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MICROBIOLOGY

The biofilm-forming ability of *Salmonella enterica* subsp. *enterica* isolated from swine-feed mills

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ABSTRACT: Animal feed has been considered an important vehicle for introducing *Salmonella enterica* subsp. *enterica* in pig farms. *Salmonella* survival and persistence in feed mill environments have been associated with biofilm-forming ability. This study evaluated 54 *Salmonella* isolates from swine-feed mills for: *i.* phenotypic expression of curli fimbriae and cellulose; *ii.* pellicle formation at the air-liquid interface; *iii.* adhesion on polystyrene microtiter plates; and *iv.* the presence of the main genes associated with biofilm formation. Regarding phenotypic cell morphology assays, all *Salmonella* isolates presented morphotype RDAR at 28 °C and SAW at 37 °C. Rigid pellicle formation at the air-liquid interface was observed in 51.85% (28/54), while fragile pellicle was seen in 18.52% (10/54), and 29.62% (16/54) were not able to produce pellicle. Biofilm quantification on polystyrene microtiter plates showed that 98.15% (53/54) of *Salmonella* isolates were able to form biofilms at 28 °C, while 83.33% (45/54) of the isolates were classified as non-adherent at 37 °C. The genes *csgD*, *fimA*, *adrA,* and *bapA* were found in all isolates evaluated. These results indicated that *Salmonella* serovars from swine-feed mills have the biofilm-forming ability.
Key words: biofilm, *Salmonella*, polystyrene, RDAR, feed mill, swine.

Capacidade de formação de biofilme de *Salmonella enterica* subsp. *enterica* proveniente de fábricas de rações para suínos

RESUMO: A ração animal tem sido considerada um importante veículo para a introdução de *Salmonella enterica* subsp. *enterica* em granjas de suínos. A sobrevivência e persistência de *Salmonella* em ambientes de fábricas de rações têm sido associadas a capacidade de formação de biofilme. Neste sentido, o objetivo deste estudo foi avaliar 54 isolados de *Salmonella* provenientes de fábricas de rações para suínos quanto: *i.* expressão fenotípica de fimbria *curli* e celulose; *ii*. formação de película na interface ar-líquido; *iii.* adesão em microplacas de poliestireno e *iv*. a presença dos principais genes associados a formação de biofilme. Quanto aos ensaios fenotípicos de morfologia celular, todos os isolados de *Salmonella* apresentaram o morfotipo RDAR a 28 °C e SAW a 37 °C. A formação de uma película rígida na interface ar-líquido foi observada em 51,85% (28/54) dos isolados, enquanto uma película frágil foi observada em 18,52% (10/54) e 29,62% dos isolados não foram capazes de produzir película. A quantificação de biofilme em microplacas de poliestireno mostrou que 98,15% (53/54) dos isolados de *Salmonella* foram capazes de formar biofilme a 28 °C, enquanto que 83,33% (45/54) dos isolados foram classificados como não aderentes a 37 °C. Os genes *csgD*, *fimA*, *adrA* e *bapA* foram encontrados em todos os isolados estudados. Esses resultados indicam que os sorovares de *Salmonella* oriundos de fábricas de rações para suínos possuem capacidade de formação de biofilme.
Palavras-chave: biofilme, *Salmonella*, poliestireno, RDAR, fábrica de ração, suínos.

INTRODUCTION

Animal feed is considered a risk factor for *Salmonella* infection in pigs and poultry (MAGOSSI et al., 2020; SHARIAT et al., 2020; GOSLING et al., 2022). *Salmonella* can survive in the environment for long periods because this microorganism can live in low water activity conditions and adapt to different temperatures (SHARIAT et al., 2020). Animal feed can become infected with *Salmonella* through contaminated ingredients of animal and plant origin, but contamination of final products can also occur during the processing and handling of the feed (WIERUP & HÄGGBLOM, 2010; MINHA et al., 2020). Although, common *Salmonella* serovars have been frequently identified in ingredients and at several points in feed processing worldwide, there is still a lack of data concerning the contamination and distribution of *Salmonella* in Brazilian feed mills. Previous studies reported that the prevalence of *Salmonella* ranges from 0% to 4.94% in feed samples (PELLEGRINI et al., 2015; VIANA et al., 2019).

