Investigations on some aerobic bacterial agents isolated from heifers at the beginning of the first lactation

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Abstract: In this study, it was aimed to identify the predominant coagulase-negative staphylococci (CNS) species in subclinical, clinical, and persistent infections in Konya province, to determine various virulence and resistance mechanisms of CNS species within the age-lactation level. The research has been carried out in a dairy herd that is free from brucellosis and tuberculosis and uses an automated herd management system. Thirty-two heifers at the beginning of lactation with milk yields of more than 20 liters have been observed in terms of udder infections during the first 1–4 months of lactation. The prevalence of intramammary infections (IMI) and of CNS-caused IMI has been 75% and 53%, respectively. The rate of CNS was 61% among all isolates, with the rate of the major pathogen 26%, and the rate of the other pathogen 12%. *S. chromogenes* has been the most frequently isolated CNS species (45.5%). In conclusion, CNS may cause udder infections at the herd level in heifers during the early lactation period, which may cause persistent infections with different mechanisms, and *S. chromogenes* may emerge as a new contagious mastitis agent in the future. CNSs should be identified at the species level rather than the group level in order to develop effective control programs. Based on the epidemiological data, the current udder health control programs in heifers in the early lactation period were detected to be insufficient.

Key words: Coagulase-negative staphylococci, early lactation, mastitis, heifer

1. Introduction

In recent years, there has been an increase in mastitis cases associated with calving and the early lactation period in dairy heifers previously thought to be free of udder infection [1–3]. In addition, the shorter duration of using the breeding, as well as the decrease in milk yield in future lactation periods due to persistent infections, began to be paid more attention to this period [4]. At the beginning of the first lactation, the rate of the infected udder in heifers ranges from 12% to 57%. This situation clearly threatened milk yield and udder health during the first and subsequent lactations [5]. Considering the mastitis pathogens of IMI in early lactation in dairy heifers, the percentage of quarters infected with CNS range from 22% [3], 26% [6], 35% [7]. Studies in the Asian continent have shown a change from *Staphylococcus aureus* to CNS in general mastitis epidemiology [8]. When the course of the infection (subclinical, clinical, or persistent) and its effects on the somatic cell are considered, it is seen that there are differences within the CNS group [9]. *S. chromogenes, S. haemolyticus, S. epidermidis*, and *S. simulans* have been associated with higher rates of persistent infection and

isolation [10,11]. It has been reported that *S. chromogenes*, *S. simulans*, and *S. xylosus* are associated with higher somatic cell counts (SCC), and the increases in SCC are similar to IMI caused by *S. aureus* [6,11]. Within the group, *S. chromogenes* has been reported to have the greatest mammary gland adaptation in dairy cows [9,12].

When considering the effects on IMI, it is clear that there is no agreement on the relationship between CNSs and milk yield [12]. High-yielding heifers are more susceptible to the CNSs, and it has been reported that intramammary infections caused by the CNS are common in early lactating heifer [13]. Several hypotheses have been proposed to explain this situation. First, the total milk yield of high lactating heifers may reduce losses caused by CNS-based IMI [12], or CNS may have a protective effect on IMI [7]. However, studies show that the effect on milk yield of species within the CNS group varies. Significant decreases in milk yield were observed 7 days after intramammary inoculation of *S. chromogenes*, *S simulans*, and *S. epidermidis*, particularly in the experimentally designed IMI models [14]. Biofilm-forming capacities [15,16] and beta-lactam resistance [17] are the other two

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issues frequently emphasized issues in the pathogenesis of CNS IMI, in addition to milk yield effects studies.

Biofilm formation is recognized as a significant persistence factor in intramammary infections. Although multiple external and internal factors influence the biofilm formation in *Staphylococcus* spp., an important step in biofilm formation is the production of polysaccharide intercellular adhesion. This matrix structure is synthesized by the proteins encoded of the *icaADBC* gene cluster. Furthermore, the role of biofilm formation in persistence CNS IMI draws attention [15–17].

Species-specific intramammary infections in lactating heifers are a common topic of study around the world. However, the etiology and incidence of IMI in lactating heifers in early lactation have been poorly investigated in our country [18]. In addition, it is very well known that dairy farmers and veterinarians in our country do not take this critical period into account for future lactations.

The main purpose of this study is to investigate whether CNS species exist in heifer mammary glands during early lactation. The second objective is to determine the roles of these CNS species in subclinical, clinical, and persistent infections as well as their effects on milk yield. Furthermore, by monitoring all environmental and contagious bacterial agents in the selected population, it would be possible to determine which species should be prioritized in terms of time-age-yield and management factors. In this way, it will contribute to filling the knowledge gaps for effective prevention/treatment protocols.

