55	5					26	40				
N_c[3]	278	23					278	13					278	11					165	202				
S. chromogenes			0.46	0.68	0.24	1.90			0.74	0.77	0.20	3.03			0.74	0.77	0.20	3.03			0.21	0.67	0.35	1.27
N_n	55	10					55	5					55	5					26	40				
N_c	57	7					57	4					57	4					44	45				
S. devriesei			0.02	0.26	0.08	0.87			0.12	0.26	0.05	1.38			0.12	0.26	0.05	1.38			0.88	0.95	0.51	1.78
N_n	55	10					55	5					55	5					26	40				
N_c	85	4					85	2					85	2					43	63				
S. equorum			0.16	0.42	0.13	0.43			0.44	0.42	0.08	2.28			0.44	0.42	0.08	2.28			0.40	0.75	0.38	1.48
N_n	55	10					55	5					55	5					26	40				
N_c	52	4					52	2					52	2					33	38				
S. haemolyticus			0.07	0.33	0.10	1.12			0.10	0.17	0.02	1.47			0.47	0.50	0.11	2.19			0.28	0.70	0.36	1.34
N_n	55	10					55	5					55	5					26	40				
N_c	66	4					66	1					66	3					41	44				

[1]Categorization based on De Vliegher et al. (2003), i.e. < 200,000 cells/mL vs. ≥ 200,000 cells/mL. [2]Number of uninfected quarters or non-colonized (N_n) teat apices. [3]Number of quarters infected (N_i) or teat apices colonized (N_c) with the bacteria belonging to the different subgroups. [4] *P*-value for the overall effect. [5]Odds ratio

It all, however, starts with conducting large scale studies including enormous numbers of samples from a huge number of herds.

We believe that, combined with the studies of other research groups around the world, good steps have been taken in further elucidating the differences among CNS species in many respects. The need to include strain-typing in large studies clearly exists as differences between strains within species have been revealed and can be relevant (Vanderhaeghen et al., 2015). In our work, strain-typing was not performed, although this will be done in the future to reinforce or counter several (of our) findings and hypotheses such as the association between *S. chromogenes* colonized teat apices and *S. chromogenes* IMI in the same quarters in early lactation (*Chapter 4.2, 5.1*) and the ability of CNS species to cause persistent IMI (*Chapter 5.1*: data not shown). Pulsed-field gel electrophoresis (PFGE), a highly discriminatory strain-typing method (Rabello et al., 2007; Holmes and Zadoks, 2011; Vanderhaeghen et al., 2015), able to distinguish CNS strains, can be used for that purpose. Pulsed-field gel electrophoresis is, however, labour intensive, expensive (Vanderhaeghen et al., 2015), and interlaboratory comparisons are difficult (Rabello et al., 2007), though a CNS-specific protocol exist as PFGE has already been used before (Shimizu et al., 1997; Thorberg et al., 2006; Taponen et al., 2008; Gillespie et al., 2009; Rajala-Schultz et al., 2009; Sawant et al., 2009; Feβler et al., 2010; Jaglic et al., 2010; Mørk et al., 2012; Pate et al., 2012; Bexiga et al., 2014; Fry et al., 2014b). The latter makes PFGE the most suitable and quickest method at the moment to distinguish different CNS strains obtained in 1 study. In order to determine the global epidemiology and population structure of CNS species, MLST (Smith et al., 2005a; Holmes and Zadoks, 2011; Vanderhaeghen et al., 2015) or whole genome sequencing (WGS) (Vanderhaeghen et al., 2015) are however more appropriate. In contrast to PFGE, being already applicable for all CNS species, a suitable MLST protocol only exists for bovine *S. epidermidis* (Frey et al., 2013) and differences among CNS species are expected.Currently, a MLST-protocol for bovine *S. chromogenes* isolates is being validated, partly based on some of our isolates (*Chapter 4.2, 5.1*). The same inconvenience occurs for WGS as only the genome of a bovine *S. chromogenes* (Fry et al., 2014a), a bovine *S. epidermidis* (Savijoki et al., 2014), and a bovine *S. xylosus* isolate (Harrison et al., 2013) have been sequenced. Bovine *S. aureus*-protocols can be valuable in developing WGS for other CNS species (Herron-Olson et al., 2007; Garcia-Alvarez et al., 2011; Wolf et al., 2011).

