

# Influence of sodium hypochlorite on STEC biofilm formation

## Abstract

Shiga toxin-producing *Escherichia coli* (STEC) include several serotypes isolated from cases of hemorrhagic colitis and hemolytic uremic syndrome. Bacteria form biofilms in different environments, which can contaminate food and generate infections, while protecting themselves against adverse conditions such as the use of disinfectants. The aim of this study was to evaluate the effects of sodium hypochlorite at different concentrations and exposure times on the formation of STEC biofilms. *In vitro* assays on polystyrene plates were performed and the strains were classified according to their ability to form biofilm. They were exposed to different solutions of NaClO. The results showed that biofilm formation was moderate or weak in most cases and the use of hypochlorite is effective at concentrations greater than or equal to 5% for at least 20min.

**Keywords:** STEC, biofilm, polystyrene, sodium hypochlorite

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## Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a group of foodborne pathogens that cause severe human disease such hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS).<sup>1</sup> In Argentina, HUS is an endemic disease, with 500 cases per year, a median of incidence of 8.4 cases per 100,000 children less than 5 year of age and lethality between 2 and 5% (2010-2015). It is the country with the highest worldwide incidence of this disease.<sup>2</sup> Cattle are the main reservoir of STEC, shedding the organism in their faces.<sup>3</sup> Previous studies have shown that the consumption of contaminated water, undercooked meat, contact with feces of cattle and direct contact with dairy cattle and dairy environment, unpasteurized dairy products and vegetables are some of the possible routes for human exposure to STEC.<sup>4,5</sup> Worldwide, outbreaks have been attributed to STEC O157 serogroup.<sup>6</sup> However, in recent years other STEC serogroups called "the Big Six", such as O26, O103, O111, O121, O45, and O145, were involved in several foodborne illness.<sup>7</sup> In Argentina, infections with STEC non-O157 serotypes are frequently associated with HC and HUS.<sup>8</sup>

The main virulence factors of STEC are the two phage-encoded cytotoxins, named Shiga toxin 1 and 2 (encoded by the *stx1* and *stx2* genes, respectively).<sup>9</sup> In addition, STEC present several adhesins and fimbriae that would allow to colonize both the host and different inert surfaces, such as intimin (encoded by the *eae* gene), which is responsible for the intimate attachment of the bacteria to intestinal epithelial cells,<sup>10</sup> an outer membrane protein which appears to function as an auto agglutinating adhesin of STEC (saa), fimbria type I (fim), antigen 43 (agn43),<sup>11,12</sup> and the auto transported proteins (ehaA).<sup>13</sup> Another adherence factor is Curli fimbria, which is involved in cellular aggregation, adherence and invasion of eukaryotic cells and related with the formation of biofilms by STEC.<sup>14</sup> Microbial biofilms are defined as a community of sessile cells attached to a substratum, interface or to each other. They are embedded in a matrix of extracellular polymeric substances produced by them, and exhibit an altered phenotype with respect to growth rate and gene transcription.<sup>15</sup> It has been shown that STEC can form biofilms in different food processing environments (floors, walls, pipes and drains) and in

materials commonly used for food processing equipment, such as stainless steel, aluminum, and polystyrene.<sup>14</sup> Bacterial biofilms constitute a particular problem in food processing plants and have definite food safety implications, as they could be a source of crossing contamination on food-contact surfaces.<sup>16</sup> Bacterial biofilms are usually much more tolerant to disinfecting agents than the free circulation cells allowing bacterial survival in adverse circumstances, such as industrial disinfection processes, making it difficult to completely inactivate biofilms formed in the equipment and in the environment.<sup>17</sup> One of the most used product to clean and disinfect utensils and surfaces both in the home and in the food industry, is the sodium hypochlorite (NaClO/55 g of active Cl / Lt).<sup>18</sup> It is a universally disinfecting agent, because NaClO can provide changes in the permeability of the cellular membrane.<sup>19</sup> Currently it is also used for the disinfection in hospitals with concentrations ranging from 1 to 10 %.<sup>20</sup> Considering the importance of biofilm formed by STEC, and the massive use of sodium hypochlorite as a disinfectant, the aim of this study was to evaluate the effects of sodium hypochlorite on the STEC biofilm at different concentrations and exposure times.

