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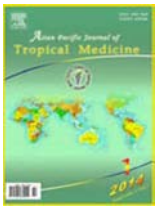

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# Assessment of the contamination potentials of some foodborne bacteria in biofilms for food products

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ABSTRACT

**Objective:** To assess biofilms formed by different bacterial strains on glass slides, and changes in biofilm mass and biofilm-associated cell populations after brief contacts between biofilms and either media agar or food products.

**Methods:** Two *Listeria monocytogenes* and *Escherichia coli (E. coli)* strains and a single *Staphylococcus aureus (S. aureus)* strain were inoculated separately in tryptic soy broth containing glass coupons incubated for 24, 48 or 72 h at 37 °C. The biofilms formed by individual bacterial strains and biofilm-associated cell populations were determined. Biofilms were subsequently allowed to have brief contacts (1-3 times), through gentle touching, with either agar, meat or soft white cheese (2 $cm^3$). Changes in biofilm mass on glass slides and cell populations embedded in biofilms were quantified.

**Results:** A nonpathogenic *E. coli* formed more biofilms than an *E. coli* O157:H7 strain. Biofilms formed by *S. aureus* and *Listeria monocytogenes* were essentially similar. The biofilm mass increased as incubation time increased within 48 h of incubation and was not positively correlated with cellulose production. Biofilm mass at 48 and 72 h of incubation was not significantly different. More frequent contacts with agar or foods did not remove more biofilms or biofilm-associated cells from glass slides. More *S. aureus* biofilms were removed followed by *Listeria* and *E. coli* biofilms. Mean contamination of agar or food models was 0.00 to 7.65 log CFU/$cm^2$. Greater contaminations in cell populations were observed with *S. aureus* and *Listeria* biofilms.

**Conclusions:** The results provide a clearer assessment of contaminating potential of foods that comes in contact with them.

## 1. Introduction

Biofilms are architecturally complex communities of microorganisms in which the cells are held together by an extracellular matrix typically containing exopolysacchrides, proteins and even nucleic acids[1]. Bacteria in biofilms are a major source of food contamination which predisposes to foodborne disease outbreak[2]. Bacterial transfer to food from biofilm can lead to food spoilage or the transmission of diseases[3]. Biofilm is a major problem in the food industry because the hygiene of the surfaces affects the overall quality and safety of the food product[4,5]. Numerous studies have shown that *Listeria monocytogenes* (*L. monocytogenes*) is capable of adhering to and forming biofilm on metal, glass or rubber surfaces[6-10]. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are also capable of forming biofilms on polypropylene mesh[11]. Studies have shown that facilities remain heavily contaminated even after hygiene operations[12,13]. Biofilms are responsible for the causation of chronic diseases that are difficult to treat and have developed resistance to cleaning and sanitation[14,15], hence, are of both public health and economic importance. *L. monocytogenes*, *E. coli* and *S. aureus* are important foodborne pathogens worldwide and many foodborne

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outbreaks have been linked to them[16].

Earlier studies did not link cellulose in bacteria to biofilm formation. However, recent studies have revealed that some species of the family Enterobacteriaceae (*e.g.*, *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp.) produce cellulose as a crucial component of the bacterial extracellular matrix[17]. Cellulose is an exopolysaccharide produced by microbial cultures and are involved in cell adhesion and biofilm formation[18].

This study therefore assessed the biofilm and contaminating potentials of foodborne bacteria in biofilms and their cellulose forming abilities.

## 2. Materials and methods

### 2.1. Bacterial strains and cultural conditions

Four laboratory stock cultures of foodborne pathogens: *L. monocytogenes*, *S. aureus*, *E. coli* (sorbitol fermenting), *E. coli* O157:H7 isolates from milk and *L. monocytogenes* (H7762) a strain from frankfurter were used in this study. Stock cultures previously stored in 8% glycerol at −20 °C were grown overnight on modified Oxford agar with antibiotic supplements (acriflavine, nalidixic acid and cycloheximide) (Becton, Dickinson and company) for *L. monocytogenes*, nutrient agar for *S. aureus* and Maconkey agar for *E. coli* and *E. coli* O157:H7. These strains were sub-cultured at least thrice.