The possibility to differentiate protective CNS species / strains from those that are more relevant for udder health should be examined using the above mentioned strain-typing techniques. Several hypotheses have been suggested to explain protection of CNS species, all demanding further investigation. Competitive exclusion or an activated immune response from pre-existing CNS IMI (Rainard and Poutrel, 1988; Matthews et al., 1990; Piepers et al., 2009), inhibiting or quickly eliminating infection and colonization of the mammary gland with major pathogens, have been proposed. This could be examined in challenge trials, i.e. inoculation of uninfected quarters with major pathogens after establishing TAC with protective CNS strains using dairy cows, although other target species such as mice could be used as well (Breyne et al., 2015). Protective CNS strains might be able to produce bacteriocins, antimicrobial peptides / proteins able of inhibiting pathogens such as *S. aureus* and *Streptococcus agalactiae* (Nascimento et al., 2005; Coelho et al., 2007). Production of bacteriocins has been reported for several staphylococci (Allgaier et al., 1985; Sashihara et al., 2000; Nascimento et al., 2005; Coelho et al., 2007; Wilaipun et al., 2008; Ceotto et al., 2010; Fagundes et al., 2011; Braem et al., 2014*).*

The contagious or opportunistic epidemiology of a number of species such as *S. chromogenes, S. devriesei*, and *S. haemolyticus* has not yet been substantiated and needs examination. A large study, sampling more parlor-associated habitats and a larger number of samples per habitat over time than in *Chapter 4.1*, is warranted to identify the role of several potential vectors, such as milking machine unit liners, and quarters having CNS IMI. In *Chapter 4.1,* swabs of unit liners and teat apices were collected before and after milking though the link with infected quarters was not studied. Analogous to *S. aureus*, studies should rely on adequate strain-typing (Zadoks et al., 2000, 2002; Smith et al., 2005a, 2005b).

The association among CNS species recovered from bulk milk samples and CNS species causing IMI in the same herd should be investigated. An extensive study, including a large number of herds and a huge number of cows and quarters within each herd is needed to do so. This would allow to compare the distribution of CNS causing IMI and CNS present in the bulk milk. Also, bulk milk might be used to screen the herd udder health status (Gillespie et al., 2012; Santos et al., 2014) and to pinpoint shortcomings in management. Possibly, the predominance of either host-adapted or environmental CNS in bulk milk is associated with the presence of certain major pathogens possessing the same ecological nature. In that respect, the

presence of several CNS species in bulk milk could reveal the predominant major pathogen in the herd, potentially not present in bulk milk due to intermittent excretion or a lower prevalence (as opposed to CNS species). Screening CNS in bulk milk could be helpful for the development of a herd-specific and effective prevention and control program without the need of sampling all high SCC-cows in the herd.

The search for an accurate (quick, cheap) CNS identification method is urgent as current routine diagnostics, i.e. bacteriological culturing followed by phenotypic identification, are too limited to differentiate among species or strains. Strains able to persist and elevate the SCC are carrying virulence factors (Vanderhaeghen et al., 2014; Zuniga et al., 2015) that other strains do not. A polymerase chain reaction (PCR), targeting genes encoding those factors should be set up allowing accurate identification of *S. chromogenes* in the first place. Probably, the combination of several genes, i.e. a genetic profile, will be associated with virulence and persistency of *S. chromogenes* rather than a single gene effect (Bertelloni et al., 2015; Zuniga et al., 2015). Multiplex PCR methods, able to detect several of these genes that are solely present in *S. chromogenes* in a single reaction, such as the commercially available real-time PCR “PathoProof Mastitis PCR Assay” (Finnzymes Oy, Espoo, Finland) (Pitkälä et al., 2007; Koskinen et al., 2009), should be adapted and validated for detection of *S. chromogenes*. Analogous to *S. aureus*, virulence gene patterns highly linking different genotypes to ecological behavior and pathogenicity should thus be searched for (Graber et al., 2009; Boss et al., 2011).

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Summary

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Worldwide, mastitis is one of the most prevalent and costly diseases in the dairy industry. Besides huge economic losses, milk quality and animal well-fare are affected. Mastitis is mainly caused by bacteria, based on their ecological nature classified into host-adapted and environmental pathogens. Currently, coagulase-negative staphylococci (CNS) are the main causative agents of intramammary infection (IMI) in dairy cows. Literature was reviewed and knowledge and assumptions concerning the CNS group were reported (*Chapter 1*). Coagulase-negative staphylococci are able to cause mild (sub)clinical mastitis and have as a group been considered as minor pathogenic for udder health. Aside from the negative impact, protective effects have been suggested as well. Differences among species within this group have been reported, emphasizing the necessity of research at the species level rather than at the group level.

