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Biofilm formation by pGFPuv labeled mutant *Cronobacter sakazakii* in the absence and presence of other bacteria

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Abstract

Cronobacter sakazakii has been reported to form biofilm on food contact surfaces thus could be a potential source of contamination in food industries. This study aimed to investigate biofilm formation by pGFPuv labeled *C. sakazakii* local strains on stainless steel (SS), Buna-N rubber (Buna-N) and polytetrafluorethylene (PTFE) surfaces at various incubation times and compare biofilm formation by *C. sakazakii* pGFPuv on SS in the presence and absence of *Staphylococcus aureus* and *Lactobacillus rhamnosus*. Two pGFPuv labeled *C. sakazakii* isolates were inoculated separately into broth medium containing SS, Buna-N or PTFE coupons and incubated for 24, 48 and 72 hrs at room temperature. Formation of biofilm by these isolates was also investigated on SS in the presence of *S. aureus* and *L. rhamnosus*. The biofilm density was enumerated by swabbing followed by plating. The results showed that pGFPuv labeled mutants formed biofilms (up to 5 Log CFU/cm^2) on all food contact surfaces. The highest biofilm density was found on PTFE while the lowest was on Buna-N surfaces. The biofilm density of *C. sakazakii* pGFPuv on SS was similar irrespective of the presence or absence of *S. aureus* and *L. rhamnosus*. This study corroborates the ability of *C. sakazakii* to form biofilms on food contact surfaces and provides evidence that the density of the biofilm is not affected by the presence of *S. aureus* and *L. rhamnosus*.

1. Introduction

The attachment of bacterial cells to surfaces may be followed by growth and biofilm formation. Biofilms are defined as aggregates of microorganisms in which cells are frequently embedded within an extracellular polymeric substance (EPS) produced by itself, usually adhering to each other and/or to a surface (Flemming *et al*., 2021). Biofilm cells are more resistant to antimicrobial agents than their free-living bacterial cells, as they have a barrier protecting them from antibiotics, sanitation processes, and external stressors (Flemming *et al*., 2016). Therefore, biofilms in food processing environments may potentially become sources of contamination which may lead to spoilage or foodborne illnesses.

Cronobacter sakazakii is an opportunistic foodborne pathogen reported to cause meningitis, septicemia, and necrotizing enterocolitis (NEC) in infants. Infants born prematurely or with low birth weight (<2.5 kg) are reported to be at the highest risk for severe infection and babies 28 days old or less are considered to be more at risk than older infants (Ling *et al*., 2020). Foods such as powdered infant formula, fresh vegetables and fruit represent potential vehicles of *C. sakazakii* infections (Henry and Fouladkhah, 2019; Chauhan *et al*., 2020). In addition, *C. sakazakii* has been reported capable of attaching and forming biofilms on polyurethane, silver-impregnated, stainless steel, and polyvinyl chloride (Ling *et al*., 2020; Abebe, 2020).

In nature, biofilm may be composed of mixed species populations with various interactions. In competitive interactions, species with similar nutrient requirements will compete for nutrients. Sometimes such competition involves one species actively inhibiting the growth of others by producing inhibitory compounds or consuming essential nutrients (Cai *et al*., 2019).

In biofilm, high cell density and low diffusion rate offer an opportunity for microorganisms to undergo various cooperations until the need for nutrient competition emerges (Sivadon *et al*., 2019). Such interactions can result in physiological and regulatory

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alterations within the biofilm which ultimately contribute to the selection of better-adapted mutants. These interactions can cause the emergence and disappearance of species and therefore play an important role in the shaping of multispecies biofilm communities (Roder *et al*., 2019). A recent study suggested that *Lactobacillus acidophilus* produces an extracellular polysaccharides matrix that could interfere with the expression of surface adhesins of Enterohemorrhagic *Escherichia coli*, while the growth is not affected. The ability of *L. acidophilus* EPS to inhibit other Gram-positive and Gram-negative biofilms was also demonstrated in *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *Listeria monocytogenes* (Kim *et al*., 2009).

Enumeration of biofilm bacteria using standard plate count requires lengthy incubation while epifluorescence microscopy can be used as an alternative method. However, epifluorescence microscopy cannot distinguish different types of bacteria. In this study, the target bacterium, i.e. *C. sakazakii* was previously transformed with Green Fluorescent Protein plasmid (pGFPuv) (Nurjanah *et al*., 2013) thus they can be distinguished from other bacteria. pGFPuv has become an especially valuable marker for nondestructively visualizing cells, particularly in biofilms (Cowan *et al*., 2000). The purposes of this research were to investigate biofilm formation by of pGFPuv labeled *C. sakazakii* on different food contact surfaces and formation of *C. sakazakii* pGFPuv mutant biofilm on SS surfaces in the presence of *S. aureus* and *L. rhamnosus*.

