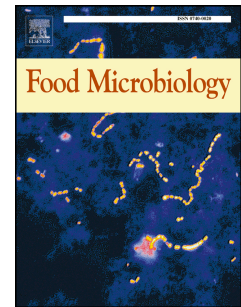


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East and West African milk products are reservoirs for human and livestock-associated *Staphylococcus aureus*

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**Title**

East and West African milk products are reservoirs for human and livestock-associated

*Staphylococcus aureus*

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## 29 **Highlights**

- 30 - 70 milk-associated *S. aureus* isolates from East and West Africa were analysed
- 31 - East and West African milk-associated *S. aureus* isolates are genetically diverse
- 32 - Both human and livestock-associated *S. aureus* lineages contaminate milk
- 33 - High prevalence of multi drug resistant *S. aureus* but no MRSA
- 34 - Isolates harbour virulence genes encoding for PVL, TSST-1 and enterotoxins

## Abstract

*Staphylococcus aureus* frequently isolated from milk products in sub-Saharan Africa (SSA) is a major pathogen responsible for food intoxication, human and animal diseases. SSA hospital-derived strains are well studied but data on the population structure of foodborne *S. aureus* required to identify possible staphylococcal food poisoning sources is lacking. Therefore, the aim was to assess the population genetic structure, virulence and antibiotic resistance genes associated with milk-derived *S. aureus* isolates from Côte d'Ivoire, Kenya and Somalia through *spa*-typing, MLST, and DNA microarray analysis. Seventy milk *S. aureus* isolates from the three countries were assigned to 27 *spa* (7 new) and 23 (12 new) MLST sequence types. Milk-associated *S. aureus* of the three countries is genetically diverse comprising human and livestock-associated clonal complexes (CCs) predominated by the CC5 (n=10) and CC30 (n=9) isolates. Panton-Valentine leukocidin, toxic shock syndrome toxin and enterotoxin encoding genes were predominantly observed among human-associated CCs. Penicillin, fosfomycin and tetracycline, but not methicillin resistance genes were frequently detected. Our findings indicate that milk-associated *S. aureus* in SSA originates from human and animal sources alike highlighting the need for an overarching One Health approach to reduce *S. aureus* disease burdens through improving production processes, animal care and hygienic measures.

## Abbreviations

ABR	Antibiotic resistance
CMT	California mastitis test
DLV	Double locus variant
CC	Clonal complex
MDR	Multi drug resistant
MLST	Multi locus sequence typing
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MSSA	Methicillin susceptible <i>Staphylococcus aureus</i>
nLV	n locus variant (n=4, 5, or 6)
PVL	Panton-Valentine leukocidin

62 SLV Single locus variant  
 63 ST Sequence type  
 64 TLV Triple locus variant  
 65

## 66 **1 Introduction**

67 *Staphylococcus aureus* can asymptotically colonize the skin and nasal cavities of humans and  
 68 animals. Roughly 20% of humans are persistent carriers while another 30% are considered to be  
 69 intermittent carriers of *S. aureus* in the nasal vestibules (van Belkum et al., 2009). However, *S. aureus*  
 70 is also responsible for a wide range of diseases in humans and animals, including skin and soft tissue  
 71 infections, mastitis, severe invasive infections, Staphylococcal Food Poisoning, and Toxic Shock  
 72 Syndrome (Tong et al., 2015). Pathogenicity, disease propagation and severity are largely influenced  
 73 by a large variety of well characterized virulence factors such as the staphylococcal enterotoxins, toxic  
 74 shock syndrome toxin (TSST-1), Panton-Valentine leukocidin (PVL), adhesins and immune evasion  
 75 factors, capsule types, accessory gene regulator (*agr*) groups, proteases, DNases and lyases (Argudín  
 76 et al., 2010; Krakauer and Stiles, 2013; Powers and Bubeck Wardenburg, 2014). However, while *S.*  
 77 *aureus* is well characterized in the industrialized world, *S. aureus* is considered to be a neglected agent  
 78 for disease in tropical and developing countries (Herrmann et al., 2013). This is despite the impact of  
 79 *S. aureus* diseases on the general population, healthcare, food and livestock production systems of  
 80 many communities in sub-Saharan Africa that in addition share a lifestyle of close daily livestock  
 81 interactions (Akindolire et al., 2015; Egyir et al., 2014a; Egyir et al., 2014b; Gitau et al., 2014; Maina  
 82 et al., 2016; Njage et al., 2013). Livestock products of cow, camel or goat origin contribute to the daily  
 83 food supply in sub-Saharan Africa. Especially milk products including fermented milk products for  
 84 increased shelf life are traditional staple food items in sub-Saharan Africa, providing important  
 85 nutrients to pastoral and sedentarized communities (Elhadi et al., 2015; Iannotti and Lesorogol, 2014).  
 86 High prevalence of zoonotic diseases, poor hygiene and production processes as well as long  
 87 transportation routes without cooling render these milk products highly susceptible to the outgrowth of  
 88 foodborne pathogens including *S. aureus* (Jans et al., 2012; Kouamé-Sina et al., 2012; Njage et al.,  
 89 2013; Noor et al., 2013). This is exemplified by a high presence of clinical and subclinical mastitis and

intramammary infections caused by *S. aureus* among dairy animals including camels in sub-Saharan Africa (Gitau et al., 2014; Issa et al., 2016; Regassa et al., 2013; Wanjohi et al., 2013). Furthermore, diarrhoea and vomiting in humans was especially associated with raw milk consumption (Kaindi et al., 2012) and possibly linked to the high prevalence of *S. aureus* in these milk products (Njage et al., 2013). Therefore, increased attention to foodborne *S. aureus* is required especially in terms of understanding the population structure, locally important lineages and their virulence factors to formulate appropriate interventions.

Population structure analysis by multi locus sequence typing (MLST) provides important epidemiological clues on emerging highly virulent lineages such as methicillin resistant *S. aureus* (MRSA) and methicillin susceptible *S. aureus* (MSSA) as well as associated clonal complexes (CCs) (Enright et al., 2000; Tong et al., 2015). There is some data available on the population structure of *S. aureus* for human and healthcare-associated settings, revealing a predominance of CC5, CC30, CC152 and typically CC88-MRSA-IV in sub-Saharan Africa (Abdulgader et al., 2015; Schaumburg et al., 2014). The emergence of MRSA in Africa is visible, but still significantly lower than in developed countries whereas PVL is highly prevalent, especially among MSSA isolates (Abdulgader et al., 2015; Schaumburg et al., 2014; Schaumburg et al., 2015). While the presence of *S. aureus* and antibiotic resistances (ABR) including multi drug resistance (MDR) is well known in humans, animals and food products in sub-Saharan Africa, knowledge on population structure and virulence factors of *S. aureus* is still mostly limited to human and healthcare associated settings.

