Article

Evaluation of the Adhesive Potential of Bacteria Isolated from Meat-Related Sources

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Abstract: Microbial adhesion constitutes the transition of microorganisms from a planktonic mode to a static one. It promotes the formation of biofilm which is responsible for spoilage, foodborne diseases, and corrosion in the food processing industry. In this study, the adhesive potential of fourteen meat-borne bacterial isolates belonging to seven different genera was investigated. All strains were found able to colonize polystyrene surfaces with different levels of firmness. Significant variations were determined in assays of bacterial hydrophobicity and motility. Among the 14 strains, *Pseudomonas fragi*, *Aeromonas salmonicida* II, *Serratia liquefaciens*, *Citrobacter braakii*, *Pseudomonas putida*, and *Aeromonas veronii* had a strong hydrophobic force, while the isolates of *Lactobacillus* genus showed the most hydrophilic property. In terms of motility, *Citrobacter braakii* and *Escherichia coli* exhibited exceptional swarming and swimming abilities, whilst conservatively weak performances were observed in the *Lactobacillus* strains. Furthermore, the majority of the isolates were predominantly electron donors and weak electron acceptors. Overall, a high level of correlation was observed between biofilm-forming ability with cell surface hydrophobicity and Lewis acid–base properties, whereas the contribution of motility in bacterial adhesion could not be confirmed. Research on the adhesive performance of foodborne bacteria is potentially conducive to developing novel control strategies, such as food processing equipment with specific surfaces, not facilitating attachment.

Keywords: biofilm; adhesion; motility; hydrophobicity; isolates

1. Introduction

Biofilms are biologically active communities characterized by cells embedded in a matrix of extracellular polymeric substances, in association with a solid surface. The existence of biofilm is ubiquitous in nature because bacteria tend to favor a community-based lifestyle compared to planktonic conditions. Biofilms are culprits of serious engineering problems as well as cross-contamination in the food industry [1]. Microbial adhesion is the first step of biofilm formation, which mainly consists of two stages: the "docking" and the "locking" phases [2,3]. Primary adhesion constitutes the reversible attachment of a planktonic bacterial cell on a conditioned surface, whose occurrence depends on the attractive and repulsive forces, including electrostatic and hydrophobic interactions between the microorganism and the contact surface. It also requires motility property generated by flagella, for movement and direction [4,5]. At the point of the secondary stage, molecularly mediated binding is employed through interactions of extracellular polymers with surface materials and/or cell receptors. This process consolidates the adhesion to an irreversible level that debuts the biofilm maturation.

LABs are mostly known for their useful technological properties in the production of fermented foods, but they could also be involved in the spoilage of anaerobically packed products [6]. Enterobacteriaceae is a capitalized group that commonly contributes to worldwide outbreaks resulting from the consumption of contaminated food, represented

by *Escherichia coli*. Further, it contains species such as *Aeromonas* and *Serratia* as frequently mentioned spoilage organisms in various food products [7,8]. *Pseudomonas*, belonging to the family Pseudomonadaceae, consists of some spoilage strains that also arouse primary attention in the food industry [9]. It is known that the bacterial effects are performed through cross-contamination caused by the presence of biofilms. Among the bacteria mentioned above, *Pseudomonas* spp. and *Escherichia coli* have been the protagonists in numerous reports regard to their adhesive ability on various contact surfaces [10,11]. However, few studies have been conducted on adhesion with the other bacteria; in addition, the relationship between biofilm-forming capacity and cell surface characteristics has not yet been evaluated. For the reasons mentioned above, fourteen isolates from meat-related sources, with *Pseudomonas*, *Aeromonas*, *Serratia* spp., and *Escherichia coli* were investigated in the present study.

The aim of this study was thus (i) to determine the adhesive performance of fourteen isolates from meat-borne sources and characterize individually their cell properties, including surface hydrophobicity, swimming and swarming motility, and electron-donor/electron-acceptor properties; (ii) to inquire into the general relationship between adhesion ability and cell surface characteristics. The study aims to enrich the theoretical framework of microbial biofilm formation and provide potential information for the further development of bacterial control strategies.