2. Materials and methods

2.1 Bacterial strains and growth media

pGFPuv labeled *C. sakazakii* FWHd16 and YRt2a (Nurjanah *et al*., 2013), *L. rhamnosus* R23, and *S. aureus* SA25923 were obtained from the bacterial culture collection of SEAFAST Center (Bogor Agricultural University, Indonesia). Microbiological media used were Brain Heart Infusion (BHI, OXOID), Druggan-Forsythe-Iversen (DFI, OXOID), Tryptic Soy Agar (TSA, OXOID), Tryptic Soy Broth (TSB, OXOID), de Man-Rogosa-Sharpe Agar (MRSA, OXOID), de Man-Rogosa -Sharpe Broth (MRSB, OXOID).

2.2 Culture preparation of pGFPuv mutants Cronobacter sakazakii FWHd16 and YRt2a

A loopful of 24 hrs pGFPuv labeled *C. sakazakii FWHd16 or YRt2a* growth on TSA slants was each inoculated into TSB and incubated at 37°C for 24 hrs. One mL of each culture was aseptically transferred into 10 mL TSB, and incubated at 37°C to reach the late log phase (ca. 10^8-10^9 CFU/mL). The cultures were then appropriately diluted for biofilm study.

2.3 Culture preparation of Staphylococcus aureus and Lactobacillus Rhamnosus

A loopful of 24 hrs growth of *S. aureus* on TSA slants was inoculated into TSB and incubated at 37°C for 24 hrs. Meanwhile, one loopful of 24 hrs growth of *L. rhamnosus* on MRSA slant was inoculated into MRS broth and incubated at 37°C for 24 hrs. One mL of *S. aureus* and *L. rhamnosus* culture was aseptically transferred into 10 mL TSB or MRSB, respectively, and incubated at 37°C to reach the late log phase (ca. 10^8-10^9 CFU/mL). The cultures were then appropriately diluted for biofilm study.

2.4 Preparation of coupons for food contact surfaces

Stainless steel (SS) type 304, Buna-N Rubber, and polytetrafuorethylene (PTFE) were made into 1 × 1 cm coupons. Before use, the coupons were cleaned, sanitized and sterilized according to Marques *et al*. (2007).

2.5 Biofilm formation by pGFPuv Cronobacter sakazakii FWHd16 and YRt2a

Two mililiters of diluted 24 hrs culture of *C. sakazakii* pGFPuv FWHd16 or YRt2a was inoculated into Erlenmeyer flask containing 1/5 TSB and coupons such that the initial number of the bacteria was ca. 10^5 CFU/mL. Incubation was carried out at room temperature (28-30°C) with 70 rpm agitation for 24, 48 and 72 h. After incubation, coupons were rinsed in sterile PSS and the biofilm density of *C. sakazakii* pGFPuv FWHd16 or YRt2a mutants was enumerated by Direct Microscopic Count (DMC) using epifluoresence microscopy based on Parizzi *et al*. (2004). The biofilm density is expressed as Log CFU/cm^2.

2.6 Formation of Cronobacter sakazakii pGFPuv biofilm in the presence of other microorganisms

Late-log phase cultures of *C. sakazakii* pGFPuv, *S. aureus* and *L. rhamnosus* were inoculated into 1/5 MRSB such that the initial count for each bacterium was 10^3 CFU/mL. SS coupons were placed in the medium, incubated at room temperature (28-30°C) and rinsed as above prior to enumeration of the attached cells. The number of attached *C. sakazakii* pGFPuv were enumerated directly under an epifluoresence microscope as the green fluorescent cells (A). The coupons were then immersed in acridine orange 0.0026% (Dewanti-Hariyadi and Wong, 1995) and all bacteria attached to SS coupons were enumerated under an epifluorescence microscope (B). The number of attached *S. aureus* and *L. rhamnosus* was calculated as (B - A), with cocci cells as *S. aureus* and rod cells as *L. rhamnosus*.