2. Materials and methods

2.1. Dairy farm and study design

This study was carried out in a commercial dairy farm in Konya province (Turkey). This farm has over 300 dairy cows and, uses an automatic milking system (AMS), and proper milking procedures. The study was conducted in tuberculosis and brucellosis-free herd. The sampling group was comprised of 32 heifers at the beginning of the first lactation with an average daily milk yield of ≥20 liters between the 1–4 months after calving. During the sampling periods and before calving, no antimicrobials were administered to the heifers.

2.2. Milk sampling and milk yield recording

Samples were taken at the end of March, April, and May, before the morning milking (3 different samplings were carried out at 30 days intervals). Each teat was cleaned with a predipping solution before sampling. Then it was wiped down with 70% alcohol and dried with a paper towel. Discarded milk samples were examined for visual abnormalities. Approximately 10 mL of milk was collected in sterile vials per quarter for bacteriological analysis. Before collecting milk samples, each quarter was tested for subclinical mastitis using the California Mastitis Test

(CMT). Milk samples were collected and transported under refrigeration to the laboratory for bacteriological isolation and identification. The milk yield records of the heifers in the sampling group were determined using AMS (DeLaval International AB, Tumba, Sweden).

2.3. Bacterial isolation and preliminary identification

First of all, each milk sample was inoculated onto blood agar (5% defibrinated sterile sheep blood), MacConkey Agar, Sabouraud dextrose Agar (SDA) (Lab-M, Lancashire, UK). Then blood agar and MacConkey agar were incubated aerobically at 37 °C for 24 h, SDA was incubated for 7–10 days at room temperature. The incubation period for fastidious microorganisms (*Streptococus* spp., *Trueperella pyogenes*, etc.) was extended to 48–60 h at 37°C. Suspected colonies were purified by passages. Quarters were considered to be infected if ≥100 cfu/mL were observed in the milk sample. Milk samples containing the growth of ≥3 different microorganisms were regarded as contaminated. For preliminary identification, Gram staining, colony morphology evaluation, and determination of various phenotypic characteristics (catalase and oxidase test) were performed. Clumping factor, hemolysis characteristics, DNase, and mannitol activities of *Staphylococcus* spp. isolates were determined [19].

2.4. Identification by matrixassisted laser desorption ionization time of flight(MALDI-TOF MS)

MALDI-TOF identification was performed using the direct transfer method. Single colonies of *Staphylococcus* spp. were subcultured onto blood agar and aerobically incubated for 18 h. Fastidious microorganisms were aerobically incubated for 48 h at 37 °C. Following passage incubation, one bacterial colony was smeared onto 96 spot steel MALDI target plates with sterile toothpicks (Bruker Daltonics Inc.). After air drying of the MALDI-TOF plate, 1 μL of matrix solution (-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid supplied by the manufacturer) was dropped into each well of the plate and dried at room temperature. A Microflex LT MALDI-TOF MS microbial identification system and Flex Control 3.0 software (Bruker Daltonics, USA) were used for identification. Scores of ≥1.7 were considered reliable at the species level [20–22].

2.5. Species identification by polymerase chain reaction (PCR)

DNA isolations were performed according to the Wizard® Genomic DNA Purification Kit protocol. PCR mixture included 5 μL Master mix (5x FIREPol® Master Mix (04-12-00115, Solis BioDyne) 0.1 μL forward primer (10 pmol/μL), 0.1 μL Reverse primer (10 pmol/μL), 2 μL DNA (100 ng/μL) and 17.9 μL sterile nuclease-free water, and the total volume was 25 μL. A 1% agarose gel was used for electrophoresis of PCR products. Thermally cycled PCR

protocols were carried out in accordance with the primer references (Table 1). *S. epidermidis* ATCC 35984 (positive control strain for *PBP*, *icaA*, and *icaD*), *S. epidermidis* ATCC 12228 (negative control biofilm strain), and *S. aureus* ATCC 25923 were used as control strains for PCR. Nuclease-free water was used as a PCR negative control.

2.6. Phenotypic detection of slime and biofilm production
The in vitro slime producing properties of all CNS isolates were determined by incubating them with Congo red agar (CRA) (BHI agar containing 5% sucrose and 0.8 g/L Congo

red) at 37 °C for up to 24 h [23]. Dry transparent black colonies were considered positive, while colonies that were pink, red, and brown colonies were considered negative [24]. *S. epidermidis* ATCC 35984 strain was used as the positive control, *S. epidermidis* ATCC 12228 strain was used as the negative control. Biofilm forming capacities of isolates were determined by microplate biofilm test (MBT). MBT was performed according to the method described by Moore [24] with minor modifications. In an aerobic environment, a single colony was incubated in 5

Table 1. Polymerase chain reaction primer sequences and estimated amplicon size were used to identify species and detect *icaA*, *icaD*, and *PBP* gene regions.

