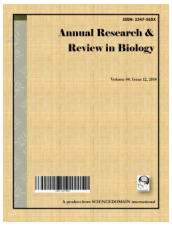

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Microbial Adhesion of *Salmonella Muenster, Salmonella Kentucky, Salmonella Newport* and *Salmonella Kiel*: Effect of Ionic Strength on Physicochemical Surface Properties

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Authors' contributions

This work was carried with collaboration between all authors. Author KE conducted all experiments protocol with help of authors ST and CZ. Authors HL, HZ and ME designed the study and consulted on the study approach. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of this study was to investigate the ability of adhesion of different *Salmonella*'s serovars (*S. Newport, S. Muenster, S. Kentucky and S. Kiel*) isolated from food surfaces under two ionic strengths (0.1M; 0.001M), in order to understand the influence of environmental characteristics on their adhesion behaviour.
Place and Duration of Study: Laboratory of bioprocesses and biointerfaces; Sciences and technologies Faculty (FST) between February 2015 and July 2015.
Methodology: Physicochemical properties (hydrophobicity, electron donor- electron acceptor) of cells surfaces and substratum surface were determined using contact angle method. The adhesion

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of *Salmonella* strains on glass was studied using optical microscope and Matlab program.
Results: *Salmonella* strains showed similar cell surface physicochemical properties under low and high ionic strength except for *S. Newport* and *S. Kentucky* at low ionic strength. In addition, all *Salmonella* strains presented strong adhesion ability at low ionic strength (0,001M) especially for *S. Newport and S. Kentucky* serovars.
Conclusion: The results presented in this work could contribute to understand and control the microbial adhesion of *Salmonella* serovars to inert surface depending on environmental conditions.

Keywords: Microbial adhesion; Salmonella serovars; S. Muenster; S. Kentucky; S. Newport and S. Kiel; physicochemical properties; ionic strength.

1. INTRODUCTION

Adhered microorganisms to solid surfaces can have the potential to act as a chronic source of microbial contamination, which may compromise food quality and represent a significant health hazard [1]. For instance, *Salmonella* spp. is able to colonize different inert food contact surfaces to form biofilms [2,3,4,5].

So, it has been recognized that a greater understanding of the interaction between microorganisms and different surfaces including the suspension medium characteristics is required to control these problems and to choose the hygienic support.

Salmonellosis has been one of the most commonly reported food-borne illnesses worldwide. In many countries, *Salmonella Enteritidis* is the most frequently isolated serotype. Epidemiological evidence has linked the majority of outbreaks in the United States to *salmonella* strains, causing an estimated one million cases of salmonellosis, 19,336 hospitalizations, and 378 fatalities per year [6]. In addition, many other outbreaks of pathogens have been found to be associated with biofilms [7,8,9].

The formation of biofilms is a complex phenomenon influenced by several factors; including the chemical and physical properties of the cell surface. The cell surface physicochemical properties can be modified depending on surface cell structures [10,11] or environmental factors such as temperature, medium composition, ionic strength and pH. Many workers have described the effects of these environmental parameters on hydrophobicity and charge [12,13,14,15,16].

Despite the fact that the environmental parameters properties play an important role in adhesion phenomenon, limited data concerning the effects of ionic strength on bacterial adhesion and subsequent biofilm formation by Salmonella strains have been published. Moreover, to our knowledge, no study has been performed on the adhesion of *Salmonella Enterica*'s serovars: *S. Newport, S. Muenster, S. Kentucky and S. Kiel* to inert surfaces under different ionic strength. Therefore, the aim of this study was to investigate the adhesion ability of different *Salmonella's* serovars (*S. Newport, S. Muenster, S. Kentucky and S. Kiel)* isolated from food surfaces under two ionic strengths (0.1M; 0.001M), in order to understand the influence of environmental characteristics on their microbial adhesion process.

2. MATERIALS AND METHODS

2.1 Bacterial Strain, Growth Conditions and Surface Characterization

Sampling and collection of strains was performed in the laboratory of microbiology, food hygiene and environment of the "Institut Pasteur – Maroc". Samples were received from different food sources (Catering, Food industries, hotels, and supermarkets). The Four species of *salmonella* strains (*S. Newport, S. Muenster, S. Kentucky and S. Kiel)* used in this study, were identified and referenced by Institut Pasteur-Maroc.

