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# Characterization of Coagulase Negative Staphylococci Isolated from Dairy Goats in Germany

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**Key words:** NAS, coagulase negative Staphylococci, Germany, goat, subclinical mastitis, transfer DNA intergenic spacer length polymorphism PCR

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**Abstract:** Over 2 years as a part of a surveillance program, 2038 one half milk samples were collected from dairy goats representing 12 German herds. Microbiological investigation of the samples revealed positive bacterial growth in 17.3% of them. Biochemical identification of the field isolates detected Non-Aureus Staphylococci (NAS) in 10.4%, Corynebacteria in 3.2%, Streptococci in 0.8%, *S. aureus* in 0.7%, other bacteria in 1.0%. Meanwhile, mixed bacterial growth was shown in 2.2% of the samples. To identify the NAS isolates at their species level the detection of their biochemical properties alone using a commercial test kit enabled the correct identification of a randomized selection of the field isolates only in 11.4%. For further identification, a combination of two other identification techniques (antibiotic resistance profiles and molecular assays) was additionally applied. By doing so, it was possible to identify 69 (58.5%) out of 118 isolated presumptive NAS field strains at their species level. The detected species included *S. caprae, S. epidermidis, S. simulans, S. hyicus, S. saprophyticus, S. chromogenes, S. lentus, S. xylosus* and *S. hominis*. Among them, *S. caprae* was detected most frequently while *S. lentus, S. xylosus* and *S. hominis* were isolated only from sporadic cases.

## INTRODUCTION

Mastitis is the most costly field problem in dairy industry. Although, the inflammation of the udder tissue is accompanied by the elevation of Somatic Cell Count (SCC)[1], the SCC in goat milk is normally much higher than in cattle milk which may lead to false diagnosis[2]. So that, goat milk is considered to be obtained from mastitis udder only if the SCC exceeds $1\times10^6$ cells per mL. However, results obtained by California Mastitis Test (CMT) should be supported by bacteriological examination as the SCC can be physiologically increased in late lactation, old ages and in low milk producers[3, 4].

In the last years, NAS turned into emerging mastitis pathogens. They replaced the coagulase positive ones (*S. aureus*) in most countries and became the most commonly isolated bacteria from mastitic milk[5]. Recently and due to the detection of some coagulase variable *Staphylococcal* species, the classification of Staphylococci into coagulase positive *S. aureus* and Coagulase Negative *S. aureus* (CNS) was changed into *S. aureus* and Non-Aureus Staphylococci (NAS)[6, 7]. So that, in the present work, the term NAS will be used for the traditionally used CNS expression.

The prevalence of NAS in goat milk samples suffering from mastitis ranges between 25-95.9% in subclinical form of the disease[3, 8] and may even reach 100% of the samples of the clinical cases in certain locations[9].

The NAS represent about 50.2-54.2% of grown colonies cultured from normal goat milk compared to 41.5% for *S. aureus*. While the *S. aureus* is responsible mainly for the induction of clinical mastitis in goats, the NAS induces subclinical mastitis in most cases. The infection with NAS usually results in subclinical mastitis which can persist during the dry period and leads to the reduction of milk production with about 5.7% in infected goats[3-5, 10].

Infection with NAS lead to the increase of SCC in the infected half compared with the healthy one. However, this increase remains less obvious than that caused by *S. aureus* infections[11-13]. This may be attributed to the lower pathogenicity of NAS compared with *S. aureus*, although, they can produce potent virulence factors, e.g., thermo-stable enterotoxins[4].

In literature, different NAS species are known to induce mastitis in goats including *S. saprophyticus*, *S. lentus*, *S. simulans*, *S. xylosus*, *S. haemolyticus*, *S. warneri*[4], *S. lugdunensis*, *S. hominis*[14], *S. arlattae S. fleurettii*, *S. nepalensis*, *S. pasteuri*, *S. sciuri*, *S. gallinarum*, *S. equorum*[5] *S. caprae*[12], *S. capitis*[4], *S. auricularis*[15], *S. cohnii*[16], *S. muscae*, *S. vitulinus*[8, 16], *S. epidermidis*, *S. chromogenes*, *S. warneri* and *S. sciuri*[17].

