Advanced Journal of Microbiology Research Vol. 2015
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

Virulence characteristics of food processing relevance in isolates of *Listeria monocytogenes* and *Escherichia coli* 0157*: H7* strains isolated from 'wara' a West African soft cheese

V. O. Adetunji

Department of Veterinary Public Health and Preventive Veterinary Medicine, University of Ibadan, Ibadan, Nigeria.
E-mail: vadetunji@gmail.com.

Accepted 13 January, 2014

Three strains of *Escherichia coli 0157:H7* and eleven strains of *Listeria monocytogenes* isolated from West African wara cheese were analysed for cellulose production, fimbriae formation, zetapotential and biofilm formation which are of relevance in food processing. *E. coli* c produced no fimbria, very low cellulose and significantly lower zetapotential (-3.1) among the three strains. The *L. monocytogenes (b)* had the highest zetapotential (-39.1) and cellulose production at 24 h of incubation. Next to it was *L. monocytogenes* (f) which also had a higher cellulose production at 72 h incubation. A positive correlation exists between zetapotential and cellulose production in the *E. coli* strains (R^2 = 0.67) while *Listeria monocytogenes* strains gave a negative correlation (R^2 = -0.08) . There was no significant difference in biofilm formation in the *L. monocytogenes* strains (P < 0.05), however the *L. monocytogenes* (a) had the highest at 48 h incubation. Weak positive correlation (R^2) values of 0.0397, 0.002 and 0.0011 were obtained for 24, 48 and 72 h incubation for *Listeria monocytogenes* counts (cfu/ml) and cellulose measurements. This study further accentuate and confirmed the presence of *E. coli* and *L. monocytogenes* with virulent factors that of relevance to the method of processing in West African soft cheese. The need for an improved hygiene during processing and sale of this product to prevent the transmission of these pathogenic organisms to humans is therefore emphasized.

Key words: West African soft cheese, *Escherichia coli, Listeria monocytogenes,* virulent strains.

INTRODUCTION

Listeria monocytogene and *Escherichia coli* 0157:H7 are two food-borne pathogens that are of concern to the cheese industry (Kovincic et al., 1991). Sporadic cases of human illness caused by *L. monocytogenes* have been linked to the consumption of cheeses such as the Mexican-style cheese (Linnan et al., 1988) and Vacherin Mont d'Or soft cheese (Bille, 1990; Margolles et al., 1996). The survival of *L. monocytogenes* and its contamination in several soft cheeses have been investigated (Frank and Marth 1978; Farber et al., 1987; Papageorgiou and Marth, 1989; Kovincic et al., 1991; Back et al., 1993).

E. coli strains are part of the normal flora of the gastrointestinal tract of humans and various animals (Kuhar et al., 1998). *E. coli* 0157: H7 has been reported in many cases of food-borne outbreaks associated with the consumption of hamburger and raw milk (Ramsaran et al., 1998). Raw milk has been identified as the second most important vehicle for the transmission of *E. coli* 0157:H7 (Reitsma and Hening, 1996) . However, outbreaks of *E. coli* 0157:H7 infections from cheese consumption have been reported (Anonymous, 1994 and Curnow, 1994).

The development of infection-resistant materials and treatments in host requires detail knowledge of the factors and forces involved in bacterial adhesion (Hanna et al., 2002). The synergistic action of several of these virulent factors is therefore needed to overcome the defense mechanisms of the host and elicit infection (Kuhar et al., 1998). Internalin InIA has been shown to interact with host cell E-cadherin to mediate invasion of mammalian epithelial cells thereby causing invasive disease (Vazquez- Boland et al., 2001; Bergmann et al., 2002; Pentecost et al., 2006).

