## 04. Biofilm formation by *Salmonella enterica* strains isolated from feed mills

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**Abstract**

Feed supplied to pigs is considered an important vehicle for *Salmonella enterica* subsp. *enterica* introduction on farm. *Salmonella* can be able to form biofilm on several abiotic surfaces, which may contribute to environmental persistence. This study aimed to evaluate the biofilm formation capacity in *Salmonella* strains isolated from four Brazilian feed mills. The biofilm formation was assessed in 54 *Salmonella* isolates belonging to different serovars by phenotypic assays: *i.* expression of curli fimbriae and cellulose in Luria-Bertani agar supplemented with Congo red, Coomassie brilliant blue and calcofluor; *ii.* adhesion on 96-well polystyrene microtiter plates. The results showed that all isolates presented the rdar morphotype (read, dry and rough colonies) on agar incubated at 28° C. From the total of isolates displaying rdar-morphotype, 14.8% (8/54) showed to be weakly adherent on polystyrene microtiter plates, and were thus considered presumptively biofilm producers. These strains were originated from ingredients and equipment samples, and were distributed among the following serovars: Montevideo (n=2), Senftenberg (n=2), Tennessee (n=1), Orion (n=1), Morehead (n=1), and *S. enterica* O: 16 (n=1). In this sense, biofilm formation might have played a role in *Salmonella* colonization of equipment in feed mills, and should be further investigated.

**Introduction**

*Salmonella* can be able to form complex communities attached to surfaces, and this biofilms may contribute to its persistence in the environment (Steenackers *et al.*, 2012). The formation of biofilms has been assessed *in vitro* mainly by phenotypic methods, such as colony morphology and adhesion to abiotic surfaces (Stepanovic *et al.*, 2000; Malcova *et al.*, 2008). Several studies demonstrated that bacterial isolates, which are originated from residual contamination of food handling environments, often display positive results in biofilm formation phenotypic tests (Stepanovic *et al.*, 2004; Vestby *et al.*, 2009).

The feed supplied to the animals is considered an important vehicle for the introduction of *Salmonella* in pig farms (Binter *et al.*, 2011). *Salmonella* contamination during feed manufacturing process has been related to the use of contaminated ingredients or the persistence of strains in the environment (Wierup & Hãggblom, 2010; Pellegrini *et al.*, 2015). In this sense, biofilm formation by *Salmonella* strains on the equipment surface may play an important role in the persistence of certain strains reported in feed mills (Pellegrini *et al.*, 2015). Thus, the aim of this study was to assess the biofilm formation ability of *Salmonella* strains isolated at various stages of feed processing.

**Material and methods**

Fifty-four *Salmonella* strains isolated during a cross-sectional study conducted in four feed mills were tested. The strains were originated from ingredient or environmental samples and belonged to sixteen serovars: Agona (n = 5), Anatum (n = 4), Cerro (n = 1), Infantis (n = 2), Mbandaka (n= 1), Montevideo (n = 18), Morehead (n = 1), Newport (n = 2), Orion (n = 3), *Salmonella enterica* O:3,10 (n = 2), *Salmonella enterica* O:16:c:- (n = 1), Schwarzengrud (n = 1), Senftenberg (n = 6), Tennessee (n = 4), Typhimurium (n = 1) and Worthington (n = 2).

Biofilm formation was assessed by two phenotypic assays: *i.* morphology of colonies formed on Luria-Bertani (LB) agar; *ii.* adhesion on polystyrene microtiter plates. *Salmonella* strains were streaked on Luria Bertani agar with low salt content (LB low salt; Sigma-Aldrich, St. Louis, USA) supplemented with 40 µg/mL of Congo Red (Sigma-Aldrich) and 20 µg/mL of Coomassie brilliant blue (Sigma-Aldrich). Cellulose production was determined on LB agar supplemented with 50 µM Calcofluor (Fluorescent brightener 28) (Sigma-Aldrich). The plates were incubated at 37°C for 24 hours or at 28°C for 96 hours. After incubation, colony morphology was classified as: rdar (presence of curli fimbriae and cellulose), pdar (presence of cellulose), bdar (presence of curli fimbriae), saw (absence of curli fimbriae and cellulose). For cellulose detection, colony fluorescence was evaluated under UV light at 366 nm (Römling *et al.*, 2003). All assays were performed in duplicate and repeated three times in different days. The adherence test was conducted in flat bottomed 96 well polystyrene plates (TPP® Techno Plastic Products AG, Switzerland) containing 20 µL of bacterial suspension (approximately 5 x $10^8$ CFU/mL) and 230 µL of Tryptic Soy Broth without glucose (TSB; Bacto®, New Jersey, USA). After incubation at 37°C for 24 hours, the microplates were processed according to the protocol described by Stepanovic *et al.* (2004), and evaluated by spectrophotometry at 570 nm. The optical density cut-off (ODc) was defined as the mean OD of the negative control (culture medium) and the isolates were classified as follows: non-adherent (OD ≤ ODc); weak adherent (ODc < OD ≤ 2xODc); moderate adherent (2x ODc < OD ≤ 4 x ODc); and strong adherent (OD > 4xODc). *Staphylococcus epidermidis* ATCC 35984 and *Salmonella* Typhimurium ATCC14028 were included in each plate as positive controls. All tests were performed in triplicate and repeated in three different days.

