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Research article

Biofilm formation by *Staphylococcus* spp. isolated from local food markets at Baghdad city

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ABSTRACT

Formation and presence of biofilm leads to rise concerning of human towards food industries, because Biofilm formation impact human health and his economic negatively Staphylococcus spp. is a pathogenic bacteria, can form biofilm when conditions are adequate, also its able to transition from human to the food and food-contact surfaces due to the nature and habit of these type of bacteria. *Staphylococcus* spp. was Isolated from different food samples in processing plant of food preparation, and identified based on 16SrRNA using T stag primer, also we used forward primer Sa442-1 and reveres primer Sa442-2 to confirm the identification of *Staphylococcus aureus,* from 36 isolates, 18 isolates was identified as *S. aureus*. The ability of bacteria to form biofilm was verified by the use of polystyrene microtiter plate assay with crystal violet staining. There were clear differences among *Staphylococcus* spp. isolates in biofilm formation, the ability was measured by determination of adhesion to polystyrene microtiter plates and compared with *Bacillus* spp. as a control positive and control negative. Isolates of *Staphylococcus* spp. showed different ability to form biofilm and adhere on polystyrene plates.

Keywords: Biofilm formation, Congo red agar, Microtiter plate, *Staphylococcus spp.*, 16SrRNA.

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INTRODUCTION

Biofilm is a phenomenon, refers to the aggregation of bacteria or that mean it's a collection of these bacteria which surrounded by exopolysaccharide matrix, were they secrete exopolysaccharide for attachment to either an inert or living surface after adhering to surfaces [1]. In recent years, the Biofilm is more popular research topic including food safety [2]. Biofilm is may include spoilage and pathogenic bacteria [3]. There are several conditions can help to form biofilm, were biofilm formation is influenced by the nature of substrate, electric charge of cell surface, presence of flagella and microbial growth phase [4]. The presence of bacteria on the production surfaces or food products can leads to serious problems, where they impacts human health also cause an economic losses due to food spoilage [5]. For example, for the population of *Staphylococcus* spp. the type *S. aureus* usually exists on the skin of human and his nasal mucus, it's often isolated in the foods directly that handled by human. *S. aureus* bacteria are distinguished with a salt-tolerance at range (10-15%), the outbreaks of food borne are usually associated with salty foods, such as dried fermented sausages and rather raw foods [6]. Bacteria that form biofilm are known to possess greater resistances to stress conditions than their planktonic counterparts that dispersed

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in the environment, including the susceptibility to sanitizers and other antimicrobials [6]. Bacteria in a biofilm community may use polymer production as a weapon to push themselves into the more nutrient and oxygen rich region at the air liquid interface [7,8]. *Staphylococcus* spp. is widespread micro-organisms that may play an important role as agent of food-borne diseases [9,10]. *S. aureus* and coagulase-negative *Staphylococcus* (CNS) are frequent bacteria on the human skin and mucous membranes, mainly as commensal flora [11].

Staphylococcus spp are the relevant biofilm-forming organisms. Biofilm well studied in dairy and meat plants process [12, 13], cross-contamination [14] and mastitis [15-18]. There are few studies regarding the biofilm production ability of *Staphylococcus* spp., which are isolated from ready- to-eat, snacks and sandwiches. Snacks are fast foods usually derived from one or more basic food items and they are often eaten between meals [19]. This study aimed to isolate and identifying *Staphylococcus* spp. in foods, and evaluate the ability of these isolates in producing biofilm.

MATERIALS and METHODS

Sample collection and preparation

Thirty six isolates of *Staphylococcus* spp. were isolated, and collected along study period from January 2014 to May 2014, from different types of food which include, cooked meat, fresh meat, cooked rice, different vegetable, salads and dairy products. Samples were collected in disposable plastic petri dishes from local Iraqi markets at different markets at Baghdad city, then after collection samples were transferred to the laboratories of Food Contamination Researches Center at Ministry of Science and Technology-Iraq.