The initial event in colonizing the host tissue is mediated

by fimbrial adhesions. The attachment by fimbriae of *E. coli* and biofilm formation of *Listeria* and other microorganisms are influenced by the physicochemical properties of the surface such as surface charge, hydrophobicity, pH, temperature and nutrient composition of the preconditioning menstrum (McGuire and Krisdhasima, 1991; Helke et al., 1993; Smoot and Pierson, 1998; Wong, 1998). Surface materials used in food processing allow differing degrees of biofilm forma-tion (Helke and Wong, 1994; Blackman and Frank, 1996). For example *Listeria* and *Salmonella* were found to adhere more to hydrophobic surfaces than hydrophilic surfaces although the attachment may be weak (Sinde and Carballo, 2000).

Extracellular polysaccharide (cellulose) for some strains is the polymers that may also influence adhesion (Abu-lail et al., 2003) . Lipopolyssarcharide molecules are anchored to the cell outer membrane through their lipid moiety. The core region of the LPS consists of negatively charged groups, such as phosphates and carboxylic groups, which usually give the LPS its negative charge (Wicken and Knox, 1980). The outer polysaccharide part of the LPS is the O-antigen, which consists of 20-70 repeating units of three to five sugars and can protrude up to 30 or more nm into the cell surroundings. In Gram-negative bacteria, the O-antigen is likely responsible for polymers interaction with surfaces. Outer-membrane proteins are less likely to interact with the solid surfaces since they are hidden behinds the O-antigen layer (Makin and Beveridge, 1996). The aim of this study was therefore to evaluate the virulence determinants of food processing relevance (biofilm, cellulose, fimbria, zeta-potential) associated with *L. monocytogenes* and *E. coli* 0157:H7 strains isolated from 'wara' cheese.

MATERIALS AND METHODS

E. coli O157 and *L. monocytogenes* isolates from 'wara' cheese were confirmed according to methods by Barrow and Feltham (1993). Cultures were purified by sub-culturing 3 times on triptose soy agar. Specific antisera were used to confirm *E. coli* O157:H7 and *L. monocytogenes.* Positive coagulation reactions were observed with *E. coli* O157:H7 antiserum and *L. monocytogenes* polyserotype antiserum in broth cultures of the test inoculum.

Preparation of special media and buffer

1.) Luria-Bertani agar on salt was prepared according to manufacturer's description and incorporated with Congo red dye (40 ug/ml of agar) and Brilliant blue dye (20 ug/ml of agar). Media 2; Luria-Bertani agar no salt was prepared according to manufacturer's description and incorporated before autoclaving with dye 200 ug/ml of the agar. Media 3; Sorbitol MacConkey agar was prepared according to manufacturer's description. Media 4; Cacofloura dye was incoporated with Tryptose soy agar at a concentration of 200 ug per ml of agar. Media 5; Minimum glucose agar (MGA) was prepared by adding 15 g Bacto agar in 1 L of deionized water (6.8 pH). To this mixture was added the following: NaH_2PO4 (6.8 g), K_2HPO4 (3.0 G), NaCl (0.5 g), NH_4Cl (1.0 g).

All the media above were then sterilized at 116°C for 15 min, cooled to 50°C and 20 mls of sterile 20% glucose solution and 2 mls of sterile 1.0 $MgSO_4$ were then added. The pH was adjusted accordingly using either 1.0 M NaOH or HCL.

Test for fimbria production of isolates

This was done by inoculating 24 h fresh cultures on Luria-Bertani agar no salt incorporated with Congo red and brilliant blue dye, and incubating for 72 h at 28°C.

Colonies with brown colouration indicates fimbria production, red colour indicates fimbria and cellulose production while pink colour indicates only cellulose production. White colour indicates no production of either fimbria or cellulose.

Measurement of fimbria production using spectrophotometer

Three test tubes (A, B, C) of 10 ml *E. coli* culture were prepared in tryptose soy broth to which 0.02% congo red was incorporated and incubated for 24, 48, and 72 h respectively. Congo red was used as blank reading against the actual reading.

The amount of fimbria produced is estimated by the amount of dye taken up by the culture, since fimbria produced takes up Congo red stain. Readings were taken at 520 nm on the spectrophotometer.