Species	Gene	Primer sequence (5'→3')	Length of amplicon (bp)	Reference/NCBI accession number
S. aureus	*nuc*	F- GCGATTGATGGTGATACGGTT R- AGCCAAGCCTTGACGAACTAAAGC	270	[49] / DQ507379.1
S. chromogenes	*sodA*	F- GCGTACCAGAAGATAAACAAACTC R- CATTATTTACAACGAGCCATGC	222	[50] / AJ343945
S. haemolyticus	*sodA*	F- CAAATTAAATTCTGCAGTTGAGG R- AGAGCCCCATTGTTCTTTGA	214	[50] / EU652775
S. epidermidis	*rdr*	F- AAGAGCGTGGAGAAAAGTATCAAG R- TCGATACCATCAAAAAGTTGG	130	[50] / CP000029
S. simulans	*gap*	F- AGCTTCGTTTACTTCTTCGATTGT R- AAAAGCACAAGCTCACATTGAC	472	[50] / DQ321698
S. dysgalactiae	*16S rRNA*	F- GGGAGTGGAAAATCCACCAT R- AAGGGAAAGCCTATCTCTAGACC	572	[50] /AB002488
S. uberis	*cpn60*	F- TCGCGGTATTGAAAAAGCAACAT R- TGCAATAATGAGAAGGGGACGAC	400	[50] /AF485804
E. coli	*phoA*	F- GGTAACGTTTCTACCGCAGAGTTG R- CAGGGTTGGTACACTGTCATTACG	468	[50] / FJ546461
Trueperella pyogenes	*Pyolysin*	F- GGCCCGAATGTCACCGC R- AACTCCGCCTCTAGCGC	270	[51] / HQ637573.1
Pasteurella multocida	*KTM1*	F- ATCCGCTATTTACCCAGTGG R- GCTGTAAACGAACTCGCCAC	460	[52] / AF016259.1
Acinetobacter lwoffii	*rpoB*	F- CCGTGTCGGTCTGGTTCGTGTA R- CCGGCGTTTCAATTGGACATAC	302	[53] / DQ207487.1
Enterococcus spp.	*16S rRNA*	F- TCAACCGGGGAGGGT R- ATTACTAGCGATTCCGG	733	[54] / LT85360
S. hyicus	*sodA*	F- GCTTATCGCGAATGTTGACCAAT R- TCGTGCTGCTGCTTTATCTGAG	205	[55] /AJ343913
Bacillus pumilus	*16S rRNA*	F- TAACTGCTTGCACCTTGACG R- TAAGGTTCTTCGCGTTGCTT	500	[56] /AY112667
S. epidermidis	*icaA*	F-TCTCTTGCAGGAGCAATCAA R-TCAGGCACTAACATCCAGCA	188	[57] / U43366.1
S. epidermidis	*icaD*	F- ATGGTCAAGCCCAGACAGAG R-ATGGTCAAGCCCAGACAGAG	198	[57] / U43366.1
S. aureus	*PBP*	F-GTAGAAATGACTGAACGTCCGATAA R-CCAATTCCACATTGTTTCGGTCTAA	310	[58] / Y00688.1

mL Tryptic soy broth (TSB) for 15 h at 37°C. The culture was diluted 1/100 with TSB after incubation. This diluted culture was transferred to a 96-well polystyrene microplate and aerobically incubated for 48 h. Each bacterial isolate was inoculated twice in at least four microplate wells. The negative control was sterile TSB, and the positive control was *S. epidermidis* ATCC 35984. To remove planktonic bacteria (free-living bacteria), the plate was washed four times with 100 μL PBS at the end of the incubation period. The microplate was fixed at 60 °C for 1 h and stained for 2 min with 100 μL of Hucker's Crystal Violet solution. Excess crystal violet solution was aspirated, the microplate was washed with PBS, and it was dried. Then, 100 μL of a solution containing 10% methanol and 7.5% acetic acid was added to each well and shaken for 1 min with an automatic microplate shaker. Microplates were measured at 563 nm absorbance value (Biotek ELx800, USA). Cutoff values were calculated for each plate and evaluated using Moore's formulas [24]. In addition to CRA and MBT, PCR was used to investigate two gene regions on the CNSs related to slime and biofilm formation. PCR protocols were carried out in accordance with the primer references (Table 1).

2.7. Scanning electron microscopy (SEM)
The biofilm structures of some MBT-positive CNS isolates were confirmed using SEM imaging. According to Sayın et al. [25], the petri dish culture method was used to form biofilms prior to imaging. Initially, 10 mL of McFarland 0.5 turbidity-adjusted bacterial culture was added to petri dishes containing 10 mL of TSB (x2) (containing 1% glucose). Following inoculation, 2×2 cm^2 sterile slides were placed in each petri dish and incubated in an aerobic environment for 48 h at 37 °C. After incubation, each slide was washed three times with 10 mL PBS to remove planktonic bacteria. Before imaging, the slides were coated with gold-palladium(Quorum SC7620, UK). The imaging was performed at the Yıldız Technical University Central Laboratory (İstanbul) using a Zeiss Evo LS 10 SEM (Germany) at magnifications of 5000kx and 10,000kx.

