FORMATION OF BIOFILMS ON FOOD CONTACT SURFACES

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ABSTRACT

Seventy five samples of food contact surfaces including stainless steel, wood and plastic (25 samples of each) were collected from food service establishments located in Ismailia and Port Said governorates. These samples were analyzed for naturally occurring biofilms. The results demonstrateed that these surfaces can harbour high attached numbers of food poisoning and spoiling microorganisms especially wood and there was no major difference in isolated organisms between surfaces. The attached microorganisms (either pioneer or secondary colonizers) include Proteus vulgaris, Proteus mirabilis, Bacillus cereus, Bacillus coagulance, Bacillus subtilis, Bacillus stearothermophilus, Bacillus megaterium, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas putida, Pseudomonas fragi, Citrobacter freundii, Clostridium perfringens, Enterobacter aerogenes, Enterobacter gergoviae, Escherichia coli, Klebsiella pneumonae, Klebsiella granulomalis, Enterococcus faecalis, Enterococcus faecium, Streptococcus pyogenes and Salmonella species. Meanwhile Listeria could not be isolated from tested surfaces. Wood surface represent higher counts than other surfaces, this may be attributed to its physicochemical characters and unsanitary measures adopted in examined establishments.

INTRODUCTION

Biofilms can be defined as phenomena that mean collections of microorganisms surrounded by the exopolysaccharide matrix they secrete which attached to either an inert or living surface. Typically, anywhere that there is a flow of water, organisms and a solid surface, a biofilm can be formed. (***Virginia, 2002***).

The microbiologists were discovered that biofilms were the predominant forms of bacterial growth. Since then, it has become apparent that organisms can behave very differently when living within a biofilm than when the same species is floating freely. Studies revealed that more than 99 % of all bacteria live in biofilm communities and they are increasingly being recognized as the preferred mode of growth of microbes in a wide range of habitats (***Yeo, 2002***). Biofilm has become a more popular research topic in many other areas in recent years including food safety (***Trachoo,2003***). Microbial attachment has been shown to occur on food contact surfaces and these attached microbes, if left nudisturbed, will form biofilms (***Zottola and Sasahara, 1994***). Growth of biofilms in food processing environments leads to increased opportunity for microbial contamination of the processed product. These biofilms may contain spoilage and pathogenic microrganisms (***Frank and Koffi, 1990; McCarthy, 1992 and Ronner and Wong, 1993***). This increases the risk of reduced shelf life and disease transmission. EPS substances associated with biofilm that are not removed by cleaning provide attachment sites for microorganisms newly arrived to the cleaned system (***Hood and Zottola, 1997***). Nowadays, the food hygienist faces a tremendous challenge in overcoming problems stemming from the formation and persistence of bacterial biofilms in food industry. So, we can say understanding biofilms and their related topics is one step toward preparing for the future, therefore this work was planned to fulfil the following:

A. Detection of naturally occurring biofilms on the food contact surfaces.

B. Confirmation of biofilms forming ability of isolated microorganisms.

MATERIALS & METHODS

A. Detection of biofilms on the food contact surfaces: Seventy five samples were taken from the washed, cleaned and stored stainless steel, wood and plastic food contact surfaces. The area was firstly measured (10 cm^2) by using a template (2X5 cm) and swabbed by 70 % alcohol to eliminate the surface contaminants. The sampling procedures were carried out according to ***Sinde and Carballo (2000)*** and ***Donald (2007)***.

MICROBIOLOGICAL TECHNIQUES:

The microbiological methods for ACC, *Staphylococcus aureus*

counts, Enterobacteriacae counts, incidence of salmonella and Listeria were carried out as reco-mmended by ***ISO.*** And for *Clostridium perfringens* (MPN/cm^2) was deter-mined according to ***Beerens et al. (1980).*** Total aerobic spore-forming and coliforms counts were carried out as recommended by *APHA (1992).*

B. Confirmation of biofilms forming ability of isolated

MICROORGANISMS:

The biofilms forming ability of 21 identified strains were confirmed. This was done by generation of experimental biofilms in vitro. The procedures were carried out as described by ***Kae et al. (2002)*** and ***Pan et al. (2006):***

Preparation of substrates for biofilms formation: Glass slide coupons were used to simulate food processing system (one coupon for each isolate). These coupons were precleaned with 1 NHCl and washed with distilled water. The treated glass slides were placed in wide mouth glass container and the slides and containers were subjected to steam sterilization at 121 °C for 15 min.

