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Note. Colonisation of Bench Cover Materials by *Salmonella typhimurium*

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Due to the increasing requirements of food safety, it is of utmost importance to know the mechanisms that can determine the occurrence of the phenomenon commonly assigned by cross contamination, which can be expressed by the transference of harmful substances or microorganisms to the human food chain. This is particularly susceptible during food preparation, because it can occur by the transference of the pathogen microorganisms from food to the surfaces where they had been prepared, and from there to foods initially not contaminated, therefore initiating the cycle. This study attempted to investigate the colonisation by *Salmonella typhimurium* ATCC 13311 of marble and granite, two materials commonly used as bench covers in kitchens of many countries. These materials were selected because there is a lack of studies in the literature about their ability for bacterial colonisation. In addition, the colonisation of stainless steel (SS) 304, a material usually studied, was also analysed in terms of comparison. Surface hydrophobicity and roughness were determined in order to explain the differences in the extent of adhesion. The results showed that SS was the material with a greater extent of colonisation by *S. typhimurium*, followed by marble and, almost to the same extent, by granite. *S. typhimurium* adheres to a greater extent to the most hydrophobic material and to the material with roughest surface.

Key Words: Salmonella typhimurium, cross-contamination, colonisation, bench cover materials

INTRODUCTION

The incidence of food-borne diseases has not decreased during the last few years (Snowdon et al., 2002). Domestic and industrial kitchens are most important focuses of attention for infection. In such environments, cross-contamination is the responsible factor for the spread of food associate diseases. Several studies have shown that cross-contamination can result from hands, sponges/clothes and utensils in domestic kitchens as well as in any food processing plant (Hilton and Austin, 2000; Kusumaningrum et al., 2002; Gorman et al., 2002; Kusumaningrum et al., 2003). Contaminated food processing surfaces may act as a potential source of transmission of pathogens to food in the food industry, catering and in the domestic environment. Exposure of pathogens on surfaces may take place either by direct contact with contaminated objects or indirectly through airborne particles (Kusumaningrum et al., 2003). To avoid contamination, knives, chopping boards and hands should be properly washed when preparing raw and then prepared foods. Thus, if efficient hygiene practices and the use of separate surfaces and equipment for raw and cooked foods can be assured, the risk of cross-contamination, and consequently, the transmission of microbiological food-borne illness will be avoided.

Salmonella spp. are pathogenic bacteria responsible for one of the most frequent food-borne diseases. Usually, they are acquired by ingestion of contaminated food items such as meat, poultry meat, eggs, and vegetables (Khuri-Bulos et al., 1994). *Salmonella enteritidis* and *S. typhimurium* are the serotypes most frequently involved in food poisoning outbreaks (Mañas et al., 2001) and, according to Zeidler (1997), it seems that the latter is taking over again. The clinically discernible syndromes of salmonellosis occurring in man and animals are enteric fever, septicaemia and acute gastroenteritis.

The purpose of the present work was to compare the colonisation on marble and granite, two materials commonly used as bench covers in kitchens of many countries, by *S. typhimurium* ATCC 13311 with the

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colonisation on SS 304, a material that has been intensively studied. The effect of material hydrophobicity and roughness on the colonisation by *S. typhimurium* was also investigated.

MATERIALS AND METHODS

Material

Media and Growth Conditions

S. typhimurium ATCC 13311 was grown in tryptic soy agar (TSA) and Tryptic Soy Broth (TSB) media (Merck, Germany) for 48 h, at 37°C. The media were prepared according to the manufacturer's instructions. Then, *S. typhimurium* was grown in 10 mL of TSB for 24 h at 25°C. An aliquot of 100 μL of this culture was transferred to 30 mL of TSB and incubated overnight at 25°C. The culture was then centrifugated at $4{,}000 \times g$ at 4°C for 15 min. The cell pellet was re-suspended in a saline solution (0.9% NaCl prepared in distilled water; Merck, Germany) to a concentration of 1×10^8 cell/mL ($OD_{600nm} \cong 0.8$ nm). These cell suspensions were used in the subsequent adhesion assays.