Isolation and identification of isolates

To isolate *Staphylococcus* spp., 25 gm of food samples were taken and homogenized with 225 mL of buffered peptone water 0.1% in a stomacher for one min, after that, homogenate was diluted serially, then 0.1 mL of each dilution were plated onto Baird-Parker agar supplemented with egg yolk solution (12.5 mL egg yolk in 25 mL 0.85 % saline solution and 1% potassium tellurite). After incubating plates at 37 °C for 48 h, colonies were grown typically and gave a jet black to dark grey with an entire margin and without a halo, then the colonies of *Staphylococcus* spp. from the appropriate dilutions are counted. The purified *Staphylococcus* spp. colonies tentatively are identified with gram staining and catalase activity [20].

DNA Extraction and PCR Condition

Staphylococcus spp. isolates were confirmed by molecular detection of *Staphylococcus* specific genes encoding 16S rRNA, bacterial DNA was extracted using a wizard Genomic DNA Purification Kit (Promega, Madison, WI). PCR amplification was performed using green master mix (Promega). The amplified products were stained with ethidium bromide after electrophoresis in a 1.5 % agarose gel. PCR primers and conditions of PCR assay are shown in **Table 1**.

Reference strains

Reference *B. subtilis*, strain PY79 as negative control was obtained from Laboratory of molecular Bacteriology intercollegiate Faculty of Biotechnology Poland, and for positive control *B*. *amyloliquefaciens* was obtained from culture maintained in the microbial culture collection of department of structure and functional biology, University of Naples, Federico II, detection of slime formation by Congo red agar method (CRA).

CRA method was used to estimate the ability of producing slime, CRA medium [50 gm sucrose (Sigma, st. Louis, Mo), brain heart in infusion broth 37 gm (Oxoid Basing stoke Hampshire UK) 10 gm agar, 0.8 g Congo red (Sigma, St. Louis, Mo) and distilled water 1000 mL] was incubated at 37 °C for 24 h, after incubation, bright black colonies were presented as slime positive, while non-producing isolates developed as a red colonies [21].

Detection of quantitative biofilm formation by the microtiter plate method (MP)

Biofilm-forming ability was estimated by determination of adhesion to polystyrene microtiter plates [22]. Briefly, isolates were inoculated in Luria-Bertani (LB) and incubated for 18 h at 37 °C. 300 µL of LB broth were distributed in flat-bottom and inoculated with 10 µL of bacterial broth, after that, plates were incubated for 18 h at 37 °C, then they were washed with adjusted Phosphate Buffer Saline (PBS) to pH 7.0, after that plates were air-dried for 1 h at 60 °C and stained with a volume of 300 µL from 0.25% crystal violet for 1 min after washing, 300 µL of 33 % acetic acid was used to destain the samples, an optical density (OD) of each well content was measured at 570 nm by using spectrophotometer (Bio TEK). Cut-off OD (ODc) was defined for microtiter- plate test as three values of standard deviation above the mean of OD for negative control, an adherence ability of tested isolates was classified based on the OD. All tested were carried out three times and the results were averaged.

Table 1. PCR primers and conditions used in PCR assays

Gene	Gene Description	Sequence (5'-3')	Size of amplified product (bp)	PCR conditions cycling
16SrRNA	Forward Reverse	GGCCTGTTGAACGTGGTCAAATCA ACCATTTCAGTACCTTCTGGTAA	370	94˚C 5 min; 37 × (94˚C 40 s, 55˚C 60 s, 72˚C 75 s); 2˚C 10 min.
Sa442-1 Sa442-2	Forward Reverse	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTTAGATAATACAACA	108	94˚C 5 min; 35 × (94˚C 1 min, 55˚C, 2 min; 72˚C 33 S

RESULTS and DISCUSSION

Bacterial identification

Isolates of *Staphylococcus* spp. were isolated from different types of food (**Table 2**), all of (36) isolates were identified as *Staphylococcus* spp. due to morphological characteristics were the colonial characters was white to golden colonies with clear zone around the colonies on Baird-parker agar with egg yolk tellurite (**Fig 1**).

Genetically, there were 18 isolates of *Staphylococcus* spp were confirmed as *S. aureus* which were isolated from minced meat, chicken meat, fried food, cocked rice, dairy products and pastry (**Table 3**).

Table 2. Source of *Staphylococcus* spp. isolated from different types of food.

