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Influence of serotype on the growth kinetics and the ability to form biofilms of *Salmonella* isolates from poultry

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ABSTRACT

The influence of the serotype on the growth behaviour and the ability to form biofilms of *Salmonella enterica* strains was investigated. The relationships between biofilm formation and growth kinetic parameters were also determined. A total of 69 strains (61 isolates from poultry and 8 reference strains from culture collections) belonging to 10 serotypes (*S. enterica* serotype Typhimurium, *S.* Newport, *S.* Paratyphi B, *S.* Poona, *S.* Derby, *S.* Infantis, *S.* Enteritidis, *S.* Virchow, *S.* Agona and *S.* Typhi) were tested. All *Salmonella* strains produced biofilms on polystyrene micro-well plates (crystal violet assay). Isolates were classified as weak (35 strains), moderate (22), or strong (12) biofilm producers. *S.* Agona and *S.* Typhi produced the most substantial ($P < 0.001$) biofilms. Growth curves were performed at 37 °C in tryptone soy broth by means of optical density ($OD_{420-580}$) measurements from 0 to 48 h. Growth kinetic parameters (Gompertz model) varied between serotypes. The maximum growth rate ($\Delta OD_{420-580}$/h) ranged from 0.030 ± 0.002 (*S.* Typhi) to 0.114 ± 0.011 (*S.* Agona). The ability of *Salmonella* strains to form biofilms was not related to their growth kinetic parameters. The formation of biofilms by *Salmonella* on polystyrene constitutes an issue of concern because plastic materials are frequently used in food facilities. The findings suggest that special efforts must be made for the effective control of *Salmonella* in food-processing environments when *S.* Agona or *S.* Typhi strains are present.

1. Introduction

Salmonella infections are recognized as one of the major public health problems in both developed and developing countries, causing millions of human and animal illnesses and significant financial losses worldwide. In the United States, more than one million human illnesses can be attributed annually to non-typhoidal salmonellae, resulting in approximately 20,000 hospitalizations and 400 deaths (CDC, 2011). In the year 2009, a total of 108,614 confirmed cases of salmonellosis were reported in the European Union, this being the second most often reported zoonotic disease in humans, after campylobacteriosis. The reported incidence of *Salmonella* infections in 2009 was 23.7 per 100,000 people (EFSA, 2011).

There are several routes for contracting salmonellosis, but more than 95% of cases of human infections caused by these bacteria are food-borne (Lestari et al., 2009). *Salmonella* represents a risk to human health when contaminated raw foods are inadequately cooked or if cross-contaminated after cooking (Capita et al., 2007). During 2009, *Salmonella* was responsible for 1722 outbreaks of food-based infection (31.0% of all reported food-borne outbreaks and 33.2% of all verified outbreaks) in the EU, remaining the most commonly recognized causative agent in the food-borne outbreaks reported (EFSA, 2011). Similarly, *Salmonella* is the most frequently reported cause of food-borne illness in Spain, causing approximately 75% of the verified food-borne outbreaks in the period 2004 to 2007 (Martínez et al., 2008).

Although several foods have been associated with salmonellosis, eggs and broiler meat are the most common food vehicles implicated in human infections, causing 49.1% and 5.2%, respectively, of the outbreaks of food-borne *Salmonella* in the European Union in 2009 (EFSA, 2011). According to the 2009 RASFF (Rapid Alert System for Food and Feed) annual report, *Salmonella* is the most frequently notified microorganism, with 70% of those related to poultry (European Commission, 2010).

Biofilms are defined as complex microbial communities embedded in a self-produced extracellular polymeric matrix that attach to surfaces and are the predominant mode of microbial growth in nature (Steenackers et al., 2011). Biofilms on surfaces in contact with food provide a reservoir of pathogenic and spoilage bacteria, increasing the risk of microbial contamination in food-processing plants and leading to critical problems in terms of

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public health and a potentially major economic impact (Shi and Zhu, 2009). It has been demonstrated that the percentage of poultry samples contaminated with *Salmonella* increases significantly during processing, as a consequence of the presence of the bacteria on surfaces with which food comes into contact (Sánchez et al., 2002).

Bacteria in biofilms are generally well protected against environmental stresses (e.g. disinfectants) and as a consequence are extremely difficult to eradicate, contributing to the resistance and persistence of bacteria on industrial surfaces and equipment. In addition, biofilms can reduce heat transfer and operating efficiency in heat exchange equipment, increasing energy consumption and the number of mechanical blockages, and accelerating the corrosion of metal surfaces (Shi and Zhu, 2009). Bacteria in biofilms have been found to be more resistant to antibiotics than planktonic cells (Capita and Alonso-Calleja, in press). Thus, factors influencing biofilm formation and ways to prevent it are currently crucial topics for poultry processors, government officials and consumers.

Bacterial adhesion and subsequent biofilm formation by *Salmonella* strains on abiotic surfaces has been evaluated in the past by a number of authors (Steenackers et al., 2011). However, very few reports compared the ability to form biofilms of *Salmonella enterica* serotypes isolated from poultry (Marin et al., 2009), and the relationship between growth kinetic parameters and a capacity for biofilm formation has not been explored. The main aim of the present study was to compare the ability to develop biofilms of strains belonging to ten different serotypes of *Salmonella*. The growth kinetics of the strains tested and the relationship between growth kinetic parameters and the ability to form biofilms were also assessed.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Sixty-nine *Salmonella* strains were used in this study. Sixty-one of these had previously been isolated from chicken carcasses in six different slaughterhouses in North-western Spain in accordance with the rules of ISO-6579-1993: *S. enterica* serotype Typhimurium (3 strains), *S.* Newport (2), *S.* Paratyphi B (2), *S.* Poona (5), *S.* Derby (1), *S.* Infantis (6), *S.* Enteritidis (36), *S.* Virchow (2), *S.* Agona (3) and *S.* Typhi (1). Serotyping was carried out at the "Laboratorio Nacional de Referencia para *Salmonella* y *Shigella* de España (LNRSSE; Instituto de Salud Carlos III, Majadahonda, Madrid, Spain)". Eight strains were obtained from the American Type Culture Collection (*S.* Typhimurium ATCC 13311 and *S.* Typhi ATCC 416), from the National Culture Type Collection (*S.* Derby NCTC 5721 and *S.* Virchow NCTC 574) and from the Spanish Type Culture Collection (*S.* Paratyphi B CECT 554, *S.* Infantis CECT 700, *S.* Enteritidis CECT 556 and *S.* Agona CECT 705).

