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Biofilm Formation in Human and Tropical Foodborne Isolates of *Listeria* Strains

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ABSTRACT

Biofilm formation in food processing is a public health concern globally. Biofilm formation in a 5 day period by various strains of *Listeria* on glass and cement was assayed using Crystal Violet Binding Assay (CVBA) and Bacteria Cell Enumeration (BCE) methods. Biofilm assessment was done in five different culture media and at two incubation temperatures (11 and 28±2°C). Scanning Electron Microscopy (SEM) was used to confirm the formation of the biofilms. *Listeria* organisms developed biofilms on food contact surfaces with greater levels significantly ($p<0.05$) formed on cement than glass. Significantly ($p<0.05$) higher biofilm formation occurred at ambient (28±2°C) than refrigeration (11°C) temperature. Biofilm production increased with additional glucose and 2% blood concentrations in the culture media. SEM confirmed the higher levels of biofilm produced on cement than glass. This study showed *Listeria* biofilms adhere easily to food contact surfaces which is influenced by nutrient and temperature. Regular evaluation of operation cleanliness in local food processing environments is highly necessary.

Key words: Biofilm, *Listeria*, surfaces, crystal violet binding assay, cell enumeration

INTRODUCTION

Foodborne illnesses arise from the consumption of food or water that has been contaminated with microbiological pathogens or toxic chemicals (Sanders, 1996, 1999). Various groups of foodborne pathogens exist, which if present in contaminated food products, can lead to significant impacts on human health and industrial economies (Sanders, 1999; Helms *et al.*, 2003). *Listeria monocytogenes* is a gram-positive food-borne pathogen, widely distributed in the environment and commonly resistant to environmental stress (Djordjevic *et al.*, 2002) and has become one of the main pathogens transmitted by food (Porta *et al.*, 2010). This organism causes among others meningitis and septicemia in the immunocompromised persons as well as abortion in pregnant women. *L. monocytogenes* has been implicated in fresh and frozen meat, poultry, seafood, ready-to-eat products, unpasteurized milk and other dairy products, especially cheeses (Kim and Frank, 1995; Adetunji *et al.*, 2003; Adetunji and Adegoke, 2008). Once introduced into the food processing environment, *Listeria* can persist for years (Unnerstand *et al.*, 1996).

Microorganisms can exist in the environment as planktonic cells or on surfaces in biofilms enclosed within a matrix predominantly made up of polysaccharide material (Gandhi and Chikindas, 2007). Biofilms can also be said to be an aggregation of microbial cells that is irreversibly associated and enclosed in a self-produced matrix of primarily polysaccharide material

having the ability to stick to surfaces (Donlan, 2002; Stepanovic *et al.*, 2004; Kokare *et al.*, 2009; Van Houdt and Michiels, 2010; Abee *et al.*, 2011). Biofilms can be encased in an exopolysaccharide matrix on both biotic and abiotic surfaces (Bose and Ghosh, 2011). Biofilm formation consists of initial attachment, micro-colony and EPS (extracellular polymeric substances) production (glycocalyx), followed by maturation stages (Davey and O'Toole, 2000; Jeyasekaran *et al.*, 2000). More than 60 years after the first report of biofilm (Zobell, 1943), they are still of concern. Bacteria live in communities for many of the same reasons that other organisms do; for example, protection from predators or other external dangers, access to resources and genetic diversity (Jefferson, 2004). *L. monocytogenes* biofilms resist cleaning and sanitation thereby promoting the transmission of *L. monocytogenes* by food (Adetunji and Adegoke, 2008). *Listeria* species can survive and grow well in various micro-niches found in food processing facilities (Chmielewski and Frank, 2003). Biofilms occur widely in nature and may become major problems in foods and processing facilities leading to food spoilage and diseases transmission (Stepanovic *et al.*, 2004; Sofos, 2009; Van Houdt and Michiels, 2010). Biofilms and have the potential to act as a persistent source of microbial contamination in the food environment (Van Houdt and Michiels, 2010). Cystic fibrosis, endocarditis, cystitis and other chronic infections that are difficult to treat have been linked to biofilms (Lynch *et al.*, 2007). In the developed world, attention has been shifted to the study of virulence factors in bacteria, not just the planktonic bacterial cells in a bid to eradicate them. 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 in Nigeria, cattle, goats, sheep and pigs are slaughtered on bare floor usually made of cement. Coupled with water insufficiency, poor facility designs and irregular inspection, blood from slaughtered animals usually pose as source of food contamination. There is perhaps no study on the pattern of biofilm production in tropical *Listeria* strains isolated from foodborne sources in Southwestern Nigeria. This study therefore assessed biofilm production on glass and cement coupons as food contact surfaces at 11 and 28±2°C in order to understand the characteristics of biofilm forming ability of tropical *Listeria* strains.

MATERIALS AND METHODS

This study was carried out between October, 2010 and June, 2011 at the Food hygiene laboratory, Department of Veterinary Public Health and Preventive Medicine, University of Ibadan.