At the time, few epidemiological investigations concerning the role of NAS in the induction of mastitis are available[5]. The available so far published data showed that the prevalence of different NAS species varies greatly and seem to depend on the region studied. However, *S. caprae*, *S. xylosus* and to less extend *S. equorum* remain among the most detectable species in most publications[18, 16].

Due to the growing importance of NAS in the dairy sector and also due to their public health importance, it is necessary to differentiate among the NAS at their species level. While most researchers prefer to use the commercial kits for biochemical identification of Staphylococci[19-21, 4], others depended on the characteristic antibiotic profile of every species[22]. Recently, various molecular tools were applied for this purpose[23].

The aim of the present work is to evaluate the epidemiological role of various species of Non-Aureus Staphylococci (NAS) isolated from subclinical mastitic milk samples from goats in Hessia state, Germany using different identification assays. Farther the diagnostic meaningfulness of the different above mentioned methods should be clarified comparatively.

## MATERIALS AND METHODS

Over 2 years, 2038 one half milk samples were collected from mastiticdairy goats in 12 German herds in Hessia state under antiseptic conditions. The Somatic Cell Count (SCC) was measured only in milk samples which were apparently normal and did not show any macroscopic abnormalities using Fossomatic® 360 (Foss Electric A/S, Denmark).

For the characterization of the present pathogens in the milk, different identification tools were applied. These included bacteriological examinations, molecular identification (PCR), biochemical identification (ID32 Staph Test) and the use of their antibiotic resistance profile as a diagnostic guide as shown later.

Bacteriological examinations were carried out where all the samples were cultured on blood agar and esculin blood agar (Merck, Darmstadt, Germany) at 37°C overnight. Only NAS field isolates from samples with medium (++/i.e., 6-15 colonies) or strong bacterial growth (+++/i.e., more than 16 colonies) in pure culture (without mixed infections) were selected for further investigation.

Macroscopically suspected grown colonies were stained with gram stain and examined microscopically and were subjected to catalase and KOH test[24].

The colonies were then tested in plasma coagulase and clumping factor test to differentiate among coagulase positive and coagulase negative *staphylococci* according to the methods described in the manual of clinical microbiology by Kloos and Bannerman. Only one colony per sample was randomly picked, fresh sub-cultured in tryptone soya broth and was then frozen in 10% glycerol until being used.

The reference strains (n = 17) used in the present work included *S. auricularis* (ATCC 33753), *S. capitis* (ATCC 27841), *S. caprae* (CCM 3573), *S. chromogenes* (ICM 7470), *S. cohnii* (GH 137), *S. epidermidis* (ATCC 14990), *S. haemolyticus* (ATCC 29970), *S. hominis* subsp. *hominis* (ATCC 27845), *S. hyicus* (ATCC 11249), *S. lentus* (ATCC 49574), *S. lugdunensis* (ATCC 43809), *S. saprophyticus* (ATCC 43867), *S. schleiferi*sub sp. *schleiferi* (ATCC 43808), *S. sciuri* (ATCC 29062), *S. simulans* (ATCC 27848), *S. warneri* (ATCC 27836) and *S. xylosus* (Kloos 162).

Biochemical identification of the isolates was performed using the ID32 staph test. The assay aims to identify various staphylococci on the base of their biochemical reactions. The ID32 staph test (BioMérieux,

Nürtingen) includes 26 wells containing dried substrates representing 26 colorimetric biochemical tests (urease, esculin hydrolyse, nitrat reduction, aceton formation, alkaline phosphatase, arginine arylamidase, arginine dihydrolase, ß-galactosidase, ß-glucuronidase, ornithinde decarboxylase, pyrridonyl-arylamidase, novobiocin resistence, acid production from arabinose, cellobiose, fructose, glucose, lactose, maltose, mannitol, mannose, raffinose, ribose, saccharose, trehalose, turanose and n-acetyl-glucosamine).

For biochemical identification of the 17 reference strains as well as a randomized selection of the the field isolates (n = 35) all tests were performed according to the manufacturer recommendation. For evaluation of the results the APILAB ID 32 computer Software was used.

Molecular identification of the isolates was done using the transfer DNA Intergenic spacer length polymorphism PCR (tDNA-ILP PCR assay). The assay was carried out for differentiation among various NAS species according to the method described by Maes *et al*.[25, 26].