Bacterial stock cultures were kept at −30 °C in tryptone soy broth (TSB, Oxoid Ltd., Hampshire, United Kingdom) containing 20% (vol/vol) glycerol. Prior to each experiment, cells were activated by transferring 20 μL of stock cultures to 5 mL of TSB and incubating overnight at 37 °C. Working cultures were kept at 4 °C on tryptone soy agar (TSA, Oxoid) plates.

2.2. Biofilm formation

Biofilm production was measured using the method described by Stepanović et al. (2004), with some modifications. The optical density of adhered cells was determined in 100-well polystyrene micro-well plates (Oy Growth Curves Ab Ltd., Helsinki, Finland) after staining with crystal violet and rinsing. Before use, strains were transferred to TSB and incubated for 5 h at 37 °C. These bacterial cultures contained approximately 10^8 cfu/mL. Three decimal dilutions were performed in TSB. Wells were filled with 20 μL of the third dilution of this bacterial culture and 230 μL of TSB to obtain a concentration of 10^4 cfu/mL in the well. This bacterial concentration was confirmed by plating. The negative controls (five in each plate) contained 250 μL of TSB only. The plates were incubated aerobically for 24 h at 37 °C. The content of the plate was then poured off and the wells washed with 300 μL of distilled water. The bacteria that remained attached were fixed by adding 250 μL of methanol to each well for 15 min. The plates were then emptied, air dried and stained with 250 μL per well of crystal violet for 5 min. Excess stain was rinsed off by placing the micro-well plate under running tap water. The plates were air dried and then the dye bound to the adherent cells was resolubilized with 250 μL of 33% glacial acetic acid per well. The optical density of each well was measured at 580 nm (OD_{580}) using a Bioscreen C MBR (Oy Growth Curves Ab Ltd.). The micro-well plates were shaken for one minute prior to the measurement of turbidity. The 69 strains and the five controls were all included on each of the micro-well plates. All experiments were replicated three times on separate days.

The cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative controls. Strains were classified into three categories: not biofilm producers, when $OD \leq ODc$, weak biofilm producers, when $ODc < OD \leq (2 \times ODc)$, moderate biofilm producers, when $(2 \times ODc) < OD \leq (4 \times ODc)$, or strong biofilm producers, when $(4 \times ODc) < OD$. Differences in the degree of biofilm formation were examined by analysis of variance (ANOVA) techniques. Mean separations were obtained using Duncan's multiple range test. Significance was determined at the $P < 0.05$ level.

2.3. Growth curves

Growth experiments were carried out on micro-well plates. The wells were filled in the same manner as described for biofilm formation (10^4 cfu/mL). Bacterial growth was monitored before incubation (at hour 0) and every 2 h until 48 h had elapsed. Growth was determined by measuring the OD at 420–580 nm ($OD_{420-580}$) using the Bioscreen C MBR (Oy Growth Curves Ab Ltd.). The micro-well plates were shaken for one minute prior to the measurement of turbidity. The model used to fit growth curves to the data obtained was the modified Gompertz equation (Garthright, 1991): $ODt = A + B^*\exp(-\exp(2.71828183^*u^*(L-t)/B+1))$, where t = the time in hours that has elapsed since inoculation, ODt = Optical Density (determined at 420–580 nm) at time t, L = the lag time when the lag period ends (hours), μ = the maximum growth rate achieved (ΔOD/h), B = the increase in $OD_{420-580}$ since inoculation to stationary phase (E), and A = the upper asymptote curve (concentration of bacteria in the stationary stage, E) − B. The time to stationary phase (T; hours) was calculated as the time elapsed to reach a concentration equal or higher than 99% of the value for E (Del Río et al., 2008).

Values for L, μ, E and T were obtained for each strain and replication by fitting a sigmoidal curve to the data set using a Marquardt algorithm that calculates those parameter values which give the minimum residual sum of squares. The goodness of fit was evaluated using the coefficient of determination (R^2). L, μ, E and T were compared for statistical significance using analysis of variance techniques. Mean separations were obtained using Duncan's multiple range test. Significance was determined at the 5% ($P < 0.05$) level.

2.4. Calibration curve

A calibration equation was performed in order to transform the optical density into plate counts. Five strains were used for this

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purpose: 4 isolates from poultry (*S.* Typhimurium, *S.* Newport, *S.* Infantis and *S.* Enteritidis) and one pattern strain (*S.* Virchow NCTC 574). The growth of the strains was determined in two ways: by measuring the $OD_{420–580}$ in a Bioscreen C MBR (Oy Growth Curves Ab Ltd.), and by enumerating viable cells in plate count agar (PCA, Oxoid Ltd.) in duplicate, using 0.1% (wt/vol) peptone water (Oxoid Ltd.) for decimal dilutions, and counting visible colonies after 48 h of incubation at 30 °C. Blank sample wells with uninoculated broth were included as a control for contamination. The degree of correlation between optical densities and microbial counts was investigated by linear regression continuous data. All data processing in this study was carried out using the Statistica® 6.0 software package (Statsoft Ltd., Chicago, Illinois, USA).

3. Results

3.1. Biofilm formation

All *S. enterica* strains tested produced biofilms on polystyrene micro-well plates, with OD_{580} values ranging from 0.126 ± 0.012 to 2.745 ± 0.033. A cut-off value (three standard deviations above the mean OD of the negative controls) of 0.118 was used for strain classification. Strains were classified as weak (35 strains; average OD_{580} = 0.185 ± 0.049), moderate (22 strains; average OD_{580} = 0.313 ± 0.120) and strong (12 strains; average OD_{580} = 1.355 ± 0.928) biofilm producers. The average OD_{580} produced by 69 tested strains was 0.428 ± 0.582.

