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Assessment of Biofilm in *E. coli* O157:H7 and *Salmonella* Strains: Influence of Cultural Conditions

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ABSTRACT

The aim of this study was to assess biofilm formation by some pathogenic strains of *E. coli* and *Salmonella* sp. using glass and cement coupons, varying nutrient-culture media and two incubation temperatures (refrigeration (11°C) and room (28±2°C)). The Crystal Violet Binding Assay (CVBA) and Bacteria Cell Enumeration (BCE) methods were used to quantify *E. coli* and *Salmonella* biofilms in 5 days while Scanning Electron Microscopy (SEM) was utilized in viewing the formed biofilms. Though, it was observed that all tested organisms did not grow at the same rate, generally, *Salmonella* sp. produced higher biofilms than *E. coli* O157:H7 strains. More biofilms were formed on cement surfaces than glass and was confirmed with SEM. Biofilms developed on the coupons were higher at room temperature and in most cases higher in media with increased nutrient concentrations. With these results, it can be deduced that there is a higher presence of biofilms formed by pathogenic organisms in our food processing plants where the principles of hygiene are not held in high esteem. Therefore, regular evaluation of operation cleanliness in the food processing environments is highly necessary in order to control these pathogens and their biofilms.

Key words: Biofilm, *E. coli* O157:H7, *Salmonella* sp., crystal violet binding assay, cell enumeration, biofilms

INTRODUCTION

Common pathogenic bacteria that may be causes of foodborne diseases include strains of *Salmonella* and *Escherichia coli* (Sockett, 1991). Many high risk pathogens that cause diseases in humans are transmitted through various food items (Hassanain, 2008). *E. coli* O157:H7 infection has considerable economic impacts. *Escherichia coli* O157:H7 has emerged with increasing frequency as a foodborne pathogen of concern over the last 20 years and is responsible for causing serious illness and severe sequelae in susceptible humans (Bacon and Sofos, 2003). *Escherichia coli* O157:H7 was first identified as being responsible for causing outbreaks of enteric infection in 1982 (Riley *et al.*, 1983). Symptoms include diarrhoea, haemorrhagic colitis and haemolytic uraemic syndrome (Doyle, 1991; Doyle *et al.*, 2001). *Salmonella* is ubiquitous geographically and zoologically (Hirsh, 2005). The occurrence of *Salmonella* can contaminate food anywhere along the farm to fork continuum (D'Aoust, 1997; Small *et al.*, 2006). Poultry products are regarded as the primary vehicles of *Salmonella* transmission (Geornaras and von Holy, 2000). Ready-to-eat products are typically contaminated during post-processing steps (Mbandi and Shelef, 2002).

Post-processing contamination is largely contributed to poor handling practices (De Cesare *et al.*, 2003). According to FDA the number of cases of salmonellosis is 2-4 millions/year in the US and the frequency is rapidly increasing. Especially *S.* enteritides is rapidly spreading in US and Europe (Enfors, 2008). All foodborne salmonellosis infections are non-typhoidal (Bailey and Maurer, 2005) and this is a major cause of food borne illness (Hassanain, 2008). Non-typhoidal salmonellosis caused more outbreaks and cases of food poisoning than all other bacteria between 1993 and 1997 (CDC, 2000). *Escherichia coli* O157:H7 is an emerging food pathogen that was first identified as a cause of illness in 1982 (Riley *et al.*, 1983). Rangel *et al.* (2005) and CDC (2000) reported *E. coli* O157:H7 to be responsible for about 73,000 illnesses, 2,000 hospitalizations and 60 deaths in the United States each year. A biofilm can be defined as an assemblage or population of microbial cells that is irreversibly associated (not removed by gentle rinsing with a surface) and enclosed in a self-produced matrix of primarily polysaccharide material having possessed the ability to stick to wet surfaces while undergoing a multi-step developmental process (Abee *et al.*, 2011; Donlan, 2002; Kokare *et al.*, 2009; Stepanovic *et al.*, 2004; Van Houdt and Michiels, 2010). Biofilm formed in food processing environments is of special importance as it has the potential to act as the chronic source of microbial contamination that may lead to food spoilage or transmission of diseases (Stepanovic *et al.*, 2004). Attachment and biofilm formation by food-borne pathogens and spoilage microorganisms on food contact surfaces in processing plants are a public health and cross-contamination concern (Ryu and Beuchat, 2005). Biofilm formation comprises a sequence of steps (Breyers and Ratner, 2004). Formation of microbial biofilms on food contact surfaces is a matter of great concern for the food processing industries (Jeyasekaran *et al.*, 2000). Biofilms formed on surfaces could develop under static conditions (i.e., on floors, walls, etc.) or under dynamic conditions (all situations in which some or all parts are moving). In general, biofilm formation involves attachment, colonization and growth of microorganisms (Forsythe, 2000). Biofilms formed by *Escherichia coli* O157:H7 on inadequately cleaned and sanitized contact surfaces may be a source of contamination of ground beef and deli meat in processing facilities as well as in food service settings (Sharma *et al.*, 2005). Food processing operations usually take place in wet environment and therefore, ideal for biofilm formation. There is little information on the presence of *Salmonella* in biofilms in food processing environments. However, various studies (Helke and Wong, 1994; Jones and Bradshaw, 1997; Joseph *et al.*, 2001) show that *Salmonella* can attach and form biofilms on surfaces found in food processing plants, including plastic, cement and stainless steel. Microaerophilic and CO_2-rich conditions provide the best environment for biofilm formation while the least biofilm was formed under anaerobic conditions (Stepanovic *et al.*, 2003). In food systems, the attachment of microorganisms leading to the formation of biofilms may be undesirable and also detrimental (Kumar and Anand, 1998). Biofilms formed in food-processing environments are of special importance as they have the potential to act as a persistent source of microbial contamination (Van Houdt and Michiels, 2010). There are not many reports on biofilm assays in developing countries especially Nigeria. Processing of food animals in most processing plants in Nigeria is far from the ideal and thus the problem of food contamination and diseases transmission are always in manifest. In most abattoirs, cattle, goats, sheep and pigs are slaughtered on bare floor usually made of cement. Coupled with water insufficiency, poor facility designs and irregular inspection and regulation, blood from slaughtered animals usually stand as source of food contamination. It is noteworthy to assay biofilms produced from food contact surfaces in order to pave a way for bacteria control. This study therefore, assessed the pattern of biofilm formation in pathogenic *E. coli* and *Salmonella* sp. from animal sources on cement and glass surfaces at 11 and 28±2°C.

