Isolation and identification of *Staphylococcus aureus* obtained from cheese samples

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ABSTRACT

Milk and dairy products including cheese are one of the most significant food commodities in terms of the food industry. However, a contaminated food product could conduce a variety of food borne bacterial infections. Although *Staphylococcus aureus* is known as normal flora members of the humans, it’s often isolated from the community and hospital-acquired infections. Therefore, investigation of *Staphylococcus aureus* from cheese samples was aimed in this study. A total of nineteen (n=19) white cheese was collected from various outdoor markets in Istanbul. All cheese samples were evaluated quantitatively. Phenotypic identification tests including Gram staining, oxidase, catalase, mannitol, and DNase were performed. The presumptive *Staphylococcus aureus* colonies (n=47) were analyzed by the 16S rRNA PCR and sequencing. And the sequences were deposited into the National Center for Biotechnology Information. According to the nucleotide BLAST analysis, a total of 47 *Staphylococcaceae* and *Enterococcaceae* members including *Staphylococcus aureus* (n=3), *Staphylococcus carnosus* (n=1), *Macrococcus caseolyticus* (n=1), *Enterococcus faecalis* (n=25), *Enterococcus faecium* (n=12), *Enterococcus durans* (n=4), and *Enterococcus gallinarum* (n=1) were identified. Regarding methicillin susceptibility testing, two of out of three *Staphylococcus aureus* were detected as methicillin-resistant.

Keywords: *Staphylococcus aureus*, 16S rRNA, cheese, PCR
Introduction

The white cheese is the most consumed cheese type in Turkey and the cheese consumption per capita was determined as 8.7 kg/person in 2017 and 9.2 kg in 2020 (Temelli et al., 2016; Ataseven, Z., 2017; www.statista.com) Cheese is such a nourishing food that could provide an environment to the bacteria for growing and multiplication including Salmonella, Escherichia, and Staphylococcus because of the contamination. From the production of cheese to the point of sale, an inadequate sanitation procedure of equipment and utensils lead to contamination of the cheese products and this affects not only food quality but also public health (Donnelly, 1990; Aguilar et al., 2016).

Staphylococcus aureus (S. aureus) is known as normal flora member of the human skin, however, some strains of the S. aureus is the main reason of the infections and intoxications in terms of consumption of the contaminated milk, dairy products and other foods (Kadiroglu et al., 2014; Bingöl and Toğay, 2017). Staphylococcal food intoxication is a gastrointestinal disease that occurs due to the toxin produced S. aureus. When food or ingredients is contaminated by the enterotoxigenic strain of Staphylococcus spp., Staphylococcal food poisoning could be induced on the occasion of Staphylococci growth and enterotoxin production (Hennekinne et al., 2012; https://www.ndhealth.gov/Disease). Moreover, pathogenic strains of S. aureus could cause skin lesions, septicaemia, and meningitis in humans and it’s responsible for bovine mastitis in animals (Younis et al., 2003; Baran et al., 2017). The transmission of S. aureus to dairy products such as milk and cheese could occur via mastitis, mammary glands or animal, skin (Saka and Gulel, 2018). There may be a risk of contamination from personnel and equipment during the production of dairy products. In other words, transmission can be occurred also by animal to animal during milking as well as by the food-handlers, human to food contamination route (Kümmel et al. 2016; Monte et al., 2018). Methicillin-resistant S. aureus (MRSA) is one of the most significant bacteria in terms of human global health due to the responsible for both community and hospital-acquired infections (Harrison et al., 2014). Moreover, livestock-associated MRSA (LA-MRSA) infections originated from livestock such as pigs, goats, and dairy cattle could transmit to the humans who is working in farms and abattoirs where raw meat processed. LA-MRSA could be occurred by handling contaminated meats. Therefore, LA-MRSA could be also the reason for human infections (Cuny et al., 2015).