Reference strains: *L. monocytogenes* strains H7762 (LM) and H8733 (LM) were obtained from Prof. Jinru Chen of the Department of Food Science and Technology, University of Georgia, USA. The strains were cultured in tryptose soya broth for 24 h at 37 C. The stock culture for this study was sub-cultured thrice on Modified Oxford Agar (fishers scientific, USA) for Listeria isolation supplemented with antibiotic supplements (acriflavine, nalidixic acid and cycloheximide) (Becton, Dickinson and company) before use.

Test strains: In addition to the reference strains three Listeria isolates from food sources (LMJ12 (cheese); LMP and LSP (poultry) were utilized for in this study (Table 1).

Table 1: Sources of strains utilized for biofilm development

	Source	
Strains	Item	Country
Listeria monocytogenes (LMH7762)	Frankfurter isolate	USA
Listeria monocytogenes (LMH8733)	Meat isolate	USA
Listeria monocytogenes (LMJ12)	Wara (soft cheese)	Nigeria
Listeria monocytogenes (LMP)	Poultry	Nigeria
Listeria spp. (LSP)	Poultry	Nigeria

Biofilm development: Methods as described by Jeyasekaran *et al*. (2000) and Joseph *et al*. (2001) with slight modifications were utilized for formation of biofilms on the food contact surfaces (cement and glass). Coupons were used as adhesion surfaces and incubation for adhesion and biofilm formation was done at ~11 and 28±2°C.

Sterilization of coupons: 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.

Media preparation: 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.

Preparation of tryptone soy broth (TSB): Tryptone Soy Broth (TSB) (LAB M, Lancashire, UK) was used and the producer's direction was followed on 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.

Tryptone Soy Broth (TSB) is being prepared by 0.5% glucose, 1% glucose, 2% sheep blood and 5% sheep blood. Two and five milliliter 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.

Preparation of broth cultures of the test strains: 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.

Inoculation and incubation of media: 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) coupons and cement (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) (in the inoculating chamber) 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.

Biofilm quantification: To quantify the biofilms formed by the *Listeria* spp. incubated in the five different broths for 5 day at refrigeration (11°C) and ambient temperatures (28±2°C), two techniques were used;

Crystal violet binding assay (CVBA): This was carried out as performed by Stepanovic *et al.* (2004) and Adetunji and Adegoke (2008) 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 curvette 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: 620 nm for the *Listeria* organisms.

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}. Tryptone 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 magnifications between 200-5000 μm.

Statistical analysis: The statistical software SPSS for Windows, version 17.0 (SPSS Inc. Chicago, IL) was used. The biofilm quantified among the various *Listeria* strains for each broth type was tested using One-way Analysis of Variance (ANOVA). LSD multiple comparisons tests were applied as post-hoc when significant differences were determined. The t-test was utilized to ascertain the significant differences between coupons types for each strain. All statistical analysis was carried out at 0.05 confidence limit.

RESULTS

Biofilm formation (CVBA) by *Listeria* spp. at refrigeration temperature (11°C) on glass and cement (day 5). *Listeria* biofilms quantified from cement were significantly higher than formed on glass surfaces for all broth types (Table 2; Fig. 1, 2). The highest biofilms formed at this temperature on cement coupons were produced by the *L. monocytogenes* (LMJ12) strains (Fig. 1) incubated in TSB+1%G (0.546±0.001 nm) and TSB+2% SB (0.543±0.002 nm). On glass coupons, the *L. monocytogenes* H8733 produced the highest biofilms (0.198±0.001 nm). Significant differences at $p<0.05$ were noticed between biofilms formed on both coupons for each strain in all