MATERIALS AND METHODS

Test strains: Two strains each of *E. coli* O157:H7 and *Salmonella* sp. laboratory stock cultures from poultry and soft cheese were used for biofilm assays (Table 1) between the months of November, 2010 and June, 2012.

Biofilm development: Methods as described by Jeyasekaran *et al*. (2000) and Joseph *et al*. (2001) with slight modifications were utilized for the formation of biofilms on food contact surfaces (cement and glass). The cement (locally made, 1×2 cm×1.5 mm, PureChem® Cement, Nig.) coupons were each scrubbed with a brush and wash with distilled water thoroughly. Thereafter they were rinsed with 95% Ethanol (AnalaR®, BDH Chemical Ltd., UK), wrapped in foil paper and allowed to dry. The glass (1×2 cm×1.2 mm, Sail brand®, CAT. NO. 7101) coupons were washed with detergent (Omo®) to remove grease, rinsed thoroughly with distilled water, wrapped in foil paper and sterilized in the Hot Air Oven (Elektro, HELIOS, Sweden) for 30 min at 120°C. Five different types of media were used for biofilm incubation and they include: Tryptone Soy Broth (TSB) (LAB M, Lancashire, UK), TSB+0.5% glucose, TSB+1% glucose, TSB+2% Sheep blood and TSB+5% Sheep blood. Tryptone Soy Broth (TSB) (LAB M, Lancashire, UK) was used and the producer's direction was followed in the preparation. Thirty grams of the broth powder was dispersed in 1000 mL of distilled water, soaked for 10 min swirled to mix and warmed to dissolve before sterilized by autoclaving at 121°C for 15 min. The broth was allowed to cool before being used. After the Tryptone Soy Broth (TSB) (LAB M, Lancashire, UK) was prepared as described above, 0.5 and 1.0 g of Glucose (D+) (M and B, UK) as well as 2 and 5 mL Sheep Blood (obtained from apparently healthy sheep from the Sheep and Goat slaughter slab Bodija abattoir) were added to 100 mL of TSB for each particular test strain. These mixtures were thoroughly stirred together. Each test strains were grown in TSB (LAB M, Lancashire, UK) for 24 h at 37°C before they were added to the various media used for incubation and biofilm development. Glass jars were used for the incubation of the test strains at both refrigeration (11°C) and room (28±2°C) temperatures. Glass (1×2 cm×1.2 mm, Sail brand®, CAT. NO. 7101) and cement coupons (locally made, 1×2 cm×1.5 mm, PureChem® Cement, Nig.), 13 each were placed in glass jars and 100 mL of the five different media were added. Two milliliter each of the test strain grown in TSB for 24 h at 37°C were then added to the glass jars containing each different media and the coupons. After incubation at refrigeration (11°C) (in the refrigerator (Haier Thermocool, HPZ, Ltd.) and ambient (28±2°C) temperatures for 48 h, the coupons were aseptically removed, washed with distilled water to remove unattached cells and freshly prepared broths were poured into the glass jar (Ren and Frank, 1993). This procedure was repeated on day 4 to complete biofilm formation on day 5. Five glass jars containing the representative media were used as control in which no test strain was added.

Biofilm quantification: To quantify the biofilms formed by the bacteria sp. incubated in the five different broths for 5 day at refrigeration (11°C) and ambient temperatures (28±2°C), the Crystal

Table 1: List, serotype and sources of strains utilized for biofilm development

Strains	Serotype	Source	Country	Codes used
E. coli C	O157:H7	Wara (soft cheese)	USA	ECH7C
E. coli S	O157:H7	Poultry	Nigeria	ECH7P
Salmonella	Enteritidis	Poultry	Nigeria	SEP
Salmonella	sp.	Poultry	Nigeria	SSP

Violet Binding Assay (CVBA) and Bacteria Cell Enumeration (BCE) methods were used to quantify the biofilms while Scanning Electron Microscopy (SEM) was used to view the growth of the biofilms on the coupons.

Crystal violet binding assay (CVBA): This was carried out as performed by Adetunji and Adegoke (2008) and Stepanovic *et al.* (2004) with some modifications. A coupon, with its replicate, was picked from each incubated broth and washed 3 times with 5 mL of distilled water. The sessile bacterial cells (biofilms) were fixed with 1 mL of 70% ethanol (AnalaR®, BDH Chemical Ltd., UK) for 15 min. The fixed samples were then air dried and stained with crystal violet. Excess stains were rinsed with running tap water and then air dried. Three milliliter of glacial acetic acid (AnalaR®, BDH Chemical Ltd., UK) was used to re-solubilize each coupon. The re-solubilized liquid was then poured into a cuvette whose wavelength was measured using the Absorption Spectrophotometer (Springfield, UK). Absorbance (OD) reading against the control for a specific media and incubation temperature type was then measured. Rate of absorbance was measured at λ: 520 and 570 nm for the *E. coli* and for *Salmonella* sp., respectively.

