

Biofilm production by coagulase-negative *Staphylococcus*: a review

Produção de biofilme por *Staphylococcus* coagulase-negativo: uma revisão

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ABSTRACT: This review aimed to describe the biofilm formation ability of coagulase-negative *Staphylococcus*, addressing its impact to the food industry. Coagulase-negative *Staphylococcus* have the ability to produce enterotoxins in food, making it an important line of study, as it constitutes a risk to public health. The biofilm formation by these microorganisms requires physicochemical processes, such as hydrophobic forces, which are essential for the first phase of fixing the biofilm on the surface. In industrial facilities, stainless steel equipment is the most associated with the formation of biofilms, due to the presence grooves and cracks. Many species of coagulase-negative *Staphylococcus* produce biofilm, but the most studied is *S. epidermidis*, as it is the most frequently isolated from food. Coagulase-negative *Staphylococcus* form biofilm on different surfaces in the food industry, and can become a source of permanent contamination, that can be present in the final product, intended for human consumption. Among other alternatives to combat the formation of biofilm in industrial food facilities, there is the implementation of Good Manufacturing Practices, which is effective in preventing bacterial adhesion, and therefore, the formation of biofilm. However, further studies are needed in order to quantify the occurrence of coagulase-negative *Staphylococcus* biofilms in the food industry.

KEYWORDS: industrial facilities; contamination; public health.

RESUMO: Esta revisão bibliográfica teve como objetivo descrever a capacidade de formação de biofilme por *Staphylococcus* coagulase-negativo, relacionando seu impacto na indústria alimentícia. Os *Staphylococcus* coagulase-negativos possuem a capacidade de produzir enterotoxina nos alimentos, tornando-se uma importante linha de estudo, pois constitui um risco para a saúde pública. A formação do biofilme por esses micro-organismos requer processos físico-químicos, como forças hidrofóbicas, essenciais para a primeira fase de fixação do biofilme na superfície. Nas indústrias, equipamentos de aço inoxidável são os mais associados à formação de biofilmes, em decorrência de possuírem ranhuras e fendas. Muitas espécies de *Staphylococcus* coagulase-negativo produzem biofilme, porém, o mais estudado é o *S. epidermidis*, por ser o mais frequentemente isolado de alimentos. Os *Staphylococcus* coagulase-negativos formam biofilme em diferentes superfícies de indústrias alimentícias, podendo se tornar uma fonte de contaminação permanente, contaminando o produto final destinado ao consumo humano. Dentre outras alternativas para combater a formação do biofilme nas plantas alimentícias, a implantação das Boas Práticas de Fabricação é eficaz para prevenir a adesão bacteriana, evitando a formação do biofilme. No entanto, são necessários estudos para quantificar a ocorrência de biofilmes de *Staphylococcus* coagulase-negativos em indústrias alimentícias.

PALAVRAS-CHAVE: indústrias; contaminação; saúde pública.

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INTRODUCTION

Bacteria of the *Staphylococcus* genus belong to the *Staphylococcaceae* family, are cocci, Gram positive, facultative anaerobic, immobile, non-spore-forming, and can produce the catalase enzyme (PRADO et al., 2015). Based on the ability to produce this enzyme, they are divided into two groups. The enzyme producers are called coagulase-positive *Staphylococcus* (CPS), while those that do not produce it are called coagulase-negative *Staphylococcus* (CNS) (OLIVEIRA, 2014).

Food poisoning by *Staphylococcus* spp. is considered the second most commonly reported foodborne illness (BRASIL, 2016), being a common cause of intoxication due to the ingestion of food contaminated with staphylococcal enterotoxins (SES) produced during bacterial multiplication (FERREIRA et al., 2018). According to WONG; BERGDOLL (2002), the amount of enterotoxin needed to cause poisoning is less than 1 µg. Staphylococcal enterotoxins are heat-resistant proteins that exhibit superantigen action and are able to remain in the food after heat treatment (BASSO, 2013). Although coagulase-negative *Staphylococcus* species produce lower amounts of SES, they have been reported as agents involved in outbreaks of food poisoning (MOTA et al., 2012).