Each strain was incubated overnight at 37°C in Liquid Luria Bertani medium (LLB) which contains tryptone, yeast extract and NaCl. After 24 h of incubation, cells were harvested by centrifugation for 15min at 8400×g and washed twice with 0.1 M or 0.001M KNO3 solution. The optical density (DO) of each bacterial suspension was adjusted to (0.7-0.8) [17].

2.2 Cleaning and Preparation of Solid Surface

The substrate used for adhesion experiments was glass. The glass samples were microscope slides (RS France, France). Before each

experiment, the glass substrates were soaked for 15 min in 90% ethanol and rinsed six times with distilled water for surface disinfection.

2.3 Contact Angle Measurements (CAM)

The physicochemical properties of the bacterial surface and the solid surface (hydrophobicity, electron donor / electron acceptor character) are determined by contact angle measurements. (GBX France). The method for measuring the contact angles on bacterial layers has been described [18]. Briefly, a suspension of cells in KNO_3 solution was deposited onto a 0.45 µm cellulose acetate filter (Sartorius) by first washing the filter with 10 ml of distilled water for wetting, and then 10 ml of the cell suspension was added obtaining a thick lawn of cells after filtration by means of negative pressure. The wet filters were placed carefully on a glass support with double-sided sticky tape and allowed to air dry until so-called stable "plateau contact angles" could be measured. For each strain, three independently grown cultures were used, from which three filters of each were prepared and measured.

Three to six contact angle measurements were made on each filter, for all probe liquids including water, formamide and diiodomethane.

2.4 Calculation of Surface Free Energy

The surface free energy cannot be measured directly. Instead, one performs contact angle measurements with test liquids deposited on solid surface to calculate surface energy. The approach of Good, van Oss and Chaudhury (acid–base theory) [19] is used in this study.

The surface energy components of a surface (γ_S^+, γ_S^- and γ_S^{LW}) were determined by performing contact angle measurements using three probe liquids (one apolar and two polar with known surface tension parameters (γ_L^+, γ_L^- and γ_L^{LW}) and employing Young's Eq:

$$\text{Cos } \theta = -1 + 2(\gamma_S^{LW}\gamma_L^{LW})^{1/2}/\gamma_L + 2(\gamma_S^+\gamma_L^-)^{1/2}/\gamma_L + 2(\gamma_S^-\gamma_L^+)^{1/2}/\gamma_L \quad (1)$$

Where θ is the measured contact angle, γ^{LW} is the van der Waals free energy component, γ+ is the electron acceptor component, γ– is the electron donor component and the subscripts (S) and (L) denote solid surface and liquid phases respectively.

The surface free energy is expressed as: $\gamma_S=\gamma_s^{LW} + \gamma_s^{AB}$ where $\gamma_s^{AB}=2(\gamma_S^+\gamma_S^-)^{1/2}$ is the acid–base free energy component.

Based on van Oss's approach [20], a microbial cell surface or solid surface is classified hydrophilic when ΔGiwi is greater than zero or hydrophobic when ΔGiwi is smaller than zero. The free energy of interaction (ΔGiwi) between surfaces molecules (i) immersed in water (w) can be expressed as:

$$\Delta Giwi = -2\gamma_{iw} = -2\left(\left((\gamma_i^{LW})^{1/2} - (\gamma_w^{LW})^{1/2}\right)^2 + 2\left((\gamma_i^+\gamma_i^-)^{1/2} + (\gamma_w^-\gamma_w^+)^{1/2} - (\gamma_i^+\gamma_w^-)^{1/2} - (\gamma_w^+\gamma_i^-)^{1/2}\right)\right) \quad (2)$$

with γiw being the interfacial tension between the surface (i) and water (w). The hydrophobicity or hydrophilicity increases with the increase of the absolute ΔGiwi value.

