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Assessment of the ability of *Listeria monocytogenes* to form Biofilm in model System simulating food stuffs

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ABSTRACT

The objective of this study is to evaluate removal biofilms using a model system simulating food stuffs. Surface tile ASIS was used for surface adhesion and biofilm formation in coplin containers containing tiles, and for determination of cells in planktonic form was carried out by plate counting of colony forming units. Results obtained in this phase of study indicate that the composition of the growth medium can dramatically effect of *Listeria monocytogenes* to form biofilm and increased adhesion of cells when nutrient availability. In this work the experiment show that *L. monocytogenes* is able to adhere to the surfaces of stainless steel, forming a microbial biofilm, the composition of the growth medium greatly affects the ability of the test organism to from biofilms and especially, a greater adhesion of cells when the availability of nutrients is low (frequent condition in a plant). May be of particular interest to the food industry stressing the need to intervene in a preventive way to avoid or at least limit for formation of biofilms on surfaces in contact with the food during their processing.

Keywords: Biofilms, *Listeria monocytogenes*, Planktonic, Stainless steel, Tile.

1. INTRODUCTION

The term biofilm was created to describe the sessile form of microbial life characterized by adhesion of microorganisms to biotic or abiotic surface [1]. *Listeria monocytogenes* attaches to and forms biofilm that are protective layers of proteins and polysaccharides surrounding the bacteria and stick onto numerous surfaces and equipments [2]. *L. monocytogenes* capable of colonizing biotic and abiotic surfaces [3,4], and persist in the environment for years[5,6] and it reported its occurrence in meat and dairy processing equipment [7,8,9]. *L. monocytogenes* was isolated from dairy industry processing plants and common sites were packing equipment, walls, floor drains, cooling pipes, hand tools or gloves and also freezers[10].The ability of *L. monocytogenes* to attach to different materials has led some authors to consider the microorganism as able to populate and colonize environmental niches [11]. The biofilm formation permits microorganisms to resist drying, UV light, and treatment with sanitizing agents. For more understanding of the interaction between microorganisms and food processing surfaces is required to control the problem. This work proposes the use of tile os stainless steel to study of adhesion of *L. monocytogenes*.

2. MATERIALS AND METHODS

2.1 Bacterial strain

In the experiment we used a strain of *L. monocytogenes* isolated from cheese, was obtained from the collection of the Laboratory of Applied Microbiology, Department of Food Science of the University of Foggia. The strain was stored at -20 °C in Tryptone Soya Broth (TSB, Oxoid, Milan) supplemented by 33% glycerol. Before use, the microorganism has been revitalized in TSB and incubated at 37 °C for 24 hours. The obtained pre-

culture was used for inoculation of different substrates. The substrates tested were TSB, TSB diluted (1:5 with distilled water) (DTSB), Bacteriological Peptone (Oxoid) , 1 % (0.1% BP) , 0.05% (0.05 BP %) and Skim Milk Powder (Oxoid) reconstituted to 1% (SMR).

2.2. Surface adhesion

As a surface for biofilm formation were used tiles in stainless steel AISI 316L (25.4 mm • 50.0 mm, thickness 0.5 mm) before each test, these tiles were washed with acetone and then immersed in a solution of NaOH 1N (JT Baker). After 60 min were rinsed with distilled water and allowed to dry for 24 h [12]. This treatment was designed to remove fingerprints, grease or oily substances that may be present on the steel.

2.3. Biofilm formation

20 ml aliquots of the different types of medium have been distributed in containers Coplin (Barloword Scientific , United Kingdom) containing 5 tiles in a vertical position in order to have the entire surface exposed to the medium (Figure 1). After sterilization by autoclaving at 121 °C for 15 min, each sample was then inoculated with the test organism (initial inoculum ~ 10^3 CFU / ml) and incubated, without shaking, at 30 °C, for 5 days.