2. Material and Methods

TSA, TSB, and MRS were purchased from Land Bridge Technology Co., Ltd. (Beijing, China). Crystal violet was purchased from Tianjin Chemical Reagent Research Institute (Tianjin, China). Other reagents and chemicals were obtained from Hope Bio-Technology Co., Ltd. (Qingdao, China) and were of analytical grade.

2.1. Bacterial Strains and Inoculation

Fourteen food-borne bacteria were tested in this study (Table 1), namely *Aeromonas veronii*, *Aeromonas salmonicida* I, *Aeromonas salmonicida* II, *Serratia liquefaciens*, *Citrobacter braakii*, *Rahnella aquatilis*, *Escherichia coli*, *Pseudomonas putida* I, *Pseudomonas putida* II, Pseudomonas fluorescecs, *Pseudomonas fragi*, *Lactobacillus plantarum*, *Lactobacillus curvatus*, and *Lactobacillus sake*. They were collected from food manipulated in Key Laboratory of Meat Products Processing, Nanjing Agricultural University, China. The strains stored at $-80\,^{\circ}$C were each revived twice on TSA plates or MRS plates for the Lactobacillus isolates. A single colony of all the strains was selected and cultured in TSB or MRS broth, and then incubated at $30\,^{\circ}$C for 18 to 24 h, allowing the concentration of bacteria to reach 10^8 CFU mL^{-1}. The activated bacterial culture was then used to prepare a bacterial suspension. An aliquot (200 μL) of each culture was transferred into fresh TSB or MRS, respectively, and incubated at $30\,^{\circ}$C for 20 to 24 h. Cells were harvested by centrifugation (Avanti J-E, Beckman Coulter, Brea, CA, USA) at $12,000\times g$ for 5 min and then washed three times with 0.1% BSP. The pellet was resuspended using 0.1% BSP to a final concentration adjusted to 10^9 CFU mL^{-1}.

Table 1. Information of 14 isolated strains and their affinity for different solvents.

Strain Number	Bacteria Isolates	Source	Affinity/%		
			Xylene	**Ethyl Acetate**	**Chloroform**
NCM1577	*L. curvatus*	Cooked ham	4.42 ± 4.64 [a]	6.90 ± 2.61 [bc]	3.88 ± 1.43 [a]
NCM1578	*L. sake*	Cooked ham	19.28 ± 2.92 [b]	4.09 ± 1.52 [abc]	4.08 ± 2.63 [a]
NCM1579	*L. plantarum*	Cooked ham	16.02 ± 3.16 [b]	0.75 ± 0.35 [a]	15.64 ± 2.97 [b]
NCM1580	*S. liquefaciens*	Cooked ham	75.64 ± 6.16 [f]	3.39 ± 1.55 [ab]	63.96 ± 2.19 [cd]
NCM1594	*C. braakii*	Chilled chicken meat	73.55 ± 6.05 [f]	3.09 ± 2.60 [ab]	15.45 ± 1.54 [b]
NCM1582	*E. Coli*	Chilled chicken meat	29.85 ± 6.35 [c]	6.44 ± 1.07 [bc]	22.77 ± 3.30 [b]
NCM1583	*R. aquatilis*	Cooked ham	56.52 ± 13.87 [e]	17.40 ± 2.71 [d]	59.45 ± 12.52 [c]
NCM1586	*P. putida* I	Chilled chicken meat	72.37 ± 8.05 [f]	31.47 ± 7.73 [f]	70.04 ± 9.51 [d]

Table 1. *Cont.*

Strain Number	Bacteria Isolates	Source	Affinity/%		
			Xylene	Ethyl Acetate	Chloroform
NCM1587	A. veronii	Chilled chicken meat	71.73 ± 5.16 [f]	8.21 ± 3.31 [c]	79.41 ± 4.67 [e]
NCM1589	A. salmonicida I	Chilled chicken meat	54.31 ± 6.08 [e]	26.11 ± 1.91 [e]	78.63 ± 7.14 [e]
NCM1590	P. fluorescens	Chicken conveyor belt surface	40.72 ± 2.69 [d]	38.15 ± 1.92 [g]	82.05 ± 8.62 [ef]
NCM1591	P. fragi	Chilled chicken meat	90.13 ± 6.77 [g]	61.77 ± 3.20 [i]	92.90 ± 1.64 [g]
NCM1592	P. putida II	Chicken conveyor belt surface	70.96 ± 17.14 [f]	57.38 ± 1.57 [h]	92.36 ± 3.41 [g]
NCM1596	A. salmonicida II	Chicken conveyor belt surface	81.14 ± 12.00 [fg]	64.96 ± 3.78 [i]	89.24 ± 5.83 [fg]

Values followed by different lowercased letters are statistically different ($p < 0.05$) ($n = 6$).