The NAS isolates were fresh cultured to extract their genomic DNA according to Reinoso *et al*.[27]. The extracted DNA was used as template for the PCR assay using the primer pair T5A (59-AGTCCGGTGCTCTAAC CAACTGAG) and T3B (59-AGGTCGCGGGTTCGA ATCC) and the amplification program (94°C×30 sec×1, followed by 40 cycles of 50°C×30 sec, 72°C×120 sec.

The obtained amplicons were separated by 1.5% agarose electrophoresis (Biorad, Germany). The gels were evaluated with the aid of the VIII marker (0.019-1.11 kbp, Boehringer Mannheim, Germany) and the Gel Documentation systems Gel Doc 1000 (Biorad, Germany).

The antibiotic resistance profiles of the reference strains (n = 17) and the NAS field isolates against novobiocin, desferioxamin, fosfomycin, polymyxin B and furazolidon were performed according to Ehrenberg. In brief, fresh sub-cultured NAS colonies (n = 3-5) were picked and inoculated into tryptone soya broth. The broth was cultured at 37°C for approximately 2-3 h (until reaching a density of the 0.5 McFarland-standard). About 0.1 mL of the suspension was swabbed over the Mueller-Hinton agar plates. The remaining suspension was withdrawn from the plates. The antibiotic discs (Novobiocin 30 µg, Desferioxamin 1000 µg, Fosfomycin 50 µg, Polymyxin B 300 iU and Furazolidon 50 µg; Oxoid, (UK) were then located with the disc dispenser (Oxoid, UK). The plates were re-incubated overnight at 37°C. The antibiotic resistance profiles could be identified by measuring the diameter of the growth inhibition zones. The assumed borderline limits between sensitive and resistant were 16 mm for novobiocin, 18 mm for desferioxamin, 30 mm for fosfomycin, 12 mm for polymyxin B and 17 mm for furazolidon[28, 29].

## RESULTS AND DISCUSSION

In the present study, 2038 one half milk samples were investigated. Out of these, 2029 milk samples (99.6%) were apparently normal and did not show any visual abnormalities clots. Only 9 (0.4%) samples showed macroscopically visible changes like divergences in consistency or blood in the milk that might indicate a clinical mastitis.

The Somatic Cell Count (SCC) was measured in apparently normal milk samples. The geometric mean of SCC per mL was 765.000 in samples from which *S. aureus* was isolated, 559.000 in case of esculin positive Streptococci, 301.000 in case of NAS and 298.000 in case of *Corynebacterium* spp.

All the samples were cultured overnight on blood agar, only 17.3% (n = 353) of the total samples showed bacterial growth. The identification of the grown bacteria revealed NAS in 10.4% (n = 211), *Corynebacterium* spp. in 3.2% (n = 65), esculin positive Streptococci in 0.8% (n = 16), *S. aureus* in 0.7% (n = 15) and finally, other bacteria in 1.0% (n = 20) of the samples. In the same time, 1.3% (n = 26) of the samples showed mixed growth of different bacteria, therefore were excluded from the evaluation.

Out of the 211 samples which reveald NAS in pure culture only 118 colonies (originating from 118 samples) met the requirements to be selected for further investigation (namely: milk-sample with elevated cell count or macroscopically visible changes, bacterial growth of NAS confirmed colonies with medium or strong bacterial growth).

For biochemical identification of the NAS isolates, the grown colonies were tested by ID32 staph test. The obtained results enabled the correct identification only of 11 (64.7%) of the ATCC-reference strains and in only 4 (11.4%) of the 35 randomely selected NAS field isolates for the performance of complete biochemical identification.

The sensitivity against the antibiotics novobiocin, desferioxamin, fosfomycin, polymyxin B and furazolidon is used as an indicator for the differentiation among various NAS strains. Although, the resistance profile of the 17 reference strains revealed the expected profile described in the literature, only 93(78,8%) out of the 118 investigated strains revealed a resistance profile similar to the reference strains.

The remaining 25 field isolates presented 6 new resistant profiles which could not be interpreted based on the available data (Table 1). Two of these profiles representing 5 NAS isolates contained a furazolidone-resistance. These field strains were proved to be Micrococci and were henceforward excluded from the study.