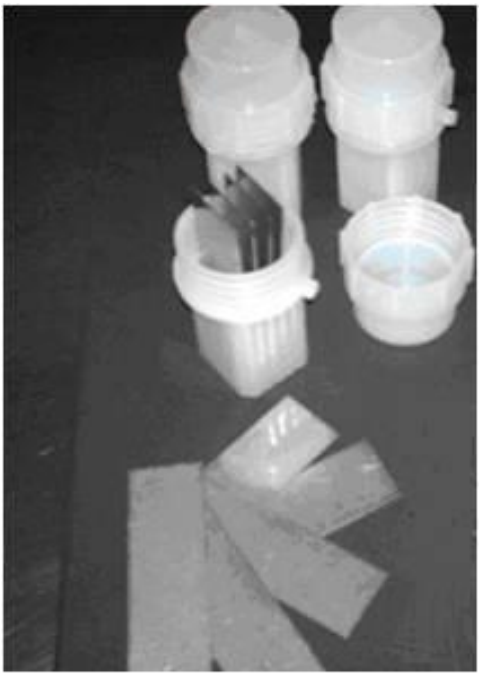

Figure 1: Example of samples prepared for the formation of biofilm.

2.4. Evaluation of the microbial biofilm

The trend of the microbial population in both plank tonic from that sessile was monitored by sampling after 8,24,48,72,96 and 120 h after inoculation, the determination of cells in planktonic form was carried out by plate counting of colony forming units (CFU) on Tryptone Soya Agar (TSA) .the plates were incubated at 37 ºC for 24 h relatively to cell in the form sessile at specified times the cards has been removed aseptically, rinsed with sterile distilled water to remove the non-adherent cells and transferred in to a disposable test tube containing 40 mL of sterile saline (0.9%NaCl). To cause the detachment of adherent cell, we used a sonicator (SONICS, Newcastle.,CT,USA). The treatment of sonication was carried out for 3 min at 20 watts. Viable cells were counted and tillable through serial dilutions of the suspensions obtained and subsequent plating on TSA, incubated at 37ºC for 24 h.

3. RESULTS AND DISCUSSION

The first phase of the experiment was proposed as an objective evaluation of capacity *L. monocytogenes* to form biofilms in model system food simulates and the determination of the influence of the composition of the growth medium on the attitude accession .As a bonding surface was tested stainless steel, material commonly present in the food industry .the substrate tested were TSB, TSB diluted (1:5) (DTSB), bacteriological peptone ,1%(0,1BP),0,05% (0.05%BP) and reconstituted skim milk 1% (SMR) . the choice of these means was prompted by the consideration that, in a system of prepared and or processed foods, the microflora can be exposed to different levels of nutrients [13], is well known also that many factors can influence the formation of biofilm and among these especially the composition of the growth medium [14 ;15] .For the foregoing reasons, the TBS and SMR were chosen because they are very rich in nutrients while the DTSB and BP (at two different concentrations) as a means extremely poor ,but closer to industrial condition because they are hardly similar to laboratory culture conditions [16] , the trend of the microbial population ,both in plank tonic from that sessile , was monitored by sampling after 8,24,48,72,96 and 120 h after inoculation.

L. monocytogenes in planktonic form grew in all conditions tested (Figure 2), after 8 h of incubation the

evolutions of the microbial load in all the substrates, the microbial load reached by the microorganism grown in TSB stood about 7,860 log CFU/ml compared to about 6.90 log CFU/ml of loads DTSB, SMR and BP 0.1%, significantly lower than was the development in BP 0.05 % with a microbial load equal to 5.813 log CFU/ml. At the end of the trial it was obvious difference between the development of microorganism grown in TSB (8.331 log CFU/ml) and grown in BP 0.05% (7.495 log CFU/ml).