Bacterial cell enumeration (BCE): To enumerate biofilm cells after 5 days of incubation, the samples, with their replicates, were washed with sterile distilled water to remove unattached cells and the biofilm cells were removed by swabbing with sterile cotton swabs. Sterile peptone water were added into the swabs, shaken vigorously and enumerated by standard spread plate technique with sterile dilution performed to 10^{-4}. Trytone Soy Agar (TSA, Scharlau, Spain) was used for enumeration and plates were incubated at 37°C for 48 h. Biofilm colonies were counted and converted to log CFU cm^{-2}.

Scanning electron microscopy (SEM): Each coupon was picked from each incubated broth and washed 3 times with 5 mL of distilled water. The sessile bacterial cells (biofilms) were fixed with 1 mL of 70% ethanol (AnalaR®, BDH Chemical Ltd., UK) for 15 min. The fixed samples were then air dried, wrapped with foil paper and transported to the, Advanced Physics Laboratory, Sheda Science and Technology Complex (SHETSCO), Abuja, Nigeria to carry out the SEM procedure. The samples for SEM analysis were placed inside the Scanning Electron Microscope (SEM) (Zeiss EVO MA10 Carl Zeiss SMT AG, Germany) and micrographs were taken at a magnification of 1000 μm.

Statistical analysis: The statistical software SPSS for Windows, version 17.0 (SPSS Inc. Chicago, IL) was used. The T-Test was utilized to ascertain the significant differences between the biofilm formed on each coupons for each strain at the two incubation temperatures in all culture types. p values less than 0.05 were considered significant.

RESULTS

Biofilm formation (crystal violet binding assay) by *E. coli* O157:H7 and *Salmonella* strains at refrigeration temperature (11°C) on glass and cement coupons (day 5): With crystal violet binding assay, pathogenic *E. coli* biofilms formed on cement were higher, significantly than produced on glass coupons for all broth types (Table 2, Fig. 1-2). There was increased biofilm formation for both *E. coli* strains with increased nutrient in the growth media (Table 2) higher than the biofilm grown using TSB medium alone. Biofilms formed best at refrigeration temperature in TSB+0.5% g on both coupons (0.285±0.001 nm in glass and

Table 2: Biofilm quantification (crystal violet binding assay): *E. coli* O157:H7 and *Salmonella* strains at refrigeration temperature (11°C) on glass and cement coupons (day 5)

	Escherichia coli O157:H7 (λ: 520 nm, n = 4)			*Salmonella* sp. (λ: 570 nm, n = 4)		
Broth	Strain codes	Glass	Cement	Strain codes	Glass	Cement
TSB	ECH7C	$0.159\pm0.002^{b\beta}$	$0.254\pm0.031^{a\alpha}$	SEP	$0.261\pm0.008^{\beta a}$	$0.430\pm0.002^{\alpha a}$
	ECH7P	$0.215\pm0.009^{a\alpha}$	$0.234\pm0.003^{b\alpha}$	SSP	$0.228\pm0.002^{\beta a}$	$0.440\pm0.002^{\alpha a}$
TSB+0.5% G	ECH7C	$0.280\pm0.001^{b\beta}$	$0.463\pm0.001^{a\alpha}$	SEP	$0.249\pm0.001^{\beta a}$	$0.379\pm0.001^{\alpha b}$
	ECH7P	$0.285\pm0.001^{a\beta}$	$0.444\pm0.001^{b\alpha}$	SSP	$0.255\pm0.001^{\beta a}$	$0.404\pm0.003^{\alpha a}$
TSB+1% G	ECH7C	$0.259\pm0.001^{b\beta}$	$0.393\pm0.001^{a\alpha}$	SEP	$0.272\pm0.004^{\beta a}$	$0.554\pm0.002^{\alpha a}$
	ECH7P	$0.264\pm0.002^{a\beta}$	$0.393\pm0.001^{a\alpha}$	SSP	$0.243\pm0.002^{\beta a}$	$0.417\pm0.001^{\alpha b}$
TSB+2% SB	ECH7C	$0.260\pm0.001^{a\beta}$	$0.322\pm0.000^{a\alpha}$	SEP	$0.233\pm0.001^{\beta a}$	$0.404\pm0.002^{\alpha a}$
	ECH7P	$0.251\pm0.001^{b\beta}$	$0.391\pm0.001^{b\alpha}$	SSP	$0.257\pm0.001^{\beta a}$	$0.413\pm0.001^{\alpha a}$
TSB+5% SB	ECH7C	$0.243\pm0.001^{b\beta}$	$0.312\pm0.001^{a\alpha}$	SEP	$0.242\pm0.001^{\beta b}$	$0.329\pm0.001^{\alpha b}$
	ECH7P	$0.244\pm0.001^{a\beta}$	$0.309\pm0.001^{b\alpha}$	SSP	$0.305\pm0.054^{\beta a}$	$0.467\pm0.001^{\alpha a}$

Values are Mean±SD, ECH7C: *E. coli* O157:H7 from soft cheese, ECH7P: *E. coli* O157:H7 from poultry, SEP: *Salmonella* Enteritidis, SSP: *Salmonella* spp. Values with the same superscripts (alphabets i.e., ^{aa}or^{bb}) between strains for a specific broth type are not statistically significant at $p<0.05$ along the columns. Values with the same superscripts (symbols i.e., $^{\alpha\alpha}$or$^{\beta\beta}$) for the coupons on the rows are not statistically significant at $p<0.05$