One of the main virulence factors of *Staphylococcus* is the ability to form biofilm (LEITE, 2013). The microbial biofilm is a community of cells embedded within a matrix of exopolysaccharides, fixed on a biological or non-biological surface (ANTUNES et al., 2011; DICICCO et al., 2012). The constituted biofilms represent a source of permanent contamination in the industry, since they can become strongly adhered to the surfaces of equipment and facilities, and can contaminate food by releasing parts of them (JOSEPH et al., 2001).

Microorganisms in biofilm become more resistant than in a planktonic state, and can endure thermal stress, mechanical forces, and sanitation processes (CHAVANT et al., 2007). Biofilm also offers protection against pH changes, osmotic shocks, desiccation, and UV radiation, representing an original source of food contamination in industrial facilities, which can cause economic losses and the spread of food poisoning (CHAVANT et al., 2007). This review aims to make a bibliographic survey on the biofilm formation ability of CNS.

The *Staphylococcus* genus is divided into 40 species, which are distributed in coagulase-positive or coagulase-negative *Staphylococcus*, depending on whether or not the coagulase enzyme is synthesized. Most of these are in the CNS group, standing out as representatives *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus* (BANNERMAN, 2007; KWOK; CHOW, 2003). In the past, it was believed that only CPS produced enterotoxins, however KÜREKCI (2016) demonstrated that CNS also had this ability. Another study carried out under laboratory conditions showed that staphylococcal enterotoxins can be produced by *S. xylosus*, *S. haemolyticus*, *S. epidermidis*,

S. cohnii, *S. warneri*, and *S. sciuri*, all belonging to the CNS group (PEREIRA et al., 2001).

Most species of CNS belong to the commensal microbiota of the skin of humans and animals (RAMOS et al., 2015), which is why contamination of food occurs. Coagulase-negative *Staphylococcus* are related to mastitis in dairy animals, impairing the quality of milk destined to the industry (MARTIN et al., 2015). RAPINI et al. (2005) detected strains of CNS that produce enterotoxins and the toxin that causes Toxic Shock Syndrome in goat cheese handlers.

However, not all species of CNS are pathogenic. Some have been used in the food industry to assist in the ripening of cheeses and meats (PRAX et al., 2013). They have also been explored as initiators for the fermentation of embedded products (PLACE et al., 2003; LEROY et al., 2010).

The biofilm consists of a monospecies or multispecies cell aggregates embedded in a polymeric matrix, containing extracellular polysaccharides, proteins, glycoproteins, and glycolipids (LAWRENCE et al., 1991; FLEMMING et al., 2007; RICKARD et al., 2003) that form a porous and hydrated structure. This structure adheres strongly to a biotic sediment (DICICCO et al., 2012), such as plant and animal tissues, or abiotic, such as various metals and polymers (BOARI et al., 2009), through organic filaments with a protein or polysaccharide structure, called glycocalyx (KASNOWSKI et al., 2010). This base is embedded in an extracellular polymer matrix capsule (MELO, 2008; BOARI et al., 2009; NEVES, 2012) and, once adhered, the microorganisms pass from the planktonic state to a sessile state, forming colonies that, when detach from the structure, return to the initial state. According to BOARI et al. (2009), monospecies biofilms commonly occur in organic tissues, resulting from infectious processes, whereas multispecies biofilms usually settle on other surfaces, such as those present in industrial food facilities.

The staphylococcal biofilm has in its composition extracellular DNA, polysaccharide compounds, teichoic acid, protein adhesives, minerals, and vitamins (HEILMANN, 2011). The bacteria within the biofilm exhibit an altered phenotype regarding growth rate and gene transcription (GIAOURIS et al., 2014). The biofilm formation impairs the action of physical and chemical agents used in cleaning and sanitizing procedures in industrial facilities (MELO, 2008; KASNOWSKI et al., 2010). It also causes difficulty in removing the biofilm from surfaces, damages on equipment by biocorrosion, and reduction of heat transfer and pressure expenditures in pipes and other equipment (DE OLIVEIRA et al., 2013), besides causing deleterious effects on the quality of the final product. In the processing of meat and dairy products, biofilms are identified as a disorder (CHEN et al., 2007), since they contain more than 90% of the contaminants present in industrial systems (LUCCHESI, 2006).