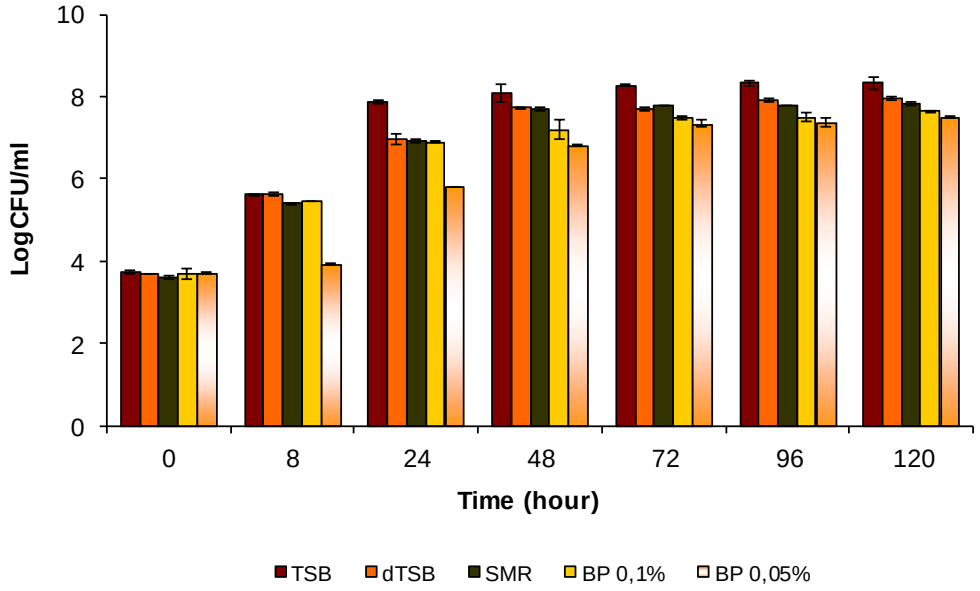

Figure 2: Evolution of microbial loads of *Listeria monocytogenes* in planktonic form observed in TSB, DTSB, SMR and BP (0.05% and 0.1%) incubated at 30 °C.

The data represent the average of two repetitions and are accompanied by the standard deviation different superscripts indicate values significantly different (one-way Anova and Tukey test, p< 0.05) .Relatively to the development in the form sessile, after 8 h of incubation, *L. monocytogenes* adhered to stainless steel only DTSB and BP, no membership is noted in the two richest substrate (Figure 3). After 24 h, the biofilm was formed in all the substrate, but the greater number of adherent cells was recorded in 0.1 % BP (3.728 Log CFU/cm^2). From 72 h onwards, the one-way analysis of variance (followed by Tukey test) showed statistically significant differences ($p<0.05$) between the loads in the form sessile microbial substrates recorded in the richest (3.60 to 3,70 Log CFU /cm^2) and the recorded in the nutrient – poor substrates (Log CFU/cm^2) and those recorded in the nutrient-poor substrates (Log CFU/cm^2 4.50 to 4.60), which, therefore, the substrate were more effective in promoting the adhesion of *L.monocytogenes*.

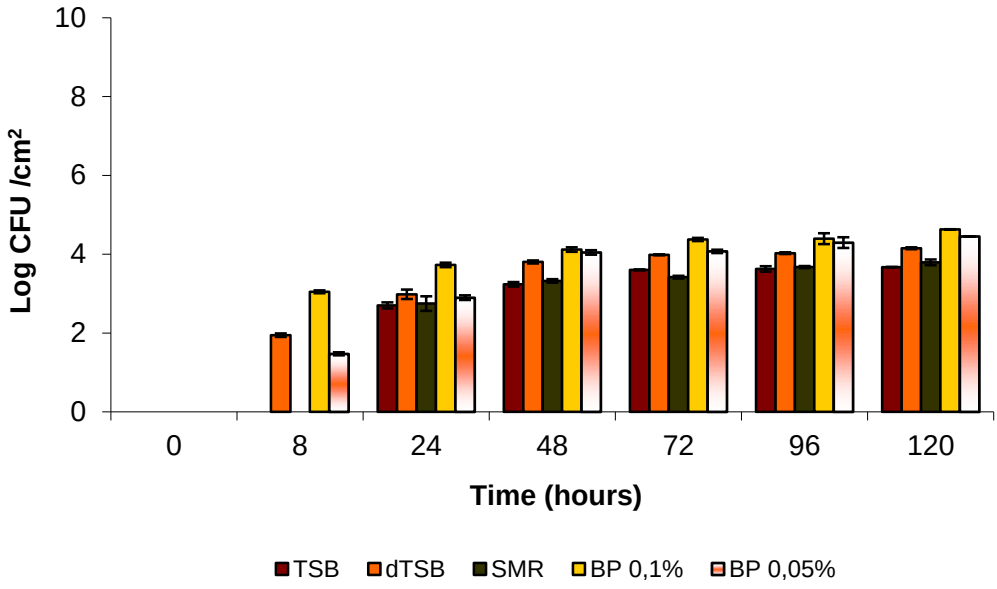

Figure 2: Evolution of microbial loads of *Listeria monocytogenes* in the form sessile observed in TSB, DTSB, SMR and BP (0.05% and 0.1%) incubated at 30°C

The data represent the average of two repetitions and are accompanied by the standard deviation different superscripts indicate values significantly different (one-way Anova and Tukey test, $p<0.05$).