2.5 Adhesion of *Salmonella*'s Serovars on Glass Surfaces

The cells are suspended in a solution of KNO_3 0.1 M and 0.001M KNO3 with an optical density between 0.7 and 0.8 (approximately 10^8 CFU ml^{-1}). Then, 10 ml of the bacterial suspension are put in contact with the substrate (Glass) for 3 hours at 25°C. After the contact period, non-adherent cells were eliminated by three consecutive rinses with sterile distilled water, [21, 22]. The glass samples were dried at room temperature, and then crystal violet binding assay was also performed and rinsed six times. The adhesion on glass was examined by using an optical microscope (G×400) coupled to a computer screen.

In this work, we have chosen to quantify the microbial adhesion by estimating the percentage of the area occupied by the bacteria as the accession of most of these bacteria is accompanied with a production of exopolymers substances. This method is based on evaluating only the percentage of the area occupied. Recently, Hamadi [23] used the Matlab program to determine the number of adherent cells and the percentage of the area occupied by the adherent cells. This percentage is determined by multiplying the ratio of the surface area compared to the total area by 100 (% of the surface area = (Surface occupied / total area) * 100).

3. RESULTS AND DISCUSSION

The bacterial adhesion is a complex phenomenon influenced by several factors; including the chemical and physical properties of the cell surface. The physicochemical interactions depend on the properties of the

surface of the microorganisms, those of the substrate and the characteristics of the suspension medium.

3.1 Bacterial and Substratum Surface Characterization

The surface hydrophobicity and the electron donor-acceptor (γ-, γ+) characteristics of *Salmonella*'s surfaces and the glass slides were determined from the measurements of contact angle and calculation using the Young–van Oss equations are presented in Table 1 and Table 2.

According to Vogler [24], when the value of the contact angle with water exceeds 65°, the surfaces are characterized as hydrophobic and hydrophilic when the value of the contact angle is less than 65°. Moreover, according to Van Oss's thermodynamical approach [25,26], a positive value of the free energy surface (ΔG_{iwi}) means that the surface is hydrophilic and a negative value indicate that it is hydrophobic. The surface free energy gives a quantitative indication of the hydrophobicity of the substrate surface; while the contact angle with water permits a qualitative assessment of hydrophobicity [27,17].

Thus, according to this approach, the obtained results (Table 1; Table 2) of the contact angle water measurements and the free energy of interaction (ΔG_{iwi}) showed that the glass surface is relatively hydrophilic (Θw = 36.1°; ΔG_{iwi} = -33.5 mJm^{-2} > 0). This result is in agreement with several studies carried out on this support [17, 23].

Also, all *Salmonella* strains with ionic strength 0.1M and 0.001M were hydrophilic with both hydrophobicities approaches except for *S. Newport* and *S. Kentucky* strains (Θw =81.2°, Θw =79.2°; ΔG_{iwi}= 38.6 mJm^{-2}, ΔG_{iwi}= -20.3 mJm^{-2}) that indicated a hydrophobic property. This finding are quite similar to Sinde and Carballo [28] who reported that *Salmonella* strains, isolated from chicken liver, fresh sausages and hamburgers, were hydrophilic with values of water contact angles ranging from 25.4° to 35.0°.

Considering the results above, the hydrophobicity of *S. Newport and S. Kentucky* serovars were significantly affected by changing ionic strength and ranging from hydrophilic to hydrophobic character. Hamadi [17], Teixeira [29] also observed a great variation of hydrophobicity among strains of the same bacterial species by changing the characteristics of the suspension medium (pH, ionic strength).

The hydrophobic character of a bacterial cell is largely influenced by the residues and the structures on cell surfaces, which can be hydrophilic or hydrophobic [17,30,31]. This means that bacteria's hydrophobicity varies among species and strains, even within the same strain, depending on the mode and the stage of growth, the composition of the growth medium and even the analysis technique [17,32,33,34].

