In situ characterization and analysis of *Salmonella* biofilm formation under meat processing environments using a combined microscopic and spectroscopic approach

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**A B S T R A C T**

*Salmonella* biofilm on food-contact surfaces present on food processing facilities may serve as a source of cross-contamination. In our work, biofilm formation by multi-strains of meat-borne *Salmonella* incubated at 20 °C, as well as the composition and distribution of extracellular polymeric substances (EPS), were investigated in situ by combining confocal laser scanning microscopy (CLSM), scanning electron microscope (SEM), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and Raman spectroscopy. A standard laboratory culture medium (tryptic soy broth, TSB) was used and compared with an actual meat substrate (meat thawing-loss broth, MTLB). The results indicated that *Salmonella* grown in both media were able to form biofilms on stainless steel surfaces via building a three-dimensional structure with multilayers of cells. Although the number of biofilm cells grown in MTLB was less than that in TSB, the cell numbers in MTLB was adequate to form a steady and mature biofilm. *Salmonella* grown in MTLB showed “cloud-shaped” morphology in the mature biofilm, whereas when grown in TSB appeared “reticular-shaped”. The ATR-FTIR and Raman analysis revealed a completely different chemical composition between biofilms and the corresponding planktonic cells, and some important differences in biofilms grown in MTLB and in TSB. Importantly, our findings suggested that the progress towards a mature *Salmonella* biofilm on stainless steel surfaces may be associated with the production of the EPS matrix, mainly consisting of polysaccharides and proteins, which may serve as useful markers of biofilm formation. Our work indicated that a combination of these non-destructive techniques provided new insights into the formation of *Salmonella* biofilm matrix.

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1. Introduction

*Salmonella* spp. is a globally widespread food-borne pathogen. In the EU, it has become the most frequently reported cause of food-borne outbreaks, resulting in a total of 95,548 confirmed cases of human salmonellosis in 2011 (EFSA-ECDC, 2013). Meanwhile, in 2013, several food recalls and outbreaks, which have been addressed by FDA and CDC, have been associated with *Salmonella* contamination in the US (www.cdc.gov/salmonella/outbreaks.html; www.fda.gov/ Safety/Recalls). Although there are many sources for contamination of food pathogens, biofilms have been demonstrated to be the principal source (Shi and Zhu, 2009), and have been associated with many outbreaks (Srey et al., 2013). A biofilm is an assemblage (aggregates) of surface-associated microbial cells that is enclosed in an extracellular polymeric substance (EPS) matrix. The ability to form biofilm has been identified as an important factor for the persistence of food-borne pathogens in food-processing facilities (Diez-Garcia et al., 2012; Vestby et al., 2009). Biofilms have become a significant problem in the food industry, in particular those sectors involving animal slaughter, poultry, red meat and dairy processing (Simoes et al., 2010).

Biofilm formation by *Salmonella* has been mostly studied in well-defined laboratory media using polystyrene micro-well assay under environmental conditions that are favorable for *Salmonella* growth (Diez-Garcia et al., 2012). However, these favorable conditions may never be encountered in food processing facilities. Additionally, the correlation between biofilm production on micro-well surfaces and biofilm formation on the surfaces of different materials in food facilities was still a controversial issue (Lianou and Koutsoumanis, 2012; Patel and Sharma, 2010; Vestby et al., 2009). Thus studying biofilm formation under laboratory favorable conditions and on micro-well surface has no, or limited, significance to the understanding of biofilm formation under actual conditions encountered in food processing plants. Therefore, knowledge on biofilm formation by multi-strain composites of *Salmonella* under unfavorable conditions encountered in chicken processing facilities, such as the likely presence of chicken meat fluid residues and stainless steel surfaces, should be investigated using direct plate count method.

The common practice in biofilm investigations is the destructive analysis of samples on which biofilms have developed. However, these methods may significantly alter the biofilm architecture from its native
form, hence making it difficult to obtain reliable information regarding the chemical composition of the biofilm matrix and their interactions. Much more information could be obtained with in situ assessment of biofilms using non-destructive analytical techniques. Confocal laser scanning microscopy (CLSM) is suitable for that purpose since it can provide a simultaneous three-dimensional image and information on different cellular and polymeric biofilm constituents (Kamjunke et al., 2012). Meanwhile, spectroscopic techniques such as Raman spectroscopy (RM) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), which also allow these limitations to be overcome and provide additional chemical information about biofilm components, are desired to enable non-destructive analysis of biofilms in situ. RM can provide fingerprint spectra and more detailed information about the chemical composition of the biofilm matrix and improve the knowledge of EPS structure (Wagner et al., 2009). ATR-FTIR can provide the opportunity for measuring biofilm in aqueous media as well as investigating the development of a biofilm in situ directly, and discriminate structural and biochemical changes in the composition of microbial cells accompanying their surface-associated growth (Bosch et al., 2006). However, to our knowledge, few studies have focused on the biofilm formation of food-borne pathogens in situ using these non-destructive techniques, in particular studying the biofilm and EPS of *Salmonella* under actual conditions encountered in food processing plants.