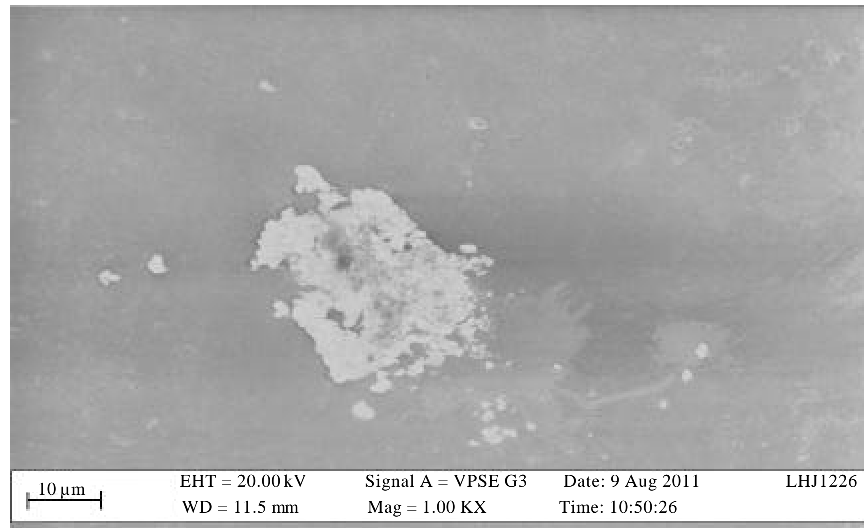

Fig. 1: *Listeria monocytogenes* (LMJ12) biofilm quantification on glass

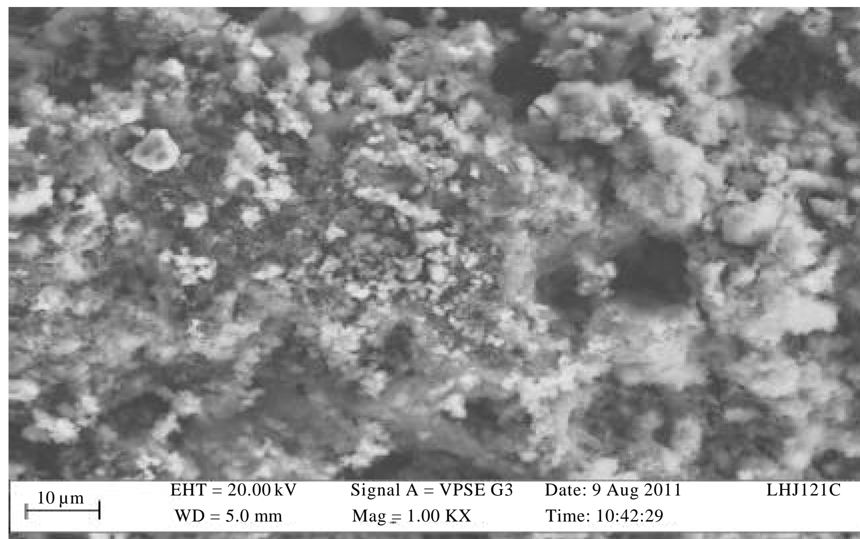

Fig. 2: *Listeria* spp. (LMJ12) biofilm quantification on cement

Table 2: Biofilm quantification (CVBA) of *Listeria* strains incubated at refrigeration temperature (11°C) on glass and cement coupons in day 5 period

Broths	Strain codes	Coupons	
		Glass	Cement
Tryptone soy broth	LMH7762	$0.172\pm0.020^{a\beta}$	$0.253\pm0.011^{a\alpha}$
	LMH8733	$0.167\pm0.017^{a\beta}$	$0.272\pm0.009^{b\alpha}$
	LMJ12	$0.156\pm0.006^{a\beta}$	$0.247\pm0.003^{a\alpha}$
	LMP	$0.174\pm0.019^{a\beta}$	$0.253\pm0.009^{a\alpha}$
	LSP	$0.165\pm0.009^{a\beta}$	$0.228\pm0.001^{c\alpha}$
Tryptone soy broth+0.5% glucose	LMH7762	$0.190\pm0.003^{a\beta}$	$0.268\pm0.008^{a\alpha}$
	LMH8733	$0.175\pm0.001^{b\beta}$	$0.286\pm0.004^{b\alpha}$
	LMJ12	$0.173\pm0.001^{b\beta}$	$0.324\pm0.001^{c\alpha}$
	LMP	$0.184\pm0.001^{c\beta}$	$0.285\pm0.001^{b\alpha}$
	LSP	$0.170\pm0.001^{d\beta}$	$0.257\pm0.001^{d\alpha}$
Tryptone soy broth+1% glucose	LMH7762	$0.169\pm0.001^{a\beta}$	$0.314\pm0.001^{a\alpha}$
	LMH8733	$0.157\pm0.001^{b\beta}$	$0.254\pm0.001^{b\alpha}$
	LMJ12	$0.167\pm0.001^{c\beta}$	$0.546\pm0.001^{c\alpha}$
	LMP	$0.195\pm0.001^{d\beta}$	$0.343\pm0.001^{d\alpha}$
	LSP	$0.181\pm0.001^{e\beta}$	$0.428\pm0.001^{e\alpha}$
Tryptone soy broth+2% sheep blood	LMH7762	$0.186\pm0.001^{a\beta}$	$0.247\pm0.001^{a\alpha}$
	LMH8733	$0.184\pm0.001^{a\beta}$	$0.222\pm0.001^{b\alpha}$
	LMJ12	$0.189\pm0.001^{b\beta}$	$0.543\pm0.002^{c\alpha}$
	LMP	$0.162\pm0.001^{c\beta}$	$0.291\pm0.001^{d\alpha}$
	LSP	$0.177\pm0.001^{d\beta}$	$0.339\pm0.001^{a\alpha}$
Tryptone soy broth+5% sheep blood	LMH7762	$0.163\pm0.001^{a\beta}$	$0.279\pm0.001^{a\alpha}$
	LMH8733	$0.198\pm0.001^{b\beta}$	$0.290\pm0.001^{b\alpha}$
	LMJ12	$0.174\pm0.001^{c\beta}$	$0.300\pm0.001^{c\alpha}$
	LMP	$0.181\pm0.001^{d\beta}$	$0.317\pm0.001^{d\alpha}$
	LSP	$0.171\pm0.001^{e\beta}$	$0.297\pm0.001^{e\alpha}$