An essential factor enabling the environmental survival of *Salmonella* is its ability

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to form biofilms (SANTOS et al., 2022). Biofilms are generally defined as structured communities of one or more species of bacterial cells locked in a self-produced extracellular matrix (ECM) attached to abiotic or living surfaces (LAMAS et al., 2018). *Salmonella* cells in the biofilm matrix are more resistant to routinely used disinfectants than their planktonic cells and more challenging to eradicate. This resistance is because of bacterial cell aggregation and exopolysaccharide production that limit the diffusion of antimicrobial agents (GALIÉ et al., 2018; MERINO et al., 2019). Biofilms may play a crucial role in the survival of *Salmonella* under unfavorable environments, such as farms, feed mills, food industries, and abattoirs. This bacterium is capable of forming a biofilm on produced food, and also in processing areas and on contact surfaces, including stainless steel, aluminum, copper, nylon, rubber, plastic, polystyrene, and glass (MERINO et al., 2019; PONTINI et al., 2021; SANTOS et al., 2022).

In this context, this study evaluated *Salmonella* isolates from swine-feed mills for: *i.* phenotypic expression of curli fimbriae and cellulose; *ii.* pellicle formation at the air-liquid interface; *iii.* adhesion on 96-well polystyrene microtiter plates; and *iv.* the presence of the main genes associated with biofilm formation phenotypes.

MATERIALS AND METHODS

Origin of the isolates

Fifty-four *Salmonella enterica* subsp. *enterica* isolates were obtained from a cross-sectional study in four feed factories in Southern Brazil, and all were selected for this study. The isolates were previously characterized according to origins, serovars, and PFGE patterns by PELLEGRINI et al. (2015). They were obtained from ingredients and the environment and belonged to 16 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), Schwarzengrund (n = 1), Senftenberg (n = 6), Tennessee (n = 4), Typhimurium (n = 1), and Worthington (n = 2). The isolates were stored at -80 °C until their reactivation in Brain Heart Infusion broth (BHI, Acumedia, USA) and incubated at 37 °C for 24 h for use.

Evaluation of biofilm-forming ability

Phenotypic detection of curli fimbriae and cellulose

For detection of curli fimbriae and cellulose, the isolates were directly streaked on Congo Red (CR) plates, which contained Luria Bertani (LB) low salt (Sigma-Aldrich, St. Louis, USA) supplemented with 1.6% agar (HiMedia Laboratories, India), 40 µg mL^{-1} of CR (Sigma-Aldrich, USA), and 20 µg mL^{-1} of Brilliant Blue G (Sigma-Aldrich, USA). Cellulose detection was also evaluated on plates containing LB low salt supplemented with 1.6% agar and 50 µmol L^{-1} of Fluorescent Brightener 28 (Sigma-Aldrich, USA) — which emits visible fluorescence under 366-nm UV light when bound to cellulose. The plates were incubated at 37 °C for 24 h, and at 28 °C for 48 to 96 h. The morphologies of the colonies were categorized as: RDAR (red, dry, and rough), PDAR (pink, dry, and rough), BDAR (brown, dry, and rough), and SAW (smooth and white). RDAR morphology means that it expresses curli fimbriae and cellulose, while PDAR expresses cellulose, BDAR expresses curli fimbriae, and SAW presents no expression of curli fimbriae and cellulose (Figure 1) (MALCOVA et al., 2008; RÖMLING et al., 2003). *Salmonella* Typhimurium ATCC 14028 and *Salmonella* Enteritidis ATCC 13076 were used as control for RDAR and SAW morphologies, respectively. All tests at both incubation temperatures were performed in duplicate and repeated at least three times.