The aim of this thesis was to get more insight in the ecological nature and epidemiological behavior of the different bovine-associated CNS species (*Chapter 2*).

Several habitats other than the mammary gland, such as teat apices and bulk milk, yield CNS species. Picturing the CNS distribution in those habitats is hampered by the presence of several bacteria other than CNS. The suitability of a (quasi-) selective medium, mannitol salt agar (MSA) was assessed for that reason (*Chapter 3*). Both reference strains and field isolates of the twenty-five predominant bovine-related CNS species were included in the study (n = 50). Also, 10 isolates of the 6 and 4 most frequently isolated species from milk and teat apices (n = 100), respectively, were tested. All isolates were plated on MSA and aerobically incubated at 37°C. Growth was examined after 24h and 48h and after another 24h incubation at room temperature. Aerobic incubation for 48h at 37°C was recommended. Additionally, the applicability of MSA for routine recovery of CNS species was assessed using bulk milk samples (n = 20) and swabs of teat apices (n = 20). All isolated CNS were identified to the species level using tDNA-PCR or sequencing of the *rpoB* or *16S rRNA* gene. Twenty and 34 CNS isolates were recovered from bulk milk and teat apices, respectively, the majority being *Staphylococcus equorum*, *S. chromogenes*, *S. fleurettii,* and *S. haemolyticus*. Advantages of the use of MSA were detected over the use of a non-selective medium as overgrowth of bacteria other than CNS was avoided and could thus not hinder us from picking up different colonies on MSA. Mannitol salt agar was considered as a highly suitable medium for

the growth and recovery of bovine-associated CNS, and was further applied in *Chapters 4.2, 5.1* and *6*.

The species-specific CNS distribution on teat apices of lactating animals was described through a cross-sectional sampling in 3 Flemish dairy herds (*Chapter 4.1*). Swabs of teat apices were taken before and after milking (n = 140). Parlour-associated habitats were explored as well and compared to CNS species causing IMI in the herds to investigate potential transmission routes. Milking machine unit liners (MMUL) and gloves or hands and elbows of milkers (MSG) were swabbed, again before and after milking (n = 252 and n = 40, respectively). All CNS were identified to the species level using tDNA-PCR or *rpoB* gene sequencing. The most frequently isolated species were *S. cohnii*, *S. haemolyticus*, *S. equorum*, *S. fleurettii*, and *S. sciuri*. A herd-specific CNS microbiota was observed and up to 4 different species were found per teat apex. The likelihood of presence of CNS on teat apices was significantly lower after milking than before and milk was thus excluded as source of teat apex colonizing species. Contamination from the cows' environment or colonization with udder-adapted species were 2 potential hypotheses that could explain the observed CNS distribution on teat apices. An efficient cleaning procedure of the MMUL in between milking was observed. The MMUL and teat apices shared a similar CNS distribution, suggesting CNS species were flushed off teat apices during milking, contaminating the MMUL. Environmental contamination of the MSG was observed, though for some species MSG appeared to act as a potential vector during milking process. A shared microbiota with human skin was also suggested. A contagious nature for the more relevant species for udder health, i.e. *S. chromogenes*, *S. simulans,* and *S. xylosus*, all favouring the mammary gland, could not be demonstrated. *Staphylococcus fleurettii* and *S. equorum* preferred the extramammary niches rather than the mammary gland.

Besides teat apices of lactating dairy cows, teat apex colonization (TAC) of non-lactating animals was examined (*Chapter 4.2*). Thirteen Flemish dairy herds were enrolled in the study and on each farm, 12 pregnant end-term heifers and dry cows (n = 156) were selected. Swabs of teat apices (n = 624) were collected 14 days prior to the expected calving date. All potential CNS isolates were genotypically identified at the species level using tDNA-PCR or *16S rRNA* gene sequencing. Logistic multilevel regression models were fit in order to identify risk factors associated with TAC prior to parturition. Seventy-two percent of all teat apices (n = 624) yielded CNS