2.2. Biofilm Assay

The biofilm assay was determined as previously described with slight modifications [12]. An aliquot (180 µL) of prepared bacterial suspension (10^9 CFU mL^{-1}) was dispensed into each well of a 96-well polystyrene microplate (Corning, New York, NY, USA). Negative controls were obtained by preparing both broths devoid of bacterial inoculum. Wells were incubated at 20 or 30 °C for 24 or 72 h, then rinsed three times with 0.1% BSP and air-dried for 45 min. Individual wells were stained with 200 µL of 0.25% (m/v) crystal violet for 30 min, then washed three times in 0.1% BSP to remove excessive staining solution. The crystal violet bound to biofilms was solubilized with 0.2 mL of 95% ethanol for 30 min. By measuring the absorbance at 570 nm via a SpectraMax M2 Microplate Reader (Molecular Device, San Jose, CA, USA), the biofilm-forming ability was determined. Five biological replicates were performed in this assay.

2.3. Motility Assay

Swimming and swarming motility were evaluated using soft-agar plate assays [13]. The media prepared for the swimming assay consisted of 10 g/L tryptone, 5 g/L NaCl, 2.5 g/L glucose, and 0.3% agar, while that of the swarming assay contained 25 g/L Luria-Bertani, 0.5 g/L glucose, and 0.5% agar. Three microliter aliquots of each bacterial suspension were spotted onto the surface of soft-agar plates, then placed at room temperature for 20 min in order to obtain a better absorption of the inoculum. After incubation at 37 °C for 8 h (swimming) or 20 h (swarming), the motility was recorded by measuring the diameter (mm) of the strain diffusion circle. Six biological replicates were performed in this assay.

2.4. Cell Surface Hydrophobicity and Electron-Donor/Acceptor Properties

Microbial adhesions to solvents were measured referring to the previous methods with some modifications [14]. Two milliliters of bacterial suspension was put into contact with an equal volume of the following solvents: xylene (apolar solvent), ethyl acetate (monopolar basic solvent and electron donor) and chloroform (monopolar acidic solvent and electron acceptor), respectively. Once mixed on a vortex for 2 min, the 2-phase system was stored at room temperature for 15 min allowing the hydrocarbon phase to rise completely. The percentage of microbial adhesion to the solvent was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the initial OD$_{600}$ of the cell suspension and A_1 represents the final OD$_{600}$ of the aqueous phase. Each parameter was performed in six biological replicates.

2.5. Statistical Analysis

Statistical significance was determined by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) using a Duncan (D)'s ANOVA at the 0.05 level of significance, and the coefficient (Table 2) was determined using Pearson correlation by SPSS 18.0. Figures were produced by Origin 8.0 (Origin Lab Corporation, Northampton, MA, USA).

**Table 2.** Correlation coefficients between adhesion with cell surface characteristics of the 14 isolates.

Index	Chloroform	Ethyl Acetate	Swarming	Swimming	24 h 20 °C Adhesion	24 h 30 °C Adhesion	72 h 20 °C Adhesion	72 h 30 °C Adhesion
Xylene	0.73 **	0.54 **	0.20	0.09	0.33 **	0.37 **	0.48 **	0.25
Chloroform		0.77 **	−0.11	−0.33 **	0.21	0.63 **	0.68 **	0.46 **
Ethyl acetate			−0.07	−0.28 *	0.23	0.50 **	0.50 **	0.27 *
Swarming				0.81 **	0.23	0.14	0.02	0.14
Swimming					0.08	−0.05	−0.17	−0.00
24 h 20 °C adhesion						0.58 **	0.50 **	0.47 **
24 h 30 °C adhesion							0.80 **	0.63 **
72 h 20 °C adhesion								0.61 **