Biofilm formation was significantly affected by the *Salmonella* serotypes, with *S.* Agona (OD_{580} = 2.573 ± 0.231) and *S.* Typhi (1.158 ± 0.098) showing significantly ($P < 0.001$) more biofilm formation that the rest of serotypes: 0.389 ± 0.246 (*S.* Typhimurium), 0.337 ± 0.053 (*S.* Newport), 0.182 ± 0.016 (*S.* Paratyphi B), 0.230 ± 0.129 (*S.* Poona), 0.227 ± 0.075 (*S.* Derby), 0.327 ± 0.195 (*S.* Infantis), 0.260 ± 0.176 (*S.* Enteritidis), and 0.199 ± 0.061 (*S.* Virchow) (Fig. 1). All strains belonging to serotypes *S.* Agona and *S.* Typhi were strong biofilm producers, while all *S.* Paratyphi B and *S.* Virchow strains were categorized as weak biofilm producers. The *S.* Newport strains tested showed a moderate capacity to form biofilms. Strains among the remaining serotypes showed higher variability in their biofilm-forming ability, being classified as weak or moderate biofilm producers (*S.* Poona and *S.* Derby), or as weak, moderate or strong producers (*S.* Typhimurium, *S.* Infantis and *S.* Enteritidis) (Table 1).

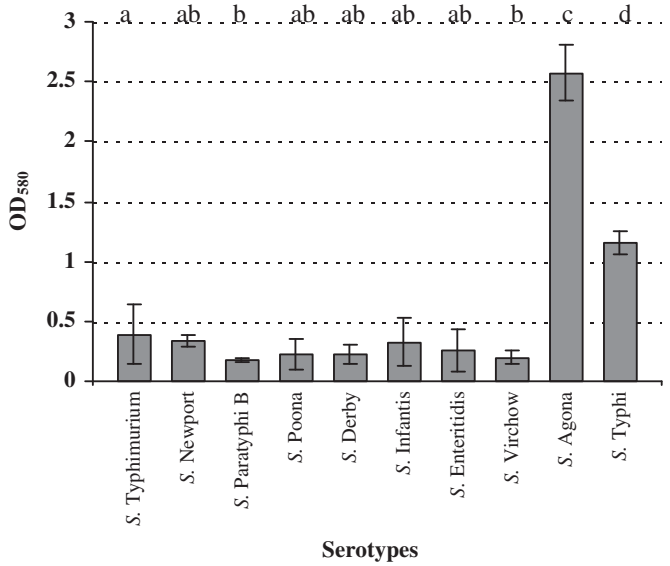

Fig. 1. Biofilm formation by different *Salmonella enterica* serotypes (optical density at 580 nm). Average values with no letters in common are significantly different ($P < 0.05$).

3.2. Growth kinetic parameters

Including the replicates, a total of 207 $OD_{420–580}$ curves were generated for the *S. enterica* strains tested and fitted to the modified Gompertz equation. The estimated growth kinetic parameters are shown in Table 2. Fig. 2 shows the comparative growth curves in TSB for ten serotypes of *S. enterica*. The R^2 values for the Gompertz model fit were high (>0.91).

Growth parameter values varied between serotypes. The lag phase (h) ranged from −2.122 ± 2.697 (*S.* Enteritidis) to 4.165 ± 0.260 (*S.* Agona). The maximum growth rate was obtained for *S.* Agona (0.114 ± 0.011), followed by *S.* Infantis (0.090 ± 0.016), *S.* Derby (0.084 ± 0.024) and *S.* Paratyphi B (0.082 ± 0.003). Strains in the above-mentioned four serotypes showed the highest OD_{max} (maximum $OD_{420–580}$) and are among those with the lowest *T* (time elapsed to stationary phase). On the other hand, *S.* Typhi showed the lowest μ and OD_{max}, as well as the highest *T*. Significant differences in OD_{24} ($OD_{420–580}$ determined at 24 h; biofilm formation was evaluated at this time) were observed between serotypes, with *S.* Paratyphi, *S.* Derby, *S.* Infantis, *S.* Virchow and *S.* Agona showing the highest figures, and *S.* Typhi the lowest.

Fig. 3 shows the regression equation and the coefficient of correlation obtained between microbial counts (log_{10} cfu/mL) and $OD_{420–580}$ values. Microbial counts were found to give linear relationship to optical density over a range of 7.5–10 log_{10} cfu/mL. An R^2 value of 0.906 was obtained over this range. This equation was used to estimate microbial counts after 24 h of incubation (the time at which biofilm formation was determined) and the maximum concentration of bacteria at the stationary phase. Thus, $OD_{24\ h}$ and OD_{max} values obtained throughout the experiment were transformed into microbial counts in accordance with this equation. The microbial counts (log_{10} cfu/mL) recorded after 24 h and the maximum microbial counts are shown in Table 2.

3.3. Relationship between biofilm formation and growth kinetic parameters

Correlations among biofilm formation by *Salmonella* and growth kinetic parameters, including lag phase (*L*; h), maximum growth rate (μ; ΔOD/h), maximum optical density determined at 420–580 nm (OD_{max}), optical density at 24 h ($OD_{24\ h}$) and time elapsed to stationary phase (*T*), are shown in Table 3. Strong correlations (>0.700; $P < 0.001$) were observed between *L*, μ and $OD_{24\ h}$. Strong negative correlations (from −0.500 to −0.860; $P < 0.001$) were obtained between the three above-mentioned parameters and T. The amount of biofilm formation was significantly correlated with *L* (0.343; $P < 0.01$), μ (0.514; $P < 0.001$), $OD_{24\ h}$ (0.351; $P < 0.001$) and *T* (−0.390; $P < 0.001$).

A scatter-plot was draw up to show the relationship between biofilm formation, μ and $OD_{24\ h}$ (Fig. 4). As can be seen, cells belonging to two serotypes (*S.* Agona and *S.* Typhi) showed a behaviour that was clearly different from the strains in the remaining serotypes. To exclude the possibility that serotype might be a confounding variable, the relationship between biofilm production and growth parameters was also evaluated for strains in each serotype. In this analysis, no significant correlations were observed, indicating that the correlation between biofilm production and growth parameters was due to the effect of serotype.

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Table 1
Classification of 69 *Salmonella enterica* isolates according to their ability to produce biofilms.