In the present work, the application of tDNA-ILP PCR assay was used to cluster the obtained genomic

Table 1: Antibiotic resistance profiles obtained by the examination of NAS isolates in the present work

| Profile No.[1)] | Species (reference strains) | Novo-biocin | Desferi-oxamin | Fosfo-mycin | Poly-myxin B | Furazo-lidon | n (field-isolates) | Field isolates (%) |
|---|---|---|---|---|---|---|---|---|
| R1 | *S. auricularis*, *S. capitis*, *S. caprae*, *S. hämolyticus*, *S. warneri* | S | R | R | S | S | 34 | 28.8 |
| R2 | *S. chromogenes*, *S. hyicus*, *S. lugdunensis*, *S. simulans*, *S. schleiferi* | S | R | S | S | S | 34 | 28.8 |
| R3 | *S. cohnii*, *S. lentus* | R | R | S | S | S | 5 | 4.2 |
| R4 | *S. saprophyticus*, *S. sciuri* | R | R | R | S | S | 7 | 5.9 |
| R5 | *S. epidermidis* | S | S | S | S | S | 10 | 8.5 |
| R6 | *S. hominis* | S | S | R | S | S | 2 | 1.7 |
| R7 | *S. xylosus* | R | R | S | R | S | 1 | 0.8 |
| R8 | | S | R | R | R | S | 9 | 7.6 |
| R9 | | S | R | S | R | S | 8 | 6.8 |
| R10 | | S | R | S | S | R[2)] | 4 | 3.4 |
| R11 | | R | R | R | R | S | 2 | 1.7 |
| R12 | | S | S | R | R | S | 1 | 0.8 |
| R13 | | E | R | R | E | R[2)] | 1 | 0.8 |

[1)]The profiles R1-R7 were recorded in control strains while R1-R13 were recorded only in investigated strains; [2)] Five isolates were addressed to be Micrococci and were excluded from further studies as they showed furazolidon-resistance

Table 2: Investigation of reference strains and field isolates with tDNA-ILP PCR assay

| PCR profile | Reference strain | Field strains (n)[1)] | Field strains (%) |
|---|---|---|---|
| A1 | *S. chromogenes, S. hyicus S. epidermidis, S. saprophyticus, S. xylosus* | 45 | 39.8% |
| A2 | *S. caprae, S. simulans* | 37 | 32.7% |
| A3 | *S. lentus, S. sciuri* | 2 | 1.8% |
| A4 | *S. auricularis, S. lugdunensis* | 0 | 0 |
| A5 | *S. hominis* | 1 | 0.9% |
| A6 | *S. capitis* | 0 | 0 |
| A7 | *S. haemolyticus* | 0 | 0 |
| A8 | *S. warneri* | 0 | 0 |
| A9 | *S. cohnii* | 0 | 0 |
| A10 | *S. schleiferi* | 0 | 0 |
| A11-A37 | - | 28 | 24.8 (0.9% each) |

[1)]Total number of investigated field isolates is 113 as 5 of the isolates reacted negatively with the PCR

fingerprints in order to identify the 17 reference strains. The assay enabled to a large extend the identification of 10 of them based on their genomic profiles (Table 2, Fig. 1). The obtained 10 profiles were nummerated from A1-A10, namely: the closely related *S. chromogenes*, *S. hyicus*, *S. epidermidis*, *S. saprophyticus* and *S. xylosus* shared the same profile (A1), *S. caprae* and *S. simulans* (A2), *S. lentus* and *S. sciuri* (A3) and *S. auricularis* and *S. lugdunensis* (A4). The profiles from A5-A10 were represented by one isolate per profile: *S. hominis* (A5), *S. capitis* (A6), *S. haemolyticus* (A7), *S. warneri* (A8), *S. cohnii* (A9) and *S. schleiferi* (A10). The application of the test to identify the field isolates of the present study released the previously mentioned profiles A1-A10 in only 85 out of the 113 isolates (75.2%), the remaining 28 isolates showed other 27 profiles (Table 2)which could not be assigned to any of the reference strains examined in the present study.

When combining both antibiotic resistance and PCR amplification profiles, a sum of 69 field isolates could be identified. In case of *S. chromogenes* (n = 3) and *S. hyicus* (n = 17) revealing the same antibiotic resistance and PCR amplicon profiles, typical cultural morphological properties[24] were additionally used for further differentiation of these two specimens. From the remaining 49 identifiable isolates, 25 could be subscribed as *S. caprae*, both *S. epidermidis* and *S. simulans* could be detected 8 times each. Meanwhile, *S. saprophyticus* (n = 4), *S. lentus* (n = 2) and finally both *S. xylosus* and *S. hominis* which could be detected only one time out of the 118 isolates (Table 3 and 4).