CVBA: Crystal violet binding assay performed at λ: 620 nm, Values are as Mean±SD with n = 4, ^{a,b}Values with the same superscripts between strains for a specific broth type are not statistically significant at p<0.05 along the columns, $^{\alpha,\beta}$Values with the same superscripts between the coupons on the rows for a specific strain are not statistically significant at p<0.05

media types. With multiple comparison, no significant differences at p<0.05 were observed for biofilms formed by all the strains incubated in TSB broth only on glass surfaces. However, within and between strains, significant differences (p<0.05) were observed with the use of TSB+0.5% G, TSB+1% G, TSB+2% SB and TSB+5% SB media. On cement surfaces, significant differences were observed in the biofilm developed between the *Listeria* spp. (LSP) from poultry and other *L. monocytogenes* strains in TSB broth, TSB+0.5% G, TSB+1% G, TSB+2% SB (except LMH8733) and TSB+5% SB media.

There was an increase in the amount of biofilm quantified on both coupons with additional increase in nutrient parameters (Table 2). With TSB+0.5%G, biofilms formed the *Listeria* strains were higher than produced with TSB broth only while a further increased was observed with TSB+1% G. Biofilms formed in TSB+2% SB were also higher than formed with TSB broth alone and a further increase was observed with TSB+5% SB though this increase was not as high as obtained with TSB+1% G.

Biofilm formation (CVBA) by *Listeria* spp. at room temperature (28±2°C) on glass and cement coupons (day 5): The *L. monocytogenes* strain isolated from poultry (LMP) which was incubated in TSB+2% SB formed the highest biofilms on glass coupon at ambient temperature (Table 3). Similarly, this strain generated the highest biofilms on cement coupon though quantified from the coupon incubated in TSB medium only. Biofilms developed on cement surfaces were significantly higher at $p<0.05$ than form on glass surfaces at this temperature as observed with all strains incubated in all broth types.

Significant differences at $p<0.05$ were observed for biofilms formed by all the strains incubated in TSB, TSB+0.5% G, TSB+1% G, TSB+2% SB and TSB+5% SB media on glass surfaces. On cement surfaces, significant differences were observed within and between all the *Listeria* strains in TSB broth, TSB+0.5% G, TSB+1% G, TSB+2% SB (except between LMH8733 and LSP) and TSB+5% SB media.

Biofilm formation (BCE) by *Listeria* strains at refrigeration temperature (11°C) on glass and cement coupons (day 5): Averagely, biofilm formed on cement contact surfaces

Table 3: Biofilm quantification (CVBA) of *Listeria* strains incubated at refrigeration temperature (28±2°C) on glass and cement coupons in day 5 period

		Coupons	
Broths	Strain codes	Glass	Cement
Tryptone soy broth	LMH7762	0.186±0.001$^{a\beta}$	0.483±0.001$^{a\alpha}$
	LMH8733	0.182±0.001$^{b\beta}$	0.538±0.001$^{b\alpha}$
	LMJ12	0.187±0.000$^{c\beta}$	0.527±0.002$^{c\alpha}$
	LMP	0.215±0.001$^{d\beta}$	0.559±0.001$^{d\alpha}$
	LSP	0.238±0.001$^{e\beta}$	0.556±0.001$^{e\alpha}$
Tryptone soy broth+0.5% glucose	LMH7762	0.275±0.000$^{a\beta}$	0.347±0.002$^{a\alpha}$
	LMH8733	0.199±0.001$^{b\beta}$	0.457±0.001$^{b\alpha}$
	LMJ12	0.249±0.001$^{c\beta}$	0.444±0.001$^{c\alpha}$
	LMP	0.271±0.000$^{d\beta}$	0.383±0.001$^{d\alpha}$
	LSP	0.269±0.001$^{e\beta}$	0.407±0.001$^{e\alpha}$
Tryptone soy broth+1% glucose	LMH7762	0.194±0.001$^{a\beta}$	0.478±0.001$^{a\alpha}$
	LMH8733	0.188±0.000$^{b\beta}$	0.464±0.001$^{b\alpha}$
	LMJ12	0.254±0.001$^{c\beta}$	0.451±0.001$^{c\alpha}$
	LMP	0.197±0.001$^{d\beta}$	0.331±0.001$^{d\alpha}$
	LSP	0.248±0.001$^{e\beta}$	0.345±0.001$^{e\alpha}$
Tryptone soy broth+2% sheep blood	LMH7762	0.269±0.001$^{a\beta}$	0.538±0.001$^{a\alpha}$
	LMH8733	0.398±0.001$^{b\beta}$	0.549±0.001$^{b\alpha}$
	LMJ12	0.308±0.001$^{c\beta}$	0.506±0.001$^{c\alpha}$
	LMP	0.469±0.001$^{d\beta}$	0.529±0.002$^{d\alpha}$
	LSP	0.429±0.001$^{e\beta}$	0.549±0.001$^{b\alpha}$
Tryptone soy broth+5% sheep blood	LMH7762	0.257±0.001$^{a\beta}$	0.487±0.001$^{a\alpha}$
	LMH8733	0.286±0.001$^{b\beta}$	0.432±0.001$^{b\alpha}$
	LMJ12	0.282±0.000$^{c\beta}$	0.468±0.001$^{c\alpha}$
	LMP	0.269±0.001$^{d\beta}$	0.450±0.000$^{d\alpha}$
	LSP	0.366±0.001$^{e\beta}$	0.407±0.001$^{e\alpha}$