with a range from 1 to 3 different CNS species per teat apex. The predominant species were *S. devriesei*, S. *chromogenes*, *S. haemolyticus,* and *S. equorum*. *Staphylococcus chromogenes* was the sole species colonizing teat apices in all herds. Large herd-dependent differences in CNS TAC distribution were observed for the other species. Dirty teat apices or teat apices swabbed in months with low precipitation were more likely to yield whatever CNS species. Teat apices of red and white Holstein Friesians, of cows dried-off without an internal teat sealer and swabbed in months with lower precipitation and higher ambient temperature had higher odds of *S. devriesei* TAC. Slightly dirty teat apices were more likely to be colonized with *S. chromogenes*. Also, low precipitation was significantly associated with *S. chromogenes* TAC. *Staphylococcus haemolyticus* TAC only depended on the ambient temperature and precipitation with teat apices swabbed in months with higher temperature and lower precipitation being more likely to yield *S. haemolyticus*. Dirty teat apices had higher odds of being colonized with S. *equorum*. High ambient temperature in combination with high precipitation increased the likelihood of *S. equorum* TAC. Our findings confirmed the environmental nature of *S. equorum* whereas a host-adapted ecology was reinforced for *S. chromogenes* and suggested for *S. devriesei* and *S. haemolyticus.*

The abovementioned herds (n = 13) and animals (n = 156) were also used for determining the species-specific prevalence and distribution of CNS IMI in fresh heifers and cows (*Chapter 5.1*). Quarter milk samples (n = 1248) were collected within four days after parturition and approximately one month later. Again, CNS were accurately identified to the species level using tDNA-PCR or *16S rRNA* gene sequencing. Several potential herd-, cow- and quarter-level risk factors were identified using logistic multilevel regression models. Twenty-six percent of all quarters were infected with 1 or 2 CNS species at parturition. The most frequently isolated species were *S. chromogenes, S. sciuri,* and *S. cohnii*. Only *S. chromogenes* was able to cause IMI in all herds, whereas other species showed a herd-dependent distribution. Quarters from heifers and with an inverted teat end had higher odds of having IMI with the for udder health more relevant CNS or with *S. chromogenes* solely. Dirty quarters were more likely to be infected with *S. cohnii, S. equorum, S. saprophyticus,* and *S. sciuri,* substantiating their environmental nature. *Staphylococcus chromogenes*, on the other hand, was reinforced as a host-adapted species. Teat apices colonized with *S. chromogenes* pre-partum had higher odds of

S. chromogenes IMI in the same quarters at parturition. Furthermore, the impact on udder health was examined. The more relevant species, i.e*. S. chromogenes, S. simulans,* and *S. xylosus* increased the quarter SCC at parturition.

Potential cow-, and quarter-level predictors associated with IMI throughout lactation were also analysed (*Chapter 5.2*). Data were obtained from a previously conducted longitudinal study in 3 Flemish dairy herds, as described in *Chapter 4.1*. Quarter milk samples were collected and CNS were identified to the species level relying on genotypic identification (i.e. tDNA-PCR or *rpoB* gene sequencing). Logistic multilevel regression models were fit. Quarters from heifers, from animals in peak lactation and with an increased quarter milk SCC were more likely to be infected with the more relevant CNS species, i.e. *S. chromogenes, S. simulans,* and *S. xylosus.* Quarters infected with species other than the aforementioned ones were also associated with an increased quarter SCC, though to a lesser extent. Especially in herds with a low bulk milk SCC, the predictors will be useful to select quarters and cows infected with the more relevant CNS species.

Identification of herd-level factors was performed to gain more insight in the variation in ecological and epidemiological nature among CNS species as well as to explain the herd-dependency in CNS distribution. Bulk milk samples (n = 300) were collected in 100 Flemish dairy herds 3 times with a 3-month interval and allowed the recovery of a sufficient number of isolates per CNS species, required for species-specific analyses (*Chapter 6*). Potential CNS species were genotypically identified to the species level using tDNA-PCR and *16S rRNA* gene sequencing. Logistic multilevel regression models, analysing potential herd-level variables, were fit. Ninety percent of all bulk milk samples yielded CNS. A wide variety of CNS species was observed, belonging to both the environmental and host-adapted subgroup. The most frequently isolated species were *S. equorum, S. haemolyticus,* and *S. epidermidis.* A seasonal effect in CNS species recovery was observed. Bulk milk from herds with lactating cows and heifers housed in a loose-pack or a tie-stall were more likely to yield whatever CNS species and subgroups of species except for *S. epidermidis*, *S. simulans,* or *S. cohnii*. Bulk milk from herds participating in a monthly veterinary udder health monitoring program were more likely to yield the more relevant CNS species. Herds constantly obtaining the milk quality premium had decreased odds of yielding *S. equorum* in their bulk milk whereas herds using dry cotton or paper towels for several cows as opposed to 1 cow during the pre-milking