Pellicle formation at the air-liquid interface

Biofilm formation was evaluated at the interface between air and the liquid medium. The isolates were inoculated in glass tubes containing 5 mL of LB low salt (Sigma-Aldrich, St. Louis, USA) and incubated at 25 °C for 96 h. Biofilm formation was visualized as a floating pellicle at the air-broth interface, which blocked the surface of the culture and could not be dispersed by shaking (SOLANO et al., 2002). The pellicle was classified as rigid (when the appearance of the pellicle was thick and could not be dispersed by shaking), fragile (when the appearance of the pellicle was thin or could easily be disrupted by shaking), and absent. *Salmonella* Typhimurium ATCC 14028 and *Salmonella* Enteritidis ATCC 13076 were used as a positive and negative control, respectively. All assays were repeated at least three times.

Biofilm quantification on polystyrene microtiter plates

The quantification of biofilm formation was performed in 230 µL of Tryptic Soya Broth (TSB) with no glucose (Becton Dickinson & Company, USA) in sterile 96-well flat-bottomed polystyrene microtiter plates (Techno Plastic Products, Germany). A quantity of 20 µL of overnight bacterial culture, adjusted at 0.5 on the MacFarland scale, was added to each well. The plates were incubated aerobically at

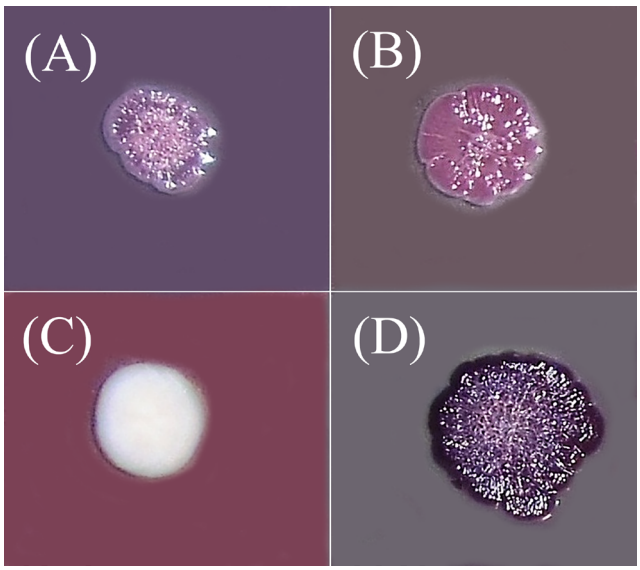

Figure 1 - Colony morphology of *Salmonella* in LB low salt agar supplemented with Congo Red and Brilliant Blue G. (A) RDAR morphology after 48 h of incubation at 28 °C; (B) PDAR morphology after 48 h of incubation at 28 °C; (C) SAW morphology after 48 h of incubation at 28 °C; and (D) RDAR morphology after 96 h of incubation at 28 °C.

37 °C for 24 h and at 28 °C for 96 h (STEPANOVIC et al., 2004). After incubation, the plate's content was drained, and the wells were washed three times with sterile distilled water. The plates were vigorously shaken to remove non-adherent cells during the washing process. The remaining attached bacteria were fixed with 250 µL of methanol per well. After 15 min, each plate was emptied and air-dried. The plates were stained with 250 µL of 2% Crystal Violet per well for 5 min. Any excess stain was rinsed off using distilled water. Subsequently, the dye bound to adherent cells was resolubilized with 250 µL of 33% (v/v) Glacial Acetic Acid per well. Each well's optical density (O.D.) was measured at 570 nm using a Strip Reader spectrophotometer (EL301, BioTek, USA). For each biofilm microtiter plate, the cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control. The isolates were classified into four categories: O.D. ≤ O.D.c = non-adherent; O.D.c < O.D. ≤ (2 x O.D.c) = weak adherent (2 x O.D.c) < O.D. ≤ (4 x O.D.c) = moderate adherent; and (4 x O.D.c) < O.D. = strong adherent. Each isolate was tested in triplicate. *Staphylococcus epidermidis* ATCC 35984 and *Salmonella* Typhimurium ATCC 14028 were used as a positive control for biofilm formation, while *Salmonella* Enteritidis ATCC 13076 was used as a negative control for biofilm formation. The wells with no inoculum were used as quality control for the medium.

