

Effect of Temperature, pH, and Water Activity on Biofilm Formation by *Salmonella enterica* Enteritidis PT4 on Stainless Steel Surfaces as Indicated by the Bead Vortexing Method and Conductance Measurements

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ABSTRACT

An assay was developed in an effort to elucidate the effect of important environmental parameters (temperature, pH, and water activity [a_w]) on *Salmonella* Enteritidis biofilm formation on stainless steel surfaces. To achieve this, a modified microbiological technique used for biofilm studying (the bead vortexing method) and a rapid method based on conductivity measurements were used. The ability of the microorganism to generate biofilm on the stainless surfaces was studied at three temperatures (5, 20, and 37°C), four pH values (4.5, 5.5, 6.5, and 7.4), and four a_w values (0.5, 1.5, 5.5, and 10.5% NaCl). Results obtained by the bead vortexing method show that maximum numbers of adherent bacteria per square centimeter (10^6 CFU/cm²) were attained in 6 days at 20°C. Biofilm formation after 7 days of incubation at 20°C was found to be independent of the pH value. In addition, the high concentration of sodium chloride (10.5% NaCl, $a_w = 0.94$) clearly inhibited the adherence of cells to the coupons. Conductance measurements were used as a supplementary tool to measure indirectly the attachment and biofilm formation of bacterial cells on stainless steel surfaces via their metabolic activity (i.e., changes in the conductance of the growth medium due to microbial growth or metabolism). Results obtained by conductance measurements corresponded well to those of the bead vortexing method. Furthermore, we were able to detect cells that remained attached on the metal surfaces even after vortexing via their metabolic activity. The results, except for demonstrating environmental-dependent *Salmonella* Enteritidis biofilm formation, indicated that traditional vortexing with beads did not remove completely biofilm cells from stainless steel; hence, conductance measurements seem to provide a more sensitive test capable to detect down to one single viable organism.

Biofilms consist of immobilized bacteria embedded in an organic polymer matrix of bacterial origin (2). Biofilms are involved in major problems associated with food industry, medicine, and everyday life. In the food industry, biofilms are responsible for significant losses of efficiency, process down time, and equipment damage, together with biofouling of pipelines, process equipment, and heat exchangers (7). In addition, the biofilms of both spoilage and pathogenic microflora that formed on food surfaces, such as that of poultry or other meat surfaces, and on food contact surfaces of the food-processing environments are also related to problems of cross-contamination, especially at postprocessing stages (3). The risk becomes even more serious, because bacteria within biofilms have been shown to have a decreased susceptibility to antimicrobial agents compared with those in the planktonic form (7).

The most frequently used material for food-processing surfaces is stainless steel. Stainless steel has been shown to be as readily cleaned as glass and more readily than polymers, aluminum, or copper. It is relatively more resistant to chemical attack by oxidizing agents and other sanitizing agents used in the food industry (9).

The different methods used for sampling and enumeration of biofilms are swabbing (13), sonication (14), surface scraping (10), shaking with beads, vortexing (17), and growing the recovered bacteria on agar plates (15). Bioluminescence (5) and microtiter plate test (19) can also be used. Microscopic techniques include mainly scanning electron microscopy and epifluorescence microscopy (12, 21).

These methods for biofilm enumeration vary significantly, and the removal of attached bacteria by such procedures depends on the efficiency of the procedure (e.g., swabbing). Some limitations arise during detachment of biofilms. Grooves, crevices, and corrosion patches are some of the areas where biofilms can grow and are hard to access. Furthermore, bacteria in biofilms are frequently subjected to various stresses (starvation, heat, cold, chemicals, disinfectants), rendering them unculturable. Conductance methods may overcome these problems and allow the detection of biofilm bacteria without disrupting the biofilm structure, since they may detect the presence of biofilms via the metabolic activity of attached cells (4, 6, 8, 11, 16).

