Running head: *SALMONELLA* BIOFILM ON BENCH COVER MATERIALS

Salmonella enterica Enteritidis Biofilm Formation and Viability on Regular and Triclosan-Impregnated Bench Cover Materials

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ABSTRACT

Contamination of food-contact surfaces by microbes as *Salmonella* is directly associated with substantial industry costs and severe foodborne outbreaks. Several approaches have been developed to control microbial attachment, namely through the development of food-contact materials incorporating antimicrobial compounds. This work assessed *Salmonella enterica* Enteritidis adhesion and biofilm formation on regular and triclosan incorporated kitchen bench stones (silestones), as well as cellular viability within biofilms. Enumeration of adhered cells on granite, marble, stainless steel and silestones revealed that all materials were prone to bacterial colonization (4 - 5 log CFU/cm^2) and no considerable effect of triclosan was observed. Conversely, results concerning biofilm formation highlighted a possible bacteriostatic activity of triclosan, since smaller amounts of *Salmonella* Enteritidis biofilms were formed on silestones and with significantly lower number of viable cells (between 10^5 to 10^6 log CFU/cm^2) than those found on the other materials (10^7 log CFU/cm^2). Summarizing, all surfaces tested failed in promoting food safety and imply a careful utilization with appropriate sanitation when used in food-processing environments. Nevertheless, triclosan confers to silestones some advantage in controlling microbial contamination due to its bacteriostatic effect.

Bacterial adhesion and subsequent biofilm formation on food contact surfaces is the major cause of economic costs in food industry and is also responsible for transmission of diseases, both from industrial and domestic environments. Nowadays it is well known the importance of good cleaning, hygiene and use of separate surfaces and equipment for raw and cooked foods to reduce the risk of cross-contamination, which is an important factor in transmission of microbiological food-borne illness (*36*). However, bacterial food poisoning continues to be an important health problem worldwide with numerous foodborne disease outbreaks and deaths being registered every year. The last report emitted by the European Food Safety Authority declares a total of 5,332 foodborne outbreaks in the European Union, causing 45,622 human cases, 6,230 hospitalisations and 32 deaths (*48*). The same document states that most of the reported outbreaks were caused by *Salmonella* (35.4%), which confirms that this bacterium is still one of the most important foodborne pathogens. As *E. coli* (*39*), *Campylobacter* (*39*), *Pseudomonas* (*4*) and *Listeria* (*21*, *32*), *Salmonella* has been reported to adhere and form biofilms that, when grown on food-contact surfaces, represent a major source of food contamination. Various food-contact surfaces, as glass, rubber, metal and plastic have been considered in studies about *Salmonella* adhesion and biofilm formation (*9*, *13*, *25*, *38*, *40*, *42, 52*) but little information is available concerning contamination of kitchen bench stones, even though these are materials commonly present in food processing environments, especially in domestic kitchens of Europe Mediterranean countries.

Like many other surfaces, kitchen bench stones are now available as regular and antimicrobial incorporated materials with granite and marble being the most frequently used regular stones, while Silestone® is now the world leader in quartz surfaces incorporating an antimicrobial agent. Silestones have the feel and the weight of a natural

stone but are synthetic materials composed of 94% quartz, available in the market worldwide, also incorporating triclosan as antibacterial agent (*11*). Among the compounds that are frequently applied to control bacterial contamination in domestic settings and during food processing, triclosan is one of the most commonly used. It is a polychloro phenoxy phenol compound with broad-spectrum antimicrobial activity (*35*) first used in the early 1970s (*10, 34*). Triclosan acts as a broad-spectrum antimicrobial agent by targeting lipid biosynthesis and inhibiting cell growth (*18*, *23*, *50*) with the minimal inhibitory concentrations for a variety of tested organisms ranging from less than one part per million to parts per thousand for *Pseudomonas* (*12*). Nowadays it is widely found in many domestic products such as shower gels, deodorants, toothpastes, hand soaps and creams (*12*), as well as in impregnated surfaces of refrigerators, chopping boards and plastic lunchboxes. Triclosan has also been used in industrial environments, such as food processing facilities, where exposed equipment, floors and walls have been treated with this compound to decrease microbial contamination (*24*).

