Biofilm Formation by *Salmonella* Enteritidis at Different Incubation Temperatures

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ABSTRACT

***Background*:** The genus *Salmonella*, associated with poultry products, is considered the leading cause of foodborne outbreaks in humans in many countries. In Brazil, *Salmonella* Enteritidis (SE) is the serovar remains as one most frequently isolated from humans, and it is also a major serovar found in animals, food, animal feed, and environmental samples, despite all the efforts to control this pathogen. Also, the bacterium is able to form biofilms on different surfaces, protecting cells from both cleaning and sanitizing procedures in the food industries. This study aimed to verify the ability of *Salmonella* Enteritidis isolates to form biofilm on polystyrene at different incubation temperatures.

***Materials, Methods & Results*:** A total of 171 SE samples were isolated from foodborne outbreaks (foods and stool cultures) and poultry products between 2003 and 2010. The biofilm-forming ability of samples was measured at four different temperatures (3°C, 9°C, 25°C, and 36°C), for 24 h, simulating temperatures usually found in poultry slaughterhouses. Later, 200 μL of each bacterial suspension was inoculated, in triplicate, onto 96-well, flat-bottomed sterile polystyrene microtiter plates, washed, after that, the biofilm was fixed with methanol. The plates were dried at ambient temperature, stained with 2% Hucker's crystal violet. Afterwards, absorbance was read using an ELISA plate reader and the optical density (OD) of each isolate was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (ODnc). The following classification was used for the determination of biofilm formation: no biofilm production, weak biofilm production, moderate biofilm production and strong biofilm production. Results demonstrated all isolates from stool cultures and foods involved in foodborne outbreaks, at least one of the four temperatures tested, were able to form biofilm, even at 3°C, undescribed as possible for the growth of SE. SE strains from poultry products also formed biofilm at least at one of the temperatures.

***Discussion*:** The prevention of biofilms formation is very important, once they can be difficult to remove from utensils and food equipment surfaces, becoming a chronic source of microbial contamination of foods, possible dissemination of diseases, and increase of resistance to cleaning and sanitization procedures. A high ability for biofilm formation on plastic surfaces was observed. We may consider that *Salmonella* has the capacity to bind to surfaces, with relevant impacts on public health. Although biofilm formation could be affected by temperature, most of the SE isolates analyzed in our study were strong biofilm producers at all temperatures, including at 3°C, a temperature used for food preservation and until then not acknowledged as worrisome regarding the development of *Salmonella* spp. There is a common sense that maintenance of food at low temperatures, particularly below 5°C, is safer to consumers as low temperatures reduce microbial multiplication. However, our results show that the growth of SE in its sessile form is possible under refrigeration. These findings lead to the assumption that the ability of SE to form biofilms, especially at low temperatures, is related to its endurance in inhospitable environments, eventually infecting humans, and that may be one of the factors associated with the high prevalence of this serovar in outbreaks of foodborne diseases. To our knowledge, this is the first publication about biofilm formation by *Salmonella* Enteritidis at 3°C.

Keywords: *Salmonella* Enteritidis, biofilm, incubation temperatures, foodborne outbreaks, poultry products.

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INTRODUCTION

Salmonellosis is one of the most common foodborne diseases and millions of human cases are reported worldwide every year, resulting in thousands of deaths [38]. In most countries, foods containing meat and eggs are the leading causes of human enteritis outbreaks involving *Salmonella* [3,14,22,36].

Salmonella Enteritidis (SE) is one of the most prevalent serotypes from human and non-human sources in the Americas, according to data from the Global Foodborne Infections Network (GFN) Country Databank [6,38,39]. Although many *Salmonella* serovars have been isolated, SE has been identified as the cause of the most cases of foodborne salmonellosis investigated in the state of Rio Grande do Sul in recent years [15,20].

Most microorganisms in their natural habitats are attached to solid surfaces [10], indicating the great advantage of growth of microorganisms on biofilms [4,5,7,8,32]. Biofilm formation by *Salmonella* on surfaces in contact with food has been recognized as a contributing factor for foodborne outbreaks. Surfaces employed in food processing wear out after repeated use and are more likely to accumulate debris and bacteria [16,25,29,33].

According to Lianou and Koutsoumanis [18], environmental parameters such as osmolarity, pH, composition of the growth medium and incubation temperature [19] are able to affect the ability of biofilm formation. Therefore, the main objective of our study was to evaluate SE biofilm formation at different temperatures usually found in food industries.