Considering the above, the present study was therefore carried out to (i) determine the biofilm formation by meat-borne multiple-isolates of *Salmonella* under a simulated meat processing environment; (ii) identify the morphology and EPS composition of *Salmonella* biofilm in situ by using multiple techniques.

2. Materials and methods

2.1. Strains and incubation medium

A six-strain cocktail of *Salmonella* (*Salmonella* Typhimurium, *Salmonella* Agona, *Salmonella* Heidelberg, *Salmonella* Derby, *Salmonella* Indiana, and *Salmonella* Infantis), previously isolated from chicken meat and processing surfaces, were tested in this study. The cocktail was prepared by individually incubating each of the six strains of *Salmonella* in tryptic soy broth (TSB) for 20 h at 37 °C. The cells were harvested by centrifugation at 12,000 g for 10 min and then washed three times with phosphate buffered saline (PBS), and pellets were resuspended in equivalent amounts of PBS. Individual suspensions of *Salmonella* were mixed to prepare the cocktail (the ratios of six strains in cocktail were 1:1:1:1:1:1).

In order to simulate contaminated surfaces in meat processing plants, chicken meat thawing-loss broth (MTLB) was used as growth medium. It was prepared as described previously (Midelet and Carpenter, 2002). The protein content of MTLB was adjusted to a final concentration of 5 mg/mL using the biuret protein assay. For comparison with MTLB, a laboratory medium (TSB) was tested as a control medium.

2.2. Biofilm formation and cells enumeration

Stainless steel plates (50 × 20 × 1 mm, grade 304, 2B finish), a material commonly used in the manufacture of meat-processing equipment, were used by Bellesi et al. (2011). For biofilm formation, plates were rinsed and cleaned prior to use, as described by Bellesi et al. (2011). For biofilm formation, 100 μl of cell suspension prepared as described above was transferred into 10 mL of MTLB or TSB containing stainless steel plates (initial cell concentrations were 2 Log CFU/mL). All plates were incubated at 20 °C for 1, 3, 5 or 7 days without agitation. Planktonic cells (those floating in the supernatant of the surfaces bearing the biofilm) in each medium were harvested at appropriate incubation times by centrifugation for 10 min at 12,000 g, and then cells were subsequently washed three times and resuspended with 0.85% NaCl solution (Dykes et al., 2003). Cell numbers of biofilm were enumerated at appropriate times. A single plate was removed for sampling and was rinsed three times with 0.85% NaCl solution to remove non-attached cells, whereas the attached biofilm cells were removed with sterile cotton swabs and the swabs were then transferred to tubes containing 0.85% NaCl solution, vortexed with beads for about 5 min, and then serial dilutions were prepared (Midelet and Carpenter, 2002). This method has been established as the most suitable method for removal of attached bacteria (Poimenidou et al., 2009). Biofilm cell counts were determined in four replicates using tryptic soybyte agar (TSA) plate. Results were expressed as Log CFU/cm².

2.3. CLSM and SEM analysis

Stainless steel plates incubated in MTLB or TSB for 1, 3, 5 and 7 days were aseptically rinsed three times with 0.85% NaCl solution to remove planktonic cells. CLSM was used for the identification of the bacteria state (viable or dead) of the biofilm. Based on the work of Li et al. (2012), biofilms were stained using LIVE/DEAD BacLight Bacterial Viability Kits (Invitrogen/Molecular Probes, Eugene, USA) for 15 min at room temperature in darkness. The staining kit contains the fluorescent nucleic acid stains SYTO 9 and propidium iodide (PI). SYTO 9 (excitation/emission: 480/500 nm) emits green fluorescence and is used to identify living microorganisms with intact membrane whereas PI (excitation/emission: 490/635 nm) emits red fluorescence and stains dead bacteria with damaged membrane. Dead and viable cells can hence be identified simultaneously. Identification of the distribution of glycoconjugates within biofilm matrix was applied following the protocol described by Quiles et al. (2012). Samples were stained with concanavalin A (ConA) conjugated with fluorescein (excitation/emission: 494/518 nm, Invitrogen/Molecular Probes, Eugene, USA) for 15 min at room temperature in darkness. Subsequently, the plates were rinsed three times with sterile de-ionized water to remove excess stain. Images of biofilm were obtained with CLSM (LSM 710, Zeiss, Germany) using a 63× oil immersion objective. The average fluorescence intensity of each image at the green or red channel was expected to be directly proportionate to the number of bacteria with live cells or dead cells, respectively. The percentage of live cells was calculated by green fluorescence intensity/(green fluorescence intensity + red fluorescence intensity) (Gu et al., 2012).