Isolates number	Samples	Isolates type
1 -3	Minced Meat	*Staphylococcus* spp.
4 -8	Chicken meat	*Staphylococcus* spp.
9-13	Fried food	*Staphylococcus* spp.
14-19	Cocked Rice	*Staphylococcus* spp.
20-25	Salads	*Staphylococcus* spp.
26-30	Dairy products	*Staphylococcus* spp.
31-36	Pastry	*Staphylococcus* spp.

Table 3. Source of *Staphylococcus aureus* isolated from different types of food, which confirmed genetically.

Isolates number	Samples	Isolates type
1 -3	Minced Meat	*Staphylococcus aureus*
4 -8	Chicken meat	*Staphylococcus aureus*
9-13	Fried food	*Staphylococcus aureus*
3	Cocked Rice	*Staphylococcus aureus*
1	Dairy products	*Staphylococcus aureus*
1	Pastry	*Staphylococcus aureus*

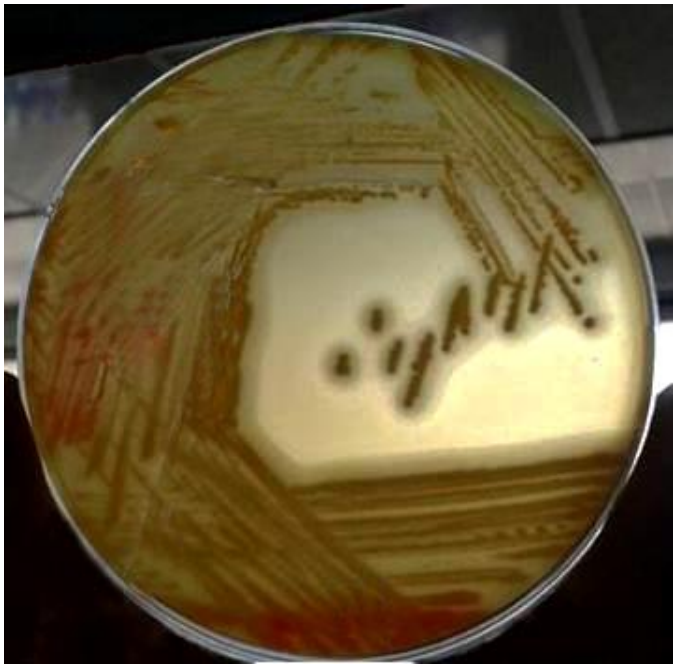

Fig 1. *Staphylococcus* spp. colonies on Baird- parker medium, 137 typical hallow clear zone around the colonies.

PCR Reaction

PCR- based detection of 16SrRNA gene was fully reproducible, results of the PCR products in lanes (1-18), which obtained with primer pairs for T stag (Staphylococci-specific 16SrRNA) (Fig. 2).

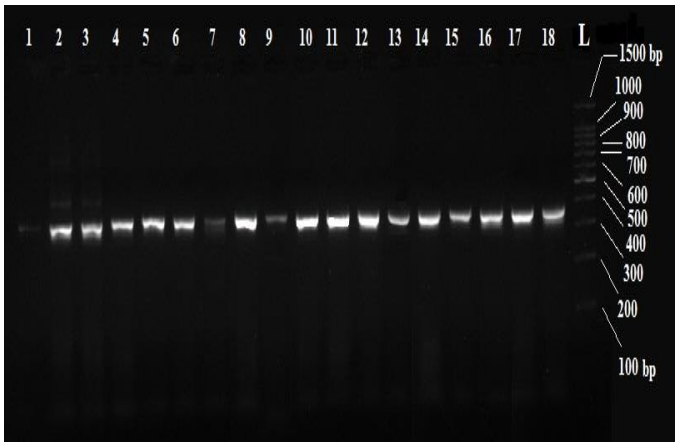

Fig 2. PCR based detection of 16SrRNA gene for *Staphylococcus* spp.

PCR reaction of forward primer Sa442-1 and reverse primer Sa442-2 to confirm the species of *S. aureus*, results of the PCR products in lanes (1-16) (**Fig. 3**).