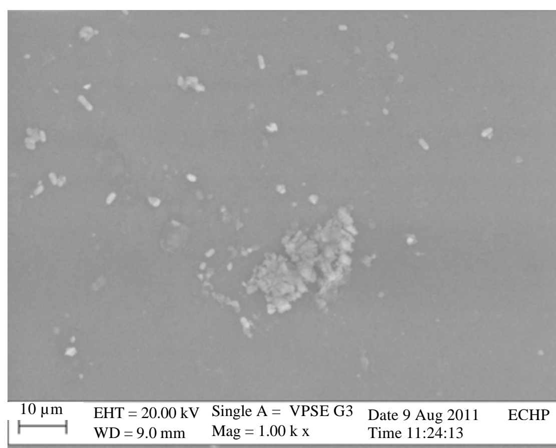

Fig. 1: *E. coli* O157:H7 biofilms quantified on glass coupon

0.463±0.001 nm in cement). For each strain, there was a difference between the biofilms formed on cement and glass coupons statistically significant a $p<0.05$ for all broths used. There was a statistical difference significant at $p<0.05$ in the biofilm quantified between strains on both coupons for each medium except for that noticed in TSB+1% g quantified on cement coupon. Salmonella biofilms developed on cement is significantly higher than generated on glass coupons for all broth types (Table 2, Fig. 3-4). Generally for the *Salmonella* strains, there is an increase in the quantity of biofilms produced with increased nutrient medium (Table 2). These strains produced biofilms higher than the *E. coli* O157:H7 strains. Of the two *Salmonella* strains, *Salmonella* sp. (SSP) (0.305±0.054 nm), incubated in TSB+5%SB medium formed the highest biofilms on glass coupon while on cement, *Salmonella* Enteritidis (0.554±0.002 nm) incubated in TSB+1%G medium was the highest biofilm former at refrigeration temperature. For each strain, there is a statistical difference between the biofilms developed on glass and cement coupons significant at $p<0.05$.

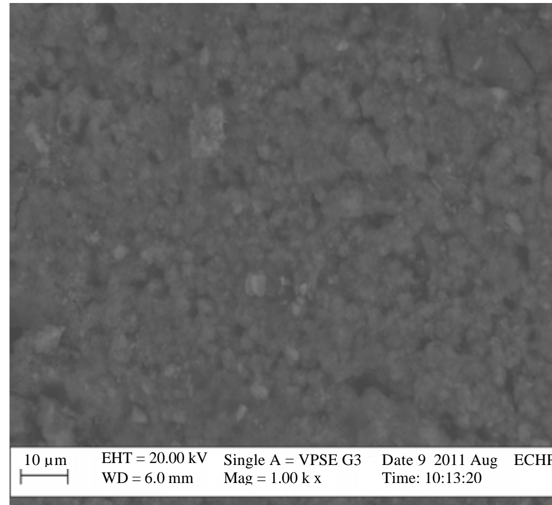

Fig. 2: *E. coli* O157:H7 biofilms quantified on cement coupon

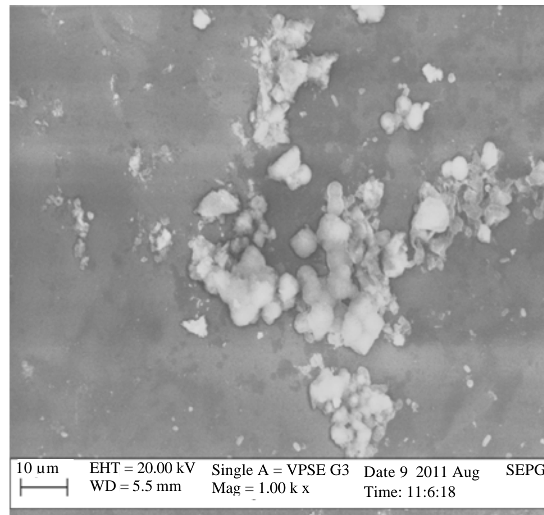

Fig. 3: *Salmonella* biofilms quantified on glass coupon

Biofilm formation (crystal violet binding assay) by *E. coli* O157:H7 and *Salmonella* strains at room temperature (28±2°C) on glass and cement coupons (day 5): At room temperature (28±2°C), higher biofilm levels for both *E. coli* O157:H7 and *Salmonella* strains were quantified than at refrigeration temperature (11°C). On glass coupons, *E. coli* O157:H7 biofilm formed in the blood media were higher than produced in TSB only on both coupons but a lower quantity was produced in media containing with glucose (Table 3). Biofilm production with increased nutrient concentrations for *E. coli* O157:H7 on the cement coupons was not consistent. For each strain, there was a difference between the biofilms formed on both coupons statistically significant a $p<0.05$ for all broths used. Statistical significance was observed between the two *E. coli* O157:H7 strains for all broth on glass coupons. This was the same as observed for cement except for the coupon incubated in TSB+1% g. *Salmonella* strains were better biofilm

Table 3: Biofilm quantification (crystal violet binding assay): *E. coli* O157:H7 and *Salmonella* Strains at room temperature (28±2°C) on glass and cement coupons (day 5)