2.8. Detection antibiotic susceptibility of CNS species
Phenotypic antibiotic susceptibilities of CNS isolates were determined using the agar disk diffusion method according to the standardized guideline [26]. Antibiotic discs (Oxoid) used in this study were as follows; Penicillin G (P-10μg), ampicillin (AMP-10μg), amoxicillin-clavulanic acid (AMC-30μg), neomycin (N-30μg), enrofloxacin (ENR-5μg), gentamicin (CN-10 μg), trimethoprim-sulfamethoxazole (SXT-25μg), oxytetracycline (OT-30μg). *S. aureus* ATCC 25923 strain was used as a quality control strain. In addition to phenotypic antimicrobial susceptibility test, PCR was used to investigate the penicillin binding protein (*PBP*) gene region associated with beta-lactam resistance (Table1).

2.9. Sequencing of gap and sodA gene for the detection of persistent CNS IMI
The Sanger method of DNA sequencing was used to determine the genetic difference/similarity of CNS identified from the same quarter over three sampling periods. PCR-derived *Gap* and *SodA* DNA fragments were used in the sequencing process (Table 1). Fifteen μL of purified PCR product + 2 μL (10 pmol/L) forward primer, Bigdye terminator v3.1 cycle (Thermo Fisher, USA) sequence kit, and ABI 3130XL (USA) device were used for sequencing. The E.Z.N.A.⁺ Gel Extraction Kit (V-spin-D2500-01, Omega Bio-Tek, USA) protocol was used to extract DNA fragments from agarose gel. MEGA-X (Proprietary freeware) software was used to analyze the data.

2.10. Statistical analysis
The IBM SPSS Statistics (Version 22.0) program was used to assess milk yield losses caused by CNS. The Levene test was used to determine the homogeneity of variances. The independent t-test was used to investigate the relationship between the two groups. One-way ANOVA, Tukey HSD test, and Bonferroni-Dunn test were used for comparisons of three or more groups. The value level of $p < 0.05$ was considered statistically significant. The noninfected animals were culture negative, and all quarters tested negative by CMT. The infected group consisted of heifers from, which only one CNS species was isolated. Heifers infected with other pathogens were not included in the statistical analysis.

3. Result
3.1. Bacteriological isolation
A total of 377 quarters of 32 lactating heifers were sampled. CMT positivity was detected to varying degrees in 20 quarters of 12 heifers during the first sampling period, 15 quarters of 11 heifers during the second sampling period, and 26 quarters of 17 heifers during the third sampling period. Two quarters, two quarters and three quarters, respectively in the first, second, and third sampling, were not eligible for sampling (inactive/nonfunctional quarters). In the first, second, and third samplings, respectively, 14, 22, and 34 agents were isolated from 377 milk samples. Total isolates were classified as 61% CNS, 26% major pathogen, 12% other pathogens (Table 2). In the sampling group, the prevalence of IMI was found to be 75%, and the prevalence of CNS-based IMI was found to be 53%. Throughout the study, two isolates were not identified and evaluated as environmental contaminants and were excluded from the study. PCR verifications revealed that 99% of all isolates were equivalent to MALDI-TOF MS typing based on genus and species. One *S. dysgalactiae* isolate was incorrectly identified as *S. canis* in the MALDI TOF MS identification. The species was identified as

Table 2. Distribution of species isolated from milk samples during 3 sampling periods.

| | S1 | S2 | S3 | | |
| | (7–50 DIM[1]) | (37–80 DIM) | (67–110 DIM) | | |
Species	n	n	n	Total	% pos. Tot.
S. chromogenes	4	9	18	31	45.5
S. simulans	1	3	1	5	7.3
S. haemoloyticus	-	-	3	3	4.4
S. hyicus	-	1	2	3	4.4
S. dysgalactiae	2	2	1	5	7.3
S. uberis	2	1	1	4	5.8
Trueperalla pyogenes	3	-	1	4	5.8
Escherichia coli	-	-	5	5	7.3
Pasteurella. multocida	-	1	-	1	1.5
Acinetobacter lwoffii	-	1	-	1	1.5
Bacillus pumilus	1	-	-	1	1.5
Enterococcus spp.*	1	2	2	5	7.3
Contaminated	-	2	-		

*Isolates identified as *Enterococcus* spp. at genus level by PCR were identified as *E. faecalis* (1), *E. columbae* (1), *E. casseliflavus* (1), *E. hirae* (1), *E. faecium* (1), by MALDI-TOF MS.
Day in milk (DIM)

S. dysgalactiae by PCR. For instance, an isolate that was initially identified as *S. canis* with a log score of 2.107 was later identified as *S. dysgalactiae* with a log score of 2.102. In another identification, a *S. dysgalactiae* isolate with a log score of 2.117 was later identified as *S. canis* with a log score of 2.063.