$* p < 0.05; ** p < 0.01.$

3. Results and Discussion

Bacterial biofilm formation is the source of microorganism cross-contamination in the food industry. This study involved 14 meat-borne bacteria for the biofilm-forming assay. Since the optimal temperature of each genus varies, an average of 30 °C was proposed to be used for coordination, while 20 °C was applied in order to simulate a lower environmental temperature that occurs during processing in some food facilities. Both short-term and long-term formations of biofilm were investigated by adjusting the incubation time to 24 h and 72 h. Results showed that all of the bacteria were able to attach to the polystyrene surface with a variation which revealed the differences among genera and within species (Figure 1). *A. salmonicida* displayed the best adhesive performance, regardless of time and temperature, with a consistency between the two isolates (*A. salmonicida* I and II), while a moderate amount of biofilm was exhibited by *A. veronii*, indicating a species-specific distinction. A similar finding was observed when studying the adhesive strength of 14 *Aeromonas* trains [15]. (Dias et al., 2018). Except *P. putida I*, all strains of *Pseudomonas* (including *P. putida* II) formed considerable amount of biofilms at 20 °C for 24 h, whereas at 72 h, a higher temperature was more favorable for attachment of these bacterial cells. The different behaviors presented by *P. putida* I and *P. putida* II were in contrast to the case of *A. salmonicida*, this phenomenon demonstrated that isolates of one species are not necessarily supposed to perform in the same way. As for the other strains tested, a relatively strong biofilm-forming capacity was obtained in both *C. braakii* and *S. liquefaciens* at 30 °C for 24 h or 72 h, respectively, while the *Lactobacillus* genus was so "modest" that it formed the least amount of biofilm whether it was on the first day or the third day. This might result from the alternation of substratum. *Lactobacillus* is recognized as inhabitants in the human gastrointestinal tract where surface proteins can take advantage of mediating adhesion with the help of mucus [16]. However, the polystyrene surface used for the present in vitro experiment is of significant difference to the epithelial one, so that related proteins could be limited to play their original utility. The performance of biofilm formation was temperature-dependent. At 24 h, nine bacteria among the fourteen displayed a high attachment under 20 °C compared to 30 °C, especially *R. aauqtilis*, *E. coli*, and the *Pseudomonas* species, showing a significant advantage. This phenomenon was probably due to the diversity of optimal temperature of the strains engaged. A large cell density in culture broths results possibly in providing a comfortable environment for bacterial communication, thus enhancing the attachment of bacteria [17]. The dynamic process of attachment evolved as time went on and 48 h later, and a great variation was observed at 72 h. More bacteria showed a stronger biofilm-forming ability at 30 °C, especially *S. liquefaciens*, *Pseudomonas* isolates *putida* II, *fluorescecs*, and *fragi*, which formed markedly improved amounts of biofilm and even exceeded that on the first day. Meanwhile, *A. veronii* maintained steady and a slightly fluctuated gap of biofilm amount between the two temperatures tested. Additionally, less biofilm of few isolates represented by *A. salmonicida* and *C. braakii* was observed at 72 h than 24 h, probably due to the biofilm dispersal at the end of incubation periods. The results above indicated that incubation