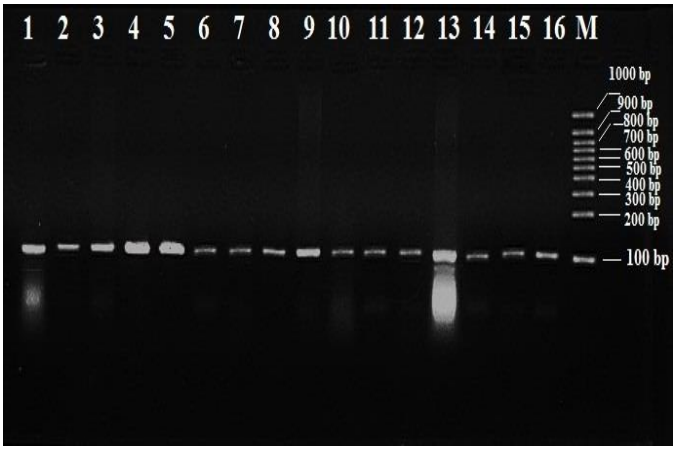

Fig 3. PCR reaction of forward primer Sa442-1 and reverse primer Sa442-2 gene for *S. aureus.*

Detection of biofilm-forming ability on Congo red agar (CRA)

Biofilm-forming ability of each isolate was detected by CRA plate test following the colorimetric scale proposed by previous study [23]. Colonies colored from very black to almost black have been described as typical of a biofilm-positive phenotype, whereas negative slime producers form colored colonies from dark red to burgundy. Some of the isolates formed black colonies after 24 h, with color changing from red to black in surrounding agar. Thus, all strains showed slime production ability after 24 h. All isolates which maintained as a positive biofilm phenotype during 48 h, most of these isolates were formed as a red colony onto CRA (**Fig 4**).

Fig 4. *Staphylococcus spp*. on Congo red agar with three different distinct color ranging from red, dark red to black

Quantification of biofilm

Biofilm formation was increased continuously during incubation period and quantified in terms of biomass by using the crystal violet staining method. Results of isolate of samples 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 were lower than positive control and higher than negative control strain. In fact the first 11 isolates formed biofilm more than other isolates were isolated from chicken meat, minced meat and fried food, whereas 25 out of 36 were less biofilm-forming ability, the less biofilm forming bacteria, were isolated from pastry, cocked rice, salads and diary product (**Fig 5**), due to the obtained results, after 18 h there were a significant among isolates to form biofilm for each isolate.

Perhaps this is due to the effect of food types were isolates of *Staphylococcus* spp. isolated, such as protein nature, complex polysaccharides nature and vegetables.

In present study, 16SrRNA gene amplification was used to characterize *Staphylococcus* spp. isolates. Primer set could be utilized for direct and rapid identification of a wide range of Staphylococci isolates at genus level. Until now, most of the PCR identification of the Staphylococcal species were isolated from food production was performed with one or two primer pairs, also in confirming species of *S. aureus* when the isolates were tested genetically. An obtained results of biofilm formation have shown that all isolates of *Staphylococcus* spp. were isolated from different food samples which have been chosen according to Iraqi people food-processing facilities, and 18 isolates was identified as *S. aureus*. In general, there were different ability of biofilm formation on polystyrene microplates was observed. The isolates of food-processing facilities does not only involve an immediate risk to food safety but more important a risk of long-term presence (even persistence) unless appropriate measurements are applied to sterile them.

Biofilm forming ability was assessed in terms of biomass using the crystal violet staining method. Thus, it could be observed that some isolates had a high biofilm-forming ability on polystyrene microplate under experimental conditions stimulating situations normally found in food. Several studies have also detected the presence of *S. aureus* biofilm on food-processing surfaces [24, 25, 26]. Biofilm biomass increased proportionally as biofilm aged. Higher variability in biofilm biomass was found among strains throughout the time course of biofilm formation, which is in accordance with previous studies [21, 3, 27]. The risk of *S. aureus* presence would be highly dependent on both the strain and the age of biofilm, as previously observed by the authors [28].