DNA extraction and PCR assays for biofilm genes

Genomic DNA was prepared using the NucleoSpin Tissue Kits (Macherey-Nagel; Düren, Germany). The genes *csgD*, *adrA*, *fimA*, and *bapA* involved in biofilm formation were detected by Polymerase Chain Reaction (PCR) assays. The oligonucleotides used are shown in table 1. The reaction mixtures were prepared in a total volume of 25 µL containing: 2.5 µL of 10x PCR buffer, 3 mM of magnesium chloride (MgCl2), 0.2 mM each dNTP (Invitrogen; Groningen; Netherlands), 1U of Taq DNA polymerase (Ludwig; Alvorada; Brazil), 20 pmol of each oligonucleotide, 2 µL of DNA (10 ng) and ultra-pure water (Milli-Q Plus; Millipore, Billerica, USA). The reaction mixtures were amplified in a thermocycler Veriti™ Thermal Cycler (Applied Biosystems; Waltham; USA).

The PCR conditions for amplification of *csgD* and *adrA* genes were: 5 min of initial denaturation at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 ºC for 30 s, and extension at 72 °C for 30 s, ending with

Table 1 - PCR primers used in this study.

Gene	Protein function	Amplicon Size (bp)	Sequence (5' – 3')	Reference
csgD	Transcription and response regulator	123	fw: TGCGGACTCGGTGCTGTTGT	OLIVEIRA et al., 2014.
			rv: CAGGAACACGTGGTCAGCGG	
adrA	Cellulose expression	92	fw: GGGCGGCGAAAGCCCTTGAT	OLIVEIRA et al., 2014.
			rv: GCCCATCAGCGCGATCCACA	
fimA	attachment	85	fw: CCTTTCTCCATCGTCCTGAA	COHEN et al., 1996.
			rv: TGGTGTTATCTGCCCGACCA	
bapA	Bacterial aggregation	667	fw: GCCATGGTGCTGGAAGGCCTGGCGGTT	BISWAS et al., 2010.
			rv: GGTCGACGGGAAGGGTAAAATGACCTTC	

fw: primer forward; rv: primer reverse.

a final extension period of 72 °C for 4 min. The PCR conditions for amplification of the *fimA* gene were: 1 min of initial denaturation at 94 °C, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 58 ºC for 30 s, and extension at 72 °C for 30 s, ending with a final extension period of 72 °C for 5 min. For amplification of the *bapA* gene, the conditions were: 5 min of initial denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 ºC for 45 s, and extension at 72 °C for 1 min, ending with a final extension period of 72 °C for 5 min.