For scanning electron microscope (SEM) analysis, biofilms were prepared as described in CLSM analysis, then the biofilm plates were air dried, and fixed in 2% glutaraldehyde (v/v) in 0.15 mol/L sodium cacodylate buffer (pH 7.2) for 12 h. After post-fixation for 90 min in 1% osmic acid (v/v), samples were rinsed in cacodylate buffer twice for 10 min each. The fixed biofilm was subjected to graded alcohol dehydration (50, 70, 80, 95 and 100% (twice); 10 min each) and coated with gold (Bonaventura et al., 2008). Images were obtained with a Hitachi S-3000N (Hitachi, Japan) scanning electron microscope at a 5000× magnification.

2.4. ATR-FTIR analysis

Biofilms incubated for 3, 5 and 7 days in MTLB or TSB were aseptically rinsed three times with 0.85% NaCl solution to remove unattached cells, and then the biofilm plates were air dried. ATR-FTIR transmission spectra from 3500 to 780 cm⁻¹ were acquired with an ATR-FTIR spectrometer (NEXUS 670, Thermo Nicolet, USA) with 2 cm⁻¹ spectral resolution (Bosch et al., 2000). To improve the signal-to-noise ratio, 256 scans were measured for each sample. Each planktonic cell suspensions was transferred to the ATR crystal, and then transmission spectra were recorded as mentioned before. Appropriate spectra were used to remove the spectral background: a NaCl solution spectrum for planktonic cell suspensions and a stainless steel plate spectrum for biofilm.
2.5. RM analysis

Each biofilm and each planktonic cell suspensions was prepared as described in ATR-FTIR analysis. All Raman spectra were acquired using a Labram HR800 spectrometer (Jobin Yvon, France) with an argon laser (514.5 nm) for excitation. The spectrometer was equipped with a grating of 600 lines/mm. During the measurement, light from the high power (maximum at 100 mW) diode laser was directed and focused onto the sample on a microscope stage through a 50× objective. The wavenumber range from 1800 to 400 cm\(^{-1}\) with a resolution of 1.5 cm\(^{-1}\), an exposure time of 60 s were collected for each sample (Sandt et al., 2009; Webb-Robertson et al., 2012). Before measurement, the wavenumber calibration of the Raman system was conducted by using a silicon wafer as reference according to the previous studies (Chao and Zhang, 2012; Ivleva et al., 2008). The spectra were pre-processed with background subtraction and baseline correction via the commercial software (NGS LabSpec, Jobin Yvon).

2.6. Statistical analysis

Statistical significance was determined by a one-way Duncan’s ANOVA procedure of SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The independent-sample T test was performed to compare the number of biofilm cells in the two different media.

The level of statistical significance was \( p < 0.05 \).

3. Results

3.1. Cell numeration of biofilm

The number of biofilm cells of multi-strains Salmonella on the stainless steel surface is shown in Fig. 1. Biofilm formation was significantly affected by type of media used. The amount of biofilm formed was time-dependent during the 7-day incubation period. Salmonella incubated in TSB reached the maximum biofilm after 5 days, with no significant changes \( (p > 0.05) \) in the following 2 days of incubation. Under the same experimental conditions, cells incubated in MTLB were significantly lower \( (p < 0.05) \) during the 7 day incubation period, suggesting that this medium may strongly affect cell-to-surface interactions, and reduce not only the growth rates and the initial cell attachment of Salmonella, but also further biofilm development.

3.2. CLSM and SEM analysis

Direct examination of the Salmonella biofilm development during a 7-day incubation period was carried out by CLSM. As shown in Fig. 2, the presence of single cells distributed over the stainless steel surface, or forming small micro-colonies, was already visible after 1 day of incubation. By day 3, small clusters of cells were detected (Fig. 2b and f). After 5 days of incubation, a well established biofilm appeared more structurally complex, with more than 80% of the substratum surface covered with large, irregularly shaped micro-colonies (Fig. 2c and g). By day 7, the images showed mature biofilm comprising large cell clusters exceeding 25 \( \mu \)m in width scattered over the surface (Fig. 2d and h). Meanwhile, we also observed important differences in the development of biofilm dependent upon the medium used. After 1 day of incubation in MTLB, there was considerably more red fluorescence (dead cells) in the visual field, compared with that observed in TSB (Table 1). Increasing of incubation period to 3 days, the percentage of live cells (green fluorescence) increased in MTLB, whereas the percentage of dead cells (red fluorescence) increased in TSB.