According to MORALES et al. (2004), the direct fixation of the biofilm on the surface results from physicochemical

properties. This connection is driven by hydrophobic or electrostatic interactions and by the action of specific adhesins, such as SSP-1 and SSP-2. The accumulation of material on surfaces forms coatings that collaborate for the development of biofilm, and bacterial cells adhered to this material serve as signals for specific receptors in other bacterial cells to stick (CHRISTENSEN et al., 1994; GELOSIA et al., 2001).

Dipole-dipole interactions, hydrogen bonds, ionic covalent bonds, and hydrophobic forces are physicochemical processes that act especially during the irreversible phase and determine the connection of bacteria with other cells or with surfaces (CHMIELEWSKI; FRANK, 2003; BERK, 2018). One of the first phases for the constitution of the biofilm is the fixation of the bacteria on the surface. A greater relevance is attributed to hydrophobicity than to electrostatic interactions, due to the generation of interaction forces that are important in the fixation of microorganisms (ARAÚJO et al., 2010). Therefore, it is possible to predict whether the adhesion is thermodynamically favorable from the determination of hydrophobicity. Physicochemical properties of the surface material, temperature, pH (MELO, 2008), expression of virulence factors (FLACH et al., 2005), and hydrophobicity (ARAÚJO et al., 2009) are factors that influence the fixation of the macromolecules. Bacterial hydrophobicity is characterized by the energy of attraction between nonpolar or vaguely polar cells immersed in an aqueous medium. Bacterial multiplication phase, growth medium composition, presence of cellular appendages, and excreted polymeric substances are conditions capable of intervening in cellular hydrophobicity. Likewise, the physicochemical properties of surfaces influence the fixation of bacteria (SIMÕES et al., 2010). The free energy of the interaction between two physical beings immersed in water is what determines the hydrophobicity of surfaces and materials. When surface molecules have greater interaction with each other than with water, these molecules are considered hydrophobic. When the interaction of surface molecules is repulsive, it is classified as hydrophilic (ARAÚJO et al., 2009). According to RODRIGUES et al. (2009), bacterial adhesion increases according to the hydrophobicity of the cell surface, as well as the adhesion surface (DONLAN; COSTERTON, 2002). Therefore, bacteria adhere more easily to hydrophobic surfaces, such as polystyrene, while glass and metal tend to be less suitable for this fixation due to their hydrophilic properties (RODRIGUES et al., 2009).

Various virulence factors related to adhesion determine the level of hydrophobicity of the cell, such as pili and fimbriae, flagella and characteristics of the bacterium's outer membrane, as well as several states of electronegativity controlled by polar functional groups, such as hydroxyls, phosphates, carboxyls, and teichoic acid (VENTURINI, 2017). The decrease in oxygen in the medium predisposes to structural changes in the lipopolysaccharide (LPS) of some bacteria, causing an increase in the hydrophobicity of the cell, a fact that indicates

that the bacteria have the ability to modify the hydrophobicity characteristics of the external membrane due to changes in the environment. Thus, the presence of LPS in the membrane tends to make cells more hydrophilic, given that when this molecule is lost, the cell becomes more hydrophobic. According to BOARI et al. (2009), Gram-negative bacteria have a competitive advantage in initial adhesion, colonization, and biofilm formation when compared to Gram-positive bacteria. Microorganisms can vary in hydrophobicity, depending on the culture conditions. The hydrophobicity of Gram-negative bacteria increases when the amount of water in the medium decreases, since its outer membrane has a large amount of lipids and a lower amount of peptidoglycan. Hydrophobicity is the thermodynamic property most easily influenced by the amount of water in the medium. Gram-positive bacteria, on the other hand, are less affected by the change in the amount of water in the medium, due to a higher content of peptidoglycan and lower lipid content in their membranes. In addition to hydrophobicity, however, other factors also influence bacterial adhesion.