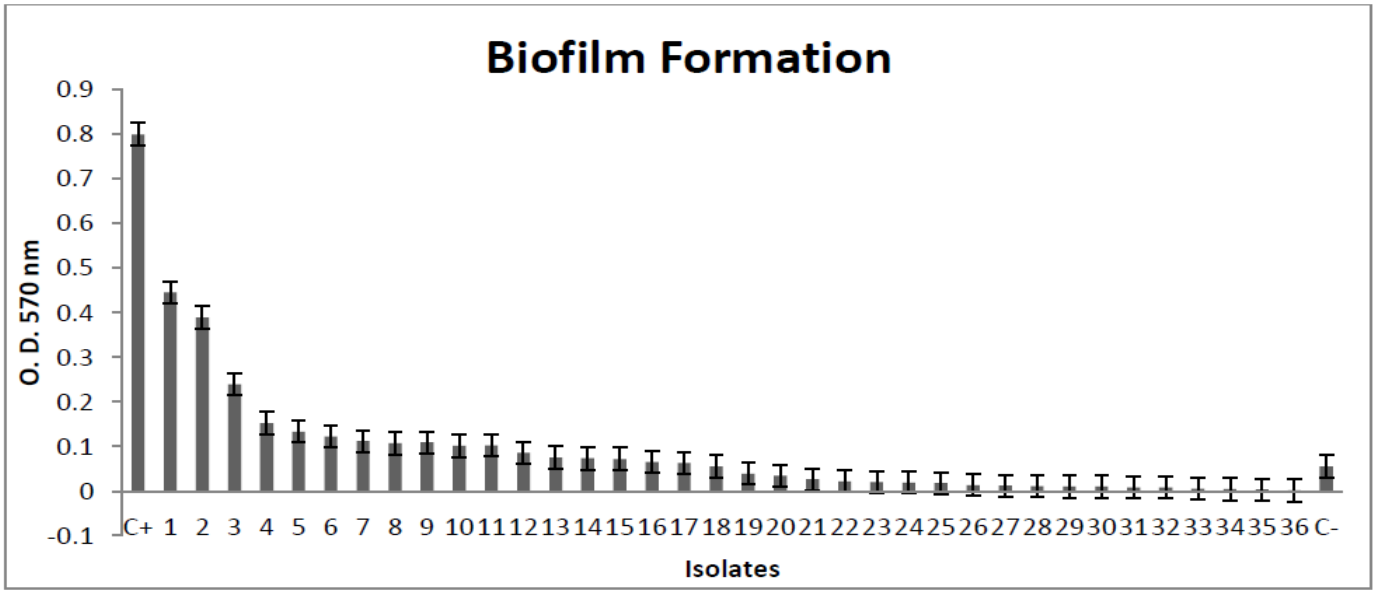

Fig 5. Isolates of *Staphylococcus* spp. with highest biofilm-forming and isolates with lowest biofilm-forming ability that isolated from different types of food. C+, control positive; C-, control negative.

Biofilm formation also was inspected by CRA plate test, which is easier to perform and less time-consuming than staining methods. This method was used successfully for detecting biofilm-forming strains of *S. epidermidis* [29, 30]. In this study, only black-colored colonies after 24 h of incubation were considered to be typical of a biofilm-positive phenotype [22] which recommended that all isolates formed black-colored colonies after 72h and biofilm-positive phenotype was maintained subsequently. These results are in accordance with those of crystal violet staining, and it contrasts with claims that CRA test results do not always correspond to biofilm production determined by staining methods [31].

The phenotypic expression of the biofilm affected conditions cultivation of the microorganism, such as the atmosphere of incubation [32]. In this study, the aerobic incubation of isolates was showed a significant differences in growth and biofilm expression, also the adoption of the chromatic scale for the reading the plates and extension of incubation at 96 h were allowed, at first case, to reduce the possible variability associated with the interpretation of operators, while in the second case allowed for us to estimate blackberries correct color of colonies above the surface of Congo red agar. In fact, the reading carried out to 24 h allowed to estimate in a comprehensive manner color of colonies only in positive isolates and in the case of negative isolates, however, the reading to 24 h allowed a correct explanation only in all of the isolates. All tested isolates in this study showed a significant ability to adhere and form biofilm on polystyrene micro-plate surfaces. In fact, some isolates showed a biofilm-forming ability moderately higher respect to a common reference strain tested, thus food-contact surfaces can be an important reservoir for *S. aureus* in the food. Also Biofilm formation detection with use of CRA plate test, is easier to perform positive results in less time-consuming than staining methods.

