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Antibiotics Susceptibility Phenotyping and Extracellular Polymeric Substances Production of *Listeria monocytogenes* Biofilm and Planktonic Cells.

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ABSTRACT

The present research aimed to compare the susceptibility of *L. monocytogenes* planktonic cells and three different ages (10, 40 and 90 days-old) of biofilm to antibiotics. A second objective was to determine the relationship between the amount of produced EPS by *L. monocytogenes* biofilm and antibiotic susceptibility of *L. monocytogenes* biofilm. The extraction of EPS was carried out by using a cation exchange resin method and exopolysaccharides content in crude EPS was determined using phenol-sulfuric acid method. Antibiotic susceptibility of *L. monocytogenes* biofilm and planktonic cells were performed by the standard disk diffusion method. The maximum amount of exopolysaccharides was determined (411.5µg/cm^2) in 90 days-old *L. monocytogenes* biofilm collected from iron pipe. While, the minimum amount (203.4µg/cm^2) was in Cu pipe. Regarding to the antibiotic susceptibility results, it was found that, *L. monocytogenes* plankton cells were more sensitive to the tested antibiotic than *L. monocytogenes* biofilm. Also, it was found that, 90 days-old *L. monocytogenes* biofilm collected from iron pipe was more resistant to all tested antibiotics than which collected from Cu pipe. The findings in this research provide useful information and background data on the role of EPS in antibiotic resistance of *L. monocytogenes* biofilm.

Keywords: Biofilm, antibiotics susceptibility, EPS, *L. monocytogenes*,

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INTRODUCTION

L. monocytogenes is a gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacterium with an optimal growth temperature range of 30 - 37˚C. *L. monocytogenes* is a food and waterborne pathogen causing listeriosis disease in human and animals (Chen *et al*., 2010; Pagadala *et al.,* 2012). In addition to that, *L. monocytogenes* is widely distributed in different environments and can be found in water, soil, animal fecal matter and sewage (El-Taweel *et al.,* 2010; Vaid *et al.,* 2010). Moreover, *L. monocytogenes* can be easily found on surfaces, particularly in the form of a biofilm (Bonsaglia *et al.,* 2014).

Biofilm is a set of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which are secreted by these microorganisms, and attached to a surface. Biofilm is a protective niche for the microorganisms, also biofilm occur, usually in wet surfaces, and their presence in the drinking water distribution system is unavoidable (Fang *et al.,* 2010). The EPS matrix provides shelter from environmental stress, such as high salinity, extreme pH, UV radiation, chlorination, antibiotics and desiccation and thus permits survival under hostile conditions (Flemming and Wingender, 2010).

Although, human listeriosis is very rare, when it occurs it can be fatal or causes serious health problems, especially in the susceptible population groups, including the elderly people, pregnant women, fetuses, neonates and immunocompromised individuals (Sofos and Georgnaras, 2010). Ampicillin or ampicillin in combination with an aminoglycoside such as streptomycin or gentamicin is the primary choice for therapy (Charpentieer and Courvalin, 1999).

The antibiotic resistance of the pathogen is a significant public health concern (Oliver *et al.,* 2011). The first antibiotic resistant *L. monocytogenes* strain was reported in 1988. Since then, an increasing number of resistant strains isolated from foods, animals and humans have been reported (Aureli *et al.,* 2003). Therefore, the objective of the current study was to compare the susceptibility of *L. monocytogenes* planktonic cells and three different ages (10, 40 and 90 days-old) of biofilm cells to antibiotics. A second objective was to determine the relationship between the amount of produced EPS of *L. monocytogenes* biofilm and antibiotic susceptibility of *L. monocytogenes* biofilm.

MATERIALS AND METHODS

Designed experimental drinking water distribution system (DWDS) model

The present study was performed using a laboratory-scale simulated distribution system. The system consisted of 6 different sets of identical drinking water distribution pipes still used till now. The pipe materials were polyvinyl chloride (PVC), Polypropylene (PP), polyethylene (PE), Iron (I), copper (Cu) and rubber (R). Tap water was pumped from the contact tank (30 liters of tap water) into the simulated drinking water distribution pipe using a peristaltic pump. *L. monocytogenes* strain ATCC 25152 culture as a planktonic cells was used as inoculum, whereas the concentration of viable cells was (10^5 CFU/ml) of pumped water in the contact tank (Figure 1).