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3. Results

3.1 Biofilm formation by pGFPuv Cronobacter sakazakii mutants

The biofilm density of pGFPuv mutant *C. sakazakii* FWHd16 on SS, Buna-N and PTFE surfaces at different incubation times is shown in Figure 1A. At 24, 48 and 72 hrs of incubation times, the number of attached cells was in the range of 5.05-4.73 Log CFU/cm^2, 4.94-4.64 Log CFU/cm^2 and 5.11-4.83 Log CFU/cm^2, respectively. Meanwhile, biofilm formation by pGFPuv mutant *C. sakazakii* YRt2a can be seen in Figure 1B. The biofilm density of pGFPuv mutant *C. sakazakii* YRt2a were in the range of 5.03-4.66 Log CFU/cm^2, 4.94-4.64 Log CFU/cm^2 and 5.15-4.76 Log CFU/cm^2 at 24, 48 and 72 hrs of incubation time, respectively. For mutant *C. sakazakii* pGFPuv FWHd16, the highest biofilm density was formed at 48 hrs, while that for YRt2a occurred at 24 hrs. At 72 hrs both mutants formed the lowest density of biofilms. Both mutants formed the highest density of the biofilm on PTFE, while those on Buna-N had the lowest density.

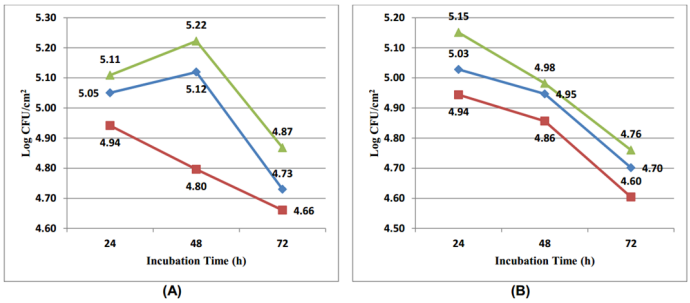

Figure 1. Biofilm density of GFPuv Mutant *C. sakazakii* FWHd16 (A) and GFPuv mutant *C. sakazakii* YRt2a (B) on SS (◆), Buna N (■) and PTFE (▲).

The results showed that GFPuv labeled *C. sakazakii* could form biofilms on all food contact surfaces at 24, 48 and 72 hrs of incubation time. The highest biofilm density was formed on PTFE at 48 hrs by GFPuv labeled *C. sakazakii* FWHd16. On Buna-N surfaces, both mutants had the highest biofilm density at 24 hrs and then decreased afterwards. These findings are similar to da Silva Meira *et al.* (2012) who showed that the number of adherent cells of *S. aureus* S28 presented two different phases i.e. initial phase, with the number of cells on SS surfaces ranging from 4.1 to 5.0 Log CFU/cm^2 at 24 and 48 hrs of incubation respectively; and a second phase comprising 48-72 hrs of incubation, when the cell counts decreased from 5.0 to 3.9 Log CFU/cm^2, respectively.

3.2 Biofilm formation by Cronobacter sakazakii pGFPuv mutants in the presence of Staphylococcus aureus and Lactobacillus rhamnosus on stainless steel surfaces

The study was designed to allow mutant *C. sakazakii* pGFPuv FWHd16 to compete with *S. aureus* and/or *L. rhamnosus* to form biofilm on SS surfaces. The results in Figure 2A show that biofilm density of mutant *C. sakazakii* pGFPuv FWHd16 on SS was not affected when in competition with other bacteria. In the presence of other bacteria, the number of attached *C. sakazakii* on SS at 24, 48 and 72 h of incubation were 5.02±0.04 Log CFU/cm^2, 4.97±0.01 Log CFU/cm^2 and 4.93±0.04 Log CFU/cm^2, respectively. These numbers were similar to those formed on SS without the presence of *S. aureus* and *L. rhamnosus*, i.e. 5.05±0.03 Log CFU/cm^2, 5.12±0.02 Log CFU/cm^2 and 4.73±0.03 Log CFU/cm^2 (Figure 1A). In the presence of *C. sakazakii*, *S. aureus* forms biofilms with densities of 5.02±0.11 Log CFU/cm^2, 5.26±0.01 Log CFU/cm^2 and 5.17±0.01 Log CFU/cm^2 at 24, 48 and 72 h whilst *L. rhamnosus* biofilms have densities of 4.94±0.06 Log CFU/cm^2, 5.17±0.01 Log CFU/cm^2 and 5.31±0.01 Log CFU/cm^2 at 24, 48 and 72 h, respectively. During the competition, an increase in incubation time correlates with increase in *S. aureus* and *L. rhamnosus* attachment while the population of pGFPuv mutant *C. sakazakii* FWHd16 biofilms tends to decrease with incubation time.