Preparation of cell suspensions: Under aseptic conditions, one colony from each isolated strain was picked from slope agar and inoculated onto plates of nutrient agar and incubated at 37 °C for 48 hours. Then one or two colonies were inoculated into 10 ml of sterile 1% peptone solution and incubating at 37 °C for 18 hours to obtain overnight cultures. Then subcultures were prepared by transferring 1 ml of each overnight culture aseptically into 500 ml flask containing 250 ml of sterile 1% peptone solution and incub-ating them at 37 °C for 18 hours.

Cell attachment and biofilms formation: The cell suspensions were added aseptically to the previously prepared containers until the slides were completely submerged. After 3 hours of incubation at 37 °C to allow cell attachment, then slides were gently removed and washed three times with sterile saline to remove loosely attached cells. The slides were then transferred into sterile flasks containing 30 ml of nutrient broth. Then the flasks were incubated at 22.5 °C for 48 hours to allow biofilms development.

Detection of attached bacteria based on light microscopy: After incubation period, each slide was picked up aseptically and then washed gently with sterile distilled water. The attached bacteria on a glass slide were detected under a light microscope after staining with 0.3% Methylene blue.

RESULTS & DISCUSSION:

The mean values of total aerobic colony counts for biofilms were $2.05 \times 10^2 \pm 1.56 \times 10^2$, $5.89 \times 10^4 \pm 5.82 \times 10^4$, and $1.53 \times 10^4 \pm 1.5 \times 10^4$ CFU/cm^2 for stainless steel, wood and plastic food contact surfaces respectively. While the mean values of total aerobic spore forming counts for biofilms were $1.23 \times 10^2 \pm 1.39 \times 10^2$, $3.75 \times 10^3 \pm 5.56 \times 10^3$, and $1.24 \times 10^2 \pm 9.1 \times 10$ CFU/cm^2 for stainless steel, wood and plastic food contact surfaces respectively. And the mean counts of Staphylococci for biofilms on different food contact surfaces were $2.11 \times 10^2 \pm 1.37 \times 10^2$, $9.14 \times 10^3 \pm 1.17 \times 10^4$ and $1.32 \times 10^2 \pm 8.8 \times 10$ CFU/cm^2 for stainless steel, wood and plastic surfaces respectively. The mean counts of coliforms on the food contact surfaces were $1.3 \times 10 \pm 1.1 \times 10$, $3.1 \times 10 \pm 5.2 \times 10$ and $3 \times 10 \pm 3.7 \times 10$ MPN/cm^2 for stainless steel, wood and plastic surfaces respectively. And the mean Enterobacteriacae counts were $5.5 \times 10 \pm 4.1 \times 10$, $5.99 \times 10^3 \pm 6.65 \times 10^3$ and $1.79 \times 10^2 \pm 1.18 \times 10^2$ CFU/cm^2 for stainless steel, wood and plastic surfaces respectively. While the mean values of *Clostridium perfringens* counts on examined food contact surfaces were $0.7 \times 10 \pm 3 \times 10$, $1.3 \times 10 \pm 3.7 \times 10$ and $0.1 \times 10 \pm 0.5 \times 10$ MPN/cm^2 for stainless steel, wood and plastic surfaces respectively. In addition, salmonella could be isolated from only one sample out of 25 samples of wood surface (4%). Meanwhile they could not be isolated from stainless steel and plastic surfaces. On the other hand, Listeria could not be detected on all examined food contact surfaces. The frequency distribution of identified strains of bacteria in biofilms and their percent on the examined stainless steel food contact surfaces were *Proteus vulgaris* (12.38%), *Bacillus coagulance* (10.31%), *Staphylococcus aureus* (10.31%), *Bacillus cereus* (8.25%), *Proteus mirabilis* (8.25%), *Bacillus subtilis* (7.21%), *Pseudomonas fragi* (6.18%), *Citrobacter freundii* (6.18%), *Clostridium perfringens* (5.15%), *Bacillus stearothermophilus* (5.15%), *Enterobacter gergoviae* (4.13%), *Bacillus megaterium* (4.13%), *Enterobacter aerogenes* (4.13%), *Pseudomonas putida* (3.09%), *Escherichia coli* (3.09%) and *Klebsiella pneumonae* (2.06%). And the frequency distribution of identified strains of bacteria in biofilms and their percent on the examined wood food contact surfaces were *Pseudomonas fragi* (10.62%), *Bacillus megaterium* (9.38%), *Staph.epidermidis* (9.38%), *Bacillus cereus* (8.12%), *Bacillus stearothermophilus* (6.88 %), *Enterobacter aerogenes* (6.88%),

