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Full Length Research Paper

Formation of biofilm by strains of *Listeria monocytogenes* isolated from soft cheese 'wara' and its processing environment

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Quantification of biofilm formation by 40 *Listeria monocytogenes* strains from wara soft cheese and its processing environment was assessed on glass vials surfaces. Attachement to glass surface was quantified using a crystal violet binding assay. All the 40 strains produced biofilms after 48 and 72 h incubation at 37°C. No biofilms were formed at 24 h incubation but biofilm formation increased with incubation period in 20 out of the 40 strains. R^2 values obtained were 0.0166 and 0.1193 respectively for biofilm formation between 24 and 48 h and 24 and 72 h incubation periods, respectively (P-values of < 0.05*). *L. monocytogenes* strains isolated from *wara* cheese were generally sensitive to augmentin, streptomycin, claforan, erythromycin, gentamycin, septrin, tarivid, and rocephine and were highly resistant to nitrofuran, fortum, zinnat, and tetracycline. The enhancement of biofilm formation in *L. monocytogenes* strains from 'wara' in this study suggests a relationship with pathogenicity in foodborne isolates. The transfer of antibiotic resistant *L. monocytogenes* to human via the food chain is a significant health concern.

Key words: biofilm, *Listeria monocytogenes*, 'wara', antibiotic sensitivity, incubation.

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium, motile by means of flagella and has been isolated from soil, silage and other environmental sources. *L. monocytogenes* is quite hardy and resists deleterious effects of freezing, drying and heat remarkably well for a bacterium that does not form spore (USFDA-CFSAN, 2006).

L. monocytogenes has become a major public health concern. During the 1980s a number of listeriosis outbreaks were linked with the consumption of contaminated foodstuffs such as coleslaw (Schlech et al., 1983), pasteurized milk (Fleming et al., 1985) and soft cheeses (Piffaretti et al., 1989). In 1983, Onyemelukwe et al. isolated *L. monocytogenes* in Northern Nigeria, from 19 patients with clinical condition characterized by meningitis, meningoencephalitics, spontaneous peritonitis, septicemia, arthritis, pelvic infection and arthritis. All isolates were type 4 serotypes. Moreover in an earlier study by Adetunji et al. (2003) the isolation rate of *L. monocytogenes* was 20% in a local cheese, wara. Wara is an unripened soft cheese used mainly as snack in Southwestern Nigeria. Its coagulant is the juice extract of Sodom apple leaves and stem (*Calotropis procera*) and pawpaw (*Carica papaya*) leaves and stem. The *C. procera* extract is the preferred coagulant. Wara cheese has a short shelf life of 2 - 3 days with presence of a variety of organisms when stored in whey (Adegoke et al., 1992; Belewu et al., 2005).

Biofilm is a factor that enhances the resistance of *L. monocytogenes* to cleaning and sanitation, thereby promoting its transmission by food. A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix of primarily polysaccharide materials and cannot be removed by gentle rinsing (Donlan, 2002). Numerous studies have shown that *L. monocytogenes* is capable of adhering and forming biofilm on metal, glass, rubber and plastic surfaces

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(Hood and Zottola, 1997; Wong, 1998; Sommer et al., 1999; Chae and Schraft, 2000, 2001; Leriche and Carpenter, 2000; Sinde and Cerballo, 2000; Joseph et al., 2001; Stepanovic et al., 2004). Some studies have also shown that nutrient-rich medium such as brain heart infusion (BWI) promotes biofilm production by *L. monocytogenes* (Hood and Zottola, 1997; Stepanovic et al., 2004).

In an earlier study by Onyemelukwe et al. (1983), most types of *L. monocytogenes* isolates were sensitive to ampicillin, crustalline, penicillin and erythromycin. All common antibiotics, except cephalosporins, has been reported to be active against *L. monocytogenes in vitro* (Hof, 1991). Experimentally, amplicillin and amoxicillin are most active (Hof, 1991), while tetracycline and chloramphenicol are reportedly not the therapeutic agents of choice (Cherubin et al., 1991). In the past, individuals who developed clinical listeriosis are usually treated with penicillin or ampicillin in conjunction with aminoglycoside (Charpentier and Courvalin, 1999). The current therapy of choice for all forms of listeriosis is a combination of ampicillin and gentamicin (Lorber 1997; Schlech, 2000).