Representative SEM images of Salmonella biofilm growing in either TSB or MTLB are shown in Fig. 3. At 1 day of incubation, a number of cells had attached to the surface (Fig. 3a and c), but they remained in monolayers and were scattered sporadically, with only a few cell aggregates observed. Interestingly, as can be seen from Fig. 3a, the cells were obvious signs of binary fission, so longer cells seem to be present in TSB, but shorter cells were present in MTLB, possibly due to the stress of MTLB medium at the initial stage of incubation. After 3 days of incubation, there were a few aggregates of cells held together by extracellular matrix, and a rudimentary biofilm was formed (Fig. 3b and f), and the morphology of most cells returned to “normal”. With prolonged incubation for 5 days and 7 days, a mature-biofilm architecture was observed, which consisted of a complex three-dimensional structure formed by many cell aggregates held together by a large quantity of extracellular matrix.

3.3. Development of glycocojugates

The development of glycocojugates in Salmonella biofilms on stainless steel surfaces was determined using ConA-fluorescein (Fig. 4). During the 7-day incubation period, the production of glycocojugates was time-dependant both in MTLB and TSB, and supported the CLSM findings, with little green fluorescence observed after 1 and 3 days of incubation, but a well established structure shown after 5 and 7 days. Notably, there were differences in the structures of glycocojugates in the mature biofilms incubated in TSB and MTLB (reticular-shaped in TSB and cloud-shaped in MTLB), which is in accord with the CLSM observations.

3.4. ATR-FTIR analysis

The ATR-FTIR spectra exhibited well defined spectral regions that correspond to the vibration of the chemical groups from Salmonella biofilm and planktonic cells (Fig. 5). A tentative assignment of the bands corresponding to the functional groups is summarized in Table 2. The peak at 3290 cm\(^{-1}\) is indicative of N\(-\)H and O\(-\)H stretching vibrations (corresponding to polysaccharides and proteins). There were two dominant peaks observed at 2930 cm\(^{-1}\) and 2850 cm\(^{-1}\), which are assigned to C\(-\)H symmetric stretch of >CH\(_2\) in fatty acids. The spectral peaks visible at 1647, 1548 and 1539 cm\(^{-1}\) are assigned to C=O stretching, C\(-\)N, –NH and \(-\)NH\(_2\) bending of protein and peptide amides (amide I and amide II), respectively. The spectral changes observed at 1453, 1402 and 1243 cm\(^{-1}\) mostly result from C\(-\)H and C\(-\)O bending, >C=O and >P=O symmetric stretching corresponding to the mixed compounds of fatty acid, proteins, and phosphorus-containing carbohydrates. For the bands in the range from 1200 cm\(^{-1}\) to 800 cm\(^{-1}\), the peaks (1084, 1056, 916 and 858 cm\(^{-1}\)) are likely to
be associated with C−O−H, C−C, P−O−P and P=O stretching, C−O−C and C−O ring vibrations in polysaccharides and glycosidic linkage deformation of carbohydrates.

Spectra of all planktonic cells displayed no noticeable signals in the range from 780 cm$^{-1}$ to 3600 cm$^{-1}$ whereas the spectra of biofilms incubated in both MTLB and TSB showed several significant peaks, suggesting that there were many compounds in the *Salmonella* biofilm matrix. Comparison of the spectra between biofilm and planktonic cells showed some important spectral differences in bands, which may be identified as the markers of biofilm formation: two bands (near 1548 and 1647 cm$^{-1}$) assigned to proteins and peptides, two bands (1056 and 1084 cm$^{-1}$) associated with carbohydrates. From the variations in biofilm spectra at 3, 5 and 7 days, we found that the development of biofilm matrix was time-dependant, and related to an increase in the carbohydrate and proteins. Thus, the progress towards a mature biofilm of *Salmonella* might be associated with the production of a polysaccharide/proteins-enriched matrix, which contributed to the biofilm architecture. Compared to the spectra of biofilm grown in MTLB, the spectra of biofilm grown in TSB showed more, and more intense peaks, particularly in the frequency range from 1100 cm$^{-1}$ to 1800 cm$^{-1}$. The less peaks in the spectra of biofilm grown in MTLB may be associated with the less number of biofilm cells in MTLB (Fig. 1), additionally, the delay growth of *Salmonella* in MTLB (this has been demonstrated in our previous study, corresponding results have not been published) may also be associated with the reduction of biofilm matrix.