Test for colanic acid production and zetapotential measurement

Isolates positive for colanic acid production were used for zeta-potential measurement. A 24 h culture was transferred to MGA and incubated at 22°C for 48 h. Cultures with milky white shiny colonies were positive for colanic acid production. The resulting positive cultures were harvested by flooding the MGA culture plates with 10 mls of sterile PBS (Phosphate buffer saline) (pH 7.43). The flooded culture plate was then agitated on an orbit shaker for 5 min. Ten microliter of liquid on flooded plate was then taken and a serial dilution to 10^{-3} in PBS (pH 7.4) in 15 ml test tube and carefully mixed. PBS was prepared for blank as a reference for the readings. Each of the dilutions was then emptied into 15 ml syringes with stoppers.

Readings on zetasizer

The readings of zeta potential of samples were done using the zetasizer equipment (3000 HSA; Malvern^R instrument). The manual sampler part of the machine was used. The machine was first of all cleansed with sterile water and test samples were subsequently injected into machine. Five readings were taken for each sample for accuracy and reported as a mean.

Fluorescence test for cellulose production

Expression of cellulose was determined by growing the cultures on LB no salt agar incorporated with cacofloura dye (200 µg/ml white stain- fluorescent brightener no. 28, ENG Scientific Inc., Clifton, NJ) for *E .coli* and tryptose soy agar + cacofloura dye were used for confirmation of cellulose production by *E. coli* and *L. monocytogenes* isolates respectively. Twenty-four hour old cultures were inoculated on appropriate media and incubated for 72 h for *E. coli* strains at 28°C and 24 h at 37°C for the *L. monocytogenes* strains. After the incubation periods colonies were observed under the UV light at excitation wavelength of 365 - 395 nm and an emission wavelength of 420 nm in a multi-purpose imaging system;

using the Kodak digital Science Imaging station 440CF (Eastman Kodak Company, Rochester, NY). Positive samples showed fluorescence brightness emanating from the surface of the colonies. Negative samples were dull or dark purple in colour.

Cellulose measurement

Positive cultures from the above showing fluorescence were now prepared for cellulose measurements at 24, 48 and 72 h of incubation. Since the preliminary studies showed variations in the growth of the different isolates, the cellulose measurement was calculated for 10^6 cfu of cells per ml. Counts of bacterial cells were made every sampling day for each culture.

Twenty-four hours fresh cultures were inoculated separately into sterile 50 ml tryptose soy broth contained in sterile 50 ml centrifuge tubes. On each sampling day 10 mls of each culture was used for quantification of cellulose and 1 ml for bacterial cell count. Serial dilutions were made to 10^{-3} for the bacterial counts. MacConkey agar was used for *E. coli* counts while Difo Oxford medium base to which was added acriflavine, nalidixic acid and cycloheximide antimicrobial supplement for *L. monocytogenes* counts. The media plates were plated using a Spiral plating machine. The plates were incubated at 37°C for 24 h. Counts were made using the electronic Q-counter. Aperture size, dilution factor were imputed into the Q-counter software before counts were made for each plate. Counts were made in duplicates and reported as means.

Quantification of cellulose produced by the isolates

On each of the sampling days for each isolate 10 mls of the broth culture was pippetted and dispensed into 15 ml centrifuge tube. The culture was then centrifuged at 3000 rpm with a centrifuge. The supernatant was decanted after centrifuge. Three milliliters of acetic nitric acid reagent was then added in two installments (1 ml then 2 mls) and mixed on the vortex on each addition. Tubes were covered with foil to reduce evaporation and create reflux and then placed in boiling water bath for 30 min. After this period of boiling the tubes were centrifuged again for 5 min at 3000 rmp. The supernatant was decanted and 10 mls of H_2SO_4 was added in 3 installments with intermittent mixing. The mixture is allowed to stand for 1 h. One milliliter of mixture is then dispensed in test tube con-taining 100mls of distilled water. This was vortexed and 1 ml of the mixture is dispensed into 4 ml of distilled water. The mixture was then placed in ice bath to cool. Ten milliliters anthrone reagent was added by layering with a pipette. This was followed subsequently by thorough mixing and placing tube back in ice bath until all tubes were mixed. The tubes were then capped and placed in boiling water for 16 min, cooled on ice bath for 2 - 3 min and allowed to stand at room temperature (22°C) for 5 - 10 min. One milliliter of each sample was placed in each cuvette for subsequent reading in the spectrophotometer. The absorbance of each sample was then read on the spectrophotometer at 620 nm wavelength against a reagent blank (Updegraff, 1969).