udder preparation were more likely to have *S. cohnii*-positive bulk milk. Pre-disinfection of the teats before attachment of the milking cluster decreased the odds of having *S. equorum* in the bulk milk. Herds where flushing with hot water or steam of the milking cluster after milking a cow with a (sub)clinical mastitis was applied, were less likely to have *S. simulans*-, *S. haemolyticus*- and *S. cohnii*-positive bulk milk. Always wearing gloves during milking process decreased the odds of having *S. devriesei* in the bulk milk and tap water being used as drinking water increased the odds of yielding *S. simulans*.

Finally, all obtained results were summarized, compared to other CNS research and discussed in *Chapter 7*.

Samenvatting

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De meest voorkomende en duurste aandoening op melkveebedrijven over de hele wereld is mastitis of een ontsteking van de melkklier. Uierontstekingen zorgen voor grote economische verliezen en een verminderde melkkwaliteit. Ook het welzijn van de dieren wordt er door aangetast. De voornaamste mastitisverwekkers zijn bacteriën. Op basis van hun ecologie kunnen deze ingedeeld worden in gastheer- (en koe-) gebonden of omgevingsgebonden kiemen. De belangrijkste bacteriële mastitispathogenen zijn momenteel de coagulase-negatieve stafylokokken (CNS). De literatuur werd bestudeerd en de huidige kennis en vooropgestelde hypotheses over deze groep stafylokokken werden weergegeven in *Hoofdstuk 1*. Coagulase-negatieve stafylokokken veroorzaken milde klinische en subklinische uierontstekingen. Als groep worden ze aanzien als minder relevante mastitisverwekkers voor de uiergezondheid in vergelijking met zeer schadelijke pathogenen zoals *Staphylococcus aureus* en *Streptococcus uberis*. Behalve hun geringe virulente karakter werden ook beschermende eigenschappen toegekend aan de CNS-groep. Recent wetenschappelijk onderzoek bracht aan het licht dat er duidelijke verschillen bestaan tussen de vele *Staphylococcus* species binnen deze groep. Studies op groepsniveau zijn dus niet langer aangewezen.

De doelstelling van deze doctoraatsthesis is meer inzicht te verwerven in de ecologie en epidemiologie van de diverse *Staphylococcus* species (*Hoofdstuk 2*).

Coagulase-negatieve stafylokokken worden ook teruggevonden in andere habitats dan de melkklier, zoals op de speentoppen en in tankmelk. Het nagaan van de prevalentie en de distributie van de verschillende CNS-species in deze habitats wordt bemoeilijkt door de aanwezigheid van vele andere bacteriën. Het gebruik van een (semi-) selectief medium, mannitol salt agar (MSA), voor het isoleren van boviene CNS uit deze habitats werd daarom onderzocht (*Hoofdstuk 3*). De groei van de vijventwintig meest voorkomende boviene CNS-species werd eerst nagegaan. Zowel referentiestammen als veldisolaten (n = 50) werden uitgeënt op MSA, alsook 10 isolaten van de 6 belangrijkste CNS-species uit melk en de 4 voornaamste CNS-species geïsoleerd van speentoppen (n = 100). De platen werden onderzocht na 24u en 48u aerobe incubatie bij 37°C en vervolgens nogmaals na 24u aerobe incubatie bij kamertemperatuur. Aerobe incubatie gedurende 48u bij 37°C bleek noodzakelijk. De praktische bruikbaarheid van MSA werd vervolgens getest met behulp van tankmelkstalen (n = 20) en swabs van speentoppen (n = 20). Alle gevonden stafylokokken werden geïdentificeerd op speciesniveau met behulp van accurate

moleculaire technieken (tDNA-PCR en *rpoB* en *16S rRNA* gensequenering). De belangrijkste CNS-species gevonden in tankmelk (20 isolaten) en van speentoppen (34 isolaten) waren *Staphylococcus equorum, S. chromogenes, S. fleurettii* en *S. haemolyticus*. Mannitol salt agar verhinderde overgroei door andere pathogenen, wat een voordeel is t.o.v. niet-selectieve media. Aangezien MSA een uiterst geschikt medium bleek te zijn voor het isoleren van boviene CNS-species werd het verder aangewend in de *Hoofdstukken 4.2, 5.1* en *6.*