Salmonella serotypes	Biofilm formation					
	Weak		Moderate		Strong	
	No. (%)	Average OD_{580} ± STD	No. (%)	Average OD_{580} ± STD	No. (%)	Average OD_{580} ± STD
S. Typhimurium ($n = 4$)	1 (25.0)	0.185 ± 0.009	2 (50.0)*	0.321 ± 0.125	1 (25.0)	0.730 ± 0.203
S. Newport ($n = 2$)			2 (100)	0.337 ± 0.053		
S. Paratyphi B ($n = 3$)	3 (100)*	0.182 ± 0.016				
S. Poona ($n = 5$)	3 (60.0)	0.182 ± 0.016	2 (40.0)	0.318 ± 0.165		
S. Derby ($n = 2$)	1 (50.0)	0.202 ± 0.051	1 (50.0)*	0.349 ± 0.001		
S. Infantis ($n = 7$)	2 (28.6)	0.172 ± 0.051	3 (42.9)*	0.295 ± 0.121	2 (28.6)	0.525 ± 0.209
S. Enteritidis ($n = 37$)	22 (59.5)	0.185 ± 0.052	12 (32.4)*	0.309 ± 0.125	3 (8.1)	0.624 ± 0.361
S. Virchow ($n = 3$)	3 (100)*	0.199 ± 0.061				
S. Agona ($n = 4$)					4 (100)*	2.573 ± 0.231
S. Typhi ($n = 2$)					2 (100)*	1.158 ± 0.098

OD_{580}, optical density at 580 nm; STD, Standard deviation. Three repetitions were performed for each strain. * One culture collection strain is included.

4. Discussion

4.1. Biofilm formation

Biofilms formed in food-processing environments are a well recognized problem for the food industry, as they have the potential to act as sources leading to the contamination of food with pathogenic and spoilage microorganisms, which may cause the transmission of diseases and potentially have a major financial impact. It has been demonstrated that pathogenic bacteria in biofilms are the principal source of food contamination (Shi and Zhu, 2009). Biofilms increase microbial resistance to chemical, physical and biological agents and the ability to form biofilms has been identified as an important factor for the persistence of bacteria in food-processing facilities (Vestby et al., 2009). Crystal violet assay is considered a suitable technique for evaluating biofilm formation because it yields reproducible results, which allows large numbers of strains and conditions to be studied at the same time. Furthermore, the method provides quantitative results by measuring the optical density of wells (Peeters et al., 2008; Pui et al., 2011). A correlation between microtitre plate assay and biofilm formation by *Salmonella* has been demonstrated (Patel and Sharma, 2010).

Results in this study revealed that all the *S. enterica* isolates tested possessed the ability to produce biofilm on polystyrene surfaces. These findings are a matter for concern because plastic materials are frequently used on farms, in slaughterhouses, in the food-processing industry and in kitchens, for the construction of tanks, pipe-work, accessories and cutting surfaces (Steenackers et al., 2011; Stepanović et al., 2004). Previous studies have shown a correlation between biofilm production *in vitro* using polystyrene micro-wells and biofilm formation on the surfaces of different materials in food facilities (Patel and Sharma, 2010; Vestby et al., 2009).

It has been demonstrated that bacteria, including *Salmonella*, adhere in larger numbers to hydrophobic (rubber and plastic) surfaces than to hydrophilic (glass and stainless steel) surfaces (Steenackers et al., 2011; Tondo et al., 2010). This fact could be a possible explanation for the considerable ability of *Salmonella* to produce biofilm on plastic surfaces, taking into consideration that adhesion is the first step in the biofilm formation process (Steenackers et al., 2011). The OD_{580} figures recorded in the present study for *Salmonella* strains (from 0.126 ± 0.012 to 2.745 ± 0.033) are in the range of figures observed by other authors on polystyrene surfaces using micro-well plate assays with crystal violet staining (Ibuchi et al., 2010; Pui et al., 2011; Stepanović et al., 2003, 2004; Vestby et al., 2009).

There are very few studies describing the capacity to form biofilms of salmonellae isolated from poultry. Ramesh et al. (2002) studied the formation of biofilms on the galvanized steel surfaces of poultry transport containers. These authors observed that the five *Salmonella* strains (*S.* Typhimurium, *S.* Thompson, *S.* Beta, *S.* Hadar and *S.* Johannesburg) had the ability to attach to these metal surfaces. Marin et al. (2009) showed that 50% of the *Salmonella* strains isolated from surfaces in housing for broiler and laying hens were able to produce biofilms.

Results in the present study show that the ability to form biofilm of *Salmonella* is serotype-dependent. Consequently, this constitutes a factor of virulence varying between different serotypes, as suggested by other authors (Oliveira et al., 2006). In agreement with the present study's findings, Patel and Sharma (2010) observed that attachment and subsequent biofilm formation by *Salmonella* strains can differ on the basis of the specific properties of the serotypes. *S.* Agona, a serotype frequently isolated from poultry meat, is in the top ten serotypes most frequently associated with human salmonellosis in the European Union in the last few years. In 2008, a total of 120,760 cases of salmonellosis were confirmed in humans, *S.*

Table 2
Growth kinetic parameters obtained for 69 *Salmonella enterica* strains.

	L	μ	$OD_{24\ h}$	OD_{max}	*T*
S. Typhimurium ($n = 4$)	1.185 ± 2.260a	0.064 ± 0.016ab	1.017 ± 0.122a (9.376 ± 0.204a)*	1.149 ± 0.084a (9.596 ± 0.140a)	36.083 ± 4.889ab
S. Newport ($n = 2$)	0.581 ± 0.979ab	0.061 ± 0.005a	1.050 ± 0.016a (9.431 ± 0.026a)	1.159 ± 0.027ab (9.613 ± 0.045ab)	40.167 ± 4.665ac
S. Paratyphi B ($n = 3$)	2.186 ± 0.372ac	0.082 ± 0.003c	1.182 ± 0.035bc (9.651 ± 0.059bc)	1.236 ± 0.052cd (9.741 ± 0.086cd)	32.889 ± 3.060b
S. Poona ($n = 5$)	−1.588 ± 4.304bd	0.064 ± 0.007ab	1.066 ± 0.079a (9.458 ± 0.131a)	1.185 ± 0.074ac (9.657 ± 0.124ac)	41.933 ± 4.862cd
S. Derby ($n = 2$)	1.649 ± 2.034ac	0.084 ± 0.024c	1.156 ± 0.108b (9.619 ± 0.180b)	1.221 ± 0.068cd (9.716 ± 0.113cd)	33.667 ± 7.554b
S. Infantis ($n = 7$)	1.969 ± 4.358ac	0.090 ± 0.016c	1.194 ± 0.091bc (9.671 ± 0.151bc)	1.252 ± 0.045d (9.768 ± 0.074d)	31.714 ± 6.944b
S. Enteritidis ($n = 37$)	−2.122 ± 2.697d	0.058 ± 0.006a	1.042 ± 0.048a (9.418 ± 0.079a)	1.210 ± 0.062bcd (9.697 ± 0.103bcd)	45.055 ± 4.016de
S. Virchow ($n = 3$)	1.557 ± 0.838ac	0.071 ± 0.005b	1.135 ± 0.024b (9.573 ± 0.039b)	1.222 ± 0.035cd (9.717 ± 0.058cd)	35.444 ± 5.028b
S. Agona ($n = 4$)	4.165 ± 0.260c	0.114 ± 0.011d	1.221 ± 0.043c (9.717 ± 0.071c)	1.237 ± 0.046cd (9.743 ± 0.077cd)	26.917 ± 6.908f
S. Typhi ($n = 2$)	−1.996 ± 2.215d	0.030 ± 0.002e	0.675 ± 0.045d (8.784 ± 0.108d)	0.969 ± 0.101e (9.317 ± 0.154e)	47.714 ± 0.756e