time had an influence on the biofilm-forming ability. One complete development cycle of biofilm formation includes dispersion as the last step, which promotes the detachment of cells from the mature biofilm [18]. Moreover, this phenomenon results from the starvation of cells in regard to limited perfusion of nutrients and restricted removal of waste [19]. The period during which bacteria showed an impaired adhesion ability in the present study might refer to this development phase. Under 20 °C, the biofilm formed by the majority of the isolates at 24 h (e.g., *A. salmonicida* and *Pseudomonas*) grew over time to mature and disperse, leading to a decrease in the amount of biofilm at 72 h. While under 30 °C, the development of biofilm might be delayed so that at 24 h, the isolates seemed only to colonize, and the biofilm was not formed in a large quantity until 72 h. This partly proved that the lower temperature was preferable for most of the involved bacteria. Nevertheless, attention should be given to the existence of particularity in the biofilm-forming pace of individual strains.
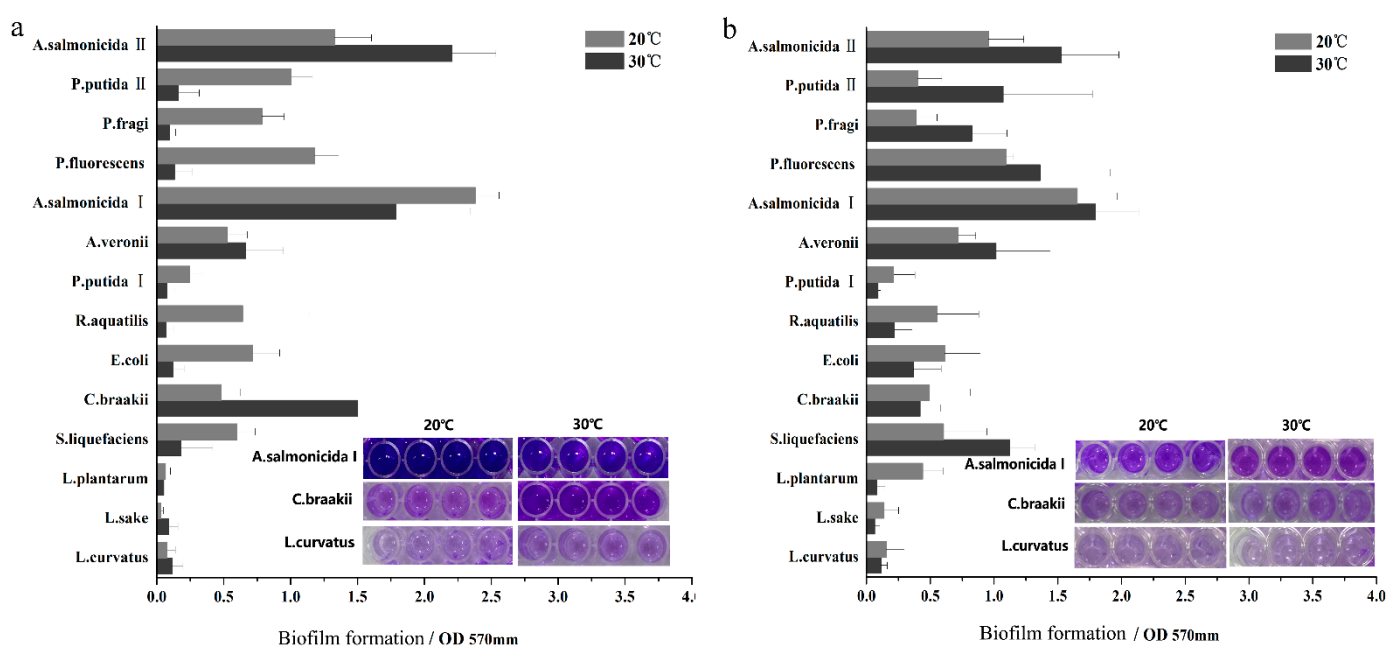

Figure 1. Biofilm formation by 14 selected isolates on 96-well polystyrene microplates under 20 or 30 °C for 24 h (**a**) and 72 h (**b**). Error bars represent standard deviations of the mean (*n* = 5). Images of the stained microplates of *A. salmonicida* I, *C. braakii* and *L. curvatus* are shown as representatives.

Cell adhesion requires contact with the bacterial cell envelope and interacting surface. Motile properties are involved in the meeting of these two objects at the docking stage during the multistep process of adhesion. Two types of motility were investigated in this study: swimming and swarming. The former is a characteristic related to individual movement while the latter shows the movement of a bacterial population. Great variability among genera was observed (Figure 2). Significant distinction existed of a certain single strain (*P. putida*) as well. *E. coli* and *C. braakii* showed a superior motility capacity. In the swimming media, they developed a relatively large strain diffusion circle whose diameter was over 75 mm, three times as large as that of the second echelon. *P. putida II*, *R. aquatilis*, and *S. liquefaciens* exhibited moderate swimming capacity with a diameter of around 20 mm. Less disparity was found in the results of the swarming assay. The five strains mentioned above also possessed a strong swarming ability but were much more reserved compared to their swimming performances. This finding suggested that the fluidity of the medium could possibly influence the diffusion of the microorganisms, a flowing state might favor the cross-contamination in food processing as subsequent biofilm development for instance, whereas no remarkable differences between the two motility-related properties appeared in *P. fluorescens*, *A. veronii*, and *P. putida I*. Both swimming and swarming motility are flagella-