	Escherichia coli O157:H7 (λ: 520 nm, n = 4)			*Salmonella* sp. (λ: 570 nm, (n = 4)		
Broth	Strain codes	Glass	Cement	Strain	Glass	Cement
TSB	ECH7C	$0.316\pm0.001^{a\beta}$	$0.537\pm0.000^{b\alpha}$	SEP	$0.435\pm0.001^{\beta b}$	$0.592\pm0.003^{\alpha a}$
	ECH7P	$0.302\pm0.001^{b\beta}$	$0.547\pm0.005^{a\alpha}$	SSP	$0.553\pm0.001^{\beta a}$	$0.571\pm0.001^{\alpha a}$
TSB+0.5% G	ECH7C	$0.277\pm0.000^{a\beta}$	$0.545\pm0.001^{a\alpha}$	SEP	$0.298\pm0.000^{\beta a}$	$0.565\pm0.001^{\alpha a}$
	ECH7P	$0.264\pm0.001^{b\beta}$	$0.492\pm0.001^{b\alpha}$	SSP	$0.273\pm0.000^{\beta a}$	$0.566\pm0.001^{\alpha a}$
TSB+1% G	ECH7C	$0.303\pm0.001^{a\beta}$	$0.564\pm0.001^{a\alpha}$	SEP	$0.404\pm0.001^{\alpha a}$	$0.483\pm0.125^{\alpha b}$
	ECH7P	$0.291\pm0.001^{b\beta}$	$0.565\pm0.001^{a\alpha}$	SSP	$0.325\pm0.000^{\beta b}$	$0.570\pm0.001^{\alpha a}$
TSB+2% SB	ECH7C	$0.359\pm0.001^{b\beta}$	$0.557\pm0.001^{a\alpha}$	SEP	$0.463\pm0.001^{\beta a}$	$0.561\pm0.001^{\alpha a}$
	ECH7P	$0.419\pm0.001^{a\beta}$	$0.517\pm0.001^{b\alpha}$	SSP	$0.473\pm0.001^{\beta a}$	$0.571\pm0.002^{\alpha a}$
TSB+5% SB	ECH7C	$0.395\pm0.001^{a\beta}$	$0.473\pm0.001^{a\alpha}$	SEP	$0.302\pm0.001^{\beta a}$	$0.603\pm0.071^{\alpha a}$
	ECH7P	$0.329\pm0.001^{b\beta}$	$0.452\pm0.001^{b\alpha}$	SSP	$0.344\pm0.001^{\beta a}$	$0.548\pm0.001^{\alpha a}$

Values are Mean±SD, ECH7C: *E. coli* O157:H7 from soft cheese, ECH7P: *E. coli* O157:H7 from poultry, SEP: *Salmonella* Enteritidis, SSP: *Salmonella* sp. Values with the same superscripts (alphabets i.e., ^{aa}or^{bb}) between strains for a specific broth type are not statistically significant at $p<0.05$ along the columns. Values with the same superscripts (symbols i.e., $^{\alpha\alpha}$or$^{\beta\beta}$) for the coupons on the rows are not statistically significant at $p<0.05$

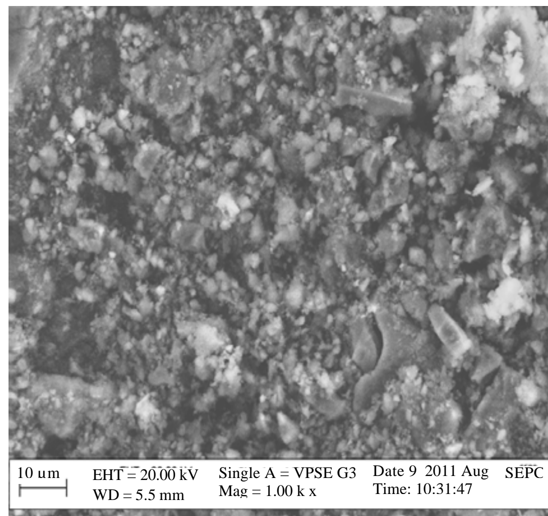

Fig. 4: *Salmonella* biofilm quantified on cement coupon

formers than *E. coli* O157:H7. *Salmonella* sp. (SSP) at room temperature (28±2°C) developed the highest level of biofilms (0.553±0.001 nm) on glass coupon in TSB broth while *Salmonella* Enteritidis developed the highest biofilms (0.603±0.071 nm) on cement coupon when incubated in TSB+SB broth (Table 3). Biofilm development increased with additional glucose levels though higher biofilm level was seen with 2% SB, this decreased when additional blood quantity was added to the TSB broth. Statistically, significant differences ($p<0.05$) were observed between both coupons for each strain in all broth types. Our results show that *Salmonella* sp. are better biofilm formers than *E. coli* O157:H7 strains.

Table 4: Biofilm quantification (Bacteria Cell Enumeration): *E. coli* O157:H7 and *Salmonella* Strains at refrigeration temperature (11°C) on glass and cement coupons (Day 5)

	Escherichia coli O157:H7 (n = 4)			*Salmonella* sp. (n = 4)		
Broth	Strain codes	Glass	Cement	Strain codes	Glass	Cement
TSB	ECH7C	$6.178\pm0.075^{a\alpha}$	$6.192\pm0.276^{a\alpha}$	SEP	$5.496\pm0.142^{a\beta}$	$6.551\pm0.055^{a\alpha}$
	ECH7P	$5.787\pm0.104^{b\alpha}$	$5.866\pm0.102^{a\alpha}$	SSP	$5.389\pm0.102^{a\alpha}$	$5.724\pm0.835^{a\beta}$
TSB+0.5% G	ECH7C	$6.562\pm0.086^{b\beta}$	$7.003\pm0.012^{a\alpha}$	SEP	$6.138\pm0.016^{a\beta}$	$6.305\pm0.047^{a\alpha}$
	ECH7P	$6.759\pm0.018^{a\alpha}$	$6.598\pm0.037^{b\beta}$	SSP	$6.153\pm0.030^{a\alpha}$	$5.906\pm0.112^{b\beta}$
TSB+1% G	ECH7C	$6.332\pm0.012^{a\alpha}$	$5.889\pm0.128^{b\beta}$	SEP	$5.500\pm0.230^{a\beta}$	$6.138\pm0.136^{b\alpha}$
	ECH7P	$5.945\pm0.157^{b\beta}$	$6.243\pm0.014^{a\alpha}$	SSP	$5.690\pm0.102^{a\beta}$	$6.485\pm0.083^{a\alpha}$
TSB+2% SB	ECH7C	$6.361\pm0.027^{b\beta}$	$6.427\pm0.121^{a\alpha}$	SEP	$5.850\pm0.981^{a\alpha}$	$6.389\pm0.010^{a\alpha}$
	ECH7P	$6.575\pm0.113^{a\alpha}$	$6.414\pm0.106^{a\beta}$	SSP	$6.247\pm0.154^{a\alpha}$	$6.097\pm0.020^{b\alpha}$
TSB+5% SB	ECH7C	$5.858\pm0.296^{a\beta}$	$6.230\pm0.029^{a\alpha}$	SEP	$5.621\pm0.717^{a\alpha}$	$6.224\pm0.089^{a\alpha}$
	ECH7P	$5.778\pm0.203^{a\beta}$	$6.543\pm0.279^{a\beta}$	SSP	$5.301\pm0.348^{a\alpha}$	$5.739\pm0.302^{b\alpha}$