L, lag phase (h), μ, maximum growth rate (ΔOD/h); OD_{max}, maximum optical density (determined at 420–580 nm); $OD_{24\ h}$, optical density after 24 h (determined at 420–580 nm); *T*, time (h) elapsed to stationary phase. Three repetitions were performed for each strain; *, data within parentheses correspond to transformation of $OD_{420-580}$ to $\log_{10}$ cfu/mL according to the regression equation obtained. Means in the same column with no letters in common are significantly different ($P < 0.05$).

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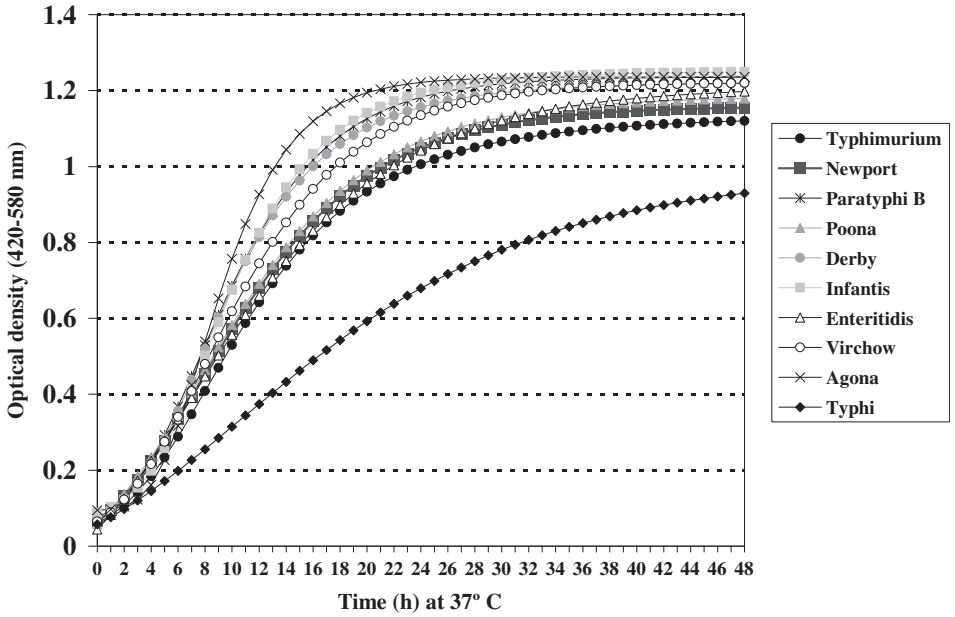

Fig. 2. Growth curves in tryptone soy broth of strains of different *Salmonella enterica* serotypes.

Agona being responsible for 636 (0.5%) (EFSA, 2011). The great ability of *S.* Agona to form biofilms has been observed previously by Vestby et al. (2009), when testing strains from fish-meal and feed factories using microtitre plates and staining with crystal violet. Among the four serotypes tested by these authors (*S.* Agona, *S.* Montevideo, *S.* Senftenberg and *S.* Typhimurium), *S.* Typhimurium showed the least capacity to produce biofilms. Bridier et al. (2010) demonstrated in another biofilm model (microtitre plates using a cell-permeant green fluorescent nucleic acid marker and image acquisition with confocal laser scanning microscopy) that *S.* Agona strains were better biofilm producers that the other serotypes tested (*S.* St Paul, *S.* Brandenburg, *S.* Derby, *S.* Dublin, *S.* Enteritidis, *S.* Hadar, *S.* Indiana and *S.* Typhimurium), which was in agreement with the present study's observations. On the other hand, Kim and Wei (2007) reported that *S.* Typhimurium (especially DT104) and *S.* Heidelberg strains were stronger biofilm formers than *S.* Agona strains.

S. Typhi is the etiological agent of typhoid fever, also called enteric fever. This illness remains a serious public health problem throughout the world, especially in developing countries, with an estimated 16 to 33 million cases and 500,000 to 600,000 deaths annually (Girard et al., 2006). Between 10 and 34 cases of human illness are recorded annually in Spain (Velasco et al., 2009). Because of the severity and considerable fatality rate (as high as 10%–20%) of typhoid fever, the ability of *S.* Typhi to form biofilms is of great public health concern. The disease is transmitted by food and water contaminated by the faeces and urine of patients and carriers. An issue for concern is that 2%–5% of patients become chronic carriers