MATERIALS AND METHODS

Sampling and collection

A total of 171 SE samples, isolated between 2003 and 2010 in the state of Rio Grande do Sul, Southern Brazil, were analyzed. Eighty samples were isolated from stool cultures or foods involved in foodborne outbreaks and 90 from poultry products (chicken cuts, poultry organs, and drag swabs) and from a standard SE strain (ATCC 13076).

Stock culture samples were stored in Brain Heart Infusion broth (BHI)[1], supplemented with 20% glycerol and maintained at -20°C. The tested microorganisms were reactivated in BHI broth, inoculated into xylose lysine deoxycholate agar (XLD)[1], and their purity was confirmed by biochemical and serological tests. Working cultures were maintained on Tryptic Soy Agar slants (TSA)[1] until analysis.

The samples were incubated under different environmental conditions at 3ºC, 9ºC, 25ºC, and 36ºC, in high-performance incubators (BOD Incubator, Tecnal TE-371)[2], with the purpose of simulating the temperatures recommended for poultry slaughterhouses: $3 \pm 1°C$ (cooling temperature, maximum of 4°C); $9 \pm 1°C$ (cutting room temperature in slaughterhouses that export to the EU, maximum of 10°C); $25 \pm 1°C$ (ambient temperature); $36 \pm 1°C$ (standard for mesophilic growth).

Using a microprocessor-based digital temperature controller (precision of $\pm 0.3°C$ and uniformity of $\pm 0.3°C$), and were monitored during incubation by digital temperature monitoring devices (Digital Thermo Hygrometer Internal Temperature and Humidity)[3].

Biofilm formation

The biofilm-forming ability of the isolates was measured by the technique described by Rodrigues *et al.* [28]. Therefore, a loopful of working cultures was transferred to TSB without dextrose (Tryptic Soy Broth without Dextrose)[1], for incubation at $36 \pm 1°C$ for 24 h. Aliquots of the cultures were then added to inoculated TSB without dextrose until a 1 McFarland standard was obtained (Nefelobac)[4]. Later, 200 μL of each bacterial suspension was inoculated, in triplicate, onto 96-well, flat-bottomed sterile polystyrene microtiter plates[5]. Inoculated TSB wells, in a triplicate, were used as negative controls. The same procedures were repeated on three independent plates, which were incubated at four different temperatures (3°C, 9°C, 25°C, and 36°C) for 24 h.

The bacterial suspension was aspirated and each well was washed three times with 250 μL of sterile physiological saline (0.9%). After that, the biofilm was fixed with 200 μL of methanol6 for 15 min and later removed. The plates were dried at ambient temperature, stained with 200 μL of 2% Hucker's crystal violet[6] for 5 min, washed in running water, and dried at ambient temperature. Afterwards, absorbance was read using an ELISA plate reader (Rosys Anthos 2010)[7] at 550 nm [28].

The optical density (OD) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (ODnc). The following classification was used for the determination of biofilm formation: no biofilm production (OD≤ODnc), weak biofilm production (ODnc<ODs≤2.ODnc), moderate biofilm production (2.ODnc<ODs≤4.ODnc), and strong biofilm production (4.ODnc<ODs) [33-35].

RESULTS

All isolates were able to form biofilm, at least at one of the four temperatures tested, even at 3°C, undescribed as possible for the growth of SE (Table 1). SE from foodborne outbreaks formed more biofilm than those of poultry origin unrelated to outbreaks, among which 4.4% of the isolates were strong biofilm producers and only 6.6% did not produce biofilms, while 52.8% of the isolates obtained from poultry products did not produce biofilms.

Table 1. Biofilm formation at 3ºC, 9ºC, 25ºC and 36ºC by *Salmonella* Enteritidis from foodborne outbreaks and poultry products.