Therefore, the aim of this study is to provide a first insight into the population structure of *S. aureus* from raw and fermented milk products as major vehicles of zoonotic infections in relation to human and livestock-associated CCs. This was combined with a detailed investigation of virulence factor and ABR gene carriage in order to understand *S. aureus* epidemiology and public health risk potential within the tightly connected system of animals, food and humans in order to contribute towards the implementation of a One Health approach in *S. aureus* and ABR control strategies.

## 2 Material and Methods

### 2.1 Milk sample origin

A total of 78 raw and fermented milk samples assigned into subsets 1-3 were incorporated into the current study to provide first insights into milk-associated *S. aureus* of geographically different regions of Africa, in particular *S. aureus* isolated from milk products from Kenya, Somalia and Côte d'Ivoire. The milk samples were collected during three independent studies designed to investigate the microflora of dairy products in these three East (Kenya and Somalia) and West (Côte d'Ivoire) African countries. General characterization of subsets 1 and 2 milk samples including sample type, location, physicochemical properties and microflora description were described in previous studies (Jans et al., 2012; Jans et al., 2013; Njage et al., 2013). Subset 1 (n=28, Kenya and Somalia) and 2 (n=15, Côte d'Ivoire) comprised 22 raw and 21 fermented milk samples collected in 2007, 2008 and 2010 (Supplementary Table S1) that originated from camel (n=27), cow (n=15) and goat (n=1) milk. Subset 3 was collected and analysed independent of subsets 1 and 2 and the samples have not been previously characterized. It comprised of raw milk samples collected from randomly selected camels from herds in Isiolo (two herds; n=10) and Marsabit (one herd; n=11), as well as milk sampled from public markets (n=14) in Garissa in Kenya during 2008. Sampling at each location was done on separate days and the samples from camel herds comprised of pooled udder milk derived. Udders were disinfected using 70% (v/v) ethanol and 3-4 strokes of foremilk were discarded prior to sample collection from all four quarters into sterile 50-mL Falcon tubes. Market raw milk samples (n=14) were collected in sterile 50-mL Falcon tubes and processed as described previously (Jans et al., 2012). pH measurements and California Mastitis Test (CMT) were performed on all samples of subset 3.

### 2.2 *Staphylococcus* spp. source, origin, isolation and enumeration in milk samples

Previously isolated *S. aureus* stored in glycerol stock at -80°C (n=52; subset 1; Njage et al., 2013) or isolated during this study from milk samples previously not screened for *S. aureus* (subset 2 and subset 3; Jans et al., 2013) were examined. Subset 2 milk samples could only be qualitatively analysed for this study since long term storage of milk samples at -20°C would have had an impact on bacterial survival to allow accurate quantitative analysis. The EN ISO 6888-2 protocol for the isolation of coagulase-positive staphylococci (ISO, 1999) was applied. Presumptive *S. aureus* isolates obtained

based on colony morphology and presence of an opaque fibrin halo on rabbit plasma fibrinogen agar (Oxoid, Basel, Switzerland) were transferred on to 5% sheep blood agar plates and incubated at 37°C for 24 h. Post incubation, *S. aureus* isolates were confirmed using the Staphaurex latex agglutination test (Oxoid, Basel, Switzerland). In case of the subset 3 milk sample analysis an identical isolation approach as previously used for subset 1 was applied during field studies in Kenya using Baird Parker agar medium supplemented with Egg yolk tellurite (BP, Biolife, Milan, Italy) and incubation under aerobic conditions at 37°C for 2 days (Njage et al., 2013). Colonies of desired presumptive *S. aureus* morphology were picked and streak purified on BP agar. Enumeration of presumptive *S. aureus* performed on the Kenyan subset 3 was performed using serial dilutions followed by plating onto BP agar. Colonies of typical *S. aureus* morphologies on BP agar were further assessed using staphylase test kit for coagulase reaction (Oxoid, Pratteln, Switzerland), Gram typing and catalase reaction.

### 2.3 DNA isolation

*S. aureus* isolates were cultivated in 10 ml Brain-Heart Infusion (BHI; Oxoid, Hampshire, UK) broth until the stationary phase was reached by incubating at 37°C and 150 rpm shaking for 18-24 h. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

### 2.4 *Spa* typing and multi locus sequence typing

*S. aureus* isolates were subjected to *spa* typing by PCR amplification and sequencing of the *spa* gene polymorphic X region as previously described (Johler et al., 2011). *Spa* types were assigned using the *spa*-server (<http://www.spaserver.ridom.de/>). *S. aureus*-specific multi locus sequence typing (MLST) was performed as previously described (Enright et al., 2000). Quality control of sequence data, assembly and comparison with the existing *S. aureus* MLST database (Jolley and Maiden, 2010) was performed using CLC Genomic Workbench (v8.5.1, Qiagen Aarhus A/S, Denmark), MEGA 5.0 and START2 (Jolley et al., 2001). Data visualization linked to phenotypes and metadata was performed in PHYLOViZ v2.0 (Francisco et al., 2012). Analysis of phylogeny and assignment to clonal complexes (CC) was performed using eBURSTv3 (Feil et al., 2004). The assignment of an ST to a CC was calculated in eBURSTv3 according to defined requirements that 6 out of 7 loci must be shared with at least one other member of the CC (Feil et al., 2004). Subgroups of CCs and the corresponding founder



ST were established if bootstrap calculations justified the subgroup by (a) 100% or (b) for ST bootstrap values between 99-100% if the suggested founder ST was not an SLV of an existing subgroup founder ST. Such subgroups were named using e.g. CC5-ST1, CC5-ST5 to avoid ambiguity (Abdulgader et al., 2015). New allele sequences, ST definitions and metadata of all corresponding isolates were submitted to the *S. aureus* MLST database. For prevalence calculations, only one isolate per ST per sample was considered.

To associate CC-STs with metadata, the complete MLST database was downloaded (pubmlst.org accession date 04-April-2016). For grouping and analysis purpose, descriptions for sample source, host and geographic origin were unified based on data entries presented in the database to use identical terminologies. Due to lack of clarity in the original database on host (animal or human) source, isolates assigned to a host with “carrier” for disease, “community acquired” or detailed medical diagnostics were interpreted as “possible human origin”.

## 2.5 DNA microarray and SplitsTree analysis

Based on *spa* types determined, 66 *S. aureus* isolates were selected and subjected to DNA microarray analysis (Monecke et al., 2008). The Staphytype genotyping kit 2.0 (Alere, Jena, Germany) was used for DNA microarray analysis in accordance with the manufacturer's instructions. An ArrayMate reader (Alere, Jena, Germany) was used for signal acquisition. Similarities in virulence and resistance gene profiles were visualized using SplitsTree4 (<http://www.splitstree.org>) (Wattiner et al., 2012).

For CC-based DNA microarray analysis of the 66 *S. aureus* isolates, CC-assignments derived from MLST were used for all isolates for which MLST was performed (n=40) due the enhanced discrimination power of MLST. Isolates without MLST data available (n=16), CCs were assigned based on the MLST-CC association with other isolates featuring the identical DNA microarray-CC designation. If the association was ambiguous, isolates were assigned to DNA microarray determine CCs only.