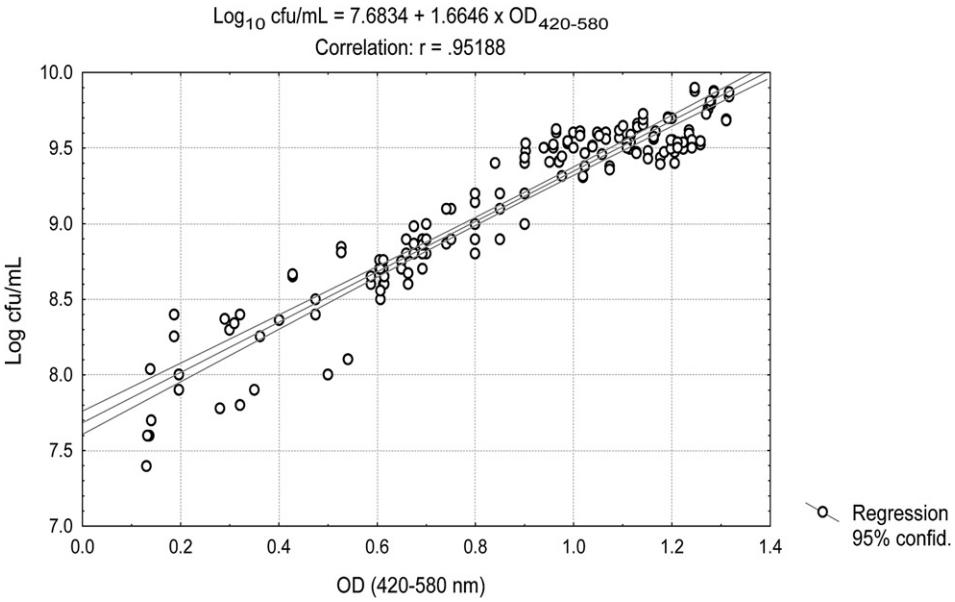

Fig. 3. Relationship between microbial counts and $OD_{420-580}$ for 69 *Salmonella enterica* strains.

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Table 3
Coefficients of correlation between growth kinetic parameters and biofilm formation by 69 *Salmonella enterica* strains.

	L	μ	OD_{max}	$OD_{24\ h}$	*T*
μ	0.760***				
OD_{max}	0.249*	0.422***			
$OD_{24\ h}$	0.740***	0.782***	0.624***		
T	−0.750***	−0.860***	−0.170	−0.500***	
Biofilm	0.343**	0.514***	−0.020	0.351***	−0.390***

L, lag phase (h), μ, maximum growth rate (ΔOD/h); OD_{max}, maximum optical density (determined at 420–580 nm); $OD_{24\ h}$, optical density after 24 h (determined at 420–580 nm); *T*, time (h) elapsed to stationary phase; Biofilm, biofilm formation (OD_{580} using the micro-well crystal violet assay). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; without asterisk, non significant.

(Steenackers et al., 2011). The *S.* Typhi strains used in this study were categorized as strong biofilm formers ($OD_{580} = 1.158 \pm 0.098$) according to the criteria suggested by Stepanović et al. (2004). Limited information about the ability of *S.* Typhi to form biofilms is currently available. In agreement with the results being reported here, Pui et al. (2011) reported that biofilm formation by *S.* Typhi on plastic cutting boards was significantly higher ($P < 0.05$) than that of *S.* Typhimurium.

4.2. Growth kinetic parameters

The modified Gompertz equation was regarded as appropriate for describing *in vitro* bacterial growth ($OD_{420–580}$) because of the good agreement observed between experimental and predicted values.

A significant result in this study originates from the fact that important differences in growth kinetic parameters were observed between *Salmonella* serotypes. It should be pointed out, however, that the 10 serotypes to which the tested strains belonged were not equally represented, with serotypes *S.* Newport, *S.* Derby and *S.* Typhi being represented by two strains. Thus, it might be wiser to restrict statements about the association of growth parameters with serotypes that were represented by more isolates in this study. Although the growth kinetics of different food-borne pathogens, and primarily *Listeria monocytogenes*, have been associated with phenotypic or genotypic characteristics (Buncič et al., 2001; Capita et al., 2001), similar findings have not been obtained for *Salmonella*. On the contrary, research comparing the growth kinetic behaviour of *S. enterica* strains belonging to various serotypes has found no serotype effects. Thus, Oscar (2000) found only minor differences in lag times and specific growth rates among 11 *S. enterica* strains representing 11 different serotypes. Juneja et al. (2003) investigated the serotype-specific differences in growth and heat resistance kinetics of 10 clinical and food isolates of *Salmonella* in brain heart infusion broth at 19 °C and 37 °C. These authors observed only a small inter-serotype variation in growth kinetics, concluding that serotype effects, if any, would be expected to be limited as compared to experimental variability. Similar findings were reported by Lianou and Koutsoumanis (2011), who investigated the growth of 60 *Salmonella* strains (9 serotypes) in TSB with different pH values and NaCl concentrations, and observed that the maximum growth rate values were not related to the serotypes of the *S. enterica* strains under any of the growth conditions that were evaluated.

Fig. 3 shows a good agreement ($r = 0.952$) between optical density and plate counts, which can be fitted using a suitable relationship. The turbidimetric method gave a linear relationship only in the range of 7.5–10 $\log_{10}$ cfu/mL when compared with plate counts. Thus, data from 7.5 to 10 $\log_{10}$ cfu/mL were included in the calibration curve, with a maximum limit of 30 h of incubation. These results concur with other studies which show this method to be unable to detect cell concentrations of less than approximately 10^7 cells/mL (Dalgaard et al., 1994; Lianou and Koutsoumanis, 2011; Rattanasomboon et al., 1999). On the other hand, after 30 h of incubation data were no longer linear and they were discarded. This is because towards the end of the exponential phase of growth, cell death occurs, with a subsequent decrease in plate counts. However, turbidimetric measurement determines both viable and non-viable