This present work provides assessment of biofilm formation on glass vial surfaces and antibiogram by *L. monocytogenes* isolates from wara cheese.

MATERIALS AND METHODS

Preparation of *L. monocytogenes* culture for attachment and biofilm formation

L. monocytogenes strains (LMd, LMe, LMf, LMg, LMh) were isolated from a wara processing environment (in Nigeria) and LM strains (WLMa, WLMb and WLMc) were isolated from wara. One colony of each *L. monocytogenes* culture on modified Oxford agar supplemented with antibiotics was transferred into 9 ml tryptose soy broth. The inoculated broth was incubated at 37°C for 24 h. The cultures were then to be used for biofilm formation for 24, 48 and 72 h incubation on glass vials (Pawar and Chen, 2005). The tryptose soy broth not inoculated with *L. monocytogenes* was used as negative control throughout the study. Approximately an equal population of cells of each culture was used. Glass vials (4.5 X 1.4 cm; Fisher Scientific) were used as glass surface. The glass vials were washed with alkaline detergent, rinsed thoroughly with deionized water, and air-dried before being autoclaved at 121°C for 30 min.

5 ml of pure cultures were placed into the glass vials after which the cells in broth, were allowed to attach to glass vials for 24, 48 and 72 h at 37°C. Lids were placed on the glass vials to prevent evaporation of the broth. At the end of each incubation period, *L. monocytogenes* attached to glass surface was quantified using a crystal violet binding assay (Pawar and Chen, 2005) with some slight modifications. The broth cultures were withdrawn on each day and the glass vials were rinsed thrice with sterile distilled water to remove loosely attached cells. The glass vials were air-dried and fixed by passing over the flame of a Bunsen burner (Fisher Scientific) 3 times. The fixed cells on the surfaces were stained with 2 ml of 1% crystal violet (Fisher Scientific) for 15 min. Excess stain was rinsed off by placing the glass vials under running tap water until the washed water contained no visible stains. The glass vials were air-dried at 60°C for 5 min. The dye bound to the adherent cells was resolubilized with 200 µl of 33% (v/v) acetic acid per vial.

The optical density (O.D.) of each glass vial and a control (optical density of control = O.D.C) was measured at 570 nm using a spectrophotometer (Novaspec II). The absorbance of the negative control was subtracted from the absorbance values to determine the actual value (Pawar and Chen, 2005).

Quantification of biofilm formation

Based on the O.D. produced by bacterial films, strains were classified into the following categories: no biofilm producers, weak, moderate or strong biofilm producers (Stepanovic et al., 2000). Strains were classified as follows: :< blank = none biofilm producers; <0.160 nm= Slight biofilm producers; 0.160 - 0.260 nm = moderate biofilm producers, 0.300 nm and above = high biofilm producers. All tests were carried out in duplicate and the results are presented as means. P-values of < 0.05 were considered significant.

Antibiotic sensitivity agar preparation

According to manufacturer's specification, 2.8 g of nutrient agar was dissolved in 100 ml of distilled water followed by sterilization in autoclave at 121°C for 15 min. The agar was then distributed into Petri dishes and 10^4 cfu/ml of inoculated broth of test cultures of 3 *L. monocytogenes* isolates from wara was then added at about 38°C and gently mixed together. After the agar – broth mixture solidified, antibiotic sensitivity discs were placed into the Petri dishes followed by incubation of the preparation at 37°C for 18 – 24 h. *L. monocytogenes* strain Scott A was used as a positive control while sterile broth was used as a negative control.

Zone of clearance around each antibiotic indicated sensitivity of the organism present in the culture to that antibiotic. A sterile meter ruler was used to measure the diameter of the zone of clearance from the tip of that antibiotic disk to the circumferences of the zone of clearance or inhibition.

RESULTS

Quantification of biofilm formation at 24, 48 and 72 h incubation

A significant difference ($P < 0.05$) in biofilm formation was observed between 24 and 48 h incubation period and between 24 and 72 h incubation periods for all the listeria strains. WLMa from *wara* cheese demonstrated the highest biofilm formation at 48 h incubation (Table 1 and Figure 1) while LMe demonstrated the highest biofilm formation at 72 h incubation (Table 1). Half of the LM strains (20 out of 40) in this study increased in biofilm formation with extension of incubation period although the correlation between the incubation periods was not significant (R^2 = 0.0166-0.1193) (Table 1 and Figures 1a and b).