The objective of this study was to determine the influence of incubation temperature, pH, and water activity (a_w) on biofilm formation by *Salmonella* Enteritidis on stainless steel. Estimates of cell counts in biofilm were achieved by

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bead vortexing and plate counting and indirectly by conductance detection times using the Rapid Automated Bacterial Impedance Technique (RABIT) apparatus. We also report the ability of RABIT to detect those bacterial cells that are strongly attached to metal surfaces within the biofilm and for this reason are impossible to remove from surfaces through vortexing with beads for enumeration by agar plating.

MATERIALS AND METHODS

Bacterial strain and preparation of inoculum. *Salmonella enterica* subsp. *enterica* serovar Enteritidis phage type 4 P167807, supplied by Division of Enteric Pathogens, Central Public Health Laboratory, London, England, was stored frozen in bead vials (Protect, Technical Service Consultants Ltd., Heywood, Lancashire, England) at -80°C . Microorganism was activated by adding one bead to 100 ml of tryptone soy broth (30 g of powder in 1 liter of deionized water, pH 7.3 ± 0.2 , LAB M, International Diagnostics Group Plc, Bury, Lancashire, England) in a conical flask and incubating at 37°C for 18 h, at which time late exponential phase was attained (master culture). Working cultures were prepared by adding a 100- μl aliquot of the master culture to 100 ml of tryptone soy broth and incubating at 37°C for 18 h.

Test surface and media. Stainless steel was the surface chosen, because it is used extensively throughout the food processing industry (AISI-316, finish no. 2B, Halyvourgiki Inc, Athens, Greece). The stainless steel coupons (2.5 by 0.8 by 0.1 cm) were initially soaked in acetone overnight. Then, the following cleaning procedure was repeated each time the coupons were required to be reused. The coupons were immersed for a minimum of 30 min in detergent solution with agitation. The coupons were then thoroughly washed in tap water followed by three washes in distilled water and were allowed to air dry. Finally, the dry coupons were autoclaved at 121°C for 15 min.

The test media used for the growth of microorganism were tryptone soy agar (37 g of powder in 1 liter of deionized water, pH 7.3 ± 0.2 , LAB M, International Diagnostics Group Plc) and brain heart (BH) broth (37 g of powder in 1 liter of deionized water, pH 7.4 ± 0.2 , Merck, Darmstadt, Germany). Ringer solution tablets (pH 6.8 ± 0.2 , Merck) were used for the preparation of 10-fold serial dilutions and the rinsing of stainless steel coupons to remove loosely attached cells.

Inoculum concentration and biofilm development. Tetraplicate stainless steel test surfaces were placed individually in test tubes that contained 3.5 ml of BH broth, and the test tubes with the coupons inside them were autoclaved afterward. Following autoclaving, aliquots (1 ml) of the working culture were inoculated in the test tubes to yield an initial population of approximately 10^8 CFU/ml. Working with bacteria in the stationary phase from the beginning of the experiment has the advantage that conditions at all treatments and tubes, regarding the physiological state and number of planktonic cells, are similar; therefore, we are able to study the effect of the environmental factors (temperature, pH, a_w) only on the adherence of cells to the coupons (phenomenon of biofilm formation) and not on the growth (and attachment) of the planktonic cells in the BH medium. If the inoculum level was as low as 10^2 CFU/ml, then the concentration of planktonic cells during incubation would depend on the growth rate of cells for each treatment; hence, it may not be the same for all treatments at a given time. Although the ability of low initial bacterial populations (10^{2-3} CFU/ml) to attach to surfaces during

growth is well established, this was not an objective of the current study.

Test tubes that contained the coupons were incubated for 7 days at three temperatures (5, 20, and 37°C) to allow biofilm development. To determine the effect of pH on biofilm production, four pH values (4.5, 5.5, 6.5, and 7.4), adjusted with 1 N HCl (before autoclaving), were tested at 20°C , and the test tubes that contained the coupons were incubated for 7 days, as before. The effect of a_w on biofilm formation was investigated at 20°C and pH 7.4 in BH broth for an incubation period of 7 days. The a_w values were adjusted by adding different concentrations of NaCl (0.5, 1.5, 5.5, and 10.5%), before autoclaving.