### 3.5. Raman analysis

Representative Raman spectra of *Salmonella* biofilms and of corresponding planktonic cells in the spectral fingerprint range of 400–1800 cm$^{-1}$ are presented in Fig. 6. The tentative peak assignments of the bands are summarized in Table 3. Two broad Raman bands at 531–542 cm$^{-1}$ and 855–899 cm$^{-1}$, as well as a weak band at 414 cm$^{-1}$ are assigned to C−C stretching and C−O−C glycosidic ring deformation of carbohydrates (polysaccharides). Two bands at 1000–1020 cm$^{-1}$ and 1500–1550 cm$^{-1}$ are related to C−CH$_3$ deformation and C==C stretching of carotenoids. The peaks at 1314−1345 cm$^{-1}$ and 1640−1688 cm$^{-1}$ were assigned, respectively, to $\delta$ (CH) and C==C, C=O stretching of protein and peptide amides (amide I and amide III). The peaks at 1020−1085, 1096 and 1161 cm$^{-1}$ can be attributed to C−C, C−O, C−O−C stretching and C−C, C−O ring asymmetrical breathing of diverse carbohydrates groups. Additionally, a signal at 1700 cm$^{-1}$ corresponding to the vibrational C==O stretching of carboxylic groups from membrane lipids and fatty acids was observed.

The spectral analysis revealed a completely different chemical composition between biofilm and the corresponding planktonic cells. Also, there were some important differences in bands of biofilms incubated in different media (TSB and MTLB). In particular, there were some large differences after 3 and 7 days of incubation. As similar to the results of ATR-FTIR, these great differences in the bands of biofilm matrix may, at least partly, be associated with the growth rates of cells grown in the two media, the growth rates of cells grown in MTLB was later than that in TSB. From the variation of biofilm spectra, we found that the peaks near the bands of 1520, 1330 and 1030 and 875 cm$^{-1}$ were present in all the biofilm spectra, but not in all planktonic cell spectra. The presence of the functional groups (carbohydrates and proteins) associated with these peaks may be used as identification of biofilm formation.

### Table 1

The percentage (%) of live cells in the biofilm of *Salmonella* incubated in TSB and MTLB.

<table>
<thead>
<tr>
<th>Media</th>
<th>Incubation time (days)</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>TSB</td>
<td>93.17 ± 5.01$^{\text{aX}}$</td>
</tr>
<tr>
<td>MTLB</td>
<td>43.22 ± 2.05$^{\text{cY}}$</td>
</tr>
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The percentage of live bacteria was calculated by green fluorescence intensity/(green fluorescence intensity + red fluorescence intensity). Values were expressed as the means ± standard deviations (n = 12); different lowercase letters (a–d) at the same medium and different capital letters (X and Y) at the same incubation time indicated a significant difference (p < 0.05).
4. Discussion

In the food industry, pathogen biofilms have become a public health issue and of economic concern due to the potential for cross contamination and the resistance to microbial cells to antimicrobial agents. In our work, we studied biofilm formation of meat-borne Salmonella by combining non-destructive microscopy and spectroscopy techniques in situ. Using a meat-based medium (MTLB) together with the meat-borne isolates for biofilm formation, would be useful for obtaining more realistic information compared with standard laboratory growth media and use of isolates from other sources. In our study, the Salmonella biofilms grown in both media easily established on stainless steel surfaces. This finding is a matter for concern, particularly for the poultry and meat processing industries using modern meat processing equipments. In these situations with mechanical and process automation, the surfaces are in repeated contact with raw meat, thus increasing the opportunities for Salmonella transfer and attachment leading to biofilm formation. Biofilms grown in MTLB showed fewer numbers and were slower to develop than when grown in TSB (Fig. 1). This difference may, at least partly, result from the different nutrients in MTLB compared to TSB and the pH stress of MTLB (approximate 6.2, the low pH mainly resulted from the lactic acid in muscle), which possibly injured the Salmonella and delayed the growth rates of cells. This supposition is in agreement with results of CLSM

![Fig. 3. Scanning electron micrographs of Salmonella biofilms grown on stainless steel surfaces after 1 (a and e), 3 (b and f), 5 (c and g) and 7 days (d and h) of incubation in TSB (a–d) or MTLB(e–h), respectively. Bar scale is 5 μm, magnification 5000×.](image1)

