BIOFILM ASSESSMENT IN BACTERIA ISOLATES FROM *CLARIAS GARIEPINUS* AND *TILAPIA SPECIE*

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ABSTRACT: Biofilms in food processing has become a global concern since it reduces the microbial safety of food. This study determined the biofilm forming ability of *Escherichia coli (EC), Listeria monocytogenes (LM)* and *Staphylococcus aureus (SA)* isolates from *Clarias gariepinus* and *Tilapia specie* and assessed their antibiotic sensitivity profile. The ability of these pathogens to form biofilm on plastic and glass surfaces was assessed at ambient temperature using crystal violet binding assay. The highest total aerobic plate count and enterobacteriace count was from skin of both fish types. Isolates of *EC (6), LM (1)* and *SA (3)* were made from samples. These strains were found to be resistant or weakly sensitive (0–12mm) to the common antibiotics tested and were also capable of forming biofilms on both surfaces. An absorbance reading range of -0.012±0.003 - 0.107±0.001 and -0.095±0.000 - 0.555±0.002 was obtained for glass and plastics respectively. For cell enumeration a count (log_{10}cfu/cm^2) range of 2.651±2.651 - 5.128±0.651 was obtained for glass while a higher count of 2.651±2.651 - 6.102±0.570 was obtained on plastic surfaces. It was concluded that glass contact surfaces is more suitable than plastic as a contact surface in fish processing. Sanitary control in fish processing and proper storage conditions and selection of appropriate contact surfaces is therefore recommended.

[Adetunji VO, Shoola AAE, Odetokun IA. BIOFILM ASSESSMENT IN BACTERIA ISOLATES FROM *CLARIA GARIEPINUS* AND *TILAPIA SPECIE*. *Nat Sci* 2012;10(10):157-161]. (ISSN: 1545-0740). http://www.sciencepub.net/nature. 22

Key words: *Clarias gariepinus, Tilapia specie, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, crystal violet*

Introduction

The cheapest source of animal protein in Africa is fish (Clucas and Ward, 1996). Fish products could incubate pathogens from the natural micro-flora of fish ponds (Clucas and Ward, 1996).

Biofilm is a population of microbial cells that are enclosed within a matrix made of primarily polysaccharide material which have the ability to stick to surfaces (Donlan, 2002; Stepanovic *et al*., 2004). There is a compromise of food safety especially in minimally processed foods and raw foods due to the presence of biofilms on food and food contact surfaces (Frank and Chmielewski, 2001).

Microorganism can colonize to form biofilms on solid surfaces when adequate nutrients, minerals, and organic matter are present in fish processing environment. Free-living bacteria are more susceptible to sanitizers and antimicrobial cleaning agents than bacteria in biofilms. Biofilms are difficult to remove from food contact surfaces and equipment by normal cleaning, thus posing a food safety risk. (Kariyawasam and Asooriya, 2006). The presence and multiplication of opportunistic pathogens such as *E. coli (EC), Listeria monocytogenes (LM)* and *Staphylococcus aureus (SA)* is a concern in food processing and storage.

The main serotype of enterohemorrhagic *E. coli* (EHEC) is *E. coli* O157:H7. *E. coli* O157:H7. is the common cause of sporadic and multiperson outbreaks of bloody diarrhoea in the U.S. (Dean-Nystrom *et al*., 2003). The ability to attach, colonize, and form biofilms on various surfaces has been shown by *E. coli* O157:H7 (Uhlich *et al.,* 2006).

The main cause of staphylococcal infections is mainly *Staphylococcus aureus.* This pathogen is responsible for mild skin infections, invasive diseases and toxin mediated diseases and it is resistant to a wide range of commonly used antibiotics (Siddiqi *et al*., 2002). Recently a report of about 40% resistance which increases yearly was made to methicillin (Akhi *et al*. 2008). The adhesion ability of *S. aureus* on catheters and other indwelling medical devices and form biofilm on polymeric surfaces have been reported (Cramton *et al*., 1999).The adhesion capacity depends production of polysaccharide intercellular adhesion(PIA) encoded by the intercellular adhesion gene cluster icaADBC (Cramton *et al*., 1999).

Listeria monocytogenes is an important hardy food borne pathogen(USFDA-CFSAN, 2006) responsible for major outbreaks associated with dairy and other food products *(*Farber and Peterkin, 1991, Fleming, 1998*,* Adetunji *et al*., 2003*).* The organism is ubiquitous throughout nature and is frequently isolated from food processing environment (Hood and Zottola, 1997; Destro et al., 1996; Johansen *et al*., 1997). It has also been reported that *Listeria monocytogenes* has ability to form biofilms on food contact surfaces (Adetunji and Adegoke, 2008; Adetunji and Isola, 2011.

Information on biofilm forming ability of bacteria isolates of fish is scarce. This study therefore assayed microbial load in *C. garipeinus* and *Tilapia spp* and assessed the ability of *E. Coli* O157:H7*(EC), Listeria monocytogenes(LM)* and *Staphylococcus aureus(SA*) isolates from these fish types to form biofilms on glass and plastic surfaces.