### 2.2. Development of biofilm on glass surfaces

Biofilm was developed on glass coupons (1 $cm^2$×2 $cm^2$) (Home Science tools, Billings, USA). Prior to biofilm development, the coupons were soaked for 24 h in 5% Ariel detergent (13.5% sodium carbonate, 3.0% sodium hydroxide, 30.0% sodium sulfate, 10.0% pentasodium tripolyphosphate, 13.5% alkyl benzenesulfonic acid sodium salt, and 7.5% sodium fatty alcohol sulfate) (Unilever, Lagos, Nigeria), and then rinsed 3 times in sterile distilled water, each for 15 min. The coupons were later sterilized in a hot air oven (Electro, Helios, Sweden) at 120 °C for 30 min.

### 2.3. Biofilm development

Biofilm development was done on glass coupons in tryptose soya broth. For biofilm formation, individual coupon was placed in separate screw-caped glass jars (Jirui Glass Products Co. Ltd., Xuzhou, China), each containing 150 mL of tryptose soya broth. Biofilm development was permitted at 37 °C for 24, 48 or 72 h.

### 2.4. Biofilm quantification

At the end of 24, 48 and 72 h incubation period, developed biofilms were quantified using the crystal violet binding assay previously described by Stepanovic *et al.*, and Adetunji and Adegoke with some modifications[19,20]. At each sampling point, coupons were collected and washed 3 times, each with 5 mL of distilled water. Biofilm mass was fixed with 1 mL of 95% ethanol (AnalaR®, BDH Chemical Ltd, UK) for 15 min at room temperature. The fixed samples were heat dried for 10 min and then stained for 5 min with 2% crystal violet (AnalaR®, BDH Chemical Ltd, UK) at room temperature. Excess stains were rinsed with running tap water, and the coupons were then air dried. Each of the dried coupons was placed on a sterile Petri dish, and 3 mL of 33% glacial acetic acid (AnalaR®, BDH Chemical Ltd, UK) was used to solubilize the crystal violet. The solubilized liquid was then pipetted into a cuvette (Bibby Scientific Limited, Staffordshire, UK). Absorbance readings (at 570 nm for *L. monocytogenes* and 488 nm for *S. aureus* and *E. coli* O157:H7) were measured using a spectrophotometer (Surgienfield Instruments, Springfield, England).

### 2.5. Preparation of food models

Tryptose soya agar was prepared in 10 disposable sterile Petri-dishes. The solid agar was then cut into equal sizes of 5 g of 2 $cm^3$ cubes using sterile knives. Food models of meat and soft white cheese which have been previously scalded in flame were also cut into cubes of 2 $cm^3$ area.

### 2.6. Testing for contamination potential due to biotransfer

Biofilms were developed as previously described on glass coupons in 4 replicates. These food models, namely: meat, soft cheese and agar prepared above were placed on biofilms to make brief contacts at various frequencies (1-3). Contacts between food models and biofilms were made gently for 30 seconds either once, twice or three in succession. An untouched biofilm on glass was used as control[21]. The residual biofilm was then quantified using a crystal violet binding assay[19].

### 2.7. Testing microbial population transferred

Cell enumeration of embedded cells in residual biofilm of

touched coupons was done using a dry sterile swab to wipe the entire surface of the glass coupons rigorously to remove the residual biofilm thrice. An untouched coupon severed as a control. The swab was then submerged in a sterile 15 mL test tube containing 10 mL of 0.1% sterile peptone water and vortexed. Serial dilutions of bacterial were made and appropriate dilutions were inoculated on tryptose soya agar. This was then incubated at optimum temperature for each bacterial growth. The contamination level was estimated in log CFU/$cm^2$ thus:

The control coupon-the test coupon at the 3 frequencies of contact.

### 2.8. Quantification of cellulose produced by isolates

To test the relationship between biofilm and cellulose production, cellulose produced by different strains was quantified according to the method of Updegraff[22]. Ten milliliters of a 24 h culture for each isolate was dispensed into 15 mL centrifuge tube. The culture was then centrifuged at 3 000 r/min with a centrifuge. The supernatant was decanted after centrifugation. Three milliliters of acetic nitric acid reagent was added in two installments (1 mL then 2 mL) and mixed on the vortex on each addition. The solution in the 15 mL centrifuge tubes were covered with foil to reduce evaporation and create reflux and then placed in boiling water bath for 30 min. After this period of boiling the tubes were centrifuged again for 5 min at 3 000 r/min. The supernatant was decanted and 10 mL of $H_2SO_4$ was added in 3 installments with intermittent mixing. The mixture was allowed to stand for 1 h. One milliliter of mixture was then dispensed into a test tube containing 100 mL of distilled water. This was mixed thoroughly by agitation and 1 mL of the mixture dispensed into 4 mL of distilled water. The mixture was then placed in ice bath to cool. Ten milliliters anthrone reagent was added by layering with a pipette. This was followed subsequently by thorough mixing and placing tube back in ice bath until all tubes were mixed. The tubes were then capped and placed in boiling water for 16 min, cooled on ice bath for 2-3 min and allowed to stand at room temperature (22 °C) for 5-10 min. One milliliter of each sample was placed in each cuvette for subsequent reading in the spectrophotometer. The absorbance of each sample was then read on the spectrophotometer (Springfield, UK) at 620 nm wavelength against a reagent blank[22]. Calculation of cellulose concentration was done against cellulose standards and reported in μg.