After this, 10 µL of PCR products were added with 1 µL of Blue Green Loading Dye 1 (LCG Biotecnologia; São Paulo; Brazil), separated on a 2% (w/v) agarose gel (Invitrogen™, USA) in a 0.5 Tris/Acetate/EDTA buffer (TAE) using a molecular weight marker of 100 bp (Ludwig, Brazil). The amplified products were visualized in a Kodak Gel Logic 2200 UV transilluminator (Rochester). *Salmonella* Typhimurium ATCC® 14028 was used as positive control.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). The phenotypic cell morphology and pellicle formation were analyzed by descriptive analysis. All tests were carried out in triplicate for the biofilm quantification in polystyrene microtiter plates, and the results were averaged. The Student's t test examined differences in the degree of biofilm formation. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Fifty-four *Salmonella enterica* subsp. *enterica* isolates from four feed factories belonging to different serovars were evaluated for their ability to form biofilm through phenotypic cell morphology assays, pellicle formation at the air-liquid interface, and adhesion on polystyrene microplates (Table 2). Regarding phenotypic cell morphology assays, all *Salmonella* isolates from equipment, feed ingredients, and finished products presented morphotype RDAR at 28 °C and morphotype SAW at 37 °C. The RDAR morphotype was expressed at 28 °C, which is considered room temperature in the Brazilian feed factories. It is suggested that the RDAR morphotype is an adaptation to survive outside the host (CHIA et al., 2011) and is generally only expressed in low-temperature conditions (below 30 °C); in addition, it can persist for long periods in the environment by providing resistance to dissection and disinfection (SEIXAS et al., 2014). According to RÖMLING et al. (2003), over 90% of *S.* Typhimurium and *S.* Enteritidis strains from human disease, food, and animals expressed the RDAR morphotype at 28 °C. In another study, six *Salmonella* serovars linked to tomato-associated outbreaks, including Anatum, Baildon, Braenderup, Montevideo, Newport, and Javiana, produced the RDAR morphotype at 28 °C commonly associated with environmental persistence (KUMAR et al., 2018). This corroborated the results obtained in this study since all strains expressed the RDAR morphotype at 28 °C, and the majority (98.15%) could form biofilm on polystyrene microplates at 28 °C. However, these isolates expressed the SAW morphotype at 37 °C and were weak or non-adherent on polystyrene microplates at 37 °C.

The RDAR morphotype is characterized by the expression of the extracellular matrix components amyloid curli fimbriae and the exopolysaccharide cellulose (RÖMLING et al., 2003; ĆWIEK et al., 2019). Curli fimbriae are considered to be expressed in response to nutrient limitation under conditions

Table 2 - Biofilm formation phenotypes among *Salmonella* serovar from feed mills.