	No biofilm Production		Weak biofilm production		Moderate biofilm Production		Strong biofilm production	
	Outbreaks	Poultry	Outbreaks	Poultry	Outbreaks	Poultry	Outbreaks	Poultry
36°C	8/80	44/90	30/80	42/90	37/80	2/90	5/80	2/90
25°C	7/80	48/90	41/80	35/90	27/80	4/90	5/80	3/90
9°C	8/80	48/90	62/80	35/90	8/80	5/90	2/80	2/90
3°C	8/80	50/90	60/80	34/90	8/80	3/90	4/80	3/90
%	9.7%	52.8%	60.3%	40.6%	25.0%	3.9%	5.0%	2.7%

DISCUSSION

Biofilms were initially quantified using a tube culture method with subsequent staining for their detection and recognition and the wells of the microtiter plates were then used as a vessel for culturing the microorganism and results were measured by spectrophotometry. Different methods can be used, such as test tubes, microtiter plates, microscopy, Congo red agar plates, biofilm formation on surfaces, cell enumeration, microscopy, among others but the microtiter plating technique remains as one of the methods most widely used in biofilm research [13,15,26,32,35,37].

Bacterial adhesion is a dynamic and complex process, regulated by several characteristics of the growth medium, of the substrate, and of the cell surface [11,17] and it may be divided into primary and secondary stages. The first stage is reversible while the secondary one is not, and may occur in food processing environments [10,11,32].

With respect to food safety, biofilms are important as they form on food, cooking utensils, and surfaces, and as they are difficult to remove. Their formation in industries is especially important because of their potential as a chronic source of microbial contamination of foods, dissemination of diseases, and increases in the resistance to cleaning and sanitization [16,32,33].

The optimal growth temperature for *Salmonella* is 37ºC [14], but growth may occur between 5°C and 45°C. Although biofilm formation could be affected by temperature [18,27], most of the SE samples analyzed in our study were strong biofilm producers at all temperatures, including at 3°C, temperature used for food preservation and until then not acknowledged as worrisome regarding the development of *Salmonella* spp. According to the Brazilian Directive 210 [2], carcasses and poultry products should be immediately cooled between 0°C and 4°C. Therefore, the temperature of the last pre-cooling stage cannot be higher than 4°C. Given the maximum variation of 1°C, allowed during incubation, the temperature used in this study was 3°C.

Studies that evaluated biofilm formation by *Salmonella* spp. and *Listeria monocytogenes*, found that these bacteria have a high capacity to form biofilm on plastic surfaces [33]. Other studies evaluated biofilm formation on polystyrene by *Salmonella* Heidelberg isolated from chicken carcasses and cloacal swabs, grown in TSB without dextrose supplemented with 0.5 to 4% glucose, and incubated at 36°C [28]. Most of the tested *Salmonella* Heidelberg strains were weak or no biofilm producers under different supplementations and only two isolates were strong biofilm producers when grown in TSB without dextrose.

Microtiter assays with *Salmonella* spp. incubated at 37°C also found different results for biofilm production [12,30,31]. A high ability for biofilm formation on plastic surfaces was observed. We may consider that *Salmonella* has the capacity to bind to surfaces, with relevant impacts on public health.

Bacteria can adhere to surfaces and form biofilms [8,21], for the benefit of the bacterial population, such as multicellular organisms, being possible to became highly resistant to stressful environments [1]. It has been well documented that the genus *Salmonella* can not grow at temperatures between 4 and 8°C and that lower temperatures can reduce its presence [24]. The growth of *S.* Typhimurium and *S.* Heidelberg at 4°C, 7°C, and 10°C in BHI and non-sterile chicken slurry and suggested that low temperatures are important factors for the survival and growth of both serovars [24]. These findings lead to the assumption that the ability of SE to form biofilms, especially at low temperatures, is related to its endurance in inhospitable environments, eventually infecting humans, and that may be one of the factors associated with the high prevalence of this serovar in outbreaks of foodborne diseases.

There is a common sense that maintenance of food at low temperatures, particularly below 5°C, is safer to consumers as low temperatures reduce microbial multiplication. However, our results show that the growth of SE in its sessile form is possible under refrigeration.

Biofilm formation can be considered a selective advantage that facilitates bacterial persistence on surfaces [23]. Both cold storage and thermal treatment might alter the physicochemical properties of SE and lead to adhesion [40]. We demonstrated the possibility of biofilm formation on polystyrene, a plastic polymer that can be used for food preservation at different temperatures.

CONCLUSION

The relevance of this manuscript is the fact that the analyzed SE, especially those involved with foodborne outbreaks, formed biofilms at refrigeration temperatures, enhancing the chances of contamination of humans through consumption of foods stored refrigerated. To our knowledge, this is the first publication about biofilm formation by SE at this temperature.

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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