The Lifshitz-van der Waals (γ^{lw}) surface tension components and electron donor (γ^-) and electron acceptor (γ^+) parameters of *Salmonella* strains and glass substrates are presented in Table 1 and Table 2. Results showed that the glass surfaces exhibit weak electron acceptor propriety (γ^+=0.7 mJm^{-2}) and strong electron donor property (γ^+=54.9 mJm^{-2}). These results are in agreement with previous works in our laboratory [17] who found a similar value of the electron donor-acceptor (γ^-, γ^+) and Lifshitz-van der Waals (γ^{lw}) components.

Furthermore, at 0.1M ionic strength, *S. Muenster, S. Newport, S. Kentucky and S. Kiel* cell surfaces were predominantly electron donors (high values of γ^-) with respectively 72.0 mJm^{-2}; 51.7 mJm^{-2}; 66.9 mJm^{-2}; 68.5 mJm^{-2}, however, the values of the electron donor properties of the same strains decreased very significantly by decreasing the ionic strength to 0.001M especially for *S. Newport, S. Kentucky* serovars (11.6 mJm^{-2}; 18.0 mJm^{-2}).

Table 1. Contact angles of water (θ_w), formamide (θ_f) and diiodomethane (θ_d), Lifshitz-van der Waals (γ^{lw}), the electron donor-acceptor (γ^-, γ^+) parameters and the quantitative hydrophobicity (ΔG_{iwi}) of glass supports

Surface	Contact angle (°)			Surface tension: Components and parameters (mJm^{-2})			ΔG_{iwi} (mJm^{-2})
	Θ_w	Θ_f	Θ_d	γ^{lw}	γ^+	γ^-	ΔG_{iwi}
Glass	36.1±2.3	46.2 ±1.5	59.5±1.8	28.7	0.7	54.9	38.6

Table 2. Contact angles of water (θ_w), formamide (θ_f) and diiodomethane (θ_d), Lifshitz-van der Waals (γ^{lw}), the electron donor-acceptor (γ^-, γ^+) parameters and the quantitative hydrophobicity (ΔG_{iwi}) of *Salmonella* strains under two ionic strength (0.1M - 0.001M)

	Contact angle (°)			Surface tension: Components and parameters (mJm^{-2})			ΔG_{iwi} (mJm^{-2})
Strains	Θ_w	Θ_f	Θ_d	γ^{lw}	γ^+	γ^-	ΔG_{iwi}
0.1M							
S. Muenster (S5)	30.8 (±1.4)	52.7 (±1.2)	29.9 (±2.1)	44.3	1.5	72.0	44.7
S. Newport (S8)	39.4 (±2.2)	46.6 (±1.9)	24.7 (±3.2)	46.3	0.5	51.7	28.1
S. Kentucky (S11)	31.1 (±1.7)	51.4 (±4.4)	70.1 (±2.6)	22.8	0.6	66.9	44.4
S. Kiel (S25)	28.1 (±1.1)	48.2 (±2.7)	50.4 (±3.3)	33.9	0.0	68.5	61.8
0.001M							
S. Muenster (S5)	46.1 (±2.7)	46.6 (±3.3)	46.8 (±1.6)	36.0	0.1	40.5	21.3
S. Newport (S8)	81.2(±2.3)	69.8 (±1.5)	23.3 (±1.3)	46.6	1.8	11.6	-33.5
S. Kentucky (S11)	79.2 (±0.9)	74.8 (±2.3)	13.3 (±2.8)	49.3	4.5	18.0	-20.3
S. Kiel (S25)	53.2 (±2.9)	59.7 (±3.3)	45.9 (±2.2)	38.5	1.0	56.7	35.4

Also, the electron acceptor (γ^+) results show that S*. Muenster, S. Newport, S. Kentucky and S. Kiel* are generally weakly electron acceptor at 0.1M ionic strength with respectively 1.5 mJm^{-2}; 0.5 mJm^{-2}; 0.6 mJm^{-2}; 0.0 mJm^{-2}. Though, the values of the electron acceptor properties of the *S. Newport, S. Kentucky and S. Kiel* strains increased very significantly by decreasing the ionic strength to 0.001M (1.8 mJm^{-2}; 4.5 mJm^{-2}; 1.0 mJm^{-2}) especially for *S. Kentucky* serovar, except for *S. Muenster* serovar which the values of the electron acceptor properties decreased very significantly by decreasing the ionic strength to 0.001M (0.1 mJm^{-2}).