Sampling schedule. On days 4, 5, 6, and 7, samples (coupons) were removed aseptically from the tubes (i) to enumerate the attached cells for each treatment by the bead vortexing method and plate counting, (ii) to quantify indirectly biofilm formation by conductance measurements, and (iii) to investigate whether biofilm cells remain on the coupons after their vortexing with beads via their metabolic activity.

Detachment procedure (removal of loosely attached cells). Following incubation, each coupon was carefully removed with sterile forceps, transferred to an empty sterile test tube, and left there for 5 min to allow air drying. The coupon was then rinsed two times with 10 ml of Ringer solution to remove loosely attached cells. Between the two rinsings, the coupon was left for 5 min to dry, as describe above. After the second rinsing and a final drying, the coupon was transferred to a new test tube that contained 9 ml of Ringer solution and 10 sterile glass beads (diameter, 3 mm) and left for 10 min. Then, the coupon was either vortexed in the test tube that contained the Ringer solution and beads for 1 min, at maximum intensity, on a vortexer (Velp Scientifica, Milano, Italy) or placed (after a final rinsing with 10 ml of Ringer solution) into the RABIT tubes for the conductance measurements.

Bead vortexing method. Enumeration of biofilm cells was performed by using the bead vortexing method of Oh and Marshall (18) and Lindsay and von Holy (15) for the removal and quantitative analysis of biofilms after modification. Quantification of biofilm production under static conditions was performed by agar plating, at two steps of the detachment procedure, termed measurements A and B. Initially, 1 ml of bacterial suspension was removed from each test tube exactly before vortexing with beads. This volume was used to measure (after 10-fold serial dilutions and agar plating) those bacteria that had remained on the coupons after the second rinsing of the detachment procedure and dislodged from test surfaces after their incubation for 10 min in Ringer solution (loosely attached bacteria, measurement A). One milliliter of bacterial suspension was also removed from each test tube exactly after vortexing with beads (loosely attached bacteria and bacterial cells removed from the biofilm structure through vortexing, measurement B). In each case, 10-fold serial dilutions were prepared in sterile Ringer solution, and bacteria were enumerated on the surface of duplicate tryptone soy agar plates after incubation for 18 h at 37°C . The difference of the two measurements ($\text{CFU/ml}_{\text{MEASUREMENT A}} - \text{CFU/ml}_{\text{MEASUREMENT B}}$) corresponded to biofilm formed on stainless steel surfaces and was expressed in CFU per square centimeter. Numbers of CFU per square centimeter were transformed to log for better representation in figures. Uncolonized test surfaces subjected to vortexing with beads were used as negative controls for bacterial attachment.

Conductance measurements. RABIT (Don Whitley Scientific Limited, Shipley, England) was used for monitoring the con-

ductance changes due to the metabolic activity of cells attached on stainless steel coupons. Two types of biofilm cells were studied: (i) cells that remained on the stainless steel surfaces after rinsings with Ringer solution and before vortexing (biofilm cells) and (ii) the strongly attached cells that remained on the stainless surfaces after vortexing.

Conductance measurements were performed by placing the stainless steel coupons into RABIT tubes that contained 4.5 ml of BH broth. RABIT tubes were incubated for 48 h at 30°C, and conductance was measured every 6 min in each tube. Changes in conductance are brought about bacterial metabolism, whereby weakly charged substrates in the growth medium are transformed into highly charged end products. Changes in conductance (microsiemens) were plotted against time (hours). When the rate of change of conductivity exceeded the detection criteria (+5 μ S), the system detected growth. The time to detection (TTD) signal appeared when there were three consecutive minimum increases in conductance by 5 μ S. TTD is apparently a function of microorganism population into the RABIT tubes (CFU per milliliter), the growth kinetics of microorganism, and the properties of the test medium. For a given test protocol, the TTD is directly proportional to the initial microbial loading of the sample. Therefore, shorter detection times correlate with higher levels of microbial planktonic populations (1).