Preparation of standard curve

Fifty milligram pure cellulose was dried for 6 h at 105°C and cooled over anhydrous alumina in 10 ml 67% H_2SO_4 with gentle heat. This was diluted to 500 ml with distilled water to contain 100 ug agar (MGA) was prepared by adding 15 g Bacto agar in 1 L of deionized water (6.8 pH). To this mixture was added the following: cellulose /ml. Then analysis was done for 0.5, 1.0 and 1.5 ml stock standard, corresponding to 50, 100 and 150 ug of cellulose /ml by taking it in a screw cap culture tube. Appropriate dilution was then made with distilled water to make a final volume of 5 ml.

The tubes were then placed in ice bath to cool and cold anthrone reagent is added. The tubes were capped and thoroughly mixed placing each tube on ice bath until all were mixed. The tubes were placed in boiling water for 16 min, cooled on ice bath for 2 - 3 min and allowed to stand at room temperature (22°C) for 5 - 10 min. The absorbance at 620 nm against a reagent blank was then read.

Calculation of cellulose concentration using absorbance readings slope measurement and constant.

Formula: $y = mx + c$

Y = absorbance, x = cellulose concentration, m = slope, c = constant,

From the slope y = 0.0207 (x) + 0.04010 in ug / concentration of cells, amount of cellulose produced per cell was then calculated thus: cellulose ug/10 ml culture

The number of cells per ml of culture

The result of the equation was then calculated for bacteria cell concentration of 10^6 cfu per ml after making the specific counts per ml of culture for each incubation period.

Biofilm formation quantification

Preparation of culture

One colony of each *L. monocytogenes* and *E. coli* cultures were transferred into 9ml of tryptose soya broth. The inoculated broths were then incubated at 37°C for 24 h. These broth cultures were used for biofilm formation on glass surfaces. The uninoculated tryptose soy broth was used as a negative control.

Attachment on glass surface

Three milliliters of each broth culture was placed in glass vials. The bacteria culture was allowed to attach to the glass surface for 24, 48 and 72 h at 37°C. Lids were placed on glass vials to prevent evaporation of the broth. At the end of each day, cells attached to glass surface were quantified using crystal violet binding assay previously described by Stepanovic et al. (2004) with some modifycations. The broth was withdrawn at the end of each day and the glass vials were washed 3 times with 5 ml of sterile distilled water. The remaining attached bacteria were fixed with 1 ml of 70% ethanol per glass vial. And after 15 min the glass vials were emptied and air dried. The glass vials were then stained with crystal violet for 5 min. Excess stain was rinsed off by placing the vials under a running tap water. After the glass plates were air dried, the dye bound to the adherent cells was re-solubilized with 1 ml of acetic -nitric acid per well. The re-solubilized liquid was then poured into a cuvette. The optical absorbance (OD) of each liquid against a blank reading without inoculation was measured at 520 and 620 nm for *E. coli* and *L. monocytogenes* respectively using a spectrophotometer. Based on the OD produced by isolates strains were classified as no biofilm producers, moderate or strong biofilm producers as previously described by (Stepanovic et al., 2000). < 0.114 =/< blank reading meant no biofilm formation, 0.160 - 0.260 = 2x blank reading meant moderate biofilm producer while > 0.300 =/<4x blank reading meant strong biofilm producer (Stepanovic et al., 2000, 2004).