Materials and methods

This study was conducted in Ibadan city located in South Western Nigeria over a period of 7 months (November, 2011 – May, 2012).

Sampling

Sixty (60) fish samples, 20 each of *Tilapia spp* and *Clarias gariepinus* where obtained from:

Eleyele river, fish farms (University of Ibadan Fish Farm, Alfa's Farm) and fish markets (Officer's Mess, Bodija market and a major frozen meat outlet all in Ibadan, Oyo state.

Fish samples were collected aseptically into sterile sample bottles and transported on ice to the Food Hygiene Laboratory (Department of Veterinary Public Health, University of Ibadan) for immediate analysis.

Enumeration of microbial counts

Two centimetres square ($2cm^2$) area of the fish samples were swabbed and placed in sterile tubes containing 10ml sterile 0.1% peptone water and vortexed. Five gram fish muscle was also cut aseptically and homogenized in 10ml sterile 0.1% peptone water. Serial dilutions of both mixture was then made to 10^{-4}, 0.1ml of the final dilution was then plated on plate count agar for total aerobic plate count and on MacConkey agar for *Enterobacteriaceae* counts. All plates were incubated at 37˚C for 24 hours. At the end of incubation discrete colonies were counted using digital colony counter (Lapiz $^{(R)}$ Med. Instr. Mfg. Co., Mumbai, India) and counts were expressed as $\log_{10}$ CFU/ gm/cm^2.

Bacteria identification

Two colonies were picked from each plate count agar and MacConkey agar plates. Colonies were purified thrice and further characterised using microscopic morphology and biochemical tests according to Barrow and Feltham, 1993. Serological assay also done using specific antisera for *Listeria spp* and *E. coli* O157:H7.

Quantification was done at ambient temperature for 72 hours incubation.

Biofilm assessment

For biofilm assessment 5 isolates (*E. coli O157:H7*(*2*), *Staphylococcus aureus* (2), and *Listeria monocytogenes (1)*) were used for biofilm assessment on glass and plastic chips. A biofilm forming human strain of *Listeria monocytogenes* (H7622) was used as a positive control. Seven (7) sterile glass jars containing 100ml of Tryptone soy broth were used for biofilm assessment of the test strains and negative/positive control) (Stepanovic *et al*., 2004).

An overnight culture of strains was inoculated into appropriate glass jars. Glass and plastic chips were then placed in 4 replicates for quantification of biofilm. Cell adhesion and biofilm were allowed to develop at ambient temperature for 72hrs incubation. Quantification of biofilm was done using Cell enumeration (Joseph *et al.,* 2001) and Crystal Violet Binding Assay (Stepanovic *et al*., 2004; Adetunji and Adegoke, 2008) methods.

The Optical density of each resolubilised liquid was measured against a blank reading without inoculum of test strains at λ of 520nm for *E. coli*, 620nm for *L. monocytogenes* and 570nm for *Staphylococcus aureus* using a spectrophotometer (Springfield, UK).

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) 17.0 statistics package for Windows. The Mean differences of variables were compared using student T-Test and analysis of variance (ANOVA). P-values of < 0.05 were considered as significant.

Results

The difference in counts in the two fish types were not significantly different ($P<0.05$). *Enterobacteriaceae* counts (EC) and Total aerobic plate counts (TAPC) were higher in skin surfaces than tissue samples. TAPC was in the range of 5.618±0.056 - 6.753± 0.064 while EC was in the range of 5.707±0.058 and 6.808±0.032 in the two fish types (Table 1). One of the samples of *Tilapia spp* tested positive for *L. monocytogenes*. Six samples tested positive for *E. coli* O157:H7 (4 from *Tilapia spp* and 2 from *C. gariepinus)* sourced from the market. Three *C. gariepinus* sourced from the market were also positive to *S. aureus*. All these isolates were from the skin samples. These strains were found to be resistant or weakly sensitive (0 – 12 mm) to the common antibiotics tested (data not shown).

Biofilm Quantification

All the pathogens produced biofilms using the crytal violet binding assay on both plastic (0.095±0.000 - 0.555±0.002) and glass (0.012±0.003 - 0.107±0.001) surfaces except for SAI (which did not produce biofilm on glass) and ET (which did not produce biofilm on both glass and pastic surfaces) (Table 2). With the use of cell enumeration, the mean

values showed no significant differences between strains and within strains, however a count range of 2.651±2.651- 5.128±0.651log$_{10}$cfu/cm^2 was obtained for glass while a higher count of 2.651±2.651 - 6.102±0.570 log$_{10}$cfu/cm^2 was on plastic surfaces. *E. coli* O157:H7 from Tilapia (ET) produced the highest biofilm (6.102±0.570), while *L. monocytogenes* produced the lowest (2.651±2.651) on plastics. Significant differences were observed between test isolates (Table 3).

