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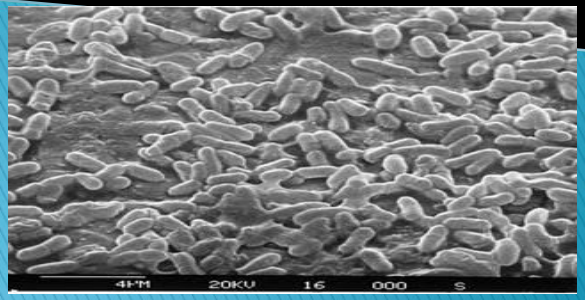
Influence of Time and Concentrations of Glucose and Sucrose on Biofilm Formation by Strains of *Listeria monocytogenes*

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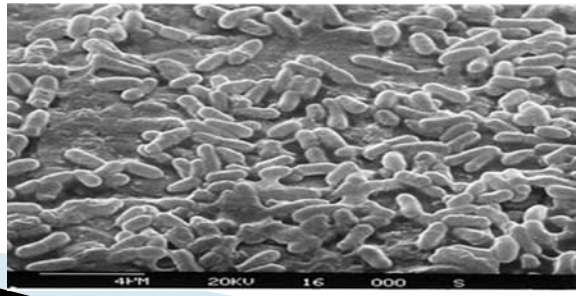


INTRODUCTION

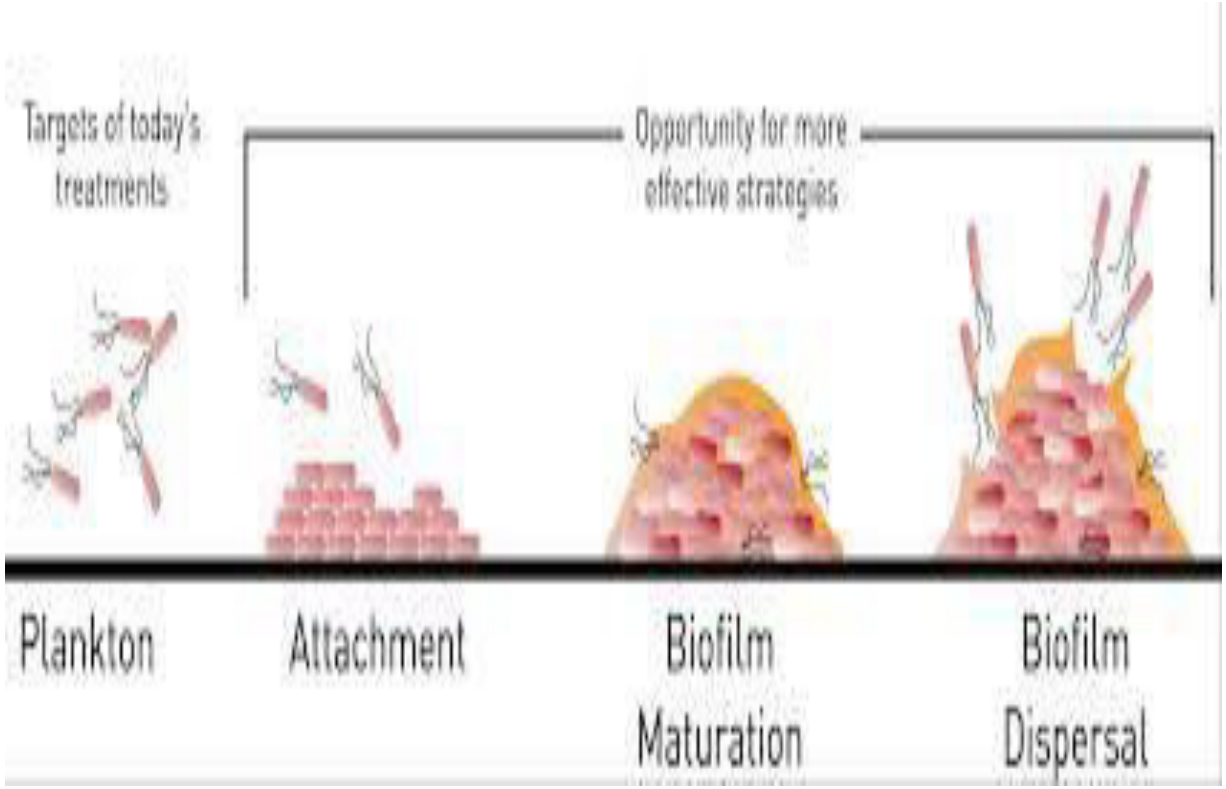
- *Listeria monocytogenes (LM)* is a major Gram positive pathogen implicated in food contamination due to their wide distribution.
- *LM* causes listeriosis in both humans and animals with high mortality.
- *LM* is usually acquired through food contamination (Taormina and Beauchat, 2002) from varieties of different raw, processed and ready-to-eat foods which have resulted in disease outbreaks (Vitas *et al.*, 2004; Aurora *et al.*, 2008; Rahimi *et al.*, 2010; Rivoal *et al.*, 2010).