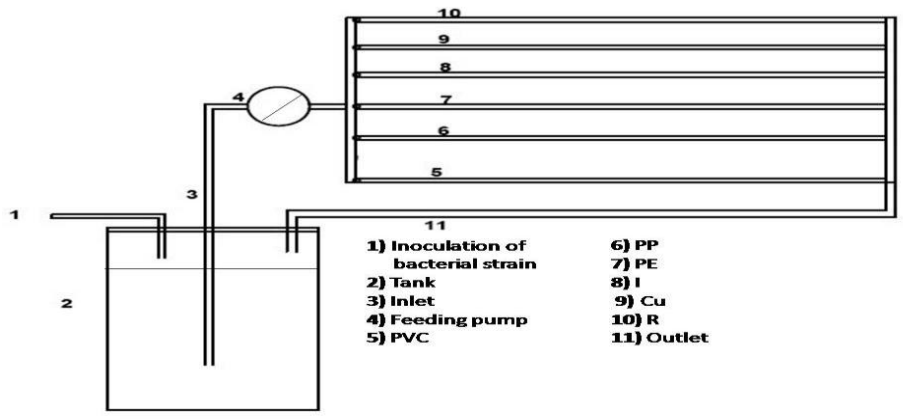

Figure1. Layout of *in vitro* simulated DWDS model.

Biofilm sampling

The biofilm samples were scraped with sterilized cotton swabs from the inner surface of tested pipe materials after 10, 40 and 90 days. Then, the samples were transferred to tubes containing 10 ml sterile water, and a vortex agitator for 2 min (Zhou *et al.,* 2009).

EPS extraction and exopolysaccharides analysis

Exopolysaccharides (EPS) of six biofilm samples which formed in different types of pipe materials in the above designed models of *L. monocytogenes* were measured each 10 days up to 90 days. Extraction of EPS was carried out according to Denkhaus *et al. (*2007); Michalowski *et al. (*2009) by using a cation exchange resin method. The exopolysaccharides content of crude EPS was determined using phenol-sulfuric acid method according to the protocol described by Dubois *et al.* (1956); Hofmann *et al.* 2009.

Antibiotic Disc Diffusion Susceptibility Test

The disk diffusion susceptibility tests were performed according to the Clinical and Laboratory Standards Institute (CLSI, 2012) by using the standard disk diffusion method on *L. monocytogenes* planktonic cells and six biofilm samples scraped from tested pipe materials (PVC, PP, PE, I, Cu and R) at three ages (10, 40 and 90 days-old). The scraped biofilm samples were transferred into 10 ml Tryptic Soy Broth (Merck, Germany). Then incubated at 37°C for 24 hrs and inoculated onto the entire surface of a dried Mueller-Hinton Agar plate (Merck, Germany) by using a sterile cotton swab.

The tested antibiotic discs (Oxoid-UK) were; amoxicillin 10 µg (AML 10), cefixime 5 µg (CFM 5), ciprofloxacin 5 µg (CIP 5), tetracycline 30 µg (TE 30), clarithromycin 15 µg (CLR 15) and streptomycin 10 µg (S10). The discs were placed on the surface of each inoculated Mueller-Hinton Agar plate. After incubation for 24 h at 37°C, the diameter (in mm) of the zone around each disk was measured by using HiAntibiotic ZoneScale (HiMidia, India). The results were interpreted in accordance with CLSI guidelines (CLSI, 2012) to classify the antibiotic sensitivity of each biofilm sample. *Staphylococcus aureus* was used as standard strain (Altuntas *et al.,* 2012).