Serovar	N°. of isolates	Origin	Feed Mill	PFGE Pulsotype	Pellicle in LB	Phenotype Morphotype 37 °C	Phenotype Morphotype 28 °C	Microtiter-plate test 37 °C	Microtiter-plate test 28 °C	Biofilm profile
Anatum	4	Equipment	B	nd[a]	Absent (1)	SAW	RDAR	Non-adherent	Moderate	P3
		Ingredient	C	nd	Fragile (1)	SAW	RDAR	Non-adherent	Weak	P6
					Rigid (2)	SAW	RDAR	Non-adherent	Moderate	P1
Cerro	1	Ingredient	C	nd	Rigid (1)	SAW	RDAR	Non-adherent	Moderate	P1
Infantis	2	Equipment	A	Ig1; Ig2	Rigid (2)	SAW	RDAR	Non-adherent	Moderate	P1
Montevideo	18	Equipment Final Product	C, D	Mt5 (2); Mt4 (1); Mt7 (2)	Absent (5)	SAW	RDAR	Non-adherent	Moderate	P3
		Equipment	D	Mt7 (1)	Absent (1)	SAW	RDAR	Weak	Moderate	P7
		Ingredient Equipment	C, D	Mt5 (1); Mt7 (1)	Fragile (2)	SAW	RDAR	Non-adherent	Moderate	P4
		Equipment	D	Mt6 (1)	Rigid (1)	SAW	RDAR	Weak	Weak	P2
		Equipment Ingredient	A	Mt1 (1); Mt2 (1); Mt3 (1); Mt7 (1); Mt8 (4)	Rigid (8)	SAW	RDAR	Non-adherent	Moderate	P1
		Equipment	D	Mt9 (1)	Absent (1)	SAW	RDAR	Non-adherent	Non-adherent	Absent*
Senftenberg	6	Equipment	D	Se3	Absent (1)	SAW	RDAR	Non-adherent	Moderate	P3
		Equipment	D	Se1	Fragile (1)	SAW	RDAR	Non-adherent	Moderate	P4
		Equipment Final product	C, D	Se2; nd	Rigid (2)	SAW	RDAR	Non-adherent	Moderate	P1
		Ingredient	D	Se3	Rigid (1)	SAW	RDAR	Weak	Weak	P2
		Ingredient	C	nd	Rigid (1)	SAW	RDAR	Weak	Moderate	P5
Tennesse	4	Ingredient	C	nd	Absent (1)	SAW	RDAR	Non-adherent	Moderate	P3
		Equipment	D	Te1; Te2	Rigid (2)	SAW	RDAR	Non-adherent	Moderate	P1
		Ingredient	C	nd	Rigid (2)	SAW	RDAR	Weak	Moderate	P5
Orion	3	Equipment	B	Or1; Or2	Absent (2)	SAW	RDAR	Non-adherent	Moderate	P3
		Ingredient	C	nd	Rigid (1)	SAW	RDAR	Weak	Moderate	P5
Morehead	1	Equipment	B	nd	Fragile (1)	SAW	RDAR	Weak	Moderate	P8
S. (O: 16: c: -)	1	Equipment	B	nd	Fragile (1)	SAW	RDAR	Weak	Moderate	P8
Agona	5	Equipment	D	Ag2	Absent (1)	SAW	RDAR	Non-adherent	Moderate	P3
		Ingredient Equipment	D	Ag3	Fragile (2)	SAW	RDAR	Non-adherent	Moderate	P4
		Equipment	D	Ag1; Ag2	Rigid (2)	SAW	RDAR	Non-adherent	Moderate	P1
Mbandaka	1	Final product	C	nd	Fragile (1)	SAW	RDAR	Non-adherent	Moderate	P4
Newport	2	Equipment	A	nd	Rigid (2)	SAW	RDAR	Non-adherent	Moderate	P1
S. (O: 3, 10)	2	Equipment Ingredient	B	nd	Absent (2)	SAW	RDAR	Non-adherent	Moderate	P3
Schwarzengrund	1	Ingredient	C	nd	Rigid (1)	SAW	RDAR	Non-adherent	Moderate	P1
Typhimurium	1	Final product	C	nd	Fragile (1)	SAW	RDAR	Non-adherent	Moderate	P4
Worthington	2	Equipment	D	Wo1	Absent (1)	SAW	RDAR	Non-adherent	Moderate	P3
					Rigid (1)	SAW	RDAR	Non-adherent	Moderate	P1
ATTCC 14028					Rigid	SAW	RDAR	Non-adherent	Moderate	

[a] No determined; [*]Absent: the isolate did not present a phenotype for biofilm formation.

of low osmolarity and low temperatures (GAVIRIA-CANTIN et al., 2022). Curli fimbriae have been reported to provide environmental persistence and ensure biofilm formation at early stages (JAIN & CHEN, 2007; SIMM et al., 2014). Conversely, cellulose keeps the structure of the matrix highly organized (VESTBY et al., 2009) and ensures resistance against environmental stresses (PROUTY et al., 2003; GUALDI et al., 2008). Both components are essential for bacterial survival in challenging environmental conditions.