Statistical analysis

Microsoft excel 2003 was use to assess the level of correlation between virulent characteristics.

Table 1. Characterization of isolates of *L. monocytogenes* and *E. coli* identified in wara cheese.

Isolates	Cellulose	Fimbrial	Colanic acid	Reaction with *E. coli* O157antiserum	Reaction with *L. monocytogenes* polyserotypes antiserum
E. coli-a	+ve	+ve	+ve	+ve	NA
E. coli-b	+ve	+ve	+ve	+ve	NA
E. coli-c	+ve	_ve	+ve	+ve	NA
L.monocytogenes-a	+ve	-ve	-ve	NA	+ve
L monocytogenes-b	+ve	-ve	-ve	NA	+ve
L monocytogenes-c	+ve	-ve	-ve	NA	+ve
L monocytogenes-d	+ve	-ve	-ve	NA	+ve
L monocytogenes-e	+ve	-ve	-ve	NA	+ve
L. monocytogenes-f	+ve	-ve	-ve	NA	+ve
L monocytogenes-g	Doubtful +ve	-ve	-ve	NA	+ve
L monocytogenes-h	Doubtful +ve	-ve	-ve	NA	+ve
L. monocytogenes-i	Doubtful +ve	-ve	-ve	NA	+ve
L. monocytogenes-j	Doubtful +ve	-ve	-ve	NA	+ve
L monocytogenes-k	Doubtful +ve	-ve	-ve	NA	+ve

+ve = positive; -ve = negative; NA = not applicable.

RESULTS

Quantification of cellulose, bacteria count (cfu/ml), fimbrial, Zeta potential and biofilm formation at 24, 48 and 72 h incubation.

E. coli c which produced no fimbria and very low cellulose (Table 1) had a significantly lower zetapotential than the other 2 strains of *E. coli*. There was no significant difference ($P < 0.05$) in the biofilm formation for the 3 *E. coli* strains (Figure 1).

For the *L. monocytogenes* the LM- b had the highest zetapotential and cellulose production at 24 h of incubation. This was closely followed by LM-f which also has a higher cellulose production at 72 h incubation (Figure 2). There is a level of correlation between zeta potential and cellulose production by these strains. But there was no significant difference in biofilm formation although the LM-a had the highest at 48 h incubation.

LM-e has the highest at 72 h incubation (Figure, 2).

Positive R^2 values obtained for 24, 48 and 72 h incubation for bacteria count (cfu/ml) and corresponding cellulose measurement were 0.0397, 0.002 and 0.0011.

DISCUSSION

The level of cellulose, fimbria, zetapotential, and biofilm formation of isolates has been reported to be virulence characteristics of such isolates (Gulsun et al., 2005; Hood and Zottola, 1997). Higher producers of these factors have higher virulence than lower producers. *E. coli* c 0157:H7 strain with significantly lower zetapotential value, lowest cellulose production and no evidence of fimbrial production when compared with other two strains is the least virulent. On the other hand, *E. coli* b 0157:H7 strain with highest cellulose, zetapotential and fimbrial production seemed to be the most virulent strain of the *E. coli* strains. The high negative values of zetapotential obtained in *E. coli* a and b stains suggested that it plays an important role in the virulence of *E. coli* 0157:H7 strains although several workers have reported that specific binding of bacteria to substrates is believed to be mediated by polymeric molecules on the bacterial cell surface, such as pilli, fimbriae, lipopolysaccharide, or capsular polyssacharides (An and Friedmann, 1998; Costerton et al., 1999; Ofek and Doyle, 1994; Ohkawa et al., 1990) . Zetapotential measurement confirmed that all the three strains of *E. coli* are negatively charged also reflecting its pathogenicity. The expression of colanic acid observed in this study has been suggested to aid *E. coli* 0157:H7 biofilm formation. This is consistent with the report by Danese et al. (2000) . The dependence of biofilm on colanic acid expression in this study was reflected in the higher biofilm values observed in the three strains of *E. coli* compared with *L. monocytogenes* strains producing no colanic acid.