CVBA: Crystal violet binding assay performed at λ: 620 nm, Values are as Mean±SD with n = 4, ^{a,b}Values with the same superscripts between strains for a specific broth type are not statistically significant at $p<0.05$ along the columns. $^{\alpha,\beta}$Values with the same superscripts between the coupons on the rows for a specific strain are not statistically significant at $p<0.05$

Table 4: Biofilm quantification (BCE) of *Listeria* strains incubated at refrigeration temperature (11°C) on glass and cement coupons in day 5 period

		Coupons (log CFU cm^{-2})	
Broths	Strain codes	Glass	Cement
Tryptone soy broth	LMH7762	$5.919\pm0.110^{\beta a}$	$6.810\pm0.012^{\alpha a}$
	LMH8733	$5.964\pm0.307^{\beta a}$	$6.735\pm0.042^{\alpha a}$
	LMJ12	$6.507\pm0.158^{\alpha b}$	$6.593\pm0.025^{\alpha a}$
	LMP	$6.531\pm0.032^{\alpha b}$	$6.587\pm0.036^{\alpha a}$
	LSP	$6.342\pm0.023^{\alpha b}$	$5.952\pm0.751^{\alpha b}$
Tryptone soy broth+0.5% glucose	LMH7762	$6.450\pm0.028^{\alpha a}$	$6.347\pm0.076^{\beta a}$
	LMH8733	$6.727\pm0.036^{\alpha b}$	$6.419\pm0.153^{\beta a}$
	LMJ12	$6.958\pm0.299^{\alpha b}$	$6.056\pm0.070^{\beta b}$
	LMP	$6.407\pm0.069^{\alpha a}$	$6.465\pm0.027^{\alpha a}$
	LSP	$6.797\pm0.016^{\alpha b}$	$6.534\pm0.035^{\beta a}$
Tryptone soy broth+1% glucose	LMH7762	$7.087\pm0.004^{\alpha a}$	$7.085\pm0.002^{\alpha a}$
	LMH8733	$5.731\pm0.844^{\alpha b}$	$6.659\pm0.282^{\alpha b}$
	LMJ12	$6.743\pm0.307^{\beta a}$	$7.181\pm0.028^{\alpha a}$
	LMP	$6.561\pm0.190^{\alpha a}$	$6.591\pm0.079^{\alpha b}$
	LSP	$7.064\pm0.022^{\alpha a}$	$6.616\pm0.076^{\beta b}$
Tryptone soy broth+2% sheep blood	LMH7762	$5.874\pm0.033^{\beta a}$	$6.465\pm0.036^{\alpha a}$
	LMH8733	$6.273\pm0.183^{\alpha b}$	$6.484\pm0.008^{\alpha a}$
	LMJ12	$5.739\pm0.046^{\beta a}$	$6.011\pm0.191^{\alpha b}$
	LMP	$5.812\pm0.039^{\beta a}$	$6.113\pm0.039^{\alpha b}$
	LSP	$6.608\pm0.095^{\beta c}$	$6.957\pm0.024^{\alpha c}$
Tryptone soy broth+5% sheep blood	LMH7762	$5.640\pm0.738^{\alpha a}$	$3.040\pm3.510^{\alpha a}$
	LMH8733	$5.540\pm0.072^{\beta a}$	$6.327\pm0.010^{\alpha b}$
	LMJ12	$5.588\pm0.679^{\alpha a}$	$5.239\pm0.275^{\alpha a}$
	LMP	$5.827\pm0.404^{\alpha a}$	$5.962\pm0.212^{\alpha c}$
	LSP	$5.602\pm0.000^{\alpha a}$	$5.639\pm0.391^{\alpha d}$

BCE: Bacteria cell count, Values are as Mean±SD with n = 4, ^{a,b}Values with the same superscripts between strains for a specific broth type are not statistically significant at $p<0.05$ along the columns, $^{\alpha,\beta}$Values with the same superscripts between the coupons on the rows for a specific strain are not statistically significant at $p<0.05$