To the authors' knowledge there is still a lack of information concerning biofilm formation on kitchen bench stones. So, this work aimed at assessing *Salmonella* Enteritidis biofilm formation ability on granite, marble and triclosan incorporated silestones. To have a comparison between different food-contact surfaces, stainless steel was also included in this study, as it has been the most used material for working surfaces and kitchen sinks because of its ease of fabrication, mechanical strength, corrosion resistance and durability (*8*). Given that microbial attachment to the surface is the first stage in biofilm formation, adhesion of *Salmonella* Enteritidis was also evaluated in order to get information on the initial interaction between bacteria and the different surfaces. Cellular viability within biofilms was also assessed to know if triclosan had any effect on biofilm-cells during biofilm development.

MATERIALS AND METHODS

Bacteria and culture conditions. In order to assess the behaviour of different strains from different sources, five *Salmonella* Enteritidis strains were used in this work: 1 food isolate (355), 3 clinical isolates (357, 358, CC) and 1 reference strain (NCTC 13349). All isolates were kindly provided by Dr. Paula Teixeira (Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal). For each assay, strains were subcultured on Luria Bertani Broth Miller agar (LBA; Sigma-Aldrich, Inc., St. Louis, Mo.) for 24–48 h at 37ºC and then grown in 30 ml of Luria Bertani Broth Miller (LB; Sigma-Aldrich) for 18 ± 2 h at room temperature with agitation at 120 rpm. Cells were harvested by centrifugation (5 min, 9000 rpm), washed twice with saline 0.9 % and cell suspensions were standardized to a concentration of approximately $1x10^9$ CFU ml^{-1} (OD $640_{nm} \approx 0.5$).

Materials. Surfaces tested were granite "Pedras Salgadas" (Vila Pouca de Aguiar, Portugal), marble (Sivec, Greece), stainless steel (SS) (304, finishing 2B, Braga, Portugal) and two kinds of silestone – white (wST) and beige (bST) (Cosentino, Spain). Squares of 2.0 by 2.0 cm^2 of each material were used after being cleaned by immersion in a 0.2% solution of a commercial detergent (Sonazol Pril, Alverca, Portugal) followed by immersion in ethanol. Each coupon was then rinsed with ultrapure water and dried at 60 ºC.

Adhesion assays and cells enumeration. Each coupon of the tested materials was placed in a well of a six-well tissue culture plate (Orange Scientific, Braine-l'Alleud, Belgium) containing 7.5 ml of LB supplemented with 0.25% (w/v) glucose (Merck, Darmstadt, Germany) and 50 µl of cell suspension. Negative controls consisted of wells filled only with culture medium without any bacterial cells. After 2 h at room temperature (22ºC) with constant shaking at 120 rpm, the coupons were rinsed three

times by soaking for 10 s in 0.9% saline in order to remove unattached cells. These washing steps were carefully performed to remove only the bacteria that were suspended in the liquid interface formed along the surface and to minimize cell detachment from the surface (*5*). Four independent assays were performed for each strain on each material with three coupons per strain per assay.

Adhered cells were scrapped from each surface with a sterile cell scraper (Orange Scientific) and collected in 1 ml of sterile Maximum Recovery Diluent (MRD; 1.0 g peptone + 8.5 g sodium chloride per litre of water, pH 7.0 ± 0.2). The efficiency of this washing procedure was confirmed by visual inspection through epifluorescence microscopy (data not shown). Each obtained suspension was serially diluted in MRD and poured on LBA plates. Colony-forming units (CFUs) were counted after 24 h incubation at 37ºC.