L. monocytogenes strains (LM-b) with the highest zetapotential and cellulose production at 24 h of incubation, followed by *L. monocytogenes* strain LM-I which also has a higher cellulose production at 72 h incubation indicated that there was a level of correlation between zetapotential and cellulose production by these strains. The production of relatively low cellulose, negative zetapotential without fimbriae in *L. monocytogenes* (LM-d, LM-e and LM-f) makes these strains to lack the ability to adhere to surfaces although invasive genes will aid the virulence of these strains. The *L. monocytogenes* strains also produce biofilms, but there was no significant difference in the biofilm formation although the *L. monocytogenes*

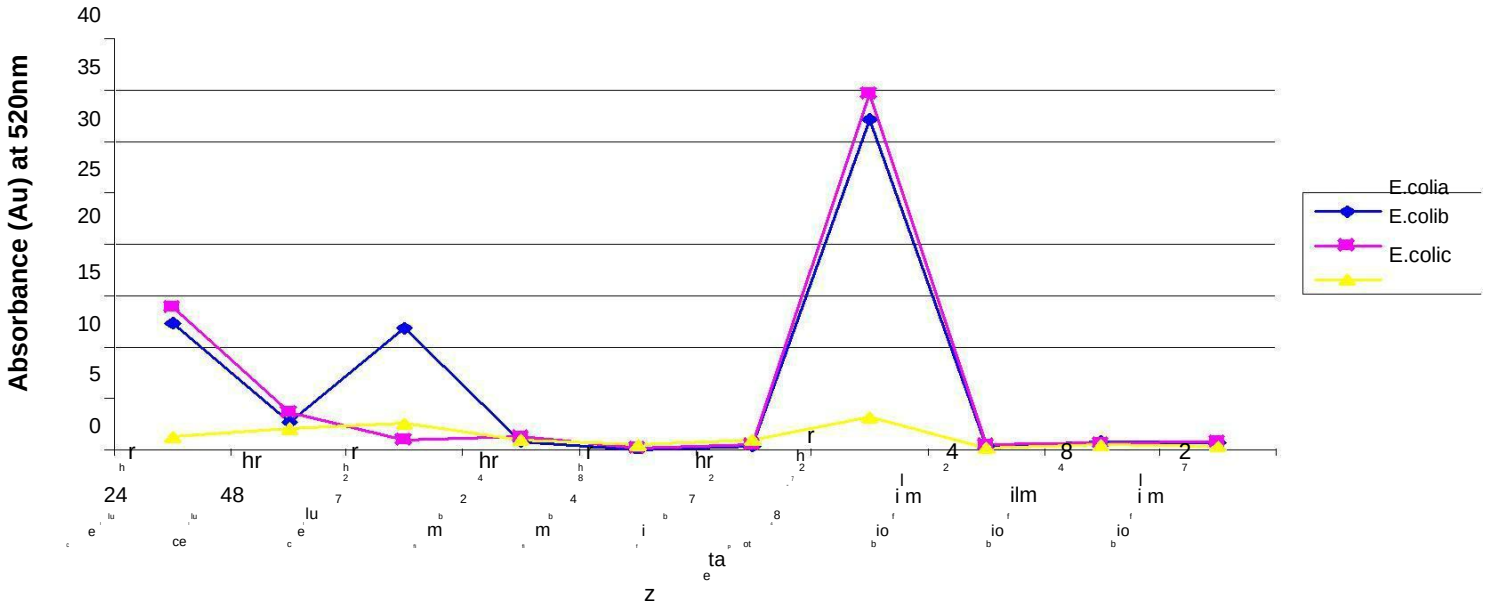

Figure 1. Chart comparing the virulence characteristics of *E. coli* O157H: 7 strains.