## 2.6 Antibiotic susceptibility testing

Isolates harbouring genes for  $\beta$ -lactam, tetracycline, aminoglycoside and chloramphenicol resistance were subjected to phenotypic antibiotic susceptibility tests using the disc diffusion test. Resistances to ampicillin, tetracycline, gentamicin, and chloramphenicol were determined based on zones of

inhibition on Mueller-Hinton Agar in accordance with Clinical and Laboratory Standards Institute guide lines (Clinical and Laboratory Standards Institute, 2016)

### 3 Results

#### 3.1 Prevalence of *S. aureus* isolates in milk samples

Eighty presumptive *S. aureus* isolates were isolated from the 78 raw and fermented milk samples that originated from Kenya (n=46) and Somalia (n=17; East Africa) and Côte d'Ivoire (n=15; West Africa). A total of 70 of these isolates were subsequently confirmed as *S. aureus* on further primary testing. Overall, *S. aureus* was isolated from 43 (55%) of the 78 milk samples with a distribution of 60% (42/70) and 40% (28/70) of the isolates being recovered from raw and fermented milk products, respectively (Fig. 1A). At specific country level there were 47 (67%), 8 (11%) and 15 (21%) isolates obtained from Kenyan, Somali and Ivorian milk products respectively. Except for one isolate from a goat milk sample, all isolates from Kenya and Somalia were of camel milk origin, whereas all Ivorian isolates were recovered from bovine milk samples (Fig. 1B).

#### 3.2 Cell counts and pH of milk samples of subset 3

This subset was comprised of 35 raw camel milk samples collected directly at herd level (Isiolo and Marsbit) as well as from public markets (Garissa) at three locations in Kenya. Mean cell counts on BP agar were  $\log_{10} 2.46 \pm 0.86$  CFU mL<sup>-1</sup> and  $\log_{10} 4.41 \pm 0.89$  CFU mL<sup>-1</sup> for the direct herd level (n=21) and public market (n=14) sampled milk, respectively. The mean pH values for these two sample groups were  $6.56 \pm 0.27$  and  $6.51 \pm 0.09$ , respectively. Four herd level and one public market collected milk sample yielded positive results in the CMT assay. A total of 33 isolates with presumptive *S. aureus* morphology isolated from these milk samples were also Gram-, catalase- and coagulase-positive. Out of this presumptive *S. aureus* isolate pool, isolates ILS-53, ILS-54 and ILS-55 confirmed as *S. aureus* using the Staphaurex test were subsequently isolated from BP agar petri dishes with 10-30 colonies and a final CFU count of  $\log_{10} 5.25$ ,  $\log_{10} 5.39$  and  $\log_{10} 4.58$  CFU mL<sup>-1</sup>, respectively.

#### 3.3 *spa* typing of *S. aureus* isolates

A total of 27 *spa* types were detected among the 70 *S. aureus* isolates including seven new *spa* types: t14847, t14869, t14892, t15154, t15155, t15156 and t15157 (Table 1 and Supplementary Table S1). Two Kenyan raw camel milk isolates could not be typed because their *spa* genes could not be

amplified by different available *spa* typing PCR primers. These *spa* types mostly showed distinct geographical distribution between and within the three countries with 18, 2 and 8 *spa* types assigned to Kenyan, Somali and Ivorian milk *S. aureus* isolates, respectively. The most frequent *spa* type from Kenyan isolates was t6521, whereas *spa* types t14847 and t289 predominated among the Somali and Ivorian isolates, respectively. Of the new *spa* types, five (t14869, t14892, t15155, t15156 and t15157) were exclusively detected in Kenya, one (t14847) was assigned to a Somali camel milk isolate only, and there was one new *spa* type (t15154) shared among camel and bovine milk isolates recovered from Kenya (Isiolo) and Côte d'Ivoire (Songon), respectively. *Spa* type t318 was recovered in Nanyuki, Garissa, Isiolo and Marsabit, t3519 from Mandera, Garissa and Isiolo, and t6521 from Isiolo and Nanyuki milk products. In Côte d'Ivoire the isolates assigned to *spa* type t084 were isolated from Bingerville and Lievre rouge milk samples.

### **3.4 *S. aureus* population structure analysis by DNA microarray and MLST**

#### **3.4.1 Population structure assessed by DNA microarray & SplitsTree analysis**

Genetic population structure of the Kenyan, Somali and Ivorian milk *S. aureus* isolates was assessed based on the DNA microarray hybridization profiles. To achieve this, 66 isolates selected based on *spa* type distribution and sample origin were analysed. Of these isolates, 59 were clustered into 11 CCs and 2 singletons, leaving 7 isolates with previously unobserved hybridization profiles that could not be assigned to CCs or STs based on homology with the hybridization profiles of the reference database (Table 2 and Supplemental Table 1). A SplitsTree constructed based on the virulence and resistance gene profiles of the isolates showed that a genetically diverse *S. aureus* population was associated with camel and bovine milk products sampled in Kenya, Somalia and Côte d'Ivoire featuring a high diversity of CCs, *agr* types and capsule types (Fig. 2). Furthermore, a clear genetic separation of the Ivorian (West Africa) from the Kenyan and Somali (East Africa) isolates was observed, with the exception of the CC5-ST5 cluster, which included both Kenyan and Ivorian isolates.

#### **3.4.2 Population structure determined by MLST**

A total of 40 *S. aureus* isolates were selected based on *spa* types and sample origin to cover the entire population diversity of isolates (Supplementary Table S1) and processed by MLST analysis. These isolates were assigned to STs and CCs that are distributed across the entire pool of the currently

publicly available *S. aureus* STs (Figure 2). The isolates were assigned to 23 STs predominated by ST30 (n=5) and ST2957 (n=4). At CC level, most isolates belonged to CC5 (27.8%, n=10) followed by CC30 (25%, n=9). CC5 was split into ST-clusters of CC5-ST5 (n=4), CC5-ST6 (n=1), CC5-ST15 (n=2), CC5-ST72 (n=1) and CC5-ST97 (n=2). All members of CC30 were either ST30 or SLV of ST30 (Supplementary Table S1).

A total of 12 new STs were defined by 17 isolates originating from Kenya (n=12, 8 STs), Somalia (n=2, 1 ST) and Côte d'Ivoire (n=3, 3 STs). Among these new STs, ST2013 and ST2077 represented new combinations of existing alleles. ST1738, ST1741, ST1742, ST1765, ST1781, ST1952, ST2073, ST2076, ST2080 and ST2094 were defined by new *arc*, *aroE*, *glpF*, *gmk*, *pta* and *yqiL* alleles. The new STs comprised 3 singletons (ST1741, ST1952 and ST2094) (Supplementary Table S1). New STs were predominantly related to CC5-ST5 (ST1741, ST2013, ST2076 and ST2077) albeit at levels ranging from SLV to 6LV. While ST1741 and ST2013 were related to CC5-ST5 as 6LV and SLV, respectively, ST2076 and ST2077 were TLV of CC5-ST9. ST1742, ST2080 and ST2094 were associated with CC130 (SLV), CC30 (SLV) and CC88 (DLV), respectively. ST2073 and ST1765 shared a DLV association with CC425. ST1952 was closest associated with CC398 at 4LV level. ST1738 and ST1781 were closest associated with CC707 at 5LV level (Supplementary Table S1).