3.2. Phenotypic test result
All of the CNSs isolated in the study tested negative for oxidase and clumping factors. DNase enzyme activities and mannitol fermentation abilities of CNS isolates were found to differ (Table 3).

3.3. Antimicrobial susceptibility test result of CNS
At the end of the incubations, the zone diameters were measured in millimeters. The results were classified as susceptible (S), intermediate (I), or resistant (R) based on the Clinical Laboratory Standards Institute reference results [26]. *S. chromogenes* and *S. hyicus* isolates had the highest antibiotic and beta-lactam resistance among CNS isolates. In the study, the most resistance among beta-lactam antibiotics was found against penicillin. *S. haemolyticus* and *S. simulans* were found to be sensitive against beta-lactams (Table 4).

3.4. *icaA*, *icaD*, and *PBP* genes of CNS
The *icaA*, *icaD*, and *PBP* gene regions have not been detected in all CNS species (Figure 1).

3.5. Slime and biofilm formation results of CNS
A total of 42 CNS isolates were tested, with 8 (19%) showing slime positivity and 9 (21%) showing biofilm positivity. Slime and biofilm formation was not observed in *S. haemolyticus* strains. Only one *S. chromogenes* isolate formed a weak biofilm in MBT, but this strain did not form slime. Seven CNS isolates formed weak biofilm (Table 5). SEM was performed on 3 selected CNS isolates that had positive CRA and MBT results (Figure 2).

3.6. Persistent CNS IMM
Strains isolated from the same quarter of the same heifer at all three sampling periods were sequenced in the study. These intramammary infections were considered to be persistent. In the first persistent infection caused by *S. chromogenes*, 94% genetic similarity was found between the isolates isolated in the first and second periods, but no genetic similarity was found between the isolate isolated in the third period and the other two isolates. In the other two *S. chromogenes*-caused persistent infections, there were 87% and 92% genetic similarities between the isolates isolated in the second and third periods, respectively, while no significant genetic similarity could be found between the first period isolates and the other isolates. In another *S. simulans*-caused persistent infection, 87% genetic similarity was found between isolates isolated in

Table 3. DNAse and Mannitol activation of CNS.

Microorganism	Only DNase positive	Only mannitol positive	Both DNase and mannitol positive
S. chromogenes	24	2	1
S. haemolyticus	1	3	-
S. simulans	-	-	1
S. hyicus	3	-	-

the second and third periods, but no genetic similarity was found between the isolate isolated in the first period and the other two isolates.

On the other hand, *S. hyicus* was isolated from the same quarter in a heifer over two periods (2nd and 3rd periods). These *S. hyicus* strains were not sequenced because they were positive for slime and biofilm. The same quarter was detected CMT positive in the first sampling. This was interpreted as there have not been planktonic bacteria enough to detect in the first period. In the relevant quarter, IMI caused by *S. hyicus* was clinically evaluated as a persistent infection.

3.7. Effect of CNS sourced IMI on milk yield
There was no statistically significant difference in milk yield between the CNS-infected heifers and noninfected heifers during the three sampling periods. *S. chromogenes*-infected heifers produced 51 kg more milk than uninfected heifers during the first sampling period. The *S. simulans*-infected heifers had the lowest average milk yield among the CNS-infected heifers during the second sampling period. Noninfected heifers produced 74 and 130 kg more milk than the infected heifers with *S. chromogenes* and *S. simulans*,

respectively. The average milk yield of the *S. chromogenes*-infected heifers was 60 kg higher than that of the noninfected heifers in the third sampling period (Table 6).

4. Discussion
Previous studies on IMI in heifers at the beginning of lactation in our country are limited. Only one study in our country, Vural et al. [18], reported CNS isolation at 50% and *S. aureus* isolation at 35% from IMI in heifers during the first 90 days of lactation. Pieperset al. [7] reported that in clinical and subclinical IMI in heifers at the beginning of lactation, the CNS isolation rate was 72%, *Streptococcus* spp. was 4.6%, and *S. aureus* was 3.5%. Tenhagen et al. [27] reported CNS 39%, *S. aureus* 5%, *T. pyogenes* 3.3%, *E.coli* 3%, *S. dysgalactiae* 2.5%, and *S. uberis* 0.3% isolation rates from milk samples taken from clinically healthy udders in heifers at the beginning of lactation in German dairy farms. According to Naqvi et al. [2], the isolation rates from milk samples taken from heifers without clinical mastitis in the first 30 days of lactation in Canadian dairy farms were CNS 62%, *S. aureus* 4.5%, *S. dysgalactiae* 0.4%, and *S. uberis* 0.4%. Yang et al. [1] reported the prevalence of IMI in heifers beginning the first lactation in Chinese dairy farms as 22.64%. In the same study, the isolate distribution was reported as CNS 30%, *E. coli* 29.58%, *S. aureus* 16.9%, and *S. uberis* 11.27%. Clinical mastitis was not found in CNS-infected heifers in our study. While the CNS rates obtained in the current study were found to be similar to previous prevalence studies in heifers beginning the first lactation, the *S. aureus* isolation rate was not consistent [1,2,7,18,27] (Table 2). This condition has been attributed to differences in herd management practices and milking procedures, as well as to an indirect effect caused by CNS.