## Materials and methods

Seven STEC strains from different serotypes and sources were studied. They were previously analyzed in the presence of *stx1*, *stx2*, *eae*, *ehxA*, and *saa* genes by PCR and was serotyped by microagglutination test in the Laboratory of Immunochimistry and Biotechnology of the Faculty of Veterinary Sciences, UNCPBA (Table 1).<sup>3,21,22</sup> The biofilm formation assay was performed in 96-well polystyrene microtiter plates as described by Angel Villegas, Baronetti,<sup>23</sup> with modifications. Briefly, the strains were grown in LB broth at 37°C for 18 h, and the cultures were diluted (1:10). An aliquot of 100 µl was inoculated in each well by triplicate, containing 100 µl of LB, and were incubated 37°C. The medium was changed at 24h. Control wells for each strain, by triplicate (without NaClO) were used. The concentrations of NaClO (2.5 and 5%) were previously determined *in vitro* by viability studies (data not shown). Both concentrations were assayed at different times of exposition according to the following conditions:

**Table 1** Biofilm formation in STEC Strains

Serotype	Origin	Virulence factor	Biofilm formation
O20:H19	cattle burger	stx1, stx2, ehxA, saa	Condition 1: moderate biofilm formed Condition 2 and 3: weak
O117:H7	cattle burger	stx2	Condition 1: moderate biofilm formed Condition 2 and 3: strong biofilm formed.
O22:H8	cattle burger	stx1, stx2, ehxA, saa	Condition 1: moderate biofilm formed Condition 2 and 3: weak biofilm formed
O130:H11	chicken burger	stx1, stx2, ehxA, saa	Condition 1: moderate biofilm formed Condition 2 and 3: non-biofilm formed
O113:H21	chicken burger	stx2, ehxA, saa	Condition 1: moderate biofilm formed Condition 2 and 3: non-biofilm formed
O178:H19	chicken burger	stx2	Condition 1: moderate biofilm formed Condition 2 and 3: moderate biofilm formed
O157:H7	minced meat	stx2, eae, ehxA	Condition 1: moderate biofilm formed Condition 2 and 3: weak biofilm formed

**Condition 1:** Incubation of the microtiter plates 24h and then, addition of NaClO for 20 min of exposure (before washing plates).

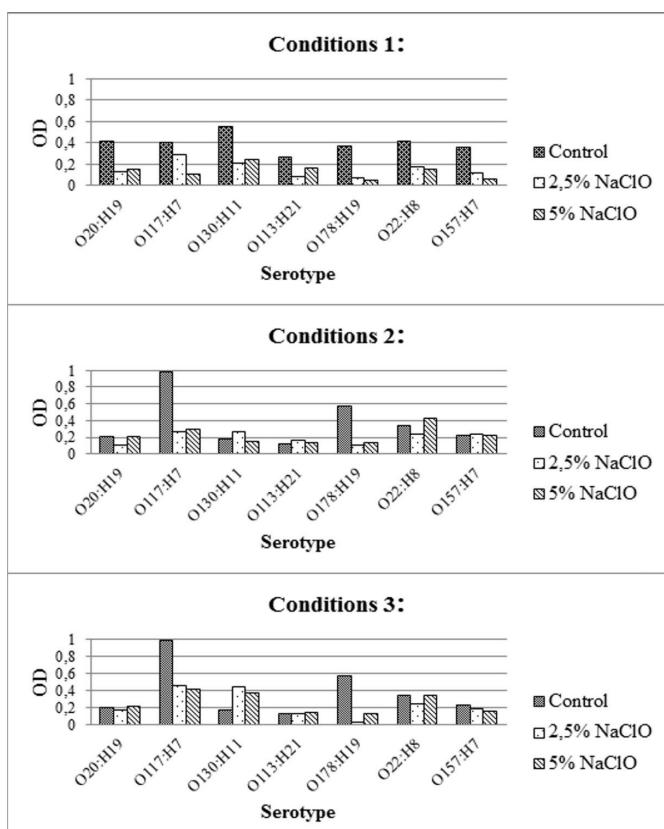
**Condition 2:** Incubation of the microtiter plates 72h and then, addition of NaClO for 20 min of exposure (before washing plates).