Antibiotic sensitivity of LM strains from wara

The *L. monocytogenes* isolates (from wara) in this study had a resistance of 23.52% to common drug of choice in the treatment of infections in Nigeria. *L. monocytogenes* was highly sensitive to Augmentin; which makes it the

Table 1. Quantification of biofilm formation after 24, 48 and 72 h incubation at 37°C.

LM strains	No. of strains	Mean absorbance (nm) at 24 h	Biofilm formation	Mean absorbance (nm) at 48 h	Biofilm Formation	Mean absorbance (nm) at 72 h	Biofilm formation
Control	Blank	0.067	؛	0.085	؛	0.077	؛
Wlma	5	0.0406	؛	0.305*	+++	0.202*	++
WLmb	5	0.0365	؛	0.164*	++	0.155*	+
WLmc	5	0.0365	؛	0.177*	++	0.182*	++
LMd	5	0.0632	؛	0.213*	++	0.186*	++
Lme	5	0.0433	؛	0.260*	++	0.297*	++
LMf	5	0.0561	؛	0.116*	+	0.179*	++
LMg	5	0.0620	؛	0.188*	++	0.225*	++
LMh	5	0.0203	؛	0.224*	++	0.134*	+

< Blank = ؛ = none; <0.160 nm = + = slight; 0.160 - 0.260 nm = ++ = moderate; 0.300 nm and above = +++ = high biofilms.
P-values of < 0.05* were observed between 24 and 48 h and between 24 and 72 h incubation periods.

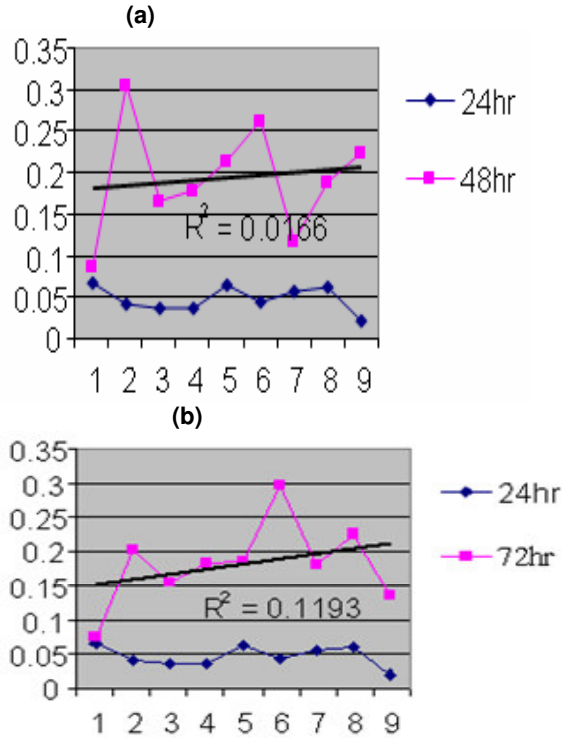

Figure 1. Showing correlation between biofilm formation (nm) of *L. monocytogenes* strains at 24 and 48 h (**a**) and 24 and 72 h (**b**) incubation periods.

most effective drug. The least sensitive drugs were Nitrofuratoin, Fortum, Zinnat and Tetraxycline (Table 2).

DISCUSSION

Wara cheese is processed traditionally in the open air thus allowing for atmospheric contamination. Previous laboratory studies have confirmed the presence of *L. monocytogenes* in milk and milk products processed in Nigeria including ice-cream, fermented milk, wara and local butter (Owhe Ureghe et al., 1993; Adetunji et al., 2003). The quality of milk used for processing, in particular its microflora coupled with unhygienic practices during processing and unstandardized processing methods are major factors that make this product have variable sensory qualities and short shelf life when compared to conventional processes (Turkoglu et al., 2003) thus making it unpopular among the urban dwellers in Nigeria.