Conflict of interest

The authors declare that they have no conflict of interests.

REFERENCE

1. **Abd El-Rahman HA, Ahmed AM, Al Harouny MS.** (2010) Formation of Bio films on Food Contact Surfaces. *SCVMJ, XV* **1**: 87-98.
2. **Trachoo N.** (2003) Biofilms and the food industry. *Songklanakarin J Sci Technol* **25**: 807-815.
3. **Rode TM, Langsrud S, Holck A, and Møretrø T.** (2007) Different patterns of Biofilm Formation in *Staphylococcus aureus* under food-related stress conditions. *Int J Food Microbiol* **116**: 372-383.
4. **Zgair AK.** (2013) Role of *Stenotrophomonas* flagella in bacterial adhesion on human epithelial cells. *World J Exp Biosci* **1**: 19-21.
5. **Zgair AK, Chhibber S.** (2012) *Stenotrophomonas maltophilia* flagellin restricts bacterial colonization in BALB/c mouse lung in vivo. *FEMS Immunol Med Microbiol* **66**:191– 200.
6. **D'Souza EL, Meria QGS, Barbosa IM, Athayde AJAA, et al.,** (2014) Biofilm formation by *Staphylococcus aureus* from food contact surfaces in a meat-based broth and sensitivity to sanitizers. *Braz J Microbiol* **45**(1):67-75.
7. **O'Toole GA, Kolter R.** (1998) Initiation of biofilm formation in Pseudomonas fluo-rescens WCS365 proceeds via multiple, convergent signalling pathways: agenetic analysis. *Mol Microbiol* **28**: 449-61.
8. **Miwa N, Kawamura A, Masuda T, Akiyama M.** (2001) An outbreak of food poisoning due to egg yolk reaction-negative Staphylococcus aureus. *Int J Food Microbiol* **64**: 361-6.
9. **Lee SHI, Camargo CH, Miranda ES, Fernandes Junior A, Rall VLM.** (2013) In vitro biofilm production by Staphylococcus spp. strains isolated from finger-foods and snacks. *Rev Inst Adolfo Lutz* **72**: 138-4.
10. **Beckers HJ, Vanleusden FM, Bindschedler O, Guerraz D.** (1984) Evaluation of a pour plate system with arabbit plasma bovine fibrinogen agar for the enumeration of Staphylococcus aureus in food. *Can J Microbiol* **30**: 470–474.
11. **Noble WC. (1990)** Systematics and the natural history of Staphylococci. *J Appl Microbiol* **69**:39-48.
12. **Somers EB. Wong AC.** (2004) Efficacy of two cleaning and sanitizing combinations on Listeria monocytogenes biofilms formed at low temperature on a variety of materials in the presence of ready-to-eat-meat residue. *J Food Prot* **67**: 2218-29.
13. **Fey PD. Olson ME.** (2010) Current concepts in biofilm formation of Staphylococcus epidermidis. *Future Microbiol* **5**: 917-33.
14. **Harraghy N, Seile S, Jacobs K, Hannig M, Menger MD, Herrmann M.** (2006) Advances in vitro and in vivo models for studying the staphylococcal factors involved in implant infections. *Int J Artif Organs* **29**: 368-78.
15. **Melchior MB, Fink-Gremmels J, Gaastra W**. (2006a) Comparative assessment of the antimicrobial susceptibility of *Staphylococcus aureus* isolates from bovine mastitis in biofilm versus planktonic culture. *J Vet Med B Infect DisVet Public Health* **53**: 326-32.
16. **Melchior MB, Vaarkamp H, Fink-Gremmels J.** (2006b) Biofilms: a role in recurrent mastitis infection?. *Vet J* **171**: 398-407.
17. **Clutterbuck AL, Woods EJ, Knottenbelt DC, Clegg PD, Percival SL.** (2007) Biofilms and their relevance to veterinary medicine. *Vet Microbiol* **121**:1-17.
18. **Alippi AM, Lopez AC, Aguilar OM.** (2002) Differentiation of Paenibacillus larvae subsp. larvae, the cause of American Foulbrood of honeybees, by using PCR and restriction fragment analysis of genes encoding 16SrRNA. *Appl Environ Microbiol* **68**: 3655–3660.