It was not possible to understand how the molecular data was combined with antimicrobial susceptibility results to give the final identification of the 69 isolates. For example: in the profile A1 there were the following species: *S. chromogenes*, *S. hyicus*, *S. epidermidis*, *S. saprophyticus*and *S. xylosus*, sharing the same profile. A total of 45 field isolates showed this pattern (Table 2). In Table 3, only 32 isolates were classified in this profile. *S. caprae* was detected in 6 herds. *S. epidermidis* and *S. chromogenes* were detected in one herd, both *S. simulans* and *S. hyicus* in 3 herds, both *S. saprophyticus*

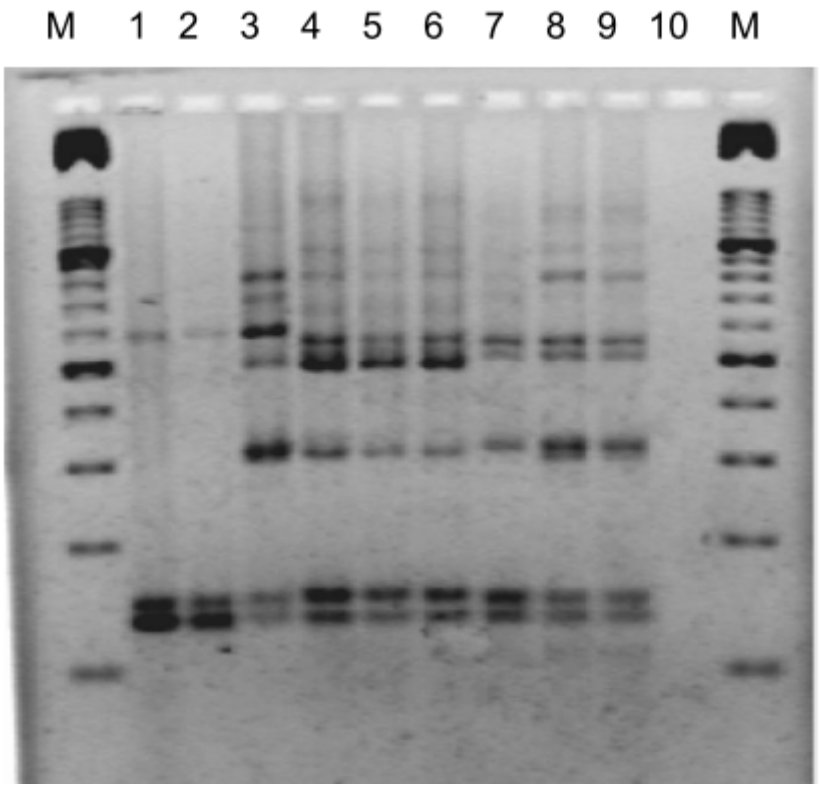


Fig. 1: t-DNA-PCR patterns obtained from reference strains. M. Marker, line 1-3 (*S. simulans*), line 4-6 (*S. xylosus*), line 7-9 (*S. epidermidis*), line 10 (Aqua dest.)

Table 3: The profiles of the 17 different NAS species isolates which were obtained after combining both antibiotic resistance and PCR amplification profiles

| Reference strain | Amplicon profile | Resistance profile | Field isolates (n) | Field isolates (%) |
|---|---|---|---|---|
| *S. chromogenes*[1)] | A1 | R2 | 3 | 2.5 |
| *S. hyicus*[1)] | A1 | R2 | 17 | 14.4 |
| *S. epidermidis* | A1 | R5 | 8 | 6.8 |
| *S. saprophyticus* | A1 | R4 | 4 | 3.4 |
| *S. xylosus* | A1 | R7 | 1 | 0.8 |
| *S. caprae* | A2 | R1 | 25 | 21.2 |
| *S. simulans* | A2 | R2 | 8 | 6.8 |
| *S. lentus* | A3 | R3 | 2 | 1.7 |
| *S. sciuri* | A3 | R4 | 0 | 0.0 |
| *S. auricularis* | A4 | R1 | 0 | 0.0 |
| *S. lugdunensis* | A4 | R2 | 0 | 0.0 |
| *S. hominis* | A5 | R6 | 1 | 0.8 |
| *S. capitis* | A6 | R1 | 0 | 0.0 |
| *S. haemolyticus* | A7 | R1 | 0 | 0.0 |
| *S. warneri* | A8 | R1 | 0 | 0.0 |
| *S. cohnii* | A9 | R3 | 0 | 0.0 |
| *S. schleiferi* | A10 | R2 | 0 | 0.0 |