### 2.9. Statistical analysis

One-way ANOVA was used to determine the significant differences at $P<0.05$. Separation of means was accomplished using the Fisher's least significant difference design and the general linear model of statistical analysis software (SAS; $\alpha=0.05$)[23].

## 3. Results

### 3.1. Biofilm and cellulose production

The biofilm forming abilities of strains used in this study is shown in Table 1. All strains produced biofilm. Biofilm increased until the 48 h of incubation in all strains. Interestingly by the 72 h of incubation biofilm increased in *E. coli* strain and *L. monocytogenes* (H7762) but dropped in *L. monocytogenes*, *S. aureus* and *E. coli* O157:H7. All the strains produced cellulose at 24, 48 and 72 h incubation in this study but not with a definite trend (Table 1). An inverse relationship was demonstrated between cellulose and biofilm production in all pathogens, except for *E. coli* O157:H7 which had a strong positive correlation.

**Table 1**
Mean biofilm and cellulose production by foodborne pathogens at 24, 48 and 72 h of incubation.

| Strains | Biofilm (OD) | | | Cellulose (μg) | | | Correlation between biofilm and associated cellulose parameter ($R^2$) |
|---|---|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | |
| *E. coli* | $0.45^{a}$ | $0.73^{a}$ | $0.75^{a}$ | $13.09^{a}$ | $3.19^{b}$ | $7.32^{a}$ | −0.886 |
| *L. monocytogenes* | $0.04^{bc}$ | $0.24^{bc}$ | $0.18^{bc}$ | $13.01^{a}$ | $2.51^{b}$ | $2.58^{a}$ | −0.660 |
| *S. aureus* | $0.00^{b}$ | $0.31^{bc}$ | $0.11^{b}$ | $4.32^{ab}$ | $3.75^{b}$ | $3.11^{a}$ | −0.143 |
| *E. coli* O157:H7 | $0.22^{c}$ | $0.52^{ac}$ | $0.35^{c}$ | $1.20^{b}$ | $2.12^{b}$ | $2.46^{a}$ | 0.478 |
| *L. monocytogenes* (H7762) | $0.06^{bc}$ | $0.16^{b}$ | $0.17^{b}$ | $6.32^{ab}$ | $16.61^{a}$ | $1.20^{a}$ | −0.239 |

OD: Optical density; Means with the same lower case letters in a row are not significantly different ($P<0.05$).

### 3.2. Residual biofilm and contaminating potential

Residual biofilms in this study ranged between 0.002 0 and 0.342 0 (OD). *S. aureus* had the highest residual biofilm of 0.342 0 followed by 0.286 0 in *L. monocytogenes* (H7762), although this difference was not significant at $P<0.05$. The least residual biofilms were in the *E. coli* and *E. coli* O157:H7 strains (Table 2). The contaminating bacterial population ranged between 0.00 to 7.65 log CFU/$cm^2$. *E. coli* O157:H7 did not contaminate the agar used throughout this study (Table 3). Frequency of contacts did not contribute significantly to contamination level, but significant differences were observed between the strains. Generally there were no significant differences between food model contaminations (Tables 3 and 4).