Biofilms and its treat to food safety

- A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix of primarily polysaccharide material (Costerton & Donlan, 2002).
- Biofilms protect bacteria from several challenges including desiccation, bacteriophages, amoebae and biocide used in industrial processes (Costerton *et al.*, 1999).



BIOFILM DEVELOPMENT



Listeria monocytogenes

- *LM* has the ability to form biofilms (Hood and Zottola, 1995; Adetunji, 2010)
- biofilms enable *LM* to bound together and adhere to several food contact surfaces.
- biofilms facilitate the spread of *LM* in the environment, increase their resistance to antimicrobials and sanitizers (Purkrtova *et al.*, 2010) and promote plasmid and gene transfer through quorum sensing (Jefferson, 2004).

Justification

- *LM* biofilms have thus pose a problem of control in food processing environments.
- Effects of sugars on biofilm formed by some Gram-positive and Gram-negative pathogens have been demonstrated (Pillai *et al*., 2004; Yang *et al*., 2006; Duarte *et al*., 2008; Croes *et al*., 2009; Tahmourespour *et al*., 2010; Moreiraa *et al*., 2013)
- Limited reports existing on the effect of glucose and sucrose on biofilms of pathogenic listeria species.

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PREVIOUS RESULTS

- **LISTERIA**
- Strains of *LM* isolated from West African wara cheese were analysed for cellulose production and biofilm formation.
- Weak positive correlation (R^2) values of 0.0397, 0.002 and 0.0011 were obtained for 24, 48 and 72 h incubation for *LM* counts (cfu/ml) and cellulose measurements (Adetunji, 2010).

PREVIOUS RESULTS

- **MYCOBACTERIUM**
- Revealed that both *Mycobacterium bovis* and *Mycobacterium tuberculosis* has affinity to form biofilms on steel, cement and ceramic (Contact surfaces used in Meat industry).
- Form higher biofilms on cement than on steel and ceramic.
- Revealed that cement as contact surface in meat industry will provide a good surface for biofilm formation and the degree of contamination and recontamination will be more greater in those abattoirs that lack good and regular cleaning and disinfection pro[illegible]ols (Adetunji *et al*., 2014a& b).

AIM OF STUDY

- This study assessed the ability of some food and disease outbreak strains of *LM* to form biofilms and tested the effect of glucose and sucrose taking into consideration the concentration of the sugars and period of incubation of the biofilms.

Materials and methods

Table 1: LM strains used in the study

Strain	Sources	Isolate type	Serotype
LM28	Food isolate	Laboratory stock culture	–
LM35	Food isolate	Laboratory stock culture	–
LM37	Food isolate	7764	1/2a
LM38	Food isolate	7762	4b
LM39	disease outbreak	8738	–
LM40	disease outbreak	8506	–
LM41	disease outbreak	7962	4b
LM42	disease	7869	–

Bacterial strains and growth media

- Eight strains of *LM* were used for biofilm development in the present study.
- Four of the strains (LM28, LM35, LM37, and LM38) were food isolates while the other four were disease outbreak isolates (LM39, LM40, LM41, and LM42).
- All strains were stored at -20°C in tryptic soy broth containing an equal volume of sterile 30% glycerol. Cultures were sub-cultured on tryptic soy agar and subsequently sub-cultured in tryptic soy broth. Inoculated cultures were incubated for 16 h at 37°C aerobically.