Values are Mean±SD, ECH7C: *E. coli* O157:H7 from soft cheese, ECH7P: *E. coli* O157:H7 from poultry, SEP: *Salmonella* Enteritidis, SSP: *Salmonella* sp. Values with the saine superscripts (alphabets i.e., ^{aa}or^{bb}) between strains for a specific broth type are not statistically significant at p<0.05 along the columns. Values with the saine superscripts (symbols i.e., $^{\alpha\alpha}$or$^{\beta\beta}$) for the coupons on the rows are not statistically significant at p<0.05

Biofilm formation (bacterial cell enumeration) by *E. coli* O157:H7 and *Salmonella* strains at refrigeration temperature (11°C) on glass and cement coupons (day 5): Biofilms produced by *E. coli* O157:H7 strains on glass surfaces at refrigeration temperature ranged from 5.778±0.203-6.759±0.018 log CFU cm^{-2} formed by the poultry isolate (Table 4). On cement coupons, the biofilms developed by the strains ranged from 5.866±0.102-7.003±0.012 log CFU cm^{-2}. Highest biofilms on glass and cement surfaces for *E. coli* O157:H7 were formed by ECH7P and ECH7C incubated in TSB+0.5% g, respectively. On both coupons, biofilms increased (>0.5 log CFU cm^{-2}) with the initial concentration of nutrients (TSB+0.5% g and TSB+2% SB) added to the TSB broth but with further amplified concentrations (TSB+1%G and TSB+5% SB), biofilm formation decreased. Statistically, significant differences at $p<0.05$ were noticed between biofilms formed by the two *E. coli* O157:H7 strains on glass surfaces incubated in all broth types except in TSB+5% SB. On the other hand, on cement coupons, there were no significant differences between the two strains except with coupons incubated in the media with glucose. Biofilms developed by *Salmonella* strains (SEP and SSP) on glass and cement food contact surfaces at 11°C were presented in Table 4. Averagely, biofilms quantified on cement surfaces were higher than on glass. Increased biofilm production was enumerated on coupons (higher than incubated in TSB media only) with additional nutrients (0.5% g and 2% SB). But with further increase in the concentrations of these nutrients, *Salmonella* biofilm production decreased. Biofilms formed by *Salmonella* strains on glass surfaces spanned from 5.301±0.348 (produced by SSP incubated in TSB+2% SB) to 6.247±0.154 (formed by SSP incubated in TSB+5% SB) log CFU cm^{-2}. On cement, the highest biofilm levels were recorded from both strains incubated in TSB only. No significant differences at $p<0.05$ were noticed between the two *Salmonella* strains for each broth type quantified on glass surfaces but there were significant differences ($p<0.05$) between biofilms quantified from the strains on cement coupons for each broth type except in TSB media. Statistical differences significant at 0.05 level of error were observed between food contact surfaces for each strain incubated in TSB media only (except SSP incubated in TSB) and TSB+G. No significant differences were however, seen with the media containing sheep blood.

Table 5: Biofilm quantification (bacteria cell enumeration): *E. coli* O157:H7 and *Salmonella* strains at room temperature (28±2°C) on glass and cement coupons (day 5)

	Escherichia coli O157:H7 (n = 4)			*Salmonella* sp. (n = 4)		
Broth	Strain codes	Glass	Cement	Strain codes	Glass	Cement
TSB	ECH7C	$5.389\pm0.102^{a\beta}$	$5.910\pm0.088^{a\alpha}$	SEP	$5.145\pm0.168^{a\beta}$	$6.602\pm0.000^{a\alpha}$
	ECH7P	$4.564\pm0.108^{b\beta}$	$5.628\pm0.174^{b\alpha}$	SSP	$4.350\pm0.404^{b\beta}$	$6.562\pm0.082^{a\alpha}$
TSB+0.5% G	ECH7C	$6.148\pm0.080^{a\beta}$	$7.018\pm0.013^{a\alpha}$	SEP	$6.242\pm0.025^{b\alpha}$	$6.040\pm0.046^{b\beta}$
	ECH7P	$6.114\pm0.000^{a\beta}$	$6.370\pm0.032^{b\alpha}$	SSP	$6.435\pm0.154^{a\alpha}$	$6.152\pm0.045^{a\beta}$
TSB+1% G	ECH7C	$6.073\pm0.263^{a\alpha}$	$6.121\pm0.032^{a\alpha}$	SEP	$5.000\pm0.000^{b\beta}$	$5.970\pm0.569^{a\alpha}$
	ECH7P	$5.929\pm0.029^{\beta\beta}$	$5.977\pm0.027^{b\alpha}$	SSP	$5.301\pm0.000^{a\alpha}$	$5.834\pm0.519^{a\alpha}$
TSB+2% SB	ECH7C	$6.439\pm0.027^{a\beta}$	$6.555\pm0.042^{a\alpha}$	SEP	$6.242\pm0.026^{b\alpha}$	$6.217\pm0.015^{a\alpha}$
	ECH7P	$6.347\pm0.019^{b\alpha}$	$6.461\pm0.138^{a\alpha}$	SSP	$6.417\pm0.086^{a0\alpha}$	$6.410\pm0.426^{a\alpha}$
TSB+5% SB	ECH7C	$6.067\pm0.189^{a\alpha}$	$6.167\pm0.246^{a\alpha}$	SEP	$5.389\pm0.102^{a\beta}$	$6.050\pm0.237^{a\alpha}$
	ECH7P	$5.929\pm0.174^{a\alpha}$	$5.628\pm0.725^{a\alpha}$	SSP	$5.151\pm0.174^{a\beta}$	$6.188\pm0.051^{a\alpha}$