Table 4. Antimicrobial susceptibility/resistance of CNS isolates from early lactating heifers milk samples.

	Antimicrobials						Species/N					
	S. chromogenes/31			*S. simulans*/5			*S. hyicus*/3			*S. haemolyticus*/3		
	S	I	R	S	I	R	S	I	R	S	I	R
P	24	-	7	5	-	-	2	-	1	3	-	-
AMC	30	-	1	5	-	-	3	-	-	3	-	-
OT	28	1	2	4	-	1	2	-	1	2	-	1
SXT	30	-	1	5	-	-	3	-	-	3	-	-
AMP	26	-	5	5	-	-	2	-	1	3	-	-
N	28	-	3	5	-	-	3	-	-	3	-	-
CN	31	-	-	4	1	-	3	-	-	3	-	-
ENR	29	-	2	5	-	-	3	-	-	3	-	-
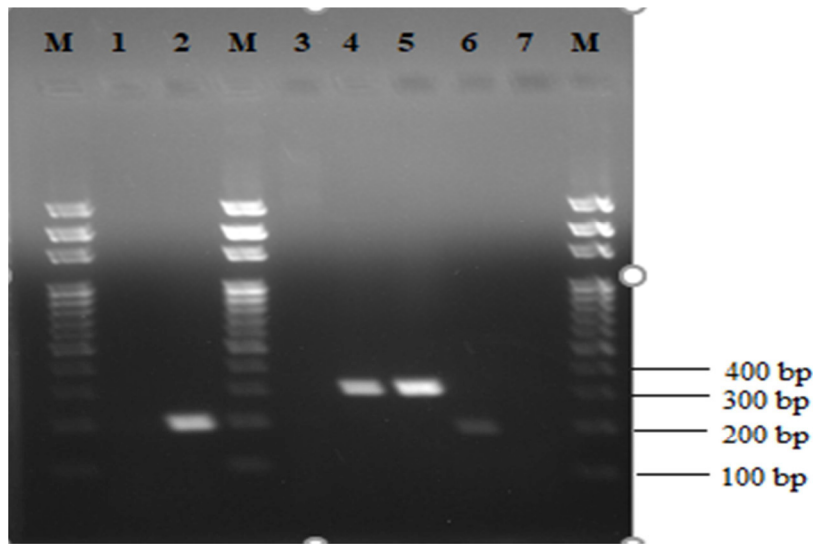
Figure 1. Images of *icaA*, *icaD* and *PBP* genes investigated on CNS in positive and negative control reference strains. M: 100 bp DNA ladder, Lane 1: *icaD* negative *S. epidermidis* ATCC 12228, Lane 2: *icaD* positive *S. epidermidis* ATCC 35984, Lane 3: *PBP* negative *S. aures* ATCC 25923, Lane 4: *PBP* positive *S. aureus* field isolate, Lane 5: *PBP* positive *S. epidermidis* ATCC 35984, Lane 6: *icaA* positive *S. epidermidis* ATCC 35984, Lane 7: *icaA* negative *S. epidermidis* ATCC 12228.

Table 5. Slime and biofilm production of CNS isolates from early lactation heifer mastitis in CRA and MBTanalysis.

| Species	Total isolate	CRA (Slime positive)	MBT (Biofilm production)	
			+	+/-
S. chromogenes	31	5	1	5
S. simulans	5	1	1	-
S. hyicus	3	2	-	2

+, Strong biofilm, +/-, weak biofilm

Previous research has shown that *S. chromogenes* can adapt to the mammary gland at a very young age [6,28]. Furthermore, some studies have shown that *S. chromogenes* and *S. simulans* are more resistant to teat postmilking disinfectants than other CNSs [16,17]. In the study, an increase was observed in the isolation rates of *S. chromogenes* from udder tissue during the sampling period. It was observed that the rate of *S. chromogenes* in persistent infections was higher than other CNS species. As a result, the ability of *S. chromogenes* to adapt to mammary glands early may have contributed to the increase in isolation rates. This high rate of *S. chromogenes* isolation in the study and previous studies supported the idea that *S. chromogenes* could be classified as contagious mastitis agents in the future. This hypothesis has been found parallel with the studies of Supré et al. [11] and De Visscher et al. [29].