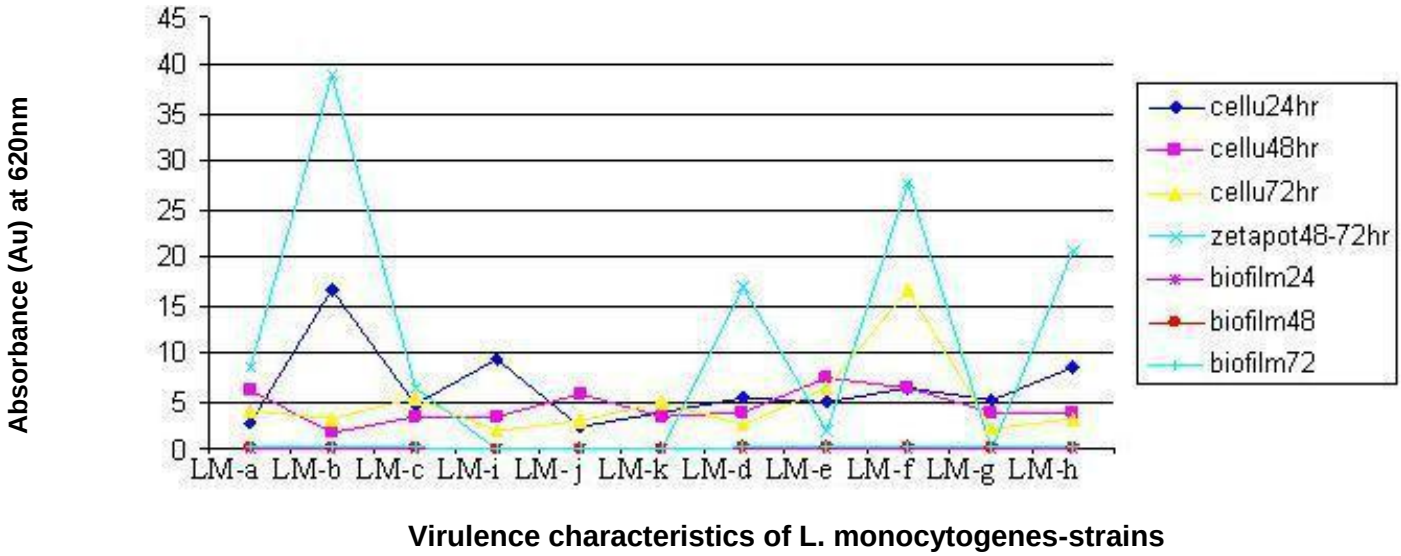

Figure 2. Chart comparing the virulence characteristics of *Listeria monocytogenes* strains.

strain LM- a has the highest at 48 h incubation. Thisimplies that the biofilm of *L. monocytogenes* will be highly resistant to removal by cleaning processes. This agrees with Jay (2000) report that protection offered to bacteria is greater in the presence of biofilm matrix. It was observed that where there were higher values for biofilms in *E. coli*-a and b, and a corresponding higher fimbrial formation for attachment. Leriche and Carpentier (1995) and Kim and Frank (1995) also reported that the attachment of a given pathogen to surfaces may be aided by the formation of a mixed-cultured biofilm which are

are different processes . The positive R^2 values showed that there was a positive correlation between the cellulose production by the isolates of *L. monocytogenes* and the bacterial growth. However, the correlation was very weak. The quantitative recovery of purified cellulose for culture also reflected some variations with extension in incubation period. Borucki et al. (2003) also reported similar variation in adhesion factor in *L. monocytogenes* strains although this study compared cellulose with the bacteria cell count.

This work confirmed the presence *E. coliO157:H7* and

L. monocytogenes strains with virulent characteristics of food processing relevance in West African soft cheese. Since wara (local soft cheese) is an important source of protein to the teaming population in Nigeria, rigorous effort should be made to improve on the microbial quality to prevent possible transmission of these pathogenic bacteria to the undiscerning public.

ACKNOWLEGDEMENT

The author wish to acknowledge Dr. Jinru Chen for assistance with the methodology.

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