(6.27 log CFU cm^{-2}) were higher than developed on glass (6.25 log CFU cm^{-2}). On glass surfaces, LMH7762 incubated in TSB+1% G developed the highest biofilms (7.087±0.004 log CFU cm^{-2}) while LMH8733 grown in TSB+5% SB formed the lowest (5.540±0.072 log CFU cm^{-2}) (Table 4). Similarly on cement coupons (Fig. 1), LMH7762 incubated in TSB+1% G formed the highest biofilms (7.085±0.002 log CFU cm^{-2}) and the lowest (5.239±0.275 log CFU cm^{-2}) produced by LMJ12 grown in TSB+5% SB. *Listeria* biofilms development at refrigeration temperature increased with the addition of glucose to the TSB media but decreased with sheep blood media (Table 4).

Between coupons for each *Listeria* strain at refrigeration temperature, there was no statistical difference in the biofilms quantified except in the following strains, TSB: LMH7762 and LMH8733, TSB+0.5% G: LMH7762, LMH8733 and LSP, TSB+1% G: LMJ12 and LSP, TSB+2% SB: LMH7762, LMJ12, LMP and LSP, TSB+5% SB: LMH8733. Statistical differences ($p<0.05$) existed between biofilms formed by LMH7762 and other *Listeria* strains (except LMH8733) on glass surfaces incubated in TSB broth only. With TSB+0.5% G, no significant differences at $p<0.05$ were noticed between LMH7762 and LMP, with TSB+1% G: LMJ12, LMP and LSP, with TSB+2% SB:

LMJ12 and LMP while with TSB+5% SB, no significant differences were not observed with all other strains. Statistically ($p < 0.05$) within strains on cement surfaces, the differences as compared to LMH7762 were as follows: TSB: LSP, TSB+0.5% G: LMJ12 and LSP, TSB+1% G: all but LMJ12, TSB+2% SB: all but LMH8733 and TSB+5% SB: All but LMJ12.

Biofilm formation (BCE) by *Listeria* strains at room temperature (28±2°C) on glass and cement coupons (day 5): Typically, higher biofilms were form on cement (6.82 log CFU cm^{-2}) surfaces than glass coupons (6.27 log CFU cm^{-2}). *Listeria* biofilms quantified on cement contact surfaces scaled from 5.827±0.404 log CFU cm^{-2} formed by LMH8733 in TSB+5% SB to 7.345±0.011 log CFU cm^{-2} produced by LMP in TSB (Table 5). With glass surfaces, biofilms generated by *Listeria* strains ranged from 5.154±0.356 log CFU cm^{-2} formed by LSP incubated in TSB to 7.103±0.028 log CFU cm^{-2} quantified from LMH7762 grown in TSB+1% G. Biofilm production increased with both concentrations of glucose but decreased with both concentrations of sheep blood.

Table 5: Biofilm quantification (BCE) of *Listeria* strains incubated at room temperature (28±2°C) glass and cement coupons in day 5 period

		Coupons (log CFU cm^{-2})	
Broths	Strain codes	Glass	Cement
Tryptone soy broth	LMH7762	$5.476\pm0.033^{\beta a}$	$7.334\pm0.006^{\alpha a}$
	LMH8733	$5.384\pm0.027^{\beta a}$	$7.297\pm0.028^{\alpha b}$
	LMJ12	$5.647\pm0.037^{\beta a}$	$7.302\pm0.001^{\alpha c}$
	LMP	$5.455\pm0.009^{\beta a}$	$7.345\pm0.011^{\alpha a}$
	LSP	$5.154\pm0.356^{\beta b}$	$7.319\pm0.008^{\alpha a}$
Tryptone soy broth+0.5% glucose	LMH7762	$7.010\pm0.014^{\alpha a}$	$6.889\pm0.023^{\beta a}$
	LMH8733	$6.199\pm0.577^{\alpha d}$	$6.859\pm0.013^{\alpha a}$
	LMJ12	$6.608\pm0.006^{\alpha c}$	$6.647\pm0.039^{\alpha b}$
	LMP	$6.782\pm0.004^{\alpha a}$	$6.607\pm0.021^{\beta b}$
	LSP	$6.484\pm0.008^{\alpha d}$	$6.529\pm0.059^{\alpha c}$
Tryptone soy broth+1% glucose	LMH7762	$7.103\pm0.028^{\alpha a}$	$7.133\pm0.016^{\alpha a}$
	LMH8733	$6.662\pm0.031^{\beta b}$	$7.288\pm0.016^{\alpha b}$
	LMJ12	$6.995\pm0.025^{\alpha a}$	$7.052\pm0.047^{\alpha c}$
	LMP	$6.588\pm0.240^{\beta b}$	$6.964\pm0.014^{\alpha d}$
	LSP	$6.704\pm0.005^{\beta b}$	$7.043\pm0.025^{\alpha c}$
Tryptone soy broth+2% sheep blood	LMH7762	$6.665\pm0.014^{\alpha a}$	$6.632\pm0.089^{\alpha a}$
	LMH8733	$6.415\pm0.073^{\beta b}$	$6.550\pm0.007^{\alpha b}$
	LMJ12	$6.405\pm0.049^{\beta b}$	$6.491\pm0.000^{\alpha b}$
	LMP	$6.672\pm0.106^{\beta a}$	$6.949\pm0.042^{\alpha c}$
	LSP	$6.217\pm0.015^{\beta c}$	$6.728\pm0.019^{\alpha d}$
Tryptone soy broth+5% sheep blood	LMH7762	$5.690\pm0.246^{\alpha a}$	$6.025\pm0.140^{\alpha a}$
	LMH8733	$6.220\pm0.185^{\alpha b}$	$5.827\pm0.404^{\alpha a}$
	LMJ12	$5.889\pm0.029^{\beta a}$	$6.199\pm0.230^{\alpha a}$
	LMP	$6.350\pm0.404^{\beta c}$	$7.011\pm0.008^{\alpha b}$
	LSP	$6.073\pm0.263^{\alpha a}$	$6.368\pm0.377^{\alpha a}$