De CNS-distributie op speentoppen van lacterende koeien en vaarzen werd beschreven op basis van de resultaten van een reeds uitgevoerde cross-sectionele studie op 3 Vlaamse melkveebedrijven (*Hoofdstuk 4.1*). Er werden zowel voor als na het melken swabs van de speentoppen genomen (n = 140). Habitats gerelateerd aan het melkproces werden ook onderzocht, i.e. swabs van speenvoeringen (n = 252) en van handen en ellebogen van de melkers (n = 40) werden verzameld. De geïsoleerde CNS-species werden vergeleken met species die intramammaire infecties (IMI) veroorzaakten op de bedrijven om mogelijke vectoren voor CNS-overdracht op te sporen. Na accurate speciesidentificatie (tDNA-PCR en *rpoB* gensequenering) bleken *S. cohnii, S. haemolyticus, S. equorum, S. fleurettii* en *S. sciuri* de voornaamste species in de onderzochte extramammaire habitats. Elk bedrijf vertoonde een eigen CNS-microbiota. Per speentop werden tot 4 verschillende CNS-species geïsoleerd. Er werden significant minder CNS-species gevonden op speentoppen na het melken dan ervoor, waardoor melk kon worden uitgesloten als oorzaak van de CNS-speentopkolonisatie. Twee hypotheses voor de CNS-species distributie op speentoppen werden naar voren gebracht: contaminatie vanuit de omgeving of kolonisatie met meer uiergebonden species. Een efficiënte reiniging van de speenbekers tussen de melkbeurten werd bevestigd op alle onderzochte bedrijven. Er werd vermoed dat tijdens het melkproces CNS-species van de speentoppen worden gespoeld en in de speenvoeringen achter blijven. Handen en ellebogen van de melkers bleken besmet vanuit de omgeving. Een mogelijke transmissie van CNS-species tijdens het melken door de handen van de melkers werd voorgesteld alsook een gedeelde microbiota tussen mens en dier. *Staphylococcus chromogenes, S. simulans* en *S. xylosus* werden meer teruggevonden als oorzaak van IMI dan in de onderzochte extramammaire habitats. De overdracht van deze koegebonden species tijdens het melkproces kon niet

aangetoond worden. *Staphylococcus fleurettii* en *S. equorum* werden daarentegen voornamelijk in de extramammaire habitats aangetroffen.

Ook de speentopkolonisatie met CNS-species bij niet-lacterende koeien en vaarzen werd grondig onderzocht (*Hoofdstuk 4.2*). Dertien Vlaamse melkveebedrijven werden geselecteerd en per bedrijf werden 12 drachtige vaarzen en droge koeien uitgekozen (n = 156). Er werden swabs genomen van alle speentoppen (n = 624) veertien dagen voor de verwachte kalfdatum. Alle geïsoleerde CNS werden moleculair geïdentificeerd met behulp van tDNA-PCR en *16S rRNA* gensequenering. Logistische regressiemodellen werden opgesteld om potentiële risicofactoren, geassocieerd met speentopkolonistie voor afkalven, te analyseren. Tweeënzeventig procent van alle speentoppen bleek gekoloniseerd met 1 tot 3 verschillende CNS-species. De voornaamste species waren *S. devriesei, S. chromogenes, S. haemolyticus* en *S. equorum. Staphylococcus chromogenes* werd als enig CNS-species terug gevonden op speentoppen in álle onderzochte bedrijven. Een grote variatie in CNS-distributie werd aangetroffen voor de andere species. Meer bevuilde speentoppen en speentoppen onderzocht in drogere maanden vertoonden vaker CNS-kolonisatie. Speentoppen van roodbonte Holstein Friesian-dieren, van koeien die drooggezet werden zonder toediening van een inwendige speenafsluiter en die werden onderzocht in drogere en warmere maanden bleken vaker gekoloniseerd te zijn met *S. devriesei.* Een lichte bevuiling van de speentop was positief geassocieerd met het voorkomen van *S. chromogenes* op deze speentop. Ook drogere weersomstandigheden bevorderden de *S. chromogenes*-kolonisatie. Speentopkolonisatie met *S. haemolyticus* werd enkel door klimatologische omstandigheden (hogere temperatuur en minder neerslag) beïnvloed. Sterk bevuilde speentoppen waren vaker gekoloniseerd met *S. equorum.* Bovendien zorgde een hogere omgevingstemperatuur en een grotere hoeveelheid neerslag voor meer *S. equorum*-positieve speentoppen. De omgevingsgebonden ecologie van *S. equorum* werd bevestigd. Een koegebonden aard werd daarentegen bekrachtigd voor *S. chromogenes* en vooropgesteld voor *S. devriesei* en *S. haemolyticus.*