Although, the isolation of the MRSA from animal and food origin were investigated frequently, the adverse effect of MRSA in dairy products illness is relatively low (Herrera et al., 2016). Hence, identification of S. aureus in cheese samples is important for both the food industry and public health. In this study, it was aimed to identify S. aureus in white cheese samples sold in outdoor markets in Istanbul.

Materials and Methods

Sample Collection and Bacteriological Analysis

A total of nineteen (n=19) white cheese was collected from outdoor markets in Istanbul in April 2018 and September 2019. The color and pH value of each cheese samples were recorded (Creamy and white, pH:6.8-7.5). The cheese samples were analyzed quantitatively by homogenizing 25 g cheese and 225 ml peptone water (Peptone:10 g/L, NaCl:5.0 g/L pH: 7.2±0.2) within 24-hour. The 10-fold serial dilutions were spread on Baird-Parker Agar Medium supplemented with Egg Yolk Enrichment (Becton Dickinson). Typical colonies (dark gray to black colonies with clear zones) were selected and counted for further identification analysis followed by the 24-h for 37 °C incubation. Phenotypic identification tests including Gram staining, oxidase testing of cytochrome oxidase with indicator (tetramethyl-p-phenylenediamine) conversion to the indophenols catalase (A slide drop with 3% H2O2 onto the presumptive S.aureus isolates on microscope slides), mannitol fermentation (mannitol-fermentation as a carbohydrate source in the presence of phenol red as a pH indicator to detect mannitol-fermenting Staphylococci), and DNase (DNA hydrolysis test composed of growing microorganism in the DNase test agar medium that produces Deoxyribonuclease when the DNA is broken down resulting with clear zone and green color fades) were performed. The presumptive (typical colonies) S. aureus colonies (n=47) were taken into consideration for further identification analysis.

Genomic DNA Isolation and 16S rRNA Sequencing

The genomic DNA isolation of the presumed S. aureus colonies (n=47) was performed by using GeneAll® (South Korea) genomic DNA isolation kit according to the manufacturer’s instructions. Isolated genomic DNA samples were stored at –20 °C until PCR analysis. The 16S rRNA PCR analysis was performed according to the Frank et al. (2008). The 16S rRNA gene were amplified in a 50 µl reaction volume including 1xPCR buffer (Maximo, GeneON), 0.2 mM of each dNTPs, 2.5 mM MgCl2, and 0.5 µM of each primer (16S rRNA:27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGTACCTTGTTACGACTT) (Suardana, 2014). The PCR reaction was performed as following conditions: 2 min initial denaturation at 95 °C, 25 cycles of denaturation 1 min at 95 °C, annealing at 55°C for 1 min, and extension at 72°C for 2 min and 10 min final extension at 72°C. The obtained PCR
amplicons (~1465 bp) were evaluated by agarose gel electrophoresis (1.0 %) and screened by a transilluminator implemented in WiseDoc Gel Doc System. The purification of 16S rRNA gene amplicons was performed by BMLabosis (Ankara, Turkey) using the ExoSap-IT (Affymetrix) kit. Later on, samples were sent to Macrogen (Amsterdam, The Netherlands) for the unidirectional sequencing via ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA, USA), and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained reads were aligned and trimmed using the SILVA (Quast et al., 2013). All 16S rRNA gene sequences (n=47) were deposited into the NCBI GenBank followed by the nucleotide BLAST analysis (NCBI Accession No: MK791580-MK79194 and MN629248-MN629279) (Table 1).

**Methicillin susceptibility testing**

In order to detect MRSA identified by 16S rRNA sequencing, the agar screening method was performed according to the Brown et al., 2008. Briefly, the density of the *S. aureus* isolates was arranged to the 0.5 McFarland standard. After that, a spot inoculation (10 µl) of *S. aureus* into the Mueller Hinton Agar medium (HiMedia) including 4% NaCl (Conda) and 6 mg/L methicillin (Sigma) was performed. Plates were incubated at 37°C for 24-hour. The growth of any single colonies on methicillin plate is evaluated as resistant (Brown and Yates, 1986; Brown et al., 2008).