BCE: Bacteria cell count, Values are as Mean±SD with n = 4, ^{a,b}Values with the same superscripts between strains for a specific broth type are not statistically significant at $p<0.05$ along the columns, $^{\alpha,\beta}$Values with the same superscripts between the coupons on the rows for a specific strain are not statistically significant at $p<0.05$

Between coupons for each *Listeria* strain at refrigeration temperature, there was no statistical difference at $p<0.05$ in the biofilms quantified except in the following strains, TSB: All strains, TSB+0.5% G: LMH8733 and LMP, TSB+1% G: LMH8733, LMP and LSP, TSB+2% SB: LMH8733, LMJ12, LMP and LSP, TSB+5% SB: LMJ12 and LMP. Statistically within strains, significance differences observed at $p<0.05$ between LMH7762 and other strains grown in all broth types on glass surfaces were as follows: TSB: LSP, TSB+0.5% G: All but LMP, TSB+1% G: All but LMJ12, TSB+2% SB: All but LMP and TSB+5% SB: All but LMJ12 and LSP. On cement surfaces, the differences ($p<0.05$) were as follows: TSB: LMP and LSP, TSB+0.5%G: All but LMH8733, TSB+1% G: All strains, TSB+2% SB: All strains and TSB+5% SB: LMP.

DISCUSSION

Biofilm quantification: All strains used in this study produced biofilms. This is as similarly noted by most previous researchers (Chae and Schraft, 2001; Joseph *et al.*, 2001; Donlan, 2002; Stepanovic *et al.*, 2004; Chae *et al.*, 2006; Adetunji and Adegoke, 2008; Kokare *et al.*, 2009; Van Houdt and Michiels, 2010; Abee *et al.*, 2011).

On glass and cement coupons: Despite that fact that cement surfaces are widely used in abattoirs, slaughter slabs and other areas in the food processing industry in Nigeria, the ability of pathogenic bacteria species to form biofilms on cement surfaces have not been reported. Very scanty reports are available locally on biofilms developed on glass (Adetunji and Adegoke, 2008; Adetunji and Isola, 2011).

For all species, the significantly higher biofilms developed on cement (also demonstrated by the Scanning Electron Micrographs) was as a result of several factors. The fact that the biofilms were developed on static surfaces facilitated the biofilms grown on the two food contact surfaces. As discussed by Donlan (2002) and Sofos (2009) and may form in all areas of food processing environments. Surfaces with high free energy or wettability are more hydrophilic (Chmielewski and Frank, 2003). Hydrophobicity of bacteria and surface are important criteria in biofilm formation (Manijeh *et al.*, 2008). Cement is more hydrophilic than glass which thus facilitates bacteria adhesion and subsequent biofilm formation. Also, the topography of cement surfaces is another important factor which facilitated the higher levels of bacteria adhesion and biofilm formation (Korber *et al.*, 1989; Donlan, 2002; Faille *et al.*, 2002; Jullien *et al.*, 2003; Simoes *et al.*, 2010). The contact time (5 days) between the bacteria cells and the coupons was sufficient enough to allow easy irreversible adhesion of the bacteria cells to coupons and subsequent biofilm formation.

Fimbriae, flagella, exopolysaccharides and curli are important extracellular surface structures produced by bacteria which may affect their attachment on food contact surfaces and consequent biofilm formation. Biofilm formation by these organisms is affected by bacterial cell surface such as flagella and cell surface appendages (Van Houdt and Michiels, 2010). Flagellar motility is critical for initial cell-to-surface contact and normal biofilm formation under stagnant culture conditions for *L. monocytogenes* (Vatanyoopaisarn *et al.*, 2000; Lemon *et al.*, 2007). High levels of extracellular carbohydrates are being produced by *L. monocytogenes* strains which may increase their capacity to generate biofilms (Chae *et al.*, 2006).