Rigid pellicle formation at the air-liquid interface was observed in 51.85% (28/54), while fragile pellicle was seen in 18.52% (10/54), and 29.62% (16/54) were not able to produce the pellicle at the air-liquid interface. Biofilm formation at the air-liquid interface has been gaining interest, as this niche allows aerobic or facultative anaerobic bacteria to get access to oxygen (SPIERS et al., 2003; MEDRANO-FÉLIX et al., 2018). According to SOLANO et al. (2002), 71% of *S.* Enteritidis isolates tested produced a rigid pellicle at the air-liquid interface of LB broth, and most of them (93%) showed RDAR morphotype on Congo Red agar. Factors leading bacteria to form biofilms at air-liquid interfaces are still unknown. Some reports imply that colonization at the air-liquid interface is due to the overproduction of a cellulosic polymer (ZOGAJ et al., 2001), which holds cells together, and results in a much more adherent structure. Studies have demonstrated that *Salmonella* strains with the RDAR morphotype can form thin to rigid pellicles when incubated in a rich medium with low osmolarity at a low temperature (25 - 28 °C) (SOLANO et al., 2002; SHATILA et al., 2021). In our study, 16 of the 54 *Salmonella* isolates with the RDAR morphotype could not produce a pellicle at the air-liquid interface.

Salmonella differs in their attachment depending on the surface and temperature conditions encountered, which may influence persistence in the processing environment. The quantities of biofilm produced on polystyrene microtiter plates showed that 53 of 54 *Salmonella* isolates were able to form biofilm at 28 °C, of which 94.34% (50/53) were classified as moderate biofilm producers and 5.66% (3/53) as weak biofilm producers. This difference was confirmed as significant ($P < 0.05$). No strongly adherent isolates were observed at 28 °C. The values of optical density (O.D.) ranged from 0.210 to 0.868, with O.D. means of 0.217 for cut-off (O.D.c), 0.210 for non-adherent isolates (O.D < O.D.c), 0.3876 for weakly adherent isolates (O.D.c > O.D < 2x O.D.c), and 0.797 for moderately adherent isolates (2x O.D.c > O.D < 4x O.D.c). At 37 °C, 84.91% (45/53) of *Salmonella* isolates were classified as non-adherent, and 15.09% as weak biofilm producers. This difference was confirmed as significant ($P < 0.05$). No moderately or strongly adherent isolates were observed at 37 °C. The values of optical density (O.D.) ranged from 0.111 to 0.207, the O.D. means of 0.154 for cut-off (O.D.c), 0.139 for non-adherent isolates (O.D < O.D.c), and 0,168 for weakly adherent isolates (O.D.c > O.D.> 2x O.D.c). Biofilm production was significantly higher at 28 °C than at 37 °C ($P < 0.05$). Our results agree with the literature, which reported *Salmonella* isolates with biofilm-forming ability at 25-28 °C, categorized as weakly or moderately adherent on polystyrene microtiter plates (YANG et al., 2016; SIMONI et al., 2022).

Eight distinct phenotypic profiles (P1 to P8) were found for phenotypic biofilm formation (Table 2). There was no relationship between the biofilm profiles and serovars since different phenotypic profiles were observed among the isolates of the same serovar. The most frequent phenotypic profiles for biofilm formation were P1, P3, and P4, representing 81.48% (44/54) of the isolates. They were moderately biofilm-forming at 28 °C, did not form biofilm at 37 °C, and differed in the pellicle production at the air-liquid. While the isolates belonging to P1 (23/54) and P4 (7/54) produced rigid and fragile pellicles, the isolates belonging to P3 (14/54) were not able to create a pellicle at the air-liquid interface. These biofilm formation profiles, which were more common, were observed in isolates from all isolation points. The P1, P3, and P4 profiles were observed in *Salmonella* isolated from equipment, feed ingredients, and finished product. Ingredients of animal or vegetal origin are considered a risk factor for introducing *Salmonella* in feed mills (WIERUP & HÃGGBLOM, 2010; MINHA et al., 2020). In addition, the intermittent flow of materials carrying *Salmonella* assists the colonization of dust and aggregate debris in equipment, which may be the source of contamination of negative feed batches (PELLEGRINI et al., 2015). Once the biofilm is established in the equipment, mechanical action is one of the main measures for its elimination. Generally, disinfectants do not penetrate the biofilm matrix after an inefficient cleaning procedure and do not destroy all the biofilm cells (MERINO et al., 2019). It's essential to evaluate and develop cleaning and sanitizing strategies to remove or prevent biofilm formation by *Salmonella*, thus minimizing contamination or recontamination of feed and feed factory environments.