dependent. It was reported that flagellar motility played an essential role in assisting microorganisms to reach proximate to the surface at the early beginning of adhesion [20]. The wide range of performances among strains in the present study might mainly result from the diversity of flagellar characteristics, but also other infectors such as biosurfactant production and QS signaling [21,22]. However, all bacteria with remarkable motile capacity were not strong in forming biofilms, taking *E. coli* and *C. braakii* as examples. Furthermore, no correlation was determined in this study between motility and biofilm formation from a general view (Table 2). This finding suggested that the role of flagella-based motility might not be of critical importance to the attachment of microorganisms and it might alter the adhesion process of different bacteria. In spite of the transition from planktonic mode to biofilm growth mode promoted by motility, it might keep cells away from the surface through active movements in the meantime. Moreover, many other factors (e.g., surface interactions) exist contributing to the adhesion process. Nevertheless, the function of flagella cannot be ignored. It was found by video microscopy that the flagellum was required for rapid attachment of *Vibrio cholerae* to a surface, and flagellar mutant was defective in initial attachment to abiotic surfaces [23]. (Watnick et al., 2001). In addition, if focusing alone on the tested *Lactobacillus* spp., all three isolates had the weakest motility capacity, which is reasonable because lactobacilli rarely carry flagella and move with them. Their consistently poor abilities displayed in both adhesion and motility assays provide a hint to the intrinsic connection between these two features.
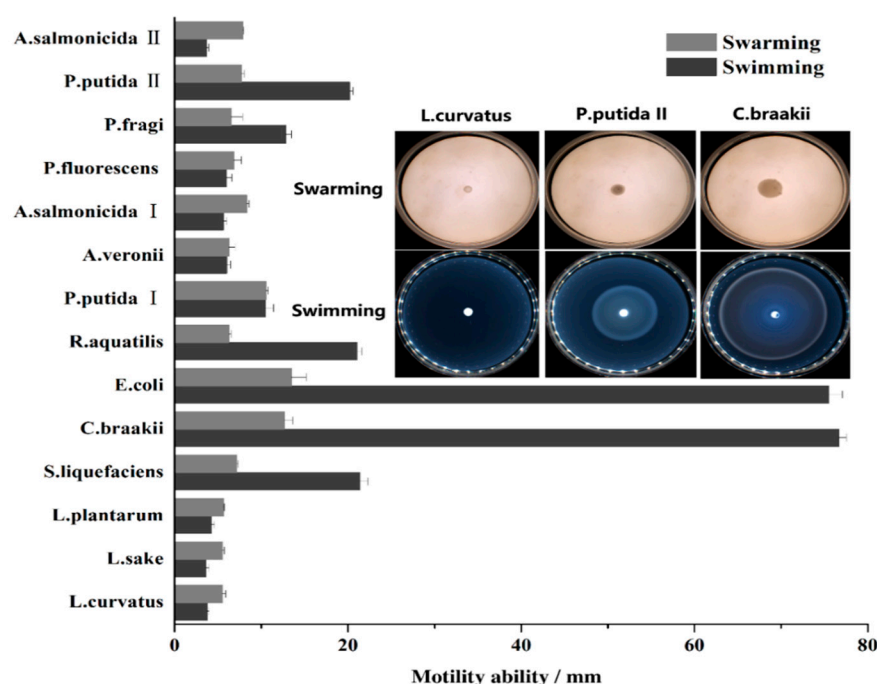
Figure 2. Motility ability evaluated by diffusion circle diameter (mm) of the 14 individual strains. Error bars represent standard deviations of the mean (*n* = 6). Colony patterns formed by *C. braakii*, *P. putida* II and *L. curvatu* were shown as representatives.

