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Influence of two different culture media on biofilm formation by *Listeria monocytogenes* isolated from a small-scale meat processing facility

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Abstract. In this study, 20 *Listeria monocytogenes* isolates detected in a food processing environment and food products were tested for biofilm-forming ability in two different culture media: Tryptone Soya Broth and Luria Bertani Broth. Statistical analysis of the data obtained was performed with the MINITAB software package, version 16.0. The two-sample t-test and confidence interval were used for data analysis. Significant differences between the isolates were observed in the ability to form biofilms.

1. Introduction

A foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a severe disease with high hospitalisation and case fatality rates. It can survive and grow in a wide range of adverse environmental conditions typical of food processing and preservation [1]. A critical point is the risk of *L. monocytogenes* persistence in food industry equipment and produce due to its ability to form biofilms [2]. Having colonised the food processing environment, *L. monocytogenes* can spread throughout the facility via aerosols, personnel, food workflows and contaminated contact materials leading to its persistent presence if sanitation procedures are insufficient [3]. In recent years, several authors investigated the relationships between biofilms and the main factors, such as surface type, temperature, and the presence of growth media and other microbes, involved in their formation, but the conclusions were often divergent [4,5,6]. Therefore, the aim of this study was to investigate the influence of two different culture media on biofilm formation by *L monocytogenes* isolates from a small-scale meat processing facility. Also, the susceptibility of the isolates to several antibiotics was assessed.

2. Materials and methods

2.1. Origin of isolates

Twenty *L. monocytogenes* isolates originating from traditional meat products and environmental swabs were studied. These samples were taken during the slaughter and preparation of dry and smoked meat products in a four-year period. The collected samples were transported to the laboratory within 2 h in a cold bin at < 4ºC.

2.2. Culture media

The culture media used in this study were Tryptone soya broth (TSB) (CM0129, Oxoid, Basingstoke, UK) and Luria Bertani (LB) broth, (Becton, Dickinson and Company, Sparks, USA).

2.3. Microplate biofilm assay

The *L. monocytogenes* isolates were examined for their ability to form biofilms using the microplate assay [7]. Each isolate was inoculated in 3 mL TSB and multiplied at 37 ºC for 18 h. On the following day, 20 µL of each isolate suspension were inoculated into four wells of sterile flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and 150 µL aliquots of the corresponding medium (TSB or LB broth) were added into each well. The plates also included a set of eight wells filled only with the tested medium as a negative control. After incubation at 30 ºC for 72 h, the plates were washed three times with sterile saline and allowed to dry at room temperature. The attached bacteria were fixed for 20 minutes at room temperature by adding 200 µL volumes of methanol into each well.

The plates were stained with 200 µL 0.3% aqueous solution of crystal violet (Crystal Violet, Fluka) for 30 minutes at room temperature. After being stained, the plates were rinsed under running water until there was no visible trace of stain. The stain bound to bacteria was dissolved by adding 200 µL of 96% ethanol. Optical density (OD) was measured spectrophotometrically (Labsystems Multiscan® MCC/340) using a 595 nm filter. Cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control.

2.4. Antibiotic susceptibility testing

The antimicrobial susceptibility of *L. monocytogenes* isolates to antibiotics was assessed using the standard disc diffusion test on Mueller Hinton agar (CM0337, Oxoid, UK), in line with the guidelines of the Clinical and Laboratory Standards Institute [9]. The following antibiotics were used: penicillin (P, 10 U), amoxicillin/clavulanic acid (AMC, 20/10 µg), ampicillin (AMP, 10 µg), ceftriaxone (CRO, 30 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (ERY, 15 µg), chloramphenicol (CHL, 30 µg), nalidixic acid (NA, 30 µg) and trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 µg).

3. Results and discussion

Our results demonstrated a strong influence of nutrient availability on biofilm production by *L. monocytogenes.* Statistical analysis was performed by the MINITAB software package, version 16.0. Concentrations were expressed as mean values, standard deviations, median and range of minimum to maximum. The two-sample t-test and confidence interval were used to examine statistical differences of transformed data between two groups of samples analysed. Significant differences between two groups of isolates were observed ($p = 0.000$; $p < 0.05$) in their ability to form biofilms. Box plots were used to illustrate both the measures of central tendency and the variability of the data on the distribution of biofilm formation by *L. monocytogenes* isolates grown in TSB and LB medium (Figure 1).