Proteus mirabilis (6.25%), *Citrobacter freundii* (5.63%), *Staph. aureus* (5%), *Clostridium perfringens* (3.75%), *Enterococcus faecalis* (3.75%), *Bacillus coagulance* (3.75%), *Pseudomonas putida* (3.12%), *Klebsiella granulomalis* (3.12%), *Enterobacter gergoviae* (2.5%), *Escherichia coli* (2.5%), *Klebsiella pneumonae* (2.5%), *Bacillus subtilis* (1.87%), *Enterococcus faecium* (1.87%), *Proteus vulgaris* (1.25%), *Streptococcus pyogenes* (1.25%) and Salmonella spp. (0.63%). While the frequency distribution of identified strains of bacteria in biofilms and their percent on the examined plastic food contact surfaces were *Staphylococcus aureus* (13.76%), *Bacillus subtilis* (12.84%), *Bacillus cereus* (11.92%), *Streptococcus pyogenes* (10.09%), *Bacillus coagulance* (9.18%), *Proteus mirabilis* (9.18%), *Citrobacter freundii* (8.26%), *Proteus vulgaris* (6.42%), *Staphylococcus.epidermidis* (4.59%), *Pseudomonas fragi* (4.59%), *Enterobacter aerogenes* (3.67%), *Klebsiella granulomalis* (2.75%) and *Clostridium perfringens* (2.75%). The identified strains isolated from the tested food contact surfaces may have a great public health importance as it contains food poisoning and spoilage micro-organisms. Their higher count in the samples may be responsible for increasing contamination probability. The isolation of Proteus spp. is indicative to environmental and faecal pollution as they inhabit the intestinal tracts of humans and animals as opportunistic pathogen. Also they can be found in soil, water and faecal matter. Pseudomonas spp. are considered as a spoilage microflora because some species can induce taste defects or produce fluorescent dyes that lead to visual defects on food and its contact surfaces. The isolation of Pseudomonas spp. seems to agree with ***Van Haecke et al. (1990), Hood and Zottola (1997), Barnes et al. (1999), Bagge et al. (2001), Giaouris and Nychas (2006)*** and ***Matthew (2009).*** Also, there are many isolated faecal coliforms including *Escherichia coli,* Enterobacter, Klebsiella and Citrob-acter. The presence of such microorganisms is thus an effective confirmation of fecal contamination. Klebsiella spp. found in the normal flora of the mouth, skin, and intestines of humans and their presence mainly indicates unhygienic handling of foods and their contact surfaces. Citrobacter spp. can be found almost every-where in soil, water, wastewater, etc. They can also be found in the human intestine and their isolation from tested surfaces indicates environmental pollution and inadequate sanitation. *E. aerogenes* is generally found in the human gastrointestinal tract and it has been found to live in various wastes, hygienic chemicals, and

soil. The Enterobacteriacae contamination may be arising from the sewage pollution of water supply or unhygienic personal habits during handling of examined surfaces. The presence of Staphylococci or their heat stable enterotoxins on the food contact surfaces are considered as indication of poor sanitation. They can lead to severe public health hazard and identified as the causative agents in many food poisoning outbreaks ***(Beckers et al., 1985 and Bennett et al., 1986)***. It is obvious that the danger of the isolated staphylococci which can be summarized in their spoilage effect resulting in shortening the shelf life in addition to the food poisoning. Especial attention to *Staphylococcus aureus,* which is frequently found as a part of the skin flora, *S. aureus* is capable of secreting several toxins. *Staphylococcus epidermidis* is part of skin flora, and consequently part of human flora. It can also be found in the mucous membranes and in animals. The isolation of Staphylococci from food contact surfaces in present study was in agreement with ***Virginia (2002), Moretro et al. (2003), Qin et al. (2007), Rode et al. (2007)*** and ***Simone et al. (2007)***. Streptococci are also part of the normal commensal flora of the mouth, skin, intestine, and upper respiratory tract of humans. *S. pyogenes* is the cause of many important human diseases ranging from mild superficial skin infections to life-threatening systemic diseases. Infections due to certain strains of *S. pyogenes* can be associated with the release of bacterial toxins. These toxins reflects the harmful effect of this microorganism on human health that can be life threatening. The isolation of Streptococci from tested food contact surfaces declared the harmful effect of mishandling of the food contact surfaces. The isolation of Enterococci in present study was in agreement with ***Gomes et al. (2008)***. The two Enterococcus species isolated from wood food contact surface considered the common commensal organisms in the intestines of humans: The Enterococcus species is closely related to Streptococci and also reflects the unsanitary precautions that conducted in tested food serving establishments. The isolation of Bacillus species is another indicator for environmental pollution and unhygienic dealing with food contact surfaces in tested food serving establishments. *Bacillus cereus* is responsible for a minority of food-borne illnesses (2–5%), causing severe nausea, vomiting and diarrhea. General speaking, Bacillus food-borne illnesses occur