**S. chromogenes* (n = 3) and *S. hyicus* (n = 17) revealed the same antibiotic resistance and PCR amplicon profiles. Because the reference strains of *S. chromogenes* and *S. hyicus* had the same amplicon and resistance profile, typical cultural morphological properties were used for further differentiation

and *S. lentus* in 2 herds. *S. hominis* and *S. xylosus* were detected once. In the present study, 17.3% of the whole collected samples showed positive bacterial growth which is the identical value stated in Swiss goat herds (17.3%) according to Schaeren and Maurer. Bacteriological examination of the grown colonies detected NAS in 10.4% of the samples versus 0.7% *S. aureus*. A good udder health in goats with a low frequency of NAS and a very low incidence of *S. aureus*-infections was already observed in previous studies in the region studied here. Moreover, it was also reported, that in last few years, NAS replaced coagulase positive Staphylococci as a major mastitis the region studied here. Moreover, it was also reported, that in last few

Table 4: The combination of both PCR amplification and resistance profiles of the 44 unidentified field isolates[1)]

| PCR profile | Resistance profile | n | (%) | PCR profile | Resistance profile | n | (%) |
|---|---|---|---|---|---|---|---|
| A1 | R1 | 2 | 1.7 | A20 | R1 | 1 | 0.8 |
| A1 | R3 | 1 | 0.8 | A21 | R1 | 1 | 0.8 |
| A1 | R6 | 1 | 0.8 | A22 | R3 | 1 | 0.8 |
| A1 | R8 | 3 | 2.5 | A23 | R2 | 1 | 0.8 |
| A1 | R9 | 3 | 2.5 | A24 | R1 | 1 | 0.8 |
| A1 | R11 | 1 | 0.8 | A25 | R1 | 1 | 0.8 |
| A1 | R12 | 1 | 0.8 | A26 | R5 | 1 | 0.8 |
| A2 | R8 | 3 | 2.5 | A27 | R5 | 1 | 0.8 |
| A2 | R11 | 1 | 0.8 | A28 | R4 | 1 | 0.8 |
| A11 | R9 | 2 | 1.7 | A29 | R9 | 1 | 0.8 |
| A12 | R4 | 1 | 0.8 | A30 | R1 | 1 | 0.8 |
| A13 | R2 | 1 | 0.8 | A31 | R1 | 1 | 0.8 |
| A14 | R9 | 1 | 0.8 | A32 | R2 | 1 | 0.8 |
| A15 | R8 | 1 | 0.8 | A33 | R4 | 1 | 0.8 |
| A16 | R2 | 1 | 0.8 | A34 | R2 | 1 | 0.8 |
| A17 | R9 | 1 | 0.8 | A35 | R2 | 1 | 0.8 |
| A18 | R8 | 1 | 0.8 | A36 | R8 | 1 | 0.8 |
| A19 | R1 | 1 | 0.8 | A37 | R3 | 1 | 0.8 |

[1)]In addition to these 44 isolates, additional 69 identifiable field strains are listed in Table 3 (total isolates 118 strains). Five isolates reacted negatively with the PCR and therefore were excluded from both tables (Table 3 and 4). These five isolated showed the antibiotic resistance profile R10 (n = 4) and R13 (n = 1) and were addressed to be Micrococci

years, NAS replaced coagulase positive Staphylococci as a major mastitis pathogen in goats in many countries worldwide[16, 30]. This may be attributed to many factors such as the vaccinal stress due to the use of anti *S. aureus* mastitis vaccines which could restrict the ability of the *S. aureus* strains to multiply and spread[31, 32].