For contact angle determinations, cells were incubated overnight at 25°C and were harvested by centrifugation ($4{,}000 \times g$ at 4°C for 15 min), washed twice in phosphate buffer saline (PBS), twice in deionised water and re-suspended in deionised water to a concentration of 1×10^8 cells/mL. This suspension was filtered through a 0.45 μm cellulose acetate membrane to obtain a homogeneous lawn of cells (Busscher et al., 1984). To standardise the moisture content, the filters were then transferred onto Petri dishes containing 1% (w/v) agar with 10% (v/v) glycerol, before being used for contact angle measurements.

Bench Cover Materials

The materials assayed were marble, granite and SS 304. The materials were cut in slides of $3.0 \times 2.0\,cm^2$. Prior to the tests, the slides were carefully washed in a 0.2% commercial detergent solution (Sonazol Pril, Alverca, Portugal) followed by ethanol and sterile water.

Bacterial and Substratum Hydrophobicity

Hydrophobicity was evaluated through contact angle measurements and using the approach of van Oss and co-workers (van Oss et al., 1987, 1988, 1989). In this approach, the degree of hydrophobicity of a given material (Equation (1)) is expressed as the free energy of interaction between two entities of that material when immersed in water (ΔG_{lwl}). If the interaction between the two entities is stronger than the interaction of each entity with water ($\Delta G_{lwl} < 0$) the material is considered hydrophobic. Conversely, if $\Delta G_{lwl} > 0$ the material is hydrophilic.

ΔG_{lwl} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{lwl} = -2\left(\sqrt{\gamma_1^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_1^{+}\gamma_w^{-}} + \sqrt{\gamma_1^{-}\gamma_w^{+}} - \sqrt{\gamma_1^{+}\gamma_1^{-}} - \sqrt{\gamma_w^{+}\gamma_w^{-}}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz–van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid–base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a solid material are obtained by measuring the contact angles of three pure liquids (one apolar and two polar), with well known surface tension components, followed by the simultaneous resolution of three equations of the form:

$$(1 + \cos\theta)\gamma_1^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_1^{LW}} + \sqrt{\gamma_s^{+}\gamma_1^{-}} + \sqrt{\gamma_s^{-}\gamma_1^{+}}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

Contact Angle Measurement

Contact angle measurements (at least 25 determinations for each liquid and for each material and microorganism) were done by the sessile drop technique on the cell lawns and on the materials, using a contact angle measurement apparatus (model OCA 15 Plus, Dataphysics Filderstadt, Germany). All the measurements were performed at room temperature. The probe liquids used were ultra-pure water, α-bromonaphthalene and formamide, both of analytical grade. Their surface tension components were obtained from literature (Janczuk et al., 1993).

Surface Topography

Atomic force microscopy (AFM) was used to perform quantitative measurements of surface topography and roughness. The AFM enables surface topography to be recorded in three dimensions by detecting the vertical deflection of an oscillating tip that rasters across the sample surface. Quantitative surface measurements such as roughness can be calculated from height data and direct comparisons can be made between samples. The measurements were carried out with a Nanoscope III controller (Digital Instruments Santa Barbara, CA) in the tapping mode under ambient conditions with untreated silicon cantilevers/tips. The Ra value (which is the arithmetical mean deviation of the profile) is the most commonly used descriptor of

surface roughness (Verran et al., 2000). AFM scans were made over $10\,\mu m \times 10\,\mu m$ areas on each surface. Surface roughness was calculated from three different readings on each surface.