**Results and Discussion**

The preparation and consumption of the cheese products with unhygienic conditions could lead to the proliferation of the *S. aureus* in cheese and it can be posing a high risk for public health. Detection, enumeration, and identification of the *S. aureus* especially coagulase positive and methicillin-resistant strains are significant. While coagulase-positive *S. aureus* strains can produce an enterotoxin, coagulase-negative isolates could able to produce enterotoxin (Nunes et al., 2015; Yildirim et al., 2019). Therefore, coagulase - negative *S. aureus* strains should be taken into consideration. In Turkey, there have been several studies that indicate the prevalence and presence of *S. aureus* strains in various cheese samples. The detection percentage were ranging from 20.2% to 92% (Yücel and Anıl, 2001; Gökmen et al., 2013; Bingöl and Toğay, 2017). The high percentage of the detection could indicate health risk in the cheese samples which has been consumed widely in Turkey. In our study, out of 19 white cheese samples, three (n=3) (15%) *S. aureus* were identified and two of them were reported as methicillin-resistant (Table 1). Similarly, the detection percentage of MRSA is not high in Turkey. For example, Saka and Gulel (2018) reported MRSA was 9 %. In another study, the detection percentage was 1.70 %, even though MRSA was investigated from 175 milk and dairy products (Ektik et al., 2017). Nevertheless, these data could show that a serious health problem.

All cheese samples were evaluated quantitatively in this study. The enumeration results were 1.6x10⁴ CFU/g (CE_1), 9.77x10¹ CFU/g (CE_2), 3.1x10² CFU/g (CE_3), 1.51x10⁶ CFU/g (CE_4), 6.35x10⁷ CFU/g (CE_5), 2.53x10⁷ CFU/g (CE_6), 1.63x10⁵ CFU/g (CE_7), 6.78x10⁴ CFU/g (CE_8), 8.05x10⁵ CFU/g (CE_9), 1.68x10⁴ CFU/g (CE_10), 1.27x10³ CFU/g (CE_11), 3.40x10⁴ CFU/g (CE_12), 2.51x10⁷ CFU/g (CE_13), 1.40x10⁷ CFU/g (CE_14), 2.34x10⁷ CFU/g (CE_15), 2.18x10⁸ CFU/g (CE_16), 1.67x10⁶ CFU/g (CE_17), 1.70x10⁶ CFU/g (CE_18), 1.30x10⁷ CFU/g (CE_20). The microbiological criteria in terms of the presence of coagulase-positive *Staphylococcus* species in cheese products established by the Food and Drug Administration (FDA) is 10²-10³ CFU/g was acceptable (https://www.fda.gov/media/74723/download). At the same time, Turkish Food Codex Microbiological Criteria takes into consideration the same reliability limits (10²-10³ CFU/g) in cheese products (Turkish Official Journal, 2011) However, the presence of *Staphylococcus* species more than 10⁴ CFU/gr in cheese product considered to be risky according to the compliance Policy Guide of FDA (Kadiroğlu et al., 2014; https://www.fda.gov/media/74723/download). In this study, 15 out of the 19 cheese samples included more than 10⁴ CFU/g presumed *Staphylococcus* species could be considered as hazardous for public health. The number of *Staphylococcus* (CFU) or concentration of enterotoxin can be shown a determining factor of risk situation. In other words, the enterotoxigenic strains of *Staphylococcus* is necessary to grow before the toxin production at detectable levels. Thereby, to cause an infection, a high dose of *Staphylococcus* is required (Food Safety Authority of Ireland 2011; Pollitt et al., 2018).
Table 1. Phenotypic characteristics and 16S rRNA genotypic identification of *S. aureus*, *S. carnosus*, *E. faecalis*, and *M. caseolyticus*, *E. faecium*, *E. durans*, and *E. gallinarum* isolates obtained from cheese samples