According to the present work, 60% of isolated pathogens from mastitic German goat milk samples belonged to one of NAS species. This ratio is similar to that reported in USA in which the NAS prevalence in goat mastitis ranged between 63.5 and 75% as reported by Tomita *et al*.[33] and Gaucher, etc., respectively. However, in opposite to the situation in USA and our findings, NAS could be isolated from up to 95.9% of mastitic goat cases and even from 46.4% of healthy udder tissues in Italy[8, 16].

One of the main mastitis diagnostic tools is the evaluation of SCC. In the present study, the highest achieved geometric mean of SCC values was detected in milk of udders when *S. aureus* was present. The second highest SCC value was recorded in samples of udder halves infected with esculin positive Streptococci. These were followed by samples containing NAS isolates and finally those harbouring *Corynebacterium* spp. These findings are expected as *S. aureus* strains might harbor more virulence factors than NAS. *S. aureus* induces the clinical form of mastitis accompanied by clinical symptom of inflammation of the udder tissues. These findings are supported by the data published by Koop *et al*.[18] and Duralioglu *et al*.[13].

The present work aimed to identify the NAS at their species level due to their important role as an emerging pathogen in the induction of goat mastitis. To achieve this goal many methods were described in literature. One of the most commonly applied tool in routine diagnostic is the use of commercial biochemical identification assays such as the ID32 staph test[19-21, 23, 4].

Generally, the commercial tests deliver accurate identification power for the characterization of *S. aureus* isolates[27]. However, according to the present findings, their application to identify NAS isolates could only identify 64.7% of the references trains and 11.4% of the selected NAS field isolates. It seems that such systems are not accurate enough for the identification of NAS species related to goat mastitis. Ehrenberg reported many cases of confusing identification of NAS species when using different commercial biochemical assays, e.g., a novobiocin sensitive NAS isolate was identified as *S. warneri* by ID32 STAPH-System and as *S. xylosus* when using the API-STAPH IDENT-System. For their use in NAS diagnosis in goat milk samples, they may need revalidation. Similar conclusion was also obtained by Sampimon *et al*.[24].

The use of antibiotic susceptibility pattern analysis for the identification of NAS species was recommended either alone[22, 29] in combination with some biochemical reactions[35, 36] or in combination with biofilm formation assay[37]. For this purpose, the sensitivity of the control NAS strains against novobiocin, desferioxamin, fosfomycin, polymyxin b and furazolidon was tested to be compared later with the resistance profiles of the 118 investigated field isolates. The selected 17 references trains of the present study released only 7 profiles. That means that not all of the included reference strains could certainly be distinguished. Out of the 118 field strains 93 of them could be assigned to these 7 susceptibility patterns. The remaining 25 filed isolates presented six unknown profiles not represented by the reference strains used in this study. Two of them, representing altogether

five isolates contained furazolidone resistance and therefore were addressed as Micrococci. Although, the antibiotic resistance assay was used in many research papers to identify the NAS at their species level, the use of this assay proved to be insufficient even for the complete differentiation of the 17 reference strains selected in the present study. The reason could be the continuous interaction among the pathogens. Bacterial evolution and the acquisition of new resistance genes may lead to dubious results[38]. This might also be the reason for the failure of the antibiotic resistance assay to identify 25 field isolates in the present work. It could also be possible that these unidentified isolates belong to other NAS species rather than those involved in the present investigation. In conclusion, the use of the antibiotic resistance profile should be combined with other identification assays to avoid false results and to improve the rate of identification.

The application of the tDNA-ILP PCR assay using the reference strains involved in the present study lead altogether to 10 amplification patterns. Eighty-five out of 118 field isolates (75.2%) could be clearly assigned to these 10 pattern. While no amplification could be seen in five isolates which has been addressed to be Micrococci in the former investigation of the antibiotic sensitivity test as being furazolidone resistant, the remaining 28 field-isolates (24,8%) delivered atypical profiles which could not be categorized. This may deal with other NAS species which are not involved in this work or may be attributed to genomic dynamism through the acquisition or loss of mobile genetic elements or genomic recombinations.