When we inserted the stainless steel coupons into the RABIT tubes, *Salmonella* cells were strongly attached to them (biofilm cells). It was confirmed (by observing no cells after plating of the run-off fluids from the coupons) that all the loosely attached cells had been removed from the coupons after the end of the detachment procedure. Thereby, at the point of insertion, we assume that coupons carry only biofilm bacteria on them (from 10^2 to 10^6 CFU/cm²), which start then to proliferate on the coupons since they are in a nutrient-rich medium (BH). During proliferation of bacteria on the coupons, three events could take place: (i) some pull away from the metal surfaces and enter into the "impedance broth," becoming planktonic; (ii) some reproduce biofilm by their reattaching to surfaces; and (iii) some stay within the biofilm consortium and strengthen it. Of course, the possibility of one of the previous events happening does not exclude another of the events occurring. Indeed, these three events could occur simultaneously. Samples are monitored for the changes that cause the conductance of BH broth every 6 min (RABIT). When the concentration of cells (biofilm and planktonic) in the test tubes reaches the conductance detection threshold (approximately 10^{5-6} CFU/ml) (1), a TTD is given by the apparatus.

RABIT tubes that contain only BH broth (without stainless surface) and RABIT tubes that contain uncolonized test surfaces in BH broth were used as negative controls.

RESULTS AND DISCUSSION

Biofilms have received considerable interest in the context of food hygiene. Of special significance is the ability of *Salmonella* Enteritidis to attach and grow on food and food contact surfaces. Biofilm formation by *Salmonella* species on stainless steel is a dynamic process, and different mechanisms are involved in their attachment and growth (20).

Several laboratory methods have been used for removal and subsequent enumeration of *Salmonella* biofilms, mainly in antimicrobial efficacy studies (5, 13). Each method is based on the measurement of different parameters, e.g., cultivation measures biomass formation of viable cells, whereas conductance measures the production of ionic mol-

ecules in the growth medium, as an indication of the metabolic activity of growing microorganisms. Since the RABIT detection time is a function of cellular activity, the obtained measurement derives from cells that may be metabolically active but not culturable and, therefore, not detectable by conventional plating techniques.

The RABIT technique presented in this article is a tool with the potential to measure indirectly the factors involved in the attachment and biofilm formation of *Salmonella* Enteritidis cells on stainless steel surfaces. RABIT measures biofilm in situ (without disrupting the biofilm structure), whereas the bead vortexing method and plate counting rely on disruption of the biofilm to release the cells into suspension for counting. The conductance measurements result in detection of bacterial cells that could not be recovered by vortexing with beads. It also provides a more sensitive test that can detect down to one single viable organism. This is extremely important because even a low number of bacterial cells may be able to reestablish biofilm quickly, and the remaining microorganisms may exhibit increased resistance to disinfectants. Advantages in using this method also include the ability to produce results faster and reduced operator fatigue compared with other techniques used so far for biofilm investigation (e.g., swabbing, sonication, vortexing).

The results obtained by the bead vortexing method of the current study indicate that the optimum conditions for *Salmonella* Enteritidis to generate biofilm on stainless surfaces are 20°C and 6 days of incubation (10^6 CFU/cm², Fig. 1A), whereas at 20°C, biofilm formation at the seventh day of incubation was found to be independent of the pH value (Fig. 2A). In addition, the high concentration of sodium chloride (10.5% NaCl, $a_w = 0.94$) clearly inhibited the adherence of cells to the coupons (Fig. 3A). These observations were also confirmed by the two-way analysis of variance presented in Table 1.