| No  | ID    | 16S rRNA       | GenBank Accession No | Gram_Reaction morphology | O | C | M | D | Methicillin (R/S) |
|-----|-------|----------------|----------------------|--------------------------|---|---|---|---|------------------|
| CE_2| CE_2  | *Staphylococcus carnosus* CE_2 | MK791585 | (+)-coccus (-) (+) (+) (+) |
| CE_3| CE_3_2| *Enterococcus faecalis* CE_3_2 | MK791587 | (+)-coccus (-) (+) (+) (-) |
| CE_1| CE_1_1| *Enterococcus faecalis* CE_1_1 | MK791580 | (+)-coccus (-) (+) (+) (+) |
| CE_1| CE_1_2| *Enterococcus faecalis* CE_1_2 | MK791581 | (+)-coccus (-) (+) (+) (+) |
| CE_1| CE_1_3| *Enterococcus faecalis* CE_1_3 | MK791582 | (+)-coccus (-) (+) (+) (+) |
| CE_1| CE_1_4| *Enterococcus faecalis* CE_1_4 | MK791583 | (+)-coccus (-) (+) (+) (+) |
| CE_1| CE_1_5| *Enterococcus faecalis* CE_1_5 | MK791584 | (+)-coccus (-) (+) (+) (+) |
| CE_4| CE_4_2| *Enterococcus faecalis* CE_4_2 | MK791592 | (+)-coccus (-) (+) (+) (+) |
| CE_4| CE_4_3| *Enterococcus faecalis* CE_4_3 | MK791593 | (+)-coccus (-) (+) (+) (+) |
| CE_4| CE_4_4| *Enterococcus faecalis* CE_4_4 | MK791594 | (+)-coccus (-) (+) (+) (+) |
| CE_3| CE_3_3| *Enterococcus faecalis* CE_3_3 | MK791588 | (+)-coccus (-) (+) (+) (+) |
| CE_3| CE_3_4| *Staphylococcus aureus* CE_3_4 | MK791589 | (+)-coccus (-) (+) (+) (+) |
| CE_3| CE_3_5| *Enterococcus faecalis* CE_3_5 | MK791590 | (+)-coccus (-) (+) (+) (+) |
| CE_4| CE_4_1| *Enterococcus faecalis* CE_4_1 | MK791591 | (+)-coccus (-) (+) (+) (+) |
| CE_3| CE_3_1| *Macrococcus caseolyticus* CE_3_1 | MK791586 | (+)-coccus (-) (+) (+) (+) |
| CE_5| CE_5_1| *Enterococcus faecium* CE_5_1 | MN629248 | (+)-coccus (-) (+) (+) (+) |
| CE_5| CE_5_3| *Enterococcus durans* CE_5_3 | MN629249 | (+)-coccus (-) (+) (+) (+) |
| CE_5| CE_5_4| *Enterococcus faecium* CE_5_4 | MN629250 | (+)-coccus (-) (+) (+) (+) |
| CE_6| CE_6_1| *Enterococcus faecium* CE_6_1 | MN629251 | (+)-coccus (-) (+) (+) (+) |
| CE_6| CE_6_2| *Enterococcus faecium* CE_6_2 | MN629252 | (+)-coccus (-) (+) (+) (+) |
| CE_6| CE_6_3| *Enterococcus faecium* CE_6_3 | MN629253 | (+)-coccus (-) (+) (+) (+) |
| CE_7| CE_7_2| *Enterococcus faecium* CE_7_2 | MN629254 | (+)-coccus (-) (+) (+) (+) |
| CE_8| CE_8_1| *Enterococcus faecium* CE_8_1 | MN629255 | (+)-coccus (-) (+) (+) (+) |
| CE   | CE_8 2 | Enterococcus durans | CE_8 2 | MN629256 | (+)-coccus | (+) | (+) | (+) | (+) |
|------|--------|---------------------|--------|----------|------------|-----|-----|-----|-----|
| CE   | CE_8 3 | Enterococcus faecium | CE_8 3 | MN629257 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_9 2 | Enterococcus faecium | CE_9 2 | MN629258 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_10 1 | Enterococcus faecium | CE_10 1 | MN629259 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_10 2 | Enterococcus faecium | CE_10 2 | MN629260 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_11 1 | Enterococcus faecium | CE_11 1 | MN629261 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_11 2 | Enterococcus durans | CE_11 2 | MN629262 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_11 3 | Enterococcus durans | CE_11 3 | MN629263 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_12 2 | Enterococcus faecalis | CE_12 2 | MN629264 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_12 3 | Enterococcus faecalis | CE_12 3 | MN629265 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_12 4 | Staphylococcus aureus | CE_12 4 | MN629266 | (+)-coccus | (+) | (+) | (+) | (+) | R |
| CE   | CE_13 1 | Staphylococcus aureus | CE_13 1 | MN629267 | (+)-coccus | (+) | (+) | (+) | (+) | R |
| CE   | CE_14 2 | Enterococcus faecalis | CE_14 2 | MN629268 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_14 3 | Enterococcus faecalis | CE_14 3 | MN629269 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_15 1 | Enterococcus faecalis | CE_15 1 | MN629270 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_15 3 | Enterococcus faecalis | CE_15 3 | MN629271 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_16 2 | Enterococcus faecalis | CE_16 2 | MN629272 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_17 1 | Enterococcus gallinarum | CE_17 1 | MN629273 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_18 1 | Enterococcus faecalis | CE_18 1 | MN629274 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_18 2 | Enterococcus faecalis | CE_18 2 | MN629275 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_18 3 | Enterococcus faecalis | CE_18 3 | MN629276 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_20 1 | Enterococcus faecalis | CE_20 1 | MN629277 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_20 3 | Enterococcus faecalis | CE_20 3 | MN629278 | (+)-coccus | (+) | (+) | (+) | (+) |
| CE   | CE_20 4 | Enterococcus faecalis | CE_20 4 | MN629279 | (+)-coccus | (+) | (+) | (+) | (+) |