Using five culture media with increased concentrations: 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. The higher biofilms levels obtained with additional nutrients on both coupons was consistent with the findings of Stepanovic *et al*. (2004), Harvey *et al*. (2007) and Pan *et al*. (2010). In a study conducted by Rodrigues *et al*. (2010), both *E. coli* and *Listeria* strains were strong biofilm formers with increased glucose concentrations. Similar results have also been observed with *Staphylococcus* species (Dobinsky *et al*., 2003; Moretro *et al*., 2003; Lim *et al*., 2004; Frank and Patel, 2007; Rodrigues *et al*., 2010). Cells form microcolonies or clusters enclosed within the hydrated matrix and pores or channels throughout the structure allow transport of oxygen, nutrients and waste (Sofos and Geornaras, 2010). In general EPS production increased under conditions where growth was extended by the high glucose content in the medium (Fleming and Wingender, 2001).

In addition to the intrinsic properties of individual strains, numerous extrinsic factors, including the physicochemical characteristics of surface materials, temperature, nutrients, pH, salt, sugar and the presence of other bacteria, have been shown to influence initial cell attachment and subsequent biofilm formation by *L. monocytogenes* (Moretro and Langsrud, 2004). Sequestration to a nutrient-rich area (colonization) is one of the reasons that drive bacteria to produce biofilms (Jefferson, 2004). Specifically, increase in glucose concentration favoured biofilm formation in *Listeria* spp. (Jensen *et al*., 2007; Pan *et al*., 2010) while more biofilms were produced with the addition of both glucose and salt to the growth media owing to the synergistic effects of these nutrients. On the contrary, trehalose and mannose which are disaccharides allow poor biofilm formation (Chmielewski and Frank, 2003). Generally, *L. monocytogenes* produce more biofilm in nutrient-rich medium (Stepanovic *et al*., 2004).

Perhaps, as far as we know, studies where blood concentrations were used as growth cultures in biofilm formation are scare. In general for all the strains, our research indicated initial increase of biofilms formed in sheep blood (2%) but decrease biofilm with further increase in sheep blood (5%) concentrations. This observation is difficult to explain. The reduced biofilm quantified might be due to the influence of blood digestion molecules after blood breakdown. Also, increase repulsive forces between bacteria cells and the surfaces might result from the actions of these molecules Hancock *et al*. (2011) stated in his research that possibly, 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. Moreover, Surface structures, i.e. proteins and other organelles with adhesive properties as well as factors merely contributing to the hydrophobicity/hydrophilicity of the cell surface, might be differently expressed depending on growth media and greatly influence the adherence properties of the strain (Hancock *et al*., 2011).

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. Generally, the optimal cultivation temperature for the production of most EPS molecules during biofilm formation by bacteria was estimated between 26 and 31°C (Lory, 1992; Gandhi *et al*., 1997). This would subsequently enhance biofilm formation at ambient temperature.

Listeria strains tested by Pan *et al*. (2010) formed higher biofilms at 37°C than at 30°C, while the least was formed at 22.5°C. This is comparable to our results as higher biofilm levels were seen at 28±2°C. Three hours after incubation at 5°C no attachment of *Listeria* strains was observed, indicating that *L. monocytogenes* requires longer incubation period when attaching to glass surface

at refrigerated temperature (Milanov *et al*., 2009). Chae and Schraft (2001), Chae *et al*. (2006) and Milanov *et al*. (2009) discovered *Listeria* strains favourably showed higher ability on attachment to glass surfaces with biofilm formation when incubation temperature of 37°C was used than at 25°C. However, Norwood and Gilmour (2001) found that *Listeria monocytogenes* strains exhibited differential, temperature-dependent, capacity for biofilm formation after 48 h incubation. They concluded that optimum temperature for biofilm formation by *L. monocytogenes* was 18°C.

Di Bonaventura *et al*. (2008) proposed that the increased hydrophobicity at high temperatures (e.g., 37°C) may enhance the initial cell adherence, contributing to a higher biofilm density. 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 (Stepanovi *et al*., 2003).

This study reveals that pathogenic *Listeria* spp. (local strains inclusive) develop high biofilms on food contact surfaces, higher on cement surfaces than glass and at ambient than refrigeration temperature. Also, higher biofilms were formed with the availability of nutrients particularly with glucose and to some extent blood. With these factors, it can be deduced that there is a higher presence of biofilms formed by pathogenic zoonotic organisms in our food processing plants where the principles of hygiene are not held in high esteem. Further studies on genotypic characteristics of isolates that aid their ability to form biofilms is necessary to elucidate other factors involved in biofilm forming abilities of these strains. In addition, since increase in biofilm formation was only observed with 2% blood concentrations but not with higher concentrations of 5% there is a need for further studies to ascertain the cause of the reduction of biofilm formation with higher concentrations of blood.

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