RESULTS AND DISCUSSION

Exopolysaccharides analysis

EPS composing of polysaccharides, proteins, DNA and lipids with different percentages, these components involve to the mechanical stability of microbial biofilm. EPS are rich with high molecular weight polysaccharides and other non-sugar compounds such as proteins (Bayles, 2007). Flemming and Wingender (2010) reported that, the EPS play an important role in the biofilm accumulation. Also, Tsuneda *et al.* (2003) recorded the polysaccharides and proteins account for 75- 89% of the biofilm EPS composition, indicating that they are the major components. The obtained results of this research represented the highest amount of exopolysaccharides of *L. monocytogenes* biofilm was recorded in I pipe. But, the lowest amount was observed in Cu pipe material in all biofilm ages from 10 to 90 days. Additionally, the amount of exopolysaccharides of PVC, PP, PE and R pipe materials were respectively: 384.2, 390.6, 375.4 and 289.3 µg/cm^2 in 90 days-old biofilm (Figure 2).

Statistically, there was a positive correlation with significant between the quantities of exopolysaccharides and with increase in biofilm ages ($P<0.01$). It means that, the direct proportion between biofilm ages and the production of exopolysaccharides amounts was recorded. This is due to the exopolysaccharides act as a source of nutrients for bacterial proliferation to biofilm formation. Also, Bayles (2007) demonstrated that the bacterial cell death is one of the important factors that can promote biofilm formation because of the release of cell contents.

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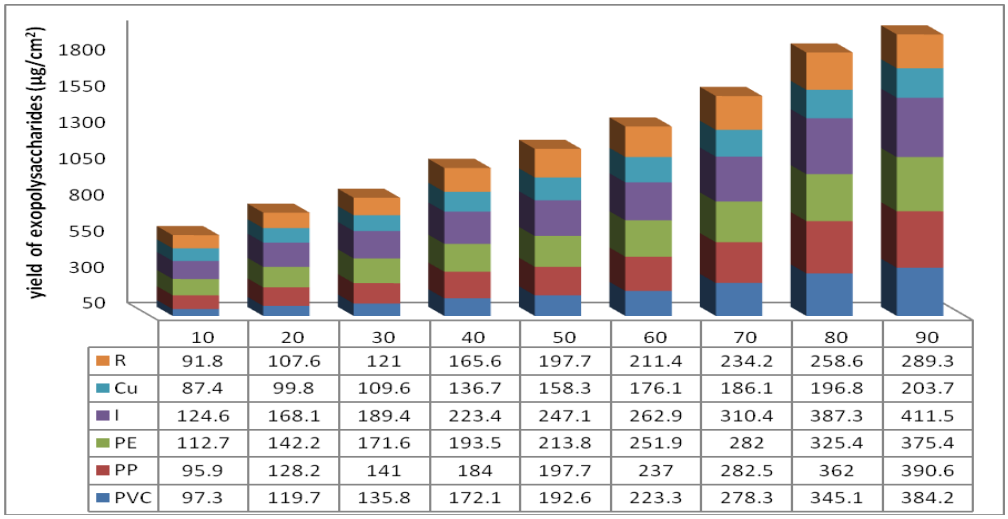

	10	20	30	40	50	60	70	80	90
R	91.8	107.6	121	165.6	197.7	211.4	234.2	258.6	289.3
Cu	87.4	99.8	109.6	136.7	158.3	176.1	186.1	196.8	203.7
I	124.6	168.1	189.4	223.4	247.1	262.9	310.4	387.3	411.5
PE	112.7	142.2	171.6	193.5	213.8	251.9	282	325.4	375.4
PP	95.9	128.2	141	184	197.7	237	282.5	362	390.6
PVC	97.3	119.7	135.8	172.1	192.6	223.3	278.3	345.1	384.2

Figure 2: Exopolysaccharides content of the extracted EPS of *L. monocytogenes* biofilm collected from six different plumping materials.