Surface characteristics such as hydrophobicity are also estimated to be able to influence the outcome of primary adhesion. Bacterial surfaces with hydrophilic properties were reported to be beneficial for electroactive biofilm formation [24]. These factors are usually species and strain-dependent. The differences in affinity may be due to the composition and content of different lipopolysaccharides, capsular material, or appendages on the cell surface. In our study, xylene, which is an apolar solvent, was used to determine cell surface hydrophobicity, and the expression of cell affinity to this solvent varied significantly among strains evaluated (Table 1). High affinity to xylene was detected in *Pseudomonas* and *Aeromonas* genera (with exception of isolates *P. fluorescens* and *A. salmonicida* I), represent-

ing an advantageous hydrophobic ability, while the *Lactobacillus* strains showed a strong hydrophilic property, with an affinity value to xylene under 20%, and even lower than 5% in *L. curvatus*. Furthermore, *S. liquefaciens* and *C. braakii* were another two competitors in hydrophobicity. The existing distinctions in this assay might be a consequence of varied envelope molecules, especially proteins and polysaccharides. Some evidence showed that the attendance of protein was likely to promote hydrophobic behavior whereas the participation of polysaccharides may result in better hydrophilic capacity [25,26]. General correlation was evident between cell surface hydrophobicity and biofilm formation (Table 2). Bacteria that possessed high hydrophobic surfaces such as *Pseudomonas* and *Aeromonas* attached readily to the model surface, while *Lactobacillus* strains that were more hydrophilic displayed poor biofilm-forming ability. It is in line with the finding of an investigation into surface characteristics of 50 *Lactococcus lactis* strains that more hydrophobic isolates were found to adhere more to polystyrene [27]. This would suggest a positive correlation between biofilm formation on polystyrene and the existence of attractive van der Waals interactions at the interface of hydrophobic cells and the inert material.

Many studies focusing on the fundamental mechanisms governing microbial adhesion have proved the importance of Lewis acid–base, i.e., electron-donor/electron-acceptor, interactions [28,29]. In the present study, apart from xylene, bacterial affinities to another two solvents, namely ethyl acetate and chloroform, were investigated to determine Lewis acid-base properties (Table 1). Among the fourteen stains, only *L. curvatus* showed a slightly stronger affinity for ethyl acetate than for chloroform, implying an electron-accepting characteristic. For *L. sake*, equal affinity was observed to both solvents. *L. plantarum* was predominantly an electron donor but a weak electron acceptor referring to its better affinity for chloroform. The three strains of *Lactobacillus* exhibited completely different behaviors in this test. However, their affinity values were maintained at a consistently low level. This is in line with all passive performances in regard to this genus through the previously mentioned assays. All other strains displayed a better affinity for chloroform than for ethyl acetate, indicating their possession of a superior ability to donate electrons and a basic character. Among them, *Pseudomonas* and *Aeromonas* spp. had an affinity value for chloroform calculated higher than 70% (surpassing 90% for *P. fragi* and *P. putida* II), which made them the strongest electron donors, and were followed by *S. liquefaciens* and *R. aquatilis* with a value of around 60%, concerning the same indicator. Different from the outstanding performances in the swarming test (Figure 2), both *C. braakii* and *E. coli* showed low potential for electron-related affinities. Taking all the 14 isolates into consideration, the electron donor/acceptor properties were correlated with the biofilm-forming process of engaged microorganisms (Table 2). On the whole, isolates with a better capacity to donate electrons (basic character) attached relatively easier to the polystyrene surface. It was demonstrated that Lewis acid–base interactions played a major role compared to electrostatic and van der Waals forces in the process of biofilm development [30]. However, these are not in line with a finding when investigating cell surface properties of marine bacteria, which suggested that the contribution of an electron-donating character in bacterial adhesion was at a low level [31]. (Grasland et al., 2003). The disagreement might result from the difference in the source of bacteria.

4. Conclusions

In conclusion, the 14 foodborne isolates tested in our study were capable of colonizing the polystyrene surface. Moreover, it was demonstrated that the biofilm formation was significantly influenced by hydrophobicity and electron-donor/electron-acceptor interactions. However, bacterial motility could not be considered as playing a constructive role in the adhesion process. Consistent performances were detected within genus or species for these cell surface characteristics. It would be advisable to conduct future research that engages in other bacteria from a food-associated source.

Abbreviations

LAB	Lactic acid bacteria
TSA	Tryptone soy agar
TSB	Tryptone soy broth
BSP	Buffered saline peptone water
CFU	Colony-forming units
QS	Quorum sensing

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