### 3.4.3 Comparison of CCs associations determined by MLST and DNA microarray

CC assignment of isolates determined as members of CC5-ST5 (n=6), CC5-ST97 (n=2), CC5-ST72 (n=1), CC5-ST6 (n=1), CC5-ST15 (n=2), CC30 (n=10), CC101 (n=2), CC152 (n=1), CC522 (n=1) were also identified as members of the corresponding CC based on DNA microarray hybridization (Supplementary Table S1). ST identification on the other hand was less reliable resulting in the ST2957 isolates ILS-08, ILS-54, ILS-58 and ILS-60 being identified as ST39 on DNA microarray data. Ambiguous identification of ST97 and ST15 but still within the same CC5 was observed for ILS-20, ILS-32, ILS-73 and ILS-79. CC130 was correctly identified for ILS-37 whereas the other two isolates ILS-12 and ILS-15 were assigned into another cluster that comprise both CC30 and CC130 strains (Fig 2). It should however be noted that such assignments were associated with low assignment scores due to limited availability of reference microarray hybridization profiles from African *S. aureus* populations that are currently available on this platform (Supplementary Table S1). New ST1738

(ILS-03 and ILS-67) and ST1781 (ILS-23, ILS-24 and ILS-26) were identified as their closest existing DLV relative ST1755. ST1765 (ILS-17) and ST2094 (ILS-78) were assigned to CC425 and CC88, respectively, which represents their closest CC founders at DLV level. Singleton ST1741 (ILS-04) was identified as CC130 member whereas ST1952 (ILS-49), ST2076 (ILS-70) and ST2077 (ILS-72) could not be identified via DNA microarrays.

#### **3.4.4 Population structure in relation to location, livestock and human association**

The complementary dataset of population structure obtained from DNA microarray and MLST was also used to assess the relationships between isolates and sample metadata. Geographical origin of isolates correlated with CC distribution. CC101, CC130, CC30, CC522-ST96 and novel CC3304, CC3305, ST3307 and ST3308 were only assigned to isolates of Kenya and Somalia, whereas CC152, CC3311 and ST3314 were only assigned to those of Côte d'Ivoire. Healthcare-associated CC5 members formed the exception and were assigned to isolates of East and West African sampling regions (Supplementary Table S1). However, CC5 when grouped by CC-STs separated also according to sampling regions into CC5-ST15 and CC5-ST72 assigned to Ivorian isolates vs. CC5-ST6 and CC5-ST97 assigned to Kenyan isolates with only CC5-ST5 still comprised of isolates of Côte d'Ivoire and Kenya (Supplementary Table S1).

Isolates were analysed with regard to human- and livestock-associated CCs. According to literature and the metadata of the MLST database, CC101, CC152, CC1781, CC30, CC5-ST5, CC5-ST15, CC5-ST72, CC88, ST1741 and ST2094 were considered human-associated CCs. In contrast, CC130, CC1765, CC2076, CC522, CC5-ST6, CC5-ST97 and ST1952 were considered livestock-associated CCs. Of the 66 DNA microarray processed isolates, 62 isolates with CC assignments were obtained of which 27 and 35 were assigned to livestock and human-associated CCs, respectively. Similarly, CC assignment also correlated to raw milk and fermented milk origin of the isolates with only CC130, CC30 and CC5-ST5 forming clusters with isolates from both sample types (Supplementary Table S1).

#### **3.5 Prevalence of selected virulence factors determined by DNA microarray**

CC association determined by MLST (n=40) was used to cluster DNA microarray data (n=66). Only 4 isolates could not be assigned to a known genetic lineage as they exhibited previously unobserved

hybridization profiles and were not among the strains processed by MLST (Table 2 and Supplementary Table S1).

### 3.5.1 Toxins

Genes associated with different leukocidins including Pantone-Valentine leukocidin (PVL) were detected among the 66 isolates analysed by DNA microarray. *lukF*, *hlga* and *lukS* were most prevalent being detected in 66 (100%), 66 (100%) and 65 (96%) isolates tested, respectively. *lukY* and *lukX* were detected in 50 (76%) and 52 (79%) isolates, respectively, whereas 40 (61%) isolates harboured *lukY* and *lukX*. *lukD* and *lukE* were harboured by 38 (58%) and 30 (45%) isolates, respectively. PVL encoding genes *lukF*-PV and *lukS*-PV were detected in 4 (6%) isolates. While PVL-encoding genes were exclusively found in human-associated CCs, *lukD*, *lukX* and *lukY* displayed a higher prevalence (85-95%) in livestock compared to (37-65%) human-associated isolates (Table 2 and Supplementary Table S1).

Genes for toxic shock syndrome toxin *tstI* were detected in 4 (6%) isolates. All isolates harboured the human *tstI* allele, however, except for CC5-ST5 (1 isolate), the other two isolates of CC522 and CC5-ST6 shared a higher association with livestock. Staphylococcal enterotoxins were detected in approximately 1/3 of the isolates analysed (Table 2 and Supplementary Table S1). The enterotoxin gene cluster (*egc*, comprising *seg*, *sei*, *sem*, *sen*, *seo*, *seu*) was most prevalent and exclusively to isolates (37%) of the human-associated CCs. *Seh* was detected as single enterotoxin in isolates (15%) of livestock-associated CCs, whereas *sea* was detected in both isolates of human and livestock-associated CCs. While isolates of human-associated CCs carried the *egc* cluster, those of livestock-associated CCs lacked it (Table 2 and Supplementary Table S1). Genes encoding for the epidermal cell differentiation inhibitor B (*edinB*) were detected in over 40% of livestock-associated CC130 and CC2076 isolates, but in only one isolate of the human-associated CC152. Two CC2076 as well as 2 isolates without CC or ST assignment harboured the *edinB* in combination with the exfoliative toxin D, while a single CC152 isolate was positive for *edinB* and PVL genes (Supplementary Table S1).