Biofilm assays and quantification. Biofilm formation methodology was the same used for adhesion assays except for the incubation time, which was extended to 48 h. After the washing procedures described above, the total amount of biofilm biomass grown on each surface was evaluated through crystal violet (CV) staining as follows. The coupons were transferred to new six-well plates and fixed by submersion in methanol (Merck) for 15 min. After methanol withdrawing, the coupons were allowed to dry at room temperature before being submerged in an aqueous 1% (w/v) CV solution (Merck) for 5 min at room temperature. They were then gently washed with 0.9% saline and transferred to a new six-well tissue culture plate. 250 µl of a 33% (v/v) acetic acid solution (Merck) was then added to each well to release and dissolve the stain. An aliquot of 250 µl of the eluted dye from each coupon was transferred to a 96-well microtitre plate and its absorbance was read in triplicate in an ELISA reader (BIO-TEK® Synergy HT, Izasa, Portugal) at 570 nm.

Bacterial viability assays. Since CV staining is a basic dye that connects to negatively charged surface molecules and polysaccharides in the extracellular matrix (*19*) and stains both living and dead cells (*30*), a different methodology was used to assess cellular viability. Biofilms formed on the surfaces were washed as described above and the number of viable cells assessed following the same procedure described for adhered cells enumeration, except that biofilm suspensions were longer and more vigorously vortexed in order to promote cells disaggregation. Once again, the efficiency of this washing procedure was confirmed by visual inspection through epifluorescence microscopy (data not shown).

Statistical analysis. Data analysis was performed using the statistical program SPSS (Statistical Package for the Social Sciences). The results were compared using the non-parametric Mann–Whitney U-test at a 95% confidence level.

RESULTS

Bacterial adhesion. Results presented in Figure 1 show that all surfaces were largely colonized by all *Salmonella* Enteritidis strains tested, with most strains achieving 10^5 CFU/cm^2 after two hours of incubation. Strains 358 and NCTC 13349 adhered in significantly higher extent to marble than to any other surface ($P < 0.05$), while the food isolate 355 and the clinical isolate 357 exhibited a greater propensity to adhere to marble and bST than to the other materials ($P < 0.05$). These data are in accordance with the average number of adhered cells of *Salmonella* Enteritidis strains to the same material, which shows that marble was more colonized than other surfaces, while granite, both silestones and SS displayed smaller and similar adhesion extents. Concerning adhesion of distinct strains to the same material, a significantly different number of adhered cells from all other strains was found only on granite and bST, with

the clinical isolate 357 displaying the lowest number of adhered cells and food isolate 355 achieving the highest value ($P < 0.05$), respectively. Nevertheless, *Salmonella* Enteritidis strains 355 and NCTC 13349 were always found to be among the strains with the highest number of adhered cells to all materials, while the clinical isolates tended to have a lower number of adhered cells. The only exception to this fact was observed on marble, where strain 358 reached an adhesion extent similar to that achieved by the food isolate and collection strain.

Biofilm formation. *Salmonella* Enteritidis biofilm formation assessed by CV staining (Table 1) showed that strains 355, 357 and NCTC 13349 formed more biofilm biomass on marble than on any other surface ($P < 0.05$). Moreover, the other two strains also displayed high biofilm amounts on this same material, with clinical isolate 358 forming significantly more biofilm on marble than on wST or SS. Mean results concerning biofilm formation by all *Salmonella* Enteritidis strains on the same material confirm marble as the material on which higher amounts of biofilm were formed ($P < 0.05$), while both silestones and SS showed similar optical density (OD) values. Biofilms on granite had less biomass than those formed on marble but significantly higher biofilm amounts were produced by strain 355 on granite than on the other three surfaces. bST was the only material where biofilm formation achieved statistically lower values, with clinical isolate 357 presenting its lowest biofilm amount on bST.