Table 1 shows the data for the initial inoculation and the accession of *L. monocytogenes* on stainless steel after 8 and 24 h of incubation, the parameter concerning the accession after 8 h again confirms the BP as the most effective in promoting the formation of biofilms, reaching values around 50%. After 8 h, no adhesion was observed in the SMR and the TSB the means in which the growth in plank tonic form was higher.

Table 1: Data for the initial inoculation and adhesione after 8 h of *Listeria monocytogenes*in TSB, DTSB, SMR and BP (0.05% - 0.1%) at 30 °C.

Listeria monocytogenes	Microbial loads				
	substrates	**Planctonic form (initial inoculum) log CFU/ml**	**Adhesions after 8 h log CFU/cm²**	**Biofilm formation after 24 h log CFU/ cm²**	**Relative adhesions after 8 h[D]**
	TSB	3.73 ± 0.057[A]	<1	2.70±0.077[A]	N.D.[E]
	DTSB	3.70 ±0.007[A]	1.94±0.44[A]	2.98±0.120[A]	31.09±0.018[A]
	SMR	3.62±0.043[A]	<1	2.75±0.186[A]	N.D.
	BP 0.1%	3.62 ±0.043[A]	3.04±0.038[B]	3.73±0.058[B]	52.06±0.042[B]
	BP 0.05%	3.72±0.035[A]	1.47±0.031[C]	2.90±0.036[A]	32.50±0.028[C]

A, to surface adhesion: stainless steel. B, the values in the same column with the same super scritte not differ significantly ($p>0.05$). C, the microbial loads are expressed in log CFU/ml or log CFU/cm^{2,} they are the average of two repetitions and are accompanied by the standard deviation. D, the relative adheison after 8 h was determined by comparing the microbial load in the form adherent after 8 h (expressed as log CFU/ml) in the microbial load planktonic form at the same time. E, not determined.

The lower tendency to the formation of biofilms shown by the facts strains develop in SMR would seem to be in agreement with what reported by [17] that observed that the skim milk, whole and chocolate milk were able to significantly reduce the adhesion of *L. monocytogenes* and *Salmonella typhimurium* to stainless steel surfaces. They attributed this effect to some components of milk, such as casein and β - lactoglobulin. Further confirmation of these results can be found in the study by [18] who reported decreases in adhering by *Pseudomonas spp*. To polystyrene surfaces in the presence of proteins such as albumin, fibrinogen and pepsin. The results obtained in this phase of the experiment indicate that the composition of the growth medium can dramatically affect the ability of *L. monocytogenes* to form biofilms and, above all, we observed an increased adhesion of cells when nutrient availability is limited. In fact, the most effective means to promote this phenomenon has shown the BP, that is poorer in nutrients [19; 20]. One possible explanation for the different response to the tested conditions may lie in the emergence of mutations in genes involved in biofilm formation [21]. The increased biofilms formation observed in BP could also be explained by changes in surface features that facilitate cell adhesion in the absence of nutrients [22]. Biofilm formation is, however, a complex process regulated by many factors, the effects of which are still poorly understood. For this reason, we can only speculate on the reasons for the different behavior of the strains tested in different nutrient media.

It also interesting to note that the level of cells present in the form sessile, upon reaching the stationary phase around 24-48 h, did not increase during of days [23] in their studies on biofilm formation by *Bacillus subtilis, Pseudomonas fragi* and *L. monocytogenes,* they observed that the levels of adherent cells never went beyond the value reached in the early hours of incubation, and after 10 days that increasing the total area on which the biofilm developed. These authors suggested that under static conditions, such as those tested by us, the number of cells in the form sessile can be very high, but not necessarily increase over time. A possible interpretation is that the surface reaches a saturation level such that a high number of cells in planktonic form does not correspond necessarily an equally high number of cells in the form sessile.

4. CONCLUSION

The results obtained in the first phase of the experiment show that *L. monocytogenes* is able to adhere to the surfaces of stainless steel, forming a microbial biofilm, the composition of the growth medium greatly affects the ability of the test organism to form biofilms and, especially, we are witnessing a greater adhesion of cells when the availability of nutrients is low.

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