In addition, the Lifshitz-van der Waals surface tension component (γ^{lw}) of all *Salmonella* strains didn't change considerably under the two ionic strength, except for *S. Kentucky* that increased very remarkably from (22,8 mJm^{-2}) to (49,3 mJm^{-2}) by decreasing ionic strength.

3.2 Adhesion of *S. Muenster, S. Newport, S. Kentucky* and *S. Kiel* on Glass Surfaces

The microbial adhesion of Salmonella strains (*S. Muenster, S. Newport, S. Kentucky and S. Kiel*) on glass with two ionic strengths (0.1M; 0.001M) are presented in Fig. 1 and quantified in Fig. 2.

The variation of microbial adhesion of *Salmonella* strains as a function of different contact times with two ionic strength showed that:

After 3 hours of contact time, the microbial adhesion of *S. Muenster, S. Newport, S. Kentucky and S. Kiel* serovars increased very significantly when the ionic strength decreased from 0.1M to 0.001M ranging respectively from (0.2%; 11%; 18%; 12.5%) to (15.8%; 65%; 70%; 29.4%). It is important to note that the bacterial adhesion is very pronounced for the strains of *S. Newport and S. Kentucky* with special organization in their adhesion behaviour (Fig. 1).

This increasing of salmonella's microbial adhesion at low ionic strength (0.001M) seems to be influenced by their physicochemical properties: The hydrophobic character and the low values of the electron donor of *S. Kentucky* and *S. Newport* at low ionic strength increases highly their adhesion ability on glass supports. This finding means that the microbial adhesion of *S. Kentucky and S. Newport strains* depend on a part of the ionic strength and on other part on factors related to the nature of the strain and its physicochemical surface properties. In addition, other factors then ionic strength seems to increase the adhesion ability of *S. Muenster and S. Kiel* serovars.

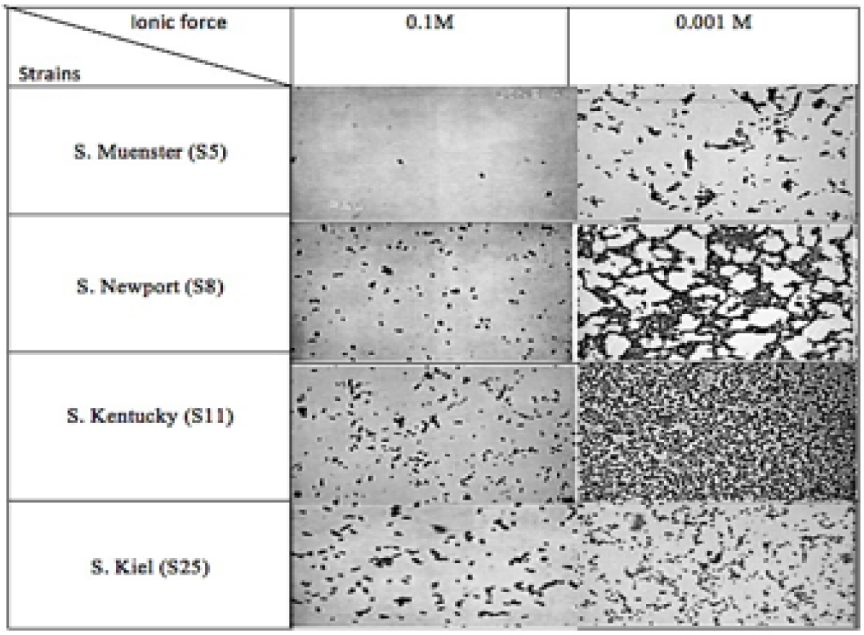

Fig. 1. Microbial adhesion of *S. Muenster (S5), S. Newport (S8), S. Kentucky (S11), S. Kiel (S25)* on glass surfaces as a function of different ionic forces (0.1M – 0.001M)