Adhesion Assays

Squares of the materials in the study were placed in 6 well tissue-culture plates containing 5 mL of a 1×10^8 cells/mL suspension in saline solution. Initial adhesion to each substratum was allowed to occur for 2 h at 25°C, in a shaker rotating at 120 rpm. Negative controls were obtained by placing the material squares in a saline solution without bacterial cells. The squares were then gently transferred to 100 mL glass beakers containing distilled water, and were allowed to rest there for approximately 10 s. Afterwards, a new transfer was made to a different 100 mL glass beaker with distilled water, followed by a third transfer 10 s later. These washing steps were carefully performed in order to remove only the cells that were suspended in the liquid interface formed along the surface, and to minimise cell detachment from the surface (Cerca et al., 2004). The substrate squares with adhered cells were dried at 37°C. All experiments were done in triplicate.

Image Analysis

Before image observation and enumeration of adhered cells, the substrate squares were stained with a 0.01% 4′,6-diamino-2-phenylindole solution (DAPI, Sigma-Aldrich Inc., St. Louis, MO) for better image contrast. Direct bacterial counts were done using an epifluorescence microscope (Zeiss, Germany) coupled to a 3 CCD video camera (Axiocam HRC, Zeiss, Germany) that acquires images with 820×560 pixels resolution and at a magnification of 1,000×. With this magnification 1 cm^2 is equivalent to 1.24×10^4 captured images (as determined by a Neubauer chamber). Cells were counted using automated enumeration software (Sigma Scan Pro, USA).

Statistical Analysis

The resulting data were analysed using the Statistical Package for the Social Sciences Software (SPSS, Inc., Chicago, USA). The comparison was performed through a one-way analysis of variance (ANOVA) by applying the Bonferroni analysis as a post hoc test. All tests were performed with a confidence level of 95%.

RESULTS AND DISCUSSION

The literature reports several adhesion studies concerning food-borne pathogens to a wide variety of surfaces, namely to food processing surfaces. Most of those studies refer to *Listeria monocytogenes* (Herald and Zottola, 1988; Mafu et al., 1990; Briandet et al., 1999; Chae and Schraft, 2000; Beresford et al., 2001; Midelet and Carpentier, 2002), with only a few reports about *S. typhimurium* (Helke et al., 1993; Helke and Wong, 1994; Hood and Zottola, 1997). These works focused on the effect of temperature and relative humidity on the behaviour of *S. typhimurium* on surfaces of SS and rubber (Helke and Wong, 1994); the effect of milk and individual milk components on the attachment of *S. typhimurium* to the same materials (Helke et al., 1993) and on the adherence to SS during growth (Hood and Zottola, 1997). There is no mention to the effect of physico-chemical surface properties on the adhesion of *S. typhimurium* to such materials. Moreover, there is no reference in the literature about bacterial adhesion to marble and granite, which are commonly used as bench covers in food processing surfaces.

Water contact angles of the materials were statistically different ($P < 0.05$, Table 1) among them. According to Vogler (1998), hydrophobic surfaces exhibited water contact angle values higher than 65°, whereas hydrophilic ones exhibit water contact angle values lower than 65°. Thus, SS and marble are hydrophobic, while granite ($\theta_W = 49.2°$) and *S. typhimurium* ($\theta_W = 17.1°$) are hydrophilic. However, with this technique it was only possible to assess hydrophobicity qualitatively (Oliveira et al., 2001). Using the approach of van Oss (1997), it is possible to determine the absolute degree of hydrophobicity of any substance *vis-à-vis* water (w). According to this criterion, *S. typhimurium* as well as granite are hydrophilic and SS 304 and marble are hydrophobic. SS was the material with the highest roughness (81.6 ± 52.8 nm), almost twice greater than granite (43.9 ± 17.9 nm), while marble presented the lower roughness (8.9 ± 0.2 nm). As stated by Flint et al. (1997) a Ra value of 0.8 μm or less is generally used to describe a hygienic surface. Thus, from the values obtained, it was evidenced that all the tested materials were hygienic surfaces (Figure 1).