Biofilm formation/formation

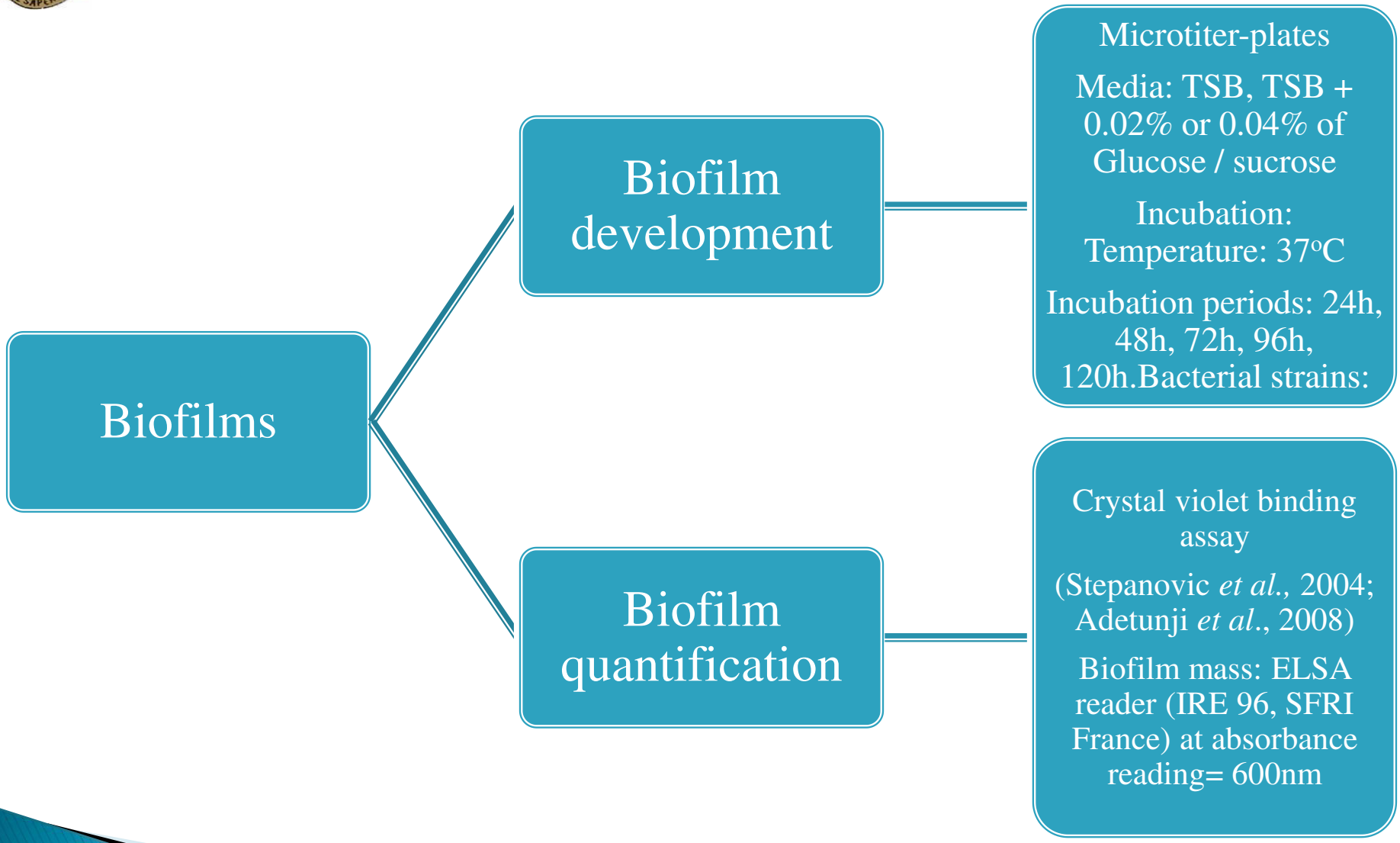
- Biofilm was developed in tryptic soy broth (TSB) in 96 well polystyrene microtiter plates. The broth was supplemented with glucose or sucrose (0.00%, 0.02% and 0.04%).
- An overnight culture of the test strains in TSB at a concentration of 10^8 bacteria cells was used for this study.
- Biofilm development was permitted at 37^{o}C for 24 h, 48 h, 72 h, 96 h and 120 h using a multifactorial study design. Un-inoculated broths and broths not supplemented with the sugar served as controls.