19. **Ezekiel CN, Kayode FO, Fapohunda SO, Olorunfemi MF, Kponi BT.** (2012) Aflatoxigenic moulds and aflatoxins in street-vended snacks in Lagos, Nigeria. *J Food Saf* **14**: 83-88.
20. **Borelli BM, Lacerda ICA, Brandão LR, Vianna CR, et al.,** (2001) Identification of *Staphylococcus* spp. isolated during the ripening process of a traditional Minas cheese. *Arq Bras Med Vet Zootec* **36**: 481-487.
21. **Melchior MB, Fink–Gremmels J, Gaastra W.** (2007) Extended antimicrobial susceptibility assay for *Staphylococcus aureus* isolates from bovine mastitis growing in biofilm. *Vet Microbiol* **125**: 141 – 149.
22. **Arciola CR, Campoccia D, Gamberini S, Cervellati M, Donati E, Montanaro L.** (2002) Detection of slime production by means of an optimised Congo red agar plate test based on a colourimetric scale in *Staphylococcus epidermidis* clinical isolates genotyped for ica locus. *Biomaterials* **23**: 4233–4239.
23. **Bagge-Ravn D, Ng Y, Hjelm M, Christiansen J, Johansen C, Gram L.** (2003) The microbial ecology of processing equipment in different fish industries—analysis of the microflora during processing and following cleaning and disinfection. *Int J Food Microbiol* **87**:239–250.
24. **Sattar SA, Springthorpe S, Mani S, Gallant M, Nair RC, et al.** (2001) Transfer of bacteria from fabrics to hands and other fabrics: development and application of a quantitative method using *Staphylococcus aureus* as a model. *J Appl Microbiol* **90**:962–970.
25. **Marino M, Frigo F, Bartolomeoli I, Maifreni M.** (2011) Safety-related properties of staphylococci isolated from food and food environments. *J Appl Microbial* **110**: 550–561.
26. **Sospedra I, Mañes J, Soriano JM.** (2012) Report of toxic shock syndrome toxin 1 (TSS1) from *Staphylococcus aureus* isolated in food handlers and surfaces from foodservice establishments. *Ecotoxicol Environ Saf* **80**: 288–290.
27. **Melchior MB, Fink-Gremmels J, Gaastra W.** (2007) Extended antimicrobial susceptibility assay for Staphylococcus aureus isolates from bovine mastitis growing in biofilms. *Vet Microbiol* **125**: 141–149.
28. **Herrera FC, Santos JA, Otero A, García-López M-L.** (2006) Occurrence of foodborne pathogenic bacteria in retail prepackaged portions of marine fish in Spain. *J Appl Microbiol* **100**: 527–536.
29. **Handke LD, Conlon KM, Slater SR, Elbaruni S. et al.** (2004) Genetic and phenotypic analysis of biofilm phenotypic variation in multiple *Staphylococcus epidermidis* isolates. *J Med Microbiol* **53**: 367–374.
30. **Oliveira M, Bexiga R, Nunes SF, Carneiro C, Cavaco LM, et al.** (2006) Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet Microbiol* **118**: 133–140.

31. **Milanov D, Lazic S, Vidic B, Petrovic J, Bugarski D, Seguljev Z.** (2010) Slime production and biofilm forming ability by Staphylococcus aureus bovine mastitis isolates. *Acta Vet Brno* **60**: 217–226.
32. **Jain A, Agarwal A.** (2009) Biofilm production, a marker of pathogenic potential of colonizing and commensal staphylococci. *J Microbiol Methods* **76**: 88–92.
33. **Baselga R, Albizu I, De La Cruz M, Del Cacho E, Barberan M, Amorena B.** (1993) Phase variation of slime production in *Staphylococcus aureus*: implication in colonization and virulence. *Infect Immun* **61**:4857-4862.

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