Fluorescence microscopy images of adhered bacteria (Figure 2) showed higher extent of bacterial adhesion on SS 304. There were statistically significant differences ($P < 0.05$) between the number of cells adhered to SS ($3.73 \times 10^5 \pm 5.66 \times 10^4$ cells/cm^2) and the number of cells adhered to the other materials: marble ($1.91 \times 10^5 \pm 1.28 \times 10^4$ cells/cm^2) and granite ($1.88 \times 10^5 \pm 2.15 \times 10^4$ cells/cm^2). However, when comparing marble and granite no significant difference was observed ($P > 0.05$) between their ability for *Salmonella* colonisation. It was verified that the extent of adhesion is higher in the most hydrophobic material (SS 304), followed by marble, which is also a very hydrophobic material. This fact was already expected since, in aqueous medium, adhesion is favoured between

Table 1. Contact angle, surface tension components and free energy of interaction between the materials immersed in water, ΔG_{lwl}

Material	Contact Angle[1] (degrees) ± σ			Surface Tension Components					ΔG_{lwl} (mJ/m²)
	θ_W	θ_F	$\theta_{\alpha-B}$	γ_S^{LW}	γ_S^+	γ_S^-	γ_S^{AB}	γ_S^{TOT}	
S. typhimurium	17.1 ± 1.1	21.7 ± 1.7	38.9 ± 1.7	35.1	1.5	54.5	18.1	53.2	32.4
SS 304	81.3 ± 2.6	62.5 ± 3.3	35.5 ± 2.5	36.5	0	6.7	0	36.5	−52.2
Marble	76.0 ± 2.7	53.8 ± 3.0	28.0 ± 1.2	39.4	0.2	7.3	2.4	41.8	−48.4
Granite	49.2 ± 2.3	43.0 ± 2.6	32.7 ± 2.3	37.6	0.3	32.9	6.3	43.9	8.1

[1] θ_W, contact angle of water; θ_F, contact angle of formamide, $\theta_{\alpha-B}$, contact angle of α-bromonaphthalene.

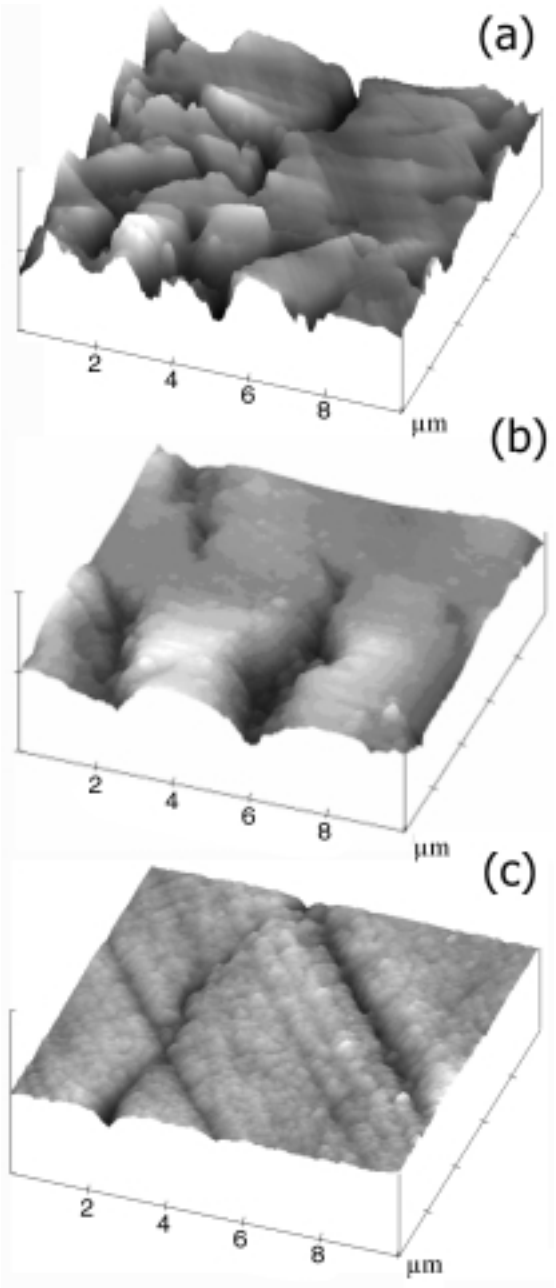

Figure 1. AFM three-dimensional images (surface topography). (a) SS; (b) granite; (c) marble (X- 2 µm/div; Z- 500 nm/div).