The comparison of biofilm formation by all tested strains of *Salmonella* Enteritidis on each material pointed out to NCTC 13349 as the strain that formed the lowest amount of biofilm on bST ($P < 0.05$). Moreover, along with food isolate 355, this strain was also one of the weakest biofilm formers on wST and SS, displaying only high biofilm amounts on marble. The three clinical isolates presented similar OD values for

all surfaces except for bST, where strain 357 formed significantly less biofilm than the other two ($P < 0.05$).

Bacterial viability within biofilms. Table 1 also presents the quantification of viable cells within *Salmonella* Enteritidis biofilms and shows that bacterial viability was significantly higher on granite and marble than on both silestones. In fact, isolates 355 and CC displayed less viable cells on wST than on any other surface, while strains 357 and NCTC 13349 presented similar amounts of viable cells on both silestones, which were lower than those found on all other materials ($P < 0.05$). An intermediate level of *Salmonella* Enteritidis viability was found on SS, with strains 357 and NCTC 13349 achieving numbers of viable cells significantly lower than those registered on both regular stones and significantly higher than those registered on both silestones. Comparing both silestones performance in terms of antimicrobial effect, bST was slightly less successful since isolates 355 and CC presented higher numbers of viable cells on this surface than on wST ($P < 0.05$).

Concerning cellular viability within biofilms formed by different *Salmonella* Enteritidis strains on the same material it is possible to see that food isolate 355 was related to low numbers of viable cells on all surfaces, with significantly lower results on granite and wST than any other strain. Conversely, clinical isolate 358 displayed always high viability values, achieving higher numbers of viable cells on wST and SS than any other strains ($P < 0.05$). Except for granite, clinical isolate 357 was one of the *Salmonella* Enteritidis strains with the lowest viability on all surfaces, while clinical isolate CC was one of the strains with higher number of viable cells on all materials except for wST and SS. Viability within biofilms formed by NCTC 13349 was higher on both regular stones than on both silestones, while SS presented intermediate numbers of viable cells of this strain ($P < 0.05$).

DISCUSSION

Since limited information is available concerning bacterial adhesion on both regular and antimicrobial incorporated stones (*26*, *37*, *46*, *47*, *49*), and no reports have been made concerning biofilm formation on any of these surfaces, the present work reports the study of the attachment and biofilm formation capability of five *Salmonella* Enteritidis strains on granite, marble and on two silestones impregnated with triclosan . SS was also included for comparative purposes, since it is widely used not only in domestic kitchens but also in the food processing industry, where working surfaces and machinery (*9*, *29*, *33*) as well as tanks and pipelines (*1*) are made of this material.

As previously reported (*27, 46*) this work showed that, although all strains were able to colonize all surfaces, *Salmonella* adhesion was strongly strain dependent and the number of adhered cells varied according to the different materials tested (Fig. 1). Marble was the stone more prone to bacterial colonization and, thus, the less advisable material in terms of food safety, while no advantage was found for silestones comparing to granite and SS since all of them presented similar amounts of adhered cells. These results are not in agreement with other studies that found higher adhesion extent on SS than on stones and no differences concerning the number of adhered cells to granite, marble and both silestones (*46*, *47*). However, both works referred had used DAPI staining and epifluorescence microscopy while in the present work CFUs enumeration was performed, which also explains the general lower amount of adhered *Salmonella* Enteritidis cells observed comparing to other reports. Since only adhered bacteria that stay viable are the actual cause of post-process contamination, CFUs enumeration seems to be more accurate for this type of studies than epifluorescence methods On the other hand, the highest levels of adhered cells to marble are in agreement with a study that suggested a correlation between the substrate electron acceptor parameter of this

material and the number of adhered cells, since marble was the surface with the highest adhesion level and the highest electron acceptor values (*37*). Porosity is another property to take into account, since it is the most important factor of absorption and fluid transport in stone material (*22*) and it influences many rock physical properties (*51*). Given that marble has higher porosity than granite, this may enhance *Salmonella* Enteritidis adherence to the former material.