### 3.5.2 Accessory gene regulator (*agr*) groups and capsule types

All 66 isolates harboured *agr* genes with clear association to clonal complexes. CC101, CC152, CC522, CC5-ST6, CC5-ST72, CC5-ST97, CC1781 and ST1952 comprising nearly 50% of isolates

from livestock-associated CCs were affiliated to *agrI* compared to only 11-37% of isolates from the human-associated CCs. CC5, CC5-ST15 and CC1765 belonged to *agrII*. *agrIII* was harboured by CC130, CC2076, CC30, CC88, ST1741 and ST2094 isolates and thus equally shared among human and livestock-associated CCs. *agrIV* was harboured by CC5-ST6, CC5-ST72, CC5-ST97, CC101, CC152, CC522 and CC1781. With the exception of *agrB-IV*, *agrII* and *agrIV* were predominantly found in isolates of human associated CCs (Supplementary Table S1). Fifty-three percent (36/66; CC5-ST72, CC5-ST97, CC5-ST5, CC5-ST8, CC30 (1 isolate only, also cap8), CC152, CC1765, CC1781, CC2076 and ST1952) of the isolates belonged to capsule type 5, whilst 47% (31/66; CC5-ST15, CC5-ST6, CC30, CC130, CC522, CC101, CC15, CC88, ST1741 and ST2094) isolates were affiliated to capsule type 8 (Supplementary Table S1). Capsule types were equally distributed between livestock and human associated CCs.

### 3.5.3 Antibiotic resistance genes

At least one antibiotic resistance gene was detected in 43 (65%) of the 66 camel and bovine milk derived *S. aureus* isolates. This included 28 (57%) Kenyan, 2 (25%) Somali and 13 (87%) Ivorian isolates (Table 2 and Supplementary Table S1). The resistance genes were associated with 87% (13/15) bovine (Côte d'Ivoire) and 53% (30/57) camel (Kenya and Somalia) milk isolates. Fifty-six percent (24/43), 30% (13/43), 12% (5/43) and 2% (1/43) of the resistance isolates harboured one, two, three and four antibiotic resistance genes, respectively, yielding 44% (19/43) as presumptive MDR *S. aureus*.

The *fosB* gene encoding fosfomycin resistance predominated being detected in 67% (29/43) of resistant isolates that included 19 (19/49; 39%) Kenyan, 2 (2/8; 25%) Somali and 8 (8/15; 53%) Ivorian isolates. The tetracycline resistance gene *tetK* was the second most frequent resistance gene harboured by 49% (21/43) of resistant isolates that represented 18% (9/49) Kenyan and 80% (12/15) Ivorian milk isolates. The  $\beta$ -lactamase resistance operon (*blaZ/I/R*) was detected in 37 % (16/43) of the resistant isolates comprising 11 (11/49; 22%) Kenyan, 1 (1/8; 13%) Somali, and 4 (4/15; 27%) Ivorian milk isolates. Resistance genes involved in aminoglycoside (*aadD*), chloramphenicol (*fexA*) and streptogramin (*vga-BM 3327*) resistance were only found in single isolates. The *fexA* gene in combination with *fosB* and  $\beta$ -lactamase operon genes occurred in a Kenyan camel milk isolate,



whereas an Ivorian bovine milk isolate harboured aminoglycoside fosfomycin, tetracycline and  $\beta$ -lactam resistance genes. The streptogramin resistance *vga-BM* 3327 was found in a single Ivorian bovine milk isolate. No isolate harboured any of the other antibiotic genes queried on the DNA microarray including the methicillin and vancomycin resistance genes. But all the 66 isolates harboured the *sdrM* gene encoding for a multidrug efflux pump. Isolates harbouring genes for tetracycline,  $\beta$ -lactam (ampicillin), chloramphenicol and aminoglycoside (gentamicin) resistance genes were confirmed to be phenotypically resistant to corresponding antibiotics using the disc diffusion test (Supplementary Table S1).

#### 4 Discussion

In this study, we have examined 70 *S. aureus* isolates recovered from raw and fermented milk sampled in Côte d'Ivoire, Kenya and Somalia. These isolates were characterized based on *spa* typing (70 isolates), DNA microarray hybridization (66 isolates) and MLST (40 isolates) providing further insights into the genetic population structure of milk-associated *S. aureus* in three African countries. Several new *spa*-types as well as MLST STs including new alleles were revealed. In agreement with previous observations from other parts of the world, the CC and *spa*-types t002, t084, t186 and t355 association were further confirmed among African isolates of CC5-ST5, CC5-ST15, CC88 and CC152, respectively (Schaumburg et al., 2015).

MSSA members of CC5-ST5, CC5-ST8, CC5-ST15, CC30, CC121 and CC152, and MRSA members of CC5-ST5, CC5-ST8, CC80, CC88 and CC5-ST239/241 occur frequently in African hospital environments with CC5 being the most prevalent CC for MSSA and MRSA across Africa (Abdulgader et al., 2015; Schaumburg et al., 2014). Genetic diversity among the tested isolate collection was large and distributed across the entire current MLST scheme including bovine, camel and human-associated lineages as well as new members for CC-ST5 (ST2013), CC30 (ST2080) and CC152 (ST152) representing the most prevalent African CCs (Abdulgader et al., 2015; Schaumburg et al., 2014) and 49% (32/66) of the analysed isolates.

Several novel STs including ST1738, ST1741, ST1781 and ST2094 detected in our study revealed could not be linked at SLV level to any existing CC, yielding singletons that required a wider analysis approach in order to obtain potential relationships. Novel ST2094 was closest related to CC88 at DLV



level. A second isolate processed by DNA microarray only also related closest to CC88 but due to the absence of MLST data, was not further typed. CC88 comprises a typical sub-Saharan African MRSA clone ST88-MRSA-III/IV (Schaumburg et al., 2014) for which the MSSA clones revealed by this study provides valuable comparative isolates. ST1738 and ST1781 were closest associated with CC707 at 5LV level. CC707 is a small CC comprised of only 9 members mainly from nasal and skin swabs originating from rural and clinical environment in Ghana (Egyir et al., 2014a; Egyir et al., 2014b) but also human samples in Côte d'Ivoire, the Gambia, China, Brazil, Canada and Europe. Interestingly, ST1738 and ST1781 were connected to CC707 via ST1755 of an MSSA isolate from the United Arab Emirates indicating close relationship and shared camel habitat between these three ST. ST1741 was closest related to CC-ST5 albeit at 6LV level, but as such still potentially related to the main human and hospital associated CC in Africa. Although not within the strict CC definitions, broader analysis can thus reveal interesting potential connections to initiate subsequent investigations. Besides human and healthcare associated CCs, several livestock-associated CCs (CC5-ST9, CC425, CC5-ST97, CC130, CC522, CC130-ST1742) were obtained (Becker et al., 2014; Fitzgerald and Holden, 2016; Graveland et al., 2011; Smith et al., 2005). Meanwhile the detection of several CC130 isolates from camel milk suggests that camels are also associated with this CC and not only small ruminants (Merz et al., 2016). Similarly, bovine mastitis relevant CC5-ST97 lineages (Fitzgerald and Holden, 2016) also seem to affect camels, likely contributing to mastitis. Direct relationships to such livestock-associated CCs were detected at SLV level for several STs. However, widened analyses of novel STs beyond SLV level suggested closest relationships of many of these novel STs to livestock-associated CCs. This included ST1765 and ST2073 at DLV level to CC425; ST2076 and ST2077 at TLV level to CC5-ST9 and ST1952 at 4LV level to the predominant livestock-associated CC398 lineage (Köck et al., 2013; Smith, 2015; Ye et al., 2016).