Bacterial biofilms present in food facilities and equipment can separate, colonize other surfaces, and start the formation of a new biofilm, or they can contaminate foods that come into contact with the surface, becoming a chronic form of contamination (KASNOWSKI et al., 2010). This biofilm growth can cause significant damage to the industry, such as contamination of the final product and biocorrosion and damaging of pipes (KASNOWSKI et al., 2010). According to ANDRADE et al. (2003), around 16% of foodborne disease outbreaks in Brazil are caused by contaminated utensils and equipment; in France, it was responsible for 59% of these outbreaks in 2001 (MIDELET; CARPENTIER, 2004). In dairy processing facilities, residues are complex, such as fat, proteins, sugars, and minerals, and therefore it is difficult to remove these compounds, which adhere to surfaces firmly (SILVA, 2006). According to CABEÇA (2006), of the surfaces used in industrial food facilities in handling, storage, or processing equipment, stainless steel is identified as the most important source of bacterial contamination. SCHMIDT et al. (2012) proved that this material has grooves and cracks and these irregularities on the surface allow colonization by microorganisms and biofilm formation.

COS et al. (2010) and TRAN; WEBSTER (2013) verified the main biofilm formation sites, associating them to the occurrence of diseases, and concluded that biofilms can be formed on equipment surfaces, inside pipes and on several surfaces. Besides stainless steel, glass, rubber, polypropylene, and iron are surfaces where biofilms can be formed. STOCCO et al. (2017) demonstrated that biofilms could also form on surfaces of carcasses and fish, causing a challenge in several sectors of fish, poultry, and red meat processing. Water, raw food, dust, equipment, and the hands of operators can be sources of contamination for food or for the colonization of

bacteria, enabling biofilm formation (MOTA et al., 2012; SIMÕES et al., 2010; SREY et al., 2013).

SHARMA; ANAND (2002) observed multispecies biofilms in components of the pasteurization lines in a dairy facility. Studies have shown that stainless steel surfaces of facilities must be treated with ion implantation techniques, which reduces the fixation of bacteria on the surface (ZHAO et al., 2008).

Staphylococcus bacteria produce adhesins, which belong to the group of surface proteins anchored covalently or anchored in the cell wall. This group also includes adhesins, autolysins, and proteins that cross the membrane. *S. aureus* is capable of expressing more than 24 surface proteins, making its adhesion easier. According to FOSTER et al. (2014), the CNS express lower amounts of these surface proteins, with *S. epidermidis* manifesting 12 proteins (NEVES, 2012).

In *Staphylococcus* spp., the formation of biofilm is a dynamic process that occurs in three phases — adhesion, proliferation, and formation — and conclude with maturation and dispersion (O'TOOLE et al., 2000; OTTO 2013; LE et al., 2014). MARSHALL et al. (1971) highlighted that the first phase of the adhesion process is reversible, due to the fixation occurring by Van der Waals forces and electrostatic attractions. In the subsequent phase, through glycocalyx, physical interaction occurs between the cell and the surface (MELO, 2008), becoming irreversible. However, other theories report that biofilm formation occurs in five distinct stages — surface conditioning by adsorption of organic material; transport of cells and nutrients to the adhesion site; reversible bacterial adhesion; cell growth; colonization and irreversible bacterial adhesion and release of cells present in the biofilm margin (MACEDO, 2001; SALUSTIANO, 2007).

The adhesion of bacteria to surfaces is mediated by the formation of the intercellular polysaccharide adhesin (PS/A). Subsequently, proliferation and the development of multi-layers occur (HEILMANN; PETERS, 2006). In *Staphylococcus* spp., the main molecule responsible for intracellular adhesion is called the intracellular adhesion polysaccharide (IAP), through which the cells bind to each other. According to VASUDEVAN et al. (2003), PS/A and IAP have similar conformation, both with a β 1-6 polyglycosamine carbon, but are distinguished by a primary substitution in the amine group. According to ADHIKARI et al. (2007) and SHAW et al. (2007), the production of PS/A is mediated by the products of the chromosomal gene *ica*, organized in a structure composed by four genes: *icaA*, *icaD*, *icaB*, and *icaC*, in addition to a regulatory gene transcribed in the opposite direction, called *icaR*.

The expression of the IAPs present in the cell wall (BALABAN et al., 2007) is also responsibility of the *icaADBC* operon, which, when activated, encodes four proteins that are essential for the synthesis of IAP. The *icaA* gene codes for the enzyme N-acetylglucosaminyl transferase, using UDP-N-acetylglucosamine as substrate. The *icaD* gene controls the expression of the capsular polysaccharide (GERKE et al., 1998).