![Fig. 4. Images of glycoconjugates (green fluorescence) in EPS of Salmonella biofilms grown on stainless steel surfaces were stained by ConA-fluorescein dyes. The biofilms were incubated in TSB (a–d) or MTLB (e–h) for 1 (a and e), 3 (b and f), 5 (c and g) and 7 days (d and h), respectively. Bar scale is 20 μm, magnification 1000×.](image2)
(Fig. 2e, more red fluorescence) and SEM (Fig. 3e) which clearly showed the altered morphology of injured cells grown in MTLB. Additionally, it has been demonstrated that low pH can affect the cell state and inhibit the formation of multicellular status of *S.* Typhimurium (Speranza et al., 2011), and the effect of pH on biofilm development also depends on the acidulant used, with organic acids (in MTLB) tending to have a stronger influence on biofilm formation compared to inorganic acids (in TSB) (Lianou and Koutsoumanis, 2012). Compared to the casein tryptone,
soy peptone and glucose as the main nutrients in TSB, there were many organic compounds in MTLB, such as myogen, myoglobin, lactic acid, amino acid, muscle enzymes and other soluble nitrogenous compounds since the MTLB was obtained from the thawing-loss of chilled chicken meat. The great differences in carbon source, nitrogen source and pH between TSB and MTLB could also result in the variety of biofilm formation (Vazquez-Sanchez et al., 2013). Another important factor is the amount of NaCl (5 g/L) present in TSB, it has been demonstrated that NaCl could promote bacterial aggregation, and enhanced the stability of biofilms (Rode et al., 2007). Meanwhile, as previously reported, the presence of glucose in TSB can promote biofilm formation (Vazquez-Sanchez et al., 2013; Speranza et al., 2011). This was further supported by the results of CLSM (Fig. 2e) and SEM (Fig. 3e), where there were less cell aggregates where cells were grown in MTLB compared with TSB. It is known that bacterial cells are able to adapt to the medium of MTLB, and an increasing of biofilm formation, since Meira et al. (2012) demonstrated that more than 5 Log CFU/cm² cells was required for biofilm formation, since it has been suggested that the ion type and concentration can modulate cell surface properties such as surface charge and membrane composition (Xu et al., 2010). After 1 day of incubation, the cell number was 4.2 Log CFU/cm² in MTLB, and cells could not form biofilm at this time, since Nilsson et al. (2011) demonstrated that NaCl could promote bacterial aggregation, and enhanced the stability of biofilms (Rode et al., 2007). Meanwhile, as previously reported, the presence of glucose in TSB can promote biofilm formation (Vazquez-Sanchez et al., 2013). Another important factor is the amount of NaCl (5 g/L) present in TSB, it has been demonstrated that NaCl could promote bacterial aggregation, and enhanced the stability of biofilms (Rode et al., 2007). Meanwhile, as previously reported, the presence of glucose in TSB can promote biofilm formation (Vazquez-Sanchez et al., 2013; Speranza et al., 2011). This was further supported by the results of CLSM (Fig. 2e) and SEM (Fig. 3e), where there were less cell aggregates where cells were grown in MTLB compared with TSB. It is known that bacterial cells are able to adapt to the medium of MTLB, and an increasing of biofilm formation were observed after 3 days of incubation (Fig. 1).

CLSM coupled with SEM was the method of choice to characterize biofilm structures. As reported with other bacteria (Bridier et al., 2010), we also observed variation in the three-dimensional structure of Salmonella biofilm. A mature biofilm architecture was observed after incubation for 5 and 7 days in both media, which consisted of a complex three-dimensional structure formed by dense aggregates of cells held together by a network of extracellular matrix (Fig. 2c and g).

This was separated from other micro-colonies by interstitial voids, which may allow the channeling of water and nutrients to the micro-colonies (Donlan, 2002). It has been suggested that biofilm formed under the influence of specific environments may represent complex selection processes to which given bacterial strains have been exposed (Nilsson et al., 2011). Interestingly, we observed that mature Salmonella biofilm showed different morphologic types when incubated in TSB and MTLB after 5 and 7 days, namely “reticular-shaped” type (Fig. 2c and d) and “cloud-shaped” type (Fig. 2g and h). This may be explained by the view that the variety of nutrients and low pH can influence the morphology of biofilm. Additionally, we evaluated the production of glycoconjugates during biofilm development (Fig. 4), and the results indicated that high concentrations of glycoconjugates were produced in the biofilm matrix. The time of production of glycoconjugates was visually supported by Fig. 4, thus suggesting that glycoconjugates play important roles in the initial attachment of Salmonella cells to stainless steel surfaces and in the maintenance of the biofilm structure.