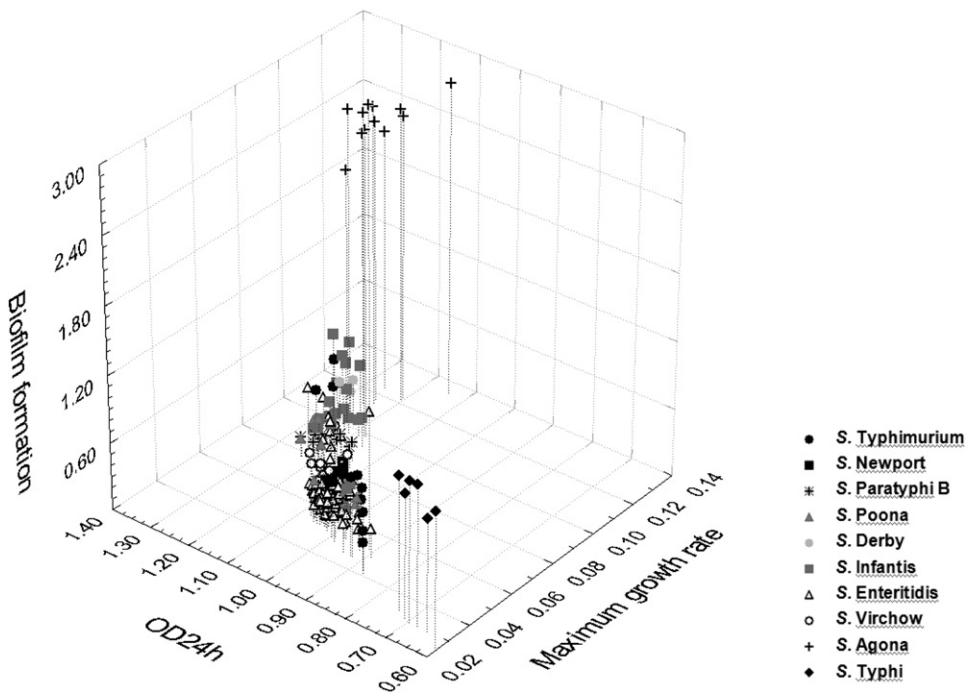

Fig. 4. Biofilm formation (OD_{580} after 24 h of incubation using the micro-well crystal violet assay) by 69 *Salmonella enterica* strains on the basis of their $OD_{24\ h}$ ($OD_{420–580}$ of the cultures after 24 h of incubation) and their maximum growth rate ($\Delta OD_{420–580}$/h).

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cells, and consequently no reductions in optical density are observed as a consequence of cell death.

4.3. Relationship between biofilm formation and growth kinetic parameters

As indicated in the previous paragraphs, an evaluation of biofilm formation by *Salmonella* has been previously performed by other authors. However, no data are available concerning the relationship between biofilm formation and growth kinetic parameters.

A bacterial biofilm is formed in various stages: (i) initial reversible adsorption of cells onto the solid surface, (ii) production of surface polysaccharides or capsular material followed by the formation of an extracellular polymeric matrix resulting in irreversible attachment, (iii) early development of biofilm architecture, and (iv) maturation and dispersal of single cells from the biofilm (Kim and Wei, 2007). Findings in the work being presented here show that the growth kinetics of the strains tested were not found to be related to their biofilm-producing capability when strains in each serotype were tested.

Some research has indicated that a linear increase in attached *Salmonella* cells occurs with respect to bacterial density in culture broths. Thus, Jain and Chen (2007) observed that the biofilm formation by *Salmonella* on polystyrene surfaces increased significantly over the incubation period, and Dunsmore and Bates (1982) noted a wide variability in the adherence of several milk-borne microorganisms over a 12 h period. In other cases, it has been reported that the number of attached cells remained constant over the period of incubation (Hood and Zottola, 1997; Stepanović et al., 2003; Tondo et al., 2010).

According to several authors (Kim and Wei, 2007; Pui et al., 2011; Rodrigues et al., 2011), the attachment of *Salmonella* to abiotic surfaces occurs within 12 h of incubation in culture broth. It is possible that after a few hours of incubation, the surface reaches a saturation level where greater numbers of planktonic cells do not entail greater numbers of attached cells. This could be one possible explanation for the absence of any relationship between biofilm formation after 24 h of incubation and the growth kinetics of the *Salmonella* strains in the present study.

In conclusion, the *S. enterica* isolates tested presented an ability to form biofilms on polystyrene surfaces. This biofilm-forming ability was considerable for the strains belonging to *S.* Agona and *S.* Typhi serotypes. Because plastic materials are widely used in food-processing and cooking facilities, the contamination of plastic surfaces by *Salmonella* strains with high biofilm productivity may have serious health and economic consequences. Thus, the need for regular sanitation programmes and the implementation of upgraded Hazard Analysis and Critical Control Point (HACCP) systems in order to avoid biofilm formation are highlighted for the poultry industry, especially when *Salmonella* strains that are strong formers of biofilms are detected. The growth kinetics of the strains tested was serotype-dependent. The variability observed between serotypes in respect of microbial growth kinetics is a feature with important implications for food safety research, underlining the importance of screening a wide range of test strains in order to obtain a representative view of the different behaviours of *Salmonella*. No relationships were observed between growth kinetic parameters and ability of *Salmonella* strains to produce biofilms, considering each serotype separately.

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References

Bridier, A., Dubois-Brissonnet, F., Boubetra, A., Thomas, V., Briandet, R., 2010. The biofilm architecture of sixty opportunistic pathogens deciphered using a high throughput CLSM method. Journal of Microbiological Methods 82, 64–70.

Buncič, S., Avery, S.M., Rocourt, J., Dimitrijevic, M., 2001. Can food-related environmental factors induce different behaviour in two key serovars, 4b and 1/2a, of *Listeria monocytogenes?* International Journal of Food Microbiology 65, 201–212.

Capita, R., Alonso-Calleja, C., Antibiotic-resistant bacteria: a challenge for the food industry. Critical Reviews in Food Science and Nutrition, in press.

Capita, R., Alonso-Calleja, C., García-Fernández, M.C., Moreno, B., 2001. Influence of strain and trisodium phosphate concentration on growth parameters of *Listeria monocytogenes in vitro*. Letters in Applied Microbiology 32, 428–432.

Capita, R., Alonso-Calleja, C., Prieto, M., 2007. Prevalence of *Salmonella enterica* serovars and genovars from chicken carcasses from slaughterhouses in Spain. Journal of Applied Microbiology 103, 1366–1375.

CDC (Centers for Disease and Control Prevention), 2011. CDC Estimates of Foodborne Illness in the United States. Available at: http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html (Date last accessed 03.12.11.).

Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K., McMeekin, T.A., 1994. Estimation of bacterial growth rates from turbidimetric and viable count data. International Journal of Food Microbiology 23, 391–404.

Del Río, E., González de Caso, B., Prieto, M., Alonso-Calleja, C., Capita, R., 2008. Effect of poultry decontaminants concentration on growth kinetics for pathogenic and spoilage bacteria. Food Microbiology 25, 888–894.

Dunsmore, D.G., Bates, P.J., 1982. Attachment of bacteria to glass surfaces immersed in milk. Australian Journal of Dairy Technology 37, 35–36.

EFSA, 2011. The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2009. EFSA Journal 9 (3), 2090–2477. Available at: http://www.efsa.europa.eu/en/efsajournal/doc/2090.pdf (Date last accessed 03.12.11.).

European Commission, 2010. The Rapid Alert System for Food and Feed (RASFF). Annual Report 2009. EU Directorate General for Health and Consumers, Luxemburg. Available at: http://ec.europa.eu/food/food/rapidalert/report2009_en.pdf (Date last accessed 03.12.11.).