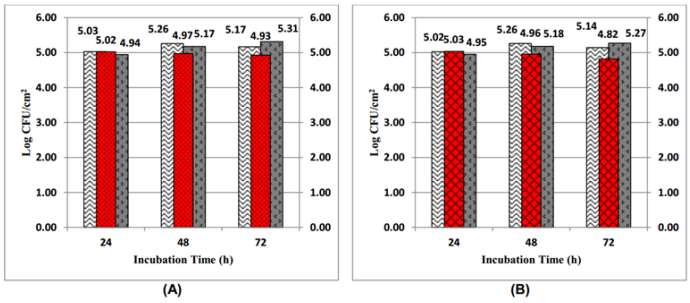

Figure 2. Biofilm formation by GFPuv Mutant *C. sakazakii* FWHd16 ( ), *S. aureus* ( ) and *L. rhamnosus* ( ) grown together in 1/5 MRSB (A) and by *C. sakazakii* YRt2a ( ), *S. aureus* ( ) and *L. rhamnosus* ( ) grown together in 1/5 MRSB (B)

A similar trend was observed with *C. sakazakii* YRt2a pGFPuv (Figure 2B). In the presence of *S. aureus* and *L. rhamnosus*, biofilm densities of this mutant were 5.04±0.04 Log CFU/cm^2, 4.96±0.03 Log CFU/cm^2 and 4.82±0.04 Log CFU/cm^2 at 24, 48 and 72 hrs, respectively. The numbers were not different from that formed by *C. sakazakii* YRt2a pGFPuv alone on SS at 24, 48 and 72 h, i.e. 5.03±0.04 Log CFU/cm^2, 4.94±0.07 Log CFU/cm^2 and 4.66±0.06 Log CFU/cm^2 (Figure 1B). Whether alone or in a competition, pGFPuv mutant *C. sakazakii* YRt2a attachment to SS seems to decrease with an increase in incubation time. On the contrary, the number of adherent *S. aureus* increased along with an increase in incubation time up to 48 hrs. In the presence of *C. sakazakii*, biofilm density of *S. aureus* was 5.03±0.03 Log CFU/cm^2, 5.26±0.02 Log CFU/cm^2 and 5.14±0.01 Log CFU/cm^2 at 24, 48 and 72 hrs, respectively. The number of attached *L. rhamnosus* also continued to increase up to 72 hrs and reached 5.27±0.02 Log CFU/cm^2. This implies that in the presence of *C.*

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sakazakii, biofilm density of *L. rhamnosus* increased steadily with increased incubation time.

4. Discussion

Mutants of *C. sakazakii* isolate FWHd16 and YRt2a formed the highest density of biofilm on PTFE surfaces and the lowest on Buna-N rubber. Higher biofilm density on PTFE resulted from the hydrophobic nature of the PTFE. Sinde and Carballo (2000) also found that *Salmonella* and *Listeria* attach in higher numbers to PTFE, which was more hydrophobic than SS or Buna-N. Cell surface hydrophobicity and the presence of extracellular filamentous appendages may influence the rate and the extent of microbial attachment. Some studies suggest that bacteria are more likely to attach to hydrophobic surfaces (Jamal *et al*., 2018). The hydrophobic interactions of bacteria to solid surfaces, when grown on media with nutrient limitation, will increase due to changes in bacterial cell size that become smaller. An increase in the hydrophobicity and aggregation of bacterial cells facilitates the bacterial transfer and attachment of bacteria on the surface (Wang *et al*., 2021). The hydrophobicity of bacterial cell surface and support surface in biofilm formation can further enhance the adhesion ability if one of them is hydrophilic (Han *et al.*, 2018). The lower density of biofilm on Buna-N surfaces was possibly due to acrylonitrile generally added to Buna-N. The agent is known to inhibit the growth of microorganisms thus affecting biofilm formation on the surface (Fasiku *et al*., 2020). Acrylonitrile used as a rubber precursor synthesis is a toxic component of the biofilm of some bacteria and fungi (Alamri *et al*., 2012). Farag *et al.* (2020) showed that the addition of acrylonitrile-based antimicrobial monomers in cross-linked terpolymer samples to cultures of *Escherichia coli, Bacillus subtilis* and *Candida albicans* had caused deformation and exhibited severe destruction to the treated cells.