The *S. aureus* population structure detected in milk products in this study with members of hospital/human-associated CCs as well as livestock-associated CCs suggests different sources and cross-contamination for *S. aureus* presence in milk. Human-related contamination of milk along the supply chain seems to play an important role in *S. aureus* dissemination from humans to milk, further to other humans and likely also to livestock described as cross-circulation of environmental, hospital

and livestock isolates (Lin et al., 2016; Schaumburg et al., 2015). In addition, the livestock-associated CCs suggest that a second source of *S. aureus* contamination of milk occurs directly from the animal *e.g.* in the case of subclinical mastitis. Our data on *S. aureus* levels in along the milk supply chain in Kenyan subset 3 samples showed low initial *S. aureus* counts of raw milk, but rising to potential health concerning levels of over  $10^5$  CFU/mL by the time the milk products reached the market. Therefore, regardless of the source of contamination, increased awareness is required to minimize contamination through diagnosis and treatment of infected animals followed by improved hygiene practises and prevention of bacterial growth during processing and storage.

ABRs are a major problem regarding the treatment of *S. aureus* infections. Consistent with the low MRSA prevalence reported in sub-Saharan Africa (Maina et al., 2016; Omuse et al., 2014; Schaumburg et al., 2015), MRSA were not detected in this study. However, all MSSA described carried a multi drug efflux pump and most carried additional ABR genes, in particular genes encoding for penicillin (27%), tetracycline (33%) and fosfomycin (42%) resistances. This corresponds with previously reported high prevalence of penicillin and tetracycline resistances in *S. aureus* of human and dairy origin including rural, urban and hospital environments in sub-Saharan Africa (Aiken et al., 2014; Egyir et al., 2014a; Egyir et al., 2014b; Schaumburg et al., 2014; Shitandi and Sternesjö, 2004). However in this study, the presence of *fosB* was detected in nearly 2/3 of all human-associated CCs isolates but only in below 15% of livestock-associated CCs. Similar findings were obtained for *tetK* that was more prevalent among human-associated isolates whereas genes encoding for penicillin-resistance displayed a higher prevalence among livestock-associated CCs. These data possibly indicate exposure to different antibiotics in human and veterinary settings to yield different selection pressures.

Although fosfomycin resistance genes at such high prevalence have been previously reported an observation that might be of concern if such genes confer phenotypic resistance is the fact that some isolates carrying these genes also harbour *pvl*-and *tstI* genes. This might be problematic considering the revived activities of promoting fosfomycin for veterinary use and as a potential treatment of MRSA infections such as skin infections (Pérez et al., 2014; Popovic et al., 2010). Therefore, ABR awareness and judicious use of antibiotic therapy in humans and livestock should be encouraged.

Virulence factors significantly differed between the two groups especially for staphylococcal enterotoxins, *tstI* and PVL, which with the exception of *tstI*, were less frequently detected among isolates of livestock-associated CCs (Cuny et al., 2015). PVL is a concerning virulence factor contributing to soft tissue and skin infections. It has mostly been detected in human or hospital-associated *S. aureus* isolates including *S. aureus* in Africa (Abdulgader et al., 2015) or in strains assigned to livestock-associated MRSA (Ünal et al., 2012). Recently, the detection of *pvl*-positive isolates in Columbian cheese (Herrera et al., 2016), in camel meat in Saudi Arabia (Raji et al., 2016) and in camel milk in the United Arab Emirates (Monecke et al., 2011) was reported. Sub-Saharan Africa is considered to be an endemic region for *pvl*-positive *S. aureus*, the majority of which are MSSA (Schaumburg et al., 2014). The *pvl*-positive *S. aureus* identified in this study belong to MSSA strains of CC5-ST15, CC30 and CC152, which represent typical PVL-CCs of sub-Saharan Africa and support the reports on high prevalence of PVL carrying MSSA in African countries (Schaumburg et al., 2014). Even though these PVL carrying isolates of our study are MSSA and might thus be considered of a lesser health risk, all isolates harbour *sdrM* and 3 isolates harbour penicillin and fosfomycin resistance genes and one strain additionally harbours a tetracycline resistance gene. The combinations of PVL, MDR, enterotoxin genes, *edinB*, *cna* and *sasG* yields critical combinations with elevated human health threats.

Although this study provides a first and novel insight into the population structure, virulence characteristics and antibiotic resistances of milk-associated *S. aureus* in three African countries, this study also features some limitations. Given the number of samples per location and the number of locations, this study provides a small but relevant snapshot of *S. aureus* in various areas of three different African countries. While not providing a complete and fully representative epidemiologic surveillance picture, such snapshots are a necessary first step to initiate the succession of epidemiologic studies of increasing complexity. The snapshot of this study provides baseline data that can be considered for the design, sample size calculations and justification of enhanced epidemiologic cross sectional studies that would provide the necessary input for intervention studies. The data provided in this study of potential mixed human and animal *S. aureus* strains in food will also help to justify a transdisciplinary One Health or Eco Health approach to enhance research output through

collaborations between food microbiologists, veterinary and human medicine as well as local stakeholders along the dairy value chain.

## 5 Conclusions

This study represents one of the first assessments of population structure, virulence and antibiotic resistance characteristics outside healthcare or human-associated settings. *S. aureus* from traditional raw and fermented dairy products in Kenya, Somalia and Côte d'Ivoire are highly diverse yielding 12 new ST from the 40 and 66 isolates processed by MLST and DNA microarray, respectively. MLST and CC-assignment suggest that *S. aureus* in milk is originating from human and animal sources alike highlighting the strong need for improved manufacturing processes, hygiene, producer and consumer awareness as well as proper animal care. As previously indicated, MRSA prevalence in Africa is still low and all isolates analysed in this study were MSSA. However, high prevalence of MDR to penicillin, tetracycline and fosfomycin was observed. In addition, we detected high viable cell counts in food products clearly and detected a broad range of genes encoding virulence factors such as PVL, TSST-1, and staphylococcal enterotoxins, indicating a health risk for humans and animals alike. The expected circulation of isolates within this highly connected environment only strengthens the clear need for an overarching One Health approach to control and mitigate the outcome of MDR *S. aureus* contamination in milk.