Since it was not possible to test silestones with no triclosan incorporated, we cannot assure that different performances between silestones and the other surfaces are reflecting triclosan action. Nevertheless, the results obtained are supported by previous findings that allow us making considerations about the possible role of this antimicrobial agent on *Salmonella* Enteritidis cells. Accordingly, the absence of significant differences between adhesion results to silestones and most of the other materials is supported by the fact that cells used for the adhesion assays were in stationary-phase, which are known to have higher resistance to triclosan than cells in log-phase (*45*). Moreover, it has been reported that polymers impregnated with high concentrations of triclosan had accomplished just some initial slowing down of bacterial growth rates through the compound released to the liquid medium, while triclosan that remained immobilized in the material did not contribute to the antibacterial character of the polymer (*16*). It is then possible to infer that the release rate of triclosan from silestones to the surrounding media was too low to achieve a significant effect on *Salmonella* Enteritidis cells after only two hours of contact.

All strains were able to form biofilm on all surfaces tested, but total biomass amount was strain dependent and different for each strain on distinct materials (Table 1). Marble was the surface on which most *Salmonella* Enteritidis strains were able to form more biofilm ($P < 0.05$). CV staining of biofilms formed on granite displayed higher OD

values than those registered for SS and silestones, which were the materials with lower biofilm amounts Differences between the extent of adhesion and total biofilm biomass results were not surprising, since it is already established that initial adhesion extent does not always correlate with biomass amount after biofilm development (*7*, *28*, *40*).

Results concerning *Salmonella* Enteritidis viability within biofilms have shown granite and marble to bear the highest numbers of viable cells and, in contrast with CV OD values, no significant differences were observed between these surfaces (Table 1). On the other hand, most biofilms formed on SS had higher cellular viability than biofilms formed on silestones, even though similar amounts of total biomass were found between these materials. Such observations confirm the importance of using different methods for biofilm analysis, as most authors have done (*2*, *14*, *15*, *17*, *20*, *31*, *41*), not only to get more information about the biofilms formed on each material but also to prevent erroneous interpretation of results and biased conclusions. It is also possible to deduce that different OD values reflect different biofilms constitution, which is in agreement with the fact that, although extracellular matrices are always present in biofilms, there is a huge diversity in their composition and in the timing of their synthesis. Furthermore, this diversity was found not only among biofilms formed by different species but also amongst biofilms formed by different strains of a single species (*3*).

Salmonella Enteritidis biofilms formed on silestones had the lowest numbers of viable cells ($P < 0.05$) indicating that, even though biofilm formation was able to take place on silestones, triclosan seems to have played a role in inhibiting or retarding this biological process. It is also important to notice that CFU enumeration does not detect viable but non-culturable (VNC) cells and that triclosan, as antimicrobial agent, might induce that kind of cellular state. So, it must be taken into account that the actual total

numbers of viable cells may be larger than those reported here. Although it was not possible to know the concentration of triclosan available at the silestones surface or within biofilm, previous works had shown that at low concentrations triclosan has a bacteriostatic effect, while at higher concentrations it becomes bactericidal regardless of the bacterial phase of population growth (*6*, *43*, *44*). Moreover, the lethal activity of triclosan was found to be concentration and contact time dependent (*6*), which allows to infer that during the 2 h adhesion period the active concentration of triclosan was too low to promote a considerable effect on *Salmonella* Enteritidis cells, while during the 48 h period of biofilm formation concentrations became high enough to affect both biofilm growth and cellular viability.

The overall results of this work show that all surfaces tested were prone to bacterial colonization and biofilm development, although different materials had distinct biofilm biomass amounts and viable cells counts. Viability results revealed granite and marble to have the poorest performances in cell viability reduction, whereas silestones had better results than both regular stones and SS. Nevertheless, it must be taken into consideration that these data refer to brand new surfaces and that the same materials worn out by use may present different results, especially as concerns to silestones as antimicrobial incorporated surfaces. Moreover, surface performance may also be affected by the kind of bacterial population present, since most biofilms are found as mixed populations that confer bacteria higher survival skills than those registered for single species communities. Summarizing, as far as food safety is concerned silestones are not completely safe materials, requiring a cautious and rigorous cleaning. In this way, the pursuing of more secure materials to improve food-safety continues to be an actual need and a demanding challenge.