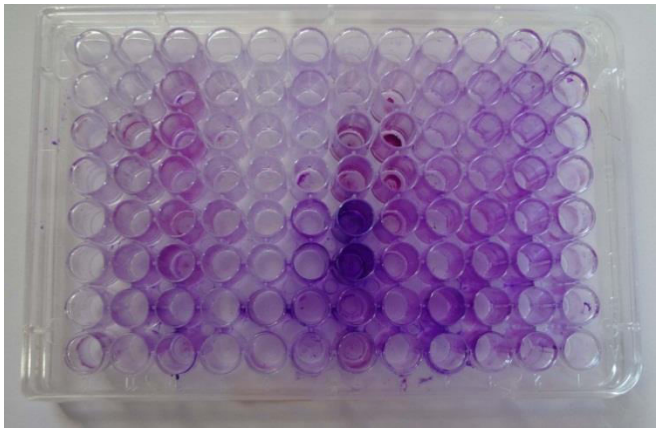
Biofilm quantification

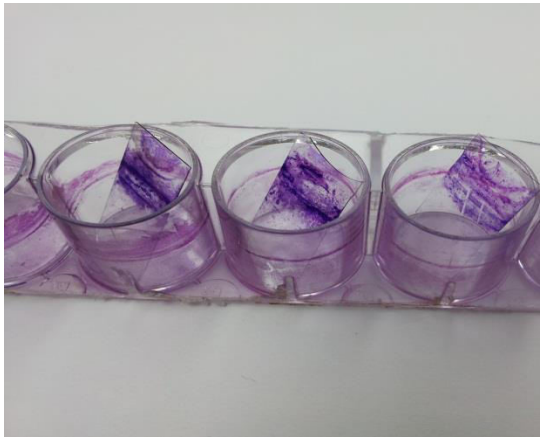
- At the end of 24 h, 48 h, 72 h, 96 h and 120 h incubation periods, developed biofilms were quantified using the crystal violet binding assay as previously described by Stepanović *et al*. (2004) and Adetunji and Adegoke (2008).
- At each sampling point, microtiter plates were emptied and washed 3 times, each with 5 mL of sterile distilled water.
- Biofilm mass was fixed with 1 mL of 95% ethanol (AnalaR, BDH Chemical Ltd., UK) for 15 min at room temperature.

Biofilm quantification

- The fixed microtiter plates were air dried for 10 min and then stained for 5 min with 2% crystal violet (AnalaR, BDH Chemical Ltd., UK).
- Excess stain was rinsed with running tap water, and the microtiter plates were then air dried.
- To each of the dried well an aliquot of 300 µl of 33% glacial acetic acid (AnalaR, BDH Chemical Ltd., UK) was used to solubilize the crystal violet.
- The absorbance of solubilized stain was read using an ELISA reader at 570 nm (Fishers Scientific, USA).


Biofilms
Biofilm development
Microtiter-plates
Media: TSB, TSB + 0.02% or 0.04% of Glucose / sucrose
Incubation: Temperature: 37°C
Incubation periods: 24h, 48h, 72h, 96h, 120h.Bacterial strains:
Biofilm quantification
Crystal violet binding assay
(Stepanovic *et al.*, 2004; Adetunji *et al.*, 2008)
Biofilm mass: ELSA reader (IRE 96, SFRI France) at absorbance reading= 600nm





Biofilm development: microtiter plate; chips

Statistical analysis

- Multifactorial design.