Values are Mean±SD, ECH7C: *E. coli* O157:H7 from soft cheese, ECH7P: *E. coli* O157:H7 from poultry, SEP: *Salmonella* Enteritidis, SSP: *Salmonella* sp. Values with the same superscripts (alphabets i.e., ^{aa}or ^{bb}) between strains for a specific broth type are not statistically significant at $p<0.05$ along the columns. Values with the same superscripts (symbols i.e., $^{\alpha\alpha}$or$^{\beta\beta}$) for the coupons on the rows are not statistically significant at $p<0.05$

Biofilm formation (bacteria cell enumeration) by *E. coli* O157:H7 and *Salmonella* strains at room temperature (28±2°C) on glass and cement coupons (day 5): Biofilms quantified from *E. coli* O157:H7 strains on glass and cement surfaces are presented in Table 5 at ambient temperature. Biofilms developed on glass surfaces ranged from 4.564±0.108 log CFU cm^{-2} (by ECH7P incubated in TSB medium only) to 6.439±0.027 log CFU cm^{-2} (by ECH7C grown in TSB+2%SB). The limit on cement coupons was from 5.628±0.725 log CFU cm^{-2} (formed by ECH7P in TSB+5%SB) to 7.018±0.013 log CFU cm^{-2} (developed by ECH7C in TSB+0.5%G). Supplementary nutrients to the TSB broth yielded increased biofilms formed by the strains on both coupons. Despite the increase, the extra concentrations for both coupons generated lower biofilms than the initial concentrations of TSB+0.5%G and TSB+2%SB. For each broth type, there were no statistical significant differences at $p<0.05$ in biofilms developed between strains on a particular glass surface except in TSB and TSB+5%SB while on cement coupons, significant differences were not observed with the sheep blood media. On the average, lower biofilms were developed on glass contact surfaces than on cement coupons for *Salmonella* strains incubated at ambient temperature (Table 5). The biofilms formed by these strains on glass coupons vary extending from 4.350±0.404 (SSP biofilms incubated in TSB) to 6.242±0.025 log CFU cm^{-2} (SEP biofilms grown in TSB+0.5%G). For cement surfaces, the span was from 5.834±0.519 (SSP biofilms quantified from TSB+1%G) to 6.602±0.000 log CFU cm^{-2} (SEP biofilms developed in TSB medium only). Generally, with the initial increase in nutrient concentrations, increased biofilm production was observed for both media containing the 0.5%G and 2%SB. The increased biofilm formation was however, reduced with further increase in the concentrations of both nutrients. There were statistical differences at $p<0.05$ between the biofilms developed by the two *Salmonella* strains on glass contact surfaces for each broth type with the exception of the strains incubated in TSB+5%SB. For the cement coupons, statistical significance ($p<0.05$) was only observed between the strains incubated in TSB+0.5%G. Between the food contact surfaces, significant differences (at 0.05 level of error) were not observed with SSP (incubated in TSB+1%SB) and both strains incubated in TSB+2%SB.

DISCUSSION

Biofilm formation on glass and cement coupons: Though cement surfaces are widely used in most processing environment in the Nigerian food industry, glass is increasingly being utilized recently, few reports are available locally on biofilms developed on glass (Adetunji and Adegoke, 2008; Adetunji and Isola, 2011) with none assayed on cement surfaces. The significantly higher biofilms ($p<0.05$) developed on cement (also demonstrated by the Scanning Electron Micrographs) noticed in all test strains was as a result of several factors. This finding is in agreement with earlier studies that reported that biofilms are easily developed on static than dynamic surfaces and may be formed in all areas of food processing environments (Donlan, 2002; Sofos, 2009). The hydrophobicity of bacteria and surface which are important criteria in biofilm formation (Manijeh *et al*., 2008) also supports the findings in this study. Cement is more hydrophilic than glass which thus facilitates bacteria adhesion and subsequent biofilm formation. The surface characteristics of the cement surfaces also support the higher biofilm levels observed than in glass (Donlan, 2002; Faille *et al*., 2002; Jullien *et al*., 2003; Ryu and Beuchat, 2005). The contact time (5 days) between the bacteria cells and the coupons was sufficient enough to allow easy irreversible adhesion of the cells and subsequent biofilm formation. The strains in this study have also been shown to possess fimbriae, flagella, exopolysaccharides and curli which are important extracellular surface structures produced by bacteria which may affect their attachment on food contact surfaces and consequent biofilm formation (Van Houdt and Michiels, 2010). Type 1 fimbriae have been demonstrated to play critical role in initial stable cell-to-surface attachment for *Salmonella enterica* serovar Enteritidis (Austin *et al*., 1998) during biofilm formation. Some poultry isolates of *Salmonella* were found to be biofilm formers on cement surfaces (Joseph *et al*., 2001) while also, other *Escherichia coli* strains such as *Escherichia coli* O_{111} have been confirmed to survive on cement surfaces forming biofilms (Ghazani *et al*., 2009). These findings also corroborate the biofilm forming ability of the strains in this study on cement.