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## 8 Tables

**Table 1: *Spa* types detected among the Kenyan, Somali and Ivorian milk-associated *S. aureus* isolates**

Country	Number of isolates	Number of <i>spa</i> types	<i>spa</i> types <sup>a</sup>
Kenya	49	18	t002, t179, t267, <b>t318</b> , t701, t714, t964, t1532, t1965, <b>t3519</b> , t4172, <b>t6521</b> , t14869, <u>t14892</u> , <u><b>t15154</b></u> , <u>t15155</u> , <u>t15156</u> , <u>t15157</u>
Somalia	8	2	t433, <u>t14847</u>
Côte d'Ivoire	15	8	<b>t084</b> , t091, <u><b>t15154</b></u> , t15157, t186, t289, t355, t442, t537

<sup>a</sup>New *spa* types are underlined, whereas *spa* types shared by more than one geographical location are designated in bold

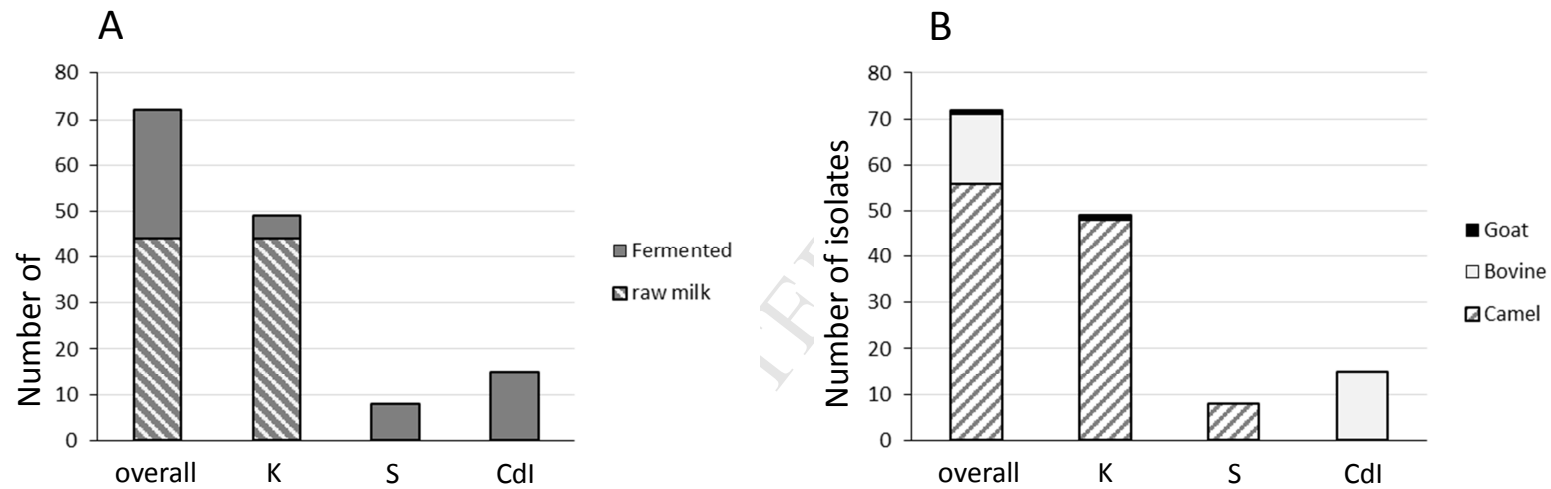
663 **Table 2: Overview of detected clonal complexes (CC), single sequence types (ST), *spa* types and the presence of selected virulence and antibiotic**  
 664 **resistance genes among *S. aureus* isolates from East African (Kenya and Somalia) camel and West African (Côte d'Ivoire) bovine milk samples. For**  
 665 **complete data per strain see Supplementary Table S1.**

CC	Isolates	<i>spa</i> -types	ST	PVL	<i>tstI</i>	Enterotoxin genes <sup>a</sup>						Antibiotic resistance genes <sup>b</sup>						Milk Type	Host	Country
						<i>sea</i>	<i>sed</i>	<i>seg</i>	<i>seh</i>	<i>sei</i>	<i>sej</i>	<i>tetK</i>	<i>blaZ1/R</i>	<i>fosB</i>	<i>aacA-aphD</i>	<i>fexA</i>	<i>vga(A)</i>			
CC101 (n=2)	ILS-55, ILS-59	t714, t4172	101	0	0	0	0	0	0	0	0	2	2	2	0	0	0	milk	camel	Kenya
CC130 (n=5)	ILS-12, ILS-13, ILS-14, ILS-15, ILS-37	t14847, t14869	1742, 130	0	0	0	0	0	4	0	0	0	0	0	0	0	0	fermented milk, milk	camel	Somalia, Kenya
CC130 chip only (n=5)	ILS-05, ILS-11, ILS-16, ILS-38, ILS-39	t1532, t14847, t14869	not done	0	0	0	0	0	0	0	0	0	0	1	0	0	0	fermented milk, milk	camel	Kenya, Somalia
CC152 (n=1)	ILS-77	t355	152	1 (human)	0	0	0	0	0	0	0	0	0	0	0	0	0	fermented milk	bovine	Côte d'Ivoire
CC1765 (n=2)	ILS-17, ILS-68	t15155, t15157	1765, 2073	0	0	0	0	0	0	0	0	0	0	2	0	0	0	milk	camel	Kenya
CC1781 (n=9)	ILS-02, ILS-03, ILS-22, ILS-23, ILS-24, ILS-25, ILS-26, ILS-27, ILS-67	t6521, t179	1738, 1781, not done	0	0	0	0	0	0	0	0	0	0	0	0	0	0	milk	camel	Kenya
CC2076 (n=2)	ILS-70, ILS-72	t091, t289	2076, 2077	0	0	0	0	0	0	0	0	1	0	0	0	0	1	fermented milk	bovine	Côte d'Ivoire
CC30 (total n=12, chip n=10)	ILS-08, ILS-09, ILS-10, ILS-47, ILS-48, ILS-54, ILS-58, ILS-60, ILS-61, ILS-64, ILS-66, ILS-76	t318, t433, t964, t3519, t15154	2957, 30, not done, 2080	2 (human)	0	2 (320E)	0	6	0	6	0	2	1	10	0	0	0	fermented milk, milk	camel	Kenya, Somalia
CC5 (n=6)	ILS-53, ILS-63, ILS-71, ILS-74, ILS-75, ILS-80	t002, t15154, t442, t15157	2013, not done, 5	0	1	2 (N315)	1	6	0	6	1	6	1	6	1	0	0	fermented milk, milk	camel, bovine	Kenya, Côte d'Ivoire
CC522 (n=2)	ILS-30, ILS-31	t15156	not done, 522	0	2	0	0	0	0	0	0	2	0	0	0	0	0	milk	camel	Kenya
CC5-ST15 (n=2)	ILS-73, ILS-79	t084	15	1	0	1	0	0	0	0	0	2	2	2	0	0	0	fermented milk	bovine	Côte d'Ivoire
CC5-ST6 (n=1)	ILS-65	t701	6	0	1	1	0	0	0	0	0	0	1	1	0	1	0	milk	camel	Kenya