Table 1: Mean microbial counts in log$_{10}$cfu/gm/cm^2 in tissue and skin samples from Tilapia and Clarias speices

Counts (log cfu/g)	Dilution levels	Tilapia tissues Mean±S.E	Tilapia skin Mean±S.E	Clarias tissues Mean±S.E	Clarias skin Mean±S.E
TAPC	10^3	5.618±0.056^{Aa}	5.715±0.036^{aA}	5.781± 0.086^{aA}	5.840± 0.062^{aA}
	10^4	6.518±0.046^{Ba}	6.536±0.034^{bA}	6.753± 0.064^{bA}	6.437± 0.229^{bA}
EC	10^3	5.707±0.058^{aA}	5.925±0.037^{aA}	5.809± 0.059^{aA}	5.940± 0.054^{aA}
	10^4	6.558±0.046^{Ba}	6.808±0.032^{bA}	6.673± 0.071^{bA}	6.772± 0.044^{bA}

TAPC=Total aerobic plate count; EC=Enterobactericea count
Different lower case letters in a column are significantly different at $p < 0.05$
Similar upper case letters in a roll are not significantly different at $p < 0.05$

Table 2. Absorbance of biofilms on glass and plastic surfaces

Strains	N	Glass (mean± SE)	Plastic (mean± SE)
***L.monocytogenes*(H7762)**	2	0.168±0.009a	0.555±0.002b
Uninoculated control	2	-0.010±0.000c	-0.051±0.000c
SA1	2	-0.073±0.001a	0.525±0.003b
SA2	2	0.105±0.000a	0.555±0.002b
ET	2	-0.012±0.003c	-0.095±0.000c
EC	2	0.050±0.000a	0.222±0.002bc
LMT	2	0.107±0.001ab	0.208±0.000bc

SA1 & SA2=*Staphylococcus aureus* from *C. gariepinus*; ET=*E. coliO157:H7* from *Tilapia specie*; EC= *E. coliO157:H7* from *C. gariepinus; LMT=Listeria monocytogenes from Tilapia specie* ; N=number; The negative values indicate no biofilm production Values with different alphabets along a row are highly significantly different($p<0.05$) Values with the same alphabets along a row are not significantly different ($p<0.05$)

Table 3. Mean counts (log$_{10}$cfu/cm^2) of biofilms cell enumeration on glass and plastic surfaces

Strains(label)	N	Glass mean±SE	Plastic mean±SE
***L.monocytogenes*(H7762)**	2	4.849±0.151^{aa}	5.000±0.301^{aa}
Uninoculated control	2	<1.0±0.00^{aa}	<1.0±0.00^{aa}
***S.aureus*(SA1)**	2	4.500±0.500^{aa}	4.500±0.500^{aa}
***S.aureus*(SA2)**	2	4.651±0.651^{aa}	5.039±1.039^{aa}
***E.coli*O157:H7(ET)**	2	2.651±2.651^{aa}	6.102±0.570^{aa}
***E.coli*(EC)**	2	4.651±0.651^{aa}	5.128±0.651^{aa}
***L.monocytogenes* LMT**	2	2.651±2.651^{aa}	2.651±2.651^{aa}

N=number; Counts with same alphabets 'aa' between coupons shows no significant difference ($p<0.05$) along the same row.

DISCUSSION

The high microbial load and pathogens isolated from these fish samples is because of the polluted water from which they were harvested (Adeyemo 2003). It also depicts a deplorable state of poor hygiene and sanitary practices employed in processing and packaging of fish and frozen fish. Similar microbial counts were made in an earlier study in fish tissues (Ola, and Oladipo, 2004). The presence of *E. coli* O157, *S. aureus* and in fish samples a public health concern since a zero tolerance level exist for all these strains(codex alimentarius). These findings agree with previous reports by Okonko *et al*., (2009) who also reported high counts of *Enterobacter* spp., *S. aureus* and *E. coli* in a study of sea food products. The highest EC and TAPC were from skin samples which might be because of the scale of *Tilapia spp* and high microbial load in water for fish culture.

All the species from this study produced biofilm. This is an indication that these pathogens

have ability to persist in the environment since pathogens in biofilm are resistance to most cleaning and disinfecting procedures. The higher biofilms formed on Plastic is in agreement with the report of Sinde and Carballo, 2000 and Donlan, 2002, who reported that glass and stainless steel are hydrophilic materials while wood and plastic are hydrophobic materials. Hydrophobic materials are reported as surfaces that provide greater bacteria adherence Djordjevic *et al*., 2002. Appropriate contact surfaces will minimize biofilm forming abilities of pathogens.

CONCLUSION

The pathogenic isolates in this study have high biofilm forming ability. These organisms formed biofilms more on plastic surfaces than on glass surfaces, therefore proper selection of fish contact surfaces can help reduce the bacteria forming biofilms in fish processing environments. This is important in food industry applications because the hygiene of the surfaces affects the overall quality and safety of the product.

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8/21/2012