- Separation of means was accomplished using Fisher's least significant difference design and the General Linear Model of Statistical Analysis Software (SAS, 2000; $\alpha = 0.05$).

RESULTS

Table 2: INFLUENCE OF TIME, GLUCOSE AND SUCROSE ON BIOFILM FORMATION BY 8 STRAINS OF LISTERIA MONOCYTOGENES

	Biofilm Mass (A_{570})
Incubation time (n =160)	
24h	0.102C
48h	0.125C
72h	0.156C
96h	0.152B
120h	0.334A
Sugar (n =320)	
Control	0.253A
glucose	0.182B
sucrose	0.126C

LM=*Listeria monocytogenes*

Table 2: INFLUENCE OF CONCENTRATIONS OF SUGAR ON BIOFILM FORMATION BY 8 STRAINS OF LISTERIA MONOCYTOGENES

	Biofilm Mass (A_{570})
Concentration of sugar (n =320)	
0%	0.253A
0.02%	0.177B
0.04%	0.131C
Bacterial strains (n=100)	
LM28	0.221B
LM35	0.207B
LM37	0.249B
LM38	0.320A
LM39	0.094C
LM40	0.111C
LM41	0.094C
LM42	0.095C

LM=Listeria monocytogenes

RESULTS

- Biofilm masses of *LM* isolates were inhibited significantly ($p<0.05$) in the presence of sucrose than glucose.
- Inhibition pattern varied among strains.
- This inhibition was higher at increased concentrations of the sugars.

RESULTS

- These confirm the biofilm inhibitory potential of sucrose and glucose on *LM* at the appropriate environmental conditions.

- Previous studies have shown that the ability of different sugars to inhibit biofilm development of pathogens depends not only on the sugar types used but also on the environment were the biofilms are developed and strain types.

RESULTS

- Different reduction patterns observed for the various *LM* biofilm masses used in this study could be due to a possible variation in the genetic backgrounds of the strains tested.

- Nutrient content of the developing medium also plays important roles in biofilm regulation (Carlson, 2000; Gilmore *et al*., 2003).

DISCUSSION

- Comparably, addition of glucose levels between 1 and 20 g/l to modified Welshimer's broth did not affect *LM* biofilm formation though mannose and trehalose enhanced biofilm formation (Kim and Frank, 1995).

- Possibly, *LM* isolates have the capability to metabolize these sugars into lactic acid which represses biofilm formation. More lactic acid could be formed with the disaccharide (sucrose) than glucose (monosaccharide).

DISCUSSION

- This could also be related to the repression of biofilm associated expression genes in *LM.*
- Earlier studies showed that gene expression affects biofilm formation in several pathogens and their serotypes (Kiska and Macrina, 1994; Li and Burne, 2001; Shemesh *et al*., 2007a).

DISCUSSION

- Shemesh *et al* (2007b) found a significant reduction in biofilm depth of *Streptococcus mutans* in Brain Heart Infusion medium supplemented with sucrose compared to Tryptone-Yeast medium supplemented with sucrose.
- This biofilm reduction is related to the down regulation and expression of most biofilm regulatory genes associated with *Streptococcus mutans*.
- Carbohydrate metabolism plays an important role in biofilm suppression.

DISCUSSION

- Notable reduction of *LM* biofilm masses observed with the use of higher sugar concentrations is expected and may be due to higher levels of sugar catabolism.
- However, varying glucose concentrations (0%, 0.1% and 0.25%) positively influenced biofilm formation in *Staphylococcus aureus* of different lineages (Croes *et al*., 2009).

CONCLUSION

- This study concludes that the use of sugars could pave a way for effective control of *LM* biofilms in the food industry.

Further studies

- Since there is limited information on the effect of sugars on biofilm associated genes in *LM*, studies analyzing the genes mostly involved and responsible for biofilm repression should be conducted.

- This would provide a better insight in control of *LM* biofilms.

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