Although both ATR-FTIR and Raman can provide extended knowledge to EPS matrix, there were some important differences between them. ATR-FTIR absorption requires a change of molecular vibrations of polar groups such as C=O, N=H, and O=H, while Raman scattering relies on changes of nonpolar functional groups such as C–C and S–S (Rasco et al., 2008).
et al., 2011; Oust et al., 2006; Naumann, 2001), therefore, they can offer complementary information. In our present study, there were many distinct peaks that appeared on ATR-FTIR and Raman spectra of biofilm and planktonic cells. These peaks represented functional group vibrations in the main biomolecular constituents such as lipids, polysaccharides, proteins, phospholipids, nucleic acids, and other carbohydrates. These materials were the main constituents of biofilm EPS. The production of EPS depends on both intrinsic and extrinsic factors. Intrinsic factors arise in accordance with the genetic profile of the cell, whereas extrinsic influences include the physicochemical environment in which the cells are located (Ivleva et al., 2009). In our study, EPS production and its processes were obviously affected by the incubation media during biofilm development and maturation (Figs. 5 and 6), which were in line with the previous study (Sutherland, 2001). Several ATR-FTIR bands could be associated with the function groups of polysaccharides in our study, indicating great amount of polysaccharides in the biofilm matrix. This finding was in agreement with the data presented in Fig. 4, where the predominant green color indicated high concentrations of glyco-conjugates in the EPS matrix. Polysaccharides are an important component during the development of biofilm, since they may consolidate cells adhesion to surfaces and aid cell–cell aggregation (Planchon et al., 2007). Meanwhile, some peaks of ATR-FTIR and Raman spectra were associated with peptides, amide and proteins, which play a key role in cell biofilm formation, since many protein fractions are responsible for intercellular aggregation and can mediate in cells adherence to surfaces (Steenackers et al., 2012). Additionally, after 7 days of incubation, there were several strong peaks in the spectra of planktonic cells (those floating in the supernatant of the surfaces bearing the biofilms), and this may be the result of dispersal EPS and the planktonic cells being released from the mature biofilm, since a few EPS may still have been located on the outside of released cell surfaces.

After 5 and 7 days of incubation, the ATR-FTIR bands of biofilm (grown in TSB) at 1243, 1402 and 2930 cm⁻¹ showed greater intensity compared to that in MTLB. The band of 1402 cm⁻¹ attributed to the stretching C=O of carboxylic groups indicated the formation of a carboxylic anion, which has been shown to cross-link with the polymer strands and provide greater binding force in the EPS development of Gram-negative cells of biofilm (Donlan, 2002). The band of 1243 cm⁻¹ associated with carbohydrates might attribute to the complex three-dimensional architecture of biofilms. The band of 1243 cm⁻¹ was associated with lipids, which might significantly influence the rheological properties and thus the stability of biofilms (Steenackers et al., 2012).

From the bands of ATR-FTIR (Fig. 5), we observed that the peaks of amide (1647, 1548 and 1539 cm⁻¹) and carbohydrates (1084 and 1056 cm⁻¹) always showed great intensity, suggesting that the carbohydrates and proteins were the fundamental structural elements of the biofilm matrix and determined the mechanical stability of biofilms. Compared to the Raman bands of biofilm in MTLB during the incubation periods (Fig. 6), we found that there was only carbohydrates compound (540 and 1096 cm⁻¹) and no other compound after 3 days of incubation, suggesting that the carbohydrates in EPS matrix was formed prior to other compounds (including protein), this finding was also in line with the ATR-FTIR spectra of biofilm grown in MTLB after 3 days of incubation. Meanwhile, after 3 days of incubation, we observed the appearance of carbohydrates (542 and 1085 cm⁻¹) in the bands of planktonic cells grown in TSB (Fig. 6), possibly suggesting that carbohydrates might detach from the EPS matrix prior to other compounds. Additionally, from the greater number of peaks of biofilm grown in TSB (Figs. 5 and 6), it can be deduced that the chemical composition of EPS matrix of biofilm grown in TSB may be more complex than that in MTLB.

It has been demonstrated that two important properties of EPS can have a marked effect on biofilms. Firstly, the composition and structure of the polysaccharides determines the primary conformation of the biofilm. For example, many bacterial EPS possess backbone structures that contain 1,3- or 1,4-glycosidic link residues that tend to be more rigid and less deformable. Secondly, EPS is not generally uniform but may vary spatially and temporally due to external and internal processes (Sutherland, 2001). These two important properties (the composition and structure of polysaccharides, and the variety of EPS) have also been reflected in our study (Tables 2 and 3, Fig. 4). From the variation of biofilm spectra of ATR-FTIR and Raman in our study, the functional groups associated with the peaks, which appeared in biofilm spectra but not in planktonic cell spectra, may be used as the identification of EPS production and biofilm formation: polysaccharides and proteins. Taking into account all the results in our present study, together with the findings described for other bacteria (Simoes et al., 2010), we believe that the biofilm formation of Salmonella under our experimental conditions occurs through a number of different stages: (1) initial cell attachment to stainless steel surfaces (about 1 day); (2) formation of small cell aggregates, a rudimentary biofilm matrix, since they may consolidate cells adhesion to surfaces and aid cell–cell aggregation (Planchon et al., 2007). Meanwhile, some peaks of ATR-FTIR and Raman spectra were associated with peptides, amide and proteins, which play a key role in cell biofilm formation, since many protein fractions are responsible for intercellular aggregation and can mediate in cells adherence to surfaces (Steenackers et al., 2012). Additionally, after 7 days of incubation, there were several strong peaks in the spectra of planktonic cells (those floating in the supernatant of the surfaces bearing the biofilms), and this may be the result of dispersal EPS and the planktonic cells being released from the mature biofilm, since a few EPS may still have been located on the outside of released cell surfaces.