The antibiotic sensitivity test was carried out for planktonic *L. monocytogenes* cells and six biofilm samples in three ages (10, 40 and 90 days-old) by using disc diffusion method. The data represented in figure (3) showed that, *L. monocytogenes* planktonic cells were sensitive to all tested antibiotics except cefixime was intermediate. The obtained results were in agreement with Altuntas *et al.,* (2012) they reported *L. monocytogenes* strains were susceptible to the antibiotics, including penicillin G, vancomycin, tetracycline, chloramphenicol, rifampicin, erythromycin, gentamicin and trimethoprim. Consequently, the emergence of resistant bacteria to conventional antimicrobials clearly shows that new biofilm control strategies are required (Simões *et al.,* 2006).

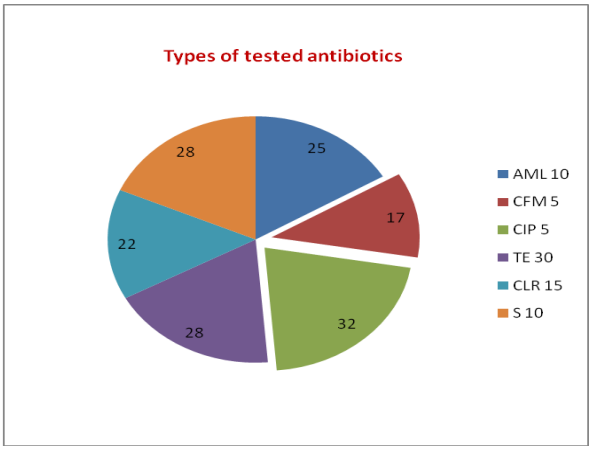

Figure 3: Estimation of antibiotic sensitivity of *L. monocytogenes* planktonic cells

L. monocytogenes is slowly becoming resistant because of the uptake of resistance genes from other Gram-positive bacteria such as *Listeria* spp., *Staphylococcus* spp. and *Enterococcus* spp. A continued surveillance of emerging antimicrobial resistance of this pathogen is therefore important to ensure an effective treatment of human listeriosis (Pesavento *et al.,* 2010). The attached bacterial cells on the surface and grow as a biofilm are protected by EPS from the killing by disinfectant (Stewart, 2002). In the present study, 10 days-old *L. monocytogenes* biofilm grown on all tested pipe materials except I pipe were sensitive to all tested antibiotic and resistant to cefixime. While, the biofilm cells scraped from I pipe were intermediate to clarithromycin (Figure 4).

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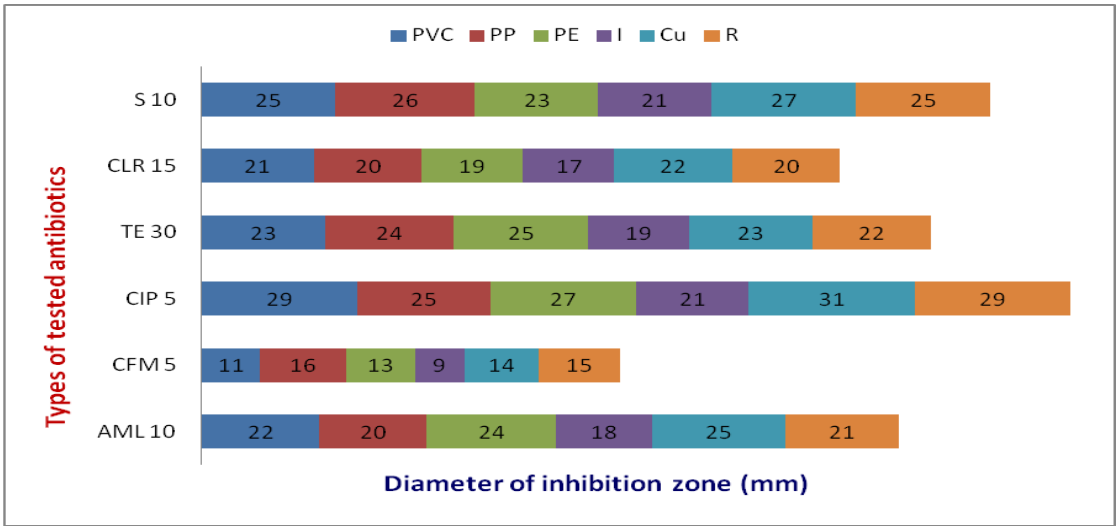