De hierboven beschreven bedrijven (n = 13) en dieren (n = 156) werden eveneens aangewend om de CNS-prevalentie en -distributie in de melk bij pas gekalfde vaarzen en koeien te bepalen (*Hoofdstuk 5.1*). Kwartiermelkstalen (n = 1248) werden genomen binnen de 4 dagen na afkalven en ongeveer 1 maand later.

Geïsoleerde CNS werden opnieuw op een accurate manier geïdentificeerd op speciesniveau (tDNA-PCR en *16S rRNA* gensequenering). Verschillende risicofactoren, zowel op bedrijfs-, koe- als kwartierniveau, werden geanalyseerd met behulp van logistische, multilevel regressiemodellen. Zesentwintig procent van alle kwartieren bleek geïnfecteerd met 1 tot 2 verschillende CNS-species bij afkalven. *Staphylococcus chromogenes, S. sciuri* en *S. cohnii* werden het vaakst terug gevonden. Opnieuw was *S. chromogenes* het enige species dat op álle bedrijven werd geïsoleerd en werden grote bedrijfsverschillen gevonden in de distributie van de andere CNS-species. Kwartieren van vaarzen en kwartieren met een ingestulpte speentop waren vaker geïnfecteerd met één van de voor uiergezondheid relevantere species, i.e. *S. chromogenes, S. simulans* en *S. xylosus* en met *S. chromogenes* afzonderlijk. Sterk bevuilde kwartieren waren vaker geïnfecteerd met *S. cohnii, S. equorum, S. saprophyticus* en *S. sciuri*, wat de omgevingsgebonden ecologie van deze species bevestigde. *Staphylococcus chromogenes* werd benoemd als koegebonden species. Speentoppen die voor afkalven gekoloniseerd waren met *S. chromogenes* bleken positief geassocieerd te zijn met IMI veroorzaakt door *S. chromogenes* bij afkalven in diezelfde kwartieren. *Staphylococcus chromogenes, S. simulans* en *S. xylosus* waren in staat het kwartiercelgetal bij afkalven te verhogen en zijn dus meer relevant dan andere species.

Voorspellende factoren op koe- en kwartierniveau, geassocieerd met IMI doorheen de lactatie, werden ook geïdentificeerd (*Hoofdstuk 5.2*). Een bestaande dataset van een longitudinale studie op drie Vlaamse melkveebedrijven werd hiervoor gebruikt, zoals in *Hoofdstuk 4.1.* Kwartiermelkstalen waren verzameld en CNS waren geïdentificeerd met behulp van de moleculaire technieken tDNA-PCR en *rpoB* gensequenering. Logistische multilevel regressiemodellen werden opgesteld. Kwartieren van vaarzen en van dieren in pieklactatie en met een verhoogd kwartiercelgetal bleken vaker geïnfecteerd met de meer relevante CNS-species, i.e. *S. chromogenes, S. simulans* en *S. xylosus.* Kwartieren geïnfecteerd met andere CNS-species hadden ook een verhoogd celgetal, maar de verhoging van het celgetal was minder uitgesproken. Voornamelijk op bedrijven met een laag tankmelkcelgetal kunnen deze voorspellende factoren aangewend worden om de koeien en vaarzen geïnfecteerd met de meer relevante CNS-species op te sporen.

Om meer inzicht te verwerven in de ecologische en epidemiologische variatie tussen de verschillende CNS-species en om de bedrijfsafhankelijke CNS-distributie