O: Oxidase, C: Catalase, M: Mannitol fermentation, D: DNAse, Methicillin: Methicillin Susceptibility, S: Susceptible, R: Resistant (-): Negative reaction, (+): Positive reaction, (+/-): Late positive
Presumptive *S. aureus* isolates (isolate IDs: CE_12_4 and CE_13_1) were compatible with the phenotypic identification tests including oxidase, catalase, mannitol fermentation, and Dnase. However, presumptive *S. aureus* isolate (ID: CE_3_4) was mannitol fermentation and DNAse tests were negative (Table 1). Although phenotypic tests for the isolate CE_3_4 were not coherent, some of strains of the *S. aureus* could show a negative reaction for the DNAse and mannitol fermentation tests (Kateete et al., 2010). According to the 16S rRNA identification results, presumptive isolates (IDs: CE_13_1, CE_12_4 and CE_3_4) were identified as *S. aureus*. In accordance with phenotypic identification tests for the isolates including CE_2, CE_1_1, CE_4_2, CE_4_4, CE_3_3, and CE_3_5 were considered as *S. aureus*. However, the 16S rRNA identification test showed that these isolates were identified as CE_2 (*S. carnosus*), CE_1_1 (*E. faecalis*), CE_4_2 (*E. faecalis*), CE_4_4 (*E. faecalis*, CE_3_3 (*E. faecalis*), and CE_3_5 (*E. faecalis*). Therefore, our results showed that some of the phenotypic identification tests did not correspond to the genotypic identification test. Considering the phenotypic results in Table 1, it was seen that only 47 of the phenotypic test results did not indicate *S. aureus*. On occasion, phenotypic tests can be variable under some conditions. For instance, *E. faecalis* is catalase-positive under the acquisition of heme however, *E. faecalis* strains are catalase negative (Frankenberg et al., 2002). The 16S rRNA analysis showed that the other *Staphylococcaceae* members including *Staphylococcus carnosus* (n=1), and *Macrococcus caseolyticus* (n=1) were reported in this study. Moreover, *Enterococcus faecalis* (n=25), *Enterococcus faecium* (n=12), *Enterococcus durans* (n=4), and *Enterococcus gallinarum* (n=1) belonging to the *Enterococcaceae* family was reported in this study (Table 1). Although *E. gallinarum* was reported from clinical samples in Turkey (Özseven et al., 2011), *E. gallinarum* can be isolated during cheese making and ripening procedure. In Italy, *E. gallinarum* was reported a low abundance in artisanal Italian goat’s cheese during ripening procedure (Suzzi et al., 2011).