Figure 4: Estimation of antibiotic sensitivity of 10 days-old *L. monocytogenes* biofilm harvested from six different pipe materials

The results of 40 days-old *L. monocytogenes* biofilm antibiotic sensitivity illustrated graphically in (Figure 5), it was showed that, all biofilm cells were resistant to cefixime and sensitive to amoxicillin and streptomycin. The biofilm grown on I pipe material was intermediate to ciprofloxacin, clarithromycin and tetracycline. While, in Cu pipe material was sensitive to ciprofloxacin and clarithromycin then intermediate to tetracycline. Additionally, in the case of PP and PE biofilm cells were sensitive to ciprofloxacin and tetracycline, then intermediate to clarithromycin. While in PVC, the biofilm cells were sensitive to all tested antibiotics except cefixime was resistant. Whereas, the biofilm cells grown on R were sensitive to ciprofloxacin and clarithromycin then intermediate to tetracycline.

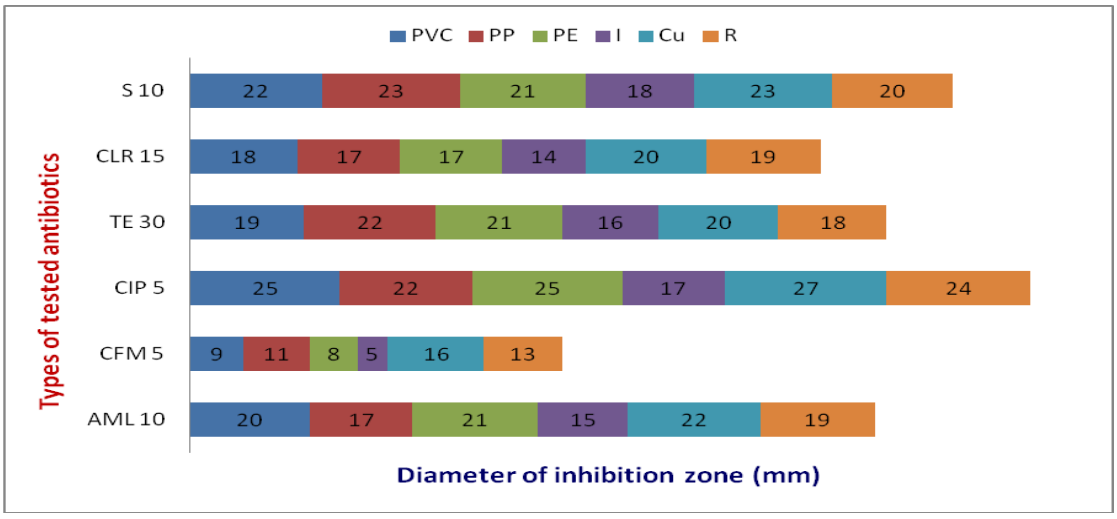

Figure 5: Estimation of antibiotic sensitivity of 40 days-old *L. monocytogenes* biofilm harvested from six different pipe materials

By regarding the results of 90 days-old *L. monocytogenes* biofilm antibiotic sensitivity, it was demonstrated the biofilm grown on all tested pipe materials was resistant to cefixime and sensitive to a amoxicillin except I and PP pipes were intermediate to amoxicillin. In case of PVC pipe material was sensitive to streptomycin, then intermediate to ciprofloxacin, clarithromycin and tetracycline. While, in PP pipe material the results found that, the biofilm cells were sensitive to tetracycline and clarithromycin then intermediate to ciprofloxacin and resistant to streptomycin. Accordingly, in the case of PE and R the results reported the biofilm cells were sensitive to ciprofloxacin and streptomycin then intermediate to clarithromycin and tetracycline. While in Cu pipe, the biofilm cells were sensitive to all tested antibiotics except cefixime was resistant (Figure 6).