The level of biofilm production of isolates has been reported to be a virulence characteristic of such isolates (Hood and Zottola, 1995; Gulsun et al., 2005). Higher producers of this factor have higher virulence than lower producers, which is the essence of this study. Several workers have reported cellulose, biofilm and fimbria production to have influenced virulence (Hood and Zottola, 1995). A significant difference ($P < 0.05$) in biofilm formation was observed between 24 and 48 h incubation period and between 24 and 72 h incubation periods for all the listeria strains. Although the WLMa from *wara* cheese demonstrated the highest biofilm formation at 48 h incubation (Table 1 and Figure 1), LMe demonstrated the highest biofilm formation at 72 h incubation (Table 1). Increase in biofilm formation with extension of incubation period demonstrated in this study has also been demonstrated by Moltz and Martin, (2005) in stainless steel chips.

Numerous studies have examined factors and growth conditions that affect biofilm formation of various surfaces. However most of these previous studies assessed only a few strains. This study has shown that there are differences in the ability of strains to attach to a glass surface. A similar report suggesting differences in biofilm forming ability is Norwood and Gilmour (1999). This report is also the first reporting biofilm ability of *L. monocytogenes* strains from the Nigerian local cheese wara. The enhancement of biofilm formation in wara strains (WLMa) may be a relationship to pathogenicity in

Table 2. Antibiogram of *L. monocytogenes* isolated from soft cheese 'wara'.

Antibiotics	No. of isolates	Mean zone of inhibition (mm)	Sensitivity
Ampicillin (PN)	3	10 ± 1.16	WS
Ciproxin (CPX)	3	11 ± 1.53	WS
Streptomycin (S)	3	15 ± 0.87	MS
Augmentin (A)	3	20 ± 0.87	S
Claforan (CTX)	3	17 ± 3.51	MS
Erythromycin (E)	3	17 ± 2.52	MS
Gentamycin (CN)	3	18 ± 1.04	MS
Rocephine (CRO)	3	15 ± 1.44	MS
Septrin (SXT)	3	15 ± 0.73	MS
Cloxacillin (CXL)	3	10 ± 1.01	WS
Nitrofurantoin (N)	3	00 ± 0	R
Fortum (CAZ)	3	00 ± 0	R
Gentamycin (CN)	3	12 ± 0.76	WS
Tarivid (OFX)	3	16 ± 0	MS
Chloramphenicol (C)	3	11 ± 1.53	WS
Zinnat (CXM)	3	00 ± 0	R
Tetracycline (T)	3	00 ± 0	R

Mm: Measurements of the zone of inhibition.
WS = Weakly sensitive; MS = moderately sensitive; S = sensitive; R = resistant.

foodborne isolates. Similar report of a potential for biofilm formation in a foodborne *L. monocytogenes* isolate was also demonstrated by Kalmokoff et al. (2001).

The resistance shown by *L. monocytogenes* strains in this study to tetraxycline agrees with an earlier report by Charpentier et al. (1993) and Poyart-Salmeron et al. (1990) who determined a new class of tetraxycline resistance gene tet(s) in *L. monocytogenes* BM4210 and 37-kb plasmid, pIP811 in *L. monocytogenes* strains isolated from a patient with meningoencephalitis, respectively. This resistance shown by *L. monocytogenes* strains from wara in this study to common drugs of choice in Nigeria may be due to abuse of drugs use in animal husbandry and appearance of new strains (Dina and Arowolo, 1991). Emergence of bacteria resistant to most commonly used antibiotics in Nigeria is of considerable medical significance because of therapeutic problems. In another study multiple resistances was observed in some Nigerian bacterial strains (Lateef et al., 2005). Contrary to this study, Poyart-Salmeron et al. (1990) reported resistance of *L. monocytogenes* strains isolated from a patient with meningoencephalitis to chloramphenicol, erythromycin, streptomycin. This suggests genetic differences in the *L. monocytogenes* strains. *L. monocytogenes* strains in this study were highly sensitive to Augmentin, making it the most effective drug at this time. Similar results were obtained by Umoh et al. (1995) who recorded high sensitivity of Streptomycin and Augmentin to *L. monocytogenes*.

It is concluded that *L. monocytogenes* strains from wara are capable of enhanced biofilm formation and drug résistance. Further studies on whether the ability to form biofilm may influence the survival within a food-processing environment should be investigated.

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