The metabolic activity of biofilm cells that remained on the surfaces after the rinsing with Ringer solution and before vortexing with beads was also studied via conductivity measurements (Figs. 1B, 2B, and 3B). RABIT does not measure cells. Instead, RABIT electrodes monitor changes in conductance due to microbial metabolism. TTD was given by the apparatus when the concentration of cells in test medium (BH) into RABIT tubes (planktonic and biofilm cells) reached the RABIT detection threshold. In this study, RABIT was used as a supplementary and comparative tool of the bead vortexing method to quantify biofilm and not for precise enumeration of biofilm cells. Flint et al. (6) and Johnston and Jones (11), who also used conductance measurements for biofilm quantification, calculated the number of cells on stainless steel surfaces by relating the TTD to the equivalent number of cells indicated on a standard calibration curve prepared from serial dilutions of planktonic cells. However, current knowledge supports that the metabolic activities of biofilm cells may vary from those of planktonic ones, since a biofilm is a microbially derived sessile community characterized by cells that attach to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substance, and exhibit

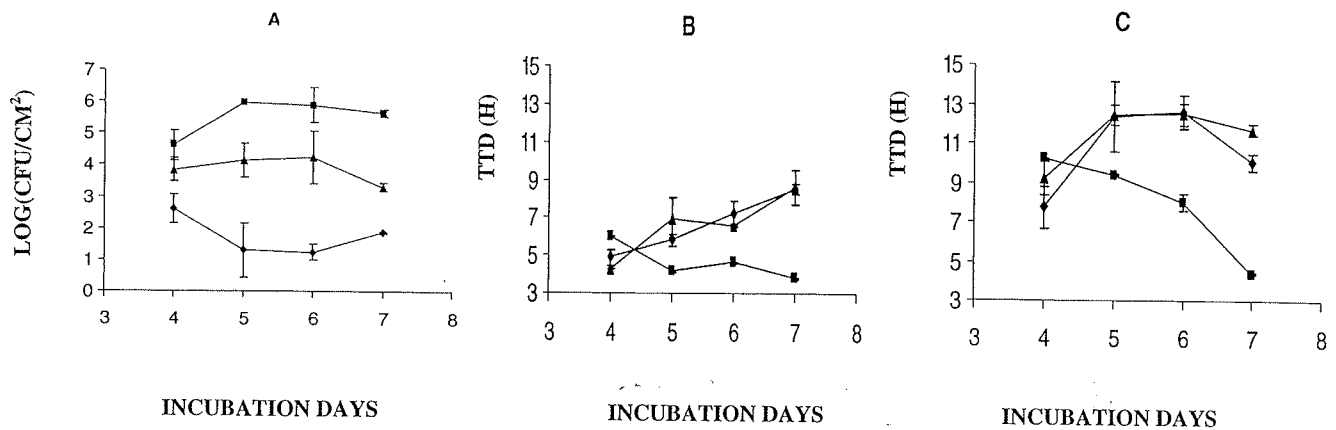


FIGURE 1. (A) Biofilm development (log CFU per square centimeter) by *Salmonella Enteritidis* on stainless steel coupons. (B) Relationship between RABIT TTD and incubation days (a TTD [in hours] is given by the apparatus when the concentration of cells in the test tubes reaches the conductance detection threshold); coupons were inserted into RABIT tubes after the end of the detachment procedure. (C) Relationship between RABIT TTD (in hours) and incubation days; coupons were inserted into RABIT tubes after bead vortexing. Coupons were incubated in BH that contained 0.5% NaCl with initial pH 7.4 (5 [◆], 20 [■], and 37°C [▲]).

an altered phenotype with respect to growth, gene expression, and protein production (2, 22). Hence, this method (6, 11) may be an effective means of indirect calculation of bacterial planktonic cell numbers; however, more evidence is needed to ensure that this method is suitable for accurate estimation of the actual biofilm population. This is due to the fact that biofilm cells may have an altered metabolism compared with planktonic cells, and such a hypothesis (22) does not seem to be taken into account by the specific method.