The combination of various phenotypic and genotypic assays in order to accurately identify the NAS isolates at their species level was also recommended by Widerstrom[39]. In the present work, combining the various data delivered from the characterization of the 17 reference strains through tDNA-ILP PCR assay, antibiotic resistance test, culture morphology enabled the identification of 69 out of the 118 field isolates (58.5%). The isolates could be arranged in 9 different NAS species. The detected 9 different NAS species in the present work were *S. caprae* (n = 25), *S. hyicus* (n = 17), *S. epidermidis* (n = 8), *S. simulans* (n = 8), *S. saprophyticus* (n = 4), *S. chromogenes* (n = 3), *S. lentus* (n = 2), *S. xylosus* (n = 1) and *S. hominis* (n = 1). These findings are in agreement with the published data dealing with the occurrence of different NAS species by Aulrich and Barth[3], Koop *et al*.[23] and Rashid *et al*.[10] who listed *S. caprae, S. simulans, S. auricularis*and *S. hyicus*as major mastitis pathogens in goats.

Although, *S. caprae* seems to be the major NAS species detectable in goat milk as reported here or recommended in earlier studies[12, 3]. It is interesting to note that *S. caprae* from goat milk was not reported in older research papers as Orden *et al*.[40] and Maisi and Riipinen[41] and Winter or was not among the most commonly isolated NAS species[42]. This may be due to its minor role in the induction of mastitis previously, different geographic distribution or due to the inaccurate diagnostic tools used previously as the researchers depended on the application of API-staph-system or other biochemical test systems alone which also in the present work was shown to be insufficient to be used for NAS diagnosis at their species level. In the present study other NAS members were also detected such as *S. hyicus* which was isolated 17 times, 12 of them from the same goat indicating a chronic subclinical mastitis possibly caused by an identical field-strain. The presence of *S. hyicus* in mastitic goat milk was previously already documented[3]. Meanwhile, *S. epidermidis*, *S. simulans*, *S. saprophyticus* and *S. chromogenes* could also be detected with a lower prevalence. The presence of these species in goat milk was reported more frequently in the past[42, 41, 17]. However, it seems that they lost their position as major NAS mastitis pathogens for the favour of other members of the NAS family. In addition to the detection in pure culture, the increased occurrence within a herd and the elevation of the cell count, especially, a significant difference between the two udder halves, the repeated detection of a NAS species in the same udder half is considered as another key criterion of possible pathogenicity of a NAS-isolate.

It is noteworthy that in the neibougher country (Swiss) and in opposite to our results, Kunz *et al*.[43] could not isolate *S. caprae* or *S. hyicus* from mastitic goats. But instead, he isolated both *S. capitis* and *S. haemolyticus* from investigated field samples. Among the most commonly isolated species in Swiss *S. saprophyticus* and *S. lentus*were reported while *S. simulans* was detected only sporadically Kunz *et al*.[43]. From the epidemiological point of view, *S. caprae* was the mostly spread NAS species among the investigated herds (6 herds) in the present study which indicates its epidemiological role in mastitis induction in goats in the investigated area.This was followed by both *S.simulans* and *S. hyicus* (3 herds) and *S. saprophyticus* and *S. lentus* in 2 herds. In opposite to *S. epidermidis*, S. *chromogenes*, *S. hominis* and *S. xylosus* which seem to be only sporadic mastitis inducers and could be detected only in one herd.

Other NAS species recorded to induce mastitis in goats from other authors could not be isolated in the present study including *S. capitis*, *S. haemolyticus*, *S. warneri*[43, 4] *S. lugdunensis*, *S. saprophyticus*, *S. hominis*, *S. cohnii*[14], *S. auricularis*. In addition *S. sciuri* which mainly is described as being a pig pathogen[44] but was accidentally isolated from goat mastitis in 1984[42]. This may attributed to their geographical distribution or possibly the presence of one or more of these species among the unidentifiable NAS group in this research.

## CONCLUSION

In the present research, different NAS field isolates originating from caprine mastitis could be characterized at their species level. Whereas *S. caprae, S. epidermidis, S. simulans, S. hyicus, S. saprophyticus, S. chromogenes, S. lentus, S. xylosus* and *S. hominis*could be isolated. The most predominant species was *S. caprae* while *S. lentus, S. xylosus* and *S. hominis*could only be isolated from sporadic cases. And seem to play a negligible role in the induction of caprine mastitis. These epidemiological data are important due to the growing role of NAS in the induction of mastitis and also due to the zoonotical public health importance of some NAS members. Regular monitoring of the filed isolates among dairy animals provides an important overview for the prevalence of mastitis, inducing pathogens and evaluates the progress of mastitis control programs.

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