Using five culture media with increased concentrations: Results of this study indicated in most cases that with additional nutrients concentrations, the biofilms quantified on the food contact surfaces increased. Higher levels of biofilms were formed with glucose than blood though higher than biofilms grown in TSB broths only. The increased biofilm proliferation was expected because biofilm production increased with nutrients. This finding is in agreement with earlier study where it was reported that sequestration to a nutrient-rich area (colonization) is one of the reasons that drive bacteria to produce biofilms (Jefferson, 2004). In general EPS production increased under conditions where growth was extended by the high glucose content in the medium (Fleming and Wingender, 2001) which is in agreement with this study. In addition *E. coli* strains have been shown to be strong biofilm formers with increased glucose concentrations (Rodrigues *et al*., 2010). Though, it has since been found that initial attachment, detachment and characteristics of biofilms of *E. coli* O157:H7 were affected by the nutrient status of the medium in which the biofilm was developed (Dewanti and Wong, 1995). The findings in this study is nearly at par with earlier report since with increase in the glucose concentration from 0.04% to 0.1 and subsequently 1% there was no significant effect on the number of adherent bacteria as quantified by plate counts (Dewanti and Wong, 1995). A positive correlation between glucose and biofilm formation in *Salmonella* Enteritidis has been established (Bonafonte *et al*., 2000) which is in agreement with findings in this study. On the contrary, trehalose and mannose which are disaccharides allow poor biofilm formation (Chmielewski and Frank, 2003). Generally, *Salmonella* sp. produced more biofilm in

nutrient-poor medium grown on plastic surfaces (Stepanovic *et al.*, 2004), this is at par with findings in this study, although the attachment surface was glass and cement in this study.

There is a strong indication that all bacteria strains do not grow at the same rate as seen in the result. The disparity in biofilm developed both in varying glucose and blood concentrations might be due to various factors. biofilm formation and adherence are not accomplished by the same mechanism in the different media; the strains probably utilize different strategies and express different biofilm-promoting genes depending on available environmental resources (Hancock *et al.*, 2011). Biofilm development in blood concentrations is scare and thus, we were unable to compare our results with other findings. The reduced biofilm quantified by some *E. coli* and *Salmonella* strains might be due to the toxic influence of blood digestion molecules after blood breakdown on the biofilms. Increase repulsive forces between bacteria cells and the surfaces might result from the actions of these molecules.

Incubating at refrigeration (11°C) and room (28±2°C) temperatures: The results generated in this study clearly demonstrated that higher biofilm levels were formed at ambient (28±2°C) than at refrigeration (11°C) temperature. Increased curli production in *E. coli* O157:H7 strains might be responsible for the higher biofilm development noticed at room than at refrigeration temperature. Curli production by *E. coli* O157:H7 enhances its ability to form biofilm (Ryu *et al.*, 2004) and these curli increases at higher temperatures. Generally, the optimal cultivation temperature for the production of most EPS molecules was estimated between 26 and 31°C (Gandhi *et al.*, 1997; Lory, 1992) thus, subsequently enhancing biofilm formation at ambient temperature. This can explain the increase in biofilm formation at 28±2°C in this study.

Higher biofilms levels were also recorded in this study at room temperature (28±2°C) for the *Salmonella* isolates tested. The capacity shown for biofilm production by *Salmonella* sp. at room temperature as a result is of particular interest to the food industry which again emphasizes the necessity for regular and appropriate cleaning (Stepanovic *et al.*, 2003). Though some observers have stated that *Salmonellae* display multicellular-characteristics in terms of biofilm formation, this multi-cellular behaviour is associated with biofilm formation at various incubation temperature (Stepanovic *et al.*, 2003) and is regulated by environmental conditions that target the agfD promoter (Gerstel and Romling, 2001; Romling *et al.*, 2000). Expression of agfD leads to the production of polymers, thin aggregative fimbriae and cellulose which form the extracellular matrix (Gerstel and Romling, 2001).

It has been proposed that the increased hydrophobicity at high temperatures (e.g., 37°C) may enhance the initial cell adherence, contributing to a higher biofilm density (Di Bonaventura *et al.*, 2008). However, biofilm cells may generate and secrete more extracellular polymeric substances in response to temperature and other factors which would also be seen as an increase in Crystal Violet (CV) absorbance in the microplate assay (Pan *et al.*, 2010). More importantly, it should be noted that the optimum temperature for a microorganism is associated with an increase in nutrient intake resulting in a rapid formation of biofilm (Stepanovic *et al.*, 2003).

CONCLUSION

In conclusion, the results of this study demonstrate that the pathogens under study did not grow at the same rate and higher incubation temperatures and nutrient influenced biofilm formation. In addition *E. coli* O157:H7 strains were observed to be lower biofilm formers when compared to *Salmonella* sp. Cement surfaces also adhered more biofilms than glass surfaces.

Of particular significance in this study is the high biofilm forming ability of these foodborne isolates, thereby increasing the risk of bacterial contamination in food processing. Further studies on the ability of these pathogens to form biofilms on ceramic are necessary since it is a more durable material than glass in food processing environments.

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