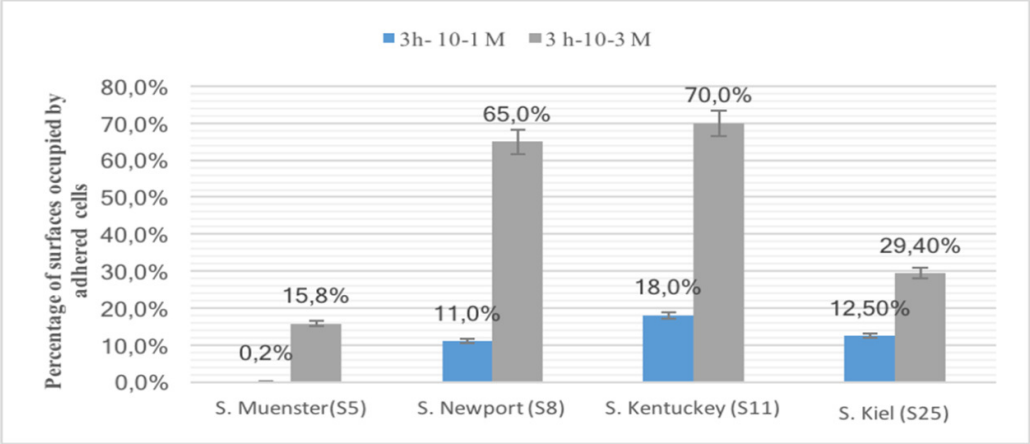

Fig. 2. Percentage of surface occupied by adherent cells of Salmonella strains with two ionic (10^{-1}M; 10^{-3}M) after 3 hrs of contact time

Several studies have shown that adhesion of bacteria partly depends upon the nature of the inert surfaces and partly upon the bacterial surface properties [35,36,4]. Moreover, Hydrophobicity and surface charge are the most important surface properties in the adhesion process as demonstrated by several studies [4, 37,38,39,40].

It is well known that bacterial surface hydrophobicity, surface charge, cell density, and the presence of exopolysaccharides are determinant factors in the adhesion process. For example, Sinde and Carballo [28] observed that differences found in the degree of attachment of S*almonella* and *L. monocytogenes* indicate that there must be other factors on the surface of the bacteria, rather than hydrophobicity, contributing to bacterial attachment to food contact surfaces.

On the other hand, Walker [41] studied the effect of pH, temperature, and contact surface on the elaboration of fimbriae (SEF21, SEF14, and SEF17) and flagella and found differences among the *salmonella* strains. Hood and Zottola [4] observed that growth media and surface conditioning were both significant factors affecting the level of adherence. In the present study, surface hydrophobicity, electron donor parameters (γ-) modified by ionic strength were determined to find an explanation for the observed differences in the extent of adhesion.

According to Van Oss [24] and Hamadi [42], the increase in ionic strength is accompanied by a decrease in the electrostatic charge. This decrease is attributed to a significant adsorption of the cations which cause a neutralization of the charged groups present on the surface. Indeed, for a weak ionic strength (0.001M), the electrostatic charge is important whereas for a high ionic strength (0.1 M) the electrostatic charge is negligible. Xiaoxia [43], shows that a decrease in the ionic strength in the solution enhanced the bacterial adhesion of *Pseudomonas sp. NCIMB 2021* and *Desulfovibrio desulfuricans ATCC* 27774 to metal surfaces due to the stronger electrostatic attraction strength.

4. CONCLUSION

The adhesion process is multifactorial phenomenon that involve several physicochemical and microbiological factors. In this study, the changing of ionic strength modifies very significantly the physicochemical properties of *S. Newport and S. Kentucky* serovars and increased highly their adhesion ability to glass surface compared to *S. Muenster and S. Kiel* serovars. For a better understanding, it would be necessary to investigate the role of surface structure such as proteins, fimbriae and flagella in their adhesion ability.

Considering all the tentative explanations based on the physicochemical properties of bacterial cells and surfaces, it was not possible to establish any direct relation to elicit the hypothesis of a reasonable model of adhesion. The main conclusion to be drawn is that *Salmonella* adhesion is strongly strain-dependent, despite the similarity in physicochemical surface properties or

environmental conditions which constitute a factor of virulence among the different serovars.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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