**Results**

Among the 54 *Salmonella* isolates tested, all presented the rdar morphotype at 28°C and saw morphotype at 37°C (Figure 1). Among them, 14.8% (8/54) showed to be weak adherent on polystyrene microtitre plates, and were considered as presumptive biofilm producers. These isolates belonged to six serovars [Montevideo (n=2), Senftenberg (n=2), Tennessee (n=1), Orion (n=1), Morehead (n=1), and *S*. *enterica* sub. *enterica* O: 16 (n=1)], and were originated from equipment (n=5) as well as ingredients delivered at the feed mills (n=3).

**Discussion**

In this study, we evaluated the phenotypic characteristics associated with biofilm formation capability in *Salmonella* isolates originated from feed mills. All isolates expressed rdar morphotype at 28°C, which is characterized by the production of an adhesive extracellular matrix consisting of curli fimbriae and cellulose (Römling *et al.*, 2003). The curli fimbriae are considered to be expressed in response to nutrient limitation, while cellulose is an extracellular component produced for mechanical and chemical protection of bacterial cells (Jain & Chen, 2010). Both components are important for survival under challenging conditions, which may be encountered by bacteria in the environment. Moreover, the expression of curli fimbriae and cellulose, leading to rdar morphotype, was temperature dependent, indicating that both components may play a much more important role at room temperature. The adherence test to abiotic surfaces revealed that 14.8% (8/54) of these isolates were weakly adherent to polystyrene plates, and were thus considered as presumptive biofilm producers. These strains were originated from ingredient delivered at the feed mills or were isolated from dust or debris samples taken from the equipment surfaces. The results indicate that *Salmonella* isolates able to produce biofilm may be introduced in feed mills through contaminated ingredients. The formation of biofilm in turn is influenced by several environmental factors (temperature, surfaces, nutrients and pH), which regulate the expression of genes responsible for biofilm formation (Linou & Koutsoumanis, 2012, Nguyen & Yuk, 2013, Simm *et al.*, 2014). In this sense, the environmental conditions found inside feed mills, such as high

moisture and temperature, may influence the formation of biofilm by certain *Salmonella* isolates, allowing their residual colonization of surfaces. The influence of these factors in the formation of biofilms needs to be further investigated.

## Conclusion

Strains isolated from feed mills can be able to form biofilm. This fact might play a role in *Salmonella* residual colonization of equipment, and should be further investigated.

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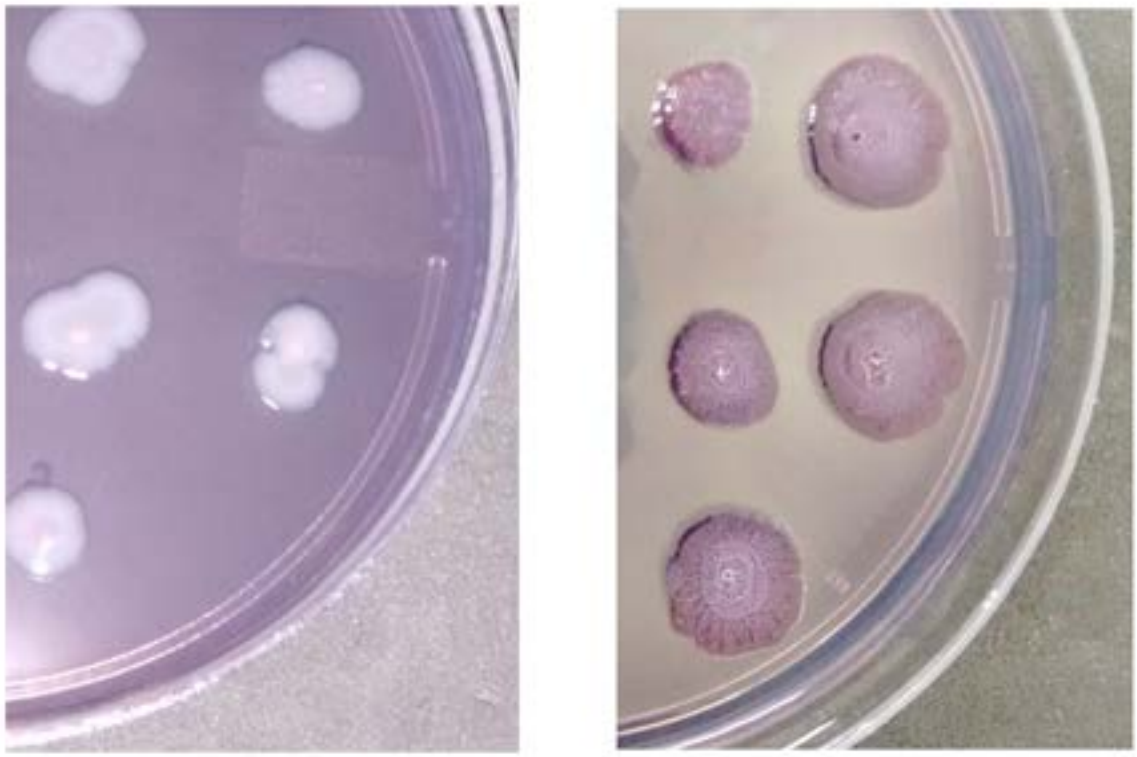

Figure 1. Morphotypes of *Salmonella* isolates. (A) saw morphotype after a 24-hour incubation at 37°C on Congo Red agar. (B) rdar morphotype after a 96-hour incubation at 28° C on Congo Red agar.