For a given test protocol, TTD (determined from the point when there is a rapid increase in metabolic activity) can be related to the number of cells present at the start of the test (1). In our study, the start of the test took place after the end of the detachment procedure; hence, the number of cells at the start of the test was equal to the number of bacterial cells that remained on the metal surfaces after the end of the detachment procedure (strongly attached to surfaces within the biofilm consortium). Changes in conductance are caused by bacterial metabolism (biofilm and planktonic). Considering that in our experimental case the

growth medium is stable (BH), the planktonic cells in RABIT tubes "derived" from biofilm ones (during the proliferation of biofilm cells on the coupons some of them most likely detach from the biofilm structure, becoming planktonic), and the generation time of *Salmonella* is constant under given experimental conditions (incubation at 30°C), it can be assumed that TTD correlates linearly with (inversely proportional to) the initial concentration of *Salmonella* bacteria on the coupons (biofilm). Results obtained by conductance measurements (Figs. 1B, 2B, and 3B) corresponded well to those of the bead vortexing technique. Shorter detection times (Figs. 1B, 2B, and 3B) correlated with higher levels of biofilm cells on coupons (Figs. 1A, 2A, and 3A).

By conductance measurements, it was also realized that biofilm may remain on stainless steel surfaces even after vortexing with beads (Figs. 1C, 2C, and 3C). Thereby, when stainless steel coupons were inserted into RABIT tubes after the vortexing detachment procedure, RABIT detected metabolic activity and TTD was given (Figs. 1C, 2C, and 3C). As expected, no microbial activity was reported

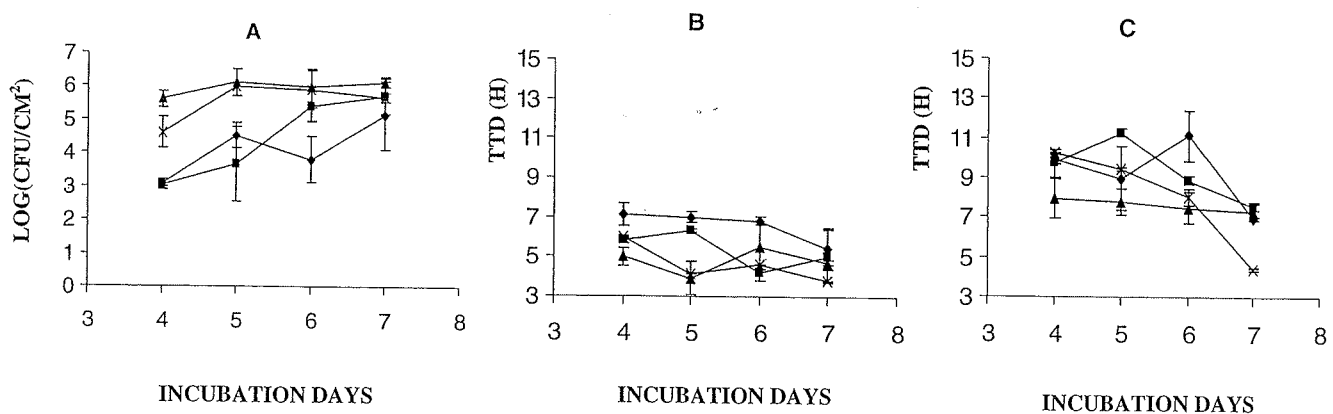


FIGURE 2. (A) Biofilm development (log CFU per square centimeter) by *Salmonella Enteritidis* on stainless steel coupons. (B) Relationship between RABIT TTD and incubation days (a TTD [in hours] is given by the apparatus when the concentration of cells in the test tubes reaches the conductance detection threshold); coupons were inserted into RABIT tubes after the end of the detachment procedure. (C) Relationship between RABIT TTD (in hours) and incubation days; coupons were inserted into RABIT tubes after bead vortexing. Coupons were incubated at 20°C in BH that contained 0.5% NaCl (pH 4.5 [◆], 5.5 [■], 6.5 [▲], and 7.4 [×]).