Garthright, W.E., 1991. Refinements in the prediction of microbial growth curves. Food Microbiology 8, 239–248.

Girard, M.P., Steele, D., Chaignat, C.-L., Kieny, M.P., 2006. A review of vaccine research and development: human enteric infections. Vaccine 24, 2732–2750.

Hood, S.K., Zottola, E.A., 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. International Journal of Food Microbiology 37, 145–153.

Ibuchi, R., Hara-Kudo, Y., Hasegawa, A., Kumagai, S., 2010. Survival of *Salmonella* on a polypropylene surface under dry conditions in relation to biofilm-formation capability. Journal of Food Protection 73 (8), 1506–1510.

Jain, S., Chen, J., 2007. Attachment and biofilm formation by various serotypes of *Salmonella* as influenced by cellulose production and thin aggregative fimbriae biosynthesis. Journal of Food Protection 70 (11), 2473–2479.

Juneja, V.K., Marks, H.M., Huang, L., 2003. Growth and heat resistance kinetic variation among various isolates of *Salmonella* and its application to risk assessment. Risk Analysis 23, 199–213.

Kim, S.H., Wei, Ch.-I., 2007. Biofilm formation by multidrug-resistant *Salmonella enterica* serotype Typhimurium phage type DT104 and other pathogens. Journal of Food Protection 70 (1), 22–29.

Lestari, S.I., Han, F., Wang, F., Ge, B., 2009. Prevalence and antimicrobial resistance of *Salmonella* serovars in conventional and organic chickens from Louisiana retail stores. Journal of Food Protection 72, 1165–1172.

Lianou, A., Koutsoumanis, K.P., 2011. Effect of the growth environment on the strain variability of *Salmonella enterica* kinetic behavior. Food Microbiology 28, 828–837.

Marin, C., Hernandez, A., Lainez, M., 2009. Biofilm development capacity of *Salmonella* strains isolated in poultry risk factors and their resistance against disinfectants. Poultry Science 88, 424–431.

Martínez, E.V., Varela, M.C., Cevallos, C., Hernández-Pezzi, G., Torres, A., Ordóñez, P., 2008. Brotes de enfermedades transmitidas por alimentos. España, 2004–2007 (excluye brotes hídricos). Boletín Epidemiológico Semanal 16, 241–252. Available at: http://www.marm.es/es/ganaderia/temas/sanidad-animal-e-higiene-ganadera/brotes_enfermadades_tcm7-6000.pdf (Date last accessed 03.12.11.).

Oliveira, K., Oliveira, T., Teixeira, P., Azeredo, J., Henriques, M., Oliveira, R., 2006. Comparison of the adhesion ability of different *Salmonella* Enteritidis serotypes to materials used in kitchens. Journal of Food Protection 69, 2352–2356.

Oscar, T.P., 2000. Variation of lag time and specific growth rate among 11 strains of *Salmonella* inoculated onto sterile ground chicken breast burgers and incubated at 25 °C. Journal of Food Safety 20, 225–236.

Patel, J., Sharma, M., 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. International Journal of Food Microbiology 139, 41–47.

Peeters, E., Nelis, H.J., Coenye, T., 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. Journal of Microbiological Methods 72, 157–165.

Pui, C.F., Wong, W.C., Chai, L.C., Lee, H.Y., Tang, J.Y.H., Noorlis, A., Farinazleen, M.G., Cheah, Y.K., Son, R., 2011. Biofilm formation by *Salmonella* Typhi and *Salmonella* Typhimurium on plastic cutting board and its transfer to dragon fruit. International Food Research Journal 18, 31–38.

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Ramesh, N., Joseph, S.W., Carr, L.E., Douglass, L.W., Wheaton, F.W., 2002. Evaluation of chemical disinfectants for the elimination of *Salmonella* biofilms form poultry transport containers. Poultry Science 81, 904–910.

Rattanasomboon, N., Bellara, S.R., Harding, C.L., Fryer, P.J., Thomas, C.R., Al-Rubeai, M., McFarlane, C.M., 1999. Growth nd enumeration of the meat spoilage bacterium *Brochothrix thermosphacta*. International Journal of Food Microbiology 51, 145–158.

Rodrigues, D., Teixeira, P., Oliveira, R., Azeredo, J., 2011. *Salmonella enterica* Enteritidis biofilm formation and viability on regular and triclosan-impregnated bench cover materials. Journal of Food Protection 74, 32–37.

Sánchez, M.X., Fluckey, W.M., Brashears, M.M., McKee, S.R., 2002. Microbial profile and antibiotic susceptibility of *Campylobacter* spp. and *Salmonella* spp. in broilers processed in air-chilled and immersion-chilled environments. Journal of Food Protection 65, 948–956.

Shi, X., Zhu, X., 2009. Biofilm formation and food safety in food industries. Trends in Food Science and Technology 20, 407–413.

Steenackers, H., Hermans, K., Vanderleyden, J., De Keersmaecker, S.C.J., 2011. *Salmonella* biofilms: an overview on occurrence, structure, regulation and eradication. Food Research International 45, 502–531.

Stepanović, S., Ćirković, I., Mijać, V., Švabić-Vlahović, M., 2003. Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. Food Microbiology 20, 339–343.

Stepanović, S., Ćirković, I., Ranin, L., Švabić-Vlahović, M., 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Letters in Applied Microbiology 38, 428–432.

Tondo, E.C., Machado, T.R.M., Malheiros, P. da S., Padrão, D.K., Carvalho, A. L. de, Brandelli, A., 2010. Adhesion and biociddes inactivation of *Salmonella* on stainless steel and polyethylene. Brazilian Journal of Microbiology 41, 1027–1037.

Velasco, L., Sobrino, L., García, M., Soler, P., Martinez, E., 2009. Infecciones por *Salmonella* Typhi y *Salmonella* Paratyphi en España. Sistema de Información Microbiológica. Años 1998–2008. Boletín Epidemiológico Semanal 17, 205–216. Available at: http://revistas.isciii.es/bes/index.php/bes/article/view/121/120 (Date last accessed 03.12.11.).

Vestby, L.K., Møretrø, T., Langsrud, S., Heir, E., Nesse, L., 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. BMC Veterinary Research 5, 20–25.

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