For most of the *Salmonella* isolates, there was no relationship between the phenotypic profile for biofilm formation and the macrorestriction profile by PFGE, except for *S.* Agona and *S.* Montevideo. The use of molecular techniques such as PFGE and MLST provides evidence that several clones of *Salmonella* serovars can persist in feed mill environments for months and even years (VESTBY et al., 2009; PRUNIĆ et al., 2016). In this study, two isolates of *S.* Agona that showed biofilm profile P4 shared the same Ag3 macrorestriction profile. These isolates produced a fragile pellicle at the air-liquid interface, showed the RDAR morphotype at 28 °C, and were moderate biofilm producers at 28 °C. In addition, four isolates of *S.* Montevideo that showed biofilm profile P1 belonged to the same macrorestriction profile (Mt8) (Table 2). These isolates produced a rigid pellicle at the air-liquid interface, showed the RDAR morphotype at 28 °C, and were moderate biofilm producers at 28 °C and non-adherent at 37 °C. The Mt8 macro restriction profile was the second most frequent among *S.* Montevideo isolates (4/18). It was observed in different sample types (ingredients and equipment) on different sampling days performed in the same feed mill (PELLEGRINI et al., 2015). Due to its ability to form a biofilm, *Salmonella* serovars can persist for long periods in the environment and pose a source of contamination for new ingredients and feed.

The genes involved in biofilm formation, *csgD*, *adrA*, *fimA,* and *bapA,* were detected by PCR in all 54 *Salmonella* isolates, amplifying DNA fragments of 123 bp, 92 bp, 85 bp, and 667 bp, respectively. Biofilm formation, in turn, is influenced by several environmental factors (temperature, surface, nutrients, and pH), which regulate the expression of the genes responsible for biofilm formation (LINOU & KOUTSOUMANIS, 2012; NGUYEN et al., 2014). The gene *csgD* is a central controlling regulator that can activate the transcription of *csgBAC* operons and encode the synthesis of curli fimbriae (SIMM et al., 2014). This gene also promotes *adrA* gene transcription, whose product interacts with *bcsABZC-bcsEFG* operons to synthesize cellulose (LIU et al., 2014). CHEN et al. (2020) revealed that *S.* Enteritidis mutants Δ*csgD*, Δ*csgA*, and Δ*bcsA*, but not Δ*adrA*, impaired biofilm formation compared with the WT strain, suggesting that biofilm formation was blocked after a single mutation of *csgD*, *csgA* or *bcsA*. Among different bacterial adhesins, type1 fimbriae (T1F) are one of the most common adhesive organelles in the members of the *Enterobacteriaceae* family, including *Salmonella* (KOLENDA et al., 2019). It was shown that T1F contributes to biofilm formation on Hep-2 cells, murine and chicken intestinal epithelium, and plastic surfaces (BODDICKER et al., 2002; LEDEBOER et al., 2006). In addition to curli and cellulose, another protein commonly found in biofilms of enteric bacteria is Bap (biofilm-associated protein). Bap is a surface protein that exhibits amyloid-like behavior (LATASA et al., 2005), and BapA is also involved in forming the bacterial pellicle (TURSI & TÜKEL, 2018).

CONCLUSION

In conclusion, *Salmonella* serovars from swine-feed mills showed a biofilm formation phenotype by evaluating colony morphology, pellicle formation at the air-liquid interface, and adhesion on the polystyrene surface. All *Salmonella* isolates in this study presented morphotype RDAR and were weakly or moderately adherent at 28 °C, except one *S.* Montevideo isolate. Biofilm-forming ability may be an important factor for the persistence of *S.* Agona and *S.* Montevideo in the environment and pose a source of contamination for new ingredients and feed.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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