due to survival of the bacterial endospores when food is improperly cooked. *Bacillus subtilis* is commonly found in soil and it is not considered a human pathogen; it may contaminate food but rarely causes food poisoning. *B. subtilis* produces the proteolytic enzyme subtilisin. *Bacillus coagulants*, it is reported that it is non pathogenic strain of genus bacillus and *Bacillus megaterium* is one of the largest eubacteria found in soil. *Bacillus stearothermophilus* is widely distributed in soil, hot springs, ocean sediment, and is a cause of spoilage in food products. It is commonly used as a challenge organism for sterilization validation studies and periodic check of sterilization cycles. The isolation of Bacillus spp. was met with ***Te Giffel et al. (1997)** and **Ryu and Beuchat** (2005).* Also *Clostridium perfringens* is ubiquitous in nature and can be found as a normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates, insects, and soil. The *C. perfringens* produce heat-labile enterotoxin mediating the food poisoning. The confirmation of biofilm forming ability revealed that *Proteus vulgaris, Proteus mirabilis, Bacillus cereus, Bacillus subtilis, Bacillus coagulance, Bacillus stearothermophilus, Bacillus megaterium, Pseudomonas putida, Pseudomonas fragi, Enterobacter gergoviae, Enterobacter aerogenes, Escherichia coli* and Salmonella were able to attach to the tested surfaces. Meanwhile, *Staphylococcus aureus, Staphylococcus epidermidis, Citrobacter freundii, Klebsiella pneumoniae, Klebsiella granulomatis, Enterococcus facealis, Enterococcus faecium* and *Streptococcus pyogenes* were not able to attach to the tested surfaces. It is evident from these data that motile strains and Bacillus species were attached easily to the surfaces. This is may be attributed to the hydrophobic character of the spores, and facilitated movement by flagellae. On the other hand, the strains that not able to attach to the surfaces may be considered secondary colonizers that trapped from the environment after primary adhesion of pioneer microorganisms.

Table (1): Statistical results of microbial counts for biofilms on different food contact surfaces.

Microorganism	Stainless steel			Wood			Plastic		
	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD
ACC	< 10	6.2×10^2	$2.05 \times 10^2 \pm 1.56 \times 10^2$	430	1.31×10^5	$5.89 \times 10^4 \pm 5.82 \times 10^4$	130	4.64×10^4	$1.53 \times 10^4 \pm 1.5 \times 10^4$
ASFC	< 10	5.4×10^2	$1.23 \times 10^2 \pm 1.39 \times 10^2$	< 10	1.9×10^4	$3.75 \times 10^3 \pm 5.56 \times 10^3$	< 10	2.6×10^2	$1.24 \times 10^2 \pm 9.1 \times 10$
Staph.aureus	< 10	5.7×10^2	$2.11 \times 10^2 \pm 1.37 \times 10^2$	370	3.8×10^4	$9.14 \times 10^3 \pm 1.17 \times 10^4$	< 10	2.6×10^2	$1.32 \times 10^2 \pm 8.8 \times 10$
Coliforms	< 3	3.8×10	$1.3 \times 10 \pm 1.1 \times 10$	3	2.4×10^2	$3.1 \times 10 \pm 5.2 \times 10$	< 3	1.6×10^2	$3 \times 10 \pm 3.7 \times 10$
Enterobact-eriacae	< 10	1.2×10^2	$5.5 \times 10 \pm 4.1 \times 10$	< 10	2×10^4	$5.99 \times 10^3 \pm 6.65 \times 10^3$	< 10	3.5×10^2	$1.79 \times 10^2 \pm 1.18 \times 10^2$
C. perfringens	< 3	1.5×10^2	$0.7 \times 10 \pm 3 \times 10$	< 3	1.6×10^2	$1.3 \times 10 \pm 3.7 \times 10$	< 3	2.7×10	$0.1 \times 10 \pm 0.5 \times 10$

Table (2): Incidence of Salmonella and Listeria on the different food contact surfaces.

Surface type	Salmonella			Listeria		
	Samples number	Positive samples	Percent	Samples number	Positive samples	Percent
Stainless steel	25	0	0 %	25	0	0 %
Wood	25	1	4 %	25	0	0 %
Plastic	25	0	0 %	25	0	0 %
Total	75	1	1.33 %	75	0	0 %

CONCLUSION:

Biofilms could be formed on food contact surfaces in food processing establishments. These biofilms harbour food poisoning and spoiling microbes that compromise the safety and quality of foods. The routine sanitary procedures in most tested establishments, which include the use of liquid soap and some times chlorine as sanitizing agent, are insufficient to control biofilm formation.

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