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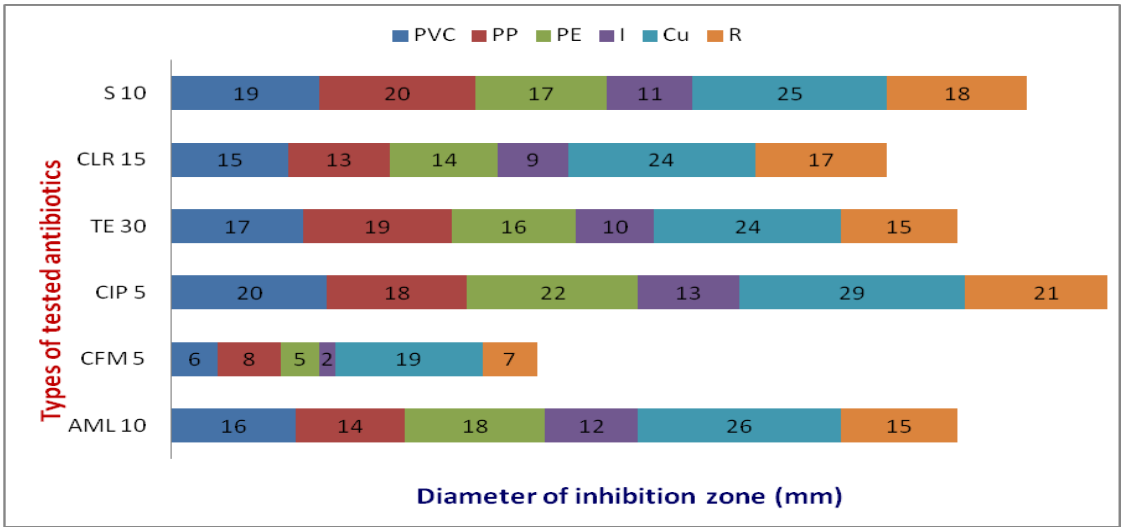

Figure 6: Estimation of antibiotic sensitivity of 90 days-old *L. monocytogenes* biofilm harvested from six different pipe materials

From the findings of the present research, it can be discussed *L. monocytogenes* planktonic cells was more sensitive to antibiotics than *L. monocytogenes* biofilm especially which formed in I pipe. This may be due to the production of large amounts of exopolysaccharides in 90 days-old than 10 days-old *L. monocytogenes* biofilm. These findings were similar with Simões and Vieira, (2009) they indicated, the biofilm cells are more resistant to antimicrobials compared to planktonic cells. Also, bacteria living in biofilm can be up to 1000 times more resistant to antibacterial compounds (such as disinfectants, antibiotics and surfactants) than planktonic cells (Davey and Otoole, 2000). Structural heterogeneity of biofilm provides an effective barrier that limit penetration of antimicrobial agents throughout the biological layer (Roeder *et al.*, 2010).

Moreover, Stewart (2002) suggested that, the EPS matrix which surrounding the attached cells produces a potent barrier that restricts the penetration of chemically reactive biocides inside the biofilm. Both structure and properties of extracellular compounds associated with solid surface cells differ from those synthesized by planktonic bacteria. These differences refer mostly to polysaccharide components of the EPS layer. Additionally, there are clear evidences that the high range of polysaccharide components increased the amount of functional groups in the EPS matrix. In addition to, it determines a lower susceptibility of biofilm populations to biocides, antibiotics and antimicrobial peptides. The functional groups of exopolysaccharides react with antimicrobial agents (Drenkard and Ausubel, 2002).

Conclusions and Recommendations

The findings in this research provide useful information and background data on the role of EPS in antibiotic resistance of *L. monocytogenes* biofilm. The *L. monocytogenes* planktonic cells are more resistant to tested antibiotics than biofilm cells. Also, there are clear direct proportion between the amount of exopolysaccharides produced by *L. monocytogenes* biofilm cells grown on all tested pipe materials and the biofilm ages. Accordingly, the obtained results, the appearance *L. monocytogenes* biofilm cells of resistant to antibiotics clearly show that new biofilm control strategies are required. There is no much available information about antibiotic sensitivity of *L. monocytogenes* particularly in biofilm state thus; the monitoring of the antibiotics sensitivity of this pathogen must be highlighted.

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