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Figure legend

FIGURE 1. Number of *Salmonella enterica* Enteritidis adhered cells per square centimetre to the different materials after 2 hours incubation. Symbols indicate statistically different values ($p<0.05$) concerning the extent of adhesion of different strains to the same material (*) and concerning the extent of adhesion of the same strain to different materials (†).

TABLE 1. *Total biomass and viability of* Salmonella *Enteritidis biofilms*

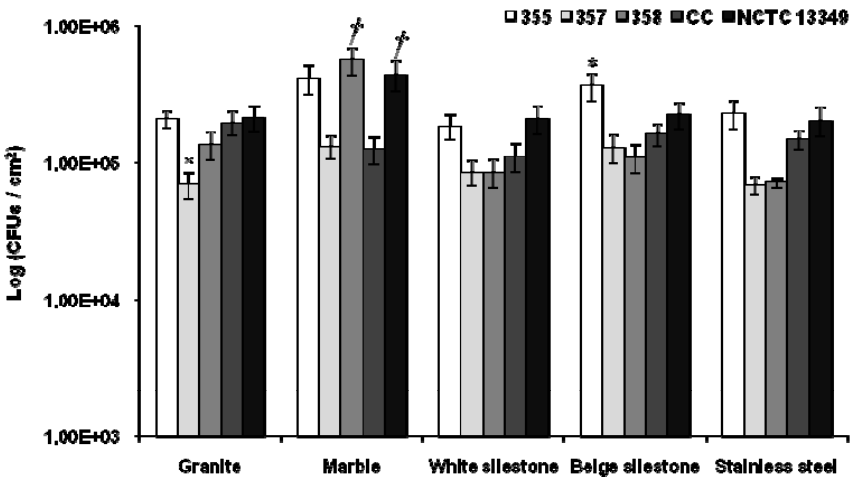
	Granite		Marble		White Silestone		Beige Silestone		Stainless steel	
Strains	Biomass[a]	Viability[b]	Biomass	Viability	Biomass	Viability	Biomass	Viability	Biomass	Viability
355	0.06±0,02†	6.78±0.12*	0.11±0.03†	6.99±0.22	0.02±0,00	5.67±0.41*,†	0.04±0.01	6.22±0.10†	0.03±0.01	6.89±0.05
357	0.08±0.02	6.98±0.10	0.16±0.04†	6.90±0.15	0.07±0.02	6.18±0.11	0.03±0.01†	6.09±0.08	0.06±0.02	6.78±0.19†
358	0.08±0.02	7.06±0.08	0.12±0.03	7.15±0.09	0.06±0.01	6.90±0.10*	0.08±0.02	6.86±0.10	0.07±0.02	7.08±0.10*
CC	0.08±0.01	7.21±0.05	0.11±0.02	7.12±0.05	0.08±0.01	6.57±0.15*,†	0.08±0.02	6.88±0.19	0.07±0.01	6.77±0.21
NCTC13349	0.04±0.01	7.12±0.05	0.15±0.03†	7.14±0.05	0.02±0.00	6.23±0.12	0.02±0.00	6.19±0.01	0.03±0.01	6.85±0.15†

[a] OD_{570nm} mean values ± SD.

[b] CFU/cm^2 mean values ± SD.

Symbols indicate statistically different values ($p<0.05$) concerning biofilms of different strains on the same material (*) and concerning biofilms of the same strain on different materials ($\dagger$).

Figure 1


355 357 358 CC NCTC 13349
1.00E+06
1.00E+05
1.00E+04
1.00E+03
Log (CFUs / cm²)
Granite
Marble
White silestone
Beige silestone
Stainless steel