trachten te verklaren, werden tenslotte risicofactoren op bedrijfsniveau geanalyseerd (*Hoofdstuk 6*). Hiervoor werden 3 maal tankmelkstalen (n = 300) verzameld op 100 Vlaamse melkveebedrijven met een interval van 3 maanden. Door het gebruik van tankmelk op een groot aantal bedrijven werden genoeg isolaten per CNS-species geïsoleerd, wat species-specifieke analyses mogelijk maakte. Er werd opnieuw gebruik gemaakt van genotypische identificatie (tDNA-PCR en *16S rRNA* gensequenering) en logistische multilevel regressiemodellen. Negentig procent van de tankmelkstalen bevatte CNS-species. Een groot aantal verschillende koegebonden en omgevingsgebonden species werden gevonden. *Staphylococcus equorum, S. haemolyticus* en *S. epidermidis* waren de meest voorkomende CNS-species in tankmelk. Een seizoensgebonden effect kwam naar voren. In tankmelk van bedrijven waar lacterende dieren in een potstal of bindstal gehuisvest zijn, werden alle geteste CNS-species, met uitzondering van *S. epidermidis, S. simulans* en *S. cohnii,* vaker teruggevonden. Het maandelijks monitoren van de uiergezondheid in samenspraak met de (bedrijfs-)dierenarts was geassocieerd met het voorkomen van de meer relevante CNS-species, i.e. *S. chromogenes, S. simulans* en *S. xylosus*, in tankmelk. Het altijd ontvangen van de premie voor een goede uiergezondheid deed daarentegen het voorkomen van *S. equorum* in tankmelk dalen. Tankmelk van bedrijven waar bij de voorbehandeling tijdens het melkproces per koe telkens minstens één droge katoenen of papieren doek werd gebruikt t.o.v. het gebruik van één doek bij meerdere dieren was minder vaak *S. cohnii*-positief. Het gebruik van een voorschuimproduct voor het melken was negatief geassocieerd met *S. equorum* in de tankmelk. Het ontsmetten met heet water of stoom van het melkstel na het melken van een dier met een klinische of subklinische uierontsteking verlaagde het voorkomen van *S. simulans, S. haemolyticus* en *S. cohnii* in de tankmelk. Tankmelk van bedrijven waar gedurende het hele melkproces steeds handschoenen gedragen werden, bevatte minder *S. devriesei.* Het gebruik van leidingwater als drinkwater voor de lacterende dieren verhoogde het voorkomen van *S. simulans* in de tankmelk.

Alle verkregen resultaten werden uiteindelijk samengevat, vergeleken met ander wetenschappelijk CNS-onderzoek en besproken in *Hoofdstuk 7.*

Curriculum Vitae and Publications

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Curriculum vitae

Anneleen De Visscher werd op 19 maart 1986 geboren te Lokeren. Zij behaalde in 2004 haar diploma hoger secundair onderwijs in de richting Latijn-Wiskunde aan het Sint-Lodewijkscollege te Lokeren. Vervolgens vatte ze de studie Diergeneeskunde aan te Gent. In 2010 studeerde ze met grote onderscheiding af als dierenarts en ontving tevens voor haar scriptie de prijs “Beste masterproef in de optie Herkauwers”. In juli 2010 trad ze in dienst als doctoraatsbursaal bij de “Mastitis and Milk Quality Research Unit” van de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde aan de Faculteit Diergeneeskunde van de Universiteit Gent. Na het behalen van een specialisatiebeurs werd haar onderzoek vanaf januari 2012 gefinancierd door het Agentschap voor Innovatie door Wetenschap en Technologie (IWT). Haar onderzoek focust op de ecologie en epidemiologie van boviene coagulase-negatieve *Staphylococcus* species met als promotoren Prof. dr. Sarne De Vliegher, Prof. dr. Freddy Haesebrouck en Dr. Sofie Piepers. Anneleen is auteur en co-auteur van meerdere wetenschappelijke publicaties. Haar onderzoeksresultaten werden ook gepresenteerd op verschillende internationale congressen waaronder de Annual Meeting van de National Mastitis Council in 2014, waarvoor zij een Scholarship ontving. Ze volgde eveneens verscheidene specialisatiecursussen en mag daardoor het diploma van de “Doctoral Schools of Life Science and Medicine” in ontvangst nemen.

Anneleen was bovendien werkzaam als dierenarts bij het M-team^{UGent} en begeleidde meerdere melkveebedrijven in Vlaanderen. Ze gaf zowel aan studenten, veehouders als dierenartsen voordrachten en opleidingen over uiergezondheid, melkkwaliteit en microbiologie. Anneleen ondersteunde, als promotor, verschillende thesisstudenten. Het belang van een goede uiergezondheid en het nut van bacteriologisch onderzoek werden meermaals neergeschreven en gepubliceerd in nationale landbouwtijdschriften. Anneleen hielp tevens in het routinelabo van het M-team^{UGent} en draaide graag mee in de dag-, nacht-, en weekenddiensten van de kliniek Verloskunde.

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