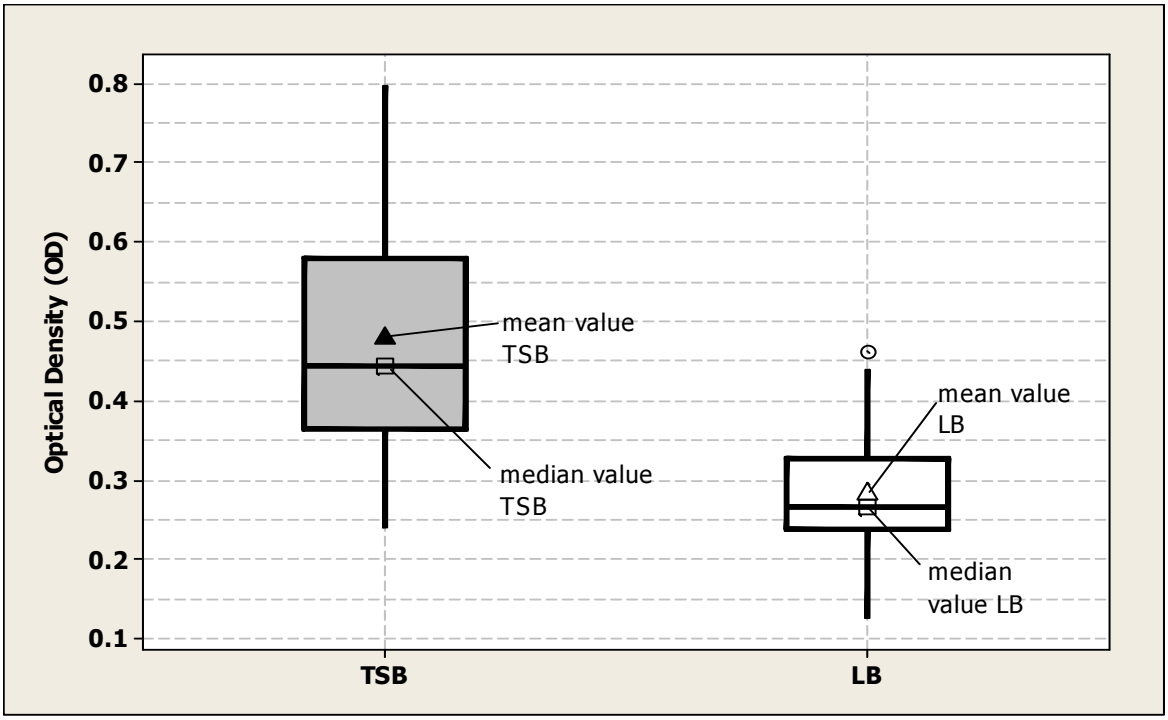

Figure 1. Box plot diagram of biofilm formation by 20 *L. monocytogenes* isolates grown in TSB and LB medium. Bacteria were incubated at 30 ºC for 72 h. The differences were considered significant if $p < 0.05$.

The ability of *L. monocytogenes* to survive in extreme conditions and to form biofilms on various surfaces is a significant challenge for food safety [9]. Biofilm formation creates major problems in the food industry because it can be an important source of food contamination [9].

All *L. monocytogenes* isolates proved susceptible to beta-lactam antibiotics (penicillin, ampicillin and amoxicillin/clavulanic acid), macrolides (erythromycin), sulphonamide (trimethoprim/sulfamethoxazole), chloramphenicol and resistant to nalidixic acid. Cefotaxime resistance was detected in 7 *L. monocytogenes* isolates obtained from minced meat samples taken from a machine, or from a mixer, dry sausage (pork), dry pancetta-sliced, pork tenderloin, pancetta or pork neck. These findings were similar to the study conducted by [10], who reported the majority of *Listeria* spp. isolated from food, clinical and environmental samples are sensitive to ordinarily used antibiotic therapy that is usually applied against Gram-positive bacteria including tetracyclines, ampicillin, penicillin G, imipenem, amoxicillin, sulphonamides, aminoglycosides, macrolides, chloramphenicol and glycopeptides.

4. Conclusion

Results of the microtitre plate based crystal violet assay revealed that all *L. monocytogenes* isolates produced biofilm on polystyrene. The nutrient-rich medium, i.e. cultivation in TSB, significantly enhanced biofilm production. Plastic materials are now more widely used in the food industry for the construction of tanks, pipework, accessories and cutting surfaces where nutrients are commonly available. Therefore, biofilms of *L. monocytogenes* are considered as a key factor contributing to the persistence of certain strains and repeated food contamination; the removal of irreversibly adhered cells is difficult and requires the application of strong mechanical force or chemical interruption of the adhesion using surfactants, sanifiers or heat. All *L. monocytogenes* isolates proved susceptible to beta lactam antibiotics, macrolides and sulphonamide, which are the first choice of antibiotics in the

therapy of listeriosis. Also, the resistance to the first generation fluoroquinolone (nalidixic acid) is considered as an intrinsic feature of this type of bacteria.

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