**Table 2**

Comparison of the residual biofilms in the strains at different frequencies of contacts (crystal violet binding assay).

| Strains | Residual biofilms (OD) | | |
|---|---|---|---|
| | 1 contact | 2 contacts | 3 contacts |
| *E. coli* | 0.002 0$^{Ca}$ | 0.002 0$^{Ca}$ | 0.002 0$^{Ca}$ |
| *L. monocytogenes* | 0.066 0$^{Ba}$ | 0.066 1$^{Ba}$ | 0.066 1$^{Ba}$ |
| *S. aureus* | 0.341 0$^{Aa}$ | 0.341 8$^{Aa}$ | 0.342 0$^{Aa}$ |
| *E. coli* O157:H7 | 0.021 0$^{Ba}$ | 0.021 0$^{Ba}$ | 0.021 0$^{Ba}$ |
| *L. monocytogenes* (H7762) | 0.285 0$^{Aa}$ | 0.286 0$^{Aa}$ | 0.285 0$^{Aa}$ |

*L. monocytogenes* (H7762): Reference strain; Means in the same column not followed by the same upper case letter are statistically significant ($P$<0.05); Means in the same row not followed by the same lower case letter are statistically significant ($P$<0.05).

**Table 3**

Mean microbial contamination (log CFU/m$^2$) levels of the various strains on the food models.

| Isolate | Food models | | |
|---|---|---|---|
| | Agar | Meat | Soft white cheese |
| *E. coli* | 7.61$^a$ | 7.39$^a$ | 7.64$^a$ |
| *L. monocytogenes* | 7.62$^a$ | 7.65$^a$ | 7.53$^a$ |
| *S. aureus* | 7.19$^b$ | 6.71$^b$ | 7.19$^a$ |
| *E. coli* O157:H7 | 0.00$^c$ | 7.61$^a$ | 7.61$^a$ |
| *L. monocytogenes* (H7762) | 7.64$^a$ | 7.63$^a$ | 7.63$^a$ |

*L. monocytogenes* (H7762): Reference strain; Means with the same lower case letters in the same row are not significantly different ($P$<0.05).

**Table 4**

Influence of various factors on the formation of biofilms and contamination by strains.

| Factors | | Biofilm mass (A600) |
|---|---|---|
| Incubation time ($n$=30) | 24 h | 0.137 4$^C$ |
| | 48 h | 0.389 4$^A$ |
| | 72 h | 0.309 4$^B$ |
| Frequency of touch ($n$=135) | 1 | 0.160 3$^A$ |
| | 2 | 0.160 3$^A$ |
| | 3 | 0.160 0$^A$ |
| Bacterial strains ($n$=135) | *E. coli* | 0.002 0$^B$ |
| | *L. monocytogenes* | 0.066 0$^B$ |
| | *S. aureus* | 0.342 0$^C$ |
| | *E. coli* O157:H7 | 0.021 0$^{AB}$ |
| | *L. monocytogenes* (H7762) | 0.029 0$^{ABC}$ |
| Food models ($n$=135) | Agar | 7.500 0$^A$ |
| | Meat | 7.340 0$^A$ |
| | Soft white cheese | 7.490 0$^A$ |

Means in the same column not followed by the same upper case letter are statistically different ($P$<0.05); *L. monocytogenes* (H7762): Reference strain.

## 4. Discussion

The biofilm forming abilities at various degrees by strains used in this study is of significance since biofilms in food processing areas are major sources of food contamination. Similar reports of biofilm forming abilities of these strains have been made by earlier researchers[1,24]. In addition, the hydrophobicity of a surface is an important criterion in biofilm formation hence the glass coupon surface which is hydrophobic facilitates more bacteria adhesion and subsequent biofilm formation[25]. The reason for the decrease in biofilm mass from 48 h to 72 h in *L. monocytogenes*, *S. aureus* and *E. coli* O157:H7 is unknown but possibly due to nutrient depletion and accumulation of bacterial waste and metabolic products in the growth media. Nutrient induced biofilm dispersion has been observed in *Pseudomonas aeruginosa* PAO1 in earlier studies[26].

A comparism of the level of cellulose (a polysacharide) produced by strains at 24, 48 and 72 h incubation in this study did not follow a clear trend. Earlier studies had shown that statistical analysis of biofilm parameters suggest that biofilm development is a more complex process than that simply described by the production of attachment and matrix components and bacterial growth[27]. Similar to findings of inverse relationship between cellulose and biofilm production in pathogens in this present study, Ude *et al*. reported a negative correlation ($R^2$) of −0.045 to −0.227 7 in wild *Pseudomonas* isolates[27]. The strong positive correlations for *E. coli* O157:H7 is expected since biofilm mass is dependent on polysaccharide production. The production of cellulose as an indication of a virulence factor in foodborne pathogens of food processing relevance has been reported. *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. isolated from the human gut were investigated for the biosynthesis of cellulose and curli fimbriae (chronic superficial gastritis). While *Citrobacter* spp. produced curli fimbriae and cellulose, *Enterobacter* spp. produced cellulose under various temperature conditions; *Klebsiella* spp. did not show pronounced expression of those extracellular matrix components[28]. However this finding is at par with the earlier report made by Midelet and Carpentier who reported a positive correlation between biofilm and cellulose production[21]. This difference may be due to the differences in surfaces used for bacteria adhesion and the fact that biofilms constitutes other components other than cellulose.