**Condition 3:** Incubation of the microtiter plates for 72h, at 24h of incubation, when replacement of LB was performed NaClO solution were incorporated, continuing the incubation for 48h.

The microplates were washed with double distilled water, fixed with methanol (Biopack) by 15 min and stained with 200 µl of violet crystal (CV) 0,1%. Finally, the plates were washed with water and the remaining dye was eluted with 200 µl of 96% ethanol. The biofilm formation was estimated by measuring of optical density at 570nm (OD570) using the microplate reader (Labsystems Multiskan MS). Each test was performed by triplicate in three separate experiments and the average of them was considered to the final analysis. According with the OD, the strains were classified as non-biofilm former (NBF), weak biofilm formers (WBF), moderate biofilm formers (MBF) and strong biofilm formers (SBF) as described Gómez, Gómez-Lus.<sup>24</sup>

## Results

Each STEC strain was classified in different categories of biofilm formation according to the incubation conditions (Table 1). Respect to the different conditions tested, data showed that with the condition 1, hypochlorite sodium decreased the biofilms formation in both concentrations (Figure 1). In the conditions 2 and 3, the biofilm formation was affected more variably. Furthermore, in condition 2, the STEC strains O130, O113 and O157 formed more biofilm with the addition of 2.5% NaClO (Figure 1). Despite the interventions carried out with the disinfectant, a complete elimination of the biofilms was not observed. It was notable that the strains could develop biofilm, although with lower OD values, even in the presence of hypochlorite during 48h (Figure 1). On the other hand, the application of the disinfectant by 20 min on a biofilm formed did not produce a total decrease in OD values, although the greatest reducing effects were observed on biofilms of 24 h of incubation (Figure 1).



**Figure 1** Effect of NaClO solutions on biofilm formation at three different conditions.

## Discussion

Because STEC may form biofilms in different surfaces of food processing environments, and they may serve as a continuous source of contamination, it is important to investigate this ability and the biofilm tolerance to sanitizers in order to provide information regarding

properly sanitizing food contact surfaces with the most effective reagents.<sup>17,25</sup> In agreement with other authors, the hypochlorite solution could reduce the formation of biofilm, but not in all strains equally, suggesting that this phenomenon depends both the strain and the surrounding environment, the serotype and their origin.<sup>17</sup> Furthermore, it has been shown that treatment with sanitizers reduced the viability of STEC, but did not completely remove the biofilm matrix (Vogeleer et al., 2015). This resistance is a multifactorial process and is mainly related to the structural and physiological characteristics of biofilms.<sup>26</sup> Previous studies have shown that in industrial systems, the formation of biofilms protected bacteria from antibacterial chemicals (including natural antibiotics). The matrix could serve as a protective barrier that prevents access of the disinfectant to the interior of the biofilm.<sup>27</sup>

Currently, the recommended concentration for disinfecting the surfaces of equipment and utensils is 1% NaClO for 20 min.<sup>20</sup> In our study it was observed that even when a 5% dilution was used for 20min, biofilm formation could be reduced but not completely eliminated, suggesting that it would be possible to found viable microorganisms for a future contamination.

In summary, STEC strains may survive on surfaces under biofilm formation. Awareness on the survival of STEC is fundamental in order to limit the risk of cross contamination and transfer of STEC to food during processing. In the food industry, efficient programs of clean and sanitization are measure to avoid the accumulation of spoilage, bacteria and the biofilm formation by pathogens.<sup>28</sup> Because there is not treatment against HUS, the prevention measures and control strategies are tools to reduce the transmission of STEC.

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## Conflicts of interest

The author declares no conflict of interest.

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