A transmembrane protein is coded by the *icaC*. This protein is formed with the externalization and elongation of the polylactide and *icaB* is responsible for the IAP deacetylation (GERKE et al., 1998). Authors report the existence of the *icaADBC* operon in CNS species other than *S. epidermidis*, such as *S. caprae*, *S. lugdunensis*, and *S. haemolyticus* (MACK et al., 2006).

The maturation phase follows the adhesion and the production of slime — which is an extracellular polysaccharide layer that preserves the adherent surface growth (ALCARÁZ et al., 2003) — occurs, balancing the associations between cells and between the surface and thus, enabling bacterial accumulation, and completing the stage of colonization of the surface. In addition, channels are formed in the biofilm structure to transport water and nutrients to the deeper layers. Molecular techniques such as the amplification of DNA sequences by Polymerase Chain Reaction (PCR) are effective for the identification of *ica* genes (MARTIN, 2015), which are responsible for coding the synthesis of slime, enabling the detection of virulent slime-producing strains. At the end of the steps, there is the detachment of superficial bacterial cells from the biofilm. BAGGE-RAVN et al. (2003) isolated microorganisms from several fish processing facilities. *Staphylococcus* spp. were among the genera of bacteria isolated by them, with the majority of these isolates having the capacity to form biofilm.

One of the main virulence factors of CNS is the ability to attach to polymeric surfaces, where biofilms can be formed, especially *S. epidermidis*, which is the most frequently isolated species (COSTA et al., 2004). MALES et al. (1985) reported the ability of *S. simulans* to form biofilm, while STEPANOVIC et al. (2003) reported this ability in *S. sciuri*, FOKA et al. (2006) in *S. haemolyticus* and FREDHEIM et al. (2009), and QU et al. (2010), in *S. capitis*.

The presence of slime in CNS isolated from human catheters was reported, with *S. epidermidis*, *S. haemolyticus*, *S. warneri*, *S. xyloso*, *S. lugdunensis*, *S. hominis*, *S. saprophyticus*, *S. schleiferi*, and *S. chromogenes* being detected. MACK et al. (2006), FREDHEIM et al. (2009) and ROHDE et al. (2010) identified the *icaADBC* operon in *S. caprae*, *S. lugdunensis*, and *S. haemolyticus*, supporting the fact that biofilm formation can be observed in these species (MALES et al., 1985). The *icaADBC* operon has not been detected very often in biofilm-forming isolates of *S. haemolyticus*. According to QU et al. (2010), *S. capitis* is capable of producing biofilm even in the absence of the *icaD* gene. In contrast, the genes of the *icaADBC* operon were detected in a non-biofilm-forming *S. warneri* isolate (BRADFORD et al., 2006; QU et al., 2010).

A study showed that 72% (59/72) of *S. haemolyticus* strains grown in Triptona Soy Broth (TSB) supplemented with glucose formed biofilm and in a medium with NaCl only 31% (22/72) (FREDHEIM et al., 2009). In another similar study, testing different biofilm formation conditions by CNS isolated from human blood, there was biofilm formation by *S. capitis*

in TSB supplemented with 4% NaCl and by *S. warneri* in TSB supplemented with 1% glucose.

Coagulase-negative *Staphylococcus* counts performed in pasteurized milk by ATAIDE (2006) presented values of 7.0×10^2 and 1.1×10^4 UFC / mL. As *Staphylococcus* spp. are sensitive to the temperatures of pasteurization, the authors concluded that this contamination could be attributed to the release of fragments of biofilm, present on some surface of the pasteurizer, on the outlet pipe of the equipment, or on the surface of the storage tank of pasteurized milk. In 2008, another study was carried out in a milk processing facility in Brazil, isolating 25 CNS from the surfaces of equipment. Four of the isolates were from the receiving tank before cleaning, of which one remained

after cleaning the equipment. One isolate from the pasteurizer outlet pipe was obtained, even after it had gone through the sanitization process. From the already packaged milk, an isolate was also obtained. These results can be attributed to the formation of biofilm in the equipment, allowing the release of fragments in the already processed milk (SANTOS et al., 2009).

It is concluded that species of CNS have the capacity to form biofilm on different surfaces of equipment and materials used in industrial facilities, which can be a source of contamination for food and generate losses for the industry and a risk to public health. Still, further studies need to be carried out in order to quantify the occurrence of CNS biofilms in the food industry.

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