The highest residual biofilm in *S. aureus* followed by *L. monocytogenes* (H7762) in this present study is in agreement with earlier studies which have shown that biofilm-forming ability in *Staphylococci* is increasingly recognized as an important virulence factor facilitating their persistence in the host. Similarly Midelet and Carpentier reported a listerial strain to be more strongly attached to polymers[21]. The difference in surfaces used for biofilm development may be the reason for this slight difference.

The inability for frequency of contact to affect contamination level can be inferred to be an indication of similar attachment strength by these pathogens, although the assessment of attachment strength was not part of the objective of this study. The amount of microbial transfer

from an inert surface to a food depends on the various characteristics or properties of the biofilm thus strengthening the findings in this study. These properties include: surface density of the microbial population, the structure of the biofilm, its capacity to produce exopolysaccharides (cellulose) and the attachment strength of the microbial population, which is probably also linked to these earlier listed properties[21]. This implies that factors other than frequency of contact may have been responsible for the level of contamination and therefore must be explored in feature studies.

Similar food model contamination in this study is possibly due to strong microbial attachment and inaccessibility due to internalization and aggregation of cells in biofilm, however, *E. coli* O157:H7 did not contaminate the agar used throughout this study. The contamination level of food models is of public health significance. This finding is in agreement with that of Oliveira *et al*. who reported biotransfer of bacterium to food substrates at all stages of biofilm formation in *L. monocytogenes*[29]. Midelet and Carpentier also reported contaminating potential of >6.00 log CFU/cm$^2$ on meat by *L. monocytogenes*[21]. *L. monocytogenes* strains produced greater microbial load contamination than *E. coli* species in this study. A similar report was made by Sommer and Odetokun in biofilms on stainless steel, and the bacterial population was higher with Gram-negative strains than with Gram-positive strains[8] . In addition to contamination level, the microbial contamination also gives a rough estimate of the attachment strengths of cells in a biofilm, this was consistent with earlier observations made by Eginton *et al.*, and Midelet and Carpentier where *Listeria* attached more strongly to polymers[21,30]. Greater than 6.0 log CFU/mL contamination levels were obtained on all the food models for the 3 species of organisms tested. A zero tolerance limit per gram exists for *L. monocytogenes* and *E. coli* O157:H7 in cheese and meat, while a limit of <100/g and <10/g exists respectively for *S. aureus* and *E. coli* (FAO, 2013). The values obtained in this study were much higher than these limits, thus showing a high level of risk of contamination of food models. Similar inferences can be made from earlier studies by Adeyemi *et al.* and Adetunji *et al*. where report of high contamination were made in ready-to-eat vended food items and in milk and milk products respectively in Ibadan, South-Western Nigeria[31,32]. Furthermore, a prevalence of 87.9% of uropathogenic *E. coli* was reported by Ponnusamy *et al*.[33], 62.2% of these strains had biofilm forming abilities thus corroborating the findings in this study.

Overall in this study there was no significant difference ($P$>0.05) in effect of food model and frequency of touch, but the influence of strain and incubation time was significant. This finding indicates the importance of strain characteristics and growth period for contamination and biofilm formation thereby raising the need for further studies in the genetic compositions and optimum growth condition for biofilm formation by these strains under study.

All the strains used in this study produced biofilms and cellulose at various degrees, this is of significance, since biofilm forming abilities of these strains also indicate that these pathogens have ability to persist in the environment and will resist most cleaning and disinfecting procedures. It can be inferred from this study that proper sanitary procedures to remove biofilms or prevent their formation will go a long way to reduce bacterial contamination from food contact surfaces. This is of particular importance in developing countries where unsuitable surfaces like wood are used for food processing in poor sanitary environment. These research results should support the development of policies, guidelines, standards and regulations in an effort to find solution to food safety problems in Nigeria and the world at large.

## Conflict of interest statement

We declare that we have no conflict of interest.

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