The study's finding on milk yield has shown that CNS-infected heifers do not have a lower milk yield curve than noninfected heifers. Previous research has shown CNS species with high adaptability to bovine mammary tissue. *S. chromogenes* has *S. aureus*-like mammary gland adhesion capacity and uses the protease enzymes it secretes to increase the chemotactic effect on neutrophils [11,30]. While this chemotactic effect significantly increases the SCC, it causes less damage to udder tissue than other contagious agents. As a result, they are unable to show the same clinical effect on udder tissue [11,30]. This effect partly explains why, despite an increase in CMT positive udder quarters, there was no effect on milk yield in the study. Another hypothesis between CNS and milk yield is that clinical infections caused by CNS are uncommon [11,31]. There was no CNS-related clinical mastitis in our study. It was concluded that this is why, over a three-month period, there was no negative effect on direct CNS-induced milk yield. In the current study, we observed that these two hypotheses were consistent with previous research [11,30,31].

According to some studies, the rate of CNS isolation has increased in recent years. There are differences in the isolation rates of major mastitis pathogens and CNS in epidemiological studies on CNS. It is arguable whether the CNS has a synergistic or antagonistic effect on IMI caused by major mastitis pathogens [32–34]. Although
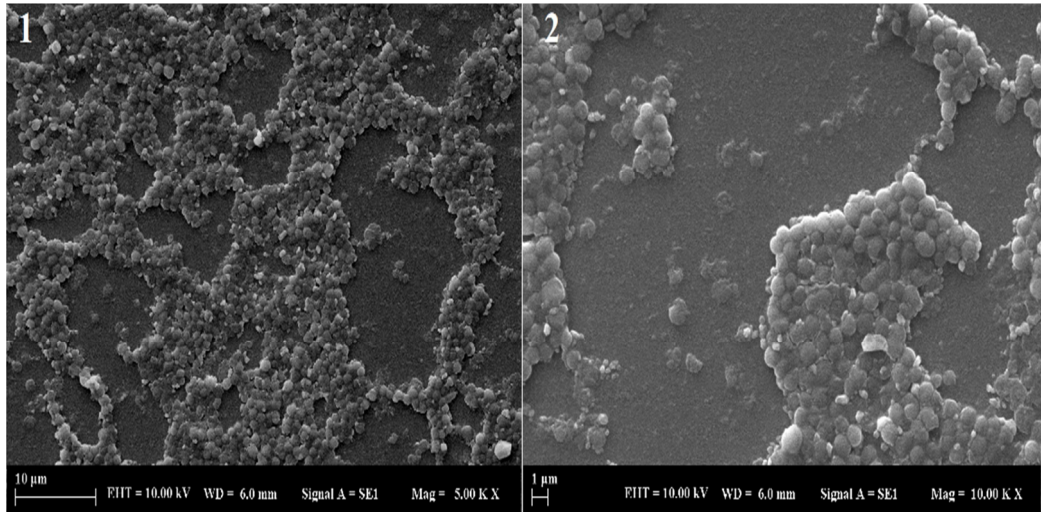
Figure 2. Scanning electron microscope image of biofilm and slime positive (weak) a SEM images magnifications of 5000 (image 1) and 10,000 (image 2) KX.

Table 6. Statistical analysis of milk yield differences between infected with CNS species and noninfected heifers at the beginning of the first lactation $p < 0.05$.

Health status	Sampling period	Heifer (n)	Mean	SE	P-value
Noninfected	1	16	863	31.53252	0.491
S. chromogenes		4	914	68.76172	
Noninfected	2	13	785	38.63531	0.285
S. chromogenes		7	711	47.39864	
S. simulans		3	655	105.14480	
Noninfected	3	11	793	48.30248	0.361
S. chromogenes		9	853	38.93635	

the differences in isolation of CNSs and other major mastitis pathogens in observational field studies cannot be interpreted as a direct protective or predisposition effect of CNS, the CNS-induced protective effect is more apparent in in-vitro and challenge studies. According to in vitro studies, bacteriocin and bacteriocin-like antibacterial peptides are the mechanisms of this effect [33–37]. De Vliegher et al. [35] reported that *S. chromogenes* isolated from a heifer's teat during the prepartum period inhibits the growth of *S. aureus*, *S. dysgalactiae*, and *S. uberis* in vitro, but not *E. coli*. Reyher et al. [34] reported an increase in intramammary infections due to *S. aureus* after CNS were isolated in milk samples in their observational field study. Furthermore, they claim that CNS-caused postpartum intramammary infections are a risk factor for *E. coli* IMI. We found no IMI due to *S. aureus* and *S. agalactiae* in heifers during the sampling period. In herds where CNS early colonization is common, CNSs may have a protective effect against IMI caused by contagious mastitis agents via various antagonistic mechanisms. Taking strict precautionary measures against contagious mastitis agents during milking practice reduces the prevalence of these contagious agents in herds. Subclinical IMI caused by environmental mastitis pathogens such as *E. coli*, *S. uberis*, *S. dysgalactiae*, and *Enteroccocus* spp. were also detected during the sampling periods previous or following isolation from the quarter from which the CNS was isolated. This finding suggests that CNSs may sensitize udder tissue to these opportunistic environmental mastitis agents, or that environmental agents may cause various udder immune system impairments that predispose to CNS placement in udder tissue. As a result of all of these hypotheses, it was determined that more research with various molecular and in vitro tests, as well as larger sample numbers, is required. Our study's findings were found to be consistent with previous research findings [33,35].