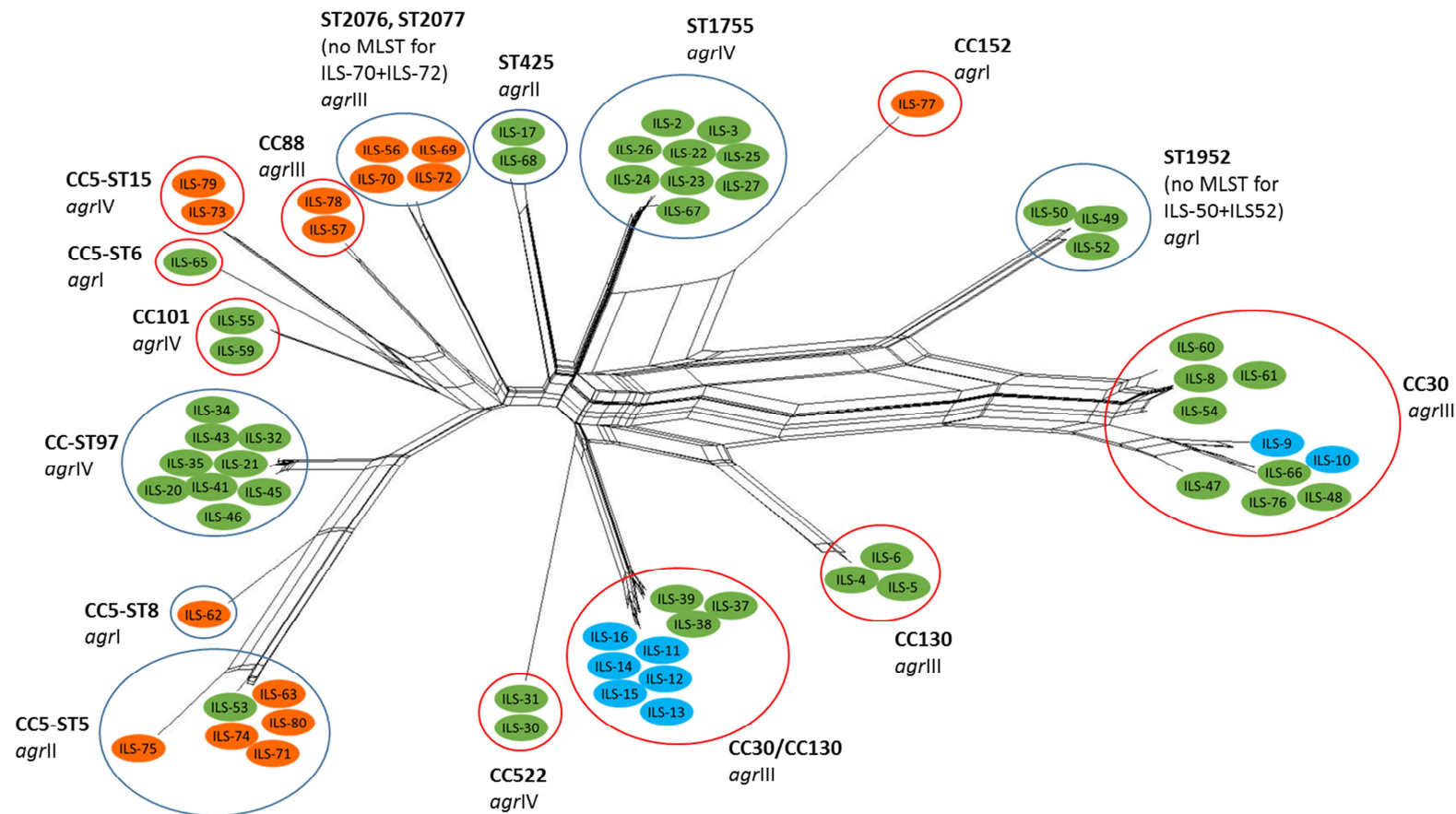
CC	Isolates	spa-types	ST	PVL	tstI	Enterotoxin genes <sup>a</sup>					Antibiotic resistance genes <sup>b</sup>							Milk Type	Host	Country
						sea	sed	seg	seh	sei	sej	tetK	blaZ/I/R	fosB	aacA-aphD	fexA	vga(A)			
CC5-ST72 (n=1)	ILS-62	t537	72	0	0	0	0	1	0	1	0	1	1	1	0	0	0	fermented milk	bovine	Côte d'Ivoire
CC5-ST97 (n=9)	ILS-20, ILS-21, ILS-32, ILS-34, ILS-35, ILS-41, ILS-43, ILS-45, ILS-46	t267, t1965	97, not done	0	0	0	0	0	0	0	0	4	9	0	0	0	0	fermented milk	camel	Kenya
CC88 chip only (n=1)	ILS-57	t186	not done	0	0	0	0	0	0	0	0	1	0	0	0	0	0	fermented milk	bovine	Côte d'Ivoire
chip only but new ST, not assigned (n=4)	ILS-50, ILS-52, ILS-56, ILS-69	t14892, t289	not done	0	0	0	0	0	0	0	0	2	0	0	0	0	0	fermented milk	camel, bovine	Kenya, Côte d'Ivoire
not done (n=12)	ILS-01, ILS-07, ILS-18, ILS-19, ILS-28, ILS-29, ILS-33, ILS-36, ILS-40, ILS-42, ILS-44, ILS-51	untyp <sup>c</sup> , t267, t14869	not done	- <sup>d</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-	fermented milk, milk	camel	Kenya
ST1741 (n=2)	ILS-04, ILS-06	t1532	1741	0	0	0	0	0	0	0	0	0	0	2	0	0	0	milk	camel	Kenya
ST1952 (n=1)	ILS-49	t14892	1952	0	0	0	0	0	0	0	0	0	0	0	0	0	0	fermented milk	camel	Kenya
ST2094 (n=1)	ILS-78	t186	2094	0	0	0	0	0	0	0	0	0	0	0	0	0	0	fermented milk	bovine	Côte d'Ivoire

<sup>a</sup>: complete list of enterotoxin genes tested is available in Supplementary Table S1. <sup>b</sup>: all isolates carried the multi drug efflux pump *sdrM*; <sup>c</sup>Untyp: no spa-type obtained; <sup>d</sup>:- assay not performed

## 9 Figures



**Fig. 1: The distribution of the Kenyan (K), Somali (S) and Côte d'Ivoire (CdI) *S. aureus* isolates based on (A) milk product and (B) animal species sources.**



**Fig. 2: Splits Tree network based on similarity of DNA microarray hybridization profiles of 66 *S. aureus* isolates derived from Kenyan, Somali and Ivorian camel and bovine milk products. ST1952, ST2076 and ST2077 isolates that could not be assigned to a CC or ST due to unique DNA microarray**

684 hybridization profiles were assigned based on MLST data. Solid spheres: Green=Kenya; Blue=Somalia; Orange=Côte d'Ivoire. Open circles: blue=capsule type  
685 5; red=capsule type 8.

686



## 10 Supplemental material

**Supplementary Table S1: Complete isolate table with isolate origin, CFU, MLST info, spa types and chip data.** Explanations: n.d.: not determined; ST: sequence type; CC: clonal complex; *n*LV: number of different loci between CC-ST and ST of the designated isolate, “=” all loci identical, SLV: single locus variant, DLV: double loci variant, TLV: triple loci variant, 4LV/5LV/6LV: 4, 5 or 6 loci variant.