*S. carnosus* is generally isolated from meat products or fish and it’s known as meat starter culture (Bückle et al., 2017). Similarly, in Turkey, *S. carnosus* was reported from Turkish fermented sausage (Nazli, 1998). Another study that was carried on in France, *S. carnosus* was detected only in dry sausage samples (Coton et al., 2010). The detection of *S. carnosus* in our study could show the contamination of cheese samples. *M. caseolyticus* was also identified in various dairy and meat food sources related to flavor development (Mazhar et al., 2018). Besides, *M. caseolyticus* can be isolated from bovine milk, chicken, and humans. In Switzerland, *M. caseolyticus* was isolated from bovine mastitis milk (Schwendener et al., 2017). However, to best our knowledge, *M. caseolyticus* has not been detected from white cheese samples in Turkey before. As distinct from *S. carnosus* and *M. caseolyticus*, *E. faecalis* is known as a flora member of the gastrointestinal tract in humans and animals (Abdeen et al., 2016). However, the presence of *E. faecalis* in food sources such as cheese could show fecal contamination and/or inadequate hygienic measures in cheese samples. Moreover, the transmission of *E. faecalis* to the human by consumption of dairy products could cause various infections (Anderson et al., 2016). Similarly, various antibiotic-resistant *Enterococci* such as *E. faecium* has been reported from nosocomial-acquired patients (Sanders et al., 2010). Along with the harmful effects of *Enterococci*, these species are also known to have probiotic potential. Because *Enterococci* has a tolerance to the salts and acids thereby, *Enterococci* could adapt to various foods and could involve the fermentation process of cheese. (Hanchi et al., 2018). And another striking feature of *Enterococci* including *E. faecalis, E. faecium*, and *E. durans* has lipolytic activity and production of aromatic compounds (Amaral et al., 2016). In Turkey, *E. faecium* has been used for cheese production as a starter culture. And they were concluded that *E. faecium* FAIR-E’198 could be used as a starter culture (Gönçioğlu et al., 2009).

**Conclusion**

In conclusion, *S. aureus, E. faecalis, E. faecium, E. durans, E. gallinarum, S. carnosus,* and *M. caseolyticus* were identified by phenotypic and genotypic identification methodologies. Phenotypic identification tests results should be validated by genotypic identification tests. The detection of MRSA in our study could show the significance of the methicillin resistance in cheese samples for public health. To prevent the transmission of *S. aureus* to cheese products, hygiene and sanitation precautions should be taken during production and sales of the cheese. Also, critical control points should be determined. According to our data, the presence of *S. aureus* and *Enterococci* in cheese products could give an opinion about transmission strategies of these bacteria needed to be studied.

**Compliance with Ethical Standard**

**Conflict of interests:** The authors declare that for this article they have no actual, potential or perceived the conflict of interests.

**Ethics committee approval:** Author declare that this study does not include any experiments with human or animal subjects.

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