DNase positivity 11.9%, mannitol positivity 66.6% were found of the total CNS in our study. We observed that mannitol fermentation and DNase activity in the preidentification of *Staphylococcus* species did not have an effect good enough to distinguish CNS from *S. aureus*. In the identification performed with MALDI-TOF MS, it is correctly identified all the CNS species. However, MALDI-TOF MS was not suitable enough for differentiating *Streptococcus* species isolated in the study. The misidentification and close log score value between *S. canis* and *S. dysgalactiae* were thought to be due to a lack of information in the MALDI TOF required database to distinguish isolates or to the protein and other cell wall structure of *S. canis* and *S. dysgalactiae* not being sufficiently different. This relationship has been shown in some streptococcal species such as *S. pneumoniae*, *S. mitis*, and *S. parasanguinis* in previous research [38,39]. We concluded that a similar relationship could also exist between *S. dysgalactiae* and *S. canis*.

The *icaA* and *icaD* genes were found to be negative in all of the CNSs in the study. Biofilm and slime formation were observed in three CNS strains isolated from persistent intramammary infections. According to this perspective, slime and biofilm formation in the CNSs played a minor role in the persistent infections, and the findings were consistent with previous Simojoki et al. [15], Piessens et al. [17] studies. The study did not specify the duration of persistent infections during the first lactation. However, it has been determined that *S. chromogenes*, *S. simulans*, and *S. hyicus* colonize udder tissue in the first and second months of lactation, and may become persistent in the subsequent period. This finding supports previous research [9,40].

In our study, we found no relationship between phenotypic methods (CRA and MBT) and two gene regions (*icaA*, *icaD*) in the CNS responsible for slime and biofilm production. This finding was thought to be due to the suppressive effects of minor differences in culture conditions and various stress conditions on gene expression, or to the presence of different adhesion gene groups that are not part of the *ica* operon. In this respect, it was found to be similar to previous studies [15,41]. In phenotypic studies on antibiotic resistance profiles of CNS isolated from multiparous cows with subclinical and clinical mastitis in our country, Hadimli et al. [42], Turutoglu et al. [43], Tel and Keskin [44] reported penicillin resistance as 52.5%, 62.5%, 74%, and 76%, respectively. Rajala-Schultz et al. [45,46] reported 26% and 22% penicillin resistances in CNS isolated from heifers at the beginning of lactation, respectively. Antibiotic resistance is lower in CNS isolated from heifers at the beginning of lactation when compared to isolated from multiparous cows. The findings of our study support that. CNSs are known to be opportunistic pathogens of udder tissue. Multiparous cows may be exposed to long-term antibiotic exposure as a result of dry period treatment and other intra-mammary infections. As a result, this exposure may contribute to the development of antibiotic resistance in the CNS. This hypothesis was concluded to be the reason for the low antibiotic resistance profile in the CNS isolated in the study. In this respect, the study was found to be consistent with the findings of Rajala-Schultz et al. [45,46].

In the study, beta-lactam resistance in the CNS was observed to vary in the phenotypic and molecular description. The failure to associate beta-lactam resistance phenotypically with the molecular method in CNS could be related to changes in *PBP* subtypes and in vitro conditions. Using phenotypic methods, no beta-lactam resistance was found in clavulanic acid. We hypothesized that differences in beta-lactamase and beta-lactam resistance could be related to beta-lactamase production. This finding was consistent with previous research [43,47,48].

5. Conclusion
Mastitis control programs in dairy herds include the practices for prevention and control of contagious and environmental mastitis agents. The presence of various mastitis agents in heifers during prepartum and early lactation suggests that the current mastitis control programs do not reduce the prevalence of IMI in this group. Defining CNS based on species, revealing its resources and transmission mechanisms, will help to develop effective struggle strategies. Because of the low prevalence of CNS causing clinical mastitis, we can still classify them as minor pathogens. We believe that long-term studies with larger sample groups are needed to reveal its effect on milk yield, its relationship with other pathogens, mechanisms in persistent infections, risk factors, and the indirect economic losses.

For future studies; it is planned to monitor the intramammary agent variety, as well as their genus-species and/or intra-species phenotypic changes, in similar cow herds raised under conditions similar to the current study design over the next three lactation periods. As a result, it is thought to be appropriate to recommend control programs based on the outcomes.

Conflict of interest
The authors do not have any interest-based financial or personal relationships.

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