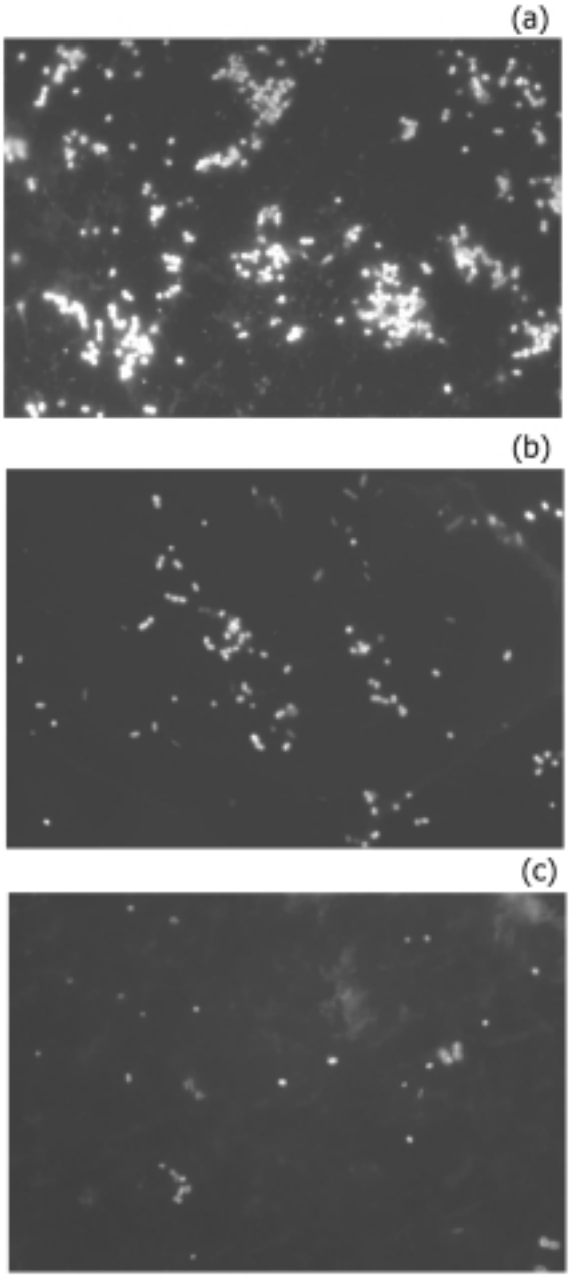

Figure 2. Images obtained by epifluorescence microscopy of cells of *S. typhimurium* adhered to: (a) SS; (b) marble; (c) granite (magnification 100×).

hydrophobic surfaces, which can enter into closer contact by squeezing the water layer between them (Oliveira et al., 2003), beside the higher roughness of SS. It is well known that roughness favours adhesion since microscopic niches can afford protection to the cells from the shear forces (Quirynen and Bollen, 1995). In the case of granite the extent of colonisation may be due to the high value of γ_S^-, which renders it a high electron donor character. In this way, *S. typhimurium* is able to establish interaction between its electron acceptor groups of cell wall and the electron-donating groups of granite, which may be determinant to the adhesion phenomenon.

In a previous work with *L. monocytogenes*, it was verified that the extent of adhesion was also higher to SS (unpublished data). Other authors, for example, Helke et al. (1993), studied the adhesion of *S. typhimurium* to SS and to other materials and also obtained similar results.

As a conclusion concerning bacterial colonisation, SS is the less advisable material to be used as bench cover material in kitchens. However, it must be stressed that these results report to materials not worn out by the use.

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