After 5 and 7 days of incubation, the ATR-FTIR bands of biofilm (grown in TSB) at 1243, 1402 and 2930 cm⁻¹ showed greater intensity compared to that in MTLB. The band of 1402 cm⁻¹ attributed to the stretching C=O of carboxylic groups indicated the formation of a carboxylic anion, which has been shown to cross-link with the polymer strands and provide greater binding force in the EPS development of Gram-negative cells of biofilm (Donlan, 2002). The band of 1243 cm⁻¹ associated with carbohydrates might attribute to the complex three-dimensional architecture of biofilms. The band of 1243 cm⁻¹ was associated with lipids, which might significantly influence the rheological properties and thus the stability of biofilms (Steenackers et al., 2012).

<table>
<thead>
<tr>
<th>Raman frequency (cm⁻¹)</th>
<th>Definition of the spectral assignment</th>
<th>References a</th>
</tr>
</thead>
<tbody>
<tr>
<td>414</td>
<td>Carbohydrates</td>
<td>a, b, c</td>
</tr>
<tr>
<td>531–542</td>
<td>C–O–C glycosidic ring deformation; S–S stretching</td>
<td>a, d</td>
</tr>
<tr>
<td>855–899</td>
<td>C–C stretching; C–O–C 1,4-glycosidic link</td>
<td>a, c</td>
</tr>
<tr>
<td>1000–1020</td>
<td>C–CH3 deformation</td>
<td>c</td>
</tr>
<tr>
<td>1020–1085</td>
<td>C–C, and C–O stretching (carbohydrates)</td>
<td>e</td>
</tr>
<tr>
<td>1096</td>
<td>C–C stretching; C–O–C glycosidic link; PO4³⁻ asymmetric stretching</td>
<td>f, g, h</td>
</tr>
<tr>
<td>1161</td>
<td>C–C stretching; C–C, C–O ring asymmetrical breathing (carbohydrates)</td>
<td>c, h, f</td>
</tr>
<tr>
<td>1314–1345</td>
<td>C=C stretching</td>
<td>c, i</td>
</tr>
<tr>
<td>1500–1550</td>
<td>C=C, C=O stretching; amide I</td>
<td>c, f, g, j</td>
</tr>
<tr>
<td>1640–1688</td>
<td>C=O stretching</td>
<td>c, f</td>
</tr>
<tr>
<td>1700</td>
<td></td>
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</tbody>
</table>

a (a) Wagner et al. (2009), (b) Schuster et al. (2000), (c) Ivleva et al. (2009), (d) Lu et al. (2011), (e) Schwartz et al. (2009), (f) Harz et al. (2005), (g) Maquelin et al. (2002), (h) Rosch et al. (2004), (i) Rosch et al. (2005), and (j) Andrews et al. (2010).
reduction of EPS (7 days), this observation was obviously supported by the Raman spectra of biofilm grown in TSB, however, this observation was not clearly present for biofilm grown in MLTB.

In conclusion, our study indicated that *Salmonella* cells grown in a meat-like substrate (MTLB) and a standard laboratory substrate (TSB) could form biofilms on stainless steel surfaces through building a three-dimensional structure with multilayers of cells. *Salmonella* showed different morphological types of mature biofilms when incubated in different media, namely "cloud-shaped" in TMB and "reticular-shaped" in TSB. The ATR-FTRR and Raman analysis revealed a completely different chemical composition between biofilm and the corresponding planktonic cells and some important differences in biofilm grown in TMB and biofilm grown in TSB. Moreover, our study found that the progress towards a mature *Salmonella* biofilm on stainless steel may be associated with the production of the EPS matrix containing mainly polysaccharide and proteins, which contribute to the biofilm architecture and may be used as identification of biofilm formation. ATR-FTRR and Raman were very promising additions to the CLSM analysis; combining application of these techniques can reveal valuable new insights into *Salmonella* biofilms in situ on food-contact surfaces.

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References