The study showed that pGFPuv mutant *C. sakazakii, S. aureus* and *L. rhamnosus* could form biofilms together on SS surfaces. Other studies have shown that in the events of competition for attachment, cell surface hydrophobicity plays an important role in bacterial attachment, colonization and biofilm formation (Heir *et al*., 2018; Dhowlaghar *et al*., 2018). Elgamoudi and Korolik (2020) suggest that D-amino acid production may also interfere with neighbors in the maturation of mixed biofilms. In this study, we found that the presence of *S. aureus* did not affect the density of the biofilm of *C. sakazakii.* Results of this study also showed a slight decrease in the density of biofilm formed by *S. aureus* on the surface after 48 hrs of incubation. Boles and Horswill (2008) reported that the release of cells from *S. aureus* biofilm was influenced by *agr* quorum-sensing system conducted internally due to the presence of other bacteria in the environment. D-amino acids produced by many bacteria at late stages of growth, including the stationary phase at the time of biofilm formation, have been reported to break down and inhibit the formation of a bacterial biofilm (Alreshidi *et al*., 2020; Khan *et al*. 2021). Fu *et al.* (2021) also stated that exogenous addition of D-amino acids could disassemble *S. aureus* biofilms. Different mechanisms of action for D-amino acids have been reported; for instance, D-amino acids inhibit the accumulation of proteins in the *S. aureus* matrix and the development of biofilms (Hochbaum *et al*., 2011).

Lactic acid bacteria are known to produce various antimicrobial compounds including organic acids, hydrogen peroxide and bacteriocins which can inhibit undesirable microorganisms or pathogenic bacteria isolated from food (Ozogul and Hamed, 2018). However, the results of this study did not show a significant effect of the presence *L. rhamnosus* on the biofilm formation by mutant *C. sakazakii* pGFPuv.

After 48 hrs of incubation, the biofilm densities of *C. sakazakii* pGFPuv were higher than when they were grown alone. Szeker (2007) reported that in the case of competitive adhesion on stainless steel, it was found that lactobacilli did not hinder but aid the adherence of *Pseudomonas fluorescens* and *L. monocytogenes* by offering attachment sites for them. Additionally, *C. sakazakii* has the ability to produce EPS thought to improve its resistance in biofilms and becomes one of the self-defense capabilities (Jung *et al*., 2013). The EPS matrix also helps the biofilm cells resist multiple stress conditions, such as desiccation, water or nutrient shortages, the presence of biocides, and other antimicrobial agents (Dufour *et al*., 2012).

Enhanced biofilm formation by *L. rhamnosus* in this study has also been previously reported in *L. rhamnosus* during the competition and exclusion study with *S.* Typhimurium and *L. monocytogenes*. The study indicated that *L. rhamnosus* has a potential growth advantage over *S.* Typhimurium and *L. monocytogenes* (Woo and Ahn, 2013).

This study concluded that *C. sakazakii* that have been inserted with a marker plasmid GFPuv can be applied to study biofilm formation on food contact surfaces. *C. sakazakii* pGFPuv mutants were able to form biofilms on all food contact surfaces, i.e. SS, Buna-N and PTFE. The highest *C. sakazakii* biofilm density was observed on PTFE, regardless of the isolates. The highest density of biofilms was observed at 48 hrs for pGFPuv labeled *C. sakazakii* FWH16. Meanwhile, biofilm of

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pGFPuv labeled *C. sakazakii* YRt2a reached maximum density at 24 hrs and decreased afterward. This study also showed that *C. sakazakii* pGFPuv, *S aureus* and *L. rhamnosus* were able to form biofilms when grown together in the same environmental conditions. Biofilm density of pGFPuv mutants of *C. sakazakii* FWHd16 and YRt2a were the lowest as compared to those of *S. aureus* and *L. rhamnosus* at 48 and 72 hrs. However, the density of *C. sakazakii* biofilm in the presence of *S. aureus* and *L. rhamnosus* was similar to that formed in the absence of the two competitors. After 48 h the number of adherent *L. rhamnosus* tended to increase while adherent *C. sakazakii* declined. It is possible that at prolonged incubation, the production of extracellular polysaccharides by the lactobacilli bacteria interfere with biofilm formation by *Cronobacter sakazakii*, similar to that in Enterohemorrhagic *Escherichia coli* (Kim *et al*., 2009).

5. Conclusion

Both strains of the wild type and pGFPuv mutants of *C. sakazakii* can form biofilm on SS, buna N and PTFE with the highest density on PTFE plastic and the lowest on Buna N rubber. At 48 h of incubation, the density of *C. sakazakii* biofilm is the highest and tends to decrease afterwards. The presence of *S. aureus* and *L. rhamnosus* did not affect biofilm formation by *C. sakazakii*, however, the number of adherent *S. aureus* and *L. rhamnosus* biofilms were higher. The results suggested that *C. sakazakii* could form biofilms in the absence and presence of other bacteria.

Conflict of interest

The authors declare no conflict of interest.

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