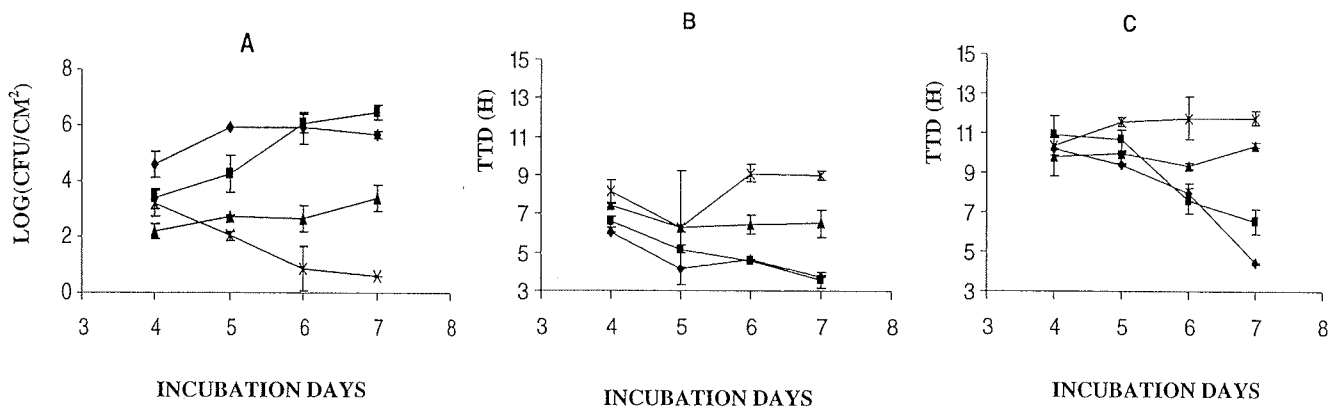


FIGURE 3. (A) Biofilm development (log CFU per square centimeter) by *Salmonella Enteritidis* on stainless steel coupons. (B) Relationship between RABIT TTD and incubation days (a TTD [in hours] is given by the apparatus when the concentration of cells in the test tubes reaches the conductance detection threshold); coupons were inserted into RABIT tubes after the end of the detachment procedure. (C) Relationship between RABIT TTD (in hours) and incubation days; coupons were inserted into RABIT tubes after bead vortexing. Coupons were incubated at 20°C in BH with initial pH 7.4 (0.5% [♦], 1.5% [■], 5.5% [▲], and 10.5% [×] NaCl).

(no TTD during the 48-h test period) while testing the two negative controls (either BH broth without stainless surface or uncolonized test surfaces in BH broth). Considering that at optimum conditions (20°C, pH 7.4, and low NaCl concentrations) formed biofilm is progressively strengthened, its removal by vortexing seems to be insufficient.

In conclusion, the present results support the efficacy of the bead vortexing method to remove attached bacteria from stainless steel surfaces, but on the other hand they also demonstrate that this method of removal is not fully efficient, since some cells may remain attached to surfaces even after vortexing with beads. It seems that the removal of biofilm cells from stainless steel by vortexing with beads and subsequent enumeration of the formed colonies by agar plating do not always provide accurate estimates of biofilm cell numbers. Indeed, such a method may be significantly improved if supplemented with conductance measurements. Such studies assist in understanding the interactions between environmental conditions and *Salmonella* biofilm

formation in the food processing operations and hence in more reliable assessment of risk.

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TABLE 1. The effect of water activity, pH, and temperature on the biofilm formation at different days^a

Source	df	F ratio	P value
Water activity			
Days	3		
NaCl	3	6.882	0.001
Day × NaCl	9	253.643	0.000
Error	45	23.487	0.000
pH			
pH	3		
Days	3	37.404	0.000
pH × days	9	24.210	0.000
Error	45	5.340	0.000
Temperature			
Days	3	0.318	0.812
T	2	213.702	0.000
Days × T	6	7.084	0.000

^a Two-way analysis of variance.

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